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Mediated enzyme electrodes

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This review summarizes the long time experience of the team at the Electrochemistry of Biocatalysts and Metal-Air Systems Department in the field of the mediated enzyme electrodes. Investigated is the redox behaviour of a mediated enzyme electrode depending on the kind of carbon materials, needed to produce the electrode (compact carbon material – pyrolytic graphite or dispersed carbon material - carbon black), the type of mediators (ferrocene derivates, nickelocene and benzoqoquinone), and the type of enzyme (glucose oxidase and lactate oxidase).

Key words: mediator, enzyme electrode, pyrolytic graphite electrode, carbon black

INTRODUCTION

The research work in the field of the biosensors has expanded very rapidly since the development of glucose enzyme electrodes, which are of particular interest in the biomedical analysis field [1, 2]. The development of electrochemical biosensors which combine the specificity and the selectivity of the enzyme catalyst with the appropriate electrochemical techniques is of a great interest for the healthcare, the environmental control, the agriculture, the food and other industries [3, 4].

The electrochemical biosensor is a complicated device which converts a biological recognition process into an electrical signal, the amplitude of which is related to the concentration of the analyte. The coupling of the electron transfer of the enzyme with the electron transfer at the electrode poses a major problem to the biosensor development. In most cases the enzyme redox centres are essentially insulated within the enzyme molecule so that a direct electron transfer to the surface of the conventional electrode does practically not occur. The electrical communication between the redox centres of the enzyme and the electrode requires either the presence of oxygen and hydrogen peroxide and their diffusion to and from the enzyme redox centres, or the presence of a redox mediator [5].

Glucose oxidase (GOD) is one of the first enzymes used in the biocatalytic electrochemical sensing elements [6, 7]. The specific substrate for this enzyme, the β-D-glucose, undergoes oxidation by oxygen producing of gluconolactone and hydrogen peroxide.

$$\text{glucose} + O_2 \xrightarrow{GOD} \text{gluconolactone} + H_2O_2 \quad (1)$$

The detection principle is based on an electrochemical reaction in which the H_2O_2 production or the O_2 consumption is detected. The produced H_2O_2 can be electrochemically oxidised on an electrode at a constant potential, and the generated anodic current is used to measure the glucose concentration. Together with the natural electron acceptor in reaction (1), oxygen, and other low-molecular-weight compounds are used as mediators between the enzyme and the electrode. The mediator is a low molecular weight redox couple, which shuttles electrons from the enzyme redox center to the surface of the electrode. The following scheme describes the reactions which take place on the mediated electrode [8]:

$$\text{glucose} + GOD^{ox} \rightarrow \text{gluconolactone} + GOD^{red} \quad (2)$$

$$GOD^{red} + 2M^{ox} \rightarrow GOD^{ox} + 2M^{red} \quad (3)$$

$$2M^{red} \rightarrow 2M^{ox} + 2e^- \quad (4)$$

where GOD^{ox} and GOD^{red} are the enzyme in oxidized and reduced form of its active center, respectively, M^{ox} and M^{red} are the oxidized and the reduced form of the electrochemical mediator. The conjugation of the enzyme catalyzed steps (2) and (3) is accomplished through the electrochemical reaction (4).

The use of the electrochemical mediator in the amperometric enzyme electrodes is connected with two basic requirements to these electrodes: the amperometric signal of the electrode must be independent of the concentration of the dissolved oxygen in the electrolyte; and the working potential

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of the enzyme electrode must be low enough so that no organic compound in the electrolyte could be oxidized simultaneously with the substrate [9].

Quinones, organic dyes such as methylene blue, phenazines, methyl violet, Alizarin yellow, prussian blue, thionin, azure A and C, toluidine blue, iron complexes such as hexacyanoferrat, ferrocene and their derivatives, act as mediators and have been widely used in a number of biosensors [8, 10–15]. Sometimes the oxidized form of the mediator is dissolved in a bulk solution, so that the concentration of the mediator on the electrode surface decreases. This results in a gradual degradation of the electrode performance. This process can be moderated by a covalent binding of the mediator on the electrode surface by some functional groups [16] or by including in a polymer film, deposited on the electrode surface [17]. This approach makes possible the stability improvement of the mediated enzyme electrode.

