GENETICALLY MODIFIED YEAST CELLS AS A BASIS FOR BIOLOGICAL RECOGNITION AND SENSING. EVALUATION OF ENZYME-BASED AND CELL-BASED ELECTRON TRANSFER PATHWAYS

Dissertation

Submitted in fulfilment of the requirements for the award of the degree Doctor of Natural Sciences to the Faculty of Chemistry and Biochemistry Ruhr-Universität Bochum

by

Halyna Shkil

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Chair of the examination board: Prof., Dr. Bernhard Hovemann
First Examiner: Prof., Dr. Wolfgang Schuhmann
Second Examiner: Prof., Dr. Raphael Stoll
PUBLICATIONS

During the course of this project, a number of papers were published which are based on the work presented in this thesis. They are listed here for reference.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>AIBN</td>
<td>azobisisobutyronitrile</td>
</tr>
<tr>
<td>Ag/AgCl</td>
<td>silver/silver chloride reference electrode</td>
</tr>
<tr>
<td>AP</td>
<td>anodic electrodeposition polymer</td>
</tr>
<tr>
<td>BMA</td>
<td>butyl methacrylate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cat</td>
<td>catalase (EC 1.11.1.6)</td>
</tr>
<tr>
<td>CP</td>
<td>cathodic electrodeposition polymer</td>
</tr>
<tr>
<td>P4VPBMA</td>
<td>poly(4-vinylpiridine-co-butyl methacrylate)</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>cyt b₅</td>
<td>cytochrome b₅</td>
</tr>
<tr>
<td>cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenolindophenol</td>
</tr>
<tr>
<td>DET</td>
<td>direct electron transfer</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>2-(dimethylamino)ethylmethacrylate</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Committee</td>
</tr>
<tr>
<td>EDP</td>
<td>electrodeposition polymer</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FdDH</td>
<td>formaldehyde dehydrogenase (EC 1.2.1.46)</td>
</tr>
<tr>
<td>FDH</td>
<td>formate dehydrogenase (EC 1.2.1.2)</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FC $b_2$</td>
<td>flavocytochrome $b_2$ (EC 1.1.2.3)</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
</tr>
<tr>
<td>Glc-Lac</td>
<td>gluconolactone</td>
</tr>
<tr>
<td>GOx</td>
<td>glucose oxidase (EC 1.1.3.4)</td>
</tr>
<tr>
<td>HOEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>ICA</td>
<td>4(5)-imidazol-carboxaldehyde</td>
</tr>
<tr>
<td>IST</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>LDH</td>
<td>NAD$^+$-dependent lactate dehydrogenase (EC 1.1.1.27)</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>sodium L-lactate</td>
</tr>
<tr>
<td>LOD</td>
<td>lactate oxidase (EC 1.1.3.4)</td>
</tr>
<tr>
<td>MOSFET</td>
<td>metal-oxide-semiconductor field-effect transistor</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide reduced</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate reduced</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>pH-SFET</td>
<td>pH-sensitive field effect transistor</td>
</tr>
<tr>
<td>PMR</td>
<td>proton magnetic resonance</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>P4VP</td>
<td>poly(4-vinylpyridines)</td>
</tr>
<tr>
<td>RE</td>
<td>reference electrode</td>
</tr>
<tr>
<td>SAM</td>
<td>self-assembled monolayers</td>
</tr>
<tr>
<td>SECM</td>
<td>scanning electrochemical microscopy</td>
</tr>
<tr>
<td>STM</td>
<td>scanning tunneling microscopy</td>
</tr>
<tr>
<td>TMPD</td>
<td>N,N,N',N'-tetramethyl-p-phenylenediamine</td>
</tr>
<tr>
<td>tPMET</td>
<td>trans plasma membrane electron transport system</td>
</tr>
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</table>
1. INTRODUCTION

1.1. Biosensing

Biosensing is a process of detectable signal generation for monitoring or identification of a biological phenomenon [Schultz et al. 2006]. Although biosensing technologies have been investigated for nearly fifty years, they only came into active use in healthcare, environmental monitoring, the food industry and defence in the last two decades.

Biosensing devices often consist of three major components:

- biological element (enzymes, antibodies, nucleic acids, cell receptors, organelles, whole cells, tissues etc.);
- transducer (optical, piezoelectric, calorimetric, electrochemical etc.);
- signal processor and display system

![Fig. 1.1 The principle of biosensing](image)

Whereas the task of the biological element is recognition of a biological phenomenon or detection of a specific analyte, the role of the transducer is conversion of the signal received from the biological element into an easily measurable output, which is easily readable by means of a computer. This information may be qualitative or quantitative, and
can be used either for detection of an analyte or for measurement of its concentration. Biosensing devices can be classified according to:

- the type of biological element used
- the nature of communication between the biological element and the transducer
- the type of transducer

The objects of this study are enzymatic and cellular biological elements, and the mechanisms of signal transduction between these elements and the transducer.

An important step in the fabrication procedure is often reliable attachment of the biorecognition element on the surface of the transducer or in its vicinity so as to provide a high stability during operation without compromising the functions of the biological element. For this purpose a variety of immobilization techniques have been developed. These will be described in detail later.

Among all the available signal transducers, electrochemical transduction seems to be the most attractive considering its numerous commercial applications. Some advantages of electrochemical transduction include:

- precise control of the electrode potential resulting into selectivity of the analyte to be quantified;
- use of electrodes of any geometry and size which allows miniaturization;
- non-manual immobilization of biological material on the transducer surface by entrapment into electropolymerized or electrodeposited polymer layers, paving a way for automatization of the process [Knopf et al. 2007].

Depending on the operation principle, electrochemical biosensing can employ potentiometric, amperometric and impedimetric transducers by converting chemical information into a measurable electrical signal [Pohanka et al. 2008]. Of all electrochemical biosensors, amperometric biosensors are the most sensitive and more
suited for mass production than potentiometric or impedimetric biosensors [Ghindilis et al. 1998]. This study therefore focussed on amperometric biosensing.

1.1.1. Principles of amperometric biosensing

Modern amperometric biosensors work in a three-electrode configuration comprised of a reference, an auxiliary (also called counter) and a working electrode. Examples of standard and miniaturized electrochemical cells are shown in Fig. 1.2. A voltage is applied between the reference and the working electrode, and current flows between the working and the auxiliary electrode.

![Diagram of amperometric biosensor](image)

Fig. 1.2 Standard (on the left) and miniaturized (on the right) electrochemical cells [Odijk et al. 2009].

The working electrode may be a noble metal, graphite or glassy carbon, covered by a layer of the biorecognition element [Wang 1999] or carbon paste embedding the biological element [Cui et al. 2005]. The operation principle of amperometric biosensors
is based on measurement of a current, resulting from the catalytic reaction in the biorecognition layer once a potential difference is applied between the reference and the working electrode. [Mehrvar et al. 2004].

1.2. Biological elements
The biological elements that have been used in biosensing devices vary widely in type and include enzymes, antibodies, nucleic acids, cell receptors, organelles, whole cells and even tissues. All the above-mentioned biological macromolecules and systems are designed by the nature to carry out specific unique functions in organisms. Mainly these are recognition, catalysis and electron transfer. Biological systems are generally capable of performing complex reactions and processes much more efficiently than any artificial system designed using modern design and synthesis approaches to mimic them. Biological macromolecules and their complexes, as well as living cells, possess subsets of all the properties that are necessary to design intelligent sensors [Knopf et al. 2007]

1.2.1. Enzymes
Enzymes are proteins, catalysing biochemical reactions in living organisms. They are usually very specific to their substrates. This feature allows the enzyme “to recognize” one specific substance even it is mixed with other substances. A high selectivity of the enzymes to their substrates is the main reason for widespread application of these biocatalysts for biosensing.

1.2.1.1. Glucose oxidase
The first enzyme, implemented in amperometric biosensing was glucose oxidase (GOx, ß-D-glucose-oxygen1-oxidoreductase, EC 1.1.3.4). This enzyme was discovered in 1928 by Muller in Aspergillus niger and Penicillium glaucum [Muller 1928] and rediscovered later by Nord and Engel in Fusarium lini [Nord et al. 1938] and by Ogura in A. oryzae [Ogura 1939]. Glucose oxidase from Aspergillus niger is a dimeric protein, composed of
two identical subunits. Each subunit folds into two domains whereby one of the domains
binds to the substrate β-D-glucose while the other domain binds non-covalently to the
cofactor flavin adenine dinucleotide (FAD). The FAD molecules stabilize the three-
dimensional structure of the enzyme and are essential for the catalytic activity of GOx,
acting as initial electron acceptors.

Fig. 1.3 Molecular structure of glucose oxidase from Aspergillus niger [Wohlfahrt et al. 1999], [RCSB PDB Protein Data Bank1]

GOx is defined as an oxidoreductase that catalyzes the reaction in such a way that all the
electrons taken from the substrate (D-glucose) are transferred to O₂ to form H₂O₂.
Catalysis by this flavoprotein depends on the redox behaviour of its flavin group.
Glucose reduces the FAD to FADH₂ without the formation of free radical semiquinone
as an intermediate to produce gluconic acid. Subsequently, oxygen, the acceptor,
oxidizes the FADH₂ back to FAD and H₂O₂ is released as a product [Raba et al. 1995].
The enthalpy change associated with this reaction is sufficiently large to be detected
thermometrically [Wilson et al. 1992].
Fig. 1.4 Enzymatic reaction of glucose oxidation by the enzyme glucose oxidase

Therefore, the above-mentioned enzymatic reaction opened several ways for biosensing of glucose and determination of its concentration (Fig.1.4). Monitoring of oxygen reduction or hydrogen peroxide oxidation on an electrode form the basis for amperometric biosensors. pH changes due to H\(^+\) formation can be detected by means of a pH-electrode or MOSFET while the associated enthalpy change can be monitored using a thermistor. The first biosensor, introduced by Clark and Lyons at the New York Academy of Sciences Symposium in 1962 determined the concentration of glucose by the measurement of the change in oxygen concentration, or change in pH, following the catalytic oxidation of glucose by glucose oxidase [Clark et al. 1962]. In 1973, Guilbaut and Lubrano [Guilbault et al. 1973] described a biosensor for measurement of blood glucose based on amperometric (anodic) monitoring of the hydrogen peroxide. The latter type of biosensor was characterized by good accuracy and precision and the advantage of being simpler, allowing for miniaturization of biosensing devices. Electrochemical oxidation of hydrogen peroxide is usually carried out using a platinum electrode at a
potential of about +0.6 V (vs. Ag/AgCl) [Wang 2008]. The working principle of a glucose biosensor, based on monitoring of hydrogen peroxide is shown in Fig. 1.5.

![Glucose biosensor diagram](image)

**Fig. 1.5 Glucose biosensor, based on monitoring of hydrogen peroxide.**

This type of the biosensor was used as a model to study the influence of the composition of immobilization matrices on the characteristics of the biosensor as described in the Section 3.1.1.

### 1.2.1.2. NAD⁺- and glutathion-dependent formaldehyde dehydrogenase

Glutathione-dependent formaldehyde dehydrogenase (FdDH, formaldehyde dehydrogenase, EC 1.2.1.46), the enzyme of molecular weight in the range of 80 to 85 kDa, belongs to the family of oxidoreductases [Schutte et al. 1976], [Allais et al. 1983], [Van Dijken et al. 1976]. Formaldehyde dehydrogenase, found in both eukaryots and prokaryots, is the main enzyme of the formaldehyde detoxication system. This enzyme requires the cofactors glutathione and NAD⁺ for catalytic activity and catalyzes the oxidation of formaldehyde to S-formylglutathion [Gellissen et al. 2005].
Application of FdDH in biosensing is hindered by insufficient activity and high costs of commercial preparations of the enzyme, isolated from the wild-type strains of Pseudomonas putida and Candida boidinii [Demkiv et al. 2007], [Evangelista 2008]. To overcome these limitations Demkiv et al. constructed mutant strains of yeast Hansenula polymorpha, which over-produce a thermotolerant formaldehyde dehydrogenase [Demkiv et al. 2005]. The enzyme, isolated from genetically engineered Hansenula polymorpha yeast cells was used for the construction of a formaldehyde-selective biosensor, described in the Section 3.1.2.1.

1.2.2. Cells versus enzymes for biosensing and biorecognition

Enzymes are the most popular biological sensing elements used in fabrication of biosensing devices. A variety of enzyme-based biosensors has been described [Turner et al. 1992], [Mulchandani et al. 1998], [Tran 1993], [Mikkelsen et al. 2004]. Although purified enzymes have very high specificity to their substrates, a few drawbacks of their application in biosensing have been recognized and include among others:

- high cost of enzyme purification
- requirement of cofactors or coenzymes
- sensitivity to extremes of pH, temperature and to presence of oxidizing agents
- enzymatic degradation

Another drawback is in using an enzyme, one is limited by the function(s) of the available biological system dictated by nature. However, thanks to progress in genetic engineering, these functional limitations can be overcome by modern approaches, allowing for design of biological macromolecules with desirable properties. An example is the method of Directed Evolution [McGregor et al. 1999], [Walmsley et al. 2000], [Anthony 1982], which involves the “repeated sequence evolution of existing native proteins coupled to a criterion-based selection protocol” and aimed at evolution of proteins or RNA with unique, desirable and artificial properties not found in nature. Such approaches not only allow to improve the properties of existing enzymes but also to design biocatalysts with new functions and hence to overcome the limitations of nature’s design [Knopf et al. 2007].

Another possibility to conquer these drawbacks is to use whole cells instead of isolated enzymes as biological elements in biosensing devices [Mulchandani et al. 1998]. Once the enzymes are in their natural surrounding in the cell, they are less sensitive to environmental changes due to the intelligent property of cells to adapt to new conditions. Therefore, better stability of cell-based biosensors is expected. The co-existence of co-factors and co-enzymes excludes the necessity to design biosensors with complicated architectures incorporating several biomolecules. The only drawback of cell-based biosensors is lack of selectivity due to a huge amount of different enzymes coexisting in the cell. However, this drawback can nowadays be surmounted by gene-engineering techniques that do not only allow for improvement of the activity of an existing enzyme, but also to express foreign enzymes in a host cell [Belkin 2003].
1.2.3. Cells
Living cells possess intelligent properties that hold great promise in the future for incorporation of specific cell types into biosensors [Knopf et al. 2007]. Among the cell candidates, the most studied and also most suitable for biosensing purposes are microorganisms [Lei et al. 2006]. Being self-regulated organisms, the microbial cells rapidly grow on a variety of different substrates, can be easily manipulated and possess the ability to adapt fast to external conditions [Jain et al. 1994], [Leung et al. 1997], [Spain et al. 1987]. In spite of the fact that most of the developed cell-based biosensors use bacteria as sensing elements, there is a growing interest to use yeast cells for this purpose. This is due to favourable advantages of using yeast cells rather than bacteria in sensing applications. Parry [McGregor et al. 1999] pointed out the physical robustness of these organisms in comparison to bacteria, with wide tolerances to pH (3 to 9), temperature (-4°C to 40°C) and osmolarity. Walmsley and Keenan [Walmsley et al. 2000] mentioned additionally “eukaryotic advantage” of yeast cells, meaning a closer relation of these organisms to human than that of prokaryotic bacteria. Furthermore, about 30% of the genes involved in human diseases have functional homologues in yeasts [Foury 1997]. This makes yeasts to model organisms for studying mammalian cells [Magera et al. 2005]. This is particularly important in estimation of the toxicity of some compounds to humans or in investigations of metabolic pathways in cells. Baronian [Baronian 2004] documented in his review about 10 different yeast species, implemented for biosensing. In this study, attention was focussed on methylotrophic yeast *Hansenula polymorpha*, to explore some of the biosensing challenges of genetically engineered organisms.

1.2.3.1. Methylotrophic yeast *Hansenula polymorpha*
Unconventional yeasts are important model eukaryotic organisms in the investigation of molecular mechanisms of living processes and at the same time prospective biotechnological objects. Different from baker’s yeast, they have been less studied in
genetic and biochemical aspects but have unique metabolic properties. For example, they are able to consume unconventional organic substrates and to over-produce a lot of important biologically active compounds, they contain specific enzymes and are able to adapt themselves to a variety of toxic compounds and to detoxicate them [Anthony 1982], [Sahm 1977], [Sahm et al. 1973], [Woodward 1989]. The availability of the methods of classic mutagenesis, gene-engineering manipulations, make it possible to modify the metabolic pathways of yeast cells thereby allowing for construction of new yeast strains with modified physio-biochemical characteristics, which are very valuable for industrial applications of microorganisms. Cells of microorganisms are the primary source of enzymes, which have became an irreplaceable instrument of modern analytical biotechnology, which combines the inventions of analytical biochemistry, enzymology, immunology, gene engineering and electronics. This has enabled the creation of a range of attractive, fast and inexpensive methods for detection and analysis of different substances in products, in the environment and for quality control in technological processes. In the last ten years, methylotrophic yeasts became an important source of biologically active compounds: low molecular metabolites, enzymes, heterologous proteins – products of expression of allogeneic genes, inserted into these organisms by methods of gene engineering.

Most methylotrophic eukaryotes are single-cell fungi, which belong to four genera: *Candida, Hansenula, Pichia* and *Torulopsis*. The last genus is often combined with the genus *Candida* and methylotrophic species of genus *Hansenula* have recently been merged with the genus *Pichia* [Kurtzman 1984]. Among approximately 600 genera of yeasts known today, about 50 of them are able to consume methanol [Goldberg et al. 1991]. The typical natural habitat of methylotrophic yeasts are soils, flowers, leaves, tree sap and cortex, spoiled vegetables and fruits. This ecological niche is characterized by the presence of pectins and lignins which are complex polymers containing methoxyl groups. Through their hydrolysis, methanol is formed, which is used as a source of carbon for methylotrophic yeasts. All methylotrophic yeasts belong to the facultative
methylotrophes, because they can also grow on polycarbonate compounds. Fermentation is not common for methylotrophic yeasts and it takes place only to a very small degree in the absence of oxygen. These yeasts are petite-negative and glucose does not repress its respiration [Spencer et al. 1997], [Zitomer et al. 1992]. Species of the Candida and Torulopsis genera are asporogenous, whereas Hansenula and Pichia species have sexual reproductive cycles and can be directly manipulated by genetic engineering techniques [Spencer et al. 1997], [Verseveld et al. 1987]. The three most genetically studied yeast species are: Pichia methanolica (pinus), Pichia pastoris and Pichia angusta (another name of Hansenula polymorpha). They are essentially haplodiploid organisms, but as a rule, haploids may be found in nature. Every strain forms three types of cells: two haploid and one diploid, which can undergo meiosis. The cell cycle of Hansenula polymorpha is very similar to that of Saccharomyces cerevisiae. Diploid cells of Hansenula polymorpha are stable in nutrient-rich media, but once switched from nutrient-rich to nutrient-poor conditions, they start producing a variety of haploid spores, which can go on to mate (conjugate), reforming the diploid.

Hansenula polymorpha is a thermotolerant organism with an optimum temperature of 37 °C. This is not common for methylotrophic yeasts, for which the optimum temperature is 28–30 °C [Anthony 1982]. Special interest in Hansenula over the last years can be explained by three particular physiological peculiarities of methanol metabolism in this type of yeasts:

1. fast growth on methanol as the only carbon and energy source [Levine et al. 1973];

2. a simple transition from diploid to haploid [Teunisson et al. 1960], [Kurtzman 1984];

3. expression of large quantities of the key enzymes in methanol metabolism (alcohol oxidase), which can make up to one-third of the whole amount of cell proteins.
1.2.3.2. L-Lactate assimilation in yeast cells

Some yeast species are capable to use lactic acid as an energy and carbon source. Nevertheless, this process has not been studied in details. The first step of lactate assimilation in yeast cells is the transport of this substrate into the cell [Brooks 2002].

The mechanisms of lactic acid transport vary among different species. *Candida utilis*, *Torulaspora delbruesckii* and *Saccharomyces cerevisiae* transport lactate into the cell using lactate-proton symport mechanism. *Kluyveromyces marxianus* perform lactate transport via a uniport mechanism. *Saccharomyces cerevisiae* is capable to use short-chain monocarboxylic acids as the only carbon and energy source under aerobic conditions. The first step for metabolism of these substrates is their transport through the plasma membrane.

For the transport of lactate into the cell, an integrated membrane protein lactate permease, encoded by the gene *JEN1* is responsible. Its absence hinders yeasts from consuming lactate. Symporter Jen1p is specifically induced by lactate and is responsible for transport of lactate, pyruvate, acetate and propionate into the cell [Branduardi et al. 2006], [Casal et al. 1999]. The activity of *JEN1* promoter is induced by glucose concentrations lower than 3 g/l and is maximum at an approximate concentration of 0.1 g/l. [Casal et al. 1999], [Lodi et al. 2002].

Lactate assimilation in most yeasts occurs through selective oxidation of L- and D-lactate that is catalysed by mitochondrial L-lactate:ferricytochrome c oxidoreductase (EC 1.1.2.3; flavocytochrome *b*<sub>2</sub>, FC *b*<sub>2</sub>) and D-lactate:ferricytochrome c-oxidoreductase (EC 1.1.2.4). These proteins are encoded by genes *CYB2* and *DLD1*, respectively.

1.2.3.3. Flavocytochrome *b*<sub>2</sub> (FC *b*<sub>2</sub>)

Cytochromes are heme containing electron transport proteins, which participate in the process of energy conversion for ATP synthesis during the oxidation of metabolites and photosynthesis. They have been isolated from cells of different organisms, including bacteria and higher organisms. All cytochromes contain protoheme IX or its derivatives
and are capable of reversible change of the oxidation state of heme iron between +2 and +3. All these proteins have at least one histidine residue attached to the iron atom, however, the type of attachment, and the nature of the sixth ligand vary. The redox potential for conversion between the +2 and +3 oxidation states of iron depends strongly on the proteine environment and pH.

Flavocytochromes b$_2$ or L-lactate:ferricytochrome c-oxidoreductase (EC 1.1.2.3) is 2-hydroxyacid dehydrogenase found in the intermembrane space of yeast mitochondria where it couples oxidation of the substrate to reduction of cytochrome c [Holzenburg 2000]. Nowadays there is no information on FC b$_2$ from Hansenula polymorpha yeast. At the same time the physical-chemical properties of this enzyme from baker’s yeast Saccharomyces cerevisiae and yeast Hansenula anomala are known [Ghrir et al. 1984], [Haumont et al. 1987], [Silvestrini et al. 1993].

Flavoytochrome b$_2$ is an essential component of the respiratory chain in yeast cells, which combines the functions of two different enzymes, L-lactate dehydrogenase and cytochrome b. In addition to providing pyruvate for the Krebs cycle, it participates in a shorter respiratory chain, directing the energy gained from L-lactate dehydrogenation (Fig. 1.7). Its production in the cell is induced by the presence of oxygen and L-lactate [Holzenburg 2000].
The peculiarity of this enzyme is its absolute specificity as an electron donor to the stereoconfiguration of a substrate, and its non-selectivity towards an electron acceptor. For instance, ferricytochrome $c$, ferricyanide, methylene blue, and 2,6-dichlorophenolindophenol to name a few are capable of accepting electrons from flavocytochrome $b_2$. Heavy metals, oxygen [Appleby et al. 1959], [Armstrong et al. 1963], as well as glycerate, oxalate, malate, fluorpyruvate [Urban et al. 1988], bromopyruvate [Mulet et al. 1977], phenylglyoxal [Ghrirt et al. 1984], phenylpyruvate and fatty acids [Appleby et al. 1959], [Nygaard 1963] can act as inhibitors for FC $b_2$.

Molecular weight (MW) of flavocytochrome $b_2$ is approximately 235 kDa. FC $b_2$ is homotetramer, and each of its subunits contains one polypeptide chain, one molecule of flavomononucleotide (FMN) and protoheme IX (Fig. 1.8) [Jacq et al. 1974], [Jacq et al. 1970], [Jacq et al. 1972], [Lederer 1974]. FC $b_2$ subunits consist of two covalently bound domains with two different functions: L-lactate dehydrogenase and cytochrome $c$-reductase domains. Structural analysis of the L-lactate dehydrogenase domain (MW 39 kDa) showed that it does not contain a heme but rather a FMN. This domain has 70 % of
ferrireductase activity of the holoenzyme, but it does not exhibit any cytochrome c reductase activity [Celerier et al. 1989], [Haumont et al. 1987], [White et al. 1993].

![Molecular structure of flavocytochrome b2 from Saccharomyces cerevisiae](attachment:image.png)

**Fig. 1.8** Molecular structure of flavocytochrome $b_2$ from *Saccharomyces cerevisiae* [Xia et al. 1990], [RCSB PDB Protein Data Bank3]

At present, not much is known about the mechanisms of the different steps of electron transfer from FMN to heme, although interest to gain its understanding has lately been actively investigated using modern methods such as fluorescence spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, proton magnetic resonance (PMR) spectroscopy and radioisotope analysis. The transfer of electrons in the catalytic cycle of FC $b_2$ occurs in the direction from L-lactate through flavin to heme with its subsequent reduction comprising several steps (Fig. 1.9) [Capeillere-Blandin 1995], [Sharp et al. 1996], [Tegoni et al. 1990]:

1) lactate oxidation to pyruvate, followed by FMN reduction;
2) transfer of one electron from the completely reduced FMN to heme, followed by subsequent formation of FMN semiquinone and heme reduction;
3) reduction of the first molecule of cytochrome during the transfer of the electron from the heme group of FC \( b_2 \);
4) transfer of the second electron from the FMN semiquinone to heme resulting in complete oxidation of FMN and heme reduction;
5) reduction of the next cytochrome \( c \) molecule by the electron, transferred from the heme, followed by complete oxidation of the enzyme.

Fig. 1.9 Catalytic cycle of FC \( b_2 \) [Rouviere et al. 1997]

F – FMN; H – heme; \( F_{sq} \) – semiquinone form of FMN

Even the heme doubtless takes part in the electron transfer to cytochrome \( c \), in the reaction with the ferricyanide (as electron acceptor) the electron transfer occurs without heme involvement.

1.2.3.4. Genetic regulation of FC \( b_2 \) synthesis in yeasts

Genetic regulation of lactate utilization in yeasts has not been thoroughly studied. Moreover, the only available information focuses on baker’s yeast. It should be noticed that regulation of FC \( b_2 \) synthesis is thought to be a convenient model for the study of mitochondrial biogenesis, which depends on concerted expression of genes located in the mitochondria and the nucleus.
In _Saccharomyces cerevisiae_, L-lactate:ferricytochrome c-oxidoreductase is localized in the intermembrane space of mitochondria and encoded by the gene _CYB2_. FC \( b_2 \) is synthesized in the cytoplasm and is imported into the intermembrane space of mitochondria. In haploid yeast cells, there is only one copy of gene _CYB2_.

Expression of the gene _CYB2_ in the nucleus of _Saccharomyces cerevisiae_ is an object of strict metabolic control at the transcriptional level. The following phenomena have been observed: a) repression of the enzyme synthesis by glucose during fermentation; b) derepression/induction once the cells are grown on non-fermentable substrates, i.e. ethanol or glycerol; c) induction by the main substrate for FC \( b_2 \) – L-lactate; d) depression of expression under anaerobic conditions, or as a consequence of defectiveness of the gene responsible for heme synthesis [Nygaard 1961].

