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Separation and Detection of Peroxynitrite Using Microchip Electrophoresis with Amperometric Detection

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Introduction

Oxidative stress and nitration are thought to be involved in the pathology of several cardiovascular diseases including stroke, myocardial infarction, hypertension, atherosclerosis, and chronic heart failure, as well as the neurodegenerative diseases multiple sclerosis, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease.[1] Peroxynitrite ($ONOO^-$) is a highly reactive, oxidizing species formed in vivo from the reaction of nitric oxide ($NO\cdot$) with the superoxide anion. The reaction between these two species occurs at a diffusion-limited rate ($6 \times 10^9\ M^{-1}s^{-1}$)[2] that can supersede the scavenging of superoxide by superoxide dismutase ($2 \times 10^9\ M^{-1}s^{-1}$).[1] In situations such as the proinflammatory state, peroxynitrite can be produced faster than it can be scavenged, resulting in oxidative insult.

The ability to monitor $ONOO^-$ production in biological environments could aid in further understanding the role that $ONOO^-$ plays in the pathology of disease. However, direct detection of peroxynitrite under physiological conditions (pH 7.4) is made difficult by its short half-life (~1 s). Most assays performed in vivo rely on indirect detection schemes such as the oxidation of fluorescent and chemiluminescent probes[3–8] as well as the detection of 3-nitrotyrosine, a known marker for the presence of $ONOO^-$.[1, 9–11] The most notable drawback of these indirect methods is that they are not selective for $ONOO^-$ alone, and other reactive nitrogen and oxygen species (i.e., H_2O_2, $NO\cdot$, NO_2^-, and NO_2Cl) can contribute to the results of the assays.[10, 12–15] Recent developments have led to a fluorescent probe that is capable of reacting exclusively with $ONOO^-$, reducing the contribution of interfering species.[8] However, the probe is not commercially available, and the technique is still plagued by the fact that introduction of any probe into a biological environment can create unwanted alterations in cell function.

Because $ONOO^-$ oxidizes at a modest potential ($E° = +270$ mV vs. SSCE), it is well-suited for electrochemical detection.[16] In addition, many other species associated with $ONOO^-$ formation and degradation (i.e., $NO\cdot$, NO_2^-, and H_2O_2) are electroactive. This makes it possible to detect changes in the presence of these species along with $ONOO^-$. Another advantage of using electrochemical detection is that individual species exhibit different voltammetric profiles; therefore, current ratios can be used to identify peaks in the electropherogram.

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SUPPORTING INFORMATION AVAILABLE Further details describing the characterization of the microfluidic device as well as additional data to support the identification of peroxynitrite are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

Previous work in Amatore's group used voltammetry at platinized carbon microelectrodes to detect $ONOO^-$ released from activated fibroblasts and macrophages,[16, 17] as well as implemented a voltammetric detection scheme on a microchip device for the general detection of reactive oxygen and reactive nitrogen species released from cells.[18] Xue et al. reported the detection of $ONOO^-$ through reduction at a poly-tetraaminophthalocyanine manganese (II) ultramicrosensor.[19] Several of these electrochemical techniques have been used to quantitate $ONOO^-$ released from single stimulated macrophages (~7.5–9.0 fmol)[17, 20] as well as the intraceullar concentrations of peroxynitrite in single aortic endothelial cells (~155–205 nM) [21], and myocardial cells (~60.5–76.3 nM).[19]

However, in these studies $ONOO^-$ is not separated and is detected simultaneously with other reactive species. Typically, only a single analyte is measured, making it impossible to monitor degradation byproducts or reactivity of the analyte with other species that are present in the sample. Also, the detection of low concentrations of analytes in the presence of a high concentration of other electroactive species is difficult.

