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Electrochemical Determination of Nitrate with Nitrate Reductase-Immobilized Electrodes under Ambient Air

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Nitrate monitoring biosensors were prepared by immobilizing nitrate reductase derived from yeast on a glassy carbon electrode (GCE, d = 3 mm) or screen-printed carbon paste electrode (SPCE, d = 3 mm) using a polymer (poly(vinyl alcohol)) entrapment method. The sensor could directly determine the nitrate in an unpurged aqueous solution with the aid of an appropriate oxygen scavenger: the nitrate reduction reaction driven by the enzyme and an electron-transfer mediator, methyl viologen, at −0.85 V (GCE vs Ag/AgCl) or at −0.90 V (SPCE vs Ag/AgCl) exhibited no oxygen interference in a sulfite-added solution. The electroanalytical properties of optimized biosensors were measured: the sensitivity, linear response range, and detection limit of the sensors based on GCE were 7.3 nA/μM, 15−300 μM (r^2 = 0.995), and 4.1 μM (S/N = 3), respectively, and those of SPCE were 5.5 nA/μM, 15-250 μM (r^2 = 0.996), and 5.5 μM (S/N = 3), respectively. The disposable SPCE-based biosensor with a built-in well- or capillary-type sample cell provided high sensor-to-sensor reproducibility (RSD < 3.4% below 250 μM) and could be used more than one month in normal room-temperature storage condition. The utility of the proposed sensor system was demonstrated by determining nitrate in real samples.

Nitrate has been widely exploited to better our lives, excessively disposed into the ecosystem, and recognized as an alarming pollutant to the environment and human health.[1−3] The excess nitrate in environmental water systems causes algal blooms, a depletion of dissolved oxygen, and possibly eutrophication.[4] Nitrate in the human body, in addition to being the cause of methemoglobinemia, may become a source of carcinogenic *N*-nitrosamines.[1−7] For these reasons, accurate, rapid, and economic determination of nitrate has attracted much attention in analytical chemistry.[1] The required limit of determination, however, still needs either time-consuming sample pretreatment or elaborate instrumentation that is not suitable for in situ monitoring.[8,9]

Many different types of biosensors have been developed for point-of-care medical testing or in situ environmental monitoring for almost a half-century since the inception of Clarke-type electrodes. Nitrate monitoring biosensors also have been constructed and applied for the detection of nitrate in various matrixes.[3−8,10−23] However, the practical utility of nitrate reductase (NaR)-based biosensors for nitrate monitoring has been severely limited because of unique structural and electrochemical properties of NaR.

NaR is a mutidomain enzyme containing flavin adenine dinucleotide (FAD), heme-Fe, and molybdopterin, which cooperatively catalyzes the 2-electron reduction of nitrate to nitrite.[24,25] The crystallographic structure of NaR and its reaction mechanism in solution and on electrodes has been actively studied.[26−31] Because the active site of NaR is deeply embedded in the protein,

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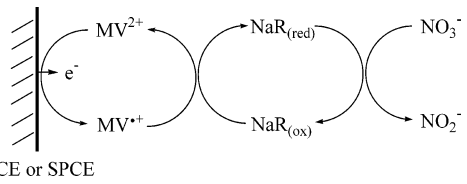

Figure 1. Detection principle of nitrate reduactase-immobilized electrode.

an electron-transfer mediator is required for efficient electron communication between electrode and NaR.[32] Figure 1 shows the overall reaction mechanism of the electrochemically driven nitrate reduction reaction via NaR and an electron-transfer mediator. Since the redox potential of FAD unit in NaR is about −400 mV (vs Ag/AgCl, pH 7.0),[25] matching mediators are selected from a group of organic dyes with low redox potentials.[8,33] The low redox potentials of NaR and its matching mediator, however, require that the dissolved oxygen be removed from the sample by an inert gas purging prior to the analysis, which is unsuitable for in situ monitoring.

In this work, we demonstrate that the yeast-originated NaR (Y-NaR)-immobilized biosensor can directly determine nitrate in an unpurged aqueous solution with simple treatment of the sample: addition of a chemical oxygen scavenger can drive the electrochemical nitrate reduction reaction without suffering from the oxygen interference.[34,35] Two different types of electrodes, glassy carbon electrode (GCE) and screen-printed carbon paste electrode (SPCE), were used to immobilize the Y-NaR, and their electrochemical properties in the presence of an oxygen scavenger have been investigated. To demonstrate the practical utility of SPCE-based biosensors for in situ monitoring, a capillary-type sample cell was built on the enzyme-immobilized SPCE for easier sampling and the biosensor was used to determine nitrate in various real samples such as river and tap waters.

