NEW MATERIAL FOR IMMOBILIZATION
OF REDOX ENZYMES ON ELECTRODES
FOR APPLICATION IN BIOFUEL CELLS

Valentina Grippo

Supervisor: Prof. dr. hab. Renata Bilewicz

Doctoral dissertation prepared in the Laboratory
of Theory and Application of Electrodes

Warsaw, 2018
Structure of the cubic phase on the front cover page prepared by dr. Przemysław Miszta, University of Warsaw, Warsaw, Poland.
This project has received funding from the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 607793; from the Swiss National Science Foundation (Sinergia Project no. CRSII2_154451/1)
Acknowledgments

First of all, and above all I want to thank my Prof Renata Bilewicz for a warm welcome in this completely new and different country. I thank her for her humanity and for her help over these years. For always having good chats with me and a warm smile any time I needed.

I thank my colleagues from the University for this Journey. I thank Agata, Jagoda and Asia P. for guiding me through the paperwork in this country – without them every single ‘podpis’ (signature) would have been impossible. I thank Maciek for making coffee every morning (almost!!!) and feeding me with chocolate and pączek. I thank Pani Ela for fruitful discussions in Italo-Anglo-Polish language. Many thanks to Ewa for introducing me to the world of the cubic phase. I thank Asia J., Dorota, Agnieszka, Krzysztof, Michał for their help. I also thank Pani Ania and Paulina for taking care of the Bioenergy grant and everything related to it. Thanks to Martina for the year together with fun and to Julia, a new just arrived friend.

I am also grateful to the Bioenergy people for the innumerable workshops together with fun, for support and solidarity. I was happy to move along this path sharing it with you all. But extraordinary thanks go to Luigi Bello (Lo Gorton), for presenting me the electrochemistry world, for making me first hate and then love this topic, for having always the perfect paper to send me and for being my friend, a good friend.

I would like to thank the people from Dropsens, especially Hussein and David, for welcoming me at their company during the secondment and providing me with their materials and cells. I would like to thank Roland Ludwig and Su Ma for prompt C1CDH solution any time I needed. I acknowledge Novozymes for providing bilirubin oxidase and professor Biernat for the nanotubes.

Special thanks to my Italian friends and family who were always ready to support me when I was desperate. Soprattutto grazie alle due Fra, a Maria, a Bea e la Kess perché in questi anni fuori non hanno mai smesso di esserci. Le nostre vite sono andate avanti ma abbiamo sempre trovato un punto di incontro per ritrovarci. Grazie a Chiara, Biske, Anna, Lorenzo, Sara, Novella, Elisa, Ela, per le risate e i momenti insieme. Grazie a Isa per i momenti di gossip sfrenato. E poi grazie ai miei genitori che mi hanno permesso di fare quel salto nel vuoto e partire per la Svezia; se sono arrivata qui è solo grazie voi (che non avete voluto che scegliessi il tecnico-industriale!!!).

E poi sopra tutto e tutti, grazie a Simo, che mi ha spronata ogni volta che ne avevo bisogno, che è sempre lì con me, che mi fa ridere (anche arrabbiare a volte!) e che mi ha aiutata a risolvere ogni problema senza ansia. Con te è tutto più semplice.

A special thanks to all of you, my friends, I really look forward to the next step and I know you will be there.

“But if you close your eyes, does it almost feel that nothing has changed at all? 
“Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza”

― Divina Commedia –

*Inferno*. XXVI, 118-120

Dante Alighieri
Abstract

Lipid cubic phase systems are excellent immobilizing agents for enzymes due to their biocompatibility and well-defined pore nanostructure. They are proposed here as a convenient matrix for incorporating enzymes and holding them on electrode surfaces in fully active forms. Sensors and biofuel cells based on cubic phase do not need additional separating membranes and can be easily miniaturized. Enzymes are highly specific for the substrate, so there is no need of membranes between the electrodes. The lipidic membrane is stable in the presence of water. Enzyme-modified electrodes show advantages when used in sensing and fuel cells because of their low cytotoxicity, biocompatibility and facile exchange of electrons between enzyme active sites and appropriately prepared electrode surfaces. A typical enzymatic biofuel cell presented in this thesis consists of an enzyme-functionalized bioanode and biocathode. Bilirubin oxidase from *Myrothecium verrucaria* (*Mv*BOd) incorporated in the mesophase shows improved stability and can be used to modify the biocathode. Synergy of Au nanoparticles, naphthyl-functionalised carbon nanotubes and this enzyme leads to improved performance of the biocathode. The stability and the catalytic performance of *Corynascus thermophilus* cellobiose dehydrogenase (*Ct*CDH) is clearly enhanced when the enzyme is trapped in the monoolein cubic phase, both when the enzyme is involved in the mediated and direct electron transfer. D-fructose dehydrogenase from *Gluconobacter sp.* (FDH) and *Ct*CDH are used as anodic enzymes and combined with the *Mv*BOD in a Thin Layer Flow Cell (TLFCL) where a solution (100 mM of sugar in 50 mM MOPS buffer, 100 mM NaCl and 30 mM CaCl₂, pH 6.0) in the presence of atmospheric air is flowed. Alternatively, a layer of lipidic cubic phase is used to separate the cathode and anode of the fuel cell instead of aqueous supporting electrolyte solution, thus, a new liquid-crystalline version of the biofuel cell is proposed.
# Table of contents

List of abbreviations: .......................................................... 1

1 Introduction ........................................................................... 3

PART I – LITERATURE .......................................................... 4

2 Enzymes as catalysts ............................................................ 4

2.1 Classification of enzymes .................................................... 6

2.2 Characteristics of enzymes selected for the thesis ...................... 7

2.2.1 Celllobiose dehydrogenase (CDH) .................................... 7

2.2.2 Fructose dehydrogenase (FDH) ....................................... 9

2.2.3 Bilirubin oxidase (BOD) ............................................... 10

2.3 Methods of enzyme immobilization on solid substrates ............... 12

2.3.1 Adsorption ....................................................................... 12

2.3.2 Nanostructuring electrode surfaces for binding and orienting enzymes .. 13

2.3.3 Self-assembled monolayers .............................................. 14

2.3.4 Polymeric matrices .......................................................... 14

2.4 Liquid crystalline mesophases (LCP) for hosting enzymes ............ 16

2.4.1 Protein crystallization with lipidic cubic phase ..................... 24

2.5 Mechanism of electron transfer between enzyme and electrode ....... 25

2.5.1 Direct electron transfer (DET) .......................................... 27

2.5.2 Mediated electron transfer (MET) ..................................... 28

2.5.3 Electrochemistry of selected enzymes .................................. 28

2.5.3.1 Electrochemical behaviour of CDH ............................... 28

2.5.3.2 Electrochemical behaviour of FDH ............................... 31

2.5.3.3 Electrochemical behaviour of BOD ............................... 32

2.6 Electrochemical applications of enzymes .................................. 33

2.6.1 Biosensors ...................................................................... 33

2.6.2 Enzymatic biofuel cells .................................................. 36
3 Methods.................................................................................................................. 38
  3.1 Cyclic Voltammetry (CV) .................................................................................. 38
  3.2 Differential Pulse Voltammetry (DPV) ............................................................... 41
  3.3 Fuel cell characterization and determination of parameters ......................... 42
  3.4 Small-angle X-ray scattering (SAXS) for cubic phase characterisation ....... 44

PART II - EXPERIMENTAL....................................................................................... 48

4 Experimental section ............................................................................................. 48
  4.1 Chemicals and Materials ................................................................................... 48
  4.2 Instrumentations and experimental set up ....................................................... 49
  4.3 Electrodes and modification schemes................................................................. 50
    4.3.1 Preparation of LCP and incorporation of enzymes ...................................... 51
    4.3.2 LCP characterization: preparation for SAXS experiments ..................... 52
    4.3.3 Synthesis of gold nanoparticles [135] ....................................................... 53
    4.3.4 Synthesis of modified carbon nanotubes ............................................... 54

5 Results and Discussion .......................................................................................... 57
  5.1 Electrochemistry of adsorbed enzymes .............................................................. 57
    5.1.1 Corynascus Thermophilus cellobiose dehydrogenase (CtCDH) .......... 57
    5.1.2 Myrothecium verrucaria bilirubin oxidase (MvBOD) ......................... 62
    5.1.3 D-fructose dehydrogenase (FDH) .......................................................... 67
  5.2 Attempt to crystallize FDH “in meso” ................................................................. 69
  5.3 Bioelectrochemical behaviour of studied enzymes in cubic phase (LCP) ....... 77
    5.3.1 SAXS measurements for the prepared cubic phases .............................. 77
    5.3.2 CtCDH in LCP ......................................................................................... 79
      5.3.2.1 Stability of the cubic phase electrode containing CtCDH ......... 83
      5.3.2.2 Catalytic lactose oxidation mediated by DCPIP ............................ 86
      5.3.2.3 Catalytic oxidation of lactose mediated by Ru(NH$_3$)$_2$Cl$_2$ .......... 93
      5.3.2.4 CtCDH- LCP for glucose catalytic oxidation ................................. 94
5.3.3  $MvBOD$ in LCP ................................................................. 95

5.4  The biofuel cell construction ............................................................... 97

5.4.1  Biobattery with $MvBOD$ on the cathode and Zn/Hopeite anode .......... 98

5.4.2  Biofuel cell with $MvBOD$ on the cathode and FDH on the anode ....... 100

5.4.3  Biofuel cell with $MvBOD$ on the cathode and $CrCDH$ on the anode .... 102

5.4.4  Thin layer flow cells (TLFCL) .................................................... 103

Concluding remarks and outlook ................................................................ 108

Summary ........................................................................................................ 112

References ...................................................................................................... 114
List of abbreviations:

- AA : Amino Acids
- ADH : Alcohol Dehydrogenase
- AFM : Atomic Force Microscopy
- ATP : Adenosine Triphosphate
- AuNPs : Gold Nanoparticles
- BFC : Biofuel Cell
- BOD : Bilirubin Oxidase
- CDH : Cellobiose Dehydrogenase
- CE : Counter Electrode (Auxiliary)
- CNT : Carbon Nanotubes
- CypCDH : *Corynascus Thermophilus* Cellobiose Dehydrogenase
- CYT : Cytochrome Domain in CDH
- Cyt c : Cytochrome c
- D : Diffusion Coefficient
- DCPIP : 2,6-Dichlorophenolindophenol,
  2,6-dichloro-4-((4-hydroxyphenyl)imino)cyclohexa-2,5-dienone
- DCPIP-SWCNTs : DCPIP modified SWCNTs
- DET : Direct Electron Transfer
- DH : Dehydrogenase Domain in CDH
- EA : Electron Acceptor
- FAD : Flavin Adenine Dinucleotide
- Fc : Ferrocene
- FCN : Ferricyanide, hexacyanoferrate(III)
- FDH : D-Fructose Dehydrogenase
- GCE : Glassy Carbon Electrode
- GOx : Glucose Oxidase
- HOPG : Highly Oriented Pyrolytic Graphite
- HRP : Horseradish Peroxidase
- IET : Internal Electron Transfer
- LB : Langmuir-Blodgett
- LPC : Lipidic Cubic Phase
List of abbreviations

- LPMO : Lytic Polysaccharide Monooxygenase
- MD : Molecular Dynamics Simulations
- MET : Mediated Electron Transfer
- MPA-gold : 3-mercaptopropionic acid modified gold
- MvBOD : Myrothecium verrucaria Bilirubin Oxidase
- MWCNTs : Multi-Walled Carbon Nanotubes
- NAD^+ : oxidised form of Nicotinamide Adenine Dinucleotide
- NADH : reduced form of Nicotinamide Adenine Dinucleotide
- NaphtMWCNTs : Naphthyl MWCNTs
- NcCDH : Neurospora Crassa CDH
- NHE : Normal Hydrogen Electrode
- NKA : Na^+/K^+-ATPase
- OCV : Open Cell Voltage
- ORR : Dioxygen Reduction Reaction
- RE : Reference Electrode
- SAM : Self Assembled Monolayer
- SAXS : Small Angle X-ray Scattering
- SE : Sucrose Stereate
- SPE : Screen Printed Electrode
- SPGE : Spectrographic Graphite
- SWCNTs : Single-Walled Carbon Nanotubes
- TB : Toluidine Blue
- TLFCL : Thin Layer Flow Cell
- TNC : Trinuclear Cluster
- WE : Working Electrode
1 Introduction

In the recent years the combination of biomolecules with electronic equipment has become a subject of interest in electrochemistry because it allows to obtain useful functional devices for many applications. The integration of enzymes, DNA and bioreceptors with electronic transducers led to the development of important specific biosensors for food [1,2], environmental analysis [3], detection of pathogens, clinical diagnosis [4,5], etc.. Moreover synergic effects of biomolecules combined with different carbon materials, metallic and semiconducting nanoparticles or nanostructures lead to advancement in the field of hybrid materials with important electronic properties. The aim of this thesis is to investigate the catalytic properties of three different enzymes (*Corynascus Thermophilus* cellobiose dehydrogenase, D-fructose dehydrogenase and *Myrothecium verrucaria* bilirubin oxidase) either adsorbed on carbon nanotubes or incorporated in liquid crystalline lipid mesophase i.e. cubic phase at electrodes. These two approaches to immobilize enzymes at the electrode surfaces will be described and modified nanostructured bioelectrodes will be used for the catalytic oxidation or reduction of analytes or as cathodes and anodes in an enzymatic biofuel cell.

The boost in the catalytic current in case of cellobiose dehydrogenase and/or gain in the enzyme stability (cellobiose dehydrogenase and bilirubin oxidase) will be shown once they are hosted in the lipidic cubic phase films at electrodes. To our knowledge, this is for the first time that these enzymes are immobilized in this biomimetic environment.
PART I – LITERATURE

2 Enzymes as catalysts

Enzymes are proteins which catalyse chemical reactions in biological systems speeding them up without being consumed themselves. They do not affect the energy balance of the reaction in which they take place, so that the final equilibrium concentrations of starting materials and products are unaffected. However, they help to decrease the activation energy needed to achieve the transition state of the process. Therefore, enzymes can be seen as agents able to speed up the approach to equilibrium. These catalysts are very selective and provide a suitable environment and a surface (the active site) where the substrate can specifically bind through non covalent forces: hydrogen and ionic bonds, dipole-dipole, ion-dipole, van der Walls or hydrophobic interactions. The amino acids present in the active site play important roles in the enzyme function for example they can be involved in the mechanism of reaction (weakening the substrate’s bonds) or they can bind the substrate orientating it correctly through immobilization of its binding groups. Enzymes are stereospecific towards chiral substrates and reactions which they catalyse because the L-amino acids, they are made of, form asymmetrical active sites. Differently from what was believed by Fisher with his ‘lock and key hypothesis’, the substrate does not fit in the active site like a ‘key fits a lock’ but the substrate induces the active site to take up the ideal shape to accommodate it [6]. This explains why enzymes have an optimal substrate which fits perfectly (geometrical specificity) and some others are catalysed with less efficiency (i.e. this is the case of cellobiose dehydrogenase, which will be described in details in the 2.2.1). Moreover, the amino acids present in the active site play important roles in the enzyme function for example they can be involved in the mechanism of reaction (weakening the substrate’s bonds) or they can bind the substrate orientating it correctly through the immobilization of its binding groups.

Generally, the Michaelis-Menten kinetics is considered as a model to describe reaction rate of many enzymatic reactions. In a typical reaction the concentration of the substrate is greater than the concentration of the enzyme of five or six order of magnitude. The enzyme E combines with the substrate S to form its product P through the enzyme-substrate ES. The enzyme-substrate ES complex can either dissociate back to E and S or form the final product P (Scheme 1).
Scheme 1

\[
\begin{array}{ccc}
E + S & \xrightarrow{k_1} & ES \\
& \xleftarrow{k_{-1}} & \\
ES & \xrightarrow{k_2} & P
\end{array}
\]

Where \(k_1, k_{-1}\) and \(k_2\) are the rate constants.

The Michaelis-Menten equation (1) relates the rate of an enzyme-catalysed reaction (\(\nu\)) to substrate concentration \([S]\):

\[
\nu = \frac{V_{\text{max}}}{K_M + [S]} \cdot [S]
\]  

(1)

where \(K_M\) and \(V_{\text{max}}\) are the Michaelis-Menten constant and the maximum velocity, respectively.

Plotting the reaction rate versus the substrate concentration gives a curve where the rate of reaction increases almost proportionally to the substrate concentration until reaching a maximum value. The substrate concentration corresponding to the half of the maximum reaction rate value is called Michaelis-Menten constant \(K_M\). The inverse of \(K_M\) defines the turnover number which expresses the affinity of the enzyme to the substrate (the higher the turnover number, the higher the affinity). Some enzymes can act as catalysts only in association with small organic molecules (called coenzymes) or metal ions (called cofactors), and those permanently associated with their proteins are called prosthetic groups (e.g. haem and FAD in cellobiose dehydrogenase). The enzyme without its cofactor is called apoenzyme while the active complex is called holoenzyme.

Scheme 2

\[
\text{apoenzyme (inactive) + cofactor} \rightarrow \text{holoenzyme (active)}
\]

In Table 1, the enzymes used in this thesis together with their substrates and prosthetic groups are shown.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Prosthetic groups</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-fructose Dehydrogenase, FDH</td>
<td>FAD, haem c</td>
<td>D-fructose</td>
</tr>
<tr>
<td>Cellobiose dehydrogenase, CDH</td>
<td>FAD, haem b</td>
<td>cellobiose, glucose, lactose</td>
</tr>
<tr>
<td>Bilirubin oxidase, BOD</td>
<td>Cu</td>
<td>O_2</td>
</tr>
</tbody>
</table>
2.1 Classification of enzymes

The name of the enzyme reflects the function it possesses within the chemical reaction catalysed. The appended suffix -ase indicates that it is an enzyme (thus an oxidoreductase catalyses the transfer of electrons from one molecule to the other, etc.). There are six major classes of reactions that enzymes can catalyse and they are divided into several subclasses. In Table 2 the enzyme classification, established by the Enzyme Commission in 1955, is shown. E.C. stands for Enzyme Commission, the first number represents the main class, and the following numbers correspond to the subclasses up to the third level.

<table>
<thead>
<tr>
<th>EC number</th>
<th>Enzyme class</th>
<th>Type of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.C.1.xxx</td>
<td>Oxidoreductase</td>
<td>Oxidations and reductions</td>
</tr>
<tr>
<td>E.C.2.xxx</td>
<td>Transferase</td>
<td>Group transfer reactions</td>
</tr>
<tr>
<td>E.C.3.xxx</td>
<td>Hydrolase</td>
<td>Hydrolysis reactions</td>
</tr>
<tr>
<td>E.C.4.xxx</td>
<td>Lyases</td>
<td>Addition or removal of groups to form double bond</td>
</tr>
<tr>
<td>E.C.5.xxx</td>
<td>Isomerases</td>
<td>Isomerization and intramolecular group transfer</td>
</tr>
<tr>
<td>E.C.6.xxx</td>
<td>Ligases</td>
<td>Joining two substrates at the expense of ATP hydrolysis</td>
</tr>
</tbody>
</table>

Proteins that catalyse electron transfer by reduction or oxidation of substrates within the redox network are so-called redox enzymes. Numerous redox enzymes exchange electrons with other biological components such as other redox-proteins, cofactors and molecular substrates or with the electrode surface. This thesis focusses on the electron transfer between three selected enzymes (C)CDH, FDH and MvBOD, Table 1) and the electrode surface; direct electron transfer between the redox centres of these enzymes and the electrode surface was reported to be not always possible and usually mediators were needed to improve the communication of the enzymes with the conductive support and to obtain good performance of the biocatalyst-modified electrode.
2.2 Characteristics of enzymes selected for the thesis

2.2.1 Cellobiose dehydrogenase (CDH)

Cellobiose dehydrogenase (CDH) is an extracellular enzyme produced by several wood-degrading fungi. A large part of wood degradation is carried out by aerobic white and soft rot fungi [8]. These fungi are able to fragment the major structural polymers of wood and other lignocellulosic materials - lignin, cellulose, and hemicelluloses – and to further metabolize the portions. White rot fungi are basidiomycetes and can mineralize lignin entirely while soft rot fungi, often ascomycetes, modify the lignin without further mineralization [9]. A third class of aerobic fungi is the brown rot and they conduct large part of the degradation in a non-enzymatic way, they are able to circumvent the lignin barrier, removing the hemicellulose and cellulose with only minor modification of the lignin [10]. While ascomycetes are less capable of rapid wood decay than basidiomycetes, they can degrade wood and other lignocellulosic material under extreme environmental conditions [11]. The enzymes secreted by these fungi and involved in the wood decay are mainly hydrolases (i.e. cellulases), peroxidases, multi-copper oxidases (i.e. laccase), hydrogen peroxide-producing oxidases and cellobiose dehydrogenase (CDH). CDH activity was first isolated by Westermark and Eriksson in 1974. They believed the enzyme was involved in a ping-pong mechanism between cellobiose oxidation to lactone while reducing quinones [12–14]. In 1999 a review on cellobiose dehydrogenases declared that a ‘true’ CDH should only oxidize cellobiose or lactose in the presence of ferricyanide, it should not oxidize glucose and should not work when oxygen is the only electron acceptor [15]. In the last twenty years, the interest in this enzyme has increased and more is known now.

The CDH enzyme belongs to a heterogeneous family of proteins with lengths of protein sequences between 749 and 816 amino acids. It is a monomeric protein consisting of two domains connected by an aminoacidic linker. The molecular mass is between 85 and 101 kDa depending on the glycosylation level [16]. It is secreted mostly by white and soft rot fungi (Ascomycota as well as Basidiomycota phyla) but at least one brown rot fungus has shown CDH activity [17]. The enzyme is divided into three classes. Class I CDHs have basidiomycete origin while class II consists only of sequences of ascomycete origin. Class III is still not well known and needs further investigations [18]. Apart from a few
differences in molecular architecture, class I and class II CDHs vary in mono and disaccharides turnover. They are both involved in biomass degradation [15,18,19] even though class I CDHs has higher turnover number toward glucose compared to class II.

CDHs oxidise β-1,4-linked di- and oligosaccharides, breakdown products of cellulose, cellobiose and cellohextrin. Lactose has a similar structure to them which made it also an eligible substrate. The natural electron acceptor is a copper-dependent lytic polysaccharide monooxygenase (LPMO). Recently, a whole variety of LPMOs, which catalyse the oxidative glycoside bond cleavage in cellulose, hemicelluloses, and starch have been identified [20].

