Development of a bienzyme system for the electrochemical determination of nitrate under ambient air

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Abstract This work reports the development of a bienzyme system consisting of salicylate hydroxylase (SHL) and nitrate reductase (NaR) for the electrochemical determination of nitrate. This method measures the concentration of nitrate directly under ambient air without suffering from oxygen interferences. The determination is based on the detection of NADH consumption, and the principle is as follows: NADH initiates the irreversible decarboxylation and hydroxylation of salicylate by SHL in the presence of oxygen to produce catechol, which results in a detectable signal due to its oxidation at the working electrode; the second enzyme NaR, in the presence of nitrate, reduced the availability of NADH, and consequently, the current difference after the injection of nitrate is proportional to its concentration. This method shows high performance characteristics for nitrate determination with a broad detection range between 10 μ M and 1000 μ M, a short measuring time of around 5 min, and a simple operation without sample pretreatment by inert gas purge or oxygen scavenger.

Keywords *Electrochemical determination · Nitrate · NADH · Nitrate reductase · Salicylate hydroxylase*

Introduction

Nitrate is a well-known alarming pollutant to both environment and human health. The excess nitrate in environmental water systems causes algal blooms, a depletion of dissolved oxygen, and possible eutrophication. Nitrate in the human body, causes methemoglobinemia (also called “baby blue syndrome”), and also may become a source of carcinogenic N-nitrosamines, some gastrointestinal cancers and teratogenic effects [1-3]. Predominantly considering human health, most countries have imposed limits for nitrate in drinking water of 25–50 mg/l (0.4–0.8 mM) [4].

Due to the high specificity of nitrate reductase (NaR) and the easy-to-handle operation of electrochemical device, various enzyme-based electrochemical detections for nitrate have been widely performed using combinations of NaR and different mediators to detect the

electron-transfers [3-19]. However, the low redox potential of this enzyme and its matching mediators suffer from oxygen interferences. Therefore, these measurements require the dissolved oxygen to be removed from the sample by an inert gas purge or an oxygen scavenger prior to the analysis.

Besides the participation of mediators in the enzymatic reaction for nitrate, NADH could also be utilized as a cofactor by NaR. To the best of our knowledge, this feature hasn't been applied to the enzyme-based electrochemical determination of nitrate by the detection of NADH. Recently, salicylate hydroxylase (SHL) is used as a signal generator and a sensing unit for NADH in an electrochemical detection, which results in high performance characteristics [20]. In this work, we present an electrochemical determination of nitrate using a bienzyme system consisting of SHL and NaR, where both enzymes utilize NADH as a cofactor. This method is performed under ambient air, it has a simple operation without sample pretreatment by inert gas purge or oxygen scavenger to avoid the oxygen interferences, it is free from the uses of mediators, and also easy to adapt for the hand-held devices.

As shown in Fig. 1, the determination principle is as follows: NADH initiates the irreversible decarboxylation and hydroxylation of salicylate by SHL in the presence of oxygen to produce catechol, which results in a detectable signal due to its oxidation at the screen-printed working electrode. The presence of nitrate reduces the availability of NADH which is achieved by using NaR, and further reduces the production of catechol at the working electrode and its oxidation current. Consequently, the stationary current difference after the injection of nitrate is proportional to the concentration of nitrate.

Experimental

Apparatus

A potentiostat EP30 (Biometria, Germany) and a computer installed with the software FIABOLO were used. The screen-printed electrode (BioSensorTrend, Germany) with a 2-electrode configuration was composed of a platinum (Pt) working electrode (diameter: 1 mm) and an Ag/AgCl reference/counter electrode. A measuring cell (volume: 1 ml), connected to a

syringe and installed with two connectors (cathode and anode) and an electrode-fitting site, was constructed for experiments.

Chemicals

Salicylate hydroxylase (SHL, EC 1.14.13.1, from *Pseudomonas* sp.) was from GDS Technology Inc. (USA). Sodium salicylate was from Nacalia Tesque Inc. (Japan). Potassium nitrate was from Fisher Scientific (USA). Nitrate reductase (NaR, EC 1.7.1.1., from corn leaves) was from Nitrate Elimination Company Inc. (USA). 2-amino-2-(hydroxymethyl)-1, 3-propanediol (TRIZMA®BASE, Tris), β -nicotinamide adenine dinucleotide hydrate reduced form (NADH), and glutaraldehyde were purchased from Sigma-Aldrich (USA). All solutions were prepared in double distilled water.