One way to circumvent these issues is to separate the species before electrochemical detection occurs. With the exception of one paper previously published by our laboratory using CE with UV detection,[22] there have been no reports of the separation of $ONOO^-$ from other reactive nitrogen and reactive oxygen species using electrophoretic methods. Here we report the first use of a microchip electrophoresis system, coupled with amperometry, to separate and detect $ONOO^-$. Microchip-based devices provide several advantages over conventional electrophoresis methods. A few such benefits include small sample/reagent volumes, on-chip sample handling, short analysis time, and the ability to implement microelectrodes using establish photolithographic techniques.[23] The small sample volume requirements and on-chip sample handling make these devices amenable for the analysis of volume or mass limited samples such as single cells[24] or dialysate samples.[25] The short analysis time is a feature that is particularly beneficial in this application. As $ONOO^-$ is highly reactive, rapid analyses allow detection to take place before significant sample degradation occurs. Similarly, being able to perform sub-minute separations allows one to track dynamic changes in the concentration that may be missed using conventional methods which require several minutes per separation.

EXPERIMENTAL SECTION

Information regarding the fabrication of the microchip device and the Pd electrode is provided in the Supporting Information.

Chemicals and Materials

The following chemicals and materials were used as received: SU-8 10 photoresist and Nano SU-8 developer (MicroChem Corp., Newton, MA); AZ 1518 photoresist and 300 MIF developer (AZ Resist, Somerville, NJ); photolithography film mask (40,000 dpi; Infinite Graphics, Minneapolis, MN); 100 mm Si wafers (Silicon, Inc., Boise, ID); glass (5 in. × 5 in. × 1.1 mm.; Telic, Valencia, CA); Sylgard 184 (Ellsworth Adhesives, Germantown, WI); Pd (99.95% purity) and Ti (99.97% purity) targets (2 in. diameter × 0.125 in. thick; Kurt J. Lesker Co., Clairton, PA); Ti etchant (TFTN; Transene Co., Danvers, MA); epoxy and Cu wire (22 gauge; Westlake Hardware, Lawrence, KS); silver colloidal paste (Ted Pella, Inc., Redding, CA); acetone, isopropyl alcohol, 30% H_2O_2, H_2SO_4, HNO_3, NaOH and HCl (Fisher Scientific, Fair Lawn, NJ); sodium nitrite, boric acid, tetradecyltrimethylammonium bromide (TTAB) (Sigma, St. Louis, MO); peroxynitrite and 3-morpholinosydnonimine (SIN-1) (Cayman Chemicals, Ann Arbor, MI). All water used was ultrapure (18.3 M Ωcm) (Labconco, Kansas City, MO).

Instrumental

A CHI 812B electrochemical analyzer with associated software (CH Instruments, Austin, TX) was used to control the three-electrode amperometric detection system. An ultravolt 30 kV power supply containing two positive and two negative polarity modules (Ronkonkoma, NY) was used to apply the separation voltages. Detection electrodes were microfabricated using a thin-layer deposition system from Kurt J. Lesker (Clairton, PA), a spin-coater from Brewer Science (Rolla, MO), a hotplate from Fisher Scientific (Allenton, PA), and an ABM near-UV flood source (Scotts Valley, CA). Channel depths were measured using a Tencor Alpha Step 200 Profilometer (San Jose, CA).

Electrochemical Detection

A three-electrode amperometric detection scheme was used for $ONOO^-$ detection. The system consisted of a Pd working electrode (50 μm wide, 200 nm profile), a platinum wire auxiliary (0.5 mm dia.), and a Ag/AgCl reference electrode. The working electrode was aligned in an end-channel configuration in order to decouple the electrode from the high voltages used for separation. The electrode alignment is further explained in the Supporting Information.

Microchip Electrophoresis

Microchip electrophoresis was carried out on a PDMS/glass hybrid device using a gated injection method (Figure S-1). Two negative high voltage leads were placed in the sample and buffer reservoirs, while two earth ground leads were placed in the sample waste and buffer waste reservoirs. The gated injection technique involved floating the high voltage at the buffer reservoir so as to allow the high voltage in the sample reservoir to deliver sample into the separation channel of the microchip. To stop an injection, the high voltage in the buffer reservoir was reestablished. All experiments discussed in this paper used 1s gated injections.

