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A new approach for nitrite determination based on a HRP/catalase biosensor

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Abstract

In this paper, a new concept of enzyme inhibition-based biosensor involving a two enzyme system was developed. The latter displays a signal increase instead of a decrease in the presence of an inhibitor. HRP and catalase were thus separately entrapped into Layered Double Hydroxides (LDH), an anionic clay, as a host matrix. The inner layer was constituted of HRP electrically wired by [$Zn_2CrABTS$] LDH and the outer layer contained catalase immobilized in [Zn_3AlCl] LDH. Both enzymes catalyzed the decomposition of H_2O_2, HRP its reduction and catalase its breakdown into oxygen and water. Nitrite was selected as a specific inhibitor of catalase. In the presence of H_2O_2, the nitrite addition blocked the H_2O_2 consumption by catalase, inducing thus an increase in the amperometric signal of the H_2O_2 reduction at 0 V by the wired HRP. The optimum configuration of the bi-enzyme biosensor displayed in aerated aqueous solutions, a nitrite sensitivity of 102 μA $M^{-1}\cdot cm^{-2}$ with a fast response time, the detective limit being 4 μM.

Keywords: Biosensor; Inhibitor determination; HRP; Catalase; Nitrite; LDH; Anionic clay

1. Introduction

Among the various types of enzymatic biosensors, a category was based on an inhibitive effect of their enzymatic activity. Water pollutants such as pesticides, insecticides and heavy metals are the main substances that were detected by amperometric biosensors [1] via an enzyme alteration process. For conventional amperometric biosensors, the functioning principle is based on the appearance or an increase in current due to the direct oxidation or reduction of the products of a biochemical reaction. In contrast, inhibitor-based biosensors are based on a decrease of their signal intensity due to the alteration of the enzyme activity. The strong initial signal is due to the amperometric detection of a defined concentration of enzyme substrate. As a consequence, the detection limit is based on the smallest observable decrease in the initial biosensor response instead of a current change on the background current for a conventional biosensor. Owing to the increase in noise from background current to high biosensor response, this may be a drawback for obtaining a sensitive detection limit.

In this paper, we report a new concept of inhibitor-based biosensor involving no initial current response (or a very weak signal) and an increase instead of a decrease in biosensor signal in the presence of an enzyme inhibitor. This strategy requires the combination of two enzymes and their immobilization in two separated layers. The first enzyme located in the outer layer, consumes totally the substrate of the second enzyme that can generate redox species at the origin of an amperometric signal at the electrode surface. The specific inhibition of the first enzyme leads thus to the appearance of an amperometric signal which increases with the increase in inhibitor concentration. This concept was illustrated with a peroxidase (HRP) and a catalase, two enzymes that catalyze the decomposition of the same substrate: hydrogen peroxide. However the mechanisms of the decomposition of hydrogen peroxide by these two enzymes are completely different. HRP reduces H_2O_2 to H_2O and catalase catalyzes the breakdown of H_2O_2 into H_2O and O_2. The concept based on a bi-enzyme electrode with HRP and catalase has been proposed to improve the upper sensing limit of H_2O_2 by a so called substrate-purging effect [2].

Our biosensor configuration consists in the immobilization of catalase in the outer layer and the immobilization and wiring of HRP in the inner layer (Fig. 1). Both enzymes were separately entrapped into Layered Double Hydroxides (LDH), an anionic clay as host matrix [3], the outer layer being [Zn_3AlCl] LDH while the inner layer [$Zn_2CrABTS$] LDH contains ABTS to connect HRP [4,5]. In the presence of H_2O_2,

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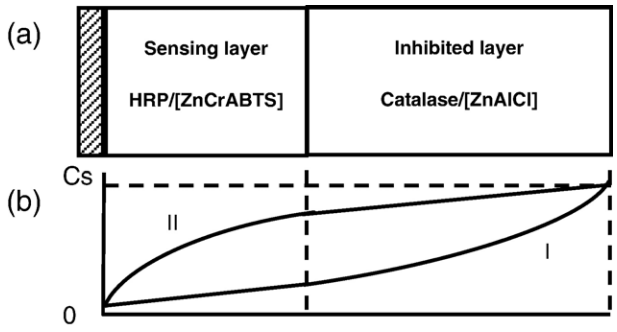

Fig. 1. (a) Schematic configuration of bi-enzyme electrode consisting of a sensing layer HRP/[ZnCrABTS] and an inhibited layer Catalase/[ZnAlCl]. (b) Substrate concentration profile in the absence of inhibitor (I) and in the presence of inhibitor (II).

the expected cathodic current due to the enzymatic reduction of H_2O_2 by the wired HRP is theoretically suppressed by the H_2O_2 decomposition by catalase. Specific inhibitors of catalase may thus be detected through the appearance and the increase of the biosensor response to H_2O_2 (Fig. 1).