Crystal structures of ascomycete CDHs have been resolved for *Myriococcum thermophilum* CDH (MtCDH), PDB: 4QI6 and *Neurospora crassa* CDH (NcCDH), PDB: 4QI7, and for the basidiomycete CDH from *Phanerochaete chrysosporium* (PDB: 1KDG and 1D7C) [20]. The enzyme contains a smaller haem b binding cytochrome (CYT) domain (~25 kDa) connected via a flexible linker containing approximately 20 amino acids to a flavin-dependent dehydrogenase domain (DH) (~65 kDa) where the FAD is not covalently bound to the protein. The structure of MtCDH was determined at a resolution of 3.2 Å and it showed a closed state between the CYT and DH where the closest edge-to-edge distance is 9 Å (well suited for efficient electron transfer). The substrate binding site is located in the flavin domain and it is accessible to substrate to entry and products to exit when the enzyme is in the closed state. When the CYT is docked onto the DH domain [20] a channel is formed leading to the enzyme surface, with sufficient size to allow substrates entry and products exit,. The haem b propionate A and prop D interact with the active site pocket in the DH, where the propionate carboxyl groups coordinate with Trp 295 (forming an anion-quadrupole interaction), with Arg 698 (in an ionic interaction) and in hydrogen bond with Tyr 99. Trp 295 has an important role as staking platform for the non-reducing end glucosyl unit of the substrate. Most of the amino-acids located in the substrate binding region in the DH (Asn 700, Asn 292, Tyr 619 and Tyr 549) have no significant direct effect on the CYT-DH internal electron transfer but they are believed to stabilize the transition state during substrate oxidation. The interface area between DH and CYT features many negatively charged amino acids (Asp, Glu), what explains the acidic isoelectric point of the enzyme and the acidic optimum pH of the internal electron transfer (IET). Electrons are generated from cellobiose oxidation catalysed by DH and shuttled via CYT to LPMO [11,16].
2.2.2 Fructose dehydrogenase (FDH)

D-fructose dehydrogenase (FDH) is a heterotrimeric-membrane-bound enzyme with a molecular weight of ca. 140 kDa, containing flavin and haems c as prosthetic groups. It is a D-fructose-ubiquinone 5-oxidoreductase functioning as primary dehydrogenase in the respiratory chain of Gluconobacter sp. [21]. It was first found in 1966 [22] in a particulate fraction of Gluconobacter cerinus var. Ammoniacus, where the enzyme was partially purified from acetic acid bacteria. The enzyme was then established to catalyse the oxidation of D-fructose to 5-keto-D-fructose by paper chromatography, and among several analogue substrates, only D-fructose is oxidised by the enzyme. Numerous dyes can act as electron acceptors, like potassium or 2,6-dichlorophenolindophenol (DCPIP) while NAD, NADP, and dioxygen are completely inactive [23]. The enzyme was solubilized in the presence of Triton X–100 and whether this was removed the enzyme underwent to a completely loss of activity due to the dissociation of its components. FDH crystal structure is not known yet but electrochemical measurements, electrophoretic studies and atomic force microscopic studies on its heterogeneous electron transfer led to a better understanding of its structure and mechanism over the last years. The height of the molecule was reported to be ca. 7 nm at pH 5 and the enzyme consists of three subunits that decompose when in neutral and alkaline pH solutions hence no oxidation could be observed [24]. Fluorescent scanning images from the SDS-gel electrophoresis of FDH indicated that the apparent molecular weights of the three subunits are 60.5 ±0.5 kDa for
the FAD containing subunit, 48.3 ±0.3 kDa for the cytochrome one and 18.7 ±0.2 kDa for subunit III [24]. FDH oxidizes fructose from subunit I and transfers the electrons from the haems containing subunit to the ubiquinone buried in the membrane. Because of this strong substrate specificity to fructose, several studies have used FDH for determination of fructose in food and diagnostic analysis [25–27].

2.2.3 Bilirubin oxidase (BOD)

Bilirubin oxidase BOD is a multi-copper oxidase catalysing the oxidation of tetrapyrroles, i.e. bilirubin to biliverdin, while molecular O₂ is reduced to water [28]. The complete X-ray structure was solved twice in literature: at 2.3 Å resolution using twinned crystals by Mizutani and his co-workers in 2010 [29] and at 2.4 Å resolution by Cracknell and co-workers in 2011 using molecular replacement with Spore Coat Protein A, a highly similar protein, as the template [30]. The DNA sequence of MvBOD contains 572 amino-acid residues; the initial 38 residues, which consist of peptides and propeptides, are cleaved in vivo prior to protein purification. The isolated protein consists of only 534 amino-acids divided in three domains. BOD catalytic centres consist of four copper ions, classified into three types of sites: one of type 1 (T1), one of type 2 (T2), and two of type 3 (T3) copper ions. In all “blue” multicopper oxidases, including BOD, the T2 and T3 sites form trinuclear clusters (TNC), where molecular O₂ is reduced to water. The T1 centre is the primary site for the oxidation of the electron donating substrate; the enzyme couples four 1-electron oxidations of the substrate to the four electrons reduction of dioxygen to water. In all the multicopper oxidases the binding sites for the copper atoms are quite identical. The T1 Cu atom has a pseudo-trigonal-bypiramidal coordination geometry, where the equatorial ligands (His-398, His 462 and Cys-457) are coordinated directly to the copper atom, while the axial residues (Met-367, Thr-397 and Asn-459) weekly coordinate to it. The T1 Cu is characterized by an intense Cys to Cu(II) charge transfer at 600 nm giving the deep blue colour generally observed in the multicopper oxidases [31]. The type 2 Cu (T2) is coordinated by two amino-acid residues (His-94 and His-401) and the type 3 copper (T3) coordinates with six amino-acid residues (His-136, His-403, His-456; His-134, His-96 and His-458) plus a water molecule.

Both the TNC and the T1 Cu lie sufficiently close (<14 Å) to the surface of MvBOD for direct electron transfer at biologically relevant rates (1.5 s⁻¹) [32].
A very narrow binding pocket for the substrate (approximately 3.5 Å diameter) formed by the interface of two domains was identified in the vicinity of the His ligands of the T1 site. The electrons coming from the substrate oxidation are transferred to the T1 and then to the TNC via a Cys-His pathway, where O₂ is reduced to water. Two well defined channels are running from the TNC to the surface [30]. One channel ends close to the T2, the other terminates close to the two Cu atoms in T3. Although all channels contain water molecules, they are distributed evenly only in the ‘T2 channel’, suggesting that this channel is more hydrophilic and is, therefore, likely to be the main channel for water transport \textit{in vivo}. The residues Asp-105 and Glu-498 located at the end of the two TNC channels are involved in H⁺ transfer and catalytic O–O splitting, respectively [33].
Cu is represented by the blue sphere. Arrows were added to show the hypothetical electron transfer, oxidation and reduction reactions. Image taken from [34]

The mechanism of bilirubin conversion to biliverdin has been widely studied by EPR (Electron Paramagnetic Resonance) spectroscopy, magnetic circular dichroism and X-ray absorption spectroscopy [28,35,36]. Type 1 Cu site (T1) accepts electrons from electron donating substrates and transfers them to the trinuclear center composed of one type 2 Cu (T2) and two type 3 Cu atoms (T3). The TNC (the T2/T3 Cu cluster) serves as the catalytic site to reduce dioxygen into water.

2.3 Methods of enzyme immobilization on solid substrates

Holding enzymes on solid substrate means to immobilize them within a few nanometres from the solid conductive surface. Independently to the kind of method chosen for the immobilization, the enzyme will have to interface with a molecular environment that can be very different from what it has in its native state. This can affect the catalytic activity for several reasons e.g. it may reduce the accessibility of the substrate to the enzyme active site, change the enzyme dynamic properties, alter the conformational integrity, lead to unfavourable orientation of the enzyme molecule or introduce substrate diffusional limitations [37]. However, different immobilization methods were successfully designed and reported in the literature.

2.3.1 Adsorption

Among many methods, the easiest way to hold an enzyme on the electrode surface is by adsorption. Adsorption makes use of the physical interactions generated between the surface and the enzyme, which include van der Waals forces, ionic interactions and hydrogen bonding. Different supports offer various physical and chemical properties (\textit{i.e.} pores size or hydrophobicity of the surface) although their differences in morphological and physical characteristics can affect the enzyme immobilization and its catalytic activities, since the direct contact between them. A wide range of available supports can be successfully used as the enzyme holder: spectrographic graphite electrodes [38] and carbon
ceramic electrodes [39], for instance, were modified by adsorption of laccase to successfully catalyse the bioelectrocatalytic reduction of oxygen.

On the other hand, direct contact between the enzyme and the electrode surface may denaturate the protein and affect its stability [37]. When laccase from *Trametes hirsuta* was physically adsorbed on bare gold electrode, by dropping the enzyme solution onto the surface, in the presence of the natural substrate, O\(_2\), the normal high potential catalytic process could not be seen. Only low redox potential process was seen, possibly from some abnormal electron transfer pathway due to the random orientation of the enzyme on the electrode surface and its partial denaturation [40].

### 2.3.2 Nanostructuring electrode surfaces for binding and orienting enzymes

To overcome the disadvantage of low amount of adsorbed enzyme on a flat-surface electrode, nanostructures are widely used as a support for enzymes’ electrochemistry. Carbon materials such as carbon nanotubes, graphite, and graphene have been utilized because of their high electrical conductivity and structural robustness. Nanoparticles, on the other hand, exhibit unique electronic, optical and catalytic properties due to their dimensions comparable to those of biomolecules. Conjugation of enzymes with nanostructures led to the development of highly efficient biosensors [41,42].

Enzymes have been effectively adsorbed or covalently bound to single- and multi-walled carbon nanotubes to allow direct electron transfer. *Myrothecium verrucaria* bilirubin oxidase (MyvBOD) was immobilised on pristine multi-walled carbon nanotubes (MWCNTs) modified glassy carbon electrode (GCE) [43] or it was bound to thiol-modified MWCNTs gold electrodes [44]. Nanotubes modified with aromatic moieties (*i.e.* aryl and biphenyl groups) were successfully used for biobatteries e.g. containing laccase *Cerrena unicolor* at the cathode [45–47]. DNA/chitosan-Fe\(_3\)O\(_4\) magnetic nanoparticles film was constructed for the immobilization of horseradish peroxidase (HRP) on a glassy carbon electrode, GCE, acting as the carrier of biological molecules. The DNA/chitosan/Fe\(_3\)O\(_4\) film can provide a microenvironment which is similar to that of the biological molecules [48]. Planar flat gold electrode modified with gold nanoparticles by their adsorption on its
surface was modified with *Trametes hirsuta* laccase to successfully catalyse the oxygen reduction [42].

Other different methods to immobilize enzymes through direct and mediated electron transfer on the electrode surface are known and commonly used: i.e. polymers, hydrogels, self-assembled monolayers and the lipidic films.

### 2.3.3 Self-assembled monolayers

*Self-assembled monolayers* (SAM) were used to covalently attach the enzyme and study direct electrochemistry. Organothiols are chemisorbed on gold or silver electrodes through metal-sulphur bridges forming a well ordered layer able to bind the enzyme through -COO\(^-\), -SH, -CH\(_2\)-OH, and other moieties. The length of the carbon chain of a thiol can affect the monolayer coverage and the immobilization efficiency. It was found [49] that thiols with short chains (n<9) form monolayer with significant amount of defects on the gold surface leading to a poor coverage. On the other hand, thiols with longer chains do form perfect layers but they also passivate the surface, making it difficult for the electron transfer and, consequently, reducing electrode sensitivity.

In a recent paper, by Al-Lolage *et al.* [50], CDH-modified electrodes were tested for direct electron transfer (DET) showing long term storage stability in a 2D system architecture: maleimide groups were coupled to the cysteine groups of CDH and built up on diamines electrografted to carbon nanotubes modified electrodes. The CDH-modified electrode was active for days however, a significant decrease in activity (50% loss) after the first week was reported and ascribed to enzyme desorption or possible loss of FAD from the flavin domain of the immobilised enzyme. A SAM of mercaptoethanol on Au electrode was used to enhance the direct electron transfer (DET) of fructose dehydrogenase (FDH)-catalysed D-fructose oxidation [51].

### 2.3.4 Polymeric matrices

Redox polymer hydrogels have been successfully used to mediate enzyme catalysis while keeping the enzyme close to the electrode surface. One example is represented by Calvo *et
al. [52] in a paper from 1996. Hydrogels were obtained by crosslinking epichlorohydrin with different redox polymers based on poly(allylamine) with covalently attached ferrocene and pyridine groups coordinating iron and ruthenium complexes. As-prepared hydrogels were used to mediate the oxidation of 20 mM glucose from GOx allowing the electrical communication of the electrode with the enzyme FADH$_2$. To facilitate the electron transfer and provide well-developed signals, a redox polymer hydrogel has to be soluble in water and should build a 3D network to allow the rapid diffusion of the substrate and a fast charge transport. It has to contain moieties (hydrophobic, charged or hydrogen-bonding domains) that can complex the enzyme, penetrate into the active centre and adsorb to the electrode. Conducting polymers, like Osmium polymers, were also widely investigated and used to incorporate enzymes and mediators in the same polymer network [53–57]. A recent paper of Lopez et al. [58] emphasized the electron transfer efficiency between enzymes (cellobiose dehydrogenase CDH and glucose oxidase GOx) and electrodes wired by conducting polymers, developing an amperometric polymer multilayer-based biosensor for the determination of lactose in the presence of high concentrations of glucose. In this paper, graphite electrodes were modified by a layer by layer (LBL) process: on the first layer, the lactose sensing one, CDH was drop casted on a positively charged polyelectrolyte poly(diallyldimethylammonium chloride) (PDADMAC)-modified electrode. The second layer was built to remove the interferences coming from glucose analyte. It consisted of glucose oxidase (GOx) and catalase (CAT) immobilized in the structure of a specifically designed hydrophilic polymer. Such amperometric sensor showed good performance providing a wide linear range, enhanced lactose saturation concentrations and causing small errors. In another paper from Urszula Salaj-Kosla et al. [56], nanoporous and planar gold electrodes were utilised as supports for the redox enzymes Aspergillus niger glucose oxidase (GOx) and Corynascus thermophilus cellobiose dehydrogenase (CtCDH). Electrodes modified with hydrogels containing enzymes, Os-redox polymers and the cross-linking agent poly(ethylene glycol)diglycidyl ether were used as biosensors for the determination of glucose and lactose in mediated electron transfer conditions.

Enzymes can also be adsorbed on a lipid bilayer surface or embedded within it. Langmuir-Blodgett (LB) method, for example, is used to transfer a protein-film from a water-air interface to an electrode, but as-prepared systems are not widely applied to enzyme electrochemistry. Another method using lipids, which is more often used with enzymes, is based on liquid crystalline phase (or mesophase), where the enzyme is accommodated in a
lipidic environment in a fully active form. The work presented in this thesis is mostly focused on this technique, which will be discussed in more details in the next paragraphs.

2.4 Liquid crystalline mesophases (LCP) for hosting enzymes

Lipidic liquid crystalline phases (LCPs), also called as mesophases, are formed by the lyotropic (water-induced) self-assembly of specific lipids, such as monounsaturated monoacylglycerols in water [59].

During the last few decades, the number of publications on lipidic cubic phases’ systems has increased exponentially, due to their wide variety of applications. More than 200 proteins have been crystallized through the use of lipidic cubic phases [60], and mesophases have been broadly applied to accommodate enzymes in biosensing systems [61–63] and for drug delivery [64]. The advantages of using such structures are multiple: lyotropic crystalline systems, such as lipidic cubic phases, are biocompatible and stable in the presence of water, and they consist of lipids spontaneously self-assembled in water, forming two layers of non-communicating channels interpenetrating each other, with well-defined channels having diameters allowing free and rapid transport of substrates and products. Furthermore the lipidic cubic phases are easy to prepare and they can be stable for months provided that they are covered with a water layer not to dry.
In a lipidic cubic phase structure, lipid molecules form a hollow framework (right) that extends to form a 3D grid around water channels (left, purple and blue). Image taken from [65]

Monoglycerides have polar head groups and non-polar hydrocarbon chains, which allow them to self-assemble into different structures under varying conditions of temperature and solvents. Monoolein (1-(cis-9-Octadecenoyl)-rac-glycerol) is the most commonly used lipid and it is used in this thesis as monoglyceride (Figure 5). It is composed of a C\textsubscript{18} hydrocarbon chain (tail) which is attached to a glycerol backbone (head group) by ester bond (Figure 5). Thanks to its amphiphilic properties, monoolein is well suited for lipidic cubic phase formation. It is a white powder, insoluble in water, which changes colour when molten (t\textsubscript{M} 36°C). It is non-toxic, biodegradable, highly oil soluble and it is often used as food emulsion [59].

Figure 5 Monoolein - (1-(cis-9-Octadecenoyl)-rac-glycerol - structure (MO)
The lipid-water systems can be conveniently described by referring to their phase diagrams, (the one for the monoolein-water system is shown in Figure 6).

From the dry state, monoolein goes to a liquid fluid isotropic (FI) phase at about 36 °C (its melting temperature) [66]. The addition of water to the molten solid leads to a number of water-induced mesophases dependent on temperature.

The driving force for this assembly is the hydrophobic effect, acting to minimize the interface between the hydrophobic part (hydrocarbon chain) and the hydrophilic one (the polar head) in water. As it can be easily recognised from the phase diagram (Figure 6), different 1, 2 or 3 dimensional self-assemblies in the presence of water are formed: lamellar, hexagonal and bicontinuous cubic phases, respectively. At low hydration level, the first liquid crystal phase to be formed is the lamellar phase (L_α and L_c). It consists of 1D bilayers separated by water; each bilayer consists of two monolayers piled over each other in a manner to minimize the exposition of the tails to water. The hexagonal phase (H_II) is formed at higher temperatures and it consists of a group of micellar cylinders packed onto a two dimensional hexagonal lattice in a tubular structure (Figure 6).
At lower temperatures and increasing water content, bicontinuous cubic phases appear. They are characterized by different symmetries: Im3m (primitive), Pn3m (double-diamond) and Ia3d (gyroid) [67]. In Im3m the water channels meet at 6-way junctions with an angle of 90°; in the Pn3m phase there is a 3D tetra-fold symmetry with a tetrahedral angle (109.51°), while the Ia3d exhibits a trifold connectivity (120°). Pn3m can exist in equilibrium with excess of water in a two phase system in a wide range of temperatures.
Figure 7 Lipid phases. Cartoon representation of the various liquid crystal phases: lamellar phase, cubic-Pn3m phase, cubic-Ia3d phase; cubic-Im3m phase, inverted hexagonal phase and fluid isotropic phase states adopted by lipids dispersed in water. Individual lipids are shown as lollipop figures with the pop and stick parts representing the polar headgroup and the non-polar acyl chain, respectively. The coloured regions represent water. Image taken from [68].

Any alterations in the composition and in the type of lipids result in phase transition or in a change in the structure [59]. So-called ‘swollen’ mesophases can be obtained by changing the lipid to a new one with increased chain splay or different conformation of the double bond: ‘cis’ double bond will result in the most wedge shaped molecule. Addition of hydration-enhancing surfactant as sucrose stearate (SE) to a monoolein based cubic phase can also raise the diameter size of the water channel. It led to a significant increase up to a 7.2 nm where more than 52% of water can be incorporated inside the water channel, comparing to Pn3m water channel size of 3.6 nm and 35% of water [69]. Considering the
average enzyme hydrodynamic diameter, of approximately 6 nm, swollen mesophases provide a much more suitable environment for the enzyme. When horseradish peroxidase (HRP) was hosted in a standard Pn3m phase, the kinetics was related to a diffusion-restricted accessibility of the substrates. The kinetics behaviour of freely diffusing enzymes can be restored with swollen mesophases. Water channel size is not the only factor determining the activity of an enzyme, also the topological connectivity plays an important role. In the same paper published by Mezzenga, three different phases Pn3m, Ia3d and HII were carefully prepared. They had the same water channel diameter of 3.4 nm and HRP performance was further investigated. The enzyme showed that the activity is related to the diffusion rates and it varies for different topological structures. The Pn3m with its tetra-fold connectivity allows faster diffusion than the tri-fold connectivity of the Ia3d which in turn allows faster diffusion than the HII phase. Nevertheless, the enzyme is catalytically active in all cases. A recent simulation study [70] by means of Brownian Dynamics simulation of small molecules diffusing into Im3m, Pn3m and Ia3d symmetries showed that the transport is entirely determined by the size of the diffusion domain (the pores size) but when bottlenecks are widely present, they become the most relevant factor for transport, regardless of the connectivity of the cubic phase. Therefore, when large amount of water is available e.g. within the Im3m phase, fast diffusion of hydrophilic molecules is observed overcoming the damping effects introduced by the large amount of geometrical constrictions (bottlenecks). Hence, the best way to increase transport is to obtain cubic phases with large water content, e.g. by introduction of additives with large hydrophilic head groups.

The typical phase behaviour for the monoolein-water system is well known, as already described above, however, there are many different additives that can alter the cubic phase behaviour. Among them, addition of lipids [71], addition of detergents [72,73] and addition of electrolytes [74], have been reported to have significant influence on the phase behaviour even leading to a phase change.

Due to the cubic symmetry, lipidic cubic phases are isotropic and can only be distinguished by their small angle X-ray scattering (SAXS) patterns – not by a polarizing microscope. NMR technique is also suitable for investigating liquid crystalline phases due to the isotropy of the structures which results in highly resolved peaks, even though it is not enough sensitive do distinguish micellar from cubic phases. To obtain a visualization of the structure in the direct space, cryo-transmission electron microscopy (cryo-TEM) and cryo-
electron tomography (CET) are also commonly used [75,76]. In standard cryo-TEM the signal results from the thickness of the vitrified sample, what limits the resolution and subsequent structural interpretations [75] but CET can be used to obtain structures having few nanometres with a high resolution [76].

Since the monoolein-water phase diagram is well known, monoolein and the lipidic cubic phases have raised enormous interests among scientists, and numerous studies were done, focusing on the lipidic interactions with biological molecules for further application as biosensors. Several proteins and enzymes have been incorporated in the cubic phases without losing the 3D structure; they are excellent immobilizing carriers for enzymes due to their membrane biomimetic properties.

An enzymatic reaction of an enzyme hosted in the lipidic cubic phase can be easily controlled using traditional electrochemical methods. The activity of an enzyme encaged in the lipidic cubic phase depends both on the size and the structure of the cubic phase [69,77,78]. Mezzenga et al. [77] discussed the enzymatic catalysis of HRP hosted in cubic phase. Since the hydrodynamic diameter of the enzyme is bigger than the water channel size, the enzyme kinetics is reflected by the diffusion of the substrate into the water channels. When two identically hydrated phases with different water channel sizes are compared, the larger the water channel size, the faster the diffusion. To induce a change in the phase at the same water content, 10% of sucrose stearate S-1670 was added into the lipid, leading to a change in the water channel width from 3.5 nm (Pn3m) to 4.2 nm (Ia3d). But when the enzyme activity in different cubic phases, with the same water channel size (3.4 nm), is compared, the Pn3m phase exhibits highest enzyme activity than Ia3d. Because of the tetra-fold symmetry the substrates and products diffuse more efficiently. Therefore, not only the size of the water channel but also the topological connectivity of the phase plays an important role. Monoolein is the most common lipid used for mesophases and for the Pn3m symmetry the water channel size is ca. 3.5 nm.

Glucose oxidase GOx, discussed in paragraph 2.6.1, has been also inserted in a Pn3m phase while the mediator, the ferrocenecarboxylic acid, was present in the buffer solution [78]. The diffusion coefficient for the mediator in the Pn3m cubic phase was estimated to be $4.9 \times 10^{-7} \text{cm}^2/\text{s}$ while for a bare GCE in the same buffer a value of $5.9 \times 10^{-6} \text{cm}^2/\text{s}$ was obtained. The diffusion coefficient decreased almost three orders of magnitude by changing the temperature from 30°C to 15°C, due to occurring structural changes of the cubic phase,
what resulted in a mixture of Pn3m cubic phase and crystal lamellar phase (already expected below 20°C).

