

NITRITE BIOSENSING WITH DISPOSABLE ELECTRODE STRIPS

A Preliminary Study

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Abstract: This paper presents the results of a preliminary study on the construction of miniaturized biosensing devices for nitrite analysis in clinical samples. Following our previous works regarding the development of amperometric nitrite biosensors using the nitrite reducing enzyme (ccNiR) from *Desulfovibrio desulfuricans* ATCC 27774, now we aim to reduce the size of the experimental set-up according to the specific needs of biomedical applications. For this, thick-film strip electrodes made of carbon conductive inks deposited on plastic supports were modified with ccNiR previously mixed with the conductive graphite ink, in the presence of propanone or methylethylketone. Then, the enzyme electrodes were dried at 40°C or 60°C, to simulate the curing procedure typically done after screen-printing. In this way, the biocompatibility of ccNiR with these organic solvents and thermal treatments was evaluated and the composition of the mix enzyme/conductive ink was optimized. The analytical performance of these electrodes was satisfactory, with a sensitivity of 52 A.µM⁻¹.cm⁻² within a linear range of 0.001 - 1 mM.

1 INTRODUCTION

The detection of nitrites in physiological fluids such as plasma and urine is commonly used for clinical diagnosis and has gained an increasing importance in biomedical research. In fact, the nitrate-nitrite-NO pathway is emerging as an important mediator of blood flow regulation, cell signaling, energetics and tissue responses to hypoxia (Bryan, 2005; Lundberg, 2009; Hord, 2009). Most of the strategies used for analytical determination of NO₃⁻ and NO converge to the quantification of NO₂⁻. However, the classical protocols for nitrite assessment lack the sensitivity and selectivity needed for the analysis of physiological samples (Almeida, 2010). For example, urine test strips are routinely used for screening nitrites in patients with infection, but results are just qualitative as they are obtained by visual comparison to a color chart. Plasma analysis is much less frequent, owing to limitations of the

analytical methods, including blood sampling and processing (Ellis, 1998). As a consequence, there is a growing demand for improved analytical tools, increasingly sensitive, reliable and, preferentially, easy-to-use and inexpensive.

An alternative approach relies on the construction of biosensing devices using stable enzymes with high catalytic activity and specificity for nitrite. Due to its high selectivity, turnover and stability, the multihemic cytochrome *c* nitrite reductase (ccNiR) from the sulphate reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774, which performs the six electron reduction of nitrite to ammonia (Almeida, 2003), has proven to be a promising candidate for the development of an electrochemical nitrite biosensor (Almeida, 2007; Chen, 2007; da Silva, 2004; Silveira 2010a,b; Zhang, 2009).

Miniaturization is critical for both health care and physiological studies. The screen-printing technology has been widely used for the large-scale

fabrication of disposable biosensors. Besides the portable dimensions, screen-printed electrodes (SPEs) are low-cost and versatile in terms of formats and materials (Jubete, 2009).

In this work, thick-film strip electrodes were fabricated using carbon based conductive pastes printed on plastic supports. The working electrodes were later modified with an extra layer of the carbon ink, previously diluted with either propanone or methyl ethyl ketone (MEK) and mixed in different proportions with ccNiR. The activity of ccNiR towards nitrite after immobilization in this harsh environment (solvents exposure and heat dry) was evaluated by cyclic voltammetry, and has proved to be highly satisfactory.

2 MATERIALS AND METHODS

2.1 Reagents

Acetone (propanone; 99%; b.p. 56°C) and propanone (methylethylketone, MEK; 99%, b.p. 79°C) were from Pronalab. The remaining chemicals were analytical grade and were used without further purification. Solutions were prepared with deionized (DI) water (18 M Ω cm) from a Millipore MilliQ purification system.

The graphite conductive ink was obtained from Acheson. Alumina slurries (0.05 and 1.0 μ m) were from Buehler.

ccNiR was purified from *Desulfovibrio desulfuricans* ATCC 27774 cells grown in nitrate, as previously described by Almeida and co-workers (Almeida, 2003).

2.2 Electrochemical Measurements

For the optimization studies, a conventional three-electrode electrochemical cell was used, with an Ag/AgCl reference electrode, a Pt counter electrode (both from Radiometer) and a home-made working electrode made of pyrolytic graphite disks (4 mm diameter) and modified with the enzyme/ink layer.

The characterization of the optimized electrode was performed after replacing the previous system by carbon paste screen-printed electrodes (CPSPEs) with a three electrode configuration (Figure 1), including an Ag/AgCl pseudo-reference, a graphite paste counter electrode and a graphite paste working electrode (3.1 mm diameter). The CPSPEs were fabricated at CIDETEC facilities, as described by Ochoteco and co-workers (Ochoteco, 2009).



Figure 1: A screen-printed three-electrode system. (1) working electrode; (2) reference electrode; (3) counter-electrode.

