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Electrochemical characterization of biosensor based on nitrite reductase and methyl viologen co-immobilized glassy carbon electrode

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Abstract

Nitrite reductase (NiR, nitric-oxide: ferricytochrome *c* oxidoreductase, EC 1.7.2.1) and methyl viologen (MV) were co-immobilized on glassy carbon electrode (GCE, $d=3$ mm) by polymer entrapment, and the electrode was tested as an electrochemical biosensor for amperometric determination of nitrite. The immobilization was performed by sequential loading and drying of a homogeneous mixture of poly(vinyl alcohol) (PVA), NiR and MV, followed by poly(allylamine hydrochloride) (PAH) solution, and finally hydrophilic polyurethane (HPU) dissolved in chloroform. The positively charged PAH layer could effectively keep immobilized cationic MV from diffusing through the membrane, holding mediator tightly near or on the electrode surface. The working principle of the biosensor was based on MV mediated electron transfer between electrode and immobilized NiR. The response time ($t_{90\%}$) of the biosensor was about 20 s and sensitivity was 11.8 nA/μM (2.5 mU NiR) with linear response range of 1.5–260 μM ($r^2=0.996$) and detection limit of 1.5 μM (S/N=3). Lineweaver–Burk plot showed that Michaelis–Menten constant ($K_{m,app}$) was about 770 μM. The biosensor showed durable storage stability for 24 days (stored in ambient air at room temperature) retaining 80% of its initial activity, and showed satisfactory reproducibility (relative standard deviation (R.S.D.)=3.8%, $n=9$). Interference study showed that chlorate, chloride, sulfite, sulfate did not interfere with the nitrite determination, however, nitrate interfered with the determination with relative sensitivity of 38% (ratio of sensitivity for nitrate to that for nitrite). In addition to the full characterization of the biosensor, kinetic study was also conducted in solution and the homogeneous rate constant (k_2) between NiR and MV were determined by chronoamperometry to be 5.8×10^5 M^{-1} s^{-1}.

Keywords: Nitrite reductase; Methyl viologen; PAH; Co-immobilization; Biosensor

1. Introduction

Nitrite is one of the well-known inorganic pollutants in environmental, food, industrial and physiological systems. The widespread nitrite pollutant becomes toxic in human body and animals; for example, nitrite is converted to carcinogenic *N*-nitrosamines in the stomach [1,2], and irreversibly reacts with hemoglobin producing methemoglobin which is incapable of binding oxygen [2]. The formation of methemoglobin is particularly hazardous for infants as it limits physical stature and neural development. Therefore, quantitative determination of nitrite is important. In addition to known nitrite determination methods [2–4], nitrite reductase (NiR) based biosensor has been prepared [5–11].

Two distinct types of NiRs are known: multi-heme containing and multi-copper containing NiRs [12,13]. The copper containing NiRs catalyze the 1-electron reduction of nitrite to nitric oxide as follows:

$$NO_2^- + 2H^+ + e^- \xrightarrow{\text{Cu–NiRs}} NO + H_2O$$

The best characterized copper containing NiR from *Achromobacter cycloclastes* contains both type 1 (T1 Cu) and type 2 copper (T2 Cu); T2 Cu is the binding and reduction site of nitrite while T1 Cu the electron transfer (ET) site [14–16]. The NiR from *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* used in this study belongs to the copper containing type, and the property of this enzyme is similar to that from *A. cycloclastes* [17]. Recently, structures of copper containing NiRs have been actively studied by X-ray crystallography [14–16,18,19], and

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their catalytic mechanism has been proposed in relation to their structures [12,13,20].

While there have been few studies on direct electrochemistry of NiR [6,21], most previous reports on NiR-based biosensors employed mediators such as viologens and some redox dyes that have sufficiently negative redox potentials [5–9,11]. It was reported that the T2 Cu sites of copper containing AxgNiR (from *Alcaligenes xylosoxidans* GIFU 1051) and AciNiR (from *A. cycloclastes* IAM 1013) are located 12–13 Å below the molecular surface [13,21], and the redox potentials of T1 and T2 Cu sites of copper containing AciNiR, AxgNiR and NiR from *Rhodobacter sphaeroides* 2.4.3 were reported to be about 0–50 mV vs. Ag/AgCl at pH 7.0 [13,20,22]. Therefore, mediators with negative redox potentials are essential for electron transfer (ET) between electrode and these enzymes; nine substances with different negative potentials ranging from −150 to −650 mV were selected and tested to construct NiR-based biosensors. The electrochemical biosensor devised in this study operates with the mechanism depicted in Fig. 1. The active components of the biosensor, NiR and MV, were entrapped in polymer matrix. Positively charged poly(allylamine hydrochloride) (PAH) layer was introduced to keep cationic MV on the electrode surface by electrostatic repulsion, resulting in enhanced ET [23]. It is shown that the NiR-based biosensor conveniently provides fast and accurate determination of nitrite.

2. Experimental

2.1. Chemicals

1,1′-Dimethyl-4,4′-bipyridinium dichloride (methyl viologen, 98%), 3,7-diamino-2,8-dimethyl-5-phenylphenaziniumchloide (safranine O, ~95%), 3,7-diamino-5-phenylphenaziniumchloride (phenosafranin, ~80%), neutral red (~60%), anthraquinone-2-sulfonic acid (97%), 2-hydroxy-1,4-naphthoquinone (97%), methylene blue (~85%), toluidine blue O (~80%), *N*-methylphenazoniummethosulfate (phenazine methosulfate, 99%), poly (allyamine hydrochloride) (PAH, M_w ~70,000), chloroform (99.95%), sodium hydrogenphosphate (99.995%), sodium dihydrogenphosphate (99.999%), potassium chloride (99.999%), sodium nitrite (99.999%) were purchased from Aldrich Chemical Co. and used as received. Poly(vinyl alcohol) (PVA, M_w ~22,000, ash ≤ 0.05%) was purchased from Fluka Chemical Co. HydroThane™: hydrophilic thermoplastic polyurethane (HPU, aliphatic type (AL-25-80A), 25% water uptake) was purchased from CardioTech International, Inc. (Wilmington, MA, USA). All other chemicals were of at least reagent grade and were used without further purification. Deionized water (18 MΩ cm) from Milli Q water purification system was used for preparing buffer and stock solutions. Sodium nitrite solution was freshly prepared just before the experiment.

