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Amperometric Biosensors for Lactate, Alcohols and Glycerol Assays in Clinical Diagnostics

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1. Introduction

Biosensors are bioanalytical devices which transform a biorecognition response into a measurable physical signal. Although biosensors are a novel achievement of bioanalytical chemistry, they are not only a subject of intensive research, but also a real commercial product (Kissinger, 2005). The estimated world analytical market is about \$20 billion per year of which 30 % is in the healthcare field. The biosensors market is expected to grow from \$6.72 billion in 2009 to \$14.42 billion in 2016 (<http://www.marketresearch.com>, Analytical Review of World Biosensors Market).

Although up to now IUPAC has not accepted an official definition of the term biosensor, its electrochemical representative is defined as “a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element” (Thevenot et al., 2001). Generally, the biosensor is a hybrid device containing two functional parts: a bioelement (an immobilized biologically active material) and a physical transducer. As bioelements pieces of tissue, microbial cells, organelles, natural biomembranes or liposomes, receptors, enzymes, antibodies and antigens, abzymes, nucleic acids and other biomolecules and even biomimetics which imitate structural and functional features of the natural analogue can be used. The bioelement is a recognition unit providing selective binding or biochemical/metabolic conversion of the analyte that result in changes in physical or physico-chemical characteristics of the transducer (Scheller et al., 1991; Schmidt & Karube, 1998; Gonchar et al., 2002; Nakamura & Karube, 2003; Sharma et al., 2003; Investigations on Sensor Systems and Technologies, 2006). The bioelement in such constructions is usually prepared in immobilized form and often covered with an outer membrane (or placed between two membranes in a sandwich manner), which either prevents the penetration of

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interfering substances into a sensitive bioselective layer and the transducer surface, or creates a diffusion barrier for the analyte. Such membrane structures increase the stability of the biorecognition element, enhance its selectivity and provide the diffusion limitations for biochemical reactions. Electrochemical, optical, piezoelectric, thermoelectric, transistor, acoustic and other elements are used as transducers in biosensor systems. Electrochemical (amperometric, potentiometric, conductometric) and optical (surface plasmon resonance) devices are the most exploited transducers in commercially available biosensors (Commercial Biosensors, 1998).

Basically, biosensors can be regarded as information transducers in which the energy of biospecific interactions is transformed into information about the nature and concentration of an analyte in the sample. The most essential advantages of biosensors are excellent chemical selectivity and high sensitivity, possibility of miniaturization and compatibility with computers. Their drawbacks are limited stability and a rather complicated procedure for preparation of the biologically active material.

Enzyme biosensors are the most widespread devices (Zhao & Jiang, 2010); many of them are produced commercially. Enzyme biosensors are characterized by their high selectivity. They also provide fast output due to high activity and high local enzyme concentration in a sensitive layer. The drawbacks of enzyme biosensors are insufficient stability and the high price of purified enzymes. Cell sensors, especially microbial ones, have been actively developed only in recent years (Shimomura-Shimizu & Karube, 2010a, 2010b; Su et al., 2011). Cell biosensors have a range of considerable advantages when compared to their enzyme analogues: availability of cells, low price and simple procedure of cell isolation, possibility to use long metabolic chains, avoiding purification of enzymes and coenzymes, advanced opportunity for metabolic engineering, integrity of the cell response (important in assaying total toxicity and mutagenic action of environmental pollutants), possibility to retain viability of sensing cells and even to provide their propagation, and, in some cases, higher stability of cell elements compared to enzyme ones. The main drawbacks of microbial biosensors are a rather low signal rate due to a lower concentration of enzymes involved in cellular response, as well as low selectivity of cell output (e.g. in the case of microbial O₂ electrode sensors due to a broad substrate specificity of cellular respiration).

These drawbacks are not absolute, taking into account recent progress in genetic engineering and the possibility to over-express the key analytical enzyme in the cell (Gonchar et al., 2002).

Most biosensors have been created for clinical diagnostics (D'Orazio, 2003; Song et al., 2006; Belluzo et al., 2008). They exploit enzymes as biocatalytic recognition elements and immunoreagents and DNA fragments as affinity tools for biorecognition of the target analytes (metabolites, antigens, antibodies, nucleic acids) coupled to electrochemical and optical modes of transduction. For simultaneous detection of multiple analytes, microarray techniques are developed for automated clinical diagnostics (Seidel & Niessner, 2008). For continuous monitoring of living processes, reagentless implantable biosensors have been developed (Wilson & Ammam, 2007).

Biosensors are regarded as very promising tools for clinical cancer testing (Rasooly & Jacobson, 2006; Wang, 2006). New genomic and proteomic approaches are being used for revealing cancer biomarkers related with genetic features, changes in gene expression, protein profiles and post-translational modifications of proteins.

Recent progress in nanobiotechnology allows using nanomolecular approaches for clinical diagnostic procedures (Salata, 2004; Jain, 2007). The most important applications are

foreseen in the areas of biomarker monitoring, cancer diagnosis, and detection of infectious microorganisms. Analytical nanobiotechnology uses different nanoscaled materials (gold and magnetic nanoparticles, nanoprobe, quantum dots as labels, DNA nanotags) for molecular detection (Baptista et al., 2008; Medintz et al., 2008; Sekhon & Kamboj, 2010). The use of nanomaterials in biosensors has allowed the introduction of many new signal transduction technologies into biosensorics and the improvement of bioanalytical parameters of the nanosensors - selectivity, response time, miniaturization of the biorecognition unit (Jianrong et al., 2004; Murphy, 2006).

2. Development of L-lactate-selective biosensors based on L-lactate-selective enzymes

Lactate, a key metabolite of the anaerobic glycolytic pathway, plays an important role in medicine, in the nutritional sector, as well as in food quality control. Amperometric biosensors offer a sensitive and selective means to monitor organic analytes like lactate. Here, different aspects of amperometric lactate biosensor construction are described: electrode materials, biorecognition elements, immobilization methods, mediators and cofactors as well as fields of application.

Biosensors for the detection of L-lactate are often based on either NAD⁺-dependent lactate dehydrogenase (LDH) from mammalian muscles or heart (EC 1.1.1.27) (Arvinte et al., 2006; Hong et al., 2002), bacterial lactate oxidase (LOX) (EC 1.13.12.4) (Hirano et al., 2002; Iwuoha et al., 1999) or bi-enzyme systems combining peroxidase (HRP) and LOX (Herrero et al., 2004; Zaydan et al., 2004; Serra et al., 1999). Some approaches let to commercially available L-lactate sensors (Luong et al., 1997; <http://www.johnmorris.com.au/html/Ysi/ysi1500.htm>; <http://www.fitnessmonitors.com/ecstore/cat111.htm>). However, due to the non-advantageous equilibrium constant of the LDH-catalysed reaction and the need to add free diffusing NAD⁺ as well as problems arising from the generally high working potentials of LOX-based amperometric biosensors, there is still a need to develop alternative sensor concepts for the determination of L-lactate. To decrease the impact of interfering compounds, related sensor's electrodes were, for example, covered with an additional permselective membrane (Madaras et al., 1996). Despite the more complex sensor preparation, this pathway is unsuitable for the development of L-lactate sensors due to the fact that negatively-charged L-lactate is simultaneously prevented from reaching the electrode surface through negatively charged membranes.

Besides LDH and LOX, another enzyme is known for participating in the lactic acid metabolism in yeasts, namely L-lactate-cytochrome *c* oxidoreductase (EC 1.1.2.3; flavocytochrome *b*₂, FC *b*₂) (Brooks, 2002), which catalyses the electron transfer from L-lactate to cytochrome *c* in yeast mitochondria. The protein can be isolated from *Saccharomyces cerevisiae* and *Hansenula anomala* (Labeyrie et al., 1978; Haumont et al., 1987; Silvestrini et al., 1993) as a tetramer with four identical subunits, each consisting of FMN- and heme-binding domains. FC *b*₂ has absolute specificity for L-lactate, moreover, it functions *in vitro* without regard to the nature of electron acceptors which makes this enzyme very promising for analytical biotechnology. However, until now application of FC *b*₂ from baker's yeast in bioanalytical devices was hampered by its instability and difficulties in purification of the enzyme (Labeyrie et al., 1978). Here, we describe the use of a purified FC *b*₂ isolated from the wild-type and recombinant thermotolerant *Hansenula polymorpha* yeast cells that overproduce this enzyme as a biological recognition element in amperometric biosensors.

2.1 Construction of biosensors using purified FC b_2 from the wild type *Hansenula polymorpha* 356

Prior to the electrochemical investigations, we have performed considerable microbiological, biochemical and analytical investigations. We have screened 16 yeast species as possible sources of the stable form of FC b_2 . The thermostability test was performed at different temperatures and time periods. The study of enzyme activity by two procedures, including our own method for FC b_2 activity visualization in PAA-gels (Gaida et al., 2003), has shown that only FC b_2 from *H. polymorpha* 356 remained as a native tetramer during a 10 min-incubation of cell-free extract at 60°C or 3 min at 70°C (Smutok et al., 2006a). For preparative purification of FC b_2 from the cells of *H. polymorpha* 356, we modified a scheme that was developed for this enzyme from the yeast *H. anomala* (Labeyrie et al., 1978). The scheme of purification includes lysis of the cell's pellet by *n*-butanol followed by extraction of cell's debris with 1% Triton X-100; ion-exchange chromatography on DEAE-Toyopearl 650M (TSK-GEL, Japan). The enzyme yield after chromatographic purification was near 80 % (Smutok et al., 2006c). The highest specific FC b_2 activity in some fractions was 20 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein (U mg^{-1}). After ammonium sulfate was added up to 70% saturation, the specific activity was increased to 30 U mg^{-1} . FC b_2 preparations have been used to develop L-lactate-sensitive biosensors (Smutok et al., 2006a).

Amperometric FC b_2 -based biosensors were evaluated using constant-potential amperometry in a three-electrode configuration with a Ag/AgCl/KCl (3 M) reference electrode and a Pt-wire counter electrode. Amperometric measurements were carried out with a bipotentiostat (EP 30, Biometra, Göttingen, Germany) or a potentiostat PGSTAT302 (Autolab, Netherlands). As working electrodes, graphite rods (type RW001, 3.05 mm diameter, Ringsdorff Werke, Bonn, Germany) were applied, which were sealed in a glass tube using epoxy, thus forming disk electrodes. Before sensor preparation, the graphite electrodes were polished on emery paper and on a polishing cloth using decreasing sizes of alumina paste (Leco, Germany). The polished electrodes were rinsed with water in an ultrasonic bath.

Although FC b_2 provides very specific electron transfer from L-lactate to cytochrome *c* in the respiratory chain of native yeast cells, theoretically, it could be supposed *in vitro* a direct electrochemical communication between the reduced heme-binding domain and the electrode surface. This hypothesis has been approved by using cyclic voltammetry (Fig. 1).

The obtained cyclic voltammogram (Fig. 1A) shows a small peak at a potential of +250 mV versus Ag/AgCl in the presence of L-lactate which can be attributed to the accessibility of the heme site of the enzyme for direct electron exchange reactions with the electrode surface. Obviously, the first monolayer of the enzyme is able to directly exchange electrons with the electrode surface, providing a favorable orientation with the accessible heme site towards the electrode.

The related hydrodynamic voltammograms at increasing L-lactate concentrations (0.5, 1 and 4 mM) are shown in Fig. 1B. The peak current is highest at a potential of about +300 mV versus Ag/AgCl; hence, all the experiments concerning direct electron transfer were performed at this working potential.

In yeast cells, mitochondrial FC b_2 catalyses the dehydrogenation of L-lactate to pyruvate, transferring the electrons from L-lactate *via* FMN as the primary electron acceptor, to the heme site of the enzyme and finally to cytochrome *c* as the terminal electron sink. No detectable direct electron transfer is possible from the reduced FMNH₂ inside the intact enzyme to cytochrome *c*, avoiding the intermediate storage of the electrons in the heme site (Ogura & Nakamura, 1966). However, it is known that a number of free-diffusing redox

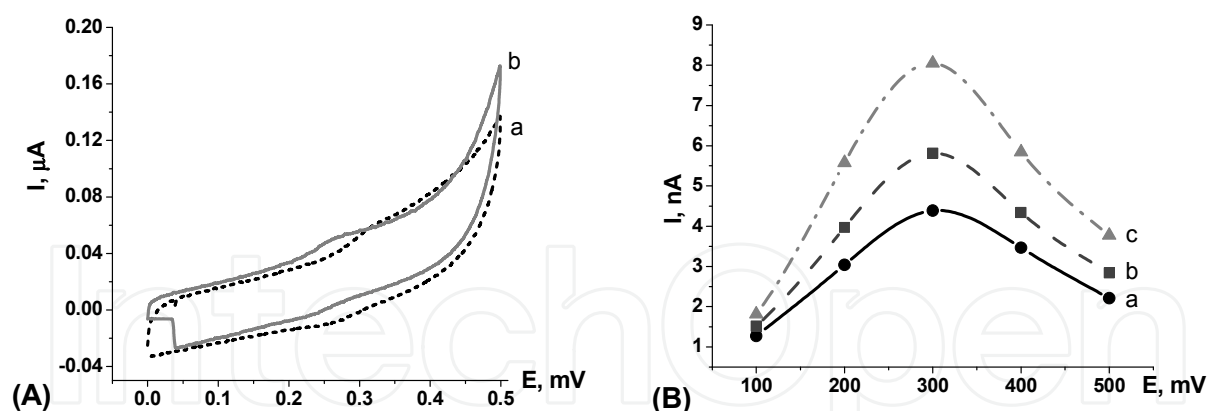


Fig. 1. (A) Cyclic voltammogram at a graphite electrode modified with adsorbed FC b_2 (a) in the absence and (b) in the presence of 20 mM L-lactate (0 to +500 mV at a scan rate of 5 mV/s in 20 mM phosphate buffer, pH 7.2). (B) Hydrodynamic voltammograms obtained from a graphite electrode modified with adsorbed FC b_2 in the presence of (a) 0.5 mM; (b) 1 mM; (c) 4 mM L-lactate in the absence of any electron-transfer mediator.

mediators can be used to transfer electrons from FC b_2 to electrodes, while direct electron transfer was not yet approved experimentally. Hence, we investigated the L-lactate-dependent current response of FC b_2 -modified electrodes in the absence and presence of various electron-transfer mediators (Figs. 2 and 3).

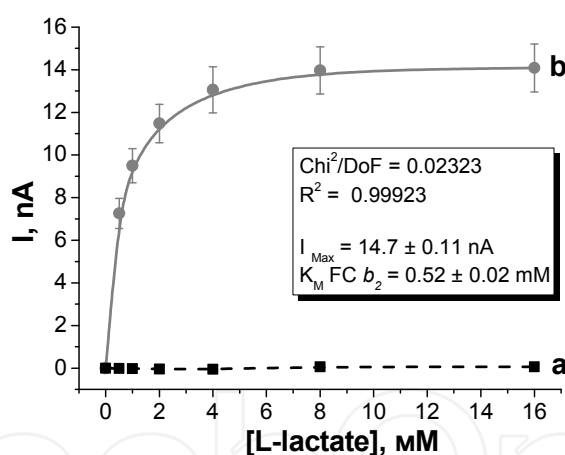


Fig. 2. Lactate calibration curve of a graphite electrode modified with adsorbed FC b_2 at a potential of +300 mV and pH 7.2 in the absence of any redox mediator (b). Control experiment with a bare graphite electrode (a).

In the absence of any free-diffusing redox mediator, direct electron transfer is only possible from those enzyme molecules which are located in a monolayer in direct contact with the electrode surface, while being orientated to allow the heme site to be situated at a productive electron-transfer distance. Although the efficiency of the direct electron transfer reaction is comparatively low, the response clearly exceeds the noise signal of the control electrode without immobilized enzyme (Fig. 2). These limitations lead to a maximum current of 14 nA at L-lactate saturation. In contrast, in the presence of the most effective free-diffusing redox mediator (phenazine ethosulfate) the response is enhanced 28 times, reaching a maximum value of 390 nA at substrate saturation.

