J. Microbiol. Biotechnol. (2006), **16**(4), 505–510

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characteristics of Mediated Enzymatic Nitrate Reduction by Gallocyanine-Bound Nanoporous Electrode

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Received: November 21, 2004
Accepted: December 2, 2004

Abstract A gallocyanine-bound nanoporous titanium dioxide electrode system was investigated to carry out a mediated enzyme reaction. Gallocyanine was bound either directly or through an aminopropylsilane linker to the film of nanoporous titanium dioxide and used as a mediator for nitrate reductase in the mediated enzymatic nitrate reduction. The electrode with the aminopropylsilane linker showed 20% higher efficiency of electron transfer at the same potential than that directly linked. The prepared electrodes showed 0.26 μmol/h nitrate reduction at a 100 mm^2 surface of the electrode, and linear current response on nitrate ion concentration up to 1.0 mM, which is very useful as a biosensor of nitrate ion in water.

Key words: Nitrate reductase, gallocyanine, amperometric, nanoporous, titanium dioxide

The direct electron transfer between an enzyme and an electrode is highly limited, because of the distance between the electrode surface and the redox active site of the enzyme that is normally inside the globular protein, and the inadequate orientation of donor to acceptor sites, which is dependent on the method of the contact of the enzyme at the electrode. Enzymes and organic molecules are not electroactive themselves and can be reduced by the mediator, and an electrochemically reduced mediator has been used fregaently as a regenerator for enzymes that involve nitrate and nitrite in their catalytic cycle [2, 11].

Titanium dioxide (TiO_2) is a low cost, widely available, non-toxic and even biocompatible substance that is widely used in commercial applications (*e.g.*, increasing paint durability, cleaning waste waters, etc.) [7]. However, in recent years it has become the semiconductor of choice because of its many advantages for electron transfer in photophysics and sensitized photo-electrochemistry, just like most of the other wide bandgaped and chemically stable oxides [4]. It is essentially easy to coat on to surfaces and possible to dope a range of dyes on TiO_2 and check their electrochemical characteristics in TiO_2 coating.

Grätzel and coworkers [4, 7] discovered that dye-sensitized nanocrystalline solar cells (DSSC) could convert visible light to electricity with high efficiency. The development of these systems was based on the preparation of rough, high surface area titanium dioxide thin-film electrodes. A solution to this problem, developed by the Grätzel group, was to use a porous nanocrystalline TiO_2 electrode structure in order to increase the internal surface area of the electrode to allow large enough amount of electron mediator to be contacted at the same time by the TiO_2 electrode and the electrolyte.

In this paper, a novel system for the mediated enzyme system was investigated using gallocyanine and nanoporous TiO_2-coated electrode with nitrate reductase as a model.

Materials and Methods

Electrochemical Cell and Cyclic Voltammetry

The electrochemical cell was maintained anaerobically by purging argon gas using a sparger and the anode was separated from the cathode in the reaction media. A commercial Ag/AgCl electrode (Fisher Scientific) was used as a reference electrode and graphite (5 mm diameter, Sigma-Aldrich) was used as the counter electrode. Both current and potential were measured in working, reference, and counter electrodes.

A cyclic voltammetry was carried out by applying a linear potential (that is, a potential that increases or decreases linearly over time) to the working electrode. As the potential was swept back and forth the formal potential in the solution, a current flowed through the electrode that

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reduced the mediator. The magnitude of this current was proportional to the extent reduction-oxidation of reaction.

Preparation of Nanoporous Titanium Dioxide Electrode System

An electrode that was coated with nanoporous titanium dioxide was prepared by coating titanium dioxide nanopaste and sintering on the surface of conducting FTO (fluorine doped tin oxide from Solaronix, Swiss) glass. Titanium dioxide nanopaste was applied as a thin layer on the FTO glass (titanium dioxide nanopaste from Solaronix) and dried at room temperature to evaporate water before sintering. Sintering was carried out at 200°C for 6 h to crosslink nanoparticles and form the nanoporous structure at the FTO electrode.

Nitrate reductase was separated from *Ochrobactrum anthropi* SY509 [8, 9] and used as the electron acceptor from the mediator.

