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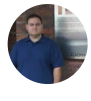

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# Simplified Nitrate-Reductase-Based Nitrate Detection by a Hybrid Thin-Layer Controlled Potential Coulometry/Spectroscopy Technique

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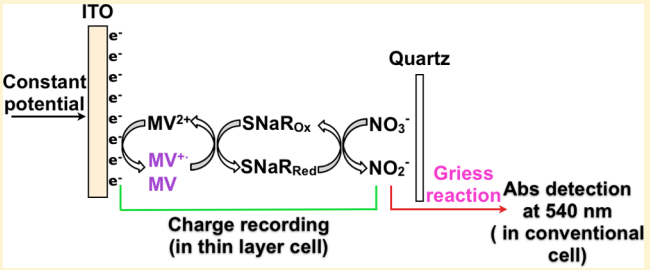
Supporting Information

**ABSTRACT:** A novel method for the detection of nitrate was developed using simplified nitrate reductase (SNaR) that was produced by genetic recombination techniques. The SNaR consists of the fragments of the Mo−molybdopterin (MO−MPT) binding site and nitrate reduction active site and has high activity for nitrate reduction. The method is based on a unique combination of the enzyme-catalyzed reduction of nitrate to nitrite by thin-layer coulometry followed by spectroscopic measurement of the colored product generated from the reaction of nitrite with Griess reagents. Coulometric reduction of nitrate to nitrite used methyl viologen ($MV^{2+}$) as the electron transfer mediator for SNaR and controlled potential coulometry in an indium tin oxide (ITO) thin-layer electrochemical cell. Absorbance at 540 nm was proportional to the concentration of nitrate in the sample with a linear range of 1−160 $\mu$M and a sensitivity of 8000 AU $M^{-1}$. The method required less than 60 $\mu$L of sample. Detection of nitrate could also be performed by measuring the charge associated with coulometry. However, the spectroscopic procedure gave superior performance because of interference from the large background charge associated with coulometry. Results for the determination of nitrate concentration in several natural water samples using this device with spectroscopic detection are in good agreement with analysis done with a standard method.




Nitrate accumulation in water supplies is detrimental to the environment and human health.[1,2] Excess nitrate in rivers is responsible for algal blooming and dissolved oxygen depletion.[3] Children under six months who intake excess nitrate may suffer from severe diseases such as methemoglobinemia, which if left untreated can be fatal.[4,5] The Environmental Protection Agency has regulated the maximum contamination level goal of nitrate (measured as nitrogen) as 10 mg/L since 1992.[5] Since then, nitrate monitoring has been a regulatory necessity. As a result, the demand for effective nitrate detection has increased dramatically, with various detection methods being reported.[6−9] Among them, nitrate-reductase (NaR)-based nitrate biosensors have drawn attention and have been largely developed during the past decade.[10−12]

Eukaryotic assimilatory NAD(P)H/ nitrate reductase (NaR. EC 1.7.7.1−3) catalyzes the first nitrate assimilation step in plants and fungi.[13] The active form of this enzyme is either a homodimer or a homotetramer.[14] Its monomer contains the cofactors flavin adenine dinucleotide (FAD), heme−iron, and Mo−molybdopterin (Mo−MPT) in a 1:1:1 ratio.[14] Nitrate reduction starts with the electron transfer from NAD(P)H to FAD and reaches the Mo−MPT nitrate reduction center through its internal electron transfer chain.[13]

The traditional methods for extracting holo−NaR from plants are complicated and difficult. Also, the obtained NaR usually has low purity and small recovery potential.[15−17] Therefore, to avoid complex extraction and purification procedures to acquire the holo−NaR, genetic recombination techniques have been used to produce the simplified NaR (SNaR) with higher accessibility and efficiency.[14] The SNaR, which originates from yeast *Pichia angusta* and consists of the fragment containing the MO−MPT binding site adjacent to the nitrate reduction active site, was shown to have high activity and specificity for nitrate reduction.[14]

Among the several kinds of NaR-based nitrate biosensors, the main ones are based on electrodes such as screen-printed carbon, carbon nanotubes, platinum, and glassy carbon.[3,18,19] The most commonly employed construction method uses conductive polymers to immobilize the NaR on the surface of the electrode to improve the electron transfer efficiency and minimize the amount of the enzyme.[18−21] Sometimes redox mediators such as Azure A, bromophenol blue, and methyl

