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Single-cell analysis by electrochemical detection with a microfluidic device

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Abstract

A novel electrochemical method with a microfluidic device was developed for analysis of single cells. In this method, cell injection, loading and cell lysis, and electrokinetic transportation and detection of intracellular species were integrated in a microfluidic chip with a double-T injector coupled with an end-channel amperometric detector. A single cell was loaded at the double-T injector on the microfluidic chip by using electric field. Then, the docked cell was lysed by a direct current electric field strength of 220 V/cm. The analyte of interest inside the cell was electrokinetically transported to the detection end of separation channel and was electrochemically detected. External standardization was used to quantify the analyte of interest in individual cells. Ascorbic acid (AA) in single wheat callus cells was chosen as the model compound. AA could be directly detected at a carbon fiber disk bundle electrode. The selectivity of electrochemical detection made the electropherogram simple. The technique described here could, in principle, be applied to a variety of electroactive species within single cells.

Keywords: Single-cell analysis; Electrochemical detection; Microfluidic chip; Ascorbic acid; Wheat callus cell

1. Introduction

Microfluidic devices have been used to perform a variety of investigations concerning cell analysis, owing to advantages such as miniaturization, multiple function integration, low reagent consumption, fast analysis speed and high detection sensitivity. Cell manipulation such as transport, docking, culturing, sorting and separation [1–7], and cell lysis [1–3] can be carried out on the mircofluidic devices. Cellular reactions have been investigated through the intracellular calcium signal within intact cells docked on the microfluidic chip [4,5]. A microfluidic device has been developed for the determination of insulin secretion from islets [8]. A microfluidic chip with a scanning thermal lens microscope has been used to determine cytochrome *c* distribution in single cells during apoptosis process [9]. However, this kind of technique without cytolysis cannot be used to quantitatively determine intracellular constituents in individual cells. More recently, Ramsey and co-workers [10] have reported a microfluidic device that integrated cell handing, lysis, electrophoresis separation and qualitative measurement of the fluorescent dyes that previously permeated into the cell prior to on-chip determination with laser-induced fluorescence (LIF) detection. However, none of the cellular constituents were quantified. Fang's group has developed another single-cell analysis system on a crossed-channel microfluidic chip with LIF detection, on which single-cell introduction, docking, lysis, and electrophoretic separation were fully integrated [11]. In their work, glutathione in human single erythrocytes was quantified after derivatization with 2,3-naphthalenedicarboxaldehyde.

Electrochemical detection (ED) coupled with capillary electrophoresis (CE) is an important detection mode for analysis of single cell [12–14], because it offers high sensitivity and selectivity for analytes. However, hitherto this detection technique has not been used for single-cell analysis based on microfluidic chips. In this work, we developed a method for

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single-cell analysis by using ED combining a microfluidic chip with a double-T injector. Ascorbic acid (AA) in single wheat callus cells was chosen as the model compound.

2. Experimental

2.1. Reagents and solutions

AA (analytical grade) was obtained from Xian Chemical Reagents Factory (Xian, China). A 1.00×10^{-3} mol/L stock solution of AA was prepared weekly by dissolving an appropriate amount of L-AA in 1.00×10^{-3} mol/L Na_2H_2EDTA deaerated. The solution of AA was stored at 4 °C. Dilute solutions were obtained by a serial dilution of the stock solutions with the running buffer. The MB cell culture medium, enzyme solution and rinse buffer for treating wheat callus cells were obtained from Institute of Life Science, Shandong University, Jinan, China. All other reagents were of analytical grade and purchased from standard reagent suppliers. All solutions were prepared with double-distilled water.

