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Dedicated to the memory of
Professor Ioan Silaghi-Dumitrescu (1950 – 2009)

BIOSENSOR BASED ON ASCORBATE OXIDASE FOR ASCORBIC ACID DETERMINATION

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A new biosensor design, consisting in ascorbate oxidase (AOx) and bovine serum albumine immobilization on the membrane of an oxygen electrode, followed by cross-linking with glutaraldehyde (GA), was proposed for ascorbic acid (AA) determination. The biosensor was optimized regarding the enzyme loading and GA concentration. The best bioelectroanalytical parameters were obtained when 1.25 % GA and 157 U/cm^2 AOx were used: sensitivity of 328 ± 67 μA/M, linear domain up to 0.4 mM and detection limit of 50 μM. The biosensor was used to estimate the AA concentration in synthetic samples and the results were in good agreement with those obtained by using the reference method based on 2,6 dichlorophenolindophenol (relative error ~ 1.1 %).

INTRODUCTION

Ascorbic acid (AA) is one of the most important bioanalytes. Thus, beyond its function in collagen and catecholamine synthesis, tyrosine degradation and bile acid biosynthesis, AA is known to increase absorption of inorganic iron, to have essential roles in the metabolism of folic acid, of amino acids, and hormones and to act as an efficient antioxidant. Consequently, the determination of AA in various natural and prepared foods, drugs and physiological fluids is very important.[1-3]

In order to measure AA in different matrices, a variety of methods has been proposed such as: titrimetric, [4-6] HPLC, [7-8] spectrophotometric, [9-10] chemiluminescent [11-13] and electrochemical methods. [14-15]

Besides these methods, those exploiting enzymatic biosensors appear particularly attractive, because enzymes are known to be versatile, highly sensitive and selective. [16] Consequently, various biosensor designs using the ability of ascorbate oxidase (AOx) to catalyze very efficiently the reaction:

$$\text{Ascorbic acid} + 1/2\ O_2 \xrightarrow{\text{AOx}} \text{dehydroascorbic acid} + H_2O \quad (1)$$

were proposed for AA detection. [16-22] The consumption of oxygen accompanying the AA oxidation was monitored using either a polarographic detector,[20] or, more often, a Clark oxygen electrode. [17-19, 21, 23] For this purpose, AOx was immobilized by covalent binding with

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glutaraldehyde to collagen, [17] gelatin, [18] nylon net [19] or by adsorption on carbon felt. [21,23]

Taking into account that enzyme immobilization in the close vicinity of the transducer is a crucial step in obtaining efficient biosensors and that a friendly microenvironment is critical for the biosensors stability, in this work a new amperometric biosensor for AA was obtained by AOx immobilization in a natural biopolymeric matrix (bovine serum albumine, BSA), cross-linked with glutaraldehyde (GA), deposited on the surface of a Clark oxygen electrode. This new design combines a gentle enzyme immobilization procedure, ensuring a long operational life of the obtained biosensor, with a sensitive and highly selective transducer, resulting in an efficient amperometric detection scheme. It is noteworthy to mention that the oxygen -permeable membrane of the transducer provides a convenient support for enzyme immobilization and, at the same time, represents a physical barrier against the free access of interfering species.

The biosensor was optimized regarding the enzyme loading and GA concentration, keeping a constant weight ratio between BSA and AOx. The biosensor was used for AA determination in synthetic samples by using standard addition method and the results were compared with those furnished by a volumetric method, based on the AA reaction with 2,6 dichlorophenolindophenol. [24]

The influence of different AOx and as well as the short-term and long-term stability of obtained biosensors were investigated. The kinetic and bioelectroanalytical parameters of the biosensor were estimated.

RESULTS AND DISCUSSION

The results presented in Figure 1 confirm the ability of the transducer included in the proposed experimental design to efficiently monitor AA concentration in the surrounding solution. Thus, the biosensor exhibits a well-shaped Michaelis-Menten behavior when the AA concentration was gradually increased in the 0-8 mM domain. The inset of Figure 1 gives qualitative information on the biosensor response rate, as well as on the signal stability. As can be seen, the biosensor response time was relatively short ($t_{95\%} \sim 50$ s).

