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Construction of effective disposable biosensors for point of care testing of nitrite

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ABSTRACT

In this paper we aim to demonstrate, as a proof-of-concept, the feasibility of the mass production of effective point of care tests for nitrite quantification in environmental, food and clinical samples. Following our previous work on the development of third generation electrochemical biosensors based on the ammonia forming *nitrite reductase* (*cc*NiR), herein we reduced the size of the electrodes' system to a miniaturized format, solved the problem of oxygen interference and performed simple quantification assays in real samples. In particular, carbon paste screen printed electrodes (SPE) were coated with a *cc*NiR/carbon ink composite homogenized in organic solvents and cured at low temperatures. The biocompatibility of these chemical and thermal treatments was evaluated by cyclic voltammetry showing that the catalytic performance was higher with the combination acetone and a 40 °C curing temperature. The successful incorporation of the protein in the carbon ink/solvent composite, while remaining catalytically competent, attests for *cc*NiR's robustness and suitability for application in screen printed based biosensors.

Because the direct electrochemical reduction of molecular oxygen occurs when electroanalytical measurements are performed at the negative potentials required to activate *cc*NiR (ca. − 0.4 V vs Ag/AgCl), an oxygen scavenging system based on the coupling of glucose oxidase and catalase activities was successfully used. This enabled the quantification of nitrite in different samples (milk, water, plasma and urine) 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 range 0.7–370 μM.

1. Introduction

This paper addresses a critical and growing need for real-time monitoring of nitrite in situ. Better analytical tools are required in clinical diagnosis, monitoring of food quality and pollution control because none of the established protocols meet all requisites we demand of an analytical assay: simplicity, sensitivity, selectivity, low detection limit, reproducibility and fast response time [1–4].

Due to their closely related chemistries, the potentially hazardous nitrite is often found in the presence of the less reactive nitrate in many different environmental, foodstuff and physiological systems. The origin these oxyanions may be either natural or due to anthropogenic activities (e.g. production of food and energy) [1]. For instance, the release of *N*-oxides into the atmosphere occurs in the course of many industrial and domestic combustion processes whereas the massive loading of lawns and agricultural fields with *N*-fertilizers is responsible for the contamination of surface waters and groundwater supplies [5]. This not only threatens the environment through the unbalance of both local and global biogeochemical

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N-cycles, but also increases the risks of human exposure to high levels of nitrite and the consequential adverse health effects (e.g. blue baby syndrome), especially via consumption of water from domestic wells that receive little or no water quality control. For these reasons, worldwide legislation sets the maximum admissible levels of nitrite in drinking water between 0.1 ppm (98/83/EC) and 3 ppm (WHO/SDE/WSH/07.01/16). Therefore, human exposure to nitrite/nitrate comes mainly from the intake of processed food like cured meats, fishes and cheeses that are treated with nitrate salts (E251, E252) and nitrite salts (E249, E250) for preservation and/or organoleptic purposes. According to the EFSA recommendations (2006/52/EC), the amounts of nitrite/nitrate added to several foodstuffs should be controlled, not exceeding 150 mg kg^{-1} of nitrite in the case of meat product [1,6,7].

In a different context, the detection of nitrite in physiological fluids such as saliva and urine is commonly used for clinical diagnosis. A good example are the colorimetric tests strips routinely used for detecting nitrite in urine, which may correlate well with urinary tract infections. But despite being easy-to-use, quick and inexpensive, results provided by the indicator strips are merely qualitative as they are obtained by visual comparison to a color chart [2]. Nitrite quantification in plasma and blood has been gaining an increasing value in biomedical research. Endogenous nitrite anions are a major intravascular storage of mammalian nitric oxide, a potent vasodilatory and signaling molecule. Even at low concentrations, nitrite regulates a number of signaling events along the (patho) physiological oxygen gradient including modulation of mitochondrial respiration and cytoprotection following ischemic insult [8–11]. Unfortunately, the actual circulating levels of nitrite in humans have been difficult to measure due to sampling problems and the poor performance of analytical assays. Despite highly sensitive methods have already been proposed, these are fairly cumbersome and not practicable for non-laboratory settings [4,12–14].

