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PAPER

Monitoring intracellular nitric oxide production using microchip electrophoresis and laser-induced fluorescence detection

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Nitric oxide (NO) is a biologically important short-lived reactive species that has been shown to be involved in a large number of physiological processes. The production of NO is substantially increased in immune and other cell types through the upregulation of inducible nitric oxide synthase (iNOS) caused by exposure to stimulating agents such as lipopolysaccharide (LPS). NO production in cells is most frequently measured *via* fluorescence microscopy using diaminofluorescein-based probes. Capillary electrophoresis with laser-induced fluorescence detection has been used previously to separate and quantitate the fluorescence derivatives of NO from potential interferences in single neurons. In this paper, microchip electrophoresis (ME) coupled to laser-induced fluorescence (LIF) detection is evaluated as a method for measurement of the NO production by Jurkat cells under control and stimulating conditions. ME is ideal for such analyses due to its fast and efficient separations, low volume requirements, and ultimate compatibility with single cell chemical cytometry systems. In these studies, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) was employed for the detection of NO, and 6-carboxyfluorescein diacetate (6-CFDA) was employed as an internal standard. Jurkat cells were stimulated using lipopolysaccharide (LPS) to produce NO, and bulk cell analysis was accomplished using ME-LIF. Stimulated cells exhibited an approximately 2.5-fold increase in intracellular NO production compared to the native cells. A NO standard prepared using diethylamine NONOate (DEA/NO) salt was used to construct a calibration curve for quantitation of NO in cell lysate. Using this calibration curve, the average intracellular NO concentrations for LPS-stimulated and native Jurkat cells were calculated to be 1.5 mM and 0.6 mM, respectively.

Introduction

Since the discovery in 1986 that nitric oxide (NO) is the endothelium-derived relaxing factor, this molecule has been shown to have numerous other physiological functions, including platelet aggregation, bronchodilation, neurotransmission, and antimicrobial activity.[1–4] Excessive NO production, along with the generation of reactive nitrogen species such as peroxynitrite, has also been linked to cancer, diabetes, and several neurodegenerative disorders, including Alzheimer's and Parkinson's diseases.[5–8]

NO is produced by a group of enzymes known as nitric oxide synthases, which generate NO through the conversion of L-arginine to L-citrulline. There are three forms of nitric oxide synthase: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS). eNOS and nNOS are both Ca^{+2}/calmodulin-dependent and continuously produce low (nanomolar) concentrations of NO over short periods of time. In contrast, iNOS produces large amounts of NO relative to the other two forms in response to tissue injury or inflammation.[5,9,10] A common method for inducing iNOS production in immune cells is stimulation with lipopolysaccharide (LPS) isolated from *E. coli*.

Due to its transient nature, the amount of NO present in biological samples such as serum, blood, or microdialysis samples is most commonly estimated through measurement of

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the more stable major NO oxidation products, nitrite and nitrate.[11,12] In addition, the ratio of arginine to citrulline has also been employed.[13,14] Hetrick *et al.* have reviewed spectroscopic and electrochemical methods for the detection of NO.[15] There are several commercially available molecular probes that react with a partially oxidized species of NO (N_2O_3) to produce fluorescent triazole derivatives.[16–18] The most popular probes are based on diaminofluorescein (DAF) and have been extensively employed for imaging NO production in live cells with microscopic techniques.[19] One drawback of DAF probes is that they can react with dehydroascorbate (DHA) to produce fluorescent derivatives that interfere with NO analysis.[20,21] Therefore, for accurate quantitation of NO in single cells or bulk cell lysates, the separation of the reaction products of the dye with NO from those with dehydroascorbate is desirable. For this reason, Sweedler's group employed capillary electrophoresis and fluorescence detection to quantitate NO production in the neurons of *Aplysia californica*.[20,21]

