Bioelectrochemistry 124 (2018) 47–56

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

A novel approach for the selective analysis of L-lysine in untreated human serum by a co-crosslinked L-lysine–α-oxidase/overoxidized polypyrrole bilayer based amperometric biosensor

Rosanna Ciriello, Francesca De Gennaro [1], Silvio Frascaro [2], Antonio Guerrieri *

Dipartimento di Scienze, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy

ARTICLE INFO

Article history:
Received 7 March 2018
Received in revised form 28 June 2018
Accepted 3 July 2018
Available online 06 July 2018

Keywords:
Biosensor
Enzyme co-crosslinking
Overoxidized polypyrrole film
L-lysine–α-oxidase
Allosteric effects
L-Lysine analysis in human serum

ABSTRACT

An amperometric biosensor based on an L-lysine-α-oxidase (LO) layer immobilized by *co*-crosslinking onto the surface of an overoxidized polypyrrole modified Pt electrode (Pt/oPPy) and able to analyse L-lysine (Lys) in untreated human serum is described. The sensing electrode has been characterised and a proper enzyme kinetics optimisation permits to use a low specific enzyme as LO from *Trichoderma viride* for the selective biorecognition of Lys in the presence of other interferent amino acids; a kinetics study of LO evidenced also the allosteric behaviour of this enzyme, a kinetic feature which was never reported before for this enzyme. The biosensor showed a sensitivity of 0.11 μA/mM mm^2, linear responses up to 4 mM and a limit of detection of 2 μM; the within-a-day coefficients of variation for replicate ($n = 5$) were 0.92% and 1.35% at 4 mM and 0.2 mM Lys levels, respectively. The permselective behaviour of Pt/oPPy modified electrode assured an interference- and fouling-free determination of Lys even in untreated serum samples. The determination of Lys in human serum from healthy donors gave Lys levels in good agreement with the expected values so that the use of the proposed biosensor appears promising in the relevant clinical fields.

1. Introduction

L-Lysine (Lys) is a well-known essential amino acid, not synthesized in man and other mammals so requiring to be assimilated through food; in fact, a Lys misbalanced diet involves severe diseases necessitating a dietary addition to foods and drug supplementation. Anyway, Lys has a clinical and a medical relevance as well. As examples, Lys levels in blood seem related to the leukemic cell proliferation [1], HIV infections [2] and ageing progression too [3]. Furthermore, impaired intestinal absorption and renal tubular reabsorption of Lys (as arginine and ornithine) due to a defective cationic amino acid transport leads to a severe disease, *i.e.* lysinuric protein intolerance (LPI), involving diarrhea, hyperammonemia, interstitial pneumonia, hepatomegaly and liver cirrhosis, osteoporosis and bone marrow involvement [4]. Oral supplementation of Lys is known to correct plasma Lys concentration in LPI [5] but large amounts of extra Lys can also contribute to the total nitrogen load, further straining the already limited capacity of the urea cycle thus inducing acute renal failure [6]. Accordingly, monitoring of Lys in blood seems essential in LPI (as well as in other Lys related diseases) for a correct pharmacological Lys supplementation thus avoiding or minimising the side effects.

Lys and, more generally, amino acid analysis in biological fluids usually requires a significant sample pre-treatment followed by liquid chromatography (LC) with chemical derivatization for its detection [7,8]; recently, more sophisticated approaches based on NMR or LC-MS have been also proposed in the relevant clinical field [9,10]. Nevertheless, even if well established, these analytical methods do not satisfy the requirements for a simple, fast, accurate and specific analysis, usually necessary in clinical and medical fields, since requiring a time-consuming sample pre-treatment and, more importantly, complex and expensive instruments. In this respect, biosensors can play certainly a significant role because of their compactness, rapidity, low cost, simplicity of use, high sensitivity and specificity ruling out any sample pre-treatment as well.

Lys sensing by biosensors has been approached till now using several Lys specific enzymes coupled to different transduction schemes (see Refs. [11–13] for update information about) but the most successful

* Corresponding author.
E-mail address: antonio.guerrieri@unibas.it (A. Guerrieri).
[1] Present address: Dipartimento di Bioscienze, Università degli Studi di Milano, Via Giovanni Celoria 26, 20133 Milano, Italy.
[2] Present address: FARMALABOR srl Stabilimento Via Pozzillo, zona ind., 76012 Canosa di Puglia (Bt), Italy.

https://doi.org/10.1016/j.bioelechem.2018.07.004
1567-5394/

approaches are the electrochemical ones using L-lysine-α-oxidase (LO) as enzyme according to the following scheme:

$$\text{L-lysine} + O_2 \rightarrow \alpha\text{-keto-}\varepsilon\text{-aminocaproate} + H_2O_2 + NH_3$$

where successively α-keto-ε-aminocaproate spontaneously dehydrates to Δ^1-piperidine-2-carboxylate. The enzyme reaction, and hence Lys, is usually monitored amperometrically by measuring or the dioxygen consumption with a Clark-type electrode or, more usefully, the hydrogen peroxide formation simply with a Pt electrode. Even if straightforwardly, this approach is not without drawbacks. For instance, LO, the enzyme used for the Lys biorecognition, is known to catalyse to varying extents also the oxidation of other L-amino acids other than Lys [14] while its selectivity towards each amino acid substrate can even differ with the method used for its immobilization [15,16]. Consequently, the interfering effect from other amino acids, particularly L-ornithine and L-arginine, is the basic drawback observed in most Lys biosensor using LO as biorecognition element: just as examples, typical interference bias in Lys analysis from L-ornithine ranges from 28% up to 90% while L-arginine biased Lys analysis of 15% [16–18].

A further drawback in the above approach comes from the electrochemical oxidation of hydrogen peroxide which requires a relatively high potential for its detection in amperometric mode: indeed, at this detection potential, endogenous electroactive compounds from biological fluids (*e.g.* ascorbate) are oxidized as well thus interfering with Lys determination in real sample. Lowering of detection potential can improve of course the selectivity of the amperometric detection but require the use of mediators like ruthenium/rhodium [11] or Prussian Blue [19] and further electrode modifications which complicate the biosensor assembly and its analytical performances. As an alternative, electrochemical interferences can be effectively reduced using permselective polymers electrosynthesized directly onto the electrode surface of the amperometric detector: indeed, this approach is surely advantageous with respect to the classical approach using up to three membranes resulting in *e.g.* a high response time [20]. Actually, permselective films from diaminobenzene monomers were used for the construction of interferent-free Lys biosensors [21–24] but their effectiveness appears questionable particularly if mediators were also coupled in some cases to reduce further the electrochemical inference effects [11,24]. Notably, none of these cited approaches were applied for the analysis of Lys in complex samples like biological fluids but limited to simpler sample analysis (*e.g.* pharmaceutical products and food samples), probably due to the limited selectivity of these biosensors.

In our laboratory, a novel amperometric biosensor able to a highly selective determination of Lys has been developed too. This achievement was obtained by immobilising LO by glutaraldehyde co-crosslinking with bovine serum albumin onto the electrode surface of the amperometric detector; a thoroughly optimization of the immobilization procedure also permitted us to achieve a fast response biosensor with high sensitivity and improved long-term stability which resulted useful also for flow-injection applications [12]. Remarkably, by switching the pH from weakly alkaline to weakly acid values and/or by regulating the hydrodynamic behaviour of the biosensor, it was possible to tune the overall kinetic control of the biosensor from merely diffusive to enzymatic or mixed mode thus improving the enzyme specificity and minimising significantly amino acid interferences in Lys analysis even using a low specific commercial enzyme like LO [13]. The so developed biosensor coupled to a permselective modified electrode proved suitable for the quantification of Lys in several cheese samples satisfying the demands for precise and sensitive detection of Lys with minimal sample preparation and clean-up [25]. In the present paper, we will show that coupling an enzyme layer based on LO immobilized by co-crosslinking with a Pt electrode previously modified by an overoxidized polypyrrole film permits the development of a highly selective biosensor for the analysis of Lys in more complex sample like human serum without any sample pre-treatment.

