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Quantification of glutathione in single cells from rat liver by microchip electrophoresis with chemiluminescence detection

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ABSTRACT

Glutathione (GSH) is a major endogenous antioxidant that has a central role in cellular defense against toxins and free radicals. Rapid and accurate detection of GSH content in single cells is important to the early diagnosis of disease and biomedical research. In this work, a novel method based on microchip electrophoresis chemiluminescence (MCE-CL) detection was developed for the quantification of glutathione (GSH) in single cells from rat liver. The detection of GSH is based on the strong sensitization of mercapto compound to luminol-H_2O_2CL system. The injection, localization, and membrane dissolution of single cell were simply and rapidly carried out on the microchip by direct electric field force, which did not require any additional membrane dissolution reagent. Under optimized experimental conditions, single cell assay was achieved within 2 min. The peak area of the GSH was taken as quantification of GSH, and a good linear relationship of GSH concentration to peak area in the range of 3.0×10^{-6} M to 6.0×10^{-4} M was obtained. The detection limit for GSH is 9.6×10^{-7} M, calculated by S/N = 3. The measured GSH content in single cells from rat liver (n = 10) ranged from 7.8 fmol to 13. fmol with a mean value of 10.8 fmol.

1. Introduction

Glutathione (GSH), a tripeptide containing mercapto group, is ubiquitous in animal and plant cells. GSH can protect the cells from oxidative damages caused by free radicals and peroxides. Moreover, it participates in a variety of physiological processes, such as maintenance of protein structure and function, amino acid transport, glycol metabolism, as well as in cells signaling and regulation [1–3]. Therefore, it is considered to be an important active substance in cell physiology. Determination of GSH content in cells is of great significance for the study of cells biology and human health [4]. Furthermore, abnormal levels of GSH have been associated with a number of diseases, including cancer, AIDS, Alzheimer's and cardiovascular disease [5–7]. Rapid and accurate detection of GSH content in biological samples is important to the early diagnosis and prevention of diseases.

Cells are the basic unit of life activity, in order to master the law of life process, cell based behavior must be explored. However, the cell is small (8–15 μm in diameter) with trace amounts of components, and the content of the tested components is often ranged from fmol to zmol [8,9]. Because the sensitivity of the detection method is not enough, it can only analyze cells population. Through the statistical analysis of the cells population obtained the average results, which hides the difference between individual cells, leading to the limitation of the development of many fields such as biology and medicine. Single cell analysis enables the detection and identification of rare or abnormal cells in a large population of cells [10], reveals a stem-cell program inhuman metastatic breast cancer cells [11], and contributes to the understanding of basic cellular functions [12,13]. Therefore, single cell analysis is of interest and significant for the early diagnosis and treatment of diseases, as well as the study of cellular physiology and pathology.

Currently, the methods for GSH detection in single cell include mainly capillary electrophoresis (CE) combined with laser induced fluorescence (LIF) detection [14–16] or electrochemistry detection [17–19], microchip electrophoresis (MCE) combined with LIF detection [20–23]. The LIF detection method can achieve high sensitivity, but it requires complicated optical systems. In addition, as GSH itself is not fluorescent, fluorescence derivatization is required before the quantitative determination of GSH with LIF detection. For electrochemical detection, although satisfactory results can be obtained, it require the fabrication of microelectrode in microfluidic chip or capillary, which is difficult to segregate the electrophoresis separation voltage and the

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electrochemical detection voltage that often results in relatively high background signal. Therefore, it is necessary to establish simple methods for the determination of GSH in single cell, which would be better to understand its physiological function.

In a previous work [24], we have demonstrated that MCE chemiluminescence (MCE-CL) detection can be applied for the determination of GSH in single cell. Although high sensitivity was achieved, the intracellular CL labeling with diazo-luminol was required, which prolonged the analysis time. In this study, by combining MCE with CL detection technology, we proposed a simple method for the determination of GSH in single cell based on the enhanced action of mercapto compounds to luminol-H_2O_2 CL system [25], and have successfully used this method for the determination of GSH in single cells from rat liver.

2. Experimental

2.1. Reagents and solutions

Luminol, cysteine (Cys), N-acetylcysteine (NAC), and GSH were purchased from Sigma (St. Louis, MO, U.S.A.)·H_2O_2, Na_3PO_4, and $NaHCO_3$ were provided by Guangzhou Chemical Reagent Second Factory (Guangzhou, China). All reagents used in this work were of analytical reagent grade, and usedas received. Milli-Q ultrapure water (18.2 MΩ) was used in all experiments. Stock solutions of Cys, NAC and GSH (1.0 mM) were prepared in ultrapure water, and stored at 4 °C. The phosphate buffer solution (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 8.0 mg NaCl, 4.0 mg KCl, 0.6 mg $Na_2HPO_4·H_2O$, 0.6 mg KH_2PO_4, 3.5 mg $NaHCO_3$, and 0.2 mg phenol red in water, and diluted to 10 mL. The chymotrypsin solution 0.25% (w/v) was prepared by dissolving chymotrypsin in D-Hanks solution, adjusted to pH 7.2 with $NaHCO_3$, and stored at −20 °C. The electrophoretic buffer was 20 mM Na_3PO_4 solution containing 1.5 mM luminol (pH 9.6), and the post-column oxidizer solution was 30 mM $NaHCO_3$ solution containing 80 mM H_2O_2 (pH 11.5).

