CONSTRUCTION OF DISPOSABLE BIOSENSORS FOR NITRITE MONITORING IN FOOD AND BEVERAGES

Tiago Monteiro[1], *Célia M. Silveira*[1,2], *Elena Jubete*[3], *M. Gabriela Almeida*[1,4]

[1] UCIBIO, REQUIMTE, Faculdade de Ciências e Tecnologia, Univ. Nova de Lisboa, 2829-516 Monte de Caparica, Portugal, tc.monteiro@campus.fct.unl.pt;
[2] Instituto de Tecnologia Química e Biológica, Univ. Nova de Lisboa, Av. da República – EAN, 2780-157 Oeiras, Portugal, c.silveira@fct.unl.pt;
[3] CIDETEC-IK4- Sensors and Photonics Unit, Parque Tecnológico de San Sebastián, Pº Miramon, 196, 20009 Donostia – San Sebastián, Spain, ejubete@cidetec.es;
[4] Centro de Investigação Interdisciplinar Egas Moniz (CiiEM), Inst. Superior de Ciências da Saúde Egas Moniz, Quinta da Granja, 2829-511 Caparica, Portugal, mg.almeida@fct.unl.pt

Abstract

Nitrite salts are regularly used as fertilizers and food preservatives. Excessive levels have raised awareness from public regulation agencies, since nitrite can have an adverse impact on public health and on ecological systems. Therefore, it is important to develop sensitive assays to monitor this ion in foodstuff products and waters. This work presents a nitrite biosensor based on a highly selective nitrite reducing enzyme incorporated on carbon ink based screen-printed electrodes. The resulting device enabled the quantification of nitrite in different samples (milk, water) in a straightforward way and with small error (1–6%). The sensitivity of the biosensor towards nitrite reduction under optimized conditions was 0.55 A M^{-1} cm^{-2} with a linear response in the range 0.7–370 µM.

Keywords: nitrites, biosensor, screen-printed electrodes, drinks

1. INTRODUCTION

Nitrites occur naturally in several green leafy vegetables and are widely used in the food industry and farming practices. For instance, nitrite potassium or sodium salts (E249, E250) are routinely employed in the manufacturing of processed meats, fishes and some cheeses, aiming at improving the organoleptic properties (color and flavor), preventing rancidity by inhibiting lipid oxidation and protecting from the growth of certain pathogenic bacteria like *Clostridium botulinum* [1,2]. The regular intake of these food products aroused public health concerns in the early 1970's, due to the potential role of nitrite in forming genotoxic *N*-nitroso compounds in the presence of secondary amines. Although the relationship between the ingestion of nitrites (or their precursors, nitrates) and the risk of cancer in humans was not unequivocally proved so far, the surveillance of nitrites in food products is critical for controlling health risks [2,3]. In Europe, the maximum authorized level of nitrites added to meat and other specific foodstuffs in which they may be used (e.g. maximum 150 mg/kg of nitrites in meat products) is regulated by 2006/52/EC directive. Another cause for concern comes from the continuous loading of lawns and agricultural fields with nitrogen containing fertilizers, which is responsible for the contamination of surface waters and groundwater supplies, thereby increasing the risks of human exposure to high levels of nitrites. Consequently, European legislation (98/83/EC) sets the maximum admissible levels of nitrites in drinking water at 0.1 ppm. Occasionally, incidents of methemoglobinemia are reported worldwide, mostly due to the consumption of water from domestic wells that are not subjected to quality control [4].

The reliable determination of nitrites in foodstuffs and waters is often a challenge. Analytical bias may be introduced at the initial stages of the procedure (sampling, storage, extraction) as well as at the final quantification step [1]. Besides, the standard protocols for nitrites measurement require sophisticated equipments (e.g. spectrophotometers, HPLC systems, gas chromatographers) that are operated by well-trained personnel at central laboratories, are expensive and time-consuming. Nitrite is a highly reactive species and its stabilization during storage could be hard to achieve. Ideally, the analysis should be performed *on site*, immediately after sampling, through a simple and cheap test, capable of providing fast readings [1,5,6]. In this regard, our group made major progresses by coupling the selective ammonia forming *nitrite reductase* enzyme, NrfA, with electrochemical transducers [7-11]. A disposable biosensor was recently implemented using screen-printed electrodes modified with a carbon ink/NrfA composite [12]. Screen-printing technology has been readily used in the fabrication of electrochemical biosensors due to the ease of production, low cost and disposable nature of the electrodes strips [13]. Herein we tested the manual printing of the enzyme/carbon ink composite on thick-film strip electrodes, which were fabricated beforehand by printing a similar conductive carbon paste on plastic supports (Fig. 1). The nitrite tests were successfully applied on real samples, like milk, drinking water.

