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Abstract

The authors describe an amperometric biosensor for the amino acid L-arginine (L-Arg). It is based on the use of a Nafion/Polyaniline (PANi) composite on a platinum screen-printed electrode (Pt-SPE) using a novel recombinant arginine deiminase isolated from *Mycoplasma hominis*. The protein was over-expressed, purified and employed as a biorecognition element of the sensor. Enzymatic hydrolysis of L-Arg leads to the formation of ammonium ions which diffuse into the Nafion/PANi layer and induce the electroreduction of PANi at a potential of -0.35 V (vs Ag/AgCl). L-Arg sensitivity was 684±32 $A \cdot M^{-1} \cdot m^{-2}$, and the apparent Michaelis-Menten constant (K_M^{app}) 0.31±0.05 mM. The calibration plot was linear over the range 3 - 200 µM L-Arg, the limit of detection was 1 µM, and the response time (for 90% of the total signal change to occur) 15 s. The sensor was selective and exhibited good storage stability (> 1 month without loss in signal). The biosensor was applied to the analysis of L-Arg in pharmaceutical samples and of ammonium and L-Arg in spiked human plasma obtained from blood of healthy volunteers and those with a hepatic disorder. Data generated was found to be in good agreement with a reference fluorometric enzymatic assay.

Keywords: L-Arginine, ammonium ions, amperometry, nafion, polyaniline, recombinant enzyme, plazma samples, pharmaceuticals

1. Introduction

L-Arginine is one of 20 amino acids found in proteins which play an important role in cellular metabolism. Its guanidine group contains five potential hydrogen bond donors that are positioned for favourable interactions with biological hydrogen bond acceptors [1]. It is dispensable in healthy individuals as it may be formed from other amino acids. However, in people, who are seriously ill or injured, higher quantities of L-Arginine are required, as the body may be unable to synthesise it in sufficient quantities [2]. Certain cancers may be auxotrophic for a particular amino acid, and amino acid deprivation is one approach used to treat these tumours. It has been suggested that arginine-degrading enzymes may be effective in the control of arginine-dependent cancers [3]. Therefore, it is important to provide a means of monitoring levels of this amino acid in biological liquids [4], nutrition supplements and most recently, cosmetics, due to the widespread use of this molecule.

A variety of detection procedures for L-Arginine analysis have been developed, most of which involve time-consuming, costly and complex techniques, such as high-performance liquid chromatography [5]. Other examples are methods based on spectrophotometry [6], fluorometry [7], atomic absorption spectrometry [8], catalytic–thermometric titrametry [9], capillary electrophoresis [10], and enzymatic assays [11, 12].

There is an ongoing demand for easy-to-use, cost effective and decentralised diagnostic tools for clinical practice. The construction of L-Arginine biosensors has already been reported and many of these are based on the use of bacterial cells [13] and enzymes [14] within a recognition layer. Earlier reports on enzyme-based L-Arginine sensors employed L-Arg oxidase co-immobilised with horseradish peroxidase on a screen-printed amperometric electrode [14, 15]. Others were based on co-immobilised arginase and urease on the surface of pH-electrode, ammonium-selective (ASE) electrodes and thin-film interdigitated conductometric electrode [16-20]. Most of these biosensors exhibited good selectivity and were responsive to the target analyte, though characterised by poor precision/insufficient stability and low sensitivity.

Non-enzymatic approaches include the use of electrocatalytic materials e.g. Martinez-Perinan et al. [21] reported the electrochemical detection of L-Arginine, L-ornithine and L-

citrulline in urine and serum, based on the use of $Ni(OH)_2$ nanoparticle-modified carbon nanotubes as a HPLC electrochemical detector. However, this method was useful only for detection of a group of amino acids and was not found to be selective for L-Arginine.

Stasyuk et al. [22a] proposed a novel L-Arg-selective amperometric bi-enzyme sensor, based on recombinant human arginase I, commercial urease and Nafion/Polyaniline (PANi) modified platinum electrodes, with sensitivity of 110 $A \cdot M^{-1} \cdot m^{-2}$ and wide linear range 0.07 – 0.6 mM. However, the main drawback of this biosensor was the insufficient working and storage stability. When multiple reactions take place in the same biolayer, there are limitations in relation to the rate of reaction and stability [23].

L-Arginine can be degraded by several enzymes including arginine deiminase (ADI), which is now commercially available, but the price of highly purified enzyme is prohibitive. ADI has high affinity for arginine and catalyses the irreversible hydrolysis of arginine to citrulline and ammonia. ADI belongs to a newly classified superfamily of guanidine-group-modifying enzymes [24]. ADI has been successfully utilised for fluorometric detection of L-Arg in beverages [12]. ADI extracted from *Pseudomonas putida* MTCC 1313 was employed as a recognition element for ASE-based sensor for detection in juices [25]. However, some inconsistencies in the analytical performance were reported.

Herein we report the construction of an original, sensitive, selective and stable amperometric biosensor for L-arginine determination. In order to realise this an ammonium-sensing PANi layer was electropolymerised via potential cycling on a Nafion-modified commercial platinum screen-printed electrode (Pt-SPE) as described previously [22a, 26]. Subsequently, a novel recombinant arginine deiminase (ADI) from *Mycoplasma hominis* was deposited on the PANi/Nafion/Pt-SPE surface and a biorecognition membrane formed by cross-linking in saturated glutaraldehyde (GA) vapour (see scheme Fig. 1S). The L-Arg biosensor was employed in quantitation studies of both pharmaceutical (food supplement) and plasma samples from 15 human volunteers.

2. Material and methods

2.1. Reagents

Arginine deiminase (EC 3.5.3.6) from *Mycoplasma hominis* with an activity of 30-35 $U \cdot mg^{-1}$ (K_M = 0.35 mM) [27] was over-expressed and purified in our laboratory [27]. Hydrochloric acid, sodium chloride, NaH_2PO_4, Na_2HPO_4, 5% Nafion® perfluorinated resin solution, glutaraldehyde (GA) (50% w/v aqueous solution), ethanol, bovine serum albumin (fraction V, 96%), and γ-aminobutyric acid (GABA) were purchased from Sigma-Aldrich (Germany, www.sigmaaldrich.com). Aniline (99%) was purchased from Sigma-Aldrich (Germany) and redistilled before use (Important: only freshly prepared aniline should be used for electropolymerisation studies). L-Arginine monohydrochloride was purchased from Sigma Aldrich (Japan, www.sigmaaldrich.com), ammonium chloride and L-lysine were obtained from Fluka (Germany, www.sigmaaldrich.com). All the chemicals and solvents used were of analytical-reagent grade and used without further purification. L-Arg containing pharmaceutical "Aminoplazmal 10% E" was purchased from Mr Braun Melsungen AG (Germany, www.bbraun.de).

20 mM phosphate buffer containing 20 mM sodium chloride (further referred to as PB) pH 7.4, was used as the supporting electrolyte during electrochemical measurements. All solutions were prepared using 18.2 MΩ·cm purified water produced using a Simplicity water purification system (Millipore, France). All glassware and polyethylene materials were rinsed with ultrapure water and dried before use.

