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Cooperative use of cytochrome cd_1 nitrite reductase and its redox partner cytochrome c_{552} to improve the selectivity of nitrite biosensing

A.S. Serra[a], S.R. Jorge[a], C.M. Silveira[a], J.J.G. Moura[a], E. Jubete[b], E. Ochoteco[b], G. Cabañero[b], H. Grande[b], M.G. Almeida[a,c,*]

[a] REQUIMTE – Dept. de Química, CQFB, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[b] CIDETEC – Centro de Tecnologías Electroquímicas, Parque Tecnológico de San Sebastián, P° Miramon, 196, 20009 Donostia – San Sebastián, Spain
[c] Escola Superior de Saúde Egas Moniz, Monte de Caparica, 2829-511 Caparica, Portugal

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ABSTRACT

In this work, a novel enzymatic biosensor for determination of nitrites constructed on an electrochemical transducing platform is proposed. The sensor is based on cytochrome-cd_1 (cyt-cd_1) nitrite reductase from *Marinobacter hydrocarbonoclasticus* strain 617 as biological recognition element, and its putative physiological redox partner cytochrome-c_{552} (cyt-c_{552}), as electron mediator. The proteins were co-immobilized using a photopolymerizable polyvinyl alcohol (PVA) derivative, onto carbon paste screen printed electrodes (CPSPEs); the optimal modification conditions were 100 μM cyt-cd_1/100 μM cyt-c_{552} and 50% PVA, after a 48 h polymerization time. Electrochemical characterization of the mediator was carried out by cyclic voltammetry. The one-electron exchange between cyt-c_{552} and the working electrode is a quasi-reversible process, without mass transport limitations. The formal potential of the mediator is 254 ± 2 mV *vs* NHE and the intermolecular electron transfer rate constant between cytochromes c_{552} and cd_1 is 9.9×10^3 M^{-1} s^{-1}. The analytical parameters of the biosensor response to nitrite as assessed by amperometric measurements were: linear range from 10 to 200 μM; detection and quantification limits of 7 and 24 μM, respectively; sensitivity of 2.49 ± 0.08 A mol^{-1} cm^2 μM^{-1}. Catalytic profiles in the presence of possible interfering species were also investigated. The interference from competitive enzymatic reduction of dissolved oxygen could be overcome by tuning the cyclic voltammograms for faster sweep rates.

1. Introduction

The physiological role of inorganic nitrites (NO_2^-) has been the subject of high controversy in the past few years. On one hand, the excessive ingestion of nitrates and nitrites via the dietary intake has potential toxic [1,2] and carcinogenic effects [3,4]. On the other hand, it has been reported that nitrite has a protective role against ischemia/reperfusion injury [5–7] and was recently recognized as an endogenous signaling species with important roles in mammalian physiology [8]. In both cases, nitrite quantification is of primordial importance for diagnosis and/or prognosis purposes. Public health security also depends on nitrites monitoring in drinking waters and food products, as regulated by the European directives 98/83/EC and 2006/52/EC, respectively.

As nitrite quantification became more important, new methodologies were developed. Most of these methods are based on sophisticated chromatographic or spectrophotometric techniques which require the use of expensive equipment operated by trained individuals and a time consuming sample preparation [9–13]. For these reasons, the application of biosensor technology for nitrite quantification is highly appealing [14]. Numerous examples of nitrite biosensors can be found in the literature, either using unspecific proteins such as hemoglobin [15], myoglobin [16,17] and cytochrome *c* [18] as biological recognition elements, or employing selective nitrite reducing enzymes, namely the multihemic cytochrome *c* nitrite reductase (*cc*NiR) [19–23], copper containing nitrite reductase (CuNiR) [24,25] and cytochrome-cd_1 nitrite reductase (cyt-cd_1 NiR) [26–29].In this work, a novel enzymatic biosensor with electrochemical transduction is presented for the determination of nitrites. This device is based on cytochrome-cd_1 (cyt-cd_1) nitrite reductase from the denitrifying bacterium *Marinobacter hydrocarbonoclasticus* strain 617 as biological recognition element. This protein is a soluble homodimer of 60 kDa whose physiological role is the one-electron reduction of nitrite (NO_2^-)

* Corresponding author at: REQUIMTE – Dept. de Química, CQFB, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica Portugal. Tel.: +351 212948550; fax: +351 212948300.
E-mail address: mga@dq.fct.unl.pt (M.G. Almeida).

