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Indirect Measurement of Nitric Oxide Production by Monitoring Nitrate and Nitrite Using Microchip Electrophoresis with Electrochemical Detection

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An indirect method for monitoring nitric oxide (NO) by determining nitrate and nitrite using microchip capillary electrophoresis (CE) with electrochemical (EC) detection has been developed. This method combines determination of nitrite by direct amperometric detection following a microchip-based CE separation and conversion of nitrate to nitrite by chemical reduction using Cu-coated Cd granules. The amount of nitrate is quantified by calculating the difference in the amount of nitrite in the sample before and after the reduction of nitrate. Optimization of the separation, injection, detection, and reduction reaction conditions, as well as studies involving integration of the reduction reaction onto the microchip, are described. It was found that nitrite can be separated and detected in ∼45 s by microchip CEEC. The reduction reaction was successfully integrated on-chip and carried out in ∼1 min following activation of the Cd granules. The usefulness of this device was demonstrated by monitoring the amount of nitrate and nitrite produced from 3-morpholinosydnonimine, a NO-releasing compound.

Nitric oxide (NO) is synthesized predominantly from L-arginine by NO synthase.[1−3] It is recognized as an important signaling compound in many physiological events, including neurotransmission, vasodilatation, and inflammation.[2−5] Depending upon the physiological system, it has been shown that high levels of NO can either be toxic or promote cell survival.[5] Therefore, regulation of NO production is a goal of therapeutic approaches to a number of different pathophysiological conditions, including autoimmune disorders, inflammation, hypertension, and neuropathic abnormalities.[2,5−9] Under aqueous conditions and in the presence of oxygen, NO has a half-life as long as 4 min.[5] However, in biological systems, NO is considerably less stable (half-life <30 s)[5] and is rapidly oxidized to nitrate and nitrite.[10−12] Due to its short half-life, direct measurement of NO is difficult; therefore, monitoring of nitrate and nitrite has been employed as a useful indicator of in vivo NO production.[13,14]

Several methods for the measurement of nitrate and nitrite in physiological fluids have been developed.[2] The most widely used is the Griess reaction,[15] in which nitrite is chemically transformed into a colored diazo compound by reaction with sulfanilamide and *N*-(1-naphthyl)ethylenediamine. Nitrate can be measured by this reaction following chemical reduction to nitrite by Cd-coated particles[16] or via enzymatic reduction by nitrate reductase.[17] This method has adequate sensitivity to monitor biological systems; however, the assays take a relatively long time to perform, because the reaction consists of two steps: the reduction of nitrate to nitrite and the derivatization and spectrophotometric detection of nitrite. Capillary electrophoresis (CE) has been proven to be an efficient technique for the separation and determination of small inorganic anions, including nitrite and nitrate, in biological samples.[18−29] The determination of nitrate and nitrite by CE with UV detection

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permits direct measurement with a single injection after minimal sample preparation but lacks sensitivity. Detection limits using CE-UV are typically in the region of 25 μM.[18−29] A rapid and sensitive method for the determination of nitrite and nitrate that integrates sample preparation and analysis has not been demonstrated.

Since the initial description of the micro-total analysis system (μ-TAS) concept,[30] use of the microchip format for CE-based separations has witnessed extensive growth.[31−34] These miniature instruments offer many potential advantages, including a high degree of automation, rapid analysis times, integration of multiple process, and negligible consumption of reagents for chemical and biological analysis. Although detection in these devices has been accomplished by a variety of methods, the most widely utilized mode has been laser-induced fluorescence (LIF).[35] Drawbacks to LIF detection include the fact that only a small number of wavelengths can be used for excitation and that pre- or postcolumn derivatization of the sample with a fluorophore is typically required.

Electrochemistry as a detection mode for microchip CE has received increased attention during the past 5 years.[35−41] Electrochemical (EC) detection provides many desirable features for a portable/disposable microchip system. EC detectors offer a high degree of selectivity and sensitivity while being amenable to miniaturization and relatively inexpensive to mass fabricate. Furthermore, microchip CE with EC detection is ideally suited for the development of a fast and sensitive method for the determination of nitrate and nitrite. Nitrite can be detected amperometrically at modest electrode potentials.[42] The ability to integrate multiple processes into the chip, including the reduction of nitrate to nitrite, makes microfabricated devices attractive for this application.

In this paper, we report on the development of a microchip CEEC device that enables the indirect determination of nitric oxide by monitoring nitrate and nitrite. The measurement of nitrite was achieved via direct amperometric detection, following separation by microchip CE. The measurement of nitrate was accomplished in a similar manner after on-chip reduction to nitrite. In that case, the signal generated corresponds to the combined amount of nitrate and nitrite in the sample. The performance of these devices was evaluated by measuring the peak efficiency, sensitivity, linearity, and reproducibility of the nitrite response. Conditions for the reduction of nitrate to nitrite were optimized to enable on-chip reaction and integration with the separation and detection components of the chip. This simple and rapid method was used to monitor the production rate of nitrate and nitrite from 3-morpholinosydnonimine (SIN-1), a metabolite of the vasodilator molsidomine and a nitric oxide-releasing compound.