Preparation of Gallocyanine-Bound Nanoporous Electrode

Gallocyanine (Sigma Aldrich) was bound to the surface of nanoporous titanium dioxide film on FTO glass by two different methods. One was direct binding and the other was indirect binding through an aminopropyltriethoxysilane (Sigma-Aldrich) linker.

The direct binding of the dye to the electrode surface usually takes place via special anchoring groups attached to the dye molecule. In the gallocyanine dye, these are one carboxylic group at the end of the rings. The carboxylic acid groups form a bond with the TiO_2 surface by donating a proton to the TiO_2. To bind gallocyanine dye to the electrode surface, the TiO_2-coated electrode was dipped into gallocyanine-saturated ethanol solution for 5 h and then thoroughly rinsed with ethanol and stored at room temperature. Prepared gallocyanine-coated electrode was dipped again into 50 mM phosphate buffer (pH=7.0) for 10 h prior to use.

Indirect binding through aminopropylsilane (linker) took place through a different method from direct binding [11]. Primary modification of the electrode surface was performed with 3-aminopropyltriethoxysilane (10% v:v solution in toluene) treatment at 70°C for 5 h. The silylated electrode was thoroughly rinsed with toluene and a HEPES-buffer solution (50 mM, pH 7.3) and dried in air at room temperature for 30 min. Then, gallocyanine was covalently linked to the aminosiloxane-functionalized electrode surface by immersing the electrode into HEPES-buffer, pH 7.3, containing gallocyanine (5 mM) and EDC (10 mM), for 2 h. The modified electrode was thoroughly rinsed with a HEPES-buffer solution and dried in air at room temperature.

Nitrate Reductase Activity and Nitrite Concentration Assay

The assay mixture (total volume, 1.5 ml) was composed of a potassium phosphate buffer (80 mM, pH 7.0), containing methyl viologen (1 mM) as an artificial electron donor, and dithionite (10 mM) as the reducing agent. The addition of excess dithionite facilitated handling of the solution without an anaerobic chamber. Enzyme was added and the mixture was stirred at 30°C. Subsequently, the reaction was started by adding potassium nitrate (10 mM). Nitrate reduction was stopped by vigorously vortexing the mixture to oxidize all the dithionite and methyl viologen. After removal of the cell extracts by centrifugation, the nitrite concentrations in the samples were measured by color change after addition of sulfanilamide and N-(1-napthyl) ethylenediamine hydrochloride.

One unit of nitrate reductase produced 1.0 μmole of nitrite-N per min·mg cell extracts in the presence of methyl viologen at pH 7.0 at 30°C. This nitrite reductase assay was performed as described for nitrate reductase. One unit of nitrite reductase reduced 1.0 μmole of nitrite-N per min·mg cell extracts in the presence of benzyl viologen at pH 7.0 at 30°C.

RESULTS AND DISCUSSIONS

Characteristics of the Nanoporous Electrode

The gallocyanine-bound nanoporous TiO_2-coated electrode was characterized using SEM to investigate the surface structure of electrode and nanoparticle, X-ray diffraction for crystallinity, and voltammetry for redox potential. The nanoporous structure of titanium dioxide formed after sintering was observed by SEM, as shown in Fig. 1. After

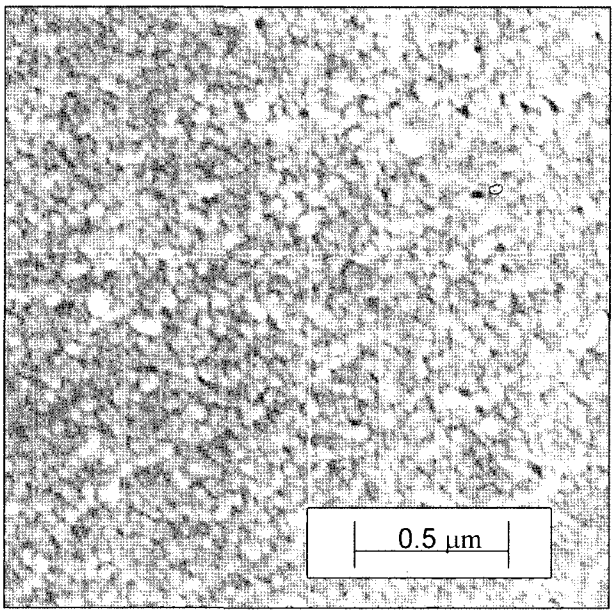

Fig. 1. Scanning electrode micrograph of a nanoporous titanium dioxide film section.