Baker’s yeast cells grown under aerobic conditions produce two different lactate cytochrome c oxidoreductases, the first one is specific to L(+)-lactate and the second one to D(-)-lactate. None of these enzymes is synthesized under anaerobic conditions. The influence of aeration on the activity of D(-)- and L(+)-lactate cytochrome c-oxidoreductase in the yeast cultures is the same. Expression of D-lactate cytochrome c-reductase is induced faster than for L-lactate:cytochrome c-oxidoreductase. However, the concentration of L(+)-lactate:cytochrome c-oxidoreductase after the lag phase is two times higher than that of D(-)-lactate:cytochrome c-oxidoreductase.

The heme group plays an important role in the regulation of FC \( b_2 \) biosynthesis. It is well known that the heme plays the role of a specific messenger in O\(_2\)-sensitive global systems of regulatory signal transduction. Heme-dependent regulation is modulated by an activating factor Hap1p, the transcriptional activity of which is directly regulated by the cofactor bound to the heme. Hap1p is the main transcription factor involved in the positive regulation of _CYB2_ transcription through its interaction with two promotor binding sites of the FC \( b_2 \) gene. Hap1p probably binds to two oppositely oriented regulatory sites of the FC \( b_2 \) gene promotor: _UASIA_ (–243/–218) and _UASIB_ (–443/–418).
CYB2 expression is fully inhibited in glucose-grown cells and is independent of oxygen availability. FC b2 is also not synthesized once the cells of baker’s yeast are grown on galactose and raffinose. It was shown that in the mutant Δhxk2 with inactivated gene of negative regulator in glucose repression – hexokinase II (H XK2) the FC b2 repression by glucose is relieved. However, it is apparent that regulation of CYB2 gene expression by carbon sources occurs by many different mechanisms, including that with complex Hap2/3/4/5p involvement, relieving a negative influence of hypothetic repressor, binding to URS-element, on the transcription of CYB2 gene.

As for other proteins, which are imported into mitochondria, for FC b2 synthesis relief from glucose repression, a full heterometric complex Hap2/3/4/5p is required. For instance, deletion of the HAP2 gene leads to disturbance of FC b2 expression.

Deletion analysis of the gene CYB2 promotor has shown that most of cis-regulatory elements are located between the positions –446/–90. Transcription start site of the gene CYB2 is in the position –44/–34. Experiments on transformation of yeast cells using plasmids containing the β-galactosidase gene under the control of different incomplete promoters of the gene CYB2, followed by analysis of the β-galactosidase activity of recombinant cells have shown that the resulting activity strongly depends on the mutations in the genes HAP1, HAP2, HAP3 and HAP4 of the recipient cells and the type of mutation in the analysed promoter.

Analysis of the nucleotide sequence of the gene CYB2 promotor uncovered that the pentanucleotide repeat CCATT (–187/–183), is often located in the middle of the binding sites of the complex Hap2/3/4/5p in a number of genes. Deletion of the –200/–137 fragment strongly enhances the expression of β-galactosidase in hybrid plasmids inserted into the cells of the wild type but not of the mutant hap1. A shorter deletion in the region –170/–137 does not break the dependence of β-galactosidase activity on the presence of the Hap1p protein in the cell, but absolutely relieves the dependence of expression of the reporter gene on the presence or absence of the Hap2/3/4/5p heterocomplex. The activity of β-galactosidase in the mutants hap2, hap3 and hap4 is
almost as high as in the wild cells for growth under the conditions of derepression. This supports the idea that the complex Hap2/3/4/5p is not a direct transcriptional activator of the gene CYB2 but relieves the effect of hypothetical repressor, which binds to the negative cis-element located in the region \(-170/–137\). The protein Hap4p is probably the limiting factor for the synthesis of the Hap2/3/4/5p activator complex and repression of CYB2 gene expression by high glucose concentrations is explained by low levels of Hap4p. The repressive effect of glucose on the synthesis of FC \(b_2\) in wild type cells decreases significantly in case of overexpression of this protein under the control of a strong constitutive promoter. The absence of the above-mentioned effect in the mutant \(hap1\), led to the conclusion that the protein Hap1p is essential for optimal functioning of the Hap2/3/4/5p complex. The mechanism of such a synergy is unknown.

Analysis of the nucleotide sequence in the position \(-137/–112\) of the regulatory region of the gene CYB2 uncovered regions very similar to the binding site of the regulatory protein Adr1p, which is involved in derepression of the glucose-repressive gene ADH2 and coordinative control of the transcription of different genes by the carbon sources in baker’s yeast. Based on the experimental data, it was concluded that the transcriptional activator Adr1p, is also essential for transcriptional regulation of the gene CYB2. Adr1p binding site of the gene CYB2 is localized 8 bp higher than the TATA box and consists of two sub-sites having opposite orientations, separated by 8 bp long ITS. Repression of Adr1p protein synthesis takes place if the cells are grown on 5 % glucose, which negatively influences expression of the gene CYB2. The mechanism of gene activation by the transcription factor Adr1p is unknown but it is supposed that it mediates chromatin rearrangement [Ramil et al. 2000]. A schematic model of the regulation of CYB2 gene expression in baker’s yeast is represented in Fig. 1.10.
Fig. 1.10 Schematic model of the regulation of CYB2 gene expression by the transcription factors Hap1p, Hap2/3/4/5p and Adr1p. Adapted from [Ramil et al. 2000]. (A) – glucose repression, (B) – depression (when the cells are grown on a mixture of ethanol and lactate).

1.2.3.5. Genetic regulation of L-lactate assimilation at high temperatures

It was found out that L-lactate assimilation at high temperatures depends on the gene CPR3, which encodes the protein cyclophilin. Cyclophilins are eukaryotic proteins firstly identified thanks to their high affinity to cyclosporin A. The physiological role of cyclophilins is unknown. *In vitro* cyclophilins have peptidyl-prolyl cis-trans isomerase activity (PPI), and therefore could be responsible for proteins assembling *in vivo*. A yeast cyclophilin gene, CPR3, hypothetically encoding a presumptive mitochondrial isoform
has been isolated. Other cyclophilin genes such as CPR1, CPR2 and gene FPR1, encoding FK506-binding protein with PPI-activity have also been discovered.

Davis et al. constructed and investigated *Saccharomyces cerevisiae* mutants with damaged CPR1, CPR2, CPR3 and FPR1 genes. All the mutants were grown under different conditions on an YPD medium, containing glucose, galactose, glycerin, pyruvate or lactate as the only carbon source. Petri dishes were incubated at 30 °C and 37 °C. All the mutants grew well on all the carbon sources at 30 °C. At 37 °C, the mutant cpr3 was not able to grow on L-lactate at all whereas on pyruvate its growth was depressed. Mutants with damaged CPR1, CPR2 and FPR1 genes grew well at 37 °C. Restoration of the cpr3-genotype revitalized the capability of the yeast cells to grow on L-lactate and pyruvate at 37 °C [Davis et al. 1992]. Since the growth of cpr3-mutants is depressed by pyruvate and inhibited by L-lactate at 37 °C, one can suppose that the product of the gene CPR3 could be involved in L-lactate methabolism on the level of it’s transport into the mitochondrial intermembrane space. However, the capability of cpr3-mutants to grow on glycerol and pyruvate proves that the product of the gene CPR3 is not necessarily important for the normal functioning of yeast mitochondria. To clarify whether the product of the gene CPR3 influences induction of the gene CYB2, encoding FC b₂, Davis et al. investigated the induction of CYB2 mRNA synthesis in wild-type and recombinant strains. Both strains were found to produce a stable mRNA of the gene CYB2, which is evidence for the absence of any influence of the product of the gene CPR3 on the induction of CYB2. As the product of CPR3 does not directly influence the gene CYB2, the only probable assumption is that the function of the gene CPR3 has something to do with maturation or posttranslational stabilization of FC b₂. Most probably, the product of the gene CPR3 participates in assembly and conformational changes of the FC b₂ precursor during its translocation into the mitochondrial intermembrane space [Davis et al. 1992].
1.2.3.6. Application of FC $b_2$ and yeast cells for L-lactate biosensing

Flavocytochrome $b_2$, thanks to its unique catalytic properties (absolute specificity to L-lactate and no necessity of exogenous cofactors) has a good prospect to substitute other enzymes in L-lactate biosensing devices. The reasons that prevent FC $b_2$ from wide application include poor stability and also the complicated procedure of its isolation from cells due to the high lability of this enzyme [Labeyrie et al. 1978]. The only real applications of the enzyme FC $b_2$ in biosensing were reported by Amine et al. [Amine et al. 1994] and Smutok et al [Smutok et al. 2005].

However the possibility to use intact cells of *Hansenula anomala* [Kulys et al. 1992], [Racek et al. 1987], [Racek et al. 1987] and *Saccharomyces cerevisiae* [Garjonyte et al. 2003], [Garjonyte et al. 2006], [Garjonyte et al. 2009] as biorecognition elements in the biosensors for L-lactate has been demonstrated. To provide a better communication of intracellular enzyme with the electrode a variety of free-diffusing mediators are used: potassium ferricyanide [Racek et al. 1987], [Racek et al. 1987], [Garjonyte et al. 2008], [Garjonyte et al. 2009]; Meldola Blue, Prussian Blue [Garjonyte et al. 2003], 2,6-dichlorophenolindophenolsodium salt hydrate [Garjonyte et al. 2008], phenazine methosulfate, 1,2-naphthoquinene-4-sulfonic acid sodium salt, p-bentoquinone [Garjonyte et al. 2009], [Garjonyte et al. 2008].

For the described sensors based on *Saccharomyces cerevisiae* cells the estimated $K_{M}^{\text{app}}$ value towards L-lactate was in the range of 1 mM to 2.45 mM, a maximum current value $I_{\text{max}}^{\text{app}}$ was 413 nA, the time for the measurement to reach complete saturation was 30 s and a linearity of up to 1 mM L-lactate. Some disadvantages of the constructed biosensors included a long stabilization time of the working electrodes (30-45 min with continuous mixing), low stability of the bioelectrodes with a half-life of the sensor being about 24 hours in a buffer and poor reproducibility [Garjonyte et al. 2003].

In the case of *Hansenula anomala* cells, $I_{\text{max}}^{\text{app}}$ was 1.2 µA, stabilization of the sensor was observed 2 min after addition of an analyte, the linear range for L-lactate was 1 mM. The biosensor was characterized by a good storage stability of about 4 weeks but
was not selective enough and showed interference for glucose, urea and other analytes [Racek et al. 1987]. Therefore, alternative biorecognition elements for L-lactate biosensing should be investigated.

**1.2.4. Genetically engineered cells**

Improved methods in genetic engineering allow for manipulation of cells at the molecular level and to design them rationally for biosensing purposes with enhanced performance. The ability to manipulate genetic mechanisms, which control cell functions and to tailor the cell toward analytes of interest is quite advanced. Engineered whole cells deployed in biosensor systems can be considered one of the practical successes of molecular-scale devices [Knopf et al. 2007]. Genetically engineered whole cells as biorecognition elements in biosensors have been employed in the past two decades for detection of a variety of analytes [King et al. 1990].

Genetically modified sensing systems are often developed by introducing a plasmid construct with a bioluminescent, chemiluminescent or fluorescent reporter gene fused to a promoter (in prokaryotic cells), or coupled with a receptor (in eukaryotic cells), which is respectively induced or activated by a target analyte (Fig 1.11).

![Fig. 1.11 Principle of biosensing using genetically-modified cells involving reporter genes. Adapted from [Daunert et al. 2000].](image-url)
The most commonly used reporter proteins include luminescent proteins such as bacterial and firefly luciferases, green fluorescent protein and its variants, and β-galactosidase. The analytes that can be detected using genetically manipulated cell biosensing devices include toxicants, cell stress factors, and specific analytes such as metals, metalloids, organic pollutants, sugars, drugs, and bacterial signalling molecules. Daunert et al. divided biosensing systems involving reporter genes into three classes namely: non-specific sensing systems for detection of toxins and carcinogens, specific sensing systems that employ metal resistance for detection of toxic heavy metals, and sensing systems based on cellular metabolism for detection of organic compounds such as sugars [Daunert et al. 2000]. Such biosensing systems have a great potential for applications in biotechnology, pharmacology, medicine and environmental monitoring [Zourob et al. 2009].

Another way to improve the characteristics of cells as biorecognition elements is to modify their metabolic pathways, often resulting into new properties which are not inherent to the native cells and which can also be used for biosensing. This is due to the complexity and interrelation of cell metabolic pathways, controlled by numerous regulatory mechanisms and the ability of the cells to adjust their metabolism to the changes. Gonchar et al. for example reported how changes in metabolism of Hansenula polymorpha yeast cells made them suitable for construction of novel biosensors (Fig. 1.12). For instance, the cells of the mutant strain 34-19, due to a formate dehydrogenase (FDH) defect, produce an enhanced acidifying output in the presence of methanol or formaldehyde, and were successfully implemented for the construction of potentiometric (based on pH-sensitive field effect transistors, pH-SFET) biosensors for detection of the above-mentioned substances [Gonchar et al. 2002]. Introducing a defect into alcohol induction in the cells of the mutant strain A3-11 resulted into disability of the cells to oxidize methanol. These cells were the basis for construction of a pH-SFET sensor, selective to formaldehyde [Gonchar et al. 2002]. The incapability of the cells of the mutant strain C-105 to decompose hydrogen peroxide inside the cell due to defective
catalase (Cat), led to the development of an extrusion mechanism for this electrochemically active compound, which can be easily detected, allowing quantification of the amount of converted methanol or ethanol. This phenomenon was used for construction of novel amperometric biosensor for alcohols [Gonchar et al. 1998], [Gonchar et al. 2002].

Fig. 1.12 Modification of methanol metabolism in the Hansenula polymorpha yeast cells for biosensing purposes.

Often researchers investigating cellular biochemistry discover such side effects of altered cell metabolism. Nevertheless, such studies also contribute to the development of new sensing systems [Sakaki et al. 2002].

Another strategy that allows improvement of cellular biorecognition elements is the overexpression of a gene, which is responsible for the synthesis of an enzyme of interest in the cell. Formally, gene overexpression was used to study the function of the gene in a cell but nowadays it is also essential in microbial biofactories to increase their productivity. Gene overexpression is defined as using a strong promoter and
neighbouring upstream activation sequences to drive high level and essentially constitutive transcription of a gene’s coding sequence, resulting in high steady state protein levels [Grotewold et al. 2003]. In an ideal case, gene overexpression leads to overproduction of a target protein and its increased content in the cell. However, one has to always keep in mind that a cell is a very complex system, and lack of relevant knowledge about the regulation mechanisms of specific protein syntheses and sorting can lead to negative or unexpected results [Snoep et al. 1995]. For instance, overexpression of the gene ADH2, that encodes the alcohol dehydrogenase isoenzyme II (EC 1.1.1.1), which is involved in ethanol metabolism in wine yeast *Saccharomyces bayanus*, does not influence the protein expression profile and has no effect on the production of the metabolites of glycolysis and alcoholic fermentation such as ethanol, acetaldehyde, and glycerol. However, it indirectly affects glucose and ammonium uptake, cell growth, and the intracellular redox potential, which lead to an altered metabolome [Maestre et al. 2008]. Alteration of expression levels of the enzymes, evolved in CO₂ fixation in *Escherichia coli* cells showed that overexpression of pyruvate carboxylase, phosphoenolpyruvate carboxylase and malic enzyme enhances succinate production, whereas overexpression of phosphoenolpyruvate carboxykinase had no detectable effect on the cell metabolism [Kim et al. 2004].

An interesting approach in genetic modification of cells was introduced by Francisco et al in 1991. They constructed a gene fusion system consisting of the signal sequence and first nine amino acids of the lipoprotein (Lpp), joined to a transmembrane domain from outer membrane protein A (OmpA) and the complete mature β-lactamase (EC 3.5.2.6) sequence. *Escherichia coli* cells, containing the plasmids with above mentioned gene fusion system were able to transport the soluble protein β-lactamase to and anchor on the outer cell membrane [Francisco et al. 1982]. Richins et al used the Lpp-OmpA gene fusion system described above to anchor organophosphorus hydrolases on the surface of *Escherichia coli* cells and suggested that immobilization of these cells onto solid supports could provide an attractive means for pesticide detoxification
[Richins et al. 1997]. Further work by the same group demonstrated successful extracellular anchoring of organophosphorus hydrolases in Moraxella sp., Pseudomonas putida JS 444 and Cyanobacteria [Chungjatupornchai et al. 2008], [Shimazu et al. 2001], [Lei et al. 2005]. Recombinant cells of Escherichia coli, Moraxella sp. and Pseudomonas putida JS 444 were used for construction of biosensors for organophosphorous pesticides detection [Mulchandani et al. 2006], [Lei et al. 2005], [Lei et al. 2005], [Mulchandani et al. 1998], [Mulchandani et al. 2001]. Zhang et al. using a similar procedure performed anchoring of another enzyme carboxylesterase from Mosquito on the outer membrane of Escherichia coli cells, demonstrating the universality of the described approach [Zhang et al. 2004] and its possible application for expression of other proteins of interest on cell surfaces. In this case, the cells can serve as a support matrix for the enzymes and the surface expressed enzymes or proteins can directly react with substrates without the necessity for substrates to enter the cell. Through this approach, the response time of cell-based biosensor can be drastically decreased whereas the sensitivity is strongly enhanced [Lei et al. 2006].

Therefore, the genetic engineering is a powerful tool for improving the biorecognition elements for biosensing [Knopf et al. 2007].

1.3. Immobilization of biological elements

The performance of biosensing devices strongly depends on the efficiency of the biological element and a crucial issue is its immobilization on the transducer surface. For this purpose a suitable surface attachment strategy must be developed, which allows the biological element to maintain in the artificial environment maximum activity and providing simultaneously a stable attachment of the biomaterial to the biosensor platform. Additionally an effective immobilization should provide access for analyte molecules to interact with the biorecognition element as well as coupling of the signal from the biological element to the transduction platform. A large variety of physical and chemical immobilization methods utilizing different materials have been developed.
aiming at fulfilling these requirements. Some can be specifically employed for the immobilization of certain biological elements while others are universal. The physical methods of immobilization include: adsorption [Taylor 1991], [Reshetilov et al. 1997], [Naessens et al. 1998], [Lee et al. 1992], microencapsulation [Ikariyama et al. 1997], [Riedel et al. 1990], matrix entrapment [Wang et al. 2000], [Kanasawud et al. 1989], [Heitzer et al. 1994], [Elasri et al. 1999], [Corbisier et al. 1999], [Peter et al. 1996], [Cassidy et al. 1996], [Vorlop et al. 1992], [Rietti-Shati et al. 1996], [Wallace et al. 1999] and electrode modification [Wang et al. 1996] among others. The chemical methods include: covalent bonding [Mello et al. 2002], crosslinking [Gusian 2006] and fixation on self-assembled monolayers (SAM) [Ferretti et al. 2000] among others (Fig. 1.13)

The adsorption technique is inexpensive and non-destructive but it is prone to some instability due to desorption processes. Microencapsulation also belongs to non-destructive methods and provides good stability of the immobilized matrix. This method
however, suffers from poor mass-transfer efficiency of substrates and products through the membrane. Matrix entrapment usually provides excellent stability though properties of the matrix material can negatively affect the entrapped biomaterial. The entrapment matrix can be natural or synthetic polymeric materials such as; agar/agarose [Kanasawud et al. 1989], alginate [Heitzer et al. 1994], [Elasri et al. 1999], [Corbisier et al. 1999], [Peter et al. 1996], carrageenan [Cassidy et al. 1996]; polyacrylamide [Vorlop et al. 1992], sol-gels [Rietti-Shati et al. 1996] and conducting polymers [Wallace et al. 1999] to mention but a few. Electrode modification is performed by simple mixing of biological elements with carbon paste containing a mediator to form the working electrode. This method of immobilization is reliable and usually provides excellent operation and storage stability of the resulting biosensors. Covalent binding is the most intensively studied method of immobilization aimed at binding of the functional groups of the biological material to those of the support surface. The major drawbacks of this method are its complication, only a small quantity of biomolecules can be immobilized and its poor reproducibility. Crosslinking allows coupling of biomolecules to each other thereby creating an insoluble net that stabilizes the immobilized biomaterial but at the same time can hinder the diffusion of the analyte and product molecules. Self-assembled monolayers (SAMs) allow controlled and oriented immobilization of enzymes on gold, platinum and silver surfaces and are often used as “a protective layer” for immobilization of cells. The choice of the immobilization method depends on the nature of the biorecognition element, the physico-chemical properties of the transducer, and the purpose and intended properties of the resulting biosensing device.

If one should think about mass production of biosensors, the possibility to automate the immobilization procedure should be considered. The earlier mentioned advantage of the possibility to non-manually form the biorecognition layer on the electrode surface of electrochemical biosensors, allows for automatization of the fabrication process. This can be performed by three techniques namely: electroadsorption, electropolymerization and electrodeposition.
Electroadsorption or electrochemically aided adsorption is based on electrophoretic movement of charged proteins to an electrode with a polarity opposite to the charge of the proteins resulting in the formation of a protein layer on the electrode. This technique was initially used for deposition of bovine serum albumine (BSA) [Suaud-Chagny et al. 1986], glucose oxidase [Johnson 1991], [Strike et al. 1993], avidine [Anzai et al. 1993] and urease [Usmani 1994]. The electroadsorption step is often followed by crosslinking with glutaraldehyde to stabilize the protein film. The merger of these two steps into one step by electroadsorption from a solution containing BSA, the active enzyme and glutaraldehyde enabled Strike et al to reduce the amount of active protein necessary for electroadsorption, and to extend the technique to immobilization of other more expensive enzymes [Strike et al. 1995]. Application of a current pulse profile during electroadsorption allows for easy control of the film properties.

Electropolymerization in the context of biosensing is the incorporation of a protein within a conducting or non-conducting polymer film, electrochemically grown on an electrode surface. In 1986, Foulds and Lowe [Foulds et al. 1986] reported the first application of this technique for enzyme immobilization. They entrapped glucose oxidase into a polypyrrole matrix on the surface of a printed platinum electrode by electropolymerization of an aqueous solution containing glucose oxidase and pyrrole monomers. Since that time, many other polymer matrices have been implemented for immobilization of various enzymes. Among them are: polycapto-p-benzoquinon [Arai et al. 1999], poly 3,4-ethylendioxythiophen [Fabiano et al. 2002], polyanilin [Garjonyte et al. 2000], poly (m-phenylendiamin) [Yang et al. 2002], polyphenol [Chen et al. 2002] and others. The electropolymerization process consists of several steps starting with diffusion of the monomer to the electrode, its oxidation at the electrode to form a radical cation, radical-radical coupling, oxidation of the formed oligomers, chain propagation, and precipitation of the polymer on the electrode surface. The properties of the polymer film are influenced by the applied potential, temperature, pH, monomer
concentration, nature of the solvent and electrolyte and properties of the electrode surface [Cass et al. 1998]. This immobilization method is simple, convenient and provides stabilization of the structure and activity of the entrapped biorecognition elements. The unique properties of conducting polymers allow efficient electronic coupling of biomolecules to the electrode, excluding the necessity of free-diffusing mediators. Additionally, the polymer backbones can be modified with redox-compounds through chemical modification of their monomers. For example, the polymers, functionalized with osmium complexes work as molecular relays and provide fast transfer of electrons from the enzyme active centre to the electrode. This happens due to the high self-exchange rates of di- and trivalent ions of osmium complexes and involves three steps: (i) electron transfer from the enzyme active site to the first osmium entity, (ii) hopping of the received electron to the next osmium redox group along the polymer chain and finally (iii) delivery of the electron to the electrode [Ohara 1995]. In spite of the above-mentioned advantages and the high popularity of this method for the construction of the variety of biosensors, loss of enzyme activity during the polymerization due to the unfavourable influence of organic solvents and radical cations is a major drawback of this method.

**Electrodeposition** or electrophoretic painting is the deposition of colloidal particles (e.g. polymers, metals, etc.) from a liquid medium onto an electrode surface under the influence of an electric field. This almost century-old technique was patented by Davey and General Electric in 1917 and was used for insulation of wires by deposition of bituminous materials [Durney 1984]. Since that time, the process became widely used for the coating of metal constructions and food can interiors in the industry. In the field of electrochemistry, this technique was introduced by Schulte and Chow, who used a commercially available electrodeposition paint for insulation of carbon-fiber microelectrodes [Schulte et al. 1996], [Schulte et al. 1998].

The electrodeposition process can be used to deposite negatively charged materials on positively charged electrodes (anodic electrodeposition) or positively
charged materials on negatively charged electrode (cathodic electrodeposition). This happens due to the influence of an electric field, which causes movement of charged colloidal particles (electrophoresis) to electrodes with a charge opposite to that of the charged colloidal particles. There are three mechanisms of electrodeposition that have been described: (i) charge destruction and decrease in solubility as for the case of acrylic polymers, (ii) concentration coagulation (onium salts) and (iii) salting out (onium salts). The first step of the electrodeposition process from aqueous solutions is the electrolysis of water, which can be represented by following reactions:

Anode: \(2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4\text{e}^-\)

Cathode: \(4\text{H}_2\text{O} + 4\text{e}^- \rightarrow 4\text{OH}^- + 2\text{H}_2\)

In the anodic process, the colloid particles carry carboxylate groups as the charge-bearing groups (Fig. 1.14a). These negatively charged anions become protonated with the hydrogen ions, produced by the oxidation of water molecules, lose their charge (charge destruction), become less soluble in water and ultimately precipitate onto the anode (Fig. 1.14b).

In cathodic deposition, the colloid particles have protonated bases as a charge-bearing group (Fig. 1.15a). The reduction of water at the cathode yields hydroxide ions. These
hydroxide ions can deprotonate the salts to restore the initial neutral bases, which are less soluble in water and can precipitate on the cathode (Fig. 1.15b).

