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Development and optimization of a novel conductometric bi-enzyme biosensor for L-arginine determination

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ABSTRACT

A highly sensitive conductometric biosensor for L-arginine determination was developed by exploiting the unique biorecognition capacities of two enzymes of urea cycle – arginase (E.C. 3.5.3.1) and urease (E.C. 3.5.1.5). The enzymes were co-immobilized in a single bioselective membrane on the working sensor, while a lysine rich bovine serum albumin (BSA) membrane was immobilized on the reference sensor, allowing differential measurements. The optimum percentage ratio of arginase and urease within the bioselective membrane was determined when the biosensor sensitivity to L-arginine and urea was optimum.

Analytical characteristics of the conductometric biosensor for L-arginine determination were compared for two types of enzyme immobilization (cross-linking with glutaraldehyde (GA) and entrapment in the polymeric membrane). The optimum features in terms of the sensitivity, the linear range, and the detection limit (4.2 μS/mM, 0.01–4 mM, and 5.0×10^{-7} M, respectively) were found for L-arginine biosensor based on enzyme cross-linking with GA. A quantitative determination of L-arginine in the real sample (a drinkable solution "Arginine Veyron") gave a satisfactory result compared to the data provided by the producer (a relative error was 4.6%). The developed biosensor showed high operational and storage stability.

1. Introduction

Nowadays, there is a considerable demand for the determination of L-arginine (2-amino-5-guanidinovaleric acid) in clinical practices and the pharmaceutical industry. The levels of L-arginine, the most basic natural amino acid, is of great interest in life science due to the fact that L-arginine and its metabolic derivatives, such as urea, ornithine, creatine, nitric oxide, and citrulline, are involved in a wide range of biological activities. These include energy metabolism, vasodilation, the urea cycle, immune response and neurotransmission in mammals [1–4].

In humans, the kidney is the major site of endogenous arginine biosynthesis (from citrulline), which contributes to the plasma pool. It is known that cells with NO synthases can reuse citrulline and synthesize arginine with enzymes arginosuccinate synthase and arginosuccinate lyase [1]. Some tumor types downregulate argininosuccinate synthase expression producing an intrinsic dependence on extracellular arginine due to an inability to synthesize endogenous arginine for their growth. This dependence on extracellular arginine is known as "arginine auxotrophy", and tumors of this type include heptocellular carcinoma, malignant melanoma, malignant pleural mesothelioma, osteosarcoma, prostate and renal cancer [1]. In health care diagnostics, L-arginine variations in blood serum is also the key indicator for origination and development of respiratory system diseases [5,6] and vascular dysfunction in diabetes [7,8].

In routine laboratory practice, a quantitative detection of L-arginine may be performed by spectrophotometry [9], capillary electrophoresis [10,11], ion exchange chromatography [12], liquid and gas chromatography [13,14]. Moreover, the determination of L-arginine based on chromatographic methods should be followed by fluorescent detection using pre-/postcolumn derivatization, as well as by electrochemical detection [15,16]. Thus, a majority of analytical procedures currently performed for L-arginine determination remains to be resource-intensive and time consuming.

Taking into account the economic demands placed on the analytical methods, it is advisable to apply ever simpler and lower costing measurement techniques. Electrochemical biosensors have been offered as a response to this challenge due to virtues of their economy, portability, simplicity, ease of mass manufacture and

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analytical capabilities. The communications over electrochemical detection of L-arginine reported coupled enzymatic systems consisting of: (a) arginase and urease, or (2) L-amino acid oxidase and horseradish peroxidase, – immobilized on the surface of inert potentiometric electrodes [17–23], SAW/conductance sensor system [24], or screen-printed three-electrode amperometric sensors [25,26].

Although some electrochemical approaches for the determination of L-arginine have been developed, the alternative designs are still valuable. In particular, the development of electrochemical enzyme biosensors for L-arginine determination in connection with a conductometric transduction mode has not been reported to date. Noteworthy that the conductometric microbiosensors offer many advantages compared to other types of electrochemical transduction: (a) thin film electrodes are suitable for miniaturization and large scale production using inexpensive technology; (b) they do not require a reference electrode and differential mode of measurements allows avoiding numerous interferences; (c) transducers are not light sensitive; (d) the driving voltage can be sufficiently lowered to significantly decrease the power consumption; (e) a large spectrum of compounds of different nature can be determined on the basis of various reactions and mechanisms.