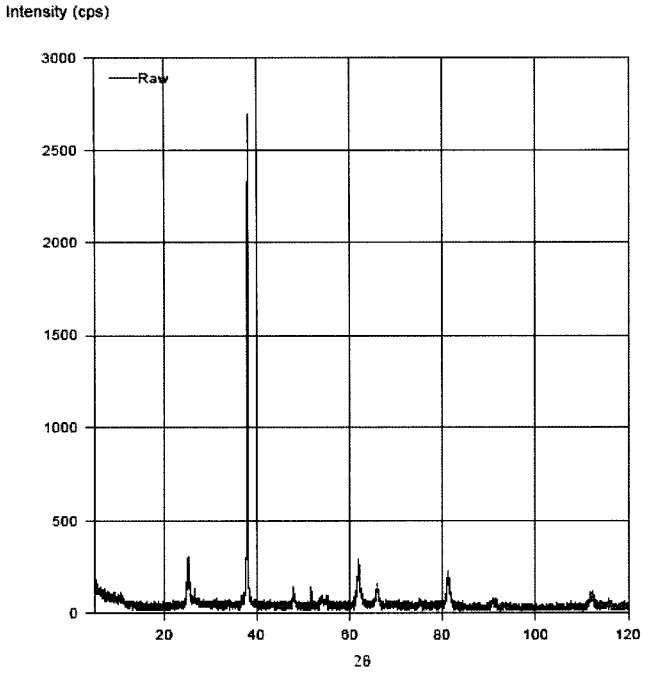

Fig. 2. XRD data for differentiating the crystallinity between anatase and rutile of formed nanoporous electrode.

sintering, the coated layer could not be stripped by water or organic solvent because of the formation of crosslinks between the titanium dioxide nanoparticles; the thickness of the film was about 20 μm based on microscopy. The surface of the film was formed evenly and the sizes of nanoparticles of the nanoporous structure in the surface was about 20–50 nm in diameter, based on SEM photography.

To confirm the crystallinity of the titanium dioxide of the formed film, X-ray diffraction data were obtained, as shown in Fig. 2. It is well known that titanium dioxide has two types of crystallinity, named anatase and rutile. Anatase has a better property of conductivity and binding dye than rutile [4, 7]. Large peaks of 25° and 38° 2theta, as shown in Fig. 2, came from anatase and a small peak of 2° 2theta came from rutile. Hence, the formed nanoporous structure was composed mainly of anatase, which is favorable for the mediated electron transfer.

Cyclic voltammography was tested in 50 mM phosphate buffer to estimate the reduction potential and reduction characteristics after gallocyanine binding, as shown in Fig. 3; electrodes prepared by the two methods showed almost the same reduction properties, and the link between nanoparticle and gallocyanine did not affect the reduction properties. The amount of bound gallocyanine could be estimated, based on Fig. 3, by comparing passed electrons before and after gallocyanine binding using Faraday's Law. About 10 nmoles were estimated as the amount of bound gallocyanine at the film (5 mm×5 mm, 20 mm thickness) from the following equation of Faraday's Law.

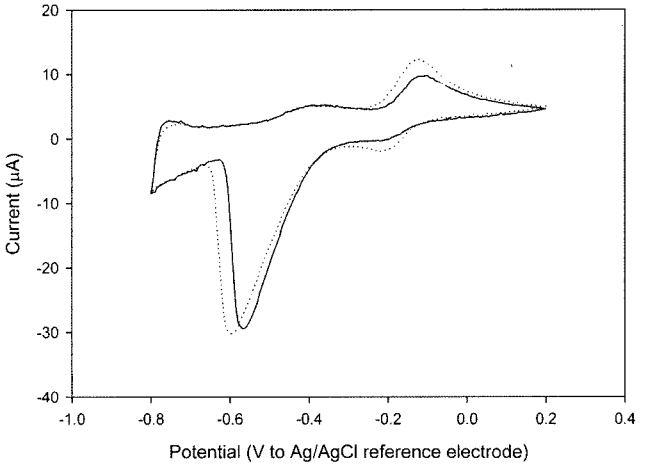

Fig. 3. Cyclic voltammograms for two different types of gallocyanine binding on nanoporous electrode (—: gallocyanine bound directly, ···: gallocyanine bound through the linker).

