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Urease-based ISFET biosensor for arginine determination

M. Sheliakina [a,b], V. Arkhypova [a], O. Soldatkin [a,c], O. Saiapina [a], B. Akata [d,e], S. Dzyadevych [a,c,*]

[a] Laboratory of Biomolecular Electronics, Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Zabolotnogo Street 150, 03143 Kyiv, Ukraine
[b] Max Planck Institute for Polymer Research, Ackermannweg 10, D-55128 Mainz, Germany
[c] Institute of High Technologies, Taras Shevchenko National University of Kyiv, Volodymyrska Street 64, 01601 Kyiv, Ukraine
[d] Micro and Nanotechnology Department, Middle East Technical University, Dumlupınar Blv. 1, 06531 Ankara, Turkey
[e] Central Laboratory, Middle East Technical University, Dumlupınar Blv. 1, 06531 Ankara, Turkey

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ABSTRACT

In this work a novel biosensor for arginine determination based on the urease inhibition effect has been proposed. Ion-selective field effect transistors were used as transducers. Urease immobilized in glutaraldehyde vapor served as a biorecognition element of the biosensor. Significant part of the work was aimed at proving the urease inhibition by arginine. Optimal concentration of urea for arginine determination was chosen. Detection limit for arginine was 0.05 mM. The biosensor selectivity towards different amino acids was studied. The results of quantitative determination of L-arginine in the real sample (a drinkable solution "Arginine Veyron") were in good agreement with the producer's data (a relative error was 5.2%). The biosensor showed a good reproducibility of arginine determination.

1. Introduction

Arginine (2-amino-5-guanidinovaleric acid) is a conditionally essential amino acid, which is a product of the living activity of the organism's healthy cells [1]. L-arginine acquired with diet is absorbed in the small intestine and transported to the liver, where it is mostly utilized in the ornithine cycle. Part of L-arginine, non-metabolized in the liver, is used as a substrate for NO production [2]. The average daily consumption of L-arginine is 5.4 g. The physiological need of tissues and organs for arginine is provided by its endogenous synthesis and/or with food [3]. Arginine is an essential precursor for the synthesis of proteins and numerous biologically important molecules, such as ornithine, proline, polyamines, creatine, and agmatine. However, the key role of arginine in the body is to serve as a substrate in the nitric oxide (NO) synthesis [4]. Nitric oxide is a gas naturally found in the body, which conveys the information between cells. One of its main functions is to increase blood flow by dilating blood vessels [5].

Arginine is one of the most effective stimulants of production of pituitary somatotropic hormone (growth hormone). Arginine also affects the muscle metabolism (can increase the muscle mass and decrease the body fatty tissue) [6]. It exhibits the antitumor activity, i.e. activates macrophages; increases the number and functional activity of T-helpers, the main component in the development of immune response; increases the number and activity of NK (natural killers) and LAK (lymphokine activated killer). Besides, arginine prevents the formation of blood clots and their adhesion to the inner walls of the arteries, thus reducing the risk of blood clots and atherosclerotic plaques [7].

Lack of arginine can lead to heart problems, hormonal and sexual disorders, obesity, and many other diseases. It is not the only cause, but other factors being equal, it promotes disease development [8]. Insufficient arginine level increases the risk of diabetes type 2 (immunity of insulin-dependent tissues to insulin). In children, a lack of arginine in the diet can stunt growth and retard sexual maturation [9].

The classical methods of arginine determination include ion exchange chromatography [10], spectrophotometry, colorimetry, and radiometry [11]. Their main disadvantage is a rather low specificity towards L-arginine. The development of biosensors can be a relevant alternative since they have a higher specificity due to the use of enzymes.

The construction of biosensors for arginine detection has already been reported [12–24] but alternative designs are still valuable. The latter used coupled enzymatic systems consisting of

* Corresponding author at: Institute of Molecular Biology and Genetics, 150 Zabolotnogo Str., 03143 Kyiv, Ukraine. Tel.: +380 442000328; fax: +380 445260759.
E-mail address: dzyad@yahoo.com (S. Dzyadevych).