The one-compartment electrochemical cell containing 0.1 M KCl in 0.05 M Tris-HCl buffer, pH 7.6 as supporting electrolyte, was thoroughly purged with Argon before each experiment. Measurements were performed with a potentiostat Autolab PSTAT 12 (Eco-Chemie) monitored by the control and data acquisition software GPES 4.9. The cyclic voltammograms (CV) were plotted at room temperature ($22 \pm 2^\circ\text{C}$), with a scan rate of 20 mV/s, in the potential window [0.0; -0.8] V (vs reference system). To evaluate the biosensors response to the analyte (0.001 - 7 mM), the cell was successively spiked with standard solutions of nitrite. After each addition, the electrochemical cell was deoxygenated and the CV was registered. The catalytic currents (I_{cat}) were measured at the inversion potential; all values were subtracted from the non-catalytic current recorded in the absence of nitrite (I_c). Each experiment was replicated two times.

2.3 Bioelectrodes Preparation

Prior to coating, the pyrolytic graphite electrodes (PGEs) were polished with alumina slurry in cloth pads. Then, the electrodes were thoroughly washed with DI water and ethanol and ultrasonicated in water for 5 min. The electrodes' surface was further washed with DI water and dried with compressed air.

CPSPEs were used as produced, with no pre-activation.

The conductive carbon inks were previously diluted with an organic solvent (acetone or MEK) in a 1:1 ratio and homogenised with the help of an ultrasound bath. The inks' suspensions were then mixed with ccNiR in different proportions (4:1, 2:1, 1:1 and 1:2 ink/enzyme). Finally, a 5 μ L drop was placed on the surface of the working electrodes which were cured for 20 min. inside an oven set at 40°C or 60°C. Control experiments were carried out with no curing treatment and/or no carbon ink; in such cases, the ccNiR layer was dried at room

temperature.

3 RESULTS

3.1 Response to Nitrite

Regardless of the composition of the electrode material and enzyme modifying layer, the CVs displayed a sigmoidal shape in the presence of nitrite (not shown), as previously observed in bare PGEs and carbon nanotubes modified electrodes (Silveira 2010a,b). This reflects the electrocatalytic reduction of nitrite into ammonium as a consequence of the direct electron transfer between the electrode and ccNiR.

In general, the plots catalytic current (I_{cat}) vs nitrite concentration could be fitted to a hyperbolic equation, denoting a Michaelis-Menten profile. The assessment of the analytical performance of each bioelectrode was based on the measurement of the following parameters: catalytic efficiency (I_{max}/I_c), sensitivity of detection (slope of the linear range), correlation coefficient (r^2) and quantification range.

3.2 Temperature and Solvent Effects

Preliminary experiments were carried out in order to check if the chemicals (organic solvents) and the thermal treatment (curing) required for printing the working electrode component in CPSPEs were compatible with nitrite reductase activity. In this regard, three different PG electrodes were modified with *i*) ccNiR only, *ii*) ccNiR mixed with carbon ink diluted in acetone and *iii*) the same as (*ii*) but with an extra curing step at 60°C. As seen in Figure 2, the maximum catalytic current (I_{max}) has increased about three times in the presence of the carbon ink. This should be related with the resultant enlargement of the electroactive area. Apparently, the carbon ink/acetone composite had no critical effect on catalytic activity.

When comparing the response profiles obtained with or without electrode curing (both in the presence of conductive ink), one can see that the thermal treatment does not have a strong influence on I_{max} . On the other hand, the sensitivity of the sensor, as given by the slope of the linear range of the plot, decreased about 55%. This indicates that partial protein denaturation has occurred.

3.3 Electrode Optimization

3.3.1 Enzyme/Carbon Ink Ratio

Different proportions of enzyme and carbon ink suspended in acetone were early tested in order to choose the best composition. The one using the highest amount of protein (1:2 ratio, corresponding to 3.3 μg of ccNiR) displayed the best results (not shown) without relevant loss of ink, and was selected for further studies.

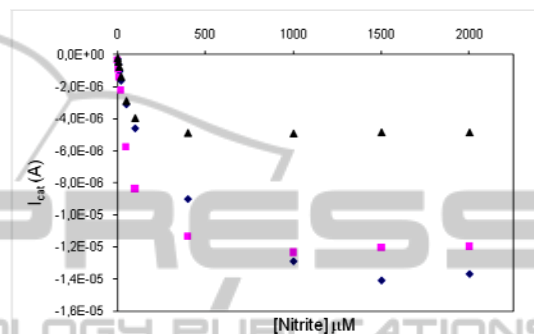


Figure 2: Electrocatalytic response to nitrite of ccNiR (3.3 μg) modified PG electrodes: (▲) without carbon conductive ink and thermal treatment (sensitivity: $32 \times 10^{-8} \text{ A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-1}$); (■) pre-mixed with the carbon conductive ink diluted in acetone (sensitivity: $73 \times 10^{-8} \text{ A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-1}$); (◆) pre-mixed with the carbon conductive ink diluted in acetone and cured at 60°C (sensitivity: $33 \times 10^{-8} \text{ A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-1}$).