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to nitric oxide (NO) (Eq. (1)) during bacterial denitrification [30].

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (1)$$

Each monomer comprises two distinct domains, one for electron transfer (ET) from donor proteins via a *c*-type heme, the other for catalysis at the active site, a d_1 heme, which is unique to this class of enzymes [31,32].

Commonly, redox mediators employed in amperometric biosensors are small synthetic molecules with no specific affinity towards the biological element. Their main advantages rely on facilitating ET and reducing the working potential [33]. In the particular case of nitrite detection, Strehlitz et al. [28] have tested several artificial electron carriers for heme containing nitrite reductases, including phenosafranin, methyl viologen and gallocyanine. Nevertheless, artificial mediators are frequently hazardous and rather general catalysts thereby affecting the selectivity of the sensor [34]. As a contrast to that, in this work, cyt-c_{552} is explored as electron mediator. This small homodimeric (20 kDa) heme protein is the putative physiological partner of cyt-cd_1 [35] and, therefore, is highly selective towards it. Furthermore, cyt-c_{552} gives a stable electrochemical response, in a low potential range (265 ± 7 mV *vs* NHE, at modified gold electrodes, pH 6.3) [35], attributed to a reversible one electron exchange, which makes it suitable for mediating purposes. Aside from monitoring the activity of cyt-cd_1 and providing a second selectivity element for the biosensor, the employment of cyt-c_{552} also reduces the risks of unspecific reductions that could arise from measurements at extreme potential values [22,36]. The efficient entrapment of two proteins with distinct molecular weights is a challenging task. This was accomplished by using a photopolymerizable polyvinyl alcohol (PVA) derivative, which provided a suitable environment maintaining the proteins simultaneously active and accessible.

The screen-printing (thick-film) technique is widely used for large-scale fabrication of disposable biosensors with several advantages including low cost, versatility, and miniaturization [38,39]. In this work, the biosensing layer was assembled onto carbon paste screen printed electrodes, increasing the chances of direct implementation of this laboratory-developed device in real life applications.

In line with our own strategy, Tepper has reported a recent work [37] where the electrochemical contact of a CuNiR was established via its natural redox partner, pseudoazurin, by conjugating the two proteins to a specific DNA tag, which was tethered to a gold electrode. In spite of not being an analytical application, the concept presented on this paper confirms a potential new trend in the design of electrochemical biosensors.

2. Materials and methods

2.1. Reagents

All chemicals were analytical grade and used without further purification. Solutions were prepared with deionized (DI) water (18 MΩ cm) from a Millipore MilliQ water purification system. Polyvinyl alcohol with azide pendant groups (PVA-AWP) was acquired from Toyo Gosei, Japan. MES sodium salt and sulphuric acid were obtained from Sigma. Sodium nitrite and potassium chloride were purchased from Merck. Alumina slurry 0.3 μm came from Buehler.

Cyt-cd_1 (375 μM, in 0.05 M Tris–HCl buffer, pH 7.6) and cyt-c_{552} (705 μM, in 0.1 M Tris–HCl buffer, pH 7.6) were purified from *M. hydrocarbonoclasticus* 617, as previously described [30], and stored at −20 °C.

2.2. Electrochemical measurements

For the optimization of the protein mixture with PVA, a conventional three-electrode electrochemical cell was used, with a Ag/AgCl reference electrode (197 mV *vs* NHE), a platinum counter electrode (both from Radiometer) and a self-made pyrolytic graphite electrode (PGE) (Ø = 4 mm) modified with the enzyme/mediator/PVA film, as working electrode. The analytical characterization of the biosensor was carried out by replacing the previous system by carbon paste screen printed electrodes (CPSPEs), following a three electrode configuration, with a graphite paste working electrode (Ø = 3.1 mm), a Ag/AgCl pseudo-reference (302 mV *vs* NHE) and a graphite paste counter electrode, prepared at CIDETEC as previously described [40]. Measurements were performed with a potentiostat Autolab PSTAT 12 (Eco-Chemie) monitored by the control and data acquisition software GPES 4.9. Unless stated otherwise, cyclic voltammograms were traced at a scan rate of 50 mV s^{-1}, at room temperature (23 ± 2 °C), using 0.15 M KCl in 0.05 M MES buffer, pH 6.3, as supporting electrolyte; before each experiment the electrochemical cell was thoroughly purged with Argon. All quoted potentials were converted to the to the NHE reference. For amperometric experiments, modified CPSPEs were poised at 0.2 V (*vs* NHE) and the electrolyte solution was magnetic stirred at the highest possible speed (with no perceptible turbulence), under an argon atmosphere. To evaluate the biosensors response to nitrite, the electrochemical cell was successively spiked with a standard solution.