EXPERIMENTAL SECTION

Materials. The following chemicals and materials were used as received: SU-8 10 photoresist (MicroChem Corp., Newton, MA); 100-mm silicon wafers (Silicon Inc., Boise, ID); 125-mm silicon wafers (MEMC Electronic Materials, St. Peters, MO); Sylgard 184, (Fisher Scientific, Pittsburgh, PA); 30-μm carbon fiber (Avco Specialty Materials, Lowell, MA); laser-grade fluorescein, sodium salt (Kodak, Rochester, NY); boric acid, anhydrous copper sulfate, glycine, sodium nitrite, sodium nitrate (Sigma, St. Louis, MO); *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), dodecyl trimethylammonium bromide (DTAB), ethylenediaminetetraacetic acid (EDTA), cadmium granules (30−80 mesh), 3-morpholinosydnonimine (SIN-1) (Aldrich, Milwaukee, WI); and sodium hydroxide (Fisher, Fair Lawn, NJ). All water used was ultrapure (Labconco, Kansas City, MO).

Conventional CE-UV Analysis. Conventional CE was carried out using a Hewlett-Packard ^{3D}capillary electrophoresis system with a diode array UV detector. Polyimide-coated fused-silica capillaries (50 μm i.d. × 360 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ), and a capillary length of 64.5 cm (effective length of 56 cm) was used. A small section of the polyimide coating was removed by flame to create a window for optical detection. The capillaries were preconditioned prior to use by flushing with 0.1 M NaOH for 30 min, water for 15 min, and buffer for 30 min. The temperature of the capillary was maintained at 20 °C, and UV detection was accomplished at 214 nm. TES (15 and 25 mM, pH 7.5 and 8.0) and boric acid (15 and 25 mM, pH 8.5 and 9.0) buffer solutions, which included DTAB (1−10 mM) as an electroosmotic flow modifier, were used for optimization of the nitrate and nitrite separation. Injections were made for 5 s at 50 mbar. Prior to injection, the capillary was flushed for 2 min with the appropriate run buffer. A separation voltage of −15 kV was used.

Microchip Fabrication. The PDMS-based microchip CE devices were fabricated by previously described procedures.[38,43] First, a negative mask containing the desired features was made by generating the design in Freehand (PC version 8.0, Macromedia, San Francisco, CA) and transferring the design onto a film using a commercial printing service (Lasergraphics, Lawrence, KS) that can provide 2400-dpi resolution. Masters were made on silicon wafers with basic photolithographic procedures using an SU-8 photoresist and the negative film as a mask. These masters

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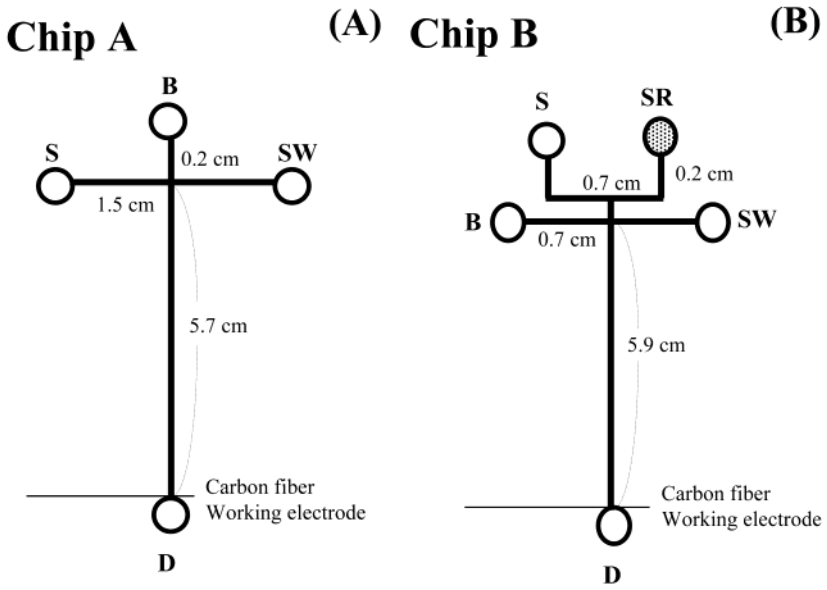

Figure 1. Schematic representation of the PDMS/carbon fiber microchip CEEC devices. Both the separation and electrode channels are 30 μm wide and 30 μm deep. The working electrode is 5 μm from the channel exit. The reference (Ag/AgCl) and auxiliary electrode (not shown) are located in the detection reservoir (D). S, sample reservoir; B, buffer reservoir; SR, sample reduction reservoir; SW, sample waste reservoir.

were cast against a mixture of PDMS oligomer and cross-linking agent (Sylagrd 184) and cured at 65 °C for at least 1 h, after which the PDMS was removed to yield a pattern of negative relief channels.