EXPERIMENTAL SECTION

Chemicals. Standard nitrate solution was purchased from SpexCertiprep (Metuchen, NJ). Sodium sulfite (99.99%), 1,1′-dimethyl-4,4′-bipyridinium dichloride (methyl viologen, 98%), 2-(*N*-morpholino)ethanesulfonic acid (MES, 99.5%), 3-(*N*-morpholino)-propanesulfonic acid (MOPS, 99.5%), and Nafion solution (5 wt % in alcohol) were purchased from Sigma-Aldrich (Milwaukee, WI) and used as received. Poly(vinyl alcohol) (PVA, M_w ~22 000, ash ≤0.05%) was purchased from Fluka (Buchs, Switzerland). Nitrate reductase (EC 1.6.6.1, purified from yeast, designated as Y-NaR by the supplier, 1 U/vial, equals to 0.048 unit/mg activity, lyophilized powder) was commercially purchased from the Nitrate Elimination Co., Inc. (NECi, Inc., Lake Linden, MI) and used as received. The enzyme solution (350 mg/mL protein concentration) was prepared by dissolving one vial of the enzyme in 60 μL of 0.05 M MOPS buffer, pH 7.0. All other chemicals were of at least reagent grade and were used without further purification. Deionized water (18 MΩ·cm) from a Milli Q water purification system was used for preparing buffer and stock solutions. Sodium sulfite solution was freshly prepared just before the experiment. The real samples were collected locally (Han River from Paldang dam, Kyungki-Do, Korea; filtration and tap water from Seoul, Korea). The samples were normally collected and used within the same day, but stored for up to 2 days at 4 °C.

Apparatus. A BAS 50W (West Lafayet, IN) or cDAQ-1604 (Elbio Co.) potentiostat was used to run cyclic voltammograms (CVs) and measure current−time responses (chronoamperometry). For the Y-NaR-immobilized GCE, glassy carbon working electrode (d = 3 mm, BAS), platinum wire counter electrode (coil), and Ag/AgCl reference electrode (3 M KCl, BAS) were used, and buffer solution in a 5-mL cell was continuously stirred by magnetic bar during the amperometric experiments. For the Y-NaR-immobilized SPCE, carbon paste working electrode (d = 3 mm), carbon paste counter electrode, and Ag/AgCl reference electrode printed all in one plate as shown in Figure 2 were clamped with a well-type 100-μL cell, and nitrate-containing solutions were dropped in the well for stationary amperometric experiments. All potentials in the text are referenced to the Ag/AgCl electrode.

Ion chromatography (IC, Dionex 500, Sunnyvale, CA) and spectrophotometry (DR 2000, HACH, Loveland, CO) were used for the comparative determination of nitrate in real samples. The IC system was equipped with an AS 40 automated sampler, a GP 40 gradient pump, an Ionpac AS14A analytical column (4 × 250 mm) with an AG 14A guard column (4 × 50 mm), an ASRS-Ultra II self-regenerating suppressor in external water flow mode using high pressurized reservoir containing ultrapure deionized water, and an ED40 electrochemical detector and operated at a flow rate of 1 mL/min with 0.1 M $NaHCO_3$/0.8 M Na_2CO_3. The spectrophotometric measurement was made with the cadmium reduction method using the reagent NitraVer from HACH. A spectrophotometric cell was filled with 25 mL of sample after filtering it through the 0.47-mm circle size glass microfiber filters (GF/C, Catalog No. 1822 047, Whatman), adding one NitraVer nitrate reagent (powder pillow) into the cell, and placing it into a spectrophotometer to read the absorbance at 500 nm.

Preparation of SPCE. A semiautomatic screen-printing machine (LS-150 type, Newlong Seimitsu Kogyo Co., Ltd.) and a set of stencils (Daeshin Co.) patterned for the electrodes shown in Figure 2 were used to prepare SPCEs. The substrate of the SPCE is a 0.5-mm-thick flexible polyester (PE, Korea 3M, Seoul, Korea) plate, which was cut in to a 10 cm × 15 cm piece and thermally treated at 150 °C for 6 h before printing to prevent further thermal shrinkage in the following process. Silver paste (LS-506J type, Asahi Chemical Research Lab. Co., Ltd.), carbon paste (TU-15ST-S type, Asahi Co.), and insulator ink (Seoul Chemical Research Laboratory, Shiheung, Korea) were sequen-