Microfluidic devices have many advantages for the analysis of cells, including the ability to grow and manipulate single or multiple cells on chip as well as the possibility to integrate on-line sample preparation and analysis.[22–24] NO production by macrophages in culture was explored by Goto *et al.* using a microfluidic device incorporating on-line reduction of nitrate by nitrate reductase, followed by reaction with the Greiss reagent and thermal lens detection.[25] Several microfluidic devices for monitoring NO release from erythrocytes, endothelial cells, and platelets using DAF-FM DA and fluorescence microscopy have been described by the Spence group.[2,26–28] The small volumes characteristic of microfluidic devices were also exploited by Amatore's group for the detection of NO release from single macrophages using amperometric detection.[29] Recently, Wang and Yin reported the use of ME-LIF using DAR-4M (a derivative of diaminorhodamines) for the quantitation of NO in blood samples.[30]

In this paper, we report a ME-based method for the measurement of intracellular NO production in Jurkat cells using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). The average intracellular NO was estimated by determining the number of viable cells using a hemocytometer followed by bulk cell lysis for measurement of total NO production. The effect of lipopolysaccharide (LPS) on intracellular NO production and the viability of Jurkat cells was also investigated. This method will be incorporated into a single cell cytometry system in the future.

Materials and methods

Reagents and materials

Boric acid and ethanol (95%) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sodium dodecyl sulfate (SDS), anhydrous dimethyl sulfoxide (DMSO), monobasic and dibasic phosphate, 0.4% Trypan blue, and lipopolysaccharide (LPS) were supplied by Sigma-Aldrich (St. Louis, MO, USA). SU-8 10 was purchased from MicroChem Corporation (Newton, MA, USA). The SU-8 developer, 2-(1-methoxy)propyl acetate, was obtained from Acros (Morris Plains, NJ, USA). Silicon wafers, 4 inches in diameter, were purchased from Silicon Inc. (Boise, ID, USA). Sylgard 184 PDMS prepolymer and curing agent was obtained from Ellsworth Adhesives (Germantown, WI, USA). Ultrapure water was generated from a Millipore Synthesis A10 system (Billerica, MA, USA). Penicillin/streptomycin antibiotic solution was obtained from American Type Culture Collection (Manassas, VA, USA). DAF-FM DA probes were purchased from Invitrogen (Carlsbad, CA, USA) or Calbiochem (Gibbstown, NJ, USA). 6-Carboxyfluorescein diacetate (6-CFDA) was obtained from Anaspec (Fremont, CA, USA). Diethylamine NONOate (DEA/NO) for NO standards was purchased from Cayman Chemical (Ann Arbor, MI, USA) and stored at –80 °C for no longer than six months.

Microchip fabrication

The fabrication of hybrid PDMS-glass microfluidic devices has been described previously by our group.[31] In these experiments, SU-8 10 negative photoresist was spin-coated onto a 4-in diameter silicon wafer with a resulting thickness of 15 ± 1 μm using a Cee 100 spin coater (Brewer Science, Rolla, MO, USA). The photoresist-coated wafer was then transferred to a hotplate (Thermo Scientific, Asheville, NC, USA) for a soft bake at 65 °C for 2 min and then 95 °C for 5 min. Microfluidic channel designs were produced using AutoCad LT 2004 (Autodesk Inc., San Rafael, CA, USA) and printed onto a transparency film at a resolution of 50,000 dpi (Infinite Graphics Inc., Minneapolis MN, USA). The coated wafer was covered with the transparency film mask and exposed to UV light (344 mJ cm^{-2}) for 16 s using an i-line UV flood source (ABM Inc., San Jose, CA, USA). Following the UV exposure, the wafer was post-baked at 65 °C for 2 min and 95 °C for 10 min. The wafer was then developed in SU-8 developer, rinsed with 2-propanol (IPA), and dried under nitrogen. A "hard bake" was performed at 200 °C for 2 h. The thickness of the raised photoresist, corresponding to the depth of the PDMS channels, was confirmed with a profilometer (Alpha Step-200, Tencor Instruments, Mountain View, CA, USA). PDMS channels were made by pouring a 10 : 1 mixture of PDMS elastomer and curing agent, respectively, onto the SU-8 patterned silicon wafer. The PDMS-covered wafer was then placed in an oven at 70 °C overnight to harden the PDMS.