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viologen are used to aid the electron transfer between electrode and NaR.[22,23]

Although polymer-modified nitrate biosensors have a large detection range and high sensitivity, their reproducibility is relatively poor because the modified surface of the electrodes may change when potential/current is applied, and the activity of NaR decreases with time, especially when it is exposed to an open environment.[19,22] Also, construction of the sensors is often time-consuming because they require several pretreatment steps for both electrode and polymer.[20−23]

Controlled potential coulometry is a technique that involves the analysis of a material from the production of charge during its complete electrolysis.[24,25] As the charge is proportional to the concentration of the analyte, it provides a path to determine the amount of nitrate being converted to nitrite during its reduction. Complete electrolysis of small sample volumes can be achieved rapidly in a thin-layer electrochemical cell.[24] A disadvantage of coulometry is the need to correct for background charge, which can become a problem at low concentrations of analyte.

The Griess diazotization reaction is a well-developed method for nitrite detection that was first introduced by J. P. Griess.[26] In this test, typically in a low pH environment, a three-step reaction (nitrosation, diazonium ion formation, and coupling) proceeds to form the diazo dye in the presence of nitrite compounds and Griess reagents.[27] Various combinations of Griess reagents (nitrosatable compounds and coupling reagents) have been systematically tested and compared in their effectiveness in forming chromophore.[27] Several of them were proposed as proper combinations in consideration of the reaction rates, stability of the diazo dye, possible interferences, and the toxicity of reagents.[27,28] Because upon two-electron reduction, nitrate produces nitrite in a 1:1 ratio, the Griess diazotization test provides an indirect but strictly proportional method for monitoring the nitrate concentration as the measurement of nitrite.

In this paper, we introduce a novel method for the determination of nitrate based on SNaR. Controlled potential coulometry using an indium tin oxide (ITO) thin-layer electrochemical cell was used for nitrate reduction to nitrite, and two modes of detection were compared: coulometry in which the charge required for reduction of nitrate to nitrite was measured and corrected for background and UV−vis absorption spectroscopy in a conventional cuvette after reaction of the coulometrically generated nitrite with Griess reagents to form a colored product. Instead of using the much more expensive nicotinamide adenine dinucleotide, methyl viologen ($MV^{2+}$) was used as the electron transfer mediator. Figure 1 (Abstract Graphic) shows the schematic of the reaction processes: A constant potential is applied to the ITO electrode

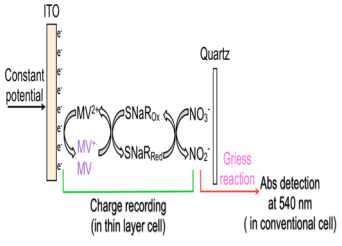


**Figure 1.** Schematic of the nitrate reduction process in a thin-layer electrochemical cell.

where electrons are transferred first to $MV^{2+}$ to form $MV^{+\bullet}$ or MV and then to SNaR, which catalyzes the reduction of nitrate to nitrite. Charge was recorded during the process of electrolysis. After that, Griess reagents were added to the collected sample to generate an absorbance proportional to the amount of nitrite produced.

In order to achieve the optimized conditions, experimental parameters of buffer and mediator concentrations as well as applied potential and time were investigated. The interferences of other anions such as phosphate, chloride, sulfate, carbonate, and bicarbonate with nitrate and nitrite were also studied. Nitrate determination in natural water samples by this device and paired testing of samples were performed with a commercially available kit.

## EXPERIMENTAL SECTION

**Reagents.** SNaR (12.2 units $mL^{-1}$, 10.2 mg $mL^{-1}$) was provided by Nitrate Elimination Co., Inc. The nitrate standard solution (100 mg $L^{-1}$ $NO_3^-$−N) was purchased from Hach Co. Potassium nitrate, sodium chloride, sodium sulfate, sodium phosphate, sodium carbonate, and sodium bicarbonate were purchased from Fisher Scientific, Inc. Other chemicals were purchased from Sigma Aldrich Co. All the chemicals were used without further purification. A Nanopure water system (Barnstead, Thermo Scientific) was the source of deionized water. Samples of ground and surface water were obtained from Cincinnati public water and the Ohio River.