With the aim to investigate this biosensor concept, nitrite, a specific inhibitor of catalase, was selected to evaluate the biosensor performance. Nitrates and nitrites are largely used as preservatives and fertilizing agents. However, a continuous exposition of these ions to humans can have serious health implications. Particularly, nitrites can react irreversibly with hemoglobin to produce methemoglobin, therefore reducing blood capacity to transport oxygen. The recognition of such a threat obliges, in most industrial countries, to nitrite monitoring in environmental, food and physiological samples. The European Community has thus established the maximum admissible levels of nitrite in drinking water at 0.1 mg/L. Besides the centralized analytical systems, like spectrophotometric and chromatographic methods [6], nitrite determination was attempted by enzymatic biosensors. The latter was mainly based on the immobilization and electrical wiring of nitrite reductase (NiR) [7–11]. However, these enzyme electrodes were faced to the fragility of NiR and the obligation to operate in the absence of oxygen for connecting the enzyme. In this context, we report here a new amperometric biosensor for nitrite determination in the presence of oxygen, based on the catalase inhibition.

2. Experimental

2.1. Reagents

Horseradish peroxidase (HRP, EC1.11.1.7, 222 U mg^{-1}) and catalase (EC 1.11.1.6, from bovine liver, 1600 U mg^{-1}solid) were obtained from Sigma-Aldrich. Layered double hydroxides (LDH) were synthesized by coprecipitation method [12]. Water used to prepare solutions was boiled–deionized–distilled water. HRP and catalase were dissolved in water (2 mg/ml). LDH were dispersed in water (2 mg/ml) and stirred at least overnight. All other reagents used were of analytical grade. Phosphate buffer solutions (PBS, pH 7.5) were prepared every three days. The hydrogen peroxide and inhibitor solutions were prepared daily in PBS.

2.2. Apparatus and experimental procedure

Amperometric measurements were performed with a Tacussel PRG-DEL potenstiostat equipped with a XY/t recorder. All experiments were carried out with a conventional three-electrode system including an Ag/AgCl reference electrode, a platinum auxiliary electrode and a working electrode. Rotating (500 rpm) platinum electrode (diameter=5 mm) or glassy carbon electrode (diameter=5 mm) were employed as working electrodes. Before use the working electrodes were polished with 1 μm diamond paste and rinsed with ethanol, acetone and distilled water. All measurements were accomplished in a thermostated 10 ml cell at 30 °C in 0.1 M phosphate buffer solution (pH 7.5).

2.3. Working electrode preparation

Pt electrode was modified with defined amounts of the catalase/[ZnAlCl] aqueous mixture. Carbon electrodes were coated first with a HRP/[ZnCrABTS] film, then covered by several layers of catalase/[ZnAlCl] exhibiting different enzyme/clay ratios. A drop (10–20 μl) of enzyme/LDH solution was spread on the electrode surface and dried in air at room temperature. This procedure was repeated in order to obtain the clay–enzyme amount. The modified electrodes were stored at 4 °C overnight for complete drying. Before use, the modified electrodes were exposed 15 min to saturated glutaraldehyde vapor (25%) in order to cross-link the entrapped molecules. Then the latter was soaked into 0.1 M PBS for 20 min under stirring to rehydrate the biofilm and remove glutaraldehyde traces.

2.4. Enzymatic activity measurements

Free enzyme activity of catalase and HRP were assayed spectrophotometrically in a 3 ml quartz cuvette by recording the variation of absorbance with time in the presence of enzyme substrate under stirring at 30 °C. All solutions were prepared in 0.1 M PBS (pH=7.5). Catalase activity was determined by mixing 1.9 ml PBS, 1 ml (0.059 M) H_2O_2 and 0.1 ml (20 μg/ml) catalase. The decomposition of H_2O_2 was followed at 230 nm. HRP activity was examined by using *o*-tolidine as reagent and H_2O_2 as substrate. The dye oxidation was measured by following at 403 nm, the absorbance of the mixture composed of 1 ml (0.2 M) H_2O_2, 2 ml (0.3 mM) *o*-tolidine and 0.050 ml (200 μg/ml) HRP. The inhibitive effect on enzymatic activity was determined by the measurement of the enzyme activity recorded under the same condition but in the presence of 1 mM of inhibitor.

