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Heterogeneous electron transfer studies with ligninolytic redox enzymes and living bacteria

Applications in biosensors and biofuel cells

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2009

Academic dissertation for the degree of Doctor in Philosophy, to be publicly defended at the Centre for Chemistry and Chemical Enginering, Sölvegatan 39, Lund, on September 25th, 2009 at 10:30 A.M. in Lecture Hall B, by permission of the Faculty of Mathematics and Natural Sciences, Lund University, Sweden. The dissertation will be held in English.

Faculty opponent: Professor Dr. Woonsup Shin, Department of Chemistry and Interdisciplinary Program of Integrated Biotechnology, Sogang University, Seoul, Republic of Korea

Heterogeneous Electron Transfer Studies with Ligninolytic Redox Enzymes and Living Bacteria. Applications in Biosensors and Biofuel Cells

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Doctoral Thesis

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Heterogeneous electron transfer studies with ligninolytic redox enzymes and living bacteria. Applications in biosensors and biofuel cells

Abstract

The catalytic properties and the inter-domain electron transfer of cellobiose dehydrogenase (CDH) from the ascomycete fungus *Myriococcum thermophilum* adsorbed on graphite and thiol (SAM) modified gold electrodes were investigated using cyclic voltammetry, flow injection amperometry and UV-Vis spectroelectrochemistry. The fabrication and characterisation of a noncompartmentalised, mediator and cofactor free glucose–oxygen biofuel cell well-operating in glucose-containing buffers and human blood serum was performed. The biofuel cell was based on adsorbed enzymes exhibiting direct bioelectrocatalysis, viz. CDH from *Dichomera saubinetii* and *Corynascus thermophilus* used as anodic bioelements and laccase (Lc) from *Trametes hirsuta* and bilirubin oxidase (BOD) from *Myrothecium verrucaria* used as cathodic bioelements, respectively.

Different bacterial strains of *E. coli* and *B. subtilis* were immobilised on the electrode surface and tested for electrical communication using soluble and polymeric mediators. In the case of *E. coli*, the introduction of cytochromes in the inner membrane facilitated the electrochemical communication when using artificial mediators, while in the case of *B. subtilis,* the results demonstrated that mediators did not have to pass the cytosolic membrane to bring about an efficient electronic communication between bacterial cells with a thick cell wall and electrodes.

Keywords: cellobiose dehydrogenase, direct electron transfer, mediated electron transfer, self-assembled monolayer, flow injection analysis, amperometric biosensor, biofuel cell, succinate:quinone reductase, *E. coli*, *B. subtilis*, whole-cell biosensor.

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Signature Vasile Coman Date 2009-08-10

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

I. **Investigation of graphite electrodes modified with cellobiose dehydrogenase from the ascomycete *Myriococcum thermophilum***
Wolfgang Harreither, **Vasile Coman**, Roland Ludwig, Dietmar Haltrich and Lo Gorton
Electroanalysis, 19, 172 (**2007**)

II. **Investigation of electron transfer between cellobiose dehydrogenase from *Myriococcum thermophilum* and gold electrodes**
Vasile Coman, Wolfgang Harreither, Roland Ludwig, Dietmar Haltrich and Lo Gorton
Chem. Anal. (Warsaw), 52, 945 (**2007**)

III. **A membrane-, mediator-, cofactor-less glucose/oxygen biofuel cell**
Vasile Coman, Cristina Vaz-Domínguez, Roland Ludwig, Wolfgang Harreither, Dietmar Haltrich, Antonio L. De Lacey, Tautgirdas Ruzgas, Lo Gorton and Sergey Shleev
Phys Chem Chem Phys, 10, 6093 (**2008**)

IV. **A direct electron transfer based glucose/oxygen biofuel cell operating in human serum**
Vasile Coman, Roland Ludwig, Wolfgang Harreither, Dietmar Haltrich, Lo Gorton, Tautgirdas Ruzgas and Sergey Shleev
Submitted to Fuel Cells (**2009**)

V. **Electrical communication of cytochrome enriched *Escherichia coli* JM109 cells with graphite electrodes**
Sergey Alferov, **Vasile Coman**, Tobias Gustavsson, Anatoly Reshetilov, Claes von Wachenfeldt, Cecilia Hägerhäll and Lo Gorton
Electrochim. Acta, 54, 4979 (**2009**)

VI. **Electrical wiring of live, metabolically enhanced *Bacillus subtilis* cells with flexible osmium-redox polymers**
Vasile Coman, Tobias Gustavsson, Arnonas Finkelsteinas, Claes von Wachenfeldt, Cecilia Hägerhäll and Lo Gorton
Submitted to J. Am. Chem. Soc. (**2009**)

My contribution to the papers

Paper I. I took part in the design and planning of the experiments. I took part in the electrochemical experiments, in the evaluation of the results and in the writing of the manuscript together with Wolfgang Harreither.

Paper II. I planned and performed all the experimental work, evaluated the results and wrote the manuscript.

Paper III. I performed the electrochemical measurements for the bioanode and for the whole biofuel cell. I participated in the data evaluation.

Paper IV. I carried out most of the electrochemical measurements. I participated in the data processing and evaluation.

Paper V. I partially performed the experimental work together with Sergey Alferov. I participated in the data evaluation and writing of the manuscript.

Paper VI. I performed most of the experimental work, evaluated the data and wrote the manuscript.

Abbreviations

ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate) diammonium salt	IR	infrared
AFM	atomic force microscopy	IUPAC	international union of pure and applied chemistry
AQDS	9,10-anthraquinone-2,6-disulphate	Lc	laccase
BFC(s)	biofuel cell(s)	LDH	lactate dehydrogenase
BOD	bilirubin oxidase	MET	mediated electron transfer
CBO	cellobiose oxidase	$NAD(P)^+$	β-nicotinamide adenine dinucleotide (phosphate)
CBQ	cellobiose:quinone oxidase	NHE	normal hydrogen electrode
CDH	cellobiose dehydrogenase	OCV	open circuit potential
CE	counter electrode	OTE	optically transparent electrode
CV	cyclic voltammetry	OTTLE	optically transparent thin-layer electrochemical cell
COx	cholesterol oxidase	PEGDGE	poly(ethylene glycol) diglycidyl ether
DCIP	dichloroindophenol	PQQ	pyrroloquinoline quinone
DCPIP	2,6-dichlorophenolindophenol	PVI	polyvinylimidazole
DET	direct electron transfer	PVP	polyvinylpyridine
DNA	deoxyribonucleic acid	QFR	quinol:fumarate reductase
EC	enzyme commission	RE	reference electrode
EPR	electron paramagnetic resonance	SAM(s)	self-assembled monolayer(s)
ET	electron transfer	SCE	saturated calomel electrode
FA	flow analysis	SPE	spectroelectrochemistry
FIA	flow injection analysis	SQOR	succinate:quinone oxidoreductases
FAD	flavin adenine dinucleotide	SQR	succinate:quinone reductase
FMN	flavin mononucleotide	STM	scanning tunnelling microscopy
GDH	glucose dehydrogenase	UV-VIS	ultraviolet-visible
GMO	genetically modified organism	WE	working electrode
GOx	glucose oxidase	WJ	wall jet

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CHAPTER 1

Introduction

1.1. Perspectives and aims of thesis

Over the past century, scientific research has been very successful in understanding fundamental processes and in improving the human living conditions but there are still many challenges ahead. In the world of today, where fossil fuel depletion and global climate change are very serious problems, threatening our very existence and future, scientific research efforts must dramatically increase with the purpose of understanding and limiting the causes of the problems and finding alternative resources of energy. The current thesis is focussed on studying heterogeneous electron transfer mechanisms between redox enzymes / bacterial cells and different electrode materials with possible applications in biosensors and biofuel cells.

This thesis work has been part of the European Union funded project BIONEL: Bioanalytical Electrochemistry and Bionanotechnology (MEST-CT-2004-514743). The multidisciplinary project was focussed on using biologically-derived material (e.g. enzymes, cells) as analytical tools (e.g. biosensors) and on making analytical measurements of biological material and living organisms. The object of the sub-project I was working on was the study of the electron transfer mechanism between (i) isolated redox enzymes and electrodes and (ii) mediators and whole living bacterial cells. As a consequence, the papers this thesis is based upon can be divided into two groups: the first set is dealing with ligninolytic redox enzymes (**Papers I to IV**) and the second one with bacterial cells (**Papers V & VI**).

After a short review of my work, the summary of the thesis will start by describing the electron transfer phenomenon (**Chapter 2**), it will continue with the electrochemical methods used in this work to study electron transfer (**Chapter 3**) and with a description of the biological materials used (**Chapter 4**) and it will come to an end with a collection of recent examples of possible applications (**Chapter 5**) involving electron transfer reactions in enzyme-based biosensors and biofuel cells (enzymatic and microbial).

1.1. Summary of research

As mentioned above, the first four papers have dealt with the use of ligninolytic redox enzymes as possible biological materials in biosensors and biofuel cells. The work from the last two papers was focussed on the use of whole bacteria as analytical tools. A short summary of the individual papers follows below.

In **Paper I** we have investigated the direct and mediated electron transfer (DET and MET) characteristics of a new cellobiose dehydrogenase – CDH (from the ascomycete fungus *Myriococcum thermophilum*) adsorbed on graphite electrodes. The effects of applied potential, pH and buffer composition were tested and optimised, and the most suitable conditions were used to evaluate the analytical characteristics of the sensor for different carbohydrates in the flow injection mode. It was found that, in contrast to the previously studied basidiomycete CDHs, glucose and several other monosaccharides were relatively good substrates for this CDH and, moreover, cellobiose did not exhibit any substrate inhibition.

In **Paper II**, the same enzyme as in **Paper I** was investigated at the surface of a thiol modified (self-assembled monolayers, SAMs) gold electrode, using cyclic voltammetry and UV-Vis spectroelectrochemistry. CDH is a monomeric protein consisting of two subdomains: a larger catalytic flavin-associated domain (DH_{cdh}) and a smaller heme-binding domain (CYT_{cdh}) connected via a protease cleavable linker region. In this study, the inter-domain electron transfer was studied at two different pH values, one acidic (pH 4.5) close to the optimum pH value, where maximal response was obtained on graphite and one neutral (pH 7), where the electrocatalysis was found to be less effective. It was found that at low pH the inter-domain electron transfer is very effective compared to at neutral pH. We think that the reason for this behaviour can be attributed to conformational changes of the two domains and the linker region.

In **Paper III**, we used CDH from *Dichomera saubinetii* as a bioanode and laccase (Lc) from *Trametes hirsuta* as a biocathode to put together a DET based, noncompartmentalised, mediator and cofactor free glucose/oxygen biofuel cell (BFC), working in an air-saturated 0.1 M citrate–phosphate buffer, pH 4.5, containing glucose. The low pH value was chosen because both enzymes work best in acidic conditions. The BFC had the following characteristics when using 5 mM glucose: an open-circuit voltage of 0.73 V; a maximum power density of 5 $\mu W\ cm^{-2}$ at 0.5 V of the cell voltage and an estimated half-life of >38 h.

In **Paper IV** we reported on the fabrication and characterisation of the very first DET based glucose/oxygen biofuel cell well-operating in neutral glucose-containing buffer and human

blood serum. The bioelements used in this study were CDH from *Corynascus thermophilus* and bilirubin oxidase (BOD) from *Myrothecium verrucaria*, which have neutral optimum pH values. The following characteristics of the mediator-, separator-, and membrane-less, and, *a priori*, non-toxic, simple, and miniature biofuel cell, were obtained: an OCV of 0.62 V and 0.58 V, a maximum power density of *ca.* 3 and 4 μW cm^{-2} at 0.37 V and 0.19 V of cell voltage, in phosphate buffer and human serum, respectively.

In **Paper V** three different strains of *Escherichia coli* (JM109 – a native "wild type" strain, JM109/ pBSD 1300 – a strain overproducing the membrane anchor domain of *Bacillus subtilis* succinate-quinone reductase (SQR), a protein that contains two transmembraneously arranged heme groups and JM109/ pLUV 1900 – a strain overproducing cytochrome c_{550} from *B. subtilis*, a protein, where the cytochrome domain is anchored to the membrane with a transmembrane helix), were immobilised on the surface of a spectrographic graphite electrode and tested for electrical communication using mediators. It was found that the introduction of the cytochromes in the bacterial membrane had great effect on the ability of the bacterial cells to effectively communicate with artificial mediators. The introduction of the transmembraneously arranged heme groups of *B. subtilis* made it possible for this strain to communicate with two flexible osmium containing redox polymers, whereas the introduction of the cytochrome c_{550} had an effect especially increasing ability of ubiquinone to act as an efficient electron acceptor.

In **Paper VI** the same osmium polymers as above were used to wire a strain of the gram-positive *B. subtilis*, which overproduces SQR, with electrodes. The results demonstrated that the mediators did not have to pass the cytosolic membrane to bring about an efficient electronic communication between bacterial cells with a thick cell wall and electrodes. It was found that the two polymers, which have different redox potentials, can be differently influenced by the presence of oxygen, most likely due to the different thermodynamic driving force, superior in the case of the high potential polymer. Moreover, wild type *B. subtilis* cells generate a much lower current response (several fold) for succinate compared to the cells, which overproduced SQR. This is expected, assuming that the source of the response is the oxidation of succinate by SQR, and demonstrates that such metabolic enhancement can greatly improve the efficacy of a microbial fuel cell.

CHAPTER 2

Electron transfer mechanisms

Electron transfer (ET) is the most ubiquitous elementary process in chemistry and biology [1-3]. ET is also the simplest chemical process because no bonds are broken or newly formed; only bond lengths and angle adjustments occur in the ET partners and the reaction medium. The fact that ET is omnipresent can be appreciated by several immediate observations: ET is the central event in inorganic and organic redox chemistry, in all electrochemical processes and it is crucial in biological metabolism, in photosynthesis and respiration.

There are several classes of electron transfer reactions, defined by the state of the two redox centres and their connectivity: ***i) inner-sphere ET*** (discovered by Henry Taube [4], Nobel prize in 1983), where the donor and acceptor are covalently linked during the ET; if this bridge is permanent the electron transfer event is termed intramolecular electron transfer and if the covalent linkage is transitory the electron transfer event is termed intermolecular electron transfer; ***ii) outer-sphere ET,*** where the chemical moieties remain separate species before, during, and after the ET event, the electrons hopping through space from the donor to the acceptor; outer-sphere ET is by definition intermolecular and it can occur between different species or between identical chemical species that differ only in their oxidation state (self-exchange); ***iii) heterogeneous ET***, where the electrons move across the boundary of two phases (e.g. liquid solid interface).

The first generally accepted theory of ET was developed by Rudolph A. Marcus (Nobel prize in 1992), elaborated in 1956 [5, 6] to explain the rates of electron transfer reactions. It was originally formulated to address outer-sphere ET reactions, but it was also extended to inner-sphere ET reactions and heterogeneous electron transfer [7-9]. As mentioned by Marcus in his Nobel lecture [10], the inspiration to develop his theory was a paper of Bill Libby [11] in which the Franck-Condon principle was used to explain why reactions between pairs of small cations in aqueous solution are relatively slow with respect to the same electron transfer reactions involving larger ions. The early experiments in the ET field (mainly self-exchange reactions) were influenced by the availability after the Second World War of many radioactive isotopes and

by the introduction of new instrumentation, which permitted to study the rates of rapid chemical reactions such the electron transfer (*e.g.* stopped-flow apparatus).

The Marcus theory, based on the transition-state theory approach, builds on the traditional Arrhenius equation for the rates of chemical reactions in two ways: ***i***) it provides a formula for the pre-exponential factor in the Arrhenius equation, based on the electronic coupling between the initial and final state of the ET reaction (*i.e.*, the overlap of the electronic wave functions of the two states); ***ii***) it provides a formula for the activation energy. The semi-classical Marcus theory [9] predicts that the ET rate is governed by the thermodynamic driving force of the reaction (ΔG^0), the nuclear reorganisation energy (λ) and the electronic coupling (H_{DA}) between the electron donor (D) and the acceptor (A) at the transition state:

$$k_{et} = \frac{2\pi}{\hbar}\frac{H_{DA}^2}{\sqrt{4\pi\lambda RT}}e^{-\frac{(\Delta G^0+\lambda)^2}{4\lambda RT}} \qquad (1),$$

where k_{et} is the direct electron transfer rate, $\hbar$ is the reduced Planck constant, R is the universal gas constant and T is the absolute temperature. The reorganisation energy (λ) includes vibrational components (inner-sphere or intramolecular reorganisational energy, λ_i) and components from the polarisation changes in the dielectric solvent environment (outer-sphere or solvent reorganisation energy, λ_0). Equation (1) partitions into nuclear (exponential) and electronic (pre-exponential) terms: ET reaches maximum when the nuclear factor is optimised (i.e. $-\Delta G^0 = \lambda$) and it only depends on the electronic factor (H_{DA}). For a better understanding of the parameters involved in the equation above, a simple model is given in **Figure 2.1**, where the reactant and product energy surfaces are treated as parabolic [3, 9].

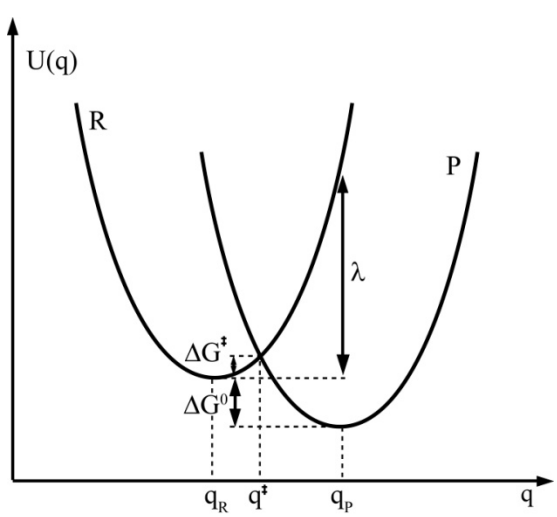


Figure 2.1. Nuclear potential (Gibbs free energy) energy curves, where q is the reaction coordinate, showing the reactants' (R) and the products' (P) states. ΔG^0 is the reaction Gibbs free energy (driving force), $\Delta G^{\ddagger}$ is the activation free energy for the reaction, λ is the reorganisation energy, q_R, $q^{\ddagger}$ and q_P are the configurations of the reactant, transition state and product, respectively.