Fig. 1.15. Cathodic electrodeposition

Kurzawa et al were the first to use the electrodeposition approach for immobilization of enzymes on electrode surfaces and fabrication of biosensors [Kurzawa et al. 2002]. In this case, glucose oxidase was entrapped in a polymer film, formed by electrodeposition of commercially available electrodeposition polymer on the surface of a platinum electrode. The constructed glucose biosensor was characterized by a fast response, good reproducibility and very good stability. Since this time, the electrodeposition technique has been used to immobilize different biological elements on conducting surfaces [Kueng et al. 2004], [Shkotova et al. 2008], [Shkotova et al. 2006]. The soft process conditions in comparison to electropolymerization, the use of an aqueous medium, no formation of radicals make this immobilization method more attractive for the construction of biosensors than the electropolymerisation approach. As in the case of electropolymerizable materials, electrodeposition polymers can be modified with osmium complexes to improve electron shuttling between the biological elements and the electrode. Particularly helpful with this immobilization method is the construction of multilayered biosensors with sophisticated architectures [Smutok et al. 2006], [Demkiv et al. 2008].
1.4. Signal transduction mechanisms between biorecognition elements and an electrode

Generally, there are three major mechanisms of signal transduction from the biorecognition element to the electrode in amperometric biosensing. Depending on the utilized electron transduction mechanism, all amperometric biosensing devices can be divided into three generations [Gorton et al. 1999], [Gorton 1995], [Habermuller et al. 2000], [Willner et al. 2005], [Gorton et al. 2005]:

- the first generation is based on monitoring of electroactive substrates or products of an enzymatic reaction or cell metabolism;
- the second generation makes use of natural or artificial mediators;
- the third generation exploit a direct electron transfer mechanism

1.4.1. Monitoring of substrates/products on an electrode

This type of signal transduction occurs via substrates or products of a biocatalytic reaction or cell metabolism. Many biosensing devices of this generation implement a class of enzymes called oxidases. These enzymes catalyze oxidation-reduction reactions, using molecular oxygen as electron acceptor and reduce it to water or hydrogen peroxide. Therefore, the signal transduction can be performed by monitoring either oxygen, the substrate or hydrogen peroxide, the product. However, only approximately a quarter of all known enzymes are oxidoreductases. The coupling of NADH or NADPH oxidase with dehydrogenases allows extension of the applicability of this transduction mechanism [Mulchandani et al. 1998] to the class of dehydrogenases.

Substrate detection

Most of the biosensors of this group are based on the monitoring of oxygen, a co-substrate for many oxidases and all types of aerobic living cells. A typical example is the glucose biosensor, with glucose oxidase immobilized on the surface of a platinum electrode [Clark et al. 1962]. At the potential of -600 mV vs. Ag/AgCl, oxygen is reduced to water on the electrode surface and a reduction current can be measured. Once
the catalytic reaction takes place, the concentration of oxygen available at the cathode for further reduction becomes lower. The corresponding drop of the reduction current is proportional to the concentration of the detected substrate.

![Diagram of signal transduction mechanism]

**Fig. 1.16.** Mechanism of signal transduction in the first generation of biosensors
BRE - biorecognition element; S-substrate; P-product

The disadvantages of this transduction mechanism include: fluctuations in the background oxygen concentration, dependence on temperature and ionic strength; strong interference from other enzymatic reactions, using oxygen as a co-substrate in case of cells; insensitivity of the electrode to small changes of oxygen concentration in case of small amount of available enzyme or substrate.

**Product detection**

Many oxidases form hydrogen peroxide as a by-product, which is also electroactive and can be oxidized on the electrode at a potential of about 600 mV vs. Ag/AgCl. A typical example of a biosensor based on the detection of the product of an enzymatic reaction is the glucose biosensor developed by Guilbault and Lubrano [Guilbault et al. 1973], [Guilbault et al. 1973], the details of which are described in section 1.2.1.1. The drawback of such biosensors is a high overpotential required to electrochemically oxidize hydrogen peroxide. A consequence of this is high interference from other
oxidizable substances (e.g. ureate, ascorbate, etc.). A number of approaches have been developed aimed at decreasing the operation potential of biosensors based on hydrogen peroxide oxidation. Among them are: use of carbon paste electrodes with dispersed metal particles [Liu et al. 2001], modification of the electrodes with Prussian Blue [Mattos et al. 2000], [Karyakin et al. 2001], [Koncki 2002], [Ricci 2005], [Karyakin et al. 2007] or implementation of a second enzyme peroxidase [Ruzgas et al. 1996], [Regalado et al. 2004], [Ferapontova et al. 2003].

1.4.2. Mediated electron transfer
In the second generation of biosensors, natural electron acceptors are substituted by artificial redox-mediators [Cass et al. 1984], [Scheller et al. 1991]. This allows to reduce the operating potential of the biosensors and to suppress the interfering influence of other substances. In case of dehydrogenases, application of artificial mediators excludes the problem of NADH or NADPH recycling at the electrode [Bartlett et al. 2008], [Bard et al. 2002]. The first biosensor of the second generation was constructed by Cass et al. in 1984. It uses ferrocene to transfer the electrons from the glucose oxidase to the electrode [Cass et al. 1984]. Degani and Heller in 1987 bound the mediator to the protein molecule and created in such a way a molecular relay, providing direct connection between the prosthetic group of the enzyme and an electrode [Degani et al. 1987]. An ideal redox-mediator should have the redox potential close to the redox potential of the enzyme’s prosthetic group. It should also be stable in its oxidized and reduced forms and capable of fast heterogeneous electron exchange between the enzyme and the electrode. A huge amount of redox compounds have been screened to act as redox mediators since the first application of ferrocene. Among them are: ferrocene derivatives, ferricyanide, quinones, conducting salts, organic dyes and others [Wang 2001], [Gorton 1995], [Chaubey et al. 2002], [Bard et al. 2002], [Habermuller et al. 2000], [Koncki 2002].
When talking about mediated electron transfer in cell-based biosensors, one has to differentiate between natural mediators produced by the cell itself and artificial redox mediators. In the first case, cells of some microbial species are capable to produce and excrete electroactive compounds, which get reduced inside the cell and can be oxidized on the electrode surface. Self-mediation has been observed for instance in *Pseudomonas* [Rabaey et al. 2004], [Rabaey et al. 2005], [Pham et al. 2008] *Shewanella* [Marsili et al. 2008] and *E.coli* [Wang et al. 2007], [Qiao et al. 2008], [Zhang et al. 2008] species. Use of artificial mediators in the case of cell biosensors aims at substituting natural electron acceptors, for example oxygen in aerobic cells, or Fe$^{3+}$ complexes in anaerobic cells. However, in case of cells, the artificial mediator has to be lipophilic to be capable to cross the cell plasma membrane and to couple the enzymatic reaction inside the cell with the electrode. Such lipophilic mediators as quinones [Tatsumi et al. 2000], menadione [Baronian et al. 2002], [Heiskanen et al. 2004], 2,6-dichlorophenolindophenol (DCPIP) and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) [Zhao et al. 2007] have been successfully used to probe intracellular redox activity. The hydrophilic mediators can be used when the enzymes to be addressed are localized in the plasma membrane [Tkac et al. 2003], [Vostiar et al. 2004], [Goldenberg et al. 1979] or as part of a double-mediator system, comprising a lipophilic and a hydrophilic mediator [Baronian et al. 2002], [Zhao et al. 2007].

The disadvantages of this approach include: leakage of the free-diffusing mediators and sample contamination, suppression of sensitivity by oxygen interference, large oxidative stress and unselectivity to the source of reducing equivalents in case of cells. A step forward to overcome the disadvantages of the electron transfer using free-diffusing mediators was covalent binding of the redox compounds (usually redox-active transition metal compounds) to a polymer backbone. In this case, redox entities function as mediators, facilitating the transfer of electrons between the biorecognition element and the electrode by the electron hopping mechanism, proposed by Kaufmann and coworkers [Kaufman et al. 1979], [Kaufman et al. 1980]. Electron hopping occurs in
three steps. Firstly, the electron is transferred from the biorecognition element to an electroactive entity of the polymer close to it, followed by sequential self-exchange steps between redox groups and final delivery of the electrons to the electrode.

Such polymers form a special class of conducting polymers and are called redox-polymers. They can be used for immobilization of biorecognition elements on the electrode surface, provide stable binding of all the components to the electrode, and form the basis for reagentless biosensors [Koncki 2002], [Schuhmann et al. 2000]. In line with this principle, Foulds et al modified pyrrole polymers with ferrocene [Foulds et al. 1988] whereas Inagaki et al. succeeded to bind ferrocene and dimethylferrocene to siloxane polymers [Inagaki et al. 1989] and used them for fabrication of glucose biosensors [Hale et al. 1990]. Heller’s group reported about “electrical wiring” of glucose oxidase to an electrode using polymers modified with osmium complexes [Heller et al. 2008; Heller 1992], [Heller 2006], [Mao et al. 2003] to measure glucose in biological fluids [Mao et al. 2003], [Mano et al. 2005], [Mano et al. 2005]. Since that time, osmium complex modified polymers have been widely applied for electrical wiring of different enzymes to the electrode [Belay et al. 1999], [Timur et al. 2006], [Ferapontova et al. 2007], [Tasca et al. 2007], [Ngounou et al. 2009], [Stoica et al. 2009], [Guschin et al. 2006], [Smutok et al. 2006], [Badura et al. 2008]. Gorton’s group also reported the successful wiring of living bacterial cells to an electrode, although the exact mechanism of electron transfer from the cellular enzymes to the osmium redox centers remains unknown [Vostiar et al. 2004], [Timur et al. 2007], [Alferov et al. 2009], [Coman et al. 2009].

1.4.3. Direct electron transfer

Biosensors of the third generation are based on direct electronic coupling of the biorecognition elements to the electrode without assistance of intermediate substances such as substrates/products in the first generation of biosensors or artificial mediators in the biosensors of the second generation [Gorton et al. 1999], [Armstrong et al. 2000],

**Enzymes**

The first reports on direct electron transfer between a redox protein and an electrode were by Eddowes and Hill [Eddowes et al. 1977], and Yeh and Kuwana [Yeh et al. 1977], who independently discovered the ability of cytochrome *c* to directly transfer electrons to gold or tin-doped indium oxide electrodes. These first reports on direct bioelectrocatalysis of cytochrome *c* were followed by the discovery that larger redox proteins such as laccase and peroxidise are also capable of direct electron transfer [Berezin et al. 1978], [Tarasevich et al. 1979; Yaropolov et al. 1979]. Since that time, direct electron transfer has been reported for catalase [Prakash et al. 2009], cytochrome P 450 [Bistolas et al. 2005], peroxidases [Yaropolov et al. 1979], [Regalado et al. 2004], [Armstrong et al. 1987], [Jönsson et al. 1989], hydrogenases [Yaropolov et al. 1984], [Vincent et al. 2007] azurin [Chi et al. 2001], [Armstrong et al. 1984]), laccases [Berezin et al. 1978], [Tarasevich et al. 1979], [Shleev et al. 2005], [Shleev et al. 2005], bilirubin oxidase [Shleev et al. 2004], [Tsujimura et al. 2004], [Tsujimura et al. 2005], [Kamitaka et al. 2007], [Ramirez et al. 2008], ascorbate oxidase [Sakurai 1996], [Santucci et al. 1998] succinate dehydrogenase [Sucheta et al. 1992], [Hirst et al. 1996], fumarate reductase [Sucheta et al. 1993], alcohol dehydrogenase [Ikeda et al. 1993], fructose dehydrogenase [Ikeda et al. 1991], [Ferapontova et al. 2005] and cellobiose dehydrogenase [Lindgren et al. 2000], [Lindgren et al. 2001], [Stoica et al. 2005], [Stoica et al. 2006], [Larsson et al. 1996], [Larsson et al. 2000]. Most of the enzymes, which are capable of direct electron transfer, contain metals such as iron or copper and have easily accessible active centres. The important factors for efficient electron transfer are orientation of the enzyme structure on the electrode [Armstrong et al. 1988], [Frew et al. 1988], distance and driving force between active center of the enzyme and electrode [Marcus et al. 1985], [Freire et al. 2003]. However, the high selectivity of
many enzymes requires deep embedding of the enzyme prosthetic group in the protein structure and thus prevents such enzymes from direct electron transfer to the electrode due to the fact that the distance between the redox centres of such enzymes and electrode exceeds the distance across which electrons can be efficiently transferred [Leger et al. 2008], [Gorton et al. 1999], [Gorton et al. 2005], [Wollenberger et al. 2008], [Ghindilis et al. 1997]. This limitation can be surmounted by chemical [Degani et al. 1987], [Degani et al. 1988], [Riklin et al. 1995] or genetic [Lambrianou et al. 2008] modification of proteins or reconstitution of the apo-enzymes with relay-cofactor units, immobilized on the electrode surface [Willner et al. 2005], [Willner et al. 1996], [Zimmermann et al. 2000]. The direct contact between enzyme redox center and the electrode can also be improved by incorporation of nanomaterials [Xiao et al. 2003], [Katz et al. 2004], [Pumera et al. 2007], [Pingarron et al. 2008].

The advantages of the DET approach include high selectivity of the constructed biosensors and absence of intermediate electron transfer steps, which eliminates interferences from other reactions.

Cells
The first evidence of direct electron transfer between living cells and an electrode appeared in 1999. Kim et al. reported the direct electrode reaction of *Shewanella putrefaciens* bacterial cells. They found that the cells of this Fe(III) reducing bacteria could not grow fermentatively on lactate, but were able to grow on an anode of a three-electrode electrochemical cell using lactate as an electron donor and the electrode as the electron acceptor. This electron transfer was believed to be due to the activity of plasma membrane cytochromes, which are involved in the reduction of the natural electron acceptor Fe(III) [Kim et al. 1999]. Later however, it was discovered that these microorganisms also produce an electron shuttle [Newman et al. 2000], which could contribute to generation of electricity on the electrode and the outer-membrane cytochromes are involved in the reduction of this electron shuttle [Lies et al. 2005]. In 2002, the group of Lovely discovered that the microorganisms of the *Geobacteracea*
family are also capable to directly transfer electrons received from the complete oxidation of organic compounds to a graphite anode [Bond et al. 2002]. The scientists proved that no soluble electron shuttle was involved in the electron transfer process [Bond et al. 2003], [Lovley 2006]. Further studies in this field revealed that some species of Aeromonas [Pham et al. 2003], Clostridium [Park et al. 2001], Rhodoferax [Chaudhuri et al. 2003], [Finneran et al. 2003], Desulfuromonas [Bond et al. 2002], Desulfobulbus [Holmes et al. 2004] and Geotrix [Bond et al. 2005] genera are also capable of electrogenesis. It was initially thought that only bacterial cells that touch the conducting surface can deliver the electrons to the electrode through the cytochromes located in the outer cell membrane. Therefore, the received current was expected to be low. However, it was observed that the current value increases when the thickness of the biofilm on the electrode becomes larger. The study of this phenomenon led to the discovery of additional mechanisms of electron transfer from bacterial cells to the electrode through conductive appendages, called pili. These conductive appendages also allow the cells, which are not in direct contact with the electrode to contribute to the electricity production [Zhang et al. 2006], [Reguera et al. 2005], [Reguera et al. 2006]. The structure and properties of the bacterial pili were characterized by scanning tunneling microscopy (STM) and atomic force microscopy (AFM) [Gorby et al. 2006].

Not much is known about direct electron transfer from yeast cells to the electrode. For a long time it was thought that it does not even exist due to the eukaryotic nature of the yeast cells. In bacteria, the electron transport chain (ETC), which seems to play the most important role in electron transfer is situated in the plasma membrane of the bacterial cells, whereas yeast cells have their ETC in the mitochondrial inner membrane (see Fig 1.17). Therefore, at the first glance it looks impossible for electrons to be directly transferred through the mitochondrial outer membrane, plasma membrane and yeast wall to an electrode surface. Nevertheless, one should not forget about the existence of the transplasma membrane electron transport system (tPMET), which has
been found in both prokaryotic and eukaryotic cells. The tPMET system as well is believed to be involved in extracellular electron transfer.

Fig. 1.17. Electron transfer pathways in bacteria and yeast. Adapted from [Schaetzle et al. 2008].

1.5. Aim of the study

Biosensor technology made great progress in the last decades. The development of this field generally proceeded in three main directions. Biologists developed increasingly selective and sensitive biorecognition elements, material scientists advanced transducer materials and immobilization matrices. This allows design of biosensors with different architectures by:

- choice and design of the biorecognition element
- choice of the immobilization method and eventual design of the immobilization matrix
- design of the transducer

This study focused on designing the biorecognition layer, including the immobilization matrix and the biorecognition element. For this, electrodeposition polymers of different structure were implemented for design of enzyme and cell-based
biosensors. As biorecognition element were used either genetically modified *Hansenula polymorpha* yeast cells or enzymes, isolated from them. The design of the transducer is out of the scope of this study.

The key to creating a perfect biosensor or the so called ”smart biosensor” is not specific to a particular material or technology but the successful synergy of all its elements and the proper exploitation of both the physical and chemical interactions of the component materials. Therefore, a modern biosensor industry pays much attention to the integration of different technologies in the design of new biosensors rather than focusing on a single technology or specific material. A prerequisite for the functional integration of different materials and technologies is the availability of knowledge about all the kind of interaction of the components of the sensory system. Hence, the scope of this thesis is also to provide the essential background to understanding the interactions between the different components of biosensors and to introduce new ideas about intelligent biosensor design.
## 2. EXPERIMENTAL

### 2.1. Materials

#### 2.1.1. Chemicals

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2.1.2. Biomaterial

Enzymes

Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (type XS) with activity 100000-250000 units/g of solid was obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

NAD$^+$- and glutathion-dependent formaldehyde dehydrogenase (EC 1.2.1.46) with specific activity 12000 units/g was obtained from the Institute of Cell Biology (Lviv, Ukraine). The enzyme was isolated from cell-free extracts of recombinant strain Tf11-6 of *Hansenula polymorpha* yeast cells.

Cells

The following yeast strains were obtained from the Institute of Cell Biology, National Academy of Sciences of the Ukraine:

*Hansenula polymorpha* strain 356 – wild type yeast cells;

*Hansenula polymorpha* tr1 (*gcr1 catX*) – impaired in glucose catabolite repression and catalase-defective mutant that has the ability to overproduce the enzyme flavocytochrome $b_2$ (EC 1.1.2.3) in a glucose-containing growth medium.

2.1.3. Mediums and buffers

YPD medium with ampicillin

10 g Bacto-yeast extract; 20 g Bacto-peptone; 20 g Dextrose; 100 mg Ampicillin, 1 l H$_2$O dest.
Liquid medium

1 g KH₂PO₄; 3.5 g (NH₄)₂SO₄; 0.5 g MgSO₄·7H₂O; 0.1 g CaCl₂; 6 g yeast extract; 10 g glucose; 2 g L-lactate; 1 l H₂O dest.

20 mM phosphate buffer pH 8.2

5.123 g/l NaH₂PO₄; 0.122 g/l Na₂HPO₄; 1 l H₂O dest.

100 mM phosphate buffer pH 8.0

47.35 ml 0.2M Na₂HPO₄; 2.65 ml 0.2M NaH₂PO₄; 50 ml H₂O dest.

50 mM phosphate buffer pH 7.8

45.75 ml 0.2M Na₂HPO₄; 4.25 ml 0.2M NaH₂PO₄; 150 ml H₂O dest.

100 M phosphate buffer pH 6.3

13.25 ml 0.2M Na₂HPO₄; 36.75 ml 0.2M NaH₂PO₄; 50 ml H₂O dest.

phosphate-citrate buffer pH 2.5

5.4 ml 0.2 M Na₂HPO₄; 44.6 ml 0.1 M citrate; 50 ml H₂O dest.

2.1.4. Consumables

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<td>Instrument</td>
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<tr>
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### 2.1.5. Instruments

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### 2.1.6. Software

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<td>G. Wittstock, Oldenburg, Germany</td>
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<td>Video camera</td>
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</table>
2.2. Methods

2.2.1. Cultivation of yeast cells
The initial strains were stored at refrigerator temperatures (4°C) in parafilm-sealed petri dishes on YPD medium and recultured monthly.

Yeast cells were cultivated to the mid-exponential phase in Erlenmeyer flasks on a shaker (200 rpm) at 37°C in an aerated medium containing: 1 g/l KH₂PO₄, 3.5 g/l (NH₄)₂SO₄, 0.5 g/l MgSO₄·7H₂O, 0.1 g/l CaCl₂ and 6 g/l of yeast extract. As carbon source was used a mixture of glucose (10 g/l) and L-lactate (2 g/l). After cultivation, the yeast cells were washed twice with 50 mM PB pH 7.8 and harvested by centrifugation.

2.2.2. Determination of cell concentration
The concentration of cell suspensions (mg of dried cells per ml of suspension) was determined spectrophotometrically at 540 nm using a preestablished gravimetrical calibration.

The concentration of the cells was calculated using the formula:

\[ C = \frac{A \cdot n}{1.33}, \]

where:

- \( A \) is the optical density at 540 nm;
- \( n \) is the dilution factor of the initial suspension;
- 1.33 is the correlation coefficient estimated by gravimetrical calibration.

2.2.3. Permeabilization of yeast cells
The cells washed from the medium were suspended in 50 mM phosphate buffer (PB) pH = 7.8 to the concentration of 50 mg/ml and mixed with the same volume of a solution containing 3 mg/ml of a permeabilizing agent cetyltrimethylammonium bromide (CTAB). The resulting mixture was incubated at 30 °C in a water bath for 15 min with occasional agitation every 2-3 min. After permeabilization, the yeast cells were
washed three times with 50 mM phosphate buffer of pH 7.8. The concentration of permeabilized yeast cells was determined in the same way as in case of intact cells.

2.2.4. Preparation of cell-free extracts

In order to collect cell debris, the freshly grown yeast cells were first washed twice with 50 mM PB of pH 7.8 and suspended in the same buffer to make a concentration of 50 mg/ml. The obtained suspension was then mixed with glass beads (d = 0.45 – 0.5 mm) whose amount was ¾ the volume of the initial suspension. The cells were destroyed in a homogenizer for 5 minutes at 1000 rpm.

2.2.5. Determination of protein concentration

The protein concentration in cell-free extracts was estimated by the method of Lowry [Lowry et al. 1951], based on the reduction of Folin-Ciocalteu reagent to heteropolytrinexylenes blue by copper-catalyzed oxidation of aromatic acids and the reactivity of the peptide nitrogens with the cupric ions under alkaline conditions. Bovine serum albumine (BSA) was used as a protein standard for calibration.

2.2.6. Determination of FC $b_2$ activity

The activity of FC $b_2$ in the yeast cells was estimated spectrophotometrically based on the degree of potassium ferricyanide reduction at $\lambda = 420$ nm, characterized by decolourization of the reagent mixture. This method is described in references [Appleby et al. 1960], [Appleby et al. 1959].

Reagents mixture:
- 0.03 M PB pH 8.0;
- 0.03 M L-lactate;
- 1 mM EDTA;
- 0.83 mM potassium ferricyanide ($K_3Fe(CN)_6$);
- 0.1 ml cell-free extract.
The specific activity was calculated using the formula:

$$activity = \frac{\Delta A/\text{min} \cdot Vr \cdot n}{\varepsilon_m \cdot Cb \cdot Vex},$$

where

- $\Delta A/\text{min}$ – optical density change per min (at $\lambda = 420$ nm);
- $Vr$ – volume of the reaction mixture, ml;
- $n$ – dilution factor of the extract;
- $\varepsilon_m$ – molar extinction coefficient, for $K_3Fe(CN)_6$, $\varepsilon_m$ is 1.04 mM$^{-1}$·cm$^{-1}$;
- $Vex$ – volume of cell-free extract, ml;
- $Cb$ – protein concentration in the extract, mg/ml.

### 2.2.7. Visualization of FC $b_2$ activity

The permeabilized cells were incubated for 15–20 min at room temperature in the absence of light with the following reagents mixture:

- 0.1 M L-lactate;
- 1 mM $K_3Fe(CN)_6$;
- 1 mM $EDTA$;
- 25 mM PB pH 8.0.

Addition of a 10 mM solution of FeCl$_3$ in 0.25 M HCl led to formation of Prussian Blue crystals, causing the colour of the suspension to turn blue. The intensity of the colour depends on the amount of Prussian Blue crystals formed and correlates with the activity of flavocytochrome $b_2$.

### 2.2.8. Synthesis of electrodeposition polymers

#### 2.2.8.1. Vinylpyridine-based polymers

As a polymer backbone commercially available poly(4-vinylpyridines) with a molecular mass 60 kDa (P4VP60) and 160 kDa (P4VP160) and copolymers of 4-vinylpyridine
with butylmethacrylate (poly(4-vinylpyridine-co-butyl) methacrylate, CP4VPBMA) were used. Firstly, P4VP60, P4VP160 and CP4VPBMA were alkylated with epichlorhydrin. Then the alkylated products were treated with 1,3-diaminopropane (DAP) or 1,8-diaminoctane (DAO) and afterwards with 4(5)-imidazol-carboxaldehyde (ICA).

2.2.8.2. Meth(acrylate)-based polymers

Before polymerization monomers were passed through an inhibitor remover column and oxygen was removed by Argon bubbling.

**Emulsion polymerization 1**

The mixture of monomers was neutralized with 3M HCl before polymerisation. As radical initiator ( tert-butyl-hydroperoxide) was used. The copolymerisation was carried out at 90°C for 5 h. The obtained copolymer was dissolved in minimal amount of methanol.

**Emulsion polymerization 2**

A radical initiator, 10% of azobisisobutyronitrile (AIBN) in benzene, was added to the mixture of monomers dissolved in 2 ml of water. The copolymerisation reaction was carried out at a temperature of 100°C. The obtained copolymer was neutralized with a corresponding amount of 10 M HCl and diluted with 3 ml methanol.

2.2.9. Polymer characterization

The overall conversion of the reaction was evaluated by gravimetry. Samples of EDP were weighed, dried at 120°C for 30 min, reweighted and then dissolved in methanol-D4. The copolymer composition was found by regression analysis of $^1$H NMR-data. $^1$H-NMR spectra were recorded on a Bruker DRX 400 spectrometer in methanol-D4 chemical shifts in ppm ($\delta$) were referenced to the residual solvent signal. NMR data were evaluated by the MestRec Lite v 4.59 software.
2.2.10. Modification of electrodeposition polymers with osmium complexes

[Os(dmbpy)$_2$Cl$_2$], [Os(bpy)$_2$Cl$_2$], [Os(bim)$_2$Cl$_2$], or [Os(ImPy)$_2$Cl$_2$] was added to an appropriate amount of the copolymer solution. The reaction mixture was heated to 75–120 °C and stirred at this temperature for 4 h to allow a ligand exchange reaction between the labile chloro ligands of the osmium complex and the polymer-bound ligand to take place.

2.2.11. Fabrication and evaluation of amperometric biosensors

2.2.11.1. Fabrication of electrodes

Graphite electrodes were fabricated by sealing graphite rods (RW001, d = 3.05 mm, Ringsdorff Werke, Bonn, Germany) into the glass tubes using epoxy glue. Platinum electrodes were fabricated from platinum wire (d = 50 µm or 250 µm) by sealing it into glass capillaries using a capillary puller. Before use, the surface of the graphite electrodes was polished with emery paper. Platinum electrodes were polished with emery cloth using aluminium pastes (Leco, Germany) with different particles size (1, 0.5 and 0.3 µm). After polishing, the electrodes were ultrasonicated in water for 10-15 min using an ultrasonic bath.

2.2.11.2. Construction of biosensors

Amperometric biosensors were constructed using a potentiostat and a standard three-electrode configuration electrochemical cell, comprising working, reference and auxiliary (counter) electrodes. As a reference electrode, a silver/silver chloride electrode (Ag/AgCl/3M KCl) was used. As working electrode, graphite disk electrodes (d = 3.05 mm), platinum disk electrodes (d = 2 mm) or gold disk electrodes (d = 2 mm) were used.