2.2. Instrumentation

All biosensors employed a three-electrode screen-printed platform (DRP-C550, DropSens, Spain) with a platinum working electrode (4 mm diameter), a platinum counter and a Ag/AgCl reference electrode. Amperometric measurements were performed using a portable potentiostat (μStat 400, DropSens, Spain), controlled by DropView 2.0 software and used in accordance with

the producer's guide. Amperometric detection was performed at -0.35 V vs. the Ag/AgCl reference electrode at room temperature in an open 3 mL vessel filled with 20 mM PB, pH 7.4 under vigorous stirring. Prior to use, the biosensors were soaked in PB for 30 min at room temperature and the sensor response to a particular substrate concentration was taken as an average of three measurements. The long-term stability of the biosensor was evaluated via repeated determination of the biosensor response every 3-4 days. In between testing the devices were stored in PB at 4 °C.

2.3. Arginine deiminase over-expression and purification

ADI expression, renaturation and purification were performed as reported previously with some modifications [27]. Cells of *Escherichia coli* BL21 (DE3) fhuA2 [lon] ompT gal (λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5) [dcm] ΔhsdS [28] were transformed with plasmid pET3d-ADI. Transformants were selected on Luria-Bertani medium at 37 °C supplemented with 18 $g \cdot L^{-1}$ of agar, 100 $mg \cdot L^{-1}$ of ampicillin, 0.2% of arginine and 0.5% of glucose. Selected transformants were employed to produce ADI using C-750501 medium supplemented with 1.25% glycerol and 0.01% lactose following autoinduction protocol modified earlier [27].

The induced cells were harvested by centrifugation (5000 × g for 15 min at 4 ºC), washed with 20 mM sodium phosphate buffer, pH 7.2, and stored at -70 ºC. To obtain a suspension of ADI inclusion bodies the bacterial pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.8 containing 1 mM EDTA and 0.2% lysozyme and treated as described earlier. The pellet containing ADI synthesised as inclusion bodies was washed with 20 mM sodium phosphate pH 7.2, 1 mM EDTA, 4% Triton X-100, re-pelleted and used for renaturation and purification of the enzyme as optimised previously [27].

The solution of purified enzyme was concentrated using an ultrafiltration spin column (Vivaspin-Turbo 15, Sartorius Stedim Biotech GmbH, Germany), filter-sterilised using 0.22 µM filters (CE 0459 Millex-gv, Millipore, USA) and stored at 4 ºC. Protein concentration was determined by the Lowry method. ADI activity was determined as described previously [29]. One unit (U) of activity was defined as the amount of enzyme that converted 1 µmol L-arginine to L-

citrulline and ammonium per min at 37 ºC.

2.4. Electrode modification with PANi-Nafion composite

An aliquot (2 µL) of 2 wt % Nafion solution (prepared from 5% sample by 96% ethanol dilution) was deposited onto the surface of a Pt-SPE and air-dried for 15 min. The aniline polymerisation was performed using cyclic voltammetry in 0.2 M aniline solution in 0.5 M HCl by sweeping the potential between -0.4 V and 1.0 V (vs. Ag/AgCl) at a scan rate of 0.02 $V \cdot s^{-1}$ at room temperature for 7 cycles. The PANi/Nafion/Pt-SPE was rinsed thoroughly with distilled water and then dried in air for 15 min. All treatments and measurements were carried out at room temperature.

2.5. Enzyme immobilisation procedure

In order to prepare the L-Arg-selective membrane 18 mL of ADI solution (300 $U \cdot mL^{-1}$ in 1 M NaCl and 20 mM PB, pH 7.2) were mixed with 2 µL of 50% BSA in 20 mM PB, pH 7.2. 2 µL of this ADI-BSA solution was drop-cast onto the PANi/Nafion/Pt-SPE surface and incubated in saturated GA (25% w/v) vapour for the cross-linking for 35 min in an exhaust fume hood at room temperature. Prior to use, the bioelectrodes were dried for 1 h at 4 ºC, following which they were washed 3 times with 0.02 M PB, pH 7.4 to remove residual GA. The electrodes with bio-selective membranes were stored in 50 mM PB, pH 7.4 at 4 ºC until use (Fig. 1S).

2.6. Preparation of real samples and standard addition assay

Plasma samples from human volunteers were obtained from the Feofaniya Clinical hospital (Kyiv, Ukraine) following the guidelines of the European Group on Ethics in Science and New Technologies of the European Commission (#17, 4.02.2003) and the statement by the Ethics Committee of the American Psychological Association. Ukrainian Government personal data protection rules as well as the Ethics Committee of the Institute of Molecular Biology and Genetics

NAS of Ukraine rules (approved on 25.04.2012 to conduct the relevant research in the frame of SMARTCANCERSENS Project, PIRSES-GA-2012-318053) were followed. This procedure was adhered to during collection, delivery and investigation of all human biological samples.

Venous blood (2 μL) was collected in a heparinised cold tube. The tube was centrifuged at 1500 g for 10 min and cell-free supernatant removed. All plasma samples were kept cold in an ice box during the time between collection and biosensor measurement. L-Arg assay in the test samples was performed by means of amperometry at -0.35 V vs. Ag/AgCl using the standard addition method. An aliquot of the plasma sample was directly injected into the measuring vessel in order to obtain a 10-fold dilution. L-Arg concentration in human plasma samples were analysed by the DMO-based fluorometric enzymatic assay reported previously [11], and used as a reference methodology.

3. Results and Discussion

3.1. Modification of platinum screen printed electrodes with Nafion/PANi

Prior to PANi deposition via electropolymerisation, the Pt-SPE working electrode surface was modified with Nafion (2% in EtOH), sulphonated tetrafluoroethylene polymer. The acidic groups on the surface of the polymer acted as charge compensator in the course of anodic polymerisation of aniline [30]. Following the drying step at room temperature, the electropolymerisation of aniline took place as per procedure 2.4 (see Fig. 2S for growth cycles), following optimisation of potential limits, scan rate and cycle number.

The profiles of cyclic voltammograms carried out during the electrodeposition of PANi onto Nafion/Pt-SPE differed slightly with respect to those reported in the literature [22]. The difference results in a ca. 0.15 V cathodic shift for the arising peaks taking into consideration the screen-printed reference electrode. The anodic scan of the first cycle was characterised by a large peak of monomer oxidation at 0.9 V. The backward scan was characterised by a nucleation loop, probably due to the new phase formation, followed by a broad cathodic wave. Upon further cycling, the monomer oxidation peak decreased with simultaneous growth of two redox processes at 0 – 0.15 V (emeraldine) and 0.5 – 0.65 V (pernigraniline), confirming the typical redox characteristics of polyaniline films which were additionally indicated visually by the colour change to dark green of the ermeraldine (half oxidised form).

3.2. Electrochemical properties of Nafion/PANi-modified electrode towards ammonium ions

The electrochemical properties of Nafion/PANi-modified electrode were examined using cyclic voltammetry at pH 7.4 (Fig. 1A). The addition of NH_4Cl to the solution led to the appearance of a voltammetric response in the form of increased cathodic currents (-0.2 to -0.4 V).