In biology, the energy conversion in the bioenergetic processes (respiration and photosynthesis) is carried out by a series of proteins and enzymes that form ET chains. In this case, phenomena like multi-step and long-range ET occur in addition to simple, single-step electron transfer. The long-range ET in biology is dominated by metalloproteins (a protein that contains a cofactor of a metal ion complex), although the phenomenon is not confined to this class (*e.g.* long-range ET between transition metal complexes intercalated with DNA [12]). In the long-range ET electrons are transferred as single entities through one or more intervening redox cofactors [13]. It is well known [14] that, in general, a protein controls the ET by changing the formal potentials of its redox active sites, defining the local environment to control the reorganisation energy and providing proper ET pathways between the redox centres. Due to the fact that ET occurs at protein-protein interfaces and that many redox proteins maintain their activity in biological membranes, a useful model system to study fundamental aspects of protein ET has proven to be ET of proteins immobilised at chemically modified electrode surfaces [15].

The redox cofactors of an enzyme can be classified into inorganic and organic cofactors. The inorganic cofactors include metal ions (Cu, Fe, Mg, Mn, Mo, W, Ni, Se, Zn) and metallic complexes (e.g. Fe-S clusters). The organic cofactors are small molecules that can be either loosely or tightly bound to the enzyme. The organic redox active centres include: quinones ($2e^-$, $2H^+$ centres), e.g. pyrroloquinoline quinone (PQQ); flavins ($2e^-$, $2H^+$), i.e. flavin adenine dinucleotide – FAD and flavin mononucleotide – FMN; NAD(P)H ($2e^-$, $1H^+$), i.e. β-nicotinamide adenine dinucleotide – NAD^+ and β-nicotinamide adenine dinucleotide phosphate – $NADP^+$; hemes ($1e^-$), e.g. heme *a*, heme *b*, heme *c* being the most common.

In the recent decades much focus has been directed into studying the ET reactions in biology, within the proteins [9, 13, 16-25] and in heterogeneous ET reactions between proteins and electrode surfaces [14, 15, 26, 27]. Different approaches have been developed to predict H_{DA} in proteins. The simplest one is the square-barrier model, where the protein is regarded as a "frozen organic glass" [13] in which H_{DA} decays exponentially with the distance between donor and acceptor:

$$H_{DA}^2 = (H_{DA}^0)^2 \exp\left(-\beta(r_{DA} - r_0)\right) \qquad (2)$$

where H_{DA}^0 is the electronic coupling at the **van der Waals** distance (r_0) and r_{DA} is the distance between the donor and acceptor. The exponential attenuation of the overlap with distance between the donor and acceptor is given by the tunnelling parameter β, which in proteins ranges between 0.8 and 1.6 $Å^{-1}$ and depends on the structure of the protein [18, 19, 25].

Apart from mechanistic aspects, the study of redox proteins coupled to electrode surfaces has implications in different fields such as biosensors, biofuel cells, bioelectronics, clinical

assays, drug screening, etc. Our motivation for studying protein (enzyme) ET at an electrode surface is, among others, directed towards "third generation" biosensors (based on direct electron transfer between the enzyme and the electrode) and biofuel cells (direct and mediated electron transfer). Applications of ET in these fields, with various examples, are discussed in **Chapter 5**.

The direct electronic coupling between the prosthetic group of the enzyme and the electrode is hard to achieve for many enzymes, since the redox centre is often deeply buried within the protein structure. Different ways to circumvent this problem address either the protein orientation on the surface (for example self-assembled monolayers - SAMs of thiols on gold surfaces) or the use of mediators (ET relays) to facilitate the electron transfer reactions.

2.1. Direct electron transfer - DET

Looking back over the past three decades, there has been a lot of interest in the study of an intriguing phenomenon called "direct electron transfer" – DET, which refers to the direct electronic coupling between the redox cofactor of a protein and an electrode surface. As mentioned above, the study of direct electrochemistry of redox proteins at surfaces involves obtaining valuable information about the thermodynamic and kinetic properties of the protein and has implications for catalytic and sensor applications. The pioneers in the field, who demonstrated a quasi-reversible, direct electrochemistry of a redox protein at a solid electrode, were Yeh and Kuwana [28] and Eddowes and Hill [29], who in 1977 independently demonstrated DET of cytochrome *c*, an essential component of the electron transport chain, at tin doped indium oxide [28] and 4,4'-bipyridyl modified gold [29] electrodes, using cyclic voltammetry. These results were reported 10 years after the first surface immobilisation of a redox enzyme (glucose oxidase - GOx) at an electrode surface – "enzyme electrode", for biosensing purposes [30]. These first reports on DET were followed in 1978/79 by publications from the Berezin group in Moscow on DET possibility for larger redox proteins with enzyme activity (redox enzymes). The scientists showed that laccase (Lc) [31, 32] and peroxidase [33] modified carbon electrodes exhibited DET in the presence of the enzymes substrates, i.e. oxygen and hydrogen peroxide.

As a conclusion from these very first reports, it can be seen that there are two different experimental approaches to prove DET between redox enzymes and electrodes: i) direct electrochemical conversion of the redox active site at the electrode surface in the absence of the enzyme substrate; ii) indirect evidence based on catalytic response generated in the presence of the enzyme substrate. The two approaches are schematically represented below in **Figure 2.2**.

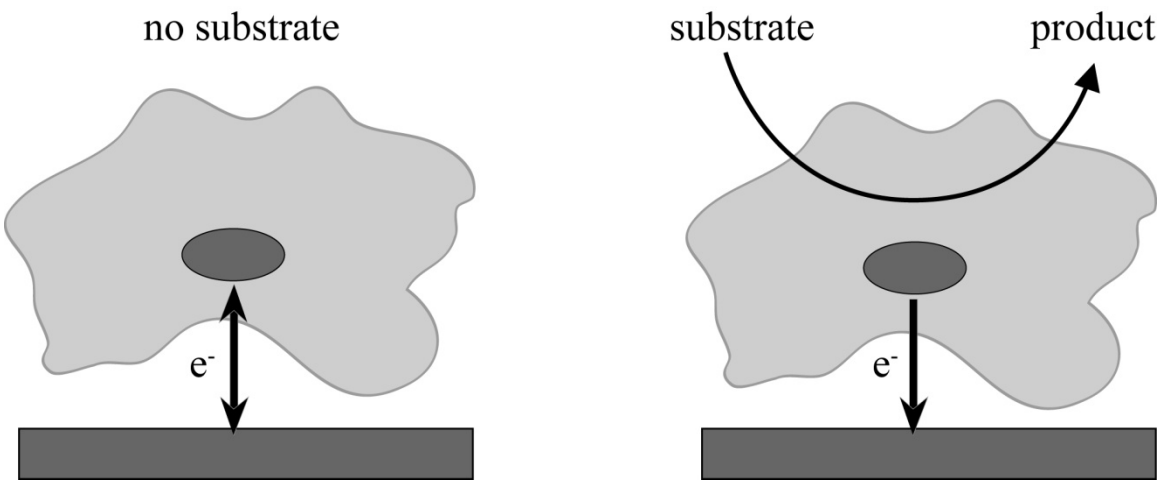


Figure 2.2. Experimental approaches for detecting DET in redox enzymes.

According to the mechanism of electron transfer, Hill and co-workers proposed that the redox enzymes can be divided in two classes [34, 35]: i) ***intrinsic***, where the electron transfer takes place within the vicinity of the prosthetic group, usually between the substrate and the enzyme, and there is apparently no need for long ET pathways to the surface of the protein; ii) ***extrinsic,*** where the primary redox site is inaccessible directly but it can be reached efficiently through a secondary redox site, situated close to the surface, which can communicate with the first one through internal ET pathways. Thus, in the extrinsic enzymes, there is a part of the surface across which the ET can occur between electrode and enzyme [35]. The biological role of the extrinsic enzymes is to transfer electrons to other proteins. In order to obtain DET with the intrinsic enzymes, Armstrong et al. [34] identified important requirements: (a) the site of the catalytic reaction has to be close to the protein surface, (b) the enzyme can deform without losing activity, (c) the electrode surface (with or without a promoter) can project into the enzyme, or (d) that electron-transfer pathways are introduced by modification.

In the case of both intrinsic and extrinsic enzymes, the orientation of the protein on the surface of the electrode is crucial for an efficient DET. According to the Marcus theory (see above), DET between a protein and electrode is dependent on three major factors: i) the distance between the redox site and the electrode; ii) the reorganisation energy, which reflects the structural rigidity of the redox site in its oxidised and reduced forms; iii) and the thermodynamic driving force of the ET, which is related to the applied potential at the surface and the redox potential of the protein [36-40]. In the case of gold electrodes, the electronic pairing between the enzyme and the electrode can be varied using different self-assembled monolayers (SAMs), managing in this way the orientation of the redox active protein on the electrode surface and the distance between the redox active site of the protein and the electrode.

2.1.1. Self-assembled monolayers of thiols on Au

The self-assembled monolayer (SAM) is an organised molecular assembly of amphiphilic molecules formed by adsorption of molecules onto a solid surface from a homogeneous solution. The organisation is given by the affinity of the head group for the surface and from the slow two-dimensional organisation of hydrophobic tail groups. The self-assembly phenomenon has been known for more than 60 years [41, 42], but it was much later before it gained popularity due to the very stable monolayers formed [43-46]. From the heterogeneous ET perspective, an important step is the discovery of the strong adsorption of thiols, sulphides, disulphides and related molecules on metal surfaces, especially gold. The credit for discovering this approach is attributed to Nuzzo and Allara [47], although it was Taniguchi et al. in 1982 [48] who already used bis(4-pyridyl)disulphide (Aldrithiol™-4) to modify an Au electrode for the study of DET with cytochrome *c*. Taniguchi and Hill [48, 49] were the pioneers in the field on the modification of Au with organic compounds that yields diffusion controlled electrochemistry of cytochrome *c*.

For a detailed description of the SAM subject, please see the dedicated literature, including review papers dealing with formation, structure, characterisation and applications of SAMs [50-55], a book chapter dedicated to the electrochemistry of SAMs on electrodes [56] and several chapters from the Volume 10 of the *Encyclopedia of Electrochemistry* [57].

The interaction between a SAM modified electrode and a particular protein can be modelled by changing the functionality of the tail-group of the modifier. For example, whereas cytochrome *c* prefers acidic groups such as -COO(H), which can interact favourably with the lysine-rich region around the exposed heme edge [49, 58-61], acidic proteins, such as plastocyanin or ferredoxins respond best if the end-group is a basic group such as $-NH_2(H^+)$ [62-64]. The modification of the electrode with alkanethiols having different end-group functionalities has proven to be a very efficient strategy for achieving efficient DET for larger redox enzymes as well, when the orientation brings the redox centre(s) of the protein close to the electrode surface, avoiding at the same time protein denaturation at surfaces [65, 66].

An important aspect worth mentioning in the case of SAMs is that the stability of the monolayer depends on the chain length as well, with the shorter chains producing less stable layers due to weaker hydrophobic interactions between the tail chains of the thiols [67]. Of course, a clean and smooth substrate, combined with a proper temperature of adsorption and a proper solvent (especially in the case of mixed SAMs) are the key ingredients for a well-organised monolayer [56]. In the case of Au substrates, a single-crystal surface, e.g. Au(111), provides the smoothness needed for a stable, well-ordered SAM [68, 69].

Another important issue for the efficient DET is, as mentioned above, the distance between the redox active site of the protein and the electrode surface. By using homogeneous

surfaces of SAMs with different chain lengths, the hypothesis that ET rates for protein SAM-modified electrode depend on the donor-acceptor distance can be easily verified. Early experiments on cytochrome *c* [59] using medium length $HS(CH_2)_nCOOH$ SAMs showed that with longer spacers the rate constants depend exponentially on the chain length, whereas, as the chain is shortened the rate constants reach a limiting value. A more recent study [70] involving azurin (blue-copper protein) on alkanethiols SAM modified Au(111) with chain lengths up to 17 CH_2-units, has shown that the rate constant of DET decays exponentially for chains longer than nine CH_2-units and that for shorter chains the rate constants are almost independent of the chain length. This type of behaviour was later demonstrated also for small-redox molecules attached in the end of alkyl-SAMs. Smalley et al. [71, 72] have shown that the limiting value of the ET rate constant is independent on both the identity of the redox couple and the nature of the linkage between the redox couple and the alkane chain.

DET reactions at SAM modified Au electrodes with redox enzymes were studied both for basic knowledge and also for possible applications in "third generation" biosensors and biofuel cells (see examples presented in **Chapter 5)**. Concerning the current thesis, heterogeneous DET phenomena were studied and exploited in our first four papers, both for mechanistic studies (**Papers I** & **II**) in the case of cellobiose dehydrogenase (CDH – described in **Chapter 4**) and for the construction of simple DET based enzymatic biofuel cells (**Papers III** & **IV**). The SAMs on Au approach has been used in two different ways. First, in **Paper II**, Au electrodes were modified with different thiol SAMs in order to investigate the DET properties of CDH from the ascomycete fungus *Myriococcum thermophilum*, using both cyclic voltammetry and UV-Vis spectroelectrochemistry. The SAM-Au approach, using CDH from different fungal sources, has been used previously in our group [36, 73-75], and one can see in these references, once again, the importance of choosing the proper thiol modification for an efficient response. Second, we used SAMs on Au when working with living bacterial cells (**Papers V** & **VI**) in order to avoid cell lysis that could occur due to strong adsorption at a naked metal surface.

2.2. Mediated electron transfer – MET

When the catalytic centre of the enzyme is deeply buried within the protein structure (intrinsic enzymes), electrical communication between this site and the electrode surface is difficult to achieve. With a few exceptions, such as heme-containing horseradish peroxidase (HRP) [36, 76-78], heterogeneous DET rates for intrinsic enzymes are very low. The reason for this resides mainly in the spatial separation between the biocatalytic redox site and the electrode

(see Marcus theory above). The electrical communication of redox enzymes that lack DET with electrodes can be established by using intermediate charge-carriers (ET mediators) between the redox centre and the electrode surface.

Almost half a century ago, Leland C. Clark Jr. marked the birth of biosensor technology with his pioneering research [79]. In the very first biosensor configuration, GOx (EC 1.1.3.4.) was physically entrapped on the surface of a Clark-oxygen electrode behind a dialysis membrane. Being the first and the most commonly used intrinsic enzyme in glucose biosensors [80, 81], GOx is the perfect example for me to try to explain the use of mediators with a redox enzyme. GOx from *Aspergillus niger* (the most commonly used GOx in biosensors) is a dimeric glycosylated protein that catalyses the oxidation of β-D-glucose into glucono-δ-lactone, which then hydrolyses into gluconic acid [82]. At the same time molecular oxygen is reduced to hydrogen peroxide (Equations 3 – 5). The enzyme contains two molecules of the coenzyme FAD tightly bound together, but not covalently attached to the apoenzyme [83].

$$\mathrm{GOx(FAD)} + \beta\text{-D-glucose} \rightarrow \mathrm{GOx(FADH_2)} + \text{glucono-}\delta\text{-lactone} \quad (3)$$

$$\mathrm{GOx(FADH_2)} + \mathrm{O_2} \rightarrow \mathrm{GOx(FAD)} + \mathrm{H_2O_2} \quad (4)$$

$$\text{glucono-}\delta\text{-lactone} + \mathrm{H_2O} \rightarrow \text{D-gluconic acid} \quad (5)$$

Being non-covalently attached, the FAD molecules can be removed from the holoenzyme without permanently denaturing the apoenzyme [84]. Moreover, when adsorbed at electrode surfaces, early reports show that a slow unfolding of the enzyme occurs with the adsorption of FAD directly at the electrode surface [85, 86]. This fact seems to be overlooked by a highly cited series of recent reports claiming efficient DET with GOx [87-89]. One elegant solution derived from the fact that FAD can be removed from the enzyme without denaturation of the apoenzyme, was adopted by Willner et al. [90], who removed the non-covalently bound FAD redox centre from the enzyme (GOx) and reconstituted the enzyme on a tether consisting of a cystamine modified Au electrode, a PQQ link and FAD [91]. The same group reconstituted GOx using FAD bound to 1.4-nm Au crystals showing that it is possible to electro-oxidise glucose when the electrons are relayed to the electrode via the FAD-Au nano-plug [92].

In the beginning of the biosensor era, the glucose concentration was determined by measuring the O_2 consumption (e.g. reduction on platinum) or the H_2O_2 production (oxidation on Pt), this approach being termed as "first generation biosensors" (for more details see **Chapter 5**). This method has a series of disadvantages, such as fluctuations in the O_2 concentration and interfering compounds in the case of H_2O_2 oxidation from real samples. Both direct O_2 reduction and H_2O_2 oxidation occur, from a sensing perspective, at rather extreme potentials, opening up

the sensing system for interfering reactions. In an attempt to limit the impact of dissolved O_2, researchers have started to employ a variety of mediation schemes. In this case reaction (4) becomes (6) in the case of using a two-electron, two-proton mediator (e.g. a quinone) or (7) in the case of a one-electron acceptor and the mediator gets re-oxidised at the electrode surface expressed in (8):

$$\mathrm{GOx(FADH_2)} + \mathrm{M_{ox}} \rightarrow \mathrm{GOx(FAD)} + \mathrm{M_{red}} \tag{6}$$

$$\mathrm{GOx(FADH_2)} + 2\mathrm{M_{ox}} \rightarrow \mathrm{GOx(FAD)} + 2\mathrm{M_{red}} + 2\mathrm{H^+} \tag{7}$$

$$\mathrm{M_{red}} \rightarrow \mathrm{M_{ox}} + x\mathrm{e^-} \tag{8}$$

In the case of immobilised GOx, the very first paper using benzoquinone as an ET mediator instead of O_2 was published in 1970 [93]. In the same paper, Williams et al. reported on using successfully ferro/ferricyanide together with the NAD^+ (nicotinamide adenine dinucleotide – two-electron, one-proton redox centre) dependent lactate dehydrogenase. Potassium hexacyanoferrate (III) was also used more recently with the complex xanthine oxidase (2 FAD, 2 Mo, 8 Fe) [94], but the most highly cited MET paper is the one reporting about using ferrocene derivatives with GOx [95], most probably because the idea was later transferred into a commercial product [96]. Various freely diffusing mediators have been used since then with different enzymes and numerous examples can be found in a series of review papers [81, 97-100]. Regarding the current thesis, we have used 1,4-benzoquinone to compare DET and MET behaviour of CDH (**Paper I**).