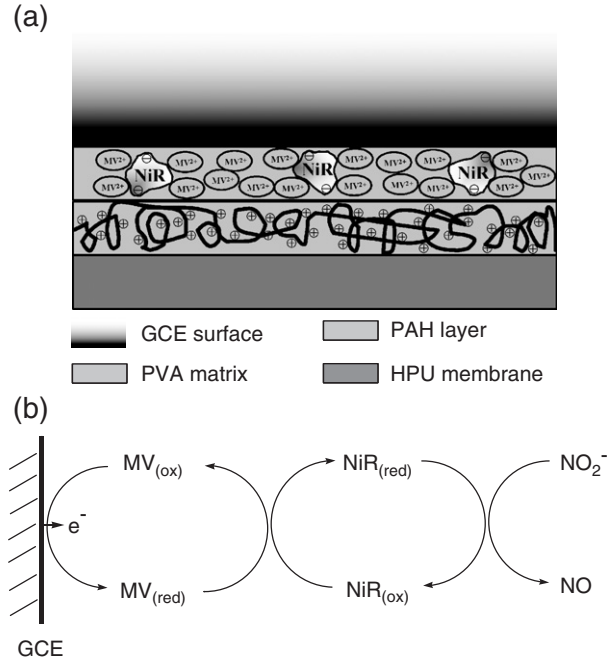

Fig. 1. Schematic of (a) the nitrite reductase and methyl viologen co-immobilized glassy carbon electrode, and (b) the operating mechanism for nitrite determination.

2.2. Enzyme

Nitrite reductase (nitric-oxide: ferricytochrome *c* oxidoreductase, EC 1.7.2.1, purified from *R. sphaeroides* forma sp. *denitrificans*) was commercially purchased from NECi, USA, and used as received. The enzyme was purified as a dimmer with molecular mass of 80 kDa [17]. The activity of the enzyme was 2.2 U/mL and protein content was 3.25 mg/mL in 0.05 M potassium phosphate buffer, pH 7.5.

2.3. Apparatus

BAS 50W or cDAQ-1604 (Elbio Co., Korea) potentiostat was used to run cyclic voltammograms (CVs) and chronoamperometry. For characterization of the enzyme electrode, glassy carbon working electrode (d=3 mm, BAS), platinum wire (BAS) counter electrode (coil), and Ag/AgCl reference electrode (3 M KCl, BAS) were used, and buffer solution in 5 mL stoppered cell was continuously stirred by magnetic bar during amperometric experiments. For kinetic study of MV and NiR pair in solution, a stoppered microcell (15 mm i.d., 30 mm in height) having a volume of 1 mL was used in order to use as little enzyme as possible. Oxygen was thoroughly driven out from solution by ultra pure nitrogen gas purging, and the experiment temperature was controlled at 25±0.2 °C by use of thermostated cell in all experiments. A continuous flow of ultra pure nitrogen gas was led over the buffer solution during the CV and amperometric experiments. All potentials in the text are referred to Ag/AgCl reference electrode.

2.4. Kinetics of MV and NiR pair in solution

The homogeneous rate constant k_2 for the reaction between NiR and MV in solution was determined by chronoamperometry [24]. The measurements were carried out first for blank solution containing 1×10^{-3} M MV and 1×10^{-2} M nitrite, and then for the solution after adding increasing amounts of NiR: 4×10^{-8},

8×10^{-8}, 1.2×10^{-7}, 1.6×10^{-7}, and 2.0×10^{-7} M to the blank solution. The chronoamperometry was recorded in the time range of 0–10 s at data collection frequency of 20 s^{-1}, and currents in the time range of 0.5–2.2 s were used to plot kinetic parameter of λ vs. time. Finally k_2 was determined from the plot of initial slope of $I_d - t$ vs. NiR concentration.

2.5. Co-immobilization of NiR and MV on GCE

Glassy carbon electrodes were polished first with 0.3 μm polishing powder (alumina, Buehler) on polishing pad (the same company), followed by sonication for 5 min in a mixed solution of water and ethanol (50 v/v %) and blow dried with N_2 gas. Next, 5 μL of enzyme solution, 5 μL of aqueous PVA (20 wt %) and 3 μL of 5×10^{-3} M MV were homogeneously mixed in 0.5 mL eppendorf tube by Vortex. Every 3 μL of the mixture (containing ~2.5 mU NiR) was added on three polished electrode surfaces by use of a Smart Dispenser (EFD 2000XL, East providence, Rhode island, USA) and allowed to dry in air. Then 2 μL of 2.5 wt% of aqueous PAH solution was added on every modified GCE and allowed to dry in air. Finally, 5 μL of 15 mg/mL of HPU solution (in chloroform) was added on every PAH modified GCE and allowed to dry in air. When not in use, the NiR and MV co-immobilized electrodes were stored in refrigerator in dry sate at 4 °C.

2.6. Electrochemical characterization of mediators

For electrochemical characterization of the selected mediators, CV and amperometry experiments were conducted with only NiR immobilized GCE and mediators dissolved solutions in 0.05 M PBS containing 0.05 M KCl, pH 7.0. Immobilization of NiR alone on GCE was performed as above mentioned with a little modification. In this case, PAH layer was not employed. CVs were recorded at 5 mV/s, and amperometries were recorded by applying working potentials of 100 mV negative than reduction peak potentials of respective mediators. The suitable mediator was selected by the magnitude of the increased catalytic reduction peak currents in CV experiments, and by the magnitude of sensitivities of the enzyme electrode in amperometric experiments.

2.7. Optimization and characterization of the biosensor

For optimization of the sensor, effects of three polymer layers of PVA, PAH and HPU, buffer concentration, amounts of enzyme and MV loading, and pH of buffer were investigated. For characterization of the sensor, sensor performance factors such as sensitivity, linear range, detection limit, storage stability, and interference were investigated. In both cases, amperometric experiments were performed, where steady-state currents were measured by applying working potential of −750 mV.

3. Results and discussion

3.1. Selection of suitable mediator

Preliminary CV experiment at slow scan rate of 5 mV/s in the presence of NiR in 0.05 M PBS (pH 7.0, contains also 0.05 M KCl) showed that direct electron transfer (DET) between electrode and NiR may not be attainable in our experimental condition. Hence, nine substances mentioned in Experimental section were selected and tested. These substances had sufficient negative potentials compared to the redox potentials of active sites (T1 Cu and T2 Cu) of copper NiRs, and might reduce the oxidized form of the enzyme (Fig. 1(b)). CVs were recoded with the NiR immobilized electrode in the presence of dissolved mediators. The obtained results showed that safranine O, phenosafranin, neutral red, anthraquinone-2-sulfonic acid, 2-hydroxy-1,4-naphathoquinone, and MV were able to reduce the oxidized form of the enzyme, while methylene blue, toluidine blue O, and phenazine methosulfate were not (Fig. 2). From these results it could be concluded that mediators having redox potentials higher than about −200 mV may not reduce the oxidized form of the enzyme, or those having potentials lower than about −350 mV may be useful for our purpose. These potentials deviated from the reported values; for example, it was reported that copper containing NiR (from *Paracoccus denitrificans*) may accept electrons from donors that had potential as high as −70 mV vs. SCE [7], and the working potential of copper containing NiR (from *Alcaligenes faecalis* S-6) immobilized gold electrode was −150 mV vs. Ag/AgCl with 1-methoxy-5-methylphenazinium methylsulfate as the mediator [9]. This deviation might be attributable to the structural differences for these enzymes.