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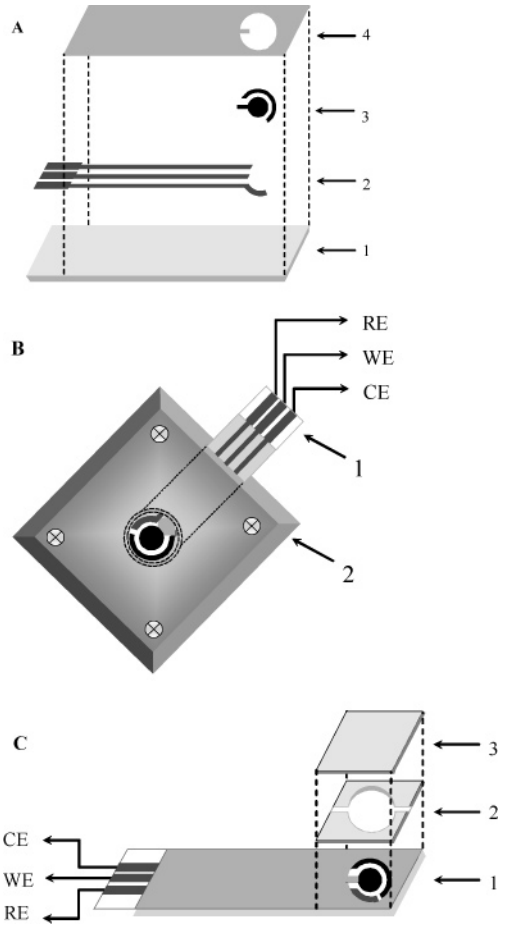

Figure 2. Screen-printed carbon paste electrode. (A) Fabrication process: 1. polyester (PE) substrate; 2, silver connectors and electrode site; 3, carbon paste electrodes; and 4, insulator. (B) SPCE with sample well: 1, SPCE; 2, 100-μL sample well block. and (C) SPCE with capillary sample cell: 1, SPCE; 2, capillary fluidic path formed on double-sided tape; and 3, cover plate.

tially screen-printed on the thermally pretreated PE plate, using the stencils corresponding to each layer pattern shown in Figure 2, and dried at 150 °C for 10 min after each printing process. The overall dimension of an individual SPCE strip was 8 mm × 34 mm. The diameter of the round-shaped working electrode was 3 mm. The Ag/AgCl reference electrode was prepared by oxidative treatment of the dried silver paste using 3 M $FeCl_3$ solution.

Y-NaR Immobilized Electrodes. The GCE electrodes were polished first with 1- and 0.3-μm alumina polishing powder on a polishing pad (Buehler), followed by sonication for 5 min in a water/ethanol solution (50 v/v %) and were air-blow dried. A 1.5-μL aliquot of Y-NaR enzyme solution was homogeneously mixed with 1.5 μL of aqueous PVA (20 wt %) in a 0.5-mL tube with cap. The mixture was then applied onto the polished electrode surface using an air pressured dispenser (2000XL, East Providence, RI) and allowed to dry in air for ~1 h. Y-NaR and the mediator methyl viologen (MV) were also coimmobilized on GCE to see the electroanalytical performance is improved: 3 μL of a Nafion solution (5 g/L in ethanol), 3 μL of 10^{-3} M MV, and 3 μL of the enzyme solution were sequentially applied and dried on the GCE. The Y-NaR-immobilized SPCEs were prepared by applying a mixture of 1.5 μL of enzyme solution and 1.5 μL of aqueous PVA (1 wt %) on the working electrode of SPCE and drying them in air. When not in use, the Y-NaR-immobilized electrodes were normally stored in refrigerator at 4 °C.

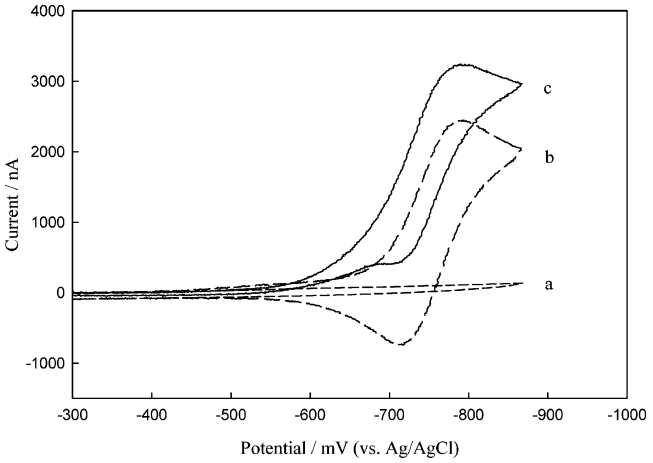

Figure 3. Cyclic voltammograms with Y-NaR-immobilized GCE in argon-purged 0.05 M MOPS buffer, pH 7.0, at scan rate of 2 mV/s: (a) in buffer only, (b) with 10^{-3} M MV, and (c) with 200 μM nitrate and 10^{-3} M MV.

