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A highly selective amperometric biosensor array for the simultaneous determination of glutamate, glucose, choline, acetylcholine, lactate and pyruvate

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ABSTRACT

The work was aimed at the development of a biosensor array for the simultaneous determination of six solutes (glutamate, glucose, choline, acetylcholine, lactate, and pyruvate) in aqueous solutions. Enzymes selective for these substrates were immobilized on the surface of amperometric platinum disc electrodes and served as bioselective elements of a biosensor array. Direct enzymatic analysis by the developed biosensors provided high sensitivity to the tested substrates (limits of detection were 1–5 μM). The linear ranges of the biosensors were from 0.001–0.01 mM to 0.2–2.5 mM. The influence of solution pH, ionic strength and buffer capacity on the biosensor responses was investigated; the conditions for simultaneous operation of all the bioselective elements were optimized. The absence of any cross-influence of the substrates of enzymatic systems used was shown as well as a high selectivity of the biosensors and the absence of any impact of interfering substances (ascorbic acid, dopamine, cysteine, paracetamol). The developed biosensor array had good response reproducibility and storage stability. The array is suitable for rapid (0.5–1 min) and simple simultaneous determination of glutamate, glucose, choline, acetylcholine, lactate, and pyruvate in aqueous (biological) samples; furthermore, the creation of a single chip with six sensitive elements is possible as well as the addition of other biosensors.
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1. Introduction

Fast, reliable and simple methods of simultaneous determination of concentration of several solutes are required for research purposes and applied tasks. Biosensors are easy-to-use and cheap devices able to provide a rapid and accurate analysis of solutes concentration [1]. If necessary, biosensors can be used for continuous monitoring in a real-time mode, for example, by combining biosensors with the microdialysis system or in vivo assays [2]. The significant advantage of biosensors is the possibility of connecting several biosensors in an array or multibiosensor, which allows simultaneous identification of several solutes in a single sample.

In biological research, the concentrations of neurotransmitters and metabolites attract considerable interest. Glutamate and acetylcholine are the most important excitatory neurotransmitters in the nervous system. Thus, the determination of their concentrations in the intercellular fluid of the brain is an urgent need for pharmacology and neurochemistry [3,4]. Choline is a precursor in the acetylcholine synthesis, so, the lack of choline can cause several nerve disorders [5,6]. The monitoring of choline and acetylcholine levels is important for the detection of neurodegenerative diseases such as neuromuscular and Alzheimer's, myasthenia, disorders of cholinergic neurotransmission [7,8].

Glucose is the main source of energy for the functioning of the brain and plays an important role in brain physiology. Disorders of glucose metabolism in the brain occurs in many diseases [9]. Pyruvate is formed from glucose as a final product of glycolysis and under aerobic conditions can be further oxidized to acetyl-coenzyme A, which enters the Krebs cycle. The level of pyruvate rises due to enhanced aerobic processes and because of its insufficient utilization by the pyruvate dehydrogenase complex. However, excessive pyruvate is rapidly converted either to lactate when anaerobic processes predominate or to acetyl-CoA at the prevalent aerobic processes [10]. The concentration of lactate is higher at hypoxia, as well as in case of disorders of the system of its utilization (liver and kidneys). Though lactate is considered to be the end metabolite in animals, there are data on the function of lactate as an energy source for the brain and its involvement in other processes, thus, the role of lactate is to be studied in more details [11,12].

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Therefore, simultaneous evaluation of the concentrations of glucose, pyruvate, and lactate is important to obtain a complete picture of the processes occurring in the body.

Determination of the glucose, glutamate and lactate concentrations is important to understand the dynamics of the energy balance of the brain [13,14]. Simultaneous monitoring of glucose, lactate, and glutamate is useful for the comprehension of energy-consuming processes and neurons communication in the brain [15]. Concentrations of the selected solutes in human blood and cerebrospinal fluid are presented in Table S1 in Supplementary information.

Therefore, the purpose of our work was to develop a biosensor array for the simultaneous measurement of the concentrations of neurotransmitters (glutamate, acetylcholine) and metabolites (glucose, lactate, pyruvate, choline) in samples of biological fluids.

Currently, the methods for determination of these solutes are usually based on high-performance liquid chromatography (usually combined with mass spectrometry) or capillary electrophoresis, as well as on spectrophotometry [16–18]. These methods are sensitive and selective, but are mainly used to analyze solutes separately. However, their modifications for multianalyte detection were recently described [19,20].

To date, several biosensor arrays and multibiosensors are known, which are capable of detecting some of the above solutes. Zhang and Wan developed an array of amperometric biosensors based on glassy carbon disk electrodes and oxidases immobilized on their surface [15]. The biosensors were designed to determine glucose, lactate, glutamate and hypoxanthine and were highly sensitive. However, their selectivity to the interferents was not investigated sufficiently (only the absence of an impact of ascorbic and uric acids was shown) and there were no data regarding the biosensor storage. In other work, Yao and Okano offered an amperometric biosensor array for the glutamate, acetylcholine and dopamine determination, which was based on platinum disk electrodes and enzymes immobilized on their surface [3]. Dopamine was determined rather non-selectively using an electrode with a Nafion® membrane. The selectivity of biosensors was well investigated, but their storage was not tested. In the work [21], a different approach was used when creating a multibiosensor for the determination of choline, glucose, glutamate, lactate, lysine and uric acid. The enzymes were immobilized on the surface of glassy carbon electrodes; luminescence arising as a result of the hydrogen peroxide oxidation was detected. However, neither the selectivity for interfering substances nor the storage conditions were investigated. Thus, none of the existing biosensor arrays can be used for the simultaneous determination of all six substances. Moreover, important analytical characteristics (crosstalk, diffusional behavior, selectivity and/or storage) of the described biosensor arrays were not investigated. The largest known array contained six biosensors and was based on optical transduction, not electrochemical.

In this paper, an amperometric biosensor array for the simultaneous determination of glucose, lactate, pyruvate, glutamate, choline and acetylcholine is presented and characterized in detail. It can be used to test the samples of blood or other physiological fluids.

2. Materials and methods

2.1. Materials

In the work, enzymes glutamate oxidase (GluOx, EC 1.4.3.11) from *Streptomyces* sp. (recombinant) with an activity of 7 U/mg from Yamasa Corporation (Japan), glucose oxidase (GOx, EC 1.1.3.4) from *Aspergillus niger* with an activity of 272 U/mg from Genzyme (UK), lactate oxidase (LOx, EC 1.1.3.2) from *Pedicoccus* sp. with an activity of 35 U/mg, pyruvate oxidase (PyrOx, EC 1.2.3.3) from *Aerococcus* sp. with an activity of 54 U/mg, choline oxidase (ChOx, EC 1.1.3.17) from *Alcaligenes* sp. with an activity of 15 U/mg, acetylcholinesterase (AChE, EC 3.1.1.7) from *Electrophorus electricus* with an activity of 426 U/mg, all from Sigma-Aldrich (USA), were used.