The aim of the work was to develop a stable and a highly sensitive L-arginine biosensor based on the conductometric transduction mode. The biosensor was based on arginase – urease system (Eqs. (1) and (2)), in which an excess of urease was added in order to ensure a complete and prompt conversion of all the urea formed to ammonium.

$$\text{L-Arginine} \xrightarrow{\text{Arginase}} \text{L-ornithine} + \text{urea} \quad (1)$$

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^- \quad (2)$$

A current paper provides the comparative studies of two techniques of enzyme immobilization in respect to the analytical characteristics of the appropriate L-arginine biosensors. The biosensor with the most promising features was optimized based on the dependencies of its analytical signal on the working buffer parameters. The selectivity, the operational and storage stability of the developed biosensor were evaluated.

2. Materials and methods

2.1. Reagents and materials

Arginase (E.C. 3.5.3.1, from bovine liver) was supplied by Sigma–Aldrich (France) and had an initial specific activity of 136 U/mg solid. Urease (E.C. 3.5.1.5, from jack beans) was also from Sigma–Aldrich and had a specific activity of 100 U/mg solid. Bovine serum albumin (BSA), glutaraldehyde (GA, 25% aq. solution), urea (60.06 g/mol), L-arginine (174.2 g/mol), other L-amino acids and their derivatives were provided by Sigma–Aldrich (France). The poly(vinyl alcohol)-styrylpyridinium (PVA-SbQ, degree of polymerization: 1700, degree of saponification: 88%, SbQ content: 1.30 mol%, solid content: 13.35 wt.%) was provided by Toyo Gosei Co., Ltd. (Japan). A phosphate buffer (PB) used was KH_2PO_4/Na_2HPO_4 (Acros Organics, Belgium) unless otherwise stated. Glycerol was purchased from Macrokhim (Ukraine). All chemicals were of analytical reagent grade and ultra-pure (UP) water used was obtained from a Millipore Milli Q purification system (France).

A multi-component buffer solution ("polymix" buffer) was prepared from the following individual solutions: 2.5 mM Tris (tris(hydroxymethyl)aminomethan, 121.14 g/mol, from Sigma–Aldrich), 2.5 mM KH_2PO_4, 2.5 mM citric acid, 2.5 mM sodium tetraborate, and 150 mM sodium chloride. The required pH values of the "polymix" buffer were adjusted by titration with either NaOH or HCl.

Tris–HCl buffer was prepared from tris(hydroxymethyl)aminomethan (121.14 g/mol) using UP water adjusting with HCl by titration. HEPES–NaOH buffer was prepared from HEPES (238.30 g/mol, from Sigma–Aldrich) using UP water adjusting with NaOH by titration.

2.2. Sensor design

Conductometric transducers were manufactured at the V.Ye. Lashkaryov Institute of Semiconductor Physics (Kyiv, Ukraine). Each of them consisted of two identical pairs of interdigitated thin film electrodes (150 nm thick), fabricated by gold vapor deposition onto a non-conducting pyroceramic substrate (5 × 30 mm). A 50 nm thick intermediate chromium layer was used to improve adhesion of gold to the substrate. Both the digit width and interdigital distance were 10 μm, and their length was ~1.5 mm. As a result, the sensitive area of each pair of electrodes was ~2.9 mm^2. The transducers' fabrication technique was previously reported in the works [27,28].

In the further manipulations, the first pair of electrodes, covered with a non-reactive BSA membrane, constituted a reference sensor. The second pair of electrodes, covered with an enzymatic membrane, was a working sensor.

2.3. Biosensor fabrication

2.3.1. Immobilization of bioselective elements of L-arginine biosensor based on cross-linking

The preparation procedure of bioselective membranes based on cross-linking with GA was partially originated from the previous works [29,30]. To improve adhesion of the biomembranes to the sensitive electrode surface, each conductometric transducer was treated with piranha solution and thoroughly rinsed in UP water. Afterwards, its surface was carefully degreased with ethanol. A bio-coating for the working sensor was elaborated by spreading a bi-enzyme solution and a solution of GA onto the sensitive electrode surface. The bi-enzyme solution contained arginase (4.45 wt.%), urease (11 wt.%), BSA (5 wt.%), glycerol (15 wt.%), and 40 mM PB, pH 7.15. A working solution of GA for cross-linking was prepared by the dilution of GA (25% aq. solution) in UP water to its final concentration of 2% (v/v). Afterwards, an enzyme-based solution (volume 0.15 μL) and the working solution of GA (volume 0.15 μL) were thoroughly homogenized and deposited onto the sensitive surface of one pair of electrodes. The reference sensor was prepared by applying the same procedure, except that arginase and urease were replaced by BSA. Time of immobilization of the membranes was 25 min.