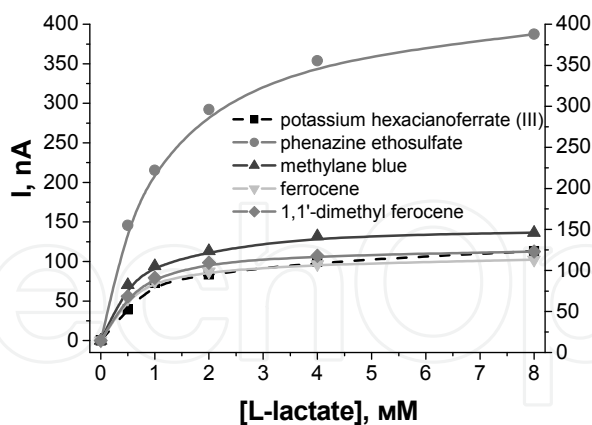


Fig. 3. Lactate calibration curves obtained with a graphite electrode modified with adsorbed FC b_2 in the presence of different free-diffusing electron-transfer mediators (+ 300 mV, pH 7.2).

As demonstrated in Fig. 3, all free-diffusing and adsorbed redox mediators used in the study accelerated the investigated oxidation of L-lactate catalyzed in the FC b_2 reaction. Phenazine ethosulfate exceeds the efficiency of the other mediators by up to 2.7–3.6 times. Hence, phenazine ethosulfate was used for all further experiments as a free-diffusing redox mediator. A variety of different immobilization techniques were applied for the preparation of the enzyme electrodes, with the aim to achieve optimal stability, the highest possible sensitivity and selectivity, and a low detection limit. FC b_2 was immobilized on graphite surfaces using some different strategies: physical adsorption; electrodeposition by anodic paint *Resydrol AY*; entrapment in a polymer layer of a precipitated cathodic paint GY 83-0270 00054; cross-linking by glutardialdehyde vapour (Smutok et al., 2005); electrodeposition by osmium-modified anodic paint (*AP-Os*); entrapment in a layer of cathodic paint (*CP-Os*) (Fig. 4).

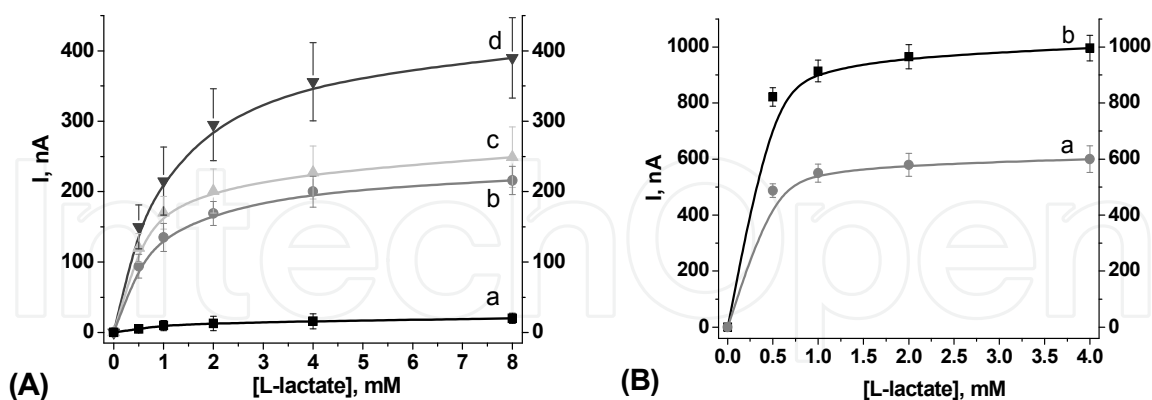


Fig. 4. (A) Lactate calibration curves obtained with graphite electrodes modified with FC b_2 using different enzyme immobilization methods in the presence of 1 mM phenazine ethosulfate as free-diffusing electron-transfer mediator (+300 mV, pH 7.6). (a) Entrapment in the anodic electrodeposition paint "Resydrol AY"; (b) entrapment in the cathodic electrodeposition paint "GY 83-0270 00054"; (c) cross-linking of the adsorbed enzyme in glutardialdehyde vapour; (d) physical adsorption. (B) electrodeposition of FC b_2 with an osmium-modified anodic paint (*AP-Os-FC b_2*) (a); entrapment of FC b_2 in a layer of a cathodic paint (*CP-Os-FC b_2*) (b).

All obtained biosensors were investigated concerning their substrate-dependent current response. As shown in Fig. 4A, the maximal responses of the different enzyme electrodes were 439 ± 3.2 , 263 ± 3.1 , and 237 ± 1.1 nA for physical adsorption, glutardialdehyde immobilization and cathodic paint precipitation, respectively. In the case of the anodic paint *Resydrol AY* the maximal current value was much lower (20 nA). The use of the electrodeposited osmium-complex modified anodic paint performed two functions: electron transfer and film immobilization. Therefore, this variant of enzyme immobilization looks most promising, especially since its signal value was 600 nA (Fig. 4B). However, we have shown that the activity of FC b_2 strongly depends on the process of electro-deposition in the presence of anodic paint. High voltage impulses of electrodeposition (+2200 mV) resulted in the inactivation of FC b_2 , probably due to the very fast generation of protons and a decrease of pH values (Fig. 5A).

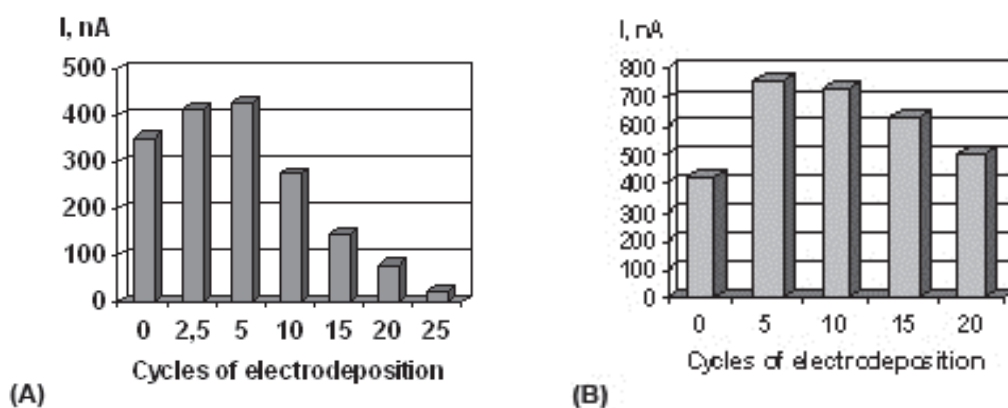


Fig. 5. (A) Dependence of sensor's output on cycles of anodic (A) and cathodic (B) schemes of electrodeposition of FC b_2 (+ 300 mV, pH 7.2 in the presence of 1 mM phenazine ethosulfate).

On the other hand, FC b_2 should have been more resistant at higher pH values according to its enzymological properties in solution (Smutok et al., 2006a). Therefore, in subsequent experiments, a cathodic paint was used as a matrix for the entrapment of FC b_2 . No remarkable negative influence on the FC b_2 activity was observed at potentiostatic pulses to potentials as low as -1200 mV. Hence, this potential was used for the cathodic paint precipitation (Fig. 5B).

The sensor with CP-*Os*-FC b_2 architecture gave the highest output; 1000 nA (Fig. 4B). Therefore, in the subsequent experiments this structure was used in conjunction with free-diffusing mediators, to cross-link immobilized FC b_2 by glutaraldehyde vapour. In the case of covalently-bound mediator, the electroinduced immobilization by CP-*Os*-FC b_2 technique has been selected as the best.

Bioanalytical characteristics of the developed FC b_2 -based biosensor in conjunction with free-diffusing mediators have been investigated. The calculated value for the apparent Michaelis-Menten constant K_M^{app} as derived from the calibration graphs in the presence of 1 mM phenazine ethosulfate for FC b_2 was about 1.0 ± 0.02 mM. The response time was rather fast: 50 % of the signal value is achieved after 3 sec and 90 % after 6 sec (Fig. 6A).

The level of selectivity was estimated in relative units (%), as a ratio to the value of L-lactate response. No interference by L-malate, pyruvate, L,D-isocitrate or acetate on FC b_2 -modified

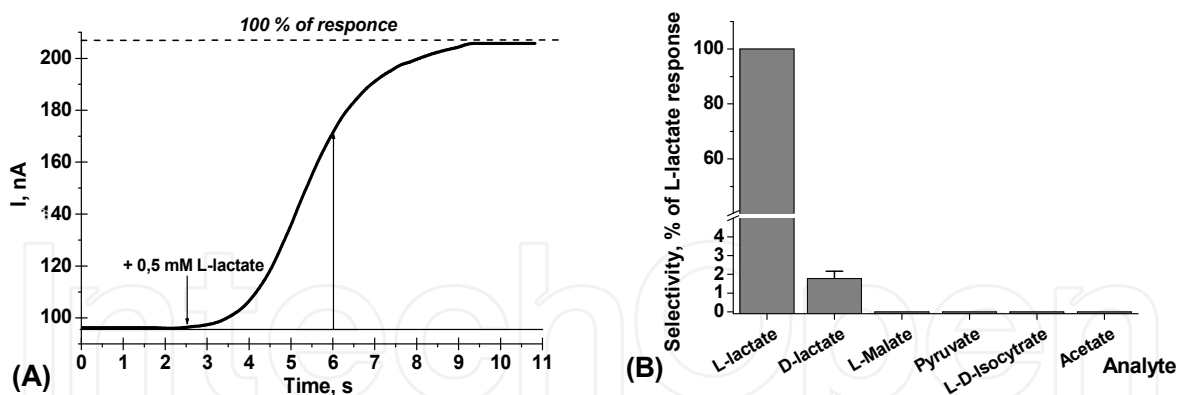


Fig. 6. (A) Development of the sensor's response with time upon addition of 5 mM L-lactate (potential +300 mV vs Ag/AgCl/3M KCl) in phosphate buffer, pH 7.2 in the presence of 1 mM phenazine ethosulfate (a). (B) Selectivity of FC b_2 -modified electrodes (response to 4 mM analyte).

electrodes was observed, but the sensor did show a low signal to D-Lactate (1.8 ± 0.3 %). This fact can be explained by the incomplete purity of the FC b_2 sample, and its possible contamination with D-lactate cytochrome *c*-oxidoreductase. In spite of this fact, the developed sensor was highly selective to L-lactate (Fig. 6B).

The temperature and pH-dependence of the obtained biosensors were evaluated and the optimal temperature of 35–38 °C at the optimal pH-value of 7.5–7.8 was derived (Fig. 7). These values are governed by the properties of the enzyme itself and are not significantly altered by the used immobilization procedure.

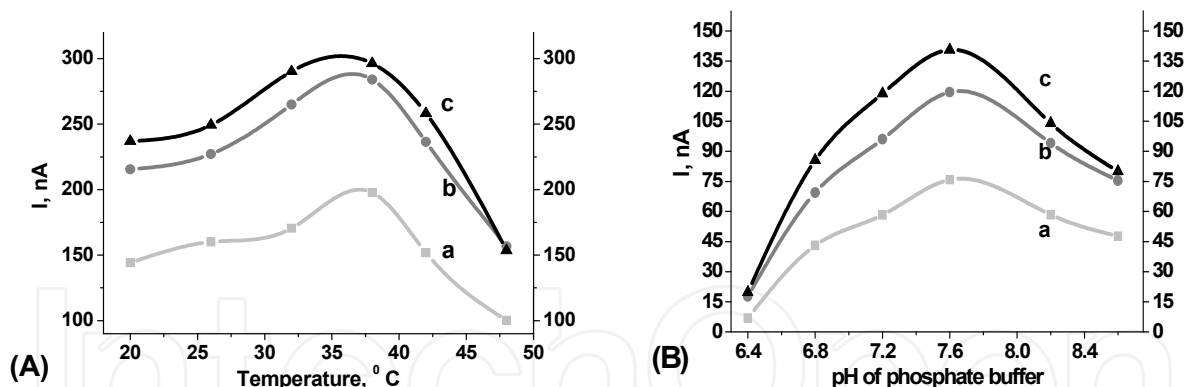


Fig. 7. Temperature- (A) and pH-dependence (B) of the biosensor's response to L-lactate: 0.5 mM (a); 2 mM (b) and 8 mM (c). Experimental conditions: +300 mV vs Ag/AgCl/3 M KCl, pH 7.6, 1 mM phenazine ethosulfate.

Simultaneous with the investigation of the sensor architecture comprising free-diffusing mediators, the main characteristics of the sensor formed by electrodeposition paint were determined. The maximal detected signal values were 1100 nA for the sensor architecture CP-Os-FC b_2 and 650 nA for AP59-Os-FC b_2 -modified electrodes (Fig. 8).

The apparent Michaelis-Menten constants (K_M^{app}) for L-lactate calculated from the calibration curves were 0.141 ± 0.001 mM and 0.135 ± 0.003 mM, respectively. The sensor response time, selectivity, optimal temperature and pH values were the same as for the biosensor based on free-diffusing redox mediators.

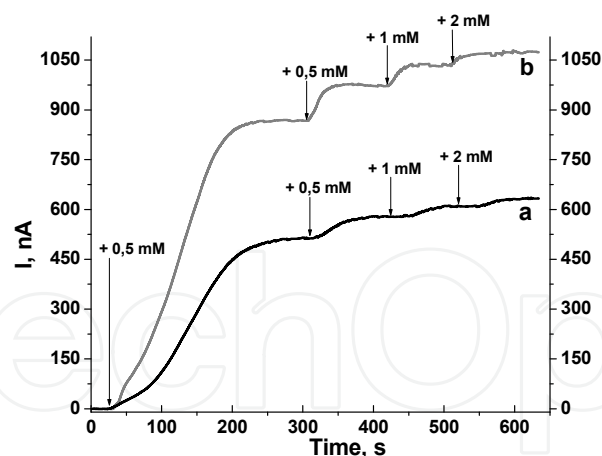


Fig. 8. Chronamperometric current response upon subsequent additions of L-lactate aliquots for sensors with $FC\ b_2$ entrapped in a layer of osmium-complex modified anodic paint ($AP-Os-FC\ b_2$) (a) and an osmium-complex containing cathodic paint ($CP-Os-FC\ b_2$) (b).

The operation and storage stabilities of the developed sensors have been evaluated. The electrodes prepared at optimal conditions were tested at 24 °C with respect to their stability. Solutions of 1 mM L-lactate (for experiments of operational stability) and 4 mM L-lactate (for the storage stability) were used in these experiments. The operational stability of the obtained microbial sensors was evaluated using a previously described automatic sequential-injection analyzer (“OLGA”) system (Schuhmann et al., 1995) and 15 measurements per hour were done (Fig. 9A).

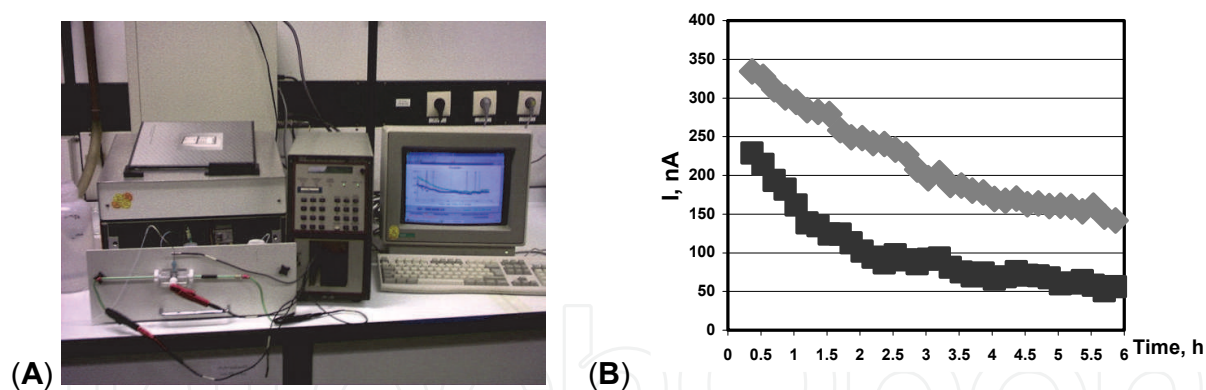


Fig. 9. Flow injection “OLGA” analyzer system with integrated bioelectrodes (A) and operation stability of the sensor obtained by “OLGA” (1 mM L-Lactate, flow-rate 5 ml min⁻¹, 24 °C and detection of results every 4 min) (B).