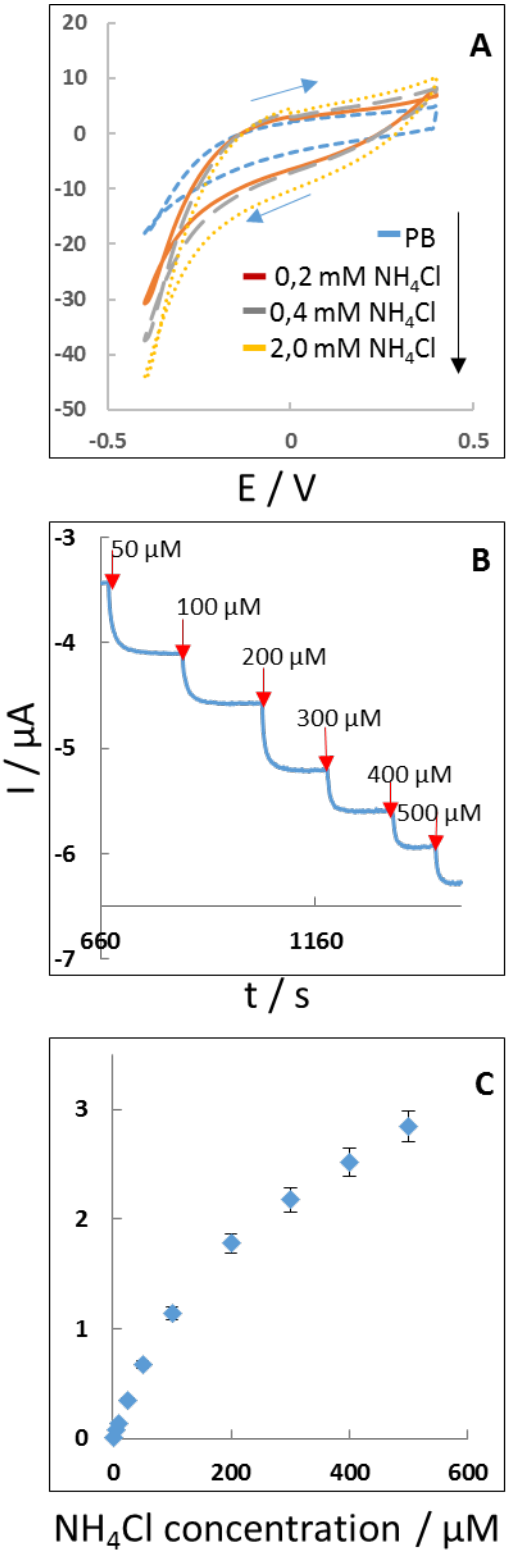


Figure 1. Amperometric ammonium detection on Pt-SPE modified with Nafion/PANi composite. **A**: Cyclic voltammograms recorded before and after addition of NH_4Cl (PB pH 7.4, scan rate 20 $mV \cdot s^{-1}$); **B**: an example of amperometric response of transducer to successive additions of NH_4Cl (-0.35 V, PB pH 7.4); **C**: calibration curve for ammonium detection obtained at PANi/Nafion/Pt-SPE (n=3).

Ammonium ions which diffused to the Nafion/PANi layer provoked the reduction of PANi on Pt-SPE to the leucoemeraldine form. If the PANi/Nafion composite is polarised at a reduction potential, the cathodic response is proportional to the ammonium ion concentration in the solution [31].

In order to determine the optimal polarisation voltage for amperometric detection, ammonium ion assays at a range of applied potentials (-0.2 to -0.4 V) were carried out with the current maximum found at -0.35 V (Fig. 3S). Hydrodynamic data for the PANi/Nafion/Pt-SPE is shown in (Fig. 1B), and exhibited a consistent increase in the steady state cathodic current in response to increased ammonium ion concentration. Under the same conditions, PANi/Nafion/Pt-SPE was completely insensitive to L-Arg (Fig. 2B). The resultant calibration curve exhibited a linear range from 0.003 to 0.2 mM and a sensitivity value within the linear range of 701±33 $A \cdot M^{-1} \cdot m^{-2}$ (Fig. 1C). The limit of detection (LOD) calculated as 3×SD was 1 µM NH_4Cl.

3.3. Immobilisation of ADI and biosensor performance in model media (PB pH 7.4)

ADI was immobilised on PANi/Nafion/Pt-SPE by cross-linking with BSA in saturated GA vapour according to experimental section 2.5. The presence of BSA ensured a high operational stability due to ideal conditions for retaining the maximal enzyme activity.

The pH optimum resulting in highest activity for the sensor was 7.2 in 20 mM PB (Fig. 4S), which is compatible with the requirements for physiological testing. These conditions were employed for further L-Arg assays. Voltammetric studies of ADI/PANi/Nafion/Pt-SPE are shown in Fig. 2A. The addition of L-Arg into the cell resulted in a significant increase in the cathodic current at potentials between -0.2 and -0.4 V (vs. Ag/AgCl).

The CV results and previous ammonium ion detection data indicated that -0.35 V was a suitable applied potential for the detection of L-Arg. In Fig. 2B, the amperometric responses of the L-Arg biosensor are reported showing a consistent increase in the cathodic current as a result of increasing L-Arg concentration. The calibration curve is shown in Fig. 2C. The dynamic range was linear between 0.003 and 0.2 mM L-Arg (Fig 2D). The K_M^{app} derived from the Lineweaver-Burk plot was 0.31±0.05 mM for L-Arg. This value is practically the same as that for ADI in solution (0.35 mM [27]). The sensitivity was calculated as 684±32 $A \cdot M^{-1} \cdot m^{-2}$, LOD was 1 µM and the response time was 15 s.

