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# Quantitation of nitric oxide-derived nitrite from activated macrophages using microdialysis sampling

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**Abstract**

An HPLC method for detecting nitrite in microdialysis samples obtained from activated RAW 264.7 macrophages in cell culture has been developed. Nitrite was quantified using a pre-column derivatization with 2,4-dinitrophenylhydrazine (2,4-DNPH) followed by HPLC–UV analysis of the azide product. For dialysates, the detection limit of nitrite was 750 nM and the quantitation limit was 2.5 μM. The microdialysis relative recovery of nitrite in the macrophage cell culture medium was determined to be 86 ± 2% ($n = 3$) at a flow rate of 0.7 μl/min. Nitrite produced from activated macrophages was measured immediately after lipopolysaccharide (LPS) stimulation using microdialysis sampling.


*Keywords:* Macrophages; Microdialysis sampling; Nitrite; Nitric oxide

## 1. Introduction

Macrophages in cell culture are a well-established in vitro model system to study inflammation caused by biomaterials [1]. Inflammation is an important area of research in biomaterials biocompatibility [2]. Macrophage cells are major phagocytic cells that release different signaling molecules to regulate inflammation. Nitric oxide is known to be an important phagocytic signal transmitter related to different inter- and intra-cellular communication processes [3]. Nitrite production from biological systems is directly related to nitric oxide release because nitric oxide is rapidly converted to nitrite and nitrate when exposed to water and oxygen in biological fluids [4]. Because nitric oxide rapidly forms nitrite and nitrate in biological fluids, it is well accepted that quantitation of nitrite serves as an indirect measurement of nitric oxide production [5].

In addition to nitric oxide production, activated macrophages produce different reactive oxygen species and reactive nitrogen species. These reactive oxygen and nitrogen species have been implicated in directing other chemical signaling processes [6]. Superoxide and nitric oxide can react to produce the reactive nitrogen species, peroxynitrite [7]. Peroxynitrite is an aggressive oxidant and is suspected in causing lipid peroxidation. The principle products of lipid peroxidation after an oxidative stress event are the aldehydes, malondialdehyde and 4-hydroxynonenal

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[8]. Lipid peroxidation products are known to induce apoptosis [9], increase chemotaxis [10] and alter cytokine production in macrophages [11].

Microdialysis is a well-known sampling technique based on diffusion across a semi-permeable membrane [12]. It has been widely applied to both in vitro and in vivo sampling of numerous analytes [13–16]. Microdialysis sampling is generally performed under non-equilibrium conditions using a perfusion fluid that matches the ionic strength and composition external to the microdialysis probe. The concentration of the analyte collected in the fluid exiting the probe ($C_{outlet}$) is a fraction of the concentration external to the probe ($C_{sample}$) [17]. This fraction is often referred to as the relative recovery, extraction fraction, or extraction efficiency for the microdialysis sampling process. Prediction of the analyte concentration external to the microdialysis probe requires knowing the correlation between $C_{outlet}$ and $C_{sample}$ [18]. The extraction efficiency, $E_d$, is expressed as shown in Eq. (1)

$$E_d = \frac{C_{outlet} - C_{inlet}}{C_{sample} - C_{inlet}} \quad (1)$$

where $E_d$ is the extraction efficiency; $C_{inlet}$ the analyte concentration in the inlet perfusate, which is often zero; $C_{outlet}$ the analyte concentration in the exiting perfusate or dialysate; and $C_{sample}$ the analyte concentration in the sample medium external to the microdialysis probe. A common analytical challenge associated with microdialysis samples is that microliter volumes, which are often much less than 50 μl, are obtained during the sampling process [19].

A broad range of spectroscopic analytical methods have been reported in the literature for nitrite quantitation [5]. The most common approach to detect nitrite is the Griess reaction which involves the formation of an azo dye that can be detected between 500 and 600 nm. Reported limits of detection range between 0.02 and 2 μM and are highly dependent upon the sample conditions and specific reagents chosen [5]. For biological samples, the detection limits for the Griess reaction have been reported between 1 and 5 μM [20]. In addition to the Griess reaction, a common fluorescence-based assay uses 2,3-diaminonapthalene as a derivatizing agent for nitrite and can achieve detection limits in the 10–30 nM range [3].

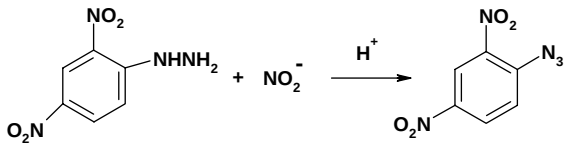


Fig. 1. The chemical reaction between 2,4-dinitrophenylhydrazine (2,4-DNPH) and nitrite.

In addition to these two common approaches for detecting nitrite, another reagent is available that allows derivatization of both nitrite and aldehyde formed during an inflammation response in the same sample. Because of our interests in both nitric oxide and aldehyde formation during an oxidative stress response, we chose to explore the use of 2,4-dinitrophenylhydrazine (2,4-DNPH) as the derivatizing agent for nitrite in microdialysis samples. 2,4-DNPH is a well-documented derivatization reagent for aldehydes and ketones [21,22]. 2,4-DNPH has been used to derivatize aldehydes from biological samples and was found to be a fast reacting derivatizing agent [23]. In addition to derivatizing aldehydes and ketones, 2,4-DNPH can derivatize $NO_2$ from environmental air samples. Recently, 2,4-DNPH has been used for the derivatization of trace amounts of nitrite from natural waters [24–26]. Nitrite reacts with 2,4-DNPH and produces 2,4-dinitrophenyl azide as shown in Fig. 1. The azide product can be separated from 2,4-DNPH and quantified using HPLC–UV.

