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Bi-enzyme L-arginine-selective amperometric biosensor based on ammonium-sensing polyaniline-modified electrode

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ABSTRACT

A novel L-arginine-selective amperometric bi-enzyme biosensor based on recombinant human arginase I isolated from the gene-engineered strain of methylotrophic yeast *Hansenula polymorpha* and commercial urease is described. The biosensing layer was placed onto a polyaniline–Nafion composite platinum electrode and covered with a calcium alginate gel. The developed sensor revealed a good selectivity to L-arginine. The sensitivity of the biosensor was 110 ± 1.3 nA/(mM mm^2) with the apparent Michaelis–Menten constant (K_M^{app}) derived from an L-arginine (L-Arg) calibration curve of 1.27 ± 0.29 mM. A linear concentration range was observed from 0.07 to 0.6 mM, a limit of detection being 0.038 mM and a response time — 10 s. The developed biosensor demonstrated good storage stability. A laboratory prototype of the proposed amperometric biosensor was applied to the samples of three commercial pharmaceuticals ("Tivortin", "Cytrarginine", "Aminoplazmal 10% E") for L-Arg testing. The obtained L-Arg-content values correlated well with those declared by producers.

1. Introduction

L-Arginine (L-Arg), the most basic amino acid, is found in particularly large amounts in protamines and histones. High concentration of free L-Arg is present in many plants including red algae, *Curcurbitaceae* and conifers where it functions as nitrogen storage and a transport form. Because of this, L-Arg can be discovered in especially high concentrations in seedlings and reserve organs. L-Arg is glucogenic and half-essential for humans and is an important metabolite of the urea cycle. L-Arg is also used as a drug in clinical therapy of endocrine diseases and hyperammonemia. Argininemia is an autosomal recessive inherited disorder of the urea cycle, caused by a deficiency in arginase I (L-arginine amidinohydrolase, EC 3.5.3.1), the enzyme catalyzing the final step in the urea cycle, i.e., the cytosolic hydrolysis of L-Arg to ornithine and urea (Holmes and Tabata, 1980). Therefore, it is important to assay the content of L-Arg in biological liquids (Kekomaki et al., 1967) and drugs (Schailer et al., 1988).

A variety of detection procedures for L-Arg analysis have been developed. The majority of the frequently used approaches are time-consuming, expensive and require skillful labor techniques, such as the high performance liquid chromatography (Farina et al., 1990). Some methods are based on spectrophotometry (Sastry and Tummuru, 1994), fluorometry (Miura et al., 1984), atom absorption spectrometry (Jin et al., 1992), catalytic–thermometric titrametry (Godinho et al., 1991), capillary electrophoresis (Hemandez et al., 1991), polarography (Li and Zhang, 1991) and enzymatic assay (Valle-Vega et al., 1980; Van Haeften and Konings, 1989). The majority of these methods are marked by poor precision, low sensitivity and selectivity. The biosensor approaches to L-Arg determination seem to be promising.

The construction of L-Arg biosensors has already been reported. All of them had the cells in bio-recognition layer (Rechnitz et al., 1977; Grobler et al., 1982) or enzymes. The first type of enzyme-based L-Arg sensors consists of L-Arg oxidase co-immobilized with horse radish peroxidase on the screen-printed amperometric electrode (Kacaniklic et al., 1994; Sarkar et al., 1999; Domínguez et al., 2001). The second type of L-Arg biosensors bases on arginase/urease co-immobilized on the surface of the pH-electrode or ammonium-selective (ASE) electrode (Koncki et al., 1996; Karacaoğlu et al., 2003; Ivnitskii and Rishpon, 1993; Komaba et al., 1998; Nikolelis and Hadjiioannou, 1983; Lvova et al., 2003; Stasyuk et al., 2011a; Kaur, http://hdl.handle.net/10603/2897; Saiapina et al., 2012).

Arginase I catalyzes the conversion of L-arginine to L-ornithine and urea. Under urease-catalyzed hydrolysis, urea produces

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ammonium ion. The scheme of the two-step enzymatic conversion of L-Arg is presented in the form of reactions:

$$L\text{-arginine} + H_2O \xrightarrow{\text{arginase I}} L\text{-ornithine} + NH_2CONH_2$$

$$NH_2CONH_2 + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^-$$

None of the products being formed in the first reaction are electroactive, and their formation gives no electrochemical response. The analytical signal is created due to the enzymatic conversion of urea to final product—ammonium ions, detectable by the classical potentiometric and conductometric sensors. Most of these biosensors exhibit good stability and an essential response to the target analyte, but, at the same time, poor precision, insufficient selectivity, low sensitivity and a long-time response (Table 1). Thus, it is important to develop a new biosensor for L-Arg determination, based on arginase and urease, with the amperometric registration of a signal.