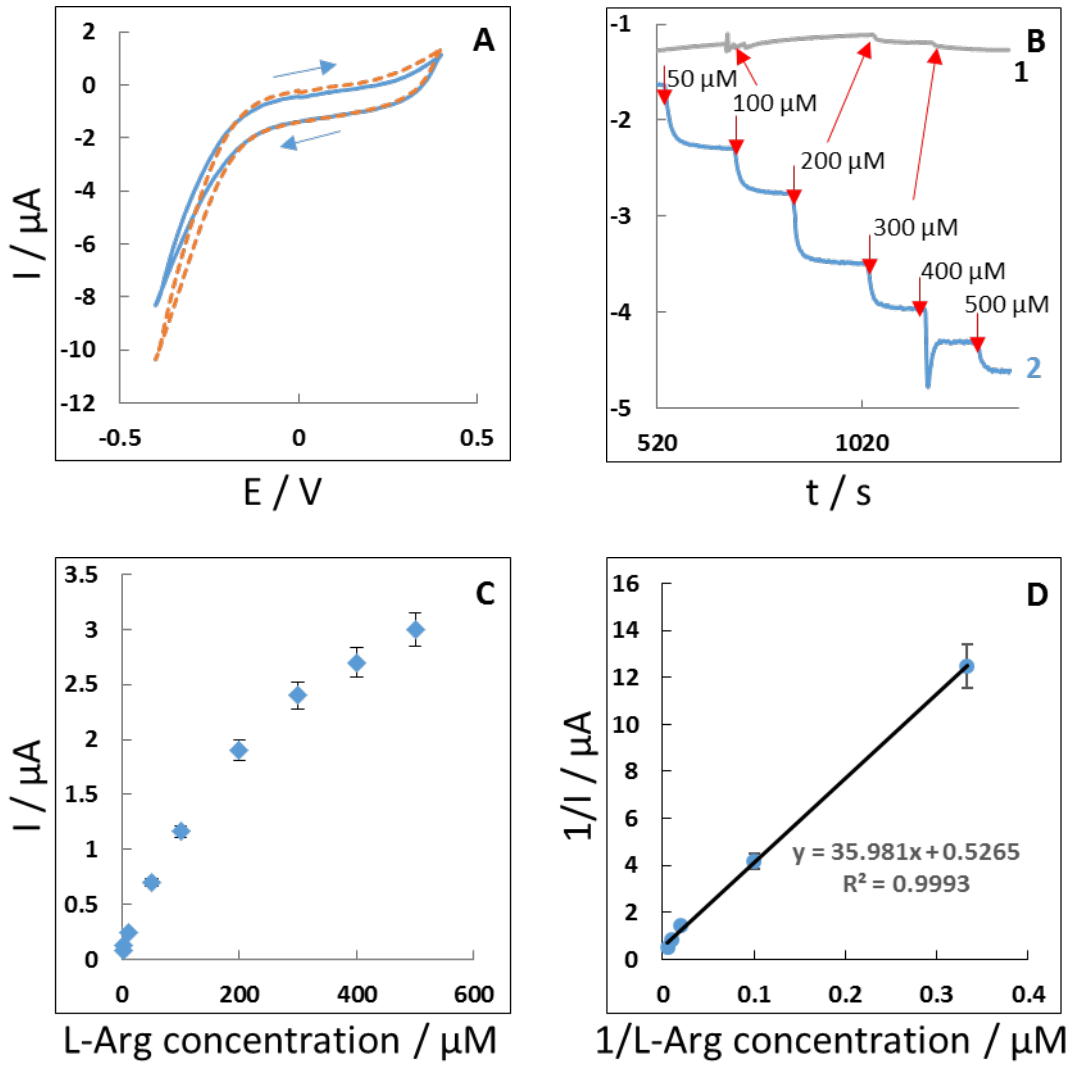


Figure 2. Amperometric L-Arg detection on a ADI/PANi/Nafion/Pt-SPE. **A**: Cyclic voltammograms recorded before and after addition of 0.5 mM L-Arg (blue and red curves, respectively; PB pH 7.4, scan rate 20 $mV \cdot s^{-1}$); **B**: an example of amperometric response of PANi/Nafion/Pt-SPE (1) and L-Arg biosensor (2) to successive additions of L-Arg (-0.35 V, PB pH 7.4); **C**: calibration curve for L-Arg detection at ADI/PANi/Nafion/Pt-SPE (n=3); **D**) Lineweaver-Burk plot for calibration curve.

The efficacy of the ADI reaction was calculated from the $I_{steady\text{-}state}/2$ values, presented in the graphs in Fig. 1C and Fig. 2C. The similarity of the calibration curves for detection of both ammonium ions and L-Arg indicates that the conversion rate of L-Arg to citrulline and ammonium ion was 97.6%. In addition, the almost ideal L-Arg conversion rate means that the procedure

chosen for ADI immobilisation on the Nafion/PANi/Pt-SPE was the most enzyme-friendly one (i.e. the active sites, conformation and catalytic activity of the ADI were not altered significantly during the immobilisation procedure).

The stability of ADI/PANi/Nafion/Pt-SPE was explored by monitoring its response to the injection of a 0.2 mM L-Arg standard solution over a period of 35 days. The biosensor preserved around 93% (SD = 6.5%) stability after 35 days storage or following 100 assays (Fig. 6S).

The comparison of the analytical performance of the L-Arg-selective biosensor with the analytical parameters of systems described in the literature (Table 1) shows that the biosensor realised here has advantageous characteristics, yielding one of the lowest limits of detection together with high sensitivity, wide linear range and long-term stability, thus making it potentially suitable for clinical sample analysis for the L-Arg levels of humans (60-100 μM in blood [32]).

The effects of some possible interfering amino acids (Ile, Lys, Thr, Orn, Ser, Val, Cys, GABA and citrulline) on the PANi/Nafion/Pt-SPE composite were investigated and the presence of amino acids in PB showed negligible response (significance level less than 5%) (Fig. 5S).

It was reported earlier, that canavanine, the substrate of arginase I, is hydrolysed by ADI and induces an analytical signal [33]. However, an impact of canavanine on the L-Arg assay is not important due to the rare presence of this compound in biological fluids (blood, serum/plasma and urine).

Notably, an addition of highly concentrated solutions of chloride salts (over 50 mM, Fig. 7S) causes a decrease in the current due to a potential shift at the internal open Ag reference electrode of the biosensor, which coincides well with the results obtained by Strehlitz et al. [31].

3.4. L-Arg assay in pharmaceutical and human plasma samples

In order to demonstrate feasibility of the L-Arg biosensor for the assessment of target analyte concentrations in real samples, some L-Arg-containing pharmaceutical and human plasma samples were collected and analysed. The results were compared with the L-Arg content estimated by the reference method (DMO-based fluorometric enzymatic method [11] (Table 2).

L-Arg assay in commercial pharmaceutical samples was performed by amperometric detection using a portable potentiostat (μStat 400), following the standard addition method (Fig. 3).

Table 1: Analytical characteristics of reported L-Arg biosensors.

Mode of signal registration	Biocomponent	LOD, mM	Linear range, mM	Response time (95%), min	Stability, days	Reference
Potentiometric, ASE*	Bacterial cells		0.05 – 1.0			[13]
Potentiometric, NH_3 gas sensor	U/A**		0.03 – 3.0	5.0		[18]
Potentiometric, ASE	U/A	0.01	0.1 – 30	1.5 – 4.0	21	[16]
Potentiometric, pH	U/A		0.025 – 0.31	10		[17]
Potentiometric, ASE	U/A	0.1	0.12 – 40	1.5 – 5.0	15	[19]
Conductometric	U/A	0.0005	0.01 – 4.0	2.0	45	[20]
Amperometric	U/A	0.038	0.07 – 0.6	0.17	3	[22a]
Amperometric	Urease/Yeast cells	0.085	0.085-0.6	1	3	[22b]
Amperometric	ADI	0.001	0.003 – 0.2	0.25	Over 35	Presented in the current paper

* ASE – ammonium-selective electrode; **U/A – Urease and arginase I.

In our study “Aminoplazmal 10% E”, an L-Arg-containing pharmaceutical was tested as it represents one of the most interesting L-Arg-containing commercially available samples (according to the manufacturer's certificate, this pharmaceutical contains a number of amino acids and other additives). The results of L-Arg assay in the “Aminoplazmal 10% E” using the biosensor were compared with those declared by manufacturer and only slight differences were observed

(RSD is less than 2.5% for n = 3) (Fig. 3).

