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# Analytical Characteristics of Electrochemical Biosensors

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## Abstract

The goal of this work is the evaluation of the analytical characteristics of the determinations performed using glucose oxidase and acetylcholinesterase based electrochemical sensors, developed applying original or optimized conventional methods of enzyme immobilization. It was found that the sensitivity of glucose determination, for example, varies from 0.048 to 3.36 mA L $mol^{-1}$ $cm^{-2}$ and the response time of the glucose oxidase based sensors – from 5 to 30 s, according to the method of the bioreceptor immobilization. The sensitivity of the analysis is affected from the activity of the immobilized biocomponent, from the composition of the solution (concentration of the substrate, of the mediator and of the inhibitor), and from the experimental conditions (pH, temperature, agitation), as well as from the kinetic parameters of the studied process. It was found that the immobilized glucose oxidase conserves its substrate specificity in the presence of a number of glucides (galactose, maltose, fructose, and saccharose) in 100 fold higher concentrations. The selectivity of glucose analysis is ensured applying a suitable potential. Interferences free glucose amperometric determination was performed at 0.00 V/SCE, in the presence of ascorbates and urates. The electrochemical quantification of enzyme inhibitors allows reaching particularly low limits of detection ($10^{-9}$-$10^{-14}$ mol $L^{-1}$).

***Keywords:*** electrochemical biosensors, analytical characteristics, glucose oxidase, acetylcholinesterase.

## Introduction

The biosensors are relatively new analytical devices developed taking advantage of the progress in the biotechnology and the material science, in particular, in association with the modern principles of transduction of the chemical information.

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They represent a variety of chemical sensors, transforming the concentration of the quantified substance into an analytically useful signal [1].
The electrochemical biosensors provide selective quantitative or semi-quantitative analytical information using a biological recognition element (enzymes, whole cells, cell organelles or particles, tissues, etc.), in direct spatial contact with an electrochemical transducer, converting the signal produced by the interaction between the bioreceptor and the analyte, into electrical one [1]. They are able to provide as reliable, sensitive, reproducible and accurate measurements as the conventional analytical instruments do, but they are less expensive and time-consuming, do not require highly trained personal and could be used for real-time, on-line and on-site determinations [2].
The rapidly expanding field of biosensors application, their diversity and the need of validation impose the systematic description of their analytical capabilities using established standard protocols for evaluation of performance criteria, in accordance with standard IUPAC protocols or definitions. These include four set of parameters: (1) calibration characteristics - sensitivity, working and linear concentration range, detection and quantitative determination limits, (2) selectivity and reliability, (3) response time, and (4) reproducibility, stability and lifetime [1].
The goal of this work is the critical evaluation of the analytical characteristics of the determinations performed using an acetylcholinesterase (Ach) based electrochemical sensor for inhibitor detection and various glucose oxidase (GOD) based electrochemical sensors for glucose quantification, developed applying different original or optimized conventional methods of enzyme immobilization and selected as typical examples of biosensors for indirect inhibitor and direct analyte monitoring.

## Experimental

### *Reagents, instrumentation and procedures*

Glucose oxidase-biotinamidocaproyl labeled (EC 1.1.3.4, from Aspergillus niger, 110 units $mg^{-1}$), glucose oxidase ((EC 1.1.3.4, from Aspergillus niger, 115 units $mg^{-1}$), glucose oxidase ((EC 1.1.3.4, from *Aspergillus niger*, 1000 units $mg^{-1}$), and horseradish peroxidase (EC 1.11.1.7, type II, 180 units $mg^{-1}$) were purchased from Sigma. Acetylcholinesterase (EC 3.1.1.7, from bovine erythrocytes, 1000 units $mg^{-1}$) was supplied from BDH Ltd., England.
All other reagents were of analytical reagent grade and were used as such without any purification pretreatment.
The electrochemical studies with amperometric registration were performed using a potentiostat EG&G Princeton Applied Research 273A and an amperometric unit PRG-DEL Tacussel.
The investigations were carried out in a conventional type electrolysis cell. Pt was used as auxiliary electrode and a saturated calomel electrode as a reference. The working electrode from spectrally pure graphite (Ringsdorf Werke, Germany) or from glassy carbon (Tokay GC 20 or Tacussel CTV 101 T), after a preliminary treatment consisting in polishing, degreasing with alcohol and

ultrasonic cleaning, was modified applying different protocols for enzyme fixation, described in details in our previous works [3-19].

