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RESEARCH PAPER

Amine oxidase-based biosensors for spermine and spermidine determination

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Abstract The present work describes the development and optimization of electrochemical biosensors for specific determination of the biogenic polyamine spermine (Spm) and spermidine (Spmd) whose assessment represents a novel important analytical tool in food analysis and human diagnostics. These biosensors have been prepared using novel engineered enzymes: polyamine oxidase (PAO) endowed with selectivity towards Spm and Spmd and spermine oxidase (SMO) characterized by strict specificity towards Spm. The current design entails biosensors in which the enzymes were entrapped in poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ), a photocrosslinkable gel, onto an electrode surface. Screen-printed electrodes (SPEs) were used as electrochemical transducers for enzymatically produced hydrogen peroxide, operating at different potential vs Ag/AgCl according to the material of the working electrode (WE): +700 mV for graphite (GP) or −100 mV for Prussian blue (PB)-modified SPE, respectively. Biosensor performances were evaluated by means of flow injection amperometric (FIA) measurements. The modified electrodes showed good sensitivity, long-term stability and reproducibility. Under optimal conditions, the PAO biosensor showed a linear range 0.003–0.3 mM for Spm and 0.01–0.4 mM for Spmd, while with the SMO biosensor, a linear range of 0.004–0.5 mM for Spm has been obtained. The main kinetic parameters apparent Michaelis constant (K_M), turnover number (K_{cat}) and steady-state current (I_{max}) were determined. The proposed device was then applied to the determination of biogenic amines in blood samples. The results obtained were in good agreement with those obtained with the GC-MS reference method.

Keywords Biosensors · Electroanalytical methods · Clinical/Biomedical analysis

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Introduction

Biogenic amines (BAs) are low molecular weight organic nitrogenous compounds, produced by the decarboxylation of amino acids in animals, plants and microorganisms. BAs can be divided into aliphatic (putrescine, cadaverine, spermidine, spermine), heterocyclic (histamine, tryptamine) and aromatic (tyramine, phenylethylamine) [1, 2]. The aliphatic BAs are the most common amine analytes in human body fluids. Polyamines have received increasing attention in recent years because of a significant biological role in growth, renovation and metabolism of every organ in the body. More recently, it has become apparent that polyamines may also have useful medical applications. Under normal conditions, levels of polyamines in physiological fluids are low whereas they may rise remarkably in the presence of several neoplastic diseases. In this framework, they have been considered as markers of tumour growth (putrescine concentrations) and cell turnover (spermidine concentrations) [3–6].

High levels of BAs can also be found in processed food as evidence of an ongoing putrefaction process, thus representing important marker substances for food freshness [7–9]. In fact, polyamines are related to the quality of the food products as they may yield carcinogenic compounds, especially when also nitrites are present [1, 10, 11].

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Real samples, upon which BAs must be determined, are complex matrices characterized by the presence of potentially interfering compounds that may hamper the simultaneous detection of specific BAs. Usually, the quantitative analysis of BAs is based on gas chromatographic methods [12, 13], thin-layer chromatography [14] and high-performance liquid chromatography with precolumn or postcolumn derivatization [15–17] or capillary electrophoresis [18, 19]. These methods require dedicated instrumentations and entail an extraction procedure or other sample pretreatments. The standard spectrophotometric detectors cannot be used as polyamines do not show distinct absorption band in the UV-Vis region [7, 20–22]. Optical methods based on fluorescence of UV-Vis spectroscopy are interesting alternatives because measurements are fast and easy to perform. A number of studies on optical spermine chemosensors have been reported over the last few years [23–25].

In the last years, electrochemical biosensors have attracted much attention for their simplicity, short analysis time, reproducibility, low cost and low detection limit and because a preceding sample preparation is not needed. Electrochemically coupled enzymatic methods, in particular, have attracted enormous attention in electroanalysis during the last decade [26]. The use of amine oxidases with various amperometric transducers has been described in the literature in recent years [1, 27, 28]. Amine oxidases are a class of enzymes catalyzing, in the presence of molecular oxygen as an electron acceptor, the oxidative deamination of primary amines, diamines and substituted amines in aldehydes, with the formation of ammonia and hydrogen peroxide. Hydrogen peroxide appears to be the most appropriate electrochemical-measuring analyte as it offers high response, accuracy and high reproducibility [1, 26]. Among the different transducers used, screen-printed electrodes have different advantages related to their disposable character and great versatility and have been used in the assembly of amperometric biosensors based on amine oxidases for the detection of BAs [26].