Electrochemical Properties of Y-NaR-Immobilized Electrodes. To characterize the Y-NaR-immobilized electrodes, the effects of MV concentration, pH, buffer concentration, scavenger concentration, amount of enzyme loading, and storage stability were examined. Activities of Y-NaR in solution, PVA matrix, and electrode surface adsorbed were also examined. The amperometric measurements with Y-NaR-immobilized GCE were carried out in a thermostated cell at 25 ± 0.2 °C with Ar purging of the sample solution and with the addition of oxygen scavenger without purging. For Y-NaR-immobilized SPCE, the apparent currents were obtained at room temperature. To increase the accuracy of measurements, corresponding background currents were also measured with the same amount of thermally inactivated Y-NaR-immobilized SPCE. The difference of the two measurements (ΔI, net currents) was used to indicate the magnitude of current responses. The applied working potentials in amperometric experiments were −850 and −900 mV for Y-NaR-immobilized GCE and SPCE, respectively. MOPS buffer or MES buffer containing 5×10^{-4} M EDTA was used in all experiments. The small amount of EDTA in buffer solution was used to keep the heavy metal ions (e.g., Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, etc.) from interacting with Y-NaR. All current responses were normalized with the mean values of the electrodes to compensate the variations resulted from different fabrications of the Y-NaR-immobilized electrodes.

RESULTS AND DISCUSSION

The electrocatalytic properties of the enzyme Y-NaR on GCE electrode in the presence of the electron-transfer mediator methyl viologen (1×10^{-3} M) were examined first in a Ar-purged 0.05 M MOPS buffer (pH 7.0) (a) without and (b) with 200 μM nitrate. The CV in Figure 3 shows that the addition of nitrate largely changed the well-defined CV trace of MV, generating a large cathodic peak current (I_{pc}) on a quasi-sigmoidal shaped CV, a typical indication of catalytic electrochemical process. Previous evidences showed that $MV^{\bullet+}$ radical does not reduce nitrate.[3,6,15] A similar electrocatalytic behavior in the presence

of MV was observed previously with the biosensor based on the NaR-immobilized ultra-thin-film composite membrane.[15] Figure 3 also suggests that the amperometric determination of nitrate with Y-NaR-immobilized GCE and diffusional MV requires at least −600 mV of applied potential or preferably −850 mV where the conversion of MV to $MV^{\bullet+}$ radical is almost complete.

The enzyme Y-NaR has been used in three different ways: (1) dissolved in a measuring buffer solution, (2) simply adsorbed on the electrode, and (3) entrapped on the electrode using PVA solution; the relative activities of Y-NaR measured by the peak currents were 100, 86, and 92%, respectively. It is worth noting that the activity of the polymer-entrapped enzyme is larger than that of the adsorbed and only a little smaller than that of the free in solution, suggesting that the polymer PVA tends to stabilize the enzyme on the GCE electrode.[36]

We then examined coimmobilized electron-transfer mediator with Y-NaR to determine whether it may result in enhanced performance. Two different immobilization methods, which are considered to be suitable for mass fabrication, have been tested: (1) a sequential stacking of negatively charged Nafion, MV^{2+}, and negatively charged Y-NaR at neutral pH,[3,19,37] and (2) coentrapment of Y-NaR and the mediator in hydrophilic polymer PVA. Of the two methods, catalytic effect could not be observed for the former, probably due to the restricted mobility of immobilized MV. On the other hand, the coentrapped enzyme and mediator exhibited almost the same CV as that with the diffusional MV. Hence, we opted to use the PVA entrapment method for immobilizing the enzyme or the mediator in this work. The electroanalytical performances of Y-NaR-immobilized GCE and SPCE have been optimized by systematically adjusting the relative compositions of the immobilizing matrix, enzyme, and mediator, and also by empirically determining the buffer type, its pH, and the concentrations of oxygen scavenger.

The GCE-based biosensor was prepared to use in batch determination of nitrate in a laboratory environment. Hence, the Y-NaR was immobilized in a relatively thick gel layer consisting of 20 wt % PVA, which provided stable amperometric responses for hours in a vigorously stirred sample solution. The enzyme layer formulated with a low concentration of PVA was easily peeled off and resulted in short-use lifetime with increased noise level. The SPCE-based biosensor was prepared essentially for disposable use: the assay is to be made on-site, and a quick and uniform mixing of the reaction layer with collected sample is necessary. Experiments showed that the enzyme layer formulated with ∼1 wt % PVA exhibited the best performance in terms of signal stability and long-term storage.

