Enzyme and Microbial Technology 52 (2013) 265–271

Contents lists available at SciVerse ScienceDirect

Enzyme and Microbial Technology

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

Immobilization of lysine oxidase on a gold–platinum nanoparticles modified Au electrode for detection of lysine

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ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form 13 January 2013
Accepted 15 January 2013

Keywords:
L-Lysine biosensor
Lysine oxidase
Gold nanoparticles
Platinum nanoparticles
3-Aminopropyltriethoxy silane

ABSTRACT

A commercial lysine oxidase (LyOx) from *Trichoderma viride* was immobilized covalently onto gold nanoparticles (AuNPs) and platinum nanoparticles (PtNPs) electrodeposited onto Au electrode using 3-aminopropyltriethoxy silane (3-APTES) and glutaraldehyde cross linking chemistry. A lysine biosensor was fabricated using LyOx/3-APTES/AuNPs-PtNPs/Au electrode as a working electrode, Ag/AgCl (3 M KCl) as standard electrode and Pt wire as auxiliary electrode connected through a potentiostat. The enzyme electrode was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The cumulative effect of AuNPs and PtNPs showed excellent electrocatalytic activity at low applied potential for detection of H_2O_2, a product of LyOx reaction. The sensor showed its optimum response within 4 s, when polarized at 0.2 V vs. Ag/AgCl in 0.1 M phosphate buffer, pH 7.5 at 30 °C. The linear range and detection limit of the sensor were 1.0–600 μM and 1.0 μM (S/N = 3), respectively. Biosensor measured lysine level in sera, milk and amino acid tablet, which correlated well with those by standard HPLC method. The enzyme electrode lost 50% of its initial activity after 200 uses over a period of 4 months.

1. Introduction

L-Lysine, an essential amino acid is used as a feed additive in the animal feed industry, which do not contain sufficient levels of lysine, the livestock's nutritional requirements, especially for single-stomach (monogastric) animals like poultry and swine [1] and as a supplement for humans, improving the feed quality by increasing the absorption of other amino acids [2–5]. It is also easily damaged by storage conditions of food and therefore may be used in the assessment of food processing techniques as well as an index of the nutritional quality of foods [6].

Among the various methods available for the determination of lysine such as liquid chromatography [7], reverse phase liquid chromatography [8], spectrophotometry [9], fluorimetry [10], flow-injection techniques combined with spectrophotometry [11] and capillary electrophoresis [12], biosensing methods are the most simple, sensitive, specific, rapid and cost effective. A variety of biosensors have been developed for lysine detection such as microbial sensors based on measurement of dissolved oxygen [13,14] and fluorescent density [15], potentiometric [16], electrochemical [17] and electro-chemiluminescent [18]. Among these biosensors, electrochemical enzyme sensors with high sensitivity, specificity, long term stability, easy detection principles and low response time have extensively reduced sampling and testing times in food monitoring [13,19–24]. However, these enzyme sensors suffer from several limitations such as leaking of biocomponent and possible diffusion barriers, which render biosensors poorly sensitive, unstable and with long response time. Covalent immobilization of enzyme not only overcomes this problem but also leads to better biomolecule activity and greater stability.

New trends focus on the development of nanomaterials based novel biosensing systems with enhanced performance of bioanalytical assay. Gold and platinum nanoparticles, in particular have been used extensively for the modifications of electrode [25,26]. Gold nanoparticles (AuNPs) provide biocompatible surface for immobilization of biomolecules, high surface area and good conductivity between electrode and biomolecules [27,28]. Platinum nanoparticles (PtNPs) not only possess similar properties of other noble metal nanoparticles but also exhibit excellent electrocatalytic property for oxidation/reduction towards H_2O_2 [29]. These metal nanoparticles in combination exhibited cumulative effect to increase the surface areas, ability to promote electron transfer and good electrocatalytic activity for H_2O_2 [30]. We describe herein construction and application of a novel amperometric lysine biosensor based on covalent immobilization of lysine oxidase (LyOx) on AuNPs and PtNPs electrodeposited onto 3-aminopropyltriethoxy silane (3-APTES) modified Au electrode.

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http://dx.doi.org/10.1016/j.enzmictec.2013.01.006

2. Materials and methods

2.1. Chemicals and reagents

Lysine oxidase (E.C. 1.4.3.14 from *Trichoderma viride*), 3-aminopropyltriethoxy silane (3-APTES) from Sigma Aldrich Co St. Louis, USA and sodium citrate dehydrate, hydrogen tetrachloroaurate trihydrate and hexachloroplatinic (IV) acid hydrate ($H_2PtCl_6 \cdot xH_2O$) from Sisco Research Laboratory, Mumbai, India were used. Alamin M Forte (amino acids with minerals capsule) manufactured by M/S Albert David Limited, Ghaziabad, India and Au wire (1.5 cm × 0.05 cm, 23 carat) were purchased from local market. All other chemicals were of analytical reagent grade. Double distilled water (DW) was used throughout the experiments.

2.2. Apparatus

Scanning electron microscopy (SEM) measurements of electrodes were carried out at Advanced Instrumentations Research Facility (AIRF), Jawaharlal Nehru University, New Delhi. Fourier transform infrared spectroscopy (FTIR) of modified electrode was carried out by FTIR spectrometer (model iS10, Thermoelectron, USA). Amperometric measurements were conducted with Potentiostat/Galvanostat (Model: Autolab, Ecochemie, The Netherland. Model: AUT83785). Ultrasonication was performed on Misonix Ultrasonic Liquid Processors (mode XL-2000 79 series).