2.2.11.3. Formation of biosensitive layer

To enable the immobilization of enzymes or cells on the surface of the working electrode, the following immobilization procedures were used:
1. Entrapment of enzyme glucose oxidase in a cathodic electrodeposition polymers: 4 µl of enzyme solution was mixed with 40 µl of EDP suspension and 30 µl of water. Electrodeposition was carried out in a miniaturized electrochemical cell by applying 20 cycles of the following sequence of potentiostatic pulses: -1.2 V for 0.2 s and 0 V for 5 s [Kurzawa et al. 2002], [Neugebauer et al. 2003]. Before use, the electrodes were washed with 20 mM PB, pH 6.3.

2. Formation of multilayered architecture of formaldehyde biosensors
A mixture of 2 µl FdDH (15 U ml⁻¹), 2 µl 25 mM NAD⁺ and 2 µl of the corresponding osmium complex-containing polymer was dropped onto the surface of a platinised graphite electrode and electrodeposited by application of 20 cycles of a potentiostatic puls sequence to -1.2 V for 0.2 s and 0 V for 5 s. Hereafter 3 µl of 50 mM solution of reduced glutathion, were dropped on the top of the modified electrode and dried for several minutes at room temperature. Finally, a Nafion membrane was formed by dropping 5 µl of 1% solution of Nafion in ethanol on the top of the electrode modified with cathodic osmium polymer, FdDH, NAD⁺ and reduced glutathion.

3. Formation of the multilayered architecture of L-lactate biosensors
A mixture of 3 µl of cell debris and 3 µl of corresponding osmium complex modified EDP was electrodeposited on the surface of a graphite electrode by application of 10 cycles of potentiostatic puls sequence to -1200 mV for 0.2 s and 0 mV for 5 s for cathodic EDPs or 2200 mV for 0.2 s and 0 mV for 5 s for anodic EDPs. Than 3 µl of 5 mM solution of cytochrome c in PB was dropped on the previous layer and dried at room temperature for 15 minutes. Finally the electrode, modified with cell debris, cyt c and osmium complex EDP was covered with the last polymer layer formed by precipitation of 3 µl of osmium complex-containing EDP with PB of pH 8.0 in case of cathodic EDPs or phosphate-citric buffer of pH 2.5 in case of anodic EDPs.

4. Entrapment under a dialysis membrane: 2-3 µl of a cell suspension (200 mg/ml) was dropped on the surface of an electrode and left to dry for 15 minutes at +4°C in the
fridge. Then the electrode was covered with a dialysis membrane and washed with 20 mM PB of pH 7.8.

5. Entrapment of intact yeast cells in an osmium complex-containing polymer: 3 µl of the polymer were dropped on the surface of the electrode and left to dry for 5 minutes at room temperature. After that, 3 µl of the cell suspension of concentration 200 mg/ml was dropped on the previous layer and left to dry for 15 minutes at +4°C. The last layer was formed by dropping 3 µl of an osmium complex modified polymer, followed by precipitation with a citrate buffer of pH 2.5 or PB of pH 8.0. Coentrapped cells were maintained on the surface of the electrode by the insoluble polymer chains. Electrodes were washed with 50 mM PB, pH 7.8.

2.2.11.4. Redox mediators
As free-diffusing mediators in this study were used diluted in the working buffer: 1 mM K₃[Fe(CN)₆], 1 mM 2,6-dichlorophenolindophenol (DCPIP), 0.5 mM methylene blue and 0.2 mM phenazine methosulfate (PMS). When the photosensitive reagent PMS was used, the electrochemical cell was covered with aluminium foil to prevent the reduction of the reagent. For estimation of ferrireductase activity of *Hansenula polymorpha* yeast cells 20 mM K₃[Fe(CN)₆] was used. For the modification of platinised graphite electrode with ferrocene 10 mM solution of ferrocene in methanol was used. Prussian Blue was electrodeposited on the electrode from the solution containing 5 mM K₃[Fe(CN)₆], 5 mM FeCl₃ and 10 mM HCl by cycling between 0.4 and 1.3 V vs. Ag/AgCl (10 cycles, scan rate 50 mV/s).

2.2.11.5. Amperometric and voltammetric measurements
Amperometric and voltammetric measurements were performed at room temperature in an electrochemical cell with a capacity of 50 ml filled with 40 ml of the working buffer solution. The biosensor was inserted into an intensively mixed electrolyte solution to which the analyte of interest was added after stabilization of background current. The
experimental data was recorded using a bipotentiostat “EP 30“ (Biometra GmbH, Göttingen, Germany) or ”Autolab“ (PGstat302, Utrecht, Netherlands) connected to a computer.

**2.2.12. Measurements using the electrochemical robotic system**

Entrapment of glucose oxidase within the electrodeposition polymers was achieved by means of a potentiostatic pulse generation of pH-shifts in front of the working electrode. The pulse profile was -1.2 V for 0.2 s followed by 0 V for 5 s. A total of 20 pulse cycles were applied. Calibrations were performed at a potential of +600 mV vs. Ag/AgCl/3M KCl by inserting the electrode bundle into the solutions of glucose in a 100 mM PB solution with a pH of 6.3. The concentration of glucose solutions were: 12; 24; 48; 90; 167; 231 mM. The working electrode was a Pt disk electrode with a diameter of 250 µm.

Entrapment of permeabilized *Hansenula polymorpha* yeast cells within electrodeposition polymers was carried out by means of potentiostatic pulse generation of pH-shifts in front of the electrode (pulse profile -1.2 V / 0.2 s; 0 V / 5s 10 pulses). Calibrations were performed at a potential of 0 mV vs. Ag/AgCl/3M KCl by dipping the electrode bundle in the 1; 2.5; 5; 10 mM solutions of L-lactate in 50 mM PB pH = 7.8 with PMS (0.2 mM) as a free-difusing mediator. The WE was a graphite disk of diameter d = 0.5 mm.

**2.2.13. Scanning electrochemical microscopy**

**2.2.13.1. Sample preparation**

Before use, the glass slide was cleaned with acetone to remove all organic impurities. Afterwards the glass slide was covered with the copolymer of HOEMA and DMAEA (1:1), kept in oven for 15 min at the temperature 90 °C and after that cooled down for 5 min at room temperature. The cell spots were fabricated on the glass slide, covered with HOEMA-DMAEMA copolymer with a capillary using 3 times diluted cell suspension (400 mg/ml). The cells were left for 20 min to adsorb on the polymer layer. After this,
the sample was washed with 1ml of 20 mM PB solution of pH 7.8 to remove the cells, which did not adsorb.

2.2.13.2. SECM set-up

SECM measurements were performed using a scanning electrochemical microscope, represented in Fig. 2.1. A typical SECM set-up consists of step-motors, allowing for precise positioning and moving of the SECM tip (WE) and the sample in x, y and z-directions and a miniaturized electrochemical cell with a three electrode-assembly (WE, RE, CE). Whereas the step-motors allow for controlling the distance between SECM tip and sample during the measurement, the three electrodes assembly connected to the potentiostat controls the applied potential and measures electrochemical activity of the sample at defined geometric points. Both, the step-motors and the potentiostat are connected to the computer and controlled by a specially developed software.

The SECM set up is usually mounted on a vibration-damping platform since the SECM experiments are sensitive to foreign vibrations. For the precise positioning of the SECM
tip before the experiment the SECM set up is equipped with an inverted microscope as shown in Fig. 2.1.

2.2.13.3. SECM measurements
Before the experiment, the glass slide with the sample was mounted on the bottom of miniaturized electrochemical cell. After positioning the working, counter and reference electrodes, the electrochemical cell was filled with 50 mM PB of pH 7.8. As WE a platinum electrode (d = 50 µm), as RE a minituarized Ag/AgCl reference electrode and as CE a platinum wire were used. The SECM tip was slowly approached to the surface using the z-positioning step-motor and piezo-element and then was withdrawed from the surface to a distance of 15 µm. All the measurements were performed in oxygen-competition mode. Therefore, the WE was polarized to the potential -600 mV vs. Ag/AgCl, at which oxygen reduction takes place. Between the measurements performed in L-lactate solutions the electrochemical cell was filled with 50 mM PB of pH 7.8 and the sample was kept in this solution for around 20 min to allow the cells to consume the rest of the intracellular L-lactate before starting a new measurement.

2.2.14. Statistical treatment of the experimental data
All the experiments were repeated 4-6 times. For each set of data, the mean (µ), standard deviation (SD) and standard error of the mean (SEM) were determined. Computation of the statistical parameters and plotting of graphs were carried out using the Origin 7.5 software. Fitting of data was performed using the least-squares method.
3. RESULTS AND DISCUSSION

3.1. Design of immobilization matrices for amperometric biosensors
This section deals with design of electrodeposition polymers (EDPs) for their application in fabrication of amperometric biosensors. A part of results, represented in Sections 3.1.1 and 3.1.2, have been published in Paper I and Paper II.

3.1.1. Electrodeposition polymers as immobilization matrices
Electrodeposition polymers can be implemented for the non-manual immobilization of biological material on electrode surfaces. In other words, EDPs can be used as immobilization matrices for biorecognition elements. The first application of electrodeposition polymers for such a purpose was introduced by Kurzawa et al in 2002 [Kurzawa et al. 2002]. The idea was derived from the process of formation of anticorrosion layers on metal surfaces in car and can industries [Beck et al. 1988]. The immobilization procedure is based on the precipitation of a polymer suspension on the electrode surface caused by electrochemically induced modulation of the polymer solubility properties. The change in polymer solubility is achieved by a protonation of the acidic (for anodic EDPs) or deprotonation of basic (for cathodic EDPs) groups of the polymer side chains. Generation of H⁺ or OH⁻ ions for protonation and deprotonation of polymer side chains results from electrochemically induced oxidation or reduction of water in the diffusion zone of the electrode and allows therefore a precipitation of the polymer film exclusively on the electrode surface [Beck et al. 1976], [Krylova 2001]. The presence of biorecognition elements in the immobilization mixture leads to their co-immobilization on the electrode surface through entrapment in the formed polymer film as shown in Fig. 3.1 [Kurzawa et al. 2002], [Neugebauer et al. 2003], [Ngounou et al. 2004]. In aqueous solutions, the immobilized polymer film forms a hydrogel structure, which stabilizes immobilized biomaterial and provides fast diffusion of substrate to the biorecognition elements.
In the first applications commercially available electrodeposition polymers Canguard (from BASF Farben und Lacken, Münster, Germany) or Resydrol (from Vianova Resins, Mainz-Castel, Germany) were used for the fabrication of biosensors for glucose [Kurzawa et al. 2002], ATP [Kueng et al. 2004], ethanol [Shkotova et al. 2006] and lactate [Shkotova et al. 2008]. Commercially available electrodeposition polymers are produced in industry with high reproducibility but are specifically optimized for the corrosion protection and not for the fabrication of the biosensors. Taking into account that some additives of commercial EDPs can negatively influence the activity of biorecognition elements, a development of synthetic routes for electrodeposition polymers, specifically adapted for biosensing applications, would be beneficial.

Since the information about the composition of commercially available electrodeposition polymers was limited, Ngounou et al. analysed the composition of Resydrol and Canguard electrodeposition polymers by means of Curie-point pyrolysis gas chromatography/mass spectrometry [Ngounou et al. 2004]. The results showed that commercial EDPs are complex systems, containing copolymers of acrylic acid or styrol
with a variety of alkyl-substituted (methyl)acrylates, binding agents and crosslinkers. Using this knowledge, Ngounou et al. developed a synthesis strategy and synthesized a library of electrodeposition polymers with different compositions. A synthesis of an initial library of electrodeposition polymers was performed by bulk or emulsion radical copolymerization of 4-5 monomers, listed in Table 3.1 with di-tert-butyl hydroperoxide (DTBP) as initiator of polymerisation. Additionally either imidazol- or pyridyl-functionalized monomers were introduced into the polymer backbone to allow further modification of obtained polymers [Ngounou et al. 2004], [Ngounou et al. 2007].

Table 3.1 Monomers, used for the synthesis of libraries of electrodeposition polymers

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<tbody>
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<td>acrylic acid</td>
<td>AA</td>
</tr>
<tr>
<td>ethyl acrylate</td>
<td>EA</td>
</tr>
<tr>
<td>2-(dimethylamino)ethyl methacrylate</td>
<td>DMAEMA</td>
</tr>
<tr>
<td>n-butyl acrylate</td>
<td>BA</td>
</tr>
<tr>
<td>methyl methacrylate</td>
<td>MMA</td>
</tr>
<tr>
<td>cyclohexyl methacrylate</td>
<td>CHMA</td>
</tr>
<tr>
<td>2-hydroxyethyl methacrylate</td>
<td>HOEMA</td>
</tr>
<tr>
<td>2-ethylhexyl methacrylate</td>
<td>EHMA</td>
</tr>
<tr>
<td>styrene</td>
<td>St</td>
</tr>
<tr>
<td>vinylacetic acid</td>
<td>VAA</td>
</tr>
<tr>
<td>itaconic acid</td>
<td>IA</td>
</tr>
<tr>
<td>vinyl imidazole</td>
<td>VI</td>
</tr>
</tbody>
</table>

From 112 members of this library 81 polymers could be successfully used for the fabrication of biosensors. Some of the remaining 31 polymers were not soluble in water at room temperatures, others were too hydrophilic and could not be precipitated by means of pH-modulation. Therefore, hydrophilic/hydrophobic balance of the polymer seems to influence its ability to be electrodeposited on an electrode surface significantly. As expected, the obtained electrodeposition polymers had different physicochemical properties and a screening of glucose biosensors fabricated by entrapment of the enzyme
glucose oxidase in the above-mentioned electrodeposition polymers on the surface of platinum electrodes showed that the properties of immobilization matrices influence the characteristics of the resulting sensors and the relationship between these parameters is quite complex [Ngounou et al. 2004], [Ngounou et al. 2007].

Generally, there are three steps in the screening of EDPs as immobilization matrices:

1. synthesis of electrodeposition polymer;
2. fabrication of the biosensor by immobilization of polymer film with entrapped biomaterial on the electrode surface;
3. evaluation of the biosensor characteristics.

A high reproducibility of all these steps is a prerequisite for the comparison of different electrodeposition polymers as immobilization matrices. Therefore, attempts to increase the reproducibility of the entire screening procedure were undertaken and will be described in the following sections.

An understanding of the relationship between the composition of electrodeposition polymers and characteristics of fabricated biosensors would allow for a rational design and optimization of electrodeposition polymers as immobilization matrices for amperometric biosensors.

### 3.1.1.1. Improvement on synthesis of electrodeposition polymers

In order to investigate the reproducibility of the synthesis procedure, developed by Ngounou et al. [Ngounou et al. 2004], [Ngounou et al. 2007] four polymers were synthesized by emulsion polymerization of monomer mixtures of exactly the same composition, containing 4.5% butyl acrylate, 36.7% dimethylaminoethylmethacrylate, 4.5% methyl methacrylate, 22.3% cyclohexyl methacrylate, and 32.5% 1-imidazolyl-hex-5-en-3-oxy-2-ol (Fig. 3.2). The polymerization reactions were carried out in reactor tubes with reflux condenser at a temperature of 90°C for 5 h with tert-butyl-hydroperoxide as radical initiator. Before the reaction, the monomers were neutralized
with a corresponding amount of 3 M HCl. The obtained copolymers were dissolved in
minimal amount of methanol.

In spite of the fact that all four polymers, shown in Fig. 3.2, were synthesized in
the same way, even optically they looked different. The polymer S2 had a white colour,
polymer S4 was yellow and polymers S3 and S5 were both cream-coloured. These
polymers were further used for the fabrication of glucose biosensors by entrapping
glucose oxidase in the polymer film, electrodeposited on the surface of platinised
graphite electrodes (d = 3.05 mm) during electrochemically induced pH modulation.
Precipitation of the EDPs on the electrode surface was achieved by application of 20
cycles of a potential pulse sequence (-1.2 V for 0.2 s and 0 V for 5 s).
Calibration of biosensors was performed by means of chronoamperometry and
oxidation of enzymatically formed H₂O₂ at the electrode (at 600 mV vs. Ag/AgCl/3M
KCl). Fig. 3.3A represents calibration graphs, obtained with four biosensors fabricated
by entrapment of glucose oxidase in the polymer S2 and represents the reproducibility of
the biosensor fabrication and evaluation procedure.
Fig. 3.3B shows chronoamperometric responses of biosensors prepared using polymers S2, S3, S4 and S5.

![Graph showing chronoamperometric responses](image)

**Fig. 3.3** Chronoamperometric calibration curves (A) of four glucose sensors fabricated using the same polymer S2 and (B) of four biosensors fabricated using four polymers S2 (blue line), S3 (green line), S4 (pink line), S5 (red line), which had been synthesized according to the same synthesis protocol using the same initial mixture of the monomers and under the same conditions. Additions 1–6: 12; 24; 48; 90; 167; 231 mM glucose

As can be clearly seen in Fig. 3.3, the variation in the response of biosensors, fabricated from the four different polymer preparations is larger than that of the biosensors, obtained from the same polymer. This suggested that the reproducibility of polymers synthesis is much lower than that of biosensors fabrication and evaluation.

In order to find the source of irreproducibility, the obtained polymers were investigated by means of $^1$H NMR spectroscopy. Instead of assumed statistical distribution of all monomers within the polymer backbone, the NMR investigation showed a significant drift of the polymer composition. The imidazolyl-functionalized monomer was obviously not included into the polymer backbone at all since no
characteristic signals of the imidazol ring were found in NMR spectrum of the precipitated polymer (at 7.5–8.0 ppm in Fig. 3.4A).

Fig. 3.4 $^1$H NMR spectra in methanol-D4 of the solid content of one of the EDPs after (A) and before (B) precipitation with diethyl ether.

There are many studies, describing emulsion copolymerisation of two monomers but only few reports mostly in the form of patents are devoted to ternary or multicomponent copolymerisation dealing mainly with properties of the resulting polymers and their application. In spite of the complexity of a multicomponent copolymerisation and the absence of reliable models describing kinetics in such systems, they are attractive due to a large variation in properties of resulting copolymers [Matyjaszewski et al. 2002]. Generally, however, the properties of the resulting copolymer are determined by intrinsic factors (monomers nature, overall yield of polymerization) and reaction conditions [Matyjaszewski et al. 2002]. Therefore, a better reproducibility of the polymer synthesis can be achieved by increasing the precision of experimental conditions control and by controlling a polymerization reaction itself.
Further improvements on EDPs synthesis, described in this section were predominantly introduced by Dr. Dimitri Guschin.

**Decrease of composition drift**

Composition drift is not a rare phenomenon in emulsion copolymerisation and is determined by reactivity ratios of monomers and monomer ratio in the polymer particles, which often differs from the overall monomer ratio in the feed.

The reactivity ratio of many acrylic monomers is, for example, higher than that of allyl- and olefin-based monomers and copolymerization of the monomers with different reactivity is usually difficult and leads often to a strong composition drift [Gu et al. 2004], [Liu et al. 2004], [Venkatesh et al. 2004]. Therefore, a decrease of the composition drift can be achieved by selecting monomers with similar reactivities.

Another factor, which has to be taken into account in order to control emulsion polymerisation is partitioning of monomers [Matyjaszewski et al. 2002]. Monomer ratio in polymerization particles is in most cases determined by the relative solubility of monomer in dispersing medium. And if the more reactive comonomer for example is the less soluble one, than increase of solvent content in the feed will lead to a larger composition drift [Schoonbrood et al. 1995]; in case of good solubility an opposite effect will be achieved [Noel et al. 1994].

According to this, a composition drift in the polymer can be decreased by selecting monomers of similar reactivity and optimization of polymerization conditions [Matyjaszewski et al. 2002]. Taking this into consideration a new set of monomers (styrene, butylacrylate, dimethylaminomethylacrylate and 2-hydroxyethylmethacrylate) was chosen for the synthesis of electrodeposition polymers in order to reduce the composition drift.

**Exclusion of handling errors**

In order to reduce pipetting errors during the preparation of the monomer mixture, an automated liquid handling workstation (Tecan RSP 9000 equipped with an XLP6000 pump) was adapted for this purpose (Fig. 3.5). The workstation is controlled by a
software module, developed in the lab and allows filling of up to eight reaction tubes with defined amounts of up to 20 different monomers. This workstation was used for the preparation of 16 mixtures of monomers for the synthesis of a new library of electrodeposition polymers.

Fig. 3.5 Automated liquid handling workstation. 1 - pipette tip rack; 2 - polymerization tubes; 3 - reagents rack; 4 - XLP6000 pump; 5 - RSP 9000.

**Improvement of temperature and homogeneity control**

Control of temperature and homogeneity of the reaction mixture is important for most of chemical reactions, including polymerization. In order to improve the control of these parameters an earlier used convenient polymerisation set-up was substituted by specifically designed parallel synthesizer, shown in Fig. 3.6. The synthesizer consists of heating, stirring and temperature monitoring modules and eight polymerization reactors with integrated reflux condenser, stirrer and thermocouples. A digital stirrer with integrated rotational-speed sensor allows stirring of high-viscous polymerization mixtures with a constant speed and an accuracy of 10 rpm. A high-precision heating module in combination with a contact thermometer provides the temperature control in
the polymerization reactors with an accuracy of 0.2 °C. Moreover, the integrated rotation-speed sensor and the thermocouples make on-line monitoring and control of the polymerisation process possible.

Fig. 3.6 Parallel polymerization reactor with improved stirring and temperature control. 1) heating module; 2) reaction tube; 3) reflux condenser; 4) combined stirring module with rotational-speed sensor; 5) temperature monitoring module with individual thermocouples; 6) individual stirrer bar with integrated 7) thermocouple

**Temperature profiles for characterization of polymerization process**

Reproducibility of the synthesis process could be estimated by comparison of the recorded temperature profiles. They are a kind of fingerprints of polymerisation reactions and provide information on the composition of monomers mixture, nature and amount of radical initiator and the presence of inhibitors.

**Exclusion of oxygen removal**

Argon purging, used in the previous synthesis procedure for the removal of molecular oxygen, was found to influence the reproducibility of the polymerization reaction negatively. This was generally due to small fluctuations in the purge rate, which are caused by variations in the argon pressure, but can not be eliminated. Therefore, the new synthesis was performed without oxygen removal by argon bubbling.
Synthesis of a library of electrodeposition polymers

An improved synthesis protocol was implemented for the synthesis of 16 electrodeposition polymers with a random content of styrene, butylacrylate, dimethylaminomethylacrylate and 2-hydroxyethylmethacrylate. The synthesis of polymers was performed by emulsion polymerization at a temperature of 100 °C using azobisisobutyronitrile (AIBN) as radical initiator. The resulting copolymers were neutralized with 10 M HCl and dissolved in methanol, which was then slowly replaced by water at 80 °C. The obtained polymer suspensions and their chemical structure are presented in Fig. 3.7.

Fig. 3.7 Photograph of a library of electrodeposition polymers and the generalized structure of EDPs.

In order to estimate a potential composition drift of the obtained electrodeposition polymers, NMR investigations were carried out. For this, aliquots of the suspensions of the electrodeposition polymers were precipitated with 10 M KOH, washed with water and dried at 120 °C for 30 min. After drying, a solid content of each polymer was weighed and re-dissolved in methanol-D4. The recorded $^1$H NMR spectra of the polymers were divided into six regions corresponding to protons of different functionality and compared with the theoretically expected NMR spectra. The relative composition of the copolymer was derived by regression analysis of the recorded $^1$H NMR spectra (Table 3.2).
Table 3.2 Relative composition of monomers in the reaction volumes (black font) and in the obtained polymers (blue font).

<table>
<thead>
<tr>
<th>Monomers mixture</th>
<th>Styrene</th>
<th>Butylacrylate</th>
<th>Dimethylaminoethylmethacrylate</th>
<th>2-Hydroxyethylmethacrylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>7.19%</td>
<td>50.98%</td>
<td>34.64%</td>
<td>7.19%</td>
</tr>
<tr>
<td>P1</td>
<td>8%</td>
<td>46%</td>
<td>40%</td>
<td>6%</td>
</tr>
<tr>
<td>P2</td>
<td>45.50%</td>
<td>5.21%</td>
<td>1.90%</td>
<td>47.39%</td>
</tr>
<tr>
<td>P2</td>
<td>43%</td>
<td>-</td>
<td>5%</td>
<td>52%</td>
</tr>
<tr>
<td>P3</td>
<td>13.57%</td>
<td>28.53%</td>
<td>27.15%</td>
<td>30.75%</td>
</tr>
<tr>
<td>P3</td>
<td>14%</td>
<td>23%</td>
<td>24%</td>
<td>39%</td>
</tr>
<tr>
<td>P4</td>
<td>23.05%</td>
<td>14.70%</td>
<td>33.72%</td>
<td>28.53%</td>
</tr>
<tr>
<td>P4</td>
<td>23%</td>
<td>3%</td>
<td>35%</td>
<td>39%</td>
</tr>
<tr>
<td>P5</td>
<td>18.04%</td>
<td>11.37%</td>
<td>28.63%</td>
<td>41.96%</td>
</tr>
<tr>
<td>P5</td>
<td>19%</td>
<td>6%</td>
<td>27%</td>
<td>48%</td>
</tr>
<tr>
<td>P6</td>
<td>23.64%</td>
<td>38.34%</td>
<td>30.03%</td>
<td>7.99%</td>
</tr>
<tr>
<td>P6</td>
<td>26%</td>
<td>33%</td>
<td>33%</td>
<td>8%</td>
</tr>
<tr>
<td>P7</td>
<td>20.21%</td>
<td>38.34%</td>
<td>1.55%</td>
<td>39.90%</td>
</tr>
<tr>
<td>P7</td>
<td>21%</td>
<td>33%</td>
<td>6%</td>
<td>40%</td>
</tr>
<tr>
<td>P8</td>
<td>18.36%</td>
<td>42.97%</td>
<td>36.33%</td>
<td>2.34%</td>
</tr>
<tr>
<td>P8</td>
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<td>28%</td>
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<td>9%</td>
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<td>P9</td>
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<td>30.74%</td>
<td>4.67%</td>
</tr>
<tr>
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<td>51%</td>
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</tr>
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<td>P10</td>
<td>26.81%</td>
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<td>29.79%</td>
<td>8.09%</td>
</tr>
<tr>
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<td>30%</td>
<td>30%</td>
<td>32%</td>
<td>8%</td>
</tr>
<tr>
<td>P11</td>
<td>40.13%</td>
<td>16.83%</td>
<td>12.30%</td>
<td>30.74%</td>
</tr>
<tr>
<td>P11</td>
<td>42%</td>
<td>6%</td>
<td>16%</td>
<td>36%</td>
</tr>
<tr>
<td>P12</td>
<td>17.73%</td>
<td>39.13%</td>
<td>10.03%</td>
<td>33.11%</td>
</tr>
<tr>
<td>P12</td>
<td>16%</td>
<td>42%</td>
<td>12%</td>
<td>30%</td>
</tr>
<tr>
<td>P13</td>
<td>-</td>
<td>15.57%</td>
<td>77.87%</td>
<td>6.56%</td>
</tr>
<tr>
<td>P13</td>
<td>-</td>
<td>25%</td>
<td>65%</td>
<td>10%</td>
</tr>
<tr>
<td>P14</td>
<td>4.47%</td>
<td>36.87%</td>
<td>29.05%</td>
<td>29.61%</td>
</tr>
<tr>
<td>P14</td>
<td>4%</td>
<td>31%</td>
<td>32%</td>
<td>34%</td>
</tr>
<tr>
<td>P15</td>
<td>30.94%</td>
<td>20.14%</td>
<td>35.25%</td>
<td>13.67%</td>
</tr>
<tr>
<td>P15</td>
<td>32%</td>
<td>26%</td>
<td>32%</td>
<td>10%</td>
</tr>
<tr>
<td>P16</td>
<td>9.40%</td>
<td>48.72%</td>
<td>41.88%</td>
<td>-</td>
</tr>
<tr>
<td>P16</td>
<td>10%</td>
<td>46%</td>
<td>44%</td>
<td>-</td>
</tr>
</tbody>
</table>
As it can be seen in Table 3.2, the drift in the polymer composition could not be completely eliminated. Nevertheless, the application of above-described equipment and a precise control of the polymerization conditions allowed for significant improvement of reproducibility of EDPs synthesis.