In this work, two separate PDMS layers were fabricated, one containing the separation and injection channels and one containing electrode channels for the carbon fiber.[43,44] Two designs for the separation layer were used (Figure 1). Chip "A" incorporated a simple single T injector. Chip "B" incorporated a gated injector with reservoirs for sample and sample plus the reduction reaction mixture. Buffer access holes were made in the PDMS separation layer with a hole punch. The PDMS electrode layer was sealed to a glass plate for increased rigidity. Both the separation and electrode channels were 30 μm deep and 30 μm wide. The carbon fiber working electrode was constructed as previously described[43] by placing a 30-μm fiber in the electrode channel. Electrical contact was made to a Cu wire with colloidal silver, and the contact point was covered with hot glue to insulate and secure the electrode. After electrode construction, the electrode was aligned 5 μm from the end of the separation channel with the aid of a light microscope, and the two layers were reversibly bonded.[43]

Microchip CEEC Analysis. Microchip-based capillary zone electrophoresis separations were carried out in uncoated channels using a Spellman CZE 1000R high-voltage power supply (Spellman High Voltage Electronics, Hauppauge, NY). For injection and separation using chip B, two power supplies, the Spellman CZE 1000R and a USB-based power supply capable of providing −2 kV (Instrumentation Design Lab, University of Kansas, Lawrence, KS), were used. The run buffer consisted of 15 mM boric acid including 7.5 mM DTAB without (chip A) or with (chip B) 1 mM EDTA, pH 9.0. Degassed and filtered buffer was introduced into the reservoirs and flushed through the channels under vacuum until air bubbles were no longer observed under a microscope. The device was conditioned with the run buffer for 15 min at field strengths between 100 and 200 V/cm.

Samples were injected by two different protocols, unpinched injection (chip A)[38,43,45] and gated injection (chip B).[46] Unpinched injections were carried out by applying −750 V to the sample reservoir for 1 s while holding the sample waste and detection reservoirs at ground. The separation was initiated by applying a high voltage (−750 V) to the buffer reservoir while holding the detection reservoir at ground. The optimized gated injection scheme (see Results and Discussion for description of optimization) was accomplished by applying −1200 V between the buffer and detection reservoirs and −960 V between the sample and sample waste reservoirs. Injection was carried out by floating the buffer reservoir for 1 s. Monitoring of the injection sequence was accomplished by fluorescence microscopy using a Zeiss Axiolab microscope (Carl Zeiss, Thorngood, NY) equipped with a 50 W Hg arc lamp assembly with appropriate fluorescein line filters. The images were monitored by a Microimage Video Systems color video camera (A209, Boyertown, PA) with camera control unit (CCU 209, Microimage Video Systems) and video capture card (CG-7 RGB color PC1, Scion Corp., Frederick, MD) coupled to a personal computer and frame grabber software (Scion Image, Scion Corp.).

Amperometric detection was performed in a three-electrode format. The carbon fiber working electrode was controlled by a LC-4CE potentiostat (Bioanalytical Systems, West Lafayette, IN). A platinum wire auxiliary electrode and a Ag/AgCl reference electrode (RE-6, Bioanalytical Systems) were placed in reservoir D (Figure 1) to complete the cell. Data collection was performed with a DA-5 analog−digital converter (Bioanalytical Systems).

Off-Chip Reduction Reaction. The method used for the chemical reduction of nitrate to nitrite was based on a previously

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described procedure.[16] Cu-coated Cd particles were prepared by rinsing 80 mg of Cd granules (previously stored in 0.1 M H_2SO_4) three times with 100 μL of water. The granules were then soaked for 1 min in 100 μL of a 5 mM $CuSO_4$ solution (prepared in 50 mM glycine−NaOH buffer, pH 9). The Cu-coated granules were drained and used within 10 min of preparation. Prolonged exposure of the granules to air diminishes their reductive ability. To carry out the reduction reaction, 100 μL of sample solution was added to the Cu-coated granules for 1 min with occasional shaking. This mixture was centrifuged at 10 000 rpm for 1 min, and the supernatant was directly injected for analysis (either by conventional or microchip CE). After use, the granules were rinsed and stored in a 0.1 M H_2SO_4 solution. The granules can be regenerated by repeating the Cu-coating procedure.

On-Chip Reduction Reaction. The on-chip reduction reaction was performed in a manner similar to the off-chip method, except that all manipulations were performed on-chip in a reservoir. Chip B (Figure 1B) was used for all on-chip reactions. Briefly, 80 mg of Cd granules were placed into the sample reduction reservoir (SR). The granules were rinsed in the reservoir with 40 μL of water. Next, 40 μL of a solution containing 5 mM $CuSO_4$ (prepared in 50 mM glycine-NaOH buffer, pH 9) was added to this reservoir. After 1 min, the solution was removed by pipetting. Rinsing with the $CuSO_4$ solution was repeated three times to ensure complete coating. The granules were then rinsed with 40 μL of the 50 mM glycine−NaOH buffer. To carry out the reduction reaction, 30 μL of sample was pipetted into the reservoir containing the Cu-coated granules and allowed to react for at least 1 min. The reaction mixture was then electrokinetically injected directly into the chip. After injection, the granules were rinsed with 40 μL of 0.1 M H_2SO_4 solution three times for regeneration.