## Results and Discussion

### *Biosensors Configurations*

The six prototypes of electrochemical biosensors evaluated in this work were:

(1) Mono-enzyme sensor of first generation with biotin modified glucose oxidase (glucose oxidase-biotinamidocaproyl labeled) immobilized onto poly(pyrrol-biotin) using the affinity system avidin-biotin, for glucose determination [3].

The proposed original procedure for enzyme immobilization included the electrogeneration of a biotinylated conducting polypyrrole film with a homogeneous distribution of the polymerized biotin groups onto the electrode surface and subsequent grafting of the biotynilated glucose oxidase via avidin-biotin bridges. The method ensures a precise and controllable formation of a polymer film on microsurfaces of complex geometry, as well as a reproducible enzyme immobilization without loss of activity.

(2) Mono-enzyme sensor of first generation with glucose oxidase entrapped in electrogenerated polymer film, for glucose determination [4].

The biosensor fabrication involved a coadsorption of an aqueous amphiphilic pyrrole monomer-enzyme mixture on the electrode surface, solvent evaporation and electropolymerization of the monomers, leading to the entrapment of the enzyme into the polypyrrole matrix. The applied technique is economical, because of the preadsorption step requiring a small quantity of enzyme and monomer; biomolecule fixation is irreversible; the amount of entrapped biocomponent can be estimated through the difference between the enzyme quantity deposited onto the electrode surface and the amount lost during the electropolymerization, performed in a solution, free of enzyme and monomer.

(3) Mono-enzyme sensor of second generation with physically adsorbed glucose oxidase, for glucose quantification [4-7].

The enzyme immobilization was performed in static conditions, applying an optimized standard procedure. The optimal operational parameters for biomolecule fixation were established according to three criteria: minimal enzyme consumption, minimal process duration, and maximal sensitivity of the determinations.

(4) Mono-enzyme sensor of second generation with covalently fixed glucose oxidase, for glucose analysis [8].

The enzyme attachment resulted from the formation of amide bonds between the enzyme amino-groups and the electrogenerated, onto the graphite electrode surface, carboxyle groups. The formation of oxygen-containing moieties led to the improvement of the hydrophilic properties of the surface and allowed its activation with carbodiimide. N-cyclohexyl-N'[2-(N-methylmorpholino)ethyl]carbodiimide-4-toluene sulphonate was used because, in contrast to the low molecular carbodiimides, it does not favor the undesirable

condensation process with protein participation. It was demonstrated that the proposed immobilization methodology ensures the formation of a mono- or quasi mono-molecular enzyme layer with an optimal molecular proximity between the glucose oxidase active sites and the electrode surface, in absence of diffusion constraints.

(5) Bienzyme sensor based on the coimobilization of glucose oxidase and peroxidase (HRP) onto laponite, for cathodic detection of glucose [9].

This new biosensor configuration is based on the adsorptive immobilization of a peroxidase (HPR) within laponite gel doped by graphite nanoparticles and a subsequent electrochemical polymerization of 3,4-dihydroxybenzaldehyde (DHB) within the composite clay film in order to stabilize the inorganic layer and electrically connect HRP. The additional immobilization of glucose oxidase in an external clay layer led to a bienzyme electrode for glucose determination.

(6) Acetylcholinesterase sensor for inhibitor detection with covalent binding of the enzyme [11-18, 20-22].

Acetylcholinesterase, like glucose oxidase, contains an important number of secondary $NH_2$-groups with little importance for the stabilization of the structure of the enzyme and for its function. Because of that, acetylcholinesterase immobilization was performed according to the described above procedure for glucose oxidase covalent binding. In addition, the covalent immobilization preserves the biological stability of the enzyme and makes the developed biosensor suitable for inhibitor assay.

***Measuring Principle***

The measuring principle of the glucose oxidase based electrochemical sensors of first generation for glucose monitoring is illustrated in Fig. 1. The enzymatically generated $H_2O_2$ in amount proportional to glucose concentration was detected amperometrically at a potential of +0.60 V/SCE.