2.2. Microfluidic chip-ED system

Microfluidic chips with the "double-T" injector was fabricated from 6 cm × 2 cm substrates of soda-lime glass using a photolithographic and wet chemical etching procedure [15]. The channel design of the microfluidic chip-ED system is depicted in Fig. 1. The channels were etched to a depth of 30 μm and a width of 80 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 [15], three 4 mm i.d. and 6 mm tall micropipet tips were epoxyed on the chip surrounding the holes, serving as reservoirs. The microfluidic chip (1) was fixed on a microscope slide (2) with epoxy glue. A Plexiglas plate of ∼4 mm thickness (3) with a ∼5 mm diameter hole as the reference electrode port (4) and a ∼0.40 cm^2 hole with a semicircular cut-off area serving as the electrochemical cell and the waste reservoir (5) was fixed on the same microscope slide, butted against the detection end of separation channel (6). Two small grooves were machined on the Plexiglas plate at the two sides of the electrochemical cell (5). Pt wires were inserted in each of the two grooves serving as the auxiliary electrode (7) and the cathode of separation high voltage (8), respectively. The cell was connected with the reference port by a magnesia rod (9). The small gap between the Plexiglas plate and the chip was filled with paraffin (10). A stainless steel tube (11) (0.40 mm i.d., 20 mm in length) was installed at the detection end of separation channel on the chip, fixed on the Plexiglas plate by epoxy glue. The working electrode (12) was inserted into the stainless steel tube, and approached the detection end of separation channel on the chip with a distance of ∼30 μm. Three Pt wires were inserted into the sample reservoir (13) as the anode of loading voltage, the sample waste reservoir (14) as the cathode of loading voltage and the buffer reservoir (15) as the anode of separation high voltage, respectively.

A high-voltage power supply (Model 9323-HVPS, Beijing Institute of New Technology, Beijing, China) coupled with a laboratory-built relay box was used to provide high voltage. ED at a constant potential was performed with an electrochemical analyzer (model CHI802, CH Instruments, Austin, TX, USA). ED was carried out with a three-electrode system that consisted of a carbon fiber disk bundle electrode (CFDBE) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a Pt wire (0.5 mm diameter) as the auxiliary electrode. The CFDBEs were constructed by the same method as that of our previous work [16]. Before use, all CFDBEs were cleaned by ultrasonication in alcohol and double-distilled water for 5 min. The microfluidic chip-ED system including the three electrodes was housed in a Faraday cage in order to minimize the interference from noise from external sources.

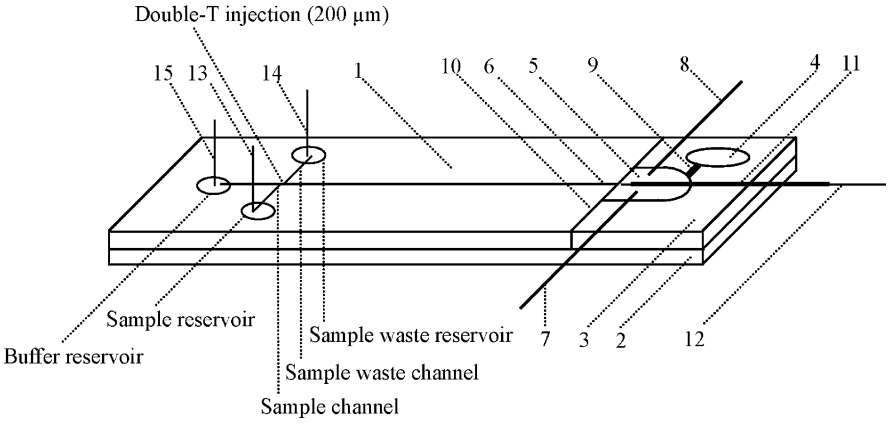

Fig. 1. Microfluidid chip-ED system: (1), microfluidic chip; (2), microscope slide; (3), Plexiglas plate; (4), reference electrode port; (5), electrochemical cell and waste reservoir; (6), separation channel; (7), auxiliary electrode; (8), cathode of separation high voltage; (9), magnesia rod; (10), paraffin; (11), stainless steel tube; (12), working electrode; (13), anode of loading voltage; (14), cathode of loading voltage; (15), anode of separation high voltage. Channel, 30-μm depth and 80-μm width; separation channel, 45-mm length. Both sample channel and sample waste channel, 5-mm length.