Two variants of the working electrodes showed some differences in the initial response values to L-lactate. There were also some differences in the kinetics of sensor inactivation. The initial sensor output for the $CP-Os-FC\ b_2$ variant of the working electrode was near 350 nA and decreased after 5.5 hours (82 measurements) to 175 nA (half-life). The $AP-Os-FC\ b_2$ variant of the sensor showed a lower initial output (250 nA) and after 5.5 hours of work revealed a lower signal (75 nA) as compared to the first sensor.

The storage stability of the constructed $CP-Os-FC\ b_2$ biosensor was found to be satisfactory over more than 7 days and a half-life activity of the sensor was observed at the 5th day of storage (Fig. 10).

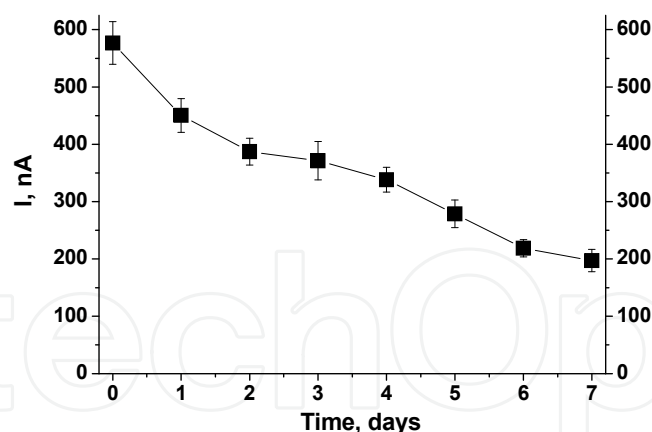


Fig. 10. Storage stability of the CP-Os-FC b_2 sensor architecture (4 mM L-Lactate, 24 °C).

2.2 Development of microbial amperometric biosensors based on the cells of flavocytochrome b_2 over-producing recombinant yeast *H. polymorpha*

Currently, four different enzymes are known as biological recognition element for L-lactate detection: lactate oxidase (LOD) (Karube et al., 1980), lactate monooxygenase (LMO) (Mascini et al., 1984), lactate dehydrogenase (LDH) (Wang & Chen, 1994) and flavocytochrome b_2 (Staskeviciene et al., 1991). However, microorganisms provide an ideal alternative to enzymes, providing certain advantages in comparison with enzyme-based biosensors: for example, avoiding isolation and purification steps for enzyme preparation; prolonged shelf-life of the sensor due to improved stability of the biorecognition element in the intact biological environment. Previous bacterial biosensors for L-lactate were successfully constructed using the whole cells of *Paracoccus denitrificans* (Kalab & Skladal, 1994), *Acetobacter pasteurianus* (Luong et al., 1989), *Alcaligenes eutrophus* (Plegge et al., 2000) and *Escherichia coli* (Adamowicz & Burstein, 1987). Physical robustness of yeasts in comparison to bacteria and superior tolerances to pH, temperature and osmolarity/ionic strength make them the preferred microorganisms, with the potential to be used as biological recognition elements for cell-based biosensors (Baronian, 2004). The application of the yeast *H. anomala* to oxidise L-lactate was investigated earlier by Racek et al. using a platinum electrode, polarised to the potential of +350 mV vs. Ag/AgCl using potassium ferricyanide as a soluble mediator (Racek & Musil, 1987a, 1987b), and later by Kulys et al. using carbon paste electrodes and different mediators (potassium ferricyanide, phenazine methosulfate, organic salt of TMPD/TCNQ, methylene green, Mendola's blue) at potentials of +50-300 mV vs. SCE (Kulys et al., 1992). Garjonyte implemented *S. cerevisiae* yeast cells for the construction of the biosensor for L-lactate using carbon paste electrodes and potassium ferricyanide, phenazine methosulfate, 2,6-dichlorophenolindophenol sodium salt hydrate, 1,2-naphthoquinone-4-sulfonic acid salt or *p*-benzoquinone as free-diffusing mediators at potentials of 0-+300 mV vs. Ag/AgCl (Garjonyte et al., 2006; Garjonyte et al., 2008).

In the meantime, the genomes of some yeast species (*S. cerevisiae*, *H. polymorpha*) were completely sequenced and gene engineering methods allowed for the tailoring of these microorganisms to enhance the activity of specific enzymes (Walmsley & Keenan, 2000). Genetically modified yeast cells of *S. cerevisiae* were successfully used for the construction of genotoxicity biosensors (Walmsley et al., 1997; Billinton et al., 1998), or biosensors for estrogen (Tucker & Fields, 2001), dibenzo-*p*-dioxins (Sakaki et al., 2002) and copper (Lehmann et al., 2000) detection. *H. polymorpha* mutants were implemented for the

development of biosensors for formaldehyde (Korpan et al., 1993) and ethanol/methanol (Gonchar et al., 1998).

Alteration of the target enzymatic pathway may cause other additional metabolic changes in the cell metabolism. Thus, cells have the ability to adjust their metabolism in order to adapt to changing conditions and to survive the imposed stress. A good example of yeast cells exhibiting such adaptation behavior was observed in genetically modified yeast cells of *H. polymorpha*, used for the detection of ethanol/methanol (Gonchar et al., 1998). A defect in the gene responsible for catalase synthesis, forced the cells to develop the mechanism of hydrogen peroxide depletion through its extrusion from the cell. Thus, the excreted hydrogen peroxide, which is a product of catalytic oxidation of alcohol in the yeast cell, was used as an analytical signal for methanol detection.

In the present study, genetically-modified yeast cells of *H. polymorpha*, over-expressing the enzyme L-lactate:cytochrome *c*-oxidoreductase (FC b_2) were used for the construction of a mediator-free biosensor for L-lactate. The recombinant cells were previously used for the construction of amperometric biosensor with improved sensitivity to L-lactate. The developed biosensor was using permeabilised cells, and the free-diffusing mediator phenazine methosulfate was used for the electrical communication between FC b_2 and the electrode surface (Smutok et al., 2007). Simultaneously we report the detection of L-lactate based on the monitoring of L-lactate-dependent respiration of intact genetically-modified cells of *H. polymorpha* with an increased content of FC b_2 within the cells of the recombinant strain (Shkil et. al., 2009).

2.2.1 Construction of free-diffusing microbial amperometric sensor using permeabilized cells of flavocytochrome b_2 over-producing recombinant yeast *H. polymorpha*

Construction of the recombinant *H. polymorpha* strain over-producing FC b_2 included several stages. The recombinant plasmid pGLG61_CYB2, which is based on the plasmid pGLG61 for multicopy integration (Fig. 11A, b), was transformed to the recipient strain *H. polymorpha* C-105 (*gcr1 catX*) avoiding glucose repression and catalase activity. The transformants were grown on YPD medium in the presence of increasing concentrations of geneticin G418. The highest concentration of G418 which allows the transformants to grow was 1 mg ml⁻¹. The transformants were stabilized by cultivation in non-selective media for ten to twelve generations with further shifting to the selective media with G418. The presence of the expression cassette in the stable transformants was examined by PCR using corresponding primers and genomic DNA of stable transformants as a template. Fragments of predictable size (~ 3.3 kb) were obtained (data not shown).

The level of FC b_2 activity in cell-free extracts of the recombinant strain "tr1" was compared with the recipient strain *H. polymorpha* C-105. As shown in Fig. 11B, the FC b_2 activity in the cell-free extracts of the transformed strain "tr1" was about 3.2 U mg⁻¹ protein, while the recipient strain *H. polymorpha* C-105 under the same growth conditions had a much lower enzyme activity of only 0.6 U mg⁻¹ protein. The transformant showed 5.2 fold higher FC b_2 activity as compared to the recipient strain. After permeabilization the cells of this FC b_2 -over-producing strain were used as biorecognition element for the construction of a microbial L-lactate-selective amperometric biosensor.

In order to evaluate the developed L-lactate biosensor based on permeabilized cells, phenazine methosulphate (PMS) was used as a free-diffusing redox mediator for establishing the electron transfer between FC b_2 located at the mitochondria membrane and

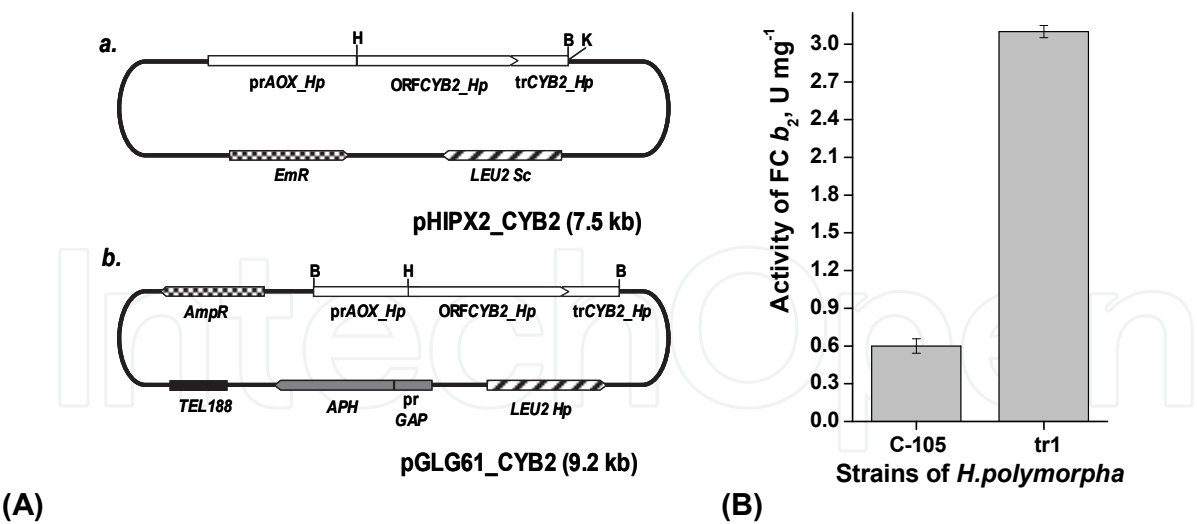


Fig. 11. (A) Circular schemes of the plasmids (a) pHIPX2_CYB2 (7.5 kb) and (b) pGLG61_CYB2 (9.2 kb). The *HpAOX* promoter and *CYB2* ORF with the terminator region are shown as open boxes. The *LEU2* genes of *S. cerevisiae* or *H. polymorpha* are shown as hatched boxes. Genes *EmR* and *AmpR* conferring resistance to erythromycin and ampicillin are shown as chequered boxes. The *H. polymorpha* truncated glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter and the geneticin resistance gene (*APH*) are shown as grey boxes, the tellomeric region (*TEL188*) as black box. Restriction sites: H - *Hind*III; B - *Bam*HI; K - *Kpn*I. (B) Comparison of FC *b*₂ activity in cell-free extracts of *H. polymorpha* C-105 (recipient strain) and of the recombinant strain “tr1”.

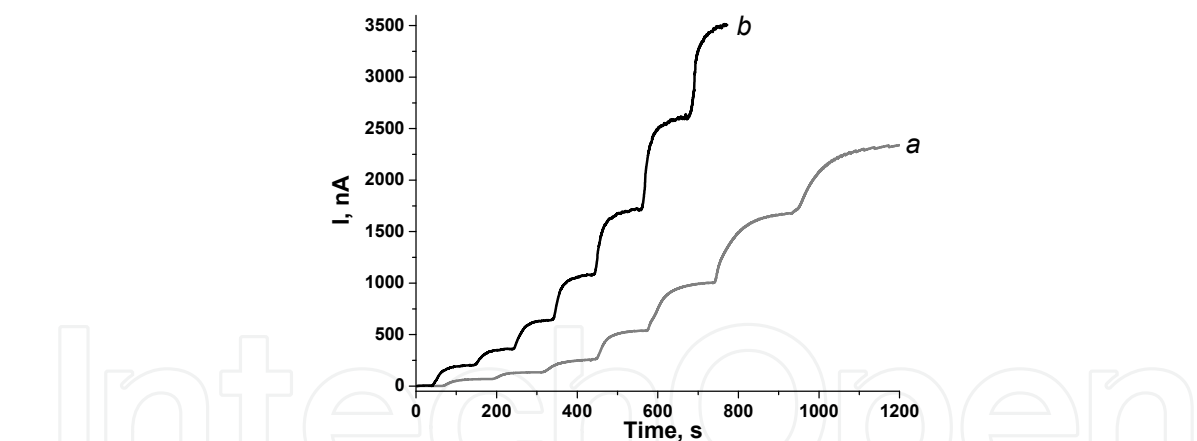


Fig. 12. Chronamperometric current response upon subsequent additions of L-lactate aliquots obtained with microbial sensors based on permeabilized cells of the recombinant strain of *H. polymorpha* (“tr1”). (a) Immobilization of cells by means of physical entrapment behind a dialysis membrane. (b) Entrapment of cells within a layer of a cathodic electrodeposition polymer CP9. (3.05 mm diameter graphite disk electrode; 0 mV vs. Ag/AgCl).

the electrode surface. It was supposed that PMS can easily diffuse into the permeabilized cell and back to the electrode. 3.05 mm graphite rod electrodes at a potential of 0.0 mV were chosen for PMS oxidation (Garjonyte et al., 2006). Since it has been shown previously that isolated FC *b*₂ exhibits only a limited stability (Smutok et al., 2005), it was suggested that the application of intact cells with the inherent ability to keep the enzyme protected in a membrane-bound state would significantly improve the properties of the sensors.

Two different immobilisation methods were evaluated, namely, the physical fixation of permeabilized cells behind a dialysis membrane and the entrapment of the cells within an electrodeposition paint layer which is formed in the presence of the cells. A typical sensor response for both sensor architectures is shown in Fig. 12. The output of the sensor prepared by electrochemically induced precipitation of the cathodic electrodeposition polymer CP9 under simultaneous entrapment of the permeabilized cells within the hydrogel layer exhibits a higher current response upon addition of L-lactate. In addition, due to the favourable diffusion of the substrate and the mediator to the polymer entrapped cells these sensors showed a faster response time. The rate of the sensors' response to 0.1 mM L-lactate is about 190 nA min^{-1} for the polymer entrapment system and 60 nA min^{-1} for the system with a dialysis membrane, respectively.

To evaluate the impact of the genetic modifications performed on the bioanalytical characteristics of the cells, L-lactate sensors based on either permeabilized cells of the recombinant strain "tr1" or cells of the recipient strain "C-105" entrapped within the cathodic electrodeposition paint were compared. Since the difference between both strains is determined mainly by the FC b_2 content, it was expected that the current response of the sensor based on the genetically engineered cells was significantly enhanced.

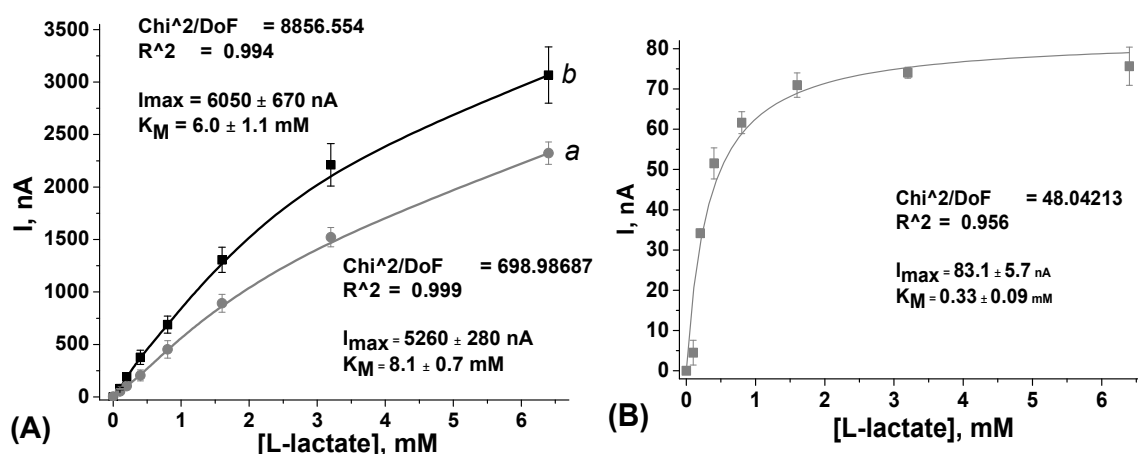


Fig. 13. (A) L-lactate calibration graphs for two types of sensors modified by genetically engineered *H. polymorpha* "tr1" cells (a - physical entrapment behind a dialysis membrane; b - electrochemically induced entrapment within a cathodic electrodeposition polymer CP9); (B) L-lactate calibration graph for sensor modified by *H. polymorpha* "C-105" cells by physical entrapment behind a dialysis membrane.

