High-Throughput Single-Cell Analysis for Enzyme Activity without Cytolysis

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2006

Impact Factor: 5.64 · DOI: 10.1021/ac060278v · Source: PubMed

6 AUTHORS, INCLUDING:

Ning Gao
Harvard University
19 PUBLICATIONS **311** CITATIONS
SEE PROFILE

Xiaoli Zhang
Shandong University
118 PUBLICATIONS **2,092** CITATIONS
SEE PROFILE

Anal. Chem. **2006,** *78,* 3213−3220

Correspondence

High-Throughput Single-Cell Analysis for Enzyme Activity without Cytolysis

Ning Gao,[†] Wenlei Wang,[†] Xiaoli Zhang,[†] Wenrui Jin,*[,†] Xuefeng Yin,[‡] and Zhaolun Fang[‡]

School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China, and Institute of Microanalytical Systems, Department of Chemistry, Zhejiang University, Hangzhou 310028, China

A novel high-throughput method without cytolysis for determination of enzyme activity inside single cells was developed by a combination of chemical cell perforation and an intracellular enzyme-catalyzed reaction. Peroxidase (PO) inside human neutrophils was chosen as the model system. Cells were perforated with digitonin to form micropores on the cell membrane. The perforated cells, with physiological buffer saline of pH 7.4 containing hydroquinone (H_2Q) and H_2O_2, were continuously propelled by pressure through a capillary as the microsampler and microreactor. Small molecules H_2Q and H_2O_2 could diffuse into the cell interior through the micropores on the cell membrane, and the large molecule PO remained in the cell interior. Intracellular PO converted H_2Q into benzoquinone (BQ). BQ diffused out from the cell interior to the cell surface through the micropores and formed a BQ zone around the cell. The process proceeded in the capillary during cell movement. The BQ zones around every moving perforated cell were continuously delivered to the capillary outlet by hydraulic flow and detected. An average detection rate of >1 cell/min was obtained.

Analysis of chemical components inside single cells is an interesting and significant technique in which the difference between cells in chemical composition, biological activity, and the responses to external stimuli can be discerned. Monitoring cellular components in individual cells can also distinguish infected cells from healthy ones, which is valuable for clinical diagnosis. A variety of techniques for the chemical analysis of single cells have been developed. In 1965, the separation of hemoglobins in single cells was achieved by electrophoresis.[1] Open-tubular liquid chromatography;[2−6] mass spectrometry;[7−9] capillary electrophoresis (CE);[10−12] and others, such as scanning microdensitometry,[13] in situ biochemical techniques,[14] and surface-enhanced resonance Raman scattering[15] have been successfully applied to single-cell analysis. The most frequently used technique for chemical analysis of single cells is CE because of its extremely small sample size; high separation speed and efficiency; biocompatible environments; and the ability to be coupled to ultrasensitive detection, such as laser-induced fluorescence (LIF) and electrochemical detection (ECD). Many CE-ECD methods for single-cell analysis for determination of electroactive neutrotransmitters and other biological molecules in single cells have been established.[16−34] CE with LIF detection has been used to determine different molecules, such

* Corresponding author. Fax +86-531-8856-5167. E-mail: jwr@sdu.edu.cn.
† Shandong University.
‡ Zhejiang University.