2. Experimental

2.1. Reagents and sample

L-Lysine, L-tyrosine, L-phenylalanine, ascorbic acid, L-lysine-α-oxidase (EC 1.4.3.14, from *Trichoderma viride*, 49.31 units per mg protein), glutaraldehyde (grade II, 25% aqueous solution) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (USA). L-cysteine, uric acid and pyrrole were purchased from Aldrich (Aldrich-Chemie, Germany). All the other chemicals were of analytical reagent grade. Before its use, pyrrole was purified by distillation under vacuum at 52 °C. The pyrrole and interferent solutions were prepared just before their use. L-Lysine stock solutions were stored at 4 °C. All aqueous solutions were prepared in double distilled-deionized water. Human serum samples from healthy donors come from a local hospital (Azienda Ospedaliera San Carlo — Potenza) and immediately frozen at −20 °C after their collection: human serum samples from healthy donors were collected and used by Azienda Ospedaliera San Carlo in accordance with its local ethics committee and informed consent was obtained from donors for experimentation and use of their serum samples; further, our Department provided us the relevant consent to use in our laboratory biological sample for research purposes in accordance with the ethics committee of our University.

2.2. Apparatus

A Gilson Minipuls 3 peristaltic pump (Gilson Medical Electronics, Villiers-Le-Bel, France) and a seven-port injection valve (Rheodyne mod. 7725, Cotati, CA, USA) equipped with a 20 μL sample loop were used for the flow-injection experiments. A PEEK tubing (0.25 mm ID, 130 cm length) was used to connect the sample injection valve to the EG&G Model 400 electrochemical detector (Princeton Applied Research, Princeton, N.J.). The detector included a thin-layer electrochemical cell with a Pt disk (3 mm diameter) working electrode and an Ag/AgCl, 3 M NaCl reference electrode; in some experiments, a dual Pt disk (both 3 mm diameter) working electrode (parallel configuration) was used. Two thin layer flow cell dual gaskets (Bioanalytical Systems, Inc., USA) of 0.004 inch thickness were used. A Kipp & Zonen (Delft BV, Holland) mod. BD 11 E Flatbed Yt recorder was used for flow injection signal chart recording.

Controlled electrochemical deposition of polypyrrole film was carried on a conventional three electrode cell equipped with a SCE reference electrode and a Pt counter electrode by using an EG&G model 263A potentiostat/galvanostat equipped with a M270 electrochemical research software (EG&G) version 4.23 for data control and acquisition.

Enzyme-layer thickness was estimated by using an Alpha-Step 200 profilometer (Tencor Instruments, Mountain View, CA, USA): in these experiments, the stylus mass was 8 mg and the scan rate was 0.2 mm s^{-1}. Environmental scanning electrode microscope (ESEM) studies were performed with an ESEM XL30 FEI (Philips Electron Optics, Eindhoven, The Netherlands).

2.3. Biosensor preparation

Each electrode modification has been preceded by a cleaning procedure consisting in dipping the Pt working electrode in hot nitric acid and then in polishing it by alumina (0.05 μm particles), extensive washing and sonication in bidistilled water.

The electropolymerisation of polypyrrole films (PPy) was performed at +0.7 V *versus* SCE by using a 0.4 M pyrrole solution in 0.1 M KCl supporting electrolyte until a deposition charge of typically 300 mC/cm^2 was achieved. The Pt/PPy modified electrode was overoxidized at +0.7 V *versus* SCE in a phosphate buffer (pH = 7.0, I = 0.1 M) until a steady-state background current was obtained

(at least 7 h). Overoxidised Pt/PPy electrodes (Pt/oPPy) were then washed and air-dried at room temperature.

Lysine biosensors were prepared by following the procedure elsewhere developed by the authors [12,13]. 26.3 units of L-lysine–α-oxidase (LO) were dissolved into 250 μL of phosphate buffer, pH 7.4, I 0.1 M: 50 μL of the enzymatic solution were then used to dissolve 2.6 mg of bovine serum albumin (BSA) and carefully mixed with 5 μL of 5% glutaraldehyde (GLU) solution (25% GLU solution diluted 1:5 with phosphate buffer pH 7.4, I 0.1 M). Four μL of the resulting solution were carefully pipetted onto the Pt/oPPy working electrode surface, avoiding air bubble formation, and carefully spread out to cover completely the electrode surface. The modified electrode was then air-dried at room temperature for few minutes. The resulting Pt/oPPy/LO modified electrode was preliminarily soaked in the background electrolyte for a few minutes just to removing weakly bound or adsorbed enzyme and to permit swelling of the enzyme layer. When not in use, the biosensor was stored in phosphate buffer, pH 7.4, I 0.1 M, at 4 °C in the dark. In dual working electrode experiments, both electrodes were modified with an equally electrodeposited and overoxidized oPPy film: successively, one electrode was modified with the enzyme solution as above described while the other was modified by carefully pipetting on it 4 μL of a solution obtained by carefully mixing 50 μL of a BSA solution (2.6 mg of BSA dissolved in 50 μL phosphate buffer pH 7.4, I 0.1 M) with 5 μL of 5% GLU solution; dual electrode measurements were then performed in parallel configuration.

2.4. Film morphology and thickness

Profilometric measurements and ESEM studies were performed on a modified Pt foil (10 × 15 × 0.127 mm) 99.99% pure (Aldrich) to measure the enzyme layer thickness and illustrate film morphology respectively. For this, the Pt foil was modified by an oPPy/LO layer exactly as described in the "Biosensor preparation" section to produce a surface modification almost identical to that prepared for modified electrodes: in particular, almost a half of the Pt foil surface was modified by an oPPy layer (deposition charge 300 mC/cm^2), so to appreciate any morphological difference between the Pt substrate and the polymer, while a LO layer of 3 mm diameter was dropped onto the oPPy layer so to reproduce exactly the sensing layer of here developed biosensor.

2.5. Electrochemical measurement

All the flow injection electrochemical measurements were performed using a detection potential of +0.7 V *versus* Ag/AgCl/NaCl (3 M) in acetate buffer (pH 5, I 0.1 M). Solutions and carrier stream were air saturated and the temperature was ambient. The flow rate in flow-injection experiments was 0.6 mL min^{-1} and the injection volume was 20 μL.

2.6. Analysis of serum sample

Just before analysis, serum samples were thawed at room temperature, diluted 1:2 with acetate buffer pH 5, I 0.1 M (to minimise any undesired artefact during detection) and 20 μL injected. Lysine analysis in serum was performed by the standard addition method.

3. Results and discussion

3.1. Biosensor construction and modified electrode characterization

The biosensor consists of a L-lysine–α-oxidase (LO) layer immobilized by co-crosslinking onto the surface of a Pt electrode previously modified by an overoxidized polypyrrole (oPPy) film.