In order to be effective as an electron acceptor with GOx, the mediator must compete efficiently with its natural electron acceptor, i.e. molecular oxygen. Most freely diffusing mediators do not meet this requirement, and exhibit parasitic effects of O_2 on their response, being effective only in deoxygenated solution, which is of little use in practical applications. Another disadvantage of the diffusing mediators is their possible toxicity in people, and because of that, they cannot be used in implantable biosensors because of the possibility of escape from the biosensor into the body fluid [101].

In order to eliminate the problem of mediators diffusing into the solution, Adam Heller came up with the idea that efficient ET with GOx could be accomplished by chemical modification of the enzyme with redox relays [102, 103]. Based on the previous results of Cass et al. [95], Degani and Heller coupled ferrocenecarboxylic acid to the lysine residues of GOx using water-soluble carbodiimide as activating agent and proved that electron collection efficiency can be higher than in most diffusionally mediated systems. The efficiency of such bound relays could be further increased by adding flexible chains between them and the

oligosaccharide periphery of GOx [104]. In these cases though, the modified enzyme exhibited efficient communication only in solution, i.e., when contained by a membrane in a small compartment near the electrode. Any cross-linking with an electrode surface resulted in a rigid structure, limiting the flexibility and efficiency of the redox relays and the proper orientation of the enzyme. On top of that, O_2 interference could not be prevented [105].

Based on the original idea of Degani and Heller, the field of enzymatic biosensors containing polymeric electron transfer systems emerged soon after [106]. Using carbon paste electrodes, Okamoto, Skotheim, Hale and co-workers reported various polymeric ET systems used together with GOx mainly, e.g. ferrocene/siloxane [107, 108], ferrocene/poly(ethylene oxide) [109] and poly(ether amine quinone) [110]. Heller and co-workers developed in the same time the concept of "wired" redox enzymes to increase biosensor precision and stability. They expanded the communication between the FAD centres of GOx and metal or carbon electrodes by wiring the enzyme with a high molecular weight cationic, however water soluble and highly flexible redox polymer, which had a segment bound to the electrodes [105]. They have selected for this purpose osmium-based redox polycations over ferrocene derivatives because they were known to be fast, more long-term stable in both its redox states and to have proper redox potentials for wiring GOx [111].

2.2.1. Osmium redox polymers

Heller and co-workers started the design of osmium containing redox polymers by following several objectives [101]: i) to assure efficient wiring of the buried redox active centre of the enzyme in order to permit efficient ET to the surface of the protein; this required that the redox polymer was soluble in water and also that it had hydrophobic, charged or hydrogen-bonding domains to bind the enzyme [112]; ii) to guarantee that only a small part of the molecular wire was bound to the electrode and most segments remained unadsorbed, remaining available to wire the enzyme [113]; this objective was fulfilled by using highly water-soluble redox polymers of high molecular weight; iii) to form a hydrophilic three-dimensional network that could incorporate a large number of enzyme molecules [114-116]; this objective was fulfilled by using a cross-linker, i.e. poly(ethylene glycol) diglycidyl ether (PEGDGE), which together with the redox polymer generated a redox hydrogel, eliminating in the same time the need for a membrane. The extent of cross-linking, combined with the molecular mass of the starting polymer, defines the mechanical properties of the hydrogel, ranging from soft to tough materials [117].

Redox hydrogels conduct electrons by self-exchange between the redox functionalities tethered to the polymeric backbones (e.g. polyvinylimidazole - PVI or polyvinylpyridine – PVP).

ET by self-exchange takes place by collisions between reduced (electron-loaded) and oxidised (hole-loaded) redox centres [81, 117-119]. The efficiency of ET by self-exchange was shown to increase when the redox functions are tethered to the polymer backbones by long and flexible spacers that are between 8 and 15 atoms long [120-123].

The redox hydrogels, tailored to be polycations, can be used to wire different redox enzymes. In order to eliminate the electrocatalytic reactions of interferences (e.g. ascorbate, urate in blood, in the case of implantable glucose biosensors) the redox potential of the hydrogel can be adjusted to be close to the one of the redox site of the enzyme. By changing the ligands of the transition metal ion, i.e. $Os^{2+/3+}$, incorporated in the electron-conductive complex, the redox potentials of the redox wire could be tuned, ranging from 0 V *vs.* NHE (for the PVP-[Os(N,N′-dialkylated-2,2′-biimidazole)$_3$] $^{2+/3+}$), best suited for GOx [120, 122] to +0.75 V *vs.* NHE (for PVP-[Os(4,4′-dimethyl-2,2′-bipyridine)$_2$(4-aminomethyl-4′-methyl-2,2′-bipyridine)Cl]$^{2+/3+}$), which is close to the redox potential of the T1 site of high-potential "blue" multi-copper oxidases (see Chapter 4 for details), e.g. Lc [121, 123].

The Os containing redox hydrogels have been proven very useful when the need to avoid the leaching of freely diffusing redox mediators is imperative, e.g. in the case of implantable glucose monitoring biosensors [124, 125]. They were also shown to be efficient in the field of membrane-less biofuel cells [126-132] and bioelectrochemical propulsion [133]. In our group, in addition to applying Os-containing polymers to wire enzymes [134-137], the redox polymers have been used in conjunction with living bacterial cells [138-140]. In these studies the Os-containing polymers were either P(VI)$_{12}$-[Os-(4,4′-dimethyl-2,2′-bipyridyl)$_2$Cl$_2$]$^{2+/+}$, denoted usually osmium redox polymer **I** or PVP-[Os-(N,N′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$, denoted as osmium redox polymer **II**. The two Os-polymers were chosen since they differ greatly in both redox potential (i.e. approx. +0.35 V *vs.* NHE for Os-polymer **I** and 0 V *vs.* NHE for Os-polymer **II**) and the length of the side chains, where the $Os^{2+/3+}$- functionalities are located at their ends. For the structures of the two Os-polymers, see **Figure 2.3** below.

With bacterial cells, initial studies in our group [138] were made by wiring Os-polymer **I** with the structurally rather simple gram-negative *Gluconobacter oxydans* [141], where redox enzymes from the cytoplasmic membrane were addressed, yielding response for glucose, fructose, ethanol and glycerol. In further studies focus was on the structurally more complex gram-negative *Pseudomonas putida* and *Pseudomonas fluorescens* [139, 140], where response currents could be obtained both for substrates being metabolised in the cytoplasmic membrane (glucose) as well as in the cytosol of the cell (phenol). As a logical continuation of the previous studies, in **Paper V** we have shown that introduction of a cytochrome to the cytoplasmic membrane of *Escherichia coli* greatly facilitated the communication between these gram-negative bacterial cells and the osmium polymers. In **Paper VI** we have demonstrated the

possibility of wiring efficiently the gram-positive *Bacillus subtilis*, having a substantially thicker peptidoglycan cell wall [142].

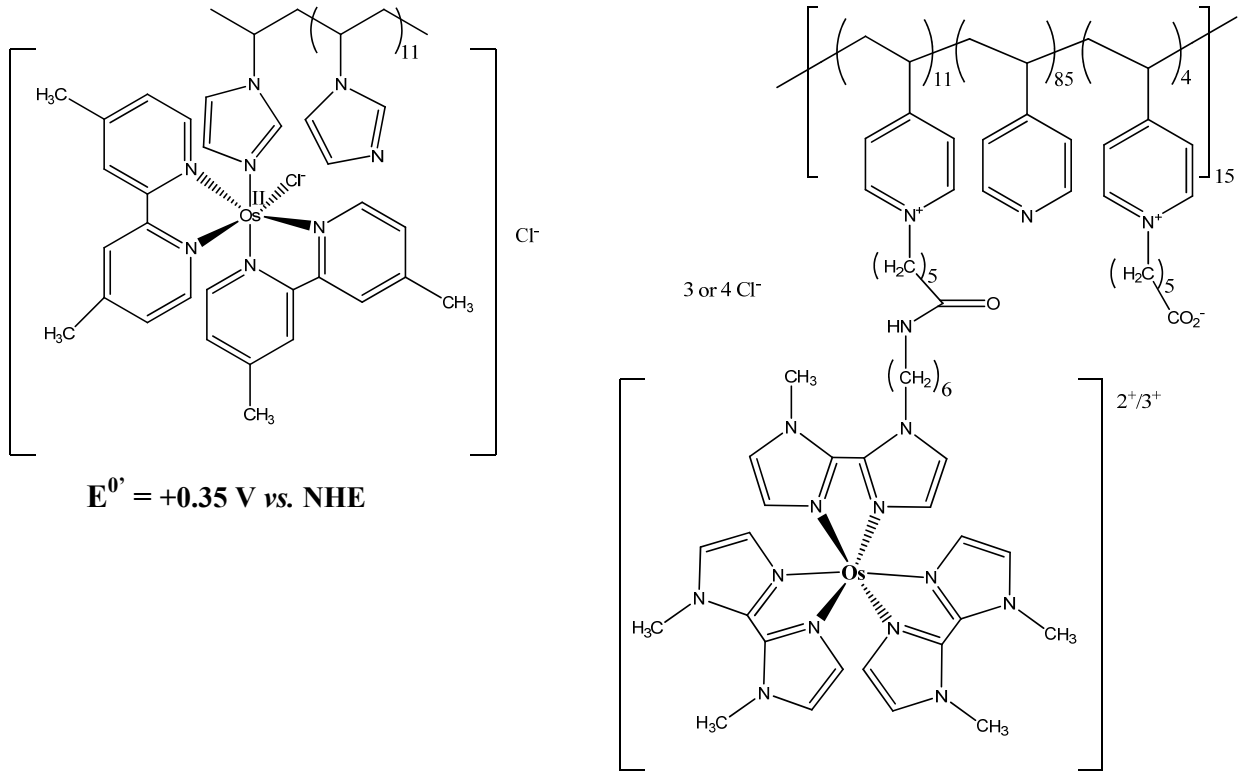


Figure 2.3. Structure of osmium redox polymer **I** (left) and **II** (right).

CHAPTER 3

Electrochemical methods for electron transfer studies

Electrochemistry covers studies of the interplay between electricity and chemistry. As a result of that, electrochemistry is a part of many processes including corrosion, batteries, electroplating, electrosynthesis, biomedical applications, etc. The basic reaction in electrochemistry is denoted as follows:

$$\mathrm{Ox} + \mathrm{ne}^{-} \leftrightarrow \mathrm{Red} \tag{9}$$

where Ox and Red are the oxidised and reduced species and n represents the number of electrons involved in the transformation. The potential of the electrode can be related to the standard potential of the redox reaction and the activities of the species involved in the conversion, by the Nernst equation [143]:

$$\mathrm{E} = \mathrm{E}^{0\prime} + \frac{\mathrm{RT}}{\mathrm{nF}} \ln \frac{\mathrm{a_{Ox}}}{\mathrm{a_{Red}}} \tag{10}$$

where $\mathrm{E}^{0\prime}$ is the formal potential of the redox reaction, R is the universal gas constant (8.314 J/mol K), T is the absolute temperature (K), n is the number of electrons involved in the reaction, F is the Faraday constant (96485 C/mol), a_{Ox} and a_{Red} are the chemical activities for the species involved in the reaction (the chemical activity is a product between concentration and activity coefficient, which is close to unity at low concentration values).

There are different approaches and techniques to study the charge transfer process. From the research perspective [144], the electrochemical methods can be divided into: potentiometric, steady-state polarisation measurements, transient measurements (steps and pulses) and impedance measurements. In the next sections, the methods more frequently used in the present research, voltammetry, amperometry and spectroelectrochemistry, will be described in detail.

3.1. Cyclic voltammetry

One of the most versatile and widely used electrochemical techniques for electron transfer studies [143-145], cyclic voltammetry - CV, or more formally "chronoamperometry with multiple triangular potential sweeps'' as IUPAC [146] recommends, was developed in the first half of the 20th century by Matheson and Nichols [147] and later advanced by Randles [148-150] and Sevcik [151] using the basis of polarography discovered by Heyrovsky in 1922 (Nobel prize, 1959).

In this method, the potential at the working electrode is linearly changed with time with a well-defined rate (scan rate), back and forth (cyclic) between a starting (E_{start}) and a "switching" (E_{λ}) potential value with respect to a reference electrode, and the resulting current is measured and analysed. A typical representation of the cyclic voltammetry is depicted in **Figure 3.1**, where plots of potential (a) and current (b) *vs.* time as well as a typical voltammogram (c, I *vs.* E) are shown.

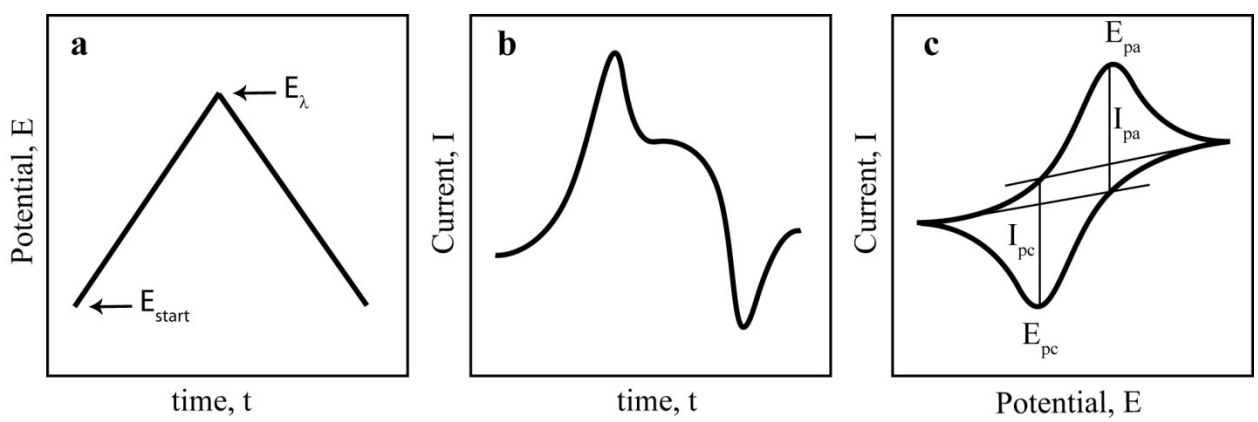


Figure 3.1. Graphical representation of (a) the excitation signal - potential, (b) response – current in time and (c) current *vs.* potential.

The important parameters determined from a voltammogram are the peak potentials (E_{pa} – anodic and E_{pc} - cathodic) and the peak currents (I_{pa} and I_{pc}).

From the peak potential values one can estimate the degree of electrochemical reversibility of the redox process by analysing the ΔE (E_{pa} - E_{pc}) value and can determine the formal potential of the redox couple involved in the reaction:

$$E^{0\prime} = \frac{E_{pa}+E_{pc}}{2} \qquad (11)$$

In the case of diffusion controlled, reversible electron transfer process, the peak height is dependent on the analyte concentration, C, as expressed by the Randles-Sevcik equation:

$$I_p = 0.4463nFAC\left(\frac{nFvD}{RT}\right)^{\frac{1}{2}} \quad (12)$$

where A is the area of the working electrode (cm^2), C is the analyte concentration (mol/cm^3), v is the scan rate (V/s), D is the diffusion coefficient of the analyte (cm^2/s) and n, F, R, T have their usual significance as mentioned in the case of the Nernst equation. At 25°C (298.15 K), the equation can be written in a more concise form:

$$I_p = (2.687x10^5)n^{3/2}v^{1/2}D^{1/2}AC \quad (13)$$

The Randles-Sevcik equation envisages that the peak current should be linearly dependent on the square root of the scan rate. This fact is important especially in the case of slow electron transfer reactions, where the peak current, normalized to $v^{1/2}$, can be increased by decreasing the scan rate [145].

In the case where the redox species is confined at the electrode surface, either by physical adsorption or covalent bonding, the diffusion dependence disappears, since there is no need for transport from and to the bulk of solution for the electrochemical reaction to take place. In the simplest case of Langmuir adsorption [152, 153], the shape of the voltammogram is a mirror symmetric one with identical areas under the two waves and the peak potentials equal to $E^{0'}$. In this case the peak current is proportional to v rather than $v^{1/2}$.

In bioelectrochemistry, voltammetry is commonly used to detect redox changes promoted by direct electron transfer reactions between redox enzymes and electrode surfaces, starting with cytochrome *c* more than 30 years ago [29, 154, 155], and continuing with multi-redox centre enzymes [15, 73, 75, 156, 157]. In the present case, CV was utilised in all the papers for determining redox potentials, following direct electron transfer, catalytic currents and polarisation curves: in **Paper I** CV was used only to determine the formal potential of 1,4-benzoquinone, which was used as a mediator for CDH; in **Paper II** CV was used to determine the formal potential of the CDH when adsorbed at different SAMs modified Au electrodes and to measure the electrocatalytic response generated by the presence of the enzyme substrate (e.g. lactose); in **Papers III** and **IV** CV was used to record polarisation curves at a low scan rate (1 mV/s) both for the separate electrodes of the BFCs (anode – CDH, cathode – Lc or BOD) and for the whole BFC; in **Papers V** and **VI** CV was used to determine the redox potentials of the mediators used with the bacterial cells and to test for any catalytic response in the presence of a substrate.