2.3. Preparation of cells

Plant cells surrounded by a cell wall mainly consisting of cellulose differ from animal cells. For single-cell analysis, their walls were removed by applying an enzymatic digestion procedure resulting in protoplasts without the cell wall. The wheat calluses were grown in a MB culture medium. Eight days later, ~0.2 g of the calluses were added into 2 mL of the enzyme solution. After vibrating lightly for 3 h at 25 °C, the enzyme solution containing a large number of protoplasts was filtered through a filter in order to remove plant material. The enzyme solution was transferred into a centrifuge tube and centrifuged for 5 min at 500 rpm. The supernatant was discarded. The enzymolysis was stopped by addition of 1 mL of rinse buffer. The protoplast mixture was disrupted by pipetting. The protoplast mixture was centrifuged for 5 min at 500 rpm to remove the residual enzyme. Then 0.5 mL of rinse buffer was added to the centrifuge tube and the protoplast suspension was disrupted until protoplasts were dispersed in the solution. The protoplast suspension was stored at 4 °C. Before use, 0.5 mL of protoplast suspension was dispersed in the running buffer with the same volume and counted using a hemocytometer. The protoplast density was adjusted to about 5×10^4 cells/mL by adding the mixture solution consisting of the running buffer and the rinse buffer with the same volume. The protoplast density was counted using a hemocytometer (Shanghai Medical Optical Instrument Plant, Shanghai, China).

2.4. Microfluidic chip-ED of AA

Microfluidic chip-ED was carried out in uncoated channels that had been flushed with 0.1 mol/L NaOH for 30 min, water for 10 min, and finally, the running buffer for 30 min by means of a syringe. Then a voltage of 1000 V was applied across the separation channel, and a detection potential of 0.90 V for the detection of AA was applied at the working electrode aligned with the detection end of the separation channel. After the electroosmotic flow reached a constant value, the standard solution was electrokinetically injected by applying 300 V to the sample reservoirs, with the sample waste reservoir grounded and both detection reservoir and buffer reservoir floating. The double-T injector was filled with the standard solution, and then the injection time of 30 s was applied. After sample loading, a separation voltage of 1000 V was applied to the buffer reservoir while the detection end was remained at the ground with the sample reservoir and the sample waste reservoir floating. At the same time, the signal was detected on the working electrode at the end of separation channel, and the electropherogram was recorded.

2.5. Loading, lysis and analysis of individual cells

The microfluidic chip was pretreated with NaOH, water and the running buffer as described above. The cell suspension was injected into the sample reservoir. The electrokinetic loading was used for analysis of single protoplasts. In the loading mode, an injection voltage of 100 V was applied at the sample reservoir with the sample waste reservoir grounded and other reservoirs floating. Protoplasts could move into the sample channel by the electrokinetic flow. When the first protoplast was seen in the sample channel under an inverted microscope with a magnification of 400×, the injection voltage was decreased to 50 V and the motion rate of protoplasts was decreased. Once a protoplast appeared in the double-T injector (see Fig. 2), the voltage at the buffer reservoir was switched to 1000 V, the voltage of the detection end of separation channel was switched to ground, and the other reservoirs

