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ORIGINAL PAPER

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Determination of reactive oxygen species in single human erythrocytes using microfluidic chip electrophoresis

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Abstract Reactive oxygen species (ROS) are known to not only mediate the damage of cellular constituents but also to regulate cellular signaling. Analysis of ROS is essential if we wish to understand the mechanisms of cellular alterations. In this paper, a microfluidic chip-based approach to the determination of ROS in single erythrocyte was developed by using a simple crossed-channel glass chip with integrated operational functions, including cell sampling, single cell loading, docking, lysing, and capillary electrophoretic (CE) separation with laser-induced fluorescence (LIF) detection. Non-fluorescent dihydrorhodamine 123 (DHR 123), which can be oxidized intracellularly by ROS to the fluorescent rhodamine 123 (Rh 123), was used as the fluorogenic reagent. The effect of pH on the migration time of Rh 123 and detection sensitivity was discussed. The present method minimized dilution of intracellular ROS during reaction with DHR 123 and determination. As a result, an extremely low detection limit of 0.8 amol has been achieved. The time required for complete analysis of one human erythrocyte was less than 3 min. A migration time precision of 4.1% RSD was obtained for six consecutively-injected cells. Upon stimulation with 4 mmol/l H_2O_2 for 10 min, the intracellular ROS concentration was found to increase on average by about a factor of 8.4.

Keywords Single-cell analysis · Microfluidic chip-based electrophoresis · Laser-induced fluorescence · Reactive oxygen species · Erythrocyte

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Introduction

Reactive oxygen species (ROS), including hydroxyl radicals (·OH), superoxide anions ($O_2^{-}\cdot$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), have been identified as important chemical mediators that regulate signal transduction pathways. ROS are generally known to be closely involved in an enormous variety of natural and pathological processes, including aging, cancer, diabetes mellitus, atherosclerosis, neurological degeneration such as Alzheimer's disease, schizophrenia, and autoimmune disorders. ROS fulfil important prerequisites for intracellular messenger molecules [1, 2]; they are easily synthesized, highly diffusible, easily degraded, and ubiquitously present within all types of cells.

ROS have been measured by chemiluminescence [3], spectrofluorometry [4], flow cytometry [5, 6] and through the determination of free radicals using a electron spin resonance technique [7]. Parmentier et al [8] reported that non-fluorescent DHR 123 can be oxidized into fluorescent Rh 123 by ROS, and developed the CE-LIF method for the measurement of ROS in cell extracts. However, these analytical procedures cannot determine the cellular ROS of individual cells, which can vary significantly from cell to cell.

The recent exploitation of microfluidic chip-based systems for biological cell studies has attracted broad interest [9, 10, 11, 12, 13, 14]. The micrometer channel dimensions of microfluidic chips are ideally suited for the sample introduction, manipulation, reaction, separation, and detection of single cells. In a previous article [12], we successfully integrated operational functions for single-cell introduction, docking, cytolysis, and CE separation of cellular constituents on a single glass microchip, and intracellular GSH was detected with laser-induced fluorescence (LIF). In this work, a method for the determination of ROS in single erythrocytes was developed that employs DHR 123 as fluorogenic reagent, achieving a detection limit of 0.8 amol. The present method for determination of ROS in individual

cells can minimize the dilution of the ROS in single cells during reaction with DHR 123 and determination, and it has been applied successfully to the monitoring of intracellular ROS variations in individual cells upon stimulation with H_2O_2.

Experimental

Instrumentation

The home-built confocal microscope LIF system used for detection has been described previously [12]. Briefly, a 488 nm argon ion laser (Model 367, 4 mW, Nanjing Electronic Equipment Corp., Nanjing, China) was coupled to an inverted microscope (Jiangnan Optics & Electronics Co., Nanjing, China), with the necessary optical components. The laser beam was reflected and focused to a 20 μm point on the separation channel from below the chip. The emitted light was collected by the same focusing system, and directed to a pinhole by the optics of the inverted microscope. A 520 nm cutoff filter was positioned directly before the window of a CR114 type PMT (Hamamatsu, Beijing) connected to a model GD-1 HV power supply and amplifier (Reike Electronic Equipment Co. Ltd., Xi'an, China). Signal output from the detector was recorded using a model XWTD-164 chart recorder (Dahua Instruments, Shanghai, China). A homebuilt multi-terminal high voltage power supply, variable in the range of 0–1,500 V, was used for sampling and CE separation.