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either arginase and urease, or L-amino acid oxidase and horseradish peroxidise. As transducers served potentiometric electrodes [12–18], conductometric electrodes [19], amperometric transducers [20–22], and the SAW/conductance sensor system [23]. Those sensors had insufficient sensitivity and selectivity; that is why this work was aimed at creating the biosensor for arginine determination based on inhibitory analysis and ion-selective field effect transistors (ISFETs).

2. Materials and methods

2.1. Materials

Enzyme arginase from bovine liver with specific activity of 136 U/mg solid, urease from jack beans with specific activity of 66 U/mg solid, butyrylcholinesterase (BuChE) from horse blood serum with specific activity of 20 U/mg solid, bovine serum albumin fraction V (BSA) were purchased from "Sigma-Aldrich" (France), and 25% glutaraldehyde aqueous solution (GA) was from "Merck" (Germany). Urea and butyrylcholine chloride (BuCh) were from "Sigma-Aldrich Chemie" (Germany) and used as substrates. 5 mM phosphate solution (KH_2PO_4–NaOH) served as a working buffer. Amino acids L-arginine, L-glycine, L-valine, L-lysine, L-cystine, L-histidine, L-threonine, L-proline were from "Sigma-Aldrich" (France). All the rest of reagents of both foreign and domestic production were of chemical grade of purity.

2.2. Design of potentiometric transducers and measuring device

We used sensor chips with a differential pair of p-channel field-effect transistors on a single crystal of a total area of 8 mm × 8 mm produced by the V.Ye. Lashkarev Institute of Semiconductor Physics of NAS of Ukraine (Fig. 1(a)). The crystal consisted of two identical transistors separated by the 50 μm wide protective n^+-region with a contact to the substrate; the p^+-diffusion busbars, which are put on the chip edge where the contacts to drain and source are placed; a wire to the integrated reference microelectrode, and two field-effect transistors with a metal gate for testing electrical parameters of the crystals produced.

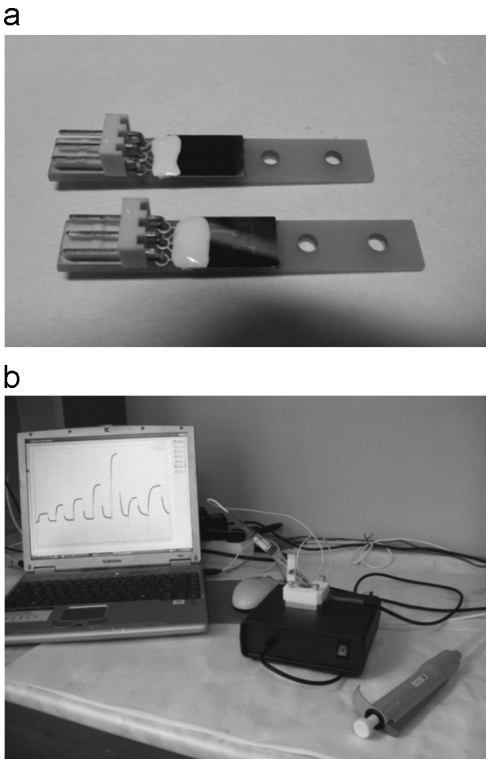


Fig. 1. General view of the transducers based on pH-sensitive field effect transistors (a) and the portative device for measurements (b).

The ion-selective properties of transistors are determined by the Si_3N_4 layer deposited on their subgate region [24]. Their pH-sensitivity was about 25 μA/pH.

The measurements were performed using a portable device, developed and manufactured at the V.Ye. Lashkarev Institute of Semiconductor Physics of NAS of Ukraine (Fig. 1(b)) [25]. The device operates due to the principle of measuring the surface potential of transistor gate. The tracking circuit was used with a negative feedback supporting a constant current value of 0.3 mA in the channel of the field-effect transistor at a constant source–drain voltage of about 2 V. The output signal corresponds to the gate potential. The device allows the operation in a differential mode (with 10- or 100-fold multiplication of the signal) as well as in a mode of monitoring the separate signals of each of the two channels. The information from the work cell with transducer is imported to the computer and processed using "MSW_32" software (V.Ye. Lashkarev Institute of Semiconductor Physics of NAS of Ukraine).