(1) Matioli, G. T.; Niewisch, H. B. *Science* **1965**, *150*, 1824−1826.
(2) Kennedy, R. T.; Oates, M. D.; Cooper, B. R.; Nickerson, B.; Jorgenson, J. W. *Science* **1989**, *246*, 57−63.
(3) Oates, M. D.; Cooper, B. R.; Jorgenson, J. W. *Anal. Chem.* **1990**, *62*, 1573−1577.
(4) Pihel, K.; Hsieh, S.; Jorgenson, J. W.; Wightman, R. M. *Anal. Chem.* **1995**, *67*, 4514−4521.
(5) Hsieh, S.; Jorgenson, J. W. *Anal. Chem.* **1997**, *69*, 3907−3914.
(6) Hsieh, S.; Dreisewerd, K.; van der Schors, R. C.; Jimenez, C. R.; Stahl-Zeng, J.; Hillenkamp, F.; Jorgenson, J. W.; Geraerts, W. P.; Li, K. W. *Anal. Chem.* **1998**, *70*, 1847−1852.
(7) Fung, E. N.; Yeung, E. S. *Anal. Chem.* **1998**, *70*, 3206−3212.
(8) Page, J. S.; Rubakhin, S. S.; Sweedler, J. V. *Anal. Chem.* **2002**, *74*, 497−503.
(9) Roddy, T. P.; Cannon, D. M., Jr.; Meserole, C. A.; Winograd, N.; Ewing, A. G. *Anal. Chem.* **2002**, *74*, 4011−4019.
(10) Chen, G.; Ewing, A. G. *Crit. Rev. Neurobiol.* **1997**, *11*, 59−90.
(11) Yeung, E. S. *J. Chromatogr., A* **1999**, *830*, 243−262.
(12) Woods, L. A.; Roddy, T. P.; Ewing, A. G. *Electrophoresis* **2004**, *25*, 1181−1187.
(13) Markovic, N. S.; Markovic, O. T.; Young, D. S. *Clin. Chem.* **1983**, *29*, 1578−1581.
(14) Gevers, E. F.; Milne, J.; Robinson, I. C.; Loveridge, N. *J. Bone Miner. Res.* **1996**, *11*, 1103−1111.
(15) Moore, B. D.; Stevenson, L.; Watt, A.; Flitsch, S.; Turner, N. J.; Cassidy, C.; Graham, D. *Nat. Biotechnol.* **2004**, *22*, 1133−1138.
(16) Chien, J. B.; Wallingford, R. A.; Ewing, A. G. *J. Neurochem.* **1990**, *54*, 633−638.
(17) Olefirowicz, T. M.; Ewing, A. G. *J. Neurosci. Methods* **1990**, *34*, 11−15.
(18) Olefirowicz, T. M.; Ewing, A. G. *Chimia* **1991**, *45*, 106−108.
(19) Bergquist, J.; Tarkowski, A.; Ekman, R.; Ewing, A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12912−12916.
(20) Kristensen, H. K.; Lau, Y. Y.; Ewing, A. G. *J. Neurosci. Methods* **1994**, *51*, 183−188.
(21) Hu, S.; Pang, D.; Wang, Z.; Cheng, J.; Li, Z.; Fan, Y.; Hu, H. *Chem. J. Chin. Univ.* **1996**, *17*, 1207−1209.
(22) Franklin, D.; Swanek, G. C.; Ewing, A. G. *Anal. Chem.* **1996**, *68*, 3912−3916.
(23) Jin, W.; Dong, Q.; Ye, X.; Yu, D. *Anal. Biochem.* **2000**, *285*, 255−259.
(24) Jin, W.; Li, W.; Xu, Q. *Electrophoresis* **2000**, *21*, 774−779.
(25) Dong, Q.; Jin, W. *Electrophoresis* **2001**, *22*, 2786−2792.
(26) Weng, Q.; Jin, W. *Electrophoresis* **2001**, *22*, 2797−2803.
(27) Jin, W.; Jiang, L. *Electrophoresis* **2002**, *23*, 2471−2476.
(28) Dong, Q.; Wang, X.; Zhu, L.; Jin, W. *J. Chromatogr., A* **2002**, *959*, 269−279.
(29) Weng, Q.; Xia, F.; Jin, W. *J. Chromatogr., B* **2002**, *779*, 347−352.
(30) Jin, W.; Li, X.; Gao, N. *Anal. Chem.* **2003**, *75*, 3859−3864.
(31) Sun, X.; Jin, W. *Anal. Chem.* **2003**, *75*, 6050−6055.