Sample Preparation. Stock solutions of nitrate and nitrite (1 mM) were prepared daily in ultrapure water. Appropriate dilutions were made with either water or run buffer to the appropriate concentrations. A fluorescein stock solution (200 μM) was prepared monthly in water. All solutions were degassed and filtered (0.2 μM Acrodisc filter, Gelman Sciences, Ann Arbor, MI) before injection. A 1 mM solution of 3-morpholinosydnonimine (SIN-1) was prepared in water just prior to use. Of this solution, 1.5 mL was incubated in a water bath at 37 °C, and 50 μL was sampled after 0, 5, 15, 30, 45, 60, 90, and 120 min. Each sample solution was immediately analyzed for the amount of nitrate and nitrite using chip B.

RESULTS AND DISCUSSION

Optimization of Separation and Reduction Reaction Conditions Using Conventional CE-UV. Conventional CE with UV detection was used to optimize the separation conditions for nitrate and nitrite and to determine the conversion efficiency of the off-chip reduction reaction. The off-chip reduction reaction takes place at pH 9; therefore, to simplify integration of the reduction reaction to the separation component of the chip, buffer systems close to this pH were investigated. In general, when using a positive polarity, the separation of nitrate and nitrite took >15 min and generated unacceptably broad peaks. To decrease the analysis time and improve peak shape, a negative polarity was employed. DTAB was added to the run buffer to reverse the electroosmotic flow (EOF).[47,48]

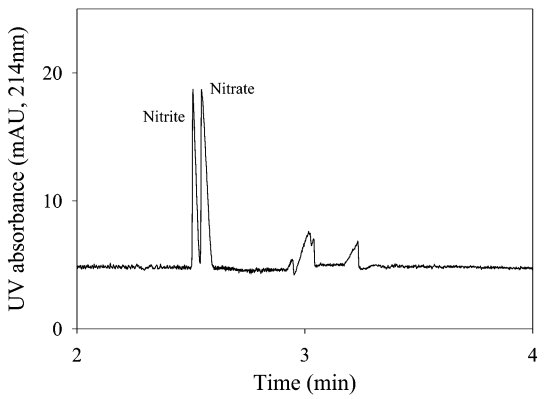

Figure 2. Separation of 500 μM nitrite and nitrate with CE-UV. Run buffer: 15 mM boric acid with 7.5 mM DTAB, pH 9.0. Separation voltage: −15 kV. UV detection at 214 nm.

Two different buffer systems were evaluated for the analysis of nitrate and nitrite by conventional CE-UV. First, TES (pH 7.5, 8.0) and boric acid (pH 8.5, 9.0) buffers of differing concentrations (15 and 25 mM) and pH values were evaluated using 1.0, 5.0, 7.5, and 10.0 mM of DTAB and negative polarity. Nitrate and nitrite were not completely resolved with DTAB concentrations less than 5 mM, whereas the background in the UV of the buffer system containing 10 mM DTAB was considerably higher than that containing 7.5 mM DTAB. There was no significant difference in the separation efficiencies achievable with the TES buffer and boric acid buffer systems; however, it was found that the more basic buffer system (boric acid) was more suitable for nitrite analysis following the reduction reaction. Therefore, the optimized buffer system consisted of 15 mM boric acid, pH 9.0, including 7.5 mM DTAB. Under these conditions, migration times of nitrite and nitrate were 2.51 and 2.55 min (Figure 2), respectively. The calibration curves for these compounds were linear over the concentration range of 25−500 μM ($r^2 > 0.999$). The limit of detection (LOD, S/N = 3) was determined experimentally to be 10 μM.

Because nitrate and nitrite both absorb in the UV (214 nm) range, CE-UV was used to monitor the conversion of nitrate to nitrite via the chemical reduction method. In this study, Cu-coated Cd, in which Cd granules were activated with a $CuSO_4$ solution, was used. Previous reports have shown that such a reduction follows pseudo-first-order reaction kinetics and that a maximum reduction (>85%) occurs after ∼2 h when carrying out the reaction on a large scale (2.5−3 g of Cd granules and 4 mL of solution).[16] We investigated the reduction efficiency of a small-scale version of the reduction reaction by CE-UV using a 500 μM nitrate solution, 80 mg of 30−80 mesh Cu-coated Cd granules, and different sampling times. It was found that once the reduction reaction was initiated, no nitrate was detected after 30 s of reaction time. Analysis of the nitrate reduction reaction over a wide range of nitrate concentrations (25−500 μM) showed that the nitrite response increased linearly as the concentration of nitrate was increased, with an average conversion efficiency of 55.7% being achieved. Therefore, it was concluded that a small-scale version of this reaction could decrease the total analysis time, convert a significant portion of nitrate to nitrite and, with appropriate standardization, be used for routine nitrate analysis.