Bovine serum albumin (BSA, fraction V), glycerol, *m*-phenylenediamine, HEPES, 25% aqueous glutaraldehyde solution, polyvinyl alcohol photopolymer containing styrylpyridine groups (PVA-SbQ), magnesium nitrate, sodium lactate, sodium pyruvate, acetylcholine chloride, choline chloride, sodium glutamate, ascorbic acid, dopamine, uric acid, and HEPES were produced by Sigma-Aldrich (USA). Thiamine pyrophosphate (TPP) (lyophilisate for preparation of injections) was obtained from Biofarma (Ukraine), and potassium dihydrogen phosphate (KH_2PO_4) – from Helicon (Russia). Other compounds used in the work were of domestic production and had reagent purity grade.

The samples of blood serum were obtained from Kyiv municipal scientific and practical center of nephrology and hemodialysis (Ukraine).

2.2. Design of amperometric transducers

All the biosensors in the array contained similar amperometric transducers based on platinum disc electrodes. The transducers were in-lab made according to the previously published algorithm [22,23]. Length of the transducer was 6.5 cm, outer diameter – 3.5 mm. The transducer consisted of a glass capillary with a platinum wire (0.4 mm in diameter) inside and a contact pad (See Scheme 1).

Sensitive part of the transducer was periodically polished with sandpaper and alumina powder (particles of 0.1 μm and 0.05 μm) to restore the sensitivity of transducer. The platinum surface was treated with ethanol wetted cotton swab before the deposition of poly(phenylenediamine) membrane.

2.3. Modification of amperometric transducers with phenylenediamine

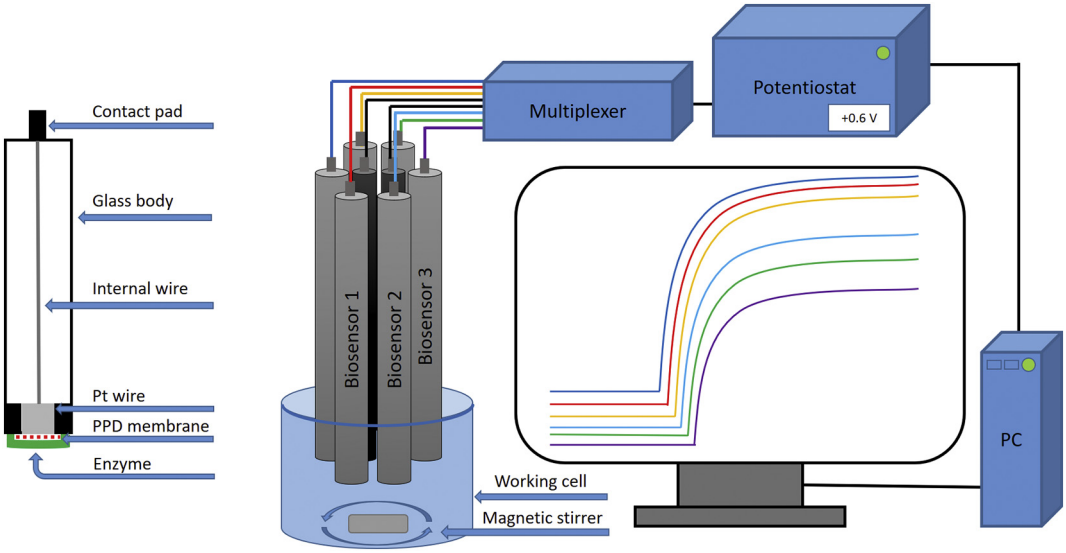
The selectivity of the amperometric transducer was improved by the deposition of an additional polymer membrane onto the sensitive region prior to the enzyme immobilization. The bare working electrode, auxiliary electrode and reference electrode were submerged in 5 mM solution of *m*-phenylenediamine in 10 mM potassium phosphate buffer, pH 7.4. Next, 10–15 cyclic voltammograms were received without stirring the solution. The voltammogram parameters were: initial potential 0 V, final potential +0.9 V, rate of the potential change 20 mV/s, step of the potential change 5 mV. The membranes were deposited on each transducer in turn, since it was impossible to get cyclic voltammograms for several electrodes simultaneously by the potentiostat used. Afterwards, the transducers were dried and the enzymes were immobilized on the poly(phenylenediamine) membrane.

2.4. Fabrication of biorecognition elements of biosensors

The biorecognition elements (except the biosensor for pyruvate determination) were obtained by covalent immobilization of enzymes and auxiliary substances on the surface of amperometric transducers. The crosslinking agent was glutaraldehyde, which forms covalent bonds with the amino groups of the enzyme and carrier proteins.

The initial solution contained 8% (hereafter – mass fraction) of GluOx, 4% of BSA, and 10% of glycerol in 100 mM phosphate buffer, pH 6.5. Glycerol was added to stabilize the enzyme during its immobilization, to prevent early drying and to improve the membrane adhesion to the transducer surface. This solution was mixed with 0.4% aqueous solution of glutaraldehyde in the ratio 1:1. Once this mixture was deposited onto the transducer surface, it was dried for 40 min in air at room temperature. The unbound components of the bioselective membrane and excessive glutaraldehyde were washed out with the working buffer solution.

The procedure of enzymes immobilization for other biosensors (except the biosensor for pyruvate determination) was similar but differed by the glutaraldehyde concentration and duration of immobilization, which were chosen in advance for each biosensor.



Scheme 1. Scheme of the single biosensor based on platinum disc electrode and scheme of the measurement setup which includes 6 biosensors, a reference and counter electrodes.

For the glucose biosensor the solution containing 5% of GOx, 3% of BSA, 10% of glycerol in 20 mM phosphate buffer, pH 6.5, was used. The GA concentration was 0.4%, the duration of immobilization - 40 min.

For the choline biosensor the solution containing 8% of ChOx, 4% of BSA, 10% of glycerol in 100 mM phosphate buffer, pH 6.5, was used. The GA concentration was 1.6%, the duration of immobilization - 10 min.

For the lactate determination the solution containing 8% of LOx, 4% of BSA, 10% of glycerol in 100 mM phosphate buffer, pH 6.5, was used. The GA concentration was 1%, the duration of immobilization - 40 min.

For the acetylcholine biosensor, two sequential immobilization procedures were carried out – first AChE was deposited, then ChOx. A solution containing 0.5% of AChE, 5% of BSA, 10% of glycerol in 100 mM phosphate buffer, pH 6.5, was mixed with 1% GA solution and deposited on the transducers for 10 min. Afterwards, a solution containing 8% of ChOx, 4% of BSA, 10% of glycerol in the same buffer, was mixed with 1.6% GA solution and immobilized for 10 min.

For the pyruvate biosensor, the biorecognition element was formed as follows. The solution containing 20% of PyrOx, 5% of BSA, 10% of glycerol in 100 mM phosphate buffer, pH 6.5, was used. It was mixed with 13.3% aqueous PVA-SbQ solution in the 1:1 ratio. Immediately afterwards, the mixture was deposited onto the sensitive surface of transducer and exposed to UV irradiation for 20 min using lamp KF-4 M.

2.5. Measurement procedure

Six amperometric biosensors, an auxiliary platinum electrode and an Ag/AgCl reference electrode (single-junction, with 3 M KCl internal filling solution) were connected to the PalmSens potentiostat (Palm Instruments BV, The Netherlands) through the 8-channel device (CH-8 multiplexer, the same manufacturer). The biosensors were located in the Teflon® holder in a circle around shared auxiliary and reference electrodes, the distance between the adjacent electrodes was 2 mm. Total diameter of the biosensor array was about 2.5 cm. Measurements were performed at room temperature in an open 4 ml cell with constant stirring and at the constant potential of +0.6 V vs Ag/AgCl reference electrode (technique “amperometric detection”, or constant potential amperometry). Scheme of a single biosensor and overall measuring setup is presented in Schematic 1.