The microchip design for all cell analysis experiments was a simple "T" design. This design consisted of a 5 cm separation channel and 0.75 cm side arms as shown in Fig. 1. An additional square guide 8.5 mm from the outlet reservoir of the separation channel was added to aid in laser alignment. The width and depth of the channels were 50 μm and 14 μm, respectively. Holes for the reservoirs were created in the PDMS using a 4 mm biopsy punch (Harris Uni-Core, Ted Pella Inc., Redding, CA, USA). The PDMS layer containing the embedded channels was reversibly sealed to a borofloat glass plate to complete the hybrid device.

Cell culture and preparation

Jurkat cells from the Jurkat clone E6-1 cell line (American Type Culture Collection) were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, L-glutamine (2 mM), penicillin (0.3 μg mL^{-1}), and streptomycin (0.3 μg mL^{-1}). The cells were maintained in a humidified environment at 37 °C and 5% CO_2 and cultured in 25 mL polystyrene culture flasks (Fisher

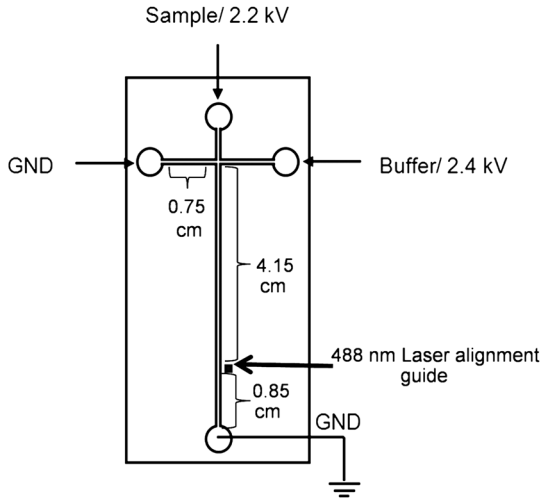

Fig. 1 A schematic of a simple "T" 5 cm microchip design with channel dimensions and applied voltages.

Scientific). Cells were passaged every 2–3 days to avoid overgrowth.

LPS stimulation protocol. Stimulation of NO production in cells was accomplished using purified LPS from the *Escherichia coli* line 0111:B4. Seven and one-half microlitres of a 1 mg mL^{-1} LPS stock solution was added to 5 mL of healthy Jurkat cells in a cell culture flask and then incubated for 3–5 h. Unstimulated (native) Jurkat cells from the same population were incubated under identical conditions and used as a control for each stimulation experiment.

Sample preparation. Jurkat cells suspended in their original medium were labeled with DAF-FM DA and 6-CFDA under dark conditions prior to bulk cell lysis and analysis. A schematic of the sample preparation steps is presented in Fig. 2A. Stock dye solutions were prepared in 99% DMSO. The dyes were loaded in a stepwise manner into the cell suspension. Ten microlitres of a 5 mM DAF-FM DA solution in DMSO were aliquoted into the culture flask and allowed to react for 15 min. Then 10 μL of 1 mM 6-CFDA was diluted in 990 μL of ultrapure water, added to the culture flasks, and reacted for an additional 20 min. Between the additions of the fluorescent probes, the flasks were returned to the incubator.

After dye loading, the Jurkat cell suspension was centrifuged at 3500 rpm for 3 min and supernatant was removed. Cells were resuspended in RPMI medium and centrifuged again to remove residual dye not sequestered by cells. The cell pellet was then lysed in 250 μL of electrophoresis buffer (10 mM boric acid, 7.5 mM SDS, pH 9.2). The lysate was filtered using a 3 kDa molecular weight cut-off centrifugal filter (VWR International, West Chester, PA, USA) to eliminate particles that could cause blockage of the microchip channels. The filtered lysate was then loaded into the sample reservoir of the microchip.