Before the measurements, the biosensor was thoroughly washed during 10–15 min in 5 mM PB (pH 6.0) to eliminate an excess of GA. The biosensor for L-arginine determination, taken as the biosensor Variant 1, was stored at 4 °C until further use.

2.3.2. Immobilization of bioselective elements of L-arginine biosensor based on enzyme entrapment in the polymeric membrane

An entrapment of arginase and urease in the polymerized PVA-SbQ matrix was performed in the following way. A working solution of PVA-SbQ was prepared by the dilution of an initial solution of PVA-SbQ in UP water to the final concentration of 20% (m/m). Afterwards, the working solution of PVA-SbQ (volume 0.15 μL) and a bi-enzyme solution (volume 0.15 μL), that contained arginase (4.45 wt.%), urease (11 wt.%), BSA (5 wt.%), glycerol (15 wt.%), and 40 mM PB, pH 7.15, were thoroughly homogenized and deposited onto the sensitive surface of one pair of pre-treated electrodes.

The reference sensor was prepared by applying the same procedure, except that arginase and urease were replaced by BSA. After that, a sensor chip was exposed under UV light for 6 min (time of irradiation was previously optimized based on the sequence of the sensitivity determination of L-arginine biosensor prepared by cross-linking).

After polymerization, the biosensor was left in a room temperature for 1 h and then, it was thoroughly rinsed in 5 mM PB (pH 6.0). The biosensor for L-arginine determination, taken as the biosensor Variant 2, was stored at 4 °C until further use.

2.4. Apparatus and electrochemical measurements

A biosensor's analyzer consisted of a sensor block and an electronic measuring block (portable four-channel biosensor's analyzer was developed in collaboration with Institute of Electrodynamics of National Academy of Sciences of Ukraine and reported in the work [31]). The sensor block consisted of a stand with fixed block of holders; each holder was connected to the fingers of an appropriate conductometric biosensor. An electronic measuring block consisted of the following modules: a module of the secondary transducers and a basic measurement-control module. A personal computer with a specific software support was an integral part of the measuring block. While working, the biosensors were applied with a sinusoidal potential, at the frequency of 30 kHz and amplitude of 10 mV. Those conditions avoided faradaic processes, double-layer charging and polarization of the microelectrodes. An illumination and temperature variations had practically no influence on characteristics of the biosensors.

Measurements were carried out in a glass cell filled with phosphate buffer (volume 3 mL), under vigorous magnetic stirring. An output potential of each conductometric transducer was proportional to an impedance difference of both sensors (namely, the working and reference sensors). Thus, a steady-state response of the biosensor was plotted as a function of the analyte concentration.

3. Results and discussion

3.1. Analytical characteristics of the conductometric biosensor for L-arginine determination

Analytical characteristics of L-arginine biosensors prepared under the protocols *2.3.1* and *2.3.2* were evaluated. In particular, the biosensor Variant 1 and the biosensor Variant 2 were compared in the same experimental conditions, in respect to the sensitivity, linear and dynamic ranges, the detection limit, an apparent Michaelis–Menten constant, and the response time (see Table 1).

According to Table 1, K_m values for both biosensors were found to be similar enough to K_m value for the solubilized arginase (namely, in the work [32], K_m value for arginase from bovine liver was found as 2.0 mM, while K_m values for the biosensors Variant 1 and Variant 2 were determined as 2.2 mM and 2.27 mM, respectively). Meanwhile, the immobilization of arginase and urease in a single bioselective membrane based on cross-linking with GA provided the biosensor with a higher sensitivity, a more extended linear concentration range and a lower detection limit compared to the corresponding features of the biosensor Variant 2. We suppose that analytical characteristics of the biosensor Variant 2 were likely attributed to the gradual release of one or both enzymes from the membrane with time.

Thus, for the further elaboration of the conductometric biosensor for L-arginine determination, immobilization of arginase and urease by means of cross-linking with GA appeared to be a relatively more promising method. However, optimization of the immobilization technique based on enzyme entrapment in the polymeric membrane should be carried out hereafter. This is because of an appreciable advantage of the biosensor Variant 2 over the biosensor Variant 1 observed in the response time.

A comparison of the previously reported biosensors for L-arginine determination and the developed conductometric L-arginine biosensor (prepared under the protocol *2.3.1*) in respect to their analytical characteristics is given in Table 2. Unfortunately, a full characterization of the previously reported biosensors was found only for potentiometric biosensor described in reference [20]. Thus, several advantages of our biosensor over the above mentioned one were found. That was the sensitivity and the response time (because of different transduction modes, the sensitivity of detection of two biosensors was conditionally accounted for their limits of detection). In addition, analytical characteristics of the conductometric L-arginine biosensor, presented in the paper, were found to be more outstanding compared to the performance of other reported L-arginine biosensors (see Table 2).