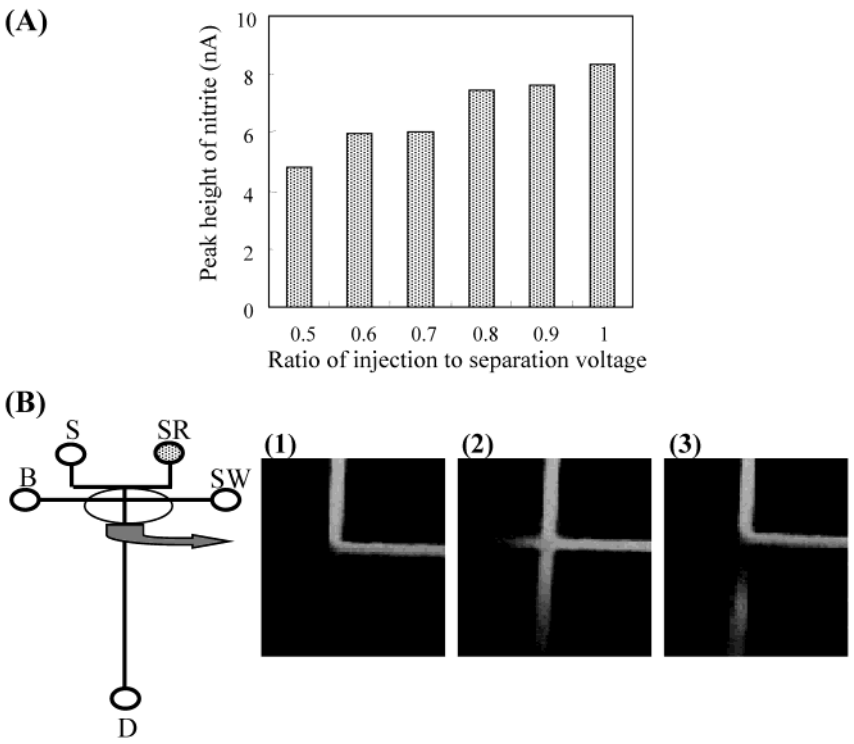

Figure 3. (A) Effect of the ratio of injection voltage (S/SW) to separation voltage (B/D, −1200 V) on the response of nitrite (peak height) using chip B. (B) Fluorescence microscope images of the optimized gated injection scheme using chip B: (1) fluorescein solution was continuously pumped from S through the injection zone to SW at −960 V, while buffer was continuously pumped from B to D at −1200 V; (2) injection by floating B for 1 s; and (3) separation. Sample: 100 μM fluorescein in 10 mM boric acid buffer with 7.5 mM DTAB and 1 mM EDTA (pH 9.0). Run buffer: 15 mM boric acid, 7.5 mM DTAB, 1 mM EDTA, pH 9.0.

Determination of Nitrite Using Microchip CEEC. Chip A (Figure 1A) was used to optimize the separation and detection conditions for nitrite. Isolation of the separation voltage from the EC detector was accomplished by employing an end-channel detection scheme.[39,44] Previous work in conventional CEEC with an end-column electrode alignment scheme using a negative polarity and reversed EOF has shown that the half-wave potential of an analyte is shifted to more negative values, and the extent of this shift is dependent upon the separation voltage.[47] Therefore, the detection potential needs to be optimized for each particular separation voltage.

The optimal detection potential was determined by hydrodynamic voltammetry using the buffer system from the CE-UV studies. A 10 μM solution of nitrite was injected, and the response at +0.9, 0.95 and 1.0 V (vs Ag/AgCl) was monitored. Although the largest current response was obtained at +1.0 V, a more stable background was obtained at +0.9 V. Therefore, all subsequent experiments used a detection potential of +0.9 V vs. Ag/AgCl. The response for nitrite was reproducible and found to be linear over the concentration range of 0.1−50 μM ($r^2 = 0.999$) with a sensitivity of 26.4 pA/μM and a LOD of 25 nM (S/N = 3). Replicate injections ($n = 6$) of a 10 μM nitrite solution led to an average of 10 200 ± 1750 theoretical plates.

Injection of Reduction Reaction. A more complex chip (chip B, Figure 1B) was designed both to enable the direct determination of nitrite and to integrate the nitrate reduction reaction. Chip B consists of a sample reservoir (S), from which nitrite can be directly analyzed, and a sample reduction (SR) reservoir, where the off-chip reduction reaction mixture can be introduced or the reduction reaction can be directly implemented (described below). In contrast to chip A, chip B uses a gated injection scheme. This is a time-based, electrokinetically biased method that operates in a continuous fill mode.[46,49] Sample is continually pumped from a sample reservoir to a sample waste reservoir through the injection zone. Concurrently, buffer is continuously pumped from a buffer reservoir to a buffer waste reservoir. A time-biased injection is performed by momentarily floating the voltage applied to the buffer reservoir, and the separation is initiated by reapplying voltage to the buffer reservoir.

To optimize the injection conditions, the effect of the ratio of injection-to-separation voltage on the response of nitrite was studied (Figure 3). At a ratio of >0.8 (injection voltage −960 V, separation voltage −1200 V), optimal response was seen. An injection voltage (−1200) equal to the separation voltage yielded the largest nitrite response; however, a voltage this high adversely affected the PDMS in the area around the sample waste reservoir. The entire reservoir became cloudy, seemingly altering the PDMS surface. Therefore, a ratio of 0.8 was employed for gated injection using chip B.