The presence of electrolytes in a buffer solution can also affect the phase. Infrared spectroscopy studies have shown that divalent ions (like Ca$^{2+}$ or Mg$^{2+}$) can penetrate the polar head group region coordinating to the hydroxyl groups and causing a partial dehydration with a complex conformational rearrangement [74]. Electrochemical impedance spectroscopy indicated that cations lead to a decrease of the ion channel diameter while increasing the membrane resistance and capacitance. High concentrations of calcium ions bound to the wall of the phase impose an electrostatic repulsion between the head group regions inducing phase transition to a more open cubic phase, where the radius of channels and lattice parameter increase (i.e. Ia3d). No changes in capacitance were observed in case of monovalent ions indicating that they prefer to stay in the water channel and do not bind to the head group region (which would lead to changes in capacitance). Changes in membrane resistance and capacitance were found not to be caused by the anions mobility.

When the membrane-bound enzyme, D-fructose dehydrogenase (FDH), was immobilized in a lipidic cubic phase film on a GC electrode covered with the single-walled carbon nanotubes (SWCNTs), direct electron transfer was observed. The maximum current density obtained for the fructose oxidation was 3.2 mA·cm$^{-2}$ and the oxidation started at ca. -0.1 V vs. Ag/AgCl [61]. This electrode was successfully used as a biosensor to evaluate the fructose concentration in selected commercially available juices, giving the results which were in good agreement with those obtained with the fructose essay kit from Sigma.

As well, small mediators can be incorporated in the cubic phase film, taking into account the fact that whether they are water soluble they will efficiently diffuse in the buffer solution filled channels. The diffusion coefficients of redox mediators used for different enzymes have been evaluated in several studies. For the determination of the diffusion coefficient (D) of hexaammine ruthenium (III) complex - [Ru(NH$_3$)$_6$]$^{3+}$ - inside the cubic phase film, linear scan voltammograms were recorded using with ultramicroelectrodes and glassy carbon electrodes modified with the mesophase [79]. The D value of [Ru(NH$_3$)$_6$]$^{3+}$ in solution is 8.86·10$^{-6}$ cm$^2$·s$^{-1}$ in phosphate buffer and it decreases to 25% of that value when it is located in the cubic phase. Interestingly, in this study other redox mediators were studied and it was found that diffusion coefficient of the mediator in a cubic
phase increases with its hydrophobicity. The more hydrophobic the compound is, the closer to the interphase with the hydrophobic regions of the lipidic layer it will be located. When derivatives of vitamin K, having long hydrophobic chain, were used as mediators in the glucose dehydrogenase catalytic oxidation reaction, they were anchored to the lipidic layer and remained in the film even after several hours [80]. To prepare this cubic phase, vitamins and monooolein were molten together and next water was added to make a mixture containing VitK/MO/H₂O at a ratio 1/64/35. In a recent study [81], a phenothiazine dye, toluidine blue (TB), was employed as the mediator for the oxidation of ethanol from alcohol dehydrogenase (ADH). The mass ratio of MO/TB/ADH was 64/1/35, where MO and TB were molten together and then aqueous enzyme solution was added.

Not only enzymes and mediators but also drugs and membrane integral proteins were immobilized in the lipidic cubic phase. Molecular dynamics simulations (MD) and electrochemical studies on doxorubicin (DOX) in LCP showed that the drug, when protonated, is located mainly in the aqueous channel (acidic pH) while the unprotonated form, present above neutral pH, is less prone to diffuse from the lipidic region to the solution [64]. The incorporation of the membrane ATP-hydrolysing sodium/potassium transporter Na⁺/K⁺-ATPase (NKA) in lipidic cubic phase allowed the protein to retain its ATP-hydrolysing activity for 7 days and after 14 days only the 40% of activity dropped, whereas the solubilized protein is inactive [82].

2.4.1 Protein crystallization with lipidic cubic phase

Crystallization of protein in lipidic cubic phases has emerged as an important tool in determining the structure of proteins. From the very first paper in 1996 [83] on in-meso crystallization of bacteriorhodopsin, huge improvements have been done. Lipidic cubic phase is a support matrix for crystals growth providing a biomimetic ‘native’ environment for the membrane protein. The main problem with the crystallization of membrane proteins is the necessity of extracting them from their native lipid bilayers by detergent solubilisation. Exposing them to solution conditions having completely different properties than those existing in native membranes may lead to denaturation, aggregation, and sometimes degradation of the protein itself. Advantages of using cubic phases are several: it is viscous – like biological membranes, it is capable of incorporating large amount of proteins, detergents and precipitants without changing its structure and it provides a flexible
matrix that can facilitate the crystal nucleation and growth. Furthermore, crystals generally appear within first weeks. As explained in the previous paragraph 2.4, typically, the protein solution (containing the detergent as well) is combined with the lipid (i.e., monoolein) according to its phase diagram. In case of in meso crystallization the protein is treated with a detergent to make it soluble and form mixed micelle. Addition of lipid will lead to reconstitution of the purified protein into the mesophase; as the system approaches the equilibrium, the cubic phase will be formed. Generally, a precipitant is added to activate nucleation and boost crystals growth. Under conditions leading to crystallization, one of the separated phases is enriched in protein, which supports nucleation and progression to a bulk crystal. It is interestingly to note that detergent might induce phase transformations. Lattice parameter of the Pn3m cubic phase in hydrated MO rises with the octyl glucoside [72] and other common detergent, i.e., DDM [73]. The sensibility of the cubic phase lattice parameters to detergent suggested the possibility of using it as a base for detergent assay.

Lipidic cubic phases were also found to being able to renature denatured integral membrane proteins, reconstituting them with 100% efficiency [84]. Diacylglycerol kinase was reconstituted in the bilayer of the mesophase, and then crystallized in situ by the in meso method providing an X-ray structure to a resolution of 2.55 Å. This structure was identical to that of the untreated reference kinase [85].

The greater the driving force for nucleation is, the more supersaturated the system is [86]. Thus, a common strategy in the area of crystallization is to work at the highest possible protein concentration to favour the nucleation.

### 2.5 Mechanism of electron transfer between enzyme and electrode

Redox enzymes might be subdivided according to the mechanism of electron transfer. The exchange of electrons between the redox centres and the electrodes plays an important role for the mechanism of numerous biosensors. Marcus theory describes how the electron transfer from a donor to an acceptor is performed [87–89]. The theory predicts the increase of the electrochemical transfer rate with increasing the applied potential. The rate constant \(k_{ET}\) for the outer sphere electron transfer is defined by:

\[
k_{ET} = kA \cdot e^{-\frac{\Delta G^*}{RT}}
\]  

(2)
Where $\kappa$ is the electronic transmission coefficient, $A$ is a collision frequency, $R$ is the gas constant, $T$ is the temperature in Kelvin, and $\Delta G^*$ is related to the free Gibbs energy ($\Delta G^0$) by:

$$\Delta G^* = \frac{(\Delta G^0 + \lambda)^2}{4\lambda} \quad (3)$$

where $\lambda$ is the reorganization energy.

The potential dependence of $k_{ET}$ arises from the relation:

$$k_{ET} \approx -nF\eta \quad (4)$$

Where $n$ is the number of electrons, $F$ the Faraday constant and $\eta$ is the overpotential and equals to $(E-E^0)$, $E$ is the applied potential.

Furthermore the rate constant $k_{ET}$ decreases exponentially with the distance of electron transfer $d$ according to equation (5):

$$k_{ET} = k_0 \cdot e^{-\beta(d-d_0)} \quad (5)$$

Where $k_0$ is the electron transfer rate at the closest distance $d_0$ and $\beta$ describes the exponential attenuation of the overlap with distance between the donor and acceptor and depends on the nature of the intervening medium through which the electron tunnels. $\beta$ is typically in the range 8.5–11.5 nm$^{-1}$ in proteins, indicating a rapid decrease of $k_{ET}$ with distance [90].

If $d$ is large enough, i.e. the redox active centre of an enzyme is hidden from the electrode surface or the enzyme has a big hydrodynamic diameter, $k_{ET}$ will decrease exponentially and the direct electron transfer (DET) will not be observed (generally if $d > 20$ Å). A way to facilitate the electron transfer from the redox centre to the electrode surface includes decreasing of $d$ by using small soluble redox molecules (called mediators). They shuttle electrons between electrode and enzyme (mediated electron transfer - MET). A good mediator has a well-defined fast and reversible voltammetry behaviour, a large heterogeneous rate constant, pH independent formal potential and its oxidized and reduced forms are stable.
2.5.1 Direct electron transfer (DET)

An effective electronic communication between the active side and the supporting material is vital for an efficient bioelectrocatalysis and not all the enzymes are able to achieve such a mediatorless electrocatalysis. Electrodes based on direct electron transfer (DET), gain in terms of selectivity and stability due to the elimination of problems related to the mediator thermodynamic losses and stability. On the contrary, current densities are lower comparing to mediated electron transfer systems and orientation of the enzyme on the solid conductive support is an important problem that has to be kept in mind while planning DET experiments. The first direct electron transfer reaction of proteins was performed on horse heart ferricytochrome c investigated on a planar gold disk electrode modified with 4,4’-bipyridyl to promote electron transfer between transition-metal complexes, in 1977 by Eddowes et al. [91]. Nowadays the majority of the methods for direct electron transfer still use films that immobilize the enzymes inhibiting their denaturation, controlling the protein orientation and avoiding further adsorption of impurities. In a review from 1988 from Hill and Frew [92], a careful investigation on the importance of binding in the direct electron transfer for redox enzymes was done. Over 50 bifunctional organic compounds were used to modify gold electrodes and they were assessed in terms of their ability to promote the direct electrochemistry of horse heart cytochrome c. The binding between enzyme and electrode surface has to resemble the interaction which takes place in the natural environment so that the binding is fast and reversible. Another important requirement for a successful DET is the electrostatic compatibility of the electrode surface with the enzyme, like the denaturation of proteins on metal surfaces [93]. When a flavoenzyme (an enzyme containing a flavin group) is adsorbed onto a metal surface, a distortion of its structure might happen causing a loss of the enzyme-bound FAD. As it was previously reported for the glucose oxidase in 1984 [94] and restated more recently in 2016 [95], it is not possible to distinguish between electron transfer from the free FAD and that from enzyme bound FAD for the reason that the presence of the soluble flavin increases the apparent direct response. Trying to avoid problems associated with the denaturation of flavoenzymes on simple metal electrodes, a number of authors have immobilised flavoenzymes onto carbon electrodes using covalent coupling and crosslinking techniques, to prevent protein destructuration while immobilizing it on the electrode surface in the correct conformation.
2.5.2 Mediated electron transfer (MET)

Some redox enzymes are too large and fragile to interact directly with a metallic electrode without being at least partly denatured; or some of their active sites are deeply buried in the protective protein matrix and direct electron exchange with an electrode can only occur under exceptional conditions. Because of this, many redox enzymes are able to utilise artificial electron acceptors, usually called mediators, they shuttle charges between the redox centres of the enzyme and the electrode surface. Finally the mediator is regenerated at the electrode surface resulting in a fast cyclic process. To be effective, a mediator must compete with the enzyme natural substrate and needs to have a fast reaction with the redox enzyme, must have a well define reversible electrochemistry response with a large heterogeneous constant and stable oxidised and reduced forms and must have a pH independent formal potential [90]. When a mediator is used to improve the communication between the solid substrate and the enzyme, the orientation of the protein is less relevant because the electrons are shuttled by the mediator. In mediated electron transfer processes only the mediator interacts with the electrode while the enzyme is in its original conformation in the bulk (far away from the electrode surface). Mediators can be present in solution as soluble mediators (i.e. ferrocene [96]) or as redox polymers [97].

2.5.3 Electrochemistry of selected enzymes

The focus in this thesis project was on three different enzymes, two of which, FDH and CtCDH, are so-called anodic enzymes because they are oxidizing the carbohydrate substrates to the corresponding lactone forms, and one cathodic enzyme, the MvBOD, able to reduce oxygen to water. More about their electrochemical behaviour will be explained in the following paragraphs.

2.5.3.1 Electrochemical behaviour of CDH

Cellulobiose dehydrogenases (CDHs) consist of two separate domains, one FAD containing dehydrogenase domain (DH) and haem b containing cytochrome domain (CYT) connected through a long flexible polypeptide linker, which facilitates the transfer of electrons from
DH via CYT to LPMO. The electron transfer via the CYT domain can also be exploited to achieve direct electron transfer to electrodes in biosensors and biofuel cells. The working pH influences the distance between the two domains and therefore the occurrence and rate of IET. When the pH increases, the two domains separate due to electrostatic repulsion and then, internal electron transfer is reduced to very small numbers and ET can only proceed via MET. At a pH close to the isoelectric point (pI), the domains do not repel each other and allow for internal electron transfer. DET directly from the DH domain of basidiomycete CDHs at pH values below the pI has only recently been observed [98] and occurs at potentials roughly 130 mV more negative than the usually observed potential of direct electron transfer of CYT. No DET from a DH of ascomycete CDHs has been observed so far.

In the catalytic process the electrons originating from the substrate oxidation will reduce the FAD cofactor to FADH$_2$ in the DH domain. The reduced FADH$_2$ is reoxidised to FAD in the oxidative cycle, which can proceed either through a single 2 e$^-$ acceptor reaction or two subsequent 1 e$^-$ acceptor reactions. Electrons can be transferred to a mediator like an osmium redox polymer [56,99] or to mediators such as quinones or 2,6-dichlorophenolindophenol (DCPIP). The substrate oxidation at the DH domain is followed by an IET reaction at the CYT domain, working as a ‘built-in’ mediator and being able to communicate with the electrode via direct electron transfer [100,101].
Figure 8 Electron transfer pathway in celllobiose dehydrogenase from *Neurospora crassa* NcCDH (PDB 4QI7): two electrons obtained from the carbohydrate oxidation are stored at the FAD in the DH domain, and are subsequently transferred to one or two electron acceptor (1 EA and 2 EA) or to the CYT domain which transfers one electron at a time to either the natural electron acceptor, LPMO, or an electrode.

Several approaches have been used to improve and optimize both direct and mediated electron transfer from CDH to the electrode surface. CDH has been directly adsorbed on pristine SWCNTs and MWCNTs modified electrodes [102], on aryl diazonium activated SWCNTs [103], on gold nanoparticles [104–106]. MET was investigated using cytochrome c as a mediator on gold electrodes [107] or osmium polymer [56,99]. A more complex system, consisting of carbon nanotubes-covered-electrodes covalently modified with maleimide groups spontaneously coupling to the cysteine groups of CDH, was also studied recently [50].
2.5.3.2 Electrochemical behaviour of FDH

As previously discussed, the crystal structure of FDH has not been solved yet but enormous improvement on its heterogeneous electron transfer pathway has been achieved recently through electrochemical studies. The enzyme comprises of three subunits. Subunit I contains a covalently bound FAD as a prosthetic group, where D-fructose is oxidised to a 5-keto-D-fructose in a $2\text{H}^+/2\text{e}^-$ oxidation. Electrons are then transferred to the tri-haems cytochrome $c$ containing subunit (subunit II), where the cytochrome cofactors act as electron acceptors. Subunit III is not directly involved in the ET but takes part in the enzyme stability.

When the enzyme is deprived of haems containing subunit, the subunit I/III subcomplex catalyse the fructose oxidation with the use of several mediators. The haem $c$ subunit in FDH is essential for direct electron transfer, while D-fructose oxidation proceeds thorough the FAD cofactor even in the absence of the haem $c$ subunit [108]. Upon further addition of subunit II independently expressed to a subunit I/III subcomplex the recovery of DET bioelectrocatalytic activity was observed [109]. The haem $c$ moiety in subunit II of FDH is responsible for anchoring the enzyme to the cytoplasmic membrane and transferring electrons to the ubiquinone [21]. After FDH adsorption on MWCNTs, four reversible redox peaks were observed with peak potentials of -0.231 V, -0.062 V, -0.054 and +0.34V vs. Ag/AgCl at pH 5.5 corresponding respectively to the FAD covalently bound in the subunit I, the haem 1, haem 2 and haem 3 in the subunit II [110]. In absence of external mediator, electrons coming from the FADH$_2$ are transferred to the electrode surface by two separated one electron transfer processes from haem 1 and haem 2. Haem 3 is too deeply buried in the protein and does not take part in the electron transfer [110]. Carbon nanotubes (CNTs) could communicate with redox centres hidden deep within protein shells as molecular wires, because of their small diameters and conductivities; the direct electron transfer reaction of FDH immobilized onto CNTs was successfully observed [111].
2.5.3.3 Electrochemical behaviour of BOD

The electrochemical behaviour of BOD from *Myrothecium verrucaria* and the redox potential of the T1 and T2/T3 sites have been investigated by Christenson et al. [28]. The midpoint potential of the T1 site was found by spectroelectrochemistry to be close to 670 mV vs. NHE, while the TNC appears at lower potential (ca. 400 mV vs. NHE). Direct (mediatorless) electron transfer between gold electrodes and BOD was also described in the same paper. *Trachyderma tsunodae* bilirubin oxidase was immobilised on bare spectrographic graphite (SPGE) and on 3-mercaptopropionic acid modified gold (MPA-gold) electrodes and the O₂-electroreduction was found to be completely different on the two substrates because of the different enzyme orientations. On SPGE, the BOD was mostly oriented with the T1 copper site resulting in a well-pronounced DET while on MPA-gold most of the enzyme was oriented with its T2/T3 copper cluster in proximity to the metal exhibiting very poor O₂-electroreduction. These results suggest an uphill intramolecular electron transfer from the T1 site to the T2/T3 cluster during the catalytic turnover of the enzyme [112].

Figure 9 Electron transfer pathway in FDH: two electrons obtained from the substrate oxidation are transferred to the cytochrome subunit; they are then transferred to the electrode through two 1 e⁻ processes.
Figure 10 Proposed mechanisms of DET from electrodes to BOD connected (A) via the T1 site and (B) via the T2/T3 cluster. Image taken from [112].

BOD is considered to be the best catalyst to convert $\text{O}_2$ to water because of its very low overpotential required to catalyse the reaction and for its turnover rate of $0.7 \text{ O}_2$ per Cu·s$^{-1}$ (three times lower than for Pt) [113]. Because of this, BOD has been involved in several studies to improve its stability and catalysis for a further development of an efficient cathode for a biofuel system. Different forms of carbon were widely used as electrode base materials for DET because of their chemical stability and good electrical conductivity: graphite electrodes [114], multi-walled carbon nanotubes-modified glassy carbon electrodes [43], mesoporous carbon-cryogel electrodes [115], carbon nanofibers [116], thiol modified multi-walled carbon nanotube-modified gold electrodes [44]. Bare capillary gold electrodes were used to first measure the redox potentials of the Cu sites by Gorton et al. [28]. The direct dioxygen reduction reaction (ORR), catalysed by BOD was investigated for other gold materials like nanoporous [117] and macroporous gold electrodes [118].

2.6 Electrochemical applications of enzymes

2.6.1 Biosensors

In 2009 IUPAC defined biosensor as “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect
chemical compounds usually by electrical, thermal or optical signals” [119]. In other words a biosensor is a device that transforms chemical information into analytically useful signals. The coupling between redox enzymes and electrodes defines the first, second or third generation of sensors. The electroactivity of a first generations biosensor is measured based on the electroactivity of the enzyme substrate or product. The use of a mediator defines the second generation biosensor. Third generation biosensors contain enzymes able to communicate directly – mediatorless – with the electrode surface and only limited number of enzymes has been reported.

**First generation**

![First generation biosensor schematic](image1)

**Second generation**

![Second generation biosensor schematic](image2)

**Third generation**

![Third generation biosensor schematic](image3)

Figure 11 First, second and third generation biosensors schematic pathways

First and second generation biosensors can be better explained by describing glucose biosensors using glucose oxidase [4]. Glucose oxidase (GOx) is a homodimeric glycoprotein of molecular weight equal to 160 kDa, with one tightly but non-covalently bound flavin adenine dinucleotide (FAD) cofactor per monomer [120]. Glucose gets oxidised to gluconolactone by the oxidised form of glucose oxidase denoted as GO(FAD); which is then reduced to GO(FADH₂), according to equation (6). GO(FADH₂) is oxidised back to GO(FAD) by O₂ producing H₂O₂, which can be finally detected by the electrode. First generation glucose biosensors rely on the use of the natural oxygen as co-substrate for generation and detection of hydrogen peroxide. Measurement of H₂O₂ has the advantage of being simple nevertheless it requires application of relatively high potential which can cause the oxidation of other biological component interfering with the result and
compromising the accuracy of the experiment. Furthermore, there is a discrepancy between oxygen and glucose concentration in the physiological body condition.

\[
\text{Glucose} + \text{GO(FAD)} + 2\text{H}^+ \rightarrow \text{gluconolactone} + (\text{GO(FADH}_2) )
\]

\[
\text{GO(FADH}_2) + \text{O}_2 \rightarrow \text{GO(FAD)} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2e^- \text{(at electrode)}
\]

Several ways have been proposed for addressing this oxygen limitation. One approach was to replace the oxygen electron acceptor with a mediator capable of shuttle electrons from the redox centre of the enzyme to the electrode surface (second generation biosensor). The schematic pathways can be summarised as follows:

\[
\text{Glucose} + \text{GO(FAD)} + 2\text{H}^+ \rightarrow \text{gluconolactone} + (\text{GO(FADH}_2) )
\]

\[
\text{GO(FADH}_2) + \text{M}_{ox} \rightarrow \text{GO(FAD)} + \text{M}_{red}
\]

\[
\text{M}_{red} \rightarrow \text{M}_{ox} + 2e^- \text{(at electrode)}
\]

where \(\text{M}_{red}\) and \(\text{M}_{ox}\) represent the reduced and oxidized form of the redox mediator, respectively. The reduced form is reoxidized at the electrode, giving a current signal (proportional to the glucose concentration). The second generation biosensor developed by Cass et al. [96] contained ferrocene derivative as soluble mediator. The voltammograms associated to the glucose oxidase catalysis are shown in Figure 12. In the absence of substrate, the reversible electrode process of ferrocene is seen in the voltammogram but upon addition of glucose the catalysis starts. The oxidation signal increases while the cathodic peak corresponding to \(\text{Fc}^+\) reduction disappears because the whole mediator is used in the catalytic regeneration of \(\text{GO(FAD)}\).
If the mediator could be eliminated a third generation glucose biosensor would be developed in the case of which the electrons would be transferred directly from glucose active site of the enzyme to the electrode at a potential close to the redox potential of the enzyme. The absence of mediators would be the main advantage of third generation biosensors, leading to very high selectivity and low overpotential. However GOx does not undergo direct electron transfer (DET) [95].

2.6.2 Enzymatic biofuel cells

Fuel cells convert the energy of a chemical reaction directly into electrical power with very high efficiency. All the fuel cells have a similar configuration. They consist of two semicells separated by ion selective membrane which keeps them in two different electrochemical environments. Opposite reactions occur in the two compartments. The oxidation of the fuel takes place at the anodic electrode while reduction of dioxygen happens to the cathodic one. The two electrodes are connected externally through a circuit.
with an electron flux from anode to cathode. The purposes of using the ion-selective barrier are several: it separates the cathode from the anode, it does not allow mixing of the fuel with the oxidant, and it controls the flow of positive ions. When biological materials are used, such cells become biofuel cells and the fuel is then called a substrate. In biofuel cells that use micro-organisms as catalytic elements, carbohydrates are used as substrates and the two chambers are separated by a membrane (a PEM-Proton Exchange Membrane-like Nafion, for example [121,122]). Because of big availability of carbohydrates in nature, they are generally used in wastewater treatment [123–125]. In contrast to microorganisms, using enzymes as biological materials simplifies the design of the cells themselves. Because of the high specificity and selectivity of their catalytic properties, there is generally no need of membranes because there will not be side-reactions and in this case the cells consist only of one compartment where electrodes are plunged in. Similar to fuel cells, the anodic enzyme (an oxidoreductase) will oxidase the substrate (natural as in microbial fuel cells), the electrons will flow to the cathode where another oxidoreductase will catalyse the reduction of dioxygen which will be reduced to water. Redox mediators can also be involved in the enzymatic process even though direct electron transfer (DET) reactions are preferred. Mediators can cause thermodynamic loss and they could be harmful for compound. Biofuel cells are very attractive but unfortunately the power output needs to be increased to employ them on a macroscale and not only on microscale.