Amperometric biosensors are not only limited to the redox enzyme but also related to the biocatalyst reaction and interaction of the reaction product with conducting polymer to induce change in current. Urea biosensor is the typical example of biocatalytic amperometric biosensor where a product of biocatalytic reaction (ammonium ion) interacts with polymer to induce a change in conductivity of the polymer (Gupta et al., 2010).

The electron conducting polymers, possessing a low electrical resistance and high conductivity, are attractive materials for biosensors construction. It was reported that some urea bioelectrodes were fabricated with urease immobilized on different polymeric films: polypyrrole (Adeloju and Wallace, 1996), polyvinylferrocene (Kuralay et al., 2005), poly(N-3-aminopropylpyrrole)-co-pyrrole (Rajesh et al., 2005), PANi (Gill et al., 2007), PANi–Nafion (Strehlitz et al., 2000; Luo and Do, 2004), polyethylenimine (Lakard et al., 2004) and other electrochemically prepared films (Gerard et al., 2002; Gupta et al., 2010). The use of nanoparticles in urea electroanalysis is continually expanding over the last few years (Grieshaber et al., 2008; Gabrovska et al., 2011).

Polyaniline (PANi) is the most promising and well-studied conducting polymer in terms of pH-sensing applications. Due to considerable changes in conductivity of PANi films under different pH, they have been reported as part of the sensing layers of many devices for detecting bacteria and other potentially harmful organisms (Muhammad-Tahir and Alocilja, 2003), for estimation of glucose (Setti et al., 2005), creatinine (Shih and Huang, 1999; Chen and Lin, 2012), lactate (Chaubey et al., 2003) and urea concentrations (Liu et al., 1995a, 1995b; Gupta et al., 2010). The main reason for PANi's being a popular choice for such applications is its wide conductivity range (doping dependent) and the simplicity of aniline transformation into a film.

Recently we have reported creating the bi-enzyme L-Arg-selective sensor (Stasyuk et al., 2011a) based on the highly purified recombinant human liver arginase I and commercial urease with a potentiometric mode of the signal registration (Table 1).

In this paper we describe the construction of a more sensitive and selective amperometric bioelectrode on L-Arg. To construct this biosensor, the ammonium-sensing PANi layer was electropolymerized by the cyclic voltammetry method on the Nafion-modified Pt electrode; then enzymes urease and arginase I were dropped on the PANi–Nafion/Pt surface and covered with a calcium alginate gel (CAG). The principal scheme of L-Arg determination (see Supplementary information, Fig. SI.1) consists of the modification of the scheme proposed earlier for the biosensor urea sensing in a flow system (Luo and Do, 2004). Unfortunately, the details of these sensing mechanisms are still unclear.

The developed bioelectrode was applied as a working electrode, and its sensing on the final product of the bi-enzyme reaction (ammonium ion) was proved. High sensitivity, the low detection limit and high selectivity of this biosensor were promoted by the modification of the platinum electrode by the Nafion and polyaniline (Nafion–PANi) layer. The resulting NH_4^+ ions diffuse further to the PANi–Nafion film and trigger the reduction of PANi on the Pt-electrode. The developed amperometric bi-enzyme PANi–Nafion/Pt electrode was applied for L-Arg assay in the samples of some pharmaceuticals.

2. Material and methods

2.1. Reagents

Urease (EC 3.5.1.5, type IX from Jack Beans, 26,100 U g^{-1}), sodium alginate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), urea, sodium ethylenediaminetetraacetate (EDTA), $CaCl_2$, NaCl, KH_2PO_4, Na_2HPO_4, NH_4Cl, phenylmethanesulfonylfluoride (PMSF), aniline (99%), 5% Nafion® perfluorinated resin solution, chloroform and Butvar solution B-98 were purchased from Sigma-Aldrich. Amino acids were obtained from Merck (Darmstadt, Germany). All buffers and standard solutions were prepared using the water purified by the Milli-Q system (Millipore).

2.2. Apparatus

A three-electrode system was used for the electropolimerization of aniline and electrochemical studies. A piece of Pt wire and an Ag/AgCl/3 M KCl electrode were used as the counter

Table 1
Comparison of different types of developed biosensors on L-Arg.