The reduced forms of the mediators can be electrochemically oxidized on an electrode at potentials lower than that of the H_2O_2. This gives a possibility to design biosensors, operating at potentials lower than those based on the natural enzymatic reaction (1). It must be noticed that a low working potential of the biosensor is preferable in order to decrease the rate of oxidation of some compounds, usually present in physiological fluids. The oxidation of this compound results in an electric signal, disturbing the signal, obtained by the glucose oxidation. In this way the possible interference with the amperometric signal of the biosensor is reduced [5].

EXPERIMENTAL

A disk of pyrolytic graphite with a 5 mm diameter is press – fitted into a Teflon holder. Prior to use, the electrode was polished on fine emery paper. After washing with distilled water, the electrode is electrochemically activated in 0.1 M phosphate buffer by consecutive anodic polarization at + 0,2 V (vs. Ag/AgCl) for 2 minutes and cathodic polarization at – 0.5 V for 5 minutes. Under this procedure, the pyrolytic graphite surface is oxidized, and oxygen containing quinoidalic groups are generated on it [18]. The presence of such groups promotes a strong adsorption of the enzyme and of the mediator on the pyrolytic graphite electrode surface.

The enzyme electrode is prepared by the application of mediator solution on the activated pyrolytic graphite surface, and after drying in the air, the electrode surface is covered with a GOD or LOD solution of the enzyme in a phosphate buffer (pH 7). After drying at a room temperature, the electrode is washed in a buffer solution. Between measurements the enzyme electrode is kept at 4 ºC.

The enzyme electrodes are prepared also by consecutive application of a mediator and an enzyme on the porous supporting layer of carbon black-PTFE (polytetrafluorethylene) material. In other cases, a porous matrix of carbon material, wet proofed with PTFE, is embedded in a plastic tube and covered by a thin layer of a mixture of the same wet proofed material and mediator (p-benzoquinone). This thin layer forms the face of the electrode onto which the enzyme is immobilized.

The electrochemical measurements are performed in a two- or three-electrode cell with an Ag/AgCl reference electrode and Pt counter electrode. The electrolytes comprise of a 0.1 M phosphate buffer solution and a 0.1 M phosphate buffer solution, containing 0.1M KCl.

RESULTS AND DISCUSSION

Mediated enzyme electrodes, prepared from electrochemically activated pyrolytic graphite electrode

The quasi-redox potentials of the investigated ferrocene derivatives are obtained from the cyclic voltammograms of an electrochemically activated pyrolytic graphite electrode, modified with the corresponding compound. The obtained experimental values of the quasi-redox potentials of the investigated ferrocene derivatives are in a good coincidence with the literature data, as well as with our data, previously obtained with the same ferrocene derivatives, adsorbed on another type of carbon electrode [19, 20, 21]. The ferrocene derivatives whose substituent displays a positive (electron donor) induction effect ($-CH_3$, $-CH_2OH$, $-CH_2COOH$), possess a more negative quasi-redox potential than that of the ferrocene. Ferrocene derivatives, where the substituents have a negative induction effect, posses a more positive quasi-redox potential than that of the ferrocene. According to the electron structure of the substituents in the pentadienyl ring, the quasi-redox potential of the corresponding ferrocene derivative can be prognosticated, and its suitability to be used as a mediator in enzyme electrodes can be eventually estimated.

The methanol-ferrocene possesses a more negative quasi-redox potential than that of the ferrocene, so that it is suitable to be used as a mediator in glucose and lactate electrodes. The

cyclic voltammogram is measured in a 0.1M phosphate buffer (Fig. 1, full line) and in the same

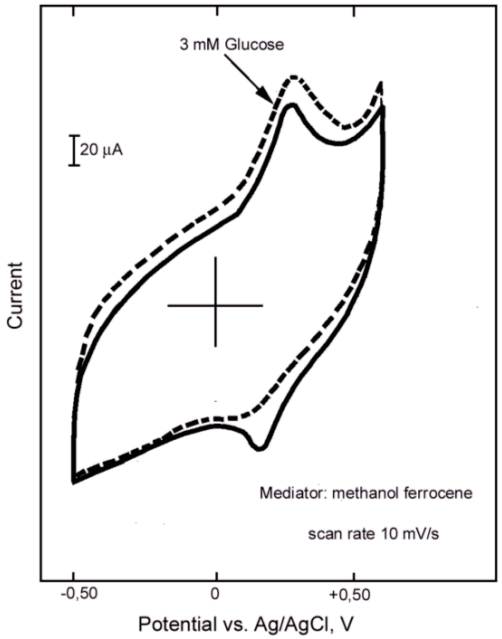

Fig. 1. Cyclic voltammograms of activated pyrographite electrodes with adsorbed GOD and methanolferrocene: in 0,1 M phosphate buffer (full line); in 0,1 M phosphate buffer containing 3 mM glucose (dotted line).

