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Microchip electrophoresis with chemiluminescence detection for assaying ascorbic acid and amino acids in single cells

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ABSTRACT

A method based on microchip electrophoresis (MCE) with chemiluminescence (CL) detection was developed for the determination of ascorbic acid (AA) and amino acids including tryptophan (Trp), glycine (Gly) and alanine (Ala) present in single cells. Cell injection, loading, lysing, electrophoretic separation and CL detection were integrated onto a simple cross microfluidic chip. A single cell was loaded in the cross intersection by electrophoretic means through applying a set of potentials at the reservoirs. The docked cell was lysed rapidly under a direct electric field. The intracellular contents were MCE separated within 130 s. CL detection was based on the enhancing effects of AA and amino acids on the CL reaction of luminol with $K_3[Fe(CN)_6]$. Rat hepatocytes were prepared and analyzed as the test cellular model. The average intracellular contents of AA, Trp, Gly and Ala in single rat hepatocytes were found to be 38.3, 5.15, 3.78 and 3.84 fmol ($n = 12$), respectively.

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1. Introduction

High throughput analysis of single cells is very important for molecular biology, physicochemical modelling of biological processes and clinical diagnosis of disease. The reason for this significance is that seemingly identical cells are often quite heterogeneous in their chemical composition and biological activity and in the timing and magnitude of their responses to external stimuli [1–4]. Therefore, quantification of intracellular constituents is essential for a better understanding of basic cellular functions and intra- and intercellular communications [2,5]. Such demand has promoted the development of analytical techniques for single cell analysis. The methods developed so far were based on flow cytometry [6–7], open tubular liquid chromatography [8], electrochemical method [9], fluorescence microscopy [10], mass spectrometry [11] and capillary electrophoresis (CE) [12–17]. Microchip electrophoresis (MCE), which is considered as a miniaturized version of classical CE, is one of the most successful applications of microfluidics in analytical chemistry [18]. High separation efficiency of MCE allows fast separations of many constituents present in single cells [19–22]. Ramsey and co-workers used a glass MCE device to achieve high throughput analysis of Jurkat cells [2]. Klepárník and Horký reported an MCE device on a plastic disk for the detection of DNA fragmentation in a single apoptotic cardiomyocyte [23]. Fang and co-workers developed a MCE system for the analysis of single cells with functional integration of cell sampling, single cell loading, docking, lysing, separation and laser induced fluorescence (LIF) detection. Using this system, glutathione (GSH) and several reactive oxygen species (ROS) in individual human erythrocytes were determined [24–26]. Zare and co-workers reported the determination of amino acids in single Jurkat cells using an integrated MCE with LIF detection [27]. Reviews on MCE for single cell analysis have been given [28,29]. In most of these MCE applications, LIF was employed as the detection scheme. Although LIF is the most widely used detection scheme due to its high sensitivity, a conventional LIF detector is sophisticated in instrumentation, expensive in cost, and difficult to be miniaturized. Diode laser-based LIF detectors can be small, but only work at a limited number of wavelengths. Furthermore, pre-column derivatization of the analytes with a fluorophore is often necessary. CL detection is considered one of the most sensitive detection schemes. Its convincing advantage over fluorescence detection is that it does not require a bulky light source. This is attractive, particularly when multiple functions are integrated into a small microfluidic chip. In addition, since CL background signal is usually very low the detector can be operated at its maximum sensitivity. However, as far as we know few MCE–CL methods have been reported so far for single cell analysis. This is likely because CL detection integrated onto MCE chips was not sensitive enough. Recently, we developed MCE–CL assays of glutathione present in single human red blood cells [30] and of biogenic amines

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in human physiological fluids [31]. In both methods, samples were pre-column derivatized with a CL tagging reagent to achieve high assay sensitivity. In the present study, we extended our previous work by demonstrating analysis of underivatized ascorbic acid and amino acids in single cells using CL detection following electrophoretic separation on chips.

Ascorbic acid (AA) and amino acids are important molecules in biological systems. AA, as one of the most important cellular antioxidants [32] and a valuable biomarker of oxidative stress [33,34], is an analyte of great importance. At the cellular level, AA mitigates reactive oxygen specie production triggered by lipopolys accharide and, therefore, prevents the induction of nitric oxide synthase and excessive production of nitric oxide that worsens oxidative stress in hepatocytes [35,36]. Tryptophan (Trp) is an essential amino acid. It exists in liver cells and participates in the synthesis of many other important molecules such as niacin and serotonin [37]. Glycine (Gly) and alanine (Ala) are amino acid neurotransmitters. Therefore, quantification of these compounds in individual hepatocytes is significant for studying various biological processes in the liver.

The aim of this work was to develop an MCE method with CL detection for simultaneous determination of AA, Trp, Gly and Ala present in individual rat hepatocytes. Integration of cell loading and lysing, electrophoretic separation and CL detection onto a microfluidic chip was investigated. Conditions for MCE separation and CL detection of the targeted compounds were studied. Finally, MCE–CL quantification of intracellular contents of AA, Trp, Gly and Ala in individual rat hepatocytes was demonstrated for the first time in this work.