2.3. Preparation of the AuNPs and PtNPs modified Au electrode

An Au wire was polished before its modification with 0.05 μm alumina powder, sonicated in a mixture of concentrated HNO_3 and ethanol in 1:1 ratio and finally washed in DW and dried at room temperature. The cleaned Au electrode was scanned between the potential ranging from +0.30 V to +1.25 V and +0.3 V to −1.3 V vs. Ag/AgCl. Au electrode was modified by immersing it into 0.5 M H_2SO_4 solution containing 1 mM $HAuCl_4$ and 1 mM H_2PtCl_6. AuNPs–PtNPs were electrodeposited with potentiostatic method at −0.2 V vs. Ag/AgCl for 400 s. After the electrodeposition of AuNPs–PtNPs, the modified electrode was gently washed with DW and dried [30].

2.4. Immobilization of enzyme

LyOx was immobilized on the AuNPs–PtNPs modified electrode by amino-glutaraldehyde cross-linking chemistry. 3-APTES solution (3-APTES + DW + 95% ethanol in 2:3:95 ratio, v/v) (100 μl) was smeared on the AuNPs–PtNPs modified electrode and kept it for 1 h at room temperature, followed by rinsing it with DW and drying it in air. The modified electrode was activated by glutaraldehyde by immersing it into 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer pH 7.0 for 2 h at room temperature (25 °C), followed by washing with DW [30]. The enzyme solution containing 1.5 U LyOx in 0.1 M sodium phosphate buffer pH 7.4 (50 μl) was layered on to surface of glutaraldehyde activated AuNPs–PtNPs modified electrode surface and kept overnight at 4 °C for glutaradehyde coupling of enzyme (Scheme 1). Finally, the enzyme electrode was washed 3–4 times with 0.1 M sodium phosphate buffer (pH 7.4) and stored dry at 4 °C.

2.5. Construction and response measurements of amperometric lysine biosensor

Amperometric biosensor for measurement of lysine was prepared using modified Au electrode (LyOx/3-APTES/AuNPs–PtNPs/Au) as working electrode, Ag/AgCl as reference and Pt wire as auxiliary electrode connected through Potentiostat/Galvanostat. EIS and cyclic voltammetric studies of electrode were carried out at different stages of its modification. To measure the response of biosensor, the three electrode system was dipped into 20 ml of 50 mM sodium phosphate buffer pH 7.5. The reaction was started by adding 0.1 ml of 0.1 mM lysine and the current (mA) generated was recorded at different voltages. Cyclic voltammograms of modified electrodes were recorded after applying a suitable voltage.

The maximum current was obtained at +0.2 V vs. Ag/AgCl, hence in subsequent studies, the electrodes were polarized at this voltage to generate current. The following electrochemical reaction occurred during the sensor response measurement.

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

$$H_2O_2 \xrightarrow{+0.2\,V} 2H^+ + O_2 + 2e^-$$

2.6. Optimization of LyOx/3-APTES/AuNPs–PtNPs modified Au electrode

To determine the optimum working conditions of the enzyme electrode, the pH of reaction buffer (0.1 M) was varied from pH 3.5–8.0 at an intervals of pH 0.5 using acetate buffer in acidic pH range (pH 3.5–5.5) and phosphate buffer in pH range 6.0–8.0. Similarly, to determine optimum temperature, the reaction mixture was incubated at 15–55 °C at an interval of 5 °C. To determine the optimum response time, the current response was recorded between 2 s and 20 s at an interval of 2 s. To study the effect of substrate concentration, different lysine concentration, ranging from 1 μM to 600 μM were used. Apparent K_m for lysine was calculated from Lineweaver Burk plot, based on following equation:

$$\frac{1}{I} = \frac{K_m}{I_{max}[S]} + \frac{1}{I_{max}}$$

where I = current, K_m = Michaelis–Menten constant, I_{max} = maximum current and [S] = lysine concentration.

2.7. Amperometric determination of lysine in milk, serum and pharmaceutical tablet

LyOx/3-APTES/AuNPs–PtNPs modified Au electrode was employed for detection of lysine in real samples e.g. milk, serum and pharmaceutical tablets. Protein samples from milk were digested in 6 M HCl for 15 min. Prior to assay, the samples were brought to pH 7.0 with 1 M KOH. The samples were then assayed for lysine content. To measure lysine content in pharmaceutical tablet namely Alamin M Forte (amino acids with minerals capsule), 1000 mg tablet was dissolved in the sodium phosphate buffer (0.1 M, pH 7.0) after grinding it in a pestle mortar and then analyzed for lysine. Fresh serum samples were collected from apparently healthy adults at hospital of local Pt BDS P.G. Institute of Medical Science, Rohtak and analyzed for lysine using the present biosensor. The procedure of measurement of lysine in these samples was same as described for response measurement of electrode except that lysine was replaced by sample. The content of lysine in these samples was determined from standard curve between lysine concentrations vs. current in mA.