Twenty-five mM HEPES, pH 7.4, was utilized as a working buffer. For the pyruvate biosensor it was necessary to add also cofactors of PyrOx – magnesium ions (120 μM), TPP (500 μM), and phosphate ions (20 mM KH_2PO_4). The substrate concentrations in the working cell were obtained by addition of the aliquots of stock solutions (50 mM – 1 mM). All measurements were carried out in three replications; points on the graphs represent mean values of results.

3. Results and discussion

3.1. Principle of the biosensor array

The operation of the biosensor array is based on enzymatic reactions in the biorecognition elements of biosensors: reaction 1 - for glutamate determination; reaction 2 – for glucose determination; reactions 3 and 4 - for acetylcholine determination; reaction 4 - for choline determination; reaction 5 - for lactate determination; reaction 6 - for pyruvate determination. The reactions 1–6 result in oxidation of substrates (glutamate, glucose, acetylcholine, choline, lactate, and pyruvate) and the formation of electrochemically active hydrogen peroxide. At a positive potential (+0.6 V) on the electrode, the decomposition of hydrogen peroxide occurs (reaction 7), which results in the formation of electrons. These electrons are directly registered by the amperometric transducer:

$$\text{Glutamate} + O_2 \xrightarrow{\text{GluOx}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2 \quad (1)$$

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOx}} \text{D-gluconolactone} + H_2O_2 \quad (2)$$

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + CH_3COO^- + H^+ \quad (3)$$

$$\text{Choline} + O_2 \xrightarrow{\text{ChOx}} \text{betaine aldehyde} + H_2O_2 \quad (4)$$

$$\text{Lactate} + O_2 \xrightarrow{\text{LOx}} \text{pyruvate} + H_2O_2 \quad (5)$$

$$\text{Pyruvate} + PO_4^{3-} + H_2O + O_2 \xrightarrow{\text{PyrOx}} \text{acetyl phosphate} + H_2O_2 + CO_2 \quad (6)$$

$$H_2O_2 \xrightarrow{+600\ \text{mV}} 2H^+ + O_2 + 2e^- \quad (7)$$

Since working electrodes have an intermediate size between macroelectrodes and microelectrodes (0.4 mm), we examined whether the electrodes exhibited hemispherical diffusion behavior (typical for microelectrodes) or linear diffusion (typical for conventional macroelectrodes). Linear diffusion occurs when the diffusion layer is much thinner than the smallest dimension of the electrode, otherwise

hemispherical (radial) diffusion (or both) takes place [24]. Types of diffusion can be distinguished by cyclic voltammetry in a solution with redox species at slow scan rate – the oxidation and reduction peaks followed by a decrease of current are observed in case of linear diffusion, whereas no clear peak (e.g., steady-state current with a plateau) occurs in case of hemispherical diffusion [25]. Hemispherical diffusion is considered more effective, but in this case individual diffusion layers of the electrodes can overlap and cause crosstalk between the electrodes.

To examine the contribution of linear and hemispherical diffusion to the total response of the working electrodes, the cyclic voltammograms were obtained in 2 mM $K_4Fe(CN)_6$ with 1 M KCl prepared in purified water (Fig. S1 in Supplementary information). Clear redox peaks were observed at most scan rates, which indicates that linear diffusion is predominant. Visible peak disappears at scan rates < 5 mV/s, showing a transition from linear diffusion to hemispherical diffusion caused by increased thickness of the diffusion layer in this case. Thus, the working electrodes demonstrate macroelectrode behavior at high scan rates and microelectrode behavior at slow scan rates, which is typical for electrodes with intermediate size and this effect can be used for improvement of the biosensor sensitivity by exploiting improved mass transport at slow scan rates [26]. For comparison, voltammograms at the platinum microelectrode (12.5 μm radius) demonstrated redox peak only at scan rate ≥ 200 mV/S (Fig. 3 in [27]). Furthermore, it should be taken into account that during long-term experiments (e.g., 10 min) and without stirring of the working buffer, diffusion layers of the electrodes can overlap and thus electrode crosstalk can occur.

3.2. Influence of buffer pH on the biosensors operation

Any enzyme is characterized by the working range of pH and optimum pH value. For the enzymes used in this work, the manufacturers claim optimum pH for GluOx of about 7–9; GOx −5.5 (working range 4–7); ChOx – 8.0–8.5; AChE – 8.0; LOx – 6.5; PyrOx – pH 6.7. However, optimal pH values often change after enzyme immobilization and shift to either alkaline or acidic region. Occasionally, immobilization results in a significantly wider working range of pH of the enzyme [28]. Since in our case the enzymes with different optimum pH are used, it was necessary to determine the pH value, which would be suitable for the simultaneous operation of all biosensors.

In the experiment, a universal ("polymix") buffer was used, which contained tris-HCl, KH_2PO_4, citric acid and sodium tetraborate (5 mM each). This buffer has approximately the same buffer capacity in a wide range of pH. The biosensors responses to substrates at different pH are shown in Fig. 1. The optimum range of pH was: for the determination of glutamate – 7–9; glucose – 7.5–9; choline – 7.5–9.5; acetylcholine – 8–8.5; lactate – 7.3–8.5; pyruvate – 7–8. For further work, pH 7.4 was selected, as this value is within optimal working ranges of all (except acetylcholine) biosensors; additionally, it corresponds to pH of blood and other biological fluids (Fig. 1).

3.3. Effect of buffer capacity on the biosensor responses

The properties of the solution, in which the measurements are made, affect the biosensor operation. In particular, changes in the concentration of working buffer or addition of a biological sample to the biosensor cell cause the change in buffer capacity, which may affect the biosensor work. Therefore, the responses of the biosensor array were measured at different concentrations of the buffer solution (Fig. 2).

As seen, the responses of all biosensors changed insignificantly at different buffer concentration. The response time also remained the same. Therefore, the proposed biosensor array can be used in biological samples, which are characterized by different buffer capacities.

3.4. An influence of ionic strength of buffer solution on the biosensors work

Ionic strength is one of the basic characteristics of the sample, which can also affect the performance of biosensors. The solution can contain metal ions, as well as ions of organic and inorganic acids, which are present in biological fluids. The ionic strength of the solution also varies depending on the concentration of the buffer solution.

Therefore, the biosensor work was investigated at various values of ionic strength. The NaCl solution as a source of ions was added to the working cell in different aliquots to obtain concentrations in the range of 0 to 50 mM. Then, the responses to corresponding substrates were measured.

The results presented in Fig. 3 show that no significant changes in the biosensor responses were observed, which is typical for amperometric biosensors. This indicates the possibility of using the biosensor array in biological samples with different ionic strength.