Therefore, we have set out to establish a new, improved and simple to use point of care tests (POCT) for nitrite quantification in real matrices like beverages, potable waters and physiological fluids. We developed electrochemical biosensors based on the stable redox enzyme cytochrome *c* nitrite reductase (*cc*NiR) from *Desulfovibrio desulfuricans* (*D. desulfuricans*) ATCC 27774, which converts nitrite into ammonia with both high turnover and selectivity [1,15]. Since miniaturization is critical for point of care testing, herein we immobilized the protein on screen printed electrodes (SPE), which are the ideal candidates to produce in a straightforward and large scale way, small, disposable, economical, and easy to use biosensors [16]. Because *cc*NiR has shown a direct electron transfer with graphite based materials, a carbon paste conductive ink was chosen for the working electrode [17–19], which is also a cheaper, easy to modify and chemically inert paint [20]. Although carbon paste screen printed electrodes (SPE) can be manufactured by automated systems, the mass production of a nitrite biosensor can be limited by the extra steps required for enzyme immobilization. Typically, the delicate biological component is applied at the last stage of the fabrication process to avoid exposure to the detrimental chemical and thermal conditions initially used [16,21]. Aiming at simplifying the construction of a disposable nitrite biosensor, we incorporated the robust *cc*NiR in the same carbon paste used for printing the SPE. In an early stage, pyrolytic graphite electrodes (PGE) were modified with a layer of this enzyme/ink, either diluted in acetone (propanone) or butanone (methylethylketone, MEK). The enzyme activity after immobilization in such harsh environment was evaluated by cyclic voltammetry and has proven to be highly satisfactory. The electrode preparation was further optimized and transferred to thick-film strip electrodes that were fabricated beforehand.

Prior to nitrite detection, *cc*NiR needs to be electrochemically reduced at negative potentials [18], which can promote the reduction of dissolved oxygen directly at the electrode and interfere in analytical measurements. Since the ultimate goal is to create a POCT for nitrite, we decided on employing an oxygen scavenger system based on glucose oxidase, catalase and glucose [22]. This allows avoiding the inconvenient degassing process before and while performing analysis in real samples, stepping towards the establishment of a novel disposable methodology for on-site nitrite monitoring.

2. Experimental section

2.1. Reagents and solutions

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

Glucose oxidase (Type II from *Aspergillus niger* 17.3 U mg^{-1}) and catalase (from bovine liver, 2–5 kU mg^{-1}) were purchased as lyophilized powders from Sigma and used as received. *cc*NiR (in 0.05 M phosphate buffer, pH 7.6) was purified from *D. desulfuricans* ATCC 27774 cells grown in a nitrate containing medium, as described in Ref. [23]. The specific activity was 300 U mg^{-1} (1 U–1 μmol nitrite reduced per minute), the turnover number was 340 s^{-1} (20 °C) and the protein concentration was 3.0 mg mL^{-1}.

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

2.2. Bioelectrodes preparation

Prior to coating, the pyrolytic graphite electrodes (PGE) 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. The SPE were used as provided with no pre-activation.

The conductive carbon inks were previously diluted (1:1 ratio) in acetone or MEK and sonicated in an ultrasound bath for homogenization. The carbon ink suspensions were then mixed with *cc*NiR in a 1:2 ink/enzyme ratio. Finally, a 5 μL drop was placed on the surface of the working electrodes (PGE or SPE) which were cured for 20 min inside an oven, at 40 °C or 60 °C. Control experiments were carried out with the same enzyme amount and no carbon ink. When not in use, the working electrodes were stored dry at 4 °C.

2.3. Electrochemical measurements

For the optimization of the biosensor preparation (i.e. organic solvent and curing process) a conventional one compartment electrochemical cell, composed by a three-electrode system, was used. The reference was an Ag/AgCl electrode and the counter electrode was a Pt wire (both from Radiometer). A home-made pyrolytic graphite working electrode (Φ=3 mm) was modified with the enzyme/carbon ink layer composite.

Once optimized, the analytical characterization of the bioelectrodes was performed in SPE made of carbon conductive inks deposited on plastic supports following the three electrode configuration shown in Fig. 1. The SPE were fabricated at CIDETEC facilities, as previously described [24,25] and included an Ag/AgCl pseudo-reference (0.302 V vs NHE), a working electrode (Φ=4.4 mm) and a counter electrode, both made of graphite paste.