3.2. Optimization of the parameters of a working buffer solution for the performance of the conductometric biosensor for L-arginine determination

An influence of buffer solution parameters (its pH, buffer capacity, and ionic strength) on the performance of the biosensor for L-arginine determination (the biosensor Variant 1) was investigated. In all experiments, the buffer solution was taken as a medium for all stock solutions with aim to provide the same conductivity baseline for analyses.

An optimization of each parameter of the buffer solution was carried out by changing a single parameter, while other parameters remained constant.

3.2.1. pH

As the biosensor comprised biological catalysts, its activity had to be pH dependent. The specific activities of arginase and urease, used in the work, were maximal at pH 9.5 and pH 8.0, respectively (as quoted by the manufacturer, Sigma–Aldrich). However, an optimum pH for the enzymatic system arginase–urease, immobilized in a single membrane of the biosensor, might differ enough from the optimum pH values of individual enzymes immobilized in the same conditions. To determine the pH optimum of the biosensor, at first we used a multi-component buffer solution since it was capable to maintain a stable buffer capacity over a wide range of pH including the range from 5 to 9.

According to Fig. 1, the maximal response of the biosensor in "polymix" buffer was found at pH 8.0 (see Fig. 1, curve 1). That value enabled us to select the phosphate buffer, Tris–HCl and HEPES–NaOH buffers, as the potential buffering systems for the further measurements. That was because the working pH ranges of above mentioned buffers were pH 4.8–8.0, pH 6.8–8.2, and pH 7.0–9.0, respectively.

The corresponding studies revealed that phosphate buffer was the only possible buffering system for L-arginine biosensor since the appropriate dependences in Tris–HCl and HEPES–NaOH buffers were not demonstrative (they are not presented in the paper). In the phosphate buffer, the pH optimum of the biosensor for both L-arginine and urea was found at pH 6.0 (see Fig. 1, curves 2 and 3, respectively).

When studying the results of appropriate investigations on the previously reported L-arginine biosensors, the authors there suggested to operate in glycine–NaOH buffer [19], Tris–HCl buffer, [17,18,20,22–24] and in phosphate buffer [25]. Considering the potentiometric biosensors, it should be mentioned that a choice of the optimal pH for the measurements, with use of bienzymatically modified ion-selective electrodes, depended on three factors: the optimum pH range for the ion-selective electrodes, the pH

Table 1
Analytical characteristics of the conductometric biosensor for L-arginine determination (measurements were carried out in 5 mM phosphate buffer, pH 6.0).

Type of immobilization	Response time (s)	Sensitivity (μS/mM)	Linear range (mM)	Dynamic range (mM)	LOD[a] (M) ($\times 10^{-7}$)	K_m (M)	CV[b] (%)
Cross-linking with GA	120 ± 5	4.2	0.01–4	0.01–8	5	2.20	3.9
Entrapment in PVA-SbQ	30 ± 3	1.2	0.1–1.4	0.1–8	250	2.27	3.9

[a] LOD, limit of detection;
[b] CV, coefficient of variation.

Table 2
Analytical characteristics of the enzymatic L-arginine biosensors reported previously and the conductometric L-arginine biosensor presented in the paper.

Type of transducer	Linear range (M)	Dynamic range (M)	LOD[a] (M)	Response time (s)	Ref.
Potentiometric	3.0×10^{-5}–3.0×10^{-3} (slope 45 mV/decade)	–	–	300	[17]
Potentiometric	2.5×10^{-5}–3.1×10^{-4}	–	–	600	[18]
Potentiometric	1.0×10^{-4}–1.0×10^{-3}	–	–	–	[19]
Potentiometric	1.0×10^{-4}–3.0×10^{-2} (slope 26.3 mV/pC)	1.0×10^{-4}–1.0×10^{-1}	1.0×10^{-5}	90–240	[20]
Potentiometric	3.2×10^{-5}–1.0×10^{-3} (slope 50 mV/decade)	1.0×10^{-5}–1.0×10^{-3}	–	–	[22]
Potentiometric	8.0×10^{-6}–1.0×10^{-3} (slope 59.0 ± 3 mV/decade)	–	–	360–480	[23]
SAW/conductance	0–4.0×10^{-4}	–	2.0×10^{-6}	–	[24]
Conductometric	1.0×10^{-5}–4.0×10^{-3}	1.0×10^{-5}–8.0×10^{-3}	5.0×10^{-7}	120 ± 5	Presented in the current paper

[a] LOD, limit of detection.

value for the maximal efficacy and the stability of both enzymes used. Since in our research the conductometric transduction mode was applied, the pH optimum and buffer composition for the developed biosensor and the reported biosensors [17–20,22–24] distinguished considerably. That took place because of the different nature of electrodes and enzyme immobilization techniques used. For instance, Komaba et al. [22] stated that Tris–HCl, pH 7.5 provided "the shortest measuring period" for the potentiometric biosensor based on enzyme entrapment in polypyrrole (PPy). The authors there explained that as follows: "Because Tris is an amine compound, it is likely that PPy is deprotonated by Tris during the FIA measurement" [22].