As clearly shown in Figure 13, a significantly increased current response is observed upon L-lactate addition for the sensors based on the genetically engineered cells "tr1" as compared with the wild-type strain "C-105". The maximal current I_{max} at substrate saturation for the "C-105" cells-based sensor was $83.1 \pm 5.7 \text{ nA}$, while the "tr1" cell-based electrode showed an I_{max} of $5260 \pm 280 \text{ nA}$ under the same conditions. These results indicate that the maximum contribution of other L-lactate oxidizing enzymes within the cells, which should be similarly present in both the "tr1" and "C105" cells, does not exceed 80 nA at substrate saturation. This value represents only about 1.5 % of the maximum sensor output of 5260 nA. It could be hence concluded that most of the contribution to the sensor current originates from the added FC b_2 activity of the recombinant strain.

In addition, sensors based on genetically engineered “tr1” cells exhibit a 20 to 25 times higher K_M value irrespective of the immobilization method. The calculated values for K_M^{app} derived from the calibration plots are 0.33 ± 0.09 mM for sensors based on “C-105” cells and 8.0 ± 0.66 mM for “tr1” cells. To investigate whether the K_M values of the cell-based sensors depend on the differences in properties of FC b_2 itself or are dominated by the significantly higher enzyme loading in the case of the “tr1” cells, a L-lactate sensor based on FC b_2 isolated from “tr1” cells was prepared using physical entrapment of the enzyme behind a dialysis membrane for enzyme immobilization (Fig. 14).

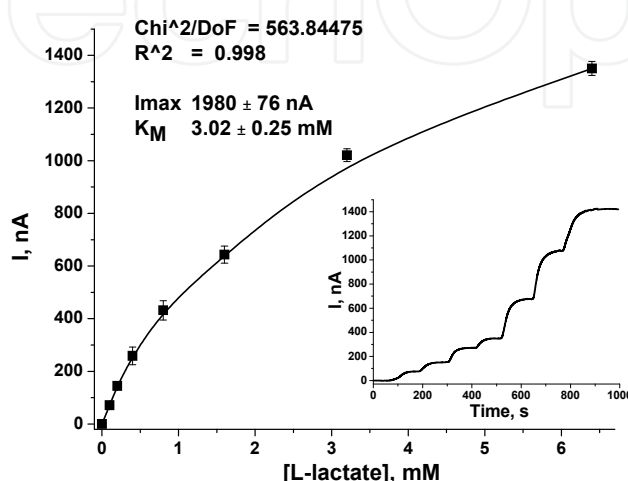


Fig. 14. L-lactate calibration curve and chronoamperogram (insert) of a biosensor based on isolated FC b_2 isolated from recombinant “tr1” cells.

The K_M^{app} of FC b_2 purified from recombinant “tr1” cells was determined from the calibration graph as 3.02 ± 0.25 mM (toward L-lactate) which is about 10 times higher than the K_M^{app} value of the sensor using permeabilized “C-105” cells. Obviously, restriction of the diffusion mass transfer through the permeabilized cells cannot be the reason for the observed increase in the K_M^{app} value. Hence, the structure of FC b_2 produced by the recombinant cells must be modulated, leading to a significantly decreased affinity of the active site of the enzyme for complementary binding of L-lactate. This may be due to errors caused by the *Taq* DNA-polymerase *in vitro*, which may lead to minor changes in the primary structure of the PCR product and consequently may result in amino acid substitutions in the FC b_2 molecule. The observed increased K_M^{app} value for recombinant cells and the enzyme isolated from these cells had a positive impact on the biosensor properties due to the increase of the linear range for L-lactate determination, up to 1.6 mM. The increased linear detection range is better adapted to the typical concentration range of L-lactate in real samples, thus avoiding dilution steps. The detection limit of the sensor is low (about 6 μ M L-lactate). The optimal pH-value for the developed L-lactate biosensor based on the recombinant cells is in the range of 7.5 to 8.2, with an optimal temperature between 36 and 42 $^{\circ}$ C.

For the application of the L-lactate biosensor in real samples, the selectivity with respect to potential substrates/interferents such as D-lactate, pyruvate, succinate, L-malate, citrate, α -ketoglutarate, urate, D-glucose and ethanol is of great importance. Hence, the amperometric current response of the cell-based L-lactate sensor was evaluated with respect to the above mentioned compounds (Fig. 15).

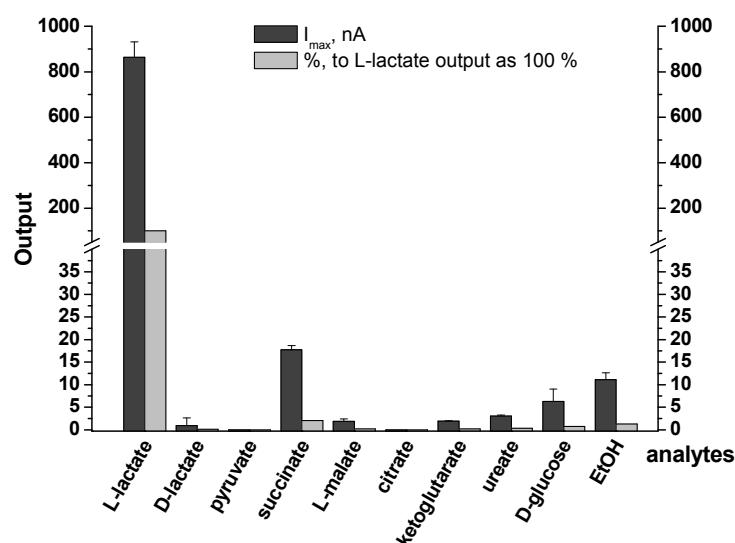


Fig. 15. Selectivity of the L-lactate biosensor (863 nA; 100 %) based on electrochemically immobilized *H. polymorpha* "tr1" cells. The current and the relative response of 1 mM solutions of D-lactate (0.92 nA; 0.1 %), pyruvate (0.0 nA; 0 %), succinate (17.7 nA; 2 %), L-malate (1.88 nA; 0.22 %), citrate (0.0 nA; 0 %), α -ketoglutarate (1.9 nA; 0.22 %), urate (3.07 nA; 0.36 %), D-glucose (6.29 nA; 0.73 %), and ethanol (11.1 nA; 1.29 %) are displayed.

The cell-based biosensor exhibits minor cross-sensitivity to most of the tested compounds. With the exception of succinate, D-glucose and ethanol the relative cross-sensitivity is below 1 %. Thus, the impact of these compounds will be insignificant for the determination of L-lactate in the real samples where the above mentioned components may be present.

The operational and storage stabilities of the L-lactate sensor were investigated. Biosensors were prepared following the optimal preparation protocol for both immobilization techniques of the permeabilized "tr1" cells. The stability tests were performed at a constant temperature of 24 °C using 1 mM solutions of L-lactate in 50 mM phosphate buffer, pH 7.8, for the investigation of the operational stability, and a 3 mM L-lactate solution - for the evaluation of the storage stability. The operational stability was evaluated using an automatic sequential injection analyzer (W. Schuhmann et al., 1995). The sensors were integrated into a flow-through electrochemical cell, and 15 injections of 500 μ l of a 1 mM L-lactate standard solution per hour were performed automatically. Fig. 16 shows the peak currents of the different L-lactate sensors upon sample injection over a period of about 24 hours (approximately 360 individual measurements).

Both types of cell-based sensors demonstrated a linear decay of the current response during continuous operation over 24 hours (360 injections of 1 mM L-lactate solutions). In another experiment the peak currents decayed to 50 % of the initial sensor output after about 26-30 hours of continuous operation. Despite the continuous decrease of the sensor output the developed biosensors exhibited satisfactory operational stability for L-lactate determination in model samples, especially when integrated in the sequential injection analyser that allows for a repetitive recalibration.

Next, the storage stability of the different sensor configurations was tested. For this, the sensors were stored between amperometric L-lactate determinations at + 4°C (Table 1). The measurements were performed at room temperature in 50 mM phosphate buffer, pH 7.8,

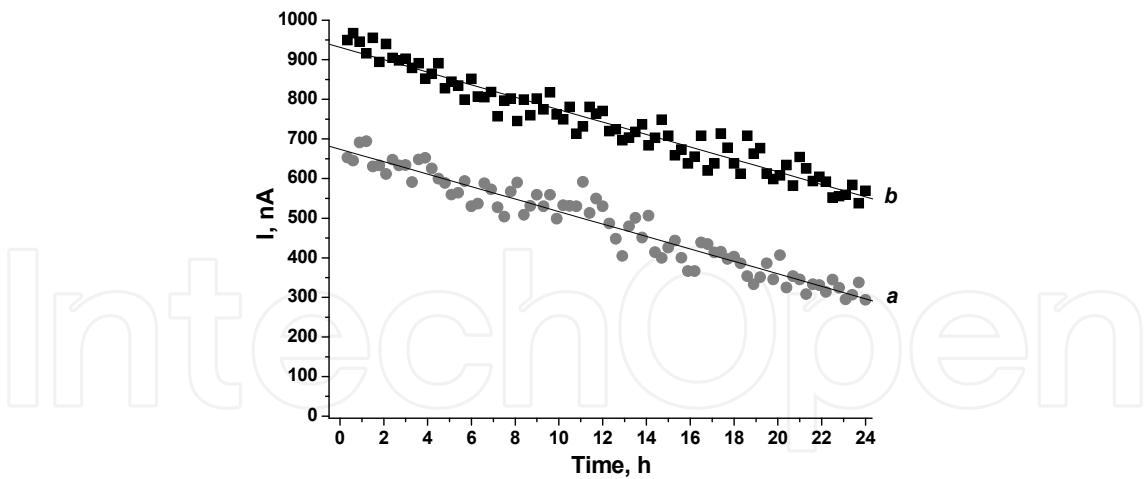


Fig. 16. Operational stability of the *H. polymorpha* “tr1” cells based L-lactate biosensors tested in an automatic sequential injection analyser (flow-rate 5 ml min⁻¹; sample injection every 4 min). (a) permeabilized cells physically entrapped behind a dialysis membrane; (b) permeabilized cells entrapped within a cathodic electrodeposited polymer.

Remaining response, % from the initial output	Time of storage, days									
	0	2	4	6	8	10	12	14	24	40
Physical entrapment behind a dialysis membrane	100	109	103	98.6	93.8	90.7	86.2	83	76.8	----
Entrapment within a cathodic electrodeposition polymer	100	99.8	98.3	98.8	97	94.4	90.5	89.3	80.1	67.2

Table 1. Storage stability of the recombinant cells-based biosensors

containing 0.2 mM PMS and 3 mM L-lactate. Both types of sensors proved to be stable for more than 24 days under storage conditions. After one day of storage in buffer solution, the detected signal of dialysis membrane-based sensor was increased by about 10 % over the initial response. This effect is often observed for amperometric biosensors and can be explained by the equilibration of sensor architecture and, especially, by a final swelling of the dialysis membrane facilitating diffusional access of the substrate to the immobilized cells. After about 24 days of storage, sensors of this type failed due to damage of the dialysis membrane. The sensors based on entrapment of the permeabilized cells within a cathodic electrodeposition polymer could be used for more than 40 days, with intermittent storage in buffer in the refrigerator.

The significantly improved stability of the developed L-lactate sensors as compared with similar sensors using the isolated enzyme (Smutok et al., 2005) may be accounted for by the use of the thermotolerant yeast *H. polymorpha* for the genetic modification and overexpression of FC *b*₂. Moreover, during cell permeabilization and the following washing steps, low molecular weight proteinases may be at least partly removed from the cells, thus

preserving FC b_2 activity. In addition, the overexpressed holoenzyme FC b_2 may be stabilized in its native environment in the mitochondria membrane.

2.2.2 Mediator-less L-lactate-selective microbial sensor based on modified *H. polymorpha* yeast cells overexpressing flavocytochrome b_2

A typical approach to target redox enzymes of bacterial or yeast cells involves the addition of a shuttle molecule, called a mediator that is recycled between the electrode and the target enzyme inside the cells. However this method requires the permeabilization of the cell membrane to facilitate the diffusion process. The usage of such soluble mediators restricts the practical application of the biosensor.

Another way to monitor the cells metabolism and specific internal processes is based on examining cell respiration, performed using an oxygen electrode, typically a Pt electrode (polarized at -600 mV vs. Ag/AgCl), a technique known to be used in first-generation biosensors. However, this method has serious drawbacks, mainly, the limited availability of soluble oxygen and an inverse relationship between the concentration of the analyte and the sensor response. Moreover, the involvement of the oxygen consumption processes within the cells (mainly through the respiratory chain) limits the selectivity of the method in detecting the analyte of interest, since there are multiple pathways that might be used to pump electrons through Complex IV, the site where the oxygen reduction process takes place. In the present study, some of the above mentioned drawbacks are overcome by specific approaches: i) using a potential of -300 mV; ii) over-expression of FC b_2 for increasing the selectivity; iii) using yeast cells instead of bacterial cells as done previously (Kalab & Skladal, 1994; Luong et al., 1989; Plegge et al., 2000; Adamowicz & Burstein, 1987) for the construction of biosensors for L-lactate detection which improves the tolerance to external conditions.

Since the method of L-lactate detection presented herein is based on the competition for oxygen consumption between a polarized electrode and the respiration process in yeast cells, the reaction at the electrode equally influences the sensor performance. Therefore, an evaluation of the oxygen reduction reaction using various types of electrode materials, such as gold, platinum and graphite was considered. Additionally, the biocompatibility of yeast cells with a specific electrode material has to be taken into consideration in order to provide good stability and reproducibility of the sensor.

The sensor's response in dependence from the applied electrode potential was evaluated in the presence and absence of L-lactate, as shown in Fig. 17.

The three different electrode materials show specific particularities in terms of on-set potential and reaction rate for oxygen reduction, as well as the sensitivity of L-lactate detection. The platinum electrode exhibits a high on-set potential for oxygen reduction, -200 mV, which is seen as a drawback due to the generation of high background noise. On the other hand, both graphite and gold electrodes exhibit on-set potentials of -100 mV, thus limiting the contribution of possible interferences to the overall reduction current. Despite this decrease in background current, the gold electrode, upon addition of L-lactate, leads to a minimum variation of the sensor response, meaning it rates poorly for sensitivity. The graphite electrode, in addition to its high sensitivity to variation in oxygen concentration, represented in Fig. 17 by the differences between opened and filled symbols (see corresponding arrows), is additionally preferred over other electrodes due to its unspecific reduction of oxygen, whereas the gold electrode is known to reduce oxygen mainly to H_2O_2 ,

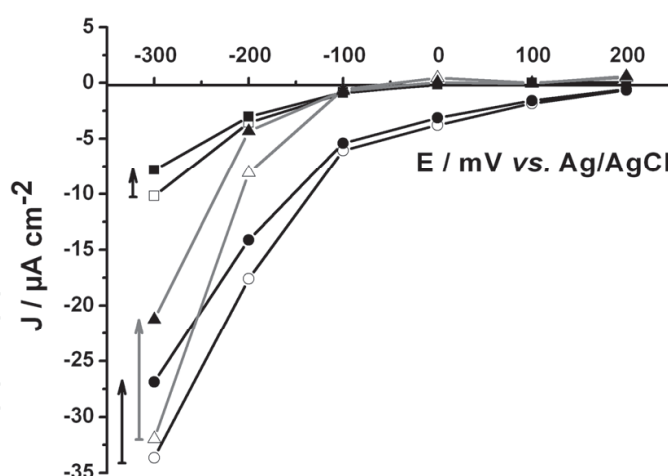


Fig. 17. Hydrodynamic voltammograms presenting the dependence of the sensor response for different electrode materials (gold - square, platinum - circles and graphite - triangles) modified with *H. polymorpha* "tr1" cells, recorded in the presence (filled symbols) and in the absence (opened-symbols) of L-lactate.

and Pt efficiently reduces oxygen to H_2O , however at the expense of a much lower reduction potential, usually at around -600 mV. Tuning the competition between electrode and yeast cells by varying the applied potential leads finally to improved selectivity and sensitivity, with an emphasis on the decrease of signal interference. Thus, as a compromise between background noise, sensor sensitivity and biocompatibility of yeast cells to the electrode material and their tolerance to exposure to products of oxygen reduction, a graphite electrode at an applied potential of -300 mV was selected for further study.