Mode of signal registration	Biocomponent	LOD, mM	Linear range, mM	Response time (95%), min	Stability, days	Reference
Potentiometric, ASE[a]	Bacterial cells		0.05–1.0			Rechnitz et al. (1977)
Potentiometric, NH_3 gas sensor	Bacterial cells		0.008–1.0			Grobler et al. (1982)
Potentiometric, NH_3 gas sensor	U/A[b]		0.03–3.0	5.0		Nikolelis and Hadjiioannou (1983)
Potentiometric, pH	U/A		0.1–1.0			Ivnitskii and Rishpon (1993)
Potentiometric, ASE	U/A	0.01	0.1–30	1.5–4.0	21	Koncki et al. (1996)
Potentiometric, pH	U/A		0.01–1.0			Komaba et al. (1998)
Potentiometric, pH	U/A		0.025–0.31	10.0		Karacaoğlu et al. (2003)
Potentiometric, ASE	U/A		0.03–0.05	5.0–7.0		Lvova et al. (2003)
Potentiometric, ASE	U/A		10^{-6}–10^{3}	0.7–5.0	60	Kaur, http://hdl.handle.net/10603/2897
Potentiometric, ASE	U/A	0.1	0.12–40	1.5–5.0	15	Stasyuk et al. (2011a, 2011b)
Conductometric	U/A	0.0005	0.01–4.0	2.0	45	Saiapina et al. (2012)
Amperometric	U/A	0.038	0.07–0.6	0.17	3	Presented in the current paper

[a] ASE—ammonium-selective electrode.
[b] U/A—Urease and arginase I.

and reference electrodes, respectively. A platinum rod electrode (ALS Co., Tokyo, Japan) was applied as a working electrode (3.0 mm diameter). Amperometric measurements were carried out with a potentiostat CHI 1200 A (IJ Cambria Scientific, Burry Port, UK) and performed in batch mode under continuous stirring in a standard 40 ml cell at room temperature.

A Scanning Electron Microscope (SEM-microanalyser REMMA-102-02, Lviv, Ukraine) and an Atomic Force Microscope (AFM-microanalyser Solver P47-PRO, NT-MDT, Netherlands) were used for morphological analyses of the PANi film on the surface of a Pt electrode.

2.3. Arginase I isolation

A recombinant human liver arginase I (further—arginase I) was isolated from a cell-free extract of the recombinant yeast *Hansenula polymorpha pGAP1–HsARG1(leu2car1 Sc:LEU2)*, an overproducer of the target enzyme according to the own developed technology (Stasyuk et al., 2011a, 2011b).

The cultivation of the recombinant yeast cells was performed in flasks on a shaker (200 rpm) at 30 °C, for 48 h, in a medium containing (g/L): glucose (10.0); $(NH_4)_2SO_4$ (3.5); KH_2PO_4 (1.0); $MgSO_4 \cdot 7H_2O$ (0.5); $CaCl_2$ (0.1); yeast extract (2.0) and supplemented with 1 mM L-arginine. To obtain the cell-free extract, washed cells were suspended in 30 mM Hepes buffer, pH 7.5 (HB), containing 1 mM PMSF and 1 mM EDTA, disrupted with glass beads (d=0.45–0.5 mm) in a planetary disintegrator at 1000 rpm (r=10 cm) at 4 °C for 6 min and centrifuged at 15,000 rpm (r=8 cm) for 40 min. The enzyme was purified from a cell-free supernatant by the one-step column affinity chromatography on the own-synthesized L-Arg-modified affinity sorbent. Arginase I was eluted with 0.5–2.0 M NaCl/HB under control of a specific enzymatic activity in each fraction (Stasyuk et al., 2011a, 2011b). Homogeneity of the purified enzyme was proved by SDS-electrophoresis in PAG. An electrophoretically homogeneous enzyme with specific activity 900 U mg^{-1} was used for the development of the L-Arg-specific amperometric biosensor.

2.4. Preparation of the PANi–Nafion/Pt electrode

Preparation of the PANi–Nafion/Pt electrode was carried out according to Luo and Do (2004). A 5 µl aliquote of 0.5 wt% neutralized Nafion solution (prepared from 5% sample by 90% ethanol/water dilution) was applied onto a working Pt electrode and was air-dried for 10 min. The PANi film was deposited by immersing the electrode in a water solution containing 0.2 M aniline in 0.5 M HCl with a sweeping potential between −200 and 1000 mV (vs. the Ag/AgCI reference electrode) with a scan rate of 50 mV s^{-1} at 22 °C for the desired cycle number. The PANi–Nafion coated electrode was rinsed with water and conditioned in 30 mM K,Na-phosphate buffer (PB), pH 7.5 before using.