3. Results and discussion

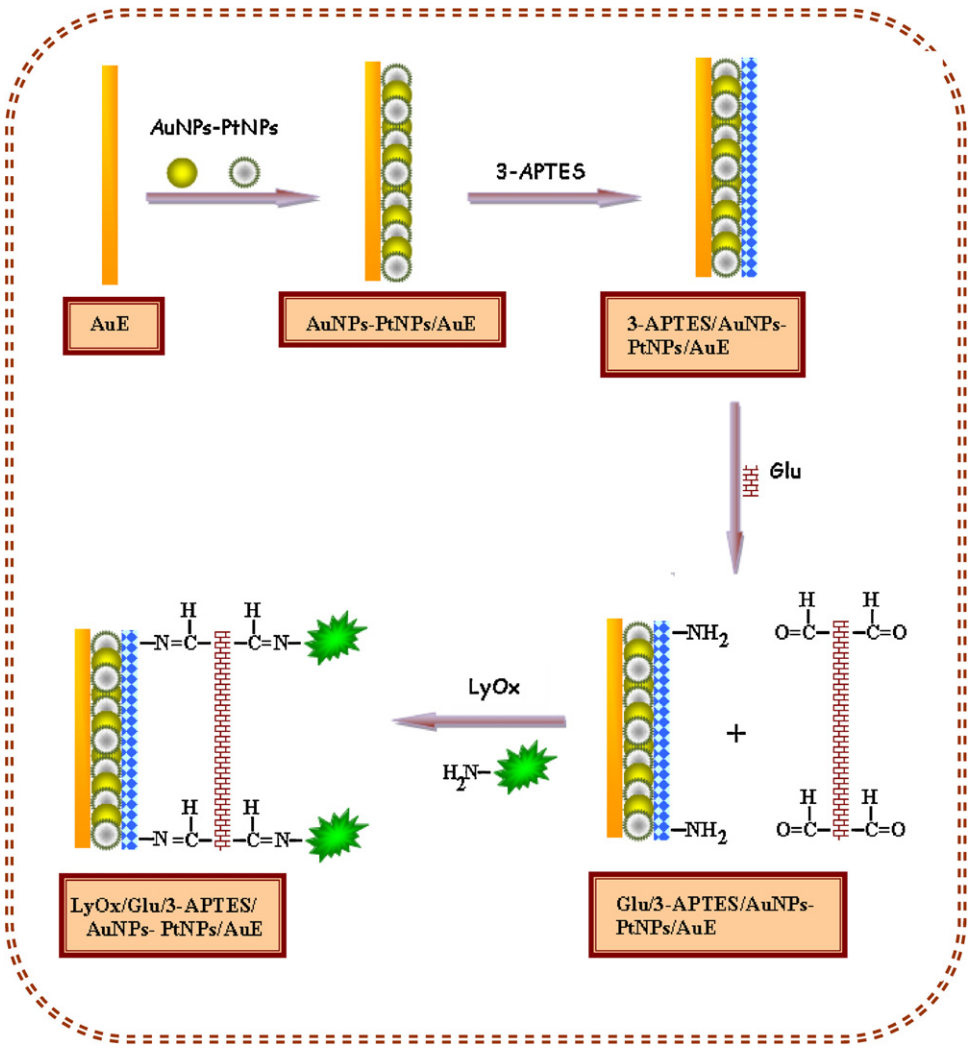
3.1. Electrode surface characterization by SEM and FTIR

Fig. 1a shows the SEM image of the AuNPs–Pt NPs on the Au electrode. The AuNPs–PtNPs were rough and consisted of a great number of nanoparticles resulted in rough surface texture. Globular shapes on the electrode surface display presence of enzyme layer after immobilization (Fig. 1b).

To investigate the anchoring of terminal amino groups in 3-APTES on nanoparticles modified Au electrode, FTIR spectroscopy of 3-APTES modified Au electrode was carried out, which gave a broad peak at 3451 cm^{-1} due to {NH stretching (Fig. 2A curve i) [31]. IR spectra of electrode showed a new and distinct peak at 1028 cm^{-1}, attributable to Si—O stretching of the silane group. Silanes, which are useful compounds for anchoring an organic layer to an inorganic surface and contain Si—O bonds, react with surface hydroxides and pendant hydrocarbon chains to link with organic over layers, such as a polymer or protein molecules. Curve ii showed the absorbance at 1725 cm^{-1} related to —CO bond of aldehyde, while the peak at 2720 cm^{-1} also indicated the presence of free aldehyde group, which reveals that glutaraldehyde gets coupled with 3-APTES. Curve iii showed a peak of —N=C— bond at 1630 cm^{-1}, while the absence of peak at 1725 cm^{-1} confirms that —CO— group of glutaraldehyde got combined with $—NH_2$ groups on surface of enzyme to form —N=C bond. It also revealed that there was no free aldehyde group of glutaraldehyde, as it got cross-linked with enzyme. These studies confirmed that the enzyme was immobilized through glutaraldehyde coupling on the surface of 3-APTES modified Au electrode.

3.2. Electrochemical impedance spectroscopy (EIS)

Fig. 2B shows electrochemical impedance spectra (EIS) of (i) bare Au electrode, (ii) 3-APTES/AuNPs–PtNPs/Au electrode and (iii) LyOx/3-APTES/AuNPs–PtNPs/Au bioelectrode, respectively. The charge transfer process in LyOx/3-APTES/AuNPs–PtNPs/Au bioelectrode was studied by monitoring charge transfer resistance (RCT) at the electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) depends on the dielectric and insulating features at the electrode/electrolyte interface. The RCT values for the Au electrode, 3-APTES/AuNPs–PtNPs/Au electrode and LyOx/3-APTES/AuNPs–PtNPs/Au electrodes were obtained as 675 Ω, 452 Ω and 929 Ω, respectively. There is a



Scheme 1. Chemical sequence of electropolymerization of AuNPs–PtNPs onto Au electrode and chemical reaction of immobilization of enzyme onto modified Au electrode (3-APTES: 3-aminopropyltriethoxy silane; Glu: glutaraldehyde).

decrease in RCT value of AuNPs–PtNPs/Au electrode compared to bare Au electrode. The RCT value depends on the dielectric and insulating features at the electrode/electrolyte interface. The deposition of two metallic nanoparticles onto the electrode increases the electron flow/transfer, which possible when the RCT value is decrease. The increased RCT value of LyOx/3-APTES/AuNPs–PtNPs/Au electrode was due to the immobilization of LyOx onto 3-APTES/AuNPs–PtNPs/Au surface. The electron transfer via redox couple was hindered by the presence of LyOx on the electrode surface.

3.3. Electrochemical characterization of LyOx/3-APTES/AuNPs–PtNPs/Au electrode by cyclic voltammetry

Fig. 3A shows the relative CV responses for four different types of electrodes i.e. bare Au, AuNPs/Au, PtNPs/Au and Au–PtNPs/Au in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl solution, respectively. In case of bare Au electrode, a well-defined oxidation peak (current 0.055 mA and potential 0.034 V) was observed (curve a). However, PtNPs/Au and AuNPs/Au electrodes showed an enhancement in oxidation peak current from 0.055 mA to 0.100 mA and 0.130 mA at potential 0.142 V and 0.150 V (curves b and c), respectively. The increase in peak currents can be attributed to the increase of effective surface area due to the electrodeposited PtNPs and AuNPs. The oxidation peak current at AuNPs–PtNPs/Au (curve d) was increased up to 0.300 mA but at a higher potential 0.212 V compared to PtNPs/Au and AuNPs/Au electrodes. The remarkably enhanced signal at AuNPs–PtNPs/Au electrode was due to cumulative effects of AuNPs and PtNPs. These results show that nanoparticles play an important role to accelerate the electron transfer and increase the oxidation current.