Co-crosslinking [26], *i.e.* an inert protein-assisted enzyme crosslinking, is a valuable and easy enzyme immobilization procedure for biosensor construction: by simply casting a small amount of the proper [12] co-crosslinking enzyme solution onto the electrode surface, an immobilized enzyme layer was realised thus allowing the construction of a biosensor displaying a fast-response time, high sensitivity and stability. In agreement with previous findings [27–29], LO co-crosslinking produced a very thin enzyme layer strongly adherent to the electrode surface and mechanically very stable in stirred or flowing solutions thus permitting also in the present case a flow-injection analysis of L-lysine (Lys) sample. Notably, co-crosslinking was so effective for LO immobilization that enzyme leaking was never observed as proved (*vide infra*) from the operational and storage stability of the biosensor and from dual working electrode experiments where no enzymatic responses were observed at the unmodified working electrode due to *e.g.* enzyme release from the adjacent enzyme layer: these last

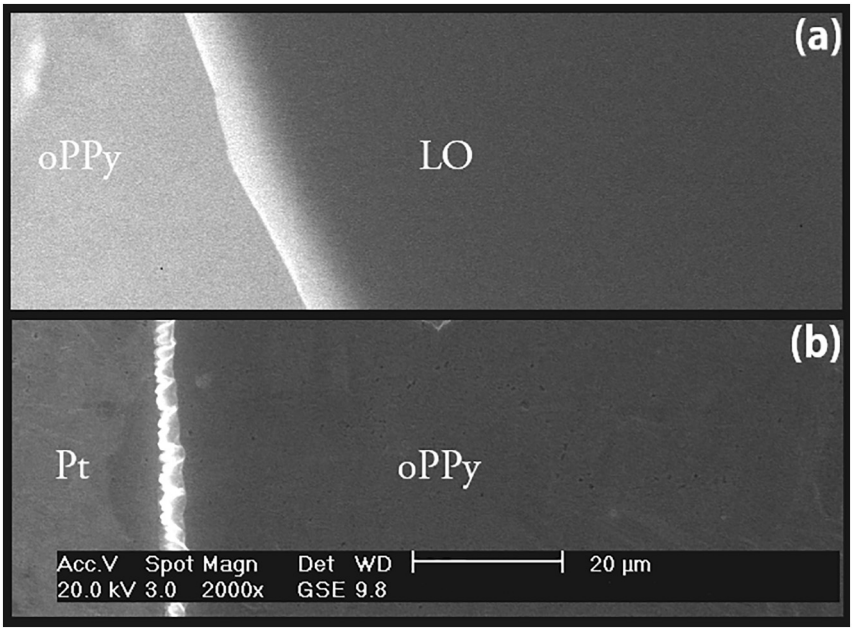

Fig. 1. Typical ESEM images of the LO enzymatic layer onto the oPPy film (a) and of oPPy film onto the Pt substrate (b). Experimental conditions as described in the Experimental section.

features are in agreement with previous findings where the flow pressures in the electrochemical cell were surely more notable [28,29].

oPPy films have been successfully used for the construction of interference and fouling-free biosensors [27,29]. Conducting polymers like PPy display poor selectivity but PPy can attain successful permselective behaviours by simply discharging the pristine PPy modified electrode for a few hours at +0.7 V *versus* SCE [30], an overoxidation process of the polymer which render PPy in its non-conducting form, *i.e.* oPPy. Permselective polymers like those derived from diaminobenzene monomers [21–24] grow as insulating, thin (nearly monolayer) film and cannot grow more since insulating: on the contrary, conducting, pristine PPy film can further grow until the desired thickness thus permitting a further control on the permeation of interfering species and, hence, a modulation of the permselective properties of the resulting overoxidized polymer.

The chemical composition and structure of PPy and oPPy films have been already described elsewhere ([30,31] and references therein). Briefly, the overoxidation process of PPy generates on the polymer an increment of some oxygenated carbon species and the formation of COOH groups, which are particularly evident onto its outer surface with respect to bulk; furthermore, the overoxidation process is followed by an iminic transformation of nitrogen groups influencing further the nature of the film. This picture, thus, should be explain the permselective behaviour of oPPy films with respect to PPy ones.

The morphology of the oPPy film and the overlying enzyme layer have been investigated by environmental scanning electrode microscope (ESEM). ESEM was here used instead of classical SEM approach since it is known to permit the collecting of electron micrographs of wet specimens in a gaseous environment thus eventually minimising any drastically alteration of the enzyme immobilising gel. Fig. 1 compares some typical ESEM images of oPPy and LO layer with respect to the Pt substrate. As can be seen, both oPPy and, particularly, the enzyme layer showed a substantial homogeneity of their surface without any structuration or holes; incidentally, those tiny imperfections visible onto the oPPy surface as "black points" (see Fig. 1b) were similarly found onto the Pt surface thus suggesting that the used Pt substrate was not perfectly flat at that magnification and its imperfections were also translated to the polymer due to the deposition process.

ESEM evaluation of oPPy thickness gave values in the range 0.8–1.33 μm which compares well with the estimated thickness value of about 0.67 μm, based on the assumption that a density charge of 45 mC/cm^2 during the electrodeposition process produces a film thickness of 0.1 μm [30]; please note however that the observed uncertainty from ESEM measurements was due to the oPPy deposit of our specimen

