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Conductometric nitrate biosensor based on methyl viologen/Nafion®/nitrate reductase interdigitated electrodes

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Abstract

A highly sensitive, fast and stable conductometric enzyme biosensor for determination of nitrate in water is reported for the first time. The biosensor electrodes were modified by methyl viologen mediator mixed with nitrate reductase (NR) from *Aspergillus niger* by cross-linking with glutaraldehyde in the presence of bovine serum albumin and Nafion® cation-exchange polymer. The process parameters for the fabrication of the enzyme electrode and various experimental variables such as pH, the enzyme loading and time of immobilization in glutaralaldehyde vapor were investigated with regard to their influence on sensitivity, limit of detection, dynamic range and operational and storage stability. The biosensor can reach 95% of steady-state conductance value in about 15 s. Linear calibration in the range of 0.02 and 0.25 mM with detection limits of 0.005 mM nitrate was obtained with a signal-to-noise ratio of 3. When stored in 5 mM phosphate buffer (pH 7.5) at 4 °C, the sensor showed good stability over 2 weeks.
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1. Introduction

During the past two centuries, the human species has substantially altered the global nitrogen cycle, increasing both the availability and the mobility of nitrogen over large regions of Earth [1–3]. Consequently, in addition to natural sources, inorganic nitrogen can nowadays enter aquatic ecosystems via anthropogenic input such as animal farming, urban and agricultural runoff, industrial wastes and sewage effluents (including effluents from sewage treatment plants that are not performing tertiary treatments). As a result, concentrations of nitrate in ground and surface waters are increasing around the world, causing one of the most prevalent environmental problems on a worldwide scale. Water containing high concentrations of nitrate can create serious problems, such as eutrophication of rivers, deterioration of water quality and potential hazard to human health, because nitrate in the gastrointestinal tract can be reduced to nitrite ions. In addition, nitrate and nitrite have the potential to form N-nitrous compounds, which are potential carcinogens [4]. Another toxic affect of high concentrations of nitrites is the rare disease of methemoglobinema or "baby blue syndrome" [5]. Predominantly on the ground of human health most countries have imposed limits for nitrate in drinking water of 25–50 mg/l (0.4–0.8 mM) [6].

Spectro photometric methods for the determination of nitrate have been developed over the past several decades [7–10]. Ion exchange chromatography combined with spectrometric, conductimetric or electrochemical detection has recently become popular for the detection of nitrate [4,11–13]. However, even though they have high sensitivity and good reproducibility, these methods require large and expensive instrument and extensive pre-treatment of the sample. Nitrate ion-selective electrodes

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(ISEs) and ion-sensitive field-effect transistors (ISFETs), which provide fast response time, simplicity, low cost, and can be easily adapted to flowing streams and in situ measurements but suffer from poor stability and severe interference effect caused by other anions present in the sample [14].

Biosensors incorporating enzymes have also been used for nitrate analysis in real samples with reasonable sensitivity and selectivity. The majority of biosensors reported for the measurement of nitrate ions are molecular-based systems using a nitrate reductase enzyme purified from plant, fungal or bacterial sources. Both electrochemical and optical biosensors have been reported [15–19].

Nitrate reductases (NR) are produced by a variety of animals, plants, and microorganisms including fungi. The enzyme is a homodimer composed of two identical subunits of approximately 100 kDa, each of which contains three cofactors, flavin adenine dinucleotide (FAD) which is the site for NAD(P)H oxidation, heme-iron (heme-Fe) and Mo-molybdopetrin (Mo-MPT) which is the site for nitrate reduction in a 1:1:1 ratio [20]. NR can be reduced by NADH, NADPH or both nucleotides, in the case of abispecific enzyme, which catalyzes the reduction of NO_3^- to NO_2^-:

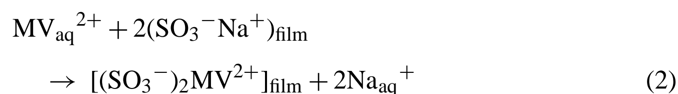
$$\mathrm{NAD(P)H} + \mathrm{NO_3^-} + \mathrm{H^+} \rightarrow \mathrm{NAD(P)^+} + \mathrm{NO_2^-} + \mathrm{H_2O} \quad (1)$$

While the NAD(P)H dependent activity of assimilatory enzymes is more rapidly lost than its nitrate reduction activity, it was shown that assimilatory nitrate reductases can be supplied with redox equivalents by redox mediators like methyl viologen, that work as artificial electron donor [21]. Methyl viologen can be reductively regenerated with sodium dithionite or electrochemically [22].