**Enzyme Assays.** The activity of SNaR was determined with a nitrate reductase activity assay kit provided by Nitrate Elimination Co., Inc. A Pierce BCA protein kit from Thermo Scientific was used for SNaR concentration determination.

**Thin-Layer Electrochemical Cell Construction and Instrumentation.** The construction of the thin-layer electrochemical cell used in this study has been described previously.[25] It consists of an indium tin oxide (ITO) working electrode (4.0 cm in length, 1.0 cm in width) and a quartz slide (2.5 cm in length, 1.0 cm in width), separated by a silicone spacer (~0.018 cm). A self-designed cell holder was used to hold the cell and separate the Ag/AgCl 3 M KCl miniature reference electrode (EDAQ, ET073) and the Pt wire auxiliary electrode. All potentials reported herein are relative to this reference electrode. Typically, the cell contains a 50 μL sample solution. All electrochemical measurements were carried out in the thin-layer cell.

Cyclic voltammetry was done with a BASi 100B Electrochemical Analyzer (Bioanalytical Systems). Controlled potential coulometry was carried out with a Gamry Reference 600 potentiostat (Gamry Instruments, Inc.) either in a glovebag or under the ambient atmosphere, depending on the experiments. After coulometry, 600 μL of pH 7.0, 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer was added to the open top of the cell, and the contents were transferred to a 1.5 mL centrifuge tube for Griess reaction. Selected Griess reagents—58 mM sulfanilamide (SAN, 100 μL) in 3 M HCl and 0.77 mM *N*-(1-naphthyl)ethylenediamine (NED, 100 μL)—were then added to the collected solution in the centrifuge tube. After the tube was vortexed, the mixture was incubated for at least 10 min at room temperature. The absorption spectra were taken with a Cary 50 UV−vis spectrometer (Agilent Technologies), and the absorbance of the diazo dye was measured at 540 nm. All the experiments were carried out at room temperature.

**Hach Spectrometer.** A Hach DR890 colorimeter was employed for measuring the concentration of nitrate in water


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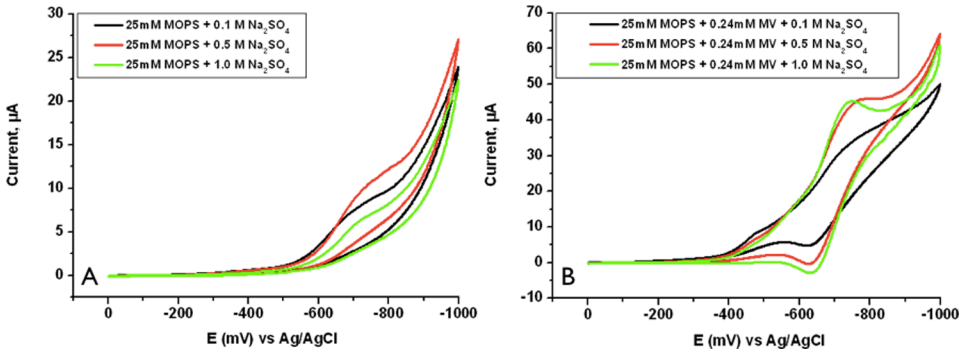


**Figure 2.** Thin-layer cyclic voltammetry of 25 mM MOPS with (A) 0.1 M, 0.5 M, 1.0 M $Na_2SO_4$ and (B) 0.24 mM $MV^{2+}$ in 0.1 M, 0.5 M, 1.0 M $Na_2SO_4$. Scan initiated from 0 mV in the negative direction to −1000 mV at a scan rate of 2 mV $s^{-1}$.

samples for comparison purposes. The cadmium reduction method was used, and the procedures were followed as described in the handbook on pp 313−315.[29]

**Safety Considerations.** Methyl viologen is toxic, and any direct contact should be avoided. Cadmium is toxic, and appropriate protections are strongly suggested.