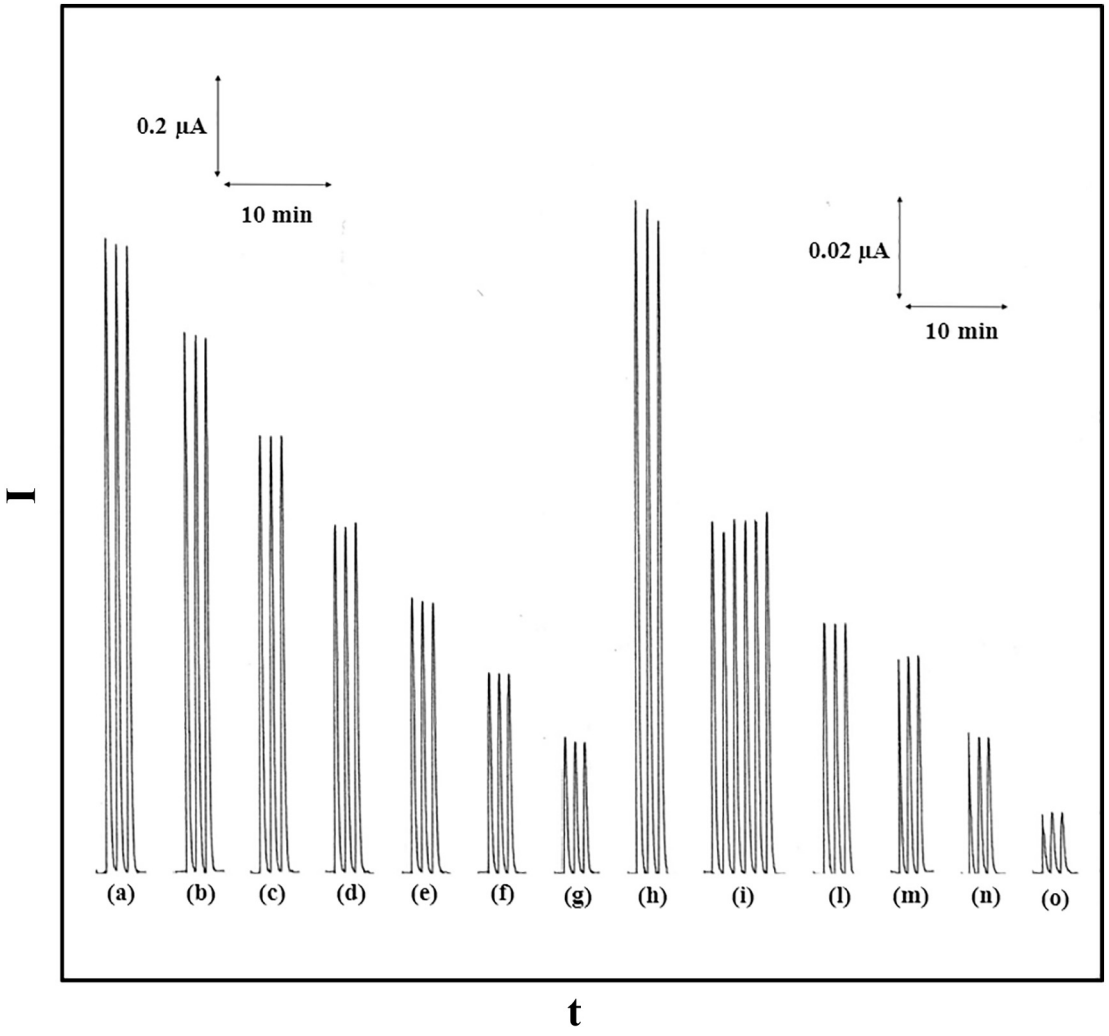

Fig. 2. Typical flow injection peaks due to repetitive injections of L-lysine standard solutions at a Pt/oPPy/LO biosensor. L-lysine concentrations: (a) 1.75, (b) 1.50, (c) 1.25, (d) 1.00, (e) 0.80, (f) 0.60, (g) 0.40, (h) 0.20, (i) 0.10, (l) 0.08, (m) 0.06, (n) 0.04 and (o) 0.02 mM. Scale legend at the left applies for (a–g) peaks while scale legend at the right applies for the (h–o) peaks in figure. Experimental conditions as described in the Experimental section.

which, at its interface with the Pt substrate, didn't grow suddenly at its interface (as expected for our electrodes) but gradually inwards as Fig. 1b shows. A similar but more lengthy curvature was also observed in the case of LO layer (see Fig. 1a) as expected for the formation of a gel deposit starting from a liquid drop (see the relevant section in the Experimental section for a description of the enzyme immobilization procedure). ESEM measurements gave LO thickness values in the range 4–8 μm which were less from those obtained from profilometric measurements in air, *i.e.* 10 ± 2 μm: quite probably, the low pressure in the ESEM camera partially dried the gel shrinking its thickness.

3.2. Kinetic behaviour of immobilized LO

The kinetic study and the following Lys analysis were performed under optimised experimental conditions (pH 5, flow rate 0.6 mL min^{-1}) assuring an enzymatic kinetic control of the biosensor, thus improving the enzyme specificity and minimising significantly amino acid interferences in Lys analysis [13].

Fig. 2 shows typical flow injection current responses relevant to repeated injections of standard Lys solutions at a Pt/oPPy/LO modified electrode. As can be seen, the responses were almost identical at each Lys level and nicely responsive peaks permitting a high sample throughput (about 1 sample/min) were observed due to the low response time of the biosensor (less than 6 s when measured as $t_{0.95}$ in batch solution [12]). The flow injection system here used for Lys analysis showed a rapid start up time (less than 5 min), no drift in signal measurements, allowed sample sizes as low as few microliters and low carrier consumption (due to the low flow rate) while being simple, flexible and cheap.

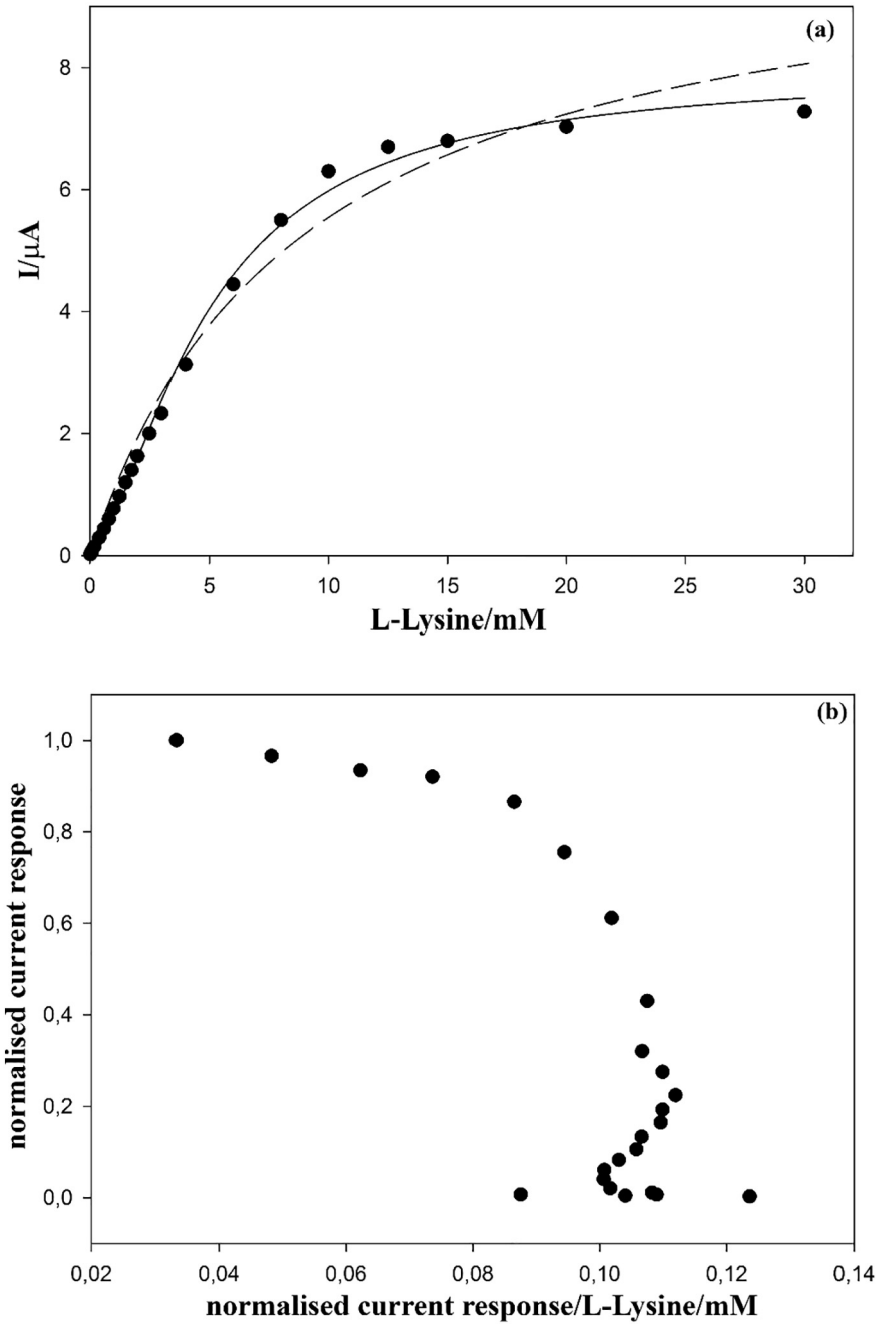

Fig. 3. Calibration curve (a) and normalised current response Eadie-Hofstee plot (b) of data in Fig. 2; continuous and dashed lines in (a) refer to Hill and Michaelis-Menten fitting, respectively.