3.4. Electrocatalytic activity of H_2O_2 on modified electrode

In the present enzymatic reaction, H_2O_2 is produced, which is detected amperometrically. Before that we checked the electrocatalytic activity of H_2O_2 on the modified electrode by cyclic voltammograms (CV), Fig. 3B displays the typical CV for 2 mM H_2O_2 at the different modified electrodes over the potential range of 0.0–0.4 V vs. Ag/AgCl. Bare Au electrode (a) showed, no significant current (−0.25 mA) while 3-APTES/AuNPs–PtNPs/Au electrode (b) had a large increase in catalytic current from −0.25 mA to +0.125 mA. The LyOx/3-APTES/Au–PtNPs/Au electrode (c) exhibited even more increase in current from +0.125 mA to +0.25 mA. These observations reveal the cumulative effect of both the nanoparticles towards electrocatalytic activity of H_2O_2. It also facilitated low-potential amperometric detection of H_2O_2, with relatively high sensitivity and wide dynamic range. To avoid any

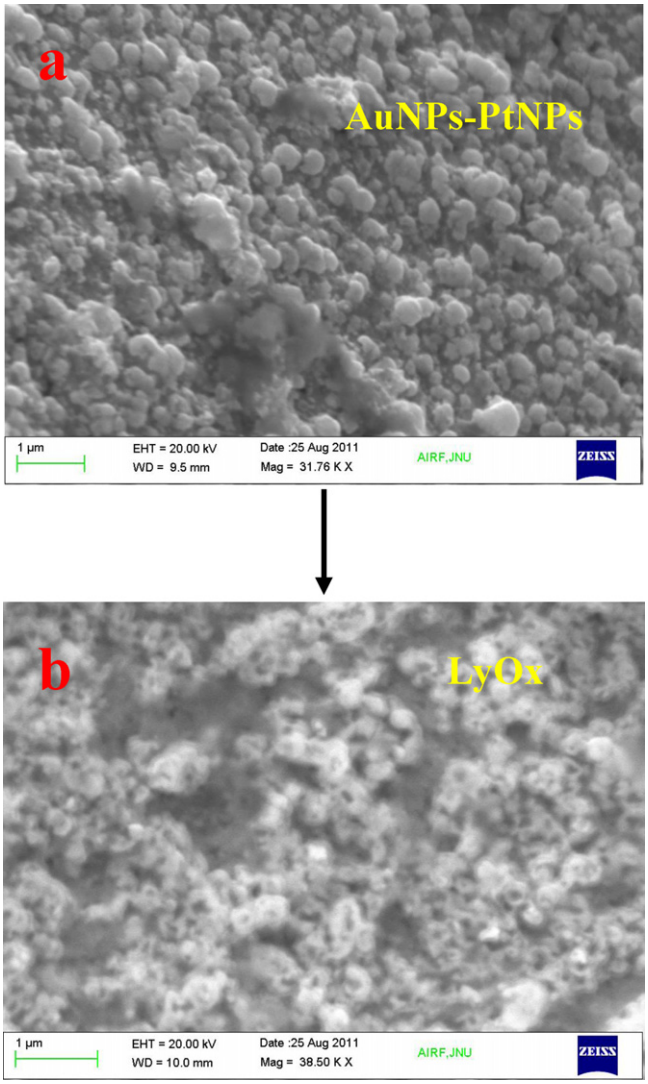


Fig. 1. Scanning electron micrographs of (a) AuNPs–PtNPs/Au electrode and (b) LyOx/3-APTES/AuNPs–PtNPs/Au electrode.

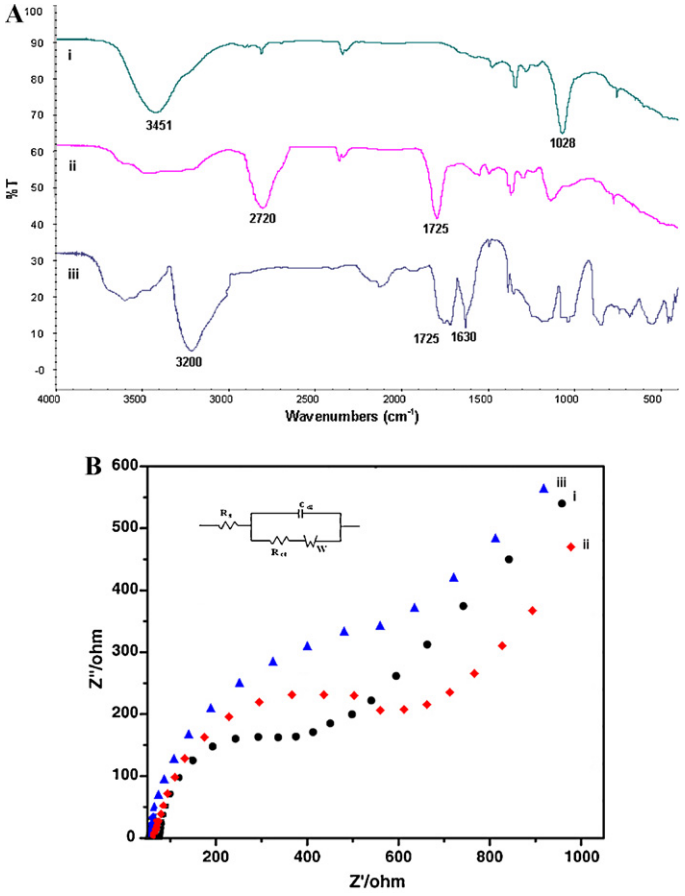


Fig. 2. (A) FTIR spectra of (i) 3-APTES/AuNPs–PtNPs/Au electrode, (ii) glutaraldehyde/3-APTES/AuNPs–PtNPs/Au electrode and (iii) LyOx/glutaraldehyde/3-APTES/AuNPs–PtNPs/Au electrode. (B) Electrochemical impedance spectra of (i) bare Au electrode, (ii) 3-APTES/AuNPs–PtNPs/Au electrode and (iii) LyOx/3-APTES/AuNPs–PtNPs/Au bioelectrode in 0.1 M sodium phosphate buffer, pH 7.5 containing 5 mM $[Fe(CN)_6]^{3-/4-}$ at a polarization potential of 0.2 V in the frequency range 0.1–10^4 Hz.