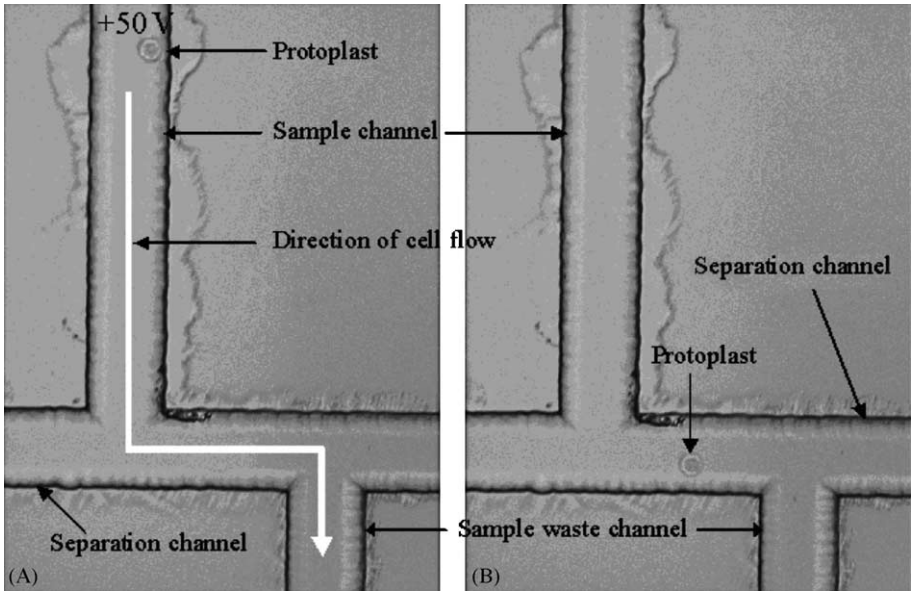

Fig. 2. The photographs of an individual protoplast docked in (A) the sample channel and (B) the double-T injector.

were kept floating. The protoplast was lysed rapidly under the high electric field. AA in the protoplast was electrically migrated to the detection end of separation channel and was detected on the CFDBE at 0.90 V.

3. Results and discussion

3.1. Detection of AA

In previous work of capillary electrophoresis with ED, it was found that AA can be oxidized at the CFDBE and the electrophoretic peak currents of AA were constant within 40 min [17]. The run time was ~3 min for the determination of AA in our experiments. Therefore, the oxidation of AA could be neglected during determination of AA. The experimental conditions for determination of AA used here were similar to our previous work [17] except for the injection electric field strength, injection time and the separation electric field strength. They were 1.88×10^{-2} mol/L Na_2HPO_4–1.20×10^{-3} mol/L NaH_2PO_4–1.00×10^{-3} mol/L Na_2H_2EDTA (pH 8.0) for the running buffer, 300 V/cm for the injection electric field strength, 30 s for the injection time, 220 V/cm for the separation electric field strength and 0.90 V for the detection potential versus SCE. Fig. 3A shows the electropherogram

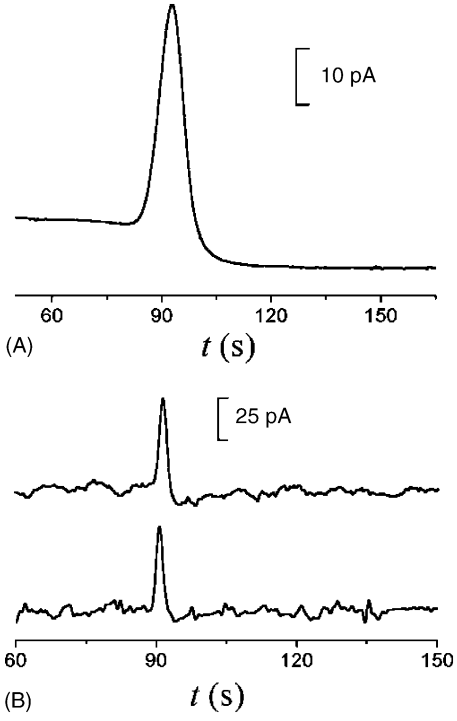

Fig. 3. Electropherograms of (A) 5.00×10^{-5} mol/L AA and (B) two individual protoplasts at the CFDBE. (A) Loading electric field strength, 30 V/cm; loading time, 30 s; (B) loading electric field strength, 5–10 V/cm. Running buffer, 1.88×10^{-2} mol/L Na_2HPO_4–1.20×10^{-3} mol/L NaH_2PO_4–1.00×10^{-3} mol/L Na_2H_2EDTA (pH 8.0); separation electric field strength, 220 V/cm; detection potential, 0.90 V vs. SCE.

of 5.00×10^{-5} mol/L AA solution. The migration time, t_m, and the width at the peak half-height, $W_{1/2}$, are 93 s and 7.0 s, respectively.