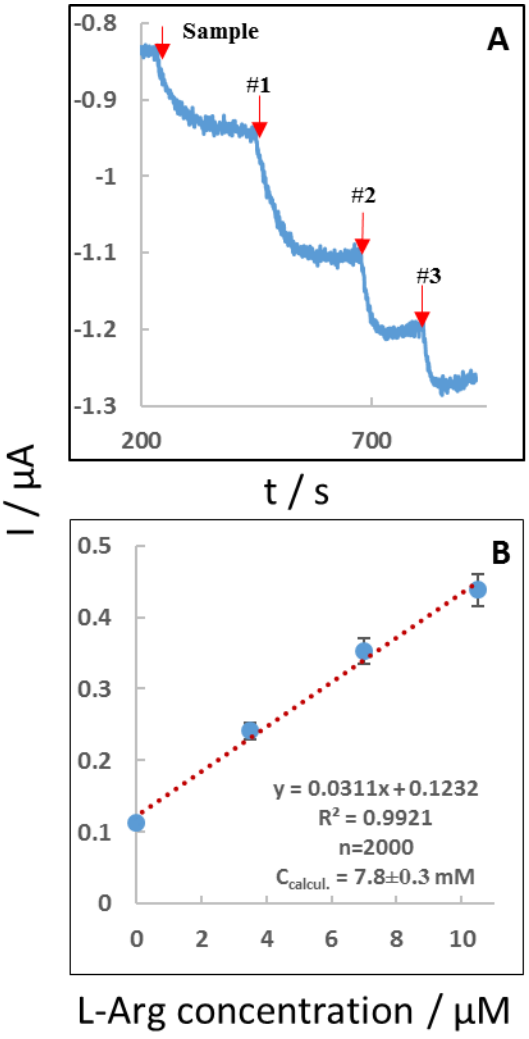
Table 2: Analytical performance of the L-Arg biosensor for real samples.

Sample	**L-Arg + NH_4^+**, mM	**NH_4^+**, mM	**L-Arg** calculated, mM	**L-Arg** reference assay, mM
	Measured by biosensor	Measured by PANi/Nafion/Pt-SPE		Fluorometric detection
Pharmaceutical	**7.8**±0.3	-	**7.8**±0.3	**8.0****
Plasma 1	**0.157**±0.022	**0.07**±0.01	**0.087**±0.014	**0.082**±0.008***
Plasma 2	**0.127**±0.018	**0.047**±0.006	**0.079**±0.012	**0.071**±0.007***
*Plasma 3**	**0.392**±0.051	**0.23**±0.02	**0.162**±0.021	**0.144**±0.015***
*Plasma 4**	**0.445**±0.063	**0.25**±0.03	**0.195**±0.027	**0.179**±0.018***

* Collected from patients with hepatic disorder

** Declared by producer

*** Fluorometric detection



The L-Arg biosensor was also tested for the L-Arg quantification in plasma samples of healthy donors and patients with hepatic disorder using the standard addition method (RSD – 14% for n = 3). Due to the fact that in serum/plasma of healthy humans the concentrations of L-Arg and endogenous ammonium ions are very close (60-100 µM [32] and 20-50 µM [34], respectively), the latter was additionally measured in plasma samples with the PANi/Nafion/Pt-SPE. A concentration of L-Arg in plasma was calculated as a difference between the signals of ADI/PANi/Nafion/Pt-SPE- and PANi/Nafion/Pt-SPE -based sensors.

Figure 3. L-Arg analyses in pharmaceutical "Aminoplazmal 10% E" by ADI sensor using method of standard additions. **A**: an example of amperometric response of L-Arg sensor to 2000-fold dissolved "Aminoplazmal" sample and subsequent standard additions (#1, #2, #3) of L-Arg (-0.35 V, PB pH 7.4); **B**: calibration curves for L-Arg detection in "Aminoplazmal" sample dissolved 2000-fold (n=3). Statistical data: parameters of linear regression; R^2 – linear regression coefficient; n – dilution factor; $C_{calcul.}$ – calculated ammonia/arginine concentration in plasma sample.

The data presented showed a strong correlation with the reference method for L-Arg measurement based on fluorometry with an RSD of 10%. The L-Arg concentration in plasma of healthy donors was 1.5-2 times lower than in plasma of patients with hepatic disorder. The results of L-Arg assays, presented in Table 2, are close to previously reported values for healthy adults [4, 32] and patients with hepatic disorder [35]. These results indicate that the L-Arg biosensor presented here can be exploited for fast and effective assay of L-Arg in simple (pharmaceuticals)

and complex (human plasma) media.

4. Conclusions

An amperometric L-Arg biosensor based on recombinant ADI has been developed and optimised for the first time. The biosensor is characterised by a low applied potential (-0.35 V), fast response time (15 s), broad linear range (0.003 to 0.2 mM), high selectivity and sensitivity (684±32 $A \cdot M^{-1} \cdot m^{-2}$), a low limit of detection (0.001 mM) and good storage stability (>35 days). The L-Arg amperometric biosensor facilitated facile analysis of real samples; there was no need for complex sample pretreatment (except sample dilution) due to the one-step L-Arg transformation by arginine deiminase. This performance cannot be achieved for bi-enzyme (arginase and urease) and for mono-enzyme (inhibitory urease) sensor formats reported previously as they will always suffer from the necessity to eliminate the endogenous urea in plasma, blood and urine samples). Measurements in real samples (pharmaceutical and human plasma) proved to be in a good agreement with reference fluorometric enzymatic assay (RSD do not exceed 10%).

5. Acknowledgement

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6. References

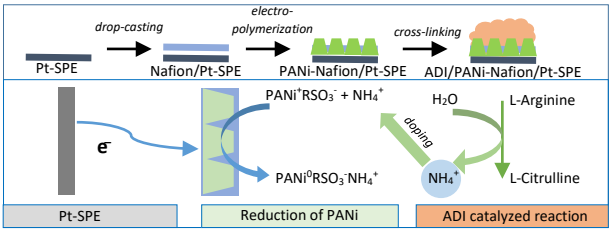
1. Yang Y, Bedford MT (2013) Protein arginine methyltransferases and cancer. Nat Rev Cancer 13: 37-50.
2. Barbul A, Lazarou SA, Efron DT, Wasserkrug H, Efron G (1990) Arginine enhances wound healing and lymphocyte immune responses in humans. Surgery 108: 331–336.
3. Feun L, You M, Wu CJ, Kuo MT, Wangpaichitr M, Spector S, Savaraj N (2008) Arginine deprivation as a targeted therapy for cancer. Curr Pharm Des 14(11): 1049-1057.
4. Vermeulen MA, van de Poll MC, Ligthart-Melis GC, Dejong CH, van den Tol MP, Boelens PG, van Leeuwen PA (2007) Specific amino acids in the critically ill patient--exogenous glutamine/arginine: a common denominator? Crit Care Med 35(9): S568-S576.
5. Farina M, Finetti G, Busnelli V (1990) High-performance liquid chromatographic method for the simultaneous assay of a new synthetic penem molecule and its salt-forming agent in injectable formulations. J Chromatogr 507: 171–176.
6. Sastry CSP, Tummuru MK (1994) Spectrophotometric determination of arginine in proteins. Food Chem 15: 257–260.
7. Miura T, Kashiwura M, Kimura M (1984) A fluorometric method for the specific determination of serum arginine with 2,3-naphthalenedicarbaldehyde. J Biochem 139: 432–437.
8. Jin LT, Zhu H, Xu TM, Tong W, Zhou WL, Fang Y (1992) Indirect determination of arginine by graphite furnace atomic absorption spectrometry after preconcentration on a Nafion chemically modified tungsten coil. Anal Chim Acta 268: 159–162.
9. Godinho OES, Aleixo LM, Neto GO (1991) Titration of proteins in dimethyl sulphoxide-water mixtures. Analyst 116: 1181–1183.
10. Hemandez L, Escalona J, Joshi N, Guzman N (1991) Laser-induced fluorescence and fluorescence microscopy for capillary electrophoresis zone detection. J Chromatogr 559: 183–196.
11. Stasyuk N, Gaida G, Gonchar M (2013) L-arginine assay with the use of arginase I. Appl Biochem Microbiol 49(5): 529-534.