10.1021/ac060278v CCC: $33.50
Published on Web 03/31/2006

as ions, amino acids, neutrotransmitters, carbohydrates, peptides, proteins, and nucleic acids for a variety of cell types.[35−59] CE with particle-counting microimmunoassay,[60] radiochemical detection,[61] and mass spectrometry[62] have been applied to single-cell analysis. Recently, microfluidic chip devices have been used to perform a variety of investigations concerning cell analysis[63−71] 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,[63−67,69,70] and cell lysis[63,64,66] can be carried out on the mircofluidic chip devices. Cellular reactions have been investigated through the intracellular calcium signal within intact cells docked on the microfluidic chip.[67,69] A microfluidic chip device has been developed for the determination of insulin secretion from islets.[71] A microfluidic chip with a scanning thermal lens microscope has been used to determine cytochrome *c* distribution in single cells during the apoptosis process.[68] A microfluidic chip device with LIF detection has been reported for separation and measurement of the fluorescent dyes that previously permeated into the cell.[72] Another single-cell analysis system on a crossed-channel microfluidic chip with LIF detection, on which single-cell introduction, docking, cytolysis, and electrophoretic separation are fully integrated, has also been developed for quantitative determination of glutathione in single human erythrocytes.[73] ECD has been used for quantitative determination of ascorbic acid in single wheat callus cells on a microfluidic device with a double-T injector.[74]

In a majority of the reported papers on single-cell analysis of chemical components for whole-cell injection by using CE or microfluidic chip-CE, the analysis rate, that is, the cell throughput, is rather low. This limits the practical application of the technique. To perform a high-throughput method for single-cell analysis, Dovichi's group has constructed a multipurpose single-cell injector to facilitate monitoring of injection, cell injection, cell lysis, and capillary reconditioning.[46] Chen and Lillard have reported a continuous cell introduction method for erythrocytes.[52] Ramsey's group has developed a microfluidic chip-based device for high-throughput single-cell analysis.[72]

In all the CE methods of single-cell analysis mentioned above, cells must be lysed before detection. In these cases, the intracellular biological macromolecules, including enzymes, are released from the detected cell into the capillary or the channel of the microfluidic device and usually adsorbed onto their wall, which can affect the next determination. Usually, the capillary or the channel must be treated before the next single-cell determination. Additionally, for ECD, the adsorption of biological macromolecules on the electrode surface can foul the electrode and causes a deterioration of amperometric response. These problems that are concerned with the adsorption of biological macromolecules make continuous determination of intracellular substances in single cells and high-throughput single-cell analysis difficult using CE in microchannels, including capillaries.

Enzymes are important biological components in cells. They control the balance of cytochemicals and actively participate in cell proliferation. Usually, measuring the activity of an enzyme is more important than measuring its amounts. CE with sensitive LIF detection[38,47,57,59,75,76] and particle-counting microimmunoassay[60] have been successfully used to determine enzyme activity in single cells. More recently, we have reported a very sensitive catalysis−electrochemical method coupled to CE with a limit of detection of 10^{-21} mol for determination of glucose-6-phosphate dehydrogenase[31] and alkaline phosphatase isoenzymes[34] in individual cells. Like all CE methods for single-cell analysis, cells are lysed before separation and detection.

