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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