Amperometric experiments were also performed with the NiR immobilized electrode and dissolved mediators. The obtained sensitivities were 11.8, 10.9, 9.7, 7.9, 6.7 and 5.3 nA/μM for MV, neutral red, safranine O, phenosafranin, 2-hydroxy-1,4-naphathoquinone and anthraquinone-2-sulfonic acid as the dissolved mediators, respectively. This order was consistent with that obtained from CV experiments, and agreed roughly with the already reported [7]. Therefore, MV was finally selected as the ET mediator for the enzyme. MV or viologens as the mediator for NiR have been already reported in the literature [5–9,11,25,26].

3.2. Homogeneous rate constant (k_2)

The homogeneous rate constant k_2 between MV and NiR in solution was determined by chronoamperometry [24]. The background $I_d - t$ curves for MV solution and the $I_d - t$ curves for increasing concentrations of NiR added solutions were recorded first in 0–10 s. Then ratios of the catalytic and diffusion currents (I_c/I_d) were plotted over the same time range. These ratios were converted into the values of kinetic parameter [24,27,28] in the same time range. The slopes in the initial time range (0.5–2.2 s, Fig. 3(a)) were estimated by linear regression and plotted with respect to NiR concentrations (Fig. 3(b)). The NiR concentrations used in the calculation were half of the actual concentrations, because two moles of reduced MV were needed to reduce one mole of NiR due to the dimer structure of the enzyme [17]. Similar rectification of the NiR concentration for calculation of k_2 value has been reported [7,24]. A k_2 value of 5.8×10^5 M^{-1} s^{-1} was calculated from the slopes of the straight line obtained by linear regression. It was reported that the different k_2 values were 7.3×10^5 M^{-1} s^{-1} for pseudoazurin–AciNiR (copper containing)

pair, 4.0×10^5 M^{-1} s^{-1} for Axg–Cyt c_{551}–AxgNiR (copper containing) [13], 2.0×10^6 M^{-1} s^{-1} for cytochrome c_{551}–cytochrome cd_1 NiR (heme containing, from *Pseudomonas aeruginosa*) pair, and 1.4×10^5 M^{-1} s^{-1} for azurin–cytochrome cd_1 NiR (same source) pair [29]. Taken into account the differences in diffusion coefficients (D_o) between reduced form of MV (8.11×10^{-6} cm^2/s, obtained in this study) and these physiological donors (0.6×10^{-6} cm^2/s for cytochrome c_3 [6]), the value obtained here was relatively small. The k_2 values of MV–NiR pairs were also reported, for example, k_2 value of MV–NiR (heme containing, from *Desulfovibrio desulfuricans*) pair was reported to be 2.2×10^8 M^{-1} s^{-1} [27], and that of MV–NiR (heme containing, from *S. deleyianum*) pair be 1.0×10^{10} M^{-1} s^{-1} [7]. These differences in k_2 value for different electron donor–NiR pairs

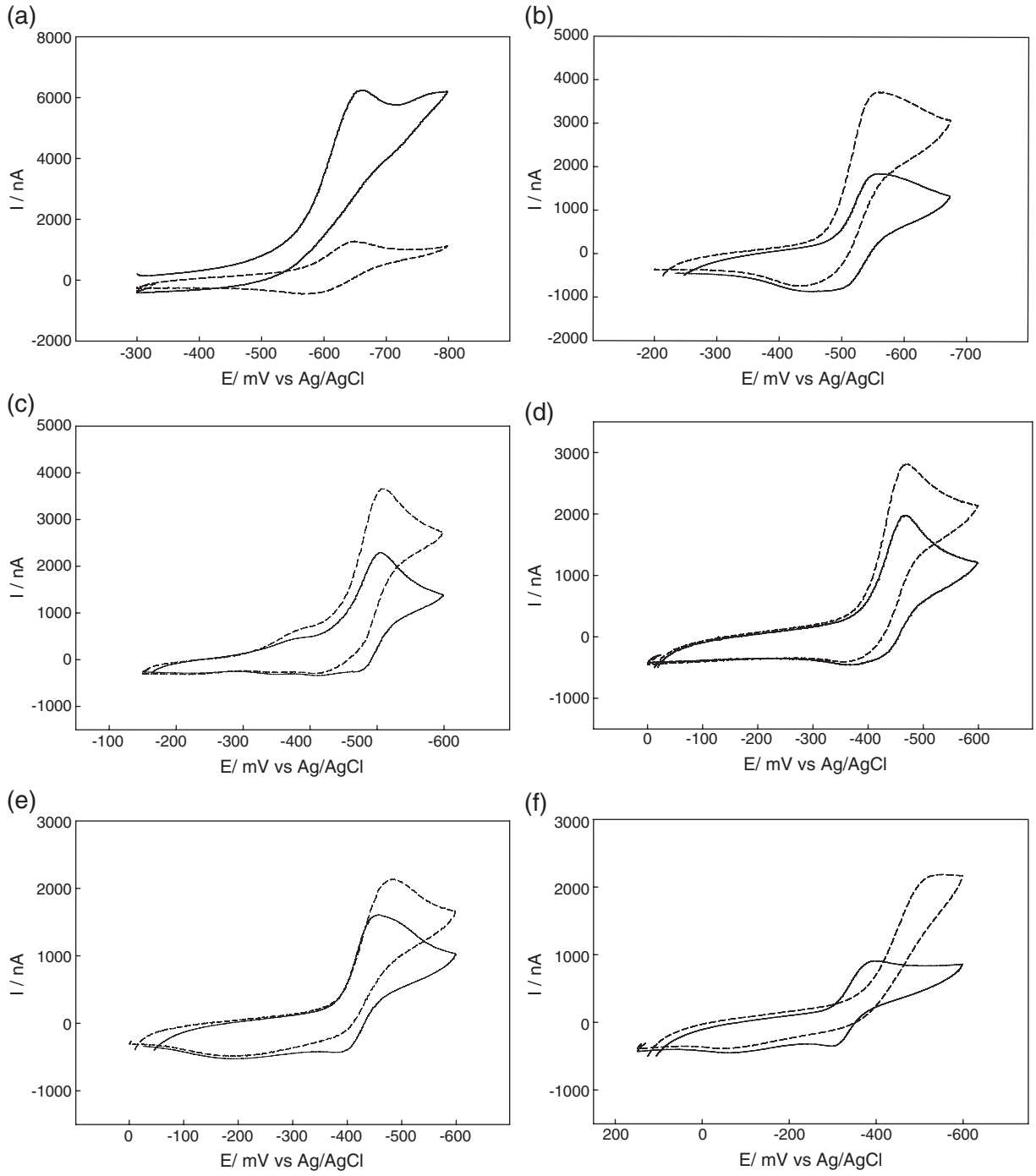

Fig. 2. CVs of dissolved mediators recorded with nitrite reductase immobilized glassy carbon electrode in 0.05 M PBS containing 0.05 M KCl, pH 7.0 under N_2 in the absence of nitrite (solid) and in the presence of 8.0×10^{-4} M nitrite (dash). Scan rate: 5 mV/s. (a) 1.0×10^{-3} M methyl viologen, (b) 1.0×10^{-4} M neutral red, (c) 1.0×10^{-4} M safranine O, (d) 1.0×10^{-4} M phenosafranin, (e) 1.0×10^{-4} M anthraquinone-2-sulfonic acid, (f) 1.0×10^{-4} M 2-hydroxy-1,4-naphthoquinone, (g) 2.0×10^{-4} M methylene blue, (h) 5.0×10^{-4} M toluidine blue O and (i) 1.0×10^{-3} M phenazine methosulfate. (*I*/nA and *E*/mV vs. Ag/AgCl in graph represent the current and electrode potential of the measurement, respectively.)