Bilirubin oxidase (BOD) has been widely used as a cathodic enzyme in the studies of biofuel cells because it can catalyse the reduction of O2 under mild conditions (room temperature, neutral pH and ambient pressure). A combination of gold nanoparticles (AuNPs) on carbon paper and bilirubin oxidase from Myrothecium verrucaria (MvBOD) as cathode and fructose dehydrogenase (FDH) from Gluconobacter sp. as anode in a fructose/O2 biofuel cell allowed to achieve 2.6 mA·cm−2 and a maximum power density of 0.66 mW·cm−2 at 360 mV of the cell voltage in quiescent solution [126]. By designing a glucose/O2 biofuel cell (BFC) made by carbon fibres working in human blood and consisting of glucose dehydrogenase from Acinotebacter calcoaceticus (GDH) and bilirubin oxidase from Magnaporthe oryzae, Mano and his co-workers [127] were able to reach a maximum power density of 129 mW·cm−2 at 380 mV vs. Ag/AgCl at 8.22 mM glucose.
3 Methods

The techniques employed to investigate the electrochemical behaviour of the studied enzymes were mainly electrochemical techniques. Properties such as redox potentials (oxidation or reduction) or electron transfer constants and reaction mechanisms are easily obtained through electroanalytical methods. Electrochemical processes generally require three-electrode systems and one electrolyte solution. One of the electrode, the working electrode - WE, responds directly to the analysed target and the output potential is measured against a second electrode, the reference - RE, which has a fixed potential kept constant by the third electrode, the counter electrode – CE or AUX, thanks to its large surface area. The potentiostat has control over the voltage across the working/counter electrodes pair and it adjusts this voltage to maintain the potential difference between the working and the reference electrodes. More about the electrochemical methods used in this work - voltammetries and biofuel cell measurements - will be described in the next sections.

Liquid crystalline lipidic cubic phases were investigated by Small Angle X-ray Scattering (SAXS). X-rays are generally used to explore the structural properties of the matter (solids, liquids or gels-like). Photons interact with electrons, and provide information about the fluctuations of electronic densities in the matter. Diffraction of X-rays by crystalline materials (X-ray crystallography) provides crystal structure identification, orientation of atomic planes in materials, and other physical information about the samples.

3.1 Cyclic Voltammetry (CV)

In voltammetric techniques the potential is varied linearly with time and the resulting flow of current is detected. A single experiment involves sweeping the potential with time and recording a plot of current (i) - or current density (j) - versus potential (E). Because of the linear relationship between potential and time (t) the E axis could be equally labelled as t therefore the potential scan rate determines the timescale of the experiment.
Cyclic voltammetry (CV) of proteins, either in solution or immobilised on electrode surface, has scan rates, $\nu$, ranging from $1 \text{ mV} \cdot \text{s}^{-1}$ to $1000 \text{ mV} \cdot \text{s}^{-1}$. A typical voltage ramp and its response curve are shown in Figure 13. The scan begins at a low potential far away from the oxidation and only nonfaradaic currents are flowing. The nonfaradaic current is present in the whole process (Figure 13 on the right – horizontal dashed lines) and it is attributed to the electrochemical double layer (redistribution of charges at the electrode surface). Scanning toward positive direction, once the potential reaches the vicinity of the potential of oxidation ($E_{\text{pox}}$), the oxidation begins and the current, due to the transferred charges in the oxidation, starts to flow. As the potential continues to grow more positive, the current increases until a maximum current, corresponding to $E_{\text{pox}}$, where the mass transfer reaches its maximum and all the molecules in front of the electrode are oxidised and the diffusion layer has sufficiently moved away from the electrode so that the flux of reactant to the electrode is not fast enough. Further increase of the potential, leads to a decrease of current. If the scan is reversed, going from positive potentials to negative ones, all the oxidised species will start to be reduced back when the potential gets to the vicinity of $E_{\text{pre}}$ (the reduction potential).

The equations that govern the electrochemistry of cyclic voltammetry are deeply described in Faulkner and Bard book [129]. Using the boundary conditions, for a planar-diffusion-controlled reversible process at temperature of 298 K where only the specie oxidised O is present in solution, the peak current is described by Randles-Ševčik equation:

Figure 13 Enzymatic reaction cyclic voltammetry. (left) CV potential ramp and (right) resulting $i-E$ curve. Lactose acts as enzyme substrate [128]
\[ i_p = 2.69 \cdot 10^5 n^{3/2} AD^{1/2} C_o v^{1/2} \quad (8) \]

Where \( i_p \) is the peak current (in A), \( n \) is number of electrons exchanged, \( A \) is the area of the electrode in cm\(^2\), \( D \) is the diffusion coefficient in cm\(^2\)·s\(^{-1}\), \( C_o \) is the initial concentration of the oxidized specie in mol·cm\(^{-3}\) and \( v \) is the scan rate in V·s\(^{-1}\).

The diffusion coefficient \( D \) can be easily extrapolated from the slope of a line passing through the origin obtained by plotting the peak currents versus the square root of the scan rates.

If the oxidised specie is adsorbed on the electrode surface, the peak current \((i_p)\) is given by:

\[ i_p = \frac{n^2F^2}{4RT} vA\Gamma \quad (9) \]

Therefore from the Faraday Law:

\[ Q = nFA\Gamma \quad (10) \]

Where \( F = 96485 \text{ C·mol}^{-1} \) is the Faraday constant, \( R = 8.3145 \text{ J·mol}^{-1}·\text{K}^{-1} \) is the ideal gas constant, \( T \) (K) is temperature, and \( \Gamma \) (mol·cm\(^{-2}\)) is the surface concentration of the electroactive component and \( Q \) (C) is the charge under the peak.

The peak potential \((E_p)\) is defined:

\[ E_p = E_{\frac{1}{2}} - 1.109 \frac{RT}{nF} = 28.5/n \text{ mV at } 25^\circ\text{C} \quad (11) \]

Where \( E_{\frac{1}{2}} \) is defined the midpoint potential \( E_{\frac{1}{2}} = E^{\circ'} + \frac{RT}{nF} \ln \left( \frac{D_R}{D_O} \right)^{1/2} \) with \( D_R \) and \( D_O \) are the diffusion coefficients of the reduced and oxidised species, respectively while \( E^{\circ'} \) is the formal potential.

In enzymatic catalysis, the redox molecule, involved in the electron transfer reaction (E), is followed by a homogeneous chemical reaction (C), where the reduced species (R) reacts with a species (Z) in solution to regenerate the oxidised specie (O) in a catalytic \((\text{EC'})\) reaction. Assuming cathodic reduction, the processes can be formulated as follows:

Scheme 3

- a) \( O + \text{ne} \rightleftharpoons R \)
- b) \( R + Z \xrightleftharpoons[k'] O + Y \)
The species Z is considered in large excess respect to O \( (C_Z^*>C_O^*) \) thus its concentration is unchanged during the experiments and diffusion of Z is not limiting the process. \( C_Z^* \) and \( C_O^* \) are the bulk concentrations of Z and O respectively. The reaction is proceeding as pseudo-first order and the dimensionless rate constant \( (\lambda) \) is defined in equation (12):

\[
\lambda = \frac{k'C_Z^*}{v (RT/F)}
\]  

(12)

Where \( k' \) is the rate constant of the transformation of R into O, \( v \) is the scan rate \( (V \cdot s^{-1}) \), \( F=96485 \text{ C} \cdot \text{mol}^{-1} \) is the Faraday constant, \( R=8.3145 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1} \) is the ideal gas constant and \( T \) (K) is the temperature.

At sufficiently negative potentials, it is assumed in a reduction process that the concentration of O \( (C_o) \) at the electrode surface \( (x=0) \) is zero and the voltammogram tends to a limiting current \( i_\infty \) where the catalytic reaction does not take place.

\[
i_\infty = nFAC_O^*(Dk'C_Z^*)^{1/2}
\]  

(13)

When \( \lambda \) become larger (large rate constant \( k' \) or small scan rate \( v \)), the limiting current enters the kinetics process region, where the rate of removal of O in Scheme 3-a is equal to the rate of production of O in Scheme 3-b and \( C_0 \) is therefore independent from the scan rate.

The \( i-E \) curve loses its peak shaped and becomes a wave.

\[
i = \frac{nFAC_O^*(Dk'C_Z^*)^{1/2}}{1 + e^{F(E-E_{\text{f}})}}
\]  

(14)

### 3.2 Differential Pulse Voltammetry (DPV)

When the nonfaradaic current on the background is too high and the faradaic current too small the peaks are not well developed and more sensitive techniques are used. Pulse voltammetries are based on the idea of applying one pulse of potential to the electrode and measuring the currents at the end of each pulse. The considerable improvement in the ratio of charging/faradaic currents compared to normal CV is a big advantage in this case. The reason is because faradaic current usually decreases with \( 1/\sqrt{t} \) which is slower that the charging current. The way the potential pulses are imposed on the electrode defines the technique. Differential pulse voltammetry (DPV) imposes increasing small-amplitude potential pulses to the electrode and the base potential is changed steadily in small increments. The corresponding voltammogram is a plot of the current difference, \( \delta(i) = i_{\text{peak}} - i_{\text{base}} \) as a function of potential.
i(τ) – i(τ) versus the base potential. The current is then sampled twice: before and after the pulse (filled circles in Figure 14). No faradaic current flows during the time when the potential is far away from the formal potential $E^{0'}$, thus $i(\tau) – i(\tau')$ is zero; only in the region of $E^{0'}$ there is a faradaic current difference that can be observed. DPV is used to detect analytes present in the solution at a concentration as low as 0.05 µM. Another advantage of this technique is that the voltammograms are peak-shaped.

Figure 14 Potential program for DPV. Image taken from [130]

3.3 Fuel cell characterization and determination of parameters

A fuel cell is a device that converts chemical energy into electrical energy. It involves redox reactions at the anode and the cathode – an oxidation and a reduction, respectively. The overall reaction must be spontaneous so the cathode is the positive electrode while the anode is the negative one. The anode is responsible for the oxidation of the fuel and the cathode is involved in the reduction of the oxidants. There are several parameters that allow to characterize and compare the efficiency of fuel cells. The performance of a fuel cell is determined by the cell voltage ($V_{cell}$) and the current (i) which define the power output according to the following equation:

$$P (W) = iV_{cell}$$  \hspace{1cm} (15)
The power output of a biofuel cell is limited by the electrochemical reactions occurring at the two electrodes.

The cell voltage depends on the differences of the equilibrium potential of anode and cathode, on their overpotentials ($\eta_a$ and $\eta_c$, respectively) and on the IR drop, according to the following equation:

$$V_{cell} = \Delta E - \eta_a - \eta_c - IR$$  \hspace{1cm} (16)

Overpotentials and IR are considered as potential inefficiency that lowers the electrical energy resulting in heat. Therefore the efficiency of a fuel cells in practise is lower than the ideal because of the system loses voltage.

The open circuit voltage (OCV) is the maximum voltage associated with the fuel cell and it theoretically described by the Nerst equation. It is determined by the onset potential at the respective electrodes.

$$E = E^o - \frac{RT}{nF} \ln Q$$  \hspace{1cm} (17)

Where Q is the activity quotient, E is the cell standard potential, n is the number of electrodes involved in the reaction, R,T,F are respectively the universal gas constant, the absolute temperature and the Faraday constant.

The magnitude of the current is defined by the rate of the reaction at the electrode and it is limited by the electrode with the smallest current density.

Generally the performance of a fuel cell is determined in term of power output, current and voltage; and for these three graph can be obtained (Figure 15)

![Figure 15](image)

Figure 15 a) Current *versus* applied potential; b) Polarization curve: voltage *versus* current density; c) power density *versus* voltage. Image taken from [131]
In general low scan rate linear voltammetry (to obtain a quasi-steady state current) can be used to build the curve in Figure 15-a. Another way is to connect the two electrodes to a reference electrode to monitor each semicell while the BFC is working. From this curve is possible to identify the OCV and which electrodes limits the electrochemical reaction. Figure 15-b represents the polarization curve. The initial voltage is lower than the ideal voltage because of the thermodynamics losses. The rapid fall in voltage at high current values resulted in kinetics losses (slow reaction rates at the electrode surface). Ohmic losses are caused by the resistance of the material to the flow of electrons and the mass transport losses are due to the change in concentration of reactants at the electrode surface. The maximum power (Figure 15-c) depends on the rate of the catalysis and can be improved with appropriate immobilization methods and efficient mass transport.

In the enzymatic biofuel cells, enzymes are used as biological catalysts and the transfer to the electrodes can appear under conditions of direct and mediated electron transfer, as previously discussed in paragraph 2.5. Mediated electron transfer biofuel cells show in general much higher current density. On the other hand, mediators are generally toxic compounds and their use in implantable devices is not desirable. The advantage of direct electron transfer biofuel cells is that there is no need of any membrane which separates the two compartments and therefore they are easy to miniaturize and implant. Enzymatic biofuel cells are biocompatible, they have higher activity under mild conditions and specific selectivity.

3.4 Small-angle X-ray scattering (SAXS) for cubic phase characterisation

Small angle X-ray scattering (SAXS) is a great method for studying the structure of biological molecules in solution. The method provides information about sizes, shapes and probably configuration of both ordered and disordered proteins in solution. SAXS experiments involve a quartz capillary filled with the compound of interest and illuminated by a collimated monochromatic X-ray beam; the intensity of the scattered X-rays is recorded by an X-ray detector [132]. The resulting scattering pattern is related to the overall shape and size of the particles under investigation. On the basis of the experimental SAXS diffraction patterns, it is possible to differentiate between the various phases according to the relative position of the first few SAXS Bragg peaks, certain space groups within the families and on the presence (or the absence) of certain peaks [133]. From the SAXS plot,
intensity (arbitrary unit, a.u.) \textit{versus} the length of the scattering vector $q$ (nm$^{-1}$), the cubic phase parameters can be evaluated following the procedure described in Kulkarni paper [59].

The lattice parameter ($a$) can be calculated with the use of following equation:

$$a = \frac{2\pi}{q_0} \sqrt{h^2 + k^2 + l^2} \quad (18)$$

Where $q_0$ is the first scattering vector and $h, k, l$ are Miller indices of the Bragg peaks. The cell lattice parameter in cubic structures can be related to the molecular characteristics of the surfactant.

To determine the aqueous channel radius ($r_w$), the water volume fraction ($\varphi_w$) was first estimated using the following equation:

$$\varphi_w = \frac{C_w}{C_w + (1 - C_w) \frac{\rho_w}{\rho_l}} \quad (19)$$

Where $\varphi_w$ is the water volume fraction, $C_w$ is the water weight fraction, $\rho_w$ is the density of water = 0.997 g cm$^{-1}$, and $\rho_l$ is the density of lipid ($\rho_{MO} = 0.942$ g ml$^{-1}$).

Next, the lipid volume fraction ($\varphi_l$) was determined from equation:

$$\varphi_l = 1 - \varphi_w \quad (20)$$

Lipid length ($\ell$) was obtained by solving equation:

$$\varphi_l = 2\sigma \ell a + \frac{4}{3} \pi \chi \left(\frac{\ell}{a}\right)^3 \quad (21)$$

Where $\ell$ is the lipid chain length/monolayer thickness, $a$ is the lattice parameter of the corresponding phase, $\sigma$ is the Euler–Poincaré characteristic, and $\chi$ is the ratio of the minimal surface in a unit cell to the quantity (unit cell volume)$^{2/3}$. For Pn3m phase $\sigma=1.919$ and $\chi=-2$; for Ia3d phase $\sigma=3.091$ and $\chi=-8$.

The aqueous channel radius ($r_w$) can be obtained from the equation:

$$r_w = \left(\frac{\sigma}{2\pi \chi}\right)^{\frac{1}{2}} a - \ell \quad (22)$$

Putting values of $\sigma$ and $\chi$ for corresponding cubic phases, equation (22) becomes:

For Pn3m phase, $r_w = 0.391 \ a - \ell \quad (23)$

For Ia3d phase, $r_w = 0.248 \ a - \ell \quad (24)$
“I think it is a sad reflection on our civilisation that while we can and do measure the temperature in the atmosphere of Venus, we do not know what goes on inside our soufflés”

Nicholas Kurti
PART II - EXPERIMENTAL

4 Experimental section

4.1 Chemicals and Materials

- 2,6-dichlorophenolindophenol (DCPIP): Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- 3-morpholinopropane-1-sulfonic acid (MOPS): Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- Ag/AgCl (KClsat) reference electrode: Eurosensor, Gliwice, Poland
- Bilirubin oxidase: Novozymes, Bagsværd, Denmark
- Bilirubin oxidase: Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- Bis-Tris buffer: Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- CaCl₂: POCh - Polish Chemicals Co., Gliwice, Poland
- Citric acid: Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- Fructose Dehydrogenase: Sorachim, Lausanne, Switzerland
- Glassy Carbon Electrode (GCE)- 3.0 mm diameter: BASi, West Lafayette, USA
- Graphene solution: DRP-GPHSOL Dropsens, Llanera (Asturias), Spain
- Hexaammineruthenium(II) chloride [Ru(NH₃)Cl₂]: Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- HOPG : Ted Pella, Inc, Redding, CA
- Horse-heart cytochrome c (cyt c): Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- Lactose: POCh - Polish Chemicals Co., Gliwice, Poland
- MgCl₂: POCh Polish Chemicals Co., Gliwice, Poland
- Monoolein (1-Oleoyl-rac-glycerol): Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- Monoolein: Hampton Research, Aliso Viejo, USA
- Multi-walled carbon nanotubes MWCNTs (NC3100): Nanocyl™ (purity >95%), Sambreville, Belgium
- Na₂HPO₄: Sigma, © 2018 Merck KGaA, Darmstadt, Germany
- NaCl: POCh - Polish Chemicals Co., Gliwice, Poland
Experimental

- NaH$_2$PO$_4$: POCh - Polish Chemicals Co., Gliwice, Poland
- SPEs: Dropsens, Llanera (Asturias), Spain
- Zn plate: GoodFellow, Huntingdon, England

All solutions were prepared using Milli Q Water (18.2 MΩ cm$^{-1}$), Millipore, Bedford, MA, USA. All used chemicals were of analytical grade.

4.2 Instrumentations and experimental set up

A three electrodes cell was used in all experiments with a Ag/AgCl (KCl$_{sat}$) as reference electrode, a platinum sheet as a counter electrode and a glassy carbon electrode (GCE) from BASi (3 mm diameter), modified with MWCNTs as working electrode. Cyclic and differential pulse voltammetry (CV and DPV, respectively) experiments were performed using an Autolab potentiostat (Metrohm Autolab, B.V., Utrecht, Netherlands).

The type of cubic phase and its structural parameters were evaluated based on small angle X-ray scattering (SAXS) measurements done using Bruker Nanostar system working with CuKα radiation, equipped with Vantec 2000 area detector. Samples were loaded into 1.5 mm capillaries and left to equilibrate for 12h. Before measurement, the samples were equilibrated for 20 min at appropriate temperature. Data were then collected for 30 min.

The equipment used during this thesis to measure the efficiency of a BFC is shown in Figure 16. The BFC was tested in a three-electrode arrangement: anode, cathode and a reference electrode - Ag/AgCl (KCl$_{sat}$). The system consisted of a cell connected to a digital multimeter (Picotest Phoenix, AZ) associated with a resistor (Warsaw University, Warsaw, Poland). Variable loads, in the range from 1 kΩ to 10 MΩ, were applied between the anode and the cathode to determine the power (P), the cell voltage ($V_{cell}$), the voltage between anode and reference electrode ($V_a$), and the voltage between cathode and reference electrode ($V_c$). The cell was eventually connected to a peristaltic pump (Aqua-Trend, Łódź, Poland) when working in the flow mode.
4.3 Electrodes and modification schemes

For the modification of the GCEs with nanotubes, a suspension of nanotubes in ethanol (0.7 mg · mL\(^{-1}\)\(_{\text{EtOH}}\)) was sonicated for 30 min, then 10 µL (0.007 mg) of the solution was deposited on the GCE surface by drop-casting. Nanotubes used in this project were pristine multi-walled carbon nanotubes (referred as MWCNTs), pristine single-walled carbon nanotubes (SWCNTs) and Naphthyl MWCNTs (referred as NaphtMWCNTs). When the electrodes got dry, they were covered with a film of a cubic phase (approx. 10 mg) and immediately immersed in the buffer solution, to prevent any change of phase, or 4 µL of the corresponding enzyme solution (ca. 10 mg·mL\(^{-1}\)) was drop casted onto the nanotubes modified electrode surface. When the cubic phase is present on the electrode surface, it will be referred to as ‘LCP’, and when the enzyme alone is adsorbed at the electrode, it will be referred as ‘ads’.

- Preparation of Zn anode

A Zn plate was used for the MvBOD based biobattery. The Zn plate was strongly rinsed with MilliQ water and EtOH to remove all the impurities. The surface was connected through a crocodile clip to the system without any further modification. A hopeite layer was formed during the Zn electrode oxidation.

Figure 16 Schematic representation of the fuel cell circuit system.
Preparation of CtCDH anode

Cellobiose dehydrogenase from *Corynascus thermophilus* was recombinantly expressed in *Trichoderma reesei*, chromatographically purified [134] and used without any further processing. The protein concentration was 10 mg·mL\(^{-1}\) according to the Bradford protein assay using bovine serum albumin as standard, and the specific activity of 6.2 U·mg\(^{-1}\) was measured with the cyt c assay.

Preparation of FDH anode

Fructose dehydrogenase from *Gluconobacter* sp. was bought from Sorachim SA and used without any further processing. A solution of 10 mg·mL\(^{-1}\) was prepared for further use in the electrode modification.

Preparation of MvBOd cathode

Bilirubin oxidase from *Myrothecium verrucaria* was initially purchased from Sigma with an activity of 25 U·mg\(^{-1}\) (one unit is defined to oxidize 1.0 μmol of bilirubin per min at pH 8.4 at 37°C). The lyophilized powder was dissolved in 10 mM PBS buffer (pH 7) to concentration of 10 mg·mL\(^{-1}\), aliquoted and stored at -20°C.

In the second part of this thesis project bilirubin oxidase, from *Myrothecium verrucaria*, was provided by Novozymes (Bagsværd, Denmark). The enzyme concentration was 3.61 mg·mL\(^{-1}\) (60.2 μL) in 20 mM Tris buffer, 100 mM Na₂SO₄ (pH 8). The enzyme solution was aliquoted, stored frozen at -20°C and used as received without any further processing.