When utilizing a gated injection scheme, it has been our experience that the ionic strengths of the sample and run buffer need to be similar to avoid leakage at the injection T. To evaluate the effect of the ionic strength on the amount of possible leaking at the injection T and the shape of injection plug, fluorescence

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microscopy was used to monitor the flow profile of fluorescein at the injection zone. A fluorescein stock solution was diluted with water or with 5, 10, or 15 mM boric acid buffer (pH 9.0) so that the final fluorescein concentration was 100 μM. All samples included 7.5 mM DTAB and 1 mM EDTA. Each of these sample solutions was separately placed in the sample reservoir (S, Figure 1B), and gated injection was performed, as described above, with a run buffer of 15 mM boric acid buffer (pH 9.0), including 7.5 mM DTAB and 1 mM EDTA. When the fluorescein was dissolved in water and the 5 mM buffer solution, fluorescein leaked toward the detection reservoir (D, Figure 1B). No leakage was observed using the 10 and 15 mM buffer solutions. Later analysis of nitrite under these conditions showed that a larger nitrite response (peak height) was obtained using the 10 mM buffer solution because of a stacking effect (data not shown), so this buffer solution was employed for all further studies.

The effect of the ionic strength of the reduction reaction solution on gated injection was also investigated using fluorescein. The fluorescein stock solution was diluted with the nitrate reduction solution (solution that results following reduction of nitrate) and either water or boric acid buffer (5, 10, or 15 mM), pH 9.0, including 7.5 mM DTAB and 1 mM EDTA. The final fluorescein concentration was 100 μM, and injections were performed as described above. When analyte solutions contained boric acid buffer concentrations of 5 mM or greater, no leakage of fluorescein at the injection zone was observed. This finding is explained by the fact that the reduction reaction solution includes many salts that raise the overall ionic strength. In this case, the ionic strengths of the sample solution and run buffer can be closely matched by diluting with a buffer that is not as concentrated as the run buffer. Consequently, a 5 mM boric acid buffer solution was used for injection of the reduction reaction solution. Figure 3B shows the optimized injection scheme. Under these conditions, there was no leakage of fluorescein at the injection zone, and a well-defined sample plug was introduced into the separation channel.

Analysis of Off-Chip Reduction Reaction. Initially, the reduction reaction was performed off-chip and analyzed with Chip B. As mentioned above, it was found that it was necessary to add EDTA to the run buffer. When carrying out the reduction step without any EDTA present in the run buffer, a huge peak was detected following the nitrite peak (Figure 4A). This peak continued well past the time frame shown in the figure, with a stable baseline occurring at ~4 min, severely delaying subsequent analysis. We postulate that this peak corresponds to a Cu−glycine complex. This is because the reduction reaction solution contains glycine that may not have been completely eliminated from the sample reduction reservoir (after activation of the Cd granules) as well as trace levels of Cu^{2+} ions produced from the reduction reaction. Injections of a 1 mM glycine buffer solution did not lead to such a peak, but injections of glycine buffer solution containing 5 μM $CuSO_4$ did produce the same peak (data not shown). To eliminate interference from the Cu−glycine complex, 1 mM EDTA was added to the run buffer. As a result, the broad peak disappeared and only the nitrite peak was observed (Figure 4B).

Using chip B and the optimized buffer and injection conditions, the migration time of nitrite was 45 s (Figure 5A). The response of nitrite was reproducible and found to be linear over the