potential interference from other electroactive species at higher potential, +0.2 V vs. Ag/AgCl was chosen for amperometric detection of H_2O_2.

3.5. Response of LyOx/3-APTES/AuNPs–PtNPs/Au electrode to lysine

To evaluate the catalytic activity of LyOx at the 3-APTES/AuNPs–PtNPs/Au electrode, the modified electrode was characterized by a cyclic voltammogram in the presence of lysine in the potential range, from 0 V to +0.4 V. Fig. 3C shows cyclic voltammograms of the LyOx/3-APTES/AuNPs–PtNPs/Au electrode in an unstirred 0.1 M KCl solution (20 ml) and 0.1 M sodium phosphate buffer, pH 7.5 (5 ml), with (curve a) and without (curve b) 0.1 mM lysine solution at a scan rate of 50 mV s^{-1}. When 0.1 mM lysine was added, well-defined oxidation (0.14 V) and reduction peaks (0.03 V) (curve a) were observed, which clearly indicate the catalytic properties of the modified electrode.

3.6. Optimization of experimental conditions of lysine biosensor

The biosensor showed optimal current (S/N) within 4 s at pH 7.5 (0.1 M sodium phosphate buffer), which is slightly higher than that of free enzyme (pH 7.4 for *T. viride*) [32]. The slight change in optimal pH of enzyme after immobilization might be due to an improved stability of enzyme at that pH. The biosensor exhibited optimum response at 30 °C, which is slightly lower than that of free enzyme (37 °C) in case *T. viride* [32]. LyOx/3-APTES/AuNPs–PtNPs/Au electrode achieved 95% of steady state currents within 4 s, which is definitely lower/better than earlier reported response attention: time such as 2 min [33], 42 s [13], 180 s [18], 60 s [23] and 6 s [34]. The differential pulse voltammogram of the biosensor showed the increase in oxidation peak i.e. current response as the concentration of lysine was increased from 1.0 μM to 600 μM (Fig. 4). When the current (*y*-axis) was plotted against the lysine concentration (*x*-axis) it showed linear relationship between biosensor response and lysine concentration but it did not pass through the intersection rather passed through *y*-axis, which may be due to background current at zero added substrate (Fig. 5). To overcome this problem, we used straight line equation ($y = mx + c$) to calculate the lysine concentration in various samples. The Lineweaver–Burk plot between 1/*I* (mA) and 1/[lysine] (Supplementary Fig. 1) yielded an apparent K_m of 333.33 μM, which is higher than that for free enzyme (40 μM) [32] indicating decreased affinity of enzyme towards substrate (lysine) after immobilization, which might be due to restricted diffusion of lysine through 3-APTES/AuNPs–PtNPs composite.

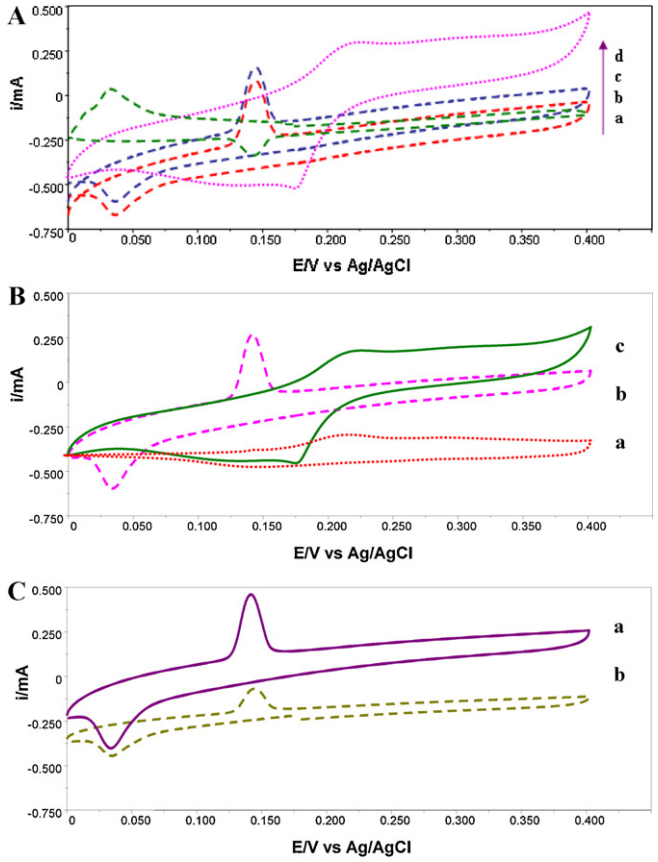


Fig. 3. (A) Cyclic voltammograms measured with bare Au electrode (a), PtNPs/Au (b), AuNPs/Au (c) and AuNPs–PtNPs/3-APTES/Au electrode (d) in a solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl; scan rate 50 mV s^{-1}. (B) Cyclic voltammograms measured with bare Au electrode (a), 3-APTES/AuNPs–PtNPs/Au electrode (b) and LyOx/3-APTES/AuNPs–PtNPs/Au electrode (c) in a solution of 2 mM H_2O_2; scan rate 50 mV s^{-1}. (C) Cyclic voltammetric (CV) curves of LyOx/3-APTES/AuNPs–PtNPs/Au electrode in 0.1 M sodium phosphate buffer (pH 7.5) (a) with and (b) without 0.1 mM lysine solution. Supporting electrolyte: 0.1 M KCl solution; scan rate: 50 mV s^{-1}.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2013.01.006.