4.3.1 Preparation of LCP and incorporation of enzymes

The cubic phase was prepared by melting monoolein at 45°C in a glass vial in a water bath and within few minutes an appropriate amount of buffer or enzyme solution was added to the molten lipid. The ratio was chosen according to the phase diagram for the monoolein–water system for the formation of a diamond type cubic phase (Pn3m) [86]. At room temperature of 25°C, the system consisted of monoolein/buffer or monoolein/enzyme solution in a ratio of 60%:40%. The glass vial was tightly sealed with Parafilm and left to equilibrate in a water bath at 25 °C for 24 h. The formation of a transparent and highly
viscous cubic phase was confirmed by macroscopic observation of the sample and by SAXS measurements [59].

Sometimes, mediators were added to the mesophase in the form of a freshly prepared solution, mixed with the enzyme solution to make a cubic phase containing the 60 % of lipid.

In Table 3 a summary of the ratios of lipidic to aqueous parts in different cubic phases prepared in this project is shown.

<table>
<thead>
<tr>
<th>LCP name</th>
<th>lipidic part</th>
<th>aqueous part</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;empty&quot;</td>
<td>60% MO</td>
<td>40% buffer a,b,c</td>
</tr>
<tr>
<td>MvBOD</td>
<td>60% MO</td>
<td>40 % MvBOD (10 mg/mL) a</td>
</tr>
<tr>
<td>MvBOD + AuNPS</td>
<td>60% MO + AuNPS</td>
<td>40 % MvBOD (10 mg/mL) a</td>
</tr>
<tr>
<td>CtCDH</td>
<td>60% MO</td>
<td>40 % CtCDH (10 mg/mL) b</td>
</tr>
<tr>
<td>CtCDH + DCPIP</td>
<td>60% MO</td>
<td>24 % CtCDH (10 mg/mL) + 16 % DCPIP (18 mM) b</td>
</tr>
<tr>
<td>cyt c</td>
<td>60% MO</td>
<td>40 % cyt c (10 mg/mL) b</td>
</tr>
<tr>
<td>DHCtCDH</td>
<td>60% MO</td>
<td>40 % DHCtCDH (9 mg/mL) b</td>
</tr>
<tr>
<td>DHCtCDH + cyt c</td>
<td>60% MO</td>
<td>20 % DHCtCDH (19 mg/mL) + 20 % cyt c (20 mg/mL) b</td>
</tr>
<tr>
<td>FDH</td>
<td>60% MO</td>
<td>40 % FDH (9-18 mg/mL) c</td>
</tr>
</tbody>
</table>

a) 0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl₂; b) 0.05 M MOPS pH 7.5 with 0.1 M NaCl and 0.03 M CaCl₂; c) 0.05 M Bis-Tris pH 5.8.

4.3.2 LCP characterization: preparation for SAXS experiments

The X-ray diffraction measurements were done to identify the structure of liquid crystalline phase after doping it with enzyme, mediators or nanoparticles. Data were collected for samples prepared in buffer with and without enzymes. According to the literature, PBS should not have any influence on the mesophase structure compared with that obtained with pure water [80] but cations are known to bridge between the monoolein head groups inducing to an intensification in the lattice parameter leading to a more ‘open’ phase [74]. After cubic phase preparation, glass capillaries were filled with ca. 20 mg of corresponding phase, left to equilibrate for 24 h and measured at 25°C.
4.3.3 Synthesis of gold nanoparticles [135]

Gold nanoparticles (AuNPs) were prepared in collaboration with dr. Asia Pawłowska, Warsaw University, using a lightly modified Brust-Schiffrin method [136]. A tetraoctylammonium bromide (80 mL, 2.19 g, 5 mmol) solution in toluene was used to extract hydrogen tetrachloroaurate (III) in aqueous solution (30 mL, 30 mM). The tetrachloroaurate ions were transferred to the organic layer bleaching the yellow aqueous layer to colourless. 1.44 μL of 1-octanethiol was added to the organic phase. Then, solution of borohydride (378 mg, 200 mmol in 25 mL of deionized water), freshly prepared, was very slowly added under energetic stirring to reduce the organic mixture. The solution was left under vigorous stirring for 3 h and successively the organic phase was separated, evaporated to 10 mL in a rotary evaporator, and mixed with 400 mL of absolute ethanol to precipitate the NPs. The mixture was then kept for 12 h at -4°C until precipitation of dark brown solid. The mixture was sonicated for 60 s and centrifuged (5 min, 13 000 rpm), the pellets were dissolved again in a small amount of toluene (10 mL), precipitated again with ethanol (400 mL) and centrifuged for four times. Finally, all samples were dissolved in 10 mL of toluene. The final concentration was estimated to be 5 mg/mL. The obtained nanoparticles were characterized by TEM and DLS.

- TEM measurements

The morphology and size of the obtained gold nanoparticles were determined using a Libra TEM (Carl Zeiss) operating at 120 kV. One or two droplets of the nanoparticle solutions prepared as described above were diluted with toluene to a very low concentration and placed on a standard copper grid (400-mesh) coated with carbon. The grid was left to dry overnight. TEM images of the synthesized AuNPs are shown below (Figure 17). The dispersed nanoparticles are estimated to have an average diameter of 4.7 nm.
Dynamic Light Scattering

The dynamic light scattering measurements were performed using ZetasizerNano ZS from Malvern Instruments. The size of the octanethiol@AuNPs from the DLS measurements is 5.3 nm, which is in excellent agreement with the TEM results (4.7 nm of the gold core). It is known that DLS size are always larger since not only the core but the whole nanoparticle is measured.

4.3.4 Synthesis of modified carbon nanotubes

Modified carbon nanotubes were obtained from our collaborators from professor Biernat’s group from the Faculty of Chemistry of Gdansk University of Technology, Gdansk, Poland.

- Synthesis of Naphthyl-functionalised MWCNTs (NapthMWCNTs) was already published in previous papers [46,47,137] and a summary of the synthetic procedure is described. After 15 min of sonication of a mixture of pristine MWCNTs, 40 mg, (from CheapTube, Brattleboro, USA), 2-naphthylamine hydrochloride (936 mg), pyridine (0.45 mL), 1,2-dichlorobenzene (5 mL), acetonitrile (5 mL), and isopentyl nitrite (0.72 mL) were added. The mixture was further sonicated at 65°C for 6 h. The solid was then collected by centrifugation, washed in sequence with dimethylformamide (DMF), methanol (MeOH), and chloroform (CHCl₃) until the
chemicals used for washing became colourless. Each washing was accompanied by sonication. Finally, the product was dried in vacuum. The product contained not only naphthyl residues bonded to nanotubes, but also some side products of further naphthylation.

![Figure 18 Synthesis of NaphtMWCNTs](image)

- The procedure for the synthesis of 2,6-dichlorophenolindophenol (DCPIP) modified carbon nanotubes is still unpublished but a brief abstract is provided below. The synthesis was modelled on the basis of few papers [138–142] and the key idea was to make the dichlorophenolindophenol (DCPIP) more reactive using ascorbic acid as reducing agent (reagent 1). Pristine SWCNTs (from CheapTube, Brattleboro, USA) were alkylated to N,N-(dihydroxymethyl) aminoethyl modified nanotubes in two steps process using chlorobenzene and acetonitrile as solvents. The first step generated the aminoethyl-modified nanotube (using ethylenediamine and isopentyl nitrite); secondly formaldehyde and acetic acid were used to alkilate the nanotubes in a kind of one-pot Mannich Base synthesis forming the final product (reagent 2). During mixing of the two reagents the pH was carefully maintained at 10 and the solution was oxidised with NaOH and iodine.

The final product is suggested to be the 2,6-dichloro-4-((2-((ethyl(hydroxymethyl)amino)methyl)-4-hydroxyphenyl)imino)cyclohexa-2,5-dienone-modified nanotube – referred as DCPIP-SWCNTs.
Figure 19 Scheme of the procedure for the synthesis of DCPIP-SWCNTs
5 Results and Discussion

5.1 Electrochemistry of adsorbed enzymes

First, the enzymes activities were checked measuring the electrochemical behaviour of the protein adsorbed on the modified electrode surface through drop casting technique.

5.1.1 Corynascus Thermophilus cellubiose dehydrogenase (CtCDH)

At a first approach to the cellubiose dehydrogenase enzyme, Highly Oriented Pyrolytic Graphite (HOPG) was used, because of its unique structure. HOPG electrodes were prepared by prof. J. Golimowski from Warsaw University starting from a HOPG plate (Ted Pella, Inc - Redding, Ca) with ZYB grade and surface area of 0.54 cm$^2$. The electrodes were cleaned by pressing a piece of ScotchTM tape on the surface of HOPG and then carefully lifting the tape to peel away graphene sheets. The enzyme solution (10 µL of a solution containing enzyme in concentration equal to 10 mg·mL$^{-1}$) was drop casted onto the electrode surface previously peeled and once dried it was immersed in 10 mM Phosphate buffer, pH 7.4, 10 mM NaCl and 5 mM MgCl$_2$. The enzyme catalysis was measured using 100 mM lactose solution in buffer as the substrate. According to the literature 2,6-dichlorophenolindophenol (DCPIP) acts as redox mediator for the flavin domain of CDHs, accepting two electrons from the fully reduced FAD (2e$^-$ EA) [11,16,143]. Midpoint potential $E^{0'}$ for DCPIP is 0.071 V vs. Ag/AgCl (KCl$_{sat}$) (Figure 20).
Experimental

Figure 20 CV of HOPG electrode immersed in plain buffer (black line) and 40 µM DCPIP buffer solution (dashed black line). Electrolyte 10 mM Phosphate buffer pH 7.4, 10 mM NaCl and 5 mM MgCl₂. Scan rate 50 mV·s⁻¹.

When DCPIP is present in solution, the catalytic current produced by the enzyme receives a boost in activity thanks to the 2e⁻·EA mediating the process (Figure 21).

When glassy carbon electrodes were used, a layer of MWCNTs was needed to increase the conductivity because of the smoothness of the plain GC electrode. A solution of pristine MWCNTs was used to modify the GCE surface for the purpose of nanostructuring its surface. A suspension of MWCNT in ethanol (0.7 mg·mL⁻¹ ethanol) was sonicated for 30 min, then 10 µL (0.007 mg) of the solution were deposited on the GCE surface. Once the electrodes dried, 0.04 mg of CdCDH (4 µL of 10 mg·mL⁻¹ solution) was casted on their surface. Deposition of nanotubes, in this case MWCNTs, on the GCE surface increases the electroactive area of the electrode improving the contact between the enzyme and the electrode surface [100].
Figure 21 CV of HOPG electrode modified with 0.1 mg of CdCDH in buffer (black line), in 0.1 M lactose (grey line) and with addition of 40 µM of DCPIP (dashed black line). Electrolyte 10 mM Phosphate buffer pH 7.4, 10 mM NaCl and 5 mM MgCl₂. Scan rate 1 mV·s⁻¹

For a better determination of the potentials corresponding to the electrochemical processes of the enzyme domains, differential pulse voltammetry (DPV) experiment was done in buffer at different pHs in absence of any enzyme substrate. Figure 22 allows the comparison of DPVs recorded for the electrode covered with MWCNTs and 0.04 mg of CdCDH drop casted on the electrode and studied in solutions containing 50 mM MOPS at pH 7.5, 100 mM NaCl and 30 mM MgCl₂ or 50 mM NaAc pH 5.5 with 100 mM NaCl and 30 mM CaCl₂. The FAD electrode process is pH dependent, therefore the signal moves towards more negative potentials as the pH increases. For 2e⁻ and 2H⁺ involved in the reaction the voltammetric peak is expected to shift 59 mV/pH unit. In each curve, in the DPV, there are two peaks corresponding to the two prosthetic groups of the enzyme. The CYT domain can be observed electrochemically at ca. -0.100 V, a value that is in good agreement with the literature [144,145]. At pH 7.5, the peak appearing at -0.460 V vs. Ag/AgCl (KCl sat.) corresponds to free FAD; as a matter of fact, when 6.5 µM free FAD is dissolved in solution, the intensity of the peak increases. The same behaviour is observed when buffer of pH 5.5 is used; the peak corresponding to free FAD released by the enzyme appears at -0.340 V vs. Ag/AgCl (KCl sat.), at the same potential of free FAD dissolved in solution (6.5 µM and 13 µM). FAD in CDH is strongly but not covalently bound and may diffuse out of
Experimental

the enzyme molecules and interact with the electrode surface [146]. As it was previously published for glucose oxidase [95], the electrochemistry of enzyme-bound FAD is not detectable through electrochemical measurements because the distance from the enzyme surface to its active site is larger than 20 Å. However, most of CtCDH molecules remain intact as it can be seen in the time-stability measurement of the catalyst, shown later in this thesis (section 5.3.2). Finally, at pH 7.5 the peak potentials are equal to -0.464 ± 0.002 V for the free FAD, and -0.115 ± 0.002 V for bound haem b in the CYT domain of the protein.

Figure 22 DPV of GCE/MWCNT/CtCDH ads in a) 50 mM NaAc pH 5.5 with 100 mM NaCl and 30 mM CaCl₂, b) same buffer with further addition of 6.5 μM of free FAD; c) 50 mM MOPS pH 7.5 with 100 mM NaCl and 30 mM CaCl₂ and with further addition of d) 6.5 μM and e) 13 μM of free FAD. Conditions: step potential = 0.006 V, modulation amplitude = 0.025 V.

Cyclic voltammograms were also recorded for the MWCNTs GCE covered with 0.04 mg of drop casted CtCDH in the absence and presence of 100 mM lactose in 50 mM PBS solution, pH 7.8, containing 100 mM NaCl and 30 mM MgCl₂. If the scan rate is slow, the addition of lactose results in the formation of the catalytic wave, shown in Figure 23-a. When no substrate is added to the buffer solution, the voltammetric curve is flat and at this low scan rate, no clear faradaic processes can be resolved in the non-turnover CVs. A well-defined catalytic wave is developed at potentials close to that of the CYT domain and current continues to increase at more positive potentials. When only the dehydrogenase domain (DH) of CDH from Corynascus thermophilus recombinantly expressed in
*Escherichia coli* is used no catalytic activity was seen (Figure 23-b). The protein concentration was 10 mg·mL\(^{-1}\) according to the Bradford protein assay using bovine serum albumin as standard, and the specific activity of 14.1 U·mg\(^{-1}\) was measured with the cyt \(c\) assay at pH 5.5. The GCEs used for the experiment with DH domain were prepared the same way as for the complete enzyme.

Figure 23 Cyclic voltammograms for a) 0.04 mg of CtCDH adsorbed on GCE covered with MWCNTs (0.007 mg) and b) 0.04 mg of DHcCDH adsorbed on GCE covered with MWCNTs (0.007 mg) in the buffer (black lines) and in presence of 100 mM lactose. Electrolyte: 50 mM PBS solution pH 7.8, containing 100 mM NaCl and 30 mM MgCl\(_2\). Scan rate 1 mV/s
5.1.2 *Myrothecium verrucaria* bilirubin oxidase (*MvBOD*)

*MvBOD* is a blue multicopper oxidase with four redox active Cu atoms. The Cu atoms are divided into 3 types: type 1 (T1), type 2 (T2) and type 3 (T3) according to their spectroscopic signatures. T2 and T3 form a trinuclear cluster which is responsible for the water formation. T1 is the Cu atom which accepts electrons from the substrate, or from the electrode, and passes them on to the trinuclear centre through a Cys-2His electron transfer [29]. The midpoint potential of the T1 site is close to 670 mV vs. NHE while the T2/T3 copper sites are approximately around 400 mV vs. NHE [28].

In Figure 24, the cyclic voltammogram for *MvBOD* modified glassy carbon electrode is shown. The electrode was first covered with 10 μL of a dispersion in ethanol containing 0.007 mg of naphthyl MWCNTs (0.7 mg·mL⁻¹). In the voltammogram with PBS buffer as electrolyte (0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl₂), two redox peaks can be resolved corresponding to the enzyme active sites. The peak corresponding to the reduction of the T2/T3 sites appears at ca. 160 mV vs. Ag/AgCl (KCl sat.) (400mV vs. NHE) while the peak for the T1 site appears at ca. 430 mV vs. Ag/AgCl (KCl sat.) (670 mV vs. NHE), in good agreement with what already reported by Christenson *et al.* [28].

Figure 24 Cyclic voltammogram of *MvBOD* modified GCE in the presence of 0.007 mg of NaphthMWCNTs in 0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl₂; scan rate 50 mV·s⁻¹.
To improve the direct communication between bilirubin oxidase (MvBOD) and glassy carbon electrode surface, three different approaches were studied to find the most efficient way of nanostructurisation of the electrode surface. A suspension of pristine (approach 1) or naphthyl-functionalised (approach 2) MWCNTs in ethanol (93 µg, 140 µL of 0.7 mg·mL\(^{-1}\)\textsubscript{EtOH}) was dropped onto the glassy carbon electrode surface and left to dry. A third approach consisted of a layer by layer (LBL) modification where NaphtMWCNTs (93 µg, 140 µL of 0.7 mg·mL\(^{-1}\)\textsubscript{EtOH}) were casted onto the GCE surface alternately with C8@AuNPs, synthesised as explained earlier (20 µL, 0.1 mg). For biomodification, 10 µL of enzyme solution containing 0.1 mg MvBOD dissolved in buffer were applied onto the electrode surface in all cases. The most advantageous approach was selected based on the limiting current of catalysed dioxygen reduction in cyclic voltammetry experiments under aerobic and anaerobic (Ar purged) conditions in PBS buffer at pH 7 with scan rate of 1 mV·s\(^{-1}\). Dioxygen reduction starts at ca. 500 mV vs. Ag/AgCl (KCl\textsubscript{sat.}) what is close to the potential of T1 site of MvBOD (Figure 25). Since no mediators have been added, this indicates the DET mechanism is operative and electrons are transferred from the electrode via CNTs to the enzyme and from there to oxygen. Naphthylation of CNTs increased the catalytic current while their combination with nanoparticles doubled it. Naphthyl-substituted MWCNTs always larger catalytic current densities than unmodified MWCNTs. Similar observations were already reported earlier for dioxygen reduction catalysed by laccase Cerrena unicolor [137]. The combination of NaphtMWCNTs and C8@AuNPs in a network was best for adsorbing the enzyme and resulted in the increased value of the biocatalytic dioxygen reduction current.
Figure 25 Comparison of catalytic current densities obtained in deoxygenated (a - black line) and oxygenated 0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl₂ recorded with GCE modified with 93 µg of MWCNTs (b - blue line) and 93 µg NaphtMWCNTs in absence (c - green line) or presence of 0.1 mg of C8@AuNPS (d – red line) in the presence of 0.1 mg of MvBOD.

The optimal amount of MvBOD was selected for the electrode surface modified with 140 µL (93 µg, 0.7 mg·mL⁻¹ EtOH) of NaphtMWCNTs by increasing the amount of bilirubin oxidase from 0.033 to 0.500 mg (from 10 mg·mL⁻¹ enzyme solution). For amounts of MvBOD higher than 0.15 mg the limiting current density of the catalytic dioxygen reduction was achieved. Further increase of enzyme amount at the electrode only slightly increased the slope of the waves without changing the height of the wave.
Figure 26 Cyclic voltammograms recorded for \( MvBOD \) catalysed dioxygen reduction in 0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl\(_2\), and scan rate: 1 mV·s\(^{-1}\). (a) Varying amounts of \( MvBOD \) on glassy carbon electrode modified with 93 µg of NaphtMWCNTs: b) 0.033 mg; c) 0.067 mg; d) 0.1 mg; e) 0.15 mg; f) 0.2 mg; g) 0.5 mg.

The amount of AuNPs used in the LBL approach has been also optimized and the results are shown in Figure 27. Different amounts of AuNPs were placed either on the very top of the NaphtMWCNTs film (Figure 27- b-d) or in between the nanotube layers (Figure 27- b’- d’) to form a complex network on top of the electrode surface. The highest value of the catalytic current was observed for GCE modified with 93 µg of NaphtMWCNTs and 0.1 mg of AuNPs. Amounts higher than 0.25 mg of AuNPs blocked the communication with the electrode surface decreasing the limiting currents and the slope of the wave. This blocking effect was due to the presence of the (poorly conducting) octane thiol substituents that cover and stabilise the NPs.
Figure 27 Cyclic voltammograms recorded for MvBOD catalysed dioxygen reduction in 0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl₂; scan rate: 1 mV·s⁻¹ with 0.1 mg MvBOD and varying amounts of AuNPs on glassy carbon electrode modified with 93 μg NaphtMWCNTs. Amount of AuNPs: b, b’: 0.05 mg, c, c’: 0.1 mg and d, d’: 0.25 mg. (b-d) are for AuNPs placed on the very top of the nanotube film, dashed lines (b’-d’) are for AuNPs placed in between the NaphtMWCNTs layers. a) as in c but in Argon saturated solution.

Combining all the information obtained so far – the optimal amounts of MvBOD (0.2 mg), NaphtMWCNTs (93 μg) and gold nanoparticles (0.1 mg) deposited on GCE using the LBL procedure – allowed to obtain the largest catalytic current for dioxygen reduction, which was equal to 650 ± 39 μA·cm⁻². The limiting current of such electrode decreased by ca. 100 μA·cm⁻² after each day of work.

Previous work on MvBOD reported a maximum current of 500 μA·cm⁻² for the enzyme bound to multi-walled carbon nanotube-modified gold [44] and more recently, Pitas et al. reported a value of current equal to 100 μA·cm⁻² for an electroactive surface area increased by the deposition of AuNPs on gold electrode [147].
Figure 28 Cyclic voltammograms recorded in deoxygenated (solid line) and oxygenated (dashed line) 0.1 M PBS buffer solution, pH 7, 0.1 M NaCl, 0.03 M MgCl₂, using GCE modified with 93 µg NaphtMWCNTs, 0.1 mg of C8@AuNPs and 0.2 mg MvBOD. Scan rate 1 mV·s⁻¹.

5.1.3 D-fructose dehydrogenase (FDH)

Fructose dehydrogenase has been adsorbed on different kinds of substrates over the last years. In order to improve the performance of bioelectrochemical devices based on the DET activity of FDH, the enzyme was loaded on bare HOPG or on single and multi-walled carbon nanotubes - modified electrodes [110,111,148]. It was also adsorbed on gold electrode from a 2-mercaptoethanol (ME) solution containing FDH, giving catalytic current density two times larger than when it was casted on Au electrodes from a ME-free FDH solution [51]. Recently, the gold nanoparticles modified with glutathione were electrodeposited on reticulated vitreous carbon and FDH was adsorbed on top of them. The resulting catalytic current increased up to 700 µA·cm⁻² thanks to the three dimensional nanostructured substrate [149]. Fructose dehydrogenase was adsorbed on different kind substrates over the last years. In order to improve the performance of bioelectrochemical devices based on the DET activity of FDH, the enzyme was loaded on bare HOPG or on single and multiple-walled carbon nanotube - modified electrodes [110,111,148]. It was also adsorbed on gold electrode from a 2-mercaptoethanol (ME) solution containing FDH giving twice larger catalytic current density than when it was casted on Au electrodes from
a ME-free FDH solution [51]. Recently, the gold nanoparticles modified with glutathione were electrodeposited on reticulated vitreous carbon and FDH was adsorbed on them, the resulting catalytic current increased up to 700 μA/cm$^2$ thanks to the three dimensional nanostructured substrate [149].