(32) Wang, W.; Xin, H.; Shao, H.; Jin, W. *J. Chromatogr., B* **2003**, *789*, 425−429.
(33) Weng, Q.; Jin, W. *Anal. Chim. Acta* **2003**, *478*, 199−207.
(34) Sun, X.; Jin, W.; Li, D.; Bai, Z. *Electrophoresis* **2004**, *25*, 1860−1866.
(35) Hogan, B. L.; Yeung, E. S. *Anal. Chem.* **1992**, *64*, 2841−2845.
(36) Lee, T. T.; Yeung, E. S. *Anal. Chem.* **1992**, *64*, 3045−3051.
(37) Cheung, N. H.; Yeung, E. S. *Anal. Chem.* **1994**, *66*, 929−936.
(38) Xue, Q.; Yeung, E. S. *Anal. Chem.* **1994**, *66*, 1175−1178.
(39) Chang, H. T.; Yeung, E. S. *Anal. Chem.* **1995**, *67*, 1079−1083.
(40) Tan, W.; Parpura, V.; Haydon, P. G.; Yeung, E. S. *Anal. Chem.* **1995**, *67*, 2575−2579.
(41) Orwar, O.; Fishman, H. A.; Ziv, N. E.; Scheller, R. H.; Zare, R. N. *Anal. Chem.* **1995**, *67*, 4261−4268.
(42) Lillard, S. J.; Yeung, E. S.; McCloskey, M. A. *Anal. Chem.* **1996**, *68*, 2897−2904.
(43) Lillard, S. J.; Chiu, D. T.; Scheller, R. H.; Zare, R. N.; Rodriguez-Cruz, S. E.; Williams, E. R.; Orwar, O.; Sandberg, M.; Lundqvist, J. A. *Anal. Chem.* **1998**, *70*, 3517−3524.
(44) Sims, C. E.; Meredith, G. D.; Krasieva, T. B.; Berns, M. W.; Tromberg, B. J.; Allbritton, N. L. *Anal. Chem.* **1998**, *70*, 4570−4577.
(45) Zhang, Z.; Krylov, S.; Arriaga, E. A.; Polakowski, R.; Dovichi, N. J. *Anal. Chem.* **2000**, *72*, 318−322.
(46) Krylov, S. N.; Starke, D. A.; Arriaga, E. A.; Zhang, Z.; Chan, N. W.; Palcic, M. M.; Dovichi, N. J. *Anal. Chem.* **2000**, *72*, 872−877.
(47) Meredith, G. D.; Sims, C. E.; Soughayer, J. S.; Allbritton, N. L. *Nat. Biotechnol.* **2000**, *18*, 309−312.
(48) Han, F.; Lillard, S. J. *Anal. Chem.* **2000**, *72*, 4073−4079.
(49) Krylov, S. N.; Dovichi, N. J. *Electrophoresis* **2000**, *21*, 767−773.
(50) Hu, S.; Zhang, L.; Cook, L. M.; Dovichi, N. J. *Electrophoresis* **2001**, *22*, 3677−3682.
(51) Li, H.; Sims, C. E.; Wu, H. Y.; Allbritton, N. L. *Anal. Chem.* **2001**, *73*, 4625−4631.
(52) Chen, S.; Lillard, S. J. *Anal. Chem.* **2001**, *73*, 111−118.
(53) Zabzdyr, J. L.; Lillard, S. J. *Anal. Chem.* **2001**, *73*, 5771−5775.
(54) Hu, S.; Jiang, J.; Cook, L. M.; Richards, D. P.; Horlick, L.; Wong, B.; Dovichi, N. J. *Electrophoresis* **2002**, *23*, 3136−3142.
(55) Hu, S.; Le, Z.; Krylov, S.; Dovichi, N. J. *Anal. Chem.* **2003**, *75*, 3495−3501.
(56) Han, F.; Wang, Y.; Sims, C. E.; Bachman, M.; Chang, R.; Li, G. P.; Allbritton, N. L. *Anal. Chem.* **2003**, *75*, 3688−3696.
(57) Zarrine-Afsar, A.; Krylov, S. N. *Anal. Chem.* **2003**, *75*, 3720−3724.
(58) Hu, S.; Michels, D. A.; Fazal, M. A.; Ratisoontorn, C.; Cunningham, M. L.; Dovichi, N. J. *Anal. Chem.* **2004**, *76*, 4044−4049.
(59) Li, H.; Sims, C. E.; Kaluzova, M.; Stanbridge, E. J.; Allbritton, N. L. *Biochemistry* **2004**, *43*, 1599−1608.
(60) Rosenzweig, Z.; Yeung, E. S. *Anal. Chem.* **1994**, *66*, 1771−1776.
(61) Jankowski, J. A.; Tracht, S.; Sweedler, J. V. *TrAC* **1995**, *14*, 170−176.
(62) Hofstadler, S. A.; Swanek, F. D.; Gale, D. C.; Ewing, A. G.; Smith, R. D. *Anal. Chem.* **1995**, *67*, 1477−1480.
(63) Li, P. C.; Harrison, D. J. *Anal. Chem.* **1997**, *69*, 1564−1568.
(64) Waters, L. C.; Jacobson, S. C.; Kroutchinina, N.; Khandurina, J.; Foote, R. S.; Ramsey, J. M. *Anal. Chem.* **1998**, *70*, 158−162.
(65) Inoue, I.; Wakamoto, Y.; Moriguchi, H.; Okano, K.; Yasuda, K. *Lab Chip* **2001**, *1*, 50−55.
(66) Schilling, E. A.; Kamholz, A. E.; Yager, P. *Anal. Chem.* **2002**, *74*, 1798−1804.
(67) Yang, M.; Li, C. W.; Yang, J. *Anal. Chem.* **2002**, *74*, 3991−4001.
(68) Tamaki, E.; Sato, K.; Tokeshi, M.; Sato, K.; Aihara, M.; Kitamori, T. *Anal. Chem.* **2002**, *74*, 1560−1564.
(69) Wheeler, A. R.; Throndset, W. R.; Whelan, R. J.; Leach, A. M.; Zare, R. N.; Liao, Y. H.; Farrell, K.; Manger, I. D.; Daridon, A. *Anal. Chem.* **2003**, *75*, 3581−3586.
(70) Wolff, A.; Perch-Nielsen, I. R.; Larsen, U. D.; Friis, P.; Goranovic, G.; Poulsen, C. R.; Kutter, J. P.; Telleman, P. *Lab Chip* **2003**, *3*, 22−27.
(71) Roper, M. G.; Shackman, J. G.; Dahlgren, G. M.; Kennedy, R. T. *Anal. Chem.* **2003**, *75*, 4711−4717.
(72) McClain, M. A.; Culbertson, C. T.; Jacobson, S. C.; Allbritton, N. L.; Sims, C. E.; Ramsey, J. M. *Anal. Chem.* **2003**, *75*, 5646−5655.
(73) Gao, J.; Yin, X. F.; Fang, Z. L. *Lab Chip* **2004**, *4*, 47−52.
(74) Xia, F.; Jin, W.; Yin, X.; Fang, Z. *J. Chromatogr., A* **2005**, *1063*, 227−233.