2.5. Surface analysis of PANi deposited on the Pt electrode by Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM)

The PANi film on the surface of the Pt-electrode was formed after 11 cycles of electrodeposition in the solution of 0.2 M aniline in 0.5 M HCl with a sweeping potential between −200 and 1000 mV (vs. the Ag/AgCI reference electrode), a scan rate of 50 mV s^{-1}. The special covered film was formed on the PANi/Pt electrode with a Butvar solution B-98 in 1.5% chloroform using an ultrasound method at the resonance frequency of 24 kHz.

Both SEM and AFM were used to characterize the thickness and morphology of the PANi film on a Pt-electrode. For AFM, the tapping mode with a resonance frequency of 160 kHz, scan rate of 1 Hz/s and resolution of 256 × 256 pixels was applied.

2.6. Immobilization of the enzymes on the PANi-Nafion/Pt electrode surface

Urease and arginase I were dropped on the PANi–Nafion/Pt electrode and covered by a calcium alginate gel (CAG) for physical holding. The immobilization procedure was as following: 5 µl of urease solution with activity 150 U ml^{-1} in 30 mM Hepes buffer, pH 7.5 (HB) was placed on the top of the PANi–Nafion/Pt electrode. After drying for 2 min at room temperature, the layer of urease was covered with a 5 µl solution of arginase I in HB, with activity 95 U ml^{-1}. Dried bi-enzyme layers were covered with a 4 µl 2% sodium alginate solution followed by the next addition of 4 µl 10 mM $CaCl_2$ for CAG formation. The prepared bio-functionalized electrode was rinsed by HB, containing 10 mM $CaCl_2$ and stored at +4 °C till application.

2.7. Tested samples of commercial pharmaceuticals

The assay of L-Arg in the samples of commercial pharmaceuticals by the constructed bi-enzyme amperometric sensor was performed at room temperature using the multiple standard addition method. The samples were diluted with PB. The following L-Arg-containing pharmaceuticals were tested: "Tivortine" (Ltd. "Yuria Farm", Ukraine), "Citrarginine" ("Laphal Industria" for "Zambon France", France) and "Aminoplazmal 10% E" (Mr Brown Melzunhen AG, Germany). Due to the manufacturer's certificates, "Tivortine" contains arginine hydrochloride only; "Citrarginine" — arginine citrate, betaine chloride and additive substances; "Aminoplazmal 10% E" contains, in addition to L-Arg, a number of amino acids and some additives (Stasyuk et al., 2011a).

3. Results and discussion

3.1. The main principle of biolectrode construction

The detection of L-Arg by the developed bioelectrode is based on a bi-enzymatic layer for the generation of ammonium finally sensed by the PANi–Nafion/Pt electrode (see Supplementary information, Fig. SI.1). The first stage of biorecognition is the arginase I-catalyzed L-Arg conversion to L-ornithine and urea. The second one is a urease-dependent hydrolysis of urea to NH_4^+ and HCO_3^-. The resulting NH_4^+ ions diffuse further to the PANi–Nafion film and trigger the reduction of PANi on the Pt electrode.

3.2. The PANi–Nafion-modified Pt electrode construction and analysis

3.2.1. Construction

For construction of the PANi-based ammonium sensitive electrode, covering of Pt electrode with Nafion was previously proposed (Luo and Do, 2004). The cation-exchanged sulfonate groups of immobilized Nafion serve as a compensator of a negative charge generated during the anodic polymerization of polyaniline and polypyrrole (Hirai et al., 1988). The PANi film was formed on the surface of Nafion/Pt rod electrode by 11 cycles of electrodeposition (number of cycles was experimentally optimized). The characteristic of this process is shown in Fig. 1.