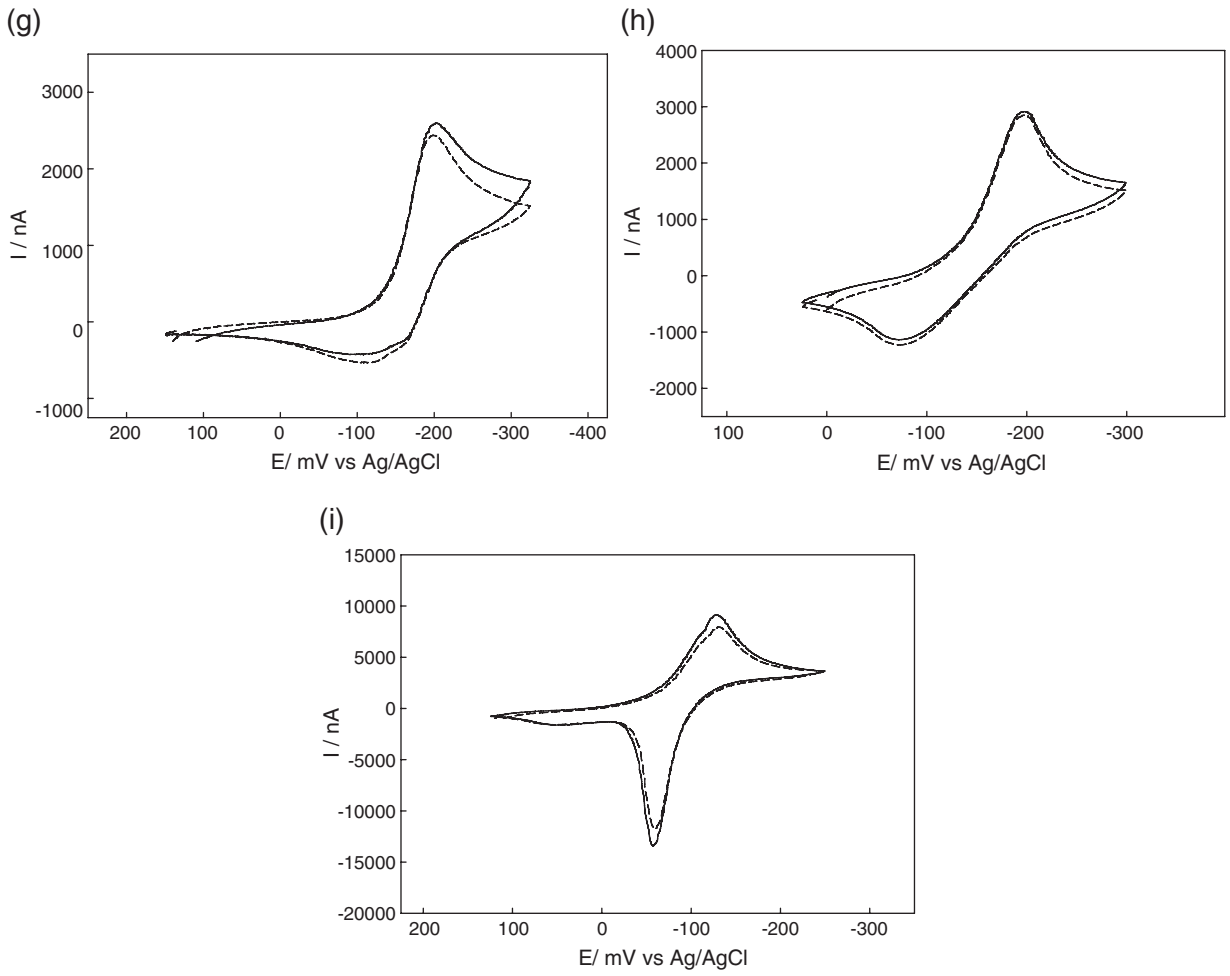

Fig. 2 (*continued*).

might be resulted mainly from the differences in structures of these enzymes, for example, one electron reduction of the substrate by copper containing NiR instead of six electron reduction by heme containing NiR.

3.3. Electrochemical characterization of the immobilized MV

To characterize the immobilized MV, CVs were recorded in 0.05 M PBS containing 0.05 M KCl, pH 7.0 at scan rate of 50 mV/s under N_2. Dissolved MV showed reversible CV characteristics, showing half potential ($E_{1/2}=(E_{p,c}+E_{p,a})/2$) of −642 mV, peak separation (ΔE_p) of 61 mV and ratio of cathodic to anodic peak current (I_c/I_a) of 0.986. Peak currents varied linearly with the square root of scan rate up to 100 mV/s, indicating a diffusion-controlled process. The corresponding data obtained with immobilized MV were −607, 66 and 1.233 mV, respectively, indicating a quasi-reversible system. It could be concluded from the value of I_c/I_a that re-oxidation of reduced form of immobilized MV ($MV^{+\cdot}$ radical) was not quantitative in PVA matrix at the electrode surface. This phenomenon might be resulted from the dimerization of $MV^{+\cdot}$ radical, restricted mobility of MV in PVA matrix, or possible leaking of MV from electrode surface.

Scan rate dependence of reduction peak current ($I_{p,c}$) of the immobilized MV was also tested at various scan rates of 5, 10, 25, 50, 100 and 200 mV/s in the same condition. As shown in Fig. 4, $I_{p,c}$ values of the immobilized MV varied linearly with scan rate up to 50 mV/s, which was one of the characteristics of a surface restricted redox material [30], indicating that the immobilized MV molecules were relatively free in PVA matrix, making it possible that further electron transfer from $MV^{+\cdot}$ to the co-immobilized NiR.