## RESULTS AND DISCUSSION

**Supporting Electrolyte and Mediator Selection.** The performance of the SNaR-based nitrate detection method was first optimized by choosing a supporting electrolyte system to provide adequate conductivity in the thin-layer cell and pH control for SNaR and a mediator to transfer electrons from the ITO electrode to SNaR. Although there is no universal buffer system (buffer and supporting electrolyte) for electrochemical experiments, several criteria such as electrochemical and chemical inertness and electrical conductivity have been used to evaluate buffer systems for typical analytes.[30] In our study, the SNaR was initially dissolved in 25 mM MOPS. Since MOPS is a commonly used buffer for biological systems at neutral pH and also has good solubility and electrochemical potential window (as shown in Figure 2A), we decided to use it as the buffer in this study. Because thin-layer electrochemical cells inherently have greater solution resistance, an additional supporting electrolyte was added to the buffer to minimize resistance in the cell. For this supporting electrolyte, preliminary experiments showed that $Na_2SO_4$ was inert at very negative potential (−1000 mV) and did not interfere with either nitrate or nitrite. Three $Na_2SO_4$ solutions of different concentrations were selected for comparison. Figure 2A shows the cyclic voltammogram of three concentrations of $Na_2SO_4$ in 25 mM MOPS. The background voltammograms show $O_2$ reduction at around −700 mV.

As is typical of many redox enzymes, an electron transfer mediator is needed to couple the enzyme to the electrode for electrochemical reduction. Several mediators including 2,6-dichlorophenolindophenol, methylene blue, 2-hydroxy-1,4-naphthoquinone, azure A, bromophenol blue (BPB), and methyl viologen ($MV^{2+}$) were evaluated. BPB and $MV^{2+}$ were found to be more effective in reducing nitrate than the other mediators. However, in the thin-layer cell, the electron transfer of BPB was irreversible and gave a narrower linear range for nitrate detection, and as a result, $MV^{2+}$ was chosen as the mediator for the following studies. The electrochemistry of $MV^{2+}$ was characterized by cyclic voltammetry with 0.1, 0.5, and 1.0 M $Na_2SO_4$ in 25 mM MOPS buffer, and its peak separation and peak current as well as redox potential were compared. As shown in Figure 2B, in 0.1 M $Na_2SO_4$, the voltammetric peaks are less well-defined compared with higher concentrations. The lower concentration of supporting electrolyte causes high solution resistance and IR drop, which lowers the current response and makes the peaks less distinguishable.[31] In 0.5 M $Na_2SO_4$, the peaks at −761 mV ($E_{pc}$) and −624 mV ($E_{pa}$) corresponded to the reduction and oxidation of $MV^{2+}$ and $MV^{+\bullet}$, respectively. The calculated redox potential of $MV^{2+}$/$MV^{+\bullet}$ from the average of the peak potentials is −692.5 mV, which agrees with the redox potential obtained by spectroelectrochemistry in a thin-layer cell (Figure S1). In 1.0 M $Na_2SO_4$, the peak potentials of −751 ($E_{pc}$), −630 ($E_{pa}$), and the redox potential of −690.5 mV were obtained in the thin-layer cell. The current responses for 0.5 and 1.0 M $Na_2SO_4$ were similar, and for solubility considerations, the lower concentration of $Na_2SO_4$ is more easily prepared. Consequently, 0.5 M $Na_2SO_4$ in 25 mM MOPS buffer was selected as the supporting electrolyte.

**Controlled Potential Coulometry.** The performance of the nitrate detection method was initially characterized by controlled potential coulometry in which the charge required for reduction of nitrate to nitrite was measured. A plot of the charge measured after 300 s versus nitrate concentration gave a very large ordinate intercept of −23 mC, which is the background charge due to the reduction of dissolved $O_2$, either directly at the electrode or indirectly by reaction with the electrochemically generated $MV^{+\bullet}$, the reduction of $MV^{2+}$, and the reduction of enzyme. Such a large background charge prevented a stable charge−[$NO_3^-$] relationship from being obtained, especially at concentrations of nitrate at the low micromolar level where the charge due to nitrate reduction was very small by comparison, causing poor reproducibility in background-corrected charges.

In order to improve performance by minimizing the background, the contribution from dissolved $O_2$ was eliminated by deoxygenating the solutions with a shlink line and performing the experiment anaerobically in a nitrogen-filled glovebag. The result was a significant decrease in background signal to about −4.5 mC, as seen from the calibration plot shown in Figure 3. As a result, a more stable charge−[$NO_3^-$] correlation was obtained that gave a linear range of 8−245 μM. Although oxygen removal improved the performance, the method still lacked the precision and detection limit expected from an electrochemical technique because the background charge was still large compared to the nitrate charge, especially at low nitrate concentrations.


