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Nitrite/nitrate detection in serum based on dual-plate generator–collector currents in a microtrench

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ABSTRACT

A dual-electrode sensor is developed for rapid detection of nitrite/nitrate at micromolar levels in phosphate buffer media and in dilute horse serum without additional sample pre-treatment. A generator–collector configuration is employed so that on one electrode nitrate is reduced to nitrite and on the second electrode nitrite is oxidised back to nitrate. The resulting redox cycle gives rise to a specific and enhanced current signal which is exploited for sensitive and reliable measurement of nitrite/nitrate in the presence of oxygen.

The electrode design is based on a dual-plate microtrench (approximately 15 μm inter-electrode gap) fabricated from gold-coated glass and with a nano-silver catalyst for the reduction of nitrate. Fine tuning of the phosphate buffer pH is crucial for maximising collector current signals whilst minimising unwanted gold surface oxidation. A limit of detection of 24 μM nitrate and a linear concentration range of 200–1400 μM is reported for the microtrench sensor in phosphate buffer and dilute horse serum. Relative standard deviations for repeat measurements were in the range 1.8–6.9% ($n=3$) indicating good repeatability in both aqueous and biological media. Preliminary method validation against the standard chemiluminescence method used in medical laboratories is reported for nitrate analysis in serum.

1. Introduction

The construction of an electrochemical nitrate sensor that functions in the presence of oxygen in biological fluids with high selectivity against common background interferents such as cells, proteins and electrolytes, is highly desired for clinical studies and opens up the possibility of point-of-care assays for use in hospitals and healthcare settings [1,2]. The use of electrochemical sensors is attractive as they are easily miniaturised, provide relatively quick results, are of good accuracy and are suitable for use in a wide range of solutions. These advantages can enable low level detection of analytes for clinical applications, as previously demonstrated by the success of electrochemical glucose biosensors [3].

Testing for the presence of nitrate in biological fluids such as serum has attracted increasing interest in recent years for clinical and sports science applications [4]. Nitrate levels produced endogenously represent the final product of nitric oxide (NO) and nitrite oxidation pathways, therefore providing an indication of NO levels and activity [4]. In clinical studies, nitrate levels have been used as a biomarker for potential diagnosis and monitoring of human health conditions such as infective and inflammatory diseases [5], cardiovascular [6] and neurological conditions [7]. Dietary or environmental exposure to nitrate has long been considered to be harmful due to risks associated with gastric cancer [8] and methemoglobinemia [9]. More recently, dietary nitrate supplementation has been shown to reduce blood pressure and lower the oxygen cost of submaximal exercise through enhancement of NO bioavailability [10–12].

Numerous methods have been established for detection of nitrate in aqueous and biological solutions including the Griess colorimetric assay [13,14], fluorometric [15] and chemiluminescence [16,17] methods, and methods based on solid-phase separation such as high-performance liquid chromatography [18], gas chromatography–mass spectrometry [14], ion chromatography [19] and capillary electrophoresis [20]. Whilst ion-selective electrodes are established for sensitive detection of nitrate in aqueous solutions [21], these electrodes are not used for nitrate analysis in biological fluids due to the presence of ionic interferents. The two best established methods for determining nitrate in biological fluids involve the reduction of nitrate to NO using vanadium (III)

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chloride, followed by either a relatively complex chemical test, the Griess reaction, coupled with colorimetric detection, or gas-phase chemiluminescent detection [13,14,17]. In general, these techniques provide very accurate results but require the use of specialist equipment, tedious procedures and take extended periods of time to perform. Furthermore, vanadium chloride and alternative reducing agents based on cadmium reagents are highly toxic. Nitrate reductase is also used as a reducing agent but requires careful handing in a controlled environment. Despite the wide range of methods available for nitrate detection in blood there is currently no rapid and convenient means of doing so for point-of-care testing.

Several electrochemical sensors and biosensors have been developed for detection of nitrate ions in aqueous solutions [22]. However, the electrochemical response is hindered by slow charge transfer kinetics leading to poor sensitivity and irreproducible measurements [22]. A wide range of electrode materials have been sought to combat this restriction including electrodeposited and bulk reactive metals [23–26], chemically-modified electrodes with metal complexes [24], and composites based on silver graphite. Excellent limits of detection and good reproducibility have been reported in aqueous solutions but a lack of selectivity of bulk and chemically modified electrodes limits their applications in biological fluids [22]. Furthermore, the presence of dissolved oxygen impedes the reduction of nitrate and hence most electrochemical sensors to date require using degassed solutions. More sophisticated approaches have been developed based on the use of biological catalysts such as reductase enzymes which enable good sensitivity and impart a greater degree of selectively [27–29]. However, these bio-sensor systems suffer from the limitations of costly biological reagents, increasing complexity, and fragility of the electrode.