To demonstrate the novelty of the present biosensor for L-lactate, two sensors were built, one based on the wild-type strain 356, and the other based on the recombinant strain "tr1". The resulting biosensor performances are compared and presented in Fig. 18.

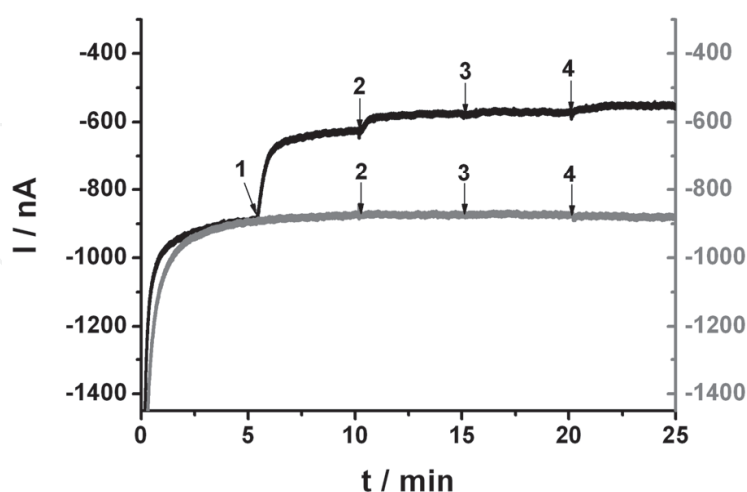


Fig. 18. Chronoamperometric response (in current density values) upon consecutive additions of L-lactate for two biosensors based on wild type (black line) and recombinant (grey line) *H. polymorpha* cells. Working electrode (graphite) polarized at -300 mV vs. Ag/AgCl 3M KCl. Additions 1-4, respectively: 1; 2.5; 5; 10 mM L-lactate.

As can be seen, the addition of L-lactate to the sensor based on the wild-type cells does not induce any effect on the level of oxygen concentration. Only the genetically modified yeast cells, overexpressing the FC b_2 , produce a drastic change in the cell respiratory process, and thus L-lactate respiration contributes significantly to the depletion of oxygen at the electrode surface, seen here as a drop of the oxygen reduction current, proportional to the concentration of L-lactate (Fig. 18). The overexpression of FC b_2 does not only result in a tremendous increase in sensitivity to L-lactate (250 nA mM⁻¹ L-lactate), but also in the modulation of the selectivity of the sensor due to the specificity of oxygen consumption of the cell almost exclusively *via* L-lactate respiration. The addition of any other cell nutrients (e.g. glucose, etc.) does not have any effect on the background current.

Thus, by performing genetic modification, it is possible not only to inducing the synthesis of a higher amount (five times higher) of the target enzyme FC b_2 , but also to influencing the metabolic pathways, in which this enzyme is involved. That makes the application of genetically modified yeast cells advantageous for L-lactate detection over wild-type cells.

3. Development of a reagentless bienzyme amperometric biosensor based on alcohol oxidase/oxidase and Os-complex modified electrodeposition paints

Determination of ethanol is important in food technology, fermentation and wine industries, as well as in clinical chemistry (Alexander et al., 1998; Esti et al., 2003; Watson et al., 1998). Classical methods such as refractometry (Pen'kovskii et al., 2004) or densitometry (Bavcar & Kosmerl, 2003) of the distilled samples, as well as gas chromatography (Schmitt & Aderjan, 2004) which are routinely used in in clinics and industry are time- and labour-consuming procedures. In order to replace these methods, a variety of enzymatic and biosensor approaches were developed based on alcohol-specific enzymes isolated from different microbial species: NAD⁺-dependent alcohol dehydrogenase (Ivanova et al., 2003; Gautier et al., 1990), quinoxinoprotein alcohol dehydrogenase (Niculescu et al., 2002), or alcohol oxidase (Gibson et al., 1992; Gonchar et al., 2001, 2002; Patel et al., 2001) were proposed and applied for the determination of ethanol in complex samples. Alcohol biosensors based on NAD⁺-dependent alcohol dehydrogenase (ADH) usually exhibit high selectivity, however, due to the need to add the coenzyme NAD⁺ to the sample, ethanol determination is quite expensive using these types of biosensors. Even more important, ADH-based sensors showed limited operational stability (Rebelo et al., 1994).

Alternatively, alcohol oxidase (AOX), a flavoprotein, was used for the construction of alcohol biosensors. AOX exhibits an inherent non-selectivity since other primary alcohols as well as hydrated formaldehyde are enzymatically converted by this enzyme. For some real samples, this property is not crucial, because of the high abundance of a single analyte, e.g. ethanol in wine (Esti et al., 2003; Niculescu et al., 2002; Hasunuma et al., 2004). However, AOX based sensors suffer also from a limited stability and from the rather high working potential (+600 mV vs Ag/AgCl) which has to be applied for the oxidation of enzymatically-generated H₂O₂. In order to overcome these limitations, bi-enzyme sensors coupling a H₂O₂ generating oxidase reaction with the peroxidase-catalyzed H₂O₂ reduction were proposed (Castillo et al., 2003; de Prada et al., 2003). The low working potential (-50 mV vs Ag/AgCl) and the possibility to wire the peroxidase via suitable redox polymers to the electrode surface in order to provoke fast electron transfer make bi-enzyme sensor architectures a target of research (Castillo et al., 2003; de Prada et al., 2003; Vijayakumar et al., 1996). However, the main problem remains the inherent instability of AOX which often leads to a dramatic

decrease of activity during the immobilization process. Although the presence of stabilizers was shown to partially overcome this problem (Esti et al., 2003), there is still a need for the development of improved alcohol sensors with significantly increased operational stability.

3.1 Development of a reagentless bienzyme amperometric biosensor based on alcohol oxidase/peroxidase and Os-complex modified electrodeposition paints

The development of an alcohol bi-enzyme biosensor is reported which is based on AOX isolated from a genetically constructed mutant strain *Hansenula polymorpha* C-105 (Smutok et al., 2006b) or mutated AOX with decreased affinity toward substrates (Dmytruk et al., 2007) coupled with commercial peroxidase (HRP). For immobilization of the enzymes, a non-manual electrochemically-induced precipitation procedure was applied using a new type of Os-complex modified electrodeposition paints (EDP) for the immobilization of horseradish peroxidase in a first layer and a cathodic EDP for the immobilization and stabilization of AOX in a second layer. The used redox EDP assures fast electron transfer between the integrated peroxidase and the electrode surface at a low working potential.

The electrochemical communication between immobilized enzymes and redox polymers was analyzed before sensor construction. There is no evidence that artificial electron acceptors other than O_2 are able to re-oxidize the reduced form of AOX in solution (Geissler et al., 1986), and hence AOX activity is strongly dependent on the availability of dissolved molecular oxygen. Nevertheless, possibilities for direct electron transfer between adsorbed AOX and the electrode surface as well as wiring of the enzyme by means of the Os-complex EDP were investigated. Formation of reduced AOX was induced by ethanol addition under strictly anaerobic conditions (after saturation of the system by argon for at least 1 hour). However, in accordance with previous results, neither direct electron transfer nor an Os-complex mediated wiring of the enzyme was observed for reduced AOX. Most probably, due to the complicated octameric structure of the enzyme, the polymer-bound Os-complexes were not able to approach the enzyme-integrated $FADH_2$ moiety to allow for fast electron transfer kinetics.

The integration of AOX into films of EDP was evaluated with respect to possible inactivation of the enzyme. Anodic deposition paints (AP) are precipitated on the electrode surface by applying potential pulses of up to +2200 mV (vs. Ag/AgCl), leading to the generation of protons in the diffusion zone in front of the electrode. After electrochemical-induced precipitation of the redox polymer AP-Os (anodic paint covalently linked with osmium-complexes) in the presence of AOX at such high potentials, no residual immobilized enzyme activity could be observed. Apparently, since AOX is unstable at pH-values below 6.5, the enzyme was denatured due to local proton generation. On the other hand, as suggested by the properties of AOX in solution (Woodward, 1990; Patel et al., 2001), AOX should be resistant to alkaline pH-values. Thus, integration of AOX into a cathodic EDP (CP) was performed using potential pulses to -1200 mV (vs. Ag/AgCl), leading to the generation of OH^- -ions close to the electrode surface. No detectable deterioration of AOX activity was observed using this immobilization procedure.

In addition, due to the fact that high H_2O_2 concentrations limit AOX stability, it is essential that enzymatically generated H_2O_2 is removed as fast as possible. Thus, we have designed a bi-enzyme sensor architecture using a first layer containing horseradish peroxidase (HRP) integrated within an Os-complex modified EDP film (AP-Os) enabling fast electron transfer between the oxidized active site of HRP and the electrode surface, concomitantly decreasing AOX inactivation by high local H_2O_2 concentrations. On top of this layer, a second layer was

precipitated, consisting of AOX integrated within an electrochemically precipitated CP9 layer. Alternatively, a mixture of HRP and AOX was integrated within the second layer. Thus, ethanol oxidation by means of AOX in the outer layer is coupled with the peroxidative oxidation of Os-complexes bound to the backbone of an anodic paint, followed by the final reduction of the mediator at the working electrode surface. The sensor architecture and the anticipated electron-transfer pathway are shown in Fig.19.

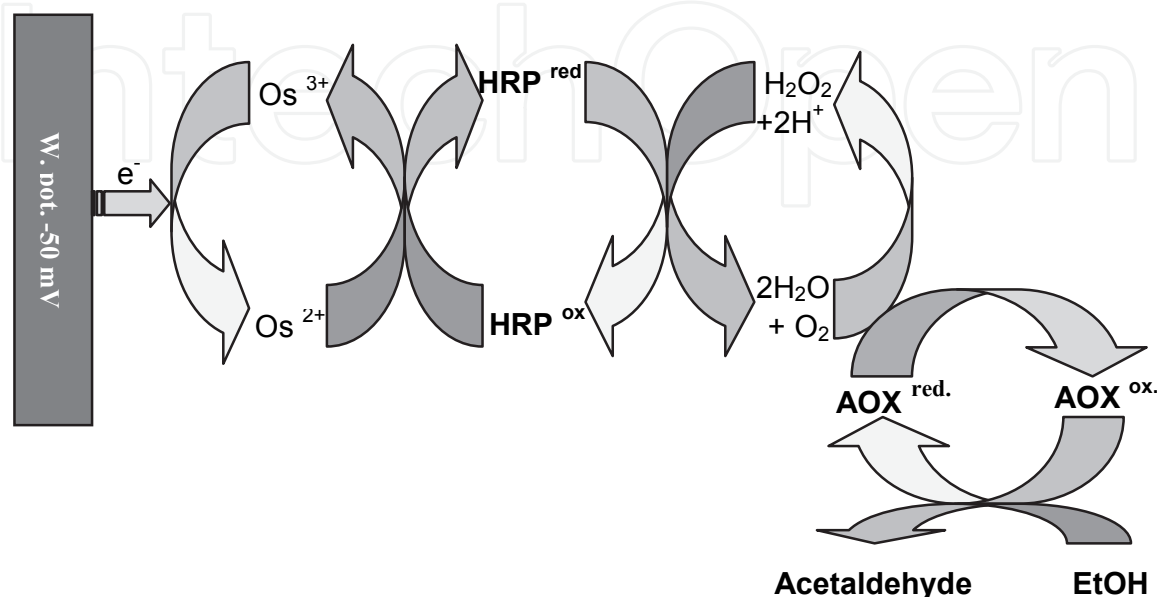


Fig. 19. Reaction scheme and electron-transfer pathway from ethanol *via* AOX, enzymatically generated H₂O₂, HRP, and polymer-bound Os-relays to the electrode.

The possibility of HRP to productively accept electrons from the Os-complexes coordinatively attached to the backbone of the anodic paint (AP-Os) was evaluated by means of cyclic voltammetry (Fig. 20).

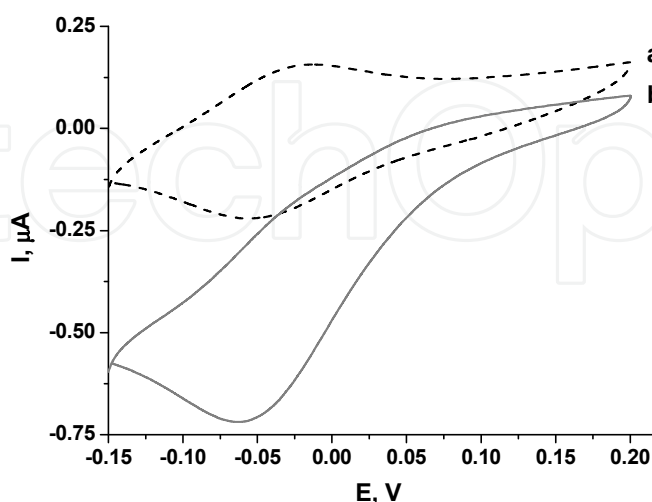


Fig. 20. Cyclic voltammogram at a graphite electrode modified with a layer containing HRP-AP-Os in the absence (a) and in the presence (b) of 1 mM H₂O₂. Potentials *vs.* SCE: -150 mV to -200 mV; scan rate 7 mV/s in 100 mM phosphate buffer, pH 7.6.

In the presence of H_2O_2 , the reduction peak at a potential of about -50 mV *vs* SCE clearly reveals the accessibility of the heme site within HRP by the polymer-bound redox relays and the electron exchange with AP-Os.

Different electrode materials were investigated in combination with the optimal bi-enzyme sensor architecture (Fig. 21).

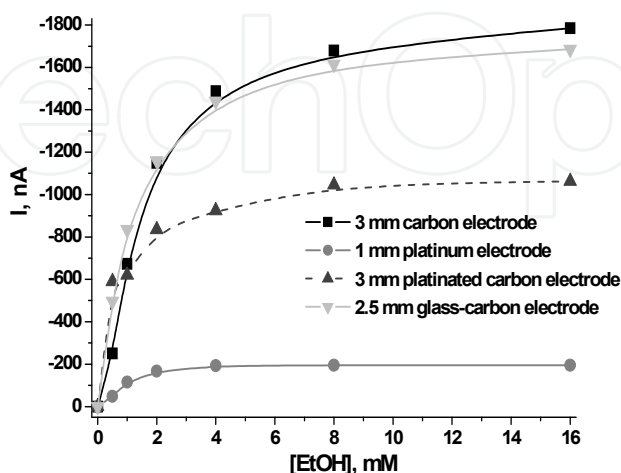


Fig. 21. Ethanol calibration graphs at HRP-AP-Os//AOX-CP9 bi-enzyme sensors on different electrode materials.

The current densities calculated by dividing the maximal current at substrate saturation by the geometric electrode area were 244.5 nA mm^{-2} for graphite electrodes, 248.6 nA mm^{-2} for Pt electrodes; 145.5 nA mm^{-2} for electrochemically platinized graphite electrodes, and 343.6 nA mm^{-2} for glassy carbon electrodes (Fig. 21). Most probably, the platinization prevents good contact between the redox EDP or the HRP with the electrode surface, thus leading to a somewhat decreased current density. Since the current density at the Pt surface is similar to the one at the graphite surface, it can be assumed that the majority of electron transfer occurs *via* the polymer-bound Os centers, with only minor contributions by direct electron transfer from the surface adsorbed HRP.

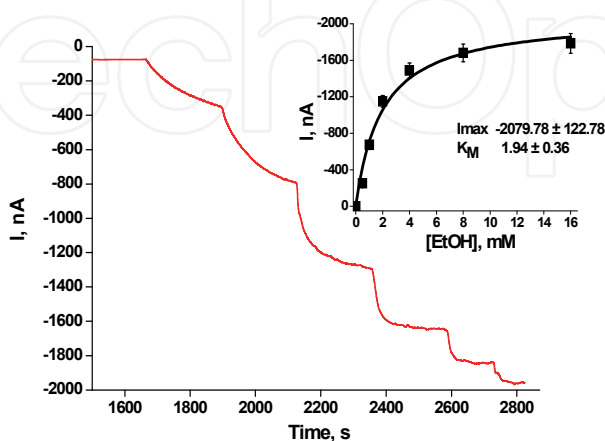


Fig. 22. Chronamperometric determination of ethanol using a HRP-AP-Os//AOX-CP bi-enzyme sensors and the derived calibration graph (graphite electrode; -50 mV *vs* Ag/AgCl).