3. Results and discussion

3.1. H_2O_2-purging effect at catalase electrode

The immobilization of catalase in the outer layer was dedicated to the total consumption of H_2O_2, to suppress the amperometric signal due to the electrically wired HRP. The effect of the immobilized amount of catalase on the H_2O_2 concentration was therefore investigated by depositing different

Table 1
Influence of catalase amount on the H_2O_2 sensitivity

Electrode (E_{app})	Catalase/[ZnAlCl]		BSA/[ZnAlCl]	
	Amount μg/μg	Sensitivity $mAM^{-1}cm^{-2}$	Amount μg/μg	Sensitivity $mAM^{-1}cm^{-2}$
Pt (0.2 V)	20/20	143	20/20	166
	40/40	55	40/40	172
	60/60	27	60/60	125
HRP/[ZnCrABTS]/ GC (0.0 V)	60/60	49	60/60	212

enzyme/LDH coatings on a platinum electrode and monitoring the H_2O_2 oxidation at+0.25 V. This applied potential was chosen to prevent the concomitant oxidation of nitrite with H_2O_2 [13].

As previously reported for the fabrication of enzyme electrodes using [ZnAlCl] LDH as immobilization matrix [3], the ratio between enzyme and LDH into the coating was fixed at 1/1 since this value corresponded to the optimum composition. The increase of the amount and hence of the coating thickness of catalase/LDH may decrease the current signal because of the efficient enzymatic substrate-purging effect but it could also be due to the hindrance of the substrate diffusion through the biofilm. To distinguish between these two effects, catalase was replaced by the identical amount of an inactive protein (BSA) to elaborate control electrodes. Current responses of both electrodes to H_2O_2 determination are compared in Table 1. As expected, the increase in catalase layers induced a decrease in H_2O_2 sensitivity. In contrast, the sensitivities determined with BSA coatings remained similar for one or two layers while a slight decrease only appeared with the third layer. For the thicker BSA/LDH coating, corresponding to three layers (60/60 μg), the current decreased (25%) illustrates the diffusion hindrance of H_2O_2.

To be sure that the H_2O_2-purging effect would act on the bi-enzyme biosensor, the same comparative experiment has also been carried out by using a glassy carbon electrode modified with a HRP/[ZnCrABTS] coating as inner sensing layer and catalase or BSA /[ZnAlCl] as the outer layer. These outer layers corresponded to the 60/60 μg biomolecule/LDH ratio (Table 1). Concerning the amperometric sensing layer, we had already proved that the biosensor modified with 40 μg HRP and 20 μg [ZnCrABTS] exhibited the best performance for H_2O_2 detection [5]. Since the biosensor configuration required the formation of two enzyme layers increasing thus the total deposited amount of LDH, the H_2O_2 sensing layer was reduced by half, namely 20 μg HRP and 10 μg [ZnCrABTS]. In this case, the electrochemical transduction corresponded to the reduction at 0.0 V of $ABTS^{+\cdot}$ that was formed by HRP in the presence of H_2O_2 [4,5]. It was clear that the same amount of catalase played almost the same role in both biosensor configurations and the same H_2O_2-purging effect occurred on both Pt electrode and HRP modified carbon electrode (Table 1).

3.2. Nitrite inhibition

As mentioned in the Introduction, the bi-enzyme electrode was applied for nitrite determination as an analytical model to evaluate the functioning principle of this new concept of inhibitor-based biosensor. As previously reported by Cohen et al. [14] and Titov and Petrenko [15], micromolar nitrite concentrations induced a considerable decrease in catalase activity, nitrite acting as a competitive inhibitor. The specific catalase inhibition by nitrite was first determined by UV–Vis spectroscopy. The presence of 1 mM $NaNO_2$ in the enzyme solution (0.66 μg/ml) caused a decrease in catalase activity of 30% whereas nitrite had no effect on HRP activity even increasing the concentration ten times. From these results on enzymatic activity, it appears that nitrite satisfied the particular requirement for this bi-enzyme device.

The inhibition effect of nitrite was also tested amperometrically at 0.0 V with the bi-enzyme configuration (60/60 μg catalase/LDH), the H_2O_2 concentration being fixed at 10 μM. As expected, the addition of nitrite induced an increase in the current intensity related to the enzymatic reduction of H_2O_2. Moreover, it should be mentioned that this biosensor presented a rapid and stable response to nitrite within 15–30 s, depending on the nitrite concentration range. Fig. 2A shows the resulting calibration curve for nitrite obtained with this bi-enzyme biosensor illustrating thus the validity of the concept.