Fig. 3a reports the calibration plot of flow injection responses illustrated in Fig. 2 while Fig. 3b shows the relevant Eadie-Hofstee plot (here plotted as normalised current response). Predictably, the calibration plot displays the well-known behaviour expected for an enzyme catalysis, with linear and saturated responses at low and high substrate concentrations, respectively: anyway, a closer and critical inspection showed a significant deviation from the anticipated Michaelis-Menten model usually adopted for enzyme catalysis. Indeed, the Eadie-Hofstee plot improperly deviates from the expected linearity normally observed for an enzyme under Michaelis-Menten kinetic while the calibration plot significantly deviated from the hyperbolic behaviour described by the Michaelis-Menten equation (compare the experimental points with the dashed line obtained with a Michaelis-Menten fitting of the data). Accordingly, a more complex kinetic behaviour is likely for the present enzyme.

Many enzymes are indeed oligomers composed of distinct subunits or monomers: LO, for example, is an enzyme with a molecular weight of about 116 kDa but consisting of two identical subunits of approximately 56 kDa [14]. Enzymes in oligomer form quite often are composed of identical subunits, each one with its proper catalytic site. If these sites are identical and independent of each other, the binding of a substrate at one site will not affect the binding of another substrate molecule on the other sites: if the enzyme is a dimer, for example, it will distributed in 3 different species, E, ES_1 and ES_2 (where E and S represents the enzyme and substrate, respectively), the velocity rate will be doubled by the presence of the 2 subunits but the velocity curve will be the usual hyperbola representing the Michaelis-Menten model. Conversely, if the presence of substrate at one site does influence the substrate binding to other sites or change the catalytic rate at other occupied sites, then the substrate itself can behave as a modifier thus giving substrate activation or inhibition: in these cases, the enzyme is classified as allosteric, the sites are cooperative, and the velocity curve will no longer follow the hyperbolic Michaelis-Menten kinetics but perhaps a sigmoidal shape. Modelling an allosteric enzyme is quite complex but a simplified version, known as Hill equation, can be used as well [32]; in fact, it can be demonstrated that for an allosteric enzyme with n equivalent subunits, the velocity equation v will be:

$$v = \frac{V_{max} \cdot [s]^n}{\left(K_{0.5}^n + [s]^n\right)} \quad (1)$$

where $[s]$ is the substrate concentration, V_{max} the maximum velocity and $K_{0.5}$ the substrate concentration at $V_{max}/2$ (which reduces to K_M, the Michaelis-Menten constant, when $n = 1$). If the cooperativity of substrate binding is not so high, the Hill equation does not apply but it can be still used to model the enzyme kinetics even if n will no longer correspond to the actual site number but it is considered a coefficient, the Hill coefficient, describing the cooperativity degree between sites: in these cases, n has usually non-integral values and will be less than the actual site number, but the next highest integer value above the

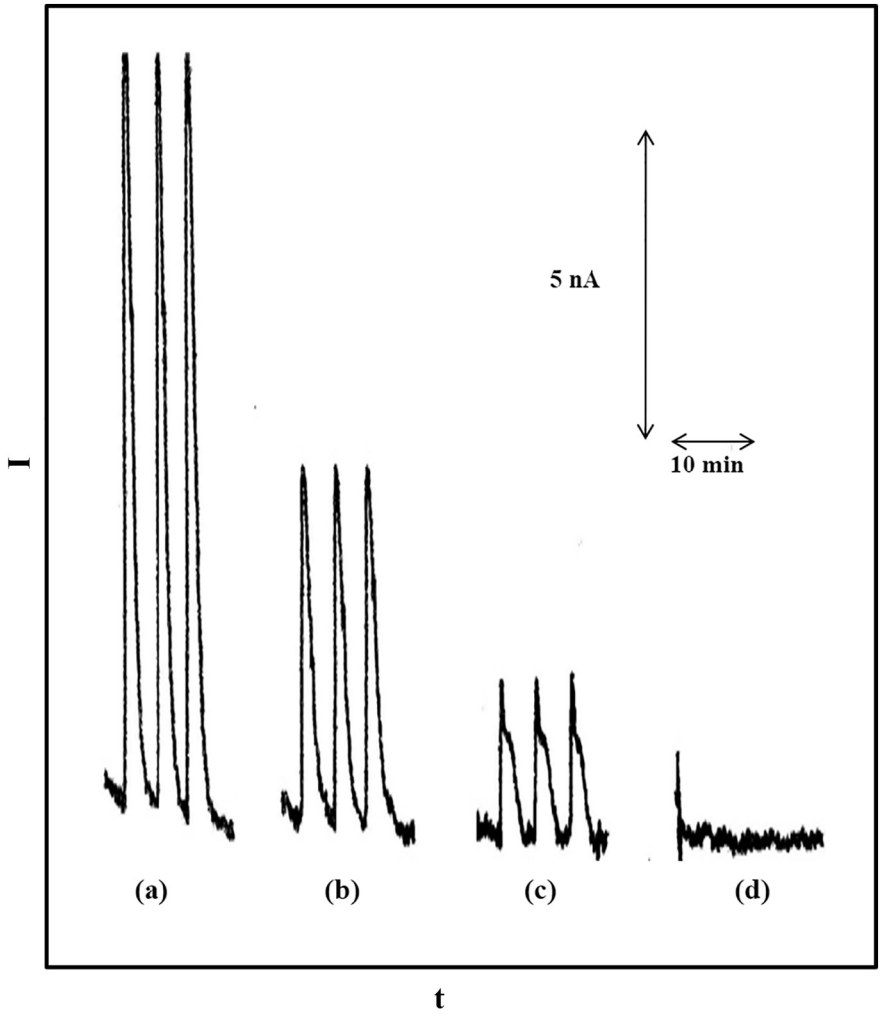

Fig. 4. Typical flow injection peaks due to repetitive injections of L-lysine standard solutions at a Pt/oPPy/LO biosensor. L-lysine concentrations: (a) 20, (b) 10 and (c) 5 μM; trace (d) refers to buffer injection. Experimental conditions as described in the Experimental section.

calculated Hill coefficient will represent the minimum number of actual sites in the enzyme [32].

According to this view, fitting of data points by Hill equation (see Fig. 3a, continuous line) gave better correlation results (correlation coefficient R better than 0.999) with respect to the classic Michaelis-Menten fitting: this suggests that LO behaves as an allosteric enzyme, a kinetic behaviour for LO never reported before, as far as we know, for this enzyme. Data fitting gave a V_{max} value of 7.9 ± 0.2 μA, a $K_{0.5}$ equal to 4.9 ± 0.2 mM and a Hill coefficient of 1.5 ± 0.1: this latter value is lower than the expected 2 suggesting that substrate binding cooperation between the two LO subunit sites is not high. Please note that these data are all apparent kinetic parameters since diffusional constrains and enzyme interaction within the immobilising layer can surely affect the overall kinetics of the immobilized enzyme: anyway, a preliminary kinetic study performed on LO in solution confirms the allosteric behaviour of the enzyme already observed with the immobilized enzyme. A detailed study of LO kinetics both in solution and in its immobilized state will be reported elsewhere.

3.3. Analytical performances of the Pt/oPPy/LO biosensor

Fitting of the linear part of the calibration curve shown in Fig. 3a gave a regression line (R better than 0.9998) with a slope of 0.79 ± 0.01 μA/mM (see Fig. S1 in Supplementary data) corresponding to a sensitivity of 0.11 μA/mM mm^2. Sensitivity values reported for similar biosensors typically refer to pH 7 or higher, where LO gives higher rates of catalysis, while the present sensitivity was observed at pH 5 and in the presence of an underlying oPPy film, which certainly lower the current response: nevertheless, sensitivity here appears higher than *e.g.* that reported for a LO biosensor using a similar enzyme immobilization approach [11]. Further, the present Pt/oPPy/LO biosensor permitted also linear Lys current responses up to 4 mM (see Fig. S1 in Supplementary data) which appears better than other biosensors using the same Lys detection approaches [11,33]. Finally, the within-a-day coefficients of variation for replicate ($n = 5$) Lys injections were 0.92% and 1.35% at 4 mM and 0.2 mM Lys levels, respectively.