Cell viability

It is well known that excessive NO production can be toxic to a cell. Therefore, it is important to know the number of viable cells at the time of lysis if one wants to determine the average intracellular concentration of NO per cell. Cell viability and cell density were measured using the Trypan blue exclusion assay and a hemocytometer cell count, respectively. To minimize flask-to-flask variability, cell viability measurements of native and stimulated populations were performed before and after their respective stimulation or incubation steps. For the cell viability assay, the Jurkat cell suspension was diluted either 1 : 1 or 1 : 3 (based on cell density) with RPMI medium and 0.4% Trypan blue solution. The number of viable cells and cell density were determined using a 4 mm^2 total area hemocytometer (Sigma Aldrich). Native Jurkat cells typically had densities of 1–5 million cells in a 5 mL flask.

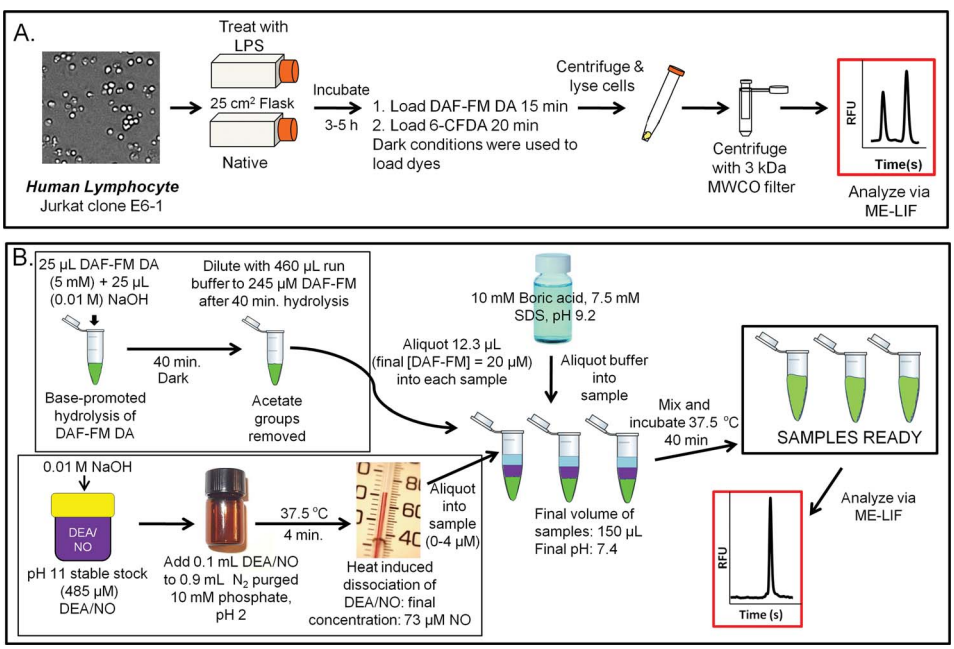

Fig. 2 The protocol used for (A) preparation of Jurkat cell lysates and (B) DEA/NO sample preparation.

Preparation of standards

An overview of the DEA/NO preparation protocol is presented in Fig. 2B. Briefly, stable stock solutions of NO donor DEA/NO were prepared in 0.01 M NaOH just prior to use (as per the vendor's instructions). NO release was facilitated by placing aliquots of the stock DEA/NO solution into deoxygenated 0.01 M phosphate buffer (pH 2, 37.5 °C) for 4 min. In a separate reaction, DAF-FM DA was incubated in 0.01 M NaOH for 40 min for the base-promoted hydrolysis of both acetate groups.[32] This served as a substitute for the esterase activity that is responsible for cleaving the acetate groups *in vivo*. Following hydrolysis in NaOH, equal concentrations of the resulting 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) were aliquoted into solutions containing 0–4 μM NO, reacted for 40 min at 37.5 °C to produce the benzotriazole derivative of DAF-FM (DAF-FM T), and analyzed *via* microchip electrophoresis.