3.1.1.2. Improvement of fabrication and evaluation procedure

Manual fabrication and evaluation of a large amount of biosensors is a time-consuming process. Screening of a large amount of different electrodeposition polymers is however indispensable for the investigation of their impact on the biosensor characteristics and the further rational design of immobilization matrices with specifically adopted properties. For this purpose an electrochemical robotic system, allowing to automate the whole screening procedure, was developed by Reiter et al. in 2004 [Reiter et al. 2004]. The electrochemical robotic system (Fig. 3.8) consists of a step-motor driven x,y-table as basis for a plastic container with an integrated shaker and microtiter plate. A glass plate, which cover a container with microtiter plate prevents the solvent from evaporation and allows if necessary to establish an inert atmosphere inside the chamber. The microtiter plate is fixed on top of a shaker and the whole chamber can be moved in x- and y-direction by x- and y- positioning step-motors. The microtiter plate contains 96 wells with a volume of about 200 µl, which are used as small electrochemical cells and can be filled with different solutions. Glass vials of a bigger volume (8 ml), located on the sides of microtiter plate (external wells) can be used for the same purpose. The three-electrode system with a capillary for the addition of solution can be moved in z-direction by a z positioning step-motor and the electrodes are positioned into the wells of microtiter plate or external glass vials through a hole in the glass plate. The three-electrode system, connected to a potentiostat, consists of a working electrode, a Pt counter electrode and a miniaturized Ag/AgCl reference electrode. Through a capillary, connected to a syringe pump, a defined quantity of reagents can be added at predefined times into the wells.
The electrochemical robotic system, controlled by an in-house-developed software is able to perform a complete library of electroanalytical techniques, including voltammetry, chronoamperometry and differential pulse voltammetry. For programming the operations in the electrochemical robotic system, an easy-to-use script language is used. A typical script is represented in Fig. 3.9. In the first two columns, the coordinates of the position of the three-electrode system are written. The third column identifies which kind of operation is performed by the robotic system once the electrode bundle is located in the pre-defined position. The next columns allow for defining the parameters of the operation, which has to be carried out once the three-electrode is in the pre-defined position.
In this study, the electrochemical system was further modified in order to improve the reproducibility of screening procedure. For this, some additional features had to be implemented, and the screening protocols had to be optimized.

**Optimization of the calibration procedure**

The most commonly used approach for biosensor calibration is a continuous measurement of the current response of the sensor upon addition of analyte aliquots to the electrolyte solution. The homogenous dissolution of the analyte in the electrolyte solution is therefore important for obtaining a correct calibration. In a standard electrochemical cell, magnetic stirrers are commonly used for this purpose. Initially it was assumed that the analyte diffusion in small volumes is fast and it is not necessary to homogenize the electrolyte solution in the wells of microtiterplate after addition of the
analyte. However, the current response was not stable after analyte additions and signal fluctuations did not allow obtaining reliable calibration curves. In order to improve the homogenization of the solution a shaker unit was integrated into the electrochemical robotic system. Figure 3.10A shows two calibration curves of glucose biosensor obtained by the continuous use approach. In the first case (blue line), the homogenization of the solution was performed by an integrated shaker. In the second case (red line) a small magnetic stirring bar was placed into the well of the microtiter plate. A nearly continuous increase of the recorded current after addition of the glucose solution into the electrolyte solution by the syringe pump in the first case did not allow constructing a reliable calibration curve. Despite a good result in the second case, when the solution was homogenized with a magnetic stirring rod, automation of this kind of stirring seems to be difficult (red line in Fig. 3.10A). As an alternative, a single use approach was tested for calibration of the biosensor. In spite of a rare use of this calibration approach in the lab research, it is the most prospective one for commercial application [Kissinger 1997]. For this, a fabricated biosensor was inserted into external vials, which were filled with glucose solutions of known concentrations and chronoamperometric measurements were run in each calibration solution. A digital I/O card made it possible to switch on the potentiostat exclusively after dipping of the biosensor into one of the calibration solutions. This allowed avoiding a potential over-swinging, which could have a negative influence on the activity of biorecognition elements of the biosensor. The recorded current decay curves in glucose solutions of different concentrations are shown in Fig. 3.10B. The current could reach a steady state approximately 60 s after the start of the measurement. Therefore, for the construction of calibration curves, current values at times higher than 60 s were used. Since the obtained values could be used for the construction of a reliable calibration curve, a single use approach was found to be suitable for the calibration of biosensors in the electrochemical robotic system.
Fig. 3.10 Chronoamperometric biosensor calibration using the robotic system. (A) Continuous use approach: red line – permanent stirring with magnetic stirring bars; blue line – mixing with the microtiter plate shaker. Addition of glucose stock solution by means of a syringe pump. (B) Single use approach. Dipping of the biosensor into vials, containing known concentrations of glucose solutions.

**Implementation of a gold microtiter plate**

Another considerable contribution to the improvement of the reproducibility of the automatic screening procedure was made through the integration of a gold microtiter plate in the electrochemical robotic system. Earlier, the same three-electrode system was used for electrodeposition of the polymer/enzyme suspension on the surface of the working electrode followed by an evaluation of the fabricated biosensor. As a result, the counter and reference electrodes were contaminated with the adsorbed electrodeposition polymer. This unknown amount of enzyme, adsorbed on the surfaces of reference and counter electrode produces hydrogen peroxide, which can contribute to the overall response of the biosensor and influence the reproducibility of the evaluation procedure negatively. To solve this problem, it was necessary to avoid any contact of the counter
and reference electrodes with the electrodeposition suspension. For this, a plastic microtiter plate was replaced by a gold microtiter plate, which was used as counter electrode during polymer electrodeposition (Fig. 3.11). The counter and reference electrodes were fixed approximately 0.8 cm higher than the working electrode, as shown in Fig. 3.11 and were used exclusively during sensor evaluation.

![Photograph of the electrochemical robotic system](image)

These changes allowed avoiding unspecific adsorption of the polymer/enzyme suspension on the surfaces of the counter and reference electrodes used for the evaluation of biosensor characteristics.

**Improvement of cleaning procedure**

Besides reproducibility of the polymer deposition procedure, a reproducible recovery of the electrode surface after each screening cycle is important for the reproducible fabrication of biosensors. Since in the electrochemical robotic system the same electrode is used for the fabrication of different biosensors the earlier polymer film had to be removed before a new polymer can be electrodeposited. Taking this into account, an electrode cleaning procedure has to fulfil two main requirements: firstly, it
should provide a reliable removal of the previous polymer film from the surface of the electrode; secondly, it should be soft enough in order not to change the properties of electrode surface. Even a small variation of surface properties of the electrode material can influence the characteristics of the resulting biosensors and becomes a source of irreproducibility of the whole screening procedure.

In a previous study [Reiter et al. 2004], cleaning of the electrode surface was performed electrochemically in acid medium. It was established that a protonation of the polymer film with simultaneous formation of gas bubbles of H₂, Cl₂ or O₂ will allow for dissolving the deposited polymer film from the electrode surface. Whereas a classical electrochemical cleaning of Pt electrodes by cycling in H₂SO₄ between -1500 and +2500 mV did not lead to complete polymer dissolution, conditioning of the electrode in 5 M HCl at a constant potential of 3 V vs. Ag/AgCl/3 M KCl allowed a removal of the polymer film from the electrode surface. Unfortunately, hydrochloric acid is known to attack a platinum surface under harsh conditions. Therefore, monitoring the electrode surface after such an aggressive cleaning procedure is necessary.

The quality of the electrode surface is usually characterized by cyclic voltammetry in the presence of a free-diffusing redox mediator such as [Ru(NH₃)₆]²⁺/³⁺. In order to monitor the quality of the electrode surface, cyclic voltammogramms of the electrode were recorded before the electrode modification, after polymer deposition and after cleaning by electrochemical formation of Cl₂ (Fig. 3.12A). The current decrease in the cyclic voltammogram, recorded after the polymer deposition is due to a hindered access of [Ru(NH₃)₆]²⁺/³⁺ towards the electrode surface and is the evidence of successful modification of the electrode with the electrodeposition polymer.
Fig. 3.12 Cyclic voltammograms from a three-electrode bundle as used for sensor preparation in the electrochemical robotic system (working electrode: 250 μm Pt disc; counter electrode: Pt; reference electrode: Ag/AgCl/3M KCl) obtained in 5 mM [Ru(NH$_3$)$_6$]Cl$_3$ in presence of 100 mM KCl as supporting electrolyte (sweep rate 50 mV/s). The cyclic voltammograms were recorded before the electrochemically induced precipitation of an electrodeposition polymer (blue line), of the polymer-modified electrode (red line) and after the cleaning process (green line). (A) Cleaning was performed electrochemically in 5 M HCl by applying a constant potential of 3 V vs. Ag/AgCl/3M KCl for 6 min. (B) Cleaning was performed mechanically on the polishing wheel integrated within the robotic system.

Cyclic voltammogram, recorded after electrochemical cleaning of the electrode in HCl indicates a successful removal of the polymer film and simultaneous increase in the active surface of the electrode as compared with the initial bare electrode (see Fig. 3.12A). Since the increase in the active surface of the electrode can not be precisely controlled and influences negatively the reproducibility of the entire screening procedure, an alternative way of electrode cleaning had to be found. For this, a polishing wheel was integrated into the electrochemical robotic system (Fig. 3.11). A
software extension allow for a controlled turning on and off the polishing wheel, as well as a precise approaching of the electrode to the surface of the polishing cloth. The cyclic voltammogram, recorded after mechanical polishing of the modified electrode was found to be identical with that, recorded with a bare electrode before the polymer deposition. This proves a successful removal of the polymer film and re-establishment of the initial electrode surface (Fig. 3.12B).

3.1.1.3. Influence of EDPs composition on the characteristics of biosensors

To compare the impact of the composition of electrodeposition polymers on the biosensor properties, they were used for the construction of two different types of biosensors: enzyme biosensors, based on glucose oxidase (for glucose detection) and cell biosensors, based on permeabilized genetically modified *Hansenula polymorpha* yeast cells (for L-lactate detection). Enzyme biosensors for glucose detection represent the first generation of biosensors, the detection principle of which is based on the electrochemical monitoring of the product of the enzymatic reaction, in this case hydrogen peroxide. In cell-based biosensors for lactate detection, a free-diffusing mediator phenazine methosulfate (PMS) is used for the establishment of the electron transfer pathway between the FC $b_2$ inside the permeabilized cells and the electrode. Fabrication and evaluation of all biosensors were performed automatically in the electrochemical robotic system. Firstly, the Pt working electrode was cleaned by polishing on the integrated polishing wheel and the clean electrode was characterized by cyclic voltammetry in 5 mM [Ru(NH$_3$)$_6$]$^{2+/-3+}$. Electrode modification was performed in a well of the gold microtiter plate containing a mixture of an electrodeposition polymer with glucose oxidase or *Hansenula polymorpha* permeabilized cells. After electrodeposition of the polymer film with entrapped biorecognition elements on the surface of the electrode, the fabricated biosensor was rinsed in phosphate buffer. Calibration of biosensors was performed in external glass vials, filled with increasing concentrations of glucose in buffer of pH 6.3 or L-lactate with 0.2 PMS in buffer of
pH 7.8, respectively. After positioning the electrode bundle into the solution, all three electrodes were connected to the potentiostat by means of a relay switch and the working electrode was polarized to a potential of +600 mV (for glucose biosensors) or 0 mV (for lactate biosensors). The catalytic current was recorded for a predefined time (e.g. 120 s) before the electrode bundle was moved to the next vial with higher glucose or lactate concentration. The current values after 60 s were used for the construction of the calibration graphs from which the apparent maximal current ($I_{\text{max}}^{\text{app}}$) and the apparent Michaelis constant ($K_{M}^{\text{app}}$) were derived. The $I_{\text{max}}^{\text{app}}$ value characterizes the sensitivity of the obtained biosensor and usually depends among others on the activity of biorecognition element in the bioselective layer of the biosensor. The $K_{M}^{\text{app}}$ value provides information on the detection limit and the linear range of the biosensor.

All glucose sensors (Fig. 3.13) showed a relatively small variation in $K_{M}^{\text{app}}$ and $I_{\text{max}}^{\text{app}}$ values of a factor of up to 10.

Fig. 3.13 $K_{M}^{\text{app}}$ and $I_{\text{max}}^{\text{app}}$ values of amperometric glucose biosensors obtained from 16 different cathodic EDPs (250 μm Pt electrode, potential pulse profile for electrodeposition: -1.2 V/0.2s and 0 V/5s; 100 mM phosphate buffer pH 6.3).
Obviously, biosensors based on polymer P7 are characterized by the best sensitivity, while the polymer P12 can be used for construction of biosensors with increased linear range and still sufficient sensitivity.

![Graph showing KM and Imax values for amperometric lactate biosensors](image)

Fig. 3.14 $K_M^{\text{app}}$ and $I_{\text{max}}^{\text{app}}$ values of amperometric lactate biosensors obtained from 16 different cathodic EDPs (500 μm graphite electrode; potential pulse profile for electrodeposition: -1.2 V/0.2s and 0 V/5s; 50 mM phosphate buffer pH 7.8).

Lactate sensors show a variation in $K_M^{\text{app}}$ by a factor of up to three and $I_{\text{max}}^{\text{app}}$ values differences are smaller than those of glucose biosensors and vary by a factor of up to four. The sensors based on polymers P3 and P9 are the best with respect to both signal height and linear range. Polymers P4 and P8 were not suitable for the construction of either glucose or of lactate biosensors.

From this comparison, it becomes clear that each biosensing system requires individual search for the optimal immobilization matrix and it is impossible to synthesize the “perfect” EDP, which would be the best immobilization matrix for all types of biosensors.
3.1.1.4. Summary

The reproducible synthesis of electrodeposition polymers was achieved by improving instrumentation for pipetting of the monomer mixtures and polymer synthesis and online control of the polymerization conditions. The NMR analysis of the obtained polymers allowed for calculation of the real composition of EDPs.

Introduction of new features in the electrochemical robotic system allowed for improvement of the reproducibility of the automatic screening procedure. The integration of a gold microtiter plate for polymer electrodeposition and a polishing wheel for electrode cleaning contributed to the improvement of the reproducibility of fabrication and evaluation procedure.

The library of electrodeposition polymers, prepared by controlled and reproducible synthesis was used for the automatic fabrication of glucose and lactate biosensors. Automatic evaluation of the obtained biosensors in the electrochemical robotic system allows for fast search of the optimal immobilization matrix for different types of biosensors and comparison of the impact of the polymer composition on the properties of the derived biosensors.

3.1.2. Osmium modified EDPs as immobilization matrices for reagentless multielement biosensors

The electrodeposition polymers described in Section 3.1.1 can be used for the fabrication of either biosensors of the first generation as in the above-described glucose biosensor based on hydrogen peroxide monitoring, or second generation biosensors, which use free-diffusing redox mediators for electron transfer between biorecognition elements and the electrode, for example, a lactate biosensor based on permeabilized Hansenula polymorpha yeast cells with PMS as a free-diffusing mediator. In order to extend the applicability of electrodeposition polymers for the construction of reagentless multilayered biosensors, the development of a strategy for binding of redox compounds to the EDP backbone was developed. Osmium and ruthenium complexes [Forster and
ferrocene derivatives [Mizutani et al. 1988], [Lange et al. 1985] or quinone-type compounds [Hendry and Turner 1988] are often used for the construction of polymer-bound redox relays. Charge transfer in redox polymer films occurs by electron hopping, as first described by Kaufman et al. [Kaufman et al. 1979], [Kaufman et al. 1980]. The scheme in Fig. 3.15 shows electron transfer in osmium complex-containing redox polymers. In the first step, the electron is transferred from the active centre of the biorecognition element to an osmium entity of the polymer close to it, followed by electron transfer to the next osmium entity and final delivery of the electron to the electrode.

Fig. 3.15 Electron transfer in osmium complex-containing redox polymers

**Functionalization of electrodeposition polymers**

In order to be able to modify electrodeposition polymers with redox compounds, the polymer backbone should carry suitable functional groups, such as imidazol, pyridyl or oxygen-containing functional groups. There are several possibilities to functionalize a polymer backbone with such functionalities.

The first strategy (see Fig. 3.16A) is based on the integration of an active precursor of the monomer (for example acryloyl chloride, methacryloyl chloride) into the polymer backbone. The monomer precursor may be later modified with the desired functionality. The main disadvantage of this method of polymer functionalization is seen
in the possible side reactions of the highly activated functional groups of the monomer precursor with functional groups of the other monomers present in the polymer backbone.

Another strategy for functionalization of the polymer backbone (Fig. 3.16B) is introduction of an already functionalized monomer into the polymer. For this, imidazolyl or pyridyl-functionalized monomer is copolymerized with other monomers. The obtained functionalized polymer can be further modified with osmium complexes by ligand exchange reactions. Ngounou et al suggested functionalization of EDPs by introduction of either copolymers of 1,2-epoxy-9-decen, 1,2-epoxy-9-hexen, glycidyl methacrylate or allylglycidyl ether with imidazole or copolymers of 1-(3-aminopropyl)imidazol or 4-picolinamin with methylacrylate into the polymer backbone [Ngounou 2005]. Unfortunately, later NMR investigations as described in section
3.1.1.1 revealed that some of the above-mentioned functionalyzed monomers were not incorporated into the backbone of most of the EDPs. A significant difference in the reactivities of the functionalyzed monomers in comparison to other monomers in the feed was thought to be the main reason for the observed phenomenon. In order to overcome this problem Dr. Dimitri Guschin synthesized a small library of functionalyzed (meth)acrylate monomers, shown in Fig. 3.17 using a method proposed earlier [Hadjikallis et al. 2002], [Cui et al. 2002]. The six members of the library were obtained by reaction of the corresponding alcohols with methacryloyl chloride in the presence of a base [Guschin et al. 2009].

Fig. 3.17 Functionalized monomers used for the synthesis of electrodeposition polymers.

The (meth)acrylate derivatives were assumed to have a reactivity similar to that of (meth)acrylic monomers. This should allow for their incorporation into the polymer backbone during polymerization. To check this assumption, the functionalyzed (meth)acrylic monomers were copolymerized with DMAEMA. The $^{13}$C NMR spectra of the obtained copolymers confirmed successful incorporation of the functionalyzed (meth)acrylic monomers into the polymer backbone [Guschin et al. 2009]. Therefore,
the functionalized monomers, shown in Fig. 3.17 were used for the synthesis of functionalized electrodeposition polymers of different compositions.

**Modification of electrodeposition polymers with osmium complexes**

The main strategy for the modification of polymers with osmium complexes is based on ligand exchange reactions. In most cases, [Os(bpy)$_2$Cl$_2$] and [Os(bim)$_2$Cl$_2$] complexes or their derivatives are used for this purpose. Attachment of the osmium complexes occurs by exchanging labile chloro ligands against ligands attached to the polymer backbone. This is possible due to higher binding constant of the ligands attached to the polymer than that of chloro ligands. The redox potential of the osmium complex depends on the properties of the ligands attached to it. Thus, design of the ligand shell allows for modulation of the redox potential of the polymer-integrated osmium complex. This property of osmium complexes allows to adapt their redox potential to the formal potential of the prosthetic group of different enzymes. To ensure effective electron transfer from the active center of the enzyme to the polymer-bound osmium complex, the potential of osmium complex should be more positive than that of the enzyme’s prosthetic group. Additionally, Mao et al have demonstrated that the efficiency of the electron transfer in redox polymers is higher if the osmium entities are attached to the polymer backbone with a linker molecule a so-called spacer arm [Mao et al. 2003].

**Library of osmium complex modified electrodeposition polymers**

Guschin et al have synthesized a library of more than 50 osmium complex modified electrodeposition polymers varying in composition of the polymer backbone, length of the spacer arms and the redox potential of the attached osmium complex [Guschin et al. 2010]. Polymer-bound osmium complexes have redox potentials in a wide range between -400 and 800 mV vs. Ag/AgCl. Such redox electrodeposition polymers have two advantages over the redox polymers described in other studies.
Firstly, they can be non-manually electrodeposited on the electrode surface and are therefore suitable for the fabrication of multilayered biosensors with complex architectures. Secondly, the availability of polymer-bound osmium complexes with a wide range of formal potentials allows for selection of an individual optimal redox EDP for wiring different biorecognition elements to an electrode. The osmium complex modified electrodeposition polymers were used in this study for construction of multielement reagentless biosensors, including: a formaldehyde-selective biosensor based on NAD$^+$- and glutathione-dependent formaldehyde dehydrogenase (FdDH), and a L-lactate-selective biosensor based on *Hansenula polymorpha* cell debris and cytochrome c.

### 3.1.2.1. A formaldehyde-selective biosensor based on formaldehyde dehydrogenase from genetically modified *Hansenula polymorpha* yeast cells

This section describes the application of osmium complex modified electrodeposition polymers for the construction and design of a formaldehyde biosensor, utilizing NAD$^+$- and glutathione-dependent formaldehyde dehydrogenase (FdDH) from genetically modified *Hansenula polymorpha* yeast cells. All the experiments, described in this section were performed together with Olha Demkiv and Solomiya Paryzhak from the Institute of Cell Biology (Lviv, Ukraine).

NAD$^+$- and glutathione-dependent formaldehyde dehydrogenase is an enzyme that catalyzes the oxidation of formaldehyde (FA) to formic acid. Fig 3.18 shows a scheme of the electron-transfer pathway of this catalytic reaction. As can be seen, the transfer of electrons from formaldehyde to the active centre of FdDH occurs via an intermediate, S-hydroxymethylglutathione, with simultaneous reduction of NAD$^+$ to NADH. For further transfer of the electrons to the electrode surface, the enzymatically generated NADH has to be reoxidised to NAD$^+$. For this purpose, addition of a redox mediator to the system is necessary since the oxidation of NADH on the bare electrode occurs only at high overpotentials [Blaedel et al. 1975].
Construction of a biosensor based on this electron transfer pathway requires besides the enzyme formaldehyde dehydrogenase and a mediator the addition of two cofactors, glutathion and NAD$^+$. This is one of the examples of multicomponent biosensors. Construction of this kind of biosensors by such method of immobilization as adsorption is difficult since not only the attachment of several components to the electrode surface has to be reliable but also the interaction of all immobilized components with each other should be ensured. Osmium complex modified electrodeposition polymers allow for immobilization of all the required components on the electrode surface and for their interaction inside the polymer network. For additional protection of low-molecular immobilized components from leakage, the modified electrode can be additionally covered with a protective layer, for instance a Nafion layer. The architecture of such a biosensor is represented in Fig. 3.19, and was used for the design of an optimised FdDH biosensor.
For this, the enzyme FdDH and its cofactors glutathione and NAD$^+$ were immobilized on the surface of a platinised graphite electrode by entrapment in an osmium complex modified electrodeposition polymer film. The reduced glutathione and NAD$^+$ can freely diffuse in the polymer network and interact with the enzyme. The modified electrode was additionally covered with a negatively charged Nafion membrane, which prevents the leakage of glutathione and NAD$^+$ due to charge interactions, but allows the diffusion of the substrate FA into the biosensitive layer.

To optimize the working characteristics of the formaldehyde biosensor, five different osmium complex-containing electrodeposition polymers (1CPOs, 2CPOs, 3CPOs, 4CPOs, 5CPOs) were tested as immobilization matrices. The synthesis of these osmium complex-containing polymers is described in section 2.2.8.1 of chapter 2. The molecular structures of the obtained osmium complex-containing redox polymers are shown in Fig. 3.20.

Fig. 3.20 Molecular structures of the osmium redox polymers 1CPOs-5CPOs. n in the side chains is 1 or 6.
The redox potentials of the polymer-bound osmium complexes were determined using differential pulse voltammetry of electrodes modified with a layer of the redox polymer (Fig. 3.21).

![Differential pulse voltammograms (DPVs) of CPOs-modified electrodes.](image)

Fig. 3.21 Differential pulse voltammograms (DPVs) of CPOs-modified electrodes.

The potentials corresponding to the peak currents in the differential pulse voltammograms (DPVs) show the optimal potentials for the Os$^{2+}$/Os$^{3+}$ redox reaction on the electrode surface. The peak of the DPV for 1CPOs can be seen at a potential of +200 mV vs. Ag/AgCl/3M KCl. The other four polymers have two distinguishable potentials with one peak at a potential of around +360 mV and another less distinct peak at a potential of about 200 mV. The presence of two peaks in the DPV usually indicates the presence of two different redox forms of the osmium complex in the redox polymer. This happens if the polymer backbone contains more than one functional group, which can substitute the chloro ligands in the osmium complex.

Table 3.3 summarizes information on structural differences and electrochemical properties of the osmium complex-containing redox polymers 1CPOs-5CPOs. 
Table 3.3 Structural differences and electrochemical properties of Os-containing redox polymers 1 CPOs-5CPOs

<table>
<thead>
<tr>
<th>№</th>
<th>Symbol</th>
<th>Polymer backbone</th>
<th>Length of the spacer arm</th>
<th>Osmium complex</th>
<th>Redox potential, mV (vs Ag/Ag Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1CPOs</td>
<td>P4VP Mr = 60kDa</td>
<td>8 atoms</td>
<td>[Os(bpy)$_2$Cl$_2$]</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>2CPOs</td>
<td>P4VP + BMA</td>
<td>8 atoms</td>
<td>[Os(bpy)$_2$Cl$_2$]</td>
<td>200; 365</td>
</tr>
<tr>
<td>3</td>
<td>3CPOs</td>
<td>P4VP Mr = 60 kDa</td>
<td>13 atoms</td>
<td>[Os(bpy)$_2$Cl$_2$]</td>
<td>200; 365</td>
</tr>
<tr>
<td>4</td>
<td>4CPOs</td>
<td>P4VP Mr = 160 kDa</td>
<td>13 atoms</td>
<td>[Os(bpy)$_2$Cl$_2$]</td>
<td>196; 360</td>
</tr>
<tr>
<td>5</td>
<td>5CPOs</td>
<td>P4VP + BMA</td>
<td>13 atoms</td>
<td>[Os(bpy)$_2$Cl$_2$]</td>
<td>195; 361</td>
</tr>
</tbody>
</table>

Abbreviations used in Table 3.3: P4VP – poly(4-vinylpyridine); BMA – butyl methacrylate.