Enzyme-electrode preparation

The screen-printed working electrode was covered by a mixture containing 0.25 U SHL and 1% glutaraldehyde (immobilization agent) in 0.5 μ l of enzyme matrix with high reproducibility, which was performed manually using an autopipet, followed by drying and storing at 4 °C overnight. The enzyme electrode was then screwed into the measuring cell, which was filled with buffer solution, and rehydrated for around 1 h at room temperature (20 °C) to allow the enzyme matrix swelling before usage.

Measurement for nitrate

One ml of Tris-HCl buffer (100 mM, pH 8.0) containing 10 mM salicylate, 1 mM NADH, and 0.5 U ml⁻¹ NaR was added into the measuring cell which had been equipped with the SHL-based electrode. Experiments were performed at room temperature by applying the specific potential for this type of screen-printed electrode at 0.42 V (the recommended potential by BioSensorTrend) and magnetically stirring the buffer solution at 300 rpm. When a constant current was obtained, the measurement for nitrate was started by adding 100 mM of standard nitrate solution (0.1–20 μ l) into the buffer, and until a stationary current occurred, the current difference was recorded for plotting the calibration curve. After the measurement, a syringe was used for sucking the buffer solution out of the measuring cell to remove nitrate,

followed by the addition of a blank buffer (containing no nitrate) for starting a new measurement.

Results and Discussion

Investigation of the enzyme electrode

The effect of the enzyme matrix on electrode was investigated. Several enzyme electrodes, with the SHL loadings from 0.10 U to 0.50 U and glutaraldehyde loadings from 0.5% to 1.5%, were compared by detecting 1 mM NADH in 100 mM Tris-HCl buffer containing 10 mM salicylate at pH 8.0 (see Electronic Supplementary Material, Fig. S1). From the investigation, a mixture containing 0.25 U SHL and 0.5% or 1% glutaraldehyde in 0.5 μ l of enzyme matrix on a screen-printed electrode showed a maximum current response, and 0.25 U SHL and 1% glutaraldehyde exhibited a better stability, which was used for further experiments.

Investigation of the buffer solution

To obtain an optimal buffer pH for the SHL-based electrode, the investigations were performed by measuring 1 mM NADH in 100 mM Tris-HCl buffer containing 10 mM salicylate at various pH values ranging from 6.5 to 10.0 (see Electronic Supplementary Material, Fig. S2). The maximum current response was obtained between pH 7.5 and pH 8.5. Therefore, pH 8.0 which in the middle of the optimal pH range for the SHL-based electrode was used for other investigations.

The effect of salicylate was investigated on the performance of the SHL-based electrode. Various molar ratios of salicylate to NADH were tested by measuring 1 mM NADH in 100 mM Tris-HCl buffer containing different amounts of salicylate at pH 8.0 (see Electronic Supplementary Material, Fig. S3), and the saturated signal response was obtained at a molar ratio of 0.5. In this work, a sufficient salicylate concentration of 10 mM in buffer was used for other investigations in order to avoid the signal saturation due to its inadequate loading.

In the investigation of NADH concentration in buffer, the measurements for NADH were performed in 100 mM Tris-HCl buffer containing 10 mM salicylate at pH 8.0 (see Electronic

Supplementary Material, Fig. S4). A linear relationship was obtained between the current response and the concentration of NADH from 5 μM to 3.6 mM. Thus, a NADH concentration in the linear detection range could be used for the determination of nitrate in order to obtain a sensitive detection range, a higher concentration of NADH resulted in a higher upper detection limit for nitrate while did not improve the lower detection limit, and 1 mM NADH in buffer was chosen for the determination of nitrate. Also, the SHL-based electrode had a fast measuring time (20 s) for the detection of NADH.

The effect of NaR was investigated by measuring 1 mM nitrate in 100 mM Tris-HCl buffer containing 10 mM salicylate, 1 mM NADH and various loadings of NaR (0.3 U mL^{-1} , 0.5 U mL^{-1} , 0.7 U mL^{-1} or 1.0 U mL^{-1}) at pH 8.0 (see Electronic Supplementary Material, Fig. S5). With the increasing of NaR loading in buffer solution from 0.3 U mL^{-1} to 1.0 U mL^{-1} , the time for achieving a steady state after the injection of nitrate decreased from around 9 min to 2 min, while the enzyme loading did not affect the current response apparently, and 0.5 U mL^{-1} NaR with a steady-state measuring time of 5 min was used for the determination of nitrate. Also, the pH 8.0 of the buffer solution was within the optimal pH range for NaR activity, which was from 7.5 to 8.5.