3.2. Amperometric flow injection analysis

Amperometry is the most common mode of electrochemical detection, where a constant potential is being applied at the working electrode and the current generated by a redox reaction at the electrode surface is measured. The potential value is usually chosen using data from cyclic voltammetry, depending on the desired transformation (oxidation or reduction) from the region where the analyte of interest gives a limiting current plateau. For example, if the formal potential of the redox couple is 0 mV *vs.* SCE (saturated calomel electrode), one needs to apply a positive potential value in order to follow the oxidation and a negative potential value for the reduction half-reaction. The actual value of the applied potential depends on the degree of electrochemical reversibility of the process and on the number of electrons involved in the reaction.

In order to control the mass transfer to/from the working electrode surface, two main strategies can be employed: moving the electrode in the solution (rotating disk electrodes or rotating ring-disk electrodes) or moving the solution with respect to the electrode surface (wall-jet cells, tubular electrodes, thin-layer cells). In our case, the most used techniques were flow analysis (FA) and flow injection analysis (FIA) using a wall-jet (WJ) cell. In this case, an inert electrolyte solution is transported toward the surface of the working electrode using a peristaltic pump with a controllable flow rate, and the analyte of interest is added and mixed (FA) or injected (FIA) into the flow stream and carried towards the electrode. Historically, the term "wall-jet" was first introduced in 1956 by Glauert [158] to describe mathematically a jet of fluid spread on a plane surface. Based on this concept, Yamada and Matsuda [159] designed in 1973 a new experimental device for hydrodynamic voltammetry called "wall-jet electrode" and they also derived an equation for limiting diffusion current. Finally "flow injection analysis", was introduced in 1975 by Ruzicka and Hansen [160, 161], discussing the influence of sample volume, tubing, sampling rate on the response generated by a detector.

Since 1975 the number of FIA publications has increased almost exponentially [162, 163] for the first 10 years and after that a linear increase with a peak in 2007 at almost 800 publications per year (data obtained from scifinder.cas.org). The electrochemical detection principle was used in more than 10% of the publications and amperometry in approximately half of these. The applications areas are various ranging from agriculture, food industry, to biochemical, clinical and environmental analyses [162, 164, 165].

A typical FIA manifold with amperometric detection consists of a pump with connection tubing, an injection valve, a flow-through electrochemical cell with electrodes controlled by a potentiostat and a recorder. A schematic picture of such a system in which the electrochemical cell is of the WJ-type, is given below in **Figure 3.2**.

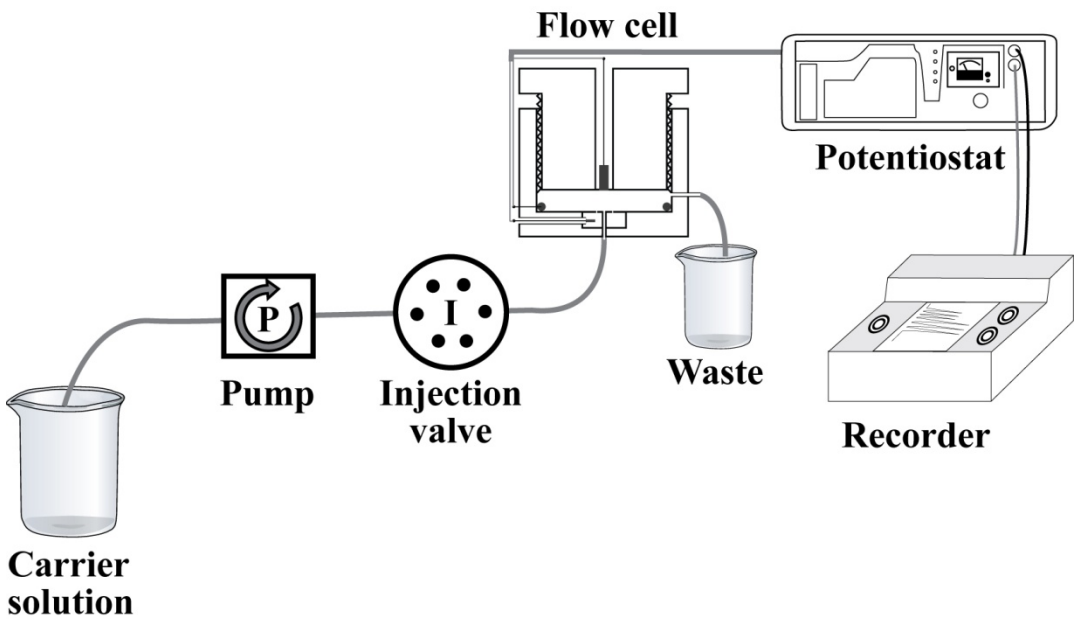


Figure 3.2. Flow-injection analysis setup using a wall-jet amperometric electrochemical cell.

A detailed description of the cell, constructed and used for the first time in our group in 1985 [166], including the flow profile at the wall-jet electrode and the shape of a typical response peak are schematically represented in **Figure 3.3**.

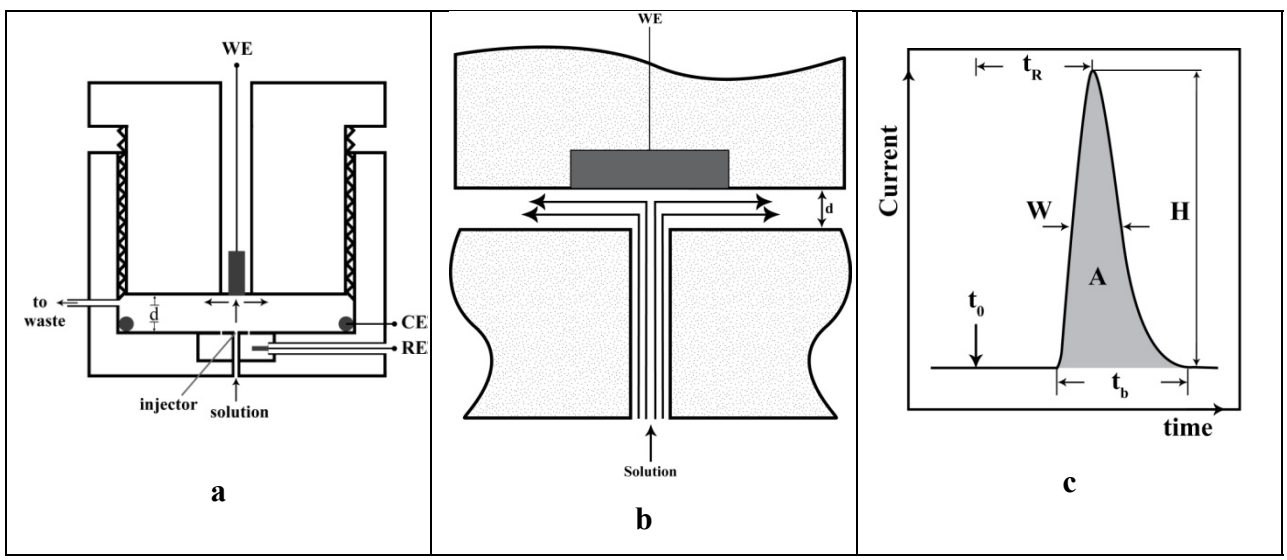


Figure 3.3. a) Amperometric wall jet cell: **WE** – working electrode, **RE** – reference electrode, **CE** – counter electrode, **d** – distance injector – WE; b) laminar flow profile at the wall-jet electrode; c) the typical peak response: $\mathbf{t_0}$ injection time, $\mathbf{t_R}$ – residence time, $\mathbf{t_b}$ – peak width at the baseline, **W** – peak width at a selected level, **H** – peak height, **A** – peak area.

As can be seen, the wall-jet cell consists of two halves that can be screwed together. The upper part contains the working electrode (WE), which can be made of various materials such as noble metals (Au, Pt) or graphite. The lower part has a Pt wire that incirculates the bottom of the cell and acts as a counter electrode (CE) and a small chamber below the end part of the inlet nozzle, which contains the Ag|AgCl reference electrode (RE) and usually 0.1 M KCl saturated with Ag^+. The chamber communicates with the sample stream through four small holes situated concentrically around the inlet. The carrier solution comes through the injector and, at an optimal combination [167] of flow rate and distance between the inlet nozzle and the working electrode (d), follows a laminated flow profile parallel to the surface of the working electrode (see **Figure 3.3. b**). The typical response of any FIA system is an asymmetric peak [162] with the height dependent on the analyte concentration. The shape of the peak is caused by the dispersion of the sample in the carrier solution during transportation from the injection valve to the detector.

The derived equation for the limiting current in a wall-jet cell, according to Yamada and Matsuda [159, 162] is given below:

$$I_{lim} = 1.60knFD^{2/3}C_0\upsilon^{-5/12}a^{-1/2}r^{3/4}V_f^{3/4} \qquad (14)$$

where k is the cell constant, C_0 the concentration of the reacting species, υ the kinematic viscosity of the solution, a the radius of the inlet capillary, r the radius of the disk electrode, and V_f is the volume flow rate of the electrolyte and n, F, D have their usual significations as mentioned above.

Dispersion in the FIA

As pointed out by Ruzicka and Hansen [162], controlled dispersion is one of the three principles that FIA is based upon, together with sample injection and reproducible timing of sample movement between injection point and detector. When the sample is injected into the flow stream it disperses into the carrier, taking a form dependent on the sample volume, the geometry of the tubing and the flow rate of the carrier. In order to properly correlate the analyte concentration with the response generated in the detector, it is important to know the dispersion factor of the system. This translates into knowing the sample concentration values before and after the dispersion and calculate a dispersion coefficient [162] using the ratio of the two concentrations. In amperometry the dispersion coefficient can be estimated using a fast redox couple (ferro/ferricyanide) and measuring the current response in FA (I_{ss}, steady-state current – where the sample is pumped continuously to the detector until a constant response with time is

obtained) and FIA (I_p, peak current) in a region where there is a linear correlation of the response with the sample concentration. The dispersion coefficient would be:

$$D = \frac{I_{ss}}{I_p} \tag{15}$$

with a value ≥ 1.

We used both FIA and FA in experiments performed for this thesis (**Papers I, V, VI**) for a rapid evaluation of a large number of enzymatic substrates. The working electrode was either spectrographic graphite electrodes (SPGE, Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm in diameter) or a disc Au electrode (BAS, West Lafayette, IN, USA, 1.6 mm in diameter); the reference electrode consisted of a Ag|AgCl 0.1 M KCl; the counter electrode consisted of a Pt wire.

3.3. UV-VIS spectroelectrochemistry

By combining spectroscopy and electrochemistry, two different techniques that can be performed simultaneously in the electrochemical cell, spectroelectrochemistry (SPE) has become an important tool [168] in the study of electrochemical reactions of inorganic, organic and biological molecules. Typically, electrochemistry is used as an excitation signal and the system changes are monitored spectroscopically. Among the variety of spectroelectrochemical techniques available, the UV-visible spectroelectrochemistry claims to be the oldest, with Kuwana and Heineman being the pioneers of the method [169, 170]. Kuwana et al. were the first to describe in 1964 an optically transparent electrode (OTE) [169] and Heineman et al. later developed the optically transparent thin-layer electrochemical cell (OTTLE) in 1967 [170]. The early developments of spectroelectrochemistry based on the OTE and OTTLE are extensively reviewed by the same authors a few decades later [171-173]. The first OTE cells were developed by using a metallic paint, giving electrical conductivity, applied to a glass cell in a thin layer in order to preserve some degree of transparency. Great improvement in terms of performance and response stability were brought by introducing the thin-layer cell with a gold minigrid between two microscope slides [170]. The advantage of these cells are the rapid redox conversion due to small diffusion distances, and the need of small sample volumes. On the downside they suffer from a low optical sensitivity due to a short optical pathway.

The evaluation of spectroelectrochemical results, in the case of a reversible or quasi-reversible system, is done by using an equation which combines the Nernst equation (10) and the Lambert-Beer's law (16).

$$A = \epsilon dc \tag{16}$$

where A is absorbance, ϵ is molecular extinction coefficient, d is path length in cm and c is molar concentration. When applying a potential, equilibrium can be achieved rapidly in a small volume cell with the bulk concentrations of the oxidised and reduced form of the molecule being the same with the ones at the surface of the electrode. In this case the equation becomes:

$$E = E^{0\prime} + \frac{RT}{nF} \ln \frac{A - A_R}{A_O - A} \tag{17}$$

where A_R is the absorbance of the reduced form, A_O is the absorbance of the oxidised form and A is the absorbance of an intermediate mixture. A plot of E as a function of $\ln(A-A_R)(A_O-A)$ gives a straight line with the intercept equal to $E^{0\prime}$and the slope directly related to n.

The first measurements were performed on small inorganic and organic compounds but they rapidly extended to more complex molecules of biological importance (proteins, respiratory complexes, etc.). In the case of redox proteins, although their UV-Vis spectra are complex and broad, it can still be attributed to the existing redox centre of the molecule (e.g. heme, metal centres, etc.). In most cases, redox proteins undergo extremely slow electron reactions at the electrode surface. In this case a low molecular weight mediator can be used to facilitate the charge transfer. Heineman et al. [174] were the first to determine the formal potential and the number of transferred electrons of cytochrome *c* in an OTTLE using 2.6-dichlorophenolindophenol (DCPIP) as mediator. A comprehensive review from 1995 [175] describes the usage of UV-Vis spectroelectrochemistry with redox proteins up to that date.

The spectroelectrochemical cell constructed in our lab and described in detail by Andreas Christenson in his doctoral thesis [176] is based on a long-optical-pathway thin capillary-type cell. The cell was constructed by improving and modifying a previous design of a gold capillary used as a working electrode in a spectroelectrochemical cell described by Larsson et al. [177]. The use of a gold working electrode allows modification with SAMs, which can improve the electron transfer by a proper orientation of the molecule of interest at the electrode/solution interface. The schematic drawings in **Figure 3.4** describe the full system and details of the spectroelectrochemical cell.

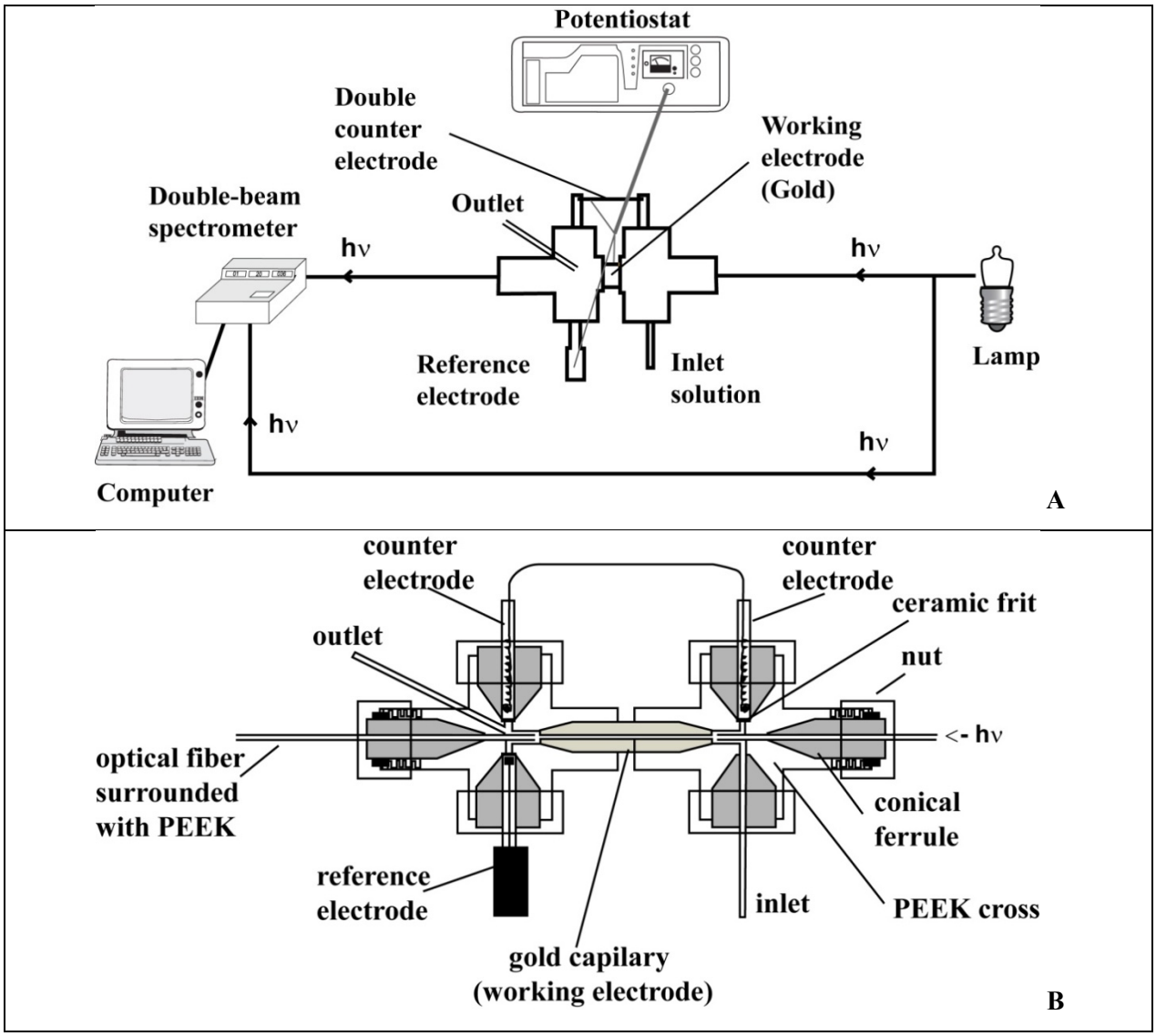


Figure 3.4. A schematic representation of the SPE system (**A**); the SPE cell (**B**).

