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Short communication

Amperometric biosensor based on diamond paste for the enantioanalysis of L-lysine

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ABSTRACT

An amperometric biosensor was proposed for the enantioanalysis of L-lysine. The biosensor is based on the impregnation of L-lysine oxidase in diamond paste. The potential used for the determination of L-lysine was 650 mV. The biosensor exhibited a linear concentration range between 1 and 100 nmol/L with a limit of detection of 4 pmol/L. The selectivity of the biosensor is high over other amino acids, such as L-serine, L-leucine, L-aspartic acid, L-glutamic acid, histamine, glycine. The proposed biosensor can be applied for the determination of L-lysine in serum samples and pharmaceutical compounds.

1. Introduction

Advanced single-crystal diamond has enabled the development of a wide range of monocrystalline diamond products to meet the exacting requirements of an amperometric transducer (Heurs, 1997). The reliability of the electrical properties of single-crystal diamond is encouraging for research in the electrochemical sensors based on monocrystalline diamond, as well, it proves that the doping of monocrystalline diamond is not necessary which minimizes the time affected for electrode's construction and also simplified the steps adapted for the design of such electrochemical sensors (Stefan and Bairu, 2003). Amperometric biosensors are a good alternative for chromatographic methods due to the fact that they can be used for the direct measurement of the enantiomers in solutions without any prior separation of the substance that has to be determined (Stefan et al., 2001). The biochemical reaction is very selective and sensitive (Stefan et al., 1999). The sensitivity of the enzymatic reaction must be correlated to the sensitivity of the transducer. Accordingly, the best electrochemical transducer for this biosensor is the amperometric one (Stefan and Nejem, 2003; Stefan et al., 2004).

L-Lysine is one of the indispensable amino acids that cannot be manufactured by the human body, but must be acquired from food sources (Hong-Ping and Bao-Shan, 1999). L-Lysine is widely available to the public as a non-prescription of oral supplement. L-Lysine can be transported into brain (Spector, 1988). L-Lysine is the precursor of acetoacetyl-CoA, which is a very important enzyme in the biosynthesis of acetylcholine in the central nervous system (Hong-Ping and Bao-Shan, 1999). L-Lysine is also the precursor in the biosynthesis of L-carnitine, which is involved in the transport of acyl groups into mitochondria for oxidation. L-Carnitine is an essential cofactor in the transfer of long-chain fatty acids across the inner mitochondrial membrane and significantly decreases the infarct volume (Fernandes et al., 1997).

Until now, the determination of lysine was carried out using liquid chromatography (Krasikov et al., 2004; Pachuski and Sherma, 2002; Misra and Pachauree, 2002; Degtiar et al., 2000), spectrometry (Danson et al., 2002), chemiluminescence (Kiba et al., 2002), flow injection analysis (Divritsioti et al., 2003), densitometry (Indrayanto et al., 2001), piezoelectric quartz crystal sensor (Cai et al., 2000), and electrochemical biosensors (Karalemas et al., 2000; Sun et al., 2000; Kelly et al., 2000; White and Guilbaut, 1978; Dempsey et al., 1992; Simonian et al., 1991; Siegler et al., 1994).

There is a great interest in developing faster, less expensive and simpler methods for analysis and furthermore enantioanalysis of L-lysine. In this paper, a diamond paste based amperometric biosensor is proposed for the enantioanalysis of L-lysine in serum

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and pharmaceutical samples. The proposed amperometric biosensor is based on physical immobilization of L-lysine oxidase in a monocrystalline natural diamond paste. Previously, the enzyme has been immobilized by cross-linking with glutaraldehyde onto a polymer film directly on the working electrode (Kelly et al., 2000), or covalently on silanized silica gel (Curulli et al., 1998), on nylon (Saurina et al., 1991), or on immobilon membranes (Romette et al., 1983). Utilization of diamond paste amperometric electrodes proved in several occasions that it enhanced the selectivity and sensitivity of the amperometric biosensor (Stefan and Nejem, 2003; Stefan-van Staden and Bokretsion, 2006).

2. Experimental

2.1. Reagents and materials

Natural diamond powder with particle size 1 μm (99.9%), β-alanine, creatine and creatinine were purchased from Aldrich (Milwaukee, USA); paraffin oil and L-lysine were purchased from Fluka (Buchs, Switzerland); L-glutamate, D-lysine and L-lysine oxidase (L-lysine oxygen oxidoreductase [deaminating]; (EC 1.4.3.14)) were purchased from Sigma (USA); asparagine and phosphate buffer (pH 7.0) were purchased from Merck (Darmstadt, Germany). D-Lysine was purchased from Sigma (Germany). L-(+)-Serin was purchased from Reidel-Haen (Germany); L-cystine from Hopkin & Williams LTD (England); L-leucine from Aldrich (Germany); histamine dihydrochloride from BDH (Poole, England); glycine from Analar, BDH (Poole, England).

De-ionized water from a Modulab System (Continental Water Systems, San Antonio, TX, USA) was used for all solution preparations. Stock solution of L-lysine (10^{-4} mol/L) was used for all solutions preparation (10^{-16}–10^{-5} mol/L), by serial dilution.