2.3. Immobilization of enzymes onto the transducer surface

A biomatrix on the surface of ISFETs was formed by the method of enzyme immobilization developed in the Laboratory of Bimolecular Electronics of Institute of Molecular Biology and Genetics, NAS of Ukraine [26]. The working enzyme-based bioselective elements were prepared as follows.

For creation of urease-based biosensor (urease-ISFET), the mixture containing 5% (w/v) urease, 5% (w/v) BSA, 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was deposited on the sensitive surface of one ISFET by the drop method, whereas the mixture containing 10% (w/v) BSA and 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was placed on the surface of the reference ISFET.

For creation of arginase–urease-based biosensor (arginase–urease-ISFET), the mixture containing 3% (w/v) arginase, 5% (w/v) urease, 2% (w/v) BSA, 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was deposited on the sensitive surface of one ISFET by the drop method, whereas the mixture containing 10% (w/v) BSA and 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was placed on the surface of the reference ISFET.

For creation of BuChE-based biosensor (BuChE-ISFET), the mixture containing 5% (w/v) BuChE, 5% (w/v) BSA, 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was deposited on the sensitive surface of one ISFET by the drop method, whereas the mixture containing 10% (w/v) BSA and 10% (w/v) glycerol in 20 mM phosphate buffer (pH 7.4) was placed on the surface of the reference ISFET.

The use of glycerol prevents from a loss in the enzyme activity during the immobilization process, and provides better homogeneity of the membrane and better adhesion to the ISFETs surface. For the membrane polymerization, the sensors were placed for 20–25 min in an atmosphere of saturated glutaraldehyde vapor. The latter reacts with available amino groups of proteins, contributing to the formation of cross binding of the schiff base type (–N═CH–). After polymerization the sensors were dried in the air and washed from glutaraldehyde excess in the buffer for 10–15 min.

2.4. Procedure of measurements by biosensor

All measurements were performed in daylight at room temperature in an open glass vessel filled with a vigorously stirred

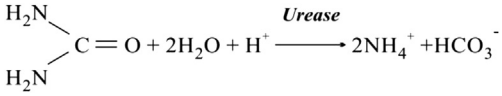
5 mM phosphate buffer solution, pH 7.4. The solutions of substrates, urea and BuCh, were prepared as 200 mM stock solutions in buffer. The substrate concentrations were varied by addition of different aliquots of standard stock solutions of the substrates into the working buffer.

To evaluate the level of urease inhibition by arginine, the biosensor was first placed in the buffer solution and the output signal (i.e., the base line) was registered. Then urea was added to the measuring cell and the steady-state signal was registered. After this, an appropriate amount of arginine was added to the work cell and the steady-state signal was registered again. The inhibition level was calculated as the ratio between value of steady-state responses before (I) and after (Ia) arginine addition (Y=100% Ia/I).

Each experiment was repeated 3 times for statistics. Nonspecific changes in the output signal associated with fluctuations of temperature, pH medium and electrical noise were avoided due to the usage of a differential measurement mode [27].

3. Results and discussion

The work of urease-ISFET biosensor is based on the cleavage reaction of the substrate urea to NH_4^+ ions with the consumption of protons [28]



This reaction changes the pH value inside the selective membrane, which is registered by the pH-sensitive field-effect transistors.

Preliminary experiments demonstrated the effect of urease inhibition by amino acid arginine. For creation of the sensor for arginine determination based on inhibitory analysis, further experiments were carried out to confirm this effect. Different procedures of measurements were primarily used (Fig. 2). In the first case, after a signal reaches the baseline we added the urea into the cell (1 mM), waited until the response was obtained, then introduced a certain aliquot of arginine and measured a response to the inhibitor injection. After washing the sensor with buffer solution, the second version of the analysis was performed as follows: the same amount of arginine was the first to be introduced into the cell, and a few minutes later 1 mM urea was added. The third version of the procedure consisted in the 10-min incubation of the arginine with 1 mM urea mixture outward of the cell and subsequent introduction into the working cell.