The dehydroascorbate derivative of DAF-FM (DAF-FM DHA) was prepared by combining DAF-FM prepared as described above with a 70-fold excess of ascorbic acid in run buffer. The synthesis of DAF-FM DHA was accomplished using following procedure: ascorbic acid (AA) was oxidized to DHA in a high pH solution (9.2) by dissolved oxygen. The DAF-FM (20 μM) was then reacted with the oxidized AA (1.45 mM) solution for about 10 min to generate DAF-FM DHA.

Microchip operation

Prior to loading a bulk cell lysate sample onto the microchip, three conditioning flushes were performed. Using vacuum, the channels were filled with IPA and the excess IPA was then removed from the reservoirs. The IPA prevented the formation of gas bubbles in the separation and sample channels. The channels were then flushed with 0.1 M NaOH followed by separation buffer prior to use. The separation buffer consisted of 10 mM boric acid and 7.5 mM SDS, pH 9.2.

Fluorescence detection was carried out using a Nikon Eclipse Ti-U inverted microscope with a 488 nm laser (diode laser, Spectra-Physics, Irvine, CA, USA) for excitation, and a photomultiplier (Hammatsu Corporation, Bridgewater, NJ, USA) was used for detection. The signal was amplified using a SR570 low noise current preamplifier at 1 μA V^{-1} (Stanford Research Systems, Sunnyvale, CA).

Sample was introduced using a 0.5 s gated injection. Separations were performed in the positive polarity mode using a 30 kV high voltage power supply (Ultravolt, Ronkonkoma, NY, USA). For all separations, voltages of 2400 V (separation) and 2200 V (sampling) were used. An in-house written LabVIEW program was used for data acquisition as well as for control of the high voltage power supply (National Instruments, Austin, TX, USA). Origin 8.1 software (OriginLab, Northampton, MA, USA) was employed for data analysis.

Results and discussion

Microchip electrophoresis

NO production in the cells was measured using DAF-FM DA, a membrane-permeable fluorogenic reagent that reacts specifically with NO (Fig. 3A). Cells were co-labelled with 6-CFDA, another fluorogenic and membrane-permeable dye, that was used as an internal standard to account for differences in cell viability, esterase activity, and volume (Fig. 3B). This reagent has been used previously in single cell chemical cytometry experiments using microchip electrophoresis.[33]

Although DAF-FM is very selective for NO, Sweedler's group has shown that it reacts with DHA to produce derivatives (DAF-FM DHAs) with a fluorescence profile similar to that of DAF-FM T.[20,32,34] Therefore, they employed capillary electrophoresis with LIF detection to separate DAF-FM T from DAF-FM DHA interference.[20] The migration time for the DAF-FM DHA peak using ME was therefore investigated. The DAF-FM DHA was separated from DAF-FM T and 6-carboxyfluorescein (6-CF) as shown in Fig. 4.

The microfluidic device was then employed for the rapid analysis of lysate collected from several million native and stimulated Jurkat cells. This Jurkat cell line has been shown to express inducible nitric oxide synthase (iNOS) following stimulation with LPS.[35] Cells of the same passage number were divided into two groups. One group was subjected to LPS stimulation and the other group was maintained under the native conditions. Several concentrations (1.5–3 μg mL^{-1}) of LPS and several incubation periods were investigated for the stimulation of NO production in the Jurkat cells. NO production was found to be greatest following incubation of the cells with 1.5 μg mL^{-1} LPS for 3 h.

Fig. 5A and B show typical electropherograms obtained for stimulated and native cells, respectively. The first large peak is DAF-FM T and the second distinct peak corresponds to 6-CF, which was used as an internal standard. Peak identification was confirmed by incubating cells individually with either DAF-FM DA or 6-CFDA. The migration times for DAF-FM T and 6-CFDA in stimulated cells were 38.1 ± 0.3 s, and 53.1 ± 0.2 s, respectively. In native cell lysate, the migration times were 36.8 ± 0.3 s, and 51.5 ± 0.6 s. Slight shifts in migration times between runs is expected with PDMS substrates. Peak height reproducibility for DAF-FM T and 6-CFDA was between 2–13% in these studies. Under our separation conditions, DAF-FM T and DAF-FM migrate in the same position; however, DAF-FM is weakly fluorescent. Since DAF-FM has been reported to be photo-oxidized to generate a fluorescent product,[32] DAF-FM solutions were protected from light during all experiments. A negligible amount of fluorescence due to DAF-FM was observed in the blank electropherogram (Fig. 6).