This paper focuses on the chromatographic and microdialysis methods development for nitrite monitoring from activated macrophages using 2,4-DNPH as a derivatizing agent for nitrite in microdialysis samples. Having the ability to measure various chemical mediators from activated macrophages with some temporal resolution will provide a greater understanding of different cell–cell interactions and signaling events during inflammatory responses.

## 2. Experimental

### 2.1. Chemicals

Sodium nitrite (purity: minimum 99.5%) was purchased from Sigma (St. Louis, MO, USA). Sodium nitrite standard solutions were made up in saline so-

lution (0.9% NaCl). 2,4-DNPH was obtained from Acros (New Jersey, USA). 2,4-DNPH was recrystallized as previously described [24]. The procedure involved recrystallization from acetonitrile–water (70:30 v/v) followed by a second recrystallization from pure acetonitrile. The reagent was dried and stored in a dessicator at room temperature. A 14.5 mM 2,4-DNPH stock solution was made up in a solution containing water–12.1 M HCl (stock HCl)–acetonitrile (50:20:10 v/v) weekly, and stored in the refrigerator before use. Each day prior to derivatization of nitrite samples, the 2,4-DNPH stock solution was diluted to 1.45 mM in a solution containing water–12.1 M HCl–acetonitrile (50:20:10 v/v). Distilled-deionized water from a Barnstead NANO pure system was used for making all aqueous solutions.

The cell culture medium contained 90% Dulbecco's modification of Eagle's medium (Fisher Scientific, Pittsburgh, PA, USA) with 10% filtered fetal bovine serum (Biowhittaker, Walkersville, MD, USA) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Fisher Scientific, Pittsburgh, PA, USA). Lipopolysaccharide (LPS), which was used to activate macrophages, was a generous gift from Dr. Daniel Loegering of Albany Medical College. All solutions used for cell culture experiments including culture media as well as microdialysis perfusion fluids were autoclaved.

### 2.2. Equipment

The HPLC assay was performed with a guard column (Columbus 5 μm C18 30 mm × 2.00 mm) followed by a separation column (Hypersil 3 μm C18 150 mm × 2.00 mm), both of which were purchased from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 0.1 M sodium phosphate (pH 2.6)–acetonitrile (50:50 v/v). The mobile phase flow rate was 0.2 ml/min. HPLC data was acquired using a Thermoseparations Products (San Jose, CA, USA) P1000 pump with a UV 1000 detector controlled by PC1000 software. Separations were performed at room temperature which ranges between 24 and 25 °C and is recorded daily in the laboratory.

To make the nitrite derivative, 10 μl of the 1.45 mM 2,4-DNPH solution was mixed with 10 μl of nitrite-containing sample. The solution was allowed to react for 5 min. The solution was then diluted by five times with the HPLC mobile phase. This diluted sample was injected into the HPLC–UV system for quantitation. The injection volume was 5 μl and the UV detector wavelength was set to 307 nm.

The microdialysis setup includes a 1 ml Hamilton non-metallic syringe (Hamilton Company, Reno, NV, USA), a CMA-102 syringe pump (CMA/Microdialysis, North Chelmsford, MA, USA) and microdialysis probes purchased from either Bioanalytical Systems (BAS-4, West Lafayette, IN, USA) or CMA Microdialysis (CMA-12, North Chelmsford, MA, USA). All membrane lengths used were 4 mm. The microdialysis perfusion fluid contained 0.9% saline and the flow rates ranged between 0.7 and 3.5 μl/min.

### 2.3. Derivative confirmation by LC-Ion Trap MS

To confirm the production of 2,4-dinitrophenyl azide, LC-MS was used. An Agilent 1100 series LC/MSD Ion Trap System (Agilent Technologies, Palo Alto, CA, USA) with an APCI source operated in negative-ion mode was used for confirmation of the azide product.

### 2.4. Calibration

To calibrate the HPLC system as well as the microdialysis probes, nitrite standards between 2 and 50 μM were made up in saline and cell-free culture medium, respectively. The precision of the HPLC assay was determined by injecting three derivatized nitrite samples from each medium (saline and cell culture). The day-to-day variation was obtained by comparing the slope of the nitrite standard calibration curve performed on three different days. Intra-day variation was determined by repeatedly injecting the same standard solution at three different times during an experimental run. To determine the limits of detection and quantitation, the peak-to-peak variation in the baseline was measured over a baseline section with 20 times width of the peak of interest.

### 2.5. Macrophage cell line

RAW 264.7 cells, a murine macrophage cell line, were used for this study. These cells were a generous gift from Dr. Michelle Lennartz of Albany Medical

College. The cells were cultured using standard techniques and were incubated at 37 °C with 5% $CO_2$. LPS stimulation was performed in a resuspended macrophage cell culture in six or twelve well plates. Cells were first resuspended in cold sterile Dulbecco's phosphate buffered saline (DPBS, pH 7.0) and then centrifuged at 800 rpm for 5 min. The supernatant was removed and the cell medium was added to achieve a cell density of 1 million/ml. LPS was added at 1 μg/ml of cell culture medium containing macrophages to stimulate the inflammatory response.

Basal nitrite concentrations were determined by removing a 1 ml aliquot of cell culture medium sample from the six-well tissue culture plate plated with macrophages. In order to separate nitrite from cells, the cell medium sample was centrifuged at 14,000 rpm for 20 min. The supernatant was saved for derivatization with 2,4-DNPH.