2.3. Bioelectrodes preparation

Prior to coating, the PGEs were polished with alumina slurry. The electrodes were then thoroughly washed with DI water and ethanol and ultrasonicated for 5 min. Then, the electrodes were washed again with DI water and dried with compressed air. Modifying mixtures were prepared by combining different ratios of water, PVA (25%, 50% and 75%), cyt-cd_1 (15, 50 and 100 μM) and cyt-c_{552} (100, 150 and 200 μM) to a final volume of 25 μL. A drop of 3 μL of the mixture was then placed onto the PGE and left to photopolymerize at 4 °C, under the beam of a 20 W halogen lamp during 24, 48 and 72 h. Before use, the modified electrodes were immersed in supporting electrolyte for 30 min at room temperature, to allow matrix hydration.

Prior to coating, the CPSPEs were activated by applying a 1.2 V potential during 120 s, in 0.1 M H_2SO_4. The electrodes were then washed with DI water, air dried, and modified with the optimal formulation selected from the previous study done with PGEs.

3. Results and discussion

3.1. System optimization on modified pyrolytic graphite electrodes

3.1.1. Enzyme/Mediator ratio

The optimum composition was selected from a group of cyt-c_{552}/cyt-cd_1 ratios, prepared with a PVA percentage of 50% and polymerization time of 48 h. The bioelectrodes were characterized by monitoring the mediator's current-potential curves, through cyclic voltammetry, as illustrated in Fig. 1. The voltammetric wave observed between 0.1 V and 0.3 V was assigned to the heterogeneous one electron reduction undergone by cyt-c_{552}.

In the presence of nitrite (Fig. 1), the enzymatic reduction of the substrate was observed and quantified by the changes on the catalytic currents, characteristic from a reversible electrochemical process followed by an irreversible chemical reaction, known as an EC' mechanism (as depicted in Fig. 2, full line arrows).

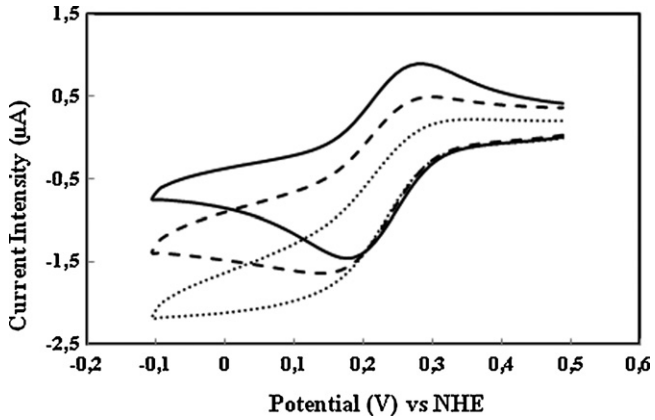

Fig. 1. Cyclic voltammograms of cyt-c_{552} (100 μM), as co-entrapped with cyt-cd_1 (100 μM), in a 50% PVA matrix (polymerization time, 48 h), on a PGE. Electrolyte: 150 mM KCl, 50 mM MES buffer, pH 6.3. Scan-rate: 50 mV s^{-1}. Nitrite concentration: 0 mM (—) 125 mM (- - -); 978 mM (…).