The cell has been recently used for biological molecules with single or multiple redox centres, going from cytochrome P450cam, to theophyllin oxidase and sulphite oxidase, diaphorase, laccases, bilirubin oxidase and even respiratory complex II (succinate:quinone oxidoreductase) from *Bacillus subtilis* [178-184]. In the experiments performed for this thesis, we used the spectroelectrochemical system in **Paper II** in order to investigate CDH from the ascomycete fungus *Myriococcum thermophilum,* both in its intact form and as separate domains (FAD and HEME domain). The technique allowed for interesting observations, unavailable when using CV for example: with the separate FAD domain the electrochemical conversion between the oxidised and the reduced form was possible at low pH; with the intact CDH, the reduction of the FAD through the HEME domain, in an uphill reaction, was shown at low pH.

CHAPTER 4

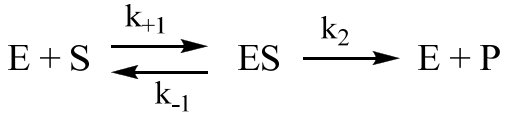
Biological materials

4.1. Enzymes

Enzymes, the catalysts of biological systems, determine the pattern of chemical transformations with high efficiency and specificity. The catalysis takes place at a particular site in the enzyme called active site. The specificity of an enzyme is due to the precise interaction of the substrate with the enzyme, generated by the complex three-dimensional structure of the enzyme. The catalytic activity of many enzymes depends on a non-amino acid component, termed cofactor, which can be a metal ion containing or a small organic molecule. The small organic molecules are called coenzymes and they can be either tightly (prosthetic group) or loosely bound to the enzyme. The enzyme without its cofactor is termed an apoenzyme, while the complete, catalytically active enzyme is called a holoenzyme [185].

Enzymes are grouped into different classes based on the reaction they catalyse. These classes are further subdivided and each enzyme has an EC (Enzyme Commission) number that allows for precise identification of all enzymes. The main classes are: 1. *Oxidoreductases*, which catalyse oxidation/reduction reactions; 2. *Transferases*, which catalyse the transfer of a group between two compounds; 3. *Hydrolases*, which deal with hydrolysis reactions; 4. *Lyases*, which catalyse the removal of a group to form double bonds; 5. *Isomerases*, which catalyse isomerisation reactions; 6. *Ligases*, which are responsible for the ligation of two substrates with the hydrolysis of a pyrophosphate bond.

To understand how enzymes function, we need a kinetic description of their mechanism. In 1913, Leonor Michaelis and Maud Menten proposed a simple model, which accounts for the kinetic properties of many enzymes [186]. In this model, an enzyme (E) combines with a substrate (S) to form an enzyme-substrate (ES) complex, which can proceed to form a product (P) or to dissociate into E and S, as shown in the reaction below:



(18)

The rate of product formation (V_0) is given by the Michaelis-Menten equation, Eq. (19):

$$V_0 = V_{max} \frac{[S]}{[S]+K_M} \tag{19}$$

where V_{max} is the reaction rate when the enzyme is fully saturated with substrate and K_M, the Michaelis constant, is the substrate concentration at which the reaction rate is half maximal. V_{max} is equal to the product of k_2 (k_{cat} – turnover number) and the total concentration of enzyme. The Michaelis constant is related to the rate constants by $K_M = (k_{-1}+k_2)/k_1$. The enzyme catalytic efficiency is best described by the ratio k_{cat}/K_M.

In the next section I will describe in more details the enzymes studied for the current thesis, i.e. cellobiose dehydrogenase and the "blue" multi-copper oxidases, laccase and bilirubin oxidase, which are all members of the oxidoreductase family.

4.1.1. Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH, cellobiose: acceptor 1-oxidoreductase, EC 1.1.99.18) is an extracellular oxidoreductase produced by many wood-degrading and phytopathogenic fungi. A number of comprehensive reviews have summarised and classified the sources of cellobiose dehydrogenase as well as the accomplishments in the field over a period of three decades [187-189]. Historically, CDH activity was first discovered in 1974 as cellobiose-dependent reduction of quinones in white-rot fungi by Westermark and Eriksson [190, 191], and the enzyme was called cellobiose:quinone oxidoreductase (cellobiose dehydrogenase). One year later, the same authors isolated an enzyme carrying a flavin group from *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) and the enzyme was called cellobiose:quinone oxidase (CBQ) [192]. Subsequently, a form containing both flavin and heme, that is now named CDH, has been isolated from the same fungus and named cellobiose oxidase (CBO) because it was incorrectly assumed to prefer O_2 as an electron acceptor [193]. CBQ was later identified as a catalytic active fragment of CBO appearing in the cultures of some fungi probably due to the action of proteases [187, 194].

More than 25 species of fungi have shown to produce CDH (http://www.brenda-enzymes.info/index.php4). All CDHs belong to two related subgroups: class I, produced by basidiomycetes (filamentous fungi) and class II, produced by ascomycetes (sac fungi). The class I CDHs have shorter sequences, work better at acidic pH and they are poor oxidisers of monosaccharides (*e.g.* glucose), whereas some class II CDHs have more complex sequences, work in neutral or basic conditions and they may additionally have high turnover numbers with monosaccharides and oligosaccharides other than cellodextrins and lactose [189]. The best studied CDH up to date is the one produced by the white-rot basidiomycete *P. chrysosporium.* A typical CDH produced by basidiomycetes is a monomeric protein comprised of two domains separated by a protease cleavable linker region [195]. The molecular weight of the holoenzyme varies, depending on the fungal source, between 80 kDa and 115 kDa [189]. CDH efficiently oxidises cellobiose, soluble cellodextrins, mannodextrins, lactose and glucose (class II) to their corresponding lactones by a ping-pong mechanism using a wide variety of electron acceptors including quinones, dichloroindophenol (DCIP), phenoxyradicals, cytochrome *c*, Fe^{3+}, Mn^{3+}, Cu^{2+}, triiodide ion, and oxygen, however, very poorly [187-189].

As already mentioned CDH has two cofactors, flavin adenine dinucleotide (FAD) and heme (see **Figure 4.1**), non-covalently bound to the enzyme, localised in the two different domains. The FAD domain is larger and belongs to the glucose-methanol-choline (GMC) family, which includes e.g. glucose oxidase (GOx) and cholesterol oxidase (COx) [196], while the cytochrome domain is smaller and hosts a heme *b* (protoheme IX) with a special ligation.

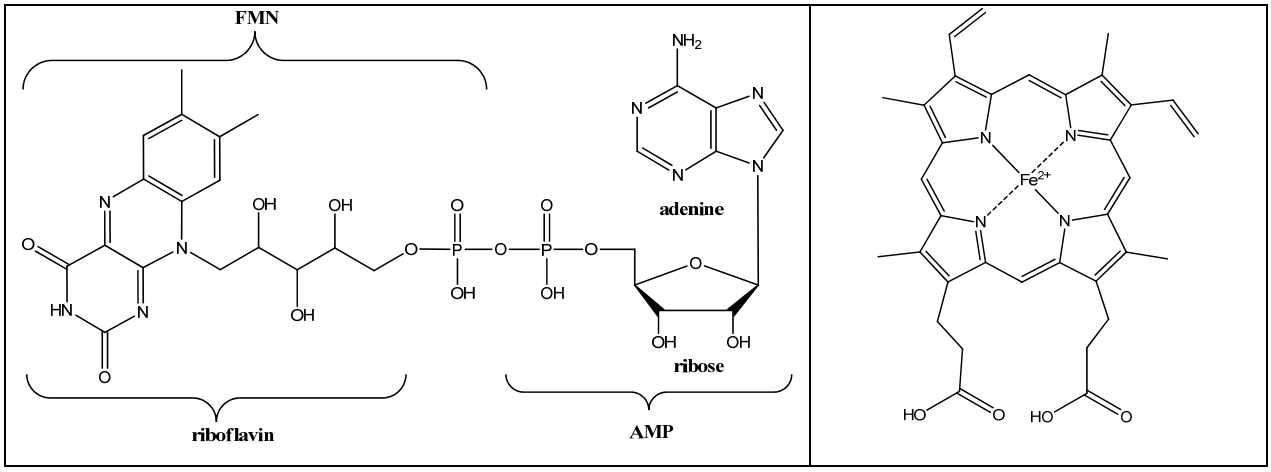


Figure 4.1. Chemical structures of FAD (left) and heme *b* (right) cofactors of CDH.

A major breakthrough in the field of CDH research was the elucidation of the crystal structures of the separate domains of *P. chrysosporium* CDH [197, 198]. The full length haemoflavoenzyme hindered crystal structure analysis studies possibly due to its inherent

flexibility. The structures of the fragments, shown in **Figure 4.2**, were modelled based on the molecular fold of the FAD and cytochrome *b* domains of *P. chrysosporium* CDH (PDBs 1D7C and 1NAA [197, 199]) using PyMol *v.* 1.1. (*http://pymol.sourceforge.net*).

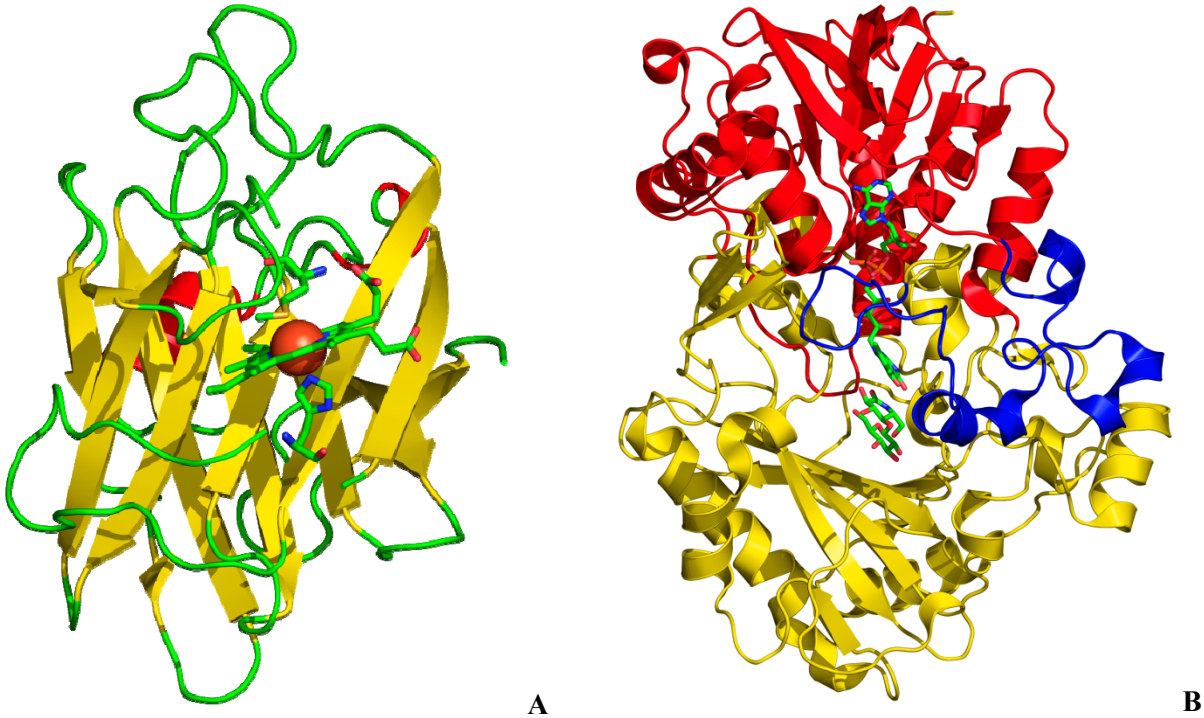


Figure 4.2. Structural representation of the separate domains of *P. chrysosporium* CDH.

A) Molecular fold of the cytochrome *b* domain with the heme group and the heme-ligating residues (His163 and Met65 – special ligation) shown as stick objects and the iron atom as a sphere; the secondary structure is depicted as follows: β-strands, arrows; α-helices, spirals.

B) Molecular fold of the flavin domain with the FAD and substrate depicted as stick objects. The flavo-subunit consists of a Rossmann domain (red) with a FAD/NAD(P)-binding domain fold and a substrate-binding domain (yellow). The active site entrance is coloured in blue. The secondary structure is depicted as follows: β-strands, arrows; α-helices, spirals.

The general reaction of CDH can be divided into a reductive and an oxidative half-reaction [189]. In the reductive part, β-D-cellobiose (the natural substrate of the enzyme) is oxidised at the C1 carbon to yield cellobionolactone, while FAD is reduced to $FADH_2$ by the 2 e^- and 2 H^+ coming from the sugar oxidation, while the oxidative half-reaction, *i.e.* the reoxidation of $FADH_2$ to FAD, can proceed either through a 2 e^- acceptor or two equivalents of a 1 e^- acceptor [187]. While the function of FAD in CDH is clear, the presence of heme, which is rather surface-exposed, has fuelled speculations concerning its function. For the catalytic role of the heme two different models have been proposed [200]: a) an “electron-chain model” where

the electrons are transported one by one from $FADH_2$ to heme and then finally to an 1 e^- acceptor; b) an "electron-sink model", where the heme has the role to store electrons in order to ensure proper conditions at the FAD site for it to reduce the electron acceptors directly. Depending on the electron acceptor, both models might be valid. In the case of cytochrome *c* reduction [201-203] and DET to electrodes [73, 75], the chain model fits better and the intramolecular ET rate is strongly pH dependent, while the direct reduction of other 1 and 2 e^- acceptors at the FAD centre favours the sink model [187, 188].

The pH dependence of the ET rate between FAD and heme is partly related to the formal potentials of the two redox centres. The experimentally determined redox potential of the isolated flavin domain of *P. chrysosporium* CDH decreased with an increase in pH from 106 mV *vs.* NHE (pH 3) to -132 mV vs. NHE (pH 7) [202], while the redox potential of the heme domain decreased from 190 mV (pH 3) to 130 mV (pH 7) [204]. In **Paper II** we have also determined the redox potential of the heme both in the separate cytochrome domain and in the intact class II CDH from *Myriococcum thermophilum*, using cyclic voltammetry and spectroelectrochemistry, and we found that the redox potential decreases from 230 mV (pH 3) to 130 mV (pH 7). When studying the nature of the FAD cofactor, certain CDH preparations revealed the presence of 6-hydroxy-FAD [205] with a slightly more negative midpoint redox potential than the unsubstituted FAD. It is well known now that especially class II CDHs have a higher content of 6-hydroxy-FAD [189, 206]. The rate for sugar oxidation decreases for CDH with 6-OH-FAD in line with the Marcus theory (see **Chapter 2**), as the thermodynamic driving force decreases.

Although CDH has been targeted by many research groups for the last 35 years, there is still no consensus on its biological functions. A detailed discussion about the possible functions can be found in the previous reviews [187-189], for example hydroxyl radicals' production used for cellulose and lignin degradation, toxic quinones reduction, antibacterial agent, etc. Gorton et al. have also suggested that the DET properties of CDH should reveal its physiological properties [179]. White rot CDH is produced in the early stage of growth on lignocellulose at the same time as three other oxidoreductases: laccase, lignin- and manganese peroxidase, which also show facile DET with electrodes. This may point to the fact that lignocellulose degradation is at least partly dependent on DET reactions between these enzymes and lignocellulose [179].

Due to its unique properties, many biotechnological and analytical applications have been proposed for CDH. In biotechnology, several studies were carried out using CDH for enzymatic treatment of paper pulp [207-209], enzymatic treatment of depolymerised biomass in non-aqueous solvent [210], production of lactobionic acid [211, 212], production of a lactose free galacto-oligosaccharide mixture [213] and for waste removal [214-216]. Regarding the analytical usage, CDH has been used in enzymatic assays [217-220] and in biosensors both for electron donors, *e.g.* lactose [221], and electron acceptors, *e.g.* diphenols [222] and catecholamines [223].

A very recent and exciting application for CDH is the use of the enzyme as an anodic element in biofuel cells, using both DET (Papers **III** & **IV**) and MET [224-227].

Concerning the current thesis, we studied several class II CDHs, *i.e. M. thermophilum* (*Mt*CDH - Papers **I** & **II**), *Dichomera saubinetii* (*Ds*CDH – Paper **III**) and *Corynascus thermophilus* (*Ct*CDH – Paper **IV**), evaluating the direct electron transfer (DET) properties on graphite (Paper **I**) and gold electrodes (Paper **II**) as well as the possibility of constructing simple, mediator and compartment-less glucose/O_2 biofuel cells working in acidic (Paper **III**) and neutral pH solutions (Paper **IV**).

4.1.2. "Blue" multi-copper oxidases; laccase and bilirubin oxidase

The multi-copper oxidases constitute an important class of enzymes which reduce molecular oxygen to water in a four-electron reduction process with concomitant one-electron oxidation of their substrate. Several recent reviews describe in detail the physico-chemical properties of these enzymes [228, 229] as well as their electrochemistry in conjunction with their biochemistry and structure [230]. There are three spectroscopically different copper centres found in multi-copper oxidases: type 1 (T1 or blue), type 2 (T2 or normal) and type 3 (T3 or coupled binuclear copper pair). The type 1 or blue copper centres exhibit an extremely intense absorption band at ~600 nm, which is responsible for the deep blue colour [228]. The T2 copper centres exhibit electron paramagnetic resonance (EPR) spectra typical for normal copper and no intense features in the visible absorption spectrum associated with ligand field transitions [228, 230]. The T3 binuclear copper site has an absorption band at ~330 nm and together with the T2 site forms a trinuclear copper cluster [228, 230]. The T1 Cu centre is at a ~13 Å distance from the trinuclear T2/T3 cluster but linked to it through a Cys-His electron transfer pathway [228, 229, 231, 232].

The principal members of the multi-copper oxidases family and the most studied to date are: laccase, ascorbate oxidase, ceruloplasmin and bilirubin oxidase. In the following paragraphs I will discuss in more detail aspects related to laccase and bilirubin oxidase since they were the subjects of the studies concerning the current thesis (**Papers III** and **IV**).