The electrochemical cells contained 0.1 M KCl in 0.05 M Tris–HCl buffer (pH 7.6) as supporting electrolyte. Unless stated otherwise, the electrolyte solution 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

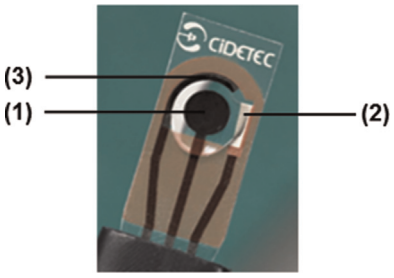

Fig. 1. The screen printed three-electrode system from ik4-CIDETEC: (1) carbon working electrode; (2) pseudo-reference electrode; and (3) carbon counter-electrode.

acquisition software GPES 4.9. The cyclic voltammograms (CV) were plotted at room temperature (22 ± 2 °C), with a scan rate of 20 mV s^{-1}, in the potential window [0.0; −0.8] V (vs reference system).

2.4. Oxygen scavenger system

The enzymatic scavenger system was adapted from Ref. [22] and was tested only with the SPE modified with the *cc*NiR/carbon conductive ink. It comprised a mix of glucose oxidase, catalase and glucose (GOx/Cat/glucose) in the following concentrations: 15 U mL^{-1}, 2000 U mL^{-1} and 50 mM, respectively. The solutions were stirred for 10 min in order to deplete dissolved oxygen.

2.5. Response to nitrite

To evaluate the biosensors response to the analyte, the electrochemical cell was successively spiked with nitrite standard solutions. After each addition, the cell was further purged with an argon flux and the CV was registered, except when the oxygen scavenger system was in use (in which case the solution was stirred after each nitrite addition). To assess the analytical parameters, the catalytic currents (ΔI_{cat}) were measured at the inversion potential (−0.8 V); all values were subtracted from the non-catalytic current recorded in the absence of nitrite (I_c).

In order to evaluate the biosensors performance in complex matrices, three different samples were tested namely tap and drinking (mineral) water, milk, plasma and urine. Molecular oxygen was removed from the cell using the scavenger system described above and a CV was recorded after nitrite addition. ΔI_{cat} were determined at the inversion potential (−0.8 V). All potentials were quoted against the Ag/AgCl pseudo-reference electrode. Each assay was replicated three times.

3. Results and discussion

3.1. Electrode optimization

Carbon inks used for screen printing are thixotropic materials containing three basic components: graphite particles, a polymeric binder and an organic solvent that is later evaporated by heating, so the ink can solidify [26,27]. The solid constituents should be dispersed homogeneously within reasonably fluid inks and with a good adhesion to substrates [28]. If other ingredients, like a protein, need to be included, one should lower the viscosity of the paint in order to facilitate the mixing and screen printing processes, while producing a firmly adhesive, uniform and conductive coating.

For this reason, prior to *cc*NiR incorporation, the carbon conductive ink was diluted with two different organic solvents, namely the frequently used MEK and the more volatile acetone. To find the most favorable curing temperature, following modification with the

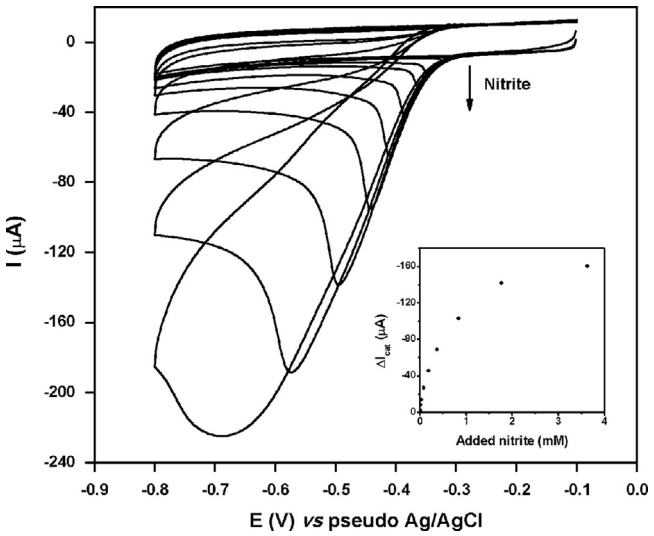

Fig. 2. Electrochemical response of *cc*NiR/carbon ink/PGE to varying nitrite concentrations (0–4 mM) in 0.1 M KCl in 0.05 M Tris–HCl buffer (pH 7.6), purged with argon. Scan rate 20 mV s^{-1}. Inset: response of the biosensor catalytic current (μA) to nitrite additions.