Solution Preparation

The separation buffer consisted of 10 mM boric acid with 2 mM tetradecyltrimethylammonium bromide (TTAB) at pH 11. Buffer was prepared once a week (or as needed). Nitrite (NO_2^-) stock solution (10 mM) was prepared weekly by dissolving $NaNO_2$ in water. Subsequent dilutions of NO_2^- for use in the microchip system were made in the appropriate run buffer. After purchase, peroxynitrite standards were stored at −80 °C for no longer than two months and thawed on ice before use. Once thawed, the commercially purchased $ONOO^-$ was diluted fourfold in cold 0.3 M NaOH (per the vendor's instructions) to give a solution of approximately 10 mM. Verification of this concentration was established by taking an absorbance measurement of the ~10 mM solution at 302 nm using an extinction coefficient of 1670 cm/M (information also provided by the vendor). SIN-1 was stored at −20 °C upon receipt. Prior to use, 500 μL of 0.05 M NaOH was added to the stock 5 mg of SIN-1 powder, yielding a concentration of 48 mM. A further dilution (1:10) of the SIN-1 solution was made in the appropriate run buffer before experiments were carried out.

RESULTS AND DISCUSSION

Detection and Identification of $ONOO^-$

As mentioned in the introduction, the half life of $ONOO^-$ at physiological pH (7.4) is ~1 s. However, it has been shown that at more alkaline conditions, $ONOO^-$ is forced to remain in its more stable anionic form, thus extending its lifetime.[22, 26] Information regarding the optimization of the separation conditions and peroxynitrite stability is provided in the Supporting Information.

The microchip system was first used to identify the migration time of the $ONOO^-$ peak from commercially purchased standards and establish a limit of detection (LOD). The literature accompanying the commercially purchased $ONOO^-$ sample claimed an assay of ≥ 90% $ONOO^-$ with the remainder consisting of NO_2^- and NO_3^-. As NO_3^- is not electrochemically active, it was expected that two peaks would be present in the $ONOO^-$ standard. As is shown in Figure 1, the $ONOO^-$ standard (100 μM) contained four peaks, two of which were considerably larger in size than the others. Peak 1 is unidentified and appears to be a product of $ONOO^-$ degradation. Peak 2 was determined to be NO_2^-. This was confirmed by injection of NO_2^- standards and further supported by voltammetric characterization. Peak 3 (the largest peak) was identified as $ONOO^-$. The identity of peak 3 as $ONOO^-$ was confirmed using three criteria. First, previous reports from our lab showed that $ONOO^-$ migrates after nitrite using conventional CE with similar run buffer conditions.[22] Second, work by Amatore's group has shown that $ONOO^-$ is electrochemically oxidized at much lower potentials than NO_2^-, a possible interfering species in the sample.[20]

Using this criterion, current ratios were obtained for each species by performing detection at +900 mV and +1100 mV (vs. Ag/AgCl). The ratios of these resulting peak heights (I_{+900} / I_{+1100}) are listed in Table S-1 in the supporting information. Peaks 3 and 4 exhibited much higher current ratios than did peaks 1 and 2. These data further support the identification of $ONOO^-$ as peak 3 since it exhibits a larger current ratio, indicating that it is easier to oxidize than peak 2 (NO_2^-). Also, NO_2^- was not detectible below +900 mV, while $ONOO^-$ was.

Third, $ONOO^-$ is a highly reactive species, whereas the other species that were reported in the assay, NO_2^- and NO_3^-, are stable. Therefore, it was expected that the peak associated with $ONOO^-$ would decay over time. To test this, the sample was left exposed to the ambient air for 7 minutes, at which point the experiment was run again. The height of peak 3 greatly diminished (decreased 64% in 7 min) while peak 1, apparently associated with $ONOO^-$ degradation, grew substantially (increased 140% in 7 min). These data serve not only to identify the $ONOO^-$ peak but also to verify that the pH 11 buffer used for the separations prolongs the lifetime of $ONOO^-$.