12. Stasyuk N, Gayda G, Fayura L, Boretskyy Y, Gonchar M, Sibirny A (2016) Novel arginine deiminase-based method to assay l-arginine in beverages. Food Chem 201: 320-326.
13. Rechnitz GA, Kobos RK, Riechel SJ, Gebauer CR (1977) A bio-selective membrane electrode prepared with living cells. Anal Chim Acta 94: 357-365.
14. Kacaniklic V, Johansson K, Marko-Varga G, Gorton I, Jonsson-Pettersson G, Csoregi E (1994) Amperometric biosensors for detection of L- and D-amino acids based on coimmobilized peroxidase and L- and D-amino acid oxidases in carbon paste electrodes. Electroanalysis 6: 381-390.
15. Domínguez R, Serra B, Reviejo AJ, Pingarrón JM (2001) Chiral analysis of amino acids using electrochemical composite bienzyme biosensors. Anal Biochem 298: 275–282.
16. Koncki R, Walcerz I, Ruckruh F, Glab S (1996) Bienzymic potentiometric electrodes for creatine and L-arginine determination. Anal Chim Acta 333: 215–222.
17. Karacaoğlu S, Timur S, Telefoncu A (2003) Arginine selective biosensor based on arginase-urease immobilized in gelatin. Artif Cells Blood Subst Immob Biotechnol 31: 357- 363.
18. Nikolelis DP, Hadjiioannou TP (1983) Construction of an arginine enzyme electrode and determination of arginine in biological materials. Anal Chim Acta 147: 33–39.
19. Stasyuk N, Smutok O, Gayda G, Gonchar M, Koval'chuk Y (2011) A new bi-enzyme potentiometric sensor for arginine analysis based on recombinant human arginase i and commercial urease. J Material Sci and Eng 1: 819-827.
20. Saiapina OY, Dzyadevych SV, Jaffrezic-Renault N, Soldatkin OP (2012) Development and optimization of a novel conductometric bi-enzyme biosensor for L-arginine determination. Talanta 92: 58-64.
21. Martínez-Periñán E, Revenga-Parra M, Zamora F, Pariente F, Lorenzo E (2016) Nanostructured electrochemical detector for the quantification of amino acids related to metabolic diseases. Sens Actuators B 236: 773-780.
22. **a)** Stasyuk N, Smutok O, Gayda G, Vus B, Koval'chuk Y, Gonchar M (2012) Bi-enzyme L-arginine-selective amperometric biosensor based on ammonium-sensing polyaniline-modified electrode. Biosensors & Bioelectronics 37(1): 46-52; **b)** Stasyuk N, Gayda G, Gonchar M (2014) l-Arginine-selective microbial amperometric sensor based on

recombinant yeast cells over-producing human liver arginase I. Sens Actuators B 204: 515-521.

23. Ricca E, Brucher B, Schrittwieser JH (2011) Multi-enzymatic cascade reactions: overview and perspectives. Advanced Synthesis & Catalysis 353(13): 2239-2262.
24. Wei Y, Zhou H, Sun Y, He Y, Luo Y (2007) Insight into the catalytic mechanism of arginine deiminase: functional studies on the crucial sites. Proteins: Structure, Function, and Bioinformatics 66(3): 740-750.
25. Verma N, Singh AK, Kaur P (2015) Biosensor based on ion selective electrode for detection of L-arginine in fruit juices. J of Anal Chem 70(9): 1111-1115.
26. Luo YC, Do JS (2004) Urea biosensor based on PANi(urease)-Nafion/Au composite electrode. Biosensors & Bioelectronics 20(1): 15-23.
27. Fayura LR, Boretsky YR, Pynyaha YV, Wheatley DN, Sibirny AA (2013) Improved method for expression and isolation of the *Mycoplasma hominis* arginine deiminase from the recombinant strain of *Escherichia coli.* Journal of Biotechnol 167: 420-426.
28. Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189(1): 113-130.
29. Misawa S, Aoshima M, Takaku H, Matsumoto M, Hayashi H (1994) High-level expression of *Mycoplasma* arginine deiminase in *Escherichia coli* and its efficient renaturation as an anti-tumor enzyme. Biotechnology 36 (2): 145-155.
30. Cho WJ, Huang HJ (1998) An amperometric urea biosensor based on a polyaniline–perfluorosulfonated ionomer composite electrode. Anal Chem 70(18): 3946-3951.
31. Strehlitz B, Gründig B, Kopinke H (2000) Sensor for amperometric determination of ammonia and ammonia-forming enzyme reactions. Anal Chim Acta 403(1-2): 11-23.
32. Böger RH, Bode-Böger SM (2001) The clinical pharmacology of L-arginine. Annu Rev Pharmacol Toxicol 41: 79-99.
33. Reczkowski RS, Ash DE (1994) Rat liver arginase: kinetic mechanism, alternate substrates, and inhibitors. Arch Biochem Biophys 312, 31-37.
34. Barsotti RJ (2001) Measurement of ammonia in blood. J Pediatr 138: S11-S19.

35. Nijveldt RJ, Siroen MPC, van der Hoven B, Teerlink T, Prins HA, Girbes ARJ, van Leeuwen PAM (2004) High plasma arginine concentrations in critically ill patients suffering from hepatic failure. Eur J Clin Nutr 58(4): 587-593.



Graphical abstract. An amperometric biosensor for sensitive and selective detection of L-Arginine based on a Nafion/polyaniline (PANi) composite at a platinum screen-printed electrode (Pt-SPE) using a novel recombinant arginine deiminase (ADI) from *Mycoplasma hominis* is reported.