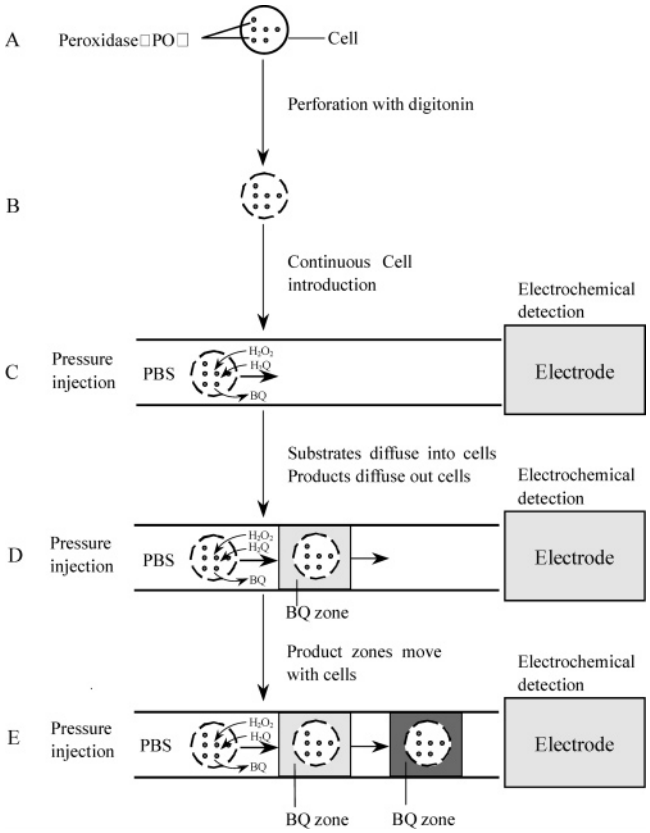

Figure 1. Schematic diagram showing the process of high-throughput ECD of PO activity in single cells.