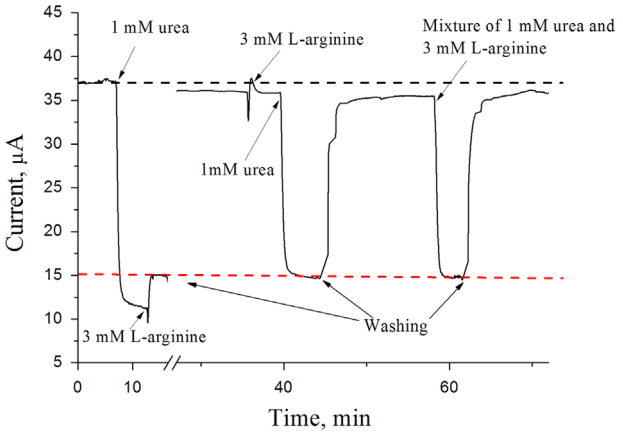


Fig. 2. Typical response curves for urease-ISFET biosensor for different protocols of measurements. Measurements conditions: base line is drawn in 5 mM phosphate buffer, pH 7.4; arrows indicate the points of different aliquots of urea and arginine.

It is seen that in all three cases the result of inhibition is the same. This indicates the presence of inhibiting urease with arginine and indirectly testifies to the competitive type of inhibition. After washing with the buffer solution the responses to urea correspond to the initial values.

To confirm that the inhibitory effect appeared due to the impact of arginine and not to any other non-specific factor (e.g., pH), the BuChE-ISFET biosensors (BuCh as a substrate) was used (Fig. 3). Just like in case of the urease biosensor, two measurement procedures were used. After receiving a response to the substrate BuCh, an arginine aliquot was added to the cell. Next, the sensor was washed and the measurement procedure was changed, i.e. arginine was added first and then – the substrate BuCh. The responses were measured both in a differential mode (Fig. 3(b)) and for separate channel of the transducer (Fig. 3(a)). When introducing both substrate and inhibitor, no significant changes in a reference membrane were revealed, and the baseline entered the previous level. The jumps observed prior to the signal stabilization are conditioned just by introduction of the solution into the cell. At the same time, in the BuChE membrane an enzymatic reaction occurs and the response to BuCh is registered. When introducing arginine, no changes in both reference and

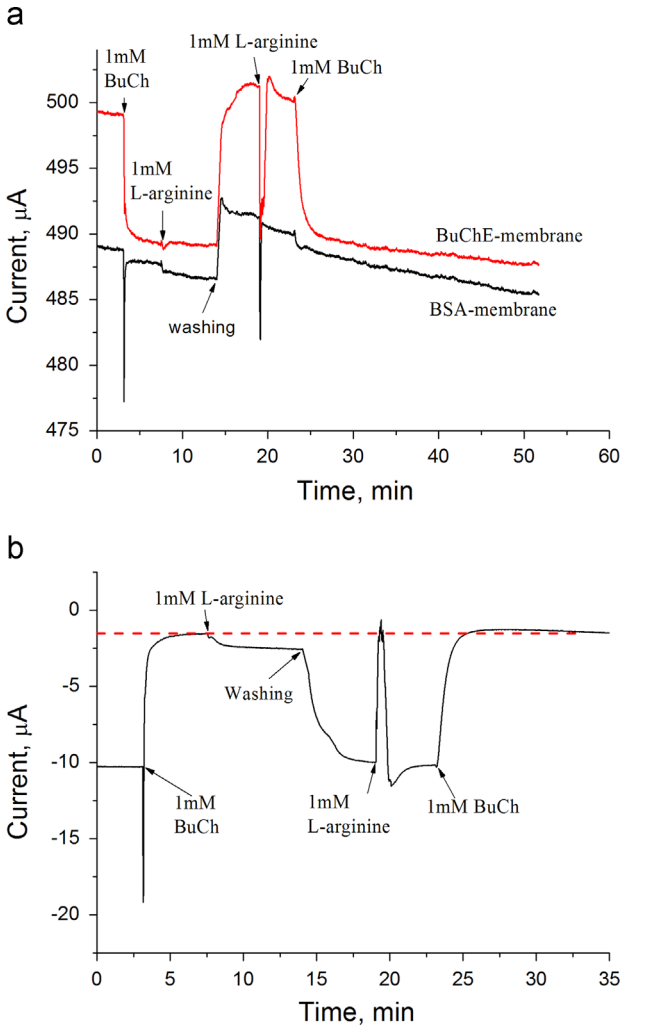


Fig. 3. Responses of the BuChE-ISFET biosensor to L-arginine and butyrylcholine for two single channels (a) and differential modes (b). Measurements conditions: base line is drawn in 5 mM phosphate buffer, pH 7.4; arrows indicate the points of different aliquots of butyrylcholine and arginine.

enzyme membranes happen. A minor change in the differential signal is a result of the change in the solution pH at adding L-arginine, the pH of stock solution of which is about 11.