3.2.2. Electrochemical characteristics

The profiles of cyclic voltammograms obtained during the electrodeposition of PANi are similar to that reported in the literature (Amalraj et al., 2010). At the first scanning cycle (peak D), presented in Fig. 1, both processes, oxidation and condensation of aniline molecule, take place. At the initial stage of the oxidation at the potential of +0.22 V, the cation-radical $C_6H_5NH_2^+$ (polaron) is

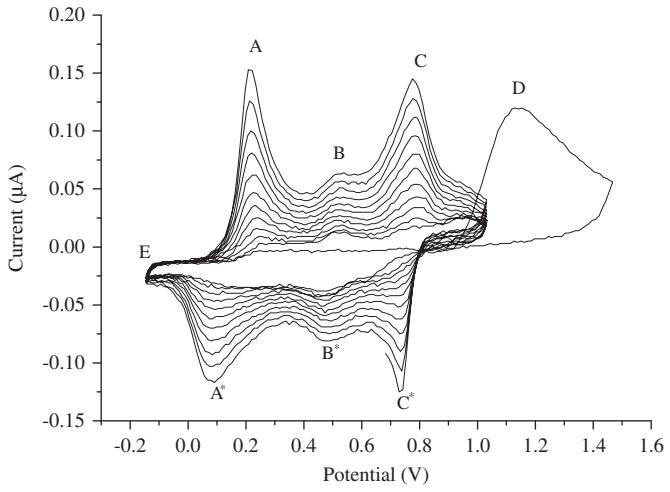

Fig. 1. Cyclic voltammogram of the PANi–Nafion film formation on the top of a 3.0 mm Pt electrode in an electrolyte solution (0.2 M aniline in 0.5 M HCl), 11 cycles of electodeposition with scan rate of 50 mV s^{-1} vs Ag/AgCl (3 M KCl) as a reference electrode at 22 °C.

formed and provides the maximum current signal (peak A). In the acid medium, a quinoidal isomeric transformation of the cation-radical results in the resonance structures formation. After arising, these molecules cooperate as the "head-to-tail" configuration and form a dimeric intermediate product, *p*-aminodyphenylaniline (PADFA), which was proved by an IR-spectroscopy (Genies et al., 1990). PADFA can be oxidized faster than aniline and produces the cation-radical which transformed at +0.75 V to a quinoidal bi-cation-radical, bipolaron (peak C). Further processes – deprotonization and oxidation of the bipolaron, followed by polymerization and the increase of its length – occur rapidly. As a result, emeraldine base is formed and reacts with the acid. The product of this reaction, emeraldine salt of an intensive green color, is represented by peaks E at −0.1 V and −0.2 V. The middle peak (B) arises at +0.5 V due to benzoquinone/hydroquinone—the products of PANi hydrolysis (Shim et al., 1990).

The cathodic current peaks follow from further electrochemical processes: reduction of the bi-polaron (peak C*) and destruction of the polyaniline, which results in the benzoquinone/hydroquinone (peak B*) arising and the polaron (peak A*) formation (Orata and Okong'o, 2000).

3.2.3. Morphological properties

The AFM and SEM micrographs of the PANi film, formed by a potentiodynamic mode of 8 min in the aniline-containing solution (Figs. 2 and 3 and Supplementary information, Fig. SI.2), show a typical characteristic of the PANi film morphology and size (Koncki et al., 1996).

The surface of the Pt-rod electrode after 11 cycles of aniline electrodeposition became dark-green in color due to the PANi film formation. The morphology of the PANi film (Fig. 2) was almost smooth and the structure of the surface was homogeneous. The Gaussian distribution by size from the AFM data (Fig. 3) proved the average thickness of the layer to be 367 nm. Statistical parameters of the electrodeposited PANi film (see Supplementary information, Table SI.1) were obtained from the AFM microphotographs (Fig. 3) by using the program Image Analysis 2.

3.2.4. Optimization of ammonium sensing

To optimize the development of the PANi–Nafion/Pt electrode, its characteristics at different conditions with varied NH_4Cl concentrations were studied (see Supplementary information,

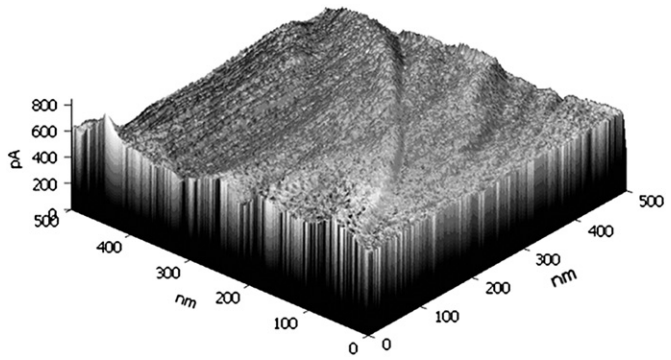

Fig. 2. Atomic Force Microscopy micrograph of the PANi film formed on the top of a 3.0 mm Pt electrode by 11 cycles of electodeposition. Structure and thickness of the PANi film on the Pt electrode was studied by the AFM method using tapping mode with a resonance frequency of 160 kHz, scan rate of 1 Hz/s and resolution of 256 × 256 pixels.