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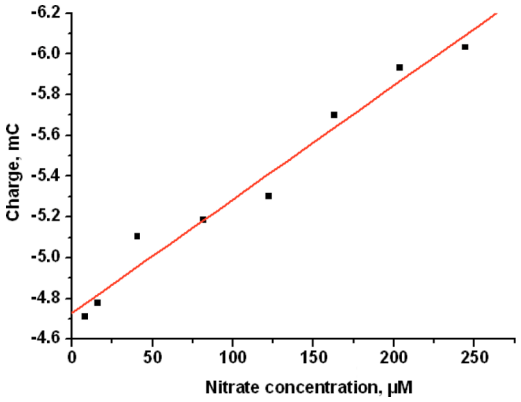


**Figure 3.** Charge responses to the concentration of nitrate under anaerobic conditions at 300, 350, and 400 s. In the experiment, 0.24 mM $MV^{2+}$ was used in 25 mM MOPS with 0.5 M $Na_2SO_4$ and 1 $\mu$M 1:1 diluted SNaR. During the reduction −1000 mV was applied .

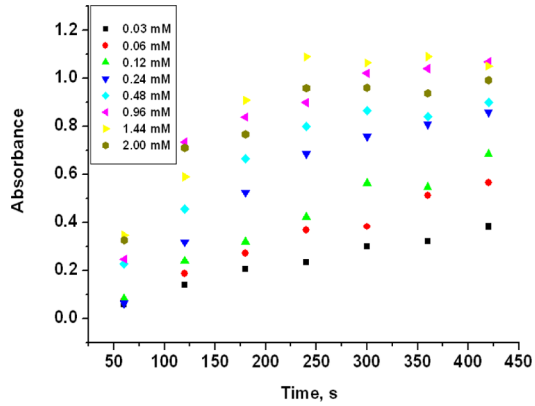


**Figure 4.** Effect of mediator concentration on nitrate reduction. Concentrations of $MV^{2+}$ were varied from 0.03 to 2 mM. Nitrate (160 $\mu$M) was used as the substrate. One microliter of 1:1 diluted SNaR was added to a 50 $\mu$L thin-layer cell. The buffer used was 25 mM MOPS with 0.5 M $Na_2SO_4$ at pH 7.0.

**Spectroscopic Detection of Nitrite after Controlled Potential Coulometry.** In order to improve precision and detection limit by further reducing the background signal, a spectroscopic method for detecting the nitrite coulometrically generated in the thin-layer cell was evaluated. Here, the contents of the thin-layer cell were transferred to a standard absorbance cuvette and mixed with Griess reaction reagents to form a pink color that could be measured by absorption spectroscopy. Switching to this combination of coulometric conversion of nitrate to nitrite and spectroscopic measurement of nitrite circumvented the problem caused by the background charge associated with coulometry. Performance of the method improved dramatically because no such background correction was necessary for the absorbance measurement. Unlike controlled potential coulometry, which requires complicated sample pretreatment to eliminate the $O_2$ effect, the amount of nitrite produced is not interfered by $O_2$. So by using the method, the samples could be prepared in ambient air. The experimental parameters of mediator concentration, applied potential, and time were also optimized.

**Evaluation of Mediator Concentration.** $MV^{2+}$ reduction, the starting point of the nitrate reduction in the thin-layer cell, is critical for a reliable nitrate detection method. In theory, as a reversible redox mediator, $MV^{2+}$ can transfer the electrons at low concentrations. However, a low mediator concentration requires a longer time to transfer the same amount of electrons to the substrate than a higher concentration. In order to find an appropriate mediator concentration, we chose a series of $MV^{2+}$ concentrations and compared their ability to reduce nitrate. In this work, 1 $\mu$L of 1:1 diluted SNaR was used, and a potential of −1000 mV was applied for times varying from 120 to 420 s. After the reduction, products were collected, and Griess reagents were added for color development. As shown in Figure 4, at low concentrations of 0.03, 0.06, and 0.12 mM, the absorbances of the diazo dye were minimal even when the potential was applied for 420 s. As the concentration of $MV^{2+}$ increased, the overall absorbance increased. For concentrations of 0.48 mM and higher, the absorbance started to reach a plateau response at 300 s, with 1.44 mM $MV^{2+}$ having the highest response. As the concentration of $MV^{2+}$ increased to 2.00 mM, the response did not increase but resulted in a slight decrease of the absorbance. One explanation for this phenomenon is as the concentrations of $MV^{2+}$ increases, the electron transfer rate to the SNaR also increases. However, at a certain mediator concentration, the system will reach the maximum electron transfer capacity, where the absorbance maximizes. Also, since low concentrations of $MV^{2+}$ were prone to have better reproducibility, both 0.24 and 1.44 mM $MV^{2+}$ were chosen as the mediator concentrations for further comparisons.