This experiment demonstrated that arginine is not an inhibitor of BuChE, and the effect of urease inhibition with arginine can be used to create the urease-ISFET biosensor for arginine determination.

Another indirect evidence of the inhibition effect was obtained by analyzing dependence of the level of urease inhibition on the L-arginine concentration in the solution for two variants of biosensors: the biosensor based on the enzyme urease (urease-ISFET, 1) and the two-enzyme biosensor based on the arginase–urease mixture (arginase–urease-ISFET, 2). The measurement procedure was identical in both cases (Fig. 4). 1 mM substrate (urea) was injected into the cell, the response was measured, then a certain aliquot of the arginine solution was added and the response was measured again. As seen, the level of inhibition in the first case is higher. It may testify to the fact that a certain part of arginine is immediately split with arginase and does not participate in the further process of inhibition. Thus, the sensor based on one enzyme only (urease-ISFET) is more sensitive to arginine. These experiments confirmed the effect of urease inhibition with amino acid arginine.

Next, a set of experiments was carried out, aimed at elimination of an influence of extreme values of pH of the tested arginine solution. The pH of working buffer solution was 7.4, the pH of stock L-arginine solution was 11. Therefore, control experiments were performed. After the response to the substrate was received, identical aliquots of the solution with pH 11 were added to the cell; their volume was equal to that of arginine with the corresponding concentration. It has been shown that the pH of added solution does not affect the response (Fig. 5).

It is well known that the choice of a buffer solution may influence the enzyme activity. Therefore, pH-dependence of the immobilized urease activity relative to urea and arginine was studied. Fig. 6 shows the urease biosensor response to the injection of 1 mM urea and the level of enzyme inhibition after contact with 1 mM arginine at different pH values.

The highest response to urea was obtained for the buffer solution with pH about 7.4, whereas the inhibition level was maximal at pH 9. These data are in good agreement with the results on urease activity toward the substrate obtained by other authors [22]. As seen, at increasing pH of the buffer we observed a higher level of inhibition, which can be explained by the fact that

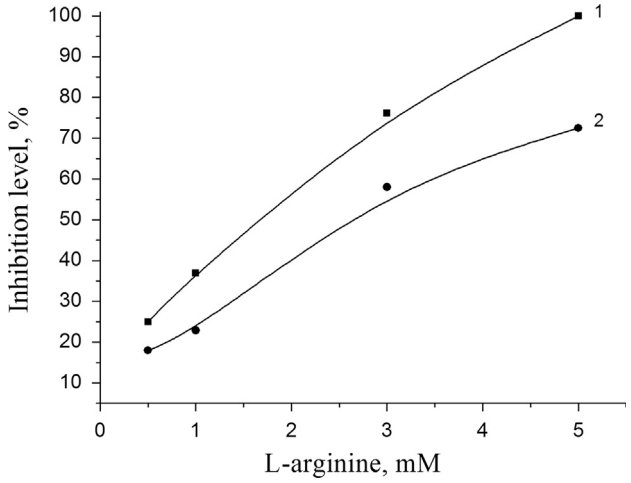


Fig. 4. Dependence of inhibition level on arginine concentration in solution: urease-ISFET based biosensor (1); arginase–urease-ISFET biosensor (2). Measurements were conducted in 5 mM phosphate buffer, pH 7.4, and 1 mM urea.