Laccase (Lc, benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is characterised by a very low substrate specificity, being able to oxidise diphenols, amino-phenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions [230, 233, 234]. According to different sources [235, 236], laccase was discovered in 1883 by Yoshida, making it one the oldest enzymes ever described. Since then many applications have been described using laccase

as it is illustrated in a few recent reviews [237, 238]. Laccases are classified according to their main production source i.e. plant and fungal laccases, although they have been found in bacteria [239, 240] and insects [241, 242] as well. Among all these, the most studied laccases are of fungal origin with more than 60 fungal strains (ascomycetes, deuteromycetes and especially basidiomycetes) showing laccase activities [243, 244].

The fungal Lc is a monomeric enzyme organised in three sequentially arranged domains with a similar β-barrel type architecture (see **Figure 4.3**, PDB 1GYC), with the dimensions of 65 x 55 x 45 Å [245].

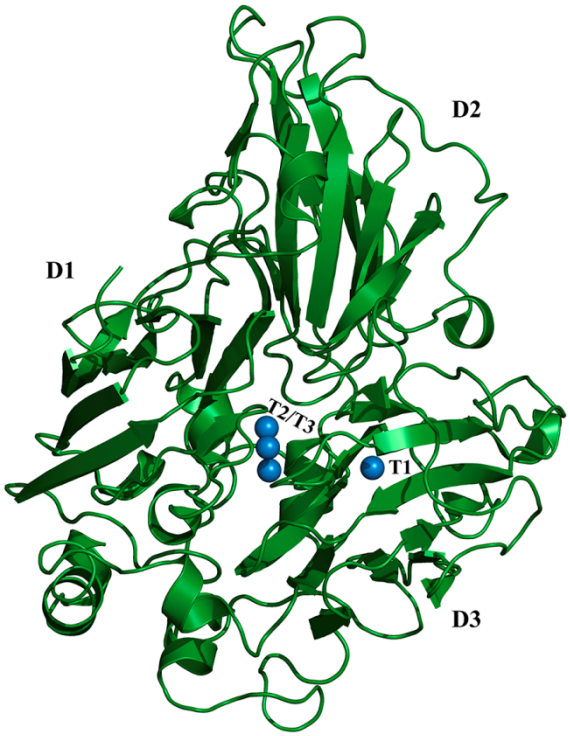


Figure 4.3. Molecular fold of *T. versicolor* laccase (ribbon diagram). The copper centres are depicted as blue spheres. The secondary structure is depicted as follows: β-strands, arrows; α-helices, spirals.

The T1 copper is situated in a cavity of the third domain, while the T2/T3 cluster sits between the first and the second domain. Although local structural differences are apparent (e.g., in the loops organising and forming the substrate-binding pocket), all of the Lc structures that have been determined to date show a significant degree of overall structural homology [230]. Similar to the heme group in CDH, the T1 copper site of Lc is situated close to the surface of the protein [228]. This pattern seems to be typical for extrinsic enzymes with built-in ET pathways, using the surface redox site to connect the active site with the surface.

The primary electron donors, which can be both organic and inorganic, are oxidised at the T1 site and the electrons are transferred to the T2/T3 cluster, where the 4 electrons reduction of molecular oxygen to water occurs [228, 229]. The key characteristic of the Lcs is the redox potential of its redox centres. Moreover, the catalytic efficiency of the enzyme towards some substrates depends on the formal potential of the T1 site [246, 247]. The value of the formal potential of the T1 copper has been determined for many laccases using mediated potentiometric titrations and was found to vary between approximately 400 and 800 mV versus NHE [183, 246, 248-251]. From an electrochemical perspective, all laccases can be divided into three groups depending on the redox potential of the T1 site: low, middle and high-redox-potential Lcs [179].

The high-redox-potential laccases (mostly originating in basidiomycetes) received very much attention in recent years, being used efficiently in bioremediation, detoxification, and especially in biofuel cell research, as will be discussed in more detail in the next chapter (see section 5.2.1). Regarding the current thesis, we used a well characterised laccase from *Trametes hirsuta* [251, 252], which exhibits efficient DET on carbon electrodes, suitable as a bioanaode in a simple, noncompartmentalised, mediatorless glucose–O_2 BFC (**Paper III**).

Bilirubin oxidase (bilirubin:oxygen oxidoreductase, EC 1.3.3.5) is a multi-copper oxidase which catalyses, unlike laccase, the reaction of bilirubin and other tetrapyrolles (**Figure 4.4**). It can also oxidise diphenols and aryl diamines [253]. Bilirubin oxidase (BOD) was discovered and characterised in the beginning of the 1980's by Murao and Tanaka [253-256]. The enzyme was purified mainly from fungi, especially from two ascomycetes: *Myrothecium verrucaria* [253-256] and *Trachyderma tsunodae* K-2593 [257]. A bacterial protein, i.e. the spore coat protein CotA from *Bacillus subtilis* has shown recently a very strong bilirubin oxidase activity [258].

BOD was found to be a monomeric enzyme with a molecular weight between 52 and 66 kDa [254, 259]. Unfortunately, no crystallographic data for BOD exists, but the enzyme has been characterised in detail biochemically, spectroscopically and electrochemically [182, 228, 230, 246, 259-265]. The catalytic site of the enzyme is very similar with the one of laccase; BOD has also a T1 copper site, which is the first electron acceptor [228, 246] which communicates with a T2/T3 copper cluster. The value of the formal potential of the T1 copper has been determined and varies at pH around 7 between 615 mV [266] and 710 mV *vs.* NHE [267] for *Trachyderma tsunodae* BOD and between 570 mV [259] and 670 mV *vs.* NHE [182] for *Myrothecium verrucaria* BOD.

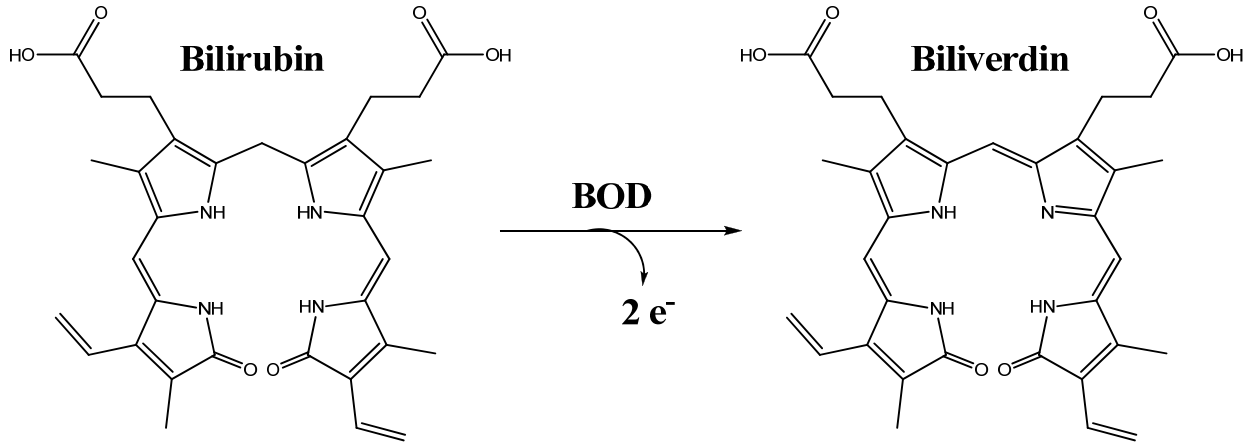


Figure 4.4. The physiologically relevant reaction catalysed by bilirubin oxidase (BOD)

Although from determinations of the formal potential, BOD can be classified as a high-redox-potential blue multi-copper oxidase, some properties of fungal BODs are very different from those of the high-redox-potential fungal Lcs. For example, BOD is not so strongly inhibited by chloride ions [126, 260, 266, 268] and it remains very active at neutral pH. On the other hand, it is quite rapidly inactivated in blood serum [269, 270], being affected by products of the reaction between urate and O_2. Considering this aspects, in **Paper IV** we used *Myrothecium verrucaria* BOD, which was proven to exhibit efficient DET on carbon electrodes [230, 261, 265] as a bioanode in a potentially implantable glucose/oxygen biofuel cell operating in neutral buffers and human serum.

4.2. Bacteria overproducing redox proteins

An alternative to studying isolated and purified enzymes is to use the source from which these proteins are isolated and purified. The use of whole bacterial cells, for example, as an intracellular source of enzymes eliminates the time consuming and expensive operation of enzyme purification, preserves the enzyme in its natural environment and protects it from inactivation due to external factors. The major limitation though of such an approach is usually the slow response, which has been attributed to diffusional limitations and the complexity of the cellular system. In order to study a certain enzyme inside a cell, it is desirable to overproduce it in order to increase the sensitivity of the system. For this purpose, a genetically modified organism (GMO), obtained using genetic engineering techniques, is used. Since the creation of the first recombinant bacterium in 1973 [271], the field has dramatically expanded and found

uses in many research fields, bacteria being now particularly important in producing large amounts of pure human proteins for use in medicine [272].

Concerning the current thesis, we have used a few recombinant *Escherichia coli* strains (**paper V**): JM109 cells harbouring plasmid pBSD 1300, which over-expressed the di-heme membrane anchor protein from *B. subtilis* succinate:quinone reductase (SQR), described in the next section and JM109 cells harbouring plasmid pLUV 1900, which over-expressed the cytochrome c_{550} from *B. subtilis*. In **paper VI** we have used *B. subtilis* 3G18/pBSD-1200 cells, which overproduce SQR (respiratory complex II).

4.2.1. Succinate:quinone oxidoreductase

Succinate:quinone oxidoreductases (SQORs; EC 1.3.5.1) are membrane-bound enzymes with remarkably similar physical and catalytical properties [273-278] present in a wide variety of organisms. They couple either the two-electron oxidation of succinate to fumarate with the two-electron reduction of quinone to quinol or the reduction of fumarate with the oxidation of quinol to quinone. Depending on the direction of the reaction the members of the super-family can be classified as succinate:quinone reductase (SQR) or quinol:fumarate reductase (QFR). SQR (respiratory complex II) belongs to the aerobic respiratory chain and serves as the only direct link between the citric acid cycle and the electron transport chain in the membrane, regulating both systems [279, 280], while QFR is involved in anaerobic respiration with fumarate as the terminal electron acceptor and it is part of an electron transport chain catalysing the oxidation of various donor substrates by fumarate [278, 281].

From a historical perspective, the reversible oxidation of succinate to fumarate in whole cells of *E. coli* has been demonstrated in 1924 [282]. Three decades later it was shown that purified preparations of mammalian SQR can perform both the oxidation of succinate and the reduction of fumarate [283], being more efficient when oxidising succinate. On the other hand, a preparation of SQR from obligate anaerobic bacteria showed the opposite behaviour, with more efficiency towards fumarate reduction [284], giving rise to a lot of questions and assumptions. It was not until 1963, when Hirsch et al. [285] have proven that in fact there are two distinct enzymes performing the two reactions.

SQRs and QFRs can be divided into three functional subclasses based on the quinone substrate and the in vivo function of the enzyme [276]: class I, SQRs that catalyse the oxidation of succinate ($E^{0'}$ = + 25 mV at pH 7 [286]) with the reduction of high potential quinones (*e.g.* ubiquinone) – exergonic reaction; class II, QFRs that catalyse the reduction of fumarate with the oxidation of a low potential quinol (*e.g.* menaquinol or rhodoquinol); class III, SQRs that

catalyse the oxidation of succinate with the reduction of low potential quinones (e.g. menaquinone or thermoplasmaquinone) – endergonic reaction. Class III is represented among others by the *B. subtilis* SQR, which was used in our studies.

In spite of the fact that they catalyse the opposite enzymatic reactions, SQR and QFR are structurally very similar [276], a fact demonstrated by the determination of the crystal structures of several members of the SQORs family: QFR of *E. coli* [287], QFR of *Wolinella succinogenes* [288], SQR of *E. coli* [289] and mitochondrial SQR from porcine heart [290]. Although no class III enzyme structure exists, it is well known that SQR from *B. subtilis* and QFR from *W. succinogenes* (see ***Figure 4.5.***) have very similar structures [276, 291, 292].

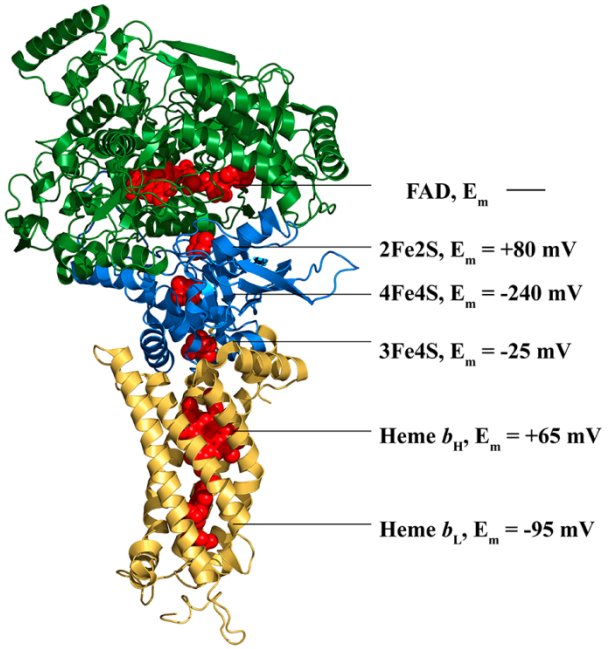


Figure 4.5. High resolution structure of *W. succinogenes* QFR (PDB 1QLA) solved at 2.2 Å resolution [288]. The enzyme comprises three protein subunits: subunit A, coloured in green, with a covalently bound FAD; subunit B, coloured in blue, containing three Fe-S clusters; subunit C, coloured in yellow, containing two heme *b* groups. All six prosthetic groups are presented as red coloured spheres, and the midpoint potentials at pH 7 correspond to *B. subtilis* SQR [292].

Succinate:quinone oxidoreductases generally contain four protein subunits, referred to as A, B, C, and D. Subunits A and B are hydrophilic, whereas the small subunits C and D are hydrophobic, membrane-integrated proteins. QFR of *W. succinogenes* and SQR of *B. subtilis* contain only one hydrophobic subunit (C), which is thought to have evolved from a fusion of the

genes for the two smaller subunits C and D [288, 293, 294]. The extrinsic part of the SQR complex, *i.e.* succinate dehydrogenase (SDH), comprises of a larger hydrophilic subunit A, which carries covalently bound flavin adenine dinucleotide (FAD) and a smaller subunit B, which contains three iron-sulphur centres: $[2Fe\text{-}2S]^{2+,\ 1+}$, $[4Fe\text{-}4S]^{2+,\ 1+}$ and $[3Fe\text{-}4S]^{1+,\ 0}$ for electron transfer between the FAD domain and the membrane anchor. The succinate/fumarate binding site is on the flavoprotein. The membrane anchor domain in the case of *B. subtilis* SQR contains two hemes of cytochrome *b* type [288, 295], a proximal heme (heme b_H) situated on the cytoplasmic side of the membrane close to the 3Fe4S cluster, and a distal heme (heme b_L) located close to the positive side of the membrane. The midpoint potentials (E_m) of the two hemes have been determined by redox titrations and were found to be +65 mV for heme b_H and -95 mV *vs*. NHE for heme b_L [184, 295, 296] when working with solubilised SQR. When the complex is situated in the membrane, the E_m values were found to be 50 mV lower (+16 and -132 mV *vs.* NHE) [295]. The redox potentials of the Fe-S centres were determined by mediator EPR titrations [295]. The FAD/$FADH_2$ E_m was not determined but assumed to be around -80 mV vs. NHE [298].

Considering the fact that in *B. subtilis* the electron transfer from succinate (E_m = +25 mV [286]) to menaquinone (E_m = -74 mV [297]) is thermodynamically unfavourable, it is believed that the electrochemical potential across the cytoplasmic membrane drives the electron transfer from heme b_H to heme b_L, which can then reduce menaquinone [294]. This theory is supported by the fact that succinate oxidation drops drastically when the *B. subtilis* cells are disrupted [300]. It has also been experimentally verified that fumarate reduction in *B. subtilis* coupled with menaquinol oxidation generates a proton motive force [301]. Thus, the electrons generated by succinate oxidation follow the path FAD, Fe-S clusters, heme b_H, heme b_L and finally menaquinone, which gets protonated on the outside of the membrane [294, 302]. Thus, the low potential heme b_L, considered unimportant for a long time, was found to be essential for the electron transfer to menaquinone [303].

In our studies, we used *B. subtilis* SQR in two different ways but for the same purpose: "wiring" live bacterial cells with different electrode materials by using mediators (*e.g.* flexible osmium polymers) for studying electron transfer from the cells to the electrode.

In **paper V** we only used the di-heme membrane anchor of *B. subtilis* SQR overproduced in *E. coli* JM109 cells and compared the "wiring" efficiency with the wild type cells and with cells overproducing cytochrome c_{550} (E_m = +178mV *vs.* NHE [304, 305]) from *B. subtilis*, a protein where the cytochrome domain is anchored on the periplasmic side of the membrane with a transmembrane helix. The overproduction of the transmembraneously arranged heme groups of *B. subtilis* SQR made it possible for the *E. coli* JM109 strain to efficiently communicate with the Os-polymers $(P(VI)_{12}\text{-}[Os\text{-}(4,4'\text{-dimethyl-}2,2'\text{-di'pyridyl})_2Cl_2]^{2+/+}$ (osmium redox polymer **I**, $E^{0'}$

= +350 mV *vs.* NHE) and PVP-[Os-(N,N′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (osmium redox polymer **II**, $E^{0'}$ = 0 mV *vs.* NHE) and the introduction of the cytochrome c_{550} had an effect in increasing the ability of ubiquinone (Q_0) to act as an efficient electron acceptor.