### 2.6. Microdialysis sampling of nitrite from LPS stimulated macrophages

Microdialysis extraction efficiency was determined in vitro in a quiescent cell-free culture (DPBS/serum) medium. The microdialysis probe was immersed in the medium that was placed in a 10 ml beaker in a 37 °C sand bath. The microdialysis perfusion flow rate was set to between 0.7 and 3.5 μl/min. Samples were collected every 30 min and six dialysate samples were obtained for each spiked nitrite concentration. Immediately after collection, the samples were subjected to the derivatization procedure. During the experiment, the nitrite concentration in the medium was quantified at the beginning, the interval between the second and third flow rate switch and the end of the experiment by obtaining a 10 μl aliquot of the sample medium. The sample medium was then mixed with 10 μl 2,4-DNPH for derivatization and quantified by the HPLC method.

The microdialysis sampling experiments with macrophages were performed in a sterile cell culture incubator. In some early experiments, six-well plates were used with 6 ml cell medium with a macrophage cell density of 1 million/ml. In later experiments, 12-well plates were used. The twelve-well plate contained 3 ml of cell medium with a macrophage cell density of 1 million/ml. The cell number and medium volume used was based on the medium plate type. An appropriate height of cell medium in the well was used to ensure the probe membrane was fully immersed in the cell medium. Macrophages were activated by addition of LPS (1 μg/ml) into the macrophage-containing wells followed by an overnight incubation. Microdialysis sampling was performed the next day.

Prior to microdialysis sampling, the plastic inlet and outlet tubing for the microdialysis probe was treated with 70% ethanol prior to being placed into the sterile cell incubator. These tubing lines were placed into the incubator through a side port that has specially drilled holes to allow for insertion of this tubing to and from the inside of the incubator. The microdialysis probe (CMA/12 4 mm probe) was prepared by immersing it in a 70% ethanol solution coupled with perfusion through the probe with autoclaved distilled water for 30 min. Then the probe was placed in and perfused with autoclaved saline for 30 min to rinse out the ethanol. Thereafter, it was inserted through the hole drilled on the well plate cover to cell medium in the tissue culture plate as shown in Fig. 2.

## 3. Results and discussion

### 3.1. LC-MS/MS of the derivative 2,4-dinitrophenyl azide

To confirm the formation of the azide product after the precolumn derivatization of nitrite with 2,4-DNPH, LC/MS and LC/MS/MS experiments were performed. The APCI mass spectra did not show molecular ion peaks for the azide (*m/z* 209). However, a fragment corresponding to a loss of 28 ($N_2$) was detected at *m/z* 181. The inability of azides to form molecular cations has been reported and is consistent with their general susceptibility to release $N_2$ [27]. MS/MS experiments were performed on mass-selected ions at *m/z* 181. The CID mass spectrum showed the most abundant ions were *m/z* 120 $[M-2NO+H]^-$ (100%), *m/z* 135 $[M-NO_2]^-$ (22%), and *m/z* 151 $[M-NO]^-$ (41%). The observation of these peaks is consistent with the proposed structure.

### 3.2. Derivative reaction time and stability

Different reaction times were performed with the derivatization process. Samples (10 μl) containing 5 μM nitrite were derivatized for 5, 10, 15 and 20 min

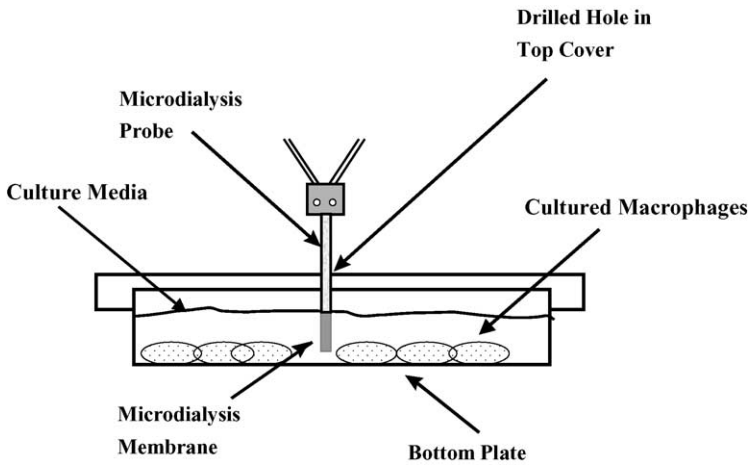


Fig. 2. Illustration of the microdialysis set-up for sampling from macrophages in cell culture.

with the procedure outlined in the experimental section. There were no statistical differences observed between the peak areas for these different reaction times as determined by a Student's *t*-test (data not shown). This observation is in agreement and consistent with the maximum reaction time being observed at 5 min for nitrite derivatization in larger volume (ml) coastal water samples [24].

The stability of the azide product was determined by derivatizing 1, 5 and 50 μM nitrite standards. The 1 and 5 μM standards were tested for stability at room temperature over a 3 h period and exhibited no statistical loss in peak area. The 50 μM nitrite standard was derivatized and was allowed to stand at room temperature protected from light overnight for 16 h. A 9% reduction in the peak area was observed after allowing the sample ($n = 3$) to stand overnight at room temperature. Kieber and Seaton have previously reported that 2,4-DNPH nitrite derivatives from coastal waters are stable for 30 days at 4 °C [24]. Our observations with respect to derivative stability are consistent with theirs.