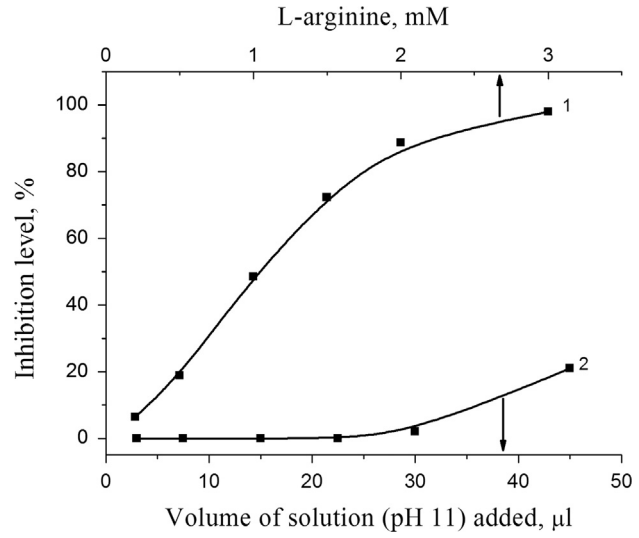


Fig. 5. Calibration curve of urease–ISFET biosensor for arginine determination (1) and the effect of solution with pH 11 added in the equal volumes (2) like for curve 1. Measurements were conducted in 5 mM phosphate buffer, pH 7.4, and 0.5 mM urea.

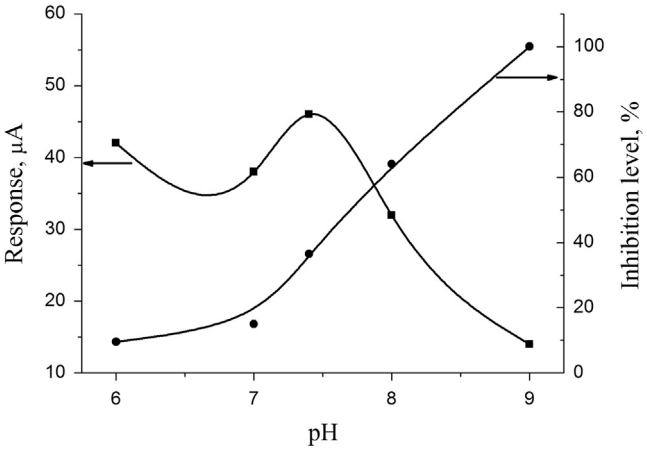


Fig. 6. Dependence of biosensor response for 1 mM urea and following inhibition levels of immobilized urease for 1 mM arginine on pH. Measurements were conducted in 5 mM phosphate buffer.

the response to substrate decreased whereas the response to inhibitor did not change. In all subsequent experiments, an optimal pH toward urea (pH 7.4) was used for measurements to obtain the best resolution for arginine determination.

Further experiments were aimed at a comprehensive study of analytical parameters of the obtained urease-ISFET biosensor and the development of measurement procedure.

The calibration curves for urea without inhibitor and with addition of 1 mM and 3 mM arginine to the solution were obtained (Fig. 7). Based on the results obtained, we have plotted a curve of dependence of the urease inhibition level on the urea concentration in the solution (by an example of the addition of 1 mM arginine).

As can be seen from Fig. 7, the highest sensitivity to arginine is observed at the urea concentration of 0.5 mM. The low urea concentrations caused a smaller inhibitory effect. At lower urea concentrations, the urease in the membrane is in excess and is involved in the substrate conversion according to the product reaction only partly (i.e. the rest of urease does not participate in the enzymatic reaction). In this case, the urease molecules linked with arginine can be compensated by involving free urease molecules in the reaction. As a result, a decrease in the biosensor

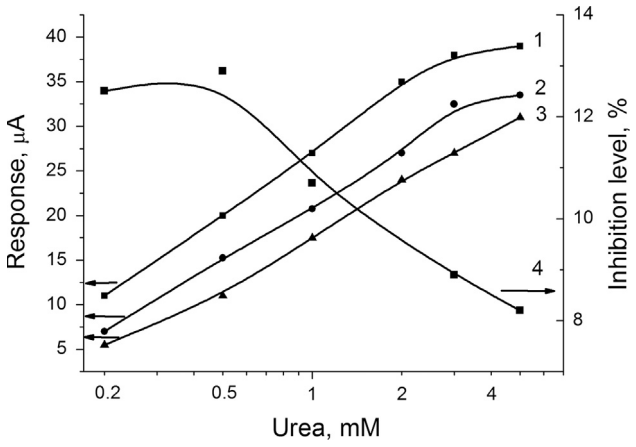


Fig. 7. Calibration curves of urease-ISFET biosensor for urea without inhibitor (1), with addition of 1 mM (2), 3 mM (3) arginine in solution; dependence of urease inhibition level on urea concentration in solution (4). Measurements were conducted in 5 mM phosphate buffer, pH 7.4.