With our collaborators from Dropsens (Asturias, Spain), fructose dehydrogenase from *Gluconobacter* sp., we tested the carbon based screen printed electrodes (SPEs) for the future application in a miniaturized amperometric fructose biosensor. Various commercially available SPEs made of carbon auxiliary electrode and silver wire as *pseudoreference* electrode were studied with different working electrodes: carbon (DRP-110), modified with MWCNTs (DRP-110CNT) or with graphene as the carbon nanomaterial (DRP-110GPH). The working electrodes had working area of 0.12 cm$^2$. A solution of 10 mg·mL$^{-1}$ of FDH in 0.1 M PBS, pH 6, 0.1 M NaCl, 0.03 M MgCl$_2$ was casted on the SPEs. The electrodes were connected to the Autolab potentiostat through their own cable and immersed in buffer once dried. Eventually, 0.1 M fructose solution was used as enzyme substrate. The results are shown in Figure 29. The *pseudoreference* electrode has a potential difference of - 0.071 V vs. Ag/AgCl (KCl sat.) [47,129].

![Figure 29 Cyclic voltammograms of FDH modified SPEs in presence (colour lines) and absence (black lines) of 100 mM fructose solution in 0.1 M PBS buffer, pH 6, 0.1 M NaCl, 0.03 M MgCl$_2$. Scan rate: 1 mV·s$^{-1}$.](image-url)
Graphene appeared to be an optimal substrate for drop-casting FDH, therefore, it was used for the modification of the screen printed electrode for the biofuel cell construction.

5.2 Attempt to crystallize FDH “in meso”

Many advantages are gained by the usage of mesophases for the immobilisation of enzymes or proteins, especially for “in meso” crystallization of proteins that could not be crystallized by conventional methods. Since the structure of FDH is still not reported, in this thesis the attempt to crystallize it in the monoolein cubic phase was also made. The enzyme was always catalytically active and stable in this mesophase as we show in this work as well. It was already mentioned (paragraph 2.2.2) that FDH consists of three domains which easily decompose in neutral or alkaline solutions [24], therefore the idea was to accommodate the enzyme in the lipidic layer without any detergents or stabilizers.

This research was possible due to a collaboration with dr Jan Kutner from Go!RNA Structural Biology Group, Biological and Chemical Research Centre at University of Warsaw. The chemicals used were:

- *D*-fructose dehydrogenase from *Gluconobacter* sp. (FCD-302 - Sorachim SA, Lausanne, Switzerland)
- Bis Tris buffer (Merck KGaA, Darmstadt, Germany)
- Monoolein (Hampton Research, California, USA)
- DDM : n-Dodecyl β-D-maltoside (Merck KGaA, Darmstadt, Germany)
- Eppendorf ThermoStat™ C
- Gas-tight syringes syringes of 100 µL (Hamilton Company, Bonaduz, GR, Switzerland)
- Dual Small Hub RN Coupler (mixing chamber) (Hamilton Company, Bonaduz, GR, Switzerland)
- Dialysis tubing: nominal MWCO 12-14 kDa (Fisherbrand, Rodano, Italy)
- Laminex Starter Kit: MD11-55 (Molecular Dimensions, Suffolk, UK)
- Laminex UV plastic base (Molecular Dimensions, Suffolk, UK)
- Lammex Plastic cover 200 micron (Molecular Dimensions, Suffolk, UK)
- Mosquito LCP needle (Molecular Dimensions, Suffolk, UK)
- CryoMount Set LihioLoop size 0.06 mm (Molecular Dimensions, Suffolk, UK)
Bis-Tris buffer was considered the most appropriate option because of its pH range, suitable for FDH, and because it is well known in the crystallographic field. The final concentration of the buffer was 0.05 M and the pH was adjusted with concentrated HCl to 5.8. Approximately 20 mg of lyophilized enzyme were dissolved in 100 µL of 0.05 M Bis-Tris at pH 5.8 buffer. The enzyme solution was spinned for 5 min with 13000 rpm speed at 4°C. The concentration was evaluated through UV measurement using the software A280 from the Thermo Scientific NanoDrop Spectrophotometer, USA. The extinction coefficient was retrieved from the Protein Identification and Analysis Tools (ProtParam) on the ExPASy Server [150,151] inserting the amino-acidic (AA) sequences noted until now for FDH [23,152]. ProtParam allows the computational studies of physical and chemical parameters (like molecular weight, theoretical pI, amino acid and atomic composition, extinction coefficient) for a given protein stored in Swiss-Prot or for a user entered protein sequence. Extinction coefficients are in units of M$^{-1}$cm$^{-1}$, measured in water at a wavelength equal to 280 nm. For the sequences in Scheme 4 with a number of amino-acids of 1213 and a molecular weight of 131.98 kDa, ε was estimated to be 170700 M$^{-1}$ cm$^{-1}$ with a theoretical pI of 6.83.

The concentration of solution of FDH was approx. 10−13 mg·mL$^{-1}$, lower than expected. However, Sorachim SA (FDH producer) reports, that 80% of the content are additives (plenty of sugars and amino-acids but without any detergent or stabilizers).
Experimental

<table>
<thead>
<tr>
<th>Scheme 4 AA sequences known for fructose dehydrogenase cytochrome subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>(UniProtKB - M1V1V5), large subunit (UniProtKB - M1VMF7), small subunit (UniProtKB - M1VB40)</td>
</tr>
</tbody>
</table>
The gel electrophoresis of the protein solution with two different markers showed presence of small molecules.

![Figure 30 SDS-PAGE](image)

Figure 30 SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gel with 60 µL of 15 mg·mL⁻¹ FDH solution performed for 1 h under constant voltage of 70V. On the right SDS–gel electrophorese of FDH from [24]. The molecular markers used were 14.3, 20.1, 29.0, 45.0, 66.4 and 97.2 kDa

In two samples (crystallization plate 907m and 9089, Table 5) a dialysis membrane was used to get rid of all small molecules below the 14 kDa weight. The enzyme was dialysed overnight under constant stirring in 1 L 0.05 M Bis-Tris buffer at pH 7.8 at 18°C, then it was immediately incorporated in the cubic phase. For the plate 9089, a detergent (10% solution of DDM, n-Dodecyl β-D-maltoside) was used together with the dialysis buffer – small amounts of detergent are tolerated by the lipidic cubic phase and the final mesophase structure was not affected by the presence of detergent [72].

The activity of the enzyme was checked. 10 µL of the dialyzed enzyme solution was casted on GCE covered with a MWCNTs layer (10 µL, 0.7 mg · mL⁻¹EtOH) and cyclic voltammetry measurements were performed in 0.05 M Bis-tris buffer of pH 5.8 at the presence of 0.1 M fructose solution. The recorded cyclic voltammograms confirmed the activity of the enzyme. Therefore, we assumed that no splitting of the three subunits happened as a result of the used pre-treatment.
Figure 31 Cyclic voltammograms of GCE modified with MWCNTs (0.007 mg) and 10 µL of dialyzed enzyme solution in the 0.05 M Bis-tris buffer pH, 5.8 (black line) or in 0.1 M fructose solution (dashed line). Scan rate: 1 mV·s⁻¹.

As a first step, a commercially available sparse matrix screen was chosen for attempt of the crystallization of FDH both, with the enzyme enclosed in LCP and with the more classic sitting-drop method. The JCSG-plus™ MD1-37-Eco Screen [153] seemed to be a good candidate, thanks to its wide pH range (from 4 to 10), the presence of acetate or citrate acids (known to work with FDH) and because of its reduced redundancy on the 96-wells conditions thus to have a complete as well as wide crystallographic screen. The first crystal appeared after one week in the well C7 with ZnAc 0.2 M, NaAc 0.1 M, PEG 10%, pH 4.5 condition (Figure 32). Successively, a customized screen was prepared in the way that each column differs from the C7 well conditions of only one parameter.

Table 4 Crystallographic screen customized.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>C7 from the Original MD1-37-ECO JCSG-plus</td>
<td>ZnAc 0.2 M NaAc 0.05 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.35 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.5 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.05 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.35 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.5 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.05 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.35 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.5 M PEG 10% pH 4.5</td>
<td>ZnAc 0.2 M NaAc 0.05 M PEG 10% pH 4.5</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Column 1 is the commercially available C7 from JCSG-plus™ MD1-37-Eco Screen, column 2 has the same condition as the first one but it is made in lab. In other columns, the condition which changed compared to the original recipe, is empathized in bold.

Both screens (original and customized) were stored in the fridge at 4°C in a deep-well block. Before usage the chosen screen was kept at room temperature for 30 minutes and spun for 5 min in 5000 rpm at 22°C.

![Overview](image1) ![Drop](image2) ![Condenser 75%](image3) ![Cross-polarisation](image4)

Figure 32 Images of the crystals growing in the C7 well - conditions (ZnAc 0.2 M, NaAc 0.1 M, PEG 10%, pH 4.5) for the JCSG-plus™ MD1-37-Eco Screen

The cubic phase was prepared as previously explained in 4.3.1. The ratio was chosen according to the phase diagram for the monoolein–water system for the formation of a diamond type cubic phase (Pn̅3m) [60]. In these experiments, the system consisted of monoolein/buffer or monoolein/enzyme solution in a ratio of 60%:40% at a temperature of 25 °C. The cubic phase was formed with 60% of lipid and 40% of protein solution (considering the monoolein unitary density - \(d = 0.9407 \, g/cm^3 \approx 1\, mg/\, \mu L\)). The Eppendorf was tightly sealed with Parafilm and left to equilibrate in the Eppendorf ThermoStat™ C set at 25 °C for 24 h. The formation of a transparent and highly viscous cubic phase was confirmed by macroscopic observation of the sample. At the beginning, the sample appeared ununiformed (non-homogenous) and cloudy (not transparent), but successively
the texture became more uniform and transparent, what confirmed the formation of the correct phase.

The phase was transferred from the Eppendorf tube, where it was prepared, to the gas-tight 100 µL syringe. The phase was then pressed with the plunger up the barrel and into the needle until it was visible from the other side, as carefully described by Caffrey [154,155]. Bubbles can appear when transferring from one syringe to the other but they easily go away. The first syringe was connected to the second syringe through the mixing chamber and the phase was transferred between them (what resulted in mixing) for about 100 times. The so-called Mosquito robot helped during the preparation of the crystallization plates, aliquoting 200 nL of LCP (or enzyme solution in case of the sitting-drop method) in the 96-well plate and immediately covering it with additional 200 nL of appropriate screen condition in Table 4. Humidity was kept at 50%. The plate was appropriately sealed with its cover, transferred into the so-called hotel where it was kept at 16°C and imaged every day.

![Figure 33 Left: Hamilton syringes filled with FDH LPC. Right: LCP plate ready for the hotel](image)

Different crystallization plates were prepared and they are summarized in the following table.

**Table 5 Summary of the crystallization plates prepared in this thesis**

<table>
<thead>
<tr>
<th>Hotel's name</th>
<th>Method</th>
<th>Conc.</th>
<th>Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>907f</td>
<td>Sitting drop method</td>
<td>1.7 mg/mL</td>
<td>JCSG-plus™ Eco Screen</td>
</tr>
<tr>
<td>907g</td>
<td>Sitting drop method</td>
<td>17.8 mg/mL</td>
<td>JCSG-plus™ Eco Screen</td>
</tr>
<tr>
<td>907j</td>
<td>Laminex LCP FDH</td>
<td>17.8 mg/mL</td>
<td>JCSG-plus™ Eco Screen</td>
</tr>
<tr>
<td>907m</td>
<td>Laminex LCP FDH dialysed</td>
<td>26.2 mg/mL</td>
<td>JCSG-plus™ Eco Screen</td>
</tr>
<tr>
<td>907x</td>
<td>Laminex LCP FDH</td>
<td>18.6 mg/mL</td>
<td>Custom screen</td>
</tr>
<tr>
<td>907y</td>
<td>Laminex LCP FDH</td>
<td>13.4 mg/mL</td>
<td>Custom screen</td>
</tr>
</tbody>
</table>
### Experimental

<table>
<thead>
<tr>
<th>Hotel's name</th>
<th>Method</th>
<th>Conc.</th>
<th>Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>907z</td>
<td>Laminex LCP FDH</td>
<td>9.2 mg/mL</td>
<td>Custom screen</td>
</tr>
<tr>
<td>9080</td>
<td>Control experiment Laminex LCP MO</td>
<td>9.2 mg/mL</td>
<td>Custom screen</td>
</tr>
<tr>
<td>9081</td>
<td>Laminex LCP FDH</td>
<td>9.2 mg/mL</td>
<td>Custom screen</td>
</tr>
<tr>
<td>9082</td>
<td>Laminex LCP FDH</td>
<td>9.2 mg/mL</td>
<td>Custom screen</td>
</tr>
<tr>
<td>9089</td>
<td>Laminex LCP FDH dialysed</td>
<td>5.4 mg/mL</td>
<td>Custom screen</td>
</tr>
<tr>
<td>908a</td>
<td>Laminex LCP FDH</td>
<td>6.1 mg/mL</td>
<td>Custom screen</td>
</tr>
</tbody>
</table>

*crystals appeared and were collected

Upon examination under the microscope and hotel imager, very tiny crystals (around 10-20 µm) were collected from the plates 907j, 907y, 907z, 9081 and 9082 according with the procedure described in literature [156]. After marking the corner where the A1 well was, the plate was taken away from the support and put under the microscope. A scalpel was used as cutting tool to open the cover around the well where the crystal was observed. As soon as the cover was lifted, 2 µL of precipitation buffer was added on top of the LPC to prevent it from drying. The crystal was harvested by scooping it directly from the LCP using the loop with the inner diameter matching the size of the crystal (loop size 0.006 mm) and was immediately immersed in a dewar with liquid nitrogen in order to flash freeze it.

![Figure 34](image)

**Figure 34** Sequence of steps illustrating the opening of an individual well for crystal harvesting. Image taken from [157]
Harvested crystals were investigated in collaboration with dr D. Trzybiński, from the Biological and Chemical Research Centre of University of Warsaw. Each loop containing a crystal was mounted on the dual source SuperNova CCD equipped with the large area Atlas and source of electromagnetic wave set to Cu wavelength (SuperNova Double Source Rigaku Oxford Diffraction). Data sets were collected with CrysAlisPro but unfortunately no cell units could be solved, apart from very small crystals ascribed to the salts present in the samples studied.

Figure 35 Hotel ID 907z, well B4: a) and b) crystals growing at day 65 under visible and polarized light. c) LCP image obtained with microscope with polarized light on the open well. Shiny and tiny crystals can be seen and were harvested. d) and e) images at the diffractometer

5.3 Bioelectrochemical behaviour of studied enzymes in cubic phase (LCP)

5.3.1 SAXS measurements for the prepared cubic phases

SAXS data for the “empty” cubic phase consisting of 60% of monoolein prepared at room temperature showed the Pn3m symmetry. When 0.04 mg of enzyme was added, the same symmetry has been preserved. This means that the enzyme is mainly located in the cubic phase’s aqueous channels and does not affect the shape of lipidic bilayer. Upon incorporation of AuNPs covered with octanethiol (of 4.7 nm diameter), the LCP still had cubic phase symmetry, but the type of the phase structure changed from cubic Pn3m to the cubic gyroid (Ia3d) phase. Most probably, the hydrophobic AuNPs were located partly in the lipidic phase and partly in the aqueous channels affecting the water channels width and
flattering the lipid membrane. When CtCDH was used, it was found that the presence of the enzyme had no influence on the mesophase formation. The SAXS spectrum indicated the existence of a cubic structure with Pn3m symmetry.

Table 6 Symmetry, lattice parameters, lipid length and aqueous channel diameters for the three monoolein based mesophases at 25°C (calculated according to equations in paragraph 3.4):

<table>
<thead>
<tr>
<th>Mesophases</th>
<th>Buffer</th>
<th>Phase symmetry</th>
<th>Lattice parameters a (nm)</th>
<th>Lipid length ℓ (nm)</th>
<th>Channel width d (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO</td>
<td>H₂O</td>
<td>Pn3m</td>
<td>9.97</td>
<td>1.70</td>
<td>4.39</td>
</tr>
<tr>
<td>MO</td>
<td>0.1 M PBS, pH 7</td>
<td>Pn3m</td>
<td>9.76</td>
<td>1.67</td>
<td>4.30</td>
</tr>
<tr>
<td>MO + MvBOD</td>
<td>0.1 M NaCl, 0.03 M MgCl₂</td>
<td>Pn3m</td>
<td>9.87</td>
<td>1.68</td>
<td>4.36</td>
</tr>
<tr>
<td>MO + MvBOD + AuNPs</td>
<td>0.05 M MOPS, pH 7.5</td>
<td>Ia3d</td>
<td>13.14</td>
<td>1.39</td>
<td>3.74</td>
</tr>
<tr>
<td>MO</td>
<td>0.1 M NaCl, 0.03 M CaCl₂</td>
<td>Pn3m</td>
<td>9.24</td>
<td>1.57</td>
<td>4.07</td>
</tr>
<tr>
<td>MO + CtCDH</td>
<td></td>
<td>Pn3m</td>
<td>9.45</td>
<td>1.61</td>
<td>4.17</td>
</tr>
</tbody>
</table>

The addition of different electrolytes did not influence the cubic phase symmetry but affected the unit cell size and the aqueous channel diameters. Incorporation of positively charged electrolytes increased the negative curvature of the membrane layer leading to more contracted Pn3m phase with smaller unit cell sizes [158].

NaCl is known to act as dehydration agent of the monoolein head group at the interface which in turn reduces its occupied effective area [59]. Incorporation of sodium chloride in the mesophase resulted in shrinking of the phase by decreasing the lattice parameter. Consequently, the larger ℓ value corresponds to the “empty” MO cubic phase made with MilliQ water. Integration of calcium ions in the lipidic membrane was also reported to lead to condensation by decreasing the radius of the aqueous channel imposing repulsion between the headgroup regions [74]. Therefore, it explains the fluctuations observed in the parameters calculations when two different buffers were used compared to those obtained when the phase was done in pure water.
Figure 36 SAXS profile at 25°C of the A) Pn3m cubic phase containing 60% of MO and 40% of PBS buffer; \(a\): 9.76; \(d\): 4.30 nm. B) Pn3m cubic phase containing 60% of MO and 40% of \(MvBOD\) in PBS buffer; \(a\): 9.87; \(d\): 4.36 nm. C) \(Ia3d\) cubic phase containing 60% of MO-AuNPs and 40% of \(MvBOD\) in PBS buffer; \(a\): 13.14; \(d\): 7.50 nm. D) Pn3m cubic phase containing 60% of MO and 40% of \(CtCDH\) in MOPS buffer; \(a\): 6.60; \(d\): 2.91 nm.

### 5.3.2 \(CtCDH\) in LCP

The catalytic behaviour of cellobiose dehydrogenase is also seen when the enzyme is encapsulated in liquid crystalline cubic phase. The electrodes were prepared as explained in paragraph 4.3. Briefly, after polishing with alumina, GCEs were modified with a layer of MWCNTs on top of which a film of lipidic cubic phase (LCP) was smeared. The electrodes were weighted before and after placing the mesophase in order to measure the amount of LCP present at the electrode.

Approximately 10 mg of LPC film covered each of the electrodes, corresponding, in case of enzyme solution of \(ca\) 10 mg·mL\(^{-1}\) to 0.04 mg of enzyme for a cubic phase prepared using 60:40 ratio of lipid to H\(_2\)O.
The voltammetric signal corresponding to the free FAD and CYT bound haem $b$ cofactors observed at $ca. -0.460$ V and $-0.100$ V vs. Ag/AgCl (KCl sat.) can be also seen (although they can be hardly recognized) in the DPV voltammograms when the enzyme was hosted in the cubic phase (figure not shown). Probably only small part of enzyme molecules was in direct electrical contact with the electrode surface, some of them residing in the bulk of the LCP film.

Even though the FAD is leaking out from the DH domain, most of the $Ct$CDH molecules keep their activity towards the substrate. Both DH and CYT domains are observed when the enzyme is entrapped in a liquid crystalline cubic phase environment (Figure 37). Electrochemical behaviour of both domains was studied in solution of pH from 3.6 to 7.5. The midpoint potentials were between $-0.20$ and $-0.42$V for DH and from $-0.11$ to $-0.14$V for the CYT site, depending on the pH value. The change of potentials with increasing pH for the cytochrome domain is due only to the change of the charge of the amino-acids located around the haem $b$ active site. The same small changes of peak location with increasing pH were also observed for cytochromes in FDH [110]. Upon addition of lactose at pH 4.6 and pH 7.8, the catalytic current onset appears at the oxidation potential of the CYT cofactor, meaning that most of $Ct$CDH molecules are still intact even though some free FAD escaped from the DH domain and that the CYT cofactor is of crucial importance for the electron transfer between the enzyme and the electrode.

The activity of the enzyme hosted in the cubic phase was investigated in two different electrolytes: 50 mM PBS buffer at pH 7.5 with 100 mM NaCl plus 30 mM MgCl$_2$ or 50 mM MOPS at pH 7.5 with 100 mM NaCl plus 30 mM CaCl$_2$. The current densities of catalysed lactose oxidation at the modified electrodes in the two buffers are compared in Figure 38. Bivalent cations have been reported to influence the activity of ascomycete CDHs [159]. Calcium was found to decrease the distance between DH and CYT domains promoting the internal electron transfer between them. The shift of catalytic potential to more negative values was observed while the current was higher. In acidic pH and in the presence of Ca$^{2+}$, the current onset potential was equal to that of the bound FAD cofactor of basidiomycete CDH [160]. The cause of it was established by performing docking simulations on both class I $Pc$CDH and class II $Mt$CDH: bivalent cations interact with the interfacial area between DH and CYT domain which contains negatively charged amino-acids. This interaction moves the two domains closer facilitating the IET and improving the current output. Such interactions are not observed in the case of monovalent ions,
nevertheless, increasing the ionic strength of the buffer leads also to an increase of the current [159–161].

![Graph a)](image)

**Figure 37** a) Cyclic voltammograms of CtCDH on MWCNTs-modified GCEs in 50 mM NaAc pH 3.6, pH 4.6, pH 5.6 with 100 mM NaCl and 30 mM CaCl₂, in 50 mM MOPS pH 6.5, pH 7.8 with 100 mM NaCl and 30 mM CaCl₂ in the absence (solid lines) and in the presence of 100 mM lactose (dashed line). Scan rate of 5 mV·s⁻¹. b) The dependence of peak potentials corresponding to the two domains on pH, and the equations of linear fitting of the data points.

GCE decorated with a layer of MWCNTs (0.007mg, 0.7 mg·mL⁻¹) modified with 10 mg of lipidic cubic phase (containing 0.04 mg of CtCDH) was used to study the effect of the presence of monovalent (Na⁺) and bivalent ions (Ca²⁺ or Mg²⁺) (Figure 38). The
resulting CVs show that the catalytic wave at 0.3 V vs. Ag/AgCl (KCl sat.) is smaller when MOPS with Ca\(^{2+}\) is used instead of PBS containing Mg\(^{2+}\) (current densities values are respectively 9.22 and 14.37 \(\mu\)A·cm\(^{-2}\)). If the current values obtained for the LCP system are compared with those obtained with the adsorbed enzyme (Figure 23), the improvement gained by the usage of LCP is clearly seen. The cubic phase is able to accommodate the enzyme improving also the communication between the electrode surface and the active centre. The internal electron transfer, IET, assisted by calcium ions seems to be more efficient because of the more negative onset potential. The importance of Ca\(^{2+}\) ions in the CDH IET was discussed in several papers by Gorton et al. [144,160,161]. Ca\(^{2+}\) ions can interact with the hydrophilic part of the monoolein, coordinating to the head group region in the aqueous channel [74]. However the membrane capacitance is larger and the resistance lower in the case of high concentrations of CaCl\(_2\).