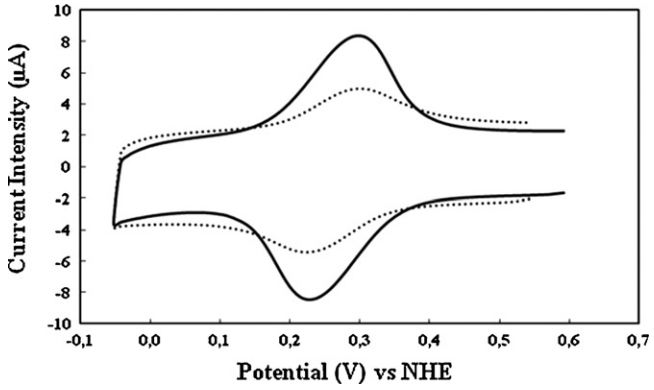

Fig. 3. Cyclic voltammograms of cyt-c_{552} (100 μM) either alone (—) or in the presence of 100 μM cyt-cd_1 (…). Proteins were entrapped in a 50% PVA film (photopolymerization for 48 h) fixed on a CPSPE. Electrolyte: 150 mM KCl, 50 mM MES buffer, pH 6.3. Scan-rate: 50 mV s^{-1}.

In control experiments using PVA modified electrodes without cyt-c_{552}, no electrochemical signals could be detected, whereas without cyt-cd_1, no catalytic reaction was observed upon substrate addition (not shown). The biosensor response to nitrite was expressed in terms of the variation of the catalytic peak, divided by the electrode coverage (Γ). In this manner it was possible to eliminate divergences due to slight differences among electrode preparations. Besides, by introducing the coverage factor, the biosensor sensitivity is translated not only by the total current (A) generated by a certain substrate concentration (μM) per unit of electrode area (cm^2), but also considers the real quantity of cyt-c_{552} (mol) that is responding on the electroactive surface of each electrode. The values for the apparent Michaelis constant (K_M^{app}) were assessed by data fittings to the Michaelis–Menten kinetic model.

Experiments performed with 15 μM cyt-cd_1 and different mediator concentrations did not display measurable catalytic currents, clearly due to an insufficient amount of enzyme. On the other hand, in the tests carried out with the highest concentration of cyt-c_{552} (200 μM) and varying concentrations of the biocatalyst, the electrochemical signal of the redox mediator decreased continuously over time, without reaching a stable cyclic voltammogram in a acceptable time interval (not shown), as required to proceed with the bioelectrode calibration; this depletion effect clearly indicates the existence of an excessive amount of the small electron carrier protein. Table 1 summarizes the results obtained for the potentially useful mediator/enzyme ratios. The analytical performance was evaluated according to the balance between sensitivity to nitrite and the amplitude of the linear range.

In this way, the optimal enzyme/mediator proportion was determined to be 100 μM cyt-cd_1/100 μM cyt-c_{552}.

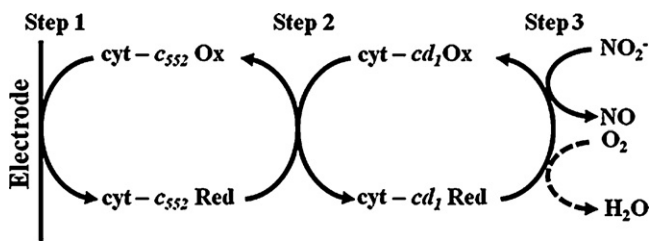

Fig. 2. Bioelectrode's reactions schemes of the one electron reduction of nitrite to nitrous oxide (—) and four electron reduction of molecular oxygen to water (…), according to a EC' mechanism.

3.1.2. PVA percentage and polymerization time

Once determined the optimal amount of both enzyme and mediator, the parameters affecting the preparation of the polymeric matrix were investigated. The PVA percentage in the mixture was altered from 50% to 25% and 75% (polymerization for 48 h), and the polymerization times were changed from 48 h to 24 h and 72 h (while fixing the PVA amount in 50%). In all cases, the device performance was negatively affected (results not shown). The electrodes modified with 25% PVA or polymerized for only 24 h displayed the worst analytical behaviour, proving to be insufficiently cross-linked for protein entrapment. At the other extreme, the high thickness of the matrices produced with 75% PVA or a 72 h polymerization time was probably responsible for the poor efficiency of the resulting bioelectrodes, preventing the proper contact among the components involved in the electron carrier system (electrode/mediator; mediator/enzyme; enzyme/substrate).