3.2.2. Buffer capacity

The dependence of responses of L-arginine biosensor on the buffer capacity was obtained in phosphate buffer (pH 6.0) with a series of concentrations in the range of 2.5–20 mM.

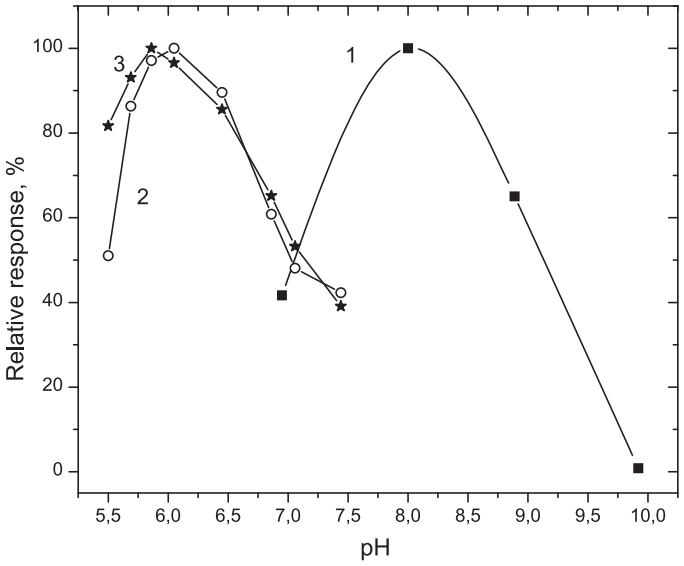

Fig. 1. pH-dependence of the conductometric biosensor for L-arginine determination obtained for L-arginine (6.7 mM) in "polymix" buffer (curve 1) and for L-arginine (6.7 mM) and urea (6.7 mM) in phosphate buffer (curves 2 and 3, respectively). Relative standard deviation of the measurements 3–5%.

According to the results (see Fig. 2), the biosensor exhibited an inversely proportional dependence of its analytical signal on the buffer capacity of phosphate buffer. That finding was quite expectable since at a low buffer capacity, the sensitivity of the biosensor response (e.g., in 2.5 mM PB) was attributed to the local pH changes within the enzymatic membrane. In addition, an increase in the buffer capacity resulted in a more extended linear and dynamic ranges of the biosensor detection. That finding could be useful for the further application of L-arginine biosensor in real samples analysis allowing regulating the sensitivity of detection depending on the composition of the formers.

3.2.3. Ionic strength

The responses of the biosensor to L-arginine in 5 mM PB (pH 6.0) were compared with those received in 5 mM PB (pH 6.0) comprising potassium chloride in the concentration range of 2.5–20 mM.

The obtained dependences (see Table 3) indicated that the sensitivity of the biosensor detection decreased, while the ionic strength of phosphate buffer increased. That observation could be accounted to the considerable growth in the global conductivity of the buffer

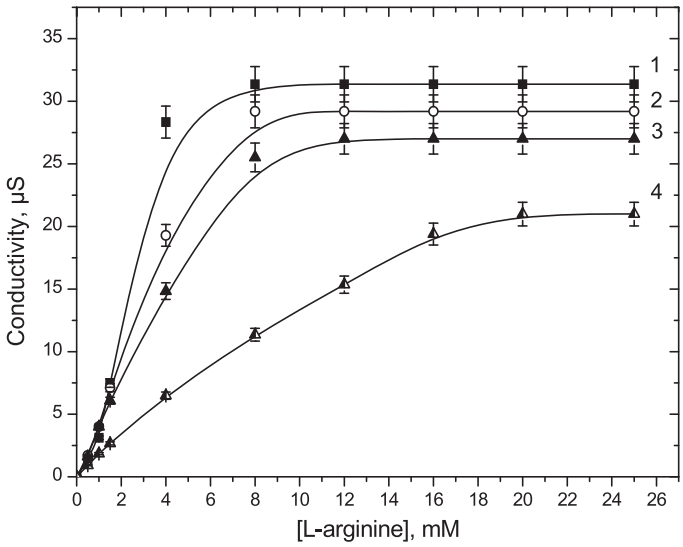

Fig. 2. The dependence of the conductometric L-arginine biosensor on the buffer capacity obtained in 2.5 mM phosphate buffer (1), 5 mM phosphate buffer (2), 10 mM phosphate buffer (3) and 20 mM phosphate buffer (4).