Fig. 4 displays the flow injection current responses at a Pt/oPPy/LO modified electrode due to repeated injections of standard Lys solutions at low concentrations. As can be seen, the present biosensor is able to provide distinct Lys responses even at micromolar concentration levels, being the artefacts due to the injection valve the main limit to Lys detection (compare Lys responses in Fig. 4 with response (d) due to buffer injection). The Lys limit of detection (LOD) estimated at signal-to-noise ratio of 3 was 2 μM corresponding to 40 pmol of Lys injected; such a LOD is surely lower that many Lys sensing devices already described elsewhere [13] and satisfactory for Lys sensing in biological fluids.

An operational stability study of the Pt/oPPy/LO biosensor was performed by repetitive injections of Lys sample at 1 mM level under the continuous run of the flow injection system. No significant differences in Lys current responses were observed during all the working day (at least 12 h) for almost all the biosensors fabricated in this study: in the worst cases, the biosensor current response decreased of about 5% at the end of the day. The storage stability was studied by discontinuously monitoring the Lys sensitivities of 3 different Pt/oPPy/LO biosensors

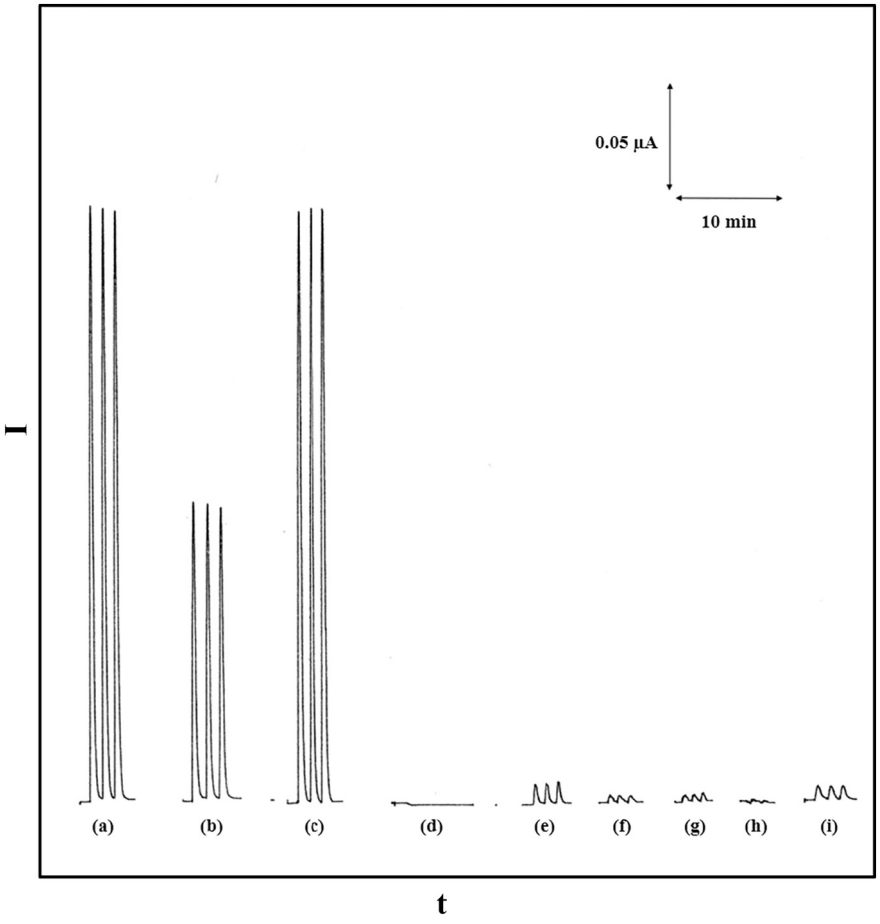

Fig. 5. Typical flow injection peaks at a Pt/oPPy/LO biosensor due to repetitive injections of (a) and (c) L-lysine 0.4 mM, (b) untreated human serum (diluted 1:2 with buffer), (d) buffer, (e) L-phenylalanine 0.4 mM, (f) L-tyrosine 0.4 mM, (g) ascorbic acid 0.1 mM, (h) cysteine 0.08 mM and (i) uric acid 0.05 mM. Experimental conditions as described in the Experimental section.

stored, when not in use, in a phosphate buffer pH 7.5 at 4 °C in the dark, without using any efforts to avoid bacterial growth in the storage buffer. No appreciable loss in Lys sensitivities were observed for all the tested biosensors while, after more than 40 days, the sensitivities were about the 90% of their initial values. Since the here proposed sensing device is under enzymatic kinetic control [12, 13], these stability studies are a clear indication of the ability of co-crosslinking (*i.e.* the immobilising procedure here employed) to retain efficiently the enzyme in membrane, even after several uses and in flow systems.

3.4. Analysis of Lys in human serum

The analytical performances of the herewith described biosensor encouraged us to investigate its application for the analysis of Lys in untreated serum sample.

As already pointed out in the Introduction section, LO from *Trichoderma viride* shows a poor selectivity towards its substrate since is able to oxidize to varying extents also several L-amino acids other than Lys; anyway, the optimization of some experimental parameters like flow rate and pH permitted us to minimise significantly the interference coming from other amino acids [13]. Indeed, using a flow rate of 0.6 mL min^{-1} and a pH value of 5, as adopted in the present work, a significant lowering of the current response of interfering amino acids was achieved therefore improving the specificity of the enzymatic catalysis towards the target analyte, *i.e.* Lys; specifically, the relevant biases, as measured at equimolar concentrations of Lys and amino acid interferent and calculated as percentage, were only 1.4% and 0.90% for ornithine and arginine, respectively [13], *i.e.* for those amino acids which surely are the most interfering species in Lys biosensors based on LO from *Trichoderma viride* [23]. The selectivity of the present biosensor results so quite appealing and surely superior with respect to other Lys biosensing devices, thus permitting, as it will be here shown, an interferent-free detection of Lys even in complex sample which endogenously contains other amino acids as well. Anyway, the flow rate and pH values here used are not strictly mandatory since an interferent-free detection of Lys has been observed even for values varying few percent from the optimal here used.