In this work, we developed a novel high-throughput method for analysis of enzyme activity in single cells without cytolysis. Peroxidase (PO) in neutrophils was chosen as the model system. In this method, neutrophils were first perforated with digitonin that bound to cholesterol on the cell membrane to form micropores (Figure 1B). The perforated cells in physiological buffer saline (PBS) containing enzyme substrates hydroquinone (H_2Q) and H_2O_2 were continuously introduced by pressure into a capillary as the microsampler and microreactor (Figure 1C). These micropores on the cell membrane were so small that only small molecules, such as H_2Q and H_2O_2, could diffuse into the cell interior, where PO converted H_2Q into benzoquinone (BQ) at a relatively high reaction rate according to the enzyme-catalyzed reaction expressed in reaction 1 (Figure 1C, D). In this case, PO still remained inside the cell, but the electroactive BQ diffused out from the cell interior to the cell surface through the micropores and formed a BQ zone around the cell (Figure 1D, E). The BQ amount on the cell surface reflected the PO activity inside the cell. The process proceeded inside moving cells, and the BQ zones generated by the intracellular enzyme-catalyzed reaction were formed around every moving perforated cell in the capillary. The BQ zones with the moving cells were continuously delivered to the capillary outlet under hydraulic flow and detected by ECD according to reaction 2.

$$H_2Q + H_2O_2 \overset{PO}{\rightleftharpoons} BQ + 2H_2O \quad (1)$$

$$BQ + 2H^+ + 2e^- \rightarrow H_2Q \quad (2)$$

EXPERIMENTAL SECTION

Chemicals. Hydroquinone (analytical grade), benzoquinone (chemical grade), H_2O_2 (content ≥ 30%, analytical grade), digitonin, and other chemicals (analytical grade) were purchased from standard reagent suppliers. 3,3,5,5-Tetramethylbenzidine (TMB_{red}, high pure grade) and spermine were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRP, R.Z ~ 3.0, 250 units/mg) was obtained from Shanghai Lizhu Dongfeng Biotechnology Co. Ltd. (Shanghai, China). PBS consisted of 0.15 mol/L NaCl, 7.6×10^{-3} mol/L Na_2HPO_4, and 2.4×10^{-3} mol/L NaH_2PO_4 (pH 7.4). All aqueous solutions were prepared with doubly distilled water. Several steps described in our previous work[31] were taken to minimize contamination from the outside environment.

Preparation and Chemical Cell-Perforation of Human Neutrophils. Human neutrophils were isolated as described previously.[27] The neutrophil suspension was counted using a hemocytometer under an inverted biological microscope, then the suspension was diluted to 1.2×10^5 cells/mL with PBS. To chemically perforate the cell membrane, 2 μL of 2.5×10^{-4} mol/L digitonin was added to 100 μL of the neutrophil suspension at a final concentration of 5.0×10^{-6} mol/L. Fifteen minutes later, the solution was diluted to 3 mL with PBS at 4 °C. The perforated neutrophils were immediately used for high-throughput analysis of PO activity in single cells. To prepare the cell extract, a part of the perforated neutrophil suspension was subjected to freeze−thawing and ultrasonicated for cytolysis. After the cells were maintained at −20 °C in a refrigerator, the cells were taken out and kept at 37 °C for defrosting, then the cells were ultrasonicated to obtain the cell extract. Before use, the cell extract was diluted with PBS.