Table 3
Influence of ionic strength of the buffer solution on the sensitivity, the linear concentration range and the dynamic range of the conductometric biosensor for L-arginine determination.

KCl (mM)	Sensitivity (μS/mM)	Linear concentration range (mM)	Dynamic range (mM)	CV[a] (%)
0	7.4		0.01–8	
2.5	5.9		0.01–9	
5	5.2	0.01–4	0.01–9	3.9
7.5	4.3		0.01–12	
10	3.8		0.01–12	
20	1.9		0.01–20	

[a] CV, coefficient of variation.

[33,34]. Moreover, an increase in ionic strength of phosphate buffer resulted in a wider dynamic range of the biosensor detection. That finding could also be useful for the application of the biosensor in real samples analysis allowing performing an accurate and the patient-specific diagnostics.

Drawing a conclusion on the dependence of the biosensor performance on the parameters of the buffer solution, it was suggested to employ 5 mM PB (pH 6.0), as a carrier solution for the determination of L-arginine in model samples; however, the further analysis of biological fluids and pharmaceutical items may require some modifications, depending on their nature.

3.3. Operational and storage stability of the conductometric biosensor for L-arginine determination

To estimate operational stability of L-arginine biosensor, the continuous measurements of its sensitivity were carried out for 10 h. An appropriate dependence (Fig. 3a) showed that L-arginine biosensor had high operational stability. An increase in the biosensor response, observed with time, might be related to the variations of the local pH due to accumulation of ammonium within the bioselective membrane. The relative standard deviation of the measurements was 3–5%.

A storage stability of L-arginine biosensor was monitored for more than 3 months. The biosensor responses as a function of a storage time are shown in Fig. 3b. Each point of the curve corresponded to an arithmetic average of between 2 and 5 consecutive measurements (one measurement corresponded to a response to 3 mM of L-arginine). The points were plotted as the values relative to the initial response of the biosensor. For that study, a number of tested biosensors was four. After each measurement, L-arginine biosensors were stored at 4 °C in a dry state.

For the conductometric biosensor for L-arginine determination, an increase of ~90% in its response was observed for 45 days after preparation. We referred that to the gradual stabilization of the conformation of bioselective membranes with time. For the next 80 days, the analytical signal of the biosensor remained relatively stable.

3.4. Selectivity studies of the conductometric bi-enzyme biosensor for L-arginine determination

To investigate a selectivity of the developed biosensor toward the detection of L-arginine, we compared the analytical signals of the biosensor to L-arginine with those to hydrophilic amino acids and some other probable substrates for arginase.

The appropriate studies (see Fig. 4) indicated that two cationic amino acids except L-arginine (namely, L-lysine and L-histidine), L-canavanine and γ-aminobutyric acid were detectable. Noteworthy, the biosensor responses to 2 mM of L-histidine and γ-aminobutyric acid (9.5 μS and 0.6 μS, respectively) were less expressed in comparison with the responses to the same concentration of L-arginine, L-canavanine and L-lysine (19.5 μS, 15.6 μS and 19.8 μS, respectively).

The biosensor response to L-canavanine was quite explainable, as structurally that non-proteinogenic α-amino acid was related to L-arginine; consequently, L-canavanine was the competitive substrate for arginase. The response to L-lysine could be explained as the response to the competitive inhibitor of arginase [35,36]. However, there were some interesting observations. In the work carried out by Xie et al. [37] it was speculated that such amino acids as L-valine and L-lysine inhibited arginase activity and switched off the