Fig. 1. Screen-printed three-electrode system, from CIDETEC. (1) working electrode; (2) reference electrode; (3) counter-electrode.

2. MATERIALS AND METHODS

2.1 Reagents

With the exception of acetone (propanone; 99%;), which was purchased to Pronalab, all chemicals were of analytical grade, from Sigma. Glucose oxidase (Type II from *Aspergillus niger*, 17.3 U mg^{-1}) and catalase (from bovine liver, 2-5 kU mg^{-1}) were obtained as lyophilized powders and used as received (Sigma). The enzyme NrfA (specific activity, 300 U mg^{-1}; 3.0 mg mL^{-1}) was purified from the sulphate reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774 [14]. Solutions were prepared with deionized water (18 MΩ . cm) from a Millipore MilliQ purification system.

2.2. Electrode modification

The screen-printed electrodes comprising a set of a Ag/AgCl *pseudo*-reference, a counter electrode and a working electrode (Φ = 4.4 mm), both made of graphite paste, were provided by CIDETEC. The latter were modified with the NrfA enzyme, previously suspended in the same carbon conductive ink used for the electrodes preparation, as below described. The carbon inks were initially diluted with acetone in a 1:1 ratio and homogenized through ultrasonication; the resulting suspensions were mixed with NrfA in a proportion of 1:2 (ink/enzyme). Then, a 5 μL drop was deposited on the surface of the working electrodes, which were cured for 20 min. at 40°C.

2.3. Electrochemical assays

Electrochemical measurements were carried out with a potentiostat Autolab PSTAT 12 (Eco-Chemie) monitored by the control and data acquisition software GPES 4.9. Cyclic voltammograms (CV) were plotted at room temperature (22 ± 2°C), using a scan rate of 20 mV/s, in the potential window [0.0; -0.8] V (vs reference system).

Because the electrochemical reduction of molecular oxygen (O_2) occurs at a negative potential close to the one required to activate the NrfA enzyme (*ca.* -0.4 V *vs* pseudo-reference Ag/AgCl), thus interfering with the electroanalytical assays, dissolved oxygen was removed from electrolyte and sample solutions prior to measurements using an enzymatic scavenger system composed of glucose oxidase (GOx), catalase (Cat) and glucose in the following concentrations: 15 U mL^{-1}, 2000 U mL^{-1} and 50 mM, respectively. In this way, while glucose is catalytically oxidized by GOx into gluconic acid, O_2 is reduced into H_2O_2, which is further depleted by the Cat action [15]. Solutions were stirred for 10 min., to assure sufficient oxygen consumption.

To evaluate the biosensors response to the analyte, the cell was successively spiked with stock solutions of nitrite; a CV was recorded after each nitrite addition. Catalytic currents (ΔI_{cat}) were measured at the inversion potential (-0.8 V vs pseudo-reference Ag/AgCl); all values were subtracted from the non-catalytic current recorded in the absence of nitrite (I_c).

The nitrite biosensors were tested in real samples (milk, mineral and tap water). Each assay was replicated three times.

3. RESULTS

3.1 Bioelectrode preparation and analytical performance

Screen-printing technology has been widely used in the mass production of disposable amperometric and/or voltammetric biosensors. Therefore, we tested the manual printing of the enzyme/carbon ink composite on the surface of SPE. Accordingly, mixtures of enzyme in acetone/ink suspension were deposited on the electrode surface and cured at 40 °C, for 20 min. Despite the chemical and thermal treatments, the resulting NrfA/carbon ink/SPE biosensors displayed a good response for nitrite reduction (Fig. 2), with sensitivity, linear range and detection limit (LOD) of 0.55 A M^{-1} cm^{-2}, 0.7-370 μM and 1.2 μM, respectively.