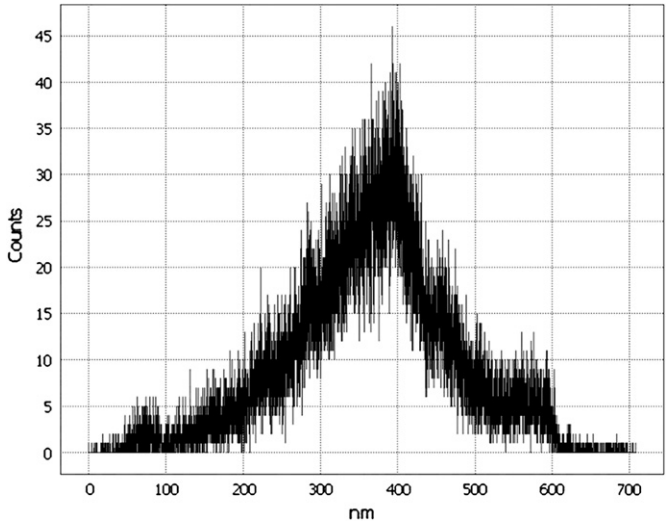

Fig. 3. The Gaussian distribution curve of the PANi film thickness resulting from the Atomic Force Microscopy (see Fig. 2). Statistical parameters are presented in Table SI.1 (Supplementary information).

Fig. SI.3). A comparison of the cyclic voltammograms (Fig. SI.3a) demonstrates that a cathodic reduction peak, contributed from NH_4^+, arises at a potential of about −200 mV (vs Ag/AgCl) for 0.5 and 3.5 mM NH_4Cl solutions. A chronamperometric analysis of ammonium ion at different working potentials is presented in Fig. SI.2b. With the injection of 0.1 mM NH_4^+ solution at an applied potential range of −100 to −300 mV, the current maximum was found at −200 mV, and only about a half of this value at −300 mV. Thus, the applied potential of −200 mV was accepted as an optimal one for NH_4^+ assay using the PANi–Nafion/Pt electrode. It is worth mentioning that no signals on the other analytes (urea, L-Arg and ornithine in the range from 0.05 to 0.5 mM with the step of 0.01 mM) were detected by the prepared electrode at these conditions. As a control, the PANi/Pt and Nafion/Pt electrodes were also tested and no amperometric signals were detected in both cases even under ammonium addition (Fig. SI.3b).

Thus, the NH_4^+-sensing layer (PANi–Nafion-containing chemosensor) of an ammonia sensitive electrode was composed and the main characteristics of this chemosensor were estimated (see Supplementary information, Fig.SI.4a). As shown, the maximal current of a chronoamperometric response of the PANi–Nafion/Pt electrode to an analyte is 2910 ± 176 nA with a constant of semi-saturation 1.26 ± 0.18 mM of NH_4Cl. The limit of detection (LOD) for the PANi–Nafion/Pt electrode (d=3.0 mm, S=7.07 mm^2) was

determined as 5.35×10^{-3} mM NH_4Cl and the sensitivity of chemosensor was estimated as 159 ± 1.2 nA/(mM mm^2).

3.3. Bi-enzyme PANi–Nafion/Pt electrode fabrication and characterization

3.3.1. Urea sensitive bioelectrode

To form a mono-enzymatic layer sensitive to urea, urease solution was dropped on the surface of the PANi–Nafion/Pt electrode and covered with a 2% calcium alginate gel (CAG) according to the experimental part. The typical calibration curve for urea biosensor (Supplementary information, Fig. SI.4b) is linear in the range between 0.03 mM and 0.3 mM urea with I_{max} 1290 ± 120 nA. The apparent Michaelis–Menten constant (K_M^{app}) derived from the calibration curve is 1.11 ± 0.22 mM and the limit of detection (LOD) is 2.91×10^{-3} mM. The typical signal rise time (95% of the steady state value at a urea concentration of 0.3 mM) is determined to be 10 s (inset of Fig. SI.4b). The sensitivity of urea the sensor was estimated as 116 ± 0.5 nA/(mM mm^2).

3.3.2. L-Arg sensitive bioelectrode

To form a bi-enzymatic layer sensitive to L-Arg, urease and arginase I were dropped on the surface of the PANi–Nafion/Pt electrode and covered with a CAG according to the experimental part. The optimal enzymes proportions were chosen empirically. The calibration curve for L-Arg biosensor and the corresponding chronamperogram (inset) are shown in Fig. 4.