A common problem of oxidase-based biosensors, using hydrogen peroxide detection, is the high operational potential that is applied (higher than +600 mV), at which many electroactive substances, which are frequently present in real sample, could also be oxidized to give interfering signals [29]. Several attempts have been made to solve this problem by modifying biosensors, at which the overvoltage for either the electrochemical oxidation or reduction reaction can be reduced thus allowing the measurements to be performed at lower potential values [29, 30]. On the other hand, in the case of amine oxidases, commonly used redox mediators are unable to shuttle electrons between the enzyme redox centre and the electrode surface. Hence, they cannot be employed in these kinds of biosensors. In contrast, redox mediators catalyzing the oxidation or reduction of hydrogen peroxide have been used, in the last decade, for the construction of oxidase-based biosensors [31–33].

In this work, the development of highly specific and sensitive biosensor for spermine (Spm) and spermidine (Spmd) using polyamine oxidase (PAO) and spermine oxidase (SMO) is reported. The used FAD-dependent PAO from *Zea mays* is an enzyme catalyzing the oxidation of Spmd and Spm at the secondary amino groups [34]. PAO enzyme has been characterized as a polyamine-specific enzyme (spermine, spermidine, acetyl spermine), whereas SMO displays an exclusive specificity for spermine [35, 36] (Fig. 1).

PAO and SMO were immobilized onto the Prussian blue-modified electrode by means of photocrosslinkable poly(vinyl alcohol) with styrylpyridinium groups (PVA-SbQ) onto the electrode surface. Prussian blue (PB) was used as redox mediator for measuring the enzymatically generated hydrogen peroxide at low applied potential (−100 mV vs. Ag/AgCl) to reduce electrochemical interferences [29, 35, 36] (Fig. 2).

The biosensors were then applied to the determination of Spm and Spmd in blood samples. Finally, obtained results were compared with those obtained with the GC-MS reference method applied on the same samples.

Experimental

Materials

Z. mays native PAO (92 U/ml) and mouse recombinant SMO (30 U/ml) were supplied by MoLiRom srl (Rome, Italy). The polymeric film employed for enzyme entrapping was a photocrosslinkable poly(vinyl alcohol) with styrylpyridinium groups (PVA-SbQ) purchased from Polysciences, Inc. (USA). Putrescine, spermidine, spermine, *N*-1-acetylspermine, 1,6-diaminohexane, KCl, HCl, $FeCl_3$, $K_3[Fe(CN)_6]$, potassium monobasic phosphate and bibasic phosphate, ethyl chloroformate (ECF) and pentafluoropropionyl anhydride (PFPA), diethyl ether, ethyl acetate and dichloromethane of pesticide grade were purchased from Sigma-Aldrich. All other chemicals were of analytical grade.

