

# MICROCHIP ELECTROPHORESIS DEVICES FOR THE DETECTION OF NITRIC OXIDE: COMPARISON OF BULK CELL AND SINGLE CELL ANALYSIS

Susan M. Lunte<sup>1\*</sup>, Dulan B. Gunasekara<sup>1</sup>, Eve C. Metto<sup>2</sup>, Matthew K. Hulvey<sup>1</sup>, Emilie R. Mainz<sup>1</sup>, Giuseppe Caruso<sup>1, 3</sup>, Jose Alberto Fracassi da Silva<sup>1, 4</sup>, Derek T. Jensen<sup>1</sup>, Anne H. Culbertson<sup>2</sup>, Ryan J. Grigsby<sup>1</sup>, and Christopher T. Culbertson<sup>2</sup>

<sup>1</sup>University of Kansas, Lawrence, KS, UNITED STATES OF AMERICA, <sup>2</sup>Kansas State University, Manhattan, KS, UNITED STATES OF AMERICA, <sup>3</sup>The University of Catania, ITALY and <sup>4</sup>State University of Campinas, São Paulo, BRAZIL

## ABSTRACT

This paper describes the development of microchip electrophoresis (ME) devices for detection of nitric oxide (NO) from bulk cell lysates and single cells. Detection of NO and other endogenous electroactive species in Jurkat cells was achieved using ME with amperometric detection. Electrochemical (EC) results were then validated by ME coupled to laser-induced fluorescence (LIF) using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FMDA), a NO-specific probe. In parallel, a single cell analysis device was developed for the determination of intracellular NO using DAF-FMDA and LIF detection.

## KEYWORDS

Nitric oxide, Microchip electrophoresis, Single cell analysis, Amperometric detection, Laser induced fluorescence detection, Bulk cell analysis

## INTRODUCTION

NO and peroxynitrite (ONOO<sup>-</sup>) have been implicated in the pathology of several cardiovascular and neurodegenerative diseases. The short half-life of NO and ONOO<sup>-</sup> at physiological pH makes their quantitation difficult in biological systems. The detection of reactive species such as NO, ONOO<sup>-</sup> and O<sub>2</sub><sup>-</sup> using chronoamperometry and fluorescent probes has been reported [1, 2]. However, interference of other electroactive species and the cross reactivity of fluorescent probes can be a concern. Electrophoresis prior to detection allows the separation of the compounds of interest from potential interferences. Recently, we developed a method that employs ME coupled to amperometric detection to monitor the production of ONOO<sup>-</sup> from 3-morpholinopyridone hydrochloride (SIN-1) *in vitro* [3]. The present work describes the use of ME-EC and ME-LIF for the detection of intracellular NO in both bulk cell lysates and single cells.

## EXPERIMENTAL

### *Microchip Fabrication*

All bulk cell analyses were carried out using a 5 cm "simple-T" PDMS/glass microchip. The fabrication of these microchips has been described previously [3]. Briefly, a soft lithography master was created on a silicon wafer (Silicon, Inc., Boise, ID) using SU-8 10 negative photoresist (MicroChem Corp., Newton, MA). To prepare the chip, PDMS is mixed with a curing agent at 10:1 ratio, poured onto the master, and cured in an oven at 70–85°C overnight. After the PDMS has hardened, it is peeled away from the master and combined with a glass chip (either blank or containing an electrode) prior to use. The separation channel was 5 cm in length, and the side and top channels were 0.75 cm long. The width and depth of the channels are 40 and 14 μm, respectively. A similar method was used to fabricate the single cell analysis device except that the chip was constructed completely from PDMS. In this case, the separation channel was 8.0 cm long, 50 μm wide, and 19 μm deep.

### *Amperometric Detection*

For the ME-EC experiments, the top PDMS layer was aligned over a glass substrate containing a Pt electrode in an in-channel configuration [4]. An electrically isolated potentiostat (Pinnacle Technologies, Lawrence, KS) was used in these studies in a two electrode configuration (Figure 1) [4]. The working and reference electrodes consisted of a 15 μm Pt band and a Ag/AgCl electrode, respectively. To fabricate the Pt working electrodes, chrome-plated glass coated with positive photoresist (Nanofilm, Inc., Westlake Village, CA) was photolithographically patterned using a photomask (Infinite Graphics, Inc., Minneapolis, MN) and UV flood source (ABM, Inc., Scotts Valley, CA). After developing and etching the exposed photoresist and chromium, respectively, the exposed glass was then wet-etched to a depth of 400 nm. A 450 nm layer of platinum was then sputtered on to the surface of the glass plate. Finally, excess platinum and photoresist was removed using acetone, and the remaining chromium layer was removed using etchant, leaving the platinum electrode imbedded into the glass surface.