The polymers, shown in Table 3.3, were modified with the same osmium complex, but differ in their composition or length of the polymer backbone and length of the spacer arms. All five redox polymers were used for fabrication of biosensors with the architecture shown in Fig. 3.19. For this, 2 µl FdDH (15 U ml$^{-1}$) and 2 µl 25 mM NAD$^+$ were mixed with 2 µl of the corresponding polymer suspension. The mixture was electrodeposited onto the surface of a platinised graphite electrode by application of 20 cycles of potentiostatic pulses with the sequence -1200 mV for 0.2 s followed by 0 mV for 5 s. After this, 3 µl of a 50 mM solution of reduced glutathione were dropped on top of the electrode modified with FdDH, NAD$^+$ and the osmium complex-containing redox polymer and dried for 2-4 min. Finally, a protective Nafion membrane was formed by
dropping 5 µl of a 1% solution of Nafion in ethanol on top of the CPOs-FdDH-NAD\(^{\pm}\)-GSH-modified electrode.

Fig. 3.22 (A) FA calibration curves for the CPOs-FdDH-NAD\(^{\pm}\)-GSH-Nafion-modified platinised graphite electrodes using different electrodeposition polymers (200 mV in 20 mM phosphate buffer, pH 8.2). (B) Cyclic voltammograms of a 1CPOs-FdDH-NAD\(^{\pm}\)-GSH-Nafion-modified platinised graphite electrode in the absence (a), and in the presence of 16 mM (b) and 32 mM FA (c) (potential sweep from 0.0 to 0.4 V at a scan rate of 5 mV s\(^{-1}\) in 20 mM phosphate buffer, pH 8.2).

Fig. 3.22A shows calibration graphs obtained by chronoamperometric experiments for the fabricated biosensors. All amperometric measurements were performed at the potential of +200 mV vs. Ag/AgCl. Higher potentials were avoided since they may induce direct oxidation of reduced glutathione.

The most promising results were obtained with the osmium complex-based redox polymers 1CPOs and 2 CPOs (see Fig. 3.22A). Taking into account the \(I_{\text{max}}^{\text{app}}\) values, 1CPOs is the most optimal for the construction of a prototype formaldehyde biosensor. Fig. 3.22B shows cyclic voltammograms (CVs) recorded with 1CPOs-FdDH-NAD\(^{\pm}\)-GSH-Nafion-modified platinised graphite electrodes in the absence and presence of FA.
The increase in the anodic current in the presence of FA proves the biocatalytic activity of the 1CPOs-FdDH-NAD^{+}-GSH-Nafion-modified electrode.

To compare the effectiveness of NADH oxidation and the electron transfer to the electrode by the polymer-bound osmium complexes with that performed by free-diffusing redox mediators the electrode modified with FdDH, NAD^{+}, glutathione and polymer, but without the osmium complex, was calibrated by sequential addition of FA to the electrolyte, containing 5 mM K_{3}[Fe(CN)_{6}], 2.5 mM Methylene Blue or 5 mM phenazine ethosulfate. Electrocatalysts such as ferrocene or Prussian Blue were deposited on the surface of a platinised graphite electrode prior to the electrodeposition of FdDH, NAD^{+}, glutathion and the polymer without the osmium complex and the modified electrodes were calibrated with FA. All calibrations, except that in a solution of phenazine ethosulfate were performed at the potential +200 mV vs. Ag/AgCl. In case of phenazine ethosulfate, a potential of 0 mV vs. Ag/AgCl was applied during the amperometric measurements. The obtained calibration curves were compared with that recorded with an electrode modified with 1CPOs-FdDH-NAD^{+}-GSH-Nafion.

![Fig. 3.23 FA calibration graphs for evaluation of different electron transfer mediators for application in a FdDH-based amperometric biosensor.](image-url)
As can be seen in Fig. 3.23, the Os-complex containing polymer (1CPOs) was the most effective electron transfer catalyst among all tested redox mediators and is therefore optimal for the construction of a prototype formaldehyde biosensor.

3.1.2.2. A L-lactate-selective biosensor based on debris of FC \( b_2 \)-overproducing *Hansenula polymorpha* yeast cells and cytochrome \( c \).

Another type of biosensor, which requires a multilayered architecture, is a multienzyme biosensor. Coupling of several enzymes often allows for improvement of the performance of biosensors [Wollenberger et al. 1993]. The construction of multienzyme biosensors is only possible by a method, which allows for simultaneous coimmobilization of several components and layer-by-layer immobilization. From this point of view, the above-described osmium complex modified electrodeposition polymers should be suitable for the construction of multienzyme biosensors. In this section, osmium complex-containing redox polymers have been utilized for the construction of an L-lactate biosensor based on the debris of FC \( b_2 \)-overproducing *Hansenula polymorpha* yeast cells and cytochrome \( c \).

In an earlier study, FC \( b_2 \)-overproducing *Hansenula polymorpha* yeast cells were already used for the construction of a biosensor for L-lactate detection [Smutok et al. 2007]. In this case, the permeabilized yeast cells were immobilized on the surface of a graphite electrode by entrapment under a dialysis membrane or in a cathodic EDP. For electron transfer between the FC \( b_2 \), located inside the permeabilized cells and the electrode, phenazine methosulfate (PMS) was used as a free-diffusing mediator. In this study, the same cells were used for construction of a reagentless biosensor for L-lactate detection using osmium complex modified EDPs as immobilization matrices.

The genetically modified cells were used as a source of the enzyme, flavocytochrome \( b_2 \). The mitochondrial FC \( b_2 \) converts L-lactate to pyruvate and transfers electrons to cytochrome \( c \). Therefore, cytochrome \( c \) is a natural electron acceptor for FC \( b_2 \) in living cells. Due to this, integration of cytochrome \( c \) into the sensor
architecture was supposed to improve the characteristics of the biosensor. Since the cell membrane prevents contact between the osmium entities of the redox EDPs and cytochrome c with the flavocytochrome b2, cell debris was used for the construction of the biosensor.

The architecture of the FC b2-cyt c-based biosensor, and a scheme for the electron transfer pathway are presented in Fig. 3.24.

![Fig. 3.24 Architecture (left) and electron transfer pathway (right) of the FC b2-cyt c-based biosensor.](image)

![Fig. 3.25 Differential pulse voltammograms of osmium complex-modified electrodeposition polymers, used for the construction of a lactate biosensor.](image)
The Fig. 3.25 shows DPVs of osmium complex modified electrodeposition polymers, selected from a library of 50 polymers for the construction of a lactate biosensor based on cell debris of genetically modified *Hansenula polymorpha* yeast as a source of flavocytochrome \(b_2\) and cytochrome \(c\). The key selection parameter was the stability of the formed polymer film. Detailed information about the electrochemical properties and structural peculiarities of the redox EDPs used for construction of the L-lactate biosensor are presented in Table 3.4.

Table 3.4 Structural differences and electrochemical properties of Os-containing redox polymers G44, G45, G112, G230 and G299.

<table>
<thead>
<tr>
<th>№</th>
<th>Symbol</th>
<th>Polymer backbone</th>
<th>Osmium complex</th>
<th>Redox potential, mV (vs Ag/Ag Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G44</td>
<td>VI + AP</td>
<td>[Os(bpy)(_2)Cl(_2)]</td>
<td>-24; 223</td>
</tr>
<tr>
<td>2</td>
<td>G45</td>
<td>BA + AA + AGE-Im</td>
<td>[Os(bpy)(_2)Cl(_2)]</td>
<td>17; 338</td>
</tr>
<tr>
<td>3</td>
<td>G112</td>
<td>BA + AA + MMA + MA-PA</td>
<td>[Os(bpy)(_2)Cl(_2)]</td>
<td>-73; 150</td>
</tr>
<tr>
<td>4</td>
<td>G230</td>
<td>BA + DMAEMA + St + PEICAME</td>
<td>[Os(bpy)(_2)Cl(_2)]</td>
<td>-45; 230</td>
</tr>
<tr>
<td>5</td>
<td>G299</td>
<td>AA + EA + BA</td>
<td>[Os(bpy)(_2)Cl(_2)]</td>
<td>-250; 133; 563</td>
</tr>
</tbody>
</table>

Abbreviations used in the Table 3.4: AA – acrylic acid; AGE-Im – 1-imidazole-1-ylhex-5-en-3-oxy-2-ol; AP – aminopenten; BA – \(n\)-butylacrylate; DMAEMA - 2-(dymethylamino)ethylmethacrylate; EA – ethyl acrylate; MA-PA – N-pyridin-4-ylmethyl-acrylamid; MMA – methyl methacrylate; VI - N-vinylimidazole; PEICAME - 1-pent-4-enyl-2H-imidazole-4-carboxylic acid methyl ester.
Fig. 3.26 Structures of osmium-modified electrodeposition polymers used for construction of the lactate biosensor.
The osmium complex modified electrodeposition polymers shown in Fig. 3.26, were selected for construction of reagentless biosensors for L-lactate detection. The first layer was formed by electrodeposition of a mixture, containing debris of recombinant yeast cells with the osmium complex modified EDP by application of potentiostatic pulses of the sequence: -1200 mV for 0.2 s followed by 0 mV for 5 s, for the cathodic EDPs G44 and G230, or +2200 mV for 0.2 s followed by 0 mV for 5 s for the anodic EDPs G45, G112 and G299. After deposition of the first layer, 3 µl of a 5 mM solution of cytochrome \(c\) was dropped on the surface of the modified electrode and dried at room temperature for 15 minutes. Finally, the electrode modified with the cell debris cyt \(c\) and the osmium complex modified EDP was covered with a polymer layer, formed by precipitation of 3 µl of the osmium complex-containing EDP with phosphate buffer (PB) of pH 8.0 in the case of the cathodic EDPs, or a citrate buffer of pH 2.5 in the case of the anodic EDPs. The formal potential of cytochrome \(c\) in a 50 mM PB of pH 7.8 was found to be 59 mV vs. Ag/AgCl. To realize the preferred electron transfer pathway across the FC \(b_2\)-cyt \(c\)-Os complex-electrode system, the redox EDP should contain a form of the polymer-bound osmium complex with a formal redox potential more positive than 59 mV vs. Ag/AgCl. The electrode has to be polarized at a potential more positive than the formal potential of the target polymer-bound osmium complex. Since the potential difference of 50 mV is considered to be optimal for effective electron transfer, the electrode was polarized at a potential of at least 50 mV more positive than the formal potential of the polymer-bound osmium complex during biosensor calibrations. Therefore, the FC \(b_2\)-cyt \(c\)-G44-modified electrode was investigated at a potential of 273 mV, the FC \(b_2\)-cyt \(c\)-G45-modified electrode at a potential of 388 mV, the FC \(b_2\)-cyt \(c\)-G112-modified electrode at a potential of 200 mV, the FC \(b_2\)-cyt \(c\)-G230-modified electrode at a potential of 280 mV and the FC \(b_2\)-cyt \(c\)-G299-modified electrode at a potential of 183 mV vs. Ag/AgCl/3 M KCl. Comparison of the calibration curves of the different biosensors is shown in Fig. 3.27.
Fig. 3.27 Calibration curves, obtained with 1- FC $b_2$-cyt c-G299-modified electrode, 2- FC $b_2$-cyt c-G44-modified electrode, 3- FC $b_2$-cyt c-G112-modified electrode, 4- FC $b_2$-cyt c-G45-modified electrode, 5- FC $b_2$-cyt c-G230-modified electrode in a 50 mM PB of pH 7.8 upon consecutive additions of L-lactate at the potentials: 183 mV, 273 mV, 200 mV, 388 mV, 280 mV vs. Ag/AgCl/3M KCl, respectively.

Taking into consideration that at high potentials interference from easily oxidized compounds usually occurs, the operation of the constructed biosensor at low potentials is preferred. From this point of view, the redox EDPs G112 and G299 are the best candidates for construction of FC $b_2$-cyt c-based biosensors. Since the FC $b_2$-cyt c-G299-modified electrode is characterized by higher $I_{\text{max app}}$ and $K_M^{\text{app}}$ values, the redox polymer G299 was used for construction of a prototype of the reagentless biosensor for L-lactate determination.

In Fig. 3.28, a comparison of the chronoamperometric responses obtained with a FC $b_2$-cyt c-based biosensor and a biosensor constructed without cytochrome c is shown. As can be seen, integration of an additional redox protein, cytochrome c, into the biosensor architecture increases the sensitivity and extends the linear range of the designed lactate biosensor.
Fig. 3.28 Chronoamperometric response (A), and calibration graphs (B) obtained using a FC $b_2$-G299-modified electrode (blue line), and a FC $b_2$-cyt $c$-G299-modified electrode (red line) upon consecutive additions of L-lactate at 183 mV vs. Ag/AgCl/3 M KCl. Additions 1–5: 1; 2.5; 5; 10; 20 mM L-lactate.

3.1.2.3. Summary

Attachment of osmium complexes to the polymer backbone of electrodeposition polymers allows to obtain redox polymers. The presence of polymer-bound redox compounds excludes the necessity for addition of free-diffusing redox mediators to support electronic communication between a biorecognition element and an electrode. Such osmium complex modified electrodeposition polymers can be used for construction of reagentless multilayered biosensors of complex architectures, thanks to their ability to undergo layer-by-layer electrodeposition. A library of redox EDPs of varying composition of the polymer backbone, length of spacer arms and redox potentials of polymer-bound osmium complexes were used for the design and optimisation of two different multielement biosensors. Successful design of electron transfer pathways for polymer-entrapped formaldehyde dehydrogenase and FC $b_2$ from
Hansenula polymorpha cell debris was demonstrated. The results clearly demonstrate that the design of suitable osmium complex modified electrodeposition polymers can open a path to new biosensor architectures with tunable properties. Increase of the complexity of the biosensor architecture was demonstrated to have a positive influence on the biosensor characteristics for the case of a L-lactate biosensor.

3.2. Study of bioanalytical properties of genetically modified Hansenula polymorpha yeast cells overexpressing FC b₂

Genetic modification of the organisms for bioanalytical purposes looks promising due to the possibility to change their metabolism in a preferred direction. Metabolic changes in the cell often result in the appearance of unusual properties, which can be successfully used for biosensing. This section focuses on the study of physiological changes in recombinant Hansenula polymorpha yeast cells caused by genetic modification and their use for bioanalytical applications.

The investigated mutant cells are impaired in glucose catabolite repression, they are catalase-defective and overproduce the enzyme flavocytochrome b₂. As shown in Fig. 3.29 the activity of flavocytochrome b₂ in recombinant cells (strain tr1) was found to be 5 times higher, compared to the wild type cells (strain 356).

![Fig. 3.29 Comparison of FC b₂ activity in recombinant (strain tr1) and wild type (strain 356) Hansenula polymorpha yeast cells.](image)
Visualisation and estimation of FC $b_2$ activity in the yeast cells was performed by the methods described in sections 2.2.6 and 2.2.7 of the experimental part.

It should be noted that the permeabilized cells of the recombinant strain tr1 were previously used by Smutok and coworkers for the construction of amperometric biosensors for L-lactate using the free-diffusing mediator phenazine methosulfate for electrochemical communication between intracellular FC $b_2$ and the electrode [Smutok et al. 2007]. The objects of this study are intact cells of the strain tr1, which are more attractive compared to permeabilized cells due to a better stability of undamaged cells. The following sections describe the investigation of metabolic changes such as enhancement of L-lactate-dependent respiration and exoelectrogenesis of genetically modified Hansenula polymorpha cells and their application in biosensing.

A part of the results, represented in sections 3.2.1 and 3.2.2 have been published in Paper III and Paper IV respectively.

### 3.2.1. Enhancement of L-lactate-dependent respiration

Cellular respiration is a sequence of metabolic reactions taking place in the cell. During these reactions biochemical energy, saved in nutrients is converted into adenosine triphosphate (ATP), the energy currency of the cell. The majority of ATP molecules are synthesized in the mitochondria of eukaryotic cells by the enzyme ATP synthase. Since the metabolic reactions leading to ATP synthesis involve oxygen, the term “respiration” is often understood as a process of oxygen consumption by the cell. In most metabolic pathways oxygen is used by the electron transport chain (ETC) of mitochondria where it becomes reduced to water (see Fig. 3.30). Oxygen reduction in ETC can be considered the driving force of the aerobic respiration since thanks to the high electronegativity of oxygen it acts as a thermodynamic sink [Babcock 1999]. Oxygen removes the low-energy electrons from the electron transport chain in the mitochondria and in this way keeps the entire mitochondrial electron transport system working.
The catabolism of most nutrients (e.g. sugars) starts in the cytoplasm and leads to the formation of intermediate energy-storing NADH or NADPH molecules, which transport the high-energy electrons to the mitochondria and deliver them further to ETC. Alcohols are catabolised in the peroxisomes and this metabolic pathway already consumes oxygen at the stage of conversion of alcohols to aldehydes. Some substrates, for instance succinate, L- and D-lactate are catabolised directly in the mitochondria, since the enzymes converting these substances are located close to the electron transport chain and can pump high-energy electrons directly into the ETC without their intermediate storage in NADH or NADPH.

![Diagram of yeast cell respiration](image)

**Fig. 3.30 Respiration in the yeast cells**

CAC- citric acid cycle (Krebs cycle), ETC – electron transport chain

The electrons obtained from succinate are further transported to the electron transport chain by ubiquinone. The electrons from L- and D-lactate are pumped into ETC through cytochrome \( c \). The four mentioned biomolecules NADH, NADPH, ubiquinone and cytochrome \( c \) act as carriers of high-energy electrons. Detailed information on the interaction of the components of the mitochondrial respiratory chain is presented in Fig. 3.31
Fig. 3.31 Electron transport chain of mitochondria

I – Complex I or NADH dehydrogenase; II – Complex II or succinate dehydrogenase; III – Complex III or cytochrome bc₁; IV – Complex IV or cytochrome c oxidase; Int, Ext – internal and external NAD(P)H dehydrogenases; Q – ubiquinone; AOX – alternative oxidase; c – cytochrome c; FC b₂ – flavocytochrome b₂; D-LCR – D-lactate ferricytochrome c oxidoreductase; Succ – succinate; Fum – Fumarate.

Since the consumption of many nutrients by the cells is followed by enhanced oxygen consumption, monitoring of the oxygen concentration in the proximity of the cells can be used to detect specific compounds. The intensity of oxygen consumption by the cells varies in the presence of different nutrients and depends usually on the preference of the cells for individual nutrient. If a nutrient is common for the cell one can usually find a high amount of the enzymes involved in the metabolic pathway utilising the preferable nutrient. However, the cells are able to adjust their metabolism to changes in their environment and if the preferable nutrient is not present in the growing medium, the cell can start to use alternative nutrients. In this case, the amount of the enzyme responsible for the conversion of the preferred nutrient can be reduced. The
example for this can be the phenomenon of glucose catabolite repression in methylotrophic yeasts.

Since the cells used in this study overexpress the enzyme FC $b_2$, the main attention was paid to L-lactate-dependent respiration. This type of respiration is carried out by a respiratory pathway including three components: enzyme flavocytochrome $b_2$, electron carrier cytochrome $c$ and Complex IV of the mitochondrial ETC (see Fig. 3.31). FC $b_2$ is usually located in the intermembrane space of mitochondrion, Complex IV is embedded in the inner mitochondrial membrane and cytochrome $c$ is loosely associated with the inner membrane, can move freely inside the inner membrane and transfer the electrons from FC $b_2$ or Complex III to Complex IV. FC $b_2$ oxidizes L-lactate to pyruvate and transfers the received electrons to Complex IV, where they are used for the reduction of oxygen to water. Therefore, the substrates for the flavocytochrome $b_2$-cytochrome $c$ - complex IV metabolic pathway are L-lactate and oxygen, the products are pyruvate and water. Since L-lactate is not a preferred nutrient for yeast cells, the amount of flavocytochrome $b_2$ and the intensity of L-lactate-dependent respiration are quite low. As a result, monitoring of oxygen consumption by the wild type cells as a bioanalytical signal for L-lactate detection is difficult. Thus, most earlier applications of yeast cells for L-lactate sensing required the use of free-diffusing mediators for direct transfer of the electrons from flavocytochrome $b_2$ to the electrode [Racek et al. 1987], [Racek et al. 1987], [Kulys et al. 1992], [Garjonyte et al. 2006], [Garjonyte et al. 2008], [Garjonyte et al. 2009], [Smutok et al. 2007]. Based on these facts, it can be concluded that the catalytic efficiency of the L-lactate-dependent respiratory pathway in the wild type yeast cells is quite low.

In the cell the activity of a multienzyme metabolic pathway is usually controlled by the enzyme catalysing the rate limiting (the slowest) reaction. It is well known that decrease in activity or quantity of the “controlling” enzyme immediately reduces flux of the metabolite through the entire metabolic pathway. An increase in the activity or quantity of “controlling enzyme” should theoretically lead to the opposite effect. Since
modern gene engineering methods allow tailoring of the cells to produce a higher amount of specific enzymes, they present opportunities to influence the activity of the whole metabolic pathway [Walmsley et al. 2000]. If one assumes that the conversion of L-lactate to pyruvate by FC \textit{b}2 is the rate-limiting reaction, the overexpression of this enzyme in the cells would increase the metabolic flux through the L-lactate-dependent respiratory pathway and provoke enhancement of oxygen consumption by the cell. In order to evaluate whether this hypothesis is true, L-lactate-dependent respiratory activity of the genetically modified \textit{Hansenula polymorpha} yeast cells was examined by electrochemical monitoring of oxygen consumption by the cells in the absence and in the presence of L-lactate.

3.2.1.1. Selection of electrode material for monitoring of L-lactate-dependent respiration

For the evaluation of L-lactate-dependent respiratory activity different electrode materials were modified with the \textit{Hansenula polymorpha} yeast cells and oxygen consumption by the cells was measured at different applied potentials in the absence and presence of L-lactate. When the yeast cells are immobilized on the electrode, which is polarized to the potential of oxygen reduction, they compete with the electrode for oxygen dissolved in the electrolyte solution and decrease the concentration of oxygen available for the reduction at the electrode surface. Therefore, the respiratory activity of the cells is proportional to the decrease of the O$_2$ reduction current at the electrode surface. In detecting the competition between these two reaction pathways for oxygen consumption none of these two processes should be dominant. For this, an optimal combination of electrode material, applied potential and amount of immobilized yeast cells has to be found to optimize sensing of L-lactate-dependent respiration of the recombinant yeast cells. Since the process of oxygen reduction at the electrode at -600 mV vs. Ag/AgCl would dominate over the biocatalytic oxygen consumption by the cells, much less negative working potentials were chosen for the investigation of this sensing
system. Chronoamperometric measurements were performed with modified and bare platinum, gold and graphite electrodes at potentials in the range from +200 to -300 mV vs. Ag/AgCl (Fig. 3.32). All three electrode materials were found to differ in the onset potential for the oxygen reduction reaction. O₂ reduction at the platinum surface already takes place at the potential of +200 mV. In spite of this, the sensitivity of the platinum electrode to L-lactate respiration, considered as the difference between the measured current values in the presence (open symbols in Fig. 3.32) and absence (filled symbols in Fig. 3.32) of L-lactate, becomes significant only at potentials lower than −100 mV.

![Graph](image)

Fig. 3.32 Oxygen reduction current at different electrode potentials for different electrode materials (gold – blue, platinum – green, graphite – red lines) modified with genetically modified yeast cells in the presence (filled symbols) and absence (open symbols) of L-lactate.

Since oxygen reduction on platinum occurs mainly through a four-electron transfer mechanism during formation of H₂O [Eckhard et al. 2007], [Song & Zhang 2008], no negative effect of the product on the immobilized cells is expected. In spite of the fact that platinum is a very active electrocatalyst for oxygen reduction, the sensitivity of the
Pt electrode to the slight changes of oxygen concentration due to enhancement of cell respiration is not the best. This is probably because oxygen reduction reaction on platinum is much faster than the biocatalytic reaction.

Oxygen reduction at a gold electrode becomes significant at potentials lower than −100 mV. In case of gold electrode only minor current changes are observed in the presence of L-lactate. Additionally, gold is known to reduce O$_2$ predominantly to H$_2$O$_2$ following a two electron transfer mechanism [Eckhard et al. 2007], [Song & Zhang 2008]. It can be assumed that the viability of the recombinant cells may be decreased in the presence of H$_2$O$_2$ since the genetically modified cells produce defective catalase and are not able to decompose this toxic compound.

The graphite electrode was characterized by low background currents and showed the highest sensitivity to L-lactate respiration of the cells (referred to the measurements at −300 mV in Fig. 3.32). Graphite usually catalyzes reduction of oxygen to hydrogen peroxide, which can be further reduced to water at more negative potentials [Song & Zhang 2008].

Monitoring of L-lactate respiration with different electrode materials at different applied potentials allowed for optimization of selectivity and sensitivity of the investigated process. Taking into account background noise and sensitivity, graphite electrodes at an applied potential of -300 mV were found to be the most suitable for monitoring of L-lactate-dependent respiration and were chosen for all further experiments. Fig. 3.33 verifies the effectiveness of oxygen reduction at a bare graphite electrode at -300 mV vs. Ag/AgCl and absence of unspecific interactions between the unmodified graphite electrode and L-lactate.
Fig. 3.33 Electrochemical behaviour of a bare graphite electrode at -300 mV vs. Ag/AgCl/3M KCl. Additions 1–4: 1; 2.5; 5; 10 mM L-lactate.