In our experiments, the length of double-T injector was 200 μm. Therefore, the injection plug length of standard solutions was much longer than the radium of cells with a diameter of ~25 μm, indicating that the electrophoretic peak width of the standard solutions was not equal to that of cells. In this case, the peak area, q, should be used to quantify AA within single cells. The linear range was 5.00×10^{-6}–1.00×10^{-4} mol/L with a correlation coefficient of 0.9997. The limit of detection (LOD) calculated from the peak area obtained for the concentration at the low end of its linear range was 5×10^{-6} mol/L, when the signal-to-noise ratio was 3. The relative standard deviations of the method for determination of AA were 1.7% for t_m and 2.5% for q.

3.2. Loading and lysis of individual cells

Usually, during loading a single cell in conventional CE for single-cell analysis, the capillary has to be immersed into the cell suspension and to wait until a cell drifts toward the injection end of the capillary. At the same time, an injection voltage is applied across the capillary to transport the cell into the capillary tip. Then the capillary is gently moved from the cell suspension into the CE running buffer. When microfluidic devices were used for single-cell analysis, the procedure for loading a cell became simple [10,11]. Ramsey and co-workers [10] used a syringe pump to transport cells into a cross intersection. Fang's group [11] used a hydrostatic pressure generated by the difference of liquid level to bring cells to the channel crossing of the crossed-channel chip. In the present work, a double-T injector was used. Both electric field loading and hydrodynamic loading could be used to drive a cell from the sample reservoir to the double-T injector. We used the electric field to load a protoplast. After the protoplast suspension was added in the sample reservoir, an injection electric field strength of 50–100 V/cm was applied to the sample reservoir with the sample waste reservoir grounded. Protoplasts moved towards the sample waste reservoir through the double-T injector by the electroosmosis of the bulk fluid. The injection electric field strength should not be too high in order to avoid cell lysis.

Cells could remain intact under 100 V/cm [1]. In our experiments, the electric field strength of 100 V/cm was used for injecting cells. When the first protoplast was seen in the sample channel, the injection voltage was switched to 50 V/cm. In this case, protoplasts moved slowly in the channel. Once one protoplast moved to the double-T injector, the injection electric field was turned off and the protoplast was docked (see Fig. 2). Using such a way, it was easy to control cell docking in the double-T injector without cytolysis. The density of cell suspension is another key parameter for docking an individual cell [10,11]. Too high cell density caused cell agglomeration within the channels, resulting in multi-cell loading and dock-

ing. The protoplast density of 5.0×10^4 cells/mL was suitable for single cell loading and docking.

After docking a cell into the double-T injector, the cell must be lysed. Usually, chemical lysis is accomplished. Erythrocytes can be lysed easily in the running buffer of pH 7.0 [18]. Other chemical reagents such as NaOH [19,20] and surfactants [1,10] such as sodium dodecyl sulfate were also used to lyse cells. However, NaOH may product a blank electrophoretic peak [19] in ED or denature some proteins, and surfactants can impair electrode performance because of adsorption on the electrode surface. Ramsey and co-workers [10] used a high electric field strength that consisted of 450 V/cm peak-to-peak square waves with a 675 V/cm dc offset and at a 50% duty cycle to lyse cells. We found that a single protoplast in the double-T injector could be lysed easily in the running buffer using a low dc electric field strength of 220 V/cm across the separation channel. The phenomenon could be seen under the inverted microscope. The short lysis time effectively minimized dilution of contents released from the single cell in the channel during lysis.

3.3. Analysis of single cells

Fig. 3B shows the electropherograms of two individual protoplasts obtained at the CFDBE. Only one electrophoretic peak appears on the electropherogram. To identify the peak, the electrophoretic behavior of other electroactive biological compounds such as tyrosine (Tyr), cysteine (Cys), histidine (His) and tryptophan (Trp), which can be directly oxidized at the carbon electrode, must be discussed under these conditions used here. Amounts of these amino acids in the wheat cells are 0.078–0.54 fmol for Trp, 0.22–0.7 fmol for Tyr, 0.068–0.50 fmol for Cys, 0.05–0.18 fmol for His [21]. Their LODs detected in the present work were 3 fmol for Trp, 6 fmol for Tyr, Cys and His. The amounts of these amino acids in wheat cells were 5–10 times lower than their LODs. This means that they could not be detected under the present experimental conditions. Therefore, the electrophoretic peak shown in Fig. 3B could be identified as the peak of AA on the basis of the migration time, by comparing with the electropherogram of the standard AA shown in Fig. 3A.