buffer which contains 3 mM of glucose (Fig. 1, dotted line). The observed increase of the anodic peak in the presence of glucose is due to the electrochemical oxidation of the reduced form of the mediator on the electrode surface. The decrease of the cathode peak is due to the consumption of part of the mediator through oxidation of the reduced form of the enzyme, obtained by the enzymatic oxidation of glucose. This effect, called the 'mediator effect', is observed also in the case of enzyme electrodes with another type of metallocene mediators [22].

The steady-state current of the investigated enzyme electrodes, as a function of the substrate concentration, is studied in both, the presence of oxygen dissolved in the electrolyte, and after the significant decrease of the dissolved oxygen concentration by blowing argon through the electrolyte. The behaviour of the lactate oxidase enzyme electrodes with mediator methanolferrocene or butylferrocene is also investigated. In order to check if the substrate (Li L-lactate) is directly oxidized on the electrode surface, electrode is prepared from electrochemically activated pyrolytic graphite, modified with a mediator and with a covering layer of Nafion. The steady-state current of these electrodes at a constant potential of + 350 mV vs. Ag/AgCl is measured in the presence of Li L-lactate in the solution, and no amperometric signal is observed, i.e. the substrate is not directly oxidized on the electrode at this potential.

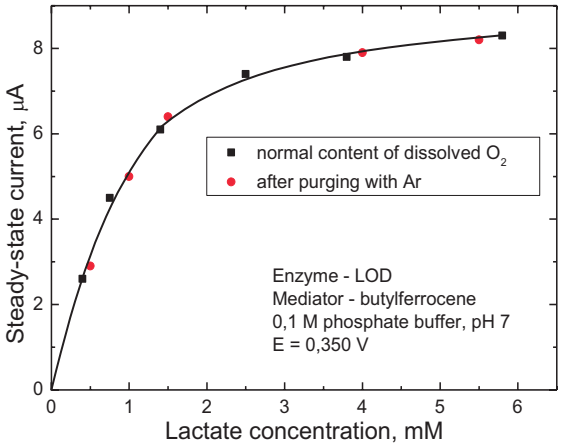

Fig. 2. Calibration curve of a lactate electrode with mediator butylferrocene: when electrolyte is with normal content of dissolved oxygen and after purging with Ar.

The calibration curve of a lactate electrode with butylferrocene is presented in Fig. 2. It is seen that the decrease in the oxygen concentration in the solution does not influence significantly the steady-state current of the electrode. This shows that the amperometric signal of the investigated electrode is generated mainly as a result of the action of the electrochemical mediator.

Application of the mediated enzyme electrode at a low working potential is preferable for the biosensing. The investigations are oriented at selecting those ferrocene derivatives which possess a redox potential lower than that of the ferrocene. Another way to achieve a mediated electrode with a low working potential is to use a structural analog of ferrocene which possesses a redox potential lower than that of the ferrocene. Investigations of the electrochemical behavior of the metallocenes in non-aqueous electrolyte have shown that the redox potential of a nickelocene/nicelocenium couple is ca. 0.4 V more negative than that of the ferrocene/ferrocenium couple [23, 24]. An investigation of nickelocene as an electrochemical mediator in GOD enzyme electrodes with low working potential is described for the first time in [25].