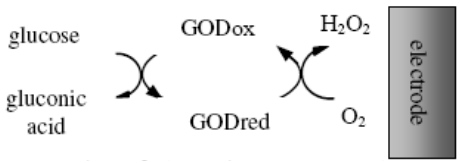


**Figure 1**. Measuring principle of the glucose oxidase sensors of first generation.

The measuring principle of the glucose oxidase based electrochemical sensors of second generation for glucose determination is presented in Fig. 2. p-benzoquinone was chosen as a mediator, because it can easily accept electrons from reduced glucose oxidase and its electrochemical behavior is well known.

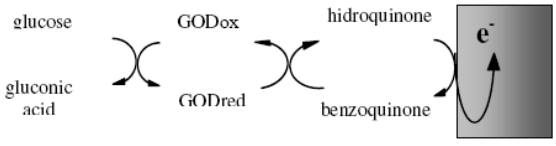


**Figure 2**. Measuring principle of the glucose oxidase sensors of second generation.

The proposed reaction mechanism of the composite clay glucose biosensor based on the coimmobilization of HRP and GOD is schematically described in Fig. 3. Since GOD catalyses the aerobic oxidation of glucose with concomitant production of $H_2O_2$, the latter was amperometrically detected via its electroenzymatic reduction at 0.00 V/SCE.

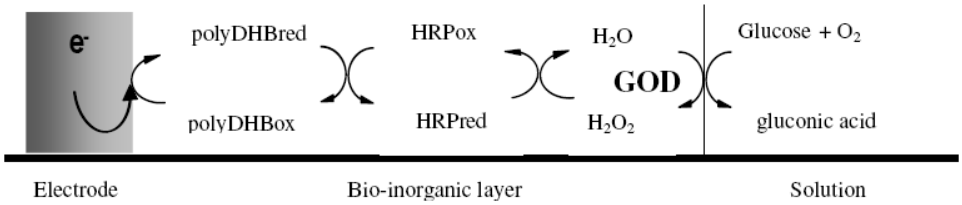


**Figure 3**. Measuring principle of the GOD/HRP sensor.

Fig. 4 illustrates the measuring principle of the acetylcholinesterase sensor for inhibitor detection. The determinations were based on the process of acetylthiocholine hydrolysis, catalyzed by the immobilized acetylcholinesterase, followed by the anodic oxidation, at a potential of +0.80 V/SCE, of the generated thiocholine. The analytical signal (the current of oxidation of thiocholine) decreased in the presence of enzyme inhibitors as a function of their concentration. This decrease resulted from the diminution of the concentration of the produced thiocholine, because of the inhibition of the enzyme activity.

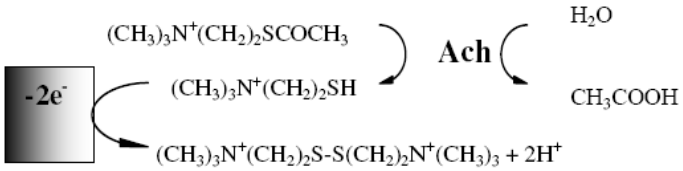


**Figure 4**. Measuring principle of the acetylcholinesterase based sensor for inhibitor detection.

***Analytical characteristics of the electrochemical biosensors***

The analytical characterization of the biosensors response included the evaluation of the following parameters: sensitivity, linear concentration range, limit of detection, specificity and selectivity, response time, stability and reproducibility, as defined by IUPAC [1]. The obtained data are presented in Table 1 and in Table 2.

The constructed calibration curves for glucose determination using the mono-enzyme sensor of first generation with biotin modified glucose oxidase were quasi-linear with glucose concentration up to 2 mmol $L^{-1}$ and curved gradually at higher concentrations. The biosensor sensitivity (determined as the slope of the initial linear part of the calibration curve) increased with the number of enzyme layers. These latter exhibited a similar kinetic behavior (the Michaelis-Menten constant kept almost the same value of 2-4 mmol $L^{-1}$). However, it was noted that the amperometric response of the biosensor was lower than the enzymatic activity. The difference was attributed to the diffusion of the major part of the enzymatically generated $H_2O_2$ into the bulk solution, rather than through the

hydrophobic polymer film. A possible approach for overcoming this problem consists of preparing an amphiphilic biotin monomer in order to confer a lesser hydrophobic character of the resulting biotinylated polypyrrole.