Microchip fabrication

The microchip was fabricated as described elsewhere [15], and it is shown in Fig. 1. The channels were etched to a depth of 12 μm and a width of 48 μm. Access holes were drilled into the etched plate with a 1.2 mm diameter diamond-tipped drill bit at the terminals of the channels. After permanent bonding by a thermal bonding procedure, four 4-mm inner diameter and 6-mm tall micropipette tips were epoxied on the chip surrounding the holes, serving as reservoirs. The channel between reservoir S and SW was used for sampling, and the channel between B and BW was used for cytolysis and separation.

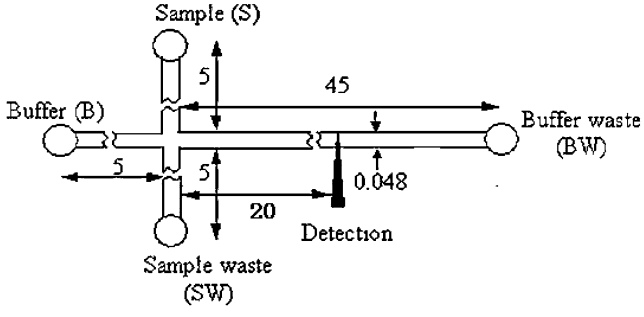

Fig. 1 Schematic diagram of the channel design of the microfluidic chip (in mm)

Reagents and chemicals

All chemicals used were of analytical reagent grade, and demineralized water was used throughout. Physiological salt solution (PSS, NaCl 0.9%, pH 7.4) was used for washing and preserving the erythrocytes. Dihydrorhodamine 123 (DHR 123) and rhodamine 123 (Rh 123) were purchased from Molecular Probes Eugene, OR, USA. A stock solution of DHR 123 was prepared at a concentration of 0.29 mM in DMF and kept in the dark at −20 °C. A stock solution of Rh 123 was prepared at a concentration of 0.35 mM in methanol and further diluted with PSS, and this was used to construct calibration curves from 0.35 to 2.8 mol/l. Borate buffer (20 mmol; pH 10.1) was used as the medium for cell lysis, as well as the working electrolyte for CE separation. H_2O_2 (4 mmol/l) in PSS was freshly prepared before use.

Sample treatment

Human blood from a healthy adult was obtained from Zhejiang Provincial Blood Center (Hangzhou). A 25 μl blood sample was centrifuged at 1,000 rpm for 5 min to separate out the erythrocytes. The supernatant was discarded and the erythrocytes were washed with PSS 3–5 times by centrifuging until a clear supernatant was obtained. After discarding the supernatant; the erythrocytes were suspended in 1 ml PSS, and 10 μl DHR 123 stock solution was added to the suspension, and reacted in the dark at room temperature for 20 min. The cells were washed with PSS again for 3–5 times and resuspended in PSS to obtain a cell population of 1.2×10^5 cells/ml. The cell population was determined using a hemocytometer (Shanghai Medical Optical Instrument Plant, Shanghai, China).

Procedures

Working electrolyte solutions of 50, 50, and 20 μl were added to the reservoirs B, BW, and SW respectively. Then 100 μl of the cell suspension (1.2×10^5 cells/ml) were added to the sample reservoir S. Owing to the differences in liquid levels in the reservoirs created by the different volumes, the cell suspension flowed from reservoir S to SW under hydrostatic pressure. When a single cell moved within the crossed section of the channels, observed under the microscope, a set of electrical potentials was applied to the four reservoirs, with B at 1,200 V, S and SW both at +700 V, and BW grounded. The sampled cell was transported towards the buffer waste reservoir (BW) by electroosmotic flow (EOF). After 0.1 s, the set of potentials was switched off,

and the sampled cell settled within the channel and adhered to its wall near the channel-crossing. After the chip was shifted from the channel-crossing viewing position to the detection point, the laser beam was re-focused. The set of electrical potentials was resumed, the docked cell was lysed immediately and the reaction product of ROS and DHR 123 (Rh 123) was released, separated by chip-based CE and detected by LIF.

Calibration

100 μl working buffer solutions were added to each of the reservoirs SW, B, and BW, while 100 μl of standard solution of Rh 123 was added to the reservoir S. The electric potentials applied to the four reservoirs at the sample loading step and the separation step are shown in Table 1. The sample injection time for repetitive injection was 1 min. The peak area was used for calibration.