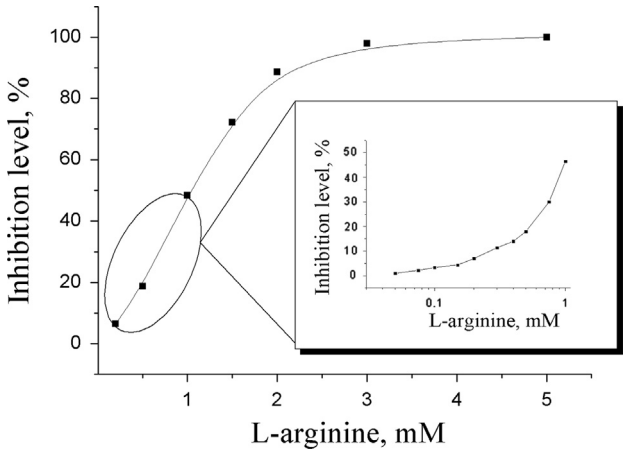


Fig. 8. Calibration curve of urease-ISFET biosensor for arginine determination used for assessment of minimum detection limit of arginine (inset presented in logarithmic scale). Measurements were conducted in 5 mM phosphate buffer, pH 7.4, and 0.5 mM urea.

response in the experiment will be lower than an actual decrease in the enzyme activity due to the inhibition. This effect is typical for immobilized enzymes irrespective of either the inhibition mechanism or the system used for detection of the enzyme activity [26]. At high substrate concentration, both urea and arginine interact with the immobilized urease simultaneously, and the sensitivity toward the arginine decreases with an increase in the urea concentration. This features only the reversible mechanism of inhibition. Finally, the 0.5 mM concentration of urea was used in further experiments. It has been also shown that inhibition does not depend on duration of the biosensor contact with arginine. At the biosensor incubation with arginine for 1–30 min no changes in the urease inhibition level were observed.

To confirm the type of urease inhibition by arginine, the calibration curves before and after inhibition were replotted using Lineweaver–Burk linearization (plots 1/response versus 1/[urea]). The typical situation for competitive inhibition was revealed when the Lineweaver–Burk double reciprocal plots showed a series of lines crossing the *y* axis at the same point.

Due to the IUPAC recommendations, the detection limit is the smallest concentration that the analyst can expect to detect with a given degree of confidence. According to this definition, the detection limit DL$=3S_b/S$, where S_b is standard deviation of the base line and S is the sensitivity (expressed as a slope of the calibration curve). In this study, arginine of different concentrations was added to the cell, beginning from 1 mM and gradually reducing until the minimum response to arginine concentration addition was reached. Fig. 8 shows the calibration curve of the sensor for L-arginine determination. The detection limit for arginine was 0.05 mM, the linear measurement range −0.1 to 2 mM.

All amino acids have a chemical structure of the type NH_2–CH(R)–COO, thus they may have identical physical and chemical properties and, therefore, participate in similar chemical reactions. This is why at the next stage of the work it was reasonable to determine the urease specificity to different amino acids. In the experiments, 200 mM solutions of different amino acids were prepared; three types of measurement procedures were used for each of them (the same as for arginine). The summary diagram for all amino acids is shown in Fig. 9. As seen, the levels of immobilized urease inhibition with amino acids arginine, lysine and cystine differ insignificantly. Glycine, valine, threonine, and proline have no inhibitory effect at all. A conclusion can be made that urease is inhibited by the amino acids, which have chemical structure and properties, similar to those of arginine.