**Table 1**. Characteristics of the glucose oxidase based amperometric sensors for glucose quantification (ambient temperature and optimal other conditions).

| Method of immobilization | Linear dynamic range, (mol $L^{-1}$) | Limit of detection, (mol $L^{-1}$) | Sensitivity*, (mA L $mol^{-1}$ $cm^{-2}$) |
|---|---|---|---|
| Affinity binding of GOD via avidin-biotin | Up to $2x10^{-3}$ (7 layers) | | 3.36 |
| Entrapment of GOD in a polymer | Up to $2.5x10^{-3}$ | $1x10^{-7}$ | 19 |
| Adsorption of GOD onto graphite | Up to $4x10^{-2}$ | $1x10^{-6}$ | 0.048 |
| Covalent binding of GOD | Up to $1.6x10^{-2}$ | $1x10^{-6}$ | 11.17 |
| Adsorption of GOD and HRP in laponite | Up to $1x10^{-4}$ | | 14.95 |
| Covalent binding of Ach | Up to $4x10^{-4}$ | $1x10^{-5}$ | 99.08 |

| Method of immobilization | Response time, (s) | Loss of activity, (%) | Reproducibility (RSD, %) |
|---|---|---|---|
| Affinity binding of GOD via avidin-biotin | 10 (7 layers) | 80 % for 15 days (4 $^{o}$C) | <1% |
| Entrapment of GOD in a polymer | 10 | 53 % for 42 days (-18 $^{o}$C) | <5% |
| Adsorption of GOD onto graphite | 20 | 50% after 8 h of use<br>50 % for 7 days (4 $^{o}$C) | 2.85 |
| Covalent binding of GOD | 30 | 8 % for 21 days (4 $^{o}$C)<br>46 % after 8 h of use | 0.48 |
| Adsorption of GOD and HRP in laponite | 5 | 55 % for 21 days (4 $^{o}$C) | 4.5 |
| Covalent binding of Ach | 15 | 50 % for 40 days (4 $^{o}$C) | 2.50% |

| Method of immobilization | Selectivity and specificity | Interferences | Analytical application |
|---|---|---|---|
| Adsorption of GOD onto graphite | For glucose, in presence of fructose, maltose, sucrose and galactose | | Foodstuffs [6]<br>Medical analysis [4] |
| Covalent binding of GOD | | $HgCl_2$, 8-oxiquinoline, semicarbazide | Foodstuffs [7] |
| Adsorption of GOD and HRP in laponite | For glucose, in presence of ascorbates and urates | | |
| Covalent binding of Ach | | Specific and non-specific inhibitors** | Foodstuffs [15]<br>Pharmaceuticals [19]<br>Environmental monitoring [20, 21] |

* at 25 $^{o}$C and optimum pH, enzyme and mediator concentrations;
** chlorofos, oxophosphol, galantamine, caffeine, atropine, nicotinic acid, eserine; $Hg^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Mn^{2+}$, $Fe^{3+}$ and $AsO_3^{3-}$.

**Table 2.** Characteristics of the acetylcholinesterase based amperometric sensor for inhibitors determination, at ambient temperature, pH 8 and optimal other conditions ($r^2$>0.98; RSD<3 %).

| Inhibitor | Limit of detection, (mol $L^{-1}$) | Calibration function ΔI=a+blg[Inhibitor] | | Linear dynamic range, (mol $L^{-1}$) |
|---|---|---|---|---|
| | | a, (μA) | b, μA/p(mol $L^{-1}$) | |
| chlorofos | $10^{-9}$ | 5.14 | 0.60 | Up to $10^{-4}$ |
| oxophosphol | $10^{-10}$ | 7.28 | 0.80 | Up to $10^{-4}$ |
| galantamine | $10^{-10}$ | 5.81 | 0.66 | Up to $10^{-4}$ |
| nicotinic acid | $10^{-10}$ | 39.66 | 3.66 | Up to $10^{-4}$ |
| tiamine | $10^{-10}$ | 58.74 | 5.23 | Up to $10^{-4}$ |
| atropine | $10^{-10}$ | 24.19 | 1.87 | Up to $10^{-5}$ |
| caffeine | $10^{-10}$ | 18.31 | 1.76 | Up to $10^{-4}$ |
| quinine | $10^{-10}$ | 22.85 | 1.99 | Up to $10^{-4}$ |
| eserine | $10^{-9}$ | 17.07 | 1.71 | Up to $10^{-5}$ |