Results and discussion

Intracellular conversion of ROS to fluorescent Rh 123

ROS are non-fluorescent in their native forms, so their conversion into fluorescent compounds is required for detection with LIF. In order to avoid diluting the ROS content in individual erythrocytes, the procedure [16] suggested by Hogan and Yeung, in which the cell itself acts as a reaction chamber, was used for intracellular conversion of ROS. DHR123 is a non-fluorescent reagent, which can partition through the lipophilic cell membrane and react irreversibly with intracellular ROS to fluorescent Rh 123. Rh 123 is more polar than DHR 123, and therefore will not migrate back through the cell membrane [8]. The reactivity of each ROS, such as hydroxyl radicals, superoxide anions, and hydrogen peroxide towards DHR 123, was studied by Parmentier et al [8]. Their results showed that DHR 123 is a non-selective ROS probe, so intracellular total ROS content can be measured via the determination of Rh 123 with LIF, as shown in Fig. 2.

During the experiments, we observed that after 0.5 h exposure in the atmosphere under daylight, colorless DHR 123 would appear pistachio in color, which was the same color as a Rh 123 solution of low concentration. CE results proved that DHR 123 could also be oxidized into Rh 123 by atmospheric oxygen. To avoid errors in the determination originating from extracellular Rh 123, a washing step was necessary. The exterior excess DHR 123 and its oxidation product Rh 123 can be completely removed by washing three times with PSS, each time followed by centrifugation.

Table 1 Applied injection and separation voltages

Reservoir	S	SW	B	BW
Injection voltage (V)	400	0	400	400
Separation voltage (V)	700	700	1,200	0

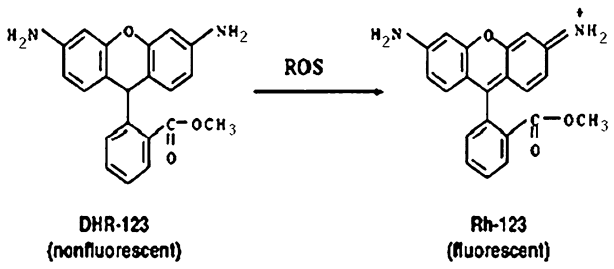

Fig. 2 Intracellular conversion of ROS to fluorescent Rh 123 by DHR 123

Effect of pH on migration time and detection sensitivity

Rh 123 is a polyfunctional acid. Figure 3a shows a schematic diagram of the protonation/deprotonation equilibrium of Rh 123. As indicated, three charged species, abbreviated as I (three positive charges), II (two positive charges), and III (one positive charges), together with the neutral species IV, exist in the electrophoretic system. The relative amounts of the four Rh 123 species vary due to the deprotonation at higher pH. Therefore, the pH of the buffer solution can affect the migration time and detection sensitivity. In order to improve the reproducibility and sensitivity, the effect of buffer pH on the electrophoretic behavior of Rh 123 was investigated using 20 mmol/l borate at different pHs, under an

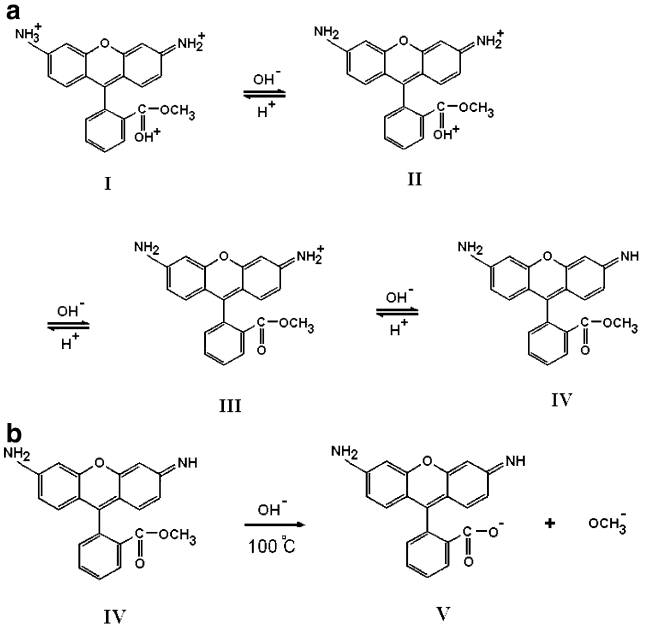

Fig. 3a–b **a** Schematic diagram of the protonation/deprotonation equilibrium of Rh 123. **b** Hydrolysis reaction equation of Rh 123 (IV)