$$\text{Reduced moles of gallocyanine} = \text{Sum of (Ampere} \times \text{time (sec))}/96{,}495 \quad (1)$$

The reduction potentials of the two prepared gallocyanine-bound electrodes were about −0.7 V to the Ag/AgCl reference electrode but could be altered depending on the scan rate and the thickness of the film, while the intrinsic reduction potential of gallocyanine was −0.3 V. The reduction potential of the bound gallocyanine from the cyclic voltammogram decreased to below −0.7 V when the scan rate increased, owing to the late depletion of reducible gallocyanine at the electrode. There was very limited reduction and oxidation at the electrode at potential higher than −0.7 V from the cyclic voltammogram, owing to the −0.7 V barrier potential of TiO_2.

Reduction and oxidation of gallocyanine at the electrode could be observed apparently by the color change from blue to transparent because reduced gallocyanine has no color whereas oxidized gallocyanine has a blue color. Transparent electrode was maintained even after cyclic voltammetry because of the irreversible reduction of gallocyanine at the electrode surface.

The electron transfer cascade from electrode to substrate in the electrochemical cell is indicated in Fig. 4. When the potential of nanoporous electrode was decreased under the barrier potential of TiO_2, the electric resistance of the nanoporous structure binding gallocyanine (mediator) became very low, and then generated electrons from the voltammetry could be transferred to the mediator by hopping because of the potential difference between electrode (−0.7 V) and gallocyanine reduction (−0.3 V). The energy state of reduced gallocyanine by a transferred electron goes to the excited state, where reduced gallocyanine (−0.3 V) can transfer its electron to a reducible enzyme

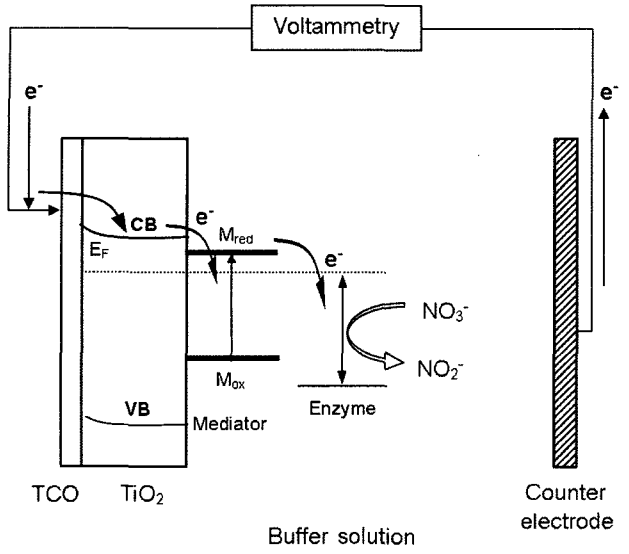

Fig. 4. Electron transfer cascade from the nanoporous electrode to nitrate via a mediator and nitrate reductase enzyme in an electrochemical cell [CB, conduction band; VB, balance band; TCO, transparent conducting oxide (=FTO)].

(0 V reduction potential of active site to Ag/AgCl reference electrode) and then be reduced again at the electrode [6].

Characteristics of Mediated Enzymatic Nitrate Reduction at the Electrode

Nitrate reduction was carried out in the electrochemical cell using reduced enzyme by the oxidation of gallocyanine, and the reaction rate was recorded by current because the reaction rate is proportional to the current passage at the electrode.

Figure 5 shows two possible binding methods of gallocyanine and the electron transfer from the electrode to nitrate via bound gallocyanine. In the absence of nitrate, the electrode can reduce the bound gallocyanine only and there is no additional current after the reduction of the bound gallocyanine. However, in the presence of nitrate, continuous electron transfer occurs through the bound gallocyanine to nitrate through the electron transfer cascade.