enzyme/carbon ink, PGE were heated during a short period of time (20 min), at 40 °C and 60 °C (although the latter is commonly employed for drying bare SPE, due to the presence of the biological component, 40 °C was also tested as the lowest possible heating temperature). Afterwards the catalytic activity towards nitrite was evaluated by cyclic voltammetry as described in the experimental section. Fig. 2 depicts typical CV registered in the absence and presence of nitrite. Following the addition of the enzyme substrate the CV adopt a sigmoid shape, reflecting the direct electron transfer between the electrode surface and *cc*NiR and the subsequent catalytic reduction of nitrite to ammonium, according to an EC mechanism. The catalytic current response (ΔI_{cat}) was plotted against nitrite concentration (Fig. 2, inset). It increased with the amount of nitrite in the cell and eventually reached a limiting value, the typical behavior of enzyme saturation kinetics.

The ΔI_{cat} values at saturating concentrations of nitrite ($\Delta I_{cat\ at\ 2\ mM}$) obtained in each tested condition were compared in Table 1. Clearly, the mildest temperature provided much better results no matter the organic phase. Regarding the solvent, acetone delivered the highest catalytic currents, proving to be less harmful to the protein. Most likely, its low boiling point facilitates solvent evaporation, thereby minimizing protein denaturation. Accordingly, the poorer performance of the MEK containing biocomposites may be associated to residual solvent.

Results obtained with control electrodes (prepared with enzyme only) show a minor decrease of catalytic activity from 40 to 60 °C that is probably due to a slight denaturation of *cc*NiR. The fact that the immobilized enzyme is highly active at elevated temperatures is in line with the previously observed maximum of activity at 57 °C, measured by homogeneous enzymatic assays in solution [29]. However, thermal inactivation is more pronounced when the enzyme is incorporated within the carbon paste. Possibly, the combined chemical and heating treatments have a negative synergistic effect on the catalytic activity. Nevertheless, compared to bare electrodes, the ΔI_{cat} values of the carbon ink modified electrodes are always higher due to enlargement of the electroactive area (both faradaic and capacitive currents increase over 20 times; data not shown) and better electrical wiring of the enzyme within the carbon ink composite relative to its immobilization on the bare PGE surface.

Other analytical parameters of the bioelectrodes were evaluated and are listed in Table 1. The sensitivity of detection, as given by the slope of the calibration curves, was similar for acetone based

Table 1
Analytical and kinetic characterization of the *cc*NiR/carbon ink/PGE response to nitrite, as obtained by cyclic voltammetry. The carbon pastes were prepared with different solvents and curing temperatures.

Conductive ink solvent	Acetone		MEK		No carbon ink	
Curing temperature (°C)	**40**	**60**	**40**	**60**	**40**	**60**
$\Delta I_{cat\ at\ 2\ mM}$ (μA)	-94 ± 6	-27 ± 3	-20 ± 4	-4.1 ± 0.04	-5.9 ± 2	-5.1 ± 2
Sensitivity ($A\ M^{-1}\ cm^{-2}$)	0.78 ± 0.1	0.87 ± 0.2	0.61 ± 0.09	0.47 ± 0.002	0.91 ± 0.1	0.83 ± 0.02
Linear range (μM)	1.7–840	1.7–180	1.7–180	3.7–37	0.20–37	0.20–37
LOD (μM)	2.0	2.0	2.2	3.9	0.29	0.29

composites and control electrodes and somewhat lower for the preparations with MEK. On the other hand, the linear ranges were considerably extended in the presence of the carbon ink due to an increased diffusion barrier to nitrite. The combination acetone/40 °C provided the widest linear range by far (1.7–840 μM). With the exception of MEK/60 °C, the limits of detection (LOD) were reasonably low with all composite biosensors (ca. 2 μM), although higher in comparison with control electrodes (ca. 0.3 μM). This was also attributed to the diffusion barrier created by the incorporation of the enzyme on the carbon ink. Taking together these results, we continued the work selecting acetone for ink resuspension and a curing temperature of 40 °C.