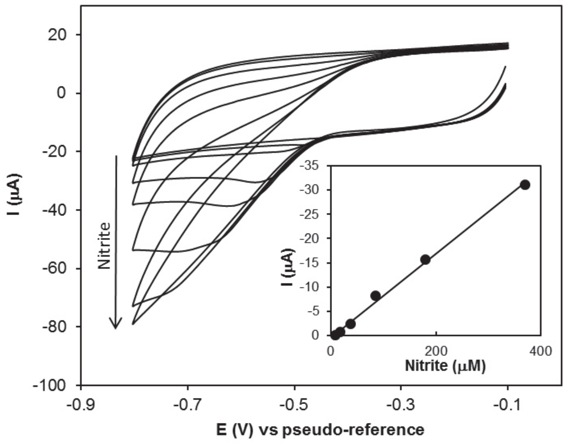

Fig. 2. Electrochemical response of NrfA/carbon ink/SPE increasing concentrations of nitrite (0-3.6 mM) in 0.1 M KCl and 50 mM tris-HCl buffer pH 7.6. Molecular oxygen was scavenged using the bi-enzyme system GOx/Cat/glucose. Scan rate, 20 mV/s. Inset: current response of the biosensor to nitrite additions.

The reproducibility of the biosensor was evaluated by comparing the slope of the calibration curves (sensitivity) of different electrode preparations. The standard deviation for 5 replicates was *ca*. 20%; this was attributed to difficulties in obtaining identical surface coverage due to the viscosity of the enzyme/carbon ink mixture and the poorly controlled deposition method. In the future, these experimental variations may be overcome by using automatized screen-printing techniques.

3.2 Stability

The storage stability of the biosensors was tested continuously, over 3 weeks. The enzyme electrodes were prepared in day 1, stored dry at 4 °C, tested a single time and discarded (3 or more independent assays were performed every day). After twenty days, the NrfA/carbon ink/SPE kept 90% of their initial activity; nonetheless, this variation falls within the experimental error (reproducibility 20%). This clearly indicates that the enzyme activity is compatible with the harsh conditions used in the electrode preparation, maintaining a stable response for a reasonable period of time.

3.3 Real Samples

The performance of the NrfA/carbon ink/SPE biosensors was evaluated in complex matrices by spiking different samples (milk, drinking and tap water) with nitrite standards and calculating the recovery percentage. As shown in Table 1, all results are close to 100%, demonstrating that the biosensors were able to detect a known concentration of nitrite in different types of matrices.

Table 1. Nitrite recovery percentage in different samples, using the NrfA/carbon ink/SPE for quantification

Sample	Nitrite added (μM)	Nitrite found (μM)	Recovery (%)
mineral water	5.0	5 ± 1	106.2
tap water	4.8	4.8 ± 0.2	99.0
milk	9.9	9.8 ± 0.3	99.3

4. CONCLUSIONS

In this paper we demonstrated the viability of the mass production of point of care tests for nitrite quantification in drinking samples such as potable waters and milk. Our previous works have demonstrated the advantages of using the mutihemic nitrite reducing enzyme NrfA, in the construction of effective nitrite biosensors that are able to overcome the typical setbacks related with sampling, processing and interferences from matrix composition [7-11].

In this work we have shown the biocompatibility of the painting inks and the electrode curing procedure with the catalytic activity of NrfA. The success of this 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 cheap and easy-to-use nitrite biosensors. One should emphasize that dissolved oxygen was easily removed from electrolyte solutions using an bi-enzymatic scavenger system composed of GOx, Cat and glucose, thereby avoiding the need of specific lab equipment for sample degassing. The immobilization of the enzyme components GOx/Cat is currently underway.

In conclusion, this approach brings us closer to succeeding in producing commercially viable nitrite point-of-care tests, easy to operate on-site (e.g. food industry) where real-time analysis of nitrites would be remarkably important.

ACKNOWLEDGMENTS

This work was supported by CiiEM - Centro de Investigação Interdisciplinar Egas Moniz, and Unidade de Ciências Biomoleculares Aplicadas-UCIBIO, which is financed by national funds from FCT/MEC (UID/Multi/ 04378/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145-FEDER-007728). C.M. Silveira and T. Monteiro acknowledge Fundação para a Ciência e Tecnologia the fellowships SFRH/BPD/79566/2011 and PD/BD/109687/2015, respectively.