In **paper VI** we used a *B. subtilis* strain which overproduces SQR (3G18/pBSD-1200 strain) and tested the "wiring" efficiency of the gram-positive cells with the two Os-polymers mentioned above. Compared to the "wild type" cells, we were able to improve the current response using succinate as substrate, in both batch and flow analysis modes, using both gold and graphite electrodes. The generated current depended on the substrate concentration, with a linear dependence in the μM range. In the case of the low potential polymer (**II**) the removal of oxygen from the solution had a great impact on the current response, a phenomenon not observed in the case of the high potential polymer (**I**). The most likely explanation for this difference is related to the superior thermodynamic driving force in the case of Os-polymer I, having an $E^{0'}$ value of +350 mV *vs.* NHE. Thus Os-polymer I efficiently competes with O_2 as electron acceptor, whereas Os-polymer II, because of its low $E^{0'}$-value (0 mV *vs.* NHE), cannot efficiently compete with O_2 as electron acceptor.

CHAPTER 5

Applications

The current section will focus on describing the principles and challenges faced when using enzymes and whole cells in biosensors and biofuel cells.

5.1. Biosensors

According to IUPAC recommendations [306], a biosensor is "an integrated receptor-transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element". The biological element, which is responsible for a selective interaction with the target analyte, can be either biocatalytic - an enzyme (or a cascade of enzymes), a whole cell (bacteria, fungi, eukaryotic cells), an organelle from the cell (*e.g.* mitochondria) or a plant or animal tissue (slice) or biocomplexing (bioaffinity) – an antibody, a receptor, etc. The transducer's role is to monitor and register the physico-chemical changes generated by the interaction between the biorecognition element and the analyte. There are four major classes of transducers including electrochemical (amperometric, potentiometric, conductometric, impedimetric, ion-charge or field effect), optical (IR, Raman, fluorescence, absorption, reflection, surface plasmon resonance), mass (piezoelectric crystals) and calorimetric (thermistors) transducers [306].

Historically, the father of biosensors is Leland C. Clark Jr., who first described in 1962 an ***enzyme electrode,*** which incorporated glucose oxidase (GOx) trapped between two membranes close to a platinum electrode surface [79]. Prior to this glucose biosensor, Clark was working on developing a sensor for oxygen in blood, by electrochemically reducing it at a platinum surface [307]. GOx was added in order to calibrate his oxygen sensor and soon he realised that the glucose concentration can be correlated with the oxygen consumption, thus the possibility of glucose quantification in the very first amperometric biosensor emerged. GOx oxidises glucose to glucono δ-lactone, while O_2 is converted to H_2O_2. The approach was further developed by Updike and Hicks in 1967 [30] when they immobilised GOx on the surface of an oxygen

electrode by using a layer of acrylamide gel. A few years later, Guilbault and Montalvo [308] developed the first potentiometric urea electrode by using immobilised urease and measuring the NH_4^+ released from the reaction with an ammonia electrode. A more detailed description of the history of biosensors with the main contributors in the field at the beginning of their development was recently published [309]. Research groups all over the world have soon joined the field of biosensor studies, and biosensors became very attractive tools in the healthcare industry, biotechnology, environmental protection and many more fields [310-314].

The basic characteristics of a biosensor [315] are: i) *selectivity*, which derives from properties of the biorecognition element (e.g. an enzyme is usually highly selective not only towards a single substrate but also towards a specific reaction); ii) *linear dynamic range*, which is dependent both on the analyte and the recognition element; iii) *sensitivity* and *detection limit*, which are influenced by the recognition process and the transducer (signal to noise ratio); iv) *response time*, determined by the overall sensor architecture, where the diffusion of the substrate to the recognition site is crucial; v) *recovery time*, which is the shortest time between two consecutive determinations and it is also influenced by the whole design of the sensor.

In the context of the current work, the focus will be on electrochemical transducers, which are the most frequently studied and applied [316], and especially on amperometric biosensors. Concerning the biorecognition element, the redox enzymes will be examined in more details. For the usage of whole-cells in biosensors, a recent review describes thoroughly the advances in the field [317].

5.1.1. Electron transfer mechanisms in amperometric enzyme based biosensors

The biorecognition element in the case of enzymatic amperometric biosensor is a redox enzyme, which is an enzyme that catalyzes the electron transfer between a donor and an acceptor. The actual measurement consists in applying a potential difference between the working electrode (modified with an enzyme) and a reference electrode and quantifying the catalytic current resulting from the reaction (see amperometry details in **Chapter 3**). The electron transfer between the enzyme and the working electrode can occur through three major mechanisms, a fact which generated the classification of amperometric biosensors into three generations: *first generation*, which is based on measuring a co-substrate or product generated in the enzymatic reaction, *second generation,* which replaces the natural co-substrate with an artificial redox mediator and *third generation,* based on direct electron transfer between the enzyme and the electrode surface. The three mechanisms have been extensively described in a number of review papers in [36, 98, 318] and their applications in a book chapter [319].

As first proposed by Clark [79], taking the example of GOx, O_2 can be reduced at the surface of a platinum electrode at a potential of -0.6 V *vs.* Ag|AgCl and the resulting current can be directly correlated with the amount of glucose in a sample. Unfortunately, for a small amount of glucose, the change in oxygen concentration is very low (it is difficult to measure a decrease in signal), and also the background oxygen concentration might fluctuate (e.g. changes in temperature, ionic strength). As an alternative, H_2O_2 can be oxidised at a platinum electrode by applying a potential around +0.6 V *vs.* Ag|AgCl, as was first proposed by Guilbault and Lubrano [320, 321]. Although this method has been widely used in many biosensors and several commercial biosensors for measuring blood glucose, the main drawback is represented by the interfering compounds that could be oxidised at this high potential value (e.g. ascorbate, urate, catecholamines, etc.). It is also well-known that the long-term stability of a platinum surface for H_2O_2 oxidation is poor [322]. Different approaches could circumvent this problem, either by using a selective membrane to remove the interferents, or by lowering the oxidation potential of H_2O_2, for example by using Prussian blue modified electrodes [323-327] or peroxidases [328-332].

Considering the previous mentioned disadvantages when using O_2 or H_2O_2, artificial redox mediators have been used in the *second generation* biosensors [95, 333]. This allows for efficient recycling of some enzymatic co-factors (e.g. NAD(P)H), which is otherwise difficult to regenerate at a bare electrode surface [334, 335]. The redox mediator has to be stable in both oxidation states, to have a formal potential close to the formal potential of the prosthetic group of the enzyme and to diffuse very fast between the enzyme and the electrode in order to compete successfully with O_2, in case of oxidases for example. Since the first description of ferrocene-mediated glucose determination using glucose oxidase [95] and its development into a commercial product [96], several reviews illustrate the vast palette of mediators used [80, 98-100, 318, 325] in biosensors. The disadvantages of the mediated approach are mainly related to contaminations of the samples due to mediator leakage and the fact that the mediators are not very selective and they act rather like general (electro)catalysts.

In order to eliminate the problem of leaking mediators, an approach which became soon popular has been adopted. This method is a step further towards “reagentless” biosensors. It consists of using a polymeric backbone, onto which mediating functionalities are incorporated, which can form a redox hydrogel as a matrix for enzyme immobilisation. These redox polymers constitute one of the most successful ways to create reagentless amperometric biosensors [325, 336]. As an example, Os polymers (described in detail in [81, 101, 117, 120]) were successfully used for “wiring” GOx initially with the main purpose of measuring glucose in biological samples [120, 122, 337], but during recent years also for making efficient bioanodes in biofuel cells (see section 5.2.1).

Amperometric biosensors based on direct electron transfer (DET) – *third generation*

Since the first reports on DET of cytochrome *c* [28, 29] and on bioelectrocatalysis for laccase and peroxidase [31-33], various reviews [36, 37, 40, 179, 230, 338-341] and book chapters [38, 311] have covered the evolution of the field. In the case of enzymes capable of DET, both extrinsic and intrinsic [34, 35], the orientation of the enzyme on the electrode surface is crucial for a favourable DET reaction (see Marcus theory, DET and SAMs in **Chapter 2**). The DET approach increases the selectivity of the biosensors and eliminates the need for mediators, which is important in the construction of reagentless devices. On the other hand, the systems based on DET offer the possibility of modulating the desired properties of an analytical device by chemical [102, 103, 342] or genetic [343] protein modification, reconstitution of the enzyme on a cofactor bound to an electrode surface [90, 91, 344] and novel interfacial technologies (e.g. the use of nanoparticles) [92, 345-347].

From the total number of roughly 4900 enzymes currently known (http://www.brenda-enzymes.org/) approximately 1300 are oxidoreductases. Among oxidoreductases, only a restricted number of enzymes have been reported to exhibit efficient DET reactions [15, 36, 38, 39, 339]. This is due to the fact that in most cases the redox active site of the enzyme is deeply buried in the polypeptide structure in order to ensure a high selectivity towards a certain substrate. Those that reveal efficient DET are mainly metal containing enzymes, especially ***heme*** (e.g. catalase [348], cytochrome P450 [349], peroxidases [33, 331, 350, 351,]), ***iron-sulphur clusters*** (e.g. hydrogenase [352, 353]) and ***copper*** (e.g. azurin [70, 354], laccases [31, 32, 230, 251], bilirubin oxidase [261-265] and ascorbate oxidase [355, 356]). Also, a number of multiple cofactor redox enzymes display efficient DET, including succinate dehydrogenase [357, 358] and fumarate reductase [359], which contain FAD and an Fe-S cluster; alcohol dehydrogenase [360] which contains PQQ and heme; fructose dehydrogenase [361, 362] and cellobiose dehydrogenase which contain FAD and heme.

In the case of CDH (described in detail in the previous chapter), direct [73-75, 221, 363] (in the absence of a substrate) and indirect [221, 364, 365] (in the presence of cellobiose) evidence of DET have been shown. Very simple DET based biosensors for lactose have been constructed using CDH from different fungal sources [221]. In the case of the current thesis, we have studied the catalytic properties of CDH from the ascomycete fungus *Myriococcum thermophilum* adsorbed directly on a graphite electrode for a large variety of carbohydrate substrates (**Paper I**) and the study of DET and inter-domain (FAD - heme) ET on SAMs

modified gold electrodes both by cyclic voltammetry and UV-vis spectroelectrochemistry (**Paper II**).

5.2. Biofuel cells

A fuel cell is an electrochemical device that converts chemical energy into electrical energy. The concept dates back from the middle of the 19th century, as mentioned in a recent review [366], which describes in detail the different types of fuel cells with their basic characteristics, efficiency, construction, etc. A fuel cell consists of two electrodes separated by electrolyte: an anode, where the oxidation of the fuel takes places and a cathode, where the reduction of the oxidant (e.g. oxygen) occurs. Compared to batteries, electrical power in a fuel cell is sustained as long as the fuel and oxidant are supplied to the electrodes. The anode and cathode are connected externally via an electric circuit and internally they are separated by an ion-selective membrane, which serves the purpose of separating the two compartments, eliminating the possibility of mixing the fuel and the oxidant and also controls the flow of positive ions from the anode to the cathode. Although the imminent depletion of fossil fuels and the energy crisis require the need for new energy sources, fuel cells are still only used in special areas mainly due to high costs for producing energy [367] ($3000 to $4000 per kW compared to $10 per kW using an internal combustion engine). This is due to the fact that electrode materials are usually made of expensive catalysts (e.g. platinum), they use highly purified fuels and they operate at high temperatures.

Biofuel cells are conceptually equivalent to all types of fuel cells in terms of converting chemical energy into electrical energy. The main differences are in the catalysts they use and as a consequence of that, the conditions under which they operate. Using biological materials as catalysts (enzymes, whole cells, etc.) make them an attractive technology for batteries in miniaturised mobile devices and even implantable electronics due to a number of reasons: operating at low temperatures, catalyst renewability, larger variety of fuels to choose from, using cheap electrode materials (carbon based) and the potential elimination of the ion-selective membrane due to their inherent selectivity. On the other hand, when using whole microbial cells, most of the time they can use “dirty” fuels like waste waters and there is no need for further fuel purification. There are still notable disadvantages compared to batteries and fuel cells; first, the very low power output they generate, and second, stability issues over long term periods.

The extractable power of a fuel cell (P) is the product of the cell voltage (V) and the cell current (I). Although the ideal cell voltage is given by the difference between the formal potentials of fuel and oxidiser, irreversible losses in the voltage (noted as overpotential) take place. There are three types of overpotentials in an electrochemical cell: activation, ohmic and

concentration overpotential [143]. The activation overpotential is given by the finite rate of the reaction at an electrode, the ohmic potential (ohmic drop) is generated by resistances associated with the electrolyte, the ion-selective membrane and the electrical connections and finally, the concentration overpotential is generated by the mass transport limitations. Similarly, the cell current is influenced by the size of the electrodes, the ion permeability and transport across the membrane, the amount of biocatalyst and the rate of electron transfer at the electrode surface [368]. Since the power is a product of voltage and current, the aim of fuel cell research is to maximise the cell voltage by minimising the three categories of overpotential and to increase the current by using high loadings of fast biocatalysts.

Usually, the biofuel cells can be divided into two major groups: enzymatic and microbial biofuel cells. This comprises the majority of research done in this field but, there are, of course exceptions from this classification (e.g. using organelles as biocatalysts [369]). The two major categories will be further described below.

5.2.1. Enzymatic biofuel cells

As the name suggests, the enzymatic biofuel cells use enzymes, especially redox enzymes, as biocatalysts either separately or both on the anode and cathode in the same time. In **Figure 5.1**., a schematic view of a biofuel cell is presented.

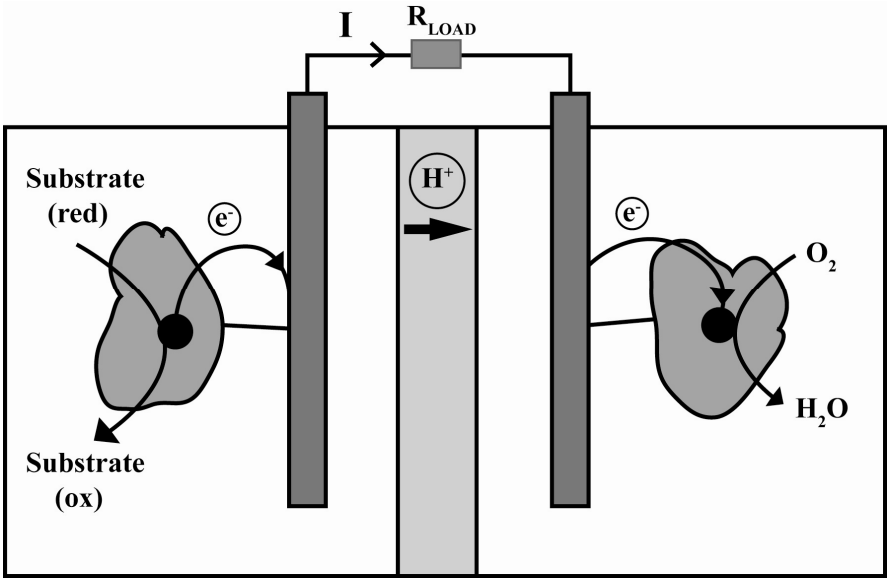


Figure 5.1. A generalised scheme of an enzymatic biofuel cell, where a fuel is oxidised at the anode and molecular oxygen is reduced at the cathode.

Historically, two early reports of using an enzyme in a biofuel cell are worth mentioning. First, in 1962, Davis and Yarborough [370] tested the glucose/GOx system together with methylene blue, obtaining an open circuit potential of 180 mV, using the reduction of oxygen on platinum as the cathode reaction. Second, in 1964 Yahiro et al. [371] used platinum foil electrodes in a compartmentalised plexiglass cell and phosphate buffer in both compartments. Three different enzymes, glucose oxidase, D-amino acid oxidase and NAD dependent alcohol dehydrogenase and their substrates, were tested in solution in the anodic compartment under stirring, without any mediators and the cathodic solution was either open to air or supplied with oxygen. The cell generated up to 350 mV in open circuit potential (OCV) and only a small current density (30 nA cm^{-2}), most probably due to the absence of electron transfer mediators. Later, in 1981 Plotkin et al. [372] reported the use of bacterial non-NAD dependent methanol dehydrogenase in a similar biofuel cell, the electron transfer being facilitated by the use of phenazine ethosulphate as mediator between the enzyme and the platinum anode. An open circuit voltage (OCV) of 0.3 V was recorded with a current density of 0.5 mA cm^{-2}. The same reaction was used later [373] in a more ambitious design where a cascade of two enzymes (methanol dehydrogenase and formate dehydrogenase) was used to complete oxidation of methanol to CO_2.

Although a reduced number of examples are present in the literature since three decades, recent years (especially after 2000) have witnessed an explosion in this research area with numerous papers being published. This research is both directed into basic research (enzyme reaction mechanism, enzyme stability) and applications (biological fluids, devices operating at body temperature, etc.). Extensive review papers and book chapters appeared on this matter and they thoroughly analyse different facets of the enzymatic biofuel cell research from electrode materials to immobilisation techniques, power generation and stability aspects [40, 367, 368, 374-389].

I will only refer in here to a few aspects, which are important and applicable to the current thesis and focus on recent examples that were possibly not covered by the aforementioned publications.

As mentioned above, the selectivity of the enzymes allows eliminating the separation membrane, if the biological elements are not harmed or influenced by the fuel or oxidiser. The first non-compartmentalised biofuel cell was developed in 1999 by the group of Willner [390] and GOx was used to oxidise glucose in the anodic compartment and microperoxidase-11 to reduce hydrogen peroxide at the cathode. Although the generated power was very small, the idea generated a lot of interest in the research community, since the membrane elimination simplifies the design and allows for further miniaturisation.

Another important aspect of the enzymatic biofuel cells is the electron transfer between the oxidoreductases and the electrode materials. Most of the enzymes used as biocatalysts have

not yet shown an efficient direct electron transfer. Usually the redox sites within the enzyme molecule are deeply buried in the protein structure. To overcome this impediment, a proper orientation and coupling with the surface is required (either covalent or electrostatic interaction) or the use of mediators could be employed [391, 392].