In order to determine the best coating configuration for the inhibitor determination, the effect of outer layer composition has been studied. The inner sensing layer of HRP/[ZnCrABTS] was fixed at the same composition (20:10 μg) as previously used. Indeed, the response to nitrite was not enhanced when the coating amount of the inner layer was increased twofold, namely nitrite sensitivity of 21 and 20 μA M^{-1} cm^{-2} for 20/10 μg and 40/20 μg HRP/[ZnCrABTS], respectively.

Fig. 2 shows the amperometric calibration curves of four biosensors exhibiting different catalase and LDH loadings. It clearly appears that the biosensor performance, especially the linear range, was markedly affected by the total amount of deposited enzyme/LDH coatings exhibiting the same ratio in mass (1:1). In order to compare these biosensor configurations, the sensitivities were measured over the same nitrite concentration range (Table 2). The nitrite sensitivity increased with the decrease in mass coating from 60/60 to 40/40 reflecting a

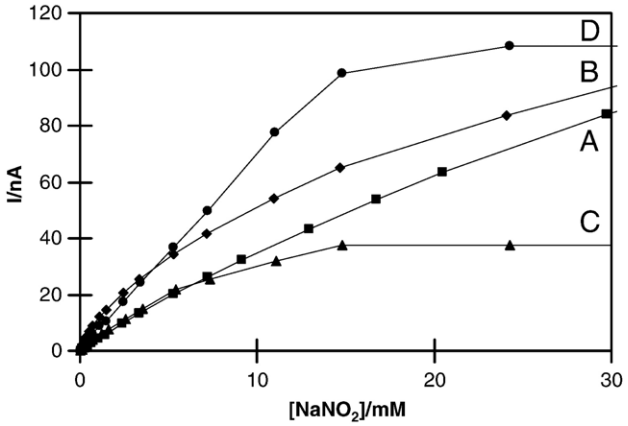

Fig. 2. Nitrite calibration curves at different configurations of bi-enzyme biosensors. Catalase/[ZnAlCl] amount. A) 60/60, B) 40/40, C) 20/20, D) 40/20 μg (10 μM H_2O_2 in PBS pH=7.5, E_{app}=0.0 V, 30 °C).

Table 2
Effect of bi-enzyme biosensor configuration on nitrite detection

Configuration	Catalase/[ZnAlCl] amount	Sensitivity [a]	Detection limit
	μg/μg	$\mu AM^{-1}cm^{-2}$	μM
A	60/60	21	40
B	40/40	68	10
C	20/20	34	20
D	40/20	46	20

[a] Sensitivities calculated in the concentration range $c \leq 0.5$ mM (30 °C, E_{app}=0.0 V).

decrease in the permeation length for H_2O_2 to reach the inner sensing layer. However, the subsequent decrease in deposited amount from 40/40 to 20/20 induced a decrease in sensitivity from 68 to 34 μA M^{-1} cm^{-2}. The beneficial effect due to the decrease in steric hindrance towards the H_2O_2 permeation was counterbalanced by the decrease in the amount of immobilized catalase. This was clearly indicated by the weak maximum current (37.5 nA) recorded for fully inhibited catalase with configuration C (Fig. 2C). Taking into account the role of catalase in the intensity of the maximum current, the deposited amount of catalase was increased from 20 to 40 μg while the LDH coating remained the same (20 μg). The comparison of configurations C (20/20) and D (40/20) clearly indicates a drastic increase in maximum current and nitrite sensitivity, corroborating thus the effect of catalase. In addition, the comparison of configurations B (40/40) and D (40/20) reflects the additional steric hindrance towards the permeation of nitrite to the sensing peroxidase layer due to the increase in clay coating from 20 to 40 μg.

Since the configuration D presented the broader linear range (up to 4 mM) (Fig. 2D), this biosensor configuration was employed to detect eight replicate standard additions of 0.25 mM nitrite. The measurement was repeated with the same electrode after a washing step in stirred phosphate buffer solution for 1 h. The relative standard deviation (RDS) of the nitrite sensitivity was 1.5%, illustrating the good reproducibility of the biosensor response associated with the reversibility of the inhibition process. The storage stability was also tested with the same electrode. The sensitivity decreased to 13% after 12 h at 4 °C. Finally, the reproducibility of the biosensor fabrication was tested with four different electrodes leading to a RDS value for the nitrite sensitivity of 4%.

Although the functioning principle of this bi-enzyme concept for nitrite determination was clearly demonstrated, the performance in term of detection limit remains weak, even for the most sensitive configuration, namely 10 μM with configuration B (Table 3). This value is higher than those reported in the literature for other nitrite biosensors (Table 4).