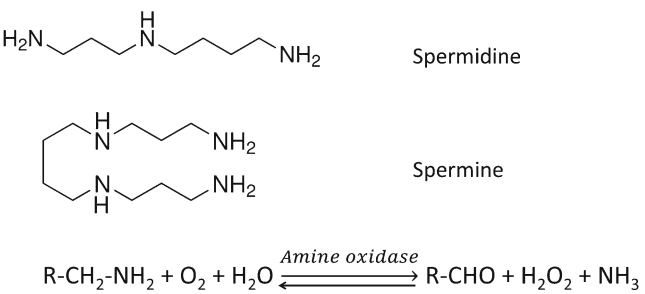

Fig. 1 General schematic representation of Spm and Spmd enzymatic oxidation

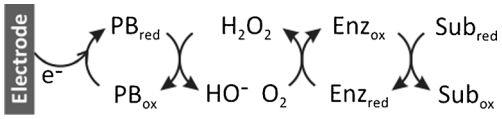

Fig. 2 Schematic representation of the sensing mechanism

Stock solutions of spermidine and spermine were prepared in phosphate buffer 50 mM, pH 5.8, with 100 mM KCl for PAO biosensor, and phosphate buffer 50 mM, pH 8.0, with 100 mM KCl for SMO biosensor. High-purity deionized water (resistance 18.2 MΩ·cm at 25 °C; TOC< 10 μg l^{-1}) obtained from Millipore (France) has been used to prepare all the solutions. Blood samples were obtained from the Central Diagnostic Laboratory at Sapienza University.

Blood samples were treated with 4 volumes of 8 % ice-cold perchloric acid, mixed thoroughly for 5 min and centrifuged at 5000*g* for 20 min. The supernatant was used for the analysis.

Apparatus

Electrochemical deposition of Prussian blue

The deposition of the redox mediator PB was obtained using the following cyclic voltammetric procedure: a graphite screen-printed electrode (GP-SPE) was immersed into a solution containing 0.1 M of KCl and 0.1 M of HCl as a supporting electrolyte, 1.9 mM of $K_3[Fe(CN)_6]$ and 1.9 mM of $FeCl_3$. The electrodeposition of PB was performed in the potential range of 0.35 to −0.1 V vs Ag/AgCl, at a scan rate of 50 mV s^{-1} for 10 cycles. After the deposition, the mediator was activated, employing the same supporting electrolyte solution, potential range and scan rate, for 40 cycles, as reported in the literature [16–18].

Biosensor preparation

Both PAO and SMO biosensors have been developed by mixing 6 μl of the enzymatic solution with 8 μl of PVA-SbQ (50 mg of PVA-SbQ was previously solubilized in 400 μl distilled water). The resulting enzyme-PVA-SbQ solution was homogenized by vortex mixing, and 4 μl of this solution was homogenously spread on the working electrode surface. The SPEs were then exposed for 20 min under a UV lamp at room temperature to allow the entrapment of the enzyme by photopolymerization. With this procedure, the final activity of the enzyme immobilized onto the electrode surface was about 0.16 U for PAO and 0.05 U for SMO. Biosensors were stored at 4 °C.

Enzymatic activity measurements

Immobilized SMO and PAO loading was determined spectrophotometrically by measuring the amount of H_2O_2 formed. In the presence of peroxidase (POD), hydrogen peroxide participates in a reaction involving *p*-hydroxybenzoic acid and 4-aminoantipyrine, and a quinone imine dye complex is formed, which is measured at 510 nm. The PVA-SbQ enzymatic layer was inserted in cuvettes containing the above-mentioned reactive solution.

Electrochemical measurements

Amperometric experiments were carried out using a μAutolab type III potentiostat (Eco Chemie, The Netherlands) controlled by means of the GPES Manager program (Eco Chemie, The Netherlands) with a conventional three-electrodes configuration. SPEs (DropSens, Oviedo, Spain), constituted of GP as a working electrode with a surface diameter of 4 mm, carbon as a counter electrode and silver/silver chloride electrode as a reference, were used. Chronoamperometric experiments were carried out in flow conditions using a flow cell (DropSens, Oviedo, Spain) connected to a sample injection valve (DropSens, Oviedo, Spain) and a Gilson's Minipuls 3 peristaltic pump with a flow rate of 0.716 ml/min. GP-SPE was able to directly detect H_2O_2 at a fixed potential of +700 mV vs Ag/AgCl, while PB-modified GP-SPE allowed the detection of H_2O_2 at −100 mV vs Ag/AgCl. Phosphate buffer 50 mM, pH 5.8, with 100 mM KCl was used as a carrier for PAO biosensor, and phosphate buffer 50 mM, pH 8.0, with 100 mM KCl for SMO. The calibration plot was obtained by adding several aliquots of substrate standard solution at different concentrations into the buffer solution. The linear regression was calculated using the software GraphPad Prism 5 from GraphPad Software Inc. (USA).