In the following sections the engineering of bioanodes (oxidative biocatalysts) and biocathods (reductive biocatalysts) will be discussed. Different strategies of improving the efficiency and lifetime of the obtained biofuel cells will be discussed.

Enzymes used for the cathodes; reduction of O_2 and H_2O_2.

Most enzymatic fuel cells use O_2 as the oxidiser, the reason being that it is freely available and it has a high reduction potential. In this case, the most used enzymes for O_2 reduction are the blue copper oxidases (see the examples in the previous cited reviews), the recent examples proving the special attention given to laccase [131, 132, 226, 251, 393-408] and bilirubin oxidase [270, 409-414]. Since we used the two enzymes for the current thesis, a detailed description of the two enzymes was carried out in the previous chapter. Most fungal laccases work optimally under acid pH (3-5) and they are inhibited by halides [415, 416]. In contrast, bilirubin oxidase works at higher pH (around 7) and it was shown to be more tolerant to chloride ions than laccases [126, 260, 266, 268]. Although both these enzymes have proven direct electron properties with the electrodes [31, 32, 230, 251, 261-265], in most of the studies mediators were used in order to increase the current density. Examples of such mediators are either monomeric, for example ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate) diammonium salt) or hydrogel forming polymers, as the ones designed and used first by Heller's group [101, 117, 120]. The polymeric mediators could be used in membrane-less biofuel cells, being attached to the surface of the electrode. A very interesting and efficient strategy of direct attachment of laccase to graphite has been developed in the group of Armstrong [393]. The researchers used 2-aminoanthracene to modify the graphite electrode and to attach laccase preferentially with the type 1 Cu centre close to the surface. This resulted in a tremendous increase in current density for O_2 reduction and in a much improved long term stability.

Instead of O_2, hydrogen peroxide has been also used in biofuel cell development. In this case highly active peroxidases have been used as biocatalysts [417, 418], and also a lot of attention has been given to microperoxidases (obtained by enzymatic digestion of cytochrome *c*) especially by the groups of Willner [390, 419, 420] and of Ramanavicius [421, 422].

Enzymes used for the anodes; oxidation of H_2, alcohols and sugars.

The first fuel and possibly the cheapest to use is hydrogen. It has a low formal potential, but the oxidation at a Pt electrode is hampered by carbon monoxide (CO) [423]. Hydrogenases have proven to be as efficient as platinum for H_2 oxidation [333, 424-426]. The reaction is catalysed at the catalytic domain containing a bimetallic active site consisting of iron (Fe-Fe). These enzymes are very active in the direction of H^+ reduction, or nickel-iron (Ni-Fe), which operate effectively in the direction of H_2 oxidation and are less damaged by the presence of O_2. This makes them more attractive for biofuel cell research. A chain of iron sulphur clusters transfers the electrons from the active site of the enzyme to the surface, making direct electron transfer possible for many types of hydrogenases. Usually, the hydrogenases are sensitive to O_2 and CO, but there are membrane-bound hydrogenases from *Ralstonia* spp. that are insensitive to CO and only partially affected by O_2 [333, 427]. The electrodes prepared with hydrogenases exhibit high current densities, but they are usually unstable [428]. Karyakin et al. have shown that electropolymerisation of pyrrole on a carbon material could improve the stability for a period of up to 6 months [429].

The use of simple alcohols as substrates was also considered. They are also rather cheap and abundant substrates for different enzymes. The enzymes used in this case are alcohol dehydrogenases and they are not damaged by O_2, which makes them suitable for anodes in membrane-less biofuel cells. A few early examples include NAD^+-dependent alcohol dehydrogenase [391], PQQ-dependent alcohol dehydrogenase [430] and methanol dehydrogenase [372, 373]. A more recent and interesting example is the use of a three enzyme cascade for the complete oxidation of glycerol [431]. The bioanode was constructed using PQQ-dependent alcohol dehydrogenase, PQQ-dependent aldehyde dehydrogenase and oxalate oxidase immobilised within a tetrabutylammonium-modified Nafion membrane and had the ability to operate at high fuel concentrations (up to 5 M glycerol).

One interesting substrate for a bioanode is lactate and the enzyme used to convert it is lactate dehydrogenase (LDH) [432, 433] or L-lactate-cytochrome *c* oxidoreductase [434].

The most extensively studied classes of compounds used as fuels in enzymatic biofuel cells are the sugars. They abound in nature and they are relatively cheap if no special purification step is required. Among the common sugars used in biofuel cells, glucose has received most attention. The enzymes that can oxidise glucose can be divided in two classes: the ones that use O_2 as electron acceptors are called oxidases and the ones which use another acceptor (e.g. cytochromes) are called dehydrogenases. Although rarely and only in special conditions these enzymes exhibit direct electron transfer with the electrodes, they are still the most used enzymes for bioanodes. Many of the recent literature examples use GOx [131, 132, 395, 396, 399, 405-

408, 412-414, 435-437], $NAD(P)^+$-dependent glucose dehydrogenases: EC 1.1.1.47 [402-404, 409, 410, 438] or the FAD, heme *c* glucose dehydrogenase from Sode's group [411, 439].

As mentioned above, GOx has two flavin adenine dinucleotide (FAD) cofactors deeply buried in the enzyme structure. Although the direct electron transfer is sluggish, different strategies for mediated electron transfer have been approached. The first approach for biofuel cells was adopted by Heller and co-workers, who used osmium containing polymers to entrap GOx and facilitate the electron transfer to/from the flavin centre [101, 117, 120, 126, 127, 131, 440]. This approach has been successfully adopted by many other research groups with satisfactory results [129, 130, 132]. A different approach has been adopted by Willner and co-workers who covalently attached FAD via a spacer to an electrode and then reconstituted GOx on top of it using the apo-enzyme [90, 92, 441]. The same approach was applied to PQQ-dependent glucose dehydrogenase, by attaching the apo-enzyme to PQQ-modified gold nanoparticles [442]. There are also reports of an efficient glucose dehydrogenase complex that contains FAD and heme *c* [439], which exhibits direct electron transfer.

One interesting example is the FAD (formerly believed PQQ) and heme containing fructose dehydrogenase, which is found in membranes of incomplete oxidisers (e.g. *Gluconobacter* sp.) [443]. This enzyme was used for the anode reaction and the combination with a multi-copper oxidase at the cathode resulted in a very efficient DET based biofuel cell [397, 444].

Finally, concerning the current study, we use a different bioelement for the anode, cellobiose dehydrogenase, which consists of one catalytic flavin containing and one heme containing domain connected through a flexible linker region. A detailed description of the enzyme was presented in the previous chapter. The presence of heme in the structure of CDH translates in efficient DET properties [73, 365]. The multiple substrates this enzyme can efficiently oxidise (**Paper I)** as well as the different optimum working pH values, which are dependent on the enzyme source (ascomycetes or basidiomycetes) [189], makes this enzyme very suitable for biofuel cells. Although with a very efficient DET, recent studies have shown that the energy output of the biofuel cells containing CDH could be further increased by using osmium polymers and carbon nanotubes [224-227]. Our purpose (see **Papers III & IV**) was to fabricate and characterise a simple, non-compartmentalised, mediator and cofactor free glucose/oxygen biofuel cell operating in buffers and human blood serum.

5.2.2. Microbial biofuel cells

The use of microbes in biofuel cells allows for multiple enzymes that reside inside the cell to be used and thus multiple substrates or even mixed substrates to be oxidised. Compared to isolated enzymes, the microorganism can oxidise more thoroughly different fuels and they also can be less susceptible to loss of activity under normal operating conditions. Disadvantages worth mentioning here are the lower turnover rate of the substrate, the fact that microorganisms ask for special cultivation conditions (when using isolated species) and the electronic communication with different electrode materials is not straightforward for the majority of microorganisms.

The earliest example of microorganisms used in a biofuel cell dates back to 1910-1911, when Potter [445, 446] tested both yeast and *E. coli* in an anodic chamber using platinum electrodes and demonstrated that they could generate electrical power. The idea was adopted much later, when Cohen demonstrated a voltage greater than 35 V for a stack of microbial fuel cells connected in series [447]. Once again, as in the case of enzymatic biofuel cells, although several notable examples were shown in publications before the '90s [370, 448-450], in recent years microbial fuel cell research has become a quickly evolving field (more than 130 publications in 2008, source: scifinder.cas.org) having a great technological support given by the study of biocatalytically modified electrodes, particularly for sensor applications.

There are three classical ways in which microorganisms can be used in biofuel cells: a) a metabolic product generated by photosynthetic or fermentation reactions could be used as a fuel in the anode compartment; b) transfer of electrons between the microorganism and the electrode with the use of mediators; c) the microorganism itself could be employed to transfer electrons directly to/from the electrode (rare cases).

In the first case, the microbe could be placed and grown directly in the anode chamber or a bioreactor could be placed in the vicinity of the biofuel cell and only the gaseous (e.g. H_2) products from the bioreactor could be brought inside the (bio)fuel cell chamber. This later configuration could eliminate problems related to temperature differences (e.g. optimal temperature for microbial proliferation compared to optimal temperature for the operation of the fuel cell), electrode poisoning and membrane fouling due to absorption of nutrients and even cells. The metabolic products generated by the microorganisms include H_2, alkanes (methane), alcohols (methanol, ethanol), aldehydes, carboxylic acids, thiols, etc. Product systems producing H_2 have been reviewed in 2001 by Das and Veziroglu [451] and the means by which biotechnology could produce other various gaseous and liquid fuels were extensively reviewed by Kosaric and Velikonja in 1995 [452].

The second case involves mediated electron transfer, which is carried out through either **i)** artificial electron shuttles added to the cell compartments or immobilised on the electrode surface or through **ii)** self-secreted mediators produced by the cell itself. When direct electron transfer is not possible, the mediated approach can be a good alternative. Interestingly enough, evolution has created a number of microbial species capable of self-mediating the electron transfer by producing and excreting small electron shuttles are oxidised at the electron surface and reduced inside the cell. For example, *Pseudomonas* species were shown to produce pyocyanin [453-455], *E. coli* K-12 was producing a self-mediator of unknown structure [456-458], and *Shewanella* species producing flavins [459].

When direct electron transfer and self-mediation are not possible, a large variety of artificial mediators can be used to improve the electron transfer across the cell membrane [373, 381, 460]. The important role of these electron shuttles in whole cell biosensors and microbial fuel cells is to replace the natural final electron acceptor (oxygen in the case of aerobic bacteria, Fe(III) oxides/complexes in the case of anaerobic organisms), thus preventing the problem of limiting concentrations of electron acceptors. They consist of organic dyes (e.g. thionin, neutral red, 2,6-dichlorophenolindophenol – DCPIP, methyl viologen, methylene blue), inorganic complexes (e.g. ferricyanide) and organometallics (osmium polymers) and they can be added directly in the anodic compartment or immobilised on the electrode surface. Problems in using artificial mediators arise from stability and toxicity concerns.

Polymeric redox active films, grown on metallic surfaces (gold, platinum, etc.) have been used in microbial fuel cells with increased efficiency, e.g. polyaniline and fluorinated polyanilines on platinum and titanium oxide [461-464]. It has also been shown that the use of a polyethyleneimine coated graphite anode modified with a derivative of 9,10-anthraquinone-2,6-disulphate (AQDS) in combination with *G. sulfurreducens* bacteria results in better stability and greater performance of the bioanode than the previous designs, where no mediator was used [443]. As shown earlier in the case of enzymatic biofuel cells, osmium containing polymers are suitable for "wiring" enzymes to electrode surfaces [101, 117, 120, 126, 127, 131, 440]. They promote a stable binding on the electrode surface, therefore reducing the problem of releasing potentially toxic compounds in the environment.

The development in the field of microbial fuel cells has been excellently summarised in a number of recent review papers [334, 336, 379, 381, 465-474], in book chapters [366, 368, 475, 476] and even in an entire book [460] as well as on a dedicated website, (http://www.microbialfuelcell.org/), put together by a number of research groups involved in microbial fuel cell work, offering a very comprehensive collection of bibliographic material, tutorials and information about different research programs.

The most used microorganisms in fuel cell applications are bacteria, although examples using yeast also exist [477-479]. The vast majority of studies in the field of microbial fuel cells target the anode compartment (*viz.* oxidation of organic materials), while oxygen is the most used electron acceptor in the cathodic compartment. Although oxygen is preferred in terms of redox potential and availability, there are a few issues to be considered when oxygen is used [480], including the high overpotential towards oxygen reduction at electrodes and the limited solubility of O_2 (~ 8 mg/L – 0.25 mM at 25 ^{0}C). Generally, the oxygen concentrations cannot be increased, but the efficiency of the catalysis can be improved by adding platinum to the cathode [481] or pyrolysed iron (II) phthalocyanine [482]. Another recent approach is to use bacteria in the cathodic compartment with the benefits of lowering the costs by using cheap electrode materials and the removal of nutrients and contaminants [483, 484]. The biocathodes can perform efficiently the reduction of oxygen [485], nitrate [486-488] or perchlorate [489].

A major breakthrough in the microbial biofuel cell research area was the discovery made by the group of Lovley (www.geobacter.org) of certain bacterial species that can directly and very efficiently electrically communicate with the anode while completely oxidise organic compounds to carbon dioxide [490]. These bacteria were named "electricigens" [467] in order to differentiate their unique features compared to the other types of microorganisms used in the biofuel cell research. They have certain advantages compared with other microorganisms [476] such as: high coulombic efficiency derived from the complete oxidation of organic matter with the electrode as the sole electron acceptor, long term sustainability, and they eliminate the need of mediators. The bacteria having the above mentioned properties are usually members of the *Geobacter* family (e.g. *G. metallireducens* and *G. sulfurreducens*) and they were intensively studied in recent years [490-497]. Other families include *Desulfuromonas* (*viz. D. acetoxidans*) [490], *Rhodoferax* (*viz. R. ferrireducens*) [498, 499], *Geothrix* (*viz. G. fermentans*) [500] and *Desulfobulbus* (*viz. D. propionicus*) [501].

The electron transfer from the cells to the electrodes is proposed to take place following two distinct mechanisms. First, *Geobacter* and *Rhodoferax* cells were proven to have the ability to produce outer membrane cytochromes [502-504] when connected to electrodes. This phenomenon has been known to take place when the cells use Fe(III) as electron acceptor [505, 506]. This implies that only the cells in direct contact with the electrode can transfer electrons and the obtained current will be low. In systems with improved power output it was observed that the cells formed thick biofilms on the anode. It was proven that in this case, conductive pili, termed "bacterial nanowires", play a major role in the biofilm formation and electron tranfer for the layers of cells, which were not in direct contact with the surface [495, 507]. The conductivity of the pili in this case was proven using atomic force microscopy (AFM). Conductive

appendages were observed and characterised using scanning tunnelling microscopy (STM) in the case of *Shewanella* and other species, by Gorby et al. [508].

Previous works from our group have shown the efficiency of the osmium containing polymers (described in **Chapter 2**) in "wiring" viable bacterial cells with electrodes [138-140]. Concerning the current thesis, the work carried out is a logical continuation of previous studies. Starting with gram-negative cells, *Gluconobacter* being the first example, where we addressed enzymes from the cytoplasmic membrane, yielding response for glucose, fructose, ethanol and glycerol [138], going to a more complex gram-negative *Pseudomonas* sp. [139, 140], where the metabolism of phenol takes place in the cytosol of the cell, we now tested the effect on electron transfer of inserting certain redox active proteins (cytochromes) in the membrane of *E. coli* (**Paper V**) and the possibility of "wiring" a gram-positive bacterium (viz. *B. subtilis*) having a substantially thicker cell wall (**Paper VI**). The insertion of the cytochromes in the inner membrane of *E. coli* increased the communication efficiency of the cells with artificial mediators, especially with the osmium containing polymers. In the case of *B. subtilis*, osmium redox polymers proved to be effective in "wiring" the gram-positive cells to electrodes.

Conclusions

This thesis summarises the results of four years of work at the Department of Analytical Chemistry at Lund University. During these years, I got acquainted with the field of Bioanalytical Electrochemistry and I learnt a lot about charge transfer mechanisms involving redox enzymes or bacteria and about biosensors and biofuel cells.

When working with redox enzymes, the focus was on class II cellobiose dehydrogenase (CDH) isolated and purified from ascomycetes fungi (e.g. *Myriococcum thermophilum, Dichomera saubinetii, Corynascus thermophilus*). In the first studies (**Papers I & II**), CDH was immobilised both on graphite and SAMs modified gold electrodes and the electron transfer properties (both direct and mediated) were tested in different pH conditions, adding numerous substrates, etc. It was found that glucose, among other monosaccharides, was a relatively good substrate for this class II CDH. Based on these results, CDH was coupled with laccase and bilirubin oxidase in simple but efficient, noncompartmentalised DET based glucose/O_2 biofuel cells (**Papers III & IV**). In this context, further studies are required with the purpose of finding more efficient electrode materials (e.g. porous graphite or gold). Moreover, genetic modifications of CDH (e.g. altering the linker region between FAD and heme, changing the surface charge) could result in a more efficient and stable enzyme.

In the case of bacterial cell, both gram-negative strains of *E. coli* (**Paper V**) and gram-positive strains of *B. subtilis* (**Paper VI**) were "wired" with mediators at different electrode surfaces. In the case of *E. coli* strains, the introduction of cytochromes in the inner membrane of cells improved, as expected, the efficiency of electron transfer, especially when connected through flexible osmium containing polymeric redox mediators. With *B. subtilis*, the same osmium polymers proved to be capable electrical connectors between bacteria and electrodes without penetrating the thick peptidoglycan layer of the cells. These results suggest that certain improvements could be made in the field of microbial biofuel cells by genetically modifying certain strains which are already efficient oxidisers of organic matter but not very efficient in transferring electrons to an electrode surface.

As a final thought, I just want to say that these years in Lund represent a great experience for me and the knowledge and skill gained here will undoubtedly function as a solid foundation for me in the future.

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