To determine the limit of detection for $ONOO^-$ using the microchip system, a calibration plot was constructed over the concentration range of 3.12 μM to 100 μM. Initially, a 100 μM concentration of $ONOO^-$ standard was made by diluting the 10 mM stock solution 1:100 in the boric acid run buffer. Each subsequent concentration of the calibration plot was created by serial dilution of the standard 1:1 from 100 μM down to 3.12μM. The plot exhibited a linear response with a $R^2 = 0.9979$ (n = 5). The peak for a 3.12 μM sample of $ONOO^-$ produced a S/N of 3.89. Using the data from the 3.12 μM sample, the calculated LOD (S/N = 3) was 2.4 μM. It is interesting to note that, had we extrapolated from the previous concentration on the calibration plot (6.25 μM; S/N = 70.16), the estimated LOD would have been 267 nM. It appears that the low μM detection limits are not inherent to the electrochemical detection method, but to the reactivity of the analyte at lower concentrations. Potential degradation pathways for $ONOO^-$ are discussed in the Supporting Information.

Monitoring the Generation of $ONOO^-$ from SIN-1

To test the capability of the microchip device to detect $ONOO^-$ production from a chemical or biological source, 3-morpholiniosydnonimine (SIN-1), a metabolite of the vasodilator moldisomine, was used. As SIN-1 degrades, it generates both nitric oxide (NO·) and superoxide simultaneously, ultimately producing $ONOO^-$ at ~1% of the original SIN-1 concentration[6] (Figure S-2).

As mentioned in the experimental section, SIN-1 was diluted in the pH 11 run buffer to a final concentration of 4.8 mM. This concentration was chosen because, at a 1% yield, the

decomposition was expected to generate an easily detectable 48μM $ONOO^-$. Figure 2 shows electropherograms of the SIN-1 injections at different time points. The peak associated with $ONOO^-$ was identified based on its migration time, voltammetry, and the fact that the peak continually grew in size during the experiment.

Using the equation from the calibration plot, the concentration of $ONOO^-$ at each of the time points was estimated. Figure 3 shows the plot of the estimated $ONOO^-$ concentration being tracked by the microchip system over time. Although not all of the data points fall into a smooth trend, it can be seen that the concentration of $ONOO^-$ increases with time, approaching a plateau near the end of the experiment. This plateau is expected as the generation of $ONOO^-$ is dependent on the concentration of SIN-1, which will eventually reach 100% decomposition. An average of the data points from each 5-injection run was also plotted vs. time. This plot, shown in Figure S-3, depicts a clear trend of the $ONOO^-$ generation from SIN-1 decay. It is also worth noting that the SIN-1 reaction appeared to yield ~12.5 μM $ONOO^-$ after 50 minutes, at which time it was estimated that the SIN-1 sample would be capable of producing ~48 μM $ONOO^-$. It is likely that the $ONOO^-$ concentration fell short of its estimated concentration due to its reactivity with CO_2 in the ambient air, as CO_2 is a known sink for $ONOO^-$ in vivo. This reaction is discussed further in the Supporting Information (Figure S-4).