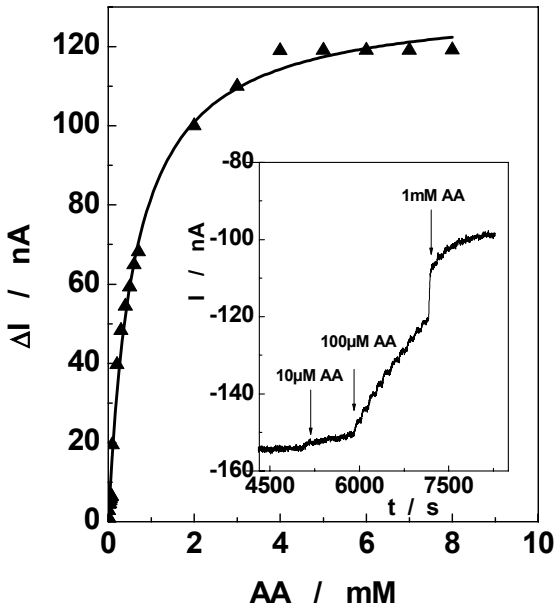

Fig. 1 – Calibration curve of the AOx based biosensor and the corresponding amperometric response to successive AA additions (inset). Experimental conditions: enzyme loading, 315 U AOx / cm^2; 2.5% GA; The solid line represents the Michaelis – Menten fitting of the experimental data.

In order to optimize the biosensor response, the influence of enzyme (AOx) loading and of the cross-linking agent (GA) concentration on the biosensor sensitivity (estimated as the ratio of maximum current intensity, I_{max} and the Michaelis–Menten apparent constant, K_M^{app}, obtained by fitting the Michaelis–Menten equation) were investigated. As can be seen from Figure 2, at constant GA concentration, the biosensor sensitivity increases when the enzyme loading decreases and, at constant AOx loading, a decrease of GA concentration resulted in a significant increase of the biosensor response. This behavior, apparently surprising, is due to the

decrease of the enzymatic membrane permeability when either the enzyme loading or GA concentration increase and it was reported also by other authors. [17, 25, 26] This explanation corroborates with the change of the biosensor linear range, induced by the enzyme loading and/or GA concentration variation (Table 1).

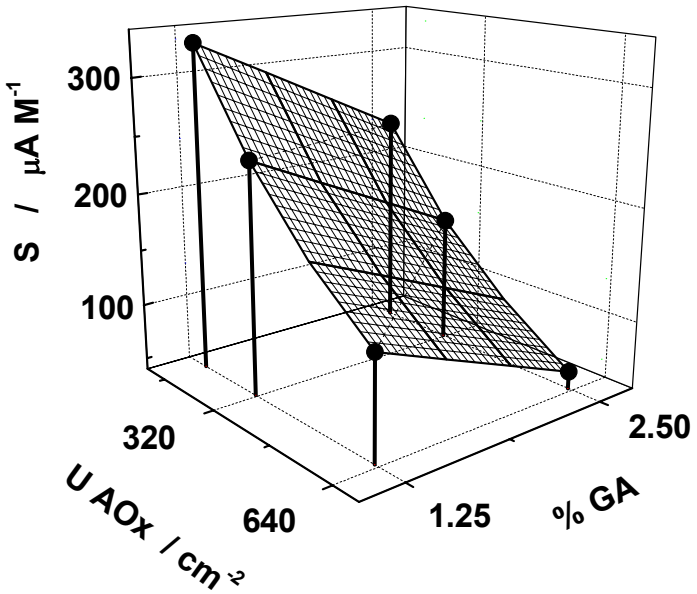

Fig. 2 – 3D representation of biosensor sensitivity as function of AOx loading and GA concentration. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer solution (pH 7); stirring rate, 650 rpm.