3.5. Reproducibility of responses of the biosensor array

Since the proposed biosensor array is designed for multiple measurements, good reproducibility of responses is important for its reliable work. To obtain accurate results of measurement of the substrates concentrations in the solution, the responses of relevant biosensors should

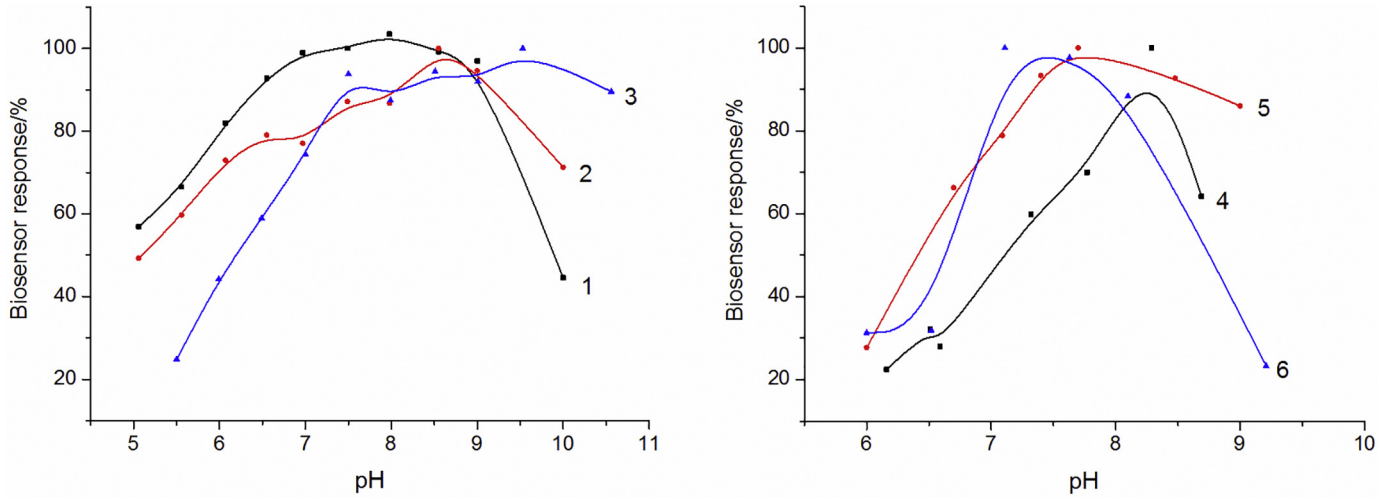


Fig. 1. Dependence of responses of biosensor array on the buffer pH. The measurements were carried out in a 5 mM universal buffer at a constant potential of +0.6 V vs Ag/AgCl reference electrode. The responses to the following substrates are shown: glutamate (1), glucose (2), choline (3), acetylcholine (4), lactate (5), pyruvate (6). The maximum value for each biosensor was taken as 100%.

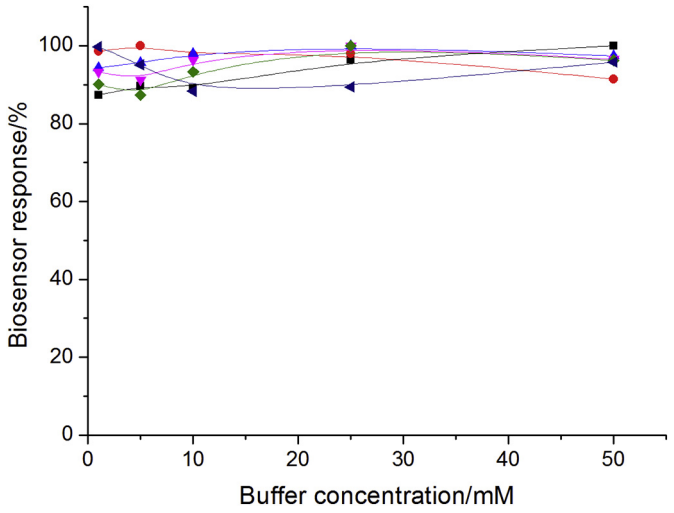


Fig. 2. Dependence of responses of the biosensor array on buffer capacity of the solution. Responses to the following substrates are shown: glutamate (black squares), glucose (red circles), choline (blue triangles), acetylcholine (pink triangles), lactate (green rhombuses), pyruvate (dark blue triangles). Measurements were carried out in HEPES buffer, pH 7.4, at different concentrations; a constant potential +0.6 V vs Ag/AgCl reference electrode. The maximum value for each biosensor was taken as 100%.

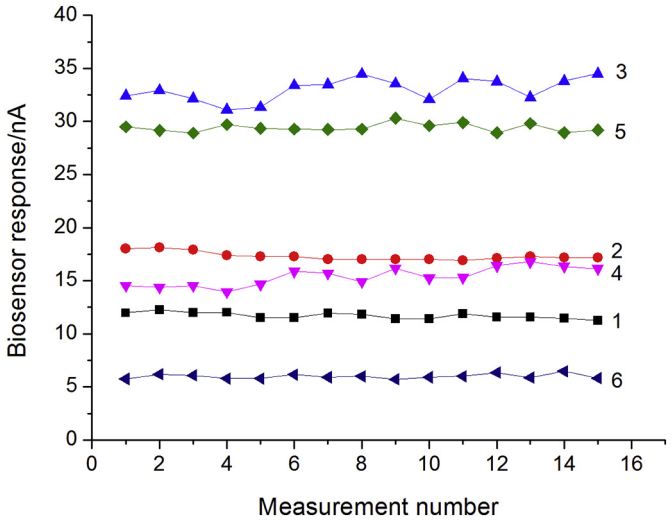


Fig. 4. Reproducibility of responses of biosensor array during 15 measurements. Concentrations of substrates in the cell: glutamate (1) - 50 μM, glucose (2) - 100 μM, choline (3) - 100 μM, acetylcholine (4) - 100 μM, lactate (5) - 50 μM, pyruvate (6) - 250 μM. The measurements were carried out in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl reference electrode.

be practically the same throughout the work, especially if small concentrations are to be measured. Therefore, the response reproducibility was investigated over several hours of continuous operation.

The biosensors all the time were in the working buffer, which was constantly stirred. Each measurement took 3 to 5 min. During the intervals between measurements (about 10 min) the biosensors were washed from substrates by the working buffer. The substrate concentrations were on the linear parts of the calibration curves for all tested biosensors.

The results are shown in Fig. 4. All biosensors demonstrated high response reproducibility; relative standard deviation did not exceed 5% (see Table 1). The responses did not change over time, which proves the possibility of efficient use of the biosensor array for multiple measurements all over the day.

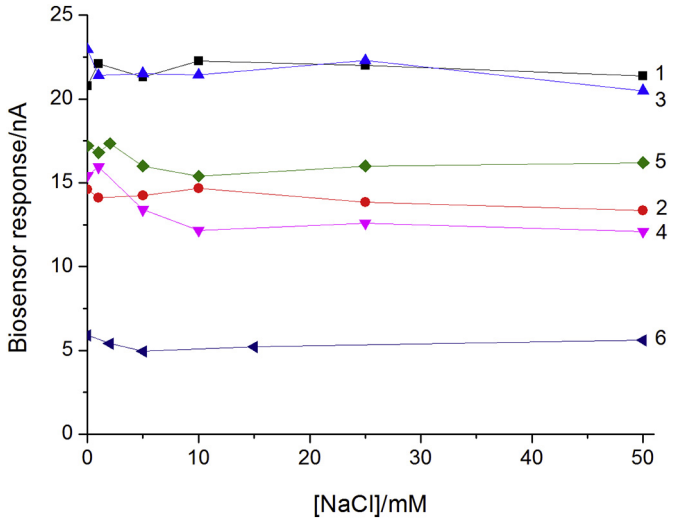


Fig. 3. Dependence of responses of biosensor array on the solution ionic strength (various NaCl concentrations). Concentrations of substrates in the cell: glutamate (1) - 100 μM, glucose (2) - 100 μM, choline (3) - 100 μM, acetylcholine (4) - 100 μM, lactate (5) - 50 μM, pyruvate (6) - 250 μM. The measurements were carried out in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl reference electrode.