The apparent Michaelis-Menten constant K_M^{app} derived from the calibration curve shown in Fig. 22 is 1.94 ± 0.37 mM for EtOH, a value which is in good agreement with the value of 2.7 mM for AOX in solution (Woodward et al., 1990). The typical signal rise time (95 % of the steady state value at an ethanol concentration of 4 mM) was determined to be 45 s. The optimal working pH value in phosphate buffer was determined to be 7.6.

The selectivity of the *HRP-Ap-Os//AOX-CP* bi-enzyme sensor was evaluated by determination of the concentration-dependent current response curves for different analytes, which are structurally similar to ethanol. The related calibration graphs for primary alcohols as well as formaldehyde are shown in Fig. 23.

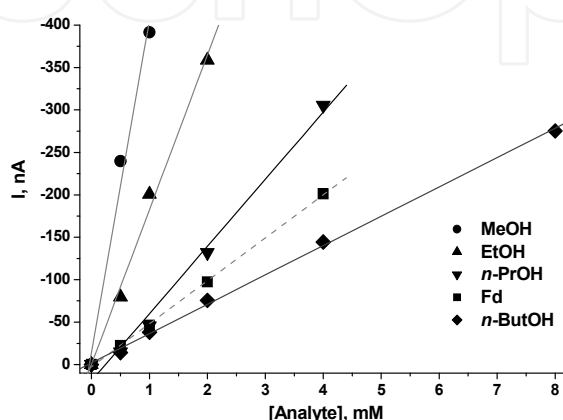


Fig. 23. Linear dynamic range of the response of *HRP-Ap-Os//AOX-CP* bi-enzyme sensors to primary alcohols and formaldehyde (graphite electrode; -50 mV vs Ag/AgCl).

The sensitivity of the sensor to the different analytes represents the affinity of AOX in solution, with the highest sensitivity for methanol ($0.48 \mu\text{A} \cdot \text{mM}^{-1}$) and ethanol ($0.2 \mu\text{A} \cdot \text{mM}^{-1}$). This sensitivity is significantly higher than that reported earlier for other AOX-based amperometric sensors, which were in the range from 0.006 to $0.03 \mu\text{A} \cdot \text{mM}^{-1}$ (Patel et al., 2001). The linear dynamic range was found to be about 1 mM for methanol, 2 mM for ethanol, 4 mM for *n*-propanol and formaldehyde, and 8 mM for *n*-butanol.

As pointed out above, the main challenge in the development of alcohol biosensors is the limited operational and storage stability of the previously reported sensors. A significant improvement in the stability of the newly constructed alcohol sensor was expected due to the use of AOX from a genetically constructed mutant strain of the thermotolerant yeast *H. polymorpha* and the fast removal of enzymatically produced H_2O_2 . The operational and storage stability of the *HRP-Ap-Os//AOX-CP* bi-enzyme sensor prepared under optimal conditions was tested at 24°C . The operational stability was evaluated using a previously described automatic sequential injection analyzer (Schuhmann et al., 1995) repetitively injecting a 2 mM ethanol solution. More than 1000 injections were performed, and the obtained current peak heights were plotted against the analysis time (Fig. 24).

For the *HRP-Ap-Os//AOX-CP9* bi-enzyme sensor, the current decreased from an initial peak height of 900 nA to 540 nA after 51 h. The inactivation constant of the sensor was $6.1 \times 10^{-4} \mu\text{A} \cdot \text{min}^{-1}$.

The storage stability of the constructed biosensor was detected by repeated sensor tests with the sensor being stored in buffer solution at 4°C . The biosensor was stable for more than 16 days, however after about 14 days of storage there was a 50 % drop in current output. The

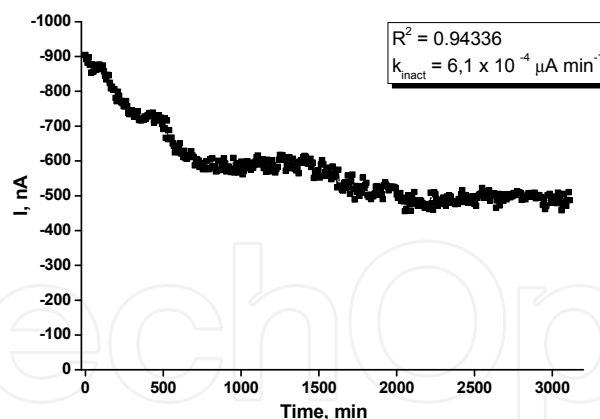


Fig. 24. Operation stability of alcohol bi-enzyme sensors evaluated by means of an automatic sequential injection analysis system.

significantly improved stability of the developed bi-enzyme sensor can be explained by their generic “sandwich” architecture using anodic and cathodic EDPs incorporating different enzymes according to their specific properties, namely, pH stability under electroprecipitation conditions.

3.2 Development an amperometric biosensor based on mutated alcohol oxidases from the yeast *H. polymorpha* with decreased affinity toward substrates

Yeast alcohol oxidase (AOX; alcohol:O₂ oxidoreductase, EC 1.1.3.13) has been extensively used for the determination of lower primary alcohols and formaldehyde (Alpeeva et al., 2005; Shkotova et. al., 2006). Significant progress in the selection of optimal transducers, and the immobilization and stabilization of the enzyme has been achieved. However, the affinity of AOX to ethanol is rather high, and direct AOX-based measurements of a target analyte, e.g. ethanol in real samples of wines, beers or fermentation cultures, is complicated and economically disadvantageous due to the necessity of diluting the samples. This is especially important in automatic electrochemical devices such as biosensors, since the additional step of sample dilution significantly increases the cost of analysis. In order to overcome this inconvenience, obtaining modified forms of AOX with a decreased affinity towards substrates and a wide range of linear responses is highly desirable.

Here, we describe the selection of methylotrophic yeast *H. polymorpha* mutants which produce a modified AOX with decreased affinity towards substrates, and the construction of an amperometric biosensor with a broadened linear range towards primary alcohols and aldehydes.

To isolate the mutant forms of AOX (mAOX) having decreased affinity to substrates, a positive selection procedure was developed. Allyl alcohol was used as the selective agent in the medium, with methanol as the sole carbon and energy source. The rationale for the selection scheme was as follows: since the oxidation of allyl alcohol by AOX is accompanied by the formation of the highly toxic molecule acrolein, in medium of methanol (0.5 %) and allyl alcohol (minimal inhibitory concentration was found to be 0.3 mM), mutants lacking AOX could not survive. Only mutants producing lower amounts of acrolein and enough formaldehyde for growth will survive and could be selected. One of the reasons for such an event would be the decreased affinity of AOX toward substrates while retaining a high enough reaction rate. This selection scheme would produce few or no mutants with a decreased rate of AOX reaction, as they would not grow efficiently due to the negligible

amount of formaldehyde produced. Altogether, 125 mutants of the *H. polymorpha* strain DL-1-356 that were able to grow in allyl alcohol containing medium were chosen for further selection. Mutants with decreased enzyme affinity towards ethanol were screened by a plate colony assay for AOX activity. Finally, two positive colonies (strains CA2 and CA4) which stained in the presence of 15 mM ethanol, but not 5 mM or 10 mM ethanol, were selected. The kinetic parameters of AOX from the selected mutant strains were evaluated. K_M values for methanol from the parental strain and the CA2 and CA4 mutants were calculated as 0.62, 2.48, and 1.10 mM, respectively (Table 2). At the same time, the values of V_{max} (recalculated per mg of protein) for AOX from the aforementioned strains were 27.4, 66.7 and 31.3 μmol of atomic O $\text{min}^{-1} \text{mg}^{-1}$ at 20 °C, respectively (Table 2). Thus, allyl alcohol/methanol-based selection is suitable for obtaining *H. polymorpha* mutants with decreased AOX affinity toward the substrate and without decreased maximal rate of the specific enzyme reaction.

<i>H. polymorpha</i> strain	K_M , mM	V_{max} , μmol of atomic O $\text{min}^{-1} \text{mg}^{-1}$ of protein at 20°C	Sigma factor, σ	Linear regression coefficient R for reciprocal plot
DL-1-356	0.62	27.4	0.084	0.992
CA2	2.48	66.7	0.040	0.958
CA4	1.10	31.3	0.130	0.990

Table 2. K_M and V_{max} values of AOX towards methanol for natural and mutant *H. polymorpha* strains

The construction of a bioselective layer of the sensor was performed according to the techniques described above. The structure of the bi-enzyme electrode was configured as *HRP-AP-Os//AOX(CA2)-CP* and included two layers: the inner, with horseradish peroxidase (*HRP*) electrochemically precipitated in the presence of a carboxylate-containing polymer modified with an osmium complex (*AP-Os*), and the outer, with mutated *mAOX(CA2)*, immobilized *via* cathodic precipitation in the presence of an amino-containing polymer (*CP*). Platinized graphite rods were used as working electrodes. The characterisation of the enzymatic

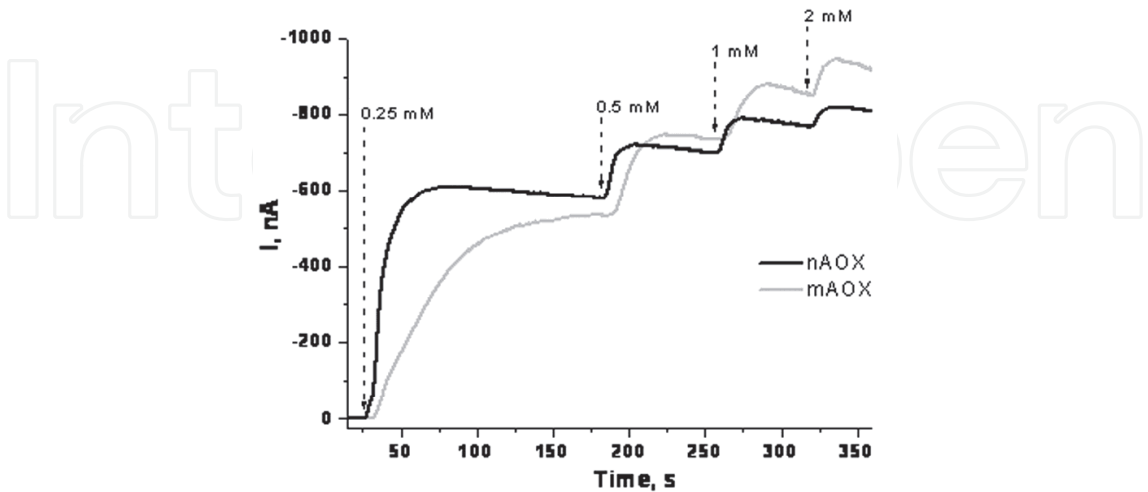


Fig. 25. Chronoamperometric assay of ethanol by *HRP/Os-Ap59//AOX/CP9* biosensors based on the natural (nAOX) and mutated (mAOX) forms of alcohol oxidase. Arrows indicate step-wise addition of ethanol in different concentrations.

properties of mAOX was performed using natural (wild type) nAOX as a reference. The typical dynamic ranges for AOX-modified sensors to ethanol are presented in Fig. 25.

In addition to alcohols (*e.g.*, ethanol or methanol), formaldehyde is also a substrate for AOX (Verduyn *et al.*, 1984). The calibration curves of the biosensors response to ethanol and formaldehyde clearly show the lower affinities toward both substrates of mAOX compared to wild-type enzyme (Fig. 26A).

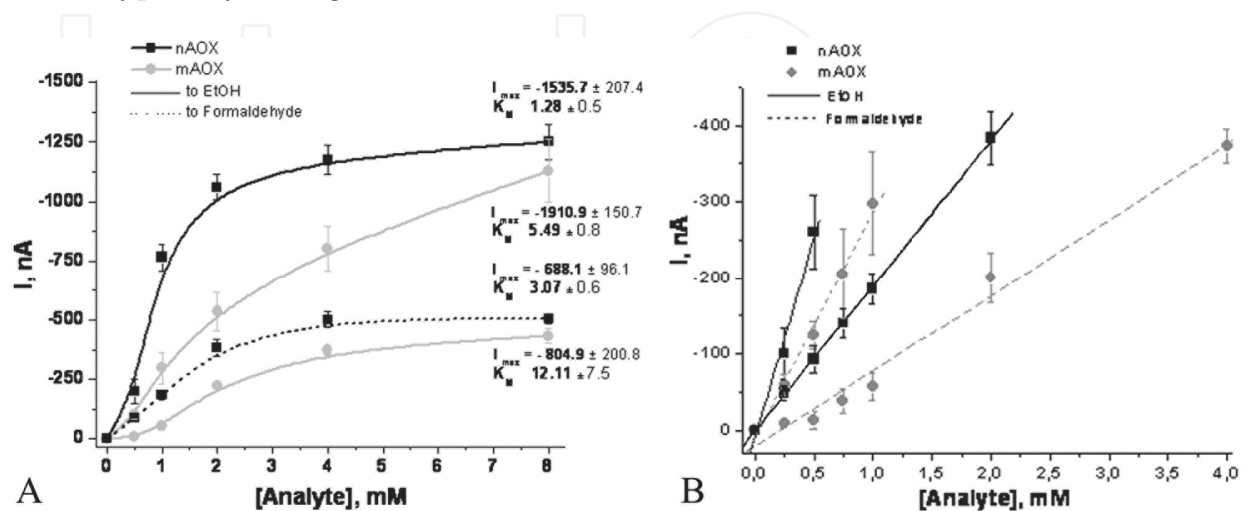


Fig. 26. Calibration curves and calculated K_M values of sensors based on the different forms of AOX toward ethanol (EtOH) and formaldehyde (A) and dynamic linear ranges of the corresponding sensors towards both analytes (B).

As previously mentioned, the natural and mutant forms of AOX differ in K_M value towards methanol in solution: the mutant enzyme possesses a 4-fold increased K_M value and thus, decreased substrate affinity (Table 2). The results obtained using amperometric sensors with immobilized AOXs show a good agreement with those obtained with AOXs in solution. The K_M values of a nAOX-based sensor were 1.28 mM for ethanol and 3.07 mM for formaldehyde, whereas these values for the mAOX-based electrode were increased to 5.40 mM for ethanol and 12.1 mM for formaldehyde. These results also show that mutant AOXs have a decreased affinity not only to the physiological substrate methanol, but also to other substrates, such as ethanol and formaldehyde. It is interesting to note that the I_{max} values for ethanol and formaldehyde of nAOX and mAOX-based electrodes were 1536/1911 nA and 688/805 nA, respectively. The I_{max} values of the sensors did not differ significantly (Fig. 26A). In addition, the linear dynamic range of the mAOX-based electrode for ethanol was 4 mM as compared to 0.5 mM for the nAOX-based electrodes (Fig. 26B).

The study of operational stability and inactivation kinetics of the constructed biosensors revealed that the sensors based on either mutant or natural (wild type) enzymes are very similar (Fig. 27).

Both sensors retained 50 % of their activity after 5 h of continuous operation in a sequential-injection analyzer. The biosensors were stable for more than 16 days, however, after 14 days of storage there was a 50 % drop in current output (data not shown). In conclusion, the constructed amperometric biosensor based on mAOX(CA2) was characterised by a decreased affinity towards the analyzed substrates without altered operational stability of the sensor.

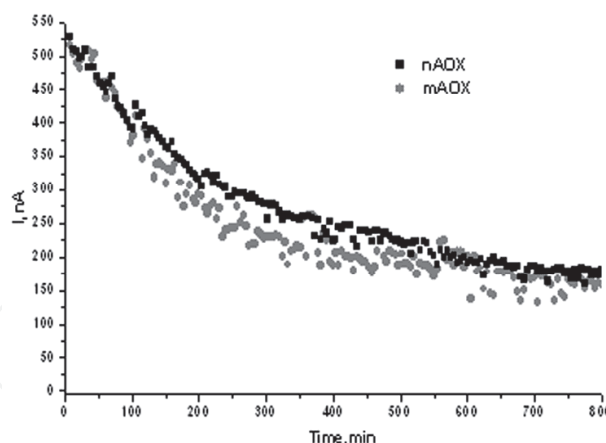


Fig. 27. Comparison of an operational stability of nAOX and mAOX-modified electrodes.