Table 1

Analytical parameters of the amperometric biosensors for AA detection

Biosensor number	AOx loading (U/cm^2)	GA conc. (%)	DL (M)	Linear domain (M)	R/N
1	630	2.5	$4*10^{-5}$	up to 10^{-3}	0.998/17
2		1.25	$5*10^{-5}$	up to $7*10^{-4}$	0.998/17
3	315	2.5	$5*10^{-5}$	up to $6*10^{-4}$	0.996/16
4		1.25	$5*10^{-5}$	up to $5*10^{-4}$	0.998/17
5	157	2.5	$5*10^{-5}$	up to $5*10^{-4}$	0.989/16
6		1.25	$5*10^{-5}$	up to $4*10^{-4}$	0.978/12

The linear domain of the investigated biosensors (Table 1) is situated roughly in the concentration range of $5*10^{-5}$ -10^{-3} M, being in good agreement with the literature data, [18] or even wider than that reported for other AOx based biosensors. [17, 21]

Irrespective to the enzyme loading or GA concentration, the detection limits (Table 1), estimated from experimental data used for calibration curves are situated around $5*10^{-5}$ M. This value is lower than those reported for other AOx based biosensors [21-22] proving the efficiency of the signal transduction in the proposed biosensor design.

The results of biosensor optimization point out that the best biosensor sensitivity was obtained when the enzyme loading was 157 U AOx/cm^2 and GA concentration was 1.25 %. Newertheless, in order to increase the lifetime of the biosensor, for all further experiments, a double enzyme loading (315 U AOx/cm^2) was preferred.

From the calibration curves, recorded for different biosensors prepared with various AOx loading and GA concentration, the Michaelis-Menten kinetic parameters (K_M and I_{max}) were estimated (Table 2). A good agreement between the values obtained by nonlinear fitting of the Michaelis-Menten equation and those calculated from the Hanes–Woolf linearization was observed.

Table 2

Kinetic parameters of the amperometric biosensors for AA detection

Biosensor number	AOx loading (U/cm^2)	GA conc. (%)	Michaelis-Menten fitting			Hanes-Woolf linearisation		
			I_{max} (nA)	K_M^{app} (mM)	R^2	I_{max} (nA)	K_M^{app} (mM)	R/N
1	630	2.5	65 ± 1.3	1.17 ± 0.08	0.991	67 ± 2.1	1.3 ± 0.2	0.997 / 27
2		1.25	110 ± 2.3	0.85 ± 0.06	0.990	105 ± 3.1	0.63 ± 0.02	0.999 / 7
3	315	2.5	139 ± 3.6	0.90 ± 0.08	0.983	130 ± 4.1	0.70 ± 0.01	0.998 / 14
4		1.25	158 ± 4.8	0.65 ± 0.07	0.973	151 ± 4.7	0.46 ± 0.02	0.999 / 10
5	157	2.5	152 ± 3.6	0.65 ± 0.06	0.984	167 ± 2.2	0.49 ± 0.01	0.989 / 9
6		1.25	181 ± 10	0.55 ± 0.08	0.963	194 ± 3.1	0.45 ± 0.08	0.999 / 16

The literature survey reveals that, for free AOx, the value of Michaelis–Menten constant varies between 0.11 mM and 1.3 mM. [17, 20, 27] The K_M^{app} values included in Table 2 are slightly higher than the inferior limit for free AOx, and higher than that reported for immobilized AOx, K_M^{app} (0.1 mM [17]). This behavior can be understood as a combined effect of the diffusion constraints existing in the enzyme layer, of the different enzyme provenience and of the peculiar immobilization procedure and proves, once again, that K_M^{app} is not an intrinsic property of a given enzyme, being strongly dependent on the microenvironment created around the immobilized enzyme.

The short-term stability of the obtained biosensor was evaluated by using two biosensors, differing by the concentration of the cross-linking agent and performing 7 consecutive measurements at 0.5 mM AA concentration (Figure 3A). A relative invariance of the biosensor response can be noticed, irrespective the GA concentration, reflecting a good short-term stability of the biosensors.

To evaluate the medium-term stability of the AA biosensors, repetitive measurements were performed with two biosensors, differing by the concentration of the cross-linking agent. The results from Figure 3B show that, during one week, both biosensors exhibit an excellent stability.