CONCLUSIONS

The work shown here has demonstrated the utility of a microchip system for the analysis of the short-lived, reactive species peroxynitrite. The rapid separation time of the microchip, compared to that of conventional CE instruments, is ideal for such short-lived species, allowing the separation and detection of the analyte to take place before significant degradation occurs. Also, the short separation time requirements yield increased temporal resolution over conventional devices (tens of seconds vs. several minutes). The increased temporal resolution, in turn, allows one to monitor changing concentrations in a dynamic system. In future applications, such a device could be used to monitor transient species generated by biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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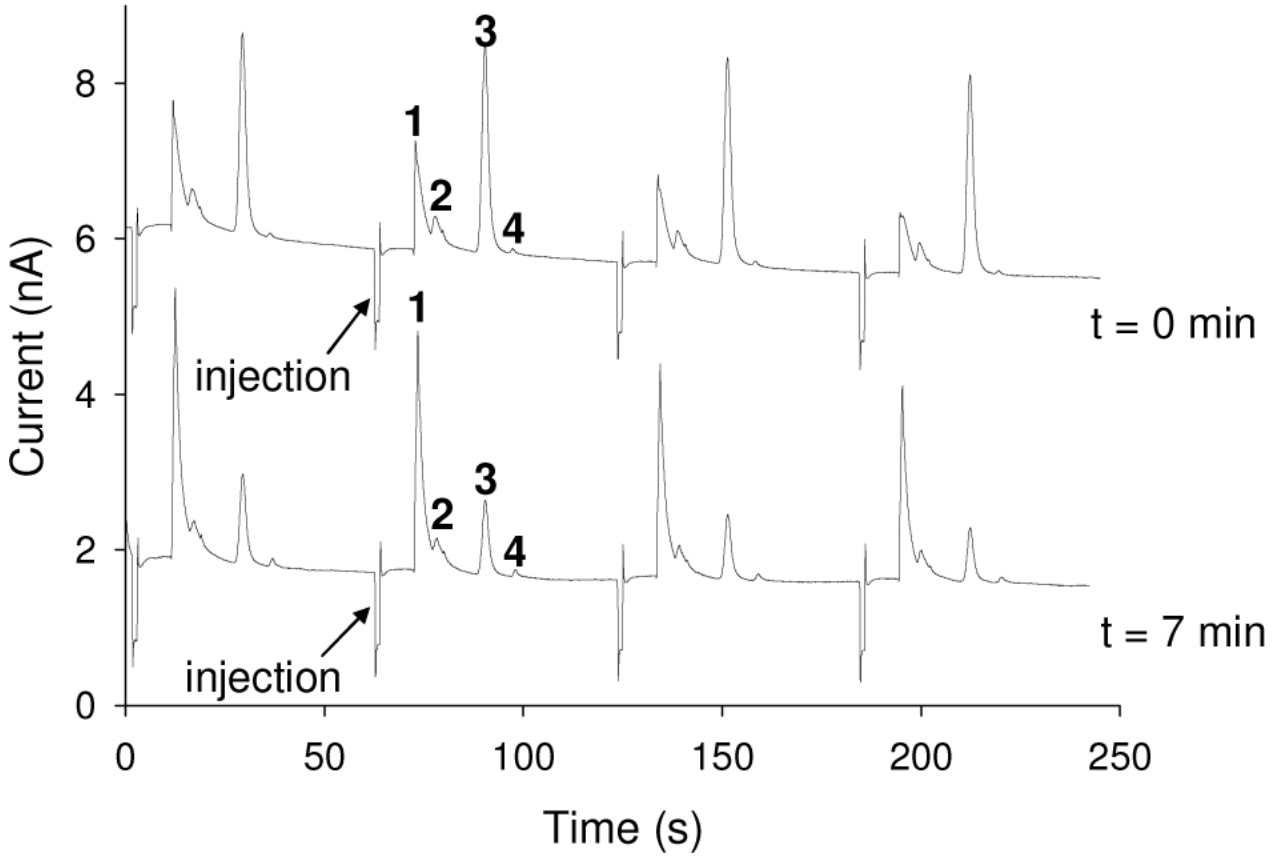

Figure 1.

Four sequential electropherograms depicting the separation and detection of $ONOO^-$ from commercially purchased standards. Peak 1 = Unidentified; Peak 2 = NO_2^-; Peak 3 = $ONOO^-$, Peak 4 = Unidentified. Peak 3 was identified as $ONOO^-$ by its abundance, voltammetry, and its decay over a period of ~7 min (bottom trace).