2. Experimental

2.1. Chemicals and solutions

Luminol, AA, Trp, Gly and Ala were purchased from Sigma Chemicals (St. Louis, MO, USA). $K_3Fe(CN)_6$, Na_2HPO_4 and $NaHCO_3$ were obtained from Guangzhou Second Chemical Reagent Factory (Guangzhou, China). Sylgard 184 (PDMS) silicone elastomer and curing agent were obtained from Dow Corning (Midland, MI, USA). All other chemicals used in this work were of analytical grade. Water was purified by employing a Milli-Q plus 185 from Millipore (Bedford, MA, USA) and used throughout the work. All solutions were filtered through a 0.22 μm membrane filter. AA and amino acid stock solutions (1.0 mM) were prepared in water. The electrophoresis buffer was 20 mM Na_2HPO_4 solution containing 2.5 mM luminol and 40 mM NaBr (pH 10.0, adjusted with 1 M NaOH solution). The CL reaction buffer was 50 mM $NaHCO_3$ solution containing 0.8 mM $K_3Fe(CN)_6$ (pH 12.5, adjusted with 1 M NaOH solution). The physiological buffer (PBS) consisted of 0.135 M NaCl and 0.02 M NaH_2PO_4–NaOH (pH 7.4). The D-Hanks solution was prepared by dissolving 0.8 g of NaCl, 0.40 g of KCl, 0.06 g of $Na_2HPO_4{\cdot}H_2O$, 0.06 g of KH_2PO_4, and 0.35 g of $NaHCO_3$ in 1 L of water with 0.02 g of phenol red (95.8%, Beijing Chemical Reagent Factory, Beijing, China) as a pH indicator. A 0.25% (w/v) trypsin (>2500 units/mg, Shanghai Chemical Reagents Co., Shanghai, China) solution was prepared by dissolving trypsin in D-Hanks solution at 4 °C. The pH of the solution was adjusted to 7.4 with $NaHCO_3$ after filtering. The trypsin solution was then sealed and stored at −20 °C.

2.2. MCE–CL system

Analysis of single cells was carried out using a laboratory-built MCE–CL system as described previously [30]. The glass/PDMS microchip assembly was mounted on the *X*–*Y* translational stage of an inverted microscope (Olympus CKX41) that also served as a platform of CL detection. Use of the *X*–*Y* translational stage allowed viewing any point of the microchannel for introducing a cell. CL signal was collected by means of a microscope objective. After passing a dichroic mirror and a lens, CL emission with a wavelength maximum at 425 nm was detected by a photomultiplier (PMT, Hamamatsu R105). The PMT was mounted in an integrated detection module including HV power supply, voltage divider and amplifier. The output signal of PMT was recorded and processed with a computer using a Chromatography Data System (Zhejiang University Star Information Technology, Hangzhou, China). A multi-terminal high voltage power supply, variable in the range of 0–8000 V (Shandong Normal University, Jinan, China), was used for cell loading, lysing and MCE separation. A valuable practical aspect of the inverted microscope setup was the possibility of visually checking all field-controlled operations on the device through the eyepiece. The inverted microscope was placed in a black box.

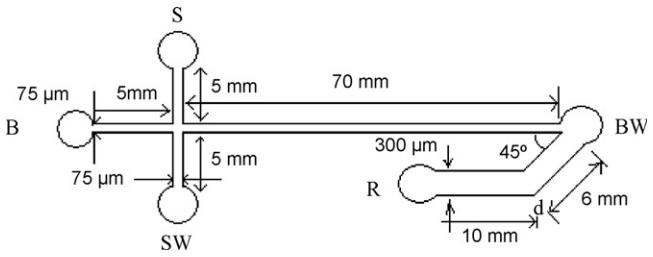

Fig. 1. Layout and dimensions of the glass/PDMS microfluidic chip used in this work. S, sample reservoir; B, buffer reservoir; SW, sample waste reservoir; BW, buffer waste reservoir and R, oxidizer solution reservoir.

2.3. Fabrication of microchip

The glass layer with microchannels was fabricated by using a standard photolithography and wet chemical etching techniques. A 9.0 cm × 2.5 cm rectangle glass (Shaoguang Microelectronics Corp., Changsha, China) with predeposit 145 nm thick Cr layer and 570 nm thick AZ1518 photoresist was exposed beneath the designed mask under an UV lamp, and was then developed and etched using a wet chemical etching procedure [38]. A smooth PDMS surface for bonding to the etched glass slide was prepared by casting the PDMS over a flat As–Ga wafer. Sylgard 184 PDMS prepolymer was mixed thoroughly with its curing agent at 10:1, w/w, and then degassed by vacuum pump. The mixture was cured against the As–Ga wafer at 80 °C for 2 h. After the replica was peeled from the mold, holes were punched as reservoirs. The PDMS covering sheet was exposed under an UV lamp for 2 h, then the glass layer and PDMS covering sheet were bonded at room temperature for 24 h. According to a work reported previously, UV exposure strengthens the bonding between the glass chip and the PDMS cover [39]. The schematic of the microfluidic chip is shown in Fig. 1. Microchannels measured 75 μm wide by 25 μm deep for cell introduction, separation and waste delivery, 300 μm wide by 25 μm deep for oxidizer introduction, respectively. The reservoirs S, B and SW were 3.5 mm in diameter and 1.5 mm deep. The reservoirs R and BW were 4.5 mm in diameter and 1.5 mm deep. The channel between reservoir S and SW was used for sampling, the channel between B and BW was used for the separation and the channel between R and BW was used for the oxidizer introduction. Join-point of the oxidizer introduction channel with the separation channel in BW was used for the collection of CL. A similar chip design was reported by Liu et al. [40]. In our work, due to the rapid CL reaction and the limited size of detection spot afforded by the microscope objective excessive band broadening was effectively prevented. The MCE peaks observed were sharp as shown below.

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