### 3.3. Nitrite quantitation

Fig. 3A and B show representative chromatograms for nitrite detection from solutions that initially contained saline or the cell culture medium (90% buffer/10% serum) without macrophage cells. A peak for the azide formed after the reaction of nitrite with 2,4-DNPH was well resolved from 2,4-DNPH. It is important to note the baseline noise level varied with the sample matrix. Nitrite standards in saline and in dialysate exhibited lower baseline noise as compared to nitrite samples from the cell-free or cell culture medium. It is likely this excess noise is due to unknown UV interferences in either the media or added serum. Therefore, the detection limit of nitrite for this assay varies as a function of the sample matrix.

The limit of detection (LOD) and limit of quantitation (LOQ) for each matrix was calculated as defined in a standard pharmaceutical analysis text [28]. The samples for each matrix, dialysate and cell culture medium, were injected. The baseline noise ($N_{p\leftrightarrow p}$) was measured over a width that was 20 times the width of the analyte peak. LOD and LOQ were calculated as: $\text{LOD} = (3N_{p\leftrightarrow p})/b$ and $\text{LOQ} = (10N_{p\leftrightarrow p})/b$, where $b$ is the slope of the calibration curve. Because there were differences in both the noise and the calibration curve slope for both matrices, there are differences between the LOD and LOQ for both matrices.

For nitrite in the microdialysis perfusion fluid (saline) the LOD was found to be 750 ± 30 nM (mean ± S.D., $n = 3$) and the LOQ was 2.5 ± 0.1 μM (mean ± S.D., $n = 3$). It should be noted that these values are based on measurements made with the most baseline noise. This detection limit is similar to that obtained by using the Griess reaction, which has reported detection limits of 500 nM in biological fluids [29]. The day-to-day variation for nitrite

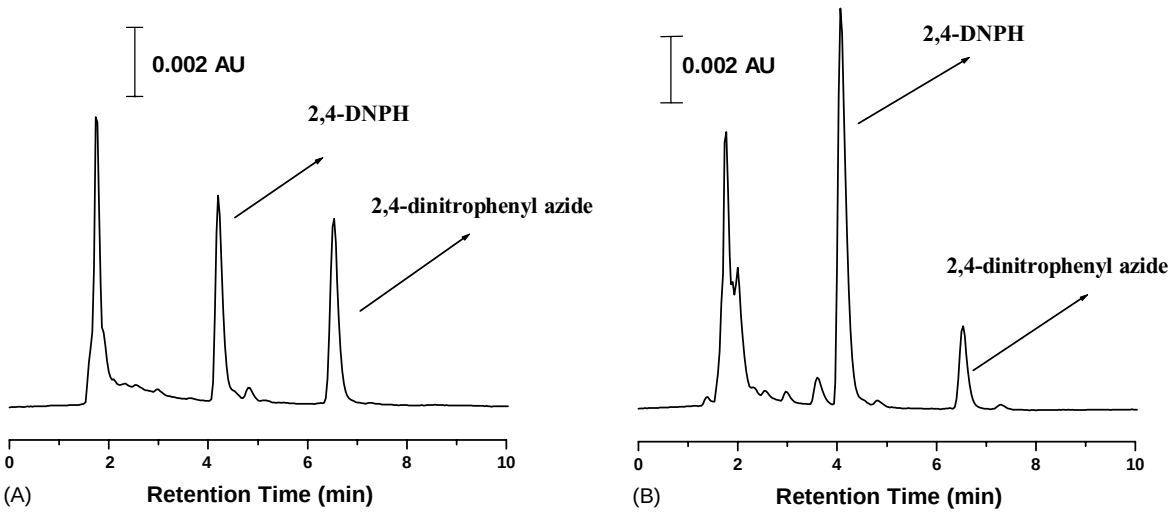


Fig. 3. Representative chromatograms of nitrite (20 μM) spiked into saline solution (A) and into the cell culture medium (10 μM) (B).

in saline solution was 4.3% ($n = 3$). The intra-day variation for cell medium standard nitrite is <8.6% ($n = 3$), while for nitrite/saline standard, the variation is <3.8% ($n = 3$). Direct sampling from cell culture gave higher chromatographic noise as discussed earlier. For samples that were quantified directly from the cell culture medium, the LOD and LOQ increased because of the noise to $1.8 \pm 0.2$ μM (LOD) and $5.8 \pm 0.5$ μM ($n = 3$) (LOQ). By using microdialysis sampling, the detection limits are greatly improved because many of the interferences from the sample medium have been removed.

The calibration curve slope for nitrite spiked into the cell medium solution was 9.6% lower than for nitrite spiked into saline. The cell culture medium is a more complex matrix than saline due to the addition of uncharacterized fetal bovine serum. For example, nitrite may react with proteins in the highly heterogeneous cell medium under the acidic conditions for derivatization, resulting in nitrite loss. Similar problems exist in most spectrometric methods for nitrite detection [5]. Therefore, two calibration curves were used to calculate nitrite concentration, one for microdialysate samples and one for cell culture containing samples.

This derivatizing method is sensitive enough to detect nitrite variation during inflammatory events since the basal concentrations were found to range between not being detected (0) and 4.1 μM giving an approximate average basal concentration of $1.5 \pm 1.5$ μM ($n = 13$). The maximum concentration of nitrite found in the activated macrophages after LPS stimulation ranged between 15 and 30 μM. These observed concentrations are similar to concentrations observed by others using this macrophage cell line and a similar dose of LPS [30]. The differences in the maximum concentrations found are due to the differences in cell passage number.