Fig. 3.34 Chronoamperometric response upon consecutive additions of L-lactate using wild type (red line) and recombinant (blue line) *Hansenula polymorpha* yeast cells. Graphite electrode polarized at -300 mV vs. Ag/AgCl/3 M KCl. Additions 1–4: 1; 2.5; 5; 10 mM L-lactate.
Fig. 3.34 compares the L-lactate-dependent respiratory activity of genetically modified and wild type *Hansenula polymorpha* yeast cells. The two graphite electrodes were modified with 3 µl of a suspension of recombinant or wild type cells, accordingly, and covered with a dialysis membrane. The measurements were performed at -300 mV vs. Ag/AgCl/3 M KCl. Since the FC$_{b2}$ content in wild type yeast cells is much lower than in recombinant cells, the intensity of the L-lactate-dependent respiration is low and at -300 mV vs. Ag/AgCl invisible. It appears that the amount of FC$_{b2}$, expressed in the wild type cells is too low to enhance the L-lactate-dependent respiration to the level at which it can compete with the graphite electrode for O$_2$. Therefore, additions of L-lactate do not provoke a decrease of the measured O$_2$ reduction current (red line in Fig. 3.34). As opposed to the wild type cells, the enhancement of respiratory activity in genetically modified cells after addition of L-lactate to the electrolyte solution is significant and can be easily detected at -300 mV vs. Ag/AgCl (blue line in Fig. 3.34). The enhancement in respiratory activity of the recombinant cells in the presence of L-lactate leads to a decrease in the concentration of oxygen available for the reduction at the electrode and a respective drop of the O$_2$ reduction current. The changed physiological properties of the genetically modified cells compared to the wild type cells can be referred to a higher content of the enzyme FC$_{b2}$ in the recombinant cells. As it was already mentioned, the genetically modified cells overexpress the enzyme FC$_{b2}$ and contain 5 times the amount of this enzyme compared to the wild type cells. The FC$_{b2}$ located in the intermembrane space of mitochondria is known to oxidise L-lactate to pyruvate and to transfer the electrons to cytochrome *c*, which donates the obtained electrons further to Complex IV of the mitochondrial respiratory chain (cytochrome *c* oxidase). There, they are used for the reduction of O$_2$ to H$_2$O. Therefore, it was supposed that a higher amount of FC$_{b2}$ generates a larger amount of electrons, which are further pumped into the mitochondrial respiratory chain by means of cytochrome *c*. Since the Complex IV of mitochondrial ETC in recombinant cells receives more electrons than that in wild type cells, it requires a larger amount of the electron acceptor O$_2$. This could
explain the observed enhanced oxygen consumption by the recombinant cells in the presence of L-lactate, which is a substrate for the enzyme FC $b_2$.

### 3.2.1.2. Proof of Complex IV involvement in L-lactate sensing

In order to prove the proposed mechanism of L-lactate sensing by the graphite electrode with immobilized genetically modified *Hansenula polymorpha* yeast cells, the O$_2$ reduction at Complex IV of the mitochondrial respiratory chain was inactivated by the inhibition of Complex IV with KCN. For this, the electrode modified with the recombinant cells was incubated in 3 mM KCN prior to the calibration with L-lactate. Fig. 3.35 compares two chronoamperometric calibration curves recorded with the same modified graphite electrode before (red line) and after incubation with KCN (blue line). Since the experiment in the absence of KCN was performed first, the equilibration of the current background was not fully established. The observed shift in the background current however does not invalidate the conclusion derived from the comparison of these two experiments. The analysis of the current responses, obtained with the modified electrode in the absence and in the presence of KCN (Fig. 3.35) suggests involvement of the entire enzyme cascade, including FC $b_2$, cyt c and cytochrome c oxidase (Complex IV) in the observed L-lactate-dependent respiration. The initial background current in the absence of L-lactate is caused by the diffusion-limited direct electrochemical O$_2$ reduction at the graphite surface polarized to a potential of -300 mV. The presence of L-lactate in the electrolyte solution induces L-lactate-dependent respiration and leads to the increase of O$_2$ consumption by the cells. This process is detected as a decrease in O$_2$ reduction current at the electrode due to a decrease in the concentration of oxygen available for the electrocatalytic reduction (Fig. 3.35, red line).
Fig. 3.35 Bioelectrochemical evidence for Complex IV involvement in the L-lactate-dependent electron transfer chain using recombinant *Hansenula polymorpha* yeast cells. Chronoamperometric response to L-lactate addition, using intact (red line) and KCN inhibited genetically modified yeast cells (blue line). Addition 1: 3 mM KCN; additions 2–5, increasing concentrations of L-lactate: 1; 2.5; 5; and 10 mM; addition 6: purging of Ar for removal of O₂.

KCN is known to inhibit Complex IV of the mitochondrial respiratory chain and to suppress the O₂ consumption by the cells via Complex IV. Therefore inhibition of L-lactate-dependent respiration with this inhibitor would prove the involvement of Complex IV in the investigated biochemical process. Fig. 3.35 shows that the addition of 3 mM KCN causes a slight shift in the recorded background current, which could be due to the change of solution conductivity [Priyantha & Bandara 1997]. A small shift in the background current, established after addition of KCN, is also observed after addition of 1 mM L-lactate to the electrolyte solution (Fig. 3.35, blue line). Further additions of L-lactate however do not lead to the concentration-dependent decrease of O₂ reduction current on the electrode as observed in the previous experiment. The absence of a
response at the modified electrode to addition of L-lactate agrees with the hypothesis of the Complex IV involvement in L-lactate-dependent respiration of the cells. A small response to L-lactate, obtained after addition of 1 mM L-lactate can be explained by incomplete inhibition of Complex IV with KCN. A time-dependent decrease in the obtained response could indicate a progressive inhibition of Complex IV with KCN. The key role of oxygen in the sensing process was demonstrated by purging Argon into the electrolyte solution, thus causing a complete removal of oxygen. As a result of oxygen removal from the system, the background current due to oxygen reduction at the electrode drops to the same level irrespective whether the yeast cell respiration was inhibited by KCN or not. Fig 3.36 represents the interactions of the immobilized recombinant cells and the electrode and electron transfer pathways inside the cells leading to L-lactate sensing. In Fig. 3.36A the measurement of background current is explained, which is a result of the competition between electrochemical O₂ reduction at the electrode and normal respiration (NR) of the immobilized cells in the absence of L-lactate. Addition of L-lactate induces activation of the L-lactate-dependent respiratory pathway in the cells. This increases the overall oxygen consumption by the cells and leads to a decrease in the concentration of oxygen available for reduction at the electrode (Fig. 3.36B). The inhibition of Complex IV with KCN shown in Fig. 3.36C results in the inactivation of not only L-lactate dependent respiration but also a significant part of normal respiration in the cells and almost does not change the concentration of oxygen available for the electrode.
Fig. 3.36  Schematic representation of L-lactate dependent electron-transfer pathways using graphite electrodes in combination with recombinant *Hansenula polymorpha* yeast cells: (A) competition for O$_2$ consumption due to electroreduction of O$_2$ at the polarized electrode and “normal”, L-lactate independent respiration (NR) of the immobilized yeast cells; (B) decrease of the available amount of O$_2$ at the electrode due to enhanced respiration involving the L-lactate-dependent electron-transfer pathway in genetically modified yeast cells overexpressing FC$_{b2}$; (C) blockage of the electron transfer pathway due to the inhibition of Complex IV leading to complete blocking of cell respiration in both NR and L-lactate respiration.
Hence, overexpression of FC $b_2$ by means of genetic modification does not only lead to a higher content of the target enzyme in the cells but also to an increase in the activity of the entire metabolic pathway of L-lactate-dependent respiration in which this enzyme is involved.

### 3.2.1.3. Stability and selectivity of bioelectrodes

Graphite electrodes modified with genetically modified yeast cells exhibited good storage stability and the L-lactate-dependent response could be recorded for more than a month. Table 3.6 represents the storage stability of the bioelectrodes. Between the measurements the modified electrodes were stored in 20 mM PB solution of pH 7.8 at 4°C.

In spite of the fact that yeast cells consume oxygen in response to the addition of a large variety of other nutrients, the intensity of L-lactate-dependent respiration in recombinant cells is still much higher compared to other types of respiration (see Fig. 3.37).

**Table 3.6 Storage stability of the electrodes, modified with genetically modified *Hansenula polymorpha* yeast cells.**

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>28</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I$, µA</td>
<td>0.56</td>
<td>0.58</td>
<td>0.55</td>
<td>0.56</td>
<td>0.57</td>
<td>0.53</td>
<td>0.5</td>
<td>0.48</td>
<td>0.47</td>
<td>0.49</td>
<td>0.47</td>
<td>0.45</td>
</tr>
<tr>
<td>% of initial response</td>
<td>100</td>
<td>104</td>
<td>98</td>
<td>100</td>
<td>102</td>
<td>95</td>
<td>89</td>
<td>86</td>
<td>84</td>
<td>87</td>
<td>84</td>
<td>80</td>
</tr>
</tbody>
</table>
The selectivity of the modified electrodes towards L-lactate could be improved in the future by introduction of defects into Complex I and Complex III of mitochondrial respiratory chain and in this way switching off a part of the normal respiration of the cells, which uses NADH, NADPH or ubiquinone for pumping electrons into the mitochondrial respiratory chain. The viability of similar mutants of *Caenorhabditis elegans* was demonstrated [Grad et al. 2005]. The exclusion of the interfering influence of ethanol requires introduction of additional mutations on the level of peroxisomal metabolism since oxygen consumption during alcohol metabolism takes place in the peroxisomes.

3.2.1.4. Visualisation of the L-lactate-dependent respiratory activity of genetically modified *Hansenula polymorpha* yeast cells using SECM

Scanning electrochemical microscopy (SECM) allows imaging of living cells and can be used for the investigation of processes occurring in living cells [Sun et al. 2007]. Monitoring of phenomena such as for example, the enhancement of L-lactate-dependent respiration in the mutant *Hansenula polymorpha* yeast cells under different conditions in
SECM could contribute to a better understanding of the peculiarities of this alternative respiration pathway. This can be useful for the development of treatment strategies for mitochondrial dysfunctions as proposed earlier [Grad et al. 2005]. The proposed strategy is based on the introduction of the yeast gene CYB2, encoding flavocytochrome b2, into the genome of the organism with a defect Complex I of mitochondrial respiratory chain. This was shown to correct deficiencies in Complex I, such as lactic acidosis and decreased NADH-dependent mitochondrial respiration. Therefore, the yeast FC b2 is considered as a candidate for gene therapy in human diseases involving Complex I.

This section describes SECM experiments aiming on visualization of L-lactate-dependent respiratory activity of the mutant Hansenula polymorpha cells. Respiratory activity of the genetically modified cells was investigated by measuring the local concentration of oxygen over the spot of the cells. The redox-competition mode of SECM [Eckhard et al. 2006] is the most suitable technique for the detection of metabolic oxygen consumption in living cells. The imaging principle of redox-competition SECM is based on the competition between two polarized electrodes, the sample and the SECM tip, for the same electrochemically active compound (in this case oxygen). Usually, for SECM experiments performed in the redox-competition mode, both the SECM tip and the sample are polarized at a potential sufficient for oxygen reduction (e.g. -600 mV vs. Ag/AgCl/3M KCl). The experiments in this work were however carried out monopotentiostatically, because O2 consumption by the sample is due to metabolic processes in the cells. It was therefore not necessary to initiate the electrochemical reduction of oxygen at the sample as in the conventional case of the redox-competition mode of SECM. The SECM measurements were performed using a Bio-SECM set up. The Bio-SECM set up, as well as the procedure for sample preparation are described in details in section 2.2.13 of the experimental part. A 50 µm Pt electrode (SECM tip) was positioned in close proximity to the cell spot with the aid of an inverted optical microscope and step-motors. A potential of -600 mV vs. Ag/AgCl/ 3M KCl was then applied to the tip, in order to initiate oxygen reduction. The tip was then slowly
approached to the surface of the sample. A sharp decrease of the oxygen reduction current at the tip indicated close approach of the tip to the surface, due to decreased concentration of oxygen, owing to diffusion limitations. After reaching the surface, the electrode was withdrawn to a distance of 15 µm from the surface, at which currents were recorded.

Fig. 3.38 Positioning of the microelectrode.

After positioning, the SECM tip was scanned over the cell spot, while simultaneously recording the oxygen reduction current at the SECM tip. The oxygen concentration was mapped by plotting the oxygen reduction current as a function of the lateral position of the tip. The oxygen reduction current signal at the SECM tip is constant unless the tip is scanned over an O₂ consuming area. At this point, the sample and the SECM tip compete for the O₂ within the gap between them. This alters the local O₂ concentration, and results in a drop of the current flowing through the SECM tip. Thus, the current at the tip is proportional to the concentration of oxygen in solution, and reflects the catalytic activity of the sample at each point in space. Fig. 3.39B shows the localisation of the area of higher oxygen consumption, which corresponds to localisation of the spot of the cells.
Fig. 3.39 Imaging of a spot of cells deposited on a glass slide, covered with a polymer. A. optical micrograph; total spot diameter 750 µm. B. SECM image with 50 µm Pt electrode, polarized at a potential E = -600 mV vs. Ag/AgCl/3M KCl at constant height 15 µm, with tilt correction.

The above-described SECM experiments were performed in L-lactate solutions of different concentrations (0 mM, 0.5 mM, 1 mM, 1.5 mM, 4 mM, 10 mM). Between the measurements, the electrochemical cell with the sample was filled with 20 mM PB pH = 7.8 and left for 20 minutes to decrease the L-lactate concentration inside the yeast cells to their initial level. In order to compare the respiratory activity of the cells in L-lactate solutions of different concentrations, the individual set of data points for a line scan at Y = 800 µm, obtained in the absence, and in the presence of different concentrations of L-lactate, were plotted. In order to devaluate the differences in background current due to unavoidable small variations in the experimental conditions during different experiments, for example, oxygen concentration and temperature among others, the area under the curve from X = 350 µm to X = 1200 µm was integrated and normalized as shown in the Fig. 3.40.
Fig. 3.40 Real and normalized line scans (baseline corrected), at Y = 800 µm in the absence of L-lactate.

The details of the performed normalization are shown in the Table 3.7.

<table>
<thead>
<tr>
<th>L-lactate, mM</th>
<th>Integration</th>
<th>Calculation</th>
<th>Normalization</th>
</tr>
</thead>
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<td>0</td>
</tr>
<tr>
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<td>0.15846</td>
</tr>
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<td>0.34245</td>
</tr>
<tr>
<td>1.5</td>
<td>355.37</td>
<td>50.93</td>
<td>0.54605</td>
</tr>
<tr>
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<td>361.54</td>
<td>57.1</td>
<td>0.6122</td>
</tr>
<tr>
<td>10</td>
<td>397.71</td>
<td>93.27</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3.7 Normalization of the line scans

Fig. 3.41 shows the normalized profiles of the line scans at Y = 800 µm, in solutions containing different concentrations of the L-lactate. In the absence of L-lactate in the solution, the decrease of the oxygen reduction current over the spot is due to the normal (L-lactate-independent) respiration of the cells and the topography of cell spot. Upon addition of L-lactate, the oxygen reduction current at the SECM tip decreases even more significantly due to the activation of the L-lactate-dependent respiration pathway
and increased consumption of oxygen by the cells. It is important to note that
topographic effects also contribute to the SECM signal. Nevertheless, since the same
sample was used for all the experiments, the contribution of the topographic effects to
the main signal is the same in all the images and does not influence the absolute values.

Fig. 3.41 Respiratory activity of a cell spot in L-lactate solutions of different
concentrations.

The results observed in this study proved the applicability of the SECM technique for
monitoring of L-lactate-dependent respiration in the mutant *Hansenula polymorpha*
yeast cells. This approach allows for fast and non-invasive screening of different
mutants and can be used for studying the influence of chemical and genetic factors on
alternative respiratory pathways in the cell and selection of suitable *Hansenula polymorpha* mutants for application in L-lactate biosensing.

3.2.1.5. Summary

Genetic modification of *Hansenula polymorpha* yeast cells results in activation of L-
lactate-dependent respiration in recombinant cells in the presence of L-lactate. This
happens due to overexpression and overproduction of the enzyme FC \( b_2 \), which is a start
point of the L-lactate-dependent respiratory pathway. An increase of the amount of electrons pumped into the mitochondrial respiratory chain through cytochrome $c$ requires a larger amount of final electron acceptor – oxygen, which acts as a sink for the low-energy electrons from ETC and becomes reduced at Complex IV to water. Enhanced oxygen consumption by genetically modified yeast cells in the presence of L-lactate allows for indirect detection of this analyte with the cathode at potentials lower than -300 mV vs. Ag/AgCl. The detection principle is based on the competition between the immobilized recombinant yeast cells and the polarized electrode for the available molecular oxygen. Graphite was found to be the most suitable electrode material for the detection of L-lactate respiration at the potential $-300\text{mV}$ vs. Ag/AgCl. The involvement of Complex IV in observed L-lactate-dependent respiration was proven by inhibition of Complex IV with KCN. The electrodes modified with recombinant cells were found to have good storage stability and showed 20% lower response compared to the initial one after storage for one month in PB at 4° C. The L-lactate-dependent respiration of the recombinant cells was visualized by means of scanning electrochemical microscopy.

3.2.2. Exoelectrogenesis

Looking for renewable energies more and more attention is paid to the energy saved in living organisms. Potential places for harvesting the electricity in living organisms are electron transport chains of chloroplasts, mitochondria and bacteria. Whereas some research groups are focusing on the direct extraction of electrons from electron transport chains by specifically designed nanoelectrodes [Ryu et al. 2010], others are trying to make the living cells to transfer the electrons to the electrode by installing a synthetic electron conduit into the plasma membrane [Jensen et al. 2010]. The last case is mimicking the natural phenomenon called exoelectrogenesis.

Exoelectrogenesis is a capability of living systems to extracellular electron transfer. Thanks to this property, some of the respiratory energy can be captured in the form of
electrons and used for electricity production. The discovery of the first exoelectrogenic bacterium *Shewanella putrefaciens* [Kim et al. 1999] and its application for the construction of a microbial fuel cell [Kim et al. 2002] waked the interest of scientific world in this research area.

![Electricity production in a microbial biofuel cell](image)

Since that time, four of the five classes of Proteobacteria as well as Firmicutes, Acidobacteria phyla and the oxygenic phototrophic cyanobacterium *Synechocystis* sp. PCC 6803 were reported to be able to generate electrical current [Logan 2009]. Further investigations revealed three different mechanisms of electron transfer from bacterial cells to the electrode: 1) by outer membrane cytochromes [Mjers & Mjers 1992]; 2) by use of excreted natural mediators [Rabaey et al. 2005a]; 3) through electrically conductive appendages, called nanowires [Gorby et al. 2006], [Reguera et al. 2005]. Since the electron transport chain in bacterial cells is located in the plasma membrane, the electrons become potentially available for the electrode positioned on the external surface of the cell membrane [Schaetzle et al. 2008].
In opposite to bacteria there are only rare reports on the exoelectrogenesis of yeast cells. This is due to the fact that yeast cells are eukaryotes and their electron transport chain, the source of electrons, is located in the mitochondrion, which is separated from the plasma membrane by cytoplasm. This makes it difficult for the electrode to harvest the electrons from the mitochondria [Schaetzle et al 2008]. Yeast cells however as well as other eukaryotic and prokaryotic cells contain trans Plasma Membrane Electron Transport systems (tPMET) also known as Plasma Membrane Oxido-Reductase systems (PMOR), which are poor studied but are supposed to be involved in extracellular electron transfer. These systems are located in the plasma membrane and can transfer the electrons from reduced cytoplasmic NADH and NADPH molecules to external electron acceptors. The electrons provided by the tPMET are usually used for the reduction of external nutrients to the form, suitable for uptake by the cells (for instance reduction of Fe III to Fe II). The number of electrons, used by the cell in this way is however much
smaller than the quantity of electrons flowing through the electron transport chain. Another hindrance for direct electric communication between the yeast cell and the electrode is a dense cell wall. This multilayered arrangement is about 100-200 nm thick and consists of glucomannoproteins, mannoproteins, glucans and chitin. Such a large distance between the exterior of the cell and the outside of the cell prevents the direct contact of the electrode with the plasma membrane and should also disturb a direct electron transfer [Schaetzle et al 2008].

In spite of all these facts, there have been also some reports about current generation by yeast cells \textit{Saccharomyces cerevisiae} in the presence of glucose [Potter 1911], [Chiao et al. 2003]. Since all the experiments, described in these two publications, were carried out without addition of artificial redox mediators, it has to be assumed that electron transfer from the yeast cells to the electrode was either direct electron transfer or involved natural redox molecules excreted by the cells. Prasad et al reported about the ability of \textit{Hansenula anomala} yeast cells to transfer electrons to graphite electrodes in the presence of glucose or lactate [Prasad et al. 2007]. The researchers explained the exoelectrogenic properties of \textit{Hansenula anomala} yeast cells by the presence of flavocytochrome $b_2$, NADH-ferricyanide reductase, NADPH-ferrireductase and cytochrome $b_5$ in their plasma membrane. These redox enzymes were suggested to act as shuttles and to mediate the transfer of electrons generated during oxidation of glucose or
lactate to the electrode in the absence of external mediators. The exact mechanism of the extracellular transfer of electrons to the electrode remained unfortunately unknown.

Nowadays, genetic engineering gives us a powerful tool to study such phenomena by overexpression of enzymes, which are assumed to participate in the investigated metabolic process. Increase of the content of target enzyme in the cell may lead to the enhancement of the cell properties, which are due to the function of this protein. The availability of the mutant cells of the yeast Hansenula polymorpha overexpressing enzyme FC $b_2$ [Smutok et al. 2007], [Shkil et al. 2009], which was suggested to be involved in exoelectrogenesis in the yeast Hansenula anomala [Prasad et al. 2007], made me curious to study the role of this enzyme in the process of electron transfer to the electrode.

Since direct communication of bacterial or yeast cells with an electrode surface is not always effective, many alternative electron shuttling systems have been used to facilitate electronic communication between cells and electrode [Kano & Ikeda 2000]. While the use of free-diffusing mediators appeared to be impractical, an alternative solution was found in use of polymer-bound mediators, for instance osmium complex-containing redox-polymers. Vostiar et al. were the first who used osmium complex modified polymers for electrical “wiring” of respiratory enzymes of intact bacterial cells. In gram-negative Gluconobacter oxydans bacterial cells, they addressed redox enzymes from the plasma membrane, yielding response for glucose, fructose, ethanol and glycerol [Vostiar et al. 2004]. Later in gram-negative Pseudomonas putida and Pseudomonas fluorescens bacterial cells [Timur et al. 2007a], [Timur et al. 2007b] the response currents could be obtained both from substrates being metabolized in the plasma membrane (glucose) as well as in the cytosol of the cell (phenol). In spite of the information available on direct electron transfer from bacterial cells to the electrode, the exact mechanism of electron transfer from bacterial cells via osmium complex modified polymers to the electrode has not been clarified. In order to define the way the osmium redox polymers capture the cellular electrons, the researchers investigated several
bacterial mutants. Alferov et al. have shown that introduction of cytochrome to the cytoplasmic membrane of *Escherichia coli* [Alferov et al. 2009] greatly facilitated the communication between these gram-negative bacterial cells and the polymer-bound osmium complexes. In a further study, Coman et al. used the gram-positive model organism *Bacillus subtilis* with a substantially thicker peptidoglycan cell wall, which was expected to be more difficult to permeate by the osmium complexes attached to the polymer backbone. Additionally the constructed *Bacillus subtilis* mutant overproduced transmembrane cytochromes and succinate:quinone oxidoreductase (EC 1.3.5.1; also known as complex II of respiratory chain) [Coman et al. 2009]. From these studies, it was concluded that membrane cytochromes and oxidoreductases participate in the osmium complex mediated electron transfer from the bacterial cells to the electrode, whereas a thicker cell wall does not influence this process negatively.

To the best of my knowledge, no work has been done on electrical wiring of eukaryotic organisms such as yeast cells with osmium complex-containing polymers. In the following section, the exoelectrogenic properties of genetically modified *Hansenula polymorpha* yeast cells overexpressing FC $b_2$ are investigated with and without involvement of osmium complex-based redox polymers in electron transfer processes between yeast cells and the electrode. I believe that the investigations described in this work contribute to the understanding of exoelectrogenic properties of yeast cells. Taking into account advantages of eukaryotic cells in comparison with prokaryotic, all the performed investigations are not only scientifically interesting but can be useful for the development of yeast cell-based biosensing and bioelectricity production devices.

### 3.2.2.1. Electrical wiring of *Hansenula polymorpha* yeast cells with a graphite electrode

To study electrogenic properties of *Hansenula polymorpha* yeast, the recombinant cells overproducing FC $b_2$ and for comparison the wild-type cells of this microorganism were separately immobilized onto disk-shaped graphite electrodes. The immobilized yeast
cells were fixed on the surface of the electrode either by a semi-permeable dialysis membrane or by entrapment into an osmium complex modified electrodeposition polymer. Both immobilization techniques provided a reliable fixation of the cells on the electrode surface and did not noticeably harm the living cells. In order to exclude unpredictable influence of potentiostatic pulses, required for the electrodeposition of osmium complex modified EDPs on the cell plasma membrane a gentle precipitation of the polymers was performed using buffer solutions of pH 8.0 or 2.5. For this 5 µl of the respective buffer was dropped on the three-layered construction, containing a layer of *Hansenula polymorpha* yeast cells encapsulated in-between layers of osmium complex modified EDP. This allowed for formation of a 3D-structure providing a good physical contact of osmium entities attached to the polymer backbone both with the electrode surface and the cells (see Fig. 3.45).

![Diagram](image)

**Fig. 3.45** Electrically wiring spatially confined cellular redox activity inside of yeast cells to the polarized electrode surface through chains of osmium complexes in the redox polymer-based immobilization matrix. Brown circles represent randomly distributed osmium complexes while blue lines are their retaining polymer backbone.
The wild-type and recombinant *Hansenula polymorpha* yeast cells entrapped either under a dialysis membrane or in the network of an osmium complex modified EDP were tested with respect to their ability to produce current in the presence of L-lactate. For this, the four sensors were calibrated by consecutive additions of L-lactate stock solution. The chronoamperometric measurements were performed at the potential 200 mV vs. Ag/AgCl, which was positive enough to provide electron transfer from the reduced osmium entities to the electrode. As can be seen in Fig. 3.46 the presence of L-lactate in the buffer solution did not induce a Faraday current when wild type or recombinant yeast cells were just entrapped behind a dialysis membrane on graphite electrodes.

![Graph showing chronoamperometric response](image)

**Fig. 3.46** Chronoamperometric response upon consecutive addition of L-lactate of:
- blue line – electrode, modified with the Os-complex-based redox polymer G390 and recombinant *Hansenula polymorpha* yeast cells;
- red line – electrode, modified with the Os-complex-based redox polymer G390 and wild type *Hansenula polymorpha* yeast cells;
- green line – electrode, modified with Os-complex-based redox polymer G390;
- orange line – electrode, modified with recombinant *Hansenula polymorpha* yeast cells and covered with a dialysis membrane. Graphite electrode polarized at 200 mV vs. Ag/AgCl/3M KCl. Additions 1-5: 1; 2.5; 5; 10; 20 mM L-lactate.
It is obvious that direct electron transfer from *Hansenula polymorpha* cells to the graphite electrode in the absence of mediators and presence of L-lactate could not be observed. The situation looks different once the cells are incorporated into the network of osmium complex-based redox polymers. In this case, a concentration dependent response upon addition of L-lactate was obtained with electrodes modified with both genetically modified and wild type cells. The control experiment with the electrode modified only with osmium complex-based redox polymer proved that the observed changes of the anodic current were due to the metabolic activity of the *Hansenula polymorpha* yeast cells and were not caused by interaction of osmium complex-based redox polymer modified electrode with L-lactate. The magnitude of the current changes provoked by the addition of substrate was higher with the electrode modified with recombinant cells as compared to the electrode modified with wild-type cells. Therefore, it was assumed that the content of FC $b_2$ in the cell correlates with the magnitude of the measured current response. Analysis of literature suggested two hypothesis of electron transfer from FC $b_2$ to the electrode via osmium entities of the redox polymer. The first hypothesis assumes the presence of excess amount of FC $b_2$ in the plasma membrane or in the cytoplasm close to the plasma membrane, where it may be wired by the polymer-bound Os complexes. One can argue against this assumption since FC $b_2$, though synthesized in the cytoplasm, is usually stored in the intermembrane space of mitochondria. In spite of this well-known fact, there have been reports, which are in favour to this hypothesis. Glick et al. [Glick et al 1993] observed the folding of some FC $b_2$ precursors outside the yeast mitochondria while Prasad et al. [Prasad et al 2007] discovered the presence of FC $b_2$ in the plasma membrane of *Hansenula anomala* yeast cells. In genetically engineered cells, overproduction of some proteins may lead to saturation of the sorting and translocation machineries. This results in accumulation of overexpressed proteins in other cellular compartments then in native cells. Wagner et al. for instance observed accumulation of overexpressed membrane proteins in cytoplasmic aggregates of recombinant *Escherichia coli* cells [Wagner et al. 2007]. Opposite to this,
the initially cytosolic protein cystein protease cathepsin B was found in the cell membrane under condition of overexpression [Leung-Toung et al. 2002], [Szpaderska et al. 2001], [Petanceska et al. 1994]. Since protein mislocalization is not a rare side effect of protein overexpression in the cell it can not be excluded that the enhanced FC \( b_2 \) synthesis in genetically modified *Hansenula polymorpha* yeast cells brought them to the limits of mitochondrial FC \( b_2 \) uptake and led to folding of this protein in the cytoplasm or plasma membrane.