**Selection of Applied Potential.** The applied potential and electrolysis time for reduction of $MV^{2+}$ were then optimized. $MV^{2+}$ is known to undergo two consecutive, one-electron reversible electron-transfer reactions. The first reduction generates the cation radical $MV^{+\bullet}$, and the second reduction generates neutral MV with redox potentials at around −650 and −1080 mV, respectively, in a conventional cell.[32] Less negative potentials are commonly applied for a one-electron transfer, and more negative potentials are used for a two-electron transfer.[32] To achieve the optimum potential and time required for effective electron transfer to SNaR, two mediator concentrations at several potentials and times were investigated and compared. Figure 5A is the absorbance of the diazo dye (as Griess reaction product) after nitrate reduction at various applied potentials at times from 120 to 480 s at an intermediate mediator concentration of 0.24 mM. For potentials of −800 and −900 mV, the absorbance increased with time, and the overall responses were lower than the potentials obtained at −1000, −1050, and −1100 mV. For potentials at −1050 and −1100 mV, the absorbance started to reach the maximum at 300 s, and it is likely that more negative potentials increase the reduction rate for $MV^{2+}$, making the production of nitrite faster than at lower potentials. At −1000 mV, the reaction was slower than −1050 and −1100 mV, but it reached a similar final absorbance when the time was sufficient (360, 420, and 480 s). Although both −1050 and −1100 mV had high and similar responses, the ITO electrode would be burned at the bottom end of the electrode after it was used for several times. When the potential applied time increased, the burning area expanded, degrading the ITO electrode. In order to maintain the active working area of the ITO electrode, −1000 mV was selected as the applied potential. When the $MV^{2+}$ concentration was 1.44 mM, applied potentials at −800, −900, and −1000 mV had almost the same response from 240 s, which is different from the result obtained with 0.24 mM $MV^{2+}$ (Figure 5B). When the $MV^{2+}$ is at low concentration, a more negative potential could provide more electrons to $MV^{2+}$ by rapidly


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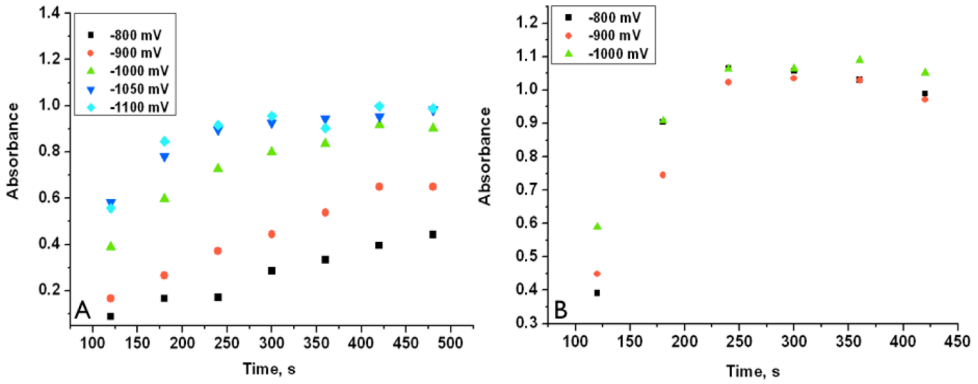


**Figure 5.** Effects of varying applied potential on nitrate reduction in (A) 0.24 mM $MV^{2+}$ from 120 to 480 s and (B) 1.44 mM $MV^{2+}$ from 120 to 420 s. Nitrate (160 $\mu$M) was used as the substrate. One microliter of 1:1 buffer diluted SNaR was added to a 50 $\mu$L thin-layer cell.

reducing it to MV rather than $MV^{+\bullet}$ within the same time period. This will contribute to a higher response, whereas when $MV^{2+}$ concentration is high, the capacity for transferring electrons will be increased even though the applied potential is less negative. As a consequence, −1000 mV was selected as the applied potential. From the absorbance versus time correlation shown in Figure 5A,B, the optimized electrolysis times for 0.24 and 1.44 mM $MV^{2+}$ were 420 and 360 s, respectively. However, the absorbance responses in 0.24 mM $MV^{2+}$ were more stable than in 1.44 mM, so in all the following experiments, 0.24 mM $MV^{2+}$ was used for further nitrate analysis.