![Cyclic voltammograms of GCE modified with MWCNTs with 10 mg of CtCDH cubic phase in (1) 50 mM MOPS, pH 7.5, 100 mM NaCl and 30 mM CaCl\(_2\) (black line) and (2) 50 mM PBS, pH 7.8, 100 mM NaCl, 30 mM MgCl\(_2\) (grey line) in the absence (solid lines) or presence (dashed lines) of 100 mM lactose. Argon saturated buffer. Scan rate 1 mV·s\(^{-1}\).](image)

Figure 38 Cyclic voltammograms of GCE modified with MWCNTs with 10 mg of CtCDH cubic phase in (1) 50 mM MOPS, pH 7.5, 100 mM NaCl and 30 mM CaCl\(_2\) (black line) and (2) 50 mM PBS, pH 7.8, 100 mM NaCl, 30 mM MgCl\(_2\) (grey line) in the absence (solid lines) or presence (dashed lines) of 100 mM lactose. Argon saturated buffer. Scan rate 1 mV·s\(^{-1}\).
5.3.2.1 Stability of the cubic phase electrode containing CtCDH

The stability of an enzyme hosted in the cubic phase increases thanks to the mesophase lipidic environment, where the enzyme is accommodated, while the unwanted side reactions are prevented. GCEs were modified with MWCNTs and covered with 0.04 mg of CtCDH incorporated in lipidic cubic phase or physisorbed directly on the nanotubes and the stabilities were investigated by measuring the changes in catalytic current over time. When the enzyme was adsorbed on the modified electrode, the activity dropped down to 60% after one week, despite the fact that the electrodes were kept in the fridge at 4°C between the measurements. When the enzyme was hosted in the cubic phase not only the stability was much better but also a slight increase of current was observed even after one week. The electrodes were kept at room temperature immersed in buffer between the measurements and the activity was measured daily for 28 days. In 2016 the stability measurements were also performed by Al-Lolage et al. [50] for CDH on graphite electrodes covered with nanotubes grafted to form a mixed monolayer with maleimide spontaneously coupled to the cysteine groups of the enzyme. These electrodes were active for one month exhibiting a significant decrease of activity within the first week due to the possible loss of FAD. In the cubic phase, CtCDH keeps its catalytic activity almost unchanged with time (Figure 39).

![Figure 39](image)

*Figure 39 Long-term stability of CtCDH adsorbed on MWCNTs/GCE (black squares) and CtCDH in LCP film on GCE covered with MWCNTs (black triangles) stored in a 100 mM lactose*
solution. Grey circles depict the results from *Ct*CDH in LCP film on GCE covered with MWCNTs but measured in pure buffer. The activity of the electrode with time is shown as the limiting catalytic current densities measured at 0.4 V in buffer: argon saturated 50 mM MOPS, pH 7.5, 30 mM CaCl$_2$ and 100 mM NaCl.

The reason for the current upsurge after six days when the enzyme is entrapped in cubic phase is more visible when the CV corresponding to 0, 6 and 28 days are plotted (Figure 40). It can be clearly noted that after six days two waves in the CV can be resolved.

![Figure 40 Changes of the voltammetric curves of GCE/MWCNTs/CtCDH in LCP with time: (1) day 0; (2) after 6 days and (3) after 28 days.](image)

The reason of the observed change of the voltamogram shape with time was interpreted as due to the slow release of FAD from the DH domain so it was assumed that some form of FAD may act as a mediator for the remaining - non-degraded *Ct*CDH. The catalytic process shifted to more positive potentials under these new mediating electron transfer conditions. The DET wave starting at *ca.* -0.150 V decreased with time while a second catalytic wave became clearly visible at *ca.* +0.125 V. Because of the mesophase matrix, the mediating species did not have possibility to escape from the film and efficiently expressed its redox functionalities. The potential of this mediated process is much more positive than that of free FAD but the mediated moiety could not be identified [162].
In another study, adenosine, which is the structural part of the FAD in the DH domain, was found to be a potential precursor of this redox active compound, since a peak at ca. 0.192 V (vs. Ag/AgCl in 0.1 M KCl) appeared both for the DH CDH and adenosine modified polyethyleneimine (PEI) electrodes [163,164].

The mediated process can be further enhanced by using an additional mediator with a positive redox potential. Cytochrome c (cyt c) is a powerful mediating compound in CDH electron transfer [107,145]. Its \( E^o \) value, measured in the absence of enzyme and substrate, is close to +0.1 V vs. Ag/AgCl (KCl sat.). Cyt c can accept electrons only from the cytochrome domain, whereas it is incapable of communicating with the DH domain [165]. An extra boost in current, starting at ca +0.1 V, was obtained when the GCE/MWCNTs/CtCDH LCP 28 days old electrode was immersed in a solution containing 40 µM cyt c with an addition of 100 mM lactose in MOPS buffer. This means that the MET via cyt c occurs at the potentials very close to those of the second catalytic wave.

Figure 41 Cyclic voltammograms of GCE/MWCNTs/CtCDH in LCP after keeping it for 8 days in the MOPS buffer solution containing 100 mM lactose in the (1) absence and (2) presence of 40 µM cyt c (3) and (4) corresponds to cyt c on GCE with and without lactose.
5.3.2.2 Catalytic lactose oxidation mediated by DCPIP

The potential of catalytic oxidation of lactose follows the standard potential of the used mediator moving toward more positive potential; thus, when 40 \( \mu M \) of DCPIP is used as mediator, \( E^{\circ} = +0.071 \) V vs. Ag/AgCl (KCl sat.) evaluated from the CV of DCPIP, the catalytic current of the process mediated by DCPIP in the cubic phase appears at the potential corresponding to \( E^{\circ} \) of DCPIP. The catalytic current of GCE modified with a layer of MWCNTS (0.007 mg, 0.7 mg \cdot mL^{-1} EtOH) with a film of CtCDH in LCP reaches 26.77 \( \mu A \) cm\(^{-2}\) (Figure 42). DCPIP acts as an efficient 2H\(^+\)/2e\(^-\) transfer mediator (2EA) for the catalytic process shifting the onset of catalytic current to more positive potentials.

The cubic phase matrix can influence the diffusion rate slowing down the transport of dissolved molecules (D value is smaller) [64,166]. The \( K_d[Fe(CN)_6] \) diffusion rate is decreased by almost a factor of 4 compared to the value obtained for the bare electrode [167]. The changes of diffusion coefficient of doxorubicin were \( 2.7 \cdot 10^{-6} \) cm\(^2\) s\(^{-1}\) in aqueous solution while the value equal to \( 0.98 \cdot 10^{-6} \) cm\(^2\) s\(^{-1}\) in the monoolein cubic phase [64].

As seen in Figure 42-1b, the redox process of DCPIP is reversible when the mediator is trapped in the cubic phase; furthermore it is clearly visible that DCPIP increases the catalytic current improving the wave development and proving facilitation of the communication between the enzyme molecules and the electrode surface. The novelty in Figure 42 is that the probe was either dissolved in solution as soluble mediator (2a) or entrapped in LCP (1a). What it is interesting, is the fact that in the case of dissolved probe it is still possible to observe the signal of the non-mediated process at more negative potentials than that of DCPIP. The differences in catalytic currents between 1 and 2a is due in part to the different concentration of the mediator (87 \( \mu g \) of DCPIP in the cubic phase versus 116 \( \mu g \) of DCPIP in the solution). The limit of the mediator concentration in the cubic phase is imposed by the monoolein phase diagram. It has to be kept smaller in order to maintain the cubic phase Pn3m structure.
Experimental

Figure 42 Cyclic voltammograms of an electrode covered with 10 mg of cubic phase containing 0.04 mg of $\text{CtCDH}$ and 87 $\mu$g of DCPIP in the cubic phase (1a) and (2a) for comparison an electrode containing 0.04 mg $\text{CtCDH}$ in cubic phase and 116 $\mu$g of DCPIP in the solution. Lactose concentration: (a) 100 mM, (b) 0 mM. Electrolyte: 50 mM PBS at pH 7.8, 100 mM NaCl, 30 mM MgCl$_2$; scan rate 1 mV/s.

Moreover, within collaboration with prof. Biernat group from Gdansk University of Technology, Poland, the mediator DCPIP was also covalently bound to SWCNTs (synthesis explained in 4.3.4). First of all, the presence of DCPIP on the SWCNTs was in these experiments confirmed by the appearance of a reversible peak on a CV curve with $E^\circ$ of ca. 0.08 V vs. Ag/AgCl (KCl sat.), performed on a GCE modified with 30 $\mu$L (0.02mg, 0.7 mg · mL$^{-1}$) of nanotubes (Figure 44).
Figure 43 Cyclic voltammograms recorded with GCE modified with 0.02 mg of DCPIP-SWCNTs (dashed line) in 50 mM MOPS, pH 7.5, 30 mM CaCl₂ and 100 mM NaCl. Black line: recorded in the same solution but on unmodified GCE. Scan rate 20 mV·s⁻¹.

GC electrodes were modified with 0.2 mg DCPIP-SWCNTs (10 µL, 2 mg·mL⁻¹ EtOH). The real electrode area of the modified GCE and the amount of DCPIP bound to SWCNTs were evaluated.

To estimate the real working surface area of the modified electrode, cyclic voltammograms were recorded in 1 M KCl solution containing the common reversible electrochemical probe undergoing 1e⁻ reversible electrode process - K₄[Fe(CN)₆]. The Randles-Ševčík equation (8) was used and the area was estimated by plotting the current intensity of the anodic peak (iₚ) vs. square root of the scan rate (ν½), using n (number of electrons exchanged) =1 , C = 5·10⁻⁶ mol·cm⁻³, D = 7.05·10⁻⁶ cm²·s⁻¹ [168].

The surface area obtained for GCE modified with 0.2 mg of DCPIP-SWCNTs was approximated to be 0.12 ± 0.02 cm². More accurate methods for the real area determination were not employed.

With the use of above surface area of the DCPIP-SWCNTs-modified electrode and a peak charge obtained from cyclic voltammetry measurements surface concentration (Γ) was evaluated, using eq. (10). It was estimated to be 1.45·10⁻¹⁰ mol·cm⁻².
In the next experiment, 0.04 mg of enzyme (4 µL of 10 mg·mL⁻¹ solution) was drop-casted on the GCE/DCPIP-SWCNTs and the catalytic current was measured in the presence of 100 mM lactose. The performance of such catalytic electrode was not satisfactory (Figure 44). To improve the performance, a different design of the catalytic film was proposed instead; the enzyme was hosted in the cubic phase layer (Figure 45).

Figure 44 Cyclic voltammograms recorded using GCE covered with 10 µL of suspension of DCPIP-SWCNTs (black dashed line) containing 0.04 mg of CtCDH ads (black line). Buffer: 50mM MOPS, pH 7.5, 30 mM CaCl₂ and 100 mM NaCl. Scan rate 1 mV·s⁻¹. Geometrical area (0.072 cm²) was used for the current density calculation.
When CtCDH is included in a lipidic cubic phase, the activity is improved and four peaks pairs appear at the slow scan rate CV. The first couple $a_1c_1$ at most negative potential, $E_{c_1} = -0.160 \text{ V vs. Ag/AgCl (KCl sat.)}$, corresponds to the haem $b$ in the cytochrome domain, the middle one $a_2c_2$ with midpoint potential of 0.053 V depicts the redox behaviour of DCPIP probe. Another pair $a_3c_3$ is clearly visible when the enzyme is hosted in the cubic
Experimental

phase (Figure 45) but not when adsorbed on the modified electrode surface (Figure 44). It was previously reported in literature [164] but was not assigned either to the intact CYT or DH domains. The first and third peak pairs belong to the enzyme since they do not appear when CtCDH is not present (*i.e.* Figure 44 - dashed line). At more negative potential at *ca.* -0.250 V the peak corresponding to free FAD appears (as previously shown in Figure 22).

In Figure 46 a comparison of enzyme activity in the cubic phase is shown when pristine single and multi-walled CNT and DCPIP-modified SWCNTs are used. Approximately the same current values are obtained when DCPIP-SWCNTs are used, but the catalytic activity is better developed what is confirmed by higher *i* values obtained at potential of 0.05 V. When the mediator is present in smaller amount (*i.e.* when it is bound to the nanotubes) the ET process goes through the haem *b* unit and in part proceeds through the DCPIP mediated pathway, similarly to what was shown in Figure 42 for the mediator dissolved in the buffer solution. Such phenomenon, that the catalytic process can follow two pathways on a single electrode, is not common and it seems that appropriate modification of electrodes can reveal similar behaviour for other oxidoreductases as well.

![Graph](image)

Figure 46 Comparison of catalytic waves for CtCDH LCP in the presence of 100 mM lactose in buffer (50mM MOPS, pH 7.5, 30 mM CaCl₂ and 100 mM NaCl) solution. GCE was modified with DCPIP-SWCNTs (red line), MWCNTs (black line) and SWCNTs (blue line). Red dashed line is like red straight line in buffer. Scan rate 1 mV·s⁻¹. Geometrical area (0.072 cm²) was used for the current density calculation.
The catalytic currents are larger in the case of the enzyme hosted in cubic phase on DCPIP- SWCNTs, but they decrease fast with time in contrary to those currents recorded using cubic phase film and pristine nanotubes (Figure 47). It is not unexpected that mediated processes are more effective that DET processes. However when cubic phase covers the electrode the catalytic activity was observed even after several weeks of work, which shows the improved stability of the modified electrode.

Figure 47 Overlap of cyclic voltammograms of DCPIP-SWCNTs-modified GCE with 0.04 mg of CtCDH in cubic phase in buffer at day 0 (blue line) and after 6 days (red line). Buffer: 50 mM MOPS, pH 7.5, 30 mM CaCl₂ and 100 mM NaCl.

The DCPIP was found to switch the catalytic process to the mediated pathway with the electrons going through DCPIP directly from the FAD, while in the presence of pristine nanotubes the process goes in a mediatorless way through the cytochrome domain. In case of DCPIP-modified nanotubes both pathways – through cytochrome DET and through DCPIP and FAD at the potentials of DCPIP – could be seen. The competition of the two pathways depends on the amount of DCPIP mediator.
5.3.2.3 Catalytic oxidation of lactose mediated by Ru(NH₃)₆Cl₂

CVs recorded for GCE/MWCNTs/CtCDH LCP in the presence of 40 µM [Ru(NH₃)₆]²⁺ dissolved in solution shows catalytic current which is four times larger than without the mediator, hence under the DET conditions (Figure 48).

![Cyclic voltammograms](image)

Figure 48 The effect of Ru(NH₃)₆Cl₂ in solution on the cyclic voltamograms recorded with the electrode modified with CtCDH hosted in the cubic phase on GCE/MWCNTs. MOPS buffer solution without (solid line) and with (dashed lines) 100 mM lactose. Ru(NH₃)₆Cl₂ in concentrations: (a) 0; (b) 40 µM. Scan rate 1 mV·s⁻¹.

The Ru(II) complex acts as 1e⁻, no H⁺ acceptor with an E°' of −0.160 V (Figure 49), and therefore accepts electrons from the DH CtCDH in its reduced state. Addition of lactose leads to a well-defined catalytic wave, starting at the mediator potential giving a current density of catalytic lactose oxidation of 32.10 µA cm⁻² in MOPS buffer at pH 7.5. The lipidic cubic phase decreases the diffusion coefficient of [Ru(NH₃)₆]²⁺ to a small extent. The D value calculated for this probe in the cubic phase is $2.17 \times 10^{-6}$ cm²·s⁻¹ while in the phosphate buffer solution it is $8.63 \times 10^{-6}$ cm²·s⁻¹ [169]. Using the mediator in cubic phase leads to increase of catalytic current of lactose oxidation and to a decrease of the overpotential. It should be, however, kept in mind that solutions of this mediator are much less stable in the presence of oxygen than those of DCPIP.
Figure 49 CV of GCE modified with MWCNTs in 40 µM Ru(NH$_3$)$_3$Cl$_2$; scan rate 50 mV·s$^{-1}$.
Electrolyte: 50 mM MOPS, 100 mM NaCl and 30 mM CaCl$_2$.

5.3.2.4 CtCDH- LCP for glucose catalytic oxidation

Ascomycetes class II CDHs (e.g. CtCDH) have high turnover rate not only for lactose but also for glucose used as the substrate in solutions of neutral pH [11,102]. Michaelis-Menten constants for glucose and lactose catalytic oxidation with CtCDH adsorbed on graphite electrodes were evaluated to be approximately 24 and 0.2 mM, respectively [170].

When MWCNTs modified GCE with LCP containing CtCDH is used as the catalytic electrode in 200 mM glucose solution containing 40 µM DCPIP, MET process takes place. This shows that the cubic phase does not affect the enzyme activity also towards the most common sugar substrate (Figure 51).
Figure 50 Cyclic voltammograms of a CtCDH-cubic phase GCE/MWCNTs electrode in 50 mM PBS, pH 7.5, 100 mM NaCl, 30 mM MgCl₂ with 40 µM DCPIP and (1) 0; (2) 200 mM glucose. Scan rate 1 mV·s⁻¹.

5.3.3 MvBOD in LCP

The electrode prepared as explained in the previous paragraph 5.1.2 with optimized amount of MvBOD (0.2 mg), 93 µg of NaphtMWCNTs and 0.1 mg of gold nanoparticles deposited on GC electrode using the LBL procedure was not stable over time. The limiting current density of such electrode decreased by ca. 100 µA·cm⁻² after each day of work. In order to improve the stability of the catalytic film at longer time scale, the NaphtMWCNTs layer was covered with a layer of lipidic liquid crystalline cubic mesophase containing 0.2 mg MvBOD and 0.1 mg of AuNPs.
Figure 51 Cyclic voltammograms recorded in deoxygenated (solid line) and oxygenated (dashed line) PBS buffer solution, pH 7, where a layer of lipid cubic phase containing 0.2 mg MvBOD and 0.1 mg of C8@AuNPs was smeared on glassy carbon electrode modified with 93 µg NaphtMWCNTs. Scan rate 1 mV·s⁻¹.

The stability of the MvBOD-based system in terms of catalytic activity was investigated by measuring the time dependence of the catalytic current of MvBOD adsorbed directly on NaphtMWCNTs/AuNPs/GCE and for the electrode covered with MvBOD/AuNPs in LCP/NaphtMWCNTs/GCE. In the former case the current density decreased by 40% after only 5 days. The experiments were repeated after storage of the electrodes at 4 °C. More than 70 % of the initial value remained after 9 days of work of the electrodes covered with NaphtMWCNTs and MvBOD/AuNPs in the cubic phase (Figure 52). In addition, the electrodes can be stored at room temperature. This implies that the GCE surface modified with NaphtMWCNTs covered with AuNPs and MvBOD entrapped in the lipidic cubic phase does significantly improve the catalytic performance of the electrode towards dioxygen reduction but the lipidic cubic phase environment makes the whole system much more stable at the longer time-scale.

The performance of bilirubin oxidase adsorbed on a multi walled carbon nanotube-modified paper on air breathing cathode was monitored over 45 days by Atanassov et al. [171]. It should be noted that the stability of their electrodes over time showed a serious
drop in current density during the first 3 days (around 50%) and further continuous decrease to finally reach 28% of its initial value after 9 days. This leads us to propose the cubic phase film as a convenient environment for retaining the activity of enzymes on AuNPs and CNTs nanostructured electrodes.

Figure 52 The long-term stability of $Mv$BOD adsorbed on NaphtMWCNTs/AuNPs/GCE (empty circles) and ($Mv$BOD+ AuNPs) in a LCP film on GCE covered with NaphtMWCNTs. (full circles). The activity of the electrode in time is shown as the catalytic current measured at 0.3 V in oxygen-saturated phosphate buffer (pH 7). The first value at 0.5 days was taken after three hours from the first measurement.

5.4 The biofuel cell construction

Bilirubin oxidase exhibits, as shown, high activity and stability at neutral/physiological pH [54]. In a research from 2015 [114], Tasca and his co-workers found that the enzyme produced by Amano Enzymes Inc. shows high catalytic currents in the temperature range between 30 and 60 °C and pH in the range of 7 to 8.5, and it loses 50% of its activity at pH 9. The bilirubin oxidase used for this part of the thesis - devoted to the construction of an enzymatic biofuel cell - was provided by Novozymes, Bagsværd, Denmark. The activity of this enzyme at different pH values was tested and the largest catalytic current was observed for pH slightly below the neutrality (Figure 53). A glassy carbon electrode was modified
with 26 µg (40 µL, 0.7 mg·mL\(^{-1}\)\(_{\text{EtOH}}\)) of NaphtMWCNTs and 0.1 mg (10 µL of 10 mg·mL\(^{-1}\)) of MvBOD. After drying, it was immersed into the oxygenated McIlvaine buffer (0.2 M citric acid and 0.1 M Na\(_2\)HPO\(_4\)) of different pH values. The advantage of using this kind of buffer is the wide range of working pH.

![Voltammetric waves recorded using GCE modified with 26 µg of NaphtMWCNTs and 0.1 mg of MvBOD in McIlvaine buffer. The pH was adjusted by combining 0.1 M solution of Na\(_2\)HPO\(_4\) and 0.2 M citric acid solution.](image)

Figure 53 The pH dependence of voltammetric waves recorded using GCE modified with 26 µg of NaphtMWCNTs and 0.1 mg of MvBOD in McIlvaine buffer. The pH was adjusted by combining 0.1 M solution of Na\(_2\)HPO\(_4\) and 0.2 M citric acid solution.

From now on the buffer used in the BFC part was chosen as a compromise between the optimal conditions for the cathodic enzyme (pH slightly acidic) and for the anodic enzyme (presence of Ca\(^{2+}\) ions). Therefore the buffer for this part of the project will be: 0.1 M MOPS pH 6.5, 0.1 M NaCl, 0.05 M CaCl\(_2\).

### 5.4.1 Biobattery with MvBOD on the cathode and Zn/Hopeite anode

The first step in a BFC construction is the development of efficient cathode and anode. First of all, MWCNTs, pristine and naphthyl-functionalised, were used for the production of a cathode in a biobattery MvBOD vs. Zn. It is known from the electrochemical studies on the enzyme (paragraph 2.5.3.3) [135], that the reduction of dioxygen on a modified GCE in the presence of bilirubin oxidase appears at ca. 0.45 V vs. Ag/AgCl (KCl\(_{\text{sat.}}\)) while zinc redox potential for the process Zn/Zn\(^{2+}\) is E\(^o\) = -0.76 V vs. NHE [129]. The open cell voltage
Experimental

(OCV) for this biobattery is expected to be higher than 1 V. In order to improve the system performance, nanotubes were covalently modified with naphthalene groups (4.3.4). The cell parameters were determined as explained in 3.3.

Glassy carbon electrodes were covered with 10 µL of pristine or naphthyl carbon nanotubes solution (0.7 mg · mL$^{-1}$$_{EtOH}$). Once the electrodes were dry, 10 µL (0.1 mg, 10 mg·mL$^{-1}$) of MvBOD solution was dropped on them and left to dry. Electrodes were placed in the cell containing Ag/AgCl (KCl$_{sat}$) as reference electrode and a zinc plate, previously cleaned as explained in 4.3, as the anode. Electrolyte buffer (0.1 M MOPS pH 6.5, 0.1 M NaCl, 0.05 M CaCl$_2$) was deoxygenated with argon or oxygenated by purging the gas for 20 minutes.
before the measurement. The efficiencies of the biobatteries that had pristine or naphthyl-modified MWCNTs are compared in Figure 54. The maximum power density of 429 µW·cm⁻² at 550 mV was obtained for pristine nanotubes while slightly lower power of 403 µW·cm⁻² at 990 mV was obtained by using NaphtMWCNTs (Figure 54-b). The OCV for this biocathode was 1.46 V for both kinds of nanotubes (Figure 54-c). Constant monitoring of the semicell voltage versus a reference electrode shows directly which electrode determines the stability of the whole system. In the biobattery, the Zn anode is highly stable compared with the bilirubin oxidase-based cathode (Figure 54-d).