3.4. Catalytic behavior of the NiR and MV co-immobilized GCE

The catalytic behavior of NiR and MV co-immobilized GCE is shown in Fig. 5. The NiR and MV co-immobilized GCE showed a quasi-reversible CV with reduction peak potential of −632 mV (Fig. 5(a)) in 0.05 M PBS containing 0.05 M KCl, pH 7.0 at scan rate of 5 mV/s under N_2. Upon addition of 8.0×10^{-4} M nitrite into the solution (Fig. 5(b)), CV exhibited a large cathodic peak current with sigmoidal shaped reverse scan, which is typical of an electrode reaction followed by an efficient catalytic reaction (EC process). This result originated from the effective electron transfer mediated by co-immobilized MV

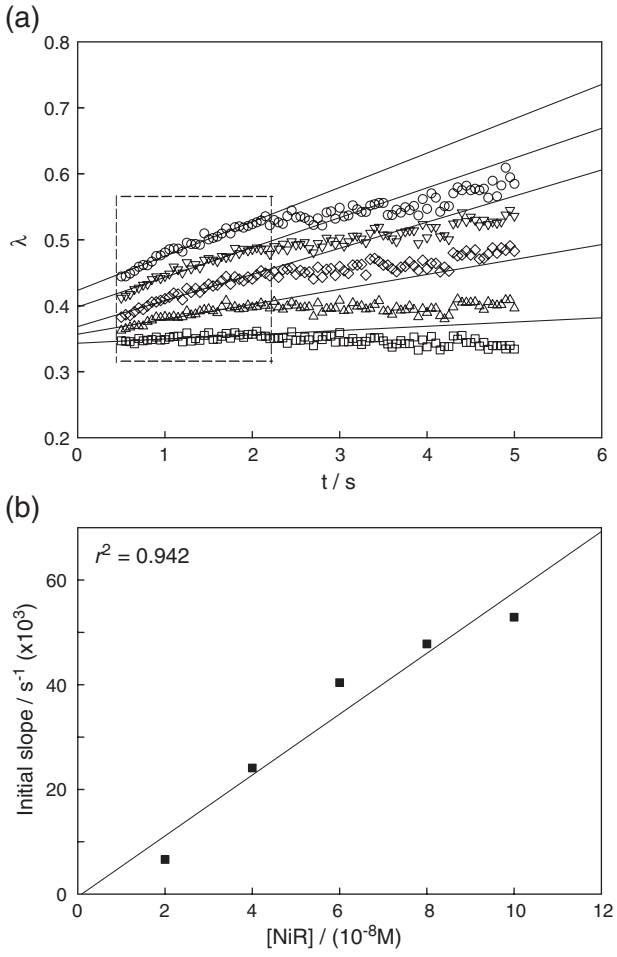

Fig. 3. Determination of homogeneous rate constant k_2 between nitrite reductase and methyl viologen in 0.05 M PBS containing 0.05 M KCl, pH 7.0 under N_2 by chronoamperometry. (a) Plot of kinetic parameter (λ) vs. time (t/s), (b) plot of initial slope of plot (a) vs. nitrite reductase concentration.

between electrode and the immobilized NiR as shown in Fig. 1 (b). Control experiments were also performed in similar conditions with the MV-immobilized electrode (without immobilized NiR); no catalytic effect was observed (Fig. 5(c)). However, it was reported that reduced MV may exhibit catalytic activity towards the reduction of nitrite to nitric oxide [26] if relatively high concentration of nitrite (2.25×10^{-1} M) were used. On the other hand, the nitrite concentrations ($<1\times10^{-3}$ M) used in this experiment may have not been sufficient to induce such effect in the presence of reduced MV. Similar electroenzymatic reductions of nitrite by NiR and mediator were also observed with the biosensors prepared with the copper and heme containing NiRs (from *R. sphaeroides* strain 2.4.3 and from *D. desulfuricans*, respectively) [6,8,11]. It was reported that isoelectric point (pI) of NiR used in this study was about 5.0 [17]. Thus, this enzyme would be negatively charged in pH 7.0 buffer, and the positively charged MV would closely surround the enzyme surface by favorable electrostatic attraction, which would be advantageous for the mediated electron transfer between electrode and immobilized NiR. Similarly, it was proposed that hydrogen bonding, salt bridge (or polar interaction), and hydrophobic contact were involved in the protein

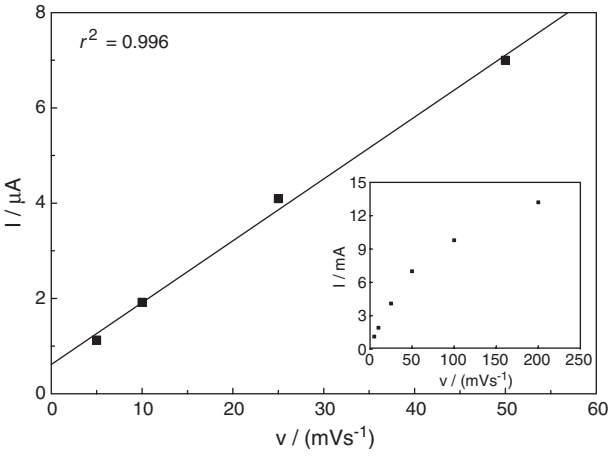

Fig. 4. Reduction peak currents ($I_{p,c}$/μA) as a function of scan rates (v/mVs^{-1}) for immobilized methyl viologen on glassy carbon electrode in 0.05 M PBS containing 0.05 M KCl, pH 7.0 under N_2. Inset: full scale of scan rates.

recognition by complex formation at specific site between small electron transfer protein (pseudoazurin) and large enzyme (NiR) prior to the intramolecular electron transfer [21,29].

When the cathodic peak current significantly increases, the catalytic reduction peak potential is negatively shifted by 125 mV; it may be due to the low conductivity of the PVA matrix used with a supporting electrolyte (0.05 M PBS containing 0.05 M KCl). A comparison experiment performed in a buffer containing higher concentration of KCl (0.4 M) showed that the catalytic reduction peak potential was almost the same as that of the immobilized MV. Buffer concentration effect will be discussed further in following sections.

3.5. Characterization of the NiR and MV co-immobilized GCE as a biosensor

In this study, as many as three polymers such as PVA, PAH and HPU were used to prepare the biosensors. The optimized compositions of these polymers were as follows: 20 wt% PVA,

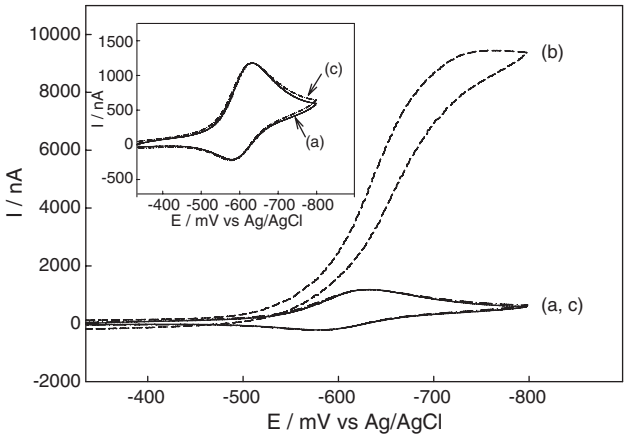

Fig. 5. CVs recorded with nitrite reductase and methyl viologen co-immobilized (a,b) and only methyl viologen immobilized (c) glassy carbon electrodes in 0.05 M PBS containing 0.05 M KCl, pH 7.0 under N_2. Scan rate: 5 mV/s. (a) In the absence of nitrite, and (b) and (c) in the presence of 8.0×10^{-4} M nitrite. Inset: enlargement of Fig. 5 (a) and (c).