As shown in Fig. 5A, gallocyanine was directly bound by an ester-like bond between the carboxylic acid of gallocyanine and the hydroxyl group of the nanoporous structure, whereas gallocyanine in Fig. 5B was bound through aminopropylsilane (linker), which links gallocyanine by an amide bond and titanium dioxide by an oxygen bond. Thus, the distance from the conductive titanium dioxide to the reducible site of gallocyanine became closer when gallocyanine was directly linked than when linked indirectly through the aminopropylsilane group, but there was less freedom of bound gallocyanine in Fig. 5A than Fig. 5B. The efficiency of electron transfer could be estimated using the cyclic voltammetry in Fig. 3 at the electrode and in Figs. 6, 7, and 8 using nitrate reductase.

Mediated enzymatic nitrate reduction in the electrochemical cell was carried out using two types of 10 mm×10 mm electrodes at various conditions to investigate the nitrate reduction performance of the prepared electrodes, and nitrite formation was estimated as shown in Figs. 6 and 7.

The effect of applied potential on the electron transfer and then the effect of barrier potential of the nanoporous electrode on the mediated enzyme reaction is shown in Fig. 6. It is well known that the barrier potential of

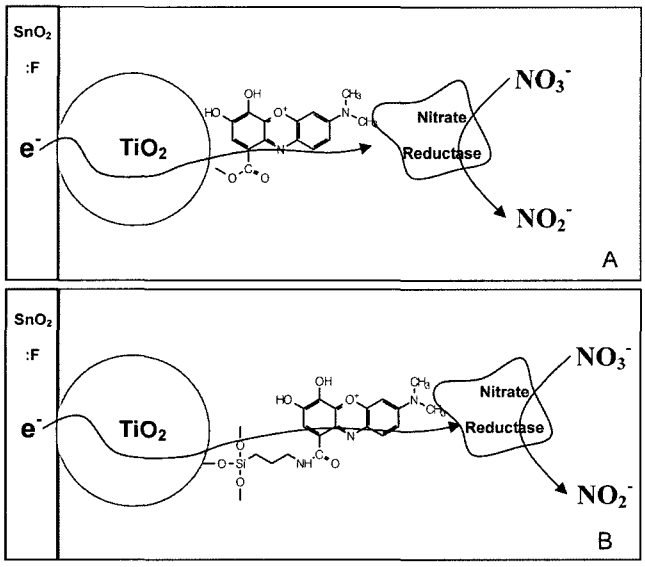

Fig. 5. Electron transfer from electrode to nitrate reductase when gallocyanine was bound directly (**A**), and when gallocyanine was bound through an aminopropylsilane linker at the nanoporous electrode (**B**).

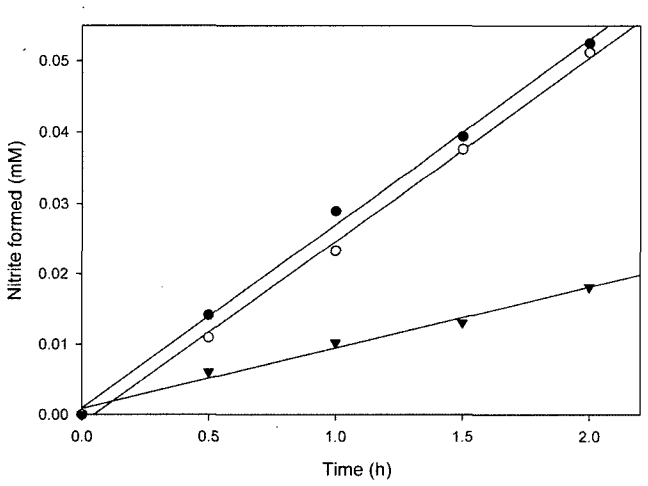

Fig. 6. Effect of applied potential on a mediated enzymatic nitrate reduction at the working electrode (▼: 0.5 V; ●: 0.7 V; and ○: 0.9 V potential to Ag/AgCl reference electrode were applied).