3.2. Miniaturization – disposable screen printed biosensors

Next, the optimized enzyme/carbon ink and solvent/curing temperature combination was implemented on a disposable SPE based on a three electrode configuration. Screen printing technology has been readily used in the fabrication of amperometric biosensors due to the ease of production, low cost and disposable nature of the resulting electrodes. Therefore, we tested the manual printing of the enzyme/carbon ink composite on the surface of SPE (made from the same carbon conductive ink) by using the procedure described earlier for the preparation of PGEs. Accordingly, mixtures of enzyme in acetone ink suspension were deposited on the electrode surface and placed at a curing temperature of 40 °C, for 20 min.

The *cc*NiR/carbon ink/SPE biosensor displayed good activity for nitrite reduction. The sensitivity, linear range and LOD of the disposable biosensors were $0.55\ A\ M^{-1}\ cm^{-2}$, 0.7–370 μM and 1.2 μM, respectively. A comparison of the results obtained on the SPE with the PGE surface showed that, despite the LOD being improved, there is a decrease of activity (30% less sensitivity to nitrite and reduced linear range) when changing the working electrode. Also, the capacitive currents are higher with the SPE due to an increase in the active surface area (data not shown). This indicates that, despite the enzyme being immobilized in similar conditions, the underlying transduction surface is important for the biosensor's response. Perhaps the interface between the enzyme/carbon ink and the PGE surface is less resistive, resulting in a better electrical connection between the two materials. Serra et al. observed a similar decrease in activity, when changing from PGE to SPE, in a nitrite biosensor based on a cd_1 type nitrite reductase. This was attributed to the lower conductivity, purity and organization of the carbon paste [30]. Further characterization of the interface should be carried out to clarify this behavior.

The reproducibility of the biosensor was evaluated by comparing the calibration curves of different electrode preparations (sensitivity and catalytic current at saturating nitrite concentrations). It was found that the standard deviation of the sensitivity for 5 replicate electrodes was around 20% (10% if compared in terms of $I_{cat\ at\ 2\ mM}$). This was attributed to difficulties in obtaining identical surface coverages, particularly due to the viscosity of the enzyme/carbon ink mixture and the poorly controlled deposition method [28]. Such experimental variation may be overcome by automatized screen printing processes.

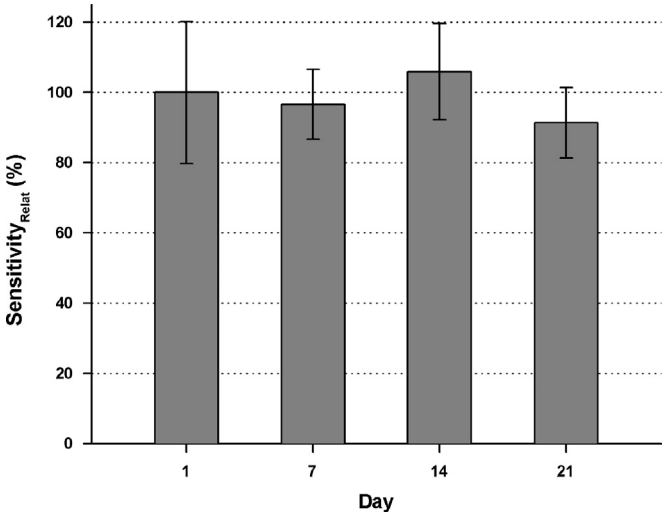

Fig. 3. Storage stability of *cc*NiR/carbon ink/SPE: time effect on the sensitivity for nitrite determination. Each determination was done with a single-use biosensor (at least 3 replicates per day), from a batch of electrodes all prepared on the first day of analysis.

The storage stability of the *cc*NiR/carbon ink/SPE was tested over a 3 weeks period. All the biosensors were prepared in day 1 of the experiment, stored dry at 4 °C, tested a single time and discarded (3 or more replicates were tested *per* day). As seen in Fig. 3, the biosensors retained 90% of their initial activity 20 days after being prepared. The 10% of variation actually falls within the experimental error (reproducibility 20%). This clearly demonstrates that the enzyme can cope with the harsh immobilization conditions/electrode preparation and keep a stable response for a considerable period of time. In fact, it has been reported previously that *cc*NiR based electrodes have long-term stability, spanning over 6 months, even with recurrent use [17].

Overall, these results highlight the robustness of the enzyme and its potential use for screen printing applications.