| Inhibitor | Limit of detection, (mol $L^{-1}$) | Calibration function ΔI=a+blg[Inhibitor] | | Linear dynamic range, mol $L^{-1}$ |
|---|---|---|---|---|
| | | a, (μA) | b, μA/p(mol $L^{-1}$) | |
| $Mn^{2+}$ | $10^{-11}$ | 2.80 | 0.24 | Up to $10^{-4}$ |
| $Cu^{2+}$ | $10^{-14}$ | 3.10 | 0.18 | Up to $10^{-2}$ |
| $Fe^{3+}$ | $10^{-10}$ | 1.66 | 0.15 | Up to $10^{-4}$ |
| $Cd^{2+}$ | $10^{-12}$ | 3.60 | 0.28 | Up to $10^{-2}$ |
| $Hg^{2+}$ | $10^{-10}$ | 3.72 | 3.72 | Up to $10^{-5}$ |
| $AsO_3^{3-}$ | $2x10^{-10}$ | 30.10 | 1.65 | Up to $2x10^{-8}$ |

ΔI – difference between the registered steady-state current in the absence and in the presence of inhibitor. p(mol $L^{-1}$) = -lg(mol $L^{-1}$)

The response time of the biosensor slightly increased from 5 to 10 s with the increase in enzyme layers. The storage stability of the electrodes modified with

2, 4 and 7 enzyme layers decreased to 50 % of its initial value, followed by stabilization in the range of 20-25 % for 15 days. The latter values remained almost constant after 38 and 64 days.

The relevant characteristics of the mono-enzyme sensor of first generation with glucose oxidase entrapped in electrogenerated polymer film for glucose determination were the fast response (10 s), the high sensitivity (19 mA L $mol^{-1}$ $cm^{-2}$) and the low limit of detection ($1x10^{-7}$ mol $L^{-1}$). The inconvenient of this sensor was the limited linear dynamic range (up to 2.5 mmol $L^{-1}$).

In contrast to the above described sensors, the mono-enzyme sensor of second generation with physically adsorbed glucose oxidase allowed glucose quantification in a large concentration range (up to $4x10^{-2}$ mol $L^{-1}$), with a sensitivity increasing with GOD and p-benzoquinone concentrations and reaching 0.048 mA L $mol^{-1}$ $cm^{-2}$ in conditions of saturation with enzyme and mediator.

The sensitivity was also affected from the experimental conditions, namely by the acidity of the solution, the optimum pH being equal to 6. The agitation had no effect, as the process was limited by the enzyme kinetics.

It was found that the immobilized glucose oxidase conserves its substrate specificity in the presence of number of glucides (galactose, maltose, fructose, saccharose) in 100 fold higher concentrations.

The response time of the biosensor was 20 s. The enzyme activity decreased with 50 % for 8 h of continuous use at ambient temperature or after one week of storage at 4 $^{o}$C. The lost of activity was attributed to the enzyme desorption.

Similar dependencies were observed applying, for glucose analysis, a mono-enzyme sensor of second generation with covalently fixed glucose oxidase. The linear concentration range was extended up to 16 mmol $L^{-1}$, with a slope of 11.15 mA L $mol^{-1}$ $cm^{-2}$ at ambient temperature and pH=5 (corresponding to the pH optimum). For similar other conditions, the sensitivity reached a maximal value at a temperature of 47 $^{o}$C.

95 % of the value of the steady-state current was attained for 30 s.