applied field strength of 240 V/cm. The electropherograms obtained are shown in Fig. 4a–d. In order to identify the major Rh 123 species at different pHs, a hydrolysis experiment was performed by heating Rh 123 at pH 10.1 for 10 min in boiling water. Figure 4e shows an electropherogram of the hydrolyzed products of Rh 123. By comparing it with the electropherogram of Rh 123 at pH 10.1 (Fig. 4d), it was observed that the migration times of peaks V and VI in both electropherograms were identical, while the peak height of IV in Fig. 4e was significantly lower, and peak V in Fig. 4e was taller owing to the hydrolysis reaction shown in Fig. 3b. Therefore, the two peaks shown in Fig. 4d were identified as the fully unprotonated form of IV (20.2 s), and its negatively charged hydrolysate V (25.2 s) was obtained in basic solution. In addition, a small peak at 35.8 s can also be distinguished in Fig. 4e, which is presumably that of the molecular aggregate of IV with its hydrolysate V. By using peak VI as a marker, the two peaks showed in Fig. 4a–c were identified as being III and IV at pH 9.3 (Fig. 4c), II and III at pH 8.3 (Fig. 4b), and I and II at pH 7.4 (Fig. 4a) due to the successive deprotonation of Rh 123 at higher pH. As pH increased, the positive charge on the Rh 123 species decreased, resulting in an increased retention time. On the other hand, increasing the pH concomitantly increased EOF, giving rise to a decreased migration time for the same Rh 123 species, which can be observed in Fig. 4: the migration time of species II is 17.3 s at pH 7.3 compared to 15.8 s at pH 8.4, while species III is 18.6 s at pH 8.4 compared to 18.3 s at pH 9.3, and species IV is 21.6 s at pH 9.3 compared to 20.2 s at pH 10.1.

The highest detection sensitivity of IV among the four species of Rh 123 can be selected from Fig. 4, so pH 10.1 buffer solution was chosen as the working solution. It has also been observed that gradual hydrolysis of Rh 123 occurs if Rh 123 standard solutions are diluted in pH 10.1 buffer solution. This resulted in decreased detection sensitivity. In order to avoid this, Rh 123 standard solutions were diluted in PSS (pH 7.4) before use.

A borate buffer containing 40% (v/v) acetonitrile was suggested by Yu [2] to improve the sensitivity of CE detection of ROS in cell extracts. However, our own experiences were that adopting such measures did not improve results, owing to unsatisfactory reproducibilities for both migration time and peak area. In the chip system, the reservoirs were open and the sample volumes in the reservoirs were only a few microliters. Owing to the relatively high volatility of acetonitrile, the concentration of acetonitrile in the buffer solution changed rapidly with time under such conditions; consequently, 20 mmol/l borate/NaOH (pH 10.1) buffer solution was chosen as the working solution for electrophoresis.

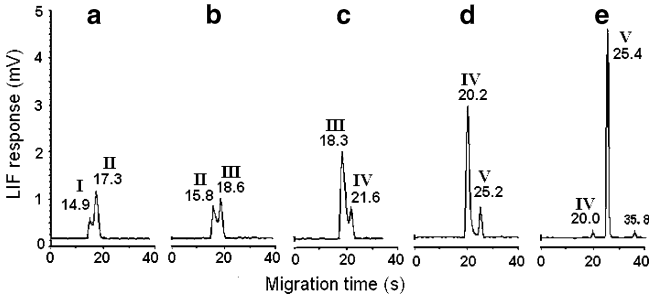

Fig. 4 Electropherograms of Rh 123 under different pH. Field strength 240 V/cm; separation length 2.0 cm. *a*, pH 7.3; *b*, pH 8.4; *c*, pH 9.3; *d*, pH 10.1; *e*, pH 10.1. Heating for 10 min at 100 °C

Quantitation

The volume of an injected standard sample under the pinched conditions used in the experiment was 14 pl [12], while the volume of a single erythrocyte is only about 0.1 pl [12]. Although the intracellular constituents would have dispersed into a larger volume during cell lysis before the separation, the time for dispersion was very short, and the volume could still be significantly smaller than an injected liquid sample. It is therefore reasonable to use the peak area for calibration when estimating the mass of ROS released for each single cell.

A series of Rh 123 standard solutions (0.35–2.8 μmol/l) (with replicates) was used to calibrate ROS. A regression equation of $y = 1.97\times10^3 c$ (c is the concentration in μmol/l, y is the peak area in μV.s, $\gamma = 0.9993$) was obtained. The detection limit of 0.053 μmol/l was determined by calculating three times the standard deviation for eight blank solutions and dividing this by the slope of the regression equation. Assuming an injected standard sample volume of 14 pl, a regression equation between peak area and absolute amount n (amol) of $y = 1.4\times10^2 n$ was obtained and the absolute detection limit was 0.8 amol, which was about two orders of magnitude lower than that (0.07 fmol) obtained by employing conventional CE [8]. The improved detection limit was a result of the much smaller sample volume and much shorter separation channel length used in the chip-based CE system.