The optimal pH condition for the Y-NaR-immobilized GCE electrode was determined over the pH range of 5.5−8.0 (MES buffer pH 5.5−6.5, and MOPS buffer pH 6.5−8.0). Figure 4 shows the pH profile of the Y-NaR-immobilized GCE with 1×10^{-3} M MV in an Ar-purged 0.05 M MOPS buffer. The highest current was found at pH 6.5, which is slightly acidic compared to those given in previous reports.[3,6,15,21,22] It may be due to the increased stability of $MV^{\bullet+}$ radical in acidic condition (pH 4.0−6.0).[21]

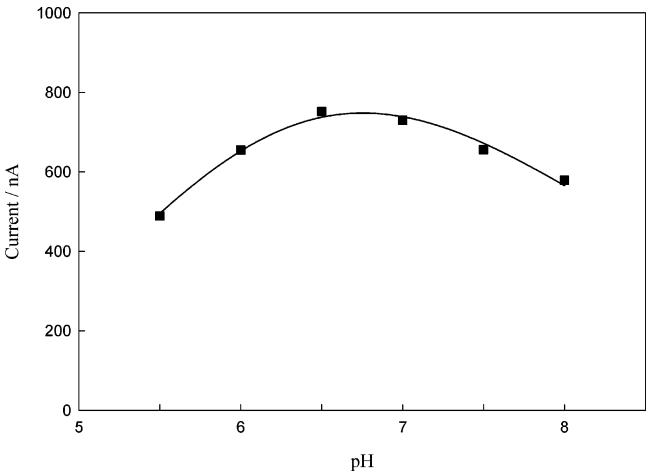

Figure 4. Effect of pH on the current response of the Y-NaR-immobilized GCE in 1 mM MV in argon-purged 0.05 M MOPS buffer (pH 6.5−8.0) and 0.05 M MES buffer (pH 5.5−6.5) containing 100 μM nitrate. Applied potential −850 mV.

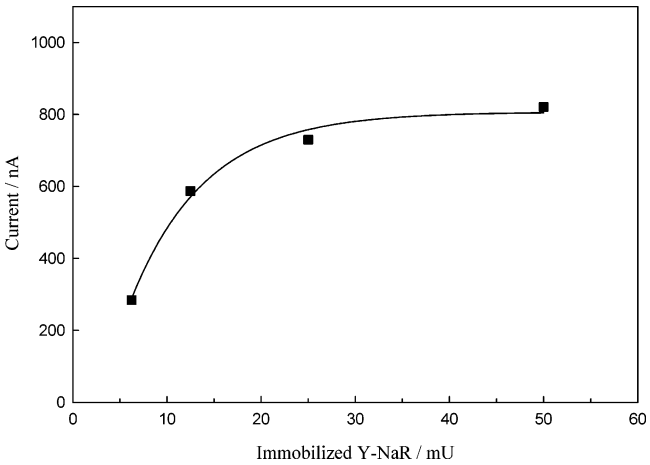

Figure 5. Effect of immobilized Y-NaR concentration on the current response of the GCE in an argon-purged 0.05 M MOPS buffer (pH 7.0) containing 1 mM MV and 100 μM nitrate. Applied potential −850 mV.

Although the highest current was found at pH 6.5, the oxygen-scavenging reagent sulfite becomes hydrogen sulfite in an acidic condition, which is a less effective scavenger than the unprotonated one.[35] For these reasons, the optimal pH for the Y-NaR-immobilized biosensors was determined at pH 7.0. Empirical examination of several buffer systems, e.g., phosphate, MES, Tris, and MOPS, showed that the Y-NaR-immobilized GCE provides stable and reproducible responses in 0.05 M MOPS buffer (pH 7.0).

Figure 5 shows the effect of immobilized enzyme concentration on the current response of the GCE-based biosensors. The magnitude of catalytic current of the electrode sharply increases with increasing Y-NaR concentration in immobilized reagent layer up to 25 mU and slowly beyond this concentration. Hence, we have optimized the enzyme layer with 25 mU (equals to 0.525 mg) of Y-NaR mixed with 20 wt % PVA for the GCE and 1 wt % for the SPCE. The effect of MV concentration on the current response of the Y-NaR-immobilized GCE and SPCE has also been examined; the increase in MV concentration from 5×10^{-4} to 5×10^{-3} M resulted in the increase in response current. However,