The calibration was performed by the stepwise addition of a standard analyte solution. To estimate the conversion level of the target analyte (L-Arg) in the biocatalytic layer, the response to the monitored analyte (ammonium) was also tested. The yields of two reactions were calculated from the I_{max} values, presented in graphs in Fig. 4 and Figs. SI.2a and SI.4b (see Supplementary information). So, the yield at the first stage of conversion (L-arginine to urea) was estimated as 99.2% and at the second stage (urea to ammonium and bicarbonate)—44.4%.

An amperometric response on L-Arg of the developed bi-enzyme electrode was tested in the range of L-Arg concentration from 0.05 to 1.8 mM. The dynamic range was linear between 0.07 mM and 0.6 mM L-Arg. The K_M^{app} derived from the calibration curve was shown to be 1.28 ± 0.29 mM for L-Arg. The maximal detected signal was found to be 1280 ± 160 nA (Fig. 4). The sensitivity was calculated as 110 ± 1.3 nA/(mM mm^2), LOD was 0.038 mM and a response time—10 s.

3.4. Characteristics of the fabricated L-Arg biosensor

The results of the study of pH dependence, the selectivity and stability of the fabricated bi-enzyme sensor are presented in Supplementary information, Fig. SI.5.

Since the pH optima for the highest activities of arginase I and urease are different (9.5 and 7.4, respectively), the pH-dependence of the sensor's output on L-Arg was investigated. The highest current response was found for a 30 mM phosphate buffer (PB), pH 7.5; this buffer therefore was chosen as optimal for L-Arg analysis by the constructed prototype of the bi-enzyme biosensor (Fig. SI.5a).

The selectivity of the constructed biosensor was estimated in relative units (%) as a ratio of the detected signal to the value of the highest current response (Fig. SI.5b): no signal to the majority of the tested amino acids (Ile, Lys, Thr, Orn, Ser, Val, Cys and citrulline) was observed. Only canavanine, the substrate of arginase I (Reczkowski and Ash, 1994), induced an essential analytical signal—slightly higher than that for L-Arg. It should be noted that possible impact of canavanine on L-Arg assay is not important due to the rare presence of this compound in biological fluids (blood, urea, wine, etc.).

Stability of the urease–arginase I–PANi–Nafion/Pt electrode was explored by monitoring its current response to the injection of a 0.25 mM L-Arg standard solution for a period of 72 h. The activity of the biosensor decreased by 80% for a period of 24 h and the half-life of the sensor was about 72 h (Fig. SI.5c). Such unsatisfactory stability can be explained by the relatively low physical stability of CAG, which was used for supporting the enzymes' layer on the PANi–Nafion/Pt electrode. It is worth mentioning that both enzymes, recombinant human arginase I and urease, are stable (Stasyuk et al., 2011a, 2011b).

3.5. Comparison of the salient features of the developed biosensor with existing L-Arg biosensors

The developed bi-enzyme arginase I-urease-based amperometric biosensor for L-Arg determination has some advantages over the conventional biosensors (Table 1). It is highly selective,

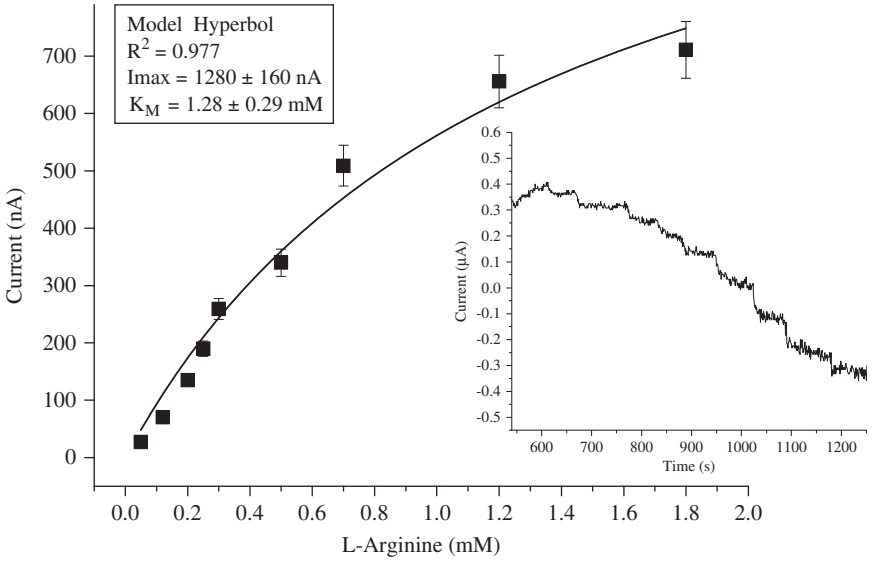

Fig. 4. Calibration curve for amperometric response on L-Arg of the developed bi-enzyme PANi–Nafion/Pt electrode. Inset: chronoamperometric current response upon subsequent additions of L-Arg. Conditions: −200 mV vs Ag/AgCl electrode in 30 mM Phosphate buffer, pH 7.5 at 22 °C.