To study the substrate specificity on the present biosensor response, the following amino acids were added in place of lysine in the reaction mixture individually at a final concentration of 1.0 mM and the biosensor response was measured under the similar assay conditions. The results as given in Table 1 showed that only DL-lysine was utilized as substrate, while DL-ornithine, L-arginine and L-cysteine were practically unutilized. This shows the absolute substrate specificity of the present biosensor.

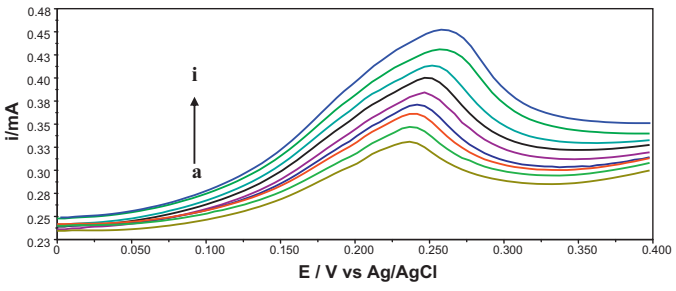


Fig. 4. DPV net current responses of LyOx/3-APTES/AuNPs–PtNPs/Au electrode in lysine concentrations (a) 1.0, (b) 10, (c) 50, (d) 100, (e) 200, (f) 300, (g) 400, (h) 500 and (i) 600 μM in 0.1 M phosphate buffer–0.1 M KCl solution (pH 7.5). DPV measuring conditions: DPV frequency 25 Hz, amplitude 0.4 V and step potential 30 mV.

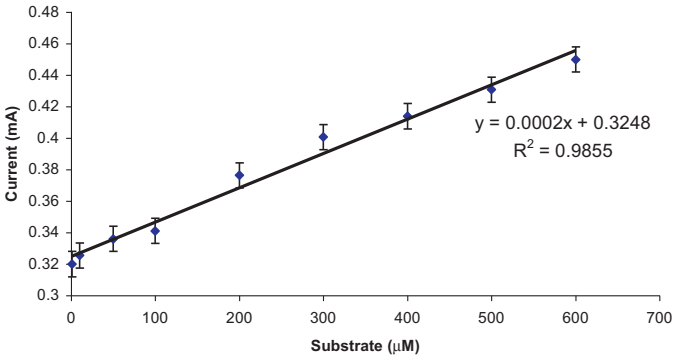


Fig. 5. Standard curve of substrate concentration (μM) vs. current (mA).

The effect of interferents (methionine, phenylalanine, proline, serine, glucose, uric acid, ascorbic acid, histidine, tyrosine, tryptophan and glutamic acid) on the biosensor response was also investigated using the solution containing lysine (1 mM) and interferrants 1 mM each in 1:1 ratio. The results indicate negligible effect of these interferrants on the electrochemical response of LyOx/3-APTES/AuNPs–PtNPs/Au electrode.

3.7. Evaluation of biosensor

The electrode response was found to be linear in the lysine concentration range of 1.0–600 μM, which is higher than 33 μM [23], 500 μM [33] and 250 μM [35] with a sensitivity of 0.2 μA/μM better than earlier reported biosensors [13,35]. The limit of detection of the present biosensor was calculated as the lowest quantity of lysine required to give a signal to a background (blank) +3 times SD of blank and found to be 1.0 μM (S/N = 3), which is lower than that for enzyme immobilized onto polyurethane hydrogel (10 μM) [35]; poly(o-phenylenediamine) membrane (100 μM) [16], aminosilylated glass (10 μM) [36]; gold-mercaptopropionic acid self assembled monolayer (100 μM) [17] and platinum electrode (3 μM) [34]. The average recoveries of lysine added to milk (at levels of 50 and 100 μM) varied from 96.63% to 97.46%, demonstrating the reliability of the present biosensor. Within-batch and between-batch coefficients of variation (CVs) for the determination of lysine in milk samples on the same day and after one week of storage were 3.3% and 4.5%, respectively indicating the good reproducibility and consistency of the present method, which could be attributed to the excellent immobilization of LyOx onto the 3-APTES/AuNPs–PtNPs/Au electrode. To study the correlation of the present biosensors, lysine level in 15 milk samples was determined by the present biosensor (x) and then compared with those by standard HPLC method (y). The value obtained by LyOx/3-APTES/AuNPs–PtNPs/Au electrode was very close to those obtained by the standard method. The correlation coefficient (r) was 0.992 with a regression equation $y = 1.10233x - 0.0639$ (Fig. 6A) indicating the high accuracy of the method.

Table 1
Substrate specificity effect on LyOx/3-APTES/AuNPs–PtNPs modified Au electrode.

Substrate	Relative rate of oxidation (%)
L-Lysine	100
DL-Lysine	87.0
DL-Ornithine	15.2
L-Arginine	10.9
L-Cysteine	10.4