3.6. Selectivity of the biosensor array

The sensitivity of biosensors to interfering species is an important factor when working with real biological fluids. In the present work, we used the mediator-free biosensor with a relatively high working potential (+0.6 V vs Ag/AgCl reference electrode), in which the oxidation of a number of electrically active compounds on the electrode surface is possible. In order to avoid this effect, a poly(phenylenediamine) film was deposited on the electrode surface. Such membrane restricts the diffusion of large molecules to the transducer surface but allows an access of hydrogen peroxide (product of enzymatic reactions, which is directly oxidized during the biosensor measurements).

A simple and effective method of deposition of additional membranes is the electropolymerization of molecules on the electrode surface at a certain potential (Fig. S2 in Supplementary information). As a result, a film with pores of definite size is formed. Of a wide class of oxy- and amino-aromatic molecules capable of electropolymerization, the phenylenediamine isomers are used in biosensors most commonly [29,30]. In these papers, a number of comparative studies were conducted on the properties of polymeric membranes based on poly (phenylenediamine) obtained from different monomers. The transducers modified by a polymeric film based on *m*-phenylenediamine were shown to have the best selectivity. Therefore, in our work this monomer was used to create an additional membrane on the platinum electrode surface (more experimental details in Section 2.3). The membrane thickness was not evaluated since it is not important for the present work. Other authors estimated the thickness of the similarly produced membrane as 15 nm [30]. To confirm the effectiveness of the film, we tested the biosensor sensitivity to such electroactive compounds as dopamine (6 μM), cysteine (300 μM), ascorbic acid (120 μM), uric acid (450 μM) and paracetamol (100 μM). The responses to all solutes were negligible (<0.1 nA) and could not interfere with the biosensor measurements. Furthermore, responses of the biosensor array to the target analytes in the presence and in the absence of the interfering substances were similar (Fig. S3 in the Supplementary information). Noteworthy, the membrane prevents oxidation of the electroactive substances only within the time scale of the experiment, but the solutes might eventually reach the electrode during a long-term incubation.

Oxidation of poly(phenylenediamine) was not observed under the working conditions. Furthermore, poly(phenylenediamine) is a

Table 1
Analytical characteristics of the biosensor array.

Characteristic	Glutamate biosensor	Choline biosensor	Acetylcholine biosensor	Glucose biosensor	Lactate biosensor	Pyruvate biosensor
Response time, min	0.5	0.5	1	0.5	0.5	1
Sensitivity to substrate, nA/mM	170–200	130–150	100–120	150–170	440–460	35–40
Linear range of substrate determination, mM	0.005–0.7	0.001–0.2	0.01–0.3	0.01–2	0.005–0.4	0.01–2.5
Limit of determination of substrate, μM	1	2	3	1	3	5
Reproducibility of responses over one working day (RSD), %	2.5	3	5	2	2	4
Storage, months	2.5	1.5	1	3	1	1

semi-conductive material and basically it can be a mediator of the electrons between enzymes and electrode, but only if it is in close contact with the enzymes (i.e., phenylenediamine polymerization takes place in the enzyme solution, so that the enzymes are captured in the polymer network). In our case only the bottom layer of the enzymatic membrane was in contact with the polymer membrane, thus direct electron transfer was insignificant.

Application of redox polymers or mediators could be an alternative approach to improve the selectivity of biosensors. The redox polymer or mediator could transfer electrons between the electrode and the enzymes at a low working potential and thus prevent oxidation of the interfering substances. However, it is known that mediators or redox polymers can effectively transfer electrons from electroactive species present in solution, and thus conditions for the interference-free electron transfer between an enzyme and an electrode should be carefully optimized [31,32]. The poly(phenylenediamine) membrane appeared to be efficient enough for the presented biosensor array and thus redox polymers or mediators were not investigated.

Furthermore, it was necessary to study the cross-effect of substrates, that is, to examine the sensitivity of every biosensor in the array to the substrates of other biosensors. The undesirable sensitivity to several substrates may occur either if the enzyme in the biorecognition element catalyzes the oxidation of a number of different compounds, or if the biorecognition element consists of several enzymes (like in the biosensor for acetylcholine determination). In the experiment, glutamate, glucose, choline, acetylcholine, lactate, and pyruvate in the concentration of 1 mM were added to the working cell. It was found that each biosensor is sensitive only to the corresponding substrate, and no response to other substrates was generated (Table S2 in Supplementary information), except for acetylcholine. Additionally, there was no cross-effect of the substrates, that is, the responses of biosensors in the array were completely independent and the presence of one substrate did not affect the determination of another substrate. Furthermore, shape of the calibration curve for each substrate did not change in the presence of 1 mM of other substrates (except for acetylcholine). The cross-sensitivity could arise if hydrogen peroxide from the biorecognition element of one biosensor diffuse to adjacent biosensor, causing the response of the latter. However, insufficient amount of hydrogen peroxide was probably formed to be registered by adjacent biosensor. The biosensor for acetylcholine detection was sensitive not only to acetylcholine but also to choline, because the biorecognition element of the biosensor contains acetylcholinesterase and choline oxidase. The response to choline was about 109% of that to acetylcholine. For this reason, for analysis of real samples the choline and acetylcholine biosensors should be used together, so that choline and acetylcholine concentrations could be determined. The rest of biosensors were highly selective to the corresponding target substrates and can be used to analyze real multicomponent samples.

3.7. Analytical characteristics of the biosensor array

Typical calibration curves for substrates determination are shown in Fig. 5, and analytical characteristics of the biosensor array – in Table 1.

According to IUPAC, response time was estimated as the time necessary to reach 90% of the steady-state response [33]. For all biosensors, it did not exceed 1 min. The response time was moderate probably due to a relatively thick manually casted enzymatic membrane and a poly (phenylenediamine) membrane, which slowed down the diffusion. It is likely that the response time can be improved by application of thinner membranes or by measuring the slope of the response instead of a steady-state value.

The biosensor for pyruvate determination had the lowest sensitivity, probably due to the loss of activity of PyrOx during immobilization. The response reproducibility of biosensors during the day was high, which allowed their multiple usages for accurate measurements (see Section 3.5).