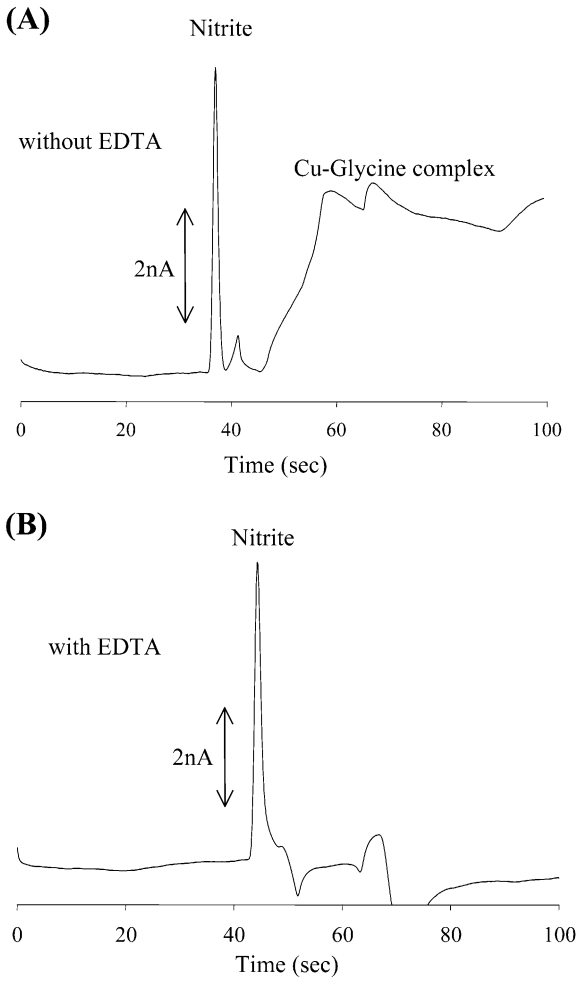

Figure 4. Electrochemical detection of nitrite produced by on-chip reduction of nitrate using chip B. Sample: nitrite produced by the reduction of 100 μM nitrate diluted with 5 mM boric acid including 7.5 mM DTAB. Separation conditions: 15 mM boric acid, pH 9.0, with 7.5 mM DTAB and (A) not including or (B) including 1 mM EDTA. Separation voltages: −1200 (B to D, 200 V/cm) and −960 V (S to SW). Injection: 1 s. E = +0.9 V vs Ag/AgCl.

concentration range 10−250 μM, with a sensitivity of 42.1 pA/μM (r^2 = 0.999). The LOD (S/N = 3) was experimentally determined to be 1.0 μM. Additionally, the response and number of theoretical plates for injection of a 50 μM nitrite solution were 1.72 ± 0.11 nA and 6650 ± 1950 plates, respectively (n = 6).

Integrated Reduction Reaction. One of the advantages of operating in the microchip format is the possibility of integrating multiple processes (sample preparation, separation, detection, etc.) onto one device. Therefore, the small-scale version of the off-chip reduction reaction was performed on-chip using chip B. The reaction was performed similarly to the off-chip method, except that the amounts and volumes of the reactants were changed to accommodate the capacity of the sample reduction reservoir (SR, Figure 1B). The Cd granules were activated with $CuSO_4$ as before, except that this was done directly on the chip. The reduction reaction was then carried out, and the resulting reaction mixture was injected directly into the chip. Comparable to the off-chip method, the amount of nitrite produced from the reduction of nitrate increased linearly as the amount of nitrate added was increased. Figure 5B shows an electropherogram of nitrite produced by the on-chip reduction of nitrate. A comparison of

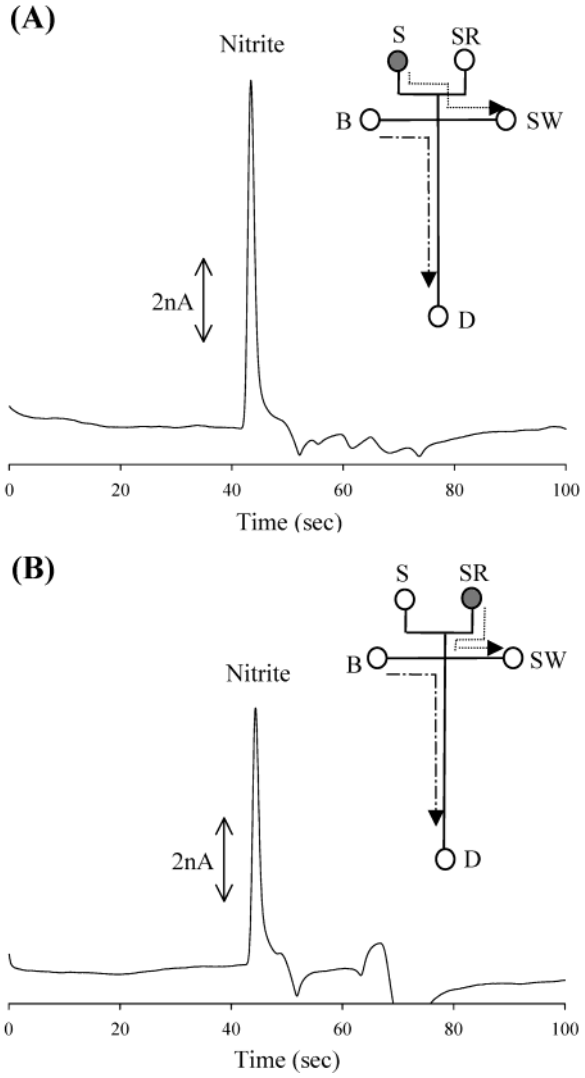

Figure 5. Electrochemical detection of nitrite and nitrate using chip B: (A) 100 μM nitrite in 10 mM boric acid including 7.5 mM DTAB and 1 mM EDTA and (B) nitrite produced by the on-chip reduction of 100 μM nitrate in 5 mM boric acid including 7.5 mM DTAB and 1 mM EDTA. Separation conditions the same as in Figure 4B.