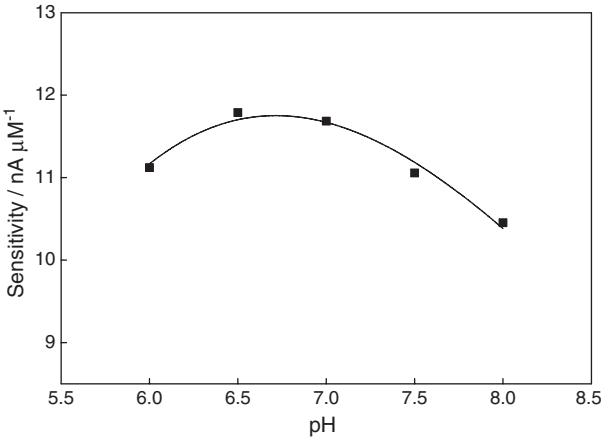

Fig. 6. Effect of pH on the sensitivity of the nitrite reductase and methyl viologen co-immobilized glassy carbon electrode in 0.05 M PBS containing 0.05 M KCl under N_2. Applied working potential was −750 mV and nitrite per injection was 20 μM.

15 mg/mL HPU and 2.5 wt% PAH. After optimization of polymer concentrations, we tested if the positively charged PAH layer effectively keeps immobilized cationic MV on the GCE surface by electrostatic repulsion. A set of experiments was conducted with NiR and MV co-immobilized electrodes with and without PAH layer. CVs showed that the catalytic reduction peak current in the presence of 8.0×10^{-4} M nitrite for the sensor without PAH layer was 4390 nA (scan rate: 5 mV/s), which was only about 46% of the sensor with PAH layer (9448 nA, Fig. 5 (b)). This result indicates an effective immobilization of MV by this PAH layer. In addition to this diffusion limiting effect, the PAH layer may have an effect of increasing the substrate concentration by acting as an anion exchanger. Amperometry experiments showed that the current from the sensor without PAH layer decayed fast with time (data not shown), while that with PAH layer was fairly stable (see also Fig. 7).

PBS was selected in this study as the supporting electrolyte for electrochemical characterization of the biosensor according to the other previously reported works [7,8]. The 0.05 M KCl was added to the buffer to enhance the conductivity of the solution because enzyme and mediator were co-immobilized in PVA matrix. The plot of response sensitivity vs. PBS concentrations showed a maximum near at 0.05 M; they were 6.1, 7.4, 8.2, 9.7, 11.8 and 11.3 nA/μM for 0.4, 0.3, 0.2, 0.1, 0.05 and 0.025 M PBS, respectively. It was thought that the electrostatic attractions between positively charged electrode surface (PAH layer) and negatively charged substrate and/or between positively charged MV and negatively charged NiR are reduced in a high ionic strength buffer. At a low concentration, the conductivity of PVA matrix might be the governing factor for the decreased current. For this reason, experiments were conducted with 0.05 M PBS.

Undoubtedly, the biosensor response will be directly dependent on the amounts of immobilized NiR and MV. In case of NiR, when increasing amounts of NiR of 1.5, 2.25, 3, 3.75 and 4.5 μg were immobilized, the maximum current response was found at 3.75 μg of NiR. When MV concentrations were varied from 1×10^{-3} to 5×10^{-3} M, the response was continuously increased beyond 5×10^{-3} M. However, we did not test beyond this range because the reduced MV tends to dimerize at high concentrations [31] or may poison the co-immobilized NiR [25]. The optimized amount and concentration of enzyme (3.75 μg) and mediator (5×10^{-3} M) were chosen based on these experiments.

As discussed in the Introduction, the overall reduction of nitrite to nitric oxide with the copper containing NiR and reduced MV involves a 1-electron/2-proton process. Thus, pH will affect the catalytic reduction of nitrite with the sensor. In order to determine the optimal pH for the sensor, experiments were carried out over the pH range of 6.0–8.0 with 0.05 M PBS. Fig. 6 shows pH profile of the sensor in the tested pH range. The acidic range was favorable than alkaline one for the sensor function, and the highest sensitivity of 11.8 nA/μM was found at pH 6.5. It was reported that the optimal pHs were 6.0 for copper containing NiR from *R. sphaeroides* strain 2.4.3 [32] and copper containing AciNiR and AxgNiR [13], 6.2 for both copper containing NiRs from *A. cycloclastes* and *Achromabacter faecalis* [16]. Compared with these optimal pHs for the free enzymes the value obtained here for the sensor was slightly alkaline range-shifted, and this shift might be ascribed to the enzyme immobilization and effects of polymer layers, especially charged PAH layer.

Fig. 7 shows the typical steady-state current response of the sensor to successive 20 μM increments in nitrite concentrations at optimal conditions under N_2. Substrate concentrations were changed by stepwise addition of a concentrated solution to a stirred buffer. The obtained current response was clear, stable and fast with response time ($t_{90\%}$) of about 20 s, which was faster than those of previously reported [7,10]. To the best of our knowledge, such clear and stable current response has rarely been reported in the literature. The relatively fast response of the sensor reported here might be attributable to the favorable electrostatic attraction between PAH layer and substrate, and/or between NiR and MV as mentioned in previous section.

Inset of Fig. 7 shows calibration curve of the sensor. The sensitivity of the sensor was 11.8 nA/μM with linear detection range of 1.5–260 μM ($r^2=0.996$) and detection limit of about 1.5 μM (S/N=3, background noise of about 5 nA). The deviation from linearity at higher concentration of the substrate

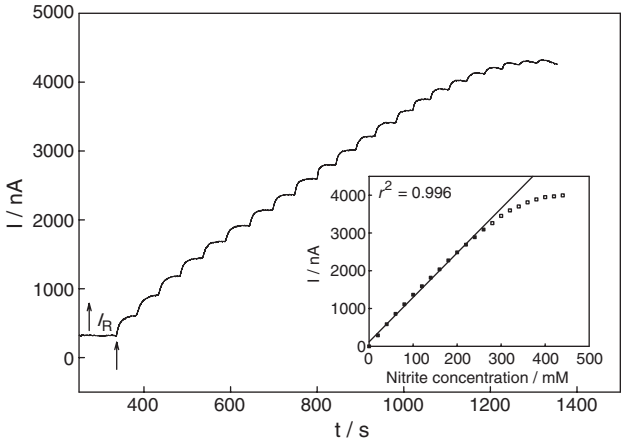

Fig. 7. Steady-state current response of the nitrite reductase and methyl viologen co-immobilized glassy carbon electrode to successive 20 μM increments of nitrite in 0.05 M PBS containing 0.05 M KCl, pH 6.5 under N_2. The applied working potential was −750 mV. Inset: calibration curve.

may have been resulted from the saturation of the immobilized enzyme, and/or inhibition of the immobilized enzyme by the enzymatic product of nitrous oxide [12]. The detection limit of the sensor was close to the maximum acceptable level (2.2 μM) in drinking water in European Union [10,11], suggesting this sensor possibly be used in water analysis. The obtained performance factors for the sensor were totally comparable to those reported in the literature. For example, the reported sensitivities were in the range of 5.6–15.6 nA/μM, linear detection ranges were up to 250 μM and detection limits were in the range of 1.0–5.4 μM [7–11]. The variations in sensitivity, linear detection range and detection limit may mainly be attributable to the different substrate specificities of NiRs from different sources and to the different methods of immobilization.