3.2. Application to carbon paste screen printed electrodes (CPSPEs)

3.2.1. Electrochemical characterization

Fig. 3 shows the cyclic voltammograms of cyt-c_{552} (100 μM) either alone or in the presence of cyt-cd_1 (100 μM), entrapped in a 50% PVA film (photopolymerization for 48 h) deposited on a CPSPE. The position of the redox waves associated to cyt-c_{552} did not change much at the thick film electrodes; though the capacitive currents are more pronounced due to an increase in the active surface area. The current intensity drops significantly in the presence of the enzyme molecules, which should partially hinder the interaction of cyt-c_{552} with the electrode surface.

The analysis of the cyt-c_{552} cyclic voltammograms traced at different scan rates (Fig. 4A) indicates that the heterogeneous ET is a quasi-reversible reaction, with peak separation values close to zero and peak intensity ratios near one. The formal potential E° was estimated to be 254 ± 2 mV *vs* NHE, which is similar to the one reported for the same protein, in solution [35].

The linear dependence of the cathodic peak currents with the scan rate (Fig. 4B) is consistent with a diffusionless process. By analysing the sensor response to a saturating concentration of substrate (2 mM), one could determine the second-order rate constant for the ET reaction between cytochromes c_{552} and cd_1. The value obtained (9.9×10^3 M^{-1} s^{-1}) is 1–2 orders of magnitude lower than the one reported in solution for the same pair of proteins (4.1×10^5 M^{-1} s^{-1}). This reduction of the intermolecular ET rate is certainly a consequence of the PVA matrix, either through diminishing the efficiency of the protein–protein

Table 1
Analytical and kinetic properties of PVA/cyt-c_{552}/cyt-cd_1 based nitrite biosensors, prepared with 50% PVA and 48 h polymerization time, in different enzyme/mediator ratios.

Parameters	Cytochrome cd_1 (μM)			Cyt-c_{552} (μM)
	50	75	100	
K_M^{app} (μM)	116 ± 13	217 ± 23	370 ± 39	50
$([\Delta I]/\Gamma)_{max}$ (A mol^{-1} cm^2)	516 ± 15	1817 ± 66	2309 ± 104	
Linear range (μM)	1–32	8–210	16–501	
Sensitivity (A mol^{-1} cm^2 μM^{-1})	3.2 ± 0.1	3.4 ± 0.1	2.32 ± 0.07	
K_M^{app} (μM)	95 ± 8	149 ± 8	493 ± 27	100
$([\mu I]/\Gamma)_{max}$ (A mol^{-1} cm^2)	655 ± 13	556 ± 9	2034 ± 53	
Linear range (μM)	16–167	8–167	1–125	
Sensitivity (A mol^{-1} cm^2 μM^{-1})	1.8 ± 0.1	1.53 ± 0.07	3.6 ± 0.1	
K_M^{app} (μM)	111 ± 5	358 ± 38	305 ± 19	150
$([\mu I]/\Gamma)_{max}$ (A mol^{-1} cm^2)	492 ± 6	1517 ± 64	1431 ± 36	
Linear range (μM)	16–167	4–125	1–63	
Sensitivity (A mol^{-1} cm^2 μM^{-1})	1.39 ± 0.07	2.8 ± 0.1	4.5 ± 0.2	

interactions or by inducing some protein conformational changes.

3.2.2. Response to nitrite

The amperometric measurements for the bioelectrode calibration were carried out at 200 mV (*vs* NHE). The response upon nitrite addition reached a steady current in 50–100 s intervals, with highly stable baselines and a small background noise (Fig. 5A).

The biosensor's response to the enzyme substrate was linear from 10 to 200 μM (Fig. 5B). The detection limit (LOD) determined as the ratio between three times the standard error of the interception and the interception, was 7 μM. The quantification limit (LOQ), calculated from the calibration curve as the ratio between ten times the standard error of the interception and the interception, was 24 μM. The sensitivity, given by the slope of the calibration curve (Fig. 5B, inset), was 2.49 ± 0.08 A mol^{-1} cm^2 μM^{-1}, and K_M^{app} was 0.53 ± 0.01 mM. In comparison with pyrolytic graphite, carbon paste is a less pure, organized and conductive material, so the strong decrease in the biosensor's sensitivity observed by swapping the working electrode material is not surprising.

3.2.3. Temperature and pH effects

The operational performance of the biosensor was evaluated in terms of temperature and pH dependences. Calibration curves obtained in a temperature range from 10 °C to 30 °C indicate that among 15 °C and 25 °C, the sensitivity variation is less than 5% (Fig. 6A).