A Rh 123 standard solution of 0.5 μmol/l Rh 123 was used to investigate the reproducibility. The average migration time was 20.1 s, with a precision of 0.3% RSD. The peak area reproducibility was 6.2% RSD ($n = 9$).

ROS contribute to the pathogenesis of several hereditary disorders of erythrocytes [5]. Amer et al. reported a flow cytometric method for measuring ROS in non-stimulated and 2 mM H_2O_2-stimulated erythrocytes. Results showed that when stimulated by H_2O_2, the fluorescence of erythrocytes increased within 30 min to 10–30-fold that of non-stimulated erythrocytes [6].

In the present study, we used chip-based electrophoresis to monitor ROS in non-stimulated and 4 mM H_2O_2-stimulated erythrocytes. The electropherograms recorded during consecutive injections of a series of single erythrocyte are shown in Fig. 5a. Only one Rh 123 peak was observed in each electropherogram,

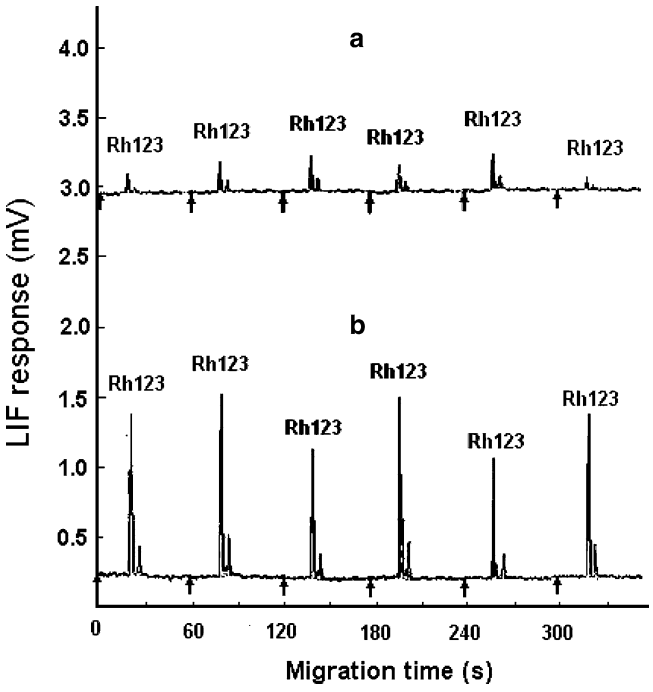

Fig. 5a–b Recorded electropherograms for a series of six erythrocytes injected consecutively. Unstimulated (**a**) and stimulated (**b**) by 4 mmol/l H_2O_2 for 10 min. Field strength 240 V/cm, separation length 2.0 cm, 20 mmol borate buffer (pH 10.1)

implying that only one cell was injected into the separation channel during the separation stage. As described previously, the EOF in the sample channel was greater than the Poiseuille flow during the separation stage, so that further cells were prevented from entering the separation channel [12]. The average migration time of the Rh 123 peaks (20.2 s), with a reproducibility of 4.1% RSD for six injected cells, agreed well with that obtained using the Rh 123 standard (20.1 s). The ROS contents in a single erythrocyte cell were 1.2, 2.1, 2.5, 1.6, 2.7, and 1.1 amol, respectively. The time required for a complete analysis, including cell injection, docking, chip shifting, laser refocusing, cell lysis, separation and detection for each erythrocyte was less than 3 min.

The recorded eletropherograms for a series of six erythrocytes stimulated by 4 mmol/l $H_2O_2$for 10 min are shown in Fig. 5b. An average content of 16 amol was obtained. Such values were 8.4 times higher than those for erythrocytes that did not undergo H_2O_2 stimulation. Compared with the flow cytometric method, the ROS increase is reasonable when considering the shorter stimulation time. The main advantages of the method are its ability to determine the cellular ROS of individual erythrocytes, the low detection limit, and the reduced operational costs.

Conclusion

The proposed microfluidic electrophoresis-LIF method provides a sensitive approach to quantitative analysis of ROS in individual cells. In single erythrocytes, ROS oxidize non-fluorescent DHR 123 to fluorescent Rh 123 by incubating living cells with DHR 123. There is almost no dilution of ROS in the cell during the intracellular reaction. The extremely low detection limit shows favorable potentials for studying intracellular ROS variations upon stimulation.

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