The stable cyclic voltammograms of the electrochemically activated pyrolytic graphite electrodes with adsorbed nickelocene and with adsorbed ferrocene are juxtaposed in Fig. 3. Two redox peaks, corresponding to the electrochemical reaction (3), are observed on each of the

voltammograms. The quasi redox potential of the nickelocene/nicelocenium couple is ca. 0,300 V more negative (cathodic) than that of the ferrocene/

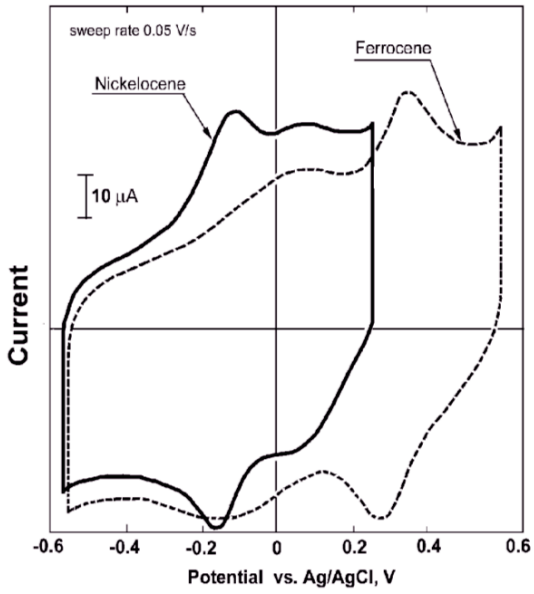

Fig. 3. Cyclic voltammograms of ferrocene and nickelocene adsorbed on activated pyrolytic electrode in 0,1 M phosphate buffer (pH 7.0).

ferrocenium couple (+ 0,230 V), obtained under the same conditions. This result is in agreement with the data for the redox potentials of the metallocenes in non-aqueous electrolytes [23, 24].

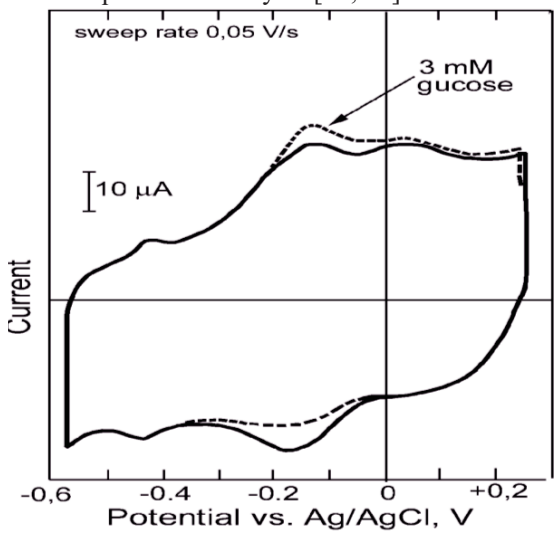

Fig. 4. Cyclic voltammograms of nickelocene GOD electrode in absence (full line) and in presence (dashed line) of 3 mM glucose in electrolyte (pH 7.0).

A nickelocene-mediated GOD electrode is prepared by adsorption of GOD on the electrochemically activated pyrolytic graphite with adsorbed nickelocene. The cyclic voltammogram of such mediated electrode is presented in Fig. 4 (full line). In the presence of glucose (dotted line) the anodic peak of nickelocene (–0,105 V) increases, whereas its cathode peak decreases. In the presence of the glucose, the oxidized form of the GOD^{ox} enzyme is reduced to GOD^{red} (2). The nickelocenium cations react with the GOD^{red} as a second substrate, which results in a decrease in their concentration (3), and correspondingly in a decrease of the cathode peak current of the electrochemical reduction of the nickelocenium to nickelocene. On the other hand, the nickelocene concentration increases due to reaction (3), which results in an increase in the anodic peak current of the electrochemical oxidation of nickelocene. The steady-state current of the nickelocene-mediated GOD electrode in 5 mM glucose solution vs. the controlled potential is presented in Fig. 5. At potentials more positive than the nickelocene redox potential (–0,105 V), the steady-state current increases significantly and reaches a plateau in the potential range between 0.0 V and + 0.2 V. In the absence of glucose (background curve), the steady-state current is very low and is practically independent of the potential up to + 0.2 V.