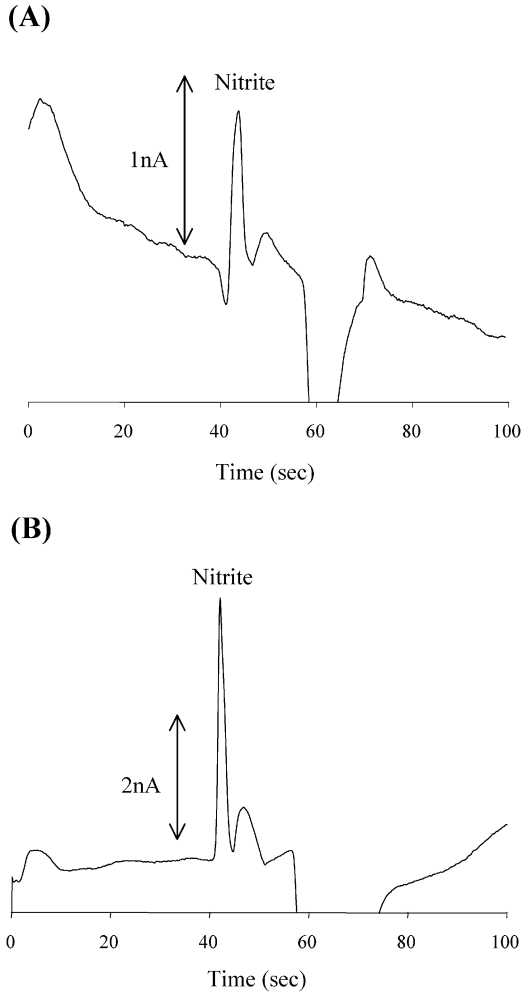

Figure 6. Electrochemical detection of products decomposed from SIN-1 using chip B. Samples were obtained 120 min after incubation of 1 mM SIN-1 in water at 37 °C. Before injection, samples were (A) not reduced and dissolved in 10 mM boric acid including 7.5 mM DTAB and 1 mM EDTA and (B) reduced on the chip in 5 mM boric acid including 7.5 mM DTAB and 1 mM EDTA. Separation conditions the same as in Figure 4B.

the data shown in Figure 5A (direct detection of 100 μM nitrite) and Figure 5B (detection of the nitrite produced from the on-chip reduction of 100 μM nitrate) shows that the conversion efficiency for the on-chip reduction of nitrate to nitrite is ~74%. This demonstrates that chip B is a device that effectively integrates sample handling with the separation and detection components of the chip and affords rapid analysis of both nitrate and nitrite.

Measurement of Nitrite and Nitrate Produced From SIN-1. SIN-1 is a metabolite of the vasodilator molsidomine and is a NO-releasing compound.[50] Using molecular oxygen, it generates superoxide and NO that together form peroxynitrite, which rapidly decomposes to nitrate and nitrite. Therefore, SIN-1 is an ideal test compound to evaluate the effectiveness of chip B in monitoring the amount of nitrite and nitrate produced from NO. To accomplish this, a 1 mM SIN-1 water solution was incubated at 37 °C, and samples were obtained after 0, 5, 15, 30, 45, 60, 90, and 120 min. Figure 6 shows electropherograms of the products produced from SIN-1 in water (120 min after incubation) using chip B without (Figure 6A) and with (Figure 6B) on-chip reduction of the sample. The amount of nitrite was determined directly (Figure 6A), and the amount of nitrate was calculated by subtracting the measured concentration of nitrite (Figure 6A) from the total amount of nitrite measured following reduction of the sample (Figure 6B). As seen in Figure 7, the production of nitrite and nitrate was determined for all samples collected over the 120 min incubation period. Both reached maximum levels (determined to be about 25 μM) at 60 min after incubation.

CONCLUSIONS

An indirect method for detecting NO using microchip CEEC to monitor nitrite and nitrate has been developed. Nitrite is measured directly while nitrate is reduced to nitrite by chemical means. Two separate measurements are made (original nitrite and original nitrite + nitrite resulting from reduction of nitrate), and the amount of nitrate present is determined from

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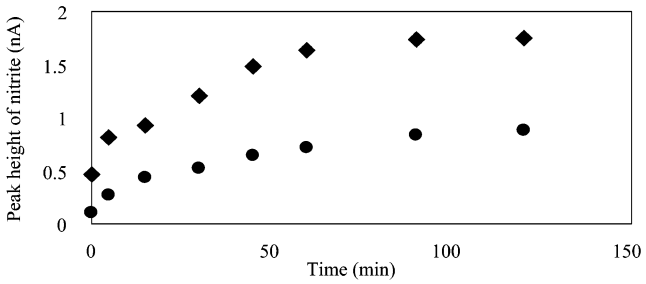

Figure 7. Measurement of the time course production of nitrite and nitrate from SIN-1 using chip B. Symbols: (●) nitrite and (◆) reduction reaction mixture (nitrite and nitrite produced from nitrate).

the difference between the two determinations. Two chip designs were evaluated. Chip A was used to optimize separation and detection conditions. Chip B was used to determine the compatibility of sample and buffer solutions with the gated injection scheme and to optimize the on-chip reduction reaction. It was found that the reduction reaction could effectively be employed on a small scale and carried out directly on-chip. The effectiveness of chip B for the rapid monitoring of nitrite and nitrate was demonstrated by the use of SIN-1, a NO-releasing compound. Future work will involve studies to activate the Cd granules on-chip, as well as biological applications of these devices, including in vitro monitoring of NO produced by bovine brain microvessel endothelial cells.

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