The Lineweaver–Burk plot showed that Michaelis–Menten constant ($K_{m,app}$) was about 770 μM. This value was in well agreement with that obtained with di-4-pyridyl disulfide modified gold electrode in copper containing AciNiR and pseudoazurin containing buffer solution, i.e. 700 μM [21]. It was also reported that K_m values were 150 μM for copper containing NiR (from *R. sphaeroides* strain 2.4.3) immobilized biosensor [8], 205 μM for tetraheme cytochrome *c* NiR (from *S. deleyianum*) immobilized biosensor [7]. Compared with these reported values, $K_{m,app}$ value reported here was relatively high. The differences in $K_{m,app}$ values may have been resulted from the difference in immobilization method employed in this study; PAH layer, acting as mass transport barrier, may limit the free approach of the substrate to enzyme layer.

The R.S.D. of the current responses was 3.8% for the sensors prepared in the same set ($n=9$). This value was comparable to 3.0% ($n=6$) for copper containing NiR (from *R. sphaeroides* strain 2.4.3) immobilized biosensor [8]. The R.S.D. for the sensors prepared in different sets ($n=5$) was 8.2%, and the relatively high R.S.D. value may be due to less reproducible enzyme, mediator and polymer loadings on the GCE surface.

Interferences from chlorate, chloride, sulfite, sulfate and nitrate were studied. The obtained results showed that chlorate, chloride, sulfite, sulfate did not interfere with the nitrite determination. However, nitrate interfered with the determination, the sensitivity of the sensor for this anion was 4.5 nA/μM, which was about 38% of that for nitrite. This interference might be originated from the selectivity of the enzyme itself, because reduced MV is not able to reduce nitrate [26]. However, contrary to this result, it was reported that nitrate did not interfere with the nitrite determination with copper containing NiR (from *R. sphaeroides* strain 2.4.3) immobilized biosensor [8], and with cytochrome *c* NiR (from *D. desulfuricans* ATCC 27774) immobilized biosensor [11]. The concentrations of nitrate and nitrite in Han River (Paldang dam, Kyunggi Do, Korea) were determined to be 107.9 and 2.9 μM by ion chromatography [33]. Even though the detection limit of the sensor (1.5 μM) was low enough to detect this low concentration of nitrite, unfortunately, the relatively high nitrate concentration and the relatively high interference effect by nitrate made it impossible to determinate nitrite concentration in this real water sample with the biosensor.

One of the obstacles for developing of NiR based biosensor was the poor stability of the enzyme [11]. However, the NiR used in this study was rather stable. It was reported that activity of NiR from the same source used in this study was not affected when the enzyme was heated even to 70 °C for 10 min, and the enzyme could be stored at −15 °C for at least 3–4 months without the loss of initial activity [17]. The obtained sensitivity of the sensor was 8.8 nA/μM after one month of storage under ambient air at room temperature. As mentioned, the sensitivity of a freshly prepared sensor was 11.8 nA/μM, if long term stability of an enzyme electrode was defined as a period in which immobilized enzyme retained 80% of initial activity, the storage stability of the sensor was estimated to be about 24 days, which were comparable to others reported in literature [7,8,11]. The reasons for the prolonged storage stability of the sensor reported here should be ascribed mainly to the relatively stable NiR itself, and might be ascribed partially to the stabilization effect by PVA matrix. It was reported that poly-ol structure tends to stabilize the immobilized enzymes [34]. The relatively long storage stability of the sensor should be useful in practical applications.

Table 1
Recovery test of the biosensor for nitrite

Added (μM)	Determined[a] (μM)	Recovery (%)	R.S.D. (%)
5	4.8	96.0	3.8
20	19.9	99.5	2.8
50	50.8	101.6	2.7
100	102.7	102.7	3.2
200	200.8	100.4	2.0

[a] An average of three determinations.

To test accuracy of the sensor, several assays were made for standard substrate solution. Substrate concentration was determined from the calibration curve in the linear range. The sensitivities of the sensor for different concentrations of the standard substrate solution were normalized by the statistical sensitivity of 11.8 nA/μM to compensate the variations resulted from the different sets of fabrications of the sensor. The linear regression equation for calculation of unknown nitrite concentration was as follows: $I=11.8\times\text{Conc.}+117.9$, where I was current in unit of nA, and Conc. was unknown nitrite concentration in unit of μM. As shown in Table 1, reliable results could be obtained. In spite of the relatively high R.S.D. values the biosensor method described here did not require expensive equipment. It is possible to develop a reliable nitrite biosensor with rapidity, durability, simplicity and convenience for actual applications.

4. Conclusions

The relatively stable copper containing NiR (from *R. sphaeroides* forma sp. *denitrificans*) was co-immobilized with methyl viologen on glassy carbon electrode surface for electroenzymatic determination of nitrite. Methyl viologen was the suitable mediator for electron transfer between electrode and NiR among the selected mediators. PAH layer could effectively prevent immobilized MV from leaking out from electrode surface. The co-immobilization method used in this study was demonstrated to be simple, rapid, and effective. Thus prepared enzyme electrode used as a nitrite biosensor showed excellent catalytic effect

for nitrite reduction and satisfactory performance factors such as high sensitivity, fast response, wide linear detection range, sufficiently low detection limit, relatively long storage stability and high reproducibility. In spite of these merits, one of the drawbacks of the sensor was the selectivity, i.e. this sensor was relatively sensitive to ubiquitous nitrate. This may be the limit of the NiR from this source used for preparation of current electrochemical biosensor for selective determination of nitrite in real samples.