Small gap sensor electrodes operating in generator–collector mode *via* bipotentiostatic control are emerging as excellent candidates for sensing redox-active analytes at low concentrations [30]. Of particular interest are nano-gap electrodes [31–34], and simple low-cost microtrench electrodes developed recently in our laboratory [35–37]. The small spacing of these electrodes combined with the ability to control both electrode potentials independently provides access to enhanced current signals. Advantages included the ability to obtain amplified currents for a given molecule by redox-cycling ions between the two electrodes, access to steady state current responses free of capacitive current, and the ability to separate desired signals from interferents by elimination of chemically irreversible processes or by size-exclusion effects. To date, electroanalytical applications of generator–collector electrodes have been demonstrated for analytes including, for example, quinones [35,37] and dopamine in the presence of known interferents [35].

In this work, we report the development of a silver-modified gold–gold microtrench electrode for nitrate detection which shows good sensitivity in generator–collector mode within a physiologically relevant concentration range (200–1400 μM). The proposed sensing mechanism is shown in Fig. 1 (the redox system nitrite/nitrate is represented by Red/Ox). Within the microtrench, nitrate is converted by reduction to nitrite at one electrode (the "generator"), while nitrite is converted back to nitrate by oxidation at the other electrode (the "collector"). A repeating – and so signal-amplifying – redox cycle is achieved as these species interconvert and diffuse across the gap between the electrodes. After development of the electrode system in aqueous buffer, the analytical parameters of the electrode were investigated in horse serum diluted in phosphate buffer. The concentration of nitrate present in the samples was determined by the method of standard addition and compared against a standard gas-phase chemiluminescence method using a chemical analyser.

2. Experimental

2.1. Reagents

Potassium nitrate ($KNO_3 \geq 99\%$), potassium nitrite ($KNO_2 \geq 96\%$), silver nitrate ($AgNO_3 \geq 99\%$), sodium hydroxide (98%), potassium chloride ($KCl \geq 99.0\%$), monosodium phosphate monohydrate (98–102%), disodium hydrogen phosphate heptahydrate (98–102%), sulphuric acid (H_2SO_4, 95–98%), hydrochloric acid (HCl, 37%), nitric acid (HNO_3, 70%), hydrogen peroxide (H_2O_2, 30 wt% in water) were all purchased from Sigma Aldrich, UK. Zinc sulphate, sodium nitrate (98+%) and sodium iodide (99+%) were purchased from Fisher. Vanadium (III) trichloride from Merck (99+%) and glacial acetic acid (100%) from VWR International. The serum was prepared from fresh horse blood obtained from a local abattoir and stored in the freezer. Aqueous solutions were made with ultrapure water at 20 °C (resistivity ≥ 18.2 MΩcm). Nitrogen (BOC) was employed for de-aerating solutions as required. Experiments were conducted at 20 ± 2 °C.

2.2. Instrumentation

Electrochemical measurements were obtained using either a CompactStat with bipotentiostat module (Ivium Technologies, Netherlands) with IviumSoft software 2.178 or a PGSTAT12 biopotentiostat system (Autolab, EcoChemie, Netherlands) with GPES 4.7 software. A conventional three or four-electrode cell was employed with a Pt wire counter electrode and saturated calomel electrode (SCE, Radiometer). The three electrode cell employed either a gold rod electrode (⌀=2 mm) or one of the two electrodes of the microtrench. The four-electrode cell set-up employed the two working electrodes of the microtrench electrode.

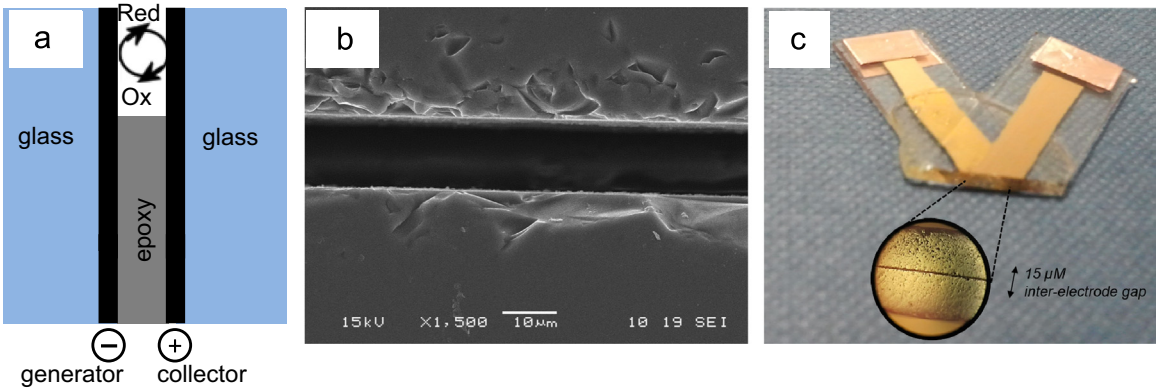

Fig. 1. (a) Schematic drawing of the nitrate sensing mechanism by coupled nitrate reduction and nitrite oxidation. (b) Scanning electron microscopy (SEM) micrograph of the microtrench. (c) Photograph of the microtrench electrode with optical image of the inter-electrode gap.