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Dual labeling for simultaneous determination of nitric oxide, glutathione and cysteine in macrophage RAW264.7 cells by microchip electrophoresis with fluorescence detection

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ABSTRACT

A simple, rapid and efficient method based on microchip electrophoresis coupled with fluorescence detection (MCE-FLD) was developed for simultaneous determination of nitric oxide (NO), glutathione (GSH) and cysteine (Cys) using dual labeling strategy. Two highly reactive fluorogenic probes, 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacene (DAMBO) and 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-*s*-indacene (TMPAB-*o*-M), were used for labeling NO and thiols, respectively, under physiological conditions. The rapid separation and sensitive detection of the derivatives were achieved on a glass microchip within 70 s in a running buffer of 20 mM H_3Cit–Na_2HPO_4 solution (pH 7.4) containing 15% (v/v) acetonitrile at a separation voltage of 2400 V. The limits of detection (S/N = 3) for NO, GSH and Cys were 7.0, 3.0 and 2.0 nM, respectively. The proposed method was validated by measuring intracellular levels of NO and biothiols in macrophage RAW264.7 cells.

1. Introduction

Nitric oxide (NO) and thiols such as glutathione (GSH) are important biological molecules which have drawn widespread attention due to their significant biological functions and the complex interactions between them in diverse physiological and pathological processes [1–3]. NO is a unique gaseous signal transduction molecule generated from the conversion of L-arginine to L-citrulline by at least three distinct isoforms of nitric oxide synthase (NOS) in biological systems [4]. NO plays a major role in maintaining normal physiological homeostasis. It not only regulates key functions in the immune, cardiovascular and nervous systems [5,6], but also protects cells against pathogen host infection or cytokine induced injury and apoptosis [7,8]. Glutathione (GSH), an ubiquitous cellular nonprotein thiol, modulates signal transduction by controlling intracellular redox state and acts as an antioxidant providing the first line of cellular defense against oxidative stress or nitrosative stress resulting from NO [9,10]. The complex relationships between the free radical NO and biologically important thiols govern their biological consequences in cellular systems. NO has been shown to modulate intracellular glutathione levels by stimulating GSH biosynthesis [11–13]. In addition, GSH may play a critical role in regulating NO bioactivity. It has been suggested that thiols can react with NO to form S-nitrosothiols such as S-nitroso-glutathione (GSNO) and S-nitrosocysteine (CySNO), which are thought to serve as NO reservoirs and vehicles to transport NO in biological systems [14,15].

In attempts to investigate their functions and relationships in biological processes, NO, and thiols such as GSH are often determined separately by different analytical methods. For the quantification of NO, available methods include Griess assay [13,16,17], electron paramagnetic resonance [18], mass spectrometry [19], chemiluminescent detection [20], electrochemical sensors [21–23], and high performance liquid chromatography with fluorescence detection [24–26]. For the analysis of GSH, the reported methods include enzyme-based method [17,27], fluorometric method [28], HPLC or CE coupled with UV absorbance [29], MS [30], electrochemical [31,32] or fluorescence detection [13,16,32]. Although the analysis of these biologically important species can be performed using these methods, the contents of NO and thiols cannot be obtained in a single sample in an assay, which will fail to reflect their state of existence and relationship truly and

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accurately. To resolve this problem, a simple and rapid analytical method capable of simultaneously measuring intracellular NO and thiols with high selectivity and sensitivity is urgently desired. As a burgeoning separation technique, microchip electrophoresis (MCE) has attracted increasing attention with the advantages of reduced reagent consumption, rapid analysis time, ease of operation, efficient resolution for compounds and increased throughput [33,34]. In particular, microchip electrophoresis coupled with fluorescence detection (MCE-FLD) has been proven to be an effective method for rapid and sensitive measurement of biologically important thiols in our pervious study [35], which makes it possible to simultaneously determine NO and thiols (*i.e.*, GSH and Cys) using appropriate fluorescent probes as labeling reagents.

In the present study, a simple, rapid and efficient MCE-FLD method for simultaneous determination of nitric oxide (NO) and thiols (GSH and Cys) has been developed on the basis of dual labeling strategy using 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacene (DAMBO) for NO and 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-*s*-indacene (TMPAB-*o*-M) for thiols. The proposed method was validated by measuring the levels of NO, GSH and Cys in macrophage RAW264.7 cells. To the best of our knowledge, this is the first example of simultaneous determination of NO, GSH and Cys in cell samples with MCE-FLD method.