Analysis of real samples by electrochemical measurements

The analysis of blood samples were performed in flow injection system. The samples appropriately diluted, according to the linear range of the calibration plot, were injected in the cell, and the resulting signal was referred to that of spermine (see the Real sample analysis section in the Results and discussion).

Analysis of real samples by GC-MS

Serum polyamines were analyzed by GC-MS according to the method of Paik et al. [37] with slight modifications.

Briefly, 0.3 ml of serum/plasma was spiked with internal standard 1,6-diaminohexane (final concentration 8.6 μM) and adjusted to pH ≥12 with 5 M NaOH. The samples were then subjected to sequential *N*-ethoxycarbonylation and *N*-pentafluoropropionylation.

N-Ethoxycarbonylation was performed in one step by adding to the aqueous-phase ethyl chloroformate (20 μl) dissolved in dichloromethane (1 ml). After vortex mixing (2 min), the mixture was saturated with NaCl and extracted with diethyl ether (3 ml) and ethyl acetate (2 ml). The organic extracts were combined and dried under reduced pressure. The sample was then subjected to the second derivatization step by adding 20 μl pentafluoropropionyl anhydride (60 °C for 60 min) and analyzed by GC-MS.

GC-MS analyses were performed with an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separations were carried out with an Agilent HP-5ms fused-silica capillary column (30 m×0.25 mm i.d.) coated with 5 % phenyl-95 % dimethylpolysiloxane (film thickness 0.25 μm) as stationary phase. Injection mode was splitless at a temperature of 260 °C. Column temperature program was 70 °C (2 min) then to 300 °C at a rate of 15 °C/min and held for 5 min. The carrier gas was helium at a constant flow of 1.0 ml/min. The spectra were obtained in the electron impact mode at 70 eV ionization energy, 280 °C ion source and 10^{-5} Torr ion source vacuum.

MS analysis was performed simultaneously in TIC (mass range scan from *m*/*z* 50 to 800 at a rate of 0.42 scans s^{-1}) and selected-ion monitoring (SIM) mode. For GC-SIM-MS, the following quantitation ions were selected for both of the two polyamines analyzed (Spmd, *m*/*z* 580; Spm, *m*/*z* 709).

Results and discussion

Both PAO and SMO biosensors were characterized in their main kinetic and electroanalytical performances. The kinetic characterization was realized by non-linear regression of the classical Michaelis-Menten kinetics using the software GraphPad Prism 5 from GraphPad Software Inc. (USA). In particular, the following kinetic apparent parameters were calculated: total enzyme concentration loading (E_T), turnover number (K_{cat}^{app}), Michaelis constant (K_m^{app}), maximum current (I_{max}) and specificity constant (K_{cat}^{app}/K_m^{app}). The biosensors were bioelectrochemically characterized in the presence of the two substrates Spm and Spmd. The kinetic parameters were calculated according to a simplified approach [38] which entails the application of Eq. 1, and it is the Lineweaver-Burk-type linearization as expressed in Eq. 2:

$$I_{lim} = \frac{I_{max}\ [S_{ox}]}{[S_{ox}] + K_m^{app}} \quad (1)$$

$$\frac{1}{I_{lim}} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max}\ [S_{ox}]} \quad (2)$$

where $[S_{ox}]$ is the concentration of the oxidized substrate, I_{lim} is the limiting current measured after every addition of substrate, K_m^{app} is the apparent Michaelis-Menten constant for the enzymatic reaction and I_{max} is the steady-state current.