3.3. Oxygen scavenging

The electrochemical biosensors based on nitrite reductases function under anaerobic conditions, at low potential ranges. Therefore there is a need to remove oxygen interference, which is usually solved by purging the solutions with an inert gas before measurements. However, this method requires laboratory-based equipment that is not suited for on-site analysis. In general, a good practical oxygen removal system must: (i) be able to sustain anaerobic conditions throughout the measurement's duration; (ii) do not interfere in any way with the electrochemical process, biorecognition element or mediators involved in the analyte quantification; (iii) present a fast oxygen reduction kinetics, to minimize the waiting time necessary to achieve anaerobic conditions; and (iv) be autonomous from any specialized equipment [22].

Herein we implemented an approach for oxygen removal based on the protocol recently proposed by Plumeré et al. for extremely low volume vessels (μL) that does not require any technical support. It is composed of a bi-enzyme system that initially uses GOx and its substrate glucose to thoroughly reduce dissolved oxygen, as schematically represented in Fig. 4. This reaction produces hydrogen peroxide that is further depleted by Cat. In this work, the proposed oxygen scavenging system was firstly tested with respect to its ability to remove oxygen and maintain anaerobic conditions in electrochemical cells containing 5 mL of supporting electrolyte. The assays were monitored by cyclic voltammetry using the *cc*NiR/carbon ink/SPE biosensor. As shown in Fig. 5, before adding glucose to the GOx–Cat containing supporting electrolyte, the electrochemical reduction of dioxygen produces a broad cathodic wave with an intense peak at ca. −0.7 V. However, 10 min after adding glucose to the bi-enzyme system, the intense cathodic signal disappears, revealing that the electrochemical interference of oxygen was efficiently eliminated. In fact, each sequential cycle of the enzymatic scavenging system decreases the oxygen concentration in solution by a factor of 2 (cf. Fig. 4); because GOx and Cat have high turnover numbers, the oxygen levels quickly become lower than the detection limit. For about an hour, the background current is stable, meaning that anaerobic conditions are maintained during the time period needed for the analytical assay. The bi-enzyme oxygen scavenging system worked efficiently in the same experimental conditions (pH 7.6) used for biosensor operation, so no further adjustments were required.

Afterwards, because none of the oxygen scavenging system components should interfere with the electroanalytical measurements [22], the catalytic activity was assessed in the presence of GOx/Cat/glucose and compared with the response obtained when using the argon purging method. The bioelectrode's sensitivity decreased 27%e and the $I_{cat\ at\ 2\ mM}$ was 47% lower, when employing the scavenging system. On the other hand, the linear range (0.2–180/370 μM) and the LOD (0.6 μM) were improved. We propose that the enzymes of the scavenging system may interact with the bioelectrode by creating a passivation/fouling layer, which can act as a diffusion barrier to nitrite, thereby diminishing the sensitivity. Also, the formation of gluconic acid and consequential small pH variation (from 7.6 to 7.1 after 1 h) may have a negative impact on *cc*NiR. Despite the somewhat decreased activity, the bioelectrode's performance is still very good in the presence of the oxygen scavenger system and in line with previously reported *cc*NiR biosensors [1]. Moreover, the biosensors' response was stable during the time required for a single calibration or the analysis of one sample.

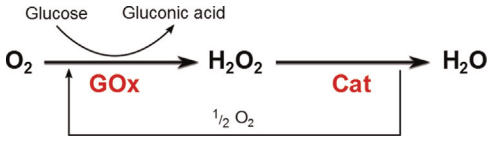

Fig. 4. Scheme of the enzymatic oxygen scavenging system based on glucose oxidase (GOx), its substrate glucose, and catalase (Cat).