**Characterization of the SNaR-Based Nitrate Detection Method.** Different concentrations of SNaR were employed to investigate the performance for nitrate determination. For each dilution ratio of SNaR, 1.0 $\mu$L was used for nitrate reduction, 25 mM MOPS was used as the dilution buffer, and 0.5 M $Na_2SO_4$ was the supporting electrolyte. Also, 0.24 mM $MV^{2+}$ was used as the mediator concentration. Figure 6 displays the best

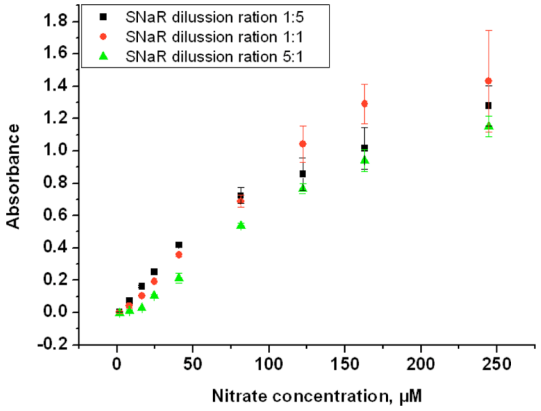


**Figure 6.** Absorbance responses of three dilution ratios of SNaR under optimized conditions at 540 nm ($n$ = 3).

absorbance responses we obtained for the diazo dye as a function of concentration of nitrate standard solutions. The dilution ratios of the SNaR were 5:1, 1:1, and 1:5, respectively. Table 1 summarizes the linear ranges and sensitivities with different dilution ratios of SNaR. Here, a 1:1 dilution ratio of SNaR to 25 mM MOPS had the largest linear range, which was from 1 to 160 $\mu$M, with a sensitivity of 8000 AU $M^{-1}$. With the 1:5 dilution ratio of SNaR, two linear relationships were obtained from 1 to 40 $\mu$M and 80−245 $\mu$M. The sensitivity decreased when the concentrations were higher. Based on the

**Table 1. Linear Range and Sensitivity of SNaR-Based Nitrate Detection Method at Different Dilution Ratios of SNaR to MOPS**

| SNaR/MOPS | linear range ($\mu$M) | sensitivity ($10^4$ AU $M^{-1}$) |
|---|---|---|
| 5:1 | 10−120 | 0.7 |
| 1:1 | 1−160 | 0.8 |
| 1:5 | 1−40/80−245 | 1.1/0.3 |

dilution factors and molar absorptivity, with the maximum conversion of nitrate to nitrite at 1:1 ratio (for example, at the maximum concentration of 160 $\mu$M), theoretically, the maximum absorbance change should be 1.1, which is close to the experimental data we obtained with 160 $\mu$M nitrate (Figure 4, 5), indicating an almost complete conversion from nitrate to nitrite in the thin-layer cell. The variances among the experimental values are caused by experimental errors because the prepared solutions and the SNaR reaction rates may be slightly different by time.

**Characterization of the Reaction Rates of Nitrate Reduction.** As with other enzymes, the catalysis rate of SNaR is substrate-concentration-dependent. The reaction rate study on a 1:1 dilution ratio of SNaR in different concentrations of nitrate showed that as the concentration of nitrate increased, the rate of the reaction increased until it reached 150 $\mu$M (Figure S2). Notably, this concentration is in agreement with the maximum detection capability of this nitrate detection method at approximately 160 $\mu$M (Table 1).