### 3.4. Microdialysis extraction efficiency in the cell culture medium

Fig. 4 shows the microdialysis nitrite $E_d$ for a CMA-12 polycarbonate probe immersed in either quiescent saline or cell culture medium at 37 °C for different perfusion fluid flow rates. The CMA probe gives a greater than 90% $E_d$ for nitrite at 0.7 μl/min. Fig. 4 shows the extraction efficiency is greatest at low perfusion fluid flow rates as expected during microdialysis sampling. Additionally, the microdialysis $E_d$ for nitrite was reduced in the culture medium which contains serum. To determine if this reduction in nitrite was due to potential protein fouling or obstruction of the membrane rather than protein binding of nitrite, a no-net flux experiment was performed [31]. The no-net flux experiment showed that the nitrite concentration to be within 2% of the expected spiked concentration of nitrite in the culture medium

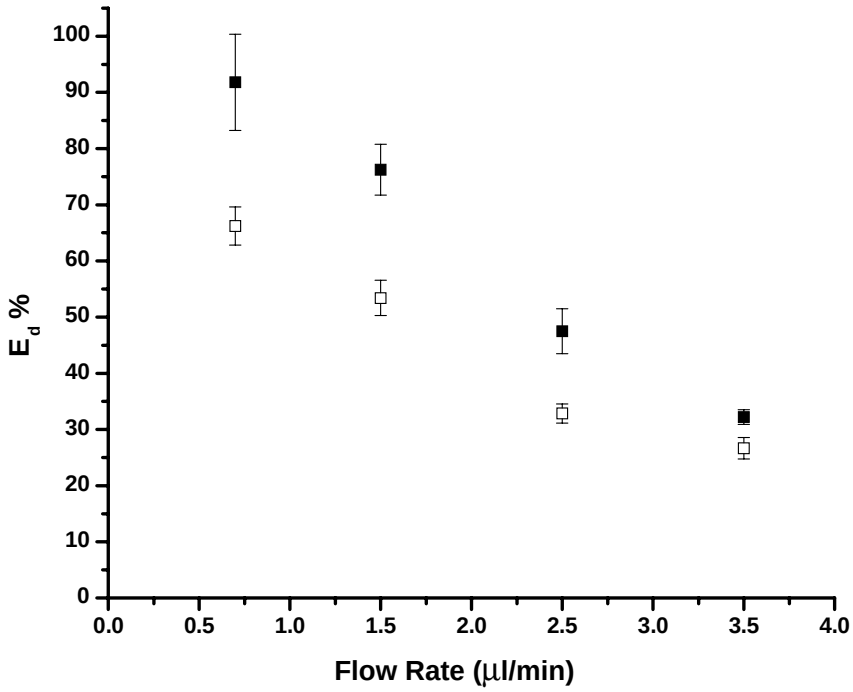


Fig. 4. Microdialysis extraction fraction ($E_d$) across a 4 mm CMA/12 microdialysis probe in quiescent saline (■) and cell-free cell culture medium (□). The samples contained nitrite spiked to a concentration of 55 μM.

(data not shown). This suggests that the lower nitrite $E_d$ is due to obstruction of nitrite mass transport into the microdialysis probe due to components, such as the serum proteins in the culture medium. This obstruction may be due to either viscosity differences between saline and culture medium as well as possible protein deposition onto the probe which may block the membrane pores.

The stability of the microdialysis $E_d$ during a long sampling period in the cell culture medium was studied by performing experiments in a quiescent cell culture medium, with serum, but without macrophages using a 37 °C sand bath. The culture medium was spiked with 22 μM nitrite and microdialysis sampling was performed over a 4 h period. Over a 4 h period the $E_d$ was found to be $104 \pm 4\%$ ($n = 6$) at 0.7 μl/min and the aliquot concentration obtained prior to microdialysis, at 2 h and at 4 h was found to be $22.4 \pm 2.0$ μM ($n = 3$). These results suggest that in the cell culture medium, the microdialysis probe $E_d$ remains stable despite being immersed in a protein-containing solution.

### 3.5. Microdialysis experiments in cell culture

Microdialysis sampling of nitrite produced by activated macrophages was performed within the sterile environment of the cell culture incubator. Fig. 5 shows representative chromatograms of nitrite from microdialysis samples obtained from the culture medium (A) as compared to the culture medium that contained the stimulated macrophages (B). The chromatogram for the dialysate shows much less baseline noise than for the culture medium. Fig. 6 shows representative chromatograms of culture medium obtained from resting macrophages (A) versus LPS-stimulated macrophages (B). The presence of nitrite in the culture medium from resting macrophages was variable, but was never greater than 4.1 μM. This variability is attributed to biological sample differences as well as the passage number for the macrophage cells.

Fig. 7 illustrates the absolute dialysate nitrite concentrations (not corrected for probe $E_d$) found from LPS (1 μg/ml) stimulated macrophages after 16 h of LPS incubation and for a 5 h observation period