All above-presented results justify an attempt of using the obtained biosensor for AA determination in synthetic samples. For this reason, the amperometric response of the bioelectrode, immersed in a synthetic sample of AA, to 3 successive additions of standard AA solution was recorded aiming to estimate the sample concentration, by using the standard addition method. Well-defined steps for each standard solution addition were observed, allowing the calculation of the AA sample concentration (Figure 4). The calculated value (1.95 ± 0.09 mM) was in good agreement (1.1% relative error) with the sample concentration (1.98 ± 0.04 mM), obtained by using the reference titrimetric method. [24] In both cases, the results are the average of three replicates and the error stands for standard deviation.

Taking into consideration the above-mentioned results and the high selectivity of the biosensor receptor, the present design recommends the obtained AOx-based biosensor for ascorbic acid determination in real samples.

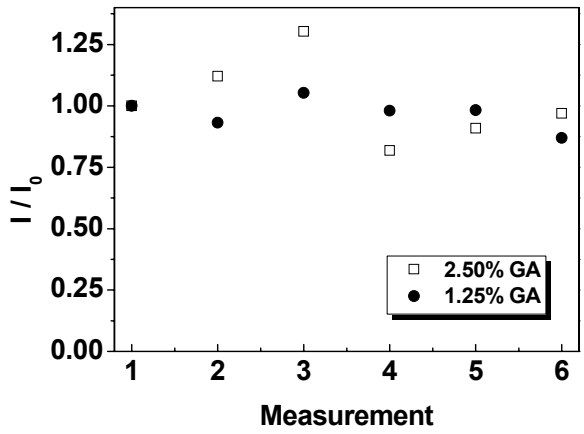

Fig. 3 A

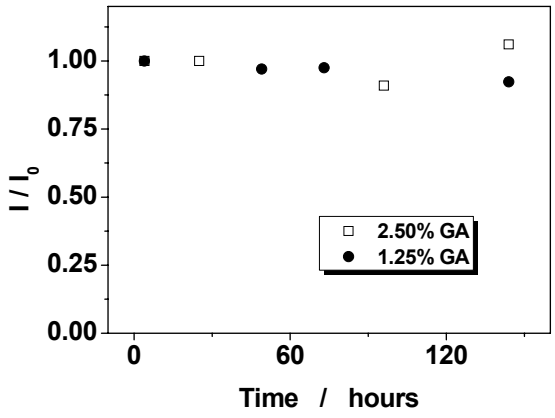

Fig. 3 B

Fig. 3 – Short-term (A) and medium-term stability (B) of the obtained biosensors. Experimental conditions: enzyme loading, 157 U AOx /cm^2; supporting electrolyte, 0.1 M phosphate buffer (pH 7); stirring rate, 600 rpm; AA concentration, 0.5 mM. I/I_0 stand for the ratio between the actual and the initial biosensor response.