**Interference Study.** In this study, the Griess reaction was employed to quantify the amount of nitrite produced in nitrate standard solutions. However, a typical application of this method is for natural water detection, which commonly contains other anions such as sulfate, phosphate, carbonate, bicarbonate, and chloride. Interferences from these anions may occur during either nitrate reduction or Griess reaction. We evaluated the possible interferences of these common anions with nitrate and nitrite individually with the Griess reagents. After adding Griess reagents, MOPS buffer, supporting electrolyte $Na_2SO_4$, $MV^{2+}$, and common anions ($Cl^-$, $SO_4^{2-}$, $PO_4^{3-}$, $HCO_3^-$, $CO_3^{2-}$) in high concentrations did not show any peak at ∼540 nm, which means that they do not couple with Griess reagents to produce diazo dye (Figure S3A). When treated the same as the nitrate sample solutions for reduction, only 0.5 M $Na_2CO_3$ exhibited some absorbance (<0.01), but it was much smaller than 2 $\mu$M of nitrate (Figure S3B). Since the concentration of $Na_2CO_3$ in real water samples is much lower than 0.5 M, no interference with nitrate would occur.


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**Characterization of Natural Water Samples.** For the application of the $MV^{2+}$-SNaR-based nitrate detection method, water samples from the Cincinnati public water system and Ohio River were tested by this method and compared with Cincinnati area water quality reports.[33] A Hach DR890 colorimeter was also employed for comparison purposes. Results summarized in Table 2 demonstrated that the nitrate concentrations for the two water supplies determined by this method were in good agreement with the Hach DR890 colorimeter (as compared with a two-sample *t*-test) and is consistent with the range of the Cincinnati annual water reports.

**Table 2. Nitrate Concentration Determinations by the SNaR-Based Nitrate Detection Method and Hach DR890 Spectrometer in Comparison with Cincinnati Water Quality Reports**

| water sample | this method (mg $L^{-1}$) | Hach (mg $L^{-1}$) | report (mg $L^{-1}$) | *p*-value |
|---|---|---|---|---|
| Cincinnati public water | 1.05 ± 0.13 | 0.97 ± 0.14 | 0.64−1.5[a] | 0.11 |
| Ohio river | 0.52 ± 0.12 | 0.47 ± 0.14 | N/A | 0.15 |

[a]The data for year 2012−2013 are not available, so the nitrate concentration reports from year 2001 to 2011 were collected and analyzed using (max + min)/2 as the approximation of the mean to predict current nitrate concentration with a 95% confidence interval. The uncertainties shown in Table 2 are the confidence intervals ($n$ = 3).

## CONCLUSION

A SNaR-based nitrate detection method was successfully developed using thin-layer controlled potential coulometry and spectroscopic detection of Griess reaction products. Controlled potential coulometry and the Griess reaction provide direct and indirect ways for nitrate detection, respectively. The volume of sample needed is much less than any conventional electrochemical-cell-based analysis. The less complicated charge-based method yields reasonable results but requires more complex sample pretreatment. On the other hand, without the need of $O_2$ removal, the spectroscopic Griess test for nitrite monitoring shows the best linear range of 1−160 $\mu$M, with high sensitivity and reproducibility.

In terms of accessibility of this method, the engineered SNaR we obtained was produced in large amounts by fermentation, which increases the accessibility of the enzyme compared with plants and yeast-extracted holo-NaR. Due to its smaller size and less complexity, SNaR is potentially more stable and more suitable for environmental applications than holo-NaR. Also, the direct use of SNaR in the thin-layer cell does not require any pretreatment and enzyme immobilization process as in nitrate biosensors.

SNaR-based nitrate reduction provides a more environmentally safe method for the nitrate reduction than the use of highly toxic Cd metal in Hach spectroscopic procedure. Also, unlike Hach spectroscopic procedure, where the presence of $Cl^-$ (higher than 2.8 mM) will dramatically reduce the rate of nitrate reduction, here as suggested in the interference study, SNaR shows extremely high selectivity to nitrate under high concentrations of other common anions.[29]

Furthermore, the use of the thin-layer cell restricts the diffusion layer (~0.018 cm thick), providing the possibility of reducing the nitrate fast and effectively. Another advantage is the small sample volume required (microliters) compared to the conventional electrochemical cell (milliliters), which also minimizes the enzyme amount.

In conclusion, this research clearly illustrates the advantages that can be gained in performance by combining two quite different techniques such as coulometry and spectroscopy. The proof-of-concept described here provides the basis for implementing this method in a way that would be more effective for routine analysis of many samples. For example, this procedure could be automated by a flow injection analysis system consisting of a coulometric flow cell that then mixes the electrochemically generated nitrite with Griess reagents before flowing through an absorbance cell for detection.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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