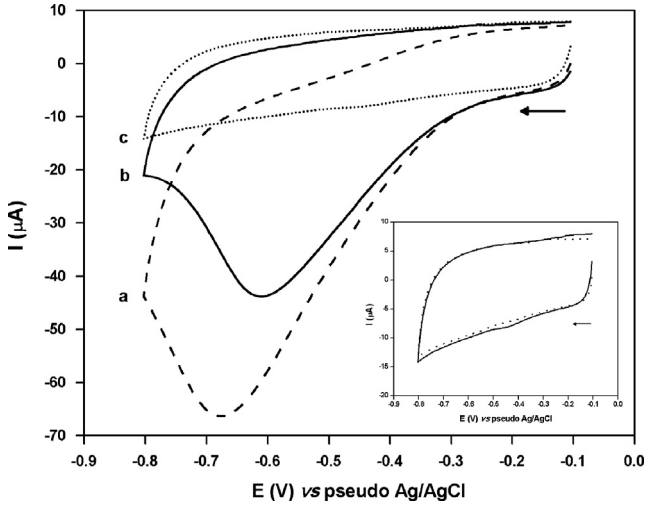

Fig. 5. Cyclic voltammograms of SPE/*cc*NiR/carbon conductive ink at 20 mV s^{-1} in 5 mL of 0.1 M KCl in 0.05 M Tris–HCl, pH 7.6: (a) GOx (12.5 μM, 15 U mL^{-1}) and Cat (16.6 μM, 2 kU mL^{-1}) in solution; (b) GOx (12.5 μM, 15 U mL^{-1}), Cat (16.6 μM, 2 kU mL^{-1}) and glucose (50 mM) in solution ($t_{0\ min}$); and (c) background current after 10 min. Inset: background current after 10 (line) and 60 (dot) minutes.

3.4. Analysis of real samples

The *cc*NiR/carbon ink/SPE biosensor performance in complex matrices was assessed by spiking different samples (drinking and tap water, milk, plasma and urine) with a nitrite solution and calculating the recovery percentage. For each sample, three independent assays ($n=3$) were performed following the procedure described in the experimental section. The obtained results, presented in Table 2, are close to 100%, showing that the employed biosensors were capable of detecting a known concentration of nitrite in all types of tested samples. This indicates that the proposed devices are able to provide reliable results in complex matrices.

4. Conclusions and future work

Herein we have demonstrated that the production of miniaturized POCT for nitrite quantification in environmental, clinical and food samples is feasible and easy to achieve. This was achieved by incorporating *cc*NiR in a conductive carbon ink composite, depositing it onto a PGE and optimizing the carbon ink solvents and curing temperatures. It has been shown that the combination of acetone/40 °C produced the better immobilization environment for *cc*NiR. With the aim of developing a miniaturized, inexpensive, portable and mass manufacturable nitrite biosensor, the optimized *cc*NiR/carbon ink biosensors were constructed using SPE. Despite being slightly inferior than the PGE based devices, the *cc*NiR/carbon ink/SPE demonstrated good activity for nitrite reduction (0.55 A M^{-1} cm^{-2}, 0.7–370 μM, and 1.2 μM). The miniaturized biosensor showed a relative standard deviation of 20%. This was due to the low reproducibility of the fabrication process, since it was difficult to obtain identical surface coverage on all electrodes with the manual deposition of the viscous paste composite. In future applications, this problem could be surpassed by the automation of electrode production (screen printing) and a better control of the paste's viscosity. As for storage stability, the SPE biosensor maintained 90% of its initial activity for the course of 3 weeks. These results attested for *cc*NiR's robustness, being able to endure the harsh immobilization process and staying active for a considerable period of time, paving the way for the employment of the enzyme in screen printing applications.

Table 2
Nitrite recovery percentage from three complex matrices using the *cc*NiR/carbon ink/SPE.

Sample	Original [nitrite] (μM)	[Nitrite] added (μM)	[Nitrite] found (μM)	Recovery (%)
Drinking water	ND[a]	5.0	5.3 ± 1	106.2
Tap water	ND	4.8	4.8 ± 0.2	99.0
Milk	ND	9.9	9.8 ± 0.3	99.3
Urine	ND	9.9	10.4 ± 1	104.7
Plasma	ND	197	190 ± 3	96.8

[a] ND – not detected.

Also, we have applied a bi-enzyme system, comprised of GOx/Cat/glucose, for removal of molecular oxygen. This approach showed to be an efficient alternative to the argon purging method, eliminating the need of specific lab equipment. Yet, the employment of additional proteins increases the cost of the final product. The immobilization of the oxygen scavenging system's components on the sensor will be envisaged in future work, turning the biosensor into a truly reagentless device, simplifying the operation (integrated biosensor) and saving biological material.

Lastly, the use of the ccNiR/carbon ink/SPE biosensor for the measurement of added nitrite in different types of real samples (water, milk plasma and urine) was proven very successful (1–6% error).

This approach has brought us closer to succeeding in producing commercially viable nitrite biosensors easy to operate on-site (e.g. clinical setting, field, and industry) and in the research laboratory, where the real time measurement of nitrite in blood and/or tissues would be remarkably important.

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