In single-cell analysis, it is important to determine the mass of analytes inside single cells rather than the concentration because the cell volumes are different. To estimate the mass linear ranges of analytes of interest, the loaded volume of standard solutions have to be obtained. When the double-T injector is used, the leakage effects allow the actual injected volume of standard solution to be larger than the solution volume between the intersections of the double-T injector [22]. Harrison and co-workers [22] have investigated the leakage effects in detail. Two main leakage phenomena must be considered. The two leakage effects depend on many experimental parameters such as the channel width and roughness, the injector symmetry, the composition of the standard solution and the running buffer, and the bias voltages to all the intersecting channels. In our work, the volume of injected analyte plug was experimentally measured by epi-fluorescent microscopy using an inverted fluorescent microscope (Model IX-81, Olympus, Tokyo, Japan) and a charge-coupled device (CCD) camera (Model Dp70, Olympus, Tokyo, Japan). The standard solution containing fluorescein (Shanghai Third Chemical Reagents Factory, Shanghai, China) was used to perform the whole injection process. All fluorescein molecules in the channels were excited by an Hg lamp. The fluorescence emitted by the fluorescein molecules in the channels was trapped by the CCD camera, obtaining a fluorescent image. The fluorescent intensity in the channels was heterogeneous because of the leakage effects. However, the converted injection volume, in which the concentration of fluorescein (i.e. analyte) was homogeneous and equal to its bulk concentration (i.e. within which the total amount of analyte was equal to that of the practical analyte plug), could be calculated from the fluorescent intensity in the taken images and the volume of unit fluorescent intensity for a homogeneous bulk concentration. Fluorescent intensity could be calculated by means of MetaMorph software (Universal Imaging Corp., PA, USA). Firstly, leakage event could be observed, when a loading voltage was applied across the sample channel and the sample waste channel for a certain time with the other reservoirs floating. During loading the standard solution from the side sample channel to the double-T injector, the standard solution extended beyond the edge of the two intersections of the double-T injector as shown in Fig. 4A. Fringing electric fields and diffusion effects depending on

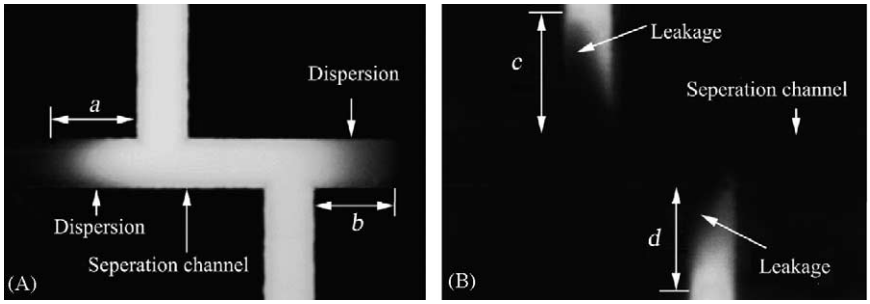

Fig. 4. Fluorescent images of (A) the dispersion of standard solution containing fluorescein loaded in the double-T injector at the end of the loading stage and (B) the leakage from both sample channel and sample waste channel into the separation channel after the sample plug leaving the double-T injector.