These results show that the bioelectrode response is very robust regarding temperature variations. Lopes et al. reported that the intermolecular electronic transfer between cyt-c_{552} and cyt-cd_1 could only be observed below pH 8.0 [35]. Hence, the biosensor activity was tested for pH values of 6, 7 and 8 in the same manner as previously described for temperature. As expected, the sensitivity has decreased about 30% from pH 6.0 to pH 7.0 (Fig. 6B) and no electrocatalytic response was observed at pH 8.0.

3.2.4. Interferences

The biosensor's response to potential interferences was studied by comparing the magnitude of the catalytic response to 100 μM nitrite with the one obtained after adding different concentrations of a second chemical species. Nitrate and nitrite are simultaneously present in various samples of both physiological and environmental nature. For this reason, the possible interference of nitrate on the bioelectrode response was investigated; though, no activity to this ion was detected up to a concentration of 1 mM. The same behaviour was observed for sulphate. However, in the presence of 1 mM sulphite, the signal dropped 15% and, by adding 50 μM hydroxylamine (an *in vitro* alternative substrate of cyt-cd_1 [41]), the bioelectrode increased its response in 18%. Thus, the presence of these compounds in real samples should be carefully evaluated.

Another documented substrate of cyt-cd_1 is molecular oxygen [42,43]. In fact, a signal increase of 20% was observed in unpurged buffer solutions. However, a strategy was developed to overcome this situation as presented ahead.

3.3. Reaction with oxygen

As previously mentioned, the amperometric measurements were vulnerable to interferences from the presence of O_2 in the medium. This is a consequence of the ability of cyt-cd_1 to catalyse,

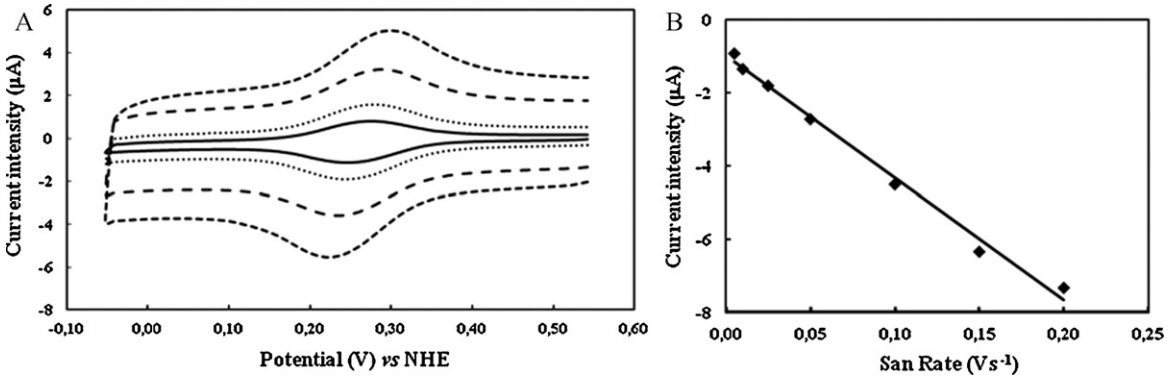

Fig. 4. Scan-rate dependence of the electrochemical response of cyt-c_{552} (100 μM) as co-entrapped with cyt-cd_1 (100 μM), in a 50% PVA matrix (polymerization time, 48 h), on a CPSPE. Electrolyte: 150 mM KCl, 50 mM MES buffer, pH 6.3. (A) Cyclic voltammograms recorded at 5 (—), 10 (…), 25 (- - -) and 50 (- - -) mV s^{-1}; (B) Linear relationship between the cathodic peak current and the scan rate ($y = -33.3x - 0.99$; $r^2 = 0.992$).