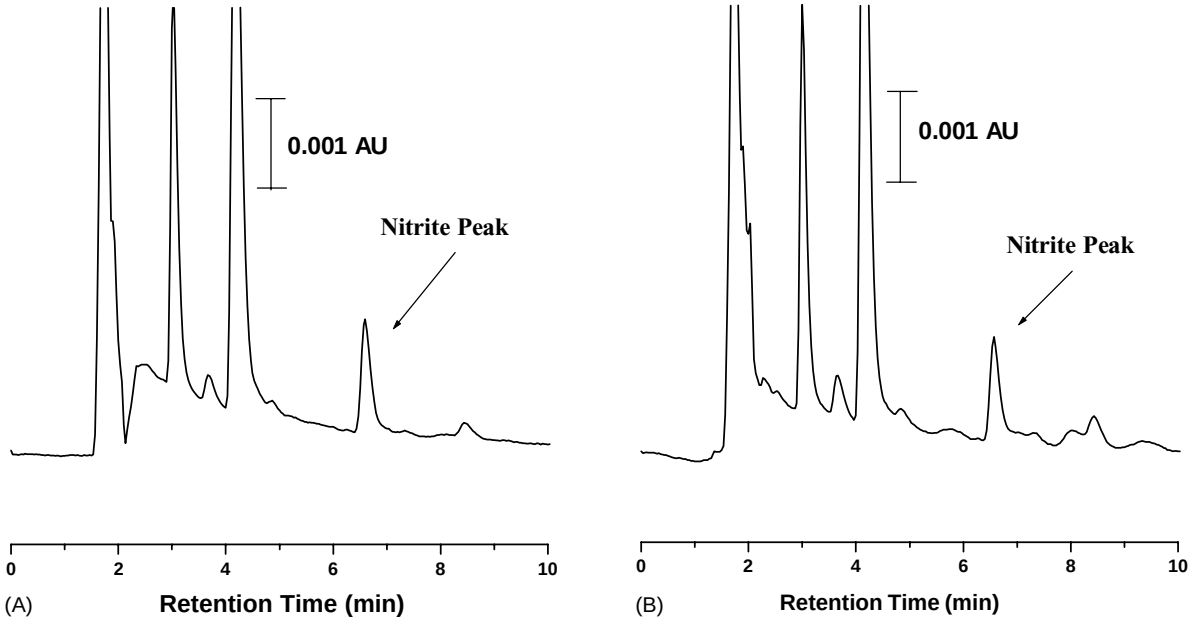


Fig. 5. Representative dialysate (A) and cell culture medium (B) chromatograms of nitrite-containing samples from activated macrophages. Macrophages were stimulated with 1 μg/ml LPS for 16 h prior to collection of samples.

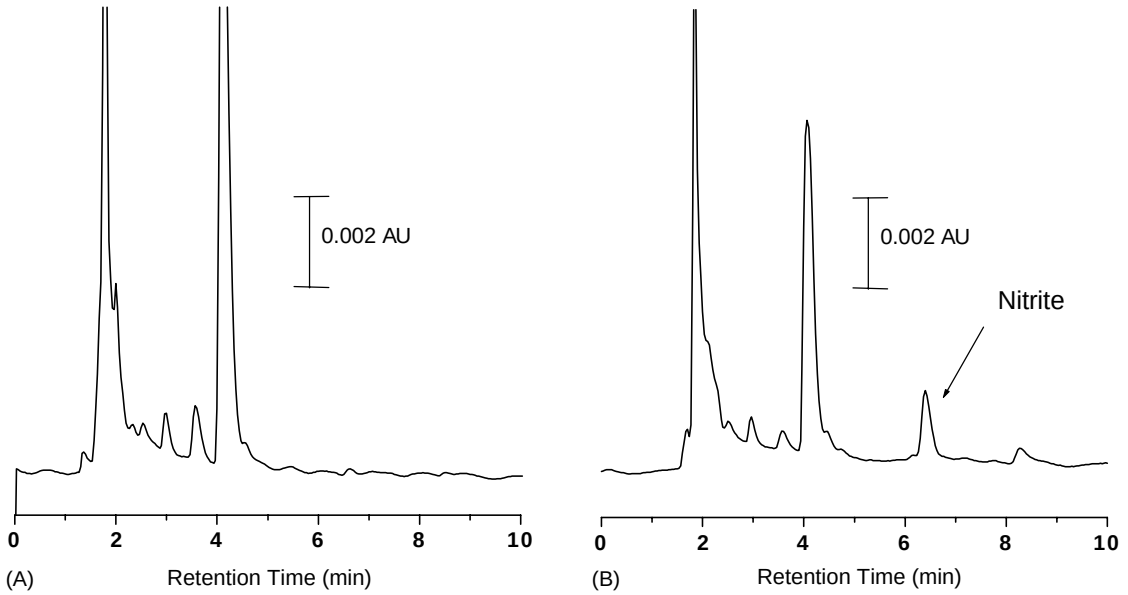


Fig. 6. Representative chromatograms from resting macrophage cell culture medium (A) and activated macrophage cell culture medium (B). Macrophages were activated with 1 μg/ml LPS.