SUPPORTING INFORMATION

Amperometric L-arginine biosensor based on a novel recombinant arginine deiminase

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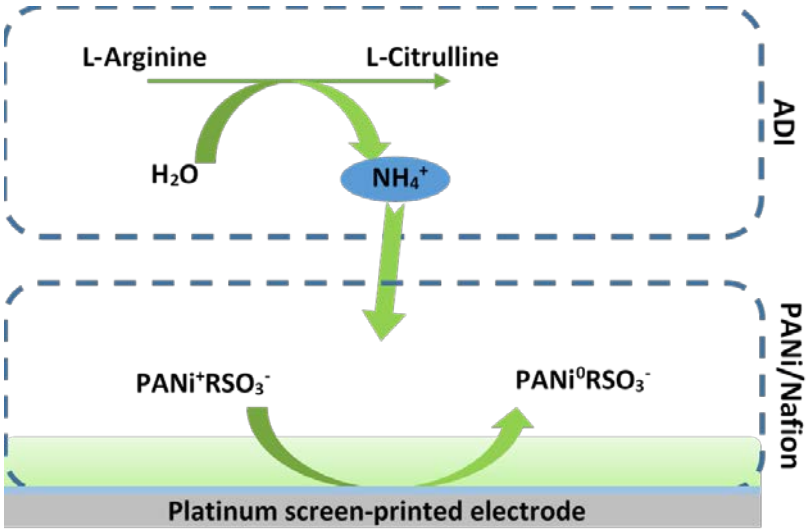

Figure 1S. Schematic representation of the biosensor. $PANi^+$ and $PANi^0$—oxidised and reduced forms of PANi, respectively; RSO_3^- - Nafion. (Stasyuk *et al*. 2012)

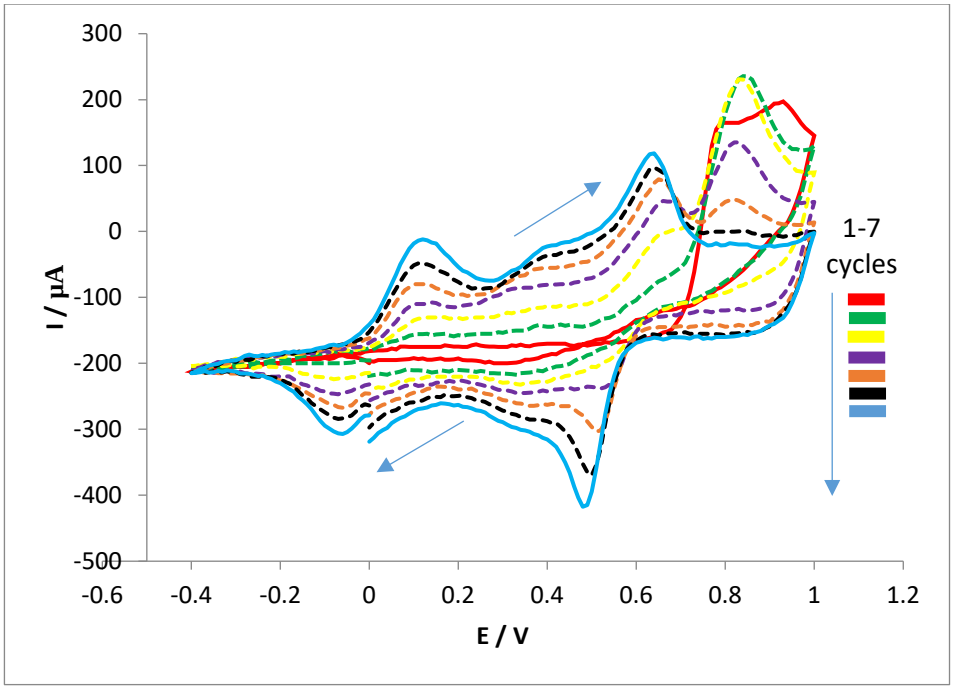

Fig. 2S. Electrochemical polymerisation of PANi onto Nafion-modified PSPE (0.5 M HCl, 0.2 M aniline, scan rate 0.02 V/s vs Ag/AgCl).

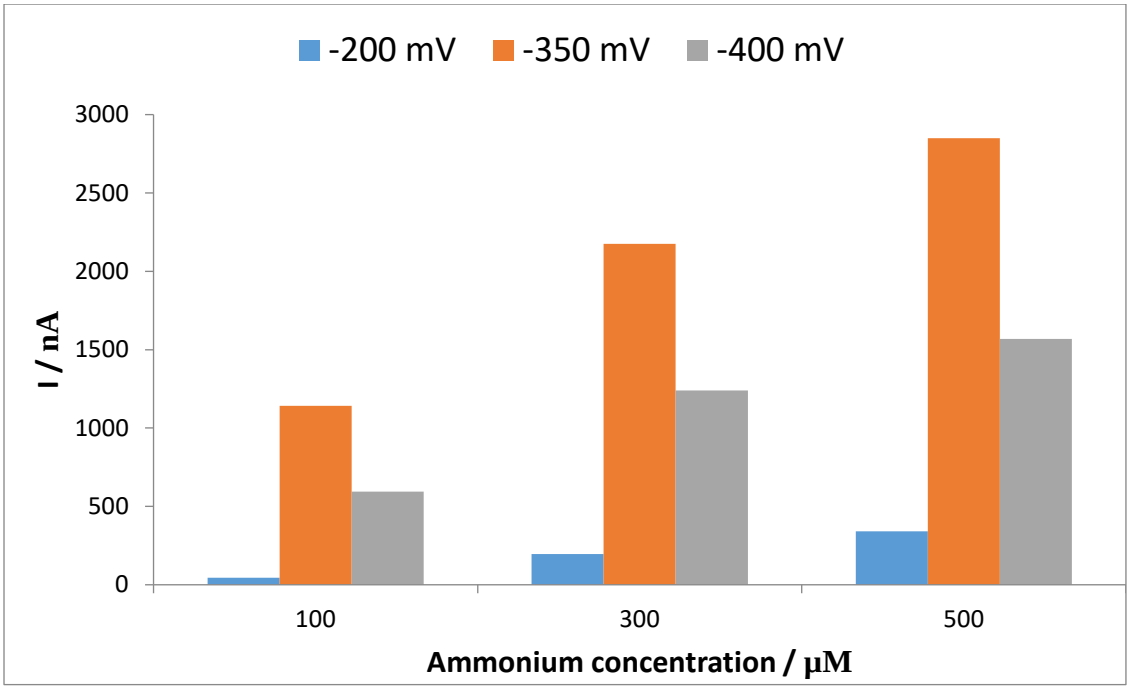

Fig. 3S. Selection of the optimal potential for ammonium ion amperometric detection (20 mM PB pH 7.4, *vs* Ag/AgCl).

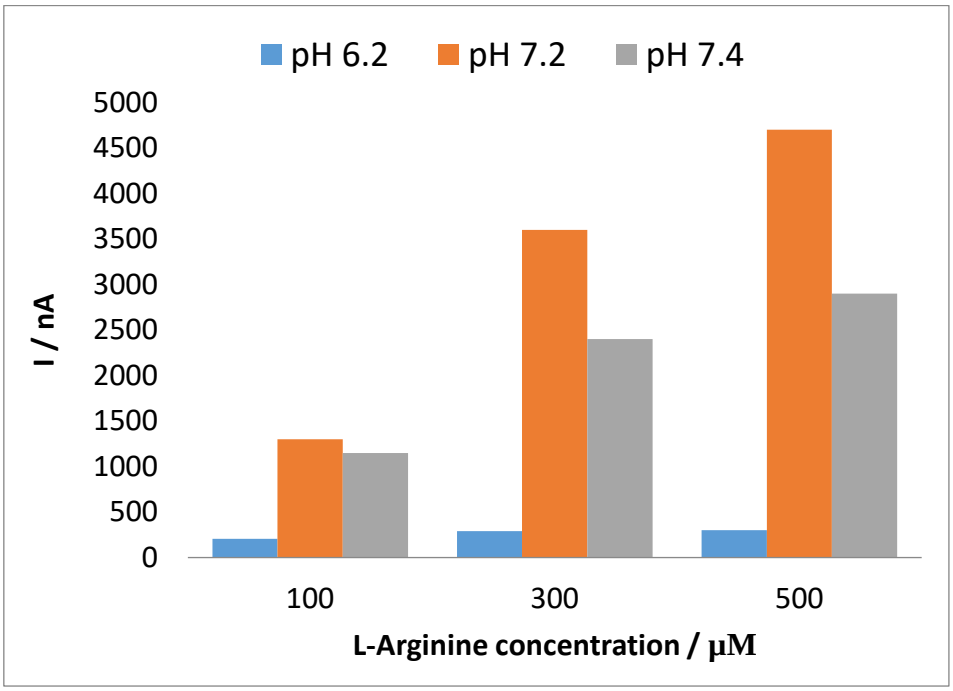

Fig. 4S. Selection of the optimal pH for L-Arg amperometric detection (-0.35 V vs Ag/AgCl in 20 mM PB, at 22 °C).