The activity of the covalently immobilized glucose oxidase decreased with 13 % for 4 h of exploitation at 23 $^{o}$C and with 46 % after 8 h of continuous use in the same conditions. Only 8 % loss of activity was observed during 24 days storage at 4 $^{o}$C.

The bienzyme electrode for glucose quantifications exhibited a limited linear part (up to $1x10^{-4}$ mol $L^{-1}$). It was suggested that a possible extension of the linearity could be obtained by increasing the thickness of the outer laponite layer containing the oxidase molecules.

The biosensor sensitivity increased with the amount of immobilized GOD, namely 0.57, 1.07 and 14.95 mA L $mol^{-1}$ $cm^{-2}$ for 5, 10 and 30 μg of GOD, respectively. This evolution seemed indicate that the limiting step in this bi-enzyme configuration is the reaction catalyzed by GOD. The sensitivity dropped to 55 % of its initial value after 3 weeks.

Most of the glucose biosensors are based on the electrooxidation of enzymatically generated $H_2O_2$. However, the amperometric detection of $H_2O_2$ requires high overpotentials and hence is faced with interferences in biological

fluids. Consequently the influence of easily oxidizable compounds present in biological fluids on the biosensor sensitivity was examined. No appreciable change in the steady-state current response of the biosensor to 100 μmol $L^{-1}$ glucose was observed for the successive injections of ascorbate and urate. This absence of interferences was attributed to the value (0.00 V/SCE) of the operating potential and represents the main advantage of this sensor.

The sensitivity of thiocholine determination applying the acetylthiocholine based sensor was found to be 99.08 mA L $mol^{-1}$ $cm^{-2}$ at conditions of saturation of the electrode surface with enzyme, ambient temperature, pH 7 (= $pH_{optimal}$), and electrode rotation speed of 1000 rpm. It varied as a function of the enumerated parameters. It was demonstrated that the process was controlled by the mass transport at low substrate concentrations (up to 1 mmol $L^{-1}$) and consequently, depended on the electrode rotation speed.

The sensitivity of the inhibitor quantification using the acetylcholinesterase based sensor was, in addition, affected by the substrate concentration. It increased with substrate concentration increase. However, in certain cases (chlorofos, galantamine), for saturated thiocholine concentrations, an inverse effect was registered. It could be explained with the competitive character of the inhibition. Because of that, at high substrate concentrations, the inhibition effect was not observed.

The upper limit of inhibitor detection was restricted in some cases, due to the enzyme activation (by thiamine, atropine, quinine, $Mn^{2+}$, $Fe^{3+}$) and the concurrent chemical ($Hg^{2+}$, $AsO_3^{3-}$), or electrochemical reactions (eserine).

The response time of the biosensor was 15 s. The loss of activity was of 50 % for 40 days when the sensor was stored at 4 $^{o}$C.

The electrochemical quantification of enzyme inhibitors allowed reaching particularly low limits of detection ($10^{-9}$-$10^{-14}$ mol $L^{-1}$).

## Conclusions

On the base of the presented results, advantageous electrochemical methods for the determination of enzyme substrates and inhibitors were developed, namely:

- a method for glucose quantification in human blood serum [5] and in foodstuffs [7, 8] in a large concentration range ($10^{-6}$-$1.6x10^{-2}$ mol/L). It possesses the same exactitude as the conventional spectrophotometric ones (relative error 0.23-3.70 %), but it is more rapid;
- a simple and rapid method for quinine quantification in soft drinks [16] with a low detection limit ($10^{-10}$ mol/L);
- a method for $AsO_3^{3-}$ quantification in waste waters [21]. It permits to solve the two main problems, related to As determination – lowering of the limit of detection ($2x10^{-10}$ mol $L^{-1}$) and distinction between its two oxidation states (AsIII and AsV);
- a sensitive method (0.15-3.72 μA/p(mol $L^{-1}$) for the determination of heavy metals in environmental samples [10, 22] with an important linear dynamic range and low detection limit ($2x10^{-10}$ – $10^{-14}$ mol $L^{-1}$);

- method for pharmaceutical (galantamine, nicotinic acid, thiamine, atropine, caffeine, eserine) analysis [13, 20];
- method for organophosphorous insecticide (chlorofos) analysis suitable for on-line and on-camp quantifications [13].

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