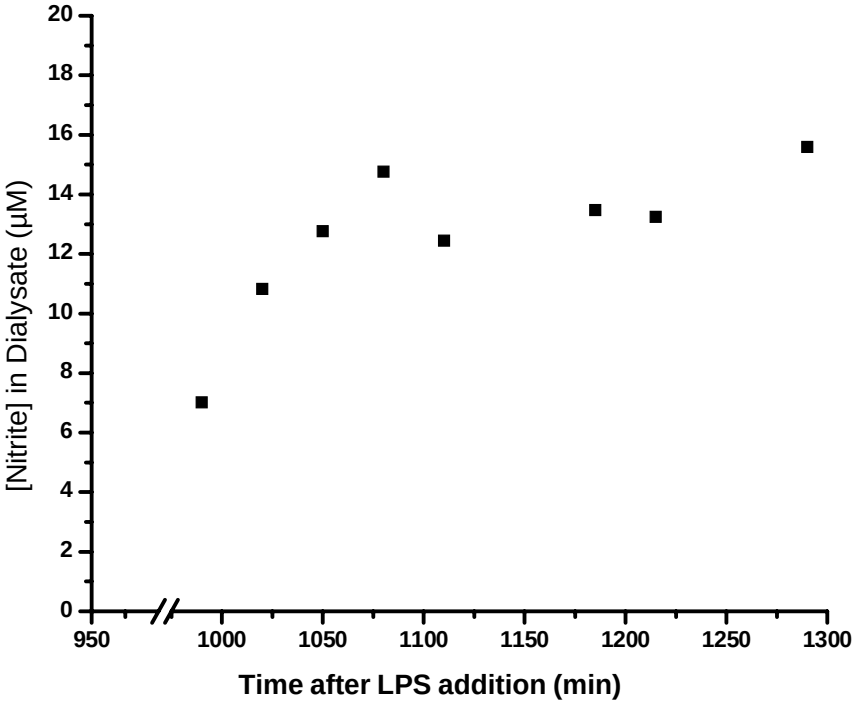


Fig. 7. Dialysate concentrations obtained from LPS-stimulated macrophages 16 h after stimulation. The break in the *x*-axis denotes that the probe was placed into the culture medium 16 h after LPS addition. The time denotes total time after LPS addition to the macrophages. The microdialysis flow rate was 0.7 μl/min and was started right before collection of samples.

thereafter. The probe was placed into the cell culture medium after the 16 h LPS incubation and prior to the 5 h observation period. The initial dialysate nitrite concentration obtained from the cell culture medium was 7 μM, and increased to 15.6 μM after 5 h. The increase in concentration over the first 75 min is most likely due to the dead volume in the exiting microdialysis tubing from the inside of the incubator to the collection vials external to the incubator. The needed FEP tubing for this distance is approximately 1 m. The tubing vendor (CMA microdialysis) reports a dead volume of 1.2 μl per 10 cm. Therefore, the dead volume between the probe outlet and the collection vial external to the incubator is >12 μl. This excludes the dead volume in the probe (∼1 μl) as well as the fittings needed between the probe and the tubing. The lag time is further exacerbated by the use of a low perfusion flow rate, 0.7 μl/min. Furthermore, since the macrophage system is quiescent rather than well stirred, the approach to steady-state concentrations in the microdialysates would be expected to take longer [18]. In retrospect, the use of such a low flow rate is probably not needed in future experiments since the nitrite concentrations obtained are significantly greater than the assay quantitation and detection limits. However, a high recovery is needed to detect nitrite formation during the first several hours of nitric oxide burst after LPS addition. Perfusing the microdialysis probe overnight rather than at the start of the experiment would significantly reduce the problem with dead volume. The concern with this approach is that sustained removal and draining of potential nutrients or other important biomolecules during an overnight perfusion may alter localized biochemistry.

Microdialysis $E_d$ was determined at the beginning and at the end of cell culture microdialysis experiment by measuring dialysate and medium nitrite concentrations. To estimate the microdialysis $E_d$, aliquots from the culture medium were removed periodically throughout the microdialysis sampling experiments. It is important to note that since the cell culture incubator needs its sterile environment to be maintained,

numerous openings into the incubator risk the possibility of introducing unwanted bacteria or other agents deleterious to the macrophage cells. Using a perfusion fluid flow rate of 0.7 μl/min, the $E_d$ obtained by comparing the first microdialysate sample to the culture medium was 64%. However, the $E_d$ found when the last collected microdialysate was compared to the concentration of nitrite in the culture medium was 84%. This large observed change in the microdialysis $E_d$ is either due to the known dead volume in the system or heterogeneity of nitrite in solution. The dead volume in the system would give a lower dialysate concentration thus causing the extraction efficiency to appear low. A potential heterogeneity in the nitrite concentration surrounding the microdialysis probe would not be unexpected because of the two dimensional geometry of the macrophages plated onto the bottom of the cell culture plate. If the sample is heterogeneously spread throughout the culture medium, it is possible that continuous aliquot removal from the sample medium agitates the sample medium enough to make it more homogenous at the end of the experiment.

In addition to these two potential reasons for differences in $E_d$, it should be noted that in most of these experiments, the nitrite concentration was still increasing from the LPS stimulation. Thus, these $E_d$ values are *approximate* and are comparable to microdialysis situations where blood samples are compared to dialysates. The collected microdialysis sample represents an average of the nitrite concentration during the collection time, and the individual aliquot from the sample medium represents the nitrite concentration at that particular time point. In order to appropriately adjust the microdialysis $E_d$, the kinetics of the nitrite increase would have to be known as discussed extensively for blood sampling by Ståhle [32].

To determine if the macrophages were activated by the presence of the microdialysis probe, a second set of experiments was performed in which the microdialysis probe was immersed into the well immediately after plating the macrophages. In a second well, the microdialysis probe was placed only after LPS stimulation. Fig. 8 shows the microdialysis results for absolute nitrite concentrations obtained in the dialysate after a