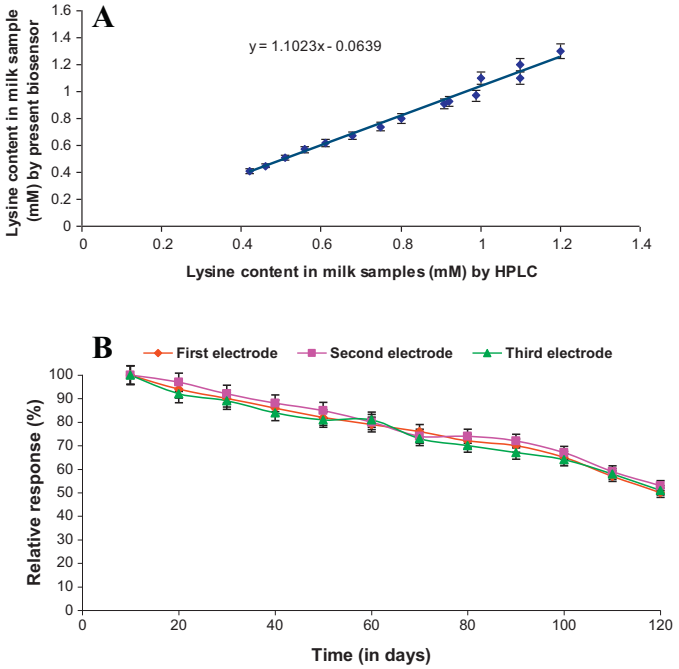


Fig. 6. A. Correlation between present biosensor employing lysine content values determined by standard HPLC method (x-axis) and LyOx/3-APTES/AuNPs–PtNPs/Au electrode method (y-axis) with regression equation $y = 1.023x - 0.0639$ with $r = 0.99$. 6B. Effect of storage at 4 °C on the response of three similar LyOx/3-APTES/AuNPs–PtNPs/Au electrodes. Error bars represent the standard deviation for three independent measurements.

3.8. Stability and reusability of biosensor

The electrode lost 50% of its initial activity after its 200 uses over a period of 4 months, when stored at 4 °C, which is better than earlier biosensors [13,16,23,33,34]. The high stability of the enzyme electrode could be attributed to the covalent coupling of enzyme onto the modified electrode. The enzyme electrode was constructed thrice and effect of storage at 4 °C was studied on the response of these three similarly constructed electrodes. The results given in Fig. 6B showed practically no variation in storage stability of the three electrodes.

3.9. Application

Lysine content of milk, sera and amino acid tablet, as determined by the present biosensor is given in Tables. The lysine content was in the range 193.7–243.1 μM in milk samples (Table 2), 211.0–273.3 μM in sera of apparently healthy persons with a mean of 247.6 μM ($n = 10$) (Table 3), which is in normal established range (150–250 μM) and 24.7 mg/tablet in amino acid tablet (Table 4).

Table 2
Determination of lysine in milk samples using LyOx/3-APTES/AuNPs–PtNPs/Au electrode.

Milk samples	L-Lysine concentration by biosensor (μM) mean ± S.D.	RSD%
Sterilized non fat dry milk	193.7 ± 1.12	0.57
Sterilized whole milk	224.5 ± 1.21	0.53
Clarified raw milk	243.1 ± 1.65	0.67

Table 3
Lysine levels in serum samples of apparently healthy adults, as determined by biosensor based on LyOx/3-APTES/AuNPs–PtNPs/Au electrode.

Serum Sample	Sex	L-Lysine (μM) mean ± S.D.
S1	M	214.0 ± 0.60
S2	F	267.0 ± 0.45
S3	F	256.8 ± 0.59
S4	F	228.9 ± 0.64
S5	M	211.0 ± 0.50
S6	M	273.3 ± 0.40
S7	F	256.5 ± 0.64
S8	F	272.7 ± 0.45
S9	F	253.8 ± 0.70
S10	M	242.8 ± 0.48

Table 4
Determination of lysine in amino acid tablets using LyOx/3-APTES/AuNPs–PtNPs/Au electrode.

Tablet	L-Lysine concentration (mg/tablet) (reported value)	L-Lysine concentration by biosensor (mg/tablet) mean ± S.D.	RSD%
Alamin M Forte	25	24.7 ± 1.43	5.78

4. Conclusion

A novel amperometric lysine biosensor was fabricated, based on covalent immobilization of LyOx onto 3-APTES/AuNPs–PtNPs/Au electrode. The resulting working electrode showed improved analytical performance in terms of fast response (4 s), limit of detection (1.0 μM), high sensitivity (0.2 μA/μM), high storage stability (120 days) and lack of interference from various potential interferrants.

References

[1] Zelder O, Klopprogge C, Kröger B, Schröder H, Haefner S, Heinzle E, Kiefer P, Wittmann C. Methods for the preparation of a fine chemical by fermentation, 2005. WO/2005/059139, 18.02.2005.
[2] Anastassiadis S. L-Lysine fermentation. Recent Patents on Biotechnology 2007;1:11–24.
[3] Carpenter KJ, Booth VH. Damage to lysine in food processing: its measurement and its significance. Nutrition Abstracts and Reviews 1973;43:423–51.
[4] Lata S, Pundir CS. L-Amino acid biosensor based on L-amino acid oxidase immobilized onto NiHCNFe/c-MWCNT/PPy/GC electrode. International Journal of Biological Macromolecules 2013;54:250–7.
[5] Chauhan N, Singh A, Narang J, Dahiya S, Pundir CS. Development of amperometric lysine biosensors based on Au nanoparticles/multiwalled carbon nanotubes/polymers modified Au electrodes. Analyst 2012;137:5113–22.
[6] Curulli A, Kelly S, O'Sullivan C, Guilbault GG, Palleschi G. A new interference free lysine biosensor using a non-conducting polymer film. Biosensors and Bioelectronics 1998;13:1245–50.
[7] Heftmann E. Chromatography: fundamentals and applications of chromatography and related differential migration methods. In: Part B, Applications. 5th ed. Amsterdam: Elsevier; 1992. p. 630.
[8] Fernández-Trapiella AC. Quantitative analysis of methionine, cysteine and lysine in feeds by reverse-phase liquid chromatography using pre-column dervatisation with 9-fluoremylmethyl chloroformate: preliminary study. Journal: Association of Official Analytical Chemists 1990;73:935–9.
[9] Alonso A, Almendral ML, Báez MD, Porras MJ, Alonso C. Enzyme immobilization on an epoxy matrix. Determination of L-arginine by flow injection techniques. Analytica Chimica Acta 1995;308:164–9.
[10] Mana H, Spohn U. Selective flow injection procedures for the determination of nitrogen containing analytes by gasdialytic–fluorimetric detection of enzymatically generated ammonia. Analytica Chimica Acta 1996;325:93–104.
[11] Pohlmann A, Stamm WW, Kusakabe H, Kula MR. Enzymatic determination of L-lysine by flow-injection techniques. Analytica Chimica Acta 1990;235:329–35.
[12] Li H, He H, Wolfbeis OS. An optical biosensor for lysine based on the use of lysine decarboxylase and a cadaverine-sensitive membrane. Biosensors and Bioelectronics 1992;7:725–32.
[13] Saurina J, Hernández-Cassou S, Alegret S, Fa'bregas E, Saurina J, Hernandez-Cassou S, et al. Amperometric determination of lysine using a lysine oxidase biosensor based on rigid-conducting composites. Biosensors and Bioelectronics 1999;14:211–20.