Fig. 5 shows and compares typical flow injection current responses due to Lys standard solution, serum (only diluted 1:2 with acetate buffer pH 5, I 0.1 M) and typical endogenous amino acids and serum compounds that can interfere in Lys analysis. Lys standard and serum samples were injected in a repeated sequence and serum sample injections were bracketed between Lys standard solution injections to test for electrode fouling due to protein adsorption from serum: as Fig. 5 demonstrates, the serum responses were practically identical, and the Lys responses remained unchanged between the set of serum injections showing that protein adsorption (which if present would have lowered the flow injection responses) is absent or at least negligible. Fig. 5 also shows that the current responses due to L-phenylalanine and L-tyrosine, *i.e.* those amino acids showing the major interference effects by the present biosensor [13], were negligible with respect to predictable Lys levels in human serum from healthy patients (181 ± 25 up to 232 ± 24 μM depending from the sex and the age of patients [3]): typical L-phenylalanine and L-tyrosine levels (58–76 μM and 58–80

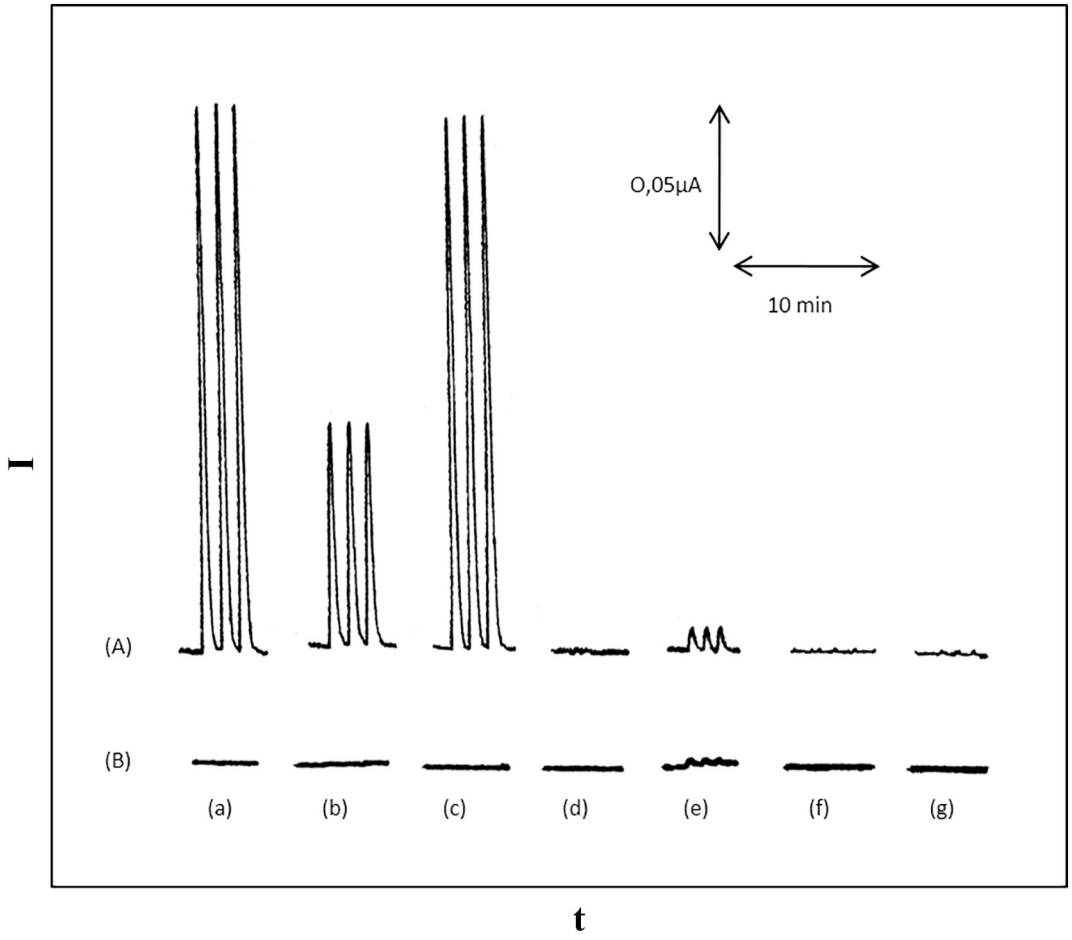

Fig. 6. Typical flow injection peaks at a (A) Pt/oPPy/LO and (B) Pt/oPPy dual electrode system due to repetitive injections of (a) and (c) L-lysine 0.4 mM, (b) untreated human serum (diluted 1:2 with buffer), (d) buffer, (e) ascorbic acid 0.1 mM, (f) cysteine 0.08 mM and (g) uric acid 0.05 mM. Experimental conditions as described in the Experimental section.

μM, respectively) are indeed 3 times lower that Lys so that negligible biases are expected in Lys analysis by the present method, even for the lower Lys levels; other endogenous amino acids are not detectable by the Pt/oPPy/LO electrode [13], even at higher concentrations, demonstrating the high selectivity of the proposed biosensor. Fig. 5 also compares the Lys current responses with common endogenous interferents (*e.g.* ascorbate) at concentration levels near the upper limits of their respective physiological concentration ranges: also for this class of compounds the current responses were negligible demonstrating that the proposed Pt/oPPy/LO electrode is also an interference-free biosensor.

To further demonstrate that the observed serum current responses were genuine Lys signals and not deriving from other compounds in the sample, dual working electrode experiments were performed where both electrodes were equally modified with an overoxidized oPPy film and only one electrode was successively modified with the enzyme layer. As Fig. 6 shows, in absence of any enzymatic catalysis (see the lower traces in Fig. 6) no current responses were observed either for Lys standard solution injections either for serum sample, demonstrating unequivocally that the observed responses at the Pt/oPPy/LO electrode are only due to Lys presence in the serum sample. Further, the absence of any fouling effect observed at the lower traces and the comparable current responses observed for both electrodes due to endogenous interferents prove that the fouling- and interference-free performance of the biosensor are due to the oPPy film and not to the enzyme layer.

Lys content in untreated serum sample from healthy donors was quantified by the standard addition method to further discriminate any matrix effect of the sample. Again, the current responses due to sample and Lys spiked sample injections were always reproducible and the relevant plot was linear as expected (see Fig. S2 and S3 in Supplementary data, respectively). The Lys concentration found in serum was 296 ± 52 μM at a confidence level of 95%, which is in good agreement with expected Lys levels in healthy individuals [3].

4. Conclusion

The present research has demonstrated that the proper coupling of a LO layer immobilized by co-crosslinking onto the surface of a Pt electrode modified by a oPPy film is effective for the development of an highly selective amperometric biosensor able to analyse Lys even in more complex sample like untreated serum other than simple ones as pharmaceutical products and food: in this respect, this study represents surely the first attempt for the analysis of Lys in human serum, a novel analytical approach never reported before. A proper tuning of the enzyme kinetics permitted us to use a low specific, commercially available enzyme as L-lysine-α-oxidase from *Trichoderma viride* for the selective biorecognition of Lys even in the presence of other interferent amino acids: a thoroughly study of LO kinetics evidenced also the allosteric behaviour of this enzyme, a kinetic feature which was never reported before for this enzyme. Preliminary results concerning the determination of Lys in untreated human serum from healthy donors gave Lys levels in good agreement with the expected values so that the use of a Pt/oPPy/LO biosensor appears promising and a good alternative in the relevant clinical fields; of course, much more studies are required to validate this novel analytic approach so work in this direction is in progress in our laboratory.

Acknowledgements

We gratefully acknowledge financial support from MIUR. We like to thank Azienda Ospedaliera San Carlo (Potenza) for donating us the serum sample and Alessandro Laurita (Università degli Studi della Basilicata, Potenza, Italy) for ESEM images. This work comes from Silvio Frascaro and Francesca De Gennaro's theses (2013).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2018.07.004.