The sensitivity of biosensors was sufficient for analysis of both blood plasma and cerebrospinal fluid except for acetylcholine, the typical concentration of which is below the limit of detection of the biosensor array (Table S1 in Supplementary information). Thus, it is necessary to increase the sensitivity of acetylcholine biosensor later on. Considering the sample volume required for the analysis (800 μl of blood serum or 2 ml of cerebrospinal fluid) it can be estimated that blood samples from a human subject can be collected with any periodicity. Cerebrospinal fluid can be analyzed with at least 2-h intervals since in adult humans the volume of cerebrospinal fluid is 125–150 ml, and it is produced at a rate of 25 ml/h [34]. It is known also that 2 ml of cerebrospinal fluid can be taken at a time from human for clinical diagnostics [35].

Concentration of dissolved oxygen can affect the biosensor performance. All oxidases that are used in this work require O_2 and thus the rate of reaction can be limited by an insufficient oxygen concentration. However, during in vitro analysis, there is enough oxygen for the reactions with low concentrations of target substances (<1 mM) and usually the potential influence of oxygen concentration is being neglected. If high concentrations of target substances in samples are expected, a saturation of working buffer and samples with oxygen can be carried out. As was shown previously with GOx-based biosensor, an increase in oxygen concentration (partial pressure) from typical blood values of 50–100 mm Hg to 150 mm Hg leads to the extension of the linear range of the biosensor, but does not affect the detection of glucose below 3 mM; the biosensor was also functional at 7 mm Hg at glucose concentrations below 3 mM [36].

Storage stability of the biosensor array was studied in different conditions. The best biosensor storage was observed at −18 °C – these results are given in the Table 1. The biosensors for glucose and glutamate were the most stable. Basically, amperometric transducers can be stored for a long time without degradation of characteristics, and the observed discrepancy can be explained by different stability of immobilized enzymes.

To demonstrate applicability of the biosensor array, three samples of blood serum from different people were analyzed (Table S3 in Supplementary information). Spectrophotometric method of determination of the substances was adapted from [37] and used as a control. The difference between biosensor and control methods did not exceed 10%. The obtained results demonstrate that the biosensor system is suitable for the analysis of real samples.

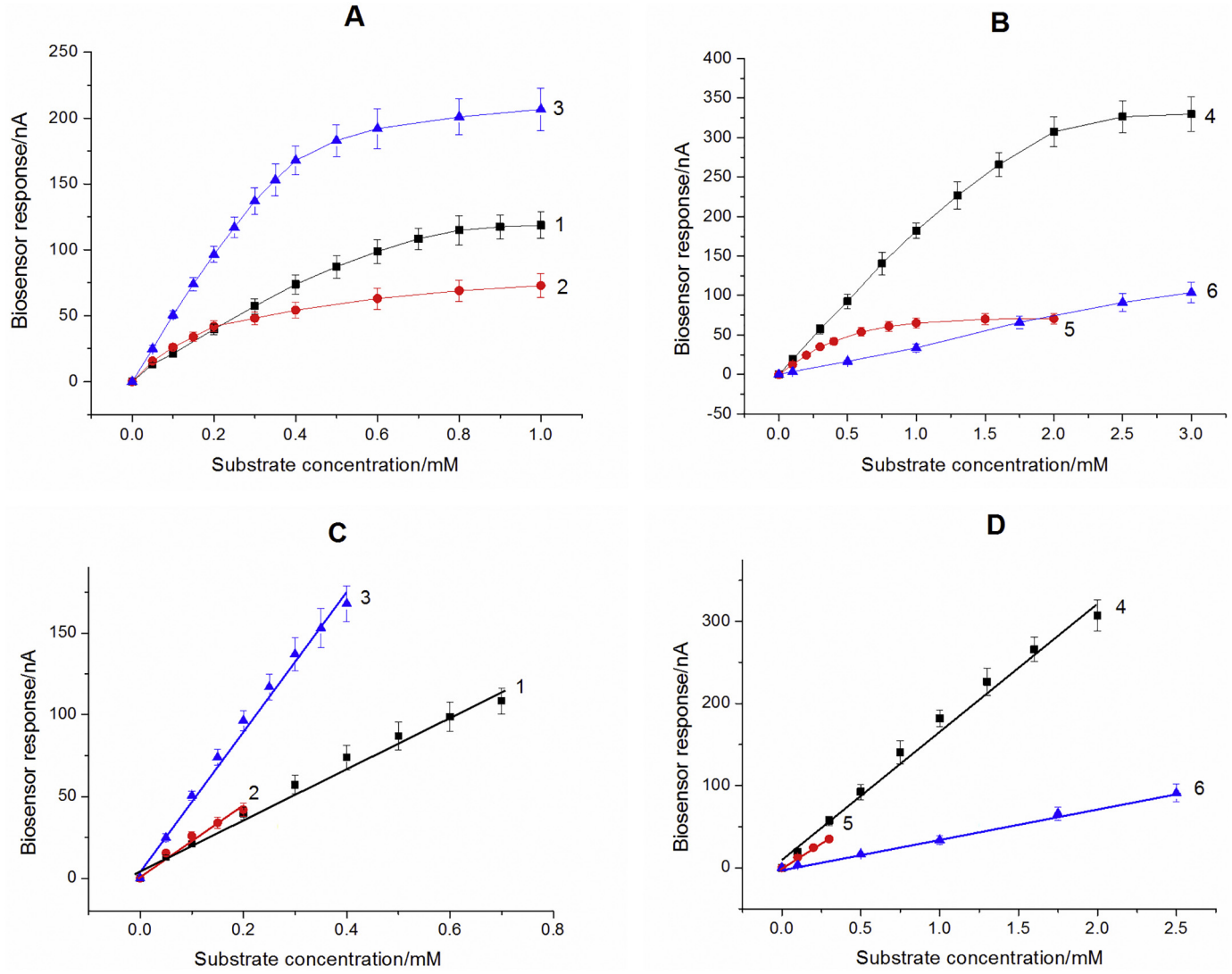


Fig. 5. Calibration curves of biosensor array for determination of: 1 – glutamate ($R^2 = 0.9862$), 2 – choline ($R^2 = 0.9699$), 3 – lactate ($R^2 = 0.9918$), 4 – glucose ($R^2 = 0.9885$), 5 – acetylcholine ($R^2 = 0.9969$), 6 – pyruvate ($R^2 = 0.9976$). Full working range of the biosensors is shown in A and B, and linear range is shown in C and D. The measurements were carried out in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl reference electrode.

Noteworthy, all biosensors are based on identical transducers; the procedures of their preparation are similar. It simplifies mass production and potentially makes it possible to produce a multibiosensor based on a single transducer with six sensitive sites. An exception was the biosensor for pyruvate determination - we failed to develop an efficient procedure of pyruvate oxidase immobilization by GA cross-linking, and had to use its encapsulation in the photopolymer. Similar negative results with PyrOx immobilization via GA are described by other authors [38]. For manufacturing it would be better to use screen-printed platinum electrodes which are much cheaper, compatible with common industry practices and do not require polishing. Furthermore, usage of constant potential amperometry could allow simultaneous deposition of PPD membranes on all electrodes in contrast to the one-by-one deposition by CV used in this work.

Pt was chosen as an electrode material since it catalyzes H_2O_2 decomposition and thus Pt-based electrodes are the most sensitive towards hydrogen peroxide [39,40]. Moreover, though relatively large amount of Pt is required, Pt disk electrode can be used repeatedly by renewing immobilized enzyme layer. To decrease the biosensor cost, other materials like graphite can be used, but metallization with Pt nanoparticles or functionalization of other kind would be required to obtain the necessary sensitivity.