4. Amperometric biosensors for glycerol determination

Glycerol determination is important in clinical diagnostics for control of the triacylglycerides (TG) level in blood. The monitoring of these components has a prognostic value to drop the risks of diseases of the cardiovascular system. An elevated TG level in blood is a strong risk factor for arteriosclerosis. At high level of TG some malignancies, namely, coronary insufficiency and infarction occur more frequently. So, the development of a quick and reliably method for TG/glycerol determination is very important.

The known and used methods for TG determination are based on the enzymatic hydrolyses of TG by means of lipases/esterases and determination of the released glycerol.

The main principles for amperometric detection of glycerol are based on the detection of the formed NADH or mediators, on the use of oxygen or hydrogen peroxide electrodes, conductive organic salts and wiring of enzymes to electrodes (Fig. 28).

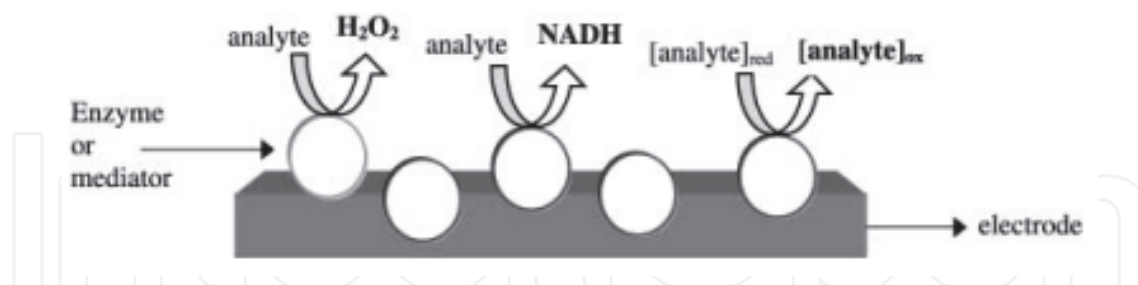


Fig. 28. Schematic representation of various types of amperometric biosensors (Prodromidis et al., 2002)

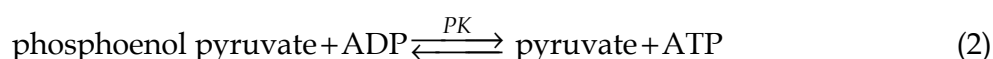
Many materials, namely, platinum (Goriushkina et al., 2010), gold (Gamella et al., 2008), glassy carbon (Guo et al., 2010), different types of graphite (Haghighi et al., 2010; Pournaghi-Azar et al., 2010), screen-printed electrodes (Ben Rejeb et al., 2007; Radoi et al., 2007; Ricci et al., 2007; Gurban et al., 2008), rigid carbon-polymer biocomposites (Alvarez-González et al., 2000; Liao et al., 2008), zeolites, clays, and polymeric membranes (Katrlik et al., 2006; Rozlosnik et al., 2009) were previously used for the construction of glycerol-selective electrodes. Various strategies of enzyme immobilization (Prodromidis et al., 2002), including physical or covalent binding, gel entrapment, electropolymerization, sol-gel techniques

(Gurban et al., 2008; Haghighi et al., 2010; Guo et al., 2010; Pournaghi-Azar et al., 2010; Liao et al., 2008) and self-assembled architectures (Gamella et al., 2008) were employed for biosensors construction.

4.1 Glycerol-selective enzymes

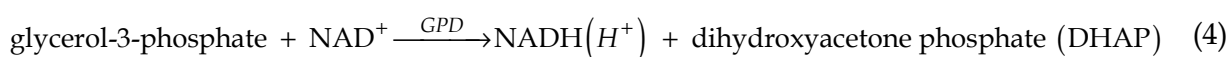
Different methods, such as liquid chromatography and spectrophotometric approach based on chemical and enzymatic reactions (West, 1998; Wu et al., 2005; Hinsch et al., 1980; Minakshi et al., 2008), in particular, oxidase-peroxidase method (Gonchar & Sybirny, 1991; Gonchar, 1998) have been proposed for glycerol determination.

The test-kits for enzymatic analysis of glycerol produced by Boehringer Mannheim and Sigma are rather expensive, because they require several enzymes and coenzymes or co-substrates. For example, the multienzymatic system (West S.I., 1998) involves three enzymes: glycerol kinase (GK), pyruvate kinase (PK) and lactate dehydrogenase (LDH), and requires the presence of the coenzyme NADH and two co-substrates, namely, ATP and phosphoenolpyruvate:

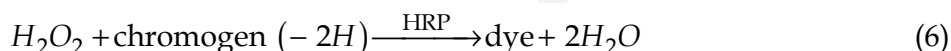
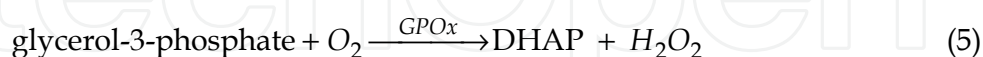


In this method, the glycerol concentration is determined by a decrease in the consumed NADH registered photometrically at 340 nm.

In the two-enzyme variant, GK (see eq. 1) and glycerol-3-phosphate dehydrogenase (GPD) are used. The reaction is also monitored photometrically by recording the generation of NADH(H⁺) at 340 nm:



In another variant of the two-enzyme method, glycerol-3-phosphate-oxidase (GPOx) and peroxidase (HRP) are used instead of GPD. This variant was applied as a basis of diagnostic kit for glycerol determination.

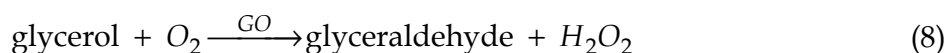


The third, dehydrogenase method, is based on the determination of NADH(H⁺) generated in the enzymatic reaction catalyzed by glycerol dehydrogenase (GDH):



The application of this method in analytical practice is problematic since the glycerol oxidation by GDH is a reversible reaction, and the selectivity of the enzyme is insufficient.

The enzyme glycerol oxidase (GO) oxidizes glycerol in the presence of oxygen under formation of hydrogen peroxide and glyceraldehyde:



GO is produced by cultivation of fungi: *Aspergillus*, *Penicillium* (Uwajima et al., 1979), *Neurospora* (Hill & Martin, 1975), *Botrytis* (Kupletskaya, 1996) and actinomycetes. GO is revealed in numerous mycelia fungi, but a highly purified enzyme preparation was isolated only from *Aspergillus japonicus* (Uwajima, 1984; Patent US 4409328(A), 1983), *Penicillium sp.* (Lin et al., 1996) and *Botrytis allii* (Patent 2117702C1, 1998; Pavlishko et al., 2004; Gayda et al., 2006).

Traditional enzymatic approaches for glycerol determination are disadvantageous due to either unsatisfactory selectivity, like in case of dehydrogenase reactions, or high cost of their everyday laboratory application. Therefore, the development of valid, selective, quick and cheap methods for glycerol analysis in clinical diagnostics is important.

4.2 Amperometric biosensors for glycerol determination

A number of biosensors for glycerol determination have been designed and investigated. They are based on the use of GDH (Kiba et al., 1996; Laurinavicius et al., 1996) and GK co-immobilized with GPOx (Compagnone et al., 1998). The latter sensor showed insufficient stability: after three days of storage in a working buffer only 10 % of the enzymes remained active. The key disadvantage of the use of GDH in amperometric biosensors is the necessity to add NAD^+ to the system which raises the price of the analysis substantially. Besides, NAD^+ can easily diffuse out of the enzyme membrane, causing a remarkable drop in biosensor sensitivity (Laurinavicius et al., 1996).

Lipase activity against TG has been measured using an amperometric GDH/NADH-based biosensor. A Prussian blue (PB) modified screen-printed electrode (SPE) was selected as a carrier for the two enzyme system. The obtained glycerol biosensor was investigated in a lipase activity assay in real samples and for detection of fatty acids. It demonstrated a good agreement with reference methods (Ben Rejeb et al., 2007).

Biosensors for NADH determination has been assembled using stable NADH oxidase (NAox) from *T. thermophilus* covalently immobilized on electrodes (Radoi et al., 2007; Serban & Murr, 2005). The NAox uses O_2 as its natural electron acceptor and produces H_2O_2 in a two-electron process. The possibility to measure glycerol with NAox-Prussian Blue bulk-modified screen-printed electrodes was investigated (Radoi et al., 2007). Various parameters: pH dependence (range of stability – pH 3.0–10.5, optimal – pH 5.0), response time (12 s) and operational stability (120 injections) were evaluated and optimised. The detection and quantification limits were 1.1×10^{-7} and 3.6×10^{-7} M, respectively, and the linear working range was comprised between 1 and 400 μM . The proposed biosensor was stable for 2 months (preserved in 50 mM phosphate buffer, pH 6.8, at 4 °C). Another high sensitive NADH amperometric biosensor based on co-immobilized NAox in combination with a redox mediator (hydroxymethyl ferrocene) and HRP was proposed (Serban & Murr, 2005) with a linear range from 5×10^{-6} M in reduction until 2×10^3 M in oxidation.

For estimation of the glycerol content in complex biological fluids two enzyme biosensor systems, based on GDH/DP and GK/GPOx/HRP, immobilized on interchangeable membranes by a prepolymer technique, were developed. A flow-injection assay of the glycerol content was performed in the presence of ferricyanide/ NAD^+ or ferrocyanide/ATP. The

biosensors were characterized by a linear range of 0.01–1 or 0.01–1.5 mM glycerol, and a sensitivity of 6.02 or 1.42 mA·M⁻¹·cm⁻² and with a signal loss of 40 % after 90 h or 30 % after 16 h during continuous operation at a sample throughput of 10 injections/h for GDH/DP or GK/GPOx/HRP-containing membranes, respectively. The operational stability of the described biosensor systems was sufficient for monitoring and control of fermentation processes for up to 24 h, with several months of storage stability (Katrlik et al., 2006).

Modified SPE for amperometric detection of H₂O₂ and NADH at low applied potential were obtained by modifying the working electrode surface with Prussian Blue (PB-SPE). The coupling of this sensor with phenazine methosulfate (PMS) in the working solution gives the possibility of measuring both NAD(P)H and H₂O₂. PMS reacts with NADH producing PMSH, which in the presence of oxygen, gives an equimolar amount of H₂O₂. This allows the measurement of both analytes with similar sensitivity (357 mA·M⁻¹·cm⁻² for H₂O₂ and 336 mA·M⁻¹·cm⁻² for NADH) and similar limit of detection (5×10^{-7} M for H₂O₂ and NADH). Results obtained with a variety of dehydrogenases (alcohol, malic, lactate, glucose, glycerol and glutamate dehydrogenase) for the detection of their substrates or the enzymatic activity were performed demonstrating the suitability of the proposed method for future biosensor applications (Ricci et al., 2007).

To improve the NADH detection biosensors based on NAOx/FMN and Prussian blue (PB) modified SPE have been developed. The catalytic effect, the sensitivity and the stability of PB-SPEs were studied at different pH values and different potentials ranges in cyclic voltammetry and amperometric measurements. NAOx and FMN were immobilised on the PB-SPEs by entrapment in a sol-gel, PVA-AWP polymer matrices or using glutaraldehyde. The amperometric detection of NADH was performed at +250 mV *versus* Ag/AgCl, and co-enzyme was entrapped with NAOx in the biocatalytic layer using sol-gel matrix. The sensitivity was 4.57 mA·M⁻¹·cm⁻² (R.S.D. = 9.2 %, $n = 4$), the linear range up to 1.61 mM and the detection limits about 1.17 µM. These biosensors revealed a good long term and operational stability, the response decreased from 3.4 % after 2 days, to 50 % of the analytical signal after 6 months (Gurban et al., 2008).

GDH and NAD⁺ were co-immobilized in a carbon paste electrode using an electropolymerized layer of non-conducting poly(*o*-phenylenediamine) (PPD). After partial oxidation of the immobilized NAD⁺, the modified electrode allows the amperometric detection of the enzymatically generated NADH at an applied potential above 0 V (Ag/AgCl). The resulting biosensor shows a fast and linear response to glycerol within the concentration range of 1.0×10^{-6} – 1.0×10^{-4} M with a detection limit of 4.3×10^{-7} M. The amperometric response remains stable for at least 3 days. The biosensor was applied to the determination of glycerol in a plant-extract syrup, with results in good agreement with those for the standard spectrophotometric method (Alvarez-González et al., 2000).

For glycerol determination two different biosensor configurations have been evaluated: one based on the GDH/DP bienzyme system, and another using GK/GPOx/HRP. Both enzyme systems were immobilized together with the mediator tetrathiafulvalene (TTF) on a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold electrode by using a dialysis membrane. After 51 days of use, the GDH/DP biosensor still exhibited 87 % of the original sensitivity, while the GK/GPOx/HRP biosensor yielded a 46 % of the original response after 8 days. Linear ranges of $1.0 \cdot 10^{-6}$ to $2.0 \cdot 10^{-5}$ or $1.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-5}$ M glycerol and sensitivities of 1214 ± 21 or 1460 ± 34 µA·M⁻¹ were obtained with GDH/DP and GK/GPOx/HRP-based biosensors, respectively. The calculated detection limits were $4.0 \cdot 10^{-7}$ and $3.1 \cdot 10^{-7}$ M, respectively (Gamella et al., 2008).

The development of nanoscience and nanotechnology has inspired scientists to continuously explore new electrode materials for constructing an enhanced electrochemical platform for sensing. A Pt nanoparticle (NP) ensemble-on-graphene hybrid nanosheet (PNEGHNs) was proposed as new electrode material. The advantages of PNEGHNs modified glassy carbon electrode (GCE) (PNEGHNs/GCE) are illustrated from comparison with the graphenes (GNs) modified GCE for electrocatalytic and sensing applications. The electrocatalytic activities toward several organic and inorganic electroactive compounds at the PNEGHNs/GCE were investigated, all of which show a remarkable increase in electrochemical performance relative to GNs/GCE. Hydrogen peroxide and trinitrotoluene (TNT) were used as two representative analytes to demonstrate the sensing performance of PNEGHNs. It is found that PNEGHNs modified GCE shows a wide linear range and low detection limit for H_2O_2 and TNT detection (Guo et al., 2010).

An iridium nanoparticle modified carbon bioelectrode for the detection and quantification of TG was successfully carried out. TG was hydrolyzed by lipase and the produced glycerol was catalytically oxidized by GDH producing NADH in a solution containing NAD^+ . Glyceryl tributyrates, a short chain TG, was chosen as the substrate for the evaluation of this TG biosensor in bovine serum and human serum. A linear response to glyceryl tributyrates in the concentration range of 0 to 10 mM and a sensitivity of $7.5 \text{ nA}\cdot\text{mM}^{-1}$ and $7.0 \text{ nA}\cdot\text{mM}^{-1}$ in bovine and human serum, respectively, were observed. The conditions for the determination of TG levels in bovine serum using this biosensor were optimized, with sunflower seed oil being used as an analyte to simulate the detection of TG in blood. The experimental results demonstrated that this iridium nano-particle modified working electrode based biosensor provided a relatively simple means for the accurate determination of TG in serum (Liao et al., 2008).

Prussian blue nanoparticles (PBNPs) immobilized on the surface of a graphite electrode was covered with a layer of Nafion. The sensor showed a good electrocatalytic activity toward H_2O_2 reduction, and it was successfully used for the amperometric detection of H_2O_2 . The calibration curve for H_2O_2 determination was linear from 2.1×10^{-6} to 1.4×10^{-4} M with a detection limit ($\text{S/N} = 3$) of 1.0×10^{-6} M (Haghighi et al., 2010). Further modification of the proposed sensor with different enzymes, namely, GO, was discussed as a perspective for the fabrication of a glycerol biosensor.

For hydrodynamic amperometry of H_2O_2 at μM concentration level, an aluminum electrode plated by a thin layer of metallic palladium and modified with Prussian blue (PB/Pd-Al) was developed. It was found that the calibration graph is linear with the H_2O_2 concentration in the range from 5×10^{-6} to 34×10^{-6} M with a correlation coefficient of 0.999. The detection limit of the method was about 4×10^{-6} M. The method was successfully used for the monitoring of H_2O_2 in saliva and environmental samples (Pournaghi-Azar et al., 2010).