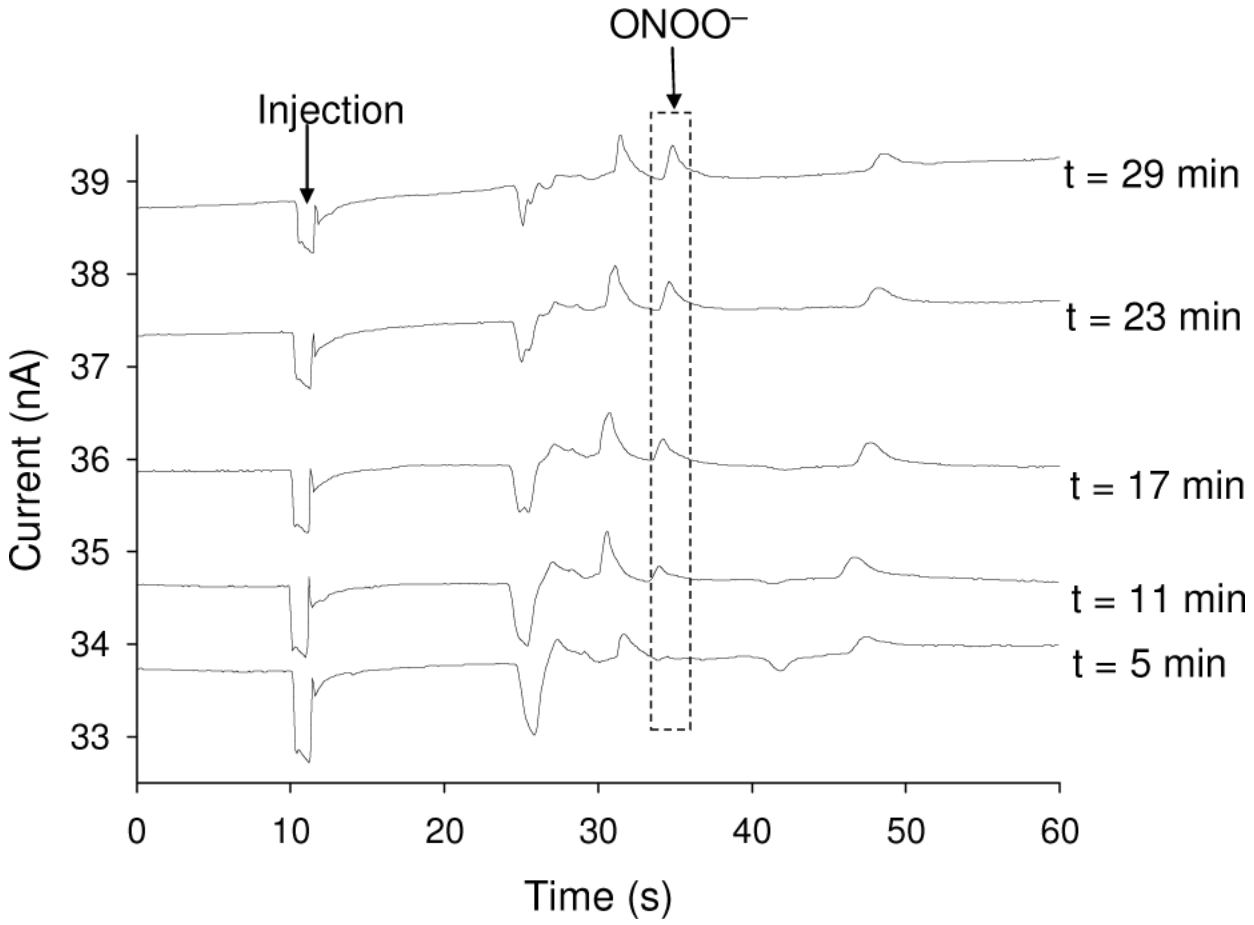

Figure 2.

Separation and detection of $ONOO^-$ from SIN-1. The electropherograms show the injection of the SIN-1 sample over increasing time points. $ONOO^-$, inscribed in the dashed box, was identified by its migration time as well as its growth over time. Separations were performed for 60 s, allowing the $ONOO^-$ concentration to be monitored every minute. The electropherograms shown represent the final injection of each 5-min run.