Table 3
Effect of temperature on nitrite detection with biosensor configuration B

Temperature	Sensitivity[a]	Detection limit	I_{max}
°C	$\mu AM^{-1}cm^{-2}$	μM	$\mu A/cm^2$
30	68	10	0.50
25	68	10	0.38
20	102	4	0.26
15	59	40	0.20

[a] Sensitivities calculated in the concentration range c=0.5 mM.

Table 4
Comparison of biosensor response for nitrite detection

Electrode type	Biological catalyst	E_{app}	Linear range	Detection limit	Reference
		(V)	μM	μM	
Platinum	Denitrifying bacteria	+0.100	4–14	4	[19]
Gold	Horse heart Mb	−1.00	8–216	0.8	[20]
Gold	NiR	−0.150	0.4–22	0.4	[11]
Glassy carbon	NiR	−0.700	5–43	5	[10]
Glassy carbon	HRP, catalase	0.00	4–50	4	This paper

Since the biosensor concept was based on two enzymes exhibiting different temperature profiles, the effect of temperature on the sensor performance was investigated between 15 and 30 ° C. Fig. 3 exhibits the different calibration curves for nitrite as a function of temperature. The best sensitivity and detection limit values were determined at 20 °C, namely 102 μAM^{-1} cm^{-2} and 4 μM (Table 3). Although most enzyme catalysed reactions exhibited a doubling of their activity for every rise of 10°C designated by a Q(10) of 2, the temperature change has a slight effect on catalase activity which displays a Q (10) of 1.15 for the enzymatic breakdown of H_2O_2 [16]. Consequently, the catalase activity should increase less rapidly than the peroxidase activity providing complex temperature dependence for the biosensor response. It should be noted that the maximum current increased with the increase in temperature. These phenomena may be ascribed to two concomitant effects. As expected, the decrease of the temperature induced a decrease in the enzyme activities that can be related to the lowering of I_{max}. Simultaneously, a decrease of temperature would facilitate the accumulation of nitrite within LDH matrix. As previously described with cyanide and arsenate, the accumulation of anionic inhibitors into the LDH layer, which

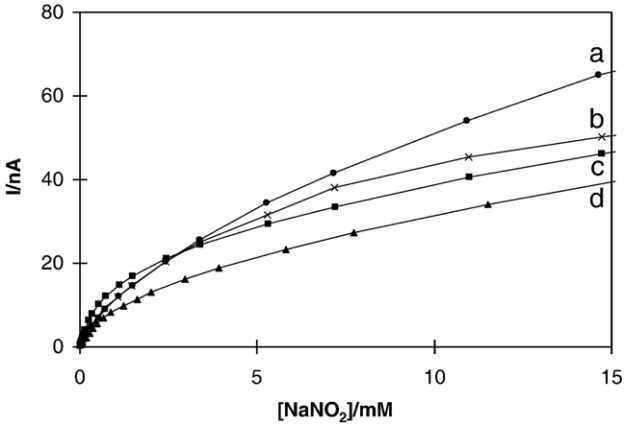

Fig. 3. Effect of temperature on nitrite calibration curves, a) 30 °C, b) 25 °C, c) 20 °C, d) 15 °C (40/40 μg catalase/[ZnAlCl], 10 μM H_2O_2 in PBS pH=7.5, E_{app}=0.0 V).

is an anionic exchanger matrix, led to a drastic enhancement of the detection limit values [17,18]. This phenomenon was more noticeable with the configuration B containing a higher amount of LDH (40 μg) than with configuration D composed with only 20 μg LDH. This increase of the LDH amount may facilitate the possible accumulation of nitrite providing thus a more sensitive detection limit.

Table 4 summarizes the present biosensor performance and those related to other nitrite biosensors. The most sensitive biosensors exhibited detection limits ranging from 0.4 to 0.8 μM [11] while other sensors presented similar analytical performance than our biosensor. However, it should be noted that the device developed by Lars Hauer Larsen et al. was rather complex bioanalytical system. The latter was composed of a biodegradation chamber for denitrifying bacteria transferring nitrite to nitrous oxide and a inside nitrous oxide microsensor to produce electrochemical signals [19]. Its main limitation was the self-reproduction of bacteria that limited the period of continuous monitoring to five days. Except this "bacterial sensor", all devices, contrarily to our biosensor, operated at negative potentials and hence require anaerobic condition to function. This constitutes a major drawback for the development of portable nitrite biosensors.

4. Conclusions

We describe herein in connection with catalase and peroxidase (HRP), an unusual enzyme combination for the determination of nitrite at low operating potential (0.0 V) in the presence of oxygen. It is expected that such original and promising concept of inhibitor-based biosensors based on the suppression of a biosensor response by enzymatic consumption and its reactivation by inhibitive effects, will be useful for the development of environmental biosensors.

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