2. Materials and methods

2.1. Reagents and solutions

Unless otherwise mentioned, all the chemicals used here were of analytical reagent grade. All aqueous solutions were prepared with ultrapure water (18.2 MΩ cm) and filtered through 0.22 μm membrane filters before use. Reduced glutathione (GSH), L-cysteine (Cys), *N*-ethylmaleimide (NEM), and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). DAMBO and TMPAB-*o*-M were synthesized in our laboratory according to Refs. [36,37], respectively. Stock solutions of DAMBO (2.0 mM) and TMPAB-*o*-M (4.0 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C in darkness. Stock solutions of Cys (3 mM) and GSH (3 mM) in ultrapure water were utilized to prepare fresh sample solutions. The electrophoretic buffer was 20 mM H_3Cit–Na_2HPO_4 buffer (pH 7.4) containing 15% (v/v) acetonitrile.

Saturated nitric oxide solution was prepared by introducing pure NO gas into the deoxygenated physiological buffer saline (PBS) solution (pH 7.4) and stored in darkness as described in Ref. [38]. The concentration of NO in PBS solution was approximately 1.8 mM at room temperature. Working solutions were obtained by diluting a certain volume of saturated NO solution in desired volume of PBS solution (pH 7.4) immediately prior to use.

2.2. Standard derivatization procedure

Into a 1.0 mL Eppendorf tube containing 760 μL PBS buffer solution (pH 7.4), 100 μL of a 4.0 mM TMPAB-*o*-M stock solution, 100 μL of a 2.0 mM DAMBO stock solution and 20 μL of the standard thiol solution (0.6 mM each) were added in succession. After incubation in a water bath at 37 °C for 6 min, the mixture was spiked with 20 μL of saturated NO solution and allowed to react at 37 °C for another 10 min. The resulting mixture was diluted 40 times with running buffer before electrophoretic analysis.

2.3. Cell culture

The macrophage RAW264.7 cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen), penicillin (100 IU/mL), and streptomycin (100 μg/mL), which were placed in a water-jacketed CO_2 incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C (Series II 3111, Thermo Scientific, USA).

2.4. Sample preparation

The cells were divided into three groups. Before probe loading, the cells of Group 1 and 2 were incubated with NEM (1.6 mM) and L-NAME (0.1 mM) for 15 min at 37 °C, respectively and then washed with fresh medium. Afterwards, the cells of three groups were incubated for 15 min at 37 °C in the medium containing TMPAB-*o*-M (10 μM) and DAMBO (1 μM), respectively. After incubation, the cells were separately suspended in PBS buffer (pH 7.4) and harvested by centrifugation at 3000 rpm for 5 min. Finally, the cells were resuspended in running buffer with a volume equal to that in PBS buffer, and then lysed for 10 min in an ultrasonic processor (VC 505, Sonics & Materials, USA). The cell lysates were centrifuged at 16,000 rpm for 10 min. The supernatants were collected and diluted to a desired concentration with running buffer before microchip electrophoretic analysis.

2.5. The MCE-FLD and CE system

The microchip-based experiments were carried out using an in-house built MCE-FLD system (as shown schematically in Fig. 1). The channels of the simple cross-section glass microchip consisted of injection channel (S-SW, 1.0 cm in length) and separation channel (B-BW, 5.0 cm in length) with the dimensions of 70 μm in width and 20 μm in depth each. The distances from reservoirs S, B, and SW to the cross-section were all 0.5 cm. The light beam delivered from a 120 W mercury lamp passed through an excitation filter (475 ± 15 nm), then was reflected by the dichromatic filter (DM 505 nM) and focused into the detection point which was 40 mm downstream from the cross-section in the separation channel through a 40× objective. The emitted fluorescence from the sample was collected by the same objective and passed through the dichromatic filter, an emission filter (530 ± 20 nm) and detected by a photon counter (H7467-01, Hamamatsu, Japan). Signal from the photon counter was recorded, processed and analyzed using a software program written in-house. Sample injection and subsequent electrophoretic separation were performed by using an eight-path high voltage power supply (0–5000 V, Dongwen High Voltage, Tianjin, China).

CE experiments were performed on a P/ACE MDQ Capillary Electrophoresis System (Beckman-Fullerton, CA, USA) incorporating a laser-induced fluorescence detector from 488 to 520 nm. Fused silica capillary of total length 60 cm × 75 μm id with an effective length of 50 cm was obtained from Yongnian Optic Fiber (Hebei, China). Prior to first use, the bare fused-silica capillary was rinsed with 1.0 M NaOH for 30 min, followed by water for 10 min, and then 1.0 M HCl for 30 min. The capillary was then rinsed with water for 10 min and finally flushed with running buffer for 10 min. Between the measurements, the capillary was flushed with running buffer for 5 min. Samples were introduced hydrodynamically by applying a pressure of 0.5 psi (3.45 kPa) for 5 s.

2.6. Microchip operation

Prior to electrophoresis, the microchannels were flushed sequentially with 1.0 M NaOH, ultrapure water and running buffer for 5 min each. After that, reservoirs B, BW, and SW were all filled with running buffer, while reservoir S was filled with sample solution. The microchip was then mounted onto the $X-Y$ translational stage of an optical microscope (IX 71, Olympus, Japan). Sample