A small peak corresponding to DAF-FM DHA was also observed in the cell lysates samples as shown in Fig. 5. The Jurkat cells used in these studies were cultured in ascorbate-free medium. It has been reported that, under these conditions, the intracellular concentration of ascorbate should be close to zero.[36,37] In a separate study using microchip electrophoresis with electrochemical detection, ascorbate was undetectable in the (underivatized) cell lysate (unpublished data).

The DAF-FM T/6-CF peak height ratio can be used to estimate the relative concentrations of NO produced by the cells. This ratio showed that there was a 120% increase in NO production in LPS-stimulated cells compared to native cells. The percent increase was calculated based on the peak height ratios for DAF-FM T and 6-CF, and was calculated as follows:

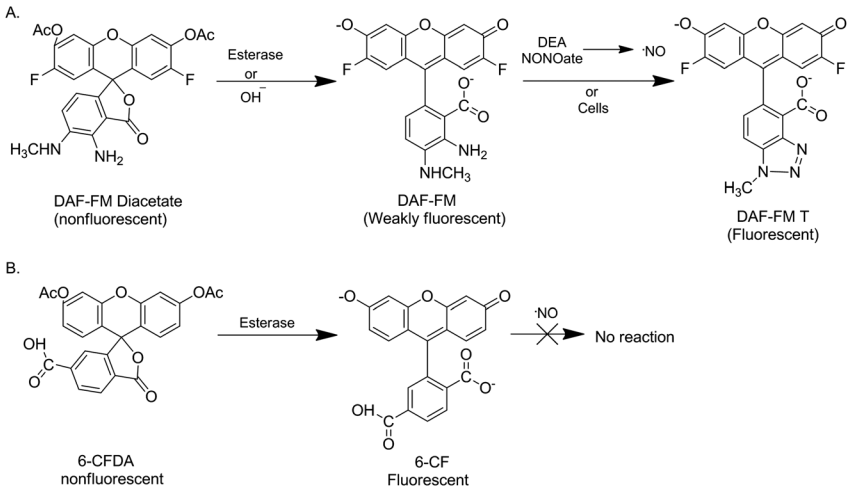

Fig. 3 Reaction schemes for (A) DAF-FM DA (B) 6-CFDA with NO and intracellular esterase. The figure was adapted from ref. 40.

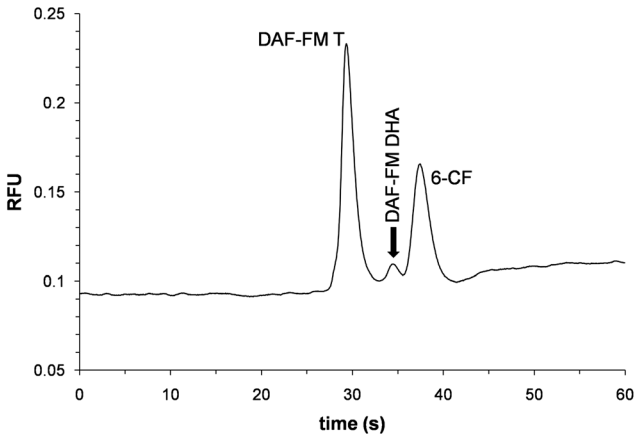

Fig. 4 Separation of DAF-FM T, DAF-FM DHA, and 6-CF using microchip electrophoresis with laser-induced fluorescence detection. Separation voltage was 2400 V (separation) and 2200 V (sampling). Run buffer consisted of 10 mM boric acid, 7.5 mM SDS, pH 9.2. Separation channel length was 5 cm. The final concentration of 6-CF was 0.024 μM. The DAF-FM DHA peak corresponds to a cellular concentration of ascorbate of 1.45 mM and the DAF-FM T signal corresponds to 200 nM NO.