3.3.2 Selection of Organic Solvent and Curing Temperature

Normally, inks for screen printing contain organic solvents that are later evaporated by heating. If other ingredients like ccNiR need to be included, it is highly recommended to lower the viscosity of the paint in order to facilitate the mixing process. For this reason, prior to enzyme incorporation, the carbon ink used in this work was diluted with two different organic solvents - MEK or acetone. It is worth noting that acetone is less commonly used for inks dilution than MEK, although it has a lower boiling point that could permit the use of lower curing temperatures. Actually, the response to nitrite was much higher when this solvent was used instead of MEK (Figure 3) and, accordingly, the linear range was also wider. Therefore, acetone has proved to be less harmful to the protein.

In order to evaporate residual organic solvents, most CPSPEs have to be dried thermally. Although a temperature of 60°C is normally selected for the curing process of those used in the present work, due

to the presence of the biocatalyst, we have also tested the lowest permitted heating temperature, i.e., 40°C. Interestingly, the differences on nitrite reducing activity were generally small, except when MEK was used for ink dilution, which generated much lower catalytic currents. Most likely, this solvent did not evaporate completely at 40°C, enhancing its detrimental effect on enzyme activity.

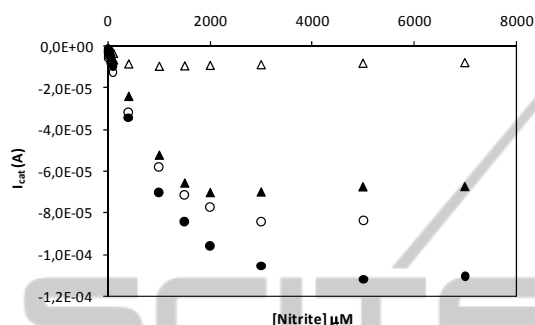


Figure 3: Electrochemical response to nitrite of ccNiR (3.3 μg) modified PG electrodes, pre-mixed with the carbon conductive ink, prepared and treated in the following ways: (Δ) diluted in MEK and cured at 40°C; (\blacktriangle) diluted in MEK and cured at 60°C; (\circ) diluted in acetone and cured at 60°C. (\bullet) diluted in acetone and cured at 40°C.

In accordance to the results obtained in this combined study, we have selected acetone for ink solubilization and a curing temperature of 40°C.

3.4 Application of Carbon Paste Screen Printed Electrodes (CPSPEs)

Following the optimization of the ccNiR containing conductive paints, the enzyme/carbon inks were deposited on CPSPEs. The CVs displayed higher background currents (not shown), which is most likely related to the roughness of the SPEs surfaces, generating higher capacitive currents. Nevertheless, the analytical parameters (sensitivity: 52 $\text{A}\cdot\mu\text{M}^{-1}\cdot\text{cm}^{-2}$; linear range: 0.001 - 1 mM) remained similar (Figure 4). The data were fitted to the Michaelis-Menten kinetic model using the software GraphPad Prism 4.0. Accordingly, the K_m^{app} is 0.9 ± 0.1 mM, which is about 250 times higher than the value previously determined by protein film voltammetry (Silveira, 2010c). This means that the diffusion of nitrite within the carbon ink is a very slow process and should be responsible for the wide linear range of the calibration curves.

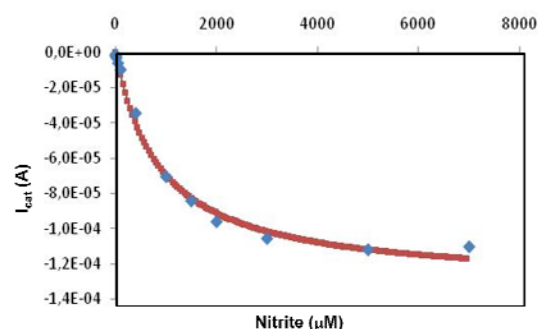


Figure 4: Nitrite response of CPSPEs modified with ccNiR in accordance with the optimized procedure (3.3 μg enzyme in a 1:1 mix of acetone:graphite ink; curing temperature = 40°C). Sensitivity: 52 $\text{A}\cdot\mu\text{M}^{-1}\cdot\text{cm}^{-2}$; linear range: 0.001 - 1 mM. The calibration curve was fitted to a hyperbolic equation according to the Michaelis-Menten using the GraphPad Prism program ($K_m^{\text{app}} = 0.9 \pm 0.1$ mM; $I_{\text{max}} = 1.32 \times 10^{-4} \pm 0.04 \times 10^{-4}$ A; $r^2 = 9.94 \times 10^{-1}$).

4 CONCLUSIONS

This R&D project was designed to address a critical and growing need for real-time monitoring of nitrites and to provide better analytical tools for its clinical diagnosis. Our previous results have demonstrated the feasibility of using ccNiR in the construction of bioelectrodes for a selective nitrite analysis (Almeida, 2007; Chen, 2007; da Silva, 2004; Silveira 2010 a,b; Zhang, 2009). Herein we have shown the biocompatibility of the painting materials and the electrode curing procedure with ccNiR activity. The success of this preliminary work opens up the possibility of including the enzyme directly in the printing paste used for the fabrication of thick-film electrodes, facilitating the mass production of easy-to-use nitrite biosensors. If coupled to a portable potentiostat, these enzyme containing disposable electrode strips will turn a long and elaborated laboratory protocol into a simple task, quickly executed onsite.

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