2.2. Instruments

Analysis of single cell was carried out using a laboratory-built MCE–CL system [24]. A cross-type glass microfluidic chip with Y-shaped CL detection reservoir was fabricated in the lab and its dimensions are listed in Fig. 1. The chip area was 9.5 × 2.5 cm. To fit the dimension of the microchannel well with the size of mammalian cells, the wide of the channels for samples injecting and separation were designed to be 60 μm, and the depth of all channels was designed to be 25 μm. To allow better mixing of chemiluminescent reagent with oxidizer, the channel from reservoir R to reservoir BW was designed to be 250 μm wide. The distance from the center of buffer reservoir B to the intersection of injecting and separation channels was 0.5 cm. The distance from the center of sample reservoir S and the sample waste reservoir SW to the separation channel was also 0.5 cm. The oxidizer solution reservoir R was 1.5 cm away from the Y-shaped intersection. The distance between the Y-shaped intersection and the buffer waste reservoir BW was 1.2 cm. The total length of separation channel was 8.9 cm.

2.3. Preparation of cells from rat liver and their extracts

Preparation of cells from rat liver was as the method proposed in literature [24]. The specific process is as follows: rats (300 g) were dissected, the liver was washed immediately 3–5 times with PBS solution, and cut into small pieces (< 1 mm). The pieces were then washed 3–5 times with PBS solution and transferred to a 5 mL centrifuge tube. A 10-fold volume of 0.25% (w/v) trypsin solution was added to the centrifuge tube. After gentle shaking, the mixture was incubated at 37 °C for enzymolysis for 20 min, and the supernatant was discarded. Then, PBS (3 mL) was added to stop the enzymatic hydrolysis, and the supernatant was discarded. The enzymolysis step was repeated three times. PBS (3 mL) was added to the centrifuge tube, and the cells were gently re-suspended. The cell solution was then centrifuged for 10 min (1000 rpm), and the supernatant was discarded. The cells were re-suspended with PBS solution, and this procedure was repeated 3–5 times to obtain the final cell suspension that would be used for single cell analysis. To prepare cell extracts, another 1 mL cell suspension was counted with a cell counter, the results showed that 1 mL of the suspension contained 4.5×10^6 cells. In the final step, 100 μL of 0.1 M NaOH solution was added into 1 mL cells suspension. The cells were dissolved by an ultrasound for 5 min at room temperature, and centrifuged for 10 min at 16,000 rpm to obtain cells extract. In this case, 1 mL of the extract contained 4.1×10^6 cells, which was then subjected to MCE analysis after dilution by running buffer.

2.4. MCE procedure

Before analysis, the microchip channel was treated sequentially with 0.1 M NaOH, ultrapure water, and electrophoretic buffer for 10 min. The channels were then filled with electrophoretic buffer by vacuum negative pressure, and each reservoir was filled with the corresponding solution. The sample solution was transported from the S reservoir to the SW reservoir in pinched mode by applying 500 V voltages to S reservoir, 220 V and 300 V voltages to B and BW reservoirs, respectively, and R reservoir was suspended. After 20 s, the voltages were switched to the B, S, SW, and R reservoirs at 2400, 1500, 1500, and 480 V, respectively, whereas the BW reservoir was grounded for separation and detection. The analytes in sample were separated in the separation channel by electrophoresis, and mixed with the post-column oxidizer solution to generate CL, which was collected by the eyepiece, transmitted to the photo multiplier tubes (PMT), and then recorded by a computer.

2.5. Single cell analysis

The channel of the microchip was rinsed sequentially with 0.1 M NaOH and ultrapure water for 20 min before analysis of each cell, and

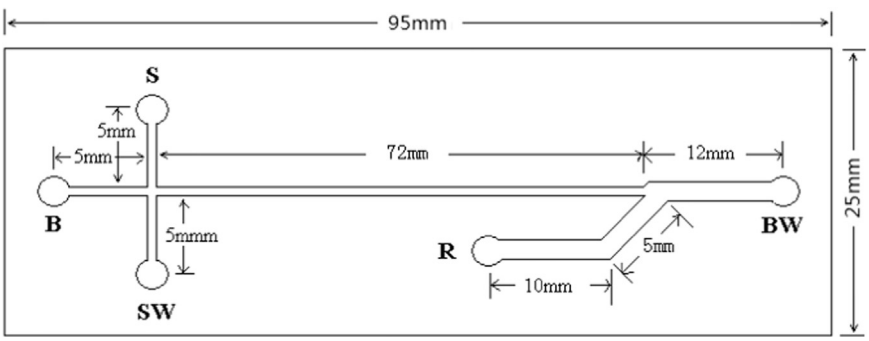

Fig. 1. Schematic of layout of the glass microchip. S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir; R: oxidizer solution reservoir.