Several reports addressed the development of amperometric biosensor devices for nitrate have also used suitable redox mediators including oxygen or nitrogen heterocycles, triphenyl methane dyes and sulphonphtaleine dyes mainly as the electron transfer between the electrode and the enzyme [6,23–25]. To our best knowledge, no previous work describing the coimmobilization of nitrate reductase enzyme and redox mediator at an electrode surface resulting in conductometric biosensors has been reported to date. Conductometric sensors for biosensing devices have been introduced by Watson et al. [26]. The device consisted of a planar glass support with interdigitated gold electrode pairs on one surface in a planar configuration. The principle of the detection is based on the fact that many biochemical reactions in solution produce changes in the electrical resistance.

Conductance measurements involve the resistance determination of a sample solution between two parallel electrodes. For the direct assaying of many enzymes and their substrates, the conductometric biosensors present a number of advantages: (a) the planar conductometric electrodes are simple and relatively cheap which suits for miniaturization and large scale production, and therefore promising for practical use, (b) they do not require a reference electrode, (c) the applied voltage can be sufficiently small to minimize substantially the sensor's power consumption, (d) large spectrum of analytes of different nature can be determined on the basis of various reactions and mechanisms.

In the present study, we present a conductometric nitrate biosensor by the coimmobilization of nitrate reductase from *Aspergillus niger* (EC 1.6.6.2) and methyl viologen in the Nafion® films at an interdigitated thin-film electrodes surface. Since viologens are highly water soluble and toxic, any practical device containing these electron mediators should be based on immobilized viologens [27,28]. Methyl viologen's electrochemical behavior involves reduction of MV^{2+}. The structure of MV^{2+} consists of a hydrophobic part that is capable of hydrophobic–hydrophobic interaction with Nafion® and two cationic pyridinium groups that undergo ion exchange with the sulphonate sites of Nafion® polymer chains, according to:



$$\mathrm{MV_{aq}}^{2+} + 2(\mathrm{SO_3}^-\mathrm{Na}^+)_{\mathrm{film}} \rightarrow [(\mathrm{SO_3}^-)_2\mathrm{MV}^{2+}]_{\mathrm{film}} + 2\mathrm{Na_{aq}}^+ \quad (2)$$

This interaction results in accumulation of MV^{2+} in Nafion® films. In the presence of dithionite as electron donor, the biocatalyzed reduction of NO_3^- to NO_2^- is stimulated. The reaction was as follows.

$$\mathrm{NO_3^-} + 2\mathrm{MV^{\bullet+}} + 2\mathrm{H^+} \rightarrow \mathrm{NO_2^-} + 2\mathrm{MV^{2+}} + \mathrm{H_2O} \quad (3)$$

The subsequent local changes of conductance inside the membrane is dependent on the reaction (3), and thus the conductometric nitrate sensor devices are assembled. The performance of the conductometric nitrate biosensor was evaluated by the detection of nitrate in water solution.

2. Experimental

2.1. Reagents

Nitrate reductase (EC 1.1.6.6.2) from *A. niger*, bovine serum albumin (BSA) and aqueous solutions (25%, w/v) of glutaraldehyde (GA), methyl viologen, Nafion® (perfluorosulfonated ion-exchange resin, 5% (w/v) solution in a solution of 80% aliphatic alcohol and 20% water mixture) and sodium hydrosulfite were purchased from Sigma–Aldrich Chemie GmbH. Sodium nitrate and sodium dithionite were from Merck. All other chemicals were of analytical grade.

2.2. Apparatus

The conductometric transducers were fabricated at the Institute of Chemo- and Bio-sensorics (Munster, Germany). Two pairs of Au (150 nm thick) interdigitated electrodes were made by the lift-off process on the pyrex glass substrate (10 mm × 30 mm). A 50 nm thick intermediate Ti layer was used to improve the adhesion of Au to the substrate. The central part of the sensor chip was closed by epoxy resin to define the electrode sensitive area. Both the digit width and interdigital distance were 10 μm, and their length was about 1.0 mm. As a result, the "sensitive" area of each electrode was about 1.0 mm^2.

The internal generator of a Stanford Research System SR 830 lock-in amplifier (Sunnyvale, CA, USA) was employed to

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