References

[1] S. Reiken, D. Briedis, The effect of lysine deprivation on leukemic blood, Amino Acids 3 (1992) 213–221.
[2] E.V. Butorov, Relationship between plasma L-lysine concentrations and levels of HIV-1 RNA, Virulence 4 (2013) 646–653.
[3] H.T. Pitkänen, S.S. Oja, K. Kemppainen, J.M. Seppä, A.A. Mero, Serum amino acid concentrations in aging men and women, Amino Acids 24 (2003) 413–421.
[4] S.M.R. Camargo, D. Bockenhauer, R. Kleta, Aminoacidurias: clinical and molecular aspects, Kidney Int. 73 (2008) 918–925.
[5] M. Lukkarinen, K. Näntö-Salonen, K. Pulkki, M. Aalto, O. Simell, Oral supplementation corrects plasma lysine concentrations in lysinuric protein intolerance, Metabolism 52 (2003) 935–938.
[6] K. Asanuma, K. Adachi, T. Sugimoto, S. Chiba, Effects of lysine-induced acute renal failure in dogs, J. Toxicol. Sci. 31 (2006) 87–98.
[7] S. Moore, D.H. Spackman, W.H. Stein, Chromatography of amino acids on sulfonated polystyrene resins: an improved system, Anal. Chem. 30 (1958) 1185–1190.
[8] B.N. Jones, J.P. Gilligan, O-phthaldialdehyde precolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolysates and physiological fluids, J. Chromatogr. A 266 (1983) 471–482.
[9] C.W. Armstrong, N.R. McGregor, J.R. Sheedy, I. Buttfield, H.L. Butt, P.R. Gooley, NMR metabolic profiling of serum identifies amino acid disturbances in chronic fatigue syndrome, Clin. Chim. Acta 413 (2012) 1525–1531.
[10] W. Tu, H. Chen, J. He, Application of LC-MS/MS analysis of plasma amino acids profiles in children with autism, J. Clin. Biochem. Nutr. 51 (2012) 248–249.
[11] S.C. Kelly, P.J. O'Connell, C.K. O'Sullivan, G.G. Guilbault, Development of an interferent free amperometric biosensor for determination of L-lysine in food, Anal. Chim. Acta 412 (2000) 111–119.
[12] A. Guerrieri, T.R.I. Cataldi, R. Ciriello, The kinetic and analytical behaviours of an L-lysine amperometric biosensor based on lysine oxidase immobilised onto a platinum electrode by co-crosslinking, Sensors Actuators B Chem. 126 (2007) 424–430.
[13] A. Guerrieri, R. Ciriello, T.R.I. Cataldi, A novel amperometric biosensor based on a co-crosslinked L-lysine-α-oxidase/overoxidized polypyrrole bilayer for the highly selective determination of L-lysine, Anal. Chim. Acta 795 (2013) 52–59.
[14] H. Kusakabe, K. Kodama, A. Kuninaka, H. Yoshino, H. Misono, K. Soda, A new antitumor enzyme, L-lysine alpha-oxidase from *Trichoderma viride*. Purification and enzymological properties, J. Biol. Chem. 255 (1980) 976–981.
[15] M.G. Lavagnini, D. Moscone, G. Palleschi, D. Compagnone, C. Cremisini, Amperometric lysine bioprobes analysis in feeds, Talanta 40 (1993) 1301–1306.
[16] A.L. Simonian, I.E. Badalian, T.T. Berezov, I.P. Smirnova, S.H. Khaduev, Flow-injection amperometric biosensor based on immobilized L-lysine-α-oxidase for L-lysine determination, Anal. Lett. 27 (1994) 2849–2860.
[17] K. Siegler, B. Weber, E. Weber, K. Tonder, E. Klingner, F. Riechert, A lysine sensor for process control, J. Chem. Technol. Biotechnol. 59 (1994) 279–287.
[18] F. Preuschoff, U. Spohn, E. Weber, K. Unverhau, K.-H. Mohr, Chemiluminometric L-lysine determination with immobilized lysine oxidase by flow-injection analysis, Anal. Chim. Acta 280 (1993) 185–189.
[19] F. Ricci, A. Amine, C.S. Tuta, A.A. Ciucu, F. Lucarelli, G. Palleschi, D. Moscone, Prussian Blue and enzyme bulk-modified screen-printed electrodes for hydrogen peroxide and glucose determination with improved storage and operational stability, Anal. Chim. Acta 485 (2003) 111–120.
[20] F. Mazzei, F. Botrè, G. Favero, E. Podestà, C. Botrè, Partially disposable biosensors for the quick assessment of damage in foodstuff after thermal treatment, Microchem. J. 91 (2009) 209–213.
[21] A. Curulli, S. Kelly, C. O'Sullivan, G.G. Guilbault, G. Palleschi, A new interference-free lysine biosensor using a non-conducting polymer film, Biosens. Bioelectron. 13 (1998) 1245–1250.
[22] I.D. Karalemas, C.A. Georgiou, D.S. Papastathopoulos, Construction of a L-lysine biosensor by immobilizing lysine oxidase on a gold-poly(o-phenylenediamine) electrode, Talanta 53 (2000) 391–402.
[23] M.H. Divritsioti, I.D. Karalemas, C.A. Georgiou, D.S. Papastathopoulos, Flow injection analysis system for L-lysine estimation in foodstuffs using a biosensor based on lysine oxidase immobilization on a gold-poly(m-phenylenediamine) electrode, Anal. Lett. 36 (2003) 1939–1963.
[24] A. Curulli, F. Valentini, S. Orlanduci, M.L. Terranova, G. Palleschi, Pt based enzyme electrode probes assembled with Prussian Blue and conducting polymer nanostructures, Biosens. Bioelectron. 20 (2004) 1223–1232.
[25] R. Ciriello, T.R.I. Cataldi, F. Crispo, A. Guerrieri, Quantification of L-lysine in cheese by a novel amperometric biosensor, Food Chem. 169 (2015) 13–19.
[26] J.F. Kennedy, C.A. White, Principles of immobilization of enzymes, in: A. Wiseman (Ed.), Handbook of Enzyme Biotechnology, Ellis Horwood Lim., John Wiley & Sons 1985, pp. 147–207.
[27] A. Guerrieri, G.E. De Benedetto, F. Palmisano, P.G. Zambonin, Electrosynthesized non-conducting polymers as permselective membranes in amperometric enzyme electrodes: a glucose biosensor based on a co-crosslinked glucose oxidase/overoxidized polypyrrole bilayer, Biosens. Bioelectron. 13 (1998) 103–112.
[28] A. Guerrieri, F. Palmisano, An acetylcholinesterase/choline oxidase-based amperometric biosensor as a liquid chromatography detector for acetylcholine and choline determination in brain tissue homogenates, Anal. Chem. 73 (2001) 2875–2882.

[29] A. Guerrieri, V. Lattanzio, F. Palmisano, P.G. Zambonin, Electrosynthesized poly(pyrrole)/poly(2-naphthol) bilayer membrane as an effective anti-interference layer for simultaneous determination of acethylcholine and choline by a dual electrode amperometric biosensor, Biosens. Bioelectron. 21 (2006) 1710–1718.
[30] D. Centonze, A. Guerrieri, C. Malitesta, F. Palmisano, P.G. Zambonin, Interference-free glucose sensor based on glucose-oxidase immobilized in an overoxidized non-conducting polypyrrole film, Fresenius J. Anal. Chem. 342 (1992) 729–733.
[31] F. Palmisano, C. Malitesta, D. Centonze, P.G. Zambonin, Correlation between permselectivity and chemical structure of overoxidized polypyrrole membranes used in electroproduced enzyme biosensors, Anal. Chem. 67 (1995) 2207–2211.
[32] I.H. Segel, Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems, John Wiley & Sons, Inc., 1993
[33] J. Saurina, S. Hernández-Cassou, E. Fàbregas, S. Alegret, Potentiometric biosensor for lysine analysis based on a chemically immobilized lysine oxidase membrane, Anal. Chim. Acta 371 (1998) 49–56.