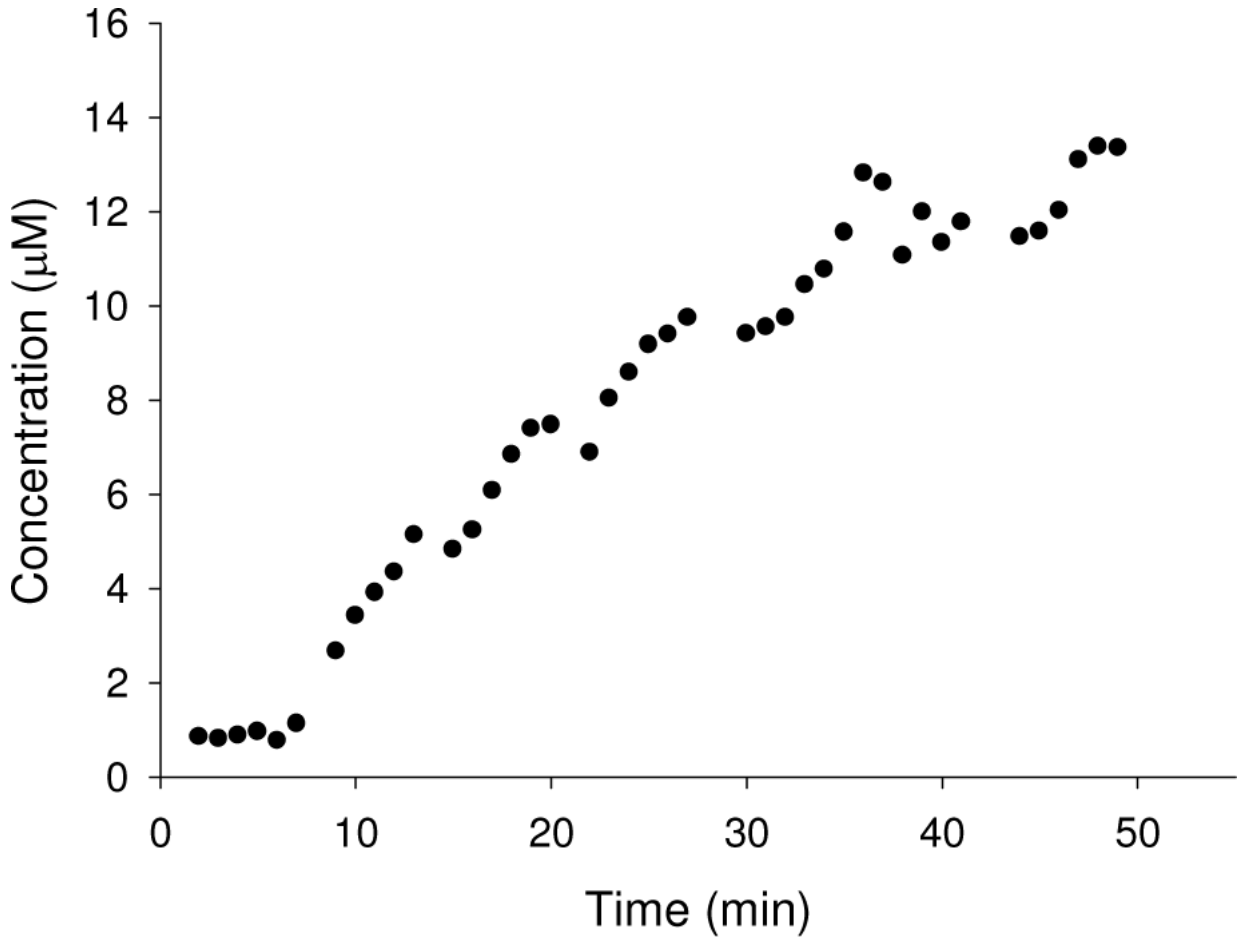

Figure 3.
Temporal resolution of the microchip device. Using data from a calibration plot, $ONOO^-$ concentration from SIN-1 was estimated for each time point in the experiment. Each data point is from a 60-s separation.