2.2. Diamond paste based amperometric biosensor

Plain diamond was prepared by mixing 0.1 g of diamond powder with 20 μL paraffin oil, and filled in a plastic pipette peak leaving an empty space of 3–4 mm in the top part to be filled with a modified diamond paste.

The diameter of the biosensor was 3 mm. Electrical contact was obtained by inserting a silver wire into the diamond paste. The biosensor tip was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensor was wetted with de-ionized water and then polished with alumina paper (polished strips 30144-011, Orion) before use. The biosensor was stored at 4 °C, when not in use. The enzyme solution used for the biosensor design was prepared in 0.1 mol/L phosphate buffer of pH 8.0.

A modified diamond paste was prepared as follows: 400 mg of natural monocrystalline diamond powder was mixed with 30 μL paraffin oil to form a diamond paste. 50 μL from the enzyme solution (1 mg enzyme/mL phosphate buffer pH 8.0) was added to the diamond paste, as described previously (Stefan-van Staden and Bokretsion, 2006). Physical mixture between paraffin oil and enzyme solution avoid the leaking of the enzyme from the membrane during the measurements.

2.3. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a PGSTAT 12 and software Ecochemie (version 4.9) was used for all chronoamperometric measurements; the pH module attached served for all pH measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

2.4. Recommended procedures

2.4.1. Direct amperometry

The chronoamperometric technique was used for the measurement of the intensity of current at 650 mV. The electrodes were dipped into a cell containing 10 mL of phosphate buffer, pH 8.0 and different volumes of L-lysine solution were added. The intensity of current measured was plotted versus the concentration of L-lysine. The unknown concentrations of L-lysine in serum and tablet solutions were determined from the calibration graph.

2.4.2. Determination of L-lysine in serum samples

Five serum samples were collected from healthy volunteers. The serum samples were buffered with (pH 8.0) in a ratio of 1:1 (v/v) and spiked with different volume of L-lysine. The pH of the final solution was checked using the pH-meter module attached to the PGSTAT. Direct amperometry was used to determine the concentration of L-lysine in serum samples.

2.4.3. Determination of L-lysine in pharmaceutical formulations

Ten tablets of L-lysine (containing 500 mg L-lysine/tablet, dicalciumphosphate, polyvinylpyrrolidone, colloidal silicon dioxide, sodium starch glycolate, magnesium stearate) sample were prepared by dissolving each tablet in 100 mL (0.1 mol/L) HCl. Further dilutions were performed using phosphate buffer (pH 8.0) in order to place the concentration of L-lysine in the working concentration range of the electrode, using automated micropipettes of high precision. The solutions were further diluted in 10 mL phosphate buffer (pH 8.0) to give a final concentration of 80 nmol/L of L-lysine. The concentration of L-lysine was determined using direct amperometry.

3. Results and discussion

3.1. Response characteristics of the amperometric biosensors

The response characteristics of the biosensors were measured at 650 mV. The reason of the selection of the potential is to obtain the maximum sensitivity and selectivity for the assay of hydrogen peroxide produced by the enzymatic reaction. The biosensor exhibits a linear concentration range from 1 to 100 nmol/L with a limit of detection of 4 pmol/L. Although reasonable signals were recorded for concentration between 100 pmol/L and 10 pmol/L of L-lysin, their values could not be placed on the calibration graph of the biosensor.

The biosensor response was highly stable and reproducible over two months, when the biosensor was intensively used everyday for measurements and was kept into the fridge at 4 °C when not in use. Within two months, the slope of the electrode was varying with ±0.09 μA/nmol L.

The low limits of detection and high reliability of the biosensor is due to the diamond paste matrix used for its design.

The response of the biosensor to different L-lysine concentrations was linear over wide concentration ranges for two months and can be described by the following equation of calibration which represents the statistical processing of the original data of calibration of the biosensor:

$$I = 0.23\,C + 2.28; \quad r = 0.9866$$

where I is the intensity of the current in μA, C is the concentration of L-lysine in nmol/L, and r is the regression coefficient.

The limit of detection was calculated using the equation proposed by Otto (1999)

$$\mathrm{DL} = \frac{I_B + 3\sigma_S - a}{S}$$

Table 1
Amperometric selectivity coefficients of the biosensor.[a]

Interfering Species (J)	pK_{sel}^{amp}
D-Lysine	2.55
L-Serine	3.36
L-Leucine	2.15
L-Aspartic acid	2.01
L-Glutamic acid	3.15
Histamine	2.09
Glycine	2.07
β-Alanine	3.15
Creatine	2.37
Creatinine	2.91
Ascorbic acid	3.50
Uric acid	3.95

[a] All measurements were made at room temperature; all values are the average of ten determinations.

where I_B is the background current, σ_S is the standard deviation for the measurement of the background current, a is the intercept of the calibration equation and S is the slope of the calibration equation. Besides the variation of the background current which was small over two months period, the variation of slope over the two months period was ±0.09 μA/mmol L while the variation of a—the intercept was ±0.9 μA over the same period of time. Accordingly, the value of the limit of detection was reliable.