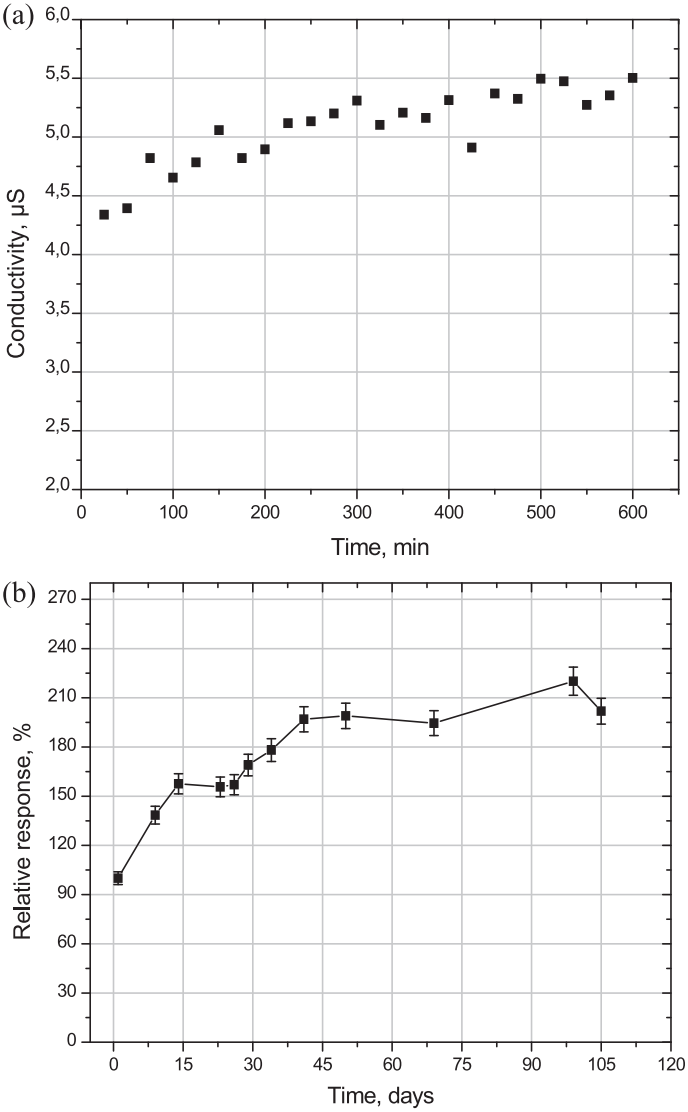

Fig. 3. (a and b) Operational stability of the conductometric biosensor for L-arginine determination. The responses of the biosensor to 1 mM of L-arginine in 5 mM phosphate buffer, pH 6.0 (a). Storage stability of the conductometric biosensor for L-arginine determination. The responses of the biosensor to 3 mM of L-arginine in 5 mM phosphate buffer, pH 6.0 (b).

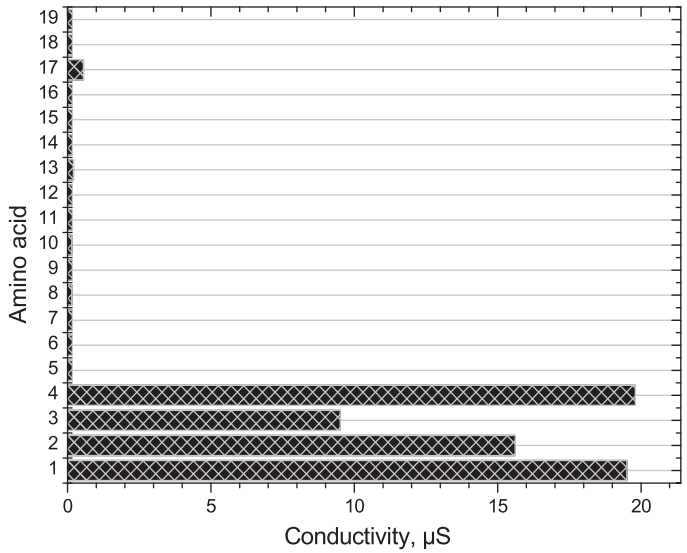

Fig. 4. The selectivity studies of the conductometric biosensor for L-arginine determination. The responses of the biosensor to 2 mM of the following amino acids: 1 – L-arginine, 2 – L-canavanine, 3 – L-histidine, 4 – L-lysine, 5 – L-glycine, 6 – L-proline, 7 – L-glutamine, 8 – L-methionine, 9 – L-threonine, 10 – L-isoleucine, 11 – L-valine, 12 – L-norvaline, 13 – trans-4-hydroxy-L-proline, 14 – L-citrulline, 15 – L-alanine, 16 – L-ornithine monohydrochloride, 17 – γ-aminobutyric acid, 18 – L-serine, 19 – L-cysteine.

EPR signal of the binuclear center by removing a bridging ligand or by increasing the inter-manganese separation. Turning to our case, if explaining the biosensor response to L-lysine by the inhibition of enzyme activity, consequently the developed arginase–urease biosensor had to give a notable response to L-valine. However, it was not confirmed through the current experiment.