Table 1 Selectivity: response of PB-SPE PAO biosensor to different biogenic amines. The concentration of substrates was 0.1 mM

Substrate	Response (%)
Spermine	100.0
Spermidine	69.0
Putrescine	1.0
Cadaverine	0.8
Histamine	0.9

Table 2 Main kinetic parameters obtained with the PB-SPE-modified PAO biosensors using Spmd and Spm as substrate

Kinetic parameters	Spmd	Spm
E_T (μM)	0.000071	0.000071
K_{cat}^{app} (s^{-1})	41183	54422
K_m^{app} (mM)	0.482	0.346
I_{max} (μA)	2.924	3.864
K_{cat}/K_m ($M^{-1}\ s^{-1}$)	8.5×10^7	9.4×10^7

Table 3 Main electroanalytical parameters obtained with the PB-SPE-modified PAO biosensors using Spmd and Spm as substrate

Electroanalytic parameters	Spmd	Spm
Response time (s)	60	60
Life time of operation (as total number of assays)	20	20
Slope (μA/mM)	2.57	5.01
Linearity range (mM)	0.01–0.4	0.003–0.3
r^2	0.9946	0.9927
LOD (mM)	0.005	0.001
Repeatability of the measurements (as pooled standard deviation in the linearity range) (%)	2.1	1.8

Table 4 Selectivity: response of SMO biosensor to different biogenic amines. The concentration of substrates was 0.1 mM

Substrate	Response (%)
Spermine	100.0
Spermidine	2.9
Putrescine	1.2
Cadaverine	1.2
Histamine	1.2

PAO biosensor

The performance of the developed biosensors towards the main BAs was then assessed as follows. As representative example, the experimental data, reported in Table 1, obtained with the PAO/GP-SPE biosensor bring out clearly its selectivity towards Spm and Spmd. In particular, the spurious response towards other considered BAs was less than 1 % with respect to Spm. These data are in full agreement with data obtained with the same engineered PAO enzyme in solution and clearly indicate the possibility to use these biodevices for determination of the total amount of Spm and Spmd in solution.

The kinetic data obtained for both the PAO/SPE biosensors with Spm and Spmd are summarized in Table 2. The table allows to evaluate the immobilization method and to compare the performance obtained in the direct (GP-SPE) and mediated (PB-SPE) detection of hydrogen peroxide.

From the analysis of the overall kinetic parameters, it is possible to assess the usefulness of the photopolymerization method in maintaining the catalytic property of the enzyme. Furthermore, the PB-SPE-based biosensor shows higher values of both K_{cat}^{app} and I_{max} than those obtained with GP-SPE-based biosensor for both the substrates under investigation (K_{cat}^{app}=1744 s^{-1}, I_{max}=0.124 μA for Spm; and K_{cat}^{app}=2113 s^{-1}, I_{max}=0.150 μA for Spmd), a finding that may be attributed to the higher efficiency of the PB-SPE transducer in the electrochemical detection of H_2O_2 with respect to the direct detection using a GP-SPE transducer [39]. About the affinity of PAO towards Spm and Spmd, the data obtained are consistent to the literature data [35] and also to the selectivity reported above.

The analytical characterization of PB-SPE-modified PAO biosensor in the presence of Spmd and Spm is reported in Table 3. The comparison with data obtained with PB-SPE PAO biosensor confirms that PB-SPE biosensor performances are better than GP-SPE PAO biosensor.

Table 5 Main kinetic and electroanalytical parameters obtained with the PB-SPE SMO biosensor using Spm as substrate

Kinetic parameters	
E_T (μM)	0.00028
K_{cat}^{app} (s^{-1})	3182
K_m^{app} (mM)	0.2941
I_{max} (μA)	0.891
K_{cat}/K_m (M^{-1} s^{-1})	1.0×10^7
Electroanalytical parameters	
Response time	80
Life time of operation (as total number of assays)	15
Slope (μA/mM)	1.11
Linearity range (mM)	0.004–0.5
r^2	0.9982
LOD (mM)	0.002
Repeatability of the measurements (as pooled standard deviation in the linearity range) (%)	1.5

Table 6 Determination of total amount of Spm+Spmd in the blood samples, obtained using the PAO-based biosensors and by GC-MS analysis, expressed as nanogram per millilitre of Spm (n=3)

Sample	PAO biosensor [Spm+Spmd] (μM)	GC-MS [Spm+Spmd] (μM)	Accuracy (%)
1	13.4	14.5	−7.2
2	17.2	17.8	−3.5
3	14.5	15.1	−3.4
4	7.8	7.9	−1.6
5	8.2	8.8	−7.3
6	22.9	23.0	−0.6
7	8.5	9.4	−9.5
8	6.2	6.6	−6.8