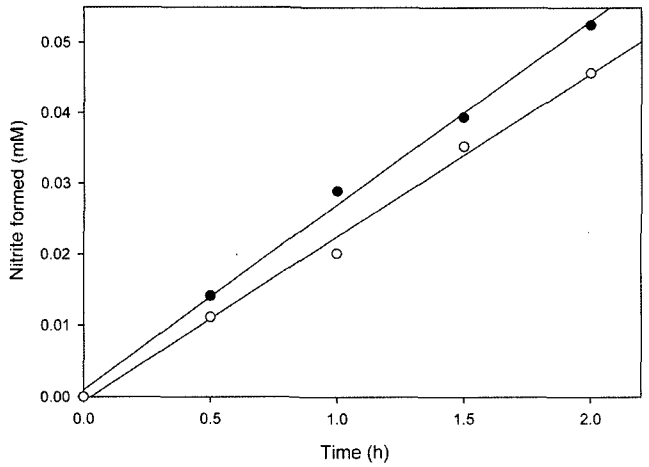

Fig. 7. Comparison between two different types of binding (○: gallocyanine bound directly; ●: gallocyanine bound through a linker).

titanium dioxide is near −0.7 V to Ag/AgCl reference electrode, and therefore −0.5 V, −0.7 V, and −0.9 V were applied and the amount of nitrite formation was estimated to confirm the existence of a barrier potential of titanium dioxide in the electrochemical cell.

The amount of the formed nitrite and patterns of the nitrite formation at −0.7 and −0.9 V were almost the same but only 35% nitrite was formed when −0.5 V potential was applied compared with the case of −0.7 V and −0.9 V. The difference of electron transfer in Fig. 6 seemed to come from an energy barrier of the nanoporous electrode, and this phenomenon could be confirmed visually by color change of electrode from blue to transparent after −0.7 V and −0.9 V potential application, while remaining blue after −0.5 V potential application.

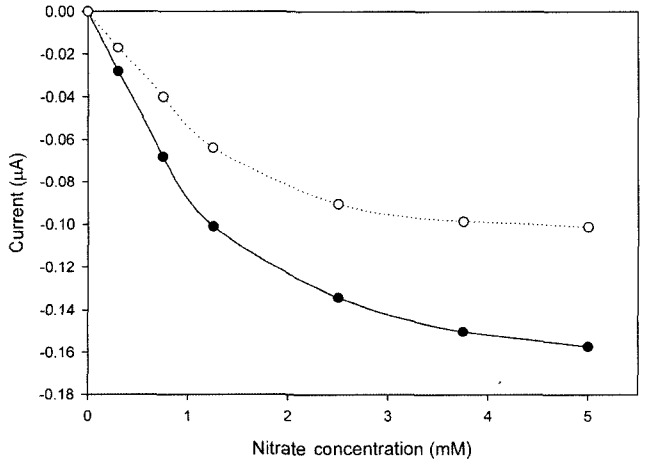

Fig. 8. Amperometric measurement of nitrate reduction at the electrode according to nitrate concentration (●: 2 unit/ml enzyme used; ○: 1 unit/ml enzyme used; each arrows indicates addition of nitrate ion by 1.25 mM).

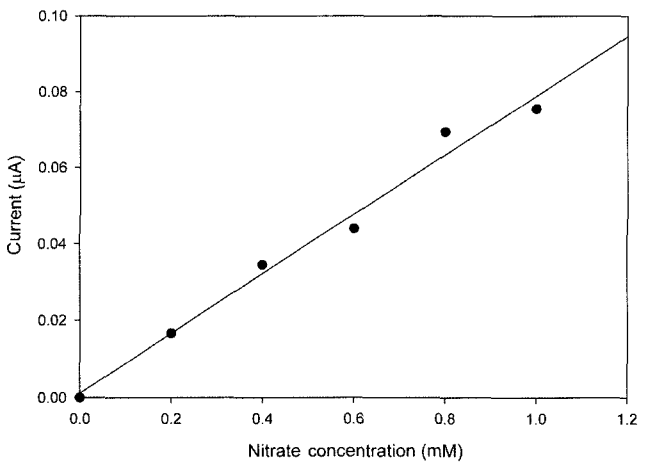

Fig. 9. Amperometirc measurement of enzyme reaction when 5 mM of nitrate ion was injected.