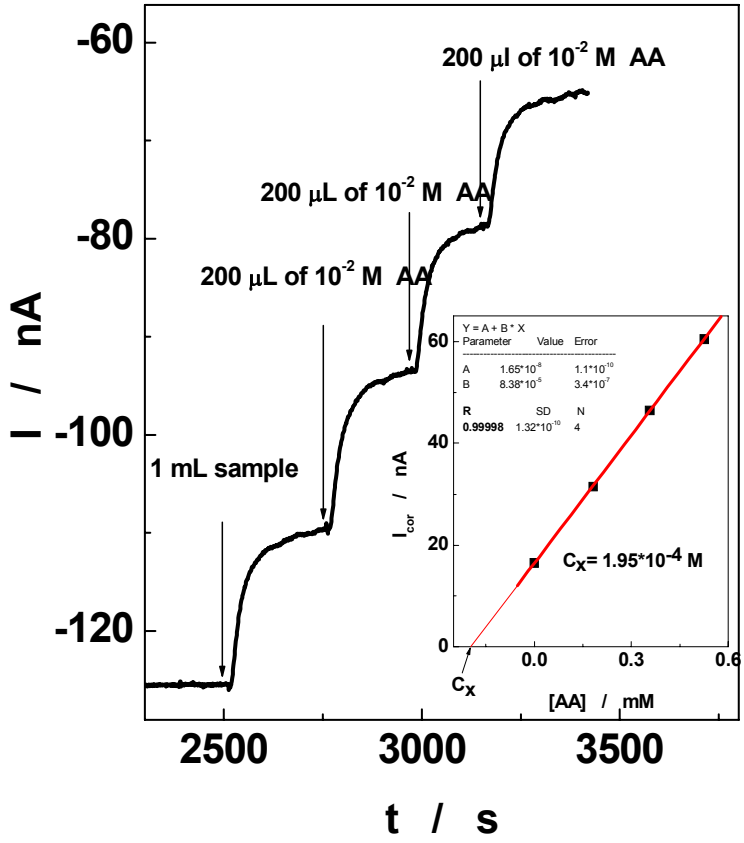

Fig. 4 – Ascorbic acid determination in synthetic sample by using standard addition method: biosensor amperometric response and its interpretation (inset). Experimental conditions: enzyme loading, 315 U AOx /cm^2; 2.5 % GA; supporting electrolyte, 0.1 M phosphate buffer (pH 7); stirring rate, 650 rpm; AA sample concentration, $1.95*10^{-4}$ M.

EXPERIMENTAL

Chemicals. Ascorbic acid (AA), ascorbate oxidase (EC 1.10.3.3; 162 U/mg solid AOx,), bovin serum albumine (BSA), glutaraldehyde (GA) and 2,6 dichlorophenolindophenol (DCPIP) were purchased from Sigma (St. Louis, MO, USA). $K_2HPO_4{\cdot}2H_2O$ and $KH_2PO_4{\cdot}H_2O$ were supplied by Merck (Darmstadt, Germany). The supporting electrolyte was a 0.1 M phosphate buffer solution (pH 7).

Electrodes preparation. The biosensor transducer was an amperometric oxygen electrode (model 5775), purchased from Yellow Springs Instrument, (Ohio, USA). The procedure used to obtain amperometric biosensors for AA consisted in deposition of AOx –BSA mixture on the surface of the gas-permeable membrane of a Clark oxygen electrode, followed by its cross-linking with GA. Because of its simplicity and efficiency, this approach was used to immobilize other enzymes in order to obtain stable electrochemical biosensors. [25]

Biosensors with different enzyme loading and cross linking degrees were prepared as follows: three aliquots of 5 µl phosphate buffer solution (pH 7) containing different amounts of AOx and BSA (the BSA / AOx weight ratio was always kept equal to 2.2) were successively deposited on the surface of the oxygen-permeable membrane (0.001'' thick Teflon® membrane). After the final drying, the probe carrying the bioactive layer was covered with 10 µl from 1.25 % or 2.5 % GA solution and left to react for 5 minutes. Finally, the obtained biosensor was carefully washed with distilled water in order to remove the excess of GA. [26] Between measurements, the biosensor was stored at 4 ^{0}C.

Electrochemical measurements. All amperometric measurements were performed using a PC-controlled electrochemical analyzer (Autolab-PGSTAT 10, EcoChemie, Utrecht, The Netherlands), in batch under magnetic stirring. The applied potential was -650 mV *vs.* an $Ag/AgCl/KCl_{1M}$ electrode, simultaneously used as reference and counter electrode.

CONCLUSIONS

The AOx immobilization procedure, consisting in its incorporation in a natural biopolymer (BSA) and subsequent cross linking with GA, was proved to be a simple and enzyme-friendly approach to obtain a stable enzymatic matrix. When this matrix was deposited in the close vicinity of a sensitive and selective amperometric transducer (Clark oxygen electrode) an efficient amperometric biosensor for AA detection was obtained.

The best bioelectroanalytical parameters were noticed when 1.25 % GA and 157 U/cm^2 AOx were used for the biosensor construction: sensitivity of 328 ± 67 µA/M, linear domain up to 0.4 mM, detection limit of 50 µM, fast response ($t_{95\%}$ < 50 sec.) and good operational and storage stability.

The results obtained using the standard addition method with this biosensor are comparable with those obtained using a standard method based on AA titration with 2,6 dichlorophenolindophenol.

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