Table 2
Comparison of different methods for L-Arg assay in the samples of pharmaceuticals.

Sample	Concentration of L-Arg, mM						
	Declared by producer	Amperometric biosensor			Potentiometric biosensor		
		Determined	CV[a], %	Dif.[b], %	Determined	CV, %	Dif., %
"Tivortin"	199.3	200.3 ± 2.5	0.8	+0.5	200.7 ± 4.5	2.1	+0.7
"Citrarginine"	475.0	479.9 ± 4.7	1.4	+1.0	447.2 ± 3.3	7.9	−5.9
"Aminoplazmal 10% E"	8.0	7.8 ± 0.3	2.2	−2.5	8.5 ± 2.5	1.3	+6.3

[a] CV—coefficient of variation.
[b] Dif.—difference between declared and determined values.

rapid, sensitive, easy-to-use, reliable and portable. When compared with response times of biosensors previously developed by other researchers (0.7–12 min), the developed biosensor response time is very fast (10 s) for the detection of L-Arg concentrations up to 0.6 mM which proves the rapidity and sensitivity of the biosensor. The biosensor is cost-effective as very small amount of biocomponent and chemicals/reagents have been used for its fabrication.

3.6. Assay of L-Arg in commercial pharmaceuticals using the bi-enzyme prototype of L-Arg biosensor

In order to demonstrate the feasibility of the constructed arginase I–urease–PANi–Nafion/Pt electrode for the analysis of some L-Arg-containing pharmaceuticals, it was applied for L-Arg assay to three commercial samples, namely: "Tivortin", "Cytrarginine" and "Aminoplazmal 10% E". The results are presented in Table 2 and Fig. SI.6 (Supplementary information). The L-Arg determination was performed using the standard addition method with different dilutions of the tested sample.

It is obvious from calibration plots that interfering effects of tested sample components (chemical background) were observed in all cases, but to a different extent. The most expressed influence was observed for "Citrarginine" (difference in the slope values for two subsequent dilutions was 24.7%). Surprisingly, for "Aminoplazmal 10% E" with the most complicated chemical composition, we observed a less expressed interference (10%). Taking into account these results, the standard addition variant is recommended for L-Arg assay using the developed biosensor. The sensitivity for each drug was calculated on the basis of the slope values (Fig. SI.6, supplementary information): "Tivortin" — 59 nA/(mM mm^2), "Citrarginine" — 42 nA/(mM mm^2), and "Aminoplasmal 10% E" — 49 nA/(mM mm^2).

The results of L-Arg assay in the samples of pharmaceuticals using the developed amperometric biosensor and the potentiometric biosensor, reported earlier by us, were compared with the L-Arg content declared by manufacturers (Table 2). As shown, only slight differences between the experimentally determined contents of L-Arg and values declared by manufacturers were observed (0.5–2.5%). These results indicate that the developed bi-enzyme PANi–Nafion/Pt electrode can be utilized for a fast and simple assay of L-Arg in pharmaceuticals.

4. Conclusions

An amperometric arginase I-urease-based on L-arginine has been developed and optimized for the first time. Co-immobilization of highly purified recombinant human liver arginase I and commercial urease on the surface of the PANi–Nafion modified Pt electrode was performed by a special doping-undoping mechanism. The constructed biosensor is characterized by a low applied potential (−200 mV), fast response to the analyte (10 s), broad linear dynamic range (0.07–0.6 mM), high selectivity and sensitivity (110 ± 1.3 nA mM^{-1} mm^{-2}) and a low limit of detection (0.038 mM). The arginase I–urease–functionalized electrode was successfully tested for L-Arg assay in some commercial pharmaceuticals using the multiple standard addition method. A high correlation between analytical results and the L-Arg content declared by manufacturers was demonstrated. Hence, the proposed laboratory prototype of an L-Arg selective biosensor would be convenient and useful in the future for routine clinical analysis and food quality control.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.04.031.

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