Another hypothesis proposed by Herst et al. suggests that the mitochondrial electron transport chain (ETC) can interact with the transplasma-membrane electron transport system (tPMET) domains through direct contact or formation of restricted domains and hence exchange reducing equivalents with it (see Fig. 3.47). In this case, a direct transfer of electrons from the components of mitochondrial ETC via the tPMET to the outside of the cell should be possible. This hypothesis is based on the observation of localization of mitochondrial tubular networks in close proximity to the plasma membrane during reduction of an impermeable water-soluble tetrazolium dye at the cell surface of *Saccharomyces cerevisiae* yeast cells. Additional screening of different mutants revealed that the extracellular reduction of tetrazolium dye by *Saccharomyces cerevisiae* yeast cells requires the presence of ubiquinone and components of Complex III and IV of the mitochondrial respiratory chain but not an active ETC of mitochondria. This suggests involvement of the mentioned components of mitochondrial ETC in the exoelectrogenic activity of the investigated yeast cells [Herst et al. 2008]. Since the role of FC \( b_2 \) in this process was not investigated in the study of Herst and co-workers and it is located between complex III and complex IV of the mitochondrial ETC the involvement of FC \( b_2 \) in the interaction with tPMET is possible.
To investigate the universality of the “cell wiring properties” of osmium complex modified polymers three osmium complex-containing polymers with different composition of the polymer backbones and length of the side chains were chosen from the library of redox polymers and tested with respect to their ability to provide an efficient electron transfer from *Hansenula polymorpha* yeast cells to graphite electrodes. The predicted structures of the utilised osmium-complex modified polymers are shown in Fig. 3.48. The procedures for synthesis and modification of these three polymers can be found in the experimental part of this thesis. Calibration curves obtained with the electrodes modified with recombinant *Hansenula polymorpha* yeast cells and the respective osmium complex based redox polymers are compared in Fig. 3.48. Despite of the differences in structure all three redox polymers were found to support electron transfer from the recombinant *Hansenula polymorpha* yeast cells to the graphite electrode in the presence of L-lactate. The calibration curves of these three bioelectrodes do not provide enough information to draw any conclusions on the relationship between the structure of the redox polymers and the effectiveness of the investigated electron transfer process but prove the universal ability of osmium complex-based redox polymers, independent of their design, to function for the purpose of cell wiring.
Fig. 3.48 Chemical structure of three Os complex modified redox polymers, namely G390, G112 and G44 (top left and right; bottom left). They were placed as immobilization matrices for yeast cells onto graphite discs. Three L-lactate calibration curves for graphite electrodes, modified with recombinant *Hansenula polymorpha* yeast cells and one of the three specified Os complex-containing polymers. For the amperometric recordings the graphite electrodes were polarized at 200 (G112 and G390) or 100 (G44) mV vs. Ag/AgCl/3M KCl, respectively.
3.2.2.2. Direct or mediated electron transfer between yeast cells and polymer-bound osmium complexes?

Knowing the key role of the redox polymer in cell-to-electrode communication, it was further investigated whether any free-diffusing natural redox mediators are involved in the process of electron transfer through the plasma membrane and cell wall as in the case of some bacteria. *Pseudomonas aeruginosa* was reported to produce pyocyanin and phenazine-1-carboxamide [Rabaey et al. 2005a], *Escherichia coli* hydroquinone derivatives [Qiao et al. 2008] providing an extracellular electron transfer. A hypothetical mechanism of extracellular electron transfer in *Escherichia coli* cells involving hydroquinone is depicted in Fig. 3.49.

![Fig. 3.49 A hypothetical mechanism for extracellular electron transport of evolved *Escherichia coli* cells [Qiao et al. 2008].](image)

*Proteus vulgaris* were found to excrete soluble electroactive species [Rawson et al. 2011] contributing to the electron transfer from the cells to single walled carbon nanotubes functionalised with osmium complexes. Recently also *Arxula adeninivorans* yeast cells were demonstrated to produce electroactive molecules participating in extracellular electron transfer [Haslett et al. 2011].
To investigate whether any free-diffusing redox molecules are involved in the electron transfer pathway between *Hansenula polymorpha* yeast cells and osmium complexes attached to the polymer backbone, a specially designed multilayered electrode of the architecture illustrated in Fig. 3.50 was constructed.

![Diagram](image)

**Fig. 3.50** The immobilization of yeast cells and an osmium complex based redox polymer on graphite electrodes in physically separated fashion; brown circles are randomly spread osmium entities that irregularly are in contact with the electrode surface, light blue lines are their fixing polymer backbones and light green lines are the chains of a polymer that does not have any attached osmium complexes or any other mediator. The broken black line corresponds to a dialysis membrane that was placed on top of the preparation to keep the living cells in place.

A layer of osmium complex modified redox polymer formed at the graphite electrode was physically separated from the upper layer of recombinant *Hansenula polymorpha* yeast cells by means of a thin film of an osmium complex-free polymer. The yeast cells forming the upper layer and adsorbed on the layer of the osmium complex-free polymer were fixed using a dialysis membrane. The electrodes with such architecture were investigated upon addition of small aliquots of L-lactate as in previous
experiments. However, as it can be seen in Fig. 3.51 (pink line), the “layered” electrode did not respond on the addition of the substrate for FC $b_2$.

Fig. 3.51 A. Blue line: Control experiment with a cell/Os complex-based redox polymer/graphite electrode assembly (Fig. 3.45). In both cases the Os redoxpolymer G390 and recombinant *Hansenula polymorpha* yeast cells were used as to make up the functional layers. For amperometry, the graphite electrode was polarized to 200 mV vs. Ag/AgCl/3M KCl. Additions 1-4: 1; 2.5; 5; 10 mM L-lactate. B. Pink line: Chronoamperometric response of cell/Os complex modified polymer/graphite electrode assemblies in a layered design as illustrated in Fig.3.50. Consecutive additions of L-lactate stock solution did not induce current flow through the polarized surface of the working electrode surface.

As a control experiment the cells from the same batch of cell culture were entrapped in an osmium complex based redox polymer on the graphite electrodes to check whereas the previously observed L-lactate-dependent response can be obtain when the cells are in direct contact with the osmium entities of the redox polymer. In this case, a calibration curve similar to that obtained earlier with the electrodes of the same architecture was recorded. The result of the control experiment allows drawing two conclusions. Firstly,
it was demonstrated that the absence of response in the layered design is not due to a potentially changed properties of the used cells but due to the new design of the “layered” electrode. Additionally, the special separation of the Os entities from the outer surface of the yeast cells was found to hinder the electron transfer from the cells to the polymer-bound osmium complexes. This suggests that the electric communication in this particular case occurs rather through a direct contact of the plasma membrane located redox systems (either FC $b_2$ or tPMET) than via free-diffusing redox molecules excreted by the yeast cells since they should have been able to move through the permeable layer of osmium complex-free polymer and deliver the electrons to the osmium entities of the redox polymer.

3.2.2.3. Electrochemical behaviour of yeast cells in the presence of free-diffusing mediators

Transplasma membrane electron transport system (tPMET) is a multicomponent system present in the plasma membrane of almost all living cells [Crane et al. 1991], [Medina et al. 1997], [Morre et al. 2004]. It consists of various cytochromes, oxidoreductases, ferrireductase, thioredoxin reductase, NADH oxidase and protein disulfide isomerase among other membrane proteins. The exact function of the tPMET in the cell is not known but it is supposed that it participates in assimilation of a number of transition metals, regulation of the redox equilibrium in the cell and the transport of metabolites. The best-studied components of the yeast tPMET are ferrireductases FRE1 [Dancis et al 1990], [Dancis et al 1992], [Shatwell & Dancis 1996] and FRE2 [Georgatsou & Alexandarski 1994]. Schatzwell et al. suggested that these two proteins represent a distinct family of membrane-bound flavocytochromes that are capable of accepting electrons from cytoplasmic NADH or NADPH molecules, transferring them across the cell membrane and deliver them to the electron acceptors such as Fe III outside the cell [Shatwell & Dancis 1996]. Due to this, the activity of the yeast tPMET is often associated with the extracellular reduction of Fe III-containing compounds such as
ferricyanide, ferric citrate, ferric EDTA, ferrioxamine B or nitroprusside [Lesuisse & Labbe 1994], [Emery 1987]. Nevertheless, the reductive systems of tPMET were found to be nonspecific towards electron acceptors and are capable of oxidoreduction of other transition metal ions such as Mn, Cu, Zn, Cd, Cr, Hg, V [Wakatsuki 1995]. Since the tPMET is not selective to electron acceptors outside the cells it can be supposed that polymer-bound Os$^{3+}$ entities interact with the components of the tPMET and accept electrons obtained from the catalytic oxidation of L-lactate in the cell. A similar mechanism of osmium complex involvement into cell-to-electrode electron transfer was proposed by Coman et al. in case of gram-positive bacterial cells Bacillus subtilis [Coman et al 2009]. It was suggested that the polymer-bound Os$^{3+}$ units penetrate the bacterial cell wall deeply enough to establish the contact with the cytochromes and oxidoreductases of the bacterial electron transport chain. The penetration of the polymer-anchored Os$^{3+}$ entities was assumed to be supported by the polyelectrolyte properties of the peptidoglycan and teicholic acid, which are components of the bacterial cell wall. I suppose that in case of Hansenula polymorpha yeast cells a similar interaction between the osmium complex-containing redox polymers with the yeast cell wall takes place.

To understand the involvement of tPMET in the electrochemical communication between the yeast cells and the electrode we studied the bioelectrochemical behaviour of the cells with the freely diffusing hydrophilic mediator ferricyanide and with double mediator system, combining the lipophilic mediator 2,6 dichlorophenolindophenol (DCPIP) and the hydrophilic mediator ferricyanide. It is commonly accepted that the hydrophobic ferricyanide is not able to cross the lipid bilayer cell membrane due to its nature and is thus unable to establish an electron exchange between intracellular redox processes and the electrode [Heiskanen et al. 2004], [Khlupova et al. 2007], [Baronian et al. 2002]. In opposite to this the lypophilic DCPIP is capable of crossing the plasma membrane and can be therefore used to probe the intracellular redox activity [Baronian et al. 2002], [Zhao et al. 2008]. The simultaneous use of hydrophilic and lypophilic
mediators (so called double-mediator system) was reported to enhance the signal obtained with a lypophylic mediator alone [Khlupova et al. 2007], [Zhao et al. 2008]. However, the nature of such signal may be complex and in some cases, it can reflect the intracellular redox activity combined with the redox activity of the tPMET.

Figures 3.52 and 3.53 show chronoamperometric curves obtained with graphite electrodes modified with either FC $b_2$-overexpressing mutant or wild-type Hansenula polymorpha yeast cells and measured in the presence of 1 mM ferricyanide or DCPIP, respectively.

Fig. 3.52 Chronoamperometric response of graphite electrodes that were immersed into 1 mM solution of $K_3[Fe(CN)_6]$ in PB pH = 7.8 and modified with: blue line - recombinant Hansenula polymorpha yeast cells; red line - wild-type Hansenula polymorpha yeast cells. The graphite electrodes were polarized to 300 mV vs. Ag/AgCl/3M KCl. Additions 1-4 correspond to 1, 2.5, 5, and 10 mM L-lactate, respectively.

The results, represented in Fig. 3.52 show a drastic difference in the behaviour of recombinant and wild-type yeast cells in the presence of ferricyanide. Whereas the genetically modified cells similarly as in the experiment with osmium redox polymer responded with increase of current on addition of L-lactate (Fig. 3.52 blue line), the
wild-type cells in most of the attempts failed to do so (Fig. 3.52 red line). Only occasionally the addition of L-lactate caused a very small current response, however, this was negligible when compared with the signal magnitude obtained with the recombinant cells. Since ferricyanide can not penetrate through the cell membrane and interact with intracellular redoxproteins, the observed increase of the current in the presence of L-lactate may only originate from the redox interaction taking place on the outer side of the plasma membrane. The drastic difference between the amperometric responses of genetically modified and wild-type cells is supposed to be due to the overexpression of FC $b_2$ enzyme in the recombinant cell provoking the increase of reductive activity of the cellular plasma membrane.

Fig. 3.53 Chronoamperometric responses of graphite electrodes immersed into 1 mM solution of DCPIP in PB pH = 7.8 and modified with: blue line – recombinant Hansenula polymorpha yeast cells; red line – wild-type Hansenula polymorpha yeast cells. The graphite surfaces were polarized to 300 mV vs. Ag/AgCl/3M KCl. Additions 1-4: correspond to 1, 2.5, 5, and 10 mM L-lactate increments, 5: the addition of 1 mM K$_3$[Fe(CN)$_6$].
In the case of the lyophilic mediator DCPIP (Fig. 3.53), both types of cells responded with a small current increase upon addition of L-lactate, whereas the addition of ferricyanide at the end of the experiment caused a multiplication of the initial signal obtained with DCPIP alone. In the case of wild-type cells, the signal amplification is due to increase of the effectiveness of electron transfer in the double-mediator system. In the case of recombinant cells the amplified signal reflects the sum of both intracellular and plasma membrane redox activity of the cells. This explains the difference in the amplification magnitude of signals obtained with recombinant and wild-type cells after addition of ferricyanide.

In order to prove the L-lactate-dependent ferrireductase activity of the plasma membrane of intact genetically modified cells and to exclude the possibility of cell disruption (as shown in Fig. 3.54A), which would make it possible for ferricyanide to reach the intracellular enzymes, ferrireductive activity of the cells was tested in a solution excluding cell immobilization on the electrode surface.

![Diagram](image)

Fig. 3.54 Ferricyanide reduction by yeast cells: A- by intracellular FC $b_2$ when the cell integrity is damaged; B - by plasma membrane integrated FC $b_2$

For this purpose, the freshly harvested cells of recombinant and wild strains of *Hansenula polymorpha* were incubated for 1 hour in ferricyanide solutions with and without L-lactate. In the first case, L-lactate enters the yeast cell, reacts with FC $b_2$, which then, directly or indirectly (via tPME), shuttles the electrons obtained from the oxidation of L-lactate to the exterior of the yeast cell. Ferricyanide molecules, dissolved
in the buffer solution can freely move to the cells and interact with the cell walls and plasma membrane reductive systems. The interaction of ferricyanide with plasma membrane embedded FC \(_b_2\) or tPMET results in ferricyanide reduction to ferrocyanide by the electrons originating from enzymatic L-lactate oxidation. Long enough incubation of the yeast cells with L-lactate and ferricyanide was expected to result in an increase in the ferrocyanide concentration in the solution to a level high enough to be detected by voltammetric measurements. Incubation of the yeast cells in ferricyanide solutions in the absence of L-lactate was performed as control experiment in order to estimate L-lactate-independent ferrireductase activity of the yeast plasma membrane systems.

Fig. 3.55 Reactions taking place in: A – solution of L-lactate and ferricyanide with genetically modified yeast cells; B – solution of L-lactate and ferricyanide with wild-type yeast cells; C – solution of ferricyanide with genetically modified yeast cells; D – solution of ferricyanide with wild-type cells.
Fig. 3.56 depicts four steady-state voltammograms, recorded with the solutions of ferricyanide with or without L-lactate, which were incubated with wild-type or genetically modified yeast cells for one hour. Before performing the voltammetric experiments all solutions were filtered from the yeast cells. In the absence of L-lactate no traces of ferrocyanide could be detected in solutions incubated with either mutant or wild-type *Hansenula polymorpha* yeast cells (Fig. 3.56, red and pink line).

![Steady-state voltammograms](image)

Fig. 3.56 Steady-state voltammograms in a solution of: blue line - 20 mM ferricyanide and 10 mM L-lactate after incubation for 1 h with genetically modified *Hansenula polymorpha* yeast cells; green line - 20 mM ferricyanide and 10 mM L-lactate after incubation for 1 h with wild-type *Hansenula polymorpha* yeast cells; red line - 20 mM ferricyanide after incubation with genetically modified *Hansenula polymorpha* yeast cells; pink line - 20 mM ferricyanide after incubation for 1 h with wild-type *Hansenula polymorpha* yeast cells.

At a potential of 425 mV vs. Ag/AgCl only a low background current could be observed. Growth of cathodic current at the potential close to the apparent redox potential (E₀’) of Fe species and its levelling to steady state indicates reduction of
ferricyanide present in the solution. A similar voltammogram was recorded in a solution of L-lactate and ferricyanide after incubation with wild-type *Hansenula polymorpha* yeast cells (Fig. 3.56, green line). From this, it can be concluded that the L-lactate-dependent ferrireductase activity of plasma membrane of the wild-type *Hansenula polymorpha* yeast cells is negligible. In opposite to this, a significant ferrireductase activity was observed with genetically modified cells in the presence of L-lactate. Appearance of a considerable anodic current in the voltammogram at potentials more positive than $E_0^′$ of the $[\text{Fe(CN)}_6]^{3-}/[\text{Fe(CN)}_6]^{3-}$ couple indicates the presence of Fe II ions in the solution after incubation with recombinant yeast cells (Fig. 3.56, blue line). At the same time, the cathodic current due to ferricyanide reduction was decreased since a part of ferricyanide was converted by the cells to ferrocyanide. This is in good agreement with the results obtained with immobilized cells.

### 3.2.2.4. Selectivity of electrical wiring of yeast cells to electron donors

Knowing the selectivity of the investigated electron-transfer pathway to different electron acceptors it is also interesting to find out which electron donors can activate it. FC $b_2$ is known for its almost exclusive selectivity to L-lactate as electron donor whereas the tPMET is assumed to accept electrons from NADH or NADPH escaping from other catalytic cycles in the cell. Fig. 3.57 summarizes the potential sources of NADH in the yeast cell. In most studies, the activity of tPMET was reported to be induced either by glucose or by ethanol. Therefore, in a set of following experiments glucose and ethanol, two key substrates for catabolism in yeast cells, were added instead of L-lactate to solutions into which graphite electrodes modified with recombinant or wild-type yeast cells were immersed.
Fig. 3.57 NADH-recycling pathways in *Saccharomyces cerevisiae*.

1. Reoxidation of mitochondrial NADH directly via the internal NADH dehydrogenase (Ndi1p), ubiquinone (CoQ6), cytochrome bc1 (bc1), cytochrome c (Cyt C) and cytochrome c oxidase (COX) [Luttik et al. 1998].

2. Reoxidation of cytosolic NADH directly via the external NADH dehydrogenases 1 and 2 (Nde1p, 2p) [Luttik et al. 1998].

3. Glucose fermentation to ethanol, reoxidation of NADH produced during glycolysis using pyruvate decarboxylases 1, 5 and 6 (Pdc1p,5p,6p) [Bakker et al. 2000].

4. Transport of ethanol across the mitochondrial membrane, reduction to acetaldehyde, NADH reoxidized via Ndi1p [Bakker et al. 2001].

5. Glycerol production, which is a net NADH-oxidizing process [Pahlman et al. 2001].

6. Electrons from glycerol-3-phosphate (G-3-P) can be transferred to ubiquinone Q6, CoQ6 via mitochondrial FAD-dependent glycerol-3 phosphate dehydrogenase (Gut2p) [Overkamp et al. 2002], [Rigoulet et al. 2004].

7. Cytosolic and mitochondrial NADH reoxidized via plasma membrane electron transport system (tPMET), [Herst et al. 2008]. Adapted from [Bakker et al. 2000] and [Herst et al. 2008]
Interestingly, neither glucose nor ethanol provoked an increase of current when the electrodes were modified with two types of yeast cells and osmium complex-based redox polymer but L-lactate added to the buffer solution at the end of the experiment did not fail to do so (Fig. 3.58). This exclusive selectivity of the cell-osmium complex-based redox polymer electron transfer pathway is in favour of the hypothesis, which supposes the involvement of plasma membrane localized FC $b_2$ in the investigated process.

![Diagram of electron transfer pathway](image)

Fig. 3.58 Chronoamperometric response upon consecutive addition of D-glucose, ethanol and L-lactate for graphite electrodes that were modified with: blue line - the Os complex modified polymer G390 and recombinant *Hansenula polymorpha* yeast cells, or, red line - the Os complex modified polymer G390 and wild-type *Hansenula polymorpha* yeast cells. The graphite electrode was polarized to 200 mV vs. Ag/AgCl/3M KCl. Additions 1-3: 1; 5; 10 mM D-glucose; 4-6: 1; 5; 10 mM ethanol; 7-9: 1; 5; 10 mM L-lactate.
The situation was more complex when the redox wiring of yeast cells to the electrode was established by free-diffusing redox mediators such as $[\text{Fe(CN}_6\text{)}]^3^-$ (Fig. 3.59) and DCPIP (Fig. 3.60).

![Diagram](image)

Fig. 3.59 Selectivity. Chronoamperometric response upon consecutive addition of D-glucose, ethanol and L-lactate of: blue line - electrode, modified with recombinant *Hansenula polymorpha* yeast cells; red line - electrode, modified with wild-type *Hansenula polymorpha* yeast cells. Graphite electrodes polarized at 300 mV vs. Ag/AgCl/3M KCl in 1 mM $K_3[\text{Fe(CN}_6\text{)}]$ solution in PB pH = 7.8. Additions 1-3: 1; 5; 10 mM D-glucose; 4-6: 1; 5; 10 mM ethanol; 7-9: 1; 5; 10 mM L-lactate.

Again, glucose absolutely failed to activate the electron transfer and induce a current flow through the working electrode regardless of the type of dissolved mediator and cell. Upon ethanol additions, however, step-like current increases were observed. The effect was more pronounced for electrodes that were modified with the wild-type *Hansenula polymorpha* yeast cells (compared to mutants). This could be due to the well-known phenomenon of catabolite repression [Gancedo 1998], which inhibits the synthesis of the enzymes participating in the conversion of the unavailable substrates and induce the
synthesis of the enzymes converting the substrate, present in the growing medium. It can be hypothesized that FC \( b_2 \) overexpression and the presence of L-lactate in the growing medium provoke stronger catabolite repression of the synthesis of ethanol-converting enzymes in the recombinant yeast cells than in wild-type cells since no response to addition of ethanol was recorded with the electrode, modified with recombinant yeast cells and free-diffusing mediators.

Fig. 3.60 Selectivity. Chronoamperometric response upon consecutive addition of D-glucose, ethanol and L-lactate of: blue line - electrode, modified with recombinant \textit{Hansenula polymorpha} yeast cells; red line - electrode, modified with wild-type \textit{Hansenula polymorpha} yeast cells. Graphite electrodes polarized at 300 mV vs. Ag/AgCl/3M KCl in 1 mM DCPIP solution in PB pH = 7.8. Additions 1-3: 1; 5; 10 mM D-glucose; 4-6: 1; 5; 10 mM ethanol; 7-9: 1; 5; 10 mM L-lactate; 10: 1mM K\(_3\)[Fe(CN)\(_6\)]]

3.2.2.5. Summary
Exoelectrogenic properties of genetically modified and wild-type \textit{Hansenula polymorpha} yeast cells were studied in the presence and absence of polymer-bound
osmium complexes. The target system was formed by immobilizing either wild-type or recombinant *Hansenula polymorpha* yeast cells overexpressing the FC b2 enzyme into osmium complex modified polymers on graphite disk electrodes. A noticeable current in the presence of L-lactate could be recorded when (1) the recombinant yeast cells were used and (2) the osmium entities of the redox polymer are in direct physical contact with the cell wall of the entrapped genetically modified yeast cells. FC b2 overexpression in the cell not only amplifies the extracellular electron transfer to polymer-bound osmium complexes in the presence of L-lactate but also induce the L-lactate-dependent ferrireductase activity of the cell plasma membrane in the recombinant yeast cells. The investigated process of electric communication between *Hansenula polymorpha* yeast cells and osmium redox polymers takes place in the presence of L-lactate but not of ethanol or glucose. This fundamental study used simply the osmium redox polymers that were readily available at the time. However, the application of specially-designed osmium complex-based polymers with both optimized structural and electrical properties and tuned redox potentials could help creating more efficient ways for the redox wiring of engineered yeast cells and thus be useful for the development of whole cell biofuel cells and biosensing devices.
4. CONCLUSIONS

Genetic engineering of the yeast cells for biosensing purposes and design of redox EDPs as immobilization matrices seem to be promising for the development of advanced biosensing and bioenergy producing devices. The intelligent design of immobilization matrices and biosensor architectures as well as investigation of genetically modified \textit{Hansenula polymorpha} yeast cells as biorecognition elements were in the scope of this thesis. Special attention was paid to the interactions of biological material with the immobilization matrix and the transducer. The main scientific results are summarized in the following conclusions:

1. The synthesis procedure of acrylic-based electrodeposition polymers used for the immobilization of biorecognition elements and automatic evaluation of the biosensors characteristics by means of electrochemical robotic system were improved. Optimal compositions of the immobilization matrices for the enzyme-based glucose and cell-based lactate biosensors were found. The influence of the properties of immobilization matrices on the performance of amperometric biosensors was analyzed.

2. An optimal osmium complex modified redox polymer for multielement formaldehyde and L-lactate biosensors with complex architecture was found and the analytical characteristics of the biosensor were optimized. The performance of FC \( b_2 \)-Os EDP biosensors could be significantly improved by implementation of cytochrome \( c \) into the biosensor architecture.

3. The genetically modified \textit{Hansenula polymorpha} cells were found to enhance their respiration in the presence of L-lactate. This property of recombinant cells was used for L-lactate detection. The L-lactate-dependent respiration of yeast cells was visualized by means of scanning electrochemical microscopy.

4. Exoelectrogenic properties of \textit{Hansenula polymorpha} yeast cells were discovered and studied in details.
5. LITERATURE


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