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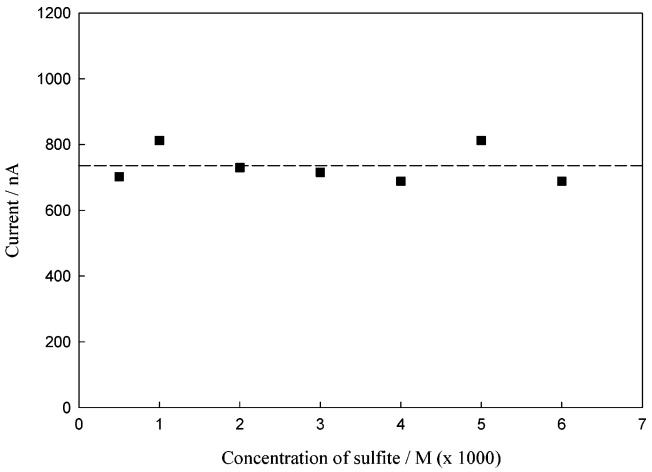

Figure 6. Effect of varying sulfite concentrations on the current responses of the Y-NaR-immobilized GCE in 0.05 M MOPS buffer (pH 7.0) containing 1 mM MV and 100 μM nitrate under ambient air conditions. Applied potential −850 mV.

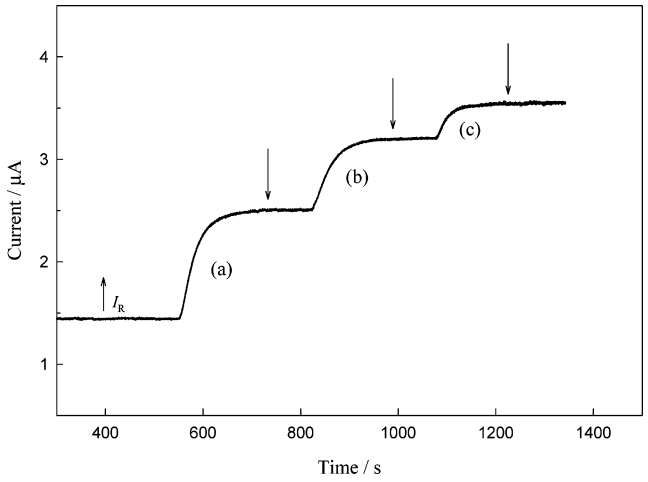

Figure 7. Steady-state current response of the Y-NaR-immobilized GCE in response to the stepwise addition of nitrate (a) 150, (b) 100, and (c) 50 μM in a 0.05 M MOPS buffer (pH 7.0) containing 1 mM MV and 1 mM sulfite under ambient air conditions. The applied potential was −850 mV. Down arrows on the current response trace indicate the injection of 500 μL of 0.05 M MOPS buffer (air saturated).

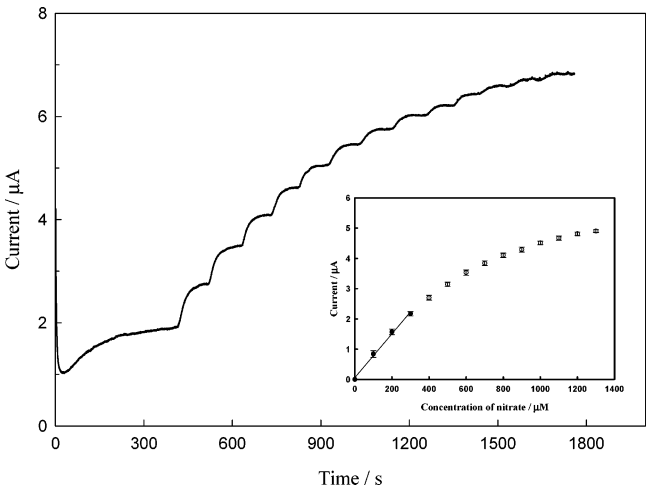

Figure 8. Current response of the Y-NaR-immobilized GCE to successive 100 μM increments of nitrate in 0.05 M MOPS buffer (pH 7.0) containing 1 mM MV and 1 mM sulfite under ambient air conditions. Applied potential −850 mV. Inset: calibration curve (each point, average of 10 measurements, is plotted after subtracting the background current measured in the buffer solution).

the increased responses beyond 1 mM for the GCE and 2.5 mM for the SPCE tend to accompany the lowered S/N ratios with deteriorated RSD values.

The use of a nitrate biosensor for in situ monitoring has not been realized to date due to the interference from dissolved oxygen. The dissolved oxygen at room temperature is $\sim 2.6 \times 10^{-4}$ M (8.3 ppm) in a buffered solution and should be removed prior to the nitrate monitoring by inert gas purging. This cumbersome process could be eliminated by adding an appropriate oxygen scavenger such as sodium sulfite to sample solution. Sulfite has been used previously as an oxygen scavenger in the analysis of phosphorescent compounds.[34,35] Since the stoichiometry of the reaction between sulfite and dioxygen is 2:1, we would need at least 5×10^{-4} M scavenger.