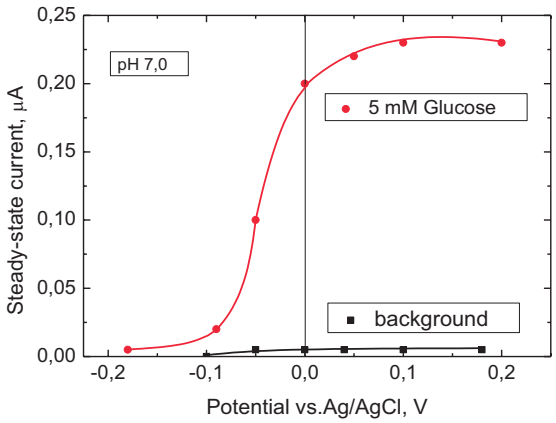

Fig. 5. Polarization curves of nickelocene-mediated GOD electrode in absence (background curve) and in presence of 5 mM glucose.

Fig. 6 presents the dependence of the steady-state amperometric response of the enzyme electrode with a nickelocene mediator at a potential of 0.0 V vs. Ag/AgCl as a function of the glucose concentration in the electrolyte. The removal of the dissolved oxygen in the cell by bubbling with Ar results in a small increase (less than 10 %) of the steady-state current in the whole investigated interval of glucose concentrations. The obtained calibration curve possesses a linear part of 0.05 mM

up to at least 1.00 mM of glucose. From the slope of the electrochemical Eadie-Hofstee plot the apparent Michaelis constant is obtained to be 1.10 mM.

The low value of the nickelocene/nicelocenium couple redox potential makes possible the use of adsorbed nickelocene as an electrochemical mediator in the amperometric glucose biosensors operation at potentials of ca. 0.00 V (vs. Ag/AgCl). This is important for the elimination of the

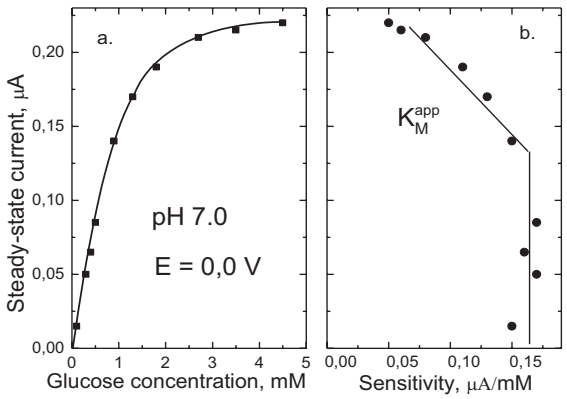

Fig. 6. (**a**) Calibration curve of nickelocene-mediated GOD electrode at E = 0,0 V vs. Ag/AgCl, (**b**) the same data in electrochemical Eadie-Hofstee coordinates.

influence of interfering species in the real glucose assay.

Mediated enzyme electrodes, prepared from PTFE modified carbon black

PTFE modified carbon blacks are prepared as a powder material by a special technology [26], which allows the use of different carbon blacks, and at a controlled carbon black/PTFE ratio. This material possesses a very high porosity, combined with an electronic conductivity, and a high hydrophobicity. Enzymes can be immobilized easily on this surface by adsorption. Mediators are mixed with the PTFE modified carbon blacks to form a powdered composite material. Porous tablet with a significant mechanical strength are pressed from this composite material without the use of any additional binder. The electrode, of different shape and dimensions, can be cut out from the tablet. [10] describes the performance of the electrodes with GOD, immobilized on a porous matrix of carbon black wet proofed with PTFE, and p-benzoquinone (BQ) as a mediator.

Fig. 7 presents the steady-state current of the electrodes with constant amount of BQ (30 wt. % in the mixture) and with increasing amounts of GOD (from 0.05 to 0.35 mg per electrode) as a function of glucose concentration. The current increases and tends to a saturation value with the increase in the glucose concentration. The saturation current and the slope of the linear part of the presented curves (at low glucose concentrations) increase with the amount of GOD in the electrolyte. The amperometric biosensor with mediated enzyme electrodes based on GOD, and mediators, adsorbed on carbon black surface, is described in [27]. The