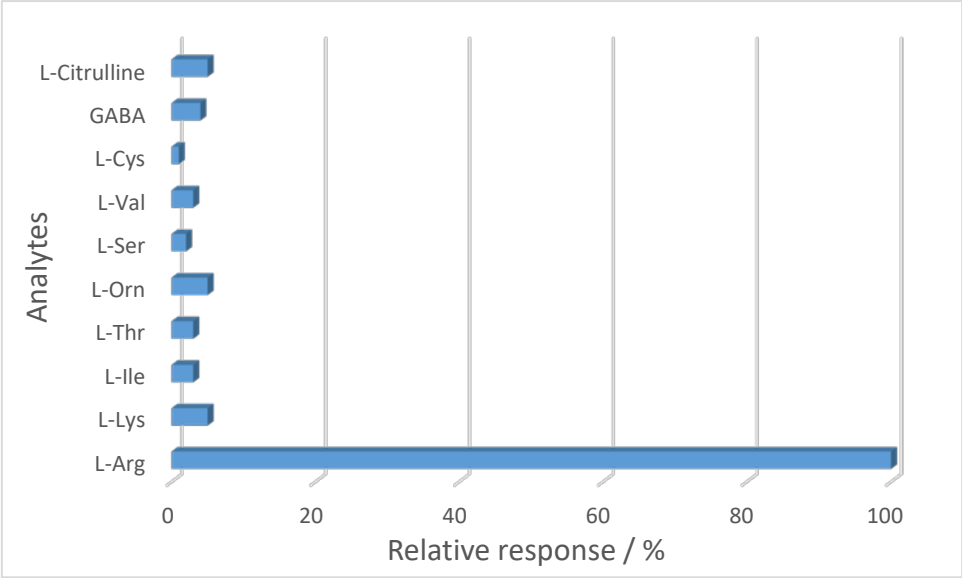

Fig. 5S. Effects of some possible interfering compounds on the L-Arg biosensor. Measurement conditions: −0.35 V vs Ag/AgCl in 20 mM PB pH 7.4. 0.1 mM L-Arg, 0.2 mM Ile, Lys, Thr, Orn, Ser, Val, Cys, GABA and citrulline.

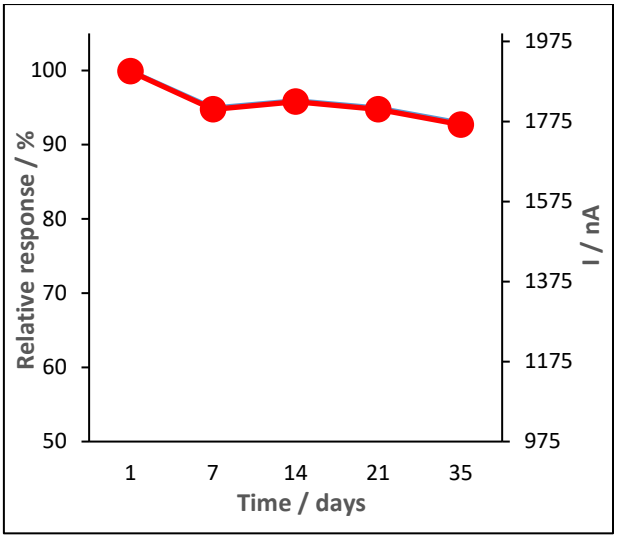

Fig. 6S. Stability of the L-Arg biosensor. Response of ADI/PANi/Nafion/PSPE to 0.2 mM L-Arg. Storage conditions: 50 mM PB pH 7.4 at + 4 ºC. Measurement conditions: -0.35 V vs Ag/AgCl in 20 mM PB pH 7.4.

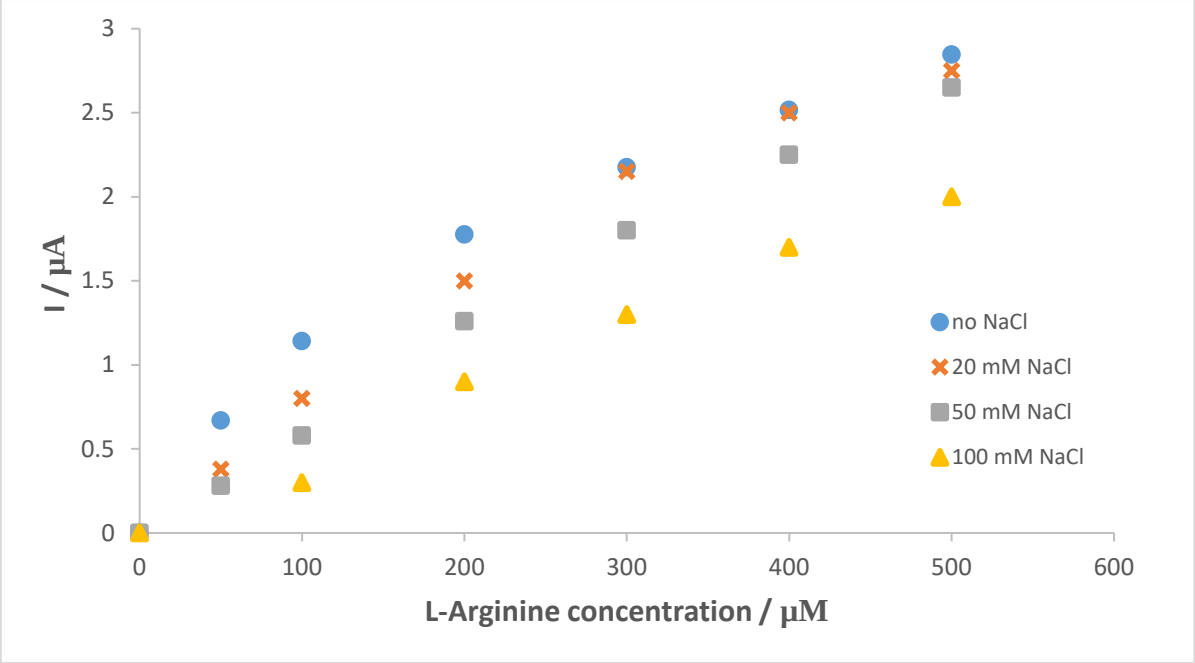

Fig. 7S. Influence of sodium chloride on the L-Arginine biosensor response (-0.35 V vs Ag/AgCl in 20 mM PB pH 7.4.)