The array is promising for utilization in a flow cell coupled with the microdialysis system, for real time measurements of the substrates concentration in tissues. In this case, it would be necessary to evaluate again cross-talk between the biosensors in array, response time and other analytical characteristics of the array since they are likely to change.

4. Conclusions

For the first time, an array of biosensors was developed for the simultaneous determination of six solutes: glutamate, glucose, choline, acetylcholine, lactate and pyruvate. The analytical characteristics of the developed biosensors – sensitivity, response time, linear range and limit of detection were investigated. High selectivity and good reproducibility of responses (the standard deviation did not exceed 5%) were shown.

The influence of parameters of the working buffer (pH, ionic strength and buffer capacity) on the biosensor array characteristics was investigated. The optimum pH for the determination of glutamate was 7–9; glucose – 7.5–9; choline – 7.5–9.5; acetylcholine – 8–8.5; lactate – 7.3–8.5; pyruvate – 7–8. pH 7.4 was selected as optimal since it corresponds to the pH value of blood and other biological fluids. It was shown that ionic strength and buffer capacity of the working buffer

did not affect the biosensors responses, which allows an application of the biosensor array for the analysis of solutions characterized by different ionic strength and buffer capacity. The biosensor array can be stored for a month, the biosensors for glutamate and glucose – 2.5–3 months. Thus, the developed biosensor array is suitable for further optimization and measurements in real biological samples. To expand the spectrum of solutes to be measured, the addition of other biosensors to the array can be considered.

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Declarations of interest

None.

Competing interests statement

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2019.03.010.