CE-ECD of Horseradish Peroxidase Activity in Standard Solution and PO Activity in Neutrophil Extract with an On-Capillary Enzyme-Catalyzed Reaction. The CE-ECD system was the same as that in our previous work.[34] Carbon fiber disk bundle electrodes (CFDBEs) as the working electrode were constructed by the same method as that of our previous work.[77] The activity of all peroxidases is defined and determined with the same method. Therefore, HRP served as a standard to quantify PO activity, and the same activity unit for both HRP and PO was used in this work. CE-ECD of both HRP activity in the standard solution and PO activity in the neutrophil extract was carried out in an uncoated capillary that had been flushed with 0.1 mol/L NaOH for 60 min and water for 5 min by means of a syringe, then the wall of the capillary was modified with PBS containing 5.00×10^{-5} mol/L spermine for 60 min. Next, CE running buffer (PBS containing 5.0×10^{-4} mol/L H_2Q, 8.0×10^{-4} mol/L H_2O_2, and 5.00×10^{-5} mol/L spermine) was introduced for 10 min. After the electroosmotic flow reached a constant value, the electromigration injection of the standard HRP or the extract was carried out by an injection voltage of 5.0 kV and an injection time of 10 s. The capillary was carefully moved from the solution into a reservoir containing the CE running buffer. A high voltage of 20 kV for 60 s was applied to mix HRP or PO with its substrates. The high voltage was turned off, and HRP or PO was incubated with the enzyme substrates for 10 min. Finally, the electroactive product BQ of the enzyme-catalyzed reaction was electromigrated

(75) Tan, W.; Yeung, E. S. *Anal. Biochem.* **1995**, *226*, 74−79.
(76) Xue, Q.; Yeung, E. S. *J. Chromatogr., B* **1996**, *677*, 233−240.
(77) Jin, W.; Yu, D.; Dong, Q.; Ye, X. *Electrophoresis* **2000**, *21*, 925−929.

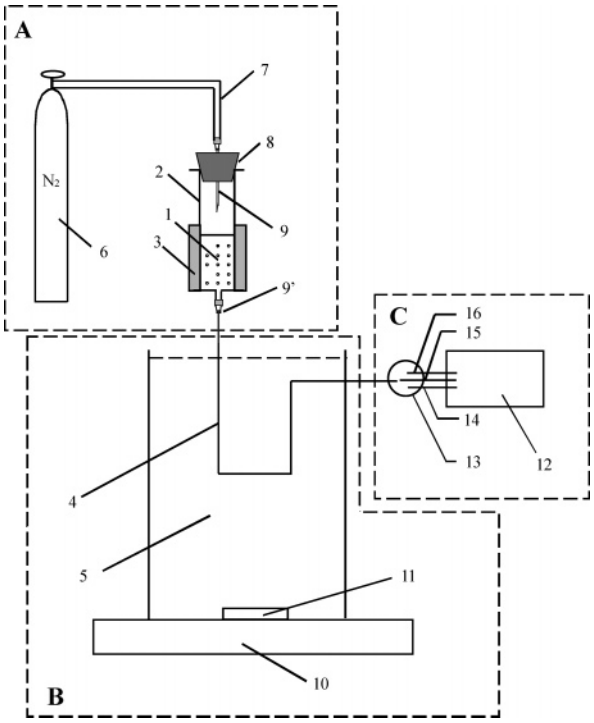

Figure 2. Overview of the high-throughput ECD system for enzyme activity inside single cells. (A) Cell injection device, (B) reaction setup, and (C) electrochemical detector. 1, cell suspension; 2, cell injector (syringe); 3, ice bath; 4, capillary; 5, warm water bath; 6, N_2 tank; 7, plastic tube; 8, rubber stopper; 9 and 9′, syringe needle; 10, electrothermal stirrer; 11, rotating magnet bar; 12, electrochemical analyzer; 13, electrochemical cell; 14, reference electrode; 15, working electrode; 16, auxiliary electrode.

to the outlet of the capillary by a high voltage of 20 kV and electrochemically detected by a CFDBE at a constant potential of −0.20 V versus SCE.