SMO biosensor

The same characterizations have been carried out also in the case of SMO-based biosensors. In Table 4, its selectivity towards the main BAs is reported. Data obtained confirm that SMO is able to detect selectively Spm, the response towards

Table 7 Determination of Spm concentration in the blood samples, obtained using the SMO-based biosensors and by GC-MS analysis, expressed as nanogram per millilitre of Spm (n=3)

Sample	SMO biosensor [Spm] (μM)	GC-MS [Spm] (μM)	Accuracy (%)
1	9.5	9.8	3.3
2	9.7	9.9	−3.2
3	9.1	9.2	−0.2
4	1.9	2.0	−4.2
5	2.9	3.1	−6.0
6	9.2	8.7	4.6
7	3.8	3.8	0.9
8	3.3	3.4	−2.9

Spmd being ≤3 % and the other BAs around 1 % with respect to the response towards Spm, confirming also in this case the data obtained in the analogous experiments exploited with the engineered SMO enzyme in solution [36] and suggesting the possibility to use these biodevices for the selective detection of Spm in solution.

Also in this case, we have performed the characterization of SMO/SPE biosensor using the PB and GP electrochemical transducer.

The main kinetic and electroanalytical data obtained for PB-SPE-modified SMO biosensor in the presence of Spm as substrate are summarized in Table 5.

Also in this case, both the kinetic and electroanalytical performances bring out the higher response of PB-SPE-based SMO biosensor with respect to the GP-SPE one.

Real sample analysis

All data presented in the previous paragraph show that PB-SPE-based PAO and SMO biosensors display higher overall electroanalytical performances in both total (Spm+Spmd) and selective (Spm) detection, thus paving the way to their use in performing the analysis of these polyamines in blood samples. Perchloric acid treatment and centrifugation were used to remove interfering factors from the plasma, with the precipitate being discarded and the supernatant used to detect small molecules such as endotoxins, pharmaceuticals or metabolites.

In addition, in order to minimize possible interferences, the analysis of real samples was carried out using the PB electrode since it provides better overall electroanalytical performances, among which is higher sensitivity, and allows a reduction of the applied potential (−100 mV), thus preventing several possible electrochemical interferences [40, 41].

The measurements were carried out on eight blood samples selected among oncologic patients. In Tables 6 and 7, the analysis of total Spm+Spmd and Spm concentrations, respectively, as obtained with two different methods, namely the flow injection system and GC-MS as reference method, is reported. In the case of the total concentration of Spm+Spmd, data obtained from GC-MS have been compared with normalized data from PAO-based biosensor, obtained by multiplying the total molar concentration by spermine molecular weight [1]. Results are in good agreement, considering that the accuracy between the two methods ranged within 10 %. This is a very remarkable finding, especially if taking into account that PAO-based biosensor shows different sensitivity towards the different substrates considered.

By comparing the data obtained with the SMO biosensor to those determined by the reference method, a good agreement (within 6 %) can be observed for spermine between the two methods.

Conclusions

Two novel amperometric biosensors, based on engineered PAO and SMO enzymes, have been characterized electrochemically in terms of selectivity towards different typical biogenic amines, main kinetic data and electroanalytical performances. The biosensors were also tested for the analysis of BAs (Spm and Spmd) in the blood samples from oncologic patients as compared and validated using a modified reference method based on GC-MS analysis. Both biosensors have been demonstrated to accurately measure the overall Spm+Spmd content expressed as Spm, in the case of PAO-based biosensors, and selectively the Spm concentration with the SMO-based biosensor. With respect to the similar papers published so far [1], the proposed biosensors have the remarkable advantage to selectively detect only spermine, thanks to the peculiar properties of SMO, while PAO enzyme is able to detect the overall amount of these two polyamines (Spm+Spmd) without being interfered by the other different biogenic amines eventually present. Moreover, analytical features of the proposed biosensors show fast response, minimal sample treatment and sensitivity of the proposed biodevices, suggesting their possible use in the point of care tests for cancer marker detection.

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