3.2. Selectivity of the amperometric biosensor

The enantioselectivity and selectivity of the biosensor was checked using both separate and mixed solution methods over D-lysine, and L-(+)-serin, L-cystine, L-leucine, L-glutamic acid, asparagine, histamine dihydrochloride, glycine, β-alanine, creatine, creatinine, ascorbic acid and uric acid. Amperometric selectivity coefficients, K_{sel}^{amp}, were determined following the method proposed by Wang (1994) for the same potential (650 mV) that was used for the determination of response characteristics of the proposed amperometric biosensor. Accordingly, the amperometric selectivity coefficient may be calculated from the equation (Wang, 1994):

$$K_{i,j}^{amp} = \left(\frac{\Delta I_t}{\Delta I_i} - 1\right) \times \frac{c_i}{c_j}$$

where $\Delta I_t = I_t - I_b$ and $\Delta I_i = I_i - I_b$, I_t and I_i are the values of the current recorded for the mixed solution (L-lysine and interfering species) and for the solution containing only L-lysine, and c_i and c_j are the concentrations of L-lysine and interfering species, respectively. I_b is the current recorded for the blank solution.

The ratio between the concentrations of L-lysine and the interfering species was 1:10. All the values of the amperometric selectivity coefficients (pK_{sel}^{amp}) obtained by using the mixed solution method, for the biosensor designed for L-lysine are higher than 2 (Table 1) demonstrating the enantioselectivity and selectivity of the proposed biosensor.

3.3. Analytical applications

The recovery test of L-lysine was conducted in the presence of its antipode, D-lysine. The results obtained (Table 2) demonstrated the suitability of the proposed biosensor for the enantioanalysis of L-lysine due to the high recovery values obtained for the assay of L-lysine in the presence of D-lysine. No significant differences in the recovery values were recorded for the ratio between L and D enantiomers varying from 1:9 to 1:99.9.

The results obtained for the analysis of L-lysine in serum samples are shown in Table 3. The results are in agreement with those obtained using a standard method used by clinical laboratories which is based on spectrometric analysis.

Table 2
Recovery of L-lysine in the presence of D-lysine.[a]

L:D	L-Lysine, recovery (%)
2:1	99.19 ± 0.07
1:1	99.89 ± 0.06
1:2	99.93 ± 0.06
1:4	99.82 ± 0.07
1:9	99.84 ± 0.06

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 3
Recovery of L-lysine in serum samples.[a]

Sample no.	% L-Lysine, recovery	
	Proposed biosensor	Standard, spectrometric method
1	99.01 ± 0.05	98.99 ± 0.15
2	98.99 ± 0.06	99.05 ± 0.20
3	99.12 ± 0.05	99.13 ± 0.10
4	99.07 ± 0.05	99.29 ± 0.15
5	99.18 ± 0.06	99.09 ± 0.13

[a] All measurements were made at room temperature; all values are the average of ten determinations.

L-lysine can be determined from its pharmaceutical formulation with an average recovery of 98.68% (RSD = 0.19%, N = 10) using the proposed biosensor; the results being in good agreement with those obtained using standard spectrometric method (97.99 ± 0.27%, N = 10).

4. Conclusions

The proposed design of the amperometric biosensor is simple and reproducible. The analytical information obtained using the proposed biosensor is highly reliable. The main advantages of the proposed biosensor over the previous designed biosensors for L-lysine analysis are the determination of L-lysine at nmol/L concentration range and high selectivity and enantioselectivity, facilitating its enantioanalysis in biological samples without any prior separation. Accordingly, comparing with previous designed sensors, diamond paste as transducer for diamond paste based biosensors proved better properties than the once used previously (Kelly et al., 2000; Curulli et al., 1998; Saurina et al., 1991; Romette et al., 1983): the lower noise and high value of the ratio between signal and noise recorded for diamond paste sensors (Stefan and Bairu, 2003) over other amperometric sensors made possible also for the proposed biosensors their utilization of low values of concentration as well as the obtaining of low detection limits. Therefore, the proposed sensor exhibited increased sensitivity, and also increased selectivity at 650 mV versus Ag/AgCl versus other amino acids as well as versus compounds like ascorbic acid and uric acid. While the proposed biosensor can be used for the measurement of L-lysine directly from the serum samples or the solution containing the pharmaceutical formulation, the other biosensors (Kelly et al., 2000; Curulli et al., 1998; Saurina et al., 1991; Romette et al., 1983) cannot be used before L-lysine was separated from the matrix. Furthermore, the proteins cannot be easily adsorbed or absorbed on the diamond paste, and immersion of the sensor in a biological fluid left its surface clean. Because for pharmaceutical samples there is a need of multiple dilutions of the initial solution, the recommendation is to use the proposed biosensors mostly for clinical analysis. Therefore, one of the main features of the biosensor is its utilization for in vivo determination of L-lysine.

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