References

[1] J. Kirsch, C. Siltanen, Q. Zhou, A. Revzin, A. Simonian, Biosensor technology: recent advances in threat agent detection and medicine, Chem. Soc. Rev. 42 (2013) 8733, https://doi.org/10.1039/c3cs60141b.
[2] O.N. Schuvailo, S.V. Dzyadevych, A.V. El'skaya, S. Gautier-Sauvigné, E. Csöregi, R. Cespuglio, A.P. Soldatkin, Carbon fibre-based microbiosensors for in vivo measurements of acetylcholine and choline, Biosens. Bioelectron. 21 (2005) 87–94, https://doi.org/10.1016/j.bios.2004.09.017.
[3] T. Yao, G. Okano, Simultaneous determination of L-glutamate, acetylcholine and dopamine in rat brain by a flow-injection biosensor system with microdialysis sampling, Anal. Sci. 24 (2008) 1469–1473http://www.ncbi.nlm.nih.gov/pubmed/18997377.
[4] T. Borisova, D. Kucherenko, O. Soldatkin, I. Kucherenko, A. Pastukhov, A. Nazarova, M. Galkin, A. Borysov, N. Krisanova, A. Soldatkin, A. El'skaya, An amperometric glutamate biosensor for monitoring glutamate release from brain nerve terminals and in blood plasma, Anal. Chim. Acta 1022 (2018) 113–123, https://doi.org/10.1016/j.aca.2018.03.015.
[5] J.A. Dani, D. Ji, F.-M. Zhou, Synaptic plasticity and nicotine addiction, Neuron 31 (2001) 349–352, https://doi.org/10.1016/S0896-6273(01)00379-8.
[6] R.J. Wurtman, M. Cansev, I.H. Ulus, Choline and its products acetylcholine and phosphatidylcholine, Handb. Neurochem. Mol. Neurobiol, Springer US, Boston, MA 2009, pp. 443–501, https://doi.org/10.1007/978-0-387-30378-9_18.
[7] D.D. Wise, T.V. Barkhimer, P.-A. Brault, J.R. Kirchhoff, W.S. Messer, R.A. Hudson, Internal standard method for the measurement of choline and acetylcholine by capillary electrophoresis with electrochemical detection, J. Chromatogr. B 775 (2002) 49–56, https://doi.org/10.1016/S1570-0232(02)00269-6.
[8] S. Upadhyay, G.R. Rao, M.K. Sharma, B.K. Bhattacharya, V.K. Rao, R. Vijayaraghavan, Immobilization of acetylcholineesterase–choline oxidase on a gold–platinum bimetallic nanoparticles modified glassy carbon electrode for the sensitive detection of organophosphate pesticides, carbamates and nerve agents, Biosens. Bioelectron. 25 (2009) 832–838, https://doi.org/10.1016/j.bios.2009.08.036.
[9] P. Mergenthaler, U. Lindauer, G.A. Dienel, A. Meisel, Sugar for the brain: the role of glucose in physiological and pathological brain function, Trends Neurosci. 36 (2013) 587–597, https://doi.org/10.1016/j.tins.2013.07.001.
[10] M. Fisher, in: David L. Nelson, Michael M. Cox (Eds.), Lehninger Principles of Biochemistry, 3rd edition, 6, Chem. Educ. 2001, pp. 69–70, https://doi.org/10.1007/s00897000455a.
[11] L.B. Gladden, Lactate metabolism: a new paradigm for the third millennium, J. Physiol. 558 (2004) 5–30, https://doi.org/10.1113/jphysiol.2003.058701.
[12] L. Riske, R.K. Thomas, G.B. Baker, S.M. Dursun, Lactate in the brain: an update on its relevance to brain energy, neurons, glia and panic disorder, Ther. Adv. Psychopharmacol. 7 (2017) 85–89, https://doi.org/10.1177/2045125316675579.
[13] M.G. Boutelle, L.K. Fellows, C. Cook, Enzyme packed bed system for the on-line measurement of glucose, glutamate, and lactate in brain microdialyzate, Anal. Chem. 64 (1992) 1790–1794, https://doi.org/10.1021/ac00041a010.
[14] N.F. Shram, L.I. Netchiporouk, C. Martelet, N. Jaffrezic-Renault, C. Bonnet, R. Cespuglio, In vivo voltammetric detection of rat brain lactate with carbon fiber microelectrodes coated with lactate oxidase, Anal. Chem. 70 (1998) 2618–2622, https://doi.org/10.1021/ac971299f.
[15] F.-F. Zhang, Q. Wan, C.-X. Li, X.-L. Wang, Z.-Q. Zhu, Y.-Z. Xian, L.-T. Jin, K. Yamamoto, Simultaneous assay of glucose, lactate, L-glutamate and hypoxanthine levels in a rat striatum using enzyme electrodes based on neutral red-doped silica nanoparticles, Anal. Bioanal. Chem. 380 (2004) 637–642, https://doi.org/10.1007/s00216-004-2804-x.
[16] J. Woitzik, N. Abromeit, F. Schaefer, Measurement of nitric oxide metabolites in brain microdialysates by a sensitive fluorometric high-performance liquid chromatography assay, Anal. Biochem. 289 (2001) 10–17, https://doi.org/10.1006/abio.2000.4893.
[17] J. Zhou, D.M. Heckert, H. Zuo, C.E. Lunte, S.M. Lunte, On-line coupling of in vivo microdialysis with capillary electrophoresis/electrochemistry, Anal. Chim. Acta 379 (1999) 307–317, https://doi.org/10.1016/S0003-2670(98)00547-9.
[18] M.A. Malone, H. Zuo, S.M. Lunte, M.R. Smyth, Determination of tryptophan and kynurenine in brain microdialysis samples by capillary electrophoresis with electrochemical detection, J. Chromatogr. A 700 (1995) 73–80, https://doi.org/10.1016/0021-9673(94)01191-G.
[19] S.P. Guiraud, I. Montoliu, L. Da Silva, L. Dayon, A.N. Galindo, J. Corthésy, M. Kussmann, F.-P. Martin, High-throughput and simultaneous quantitative analysis of homocysteine–methionine cycle metabolites and co-factors in blood plasma and cerebrospinal fluid by isotope dilution LC–MS/MS, Anal. Bioanal. Chem. 409 (2017) 295–305, https://doi.org/10.1007/s00216-016-0003-1.
[20] L. Da Silva, S. Collino, O. Cominetti, F.-P. Martin, I. Montoliu, S.O. Moreno, J. Corthesy, J. Kaput, M. Kussmann, J.P. Monteiro, S.P. Guiraud, High-throughput method for the quantitation of metabolites and co-factors from homocysteine–methionine cycle for nutritional status assessment, Bioanalysis. 8 (2016) 1937–1949, https://doi.org/10.4155/bio-2016-0112.
[21] C.A. Marquette, A. Degiuli, L.J. Blum, Electrochemiluminescent biosensors array for the concomitant detection of choline, glucose, glutamate, lactate, lysine and urate, Biosens. Bioelectron. 19 (2003) 433–439, https://doi.org/10.1016/S0956-5663(03)00225-2.
[22] I.S. Kucherenko, D.Y. Didukh, O.O. Soldatkin, A.P. Soldatkin, Amperometric biosensor system for simultaneous determination of adenosine-5′-triphosphate and glucose, Anal. Chem. 86 (2014) 5455–5462, https://doi.org/10.1021/ac5006553.
[23] O. Soldatkin, A. Nazarova, N. Krisanova, A. Borysov, D. Kucherenko, I. Kucherenko, N. Pozdnyakova, A. Soldatkin, T. Borisova, Monitoring of the velocity of high-affinity glutamate uptake by isolated brain nerve terminals using amperometric glutamate biosensor, Talanta. 135 (2015) 67–74, https://doi.org/10.1016/j.talanta.2014.12.031.
[24] R.M. Wightman, Microvoltammetric electrodes, Anal. Chem. 53 (1981) 1125A–1134A, https://doi.org/10.1021/ac00232a004.
[25] A.W. Bott, Mass transport, Curr. Sep. 14 (1996) 104–109.
[26] J. Janisch, A. Ruff, B. Speiser, C. Wolff, J. Zigelli, S. Benthin, V. Feldmann, H.A. Mayer, Consistent diffusion coefficients of ferrocene in some non-aqueous solvents: electrochemical simultaneous determination together with electrode sizes and comparison to pulse-gradient spin-echo NMR results, J. Solid State Electrochem. 15 (2011) 2083–2094, https://doi.org/10.1007/s10008-011-1399-3.
[27] C. Amatore, C. Pebay, L. Thouin, A. Wang, Cyclic voltammetry at microelectrodes. Influence of natural convection on diffusion layers as characterized by in situ mapping of concentration profiles, Electrochem. Commun. 11 (2009) 1269–1272, https://doi.org/10.1016/j.elecom.2009.04.018.
[28] N. Dizge, O. Gunaydin, F. Yilmaz, A. Tanriseven, Immobilization of invertase onto poly(3-methylthienyl methacrylate)/poly(3-thiopheneacetic acid) matrix, Biochem. Eng. J. 40 (2008) 64–71, https://doi.org/10.1016/j.bej.2007.11.017.
[29] S.C. Kelly, P.J. O'Connell, C.K. O'Sullivan, G.G. Guilbault, Development of an interferent free amperometric biosensor for determination of L-lysine in food, Anal. Chim. Acta 412 (2000) 111–119, https://doi.org/10.1016/S0003-2670(00)00767-4.
[30] S.J. Killoran, R.D. O'Neill, Characterization of permselective coatings electrosynthesized on Pt–Ir from the three phenylenediamine isomers for biosensor applications, Electrochim. Acta 53 (2008) 7303–7312, https://doi.org/10.1016/j.electacta.2008.03.076.
[31] N. Havens, P. Trihn, D. Kim, M. Luna, A.K. Wanekaya, A. Mugweru, Redox polymer covalently modified multiwalled carbon nanotube based sensors for sensitive acetaminophen and ascorbic acid detection, Electrochim. Acta 55 (2010) 2186–2190, https://doi.org/10.1016/J.ELECTACTA.2009.11.054.
[32] M. Gamella, S. Campuzano, F. Conzuelo, J.A. Curiel, R. Muñoz, A.J. Reviejo, J.M. Pingarrón, Integrated multienzyme electrochemical biosensors for monitoring malolactic fermentation in wines, Talanta. 81 (2010) 925–933, https://doi.org/10.1016/j.talanta.2010.01.038.
[33] D.R. Thevenot, K. Tóth, R.A. Durst, G.S. Wilson, Electrochemical biosensors: recommended definitions and classification, Pure Appl. Chem. 71 (1999) 2333–2348, https://doi.org/10.1351/pac199971122333.
[34] B.L.C. Wright, J.T.F. Lai, A.J. Sinclair, Cerebrospinal fluid and lumbar puncture: a practical review, J. Neurol. 259 (2012) 1530–1545, https://doi.org/10.1007/s00415-012-6413-x.
[35] A. Mlinarić, Ž. Vogrinc, Z. Drenšek, Effect of sample processing and time delay on cell count and chemistry tests in cerebrospinal fluid collected from drainage systems, Biochem. Med. 28 (2018)https://doi.org/10.11613/BM.2018.030705.

[36] Y. Zhang, G.S. Wilson, In vitro and in vivo evaluation of oxygen effects on a glucose oxidase based implantable glucose sensor, Anal. Chim. Acta 281 (1993) 513–520, https://doi.org/10.1016/0003-2670(93)85009-9.
[37] D.Y. Kucherenko, I.S. Kucherenko, O.O. Soldatkin, A.P. Soldatkin, Application of glutamate-sensitive biosensor for analysis of foodstuff, Biotechnol. Acta 11 (2018) 57–67.
[38] M. Situmorang, J.J. Gooding, D.B. Hibbert, D. Barnett, The development of a pyruvate biosensor using electrodeposited polytyramine, Electroanalysis. 14 (2002) 17–21, https://doi.org/10.1002/1521-4109(200201)14:1<17::AID-ELAN17>3.0.CO;2-O.
[39] A.L. Bereghetskyy, O.M. Schuvaylo, V.M. Arkhypova, L.V. Shkotova, J.-M. Chovelon, O. P. Soldatkin, S.V. Dzyadevych, Investigation and optimization of different transducers for creation of amperometric biosensors, Sens. Electron. Microsyst. Technol. 2 (2005) 55–62, https://doi.org/10.18524/1815-7459.2005.2.112309.
[40] S.B. Hall, E.A. Khudaish, A.L. Hart, Electrochemical oxidation of hydrogen peroxide at platinum electrodes. Part 1. An adsorption-controlled mechanism, Electrochim. Acta 43 (1998) 579–588, https://doi.org/10.1016/S0013-4686(97)00125-4.