[14] Akyilmaz E, Erdogan A, Öztürk R, Yasa I. Sensitive determination of L-lysine with a new amperometric microbial biosensor based on *Saccharomyces cerevisiae* yeast cells. Biosensors and Bioelectronics 2007;22:1055–60.
[15] Chalova VI, Zabala-Díaz IB, Woodward CL, Ricke SC. Development of a whole cell sensor-green fluorescent sensor method for lysine quantification. World Journal of Microbiology and Biotechnology 2008;24:353–9.
[16] Karalemas ID, Constantinos AG, Papastathopoulos DS. Construction of a L-lysine biosensor by immobilizing lysine oxidase on a gold-poly (o-phenylenediamine) electrode. Talanta 2000;53:391–402.
[17] Shervedani RK, Hemmatian Z, Hatefi-Mehrjardi A. Immobilisation of L-lysine α-oxidase on gold-mercaptopropionic acid self-assembled monolayer: preparation and electrochemical characterization. Bioelectrochemistry 2009;75:124–9.
[18] Marquette CA, Degiuli A, Blum LJ. Electrochemiluminescent biosensors array for the concominant detection of choline, glucose, glutamate, lactate, lysine and urate. Biosensors and Bioelectronics 2003;19:433–9.
[19] Kelly SC, O'Connell PJ, O'Sullivan CK, Guilbault GG. Development of an interferent free amperometric biosensor for determination of L-lysine in food. Analytica Chimica Acta 2000;412:111–9.
[20] Mello LD, Kubota LT. Review of the use of biosensors as analytical tools in the food and drink industries. Food Chemistry 2002;77:237–56.
[21] Vrbova E, Marek M. Biosensor for the determination of L-lysine. Analytica Chimica Acta 1992;270:131–6.
[22] Preuschoff F, Spohn U, Janasek D. Photodiode-based chemiluminometric biosensors for hydrogen peroxide and L-lysine. Biosensors and Bioelectronics 1994;9:543–9.
[23] Mazzei F, Botre F, Favero G, Podesta E, Botre C. Partially disposable biosensors for the quick assessment of damage in foodstuff after thermal treatment. Microchemical Journal 2009;91:209–13.
[24] Garcia-Villar N, Saurina J, Hernandez-Cassou S. Flow injection differential potentiometric determination of lysine by using a lysine biosensor. Analytica Chimica Acta 2003;477:315–24.
[25] Pumera M, Sanchez S, Ichimose Tang J. Electrochemical nanobiosensors. Sensors and Actuators B: Chemical 2007;123:1195–205.
[26] Wang J. Carbon-nanotubes based electrochemical biosensors: a review. Analyst 2005;130:421–6.
[27] Du D, Chen S, Cai J, Zhang A. Immobilization of acetylcholinesterase on gold nanoparticles embedded in sol–gel film for amperometric detection of organophosphorous insecticide. Biosensors and Bioelectronics 2007;23:130–4.
[28] Manso J, Luzmena M, Yanez-Sedeno P, Pingarron JM. Bienzyme amperometric biosensor using gold nanoparticle-modified electrodes for the determination of insulin in foods. Analytical Biochemistry 2008;375:345–53.
[29] Yang M, Yang Y, Liu Y, Shen G, Yu R. Platinum nanoparticles-doped sol–gel/carbon nanotubes composite electrochemical sensors and biosensors. Biosensors and Bioelectronics 2006;21:1125–31.
[30] Upadhyay S, Rao GR, Sharma MK, Bhattacharya BK, Rao VK, Vijayaraghavan R. Immobilization of acetylcholinesterase–choline oxidase on a gold–platinum bimetallic nanoparticles modified glassy carbon electrode for the sensitive detection of organophosphate pesticides, carbamates, and nerve agents. Biosensors and Bioelectronics 2009;25:832–8.
[31] Liu XD, Tokura S, Haruki M, Nishi N, Sakairi N. Surface modification of nonporous glass beads with chitosan and their adsorption property for transition metal ions. Carbohydrate Polymers 2002;49:103–8.
[32] Kusakabe H, Kodama K, Kuninaka A, Misono HYH, Soda K. A new antitumor enzyme, L-lysine alpha-oxidase from *Trichoderma viride*: purification and enzymological properties. Journal of Biological Chemistry 1980;255:976–81.
[33] Lavagnini MG, Moxone D, Pallexhi G. Amperometric lysine bioprobes analysis in feeds. Talanta 1993;40:424–30.
[34] Guerrieri A, Cataldi TRI, Ciriello R. The kinetic and analytical behaviours of an L-lysine amperometric biosensor based on lysine oxidase immobilised onto a platinum electrode by co-crosslinking. Sensors and Actuators B: Chemical 2007;126:424–30.
[35] Olschewski H, Erlenkotter A, Zaborosch C, Chemnitius GC. Screen-printed enzyme sensors for L-lysine determination. Enzyme and Microbial Technology 2000;26:537–43.
[36] Preuschoff F, Spohn U, Weber E, Unverhau K, Mohr KH. Chemiluminometric L-lysine determination with immobilized lysine oxidase by flow-injection analysis. Analytica Chimica Acta 1993;280:185–9.