Two electrodes that were prepared by different methods showed similar patterns of electron transfer (nitrite formation in Fig. 6), but showed 20% more efficiency of electron transfer when aminopropylsilane was used as a linker rather than direct binding (0.26 μmol of nitrate was reduced using the nanoporous electrode and enzyme during 1 h when gallocyanine was linked with aminopropylsilane (Fig. 5B), while 0.21 μmol nitrate was reduced when linked directly (Fig. 5A). The higher efficiency of electron transfer of linker-bound gallocyanine seemed to be caused by mobility of gallocyanine in the solution provided by the linker (~40 nmoles from cyclic voltammetry).

Amperometric measurement was carried out by adding nitrate ion consecutively to the electrochemical cell, as shown in Figs. 8 and 9. At first, all bound gallocyanine was reduced at −0.7 V and then measured current at −0.7 V to Ag/AgCl reference electrode with the addition of nitrate ion to the electrochemical cell at 50 mM phosphate buffer (pH=7.0). Current change was observed at the electrode by adding nitrate ion, which means the proceeding of a mediated reaction at the electrode.

Nitrate concentration and current relationship was investigated, as shown in Fig. 8 and Table 1 using nitrate reductase from *Ochrobactrum anthropi* SY509 [8] as the model enzyme for the mediated enzyme reaction and gallocyanine-bound electrode with linker as the working

Table 1. Kinetic parameters of Lineweaver-Burk plot for mediated enzymatic nitrate reduction from the nitrate concentration-current relationship of Fig. 6.

Enzyme activity	V_{max}*	K_m**
1 unit/ml	−0.133	1.32
2 unit/ml	−0.196	1.17

*Reaction rate was measured by current (unit: mA).

**Unit: mM.

electrode in the electrochemical cell. Figure 8 shows that the current was increased with respect to the increase of nitrate concentration and the amount of enzyme to 0.1 μA, in the case of 1 unit/ml enzyme used, and 0.16 μA in the case of 2 unit/ml enzyme. The relation between nitrate concentration and the current in Fig. 8 showed a similar pattern the of Michaelis-Menten equation. The current at the counter electrode indicates flow of electrons for the mediator reduction, thus it was proportional to the rate of nitrate reduction. Hence, current can be regarded as the velocity of nitrate reduction and can be applied to the Lineweaver-Burk plot as follows.

$$1/v(=\text{current})=1/V_{max}+K_m/V_{max}(1/[S]) \quad (2)$$

The results of the Lineweaver-Burk plot are listed in Table 1. The possible maximum current at the electrode (mV) was estimated as −0.13 μA (1 unit/ml enzyme) and −0.2 μA (2 unit/ml enzyme) and K_m as 1.3 mM (1 unit/ml) and 1.2 mM (2 unit/ml enzyme). The difference between the estimated maximum current is due to the amount of enzyme near the electrode. More current was passed by a large amount of enzyme at the electrode because more mediated enzyme reaction had occurred. From the value of K_m, the mediated enzyme system will react linearly to about 1.3 mM nitrate and this result is very useful to the application of electrode system to biosensor. In the case of nitrate, its contamination of drinking water is suspected to be responsible for cancer of the digestive system and most countries have therefore defined limits of the nitrate content of drinking water.

Typical signals and amperometric response on nitrate ion from amperometric measurement are shown in Fig. 9. Reducing potentials in the counter electrode was increased with nitrate ion addition (current reacted linearly to 1.0 mM nitrate as shown in the small figure), and the current increase diminished as nitrate concentration increased, reaching the saturation concentration of nitrate. The current at the counter electrode was changed from 0.1 to 0.2 μA according to the final concentration of nitrate, and 20–30 sec were needed for stabilization of the changed signals.

From the above results, the prepared nanoporous electrode acted efficiently as a mediator for the bioelectrochemical system. An analytical amount of reduction product was observed in the electrochemical cell, and a linear relationship was observed between current and nitrate ion concentration up to 1.0 mM.

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