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References

[1] K. Rajeshwar, J.G. Ibanez, Environmental Electrochemistry, Academic press, California, 1997, p. 29.
[2] M.J. Moorcroft, J. Davis, R.G. Compton, Detection and determination of nitrate and nitrite: a review, Talanta 54 (2001) 785–803.
[3] L.S. Clesceri, A.E. Greenberg, A.D. Eaton, Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, Washington D. C., USA, 1998, part 4000.
[4] P. McCarthy, R.W. Klusman, S.W. Cowling, J.A. Rice, Water analysis, Anal. Chem. 65 (1993) 244R–292R.
[5] C.H. Kiang, S.S. Kuan, G.G. Guilbault, A novel enzyme electrode methode for the determination of nitrite based on nitrite reductase, Anal. Chim. Acta 80 (1975) 209–214.
[6] M. Scharf, C. Moreno, C. Costa, C. van Dijk, W.P. Payne, J. LeGall, I. Moura, J.J.G. Moura, Electrochemical studies on nitrite reductase towards a biosensor, Biochem. Biophys. Res. Commun. 209 (3) (1995) 1018–1025.
[7] B. Strehlitz, B. Gründig, W. Schumacher, P.M.H. Kronack, K.-D. Vorlop, H. Kotte, A nitrite sensor based on a highly sensitive nitrite reductase mediator-coupled amperometric detection, Anal. Chem. 68 (1996) 807–816.
[8] Q. Wu, G.D. Storrier, F. Pariente, Y. Yang, J.P. Shapleigh, H.D. Abruña, A nitrite biosensor based on a maltose binding protein nitrite reductase fusion immobilized on an electropolymerized film of a pyrrole-derived bipyridinium, Anal. Chem. 69 (1997) 4856–4863.
[9] S. Sasaki, I. Karube, N. Hirota, Y. Arikawa, M. Nishiyama, M. Kukimoto, S. Horinouchi, T. Beppu, Application of nitrite reductase from *Alcaligenes faecalis* S-6 for nitrite measurement, Biosens. Bioelectron. 13 (1) (1998) 1–5.
[10] C.C. Rosa, H.J. Cruz, M. Vidal, A.G. Oliva, Optical biosensor based on nitrite reductase immobilized in controlled pore glass, Biosens. Bioelectron. 17 (2002) 45–52.
[11] S.D. Silva, S. Cosnier, M.G. Almeida, J.J.G. Moura, An efficient poly (pyrrole-viologen)-nitrite reductase biosensor for the mediated detection of nitrite, Electrochem. Commun. 6 (2004) 404–408.
[12] B.A. Averill, Dissimilatory nitrite and nitric oxide reductase, Chem. Rev. 96 (1996) 2951–2964.
[13] S. Suzuki, K. Kataoka, K. Yamaguchi, Metal coordination and mechanism of multicopper nitrite reductase, Acc. Chem. Res. 33 (2000) 728–735.
[14] J.W. Godden, S. Turley, D.C. Teller, E.T. Adman, M.Y. Liu, W.J. Payne, J. JeGall, The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*, Science 253 (1991) 438–442.
[15] M. Kukimoto, M. Nishiyama, M.E.P. Murphy, S. Turley, E.T. Adman, S. Horinouchi, T. Beppu, X-ray structure and site-directed mutagenesis of a nitrite reductase from *Alcaligenes faecalis* S-6: roles of two copper atoms in nitrite reduction, Biochemistry 33 (1994) 5246–5252.
[16] E.T. Adman, J.W. Godden, S. Turley, The structure of copper–nitrite reductase from *Achromobacter cycloclastes* at five pH values, with NO_2^- bound and with type II copper depleted, J. Biol. Chem. 270 (46) (1995) 27458–27474.
[17] W.P. Michalski, D.J.D. Nicholas, Molecular characterization of a copper-containing nitrite reductase from *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, BBA 828 (1985) 130–137.
[18] M.E.P. Murphy, S. Turley, E.T. Adman, Structure of nitrite bound to copper-containing nitrite reductase from *Alcaligenes faecalis*, J. Biol. Chem. 272 (45) (1997) 28455–28460.
[19] M.J. Ellis, F.E. Dodd, G. Sawers, R.R. Eady, S.S. Hasnain, Atomic resolution structures of native copper nitrite reductase from *Alcaligenes xylosoxidans* and the active site mutant Asp92Glu, J. Mol. Biol. 328 (2003) 429–438.
[20] F. Cutruzzolá, Bacterial nitric oxide synthesis, BBA 1411 (1999) 231–249.
[21] T. Kohzuma, S. Shidara, S. Suzuki, Direct electrochemistry of nitrite reductase from *Achromobacter cycloclastes* IAM 1013, Bull. Chem. Soc. Jpn. 67 (1) (1994) 138–143.
[22] K. Olesen, A. Veselov, Y. Zhao, Y. Wang, B. Danner, C.P. Scholes, J.P. Shapleigh, Spectroscopic, kinetic, and electrochemical characterization of heterologously expressed wild-type and mutant forms of copper-containing nitrite reductase from *Rhodobacter sphaeroides* 2.4.3, Biochemistry 37 (1998) 6086–6094.
[23] G. Decher, J. B. Schlenoff (Ed.), Multilayer thin films − sequential assembly of nanocomposite materials, WILEY-VCH Verlag GmbH and Co. KGaA, Weinheim, 2003.
[24] J.C. Hoogvliet, L.C. Lievense, C. van DIJK, C. Veeger, Electron transfer between the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) and viologens, Eur. J. Biochem. 174 (1988) 281–285.
[25] R.B. Mellor, J. Ronnenberg, W.H. Campbell, S. Diekmann, Reduction of nitrate and nitrite in water by immobilized enzymes, Nature 355 (1992) 717–719.
[26] N.F. Ferreyra, S.A. Dassie, V.M. Solis, Electroreduction of methylviologen in the presence of nitrite. Its influence on enzymatic electrodes, J. Electroanal. Chem. 486 (2000) 126–132.
[27] C. Moreno, C. Costa, I. Moura, J. Le Gall, M.Y. Liu, W.J. Payne, C. van DIJK, J.J.G. Moura, Electrochemical studies of the hexaheme nitrite reductase from *Desulfovibrio desulfuricans* ATCC 27774, Eur. J. Biochem. 212 (1993) 79–86.
[28] M.D. Ryan, J.F. Wei, B.A. Feinberg, Y.K. Lau, The chronoamperometric determination of homogeneous small molecule-redox protein reaction rates, Anal. Biochem. 96 (1979) 326–333.
[29] E. Lojou, F. Cutruzzola, M. Tegoni, P. Bianco, Electrochemical study of the intermolecular electron transfer to *Pseudomonas aeruginosa* cytochrome cd1 nitrite reductase, Electrochim. Acta 48 (2003) 1055–1064.
[30] R.W. Murray, in: A.J. Bard (Ed.), Chemically Modified Electrodes, vol. 13, Marcel Dekker, Inc., New York, 1984, pp. 191–368.
[31] P.M.S. Monk, The Viologens: Physicochemical Properties, Synthesis and Applications of the Salts of 4,4′-bipyridine, John Wiley & Sons Ltd., West Sussex, England, 1998.
[32] Y. Zhao, D.A. Lukoyanov, Y.V. Toropov, K. Wu, J.P. Shapleigh, C.P. Scholes, Catalytic function and local proton structure at the type 2 copper of nitrite reductase: the correlation of enzymatic pH dependence, conserved residues, and proton hyperfine structure, Biochemistry 41 (2002) 7464–7474.
[33] D. Quan, J.H. Shim, J.D. Kim, H.S. Park, G.S. Cha, H. Nam, Electrochemical determination of nitrate with nitrate reductase-immobilized electrodes under ambient air, Anal. Chem. 77 (14) (2005) 4467–4473.
[34] T.D. Gibson, J.R. Woodward, in: P.G. Eldman, J. Wang (Eds.), Biosensors and Chemical Sensors, ACS, Washington, D.C., 1992, pp. 40–55, Chap. 5.