Electrochemical determination of nitrate

Fig. 2 shows the calibration curve for nitrate using the bienzyme system. The measurements were performed with a SHL-based electrode containing 0.25 U SHL and 1% glutaraldehyde and a 100 mM Tris-HCl buffer containing 10 mM salicylate, 1 mM NADH and 0.5 U mL^{-1} NaR at pH 8.0. The buffer solution resulted in a constant anodic current at the working electrode due to the production of catechol from the SHL-catalyzed reaction of NADH with salicylate and oxygen, followed by the addition of nitrate into the buffer, and as the concentration of nitrate was proportional to the consumption of NADH due to the NaR-catalyzed reaction, it was further proportional to the decrease of catechol concentration at the working electrode and the decrease of the steady-state anodic current. As shown in the figure, a linear relationship was obtained between the current response (net current) and the concentration of nitrate from 10 to 1000 μM with a detection limit of 5.6 μM (slope: 487.1 nA mM^{-1} , $R^2=0.9970$, $n=3$). A sharp saturation at 1000 μM was observed due to the NADH depletion by the enzymatic reaction of NaR for nitrate.

Fig. 3 shows the current-time curve of the bienzyme system to various concentrations of nitrate. This method had a fast response of 2 s, a short steady-state measuring time of around 5 min and a quick recovery of 1 min with high reproducibility at a NaR loading of 0.5 U ml^{-1} , as shown in the figure. Hence, in this case, the determination of nitrate took around 5 min, and the total operation time for one measurement was around 6 to 7 min. The measuring time and the total operation time could be less at a higher NaR loading, and such as at a NaR loading of 1 U ml^{-1} , the determination of nitrate took around 2 min and the total operation time was around 3 min, while this would consume more enzymes and not affect the determination result.

The bienzyme system, including the enzyme electrode and the measuring buffer, was stored at 4°C with the electrode in the absence of NADH, and the system was stable for the measurement of nitrate, which could maintain around 90% of the initial response after one week's usage by performing several measurements each day. The reduction of performance was probably due to the reduced lifetimes of enzymes during storage and the electrode surface fouling during measurements.

Conclusions

In this work, we have demonstrated an electrochemical measurement of nitrate using a bienzyme system consisting of SHL and NaR under ambient air. It shows high performance characteristics with a broad detection range, a short measuring time and a simple operation without sample pretreatment by inert gas purge or oxygen scavenger. Thus, we provide a new analytical approach for the determination of nitrate that is specific, sensitive, fast, and easy to handle.

Acknowledgements

The authors gratefully acknowledge Hong Kong Research Grant Council (HKUST6253-02P and 510-0091-02-44000-0) for the financial support.

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Captions

Fig. 1 Schematic illustration of the principle for the determination of nitrate (SHL: salicylate hydroxylase, NaR: nitrate reductase. SPE: screen-printed electrode)

Fig. 2 Calibration curve for nitrate using the bienzyme system (n=3) (Enzyme electrode: 0.25 U SHL and 1% glutaraldehyde in 0.5 μ l of enzyme matrix. Buffer: 100 mM Tris-HCl buffer containing 10 mM salicylate, 1 mM NADH and 0.5 U ml^{-1} NaR at pH 8.0)

Fig. 3 Current-time curve of the bienzyme system to (a) 1000 μ M (b) 500 μ M and (c) 250 μ M nitrate with (w) washing step (n=3) (Enzyme electrode: 0.25 U SHL and 1% glutaraldehyde in 0.5 μ l of enzyme matrix. Buffer: 100 mM Tris-HCl buffer containing 10 mM salicylate, 1 mM NADH and 0.5 U ml^{-1} NaR at pH 8.0)

Figures

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Fig. 3 Current-time curve of the bienzyme system to (a) 1000 μM (b) 500 μM and (c) 250 μM nitrate with (w) washing step ($n=3$) (Enzyme electrode: 0.25 U SHL and 1% glutaraldehyde in 0.5 μl of enzyme matrix. Buffer: 100 mM Tris-HCl buffer containing 10 mM salicylate, 1 mM NADH and 0.5 U ml^{-1} NaR at pH 8.0)