the loading time caused the intrusion into other regions of the intersections. The analyte concentration in the range of the dispersion *a* and *b* indicated in Fig. 4A beyond the edges of the two intersections is heterogeneous. To estimate the converted loading volume of analyte with its bulk concentration of standard solution in the separation channel, $V_{loading}$, the fluorescent image of separation channel was taken after loading an AA standard solution containing fluorescein. When the AA standard solution did not contain fluorescein, no fluorescent panel was obtained, implying the back ground could be neglected. From the fluorescent intensity of the practical analyte plug loaded into the separation channel shown in Fig. 4A, $V_{loading}$ was calculated to be 0.57 nL. Secondly, when a separation voltage was applied across the separation channel for a certain time with the other reservoirs floating, the loaded plug left the double-T injector. At the same time, the standard solution flowed from both sample channel and sample waste channel into the separation channel in the range of *c* and *d* as shown in Fig. 4B with the standard solution loaded in the double-T injector. To estimate the converted leakage volume of analyte with its bulk concentration of standard solution in both sample channel and sample waste channel, $V_{leakage}$, the fluorescent images of both sample channel and sample waste channel were taken before and after applying the separation voltage across the separation channel. $V_{leakage}$ could be obtained to be 0.10 nL from the difference of the fluorescent intensity between the two images before and after applying the separation voltage. Thus, the converted injection volume (a sum of $V_{loading}$ and $V_{leakage}$) detected at the working electrode was 0.67 nL. From the value and the concentration linear range, the mass linear range of 3.4–67 fmol was calculated, when the peak areas were used.

In almost all CE experiments of single-cell analysis for whole-cell injection, the migration time of detected analytes was prolonged and the number of theoretical plates was decreased with increasing run numbers. The accumulation of the cellular debris and the substances in cells adsorbed on the inner surface of the capillary was probably responsible for this. This phenomenon also existed in single-cell analysis with microfluidic devices and was noted by Ramsey's group [10]. To minimize both types of adhesion, they used polymer coatings. In our previous CE work [13], we described a method of treating the detection end of capillary, to solve this problem. A similar method was used in the present work. After analyzing each cell, the separation channel was flushed with 0.1 mol/L NaOH, water and the running buffer, respectively, by using a syringe. The results of analysis for seven single protoplasts are listed in Table 1, respectively. The migration time was not prolonged and the number of theoretical plates was hardly decreased with increasing run numbers. It could be noted that the width at the peak half-height in single-cell analysis was narrower than that of the standard solution. This was because the plug length of the standard solution injected in the double-T injector was longer than the cell diameters. The external standardization could be used to quantify AA in individual cells, when the electrophoretic peak area was used. The amounts of AA in single protoplasts determined are also shown in Table 1. It was observed that the amounts of AA in single cells differed from cell to cell. The amounts of AA determined in the seven individual protoplasts were 7.3–25 fmol. On the basis of the amount of AA in one gram of wheat callus cells (100–500 μg/g) [23], the cell density (1 g/mL) [24] and the cell diameter (25 μm), the calculated mean amount of AA in a single protoplast is 5–27 fmol, which are in agreement with our values.

Table 1
Values of the migration time, t_m, the width at half peak-height, W_{12}, and the number of theoretical plates, *N*, on the electropherograms, as well as the peak area, *q*, and amount of AA in the seven single protoplasts

Protoplast	t_m (s)	W_{12} (s)	N (10^4)	q (pC)	Amount (fmol)
1	93.2	2.1	1.1	126	7.3
2	90.6	1.6	1.8	203	12
3	91.3	1.7	1.6	246	14
4	89.2	1.8	1.4	177	10
5	91.5	1.8	1.4	141	8.2
6	92.4	2.3	0.89	412	25
7	89.7	1.9	1.2	210	12

Conditions as in Fig. 3A.

4. Conclusions

The microfluidic chip with ED is a versatile technique for the analysis of biochemical electroactive substances within single cells. The selectivity of the analytes of interest detected for the different electrode material makes the electropherograms simple. The microfluidic chip-ED can integrate the whole process in single-cell analysis including single-cell loading, lysis, separation and detection of intracellular species. When the chip with a double-T injector is used, the single-cell loading is controlled easily by using electric field. The dc electric field of 220 V/cm can lyse single protoplasts docked in the double-T injector. After taking into consideration leakage effects during injection of standard solutions, the external standardization can be used to quantify the analytes of interest in individual cells. The system and the approach can be also applicable to the other chemical species. We have used the system with an Au/Hg electrode to determine glutathione in single humean hepatocarcinona cells.

Acknowledgements

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