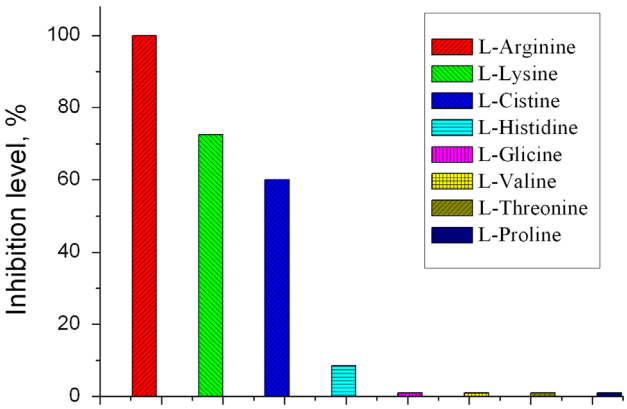


Fig. 9. Diagram of specificity of urease-ISFET biosensor towards different amino acids. Measurements were conducted in 5 mM phosphate buffer, pH 7.4, concentration of amino acids – 200 mM.

Very important characteristic of biosensors is their response reproducibility. To investigate this parameter, the responses to the same concentration of urea and arginine (1 mM) were measured throughout the day. Afterwards the level of urease inhibition by arginine was calculated (Fig. 10). During the experiment, the transducers were kept in working buffer at room temperature. As seen, the tested urease-ISFET biosensor had rather good reproducibility at arginine determination (relative standard deviation of responses was 4.5%), which allows the speculation about its availability for stable operation.

The urease-ISFET biosensor for L-arginine determination was applied in an assay of L-arginine in the commercially available drinkable solution “Arginine Veyron” (Pierre Fabre Medicament, France). The real sample analysis was performed in 4 series and the determined concentration of L-arginine was (164 ± 9)6 mg/ml. A comparison of the results obtained by the biosensor and the data provided by the producer revealed a satisfactory relative error (5.2%). However, when testing L-arginine in the pill “L-arginine” from a rather unknown producer, no response was obtained to the samples injection. It could be due to the actual absence of L-arginine in this pill, though the producer assured 500 mg of L-arginine per pill. The control tests were conducted using the HPLC system Agilent 1200 (Agilent Technologies, USA) with Zorbax Eclips AAA column and fluorescent detector. The results showed that there was only 17 mg of L-arginine per pill, i.e. less

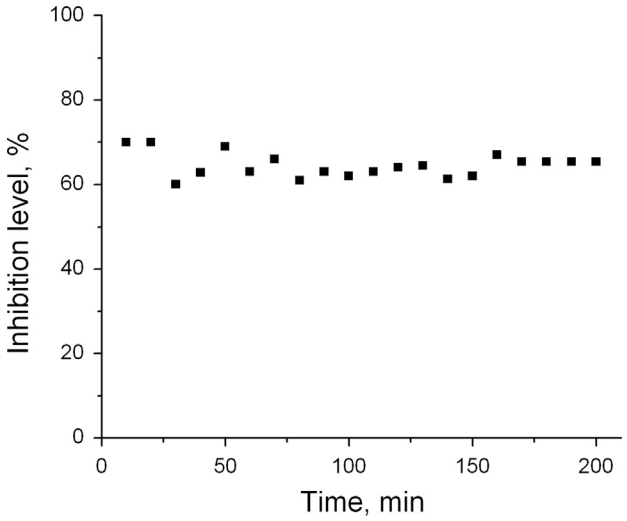


Fig. 10. Reproducibility of arginine determination by urease-ISFET biosensors. Measurements were conducted in 5 mM phosphate buffer, pH 7.4, concentration of urea – 0.5 mM, concentration of arginine – 1 mM.

than the detection limit of our urease-ISFET biosensor after dilution of our sample in the work cell. So, the producer's information appeared to be unfair.

4. Conclusions

In this work a possibility of arginine determination by the urease-ISFET biosensor was shown. Several experiments for studying urease inhibition by arginine were carried out. The obtained results indirectly demonstrated competitive inhibition of urease by arginine. The dependence of inhibition level on the pH of working buffer was studied, the optimal pH was chosen. Different concentrations of urea were used; optimal concentration of urea for inhibitory analysis was 0.5 mM. Limit of arginine detection was determined to be 0.05 mM. Different amino acids had no inhibitory effect on urease, except lysine and cistine. Urease inhibition by lysine and cistine could be explained by their physical and chemical properties. Reproducibility of arginine determination by the biosensor was 4.5%. The results of determination of L-arginine in the real sample were in good agreement with the control data. So, the proposed biosensor could be prosperous for arginine determination in real solutions.

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