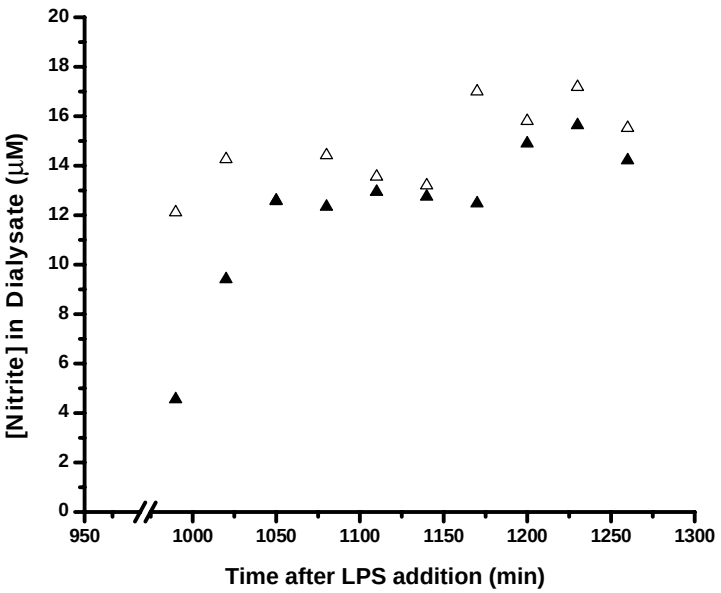


Fig. 8. Dialysate nitrite concentrations obtained from two separate cell culture wells with different conditions. 1 μg/ml LPS was added to the cells and incubated overnight. The symbols denote (△) a probe inserted into the medium the morning of the experiment and (▲) a probe placed into the culture medium after LPS stimulation and incubated overnight. The break in the x-axis denotes the time at which perfusion of the probe started 16 h after LPS addition. The time denotes total time after LPS addition to the macrophages. The microdialysis flow rate was 0.7 μl/min and was started right before collection of samples.

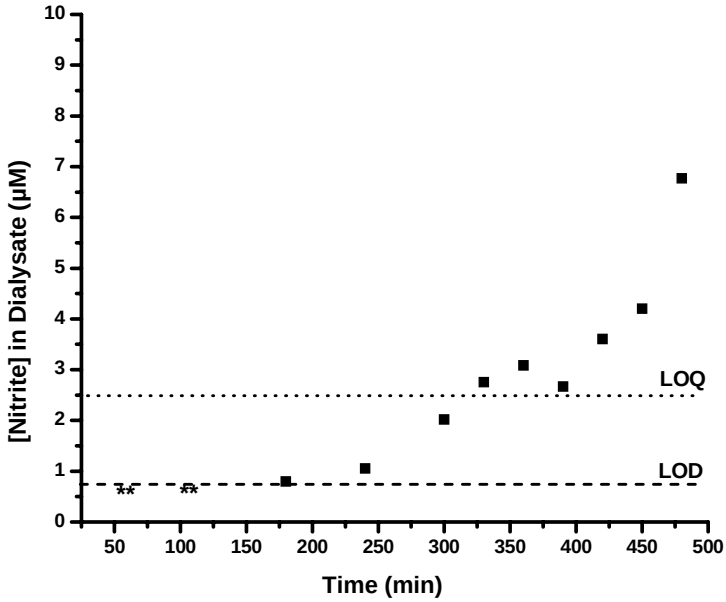


Fig. 9. Time profile of absolute nitrite concentration in the dialysate obtained from LPS (1.0 μg/ml) macrophages. The symbol (**) denotes not detected.

16 h LPS incubation. The difficulty with the microdialysis lag time due to the dead volume in the tubing is exhibited again in this figure. During these experiments, the microdialysis $E_d$ was determined to be 100% using a flow rate of 0.7 μl/min in the well containing the microdialysis probe that was only placed prior to collection of dialysates, whereas the probe that was incubated overnight exhibited a 42% $E_d$ prior to collecting dialysate. At the end of the experiment, the $E_d$ was 88 and 87%, respectively. Although the $E_d$ appears to vary throughout the experiment, in three separate activated macrophage cell culture experiments the final $E_d$ average was found to be $86 \pm 2\%$. Again these differences may be due to the sample dead volume or sample heterogeneity. However, it is significant that within three separate macrophage experiments the final microdialysis $E_d$ has a relative standard deviation of 2.3%.

Fig. 9 shows the dialysate nitrite concentration from LPS stimulated macrophages immediately after introduction of the LPS to the cell culture medium. Observable differences in the dialysate nitrite concentrations were noted within the first few dialysate samples and were significant at 4 h. The nitrite concentration continued to increase for the next 4 h. The probe was allowed to perfuse overnight (0.7 μl/min). The next day the nitrite level in the dialysate was 14.7 μM and increased to 17.7 μM during a 4 h period sampling period reaching the plateau value similar to that shown in Figs. 7 and 8. This experiment demonstrates that quantitative measurements of nitrite formation immediately following LPS addition to macrophages using microdialysis technique is feasible.

## 4. Conclusions

Microdialysis sampling has been shown to be feasible for collecting samples that reflect nitrite production from activated macrophages. The advantage of using microdialysis sampling in complex biological media is that a clean sample is obtained because the probe membrane can exclude cells and proteins from the dialysate. Therefore, extensive sample cleanup of microdialysates is avoided. An additional advantage is that microdialysis sampling prevents the need to take repeated aliquots from a sample solution, thus allowing that system to serve as its own control.

Despite the needed serum for the cell culture medium, the microdialysis sampling procedure can be used to sample from the cell culture medium for nearly 20 h. Low volume microdialysis samples containing nitrite can be derivatized and are ready for injection within 5 min. The derivatizing agent provides an additional advantage since it is possible to derivatize aldehyde and ketone inflammation products from lipids formed from reactive oxygen or reactive nitrogen (peroxynitrite) species. These activated products could potentially be detected in a single chromatographic run with nitrite. Microdialysis is suitable for studies of these types of temporal events because it can non-selectively sample multiple unrelated analytes simultaneously with minimal disruption of the site of interest.

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