Figure 6 shows the variation in response currents of GCE-based electrodes to 100 μM nitrate when the concentration of sulfite is varied from 5×10^{-4} to 6×10^{-3} M: the responses are almost the same level (mean 736 nA, RSD 7.4%) within the given scavenger concentration. We, hence, used 1×10^{-3} M sulfite, a slight excess over the dissolved oxygen in most environmental samples, throughout the experiment. Figure 7 demonstrates that the Y-NaR-modified GCE in optimized condition (25 mU of enzyme in 20 wt % PVA, 0.05 M MOPS, pH 7.0, 1 mM MV, and 1 mM sulfite) provides rapid, stable, and quantitative responses to the stepwise addition of nitrate standard solutions. The curve in Figure 7 also shows that the following additions of 500 μL of blank buffer (indicated by down arrow) to the solution do not affect the response currents, indicating that the extra sulfite efficiently consumes the additional oxygen.

Figure 8 shows a typical steady-state current response of the Y-NaR-immobilized GCE to successive 100 μM increments in nitrate concentrations at optimal conditions under ambient air. The electrode requires a presoaking time of about 7−8 min to result in a stable background current and reaches a steady-state response to the added nitrate in ~40 s ($t_{90\%}$). Electroanalytical properties of the GCE-based biosensor under ambient air are as follows: sensitivity of 7.3 nA/μM, linear response range of 15−300 μM ($r^2 = 0.995$) and detection limit of 4.1 μM (S/N = 3, background noise of ±5 nA). These parameters measured under ambient air are virtually the same as those obtained under an Ar-purging condition except for a slightly different linear dynamic range (15−250 μM). The characteristics of the GCE with PVA-entrapped Y-NaR layer are comparable to those of most other NaR-immobilized electrochemical biosensors found in the literature: the sensitivity range lies in 1.2−7.0 nA/μM, linear response range in 3−100 μM, and detection limit range in 0.4−6.7 μM under inert gas purging condition.[3,6,8,14,15,23] The Michaelis−Menten constant ($K_{m,app}$) estimated from the Lineweaver−Burk plot was ~580 μM, which is also comparable to those of reported.[3,8,21] The effect of interfering substances (nitrite, sulfate, chloride, phosphate, chlorate) was also measured. As reported previously,[6] the Y-NaR-immobilized GCE exhibits a large interfering response to chlorate (10.0 nA/μM) and a response to nitrite to some extent (2.5 nA/μM).

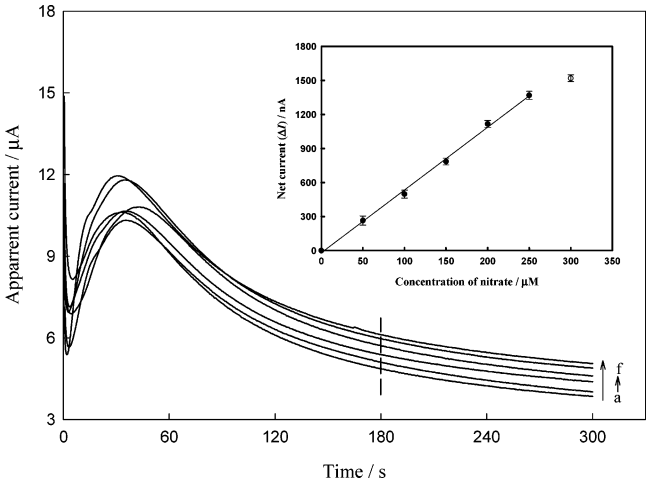

Figure 9. Current−time profiles derived from Y-NaR-immobilized SPCEs (Figure 2B) in 0.05 M MOPS buffer (pH 7.0) containing 2.5 mM MV, 1 mM sulfite, and different concentrations of nitrate: (a) 50, (b) 100, (c) 150, (d) 200, (e) 250, and (f) 300 μM. Applied potential −900 mV. Inset: calibration curve plotted from the current responses at 180 s (each point, average of 40 measurements, is plotted after subtracting the background current measured with the buffer solution, and the average RSD in linear range is less than 2.8%).