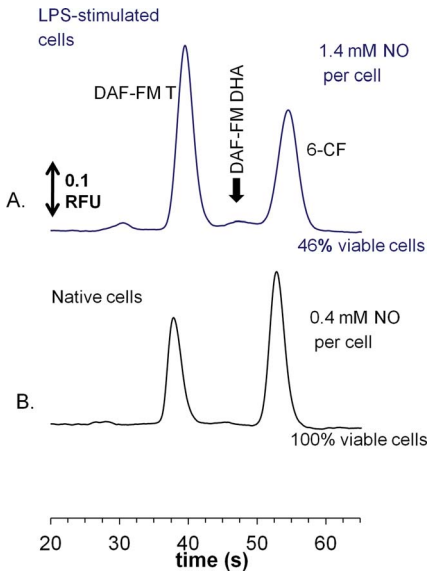

Fig. 5 Comparison of (A) LPS-stimulated and (B) native cell lysate. Separation conditions were the same as those in Fig. 4.

$$\left(\frac{\left[\frac{\text{DAF-FM T}}{\text{6-CF}}\right]_{\text{stimulated}} - \left[\frac{\text{DAF-FM T}}{\text{6-CF}}\right]_{\text{native}}}{\left[\frac{\text{DAF-FM T}}{\text{6-CF}}\right]_{\text{native}}} \right) \times 100\%$$

During these experiments, it was noted that the cell pellets obtained from the stimulated cells were consistently smaller than those obtained for native cells, indicating that the excessive NO produced during stimulation could be causing cell apoptosis.[38] In fact, Jurkat cells have been used as a model for the evaluation of the treatment of leukemic cancer cells by NO-generating drugs.[38] Realizing that the concentration of NO measured by ME-LIF for the bulk cell lysates is very likely influenced by the large amount of cell death, the number of viable cells before and after each stimulation (just prior to lysis) was measured using the Trypan blue exclusion assay. While native Jurkat cells (control experiment) typically increase their population by ~12–30% during a 3–h period, we determined that cells treated with 1.5 μg mL^{-1} LPS have a mortality rate of up to 69% during the same time frame. This substantial amount of cell death is associated with cytotoxic concentrations of NO produced by iNOS during stimulation, which greatly reduces the number of cells analysed in bulk. This can be seen in Fig. 5A. The electropherograms corresponding to the LPS-stimulated cell lysate do not show a dramatic increase of overall fluorescence response for the DAF-FM T peak compared to the native cells. However, only 46% of the cells survived after LPS stimulation; thus, the concentration of NO detected per stimulated cell was, in fact, much greater than in native cells. In addition, the comparison of the DAF-FM T/6-CF peak height ratios obtained for native and LPS-stimulated cells clearly showed increased NO production in LPS-stimulated cell lysates. The average ratio for DAF-FM

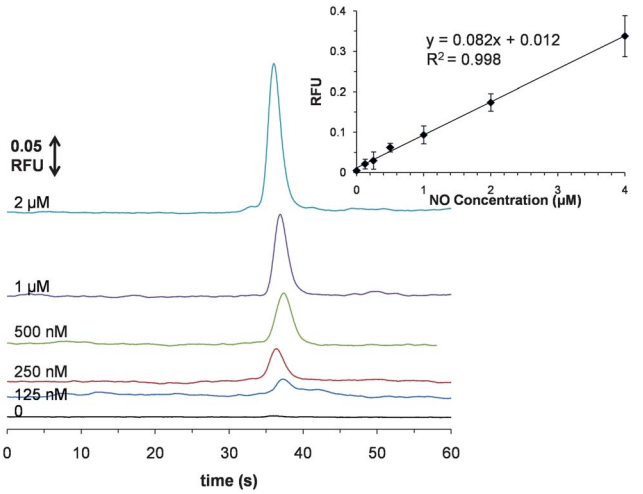

Fig. 6 Electropherograms of DAF-FM T prepared using different concentrations of NO and NO calibration curve. Separation conditions were the same as those in Fig. 4.