The cell efficiency strongly depends on the external dioxygen partial pressure. As expected, the current density is smaller when air saturated solutions are used instead of oxygen saturated, as shown in Figure 55 for the GCE/NaphtMWCNTs/MvBOD biocathode recorded during the work of the biobattery.

![Figure 55 Polarization curve of the biobattery consisting of Zn anode and GCE/ NaphtMWCNTs/MvBOD cathode under working conditions in air (empty squares) and oxygen (full squares) saturated buffer solution.](image)

### 5.4.2 Biofuel cell with MvBOD on the cathode and FDH on the anode

Once the optimal cathode was selected, the focus of the studies was on the bioanode. FDH, oxidising fructose, has been studied as the candidate for the anode biocatalyst. FDH was reported earlier to be a convenient catalyst at the anode in combination with laccase at the cathode [149]. Reticulated
Experimental

Vitreous carbon modified with ultra-small (1.8 nm) gold nanoparticles was used as electrode and power of 3.8 µW·cm⁻² as well as OCV of 740 mV were obtained.

The biocathode was prepared by modifying GCEs with 0.007 mg (10 µL, 0.7 mg·mL⁻¹EtOH) of NaphtMWCNTs and once dried, 10 µL of MvBOD (0.1 mg, 10 mg·mL⁻¹) was dropped on the electrode surface. The bioanode was made of glassy carbon electrode covered with 0.007 mg (10 µL, 0.7 mg·mL⁻¹EtOH) covered with 0.1 mg (10 µL, 10 mg·mL⁻¹) of FDH solution and left to dry at room temperature. Three electrodes arrangement was used with Ag/AgCl (KCl sat.) as reference electrode and the circuit was connected as shown in Figure 56-a. The buffer containing 100 mM fructose in 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂ was oxygenated for 20 minutes before starting the experiment. The BFC reached a maximum power density of 27 µW·cm⁻² at 336 mV and OCV was 620 mV (Figure 56-b). As showed in Figure 56-d the stability of the anode (blue line) has still to be improved.

![Figure 56 a) Schematic representation of the circuit consisting of MvBOD cathode and FDH anode. b) Dependence of power density on cell voltage and c) polarization curve for GCE/NaphtMWCNTs/MvBOD//GCE/MWCNTs/FDH cell. d) Current density vs. potential of the cathode (black line) and anode (blue line) monitored vs. Ag/AgCl (KCl sat.) reference electrode. GCEs modified with 0.007 mg of carbon nanotubes (pristine or NaphtMWCNTs) covered with](image-url)
0.1 mg of MvBOd and 0.1 mg of FDH. 100 mM fructose in oxygenated 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂.

5.4.3 Biofuel cell with MvBOd on the cathode and CdCDH on the anode

In a different type of the biofuel cell, the biocathode was prepared by modifying GCEs with 0.007 mg (10 µL, 0.7 mg·mL⁻¹ EtOH) of NaphtMWCNTs and once dried, 0.1 mg of MvBOD (10 µL of 10 mg·mL⁻¹) were dropped on the electrode surface. The bioanode was prepared using pristine MWCNTs (0.007 mg, 10 µL, 0.7 mg·mL⁻¹ EtOH) immobilised on GCE covered with 0.04 mg of CdCDH added in form of LCP layer (10 mg of LCP) (prepared as explained in section 4.3.1) or simply drop casted (4 µL, 10 mg·mL⁻¹) on the modified electrodes. The electrodes were placed in a cell and connected to a reference electrode, Ag/AgCl, (KCl sat.), as shown in Figure 57-a. The solution containing 100 mM lactose in 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂ was oxygenated for 20 minutes before measurements. The different behaviour of this BFC, comparing to that of the previous system, is shown below. When the enzyme is adsorbed on the bioanode power density of 0.41 µW·cm⁻² is obtained at 170 mV and OCV is 360 mV while for the same amount of enzyme entrapped in the lipidic cubic phase the maximal power density is more than three times larger and the OCV is larger (Pmax = 1.48 µW·cm⁻² at 324 mV, OCV 460 mV) (Figure 57).
5.4.4 Thin layer flow cells (TLFCL)

The knowledge gained until now concerning BFCs and lipidic cubic phase was used in a commercially available device developed by Dropsens (Asturias, Spain) (Figure 58-a). The cell resembles a conventional electrochemical cell with carbonous working (WE) and auxiliary (AUX) electrode and a reference (RF) made of silver (pseudoreference electrode). The diameter of the circular WE is 4 mm (area 0.12 cm$^2$) while the AUX has a bigger squared area of 0.20 cm$^2$. The cells are coated with a transparent cover that allows the
control of the sample volume by defining one thin channel (height 400 μm, and 100 μL of volume) where substrates solutions can flow over the electrodes. The cover’s transparency allows to check the formation of air bubbles inside the cell.

The changes of potential of each electrode during the work of biofuel cell were monitored against a pseudoreference Ag electrode present in the TLFCL - E = -0.071 V vs. Ag/AgCl (KCl sat.) [47,129].

Working (WE) and auxiliary electrodes (AUX) used in this system are screen-printed electrodes (SPEs) prepared using carbon ink. The WE acts as anode and it was modified as usually with 10 μL of MWCNTs (0.7 mg · mL⁻¹EtOH) and 0.04 mg of CtCDH either incorporated in LCP (10 mg of LCP made by 40 % of CtDH solution (10 mg·mL⁻¹) and 60 % of molten monoolein) or 4 μL of the same enzyme solution was directly drop casted at the WE. The biocathode was covered with 20 μL of NaphtMWCNTs (0.7 mg · mL⁻¹EtOH) and 10 μL of MvBOD (10 mg·mL⁻¹ solution). More naphthyl functionalised nanotubes were used because of the increased area of the AUX in this cell. The buffer - 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂ - contained 100 mM lactose and it was previously oxygenated for 20 minutes before it was flowed through the cell from anode to cathode with a stream rate of 1 mL·min⁻¹.

The observation that is visible at first look (in Figure 58), is the difference in OCVs when the two BFCs are compared: the cell with cubic phase has an OCV of ca. 540 mV compared to the other one whose value is less than 300 mV. Differences in maximal power densities are important, in case of the TLFCL with LCP a value of 2.22 μW·cm⁻² at 365 mV, compared to the 0.16 μW·cm⁻² at 135 mV for the adsorbed enzyme. It is also important to note (Figure 58-d) how the cubic phase helps the anode in terms of stability. The two cells are different only in the way the enzyme is immobilised on the electrode surface, therefore, it confirms that the cubic phase does not only increase the stability of the enzyme but it also helps to avoid the CtCDH to be flushed away with the flowing buffer stream.
Figure 58 a) Thin layer Flow Cell (TLFCL) with screen-printed electrodes, circular working electrode (DRP-TLFCL 110-CIR). Dependence of b) power density on cell voltage and c) polarization curve for NaphtMWCNTs/MvBOD/MWCNTs/CtCDH cell. d) Dependence of current density on potential of cathode (MvBOD – black line) and anode (CtCDH – green line) vs. Ag/AgCl (KCl sat.) reference electrode. SPEs modified with 0.007 mg of carbon nanotubes (pristine for CtCDH or NaphtMWCNTs for MvBOD) covered with 0.1 mg of MvBOD and 0.04 mg of CtCDH (drop casted ‘ads – empty circles –’ or hosted in lipidic cubic phase ‘LCP’ – full circles -). Flow rate 1 mL·min⁻¹. 100 mM lactose in oxygenated 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂.

Similar TLFC₇ were also tested in the presence of FDH as bioanode. The cells involved in this work are, as well, made by Dropsens (Asturias, Spain). They have a slightly different design in which WE and AUX electrodes are printed in parallel with the same area (0.12 cm²). Like before, they are made of carbon while the reference electrode is a silver wire. As usually, the biocathode was covered by 0.007 mg of NaphtMWCNTs (10 µL of 0.7 mg·mL⁻¹EtOH) and 0.1 mg of MvBOD (10 µL of 10 mg·mL⁻¹ solution). The bioanode was covered with 4.3 µL of graphene solution commercially available from Dropsens (DRP-GPHSOL) to create the same conditions of graphene modified SPEs (Figure 29). The
graphene solution was left in desiccator for 24 hours and successively 10 µL of FDH (10 mg·mL⁻¹) were casted on it and left to dry. In the meanwhile empty (i.e. without enzymes) LCP was prepared using 40 % of buffer (100 mM fructose in 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂) and 60 % of molten monoolein and left to equilibrate for 24 h. The characteristics of the experiments performed with the absence of flowing electrolyte, and in the LCP containing buffer and substrates which then acted as the liquid-crystalline medium instead of solution, are shown below. The electrodes are prepared as in previous experiments, but on top of them a layer of ca. 50 mg of empty LCP is smeared.

![Graphene solution](image)

**Figure 59** a) Thin layer Flow Cell (TLFCL) with screen-printed electrodes (DRP-TLFCL110). Dependence of b) power density on cell voltage and c) polarization curve for NaphtMWCNTs/MvBOD//GPH/FDH electrodes. d) Dependence of current density on potential of cathode (MvBOD -black line) and anode (FDH - green line) vs. Ag/AgCl (KCl sat.) reference electrode. SPEs modified with 0.007 mg of NaphtMWCNTs covered with 0.1 mg of MvBOD and 4.3 µL of graphene solution (GPH), covered with 0.1 mg of FDH. LCP contained 100 mM fructose in 0.1 M MOPS, pH 6.5, 0.1 M NaCl, 0.05 M CaCl₂.
From Figure 59 it is clear that when no enzymes are adsorbed on the electrodes there is no current flowing, while the maximal power density value of 1.05 µW·cm$^{-2}$ at 250 mV and OCV of approximately 400 mV are obtained when the enzymes are connected through the cubic phase layer.

Figure 60 shows the comparison of two identically prepared thin layer flow cells as described above (NaphtMWCNTs/MvBOD//GPH/FHDads), which were either immersed in the electrolyte solution or separated by the LCP medium that was used as the liquid crystalline supporting electrolyte. They have the same OCVs but the power density is higher for the one with the cubic phase, which keeps the enzyme close to the electrode surface preventing any leaking or desorbing of the enzyme into the solution as took place in the case of aqueous supporting electrolyte.

![Graph showing comparison of power density vs. potential](image)

**Figure 60**: Comparison of power density vs. potential for a TLFCL containing adsorbed FDH and aqueous supporting electrolyte under quiescent conditions (blue line) and a TLFCL containing adsorbed FDH covered with LCP containing fructose and electrolyte (black line). Cathode is composed of $M_v$BOD adsorbed on NaphtMWCNTs in both cases.
Concluding remarks and outlook

This dissertation describes the results of my studies on the redox enzymes adsorbed on the electrodes nanostructured with carbon nanotubes or hosted in lipidic cubic phase films placed on the electrodes. The best of them were used next for the construction of enzymatic biofuel cells.

The enzymes chosen for the anode were D-fructose dehydrogenase and Corynascus Thermophilus cellobiose dehydrogenase due to the negative potential at which they oxidised their substrates. For the cathode bilirubin oxidase from Myrothecium Verrucaria was chosen since it is known to reduce oxygen directly to water at positive potentials and can be used in solutions containing large concentration of chlorides as opposed to e.g. laccase.

Corynascus thermophilus cellobiose dehydrogenase (CtCDH) is studied in this work for the first time also in the environment of lyotropic lipid cubic phase. Immobilization in the cubic phase film at the electrode is shown to increase the enzyme stability and to improve catalytic performance of the electrode both when mediated and direct electron transfer mechanisms are operative. The lipidic cubic mesophase keeps the enzyme close to the electrode surface and improves the communication between enzymes and electrode surfaces, allowing easy diffusion of small substrates or mediators through the hydrophilic channels, and avoiding at the same time the leakage of the enzymes into the solution. For studies of mediated electron transfer, mediators with different formal potentials (E°') were employed: 2,6-dichlorophenolindophenol (DCPIP) and hexaaammineruthenium(II) chloride [Ru(NH₃)₆]Cl₂. These mediators are both electron acceptors from the dehydrogenase domain of the enzyme. [Ru(NH₃)₆]Cl₂, having the most negative E°' of -0.138 V vs. Ag/AgCl, gave a catalytic current density for lactose oxidation of 32.10 μA·cm⁻² in MOPS buffer at pH 7.5. The process carried out in the same solution but under direct electron transfer conditions resulted in a catalytic current density of 9.22 μA·cm⁻² hence mediation mechanism significantly improves the performance of the electrode. When DCPIP, which has more positive formal potential (E°' of 0.071 V vs. Ag/AgCl (KCl sat.)), was used as the soluble mediator the catalytic process was shifted to more positive potentials which is less favourable from the viewpoint of biofuel cell. The [Ru(NH₃)₆]Cl₂ complex is a suitable candidate for MET because of its lower formal potential but it should be kept in mind that
Concluding remarks and outlook

is also less stable in the presence of oxygen than DCPIP. An interesting observation was that using DCPIP dissolved in the solution both pathways of electron transfer could be detected: non-mediated process at more negative potentials corresponding to the direct electron transfer from the cytochrome domain and the DCPIP-mediated process. In general, mediators can be often toxic for the enzymes at longer timescale needed for biofuel cell operation, and they are often health hazards. Their usage in biofuel cells leads to lower open circuit voltage arising from the difference between the formal potential of the active site of the enzyme and that of the mediator. These factors especially stimulate studies on the direct electron transfer mechanism of enzymes. A DET based design simplifies also the construction of BFCs, since no membranes or compartments are necessary, which makes miniaturization easier.

The drawback of using solution soluble mediators was partially removed in this thesis by including the DCPIP in the cubic phase together with the enzyme and the mediated electrode transfer current density was found not significantly lower (ca. 8 μA·cm⁻²).

Electrodes covered with CtCDH hosted in a LCP film showed three times higher DET current density than those where the enzyme was drop-casted in both buffers studied (MOPS and PBS). Electrodes with CtCDH in the cubic phase retained their catalytic activity after 28 days showing a slightly increased current density after 6 days while for the adsorbed enzyme the activity dropped drastically after one week.

The presence of the enzymes (CtCDH and MvBOD) did not affect the cubic structure. It retains the Pn3m symmetry of the pure monoolein cubic phase. Both enzymes are partially included in the lipidic part of the mesophase with their hydrophilic side remaining in the water channels. The X-ray pattern obtained with different ions included in the phase indicated some changes in the electrostatic interactions while not altering the overall Pn3m symmetry. Namely, incorporation of sodium chloride in the lipidic cubic phase results in shrinking of the phase while calcium ions bridging between the lipid headgroups lead to a decrease of the radius of the aqueous channels. The unit cell size (the lattice parameter ℓ) was bigger in pure water and decreased in the presence of Mg²⁺ and Ca²⁺ (ℓ_{H₂O} > ℓ_{Mg²⁺} > ℓ_{Ca²⁺}) while the diameters of the channels changed in the opposite direction (ℓ_{H₂O} < ℓ_{Mg²⁺} < ℓ_{Ca²⁺}). Incorporation of gold nanoparticles of 5 nm diameter resulted in a phase change to a more open one (Ia3d symmetry). Such observations increase our knowledge on the lipidic cubic phases as new hosting materials for ions, molecules and nanoparticles.
The electrochemical behaviour of fructose dehydrogenase (FDH), is already known from previous studies. It is an excellent enzyme for the anode, both when the enzyme is adsorbed on the electrode surface and entrapped in the lipidic cubic phase. The drawback of this hydrophobic enzyme is that its molecular structure is still unknown, therefore, the in meso crystallization of FDH was attempted in the framework of this thesis. However, the obtained crystals were found to correspond only to small molecules – not identified as the whole protein. Further efforts in this direction would be needed especially because FDH is an important enzyme, highly catalytically active both when adsorbed on graphene, or carbon nanotubes. In this thesis the current densities of fructose oxidation reached approximately 83 µA·cm$^{-2}$ in direct electron transfer conditions and, therefore, the enzyme was used next in the enzymatic biofuel cell.

The redox enzyme employed in this thesis for the reduction of oxygen was bilirubin oxidase, $Mv$BOD. The catalytic performance of the electrode covered with this enzyme can be, as shown, improved by the synergic effect of naphthyl-functionalised MWCNTs and octanethiol modified gold nanoparticles forming a network on glassy carbon electrodes. The current density was then 650 µA·cm$^{-2}$, which is a large value for the direct electron transfer mechanism compared with the literature reports. The stability of the system was also improved when the enzyme and the nanoparticles were hosted together in the lipidic cubic phase film. The current density for the cubic phase film covered electrode was lower than in the case of enzyme simply adsorbed on the AuNPs/NaphtMWCNTs network at the electrode. On the other hand, 70 % of the dioxygen reduction current density was retained after ten days of work.

The knowledge gained through the experiments with the three enzymes was useful for the construction of a sugar/O$_2$ BFC. Either $Ct$CDH or FDH can be employed as the bioanode catalysts while $Mv$BOD is the catalyst on biocathode. The ability of the lipidic cubic phase to stabilize the systems in solutions of neutral pH makes it an enzyme-friendly matrix for the BFC application.

The BFC circuit proposed in our approach including the anode, cathode and a reference electrode allows to monitor the potential of each the electrodes against the reference electrode during the work of the BFC. Such design is not common in the biofuel cell literature and should be, therefore, advertised since it allows to determine which of the electrodes is limiting the performance of the whole biofuel cell under working conditions.
It is convenient to precede constructing the full biofuel cell by experiments performed in a hybrid BFC (sometimes called biobattery) based on a biological catalyst on the cathode and inorganic catalyst at the anode. In our case the zinc anode covered by hopeite introduced by Adam Heller was employed, due to its low and stable potential of -0.76 V vs. NHE. The best performance of such hybrid cell was obtained for the \( MvBOD \) biocathode nanostructured with naphthyl-functionalised MWCNTs instead of usually employed pristine MWCNTs and, therefore, this design of biocathode is proposed for the full biofuel cell construction with FDH or \( ClCDH \) at the bioanodes. \( ClCDH \) trapped in the lipidic cubic gained in stability and the power output increased more than three times compared with the biofuel cell based on carbon nanotubes adsorbed enzymes. Using the cubic phase under solution flow conditions, the flux of substrates towards the electrodes was maintained and the OCV was ca. 540 mV, better than that for the system with the enzymes adsorbed directly on the carbon nanotube-modified electrode surfaces, for which OCV was only 300 mV.

The liquid crystalline phase can be also used as the medium separating the electrodes instead of aqueous solution. FDH and \( MvBOD \) are in this novel design adsorbed on screen printed electrodes and covered with a LCP film. The power densities of the cell with the cubic phase between electrodes are larger than with the electrodes on which the enzymes are only physically adsorbed and aqueous solution of electrolytes flows between them. This is just the first design of a liquid crystalline biofuel cell, and a new approach proposed for the fuel cell preparation. It is important to note that it is shown here to work in case of commercially available small fuel cells and it could be easily further optimized and miniaturized.

The results of this thesis show that the electrodes with enzymes immobilized in the biocompatible lipidic cubic phase films are promising both for the applications as sugars or oxygen sensors and for the alternative fuel cell devices based on the utilization of bioelectrocatalytic reactions. Further development of this field may be expected in the coming years.
Summary

D-fructose dehydrogenase and Corynascus thermophilus cellulbiose dehydrogenase were studied as anodic enzymes and Myrothecium verrucaria bilirubin oxidase was used on biocathode. The enzymes were placed on the electrode surface by drop-casting on carbon nanotubes covered electrodes or immobilized in the liquid crystalline cubic phase film covering the carbonous electrode substrate.

CtCDH incorporated into the cubic phase was investigated under conditions of the direct and mediated electron transfer. In the latter case two mediators that interact with the enzyme flavin domain were employed giving catalytic current densities of lactose oxidation 26.77 and 32.10 μA·cm⁻² for DCPIP and [Ru(NH₃)₆]Cl₂, respectively.

The current densities of CtCDH oxidation were increased from 2.07 to 9.22 μA·cm⁻² by switching from drop casting on carbon nanotubes placed at the electrode to immobilization in the lipidic cubic phase film. The GCE/MWCNTs/CtCDH in lipidic cubic phase system showed activity for 28 days while GCE/MWCNTs/CtCDH_ads system activity dropped down after only 7 days. The increased current density of CtCDH oxidation in the LCP observed after one week of work was ascribed to partial degradation of the enzyme present in the aged film. The component obtained following degradation of the enzyme was found to play the role of mediator for the electron transfer process of the remaining intact enzyme similar to that of purposely added cyt c. Therefore, the process of lactose oxidation continued according to the mediated electron transfer mechanism in the aged film.

Fructose dehydrogenase was studied as the alternative enzyme for the anode and was found very stable and catalytically active both when adsorbed on graphene layer and on the MWCNTs. The former approach lead to current densities of fructose oxidation ca. 4 times larger.

The cathode was covered with bilirubin oxidase as the selected enzyme. The performance of the electrode was improved through the synergic effect of naphthyl-functionalised MWCNTs and thiolated gold nanoparticles placed on the electrode. The current density of oxygen reduction to water was high - reached 650 μA·cm⁻². Moreover, this enzyme incorporated into the cubic phase film retained 70 % of activity towards dioxygen reduction after ten days of work.
The NaphtMWCNTs/MvBOd biocathode was combined with FDH or CtCDH bioanodes to obtain the biofuel cell systems. The potential of each of the electrodes during the biofuel cell work was measured against the reference electrode. The biocathode potential was found more stable showing that the anode determined the overall performance of the biofuel cells. When cubic phase was used for the MWCNTs/CtCDH bioanode the power output of the BFC increased compared to that of the casted enzyme. The biofuel cell was investigated also under solution flow conditions. In the MvBOd/ FDH biofuel cell the enzymes placed in the cubic phase film remained on the electrode surface while the adsorbed enzymes were easily flushed away with the solution flow. The open circuit voltage was 540 mV. The lipidic cubic phase containing buffer and substrate was also tried in the liquid crystalline version of the graphene/FDH//NapthMWCNTs/MvBOD BFC. Its power density was ca. twice larger than using aqueous solution with substrates and adsorbed enzymes, moreover such design could be useful for further miniaturization and easier handling of the device.
References


References


References


References


References


[54] P. Ó Conghaile, M. Falk, D. MacAodha, M.E. YAKOVLEVA, C. Gonaus, C.K. Peterbauer, L. Gorton, S. Shleev, D. Leech, A fully enzymatic membrane-less glucose/oxygen fuel cell provides 0.275 mA cm–2 in 5 mM glucose operates in human


References


References


125
References


[117] U. Salaj-Kosla, S. Pöller, Y. Beyl, M.D. Scanlon, S. Beloshapkin, S. Shleev, W. Schuhmann, E. Magner, Direct electron transfer of bilirubin oxidase (Myrothecium


References


by cellobiose dehydrogenase in the presence of cytochrome c as mediator, Biochem. Soc. Trans. 28 (2000) 63–70. doi:10.1042/bst0280063.