As the biosensor selectivity was primarily determined by the selectivity of arginase, it was important to refer to a mechanism of arginase action. Ward and Dijk [38] stated that the bovine liver arginase, binuclear manganese metallohydrolase, catalyzed a hydrolysis of the guanidinium group of L-arginine to yield L-ornithine and urea. An active site of enzyme contained a Mn^{2+}–Mn^{2+} cluster bridged by a water molecule/hydroxide ion, believed to be the catalytic nucleophile. In the work carried out by Ash [39] it was also shown that fully Mn-activated arginase contained two Mn^{2+}-subunits and they formed electron paramagnetic resonance (EPR)[4] spin-coupled binuclear centers. Moreover, the author there outlined that a substrate specificity of arginase depended on: (1) the presence of an intact guanidinium group, (2) the proper length and hydrophobicity of the side chain, and (3) the stereochemistry and a nature of substituents at Cα. In addition, in the work carried out by Reczkowski and Ash [40] it was reported that D-arginine and guanidinobutyrate were not substrates for arginase, and alterations around Cα (L-argininamide, L-argininic acid, and agmatine) resulted in 170–54,000-fold reductions in k_{cat}/K_m. The authors there concluded that an exquisite substrate specificity of arginase suggested that a well-defined constellation of hydrogen bond donors had to be present at the active site.

Selectivity studies of the developed L-arginine biosensor, bare electrode and BSA-based biosensor toward L-arginine, L-lysine, L-histidine and to the mixtures of these amino acids were performed using conductometric and EIS measurements. The admittance shifts at the electrode/solution interface with immobilized arginase and urease and those at the electrode/solution interface with immobilized arginase (without urease) were compared. According to the results, bi-enzyme and monoenzyme biosensors did show significant response to L-lysine and L-histidine. Since arginase is known as extraordinarily specific enzyme, hydrolyzing the C—N bond within the guanidinium group, the responses of both biosensors to above-mentioned amino acids could be supported by the fact of formation of inhibitor complexes of arginase, studied by Bewley et al. [36]. In addition, in our work it was found that formation of the complex between arginase and appropriate amino acid caused the diminution of enzyme activity toward L-arginine (e.g., having the biosensor response to 2 mM L-arginine, 2 mM L-lysine and 2 mM L-histidine as 19.5 μS, 19.8 μS and 9.5 μS, respectively, the biosensor response to the mixture of 2 mM L-arginine and 2 mM L-lysine was 26.3 μS, and to the mixtures of 2 mM L-arginine and 2 mM L-histidine was 20.5 μS). However, activity of the biosensor was recovered after series of biosensor washing in a fresh phosphate buffer. Consequently, L-arginine biosensor was inhibited by L-lysine and L-histidine (competitive reversible inhibition), whereas the equilibrium, established at the electrode/solution interface after injection of appropriate amino acid, was recorded as the analytical signal of the biosensor. Speaking about L-canavanine, it is known that this non-protein amino acid is removed quickly from blood (there is data that there were no detectable canavanine in plasma just 3.0 h after the feeding of the canavanine sulfate-containing diet to chicks was discontinued [41]). Thus, L-canavanine could be absent at all or present in blood in trace quantities, therefore it does not influence on the accuracy of detection significantly.

3.5. Determination of L-arginine in a real sample

The biosensor for L-arginine determination was applied in assay of L-arginine in a commercially available drinkable solution "Arginine Veyron" (Pierre Fabre Médicament, France). The real sample analysis was performed in 5 series and the determined concentration of L-arginine was (964 ± 9) mM. A comparison of results obtained by the biosensor and the data provided by the producer revealed a satisfactory relative error (4.6%).

4. Conclusion

A conductometric bi-enzyme biosensor has been developed and optimized for the first time to detect L-arginine. Co-immobilization of arginase and urease based on cross-linking with glutaraldehyde provided the biosensor with the following analytical characteristics: the sensitivity 4.2 μS/mM, the linear concentration range 0.01–4 mM, the dynamic range 0.01–8 mM, the detection limit 5.0×10^{-7} M, the apparent Michaelis–Menten constant 2.2 mM, and the response time 120 ± 5 s. The sensitivity of L-arginine biosensor was optimized throughout the studies of the dependence of its analytical signal on the nature, pH, buffer capacity and ionic strength of several buffering systems. The most suitable buffering system for the biosensor performance was found, that was 5 mM PB with pH 6.0. An increase in the buffer capacity and ionic strength of phosphate buffer allowed extending the linear and dynamic ranges of the biosensor detection. The developed biosensor exhibited a remarkably stable response over 3 months.

Therefore, the conductometric bi-enzyme biosensor for L-arginine determination can be considered as a promising analytical device for biomedical purposes and the quality-control analysis of the medicaments.

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