New natural materials, such as egg shells, were proposed as enzymes carrier in bioselective membranes for triglyceride (TG)-selective amperometric biosensors. A mixture of commercial lipase, GK and GPOx was co-immobilized at an egg shell membrane through covalent coupling. Maximum current was obtained at a working potential of +400 mV. The biosensor showed optimum response within 10 sec at pH 7.0 and 35 °C. The linear range was from 0.56 to 2.25 mM TG and the detection limit was 0.28 mM. A good correlation ($r=0.985$) was obtained between the TG level determined by the standard enzyme-based colorimetric test and the proposed sensors. Serum compounds (urea, uric acid, glucose, cholesterol, ascorbic acid and pyruvic acid) did not interfere with the sensor response. The stability of enzyme electrode was determined to be 200 measurements over a period of 70

days without any considerable loss of activity, when stored at 4°C between the measurements (Narang et al., 2010).

Conducting polymer-based electrochemical sensors have shown numerous advantages in a number of areas related to human health, such as the diagnosis of infectious diseases, genetic mutations, drug discovery, forensics and food technology, due to their simplicity and high sensitivity. One of the most promising group of conductive polymers is poly(3,4-ethylenedioxythiophene); PEDOT or PEDT) and its derivatives due to their attractive properties: high stability, high conductivity (up to 400-600 S/cm) and high transparency (Rozlosnik et al., 2009; Nikolou et al., 2008). Organic transistors based on PEDT doped with poly(styrene sulfonic acid) (PEDT:PSS) offer enormous potential for facile processing of small, portable, and inexpensive sensors ideally suited for point-of-care analysis. They can be used to detect a wide range of analytes for a variety of possible applications in fields such as health care (medical diagnostics), environmental monitoring (airborne chemicals, water contamination, etc.), and food industry (smart packaging). These transistors are considered to be excellent candidates for transducers for biosensors because they have the ability to translate chemical and biological signals into electronic signals with high sensitivity. Furthermore, functionalization of PEDT:PSS films with a chemical or biological receptors can lead to high specificity (Nikolou et al., 2008).

4.3 Bioanalytical application of Glycerol oxidase (GO) as bioselective element of amperometric biosensors

The enzymatic glycerol transformation using oxidases results in generating of electrochemically active hydrogen peroxide. An amperometric GO-based biosensor is considered to be an attractive alternative over other biosensors. To construct glycerol selective biosensors, a GO preparation with a specific activity of 5.7 $\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein were used for immobilization on electrodes. The enzyme was purified from a cell-free extract of the fungus *B. allii* by anion-exchange chromatography and stabilized with 5-10 mM Mn^{2+} , 1 mM EDTA and 0.05 % polyethylene imine (Gayda et al., 2006).

4.3.1 Immobilization of GO on platinum printed electrode (Goriushkina et al., 2010)

Different methods of GO immobilization on the surface of printed platinum electrodes (SensLab, Leipzig, Germany) were compared: electrochemical polymerization in polymer PEDT, electrochemical deposition in Resydrol and immobilization using glutaraldehyde vapors.

The monomer 3,4-ethylenedioxythiophene (EDT) and poly(ethylene glycol) (MM = 1450) were used for the electrochemical polymerization. A mixture consisting of 10^{-2} M EDT, 10^{-3} M polyethylene glycol, and GO solution was prepared in 20 mM phosphate buffer, pH 6.2. EDT was polymerized by application of a potential from +200 to +1500 mV at a rate of 0.1 V/s during 15 cycles. Homogenous PEDT films were obtained on the surface of the working electrode. Film formation is enhanced in aqueous and possibly hydrophilic polymers such as polyvinyl pyrrolidone (PVP) or polyethylene glycol (PEG), which are dissolved in the electropolymerization solution. The entrapment of PVP or PEG results in an increased hydrophilicity of the deposited polymer film.

The commercial resin Resydrol (Resydrol AY 498 w/35WA) and glutaraldehyde were also used as a polymer matrix for the enzyme immobilization.

GO-based biosensors with the enzyme immobilized within a Resydrol layer or using glutaraldehyde vapor, are characterized by a narrow dynamic range and a lower response

in comparison with the biosensor based on GO immobilized in PEDT. The limit of detection for glycerol for all these biosensors is about the same (Table 3). The developed GO-PEDT-based biosensor is characterized by a linear response on the glycerol concentration in the range from 0.05 to 25.6 mM with a detection limit of 0.05 mM glycerol (Fig. 29). The stability of the GO-PEDT-based biosensor was evaluated and showed a decrease in its response value by about 2.5 % daily with almost no response after 50 days of storage. The pH optimum of the GO-PEDT-based biosensor was determined to be 7.2.

An analysis of the impact of buffer capacity and concentration of the base electrolyte showed feeble influence of their change on the response value (Fig. 30) which is typical for enzyme amperometric biosensors.

Immobilization method	Detection limit for glycerol, mM	Linear range, mM	Maximum response, nA	Storage stability
Entrapment of GO in poly(3,4-ethylenedioxythiophene) (PEDT) by electrochemical polymerization	0.05	0.05 to 25.6	1405	75% activity after 15 days, 14% after 40 days
Entrapment in Resydrol by means of electrochemically induced polymer precipitation	0.05	0.05 to 0.4	400	38% activity after 2 weeks, 13% after 40 days
Glutaraldehyde vapour	0.05	0.05 to 0.2	130	10% after 1 day

Table 3. Comparative analysis of laboratory prototypes of amperometric biosensors based on different methods of glycerol oxidase immobilization

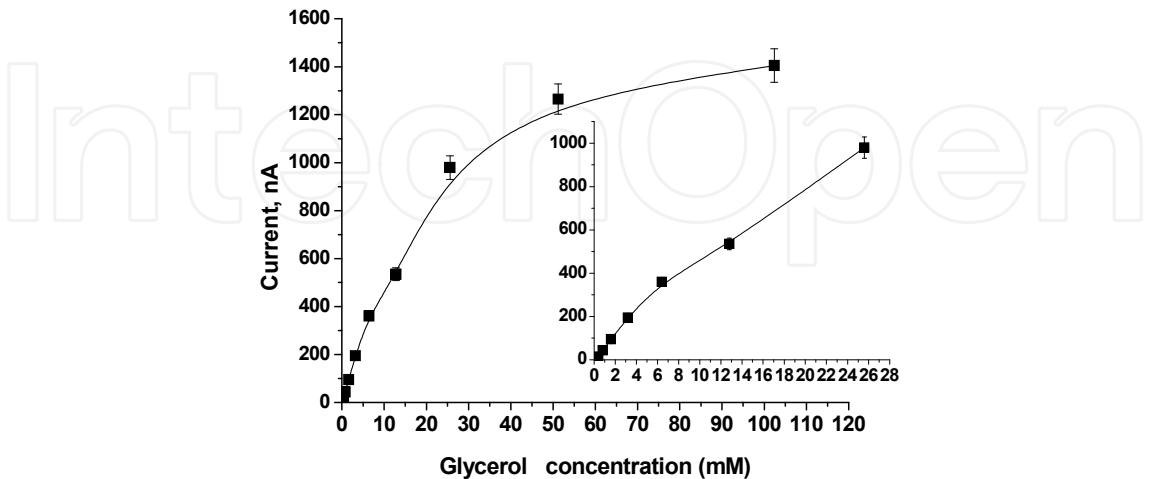


Fig. 29. The calibration curve of the GO-PEDT-based amperometric biosensor. Measuring conditions: 100 mM phosphate buffer, pH 7.2, potential of +300 mV versus the intrinsic reference electrode.

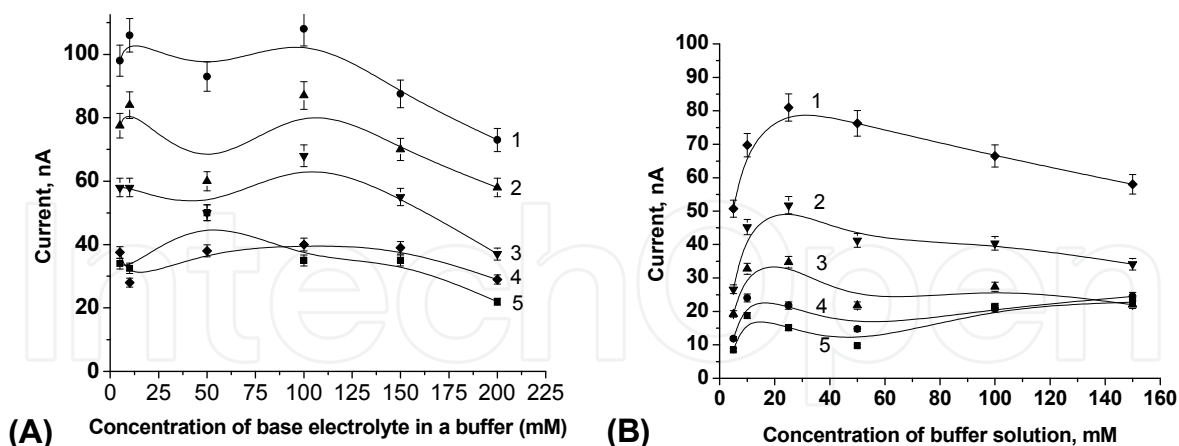


Fig. 30. Response of GO-PEDT-based amperometric biosensor on concentrations of the base electrolyte in buffer (A) and on the concentration of the buffer solution (B). Measuring conditions: 100 mM phosphate buffer, pH 7.2, potential of +300 mV *versus* the intrinsic reference electrode. Glycerol concentrations in a measuring cell: A - 6.4 mM (1); 3.2 mM (2); 1.6 mM (3); 0.8 mM (4); 0.4 mM (5); B - 1.6 mM (1); 0.8 mM (2); 0.4 mM (3); 0.2 mM (4); 0.1 mM (5).

4.3.2 Co-immobilization of glycerol oxidase and peroxidase on carbon electrode

Immobilization of glycerol oxidase (GO) in combination with horseradish peroxidase (HRP) was conducted on platinised carbon electrodes by electrodeposition in a mixture of the osmium-complex containing cathodic paint (CP-*O*s) according to the scheme which was developed by us for the immobilization of yeast alcohol oxidase (Smutok et al., 2006). Electrodeposition of the enzymes at the working electrode surface was performed in an electrochemical microcell using controlled potential pulses to -1200 mV for 0.2 sec with an interval of 5 sec for 10 cycles. The electrode was washed with 50 mM borate buffer, pH 9.0, before measurements.

Measurements were performed at room temperature in a glass cell with the volume of 50 ml, filled with 25 ml of buffer at intense stirring. After the background current was attained, glycerol was stepwise added to the measuring cell in increasing concentrations, and the amperometric signal was recorded. Fig. 31 shows current response of the bi-enzyme sensor HRP-GO-CP-*O*s upon stepwise addition of glycerol. The linear concentration range for the developed sensor was up to 5 mM of the analyte.

5. Conclusion

In this review, the development of enzyme- and cell-based amperometric biosensors is described aiming on monitoring of L-lactate, alcohols, and glycerol using genetically constructed over-producers of enzymes as well as wild type microorganisms. Novel, recombinant or mutated enzymes (L-lactate:cytochrome *c* oxidoreductase, alcohol oxidase, glycerol oxidase) were used as bioselective elements for the above mentioned biosensors. Most genetic manipulations have been done using the thermotolerant yeast *Hansenula polymorpha*. Enzymes isolated from this source demonstrated improved stability when

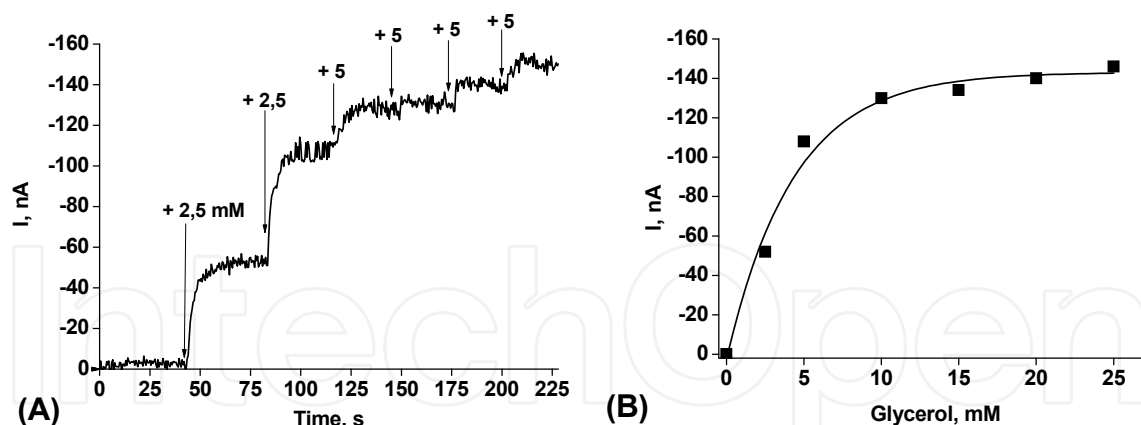


Fig. 31. Amperometric response (A) and calibration graph (B) obtained with a bi-enzyme sensor upon stepwise addition of glycerol at increasing concentrations. Experimental conditions: working potential -50 mV, 10 cycles of electrodeposition, 50 mM borate buffer, pH 9.0.

compared to non-thermotolerant yeasts. On the other hand, directed protein modification allowed increasing K_M values of the enzymes (flavocytochrome b_2 and alcohol oxidase) resulting in a wider linear range of the related biosensors. Recombinant yeast cells overproducing the target enzyme were used as the sources of the corresponding enzymes, as well as directly as microbial biorecognition elements in the sensors. For the different bioselective components (enzymes, cells or cell debris) different immobilization procedures were developed and optimized: physical adsorption, fixation behind a dialysis membrane, entrapment in a polymer layer of an anodic or cathodic electrodeposition paints, cross-linking with glutardialdehyde vapour *etc.* The developed biosensors are characterized by an in general high sensitivity, sufficient or improved selectivity, as well as improved long term operational and storage stability.

6. Acknowledgement

This work was partially supported by CRDF, project # UKB2-9044-LV-10 and in part by the Samaria and Jordan Rift Valley Regional R&D Center (Israel) and by the Research Authority of the Ariel University Center of Samaria (Israel), by NAS of Ukraine in the field of complex scientific-technical Program “Sensor systems for medical-ecological and industrial-technological needs”. Some experiments were performed by the use of equipment granted by the project “Centre of Applied Biotechnology and Basic Sciences” supported by the Operational Program “Development of Eastern Poland 2007-2013”, No. POPW.01.03.00-18-018/09.

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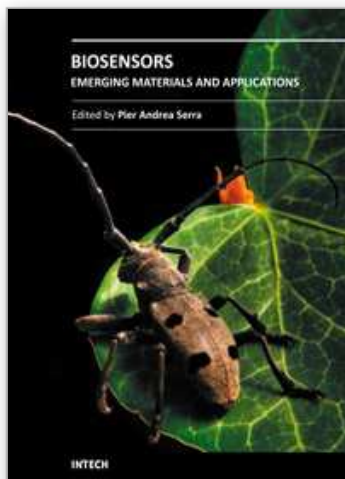
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Biosensors - Emerging Materials and Applications

Edited by Prof. Pier Andrea Serra

ISBN 978-953-307-328-6

Hard cover, 630 pages

Publisher InTech

Published online 18, July, 2011

Published in print edition July, 2011

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Oleh Smutok, Galina Gayda, Kostyantyn Dmytruk, Halyna Klepach, Marina Nisnevitch, Andriy Sibirny, Czesław Puchalski, Daniel Broda, Wolfgang Schuhmann, Mykhailo Gonchar and Vladimir Sibirny (2011). Amperometric Biosensors for Lactate, Alcohols, and Glycerol Assays in Clinical Diagnostics, Biosensors - Emerging Materials and Applications, Prof. Pier Andrea Serra (Ed.), ISBN: 978-953-307-328-6, InTech, Available from: <http://www.intechopen.com/books/biosensors-emerging-materials-and-applications/amperometric-biosensors-for-lactate-alcohols-and-glycerol-assays-in-clinical-diagnostics>

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