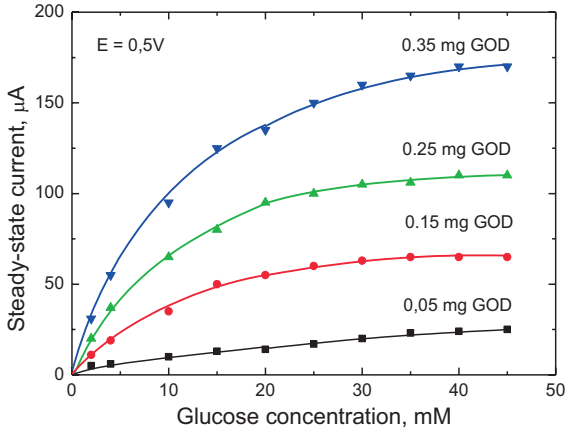

Fig. 7. Steady-state current of electrodes with 30 wt. % BQ and different amount of GOD as a function of the glucose concentration.

data, obtained with the mediator nickelocene, are juxtaposed with the results, obtained with ferrocene and its derivates. The data show similar electrochemical behaviour of the investigated ferrocenes whether applied on the carbon black electrode or applied on the graphite electrode. The obtained difference between the anodic and cathodic peak potentials of the nickelocene, ferrocene and their derivates on carbon black is bigger by 0.1V than that obtained under the same conditions on pyrolytic graphite. This can be explained by the multilayer coverage of the mediator on the carbon black surface. The adsorption of GOD on metallocene modified carbon black surface does not lead to a great change in the cyclic voltammograms of the electrodes. An insignificant decrease of the dynamic double-layer capacity of the electrode is observed, probably due to the partial blocking of the electrode surface by the adsorbed enzyme [27].

Fig. 8 presents the calibrations curves of a biosensor with ferrocene and nickelocene mediators. The figure shows that the nickelocene mediated glucose oxidase electrode exhibits a similar behaviour to that of the mediated with ferrocene. However, the quasi-redox potential of nickelocene/nickelocenium cation, which is by

0.3 V more negative than that of any of the investigated ferrocene derivatives, significantly lowers the operating potential of the biosensor to 0.00 V vs. Ag/AgCl electrode. This is important for direct glucose determination in the physiological fluids (blood, urine), where an increased selectivity of the assay is desirable. The close values of K_M^{app} for nickelocene and ferrocene-mediated electrodes are due to a similarity in the reaction mechanism.

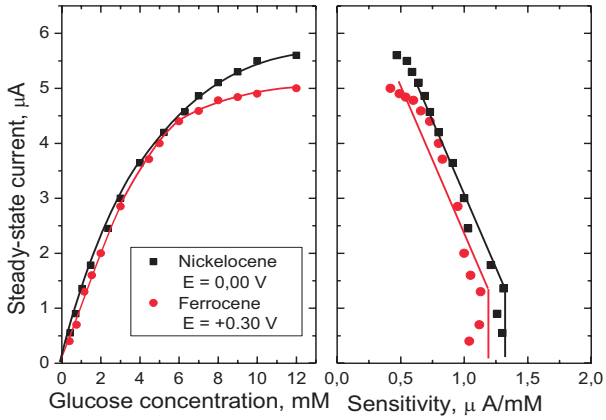

Fig. 8. (**a**) Dependence of the steady-state current of a mediated biosensor on glucose concentration; mediators ferrocene (E= + 0,300 V) and nickelocene (E = + 0,0 V). (**b**) Corresponding electrochemical Eadie-Hofstee plots.

CONCLUSIONS

Enzyme electrodes of different range of linearly dependent signal from the substrate concentration and different sensitivity can be developed using various mediators.

The results, obtained with the electrochemically modified pyrolytic graphite or the PTFE modified carbon blacks, show that the future application of mediator enzyme electrodes (ferrocene, derivatives, nickelocene, other) is possible in the field of bioreactors and biofuel cell use.

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МЕДИАТОРНИ ЕНЗИМНИ ЕЛЕКТРОДИ

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(Резюме)

В настоящата обзорна статия е обобщен дългогодишния опит на колектива от секция „Електрохимия на биокаталитичните и металовъздушни системи" в областта на медиаторните ензимни електроди. Изследвани са окислително-редукционните свойства на медиаторния ензимен електрод в зависимост от вида на въглеродните материали (компактен въглероден материал – пирографит, или дисперсен въглероден материал – въглеродни сажди); използваните медиатори (фероценови производни, никелоцен и бензохинон) и от вида на ензима (глюкозо-оксидаза и лактат-оксидаза).