### *Laser-induced Fluorescence Detection*

DAF-FMDA reacts with NO to produce a benzotriazole derivative that can be detected by LIF detection. In addition to

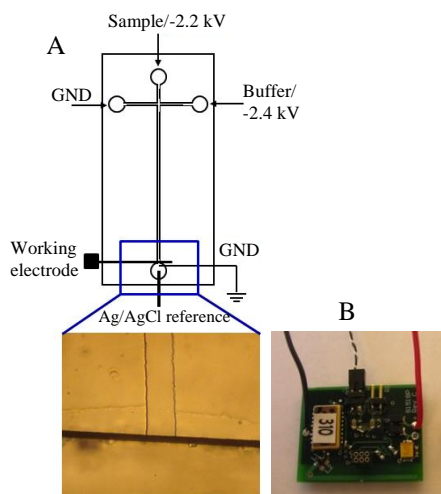


Figure 1: (A.) A PDMS / glass hybrid "simple T" design containing a Pt working electrode with in-channel configuration. (B.) Electrically isolated potentiostat (ref. 4).

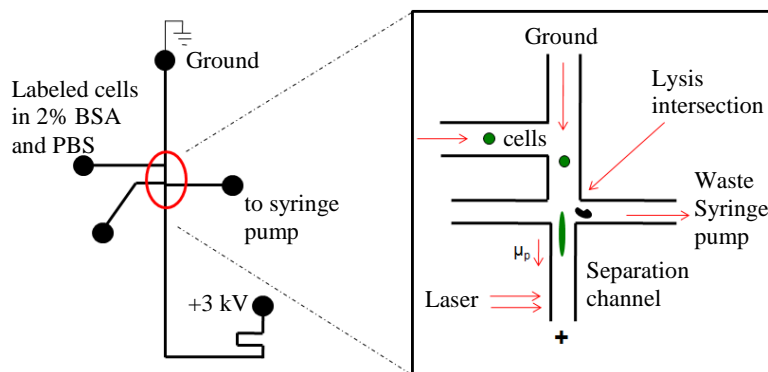


Figure 2: Microchip design for single cell analysis.

DAF-FMDA, 6-carboxyfluorescein diacetate (6-CFDA) was used in these experiments as an internal standard to account for cell-to-cell variability. Both dyes are cell permeant and are trapped in the cell following cleavage of the diacetate groups to produce negatively charged species (DAF-FM and 6-CF). In these studies, detection was accomplished using a Nikon Eclipse Ti-U inverted microscope and a 488 nm laser (Spectra-Physics, Irvine, CA) that was focused on the separation channel 85 mm from the end of the channel. A photomultiplier tube (Hamamatsu Corporation, Bridgewater, NJ) was used for collection of emission signal, and the signal was amplified by using a SR570 low noise current preamplifier at 1  $\mu\text{A/V}$  (Stanford Research Systems, Sunnyvale, CA). For single cell analysis, a 488 nm beam was selected from a multi-line argon-ion laser (Melles Griot Laser Group, Carlsbad, CA) and focused 50 mm downstream from the cell lysis intersection using a Nikon eclipse TS100 microscope (Figure 2) (Nikon Instruments, Melville, NY).

#### Electrophoresis Procedure

Reverse polarity separations for amperometric detection were performed using 10 mM boric acid (pH 11) and 2 mM TTAB. ME-LIF experiments of the bulk cell lysate were performed using normal polarity and a run buffer consisting of 10 mM boric acid (pH 9.2) and 7.5 mM SDS. The sample was injected using gated injection for both LIF and amperometric detection. Single cell analysis was accomplished using a run buffer consisting of 25 mM sodium borate, 20% v/v acetonitrile, 2% w/v bovine serum albumin (BSA), 0.6% w/v Tween-20, and 2 mM SDS. In the single cell analysis experiments, cells were transported hydrodynamically into the device using a syringe pump set at withdrawal mode at a flow rate of 0.25  $\mu\text{L}/\text{min}$