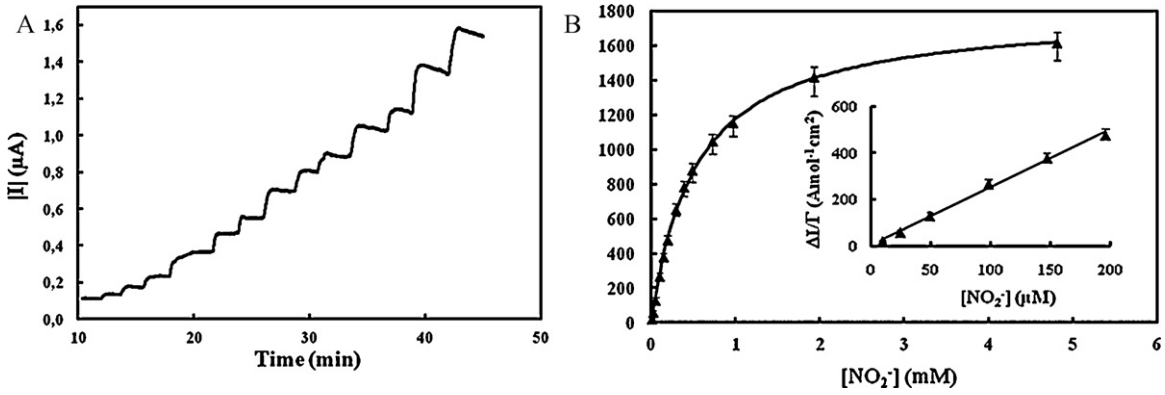

Fig. 5. Biosensor's response to nitrite at a CPSPE. (A) Representative amperomogram registered at 0.2 V *vs* NHE. (B) Data fit to a Michaelis–Menten equation, using the GraphPad Prism v4 software. Inset: calibration curve ($y = 2.49x + 3.74$; $r^2 = 0.996$). The results are expressed as the average value from three independent experiments.

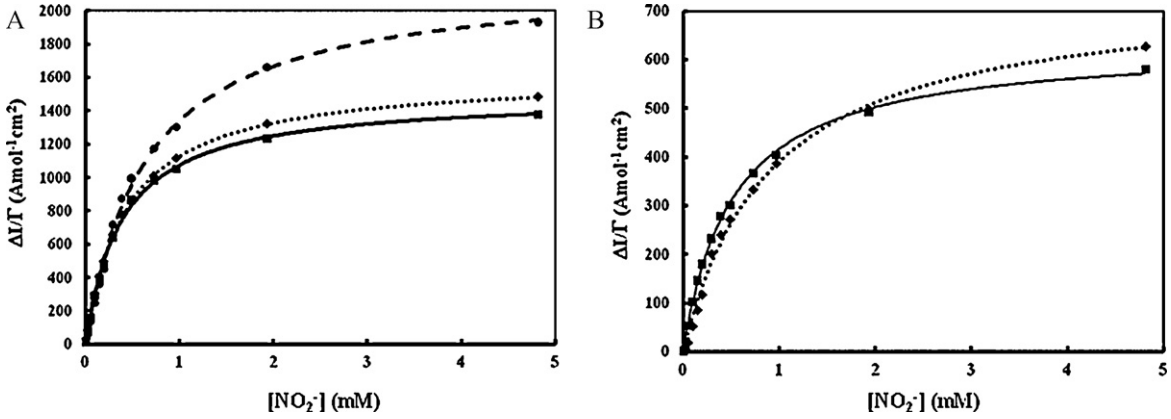

Fig. 6. Biosensor response in terms of nitrite sensitivity, at different temperature and pH conditions: (A) 15 °C (—), 20 °C (- - -) and 25 °C (…). (B) pH 6.0 (—) and pH 7.0 (…). Results were fitted to the Michaelis–Menten model using the GraphPad Prism v4 software.

in vitro, the four electron reduction of molecular oxygen to water (Eq. (2)).

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad (2)$$

Reaction schemes for the two competitive reactions are presented in Fig. 2, each one having distinct kinetic parameters. Although kinetic data for the reaction with O_2 is not available for the *M. hydrocarbonoclasticus* proteins, results obtained for cyt-cd_1 from the bacterium *Pseudomonas aeruginosa* and its corresponding physiological partner cytochrome c_{551} indicate that the turnover number for oxygen reduction [42] is approximately 4 times lower than the value obtained for the reaction with nitrite [44]. Thus, an increase of the scan rate could make the electrochemical reaction (step 1) too fast to allow the subsequent chemical reaction with O_2 (step 3). Indeed, by increasing the scan rate from 5 to 50 mV s^{-1}, it was possible to "switch off" this catalytic pathway. As shown in Fig. 7A, the cyclic voltammogram of cyt-c_{552} obtained at slow scan rates using an unpurged electrolyte solution without nitrite, has a distinctive sigmoid shape, symptomatic of electrocatalytic reduction of dissolved O_2.