Having developed the GCE-based nitrate monitoring biosensor, we extended the technology to the fabrication of disposable SPCE with built-in sample cell for in situ monitoring of nitrate in the field. Figure 2 has shown the fabrication procedures for SPCE and its capillary cell structure. The SPCE-based biosensor was used with a portable potentiostat device, and −900 mV was applied to the working electrode. The sample introduced into the sample well or capillary cell dissolved the enzyme-containing reagent layer in short time, bringing about an abrupt change in impedance and current between the working and counter electrodes. The sample and mixed reagents then underwent the diffusion-controlled nitrate reduction reaction, resulting in the reduction current proportional to the concentration of nitrate in a given time period. The current profiles in Figure 9 sequentially display the events occurring at the SPCE-based biosensor, and the calibration curve in the inset is plotted with the currents measured at 180 s after injecting the sample into the well (Figure 2b). The calibration equation obtained after subtracting the background current of the SPCE modified with inactivated enzyme in PVA layer (mean 4600 nA) was

$$\Delta I\ (\mathrm{nA}) = 5.54 C_{\mathrm{NO_3^-}}\ (\mu\mathrm{M}) - 20$$

within the linear detection range of 15−250 μM (0.9−15.5 ppm, $r^2 = 0.996$, $n = 40$, RSD < 2.8%). The sensitivity of the electrode was 5.5 nA/μM with a detection limit of 5.5 μM (0.3 ppm, S/N = 3). The detection limit of SPCE was comparable to or higher than those from other methods, which ranges from 0.05 to 20 μM in the literature.[1] The sensitivity of the Y-NaR-immobilized SPCE was ∼75% that of the same enzyme-immobilized GCE.

We may use the SPCE-based nitrate-determining biosensor in the field carrying a kit comprising a portable potentiostat, precalibrated biosensors, powdered reagent pack, and disposable sampling devices. To simulate the situation, we collected three kinds of real samples from the upper stream of Han River, a filtration plant, and water service pipes, and determined their nitrate concentrations with Y-NaR-immobilized SPCEs. The mixture of 209.2 mg of MOPS, 16.8 mg of sodium hydroxide, 12.9 mg of MV, and 2.5 mg of sodium sulfite in a reagent pack was dissolved in 20 mL of water to prepare the sample solutions with an optimal analytical condition as described in previous sections. The processed sample solutions were then transferred to the 100-μL sample well (or 15-μL capillary cell) of the SPCE-based biosensor connected to a portable monitor, and their nitrate concentrations were determined from a precalibrated curve stored in the monitor. The precision of the biosensor was estimated by measuring each sample with 40 SPCEs. Table 1 summarizes the results of the determination. The accuracy of determination was compared to the results obtained from standard methods of IC and spectrophotometry. As shown in Table 1, the nitrate concentrations determined with the SPCE-based biosensors are between those values from IC and spectrophotometric determinations. It is interesting to note that the values from IC are higher than those from the other two methods for the samples with high residual chlorine concentration. The RSD values of the Y-NaR-immobilized SPCEs were less than 3.4% for all samples, which is sufficiently precise for the purpose of in situ monitoring.

Table 1. Nitrate Concentrations in Water Samples Determined by Three Different Methods (μM)

samples	ion chromatograohy ($n = 3$)	spectrophotometry ($n = 3$)	SPCE (RSD %) ($n = 40$)
Han River	107.9	100.0	100.4 (2.7)
filtration plant	126.4	109.3	123.1 (3.4)
service pipes	126.4	100.0	106.3 (2.8)

The storage stability of the Y-NaR-immobilized SPCE was examined. The average sensitivity of the electrodes that had been stored in ambient air for 1 month was 4.4 nA/μM ($n = 5$), which corresponds to ∼80% of that of freshly prepared enzyme electrodes. The manufacturer's claimed lifetime of the lyophilized Y-NaR in desiccators at 4 °C is ∼6 months. If we optimize the immobilizing matrix other than PVA, the storage stability is expected to increase up to the lifetime of the enzyme. Nevertheless, the results presented here certainly confirm that the SPCE-based biosensors are suitable for in situ monitoring of nitrate if we adjust the analytical condition of the sample solution with appropriate reagents.

CONCLUSIONS

The results presented in this article demonstrate for the first time that the Y-NaR-immobilized electrodes can be used for the determination of nitrate under ambient air condition using appropriate oxygen scavenger. Two different types of carbon-based electrodes, GCE and SPCE, were used to immobilize the Y-NaR in PVA matrix. The electrodes were easily prepared by depositing an enzyme-containing solution on the electrodes using a commercial dispenser, implicating the possibility of mass fabrication. The composition of enzyme layer, concentration of mediator, buffering reagents, pH, and effect of oxygen scavenger concentration on electrochemical responses were optimized to result in accurate determination of nitrate. It is shown that the Y-NaR-immobilized SPCE-based biosensors are sufficiently accurate and

precise for in situ monitoring of nitrate in real samples without a cumbersome oxygen removal process by inert gas purging.

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