REFERENCES

[1] M. Hill, *Nitrates and Nitrites in Food and Water*, Woodhead Publishing Limited, Cambridge, 1996.

[2] J. J. Sindelar and A. L. Milkowski “Human safety controversies surrounding nitrate and nitrite in the diet”, *Nitric Oxide*, vol. 26, pp. 259-266, 2012.

[3] G. A. Sullivan and J. G. Sebranek “Nitrosylation of myoglobin and nitrosation of cysteine by nitrite in a model system simulating meat curing”, *J. Agric. Food Chem.*, vol. 60, pp. 1748–1754, 2012.

[4] M. G. Almeida, A. S. Serra, C. Silveira, J. J. G. Moura, “Nitrite biosensing via selective enzymes – a long but promising route”, *Sensors*, vol. 10, n. 12, pp. 11530-11555, 2010.

[5] M. J. Moorcroft, J. Davis and R. G. Compton, “Detection and determination of nitrate and nitrite: A review”, *Talanta*, vol. 54, pp. 785-803, 2001.

[6] J. Dutt and J. Davis, “Current strategies in nitrite detection and their application to field analysis”, *J. Environ. Monit.*, vol. 4, pp. 465-471, 2002.

[7] S. da Silva, S. Cosnier, M. G. Almeida and J. J. G. Moura, “An Efficient Poly(pyrrole)-Nitrite Reductase Biosensor for the Mediated Detection of Nitrite” *Electrochem. Comm.*, vol. 6, n. 4, pp. 404-408, 2004.

[8] C. M. Silveira, J. Baur, M. Holzinger, S. Cosnier and M.G. Almeida, ”Enhanced direct electron transfer of a multihemic nitrite reductase on single-walled carbon nanotube modified electrodes”, *Electroanalysis*, vol. 22, pp. 2973-8, 2010.

[9] M. G. Almeida, C. M. Silveira and J.J . G. Moura, “Biosensing nitrite using the system nitrite redutase/Nafion/Methyl viologen - a voltammetric study”, *Biosens. Bioelectron.*, vol. 22, n. 11, pp. 2485-2492, 2007.

[10] H. Chen, C. Mousty, S. Cosnier, C. Silveira, J. J. G. Moura and M. G. Almeida, “Highly sensitive ccNiR biosensor electrical-wired by [ZnCrAQS$_2$] for nitrite determination” *Electrochem. Comm.,* vol. 9, pp. 2240-2245, 2007.

[11] C. M. Silveira, S. P. Gomes, A. N. Araújo, M. C. M. Couto, M. C. B. S. M. Montenegro, R. Silva, A. S. Viana, S. Todorovic, J. J. G. Moura and M. G. Almeida, “An efficient non-mediated amperometric biosensor for nitrite determination”, *Biosens. Bioelectron.*, vol. 25, pp. 2026-2032, 2010.

[12] T. Monteiro, P. R. Rodrigues, A. L. Gonçalves, J. J. G. Moura, L. Añorga, E. Jubete, B. Piknova, A. N. Schechter, C. M. Silveira and M. G. Almeida, “Construction of effective disposable biosensors for point-of-care testing of nitrite” *Talanta*, vol. 142, pp. 246-251, 2015.

[13] E. Jubete, O. A. Loaiza, E. Ochoteco, J. A. Pomposo, H.Grande and J. Rodríguez, “Nanotechnology: A Tool for Improved Performance on Electrochemical

Screen-Printed (Bio)Sensors", *J. Sensors, pp.* 1-13, 2009.

[14] M.G. Almeida, S. Macieira, L. L. Gonçalves, R. Huber, C. A. Cunha, M. J. Romão, C. Costa, J. Lampreia, J. J. G. Moura and I. Moura, "The isolation and characterization of cytochrome *c* nitrite reductase subunits (NrfA and NrfH) from *Desulfovibrio desulfuricans* ATCC 27774. Re-evaluation of the spectroscopic data and redox properties", *Eur. J. Biochem.*, vol. **2**70, pp. 3904-3915, 2003.

[15] N. Plumeré, J. Henig and W. H. Campbell, "Enzyme-catalyzed O_2 removal system for electrochemical analysis under ambient air: application in an amperometric nitrate biosensor", *Anal. Chem.,* vol. 84, pp. 2141–2146, 2012.