However, a comparable voltammogram traced at 50 mV s^{-1} did not display such catalytic features and, in these experimental conditions, it was possible to plot a good calibration curve ($r^2 = 0.993$) for NO_2^-, with no O_2 interference; although the corresponding analytical parameters could not be directly compared to the ones obtained by amperometric measurements in deaerated solutions (Fig. 5), they are all in the same order of magnitude and generally similar (e.g., sensitivity = 1.6 A mol^{-1} cm^2 μM^{-1}; LOD = 1.1 μM).

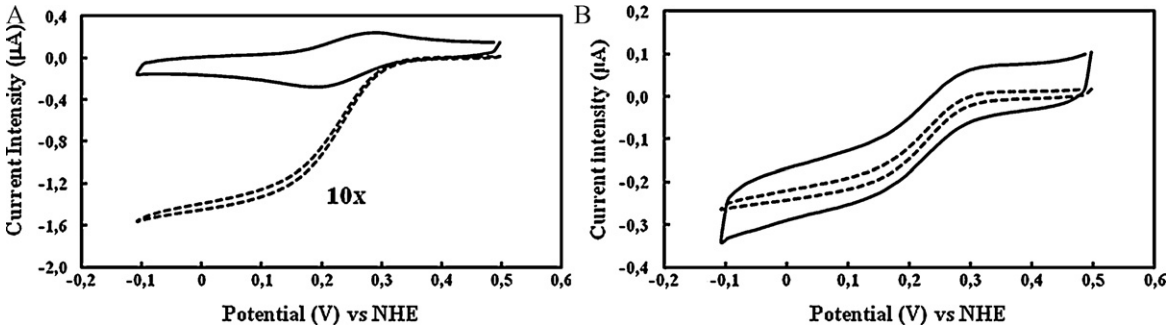

Fig. 7. Cyclic voltammograms of modified (100 μM cyt-c_{552}/100 μM cyt-cd_1 in a 50% PVA matrix; polymerization time, 48 h) CPSPEs in 150 mM KCl, 50 mM MES buffer, pH 6.3. (A) Unpurged supporting electrolyte solution, no nitrites; (B) Purged supporting electrolyte solution containing 1 mM of nitrites. Scan rate: 5 mV s^{-1}(- - -) and 50 mV s^{-1} (—).

As expected, the cyclic voltammogram of cyt-c_{552} obtained in the absence of cyt-cd_1, in a non-deoxygenated solution, without nitrite, did not display any electrocatalytic activity (not shown). Nonetheless, one should not rule out the potential contribution of mass transport of the two different enzyme substrates (NO_2^- and O_2) through the PVA film for the distinct behaviour.

It should be mentioned that, for this system, the strategy of tuning the scan rate is not applicable for the production of an oxygen sensor capable of working in the presence of nitrite. Actually, catalytic currents were always observed with saturating concentrations of NO_2^-, regardless the scan rate (Fig. 7B).

4. Conclusions

A biosensor for nitrite determination based on a modern concept was presented. For the first time, the use of an enzyme's physiological partner as the electronic mediator and its successful co-entrapment onto a single matrix, while maintaining the electron transfer activity, is reported. Cyt-c_{552} proved to be a particularly good mediator: the redox process is electrochemically quasi-reversible and takes place at a low potential, which in conjugation to the preferential electron transfer to cyt-cd_1 NiR, minimizes the risks of unspecific redox reactions. Very importantly, the interference from the enzyme response to oxygen may be overcome if signal transduction is obtained by cyclic voltammetry at moderate scan-rates.

It was also demonstrated that the modified photopolymerizable PVA matrix is well suited to this type of application, without requiring further electrode modifications or the use of expensive doping materials. By transferring the optimized conditions from PG working electrodes to carbon paste screen printed electrodes, we built up a device characterized by high operational simplicity, low cost fabrication and portability.

Although the sensitivity of the proposed sensor is lower than those from other reported systems, it is sufficient for applications in complex samples where high nitrite contents are expected.

Acknowledgments

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