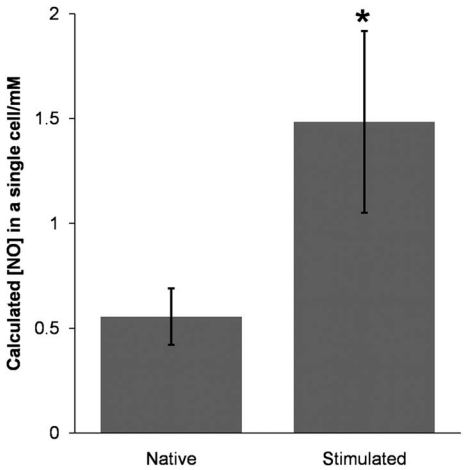

Fig. 7 Comparison of calculated average NO concentration in a single LPS-stimulated and a native Jurkat cell (n = 3, $p < 0.05$). For these calculations, the volume of a Jurkat cell was assumed to be 0.5 pL.

T/6-CF for native lysates was 0.69, while that for LPS-stimulated cells was 1.61 in the electropherograms shown in Fig. 5. The higher ratio in stimulated cells is due to an increase in NO production, resulting in a DAF-FM T peak of greater intensity. For three different sets of LPS-stimulated and native cell lysates, there was 120% increase or 2.2 ± 0.2 times higher NO production in LPS-stimulated cells.

To estimate the intracellular concentrations of NO in a single cell, a calibration curve for NO, using DEA/NO as an NO donor, was constructed. The NO standards were reacted with DAF-FM under conditions similar to the cell experiments (pH 7.4 and 37 °C). A calibration curve was constructed by reacting appropriate concentrations of NO released by DEA/NO with DAF-FM and monitoring the response using the microchip electrophoresis device (Fig. 6). A linear response was observed from 0.125 to 4 μM with a correlation coefficient of 0.998. The limit of detection was estimated to be 40 nM at S/N = 3 (based on a 125 nM NO standard solution at a S/N of 9 in Fig. 6).

Using the cell counts obtained for each set of native and stimulated cells (the same three LPS-stimulated and native cell lysate data sets used for DAF-FM T/6-CF ratio calculations were used), the concentration of NO produced per cell could be estimated. Fig. 7 shows bar graphs for the average intracellular concentration of NO in native *versus* LPS-stimulated cells. As can be seen in Fig. 7, there was an approximately 2.5-fold increase in NO production in a single LPS-stimulated cell 1.5 ± 0.4 mM *versus* native cell 0.6 ± 0.1 mM. The calculated NO concentration in a LPS-stimulated cell is statistically significant compared to the calculated NO concentration in a native cell (paired t-test, $p < 0.05$). These values for NO production are very similar to those reported by Goto *et al.* for macrophages stimulated with LPS.[25] The concentrations are much higher than values reported for platelets and erythrocytes where eNOS (not iNOS) is the primary source of NO.[26,39] These results confirmed our previous observations of elevated concentrations of NO in LPS-stimulated cells along with an increased mortality rate.

Conclusions

In this report, a microchip electrophoresis method using DAF-FM DA and 6-CFDA with LIF detection was described for the quantitation of intracellular NO concentrations in bulk cell lysates. This method was used to quantitate intracellular concentrations of NO in native and stimulated Jurkat cells. It was found that the average intracellular NO concentrations for LPS-stimulated and native Jurkat cells were 1.5 mM and 0.6 mM, respectively. These results provide an average value of NO production per cell that will be used for future comparison with results obtained with single cell chemical cytometry that employ a similar microfluidic separation format.

Acknowledgements

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