#### Cell Culture and Preparation

The Jurkat clone E-6 was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured using the recommended procedure and passaged every 3–4 days to avoid overgrowth. The cells then were stimulated to produce NO by the introduction of 1.5–3  $\mu\text{g}/\text{mL}$  lipopolysaccharide (LPS) into the flask. The cells were incubated for 3–4 hr to allow the expression of inducible nitric oxide synthase. A separate group of cells with the same passage number (native cells) was used as a control. For ME-LIF experiments, DAF-FMDA was added to the cells following stimulation and they were incubated for another 15 min. Then 6-CFDA was added and the cells were incubated for an additional 20 min. The cells were then centrifuged, washed with medium, and centrifuged again to produce a cell pellet. The cells were then lysed in run buffer and centrifuged using a 3 kDa molecular weight cut-off filter, and the filtrate was isolated for analysis. For amperometric detection, a similar isolation method was employed, but no dye was added. For single cell analysis DAF-FMDA and CFDA-labeled cells were resuspended in PBS and injected into the chemical cytometry system.

## RESULTS AND DISCUSSION

During the ME-EC analysis of the bulk cell lysates, a quickly decaying species was observed in substantially higher concentrations in LPS-stimulated cells than in the native cells (Figure 3). This species migrated with the electroosmotic flow, suggesting that it is a neutral compound. Diethylamine NONOate (DEA/NO) was used to confirm that this species was NO. In addition to NO, several other electroactive species such as nitrite, tyrosine, and glutathione were also observed in the bulk

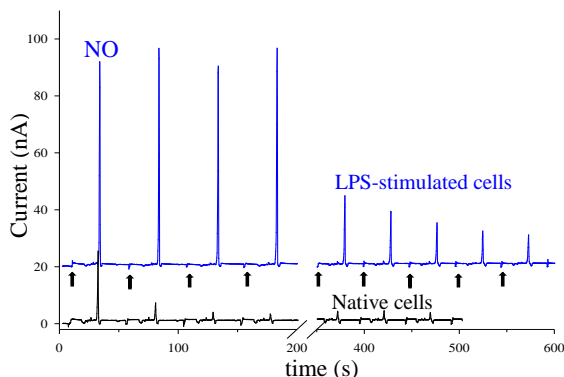


Figure 3. Comparison of production of NO using amperometric detection of LPS-stimulated and native Jurkat cell lysates. Arrows indicate injection time.

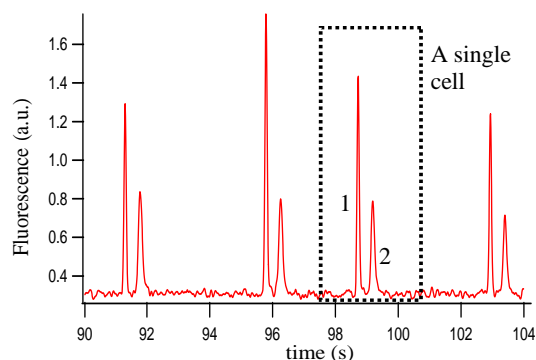


Figure 4: An electropherogram of LPS-stimulated single Jurkat cell analysis. 1. Hydrolyzed 6-CFDA (6-CF) and 2. Benzotriazole derivative of hydrolyzed DAF-FMDA

cell lysate [4]. The production of NO by the Jurkat cells was further confirmed using ME with LIF detection. Measurements using ME-LIF showed that there was a 109% increase in NO production in the LPS-stimulated compared to native bulk cell lysate. Quantitative analysis of NO present in Jurkat cells was performed using a NO standard prepared by DEA/NO. The intracellular NO concentration of a single Jurkat cell was estimated by dividing the total amount of NO produced by the total number of viable cells. The average intracellular NO concentration was estimated to be 0.51 ( $\pm 0.24$ ) mM for native cells while the intracellular concentration of stimulated cells was estimated to be 2.00 ( $\pm 0.83$ ) mM.

To compare the average intracellular NO production obtained from measurements using bulk cell analysis with those obtained for individual cells, a single cell chemical cytometry device was developed (Figure 2) [5]. Figure 4 shows four sequential electropherograms obtained for individual LPS-stimulated cells. In agreement with the results obtained for the bulk cell lysate, a higher intracellular concentration of NO was observed in LPS-stimulated cells than in native cells.

## CONCLUSION

The production of NO by several million Jurkat cells was observed using microchip electrophoresis with both amperometric and LIF detection. The average intracellular NO concentration of a single Jurkat cell was calculated in these studies. In parallel, a single cell analysis device was employed for direct comparison of the intracellular NO concentration of individual cells versus the average intracellular NO production estimated using bulk cell analysis.

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## CONTACT

\*S. M. Lunte, tel: +1-785-864-3811; slunte@ku.edu