High-Throughput ECD of PO Activity inside Single Cells. The high-throughput ECD system for determination of PO activity inside single cells is illustrated in Figure 2. It consisted of three parts: a cell injection device (A), a reaction setup (B), and an electrochemical detector (C). In this cell injection device, a plastic syringe of 5 mL served as the cell injector (2), in which the piston was replaced by a rubber stopper (8) with a hole. A syringe needle (9) was inserted into the hole of stopper for introduction of nitrogen. The syringe needle was connected to the nitrogen tank through a plastic tube (7). The needle (9′) of the syringe (2) was connected with the capillary (4) (50 μm i.d., 45 cm in length, from Yongnian Optical Conductive Fiber Plant, Yongnian, China) as the reactor. The syringe (2) was placed in an ice bath (3). The capillary (4) was passed through the warm bath of 37 °C (5). ECD was performed with an electrochemical analyzer (12) (model CHI800, CH Instruments, Austin, TX). ECD was carried out with a three-electrode system that consisted of a CFDBE (15) as the working electrode, a saturated calomel electrode (SCE) (14) as the reference electrode, and a coiled Pt wire (16) (0.5-mm diameter, 4 cm in length) as the auxiliary electrode. The arrangement of the electrochemical detection cell with the three electrodes was previously illustrated in detail.[78] The electrochemical detection cell (13) was housed in a Faraday cage to minimize the interference from noise from external sources. For the high-throughput ECD of PO activity inside single cells, the suspension of perforated neutrophils was placed in the cell injector containing PBS with 5.0×10^{-4} mol/L H_2Q and 8.0×10^{-4} mol/L H_2O_2 at 4 °C. To lower the enzyme-catalyzed reaction rate, the cell injector was put in an ice bath. The perforated neutrophils were continuously propelled by nitrogen through the capillary immersed in the warm water bath of 37 °C, and the BQ zones around every moving cell were detected by the CFDBE at −0.20 V versus SCE at the capillary outlet.

Microfluidic Chip-ECD of PO Activity inside Individual Neutrophils. To obtain the concentration ratio of the product of the enzyme-catalyzed reaction generated by a perforated cell and by the "released" PO from the same cell after cytolysis, microfluidic chip-ECD was used. The microfluidic chip-ECD system with a double-T injector was the same as that in our previous work.[74] The experimental process is illustrated in Figure 3. The microfluidic chip was first pretreated with NaOH, water, and the running buffer. After 50 μL of the perforated neutrophil suspension was injected into the sample reservoir by a syringe, the electrokinetic loading of a single perforated neutrophil was immediately performed. In the loading mode, an electric field strength of 100 V/cm was applied at the sample reservoir with the sample waste reservoir grounded and other reservoirs floating. Neutrophils could move into the sample channel by electrokinetic flow. When a cell was seen in the cross between the sample channel and the separation channel under an inverted microscope with a magnification of 400×, the injection electric field was turned off, and the motion rate of the cell was decreased. At the same time, the liquid level in the buffer reservoir was lowered by removing the buffer to allow the cell to load in the double-T injector (Figure 3B). Then the liquid level of the buffer reservoir was restored by adding the buffer. After a perforated cell was attached on the channel filled with PBS containing 5.0×10^{-4} mol/L H_2Q and 8.0×10^{-4} mol/L H_2O_2, the perforated cell was incubated for 5 min with the substrates in PBS (Figure 3C) to form a BQ zone around the cell (Figure 3D). To separate the attached cell and the BQ zone around the cell (BQ zone I), an electric field strength of 100 V/cm was applied to the buffer reservoir for 30 s with the detection reservoir grounded and both sample reservoir and sample waste reservoir floating (Figure 3E). Then the electric field strength at the buffer reservoir was switched to 400 V/cm. The cell was lysed rapidly under the high electric field strength. The electric field was turned off, and the "released" PO from the lysed cell was incubated for 5 min with the substrates, forming a BQ zone II (Figure 3F). An electric field strength of 100 V/cm was applied, and both BQ zones I and II were electromigrated to the outlet of separation channel and detected (Figure 3G). Thus, an electropherogram with two peaks corresponding to zones I and II was obtained (Figure 3H). The concentration ratio of BQ for both situations without and with cytolysis could be calculated from both electrophoretic peak areas of BQ zone II and I.

RESULTS AND DISCUSSION

Chemical Cell Perforation. Chemical cell perforation with digitonin is often used to improve the cell permeabilization. Many biochemical processes, such as tyrosine hydroxylase activity and phosphorylation,[79,80] import and export of the nuclear protein,[81−84] and nuclear permeability in apoptosis,[85] are investigated by using

(78) Jin, W.; Weng, Q.; Wu, J. *Anal. Chim. Acta* **1997**, *342*, 67−74.

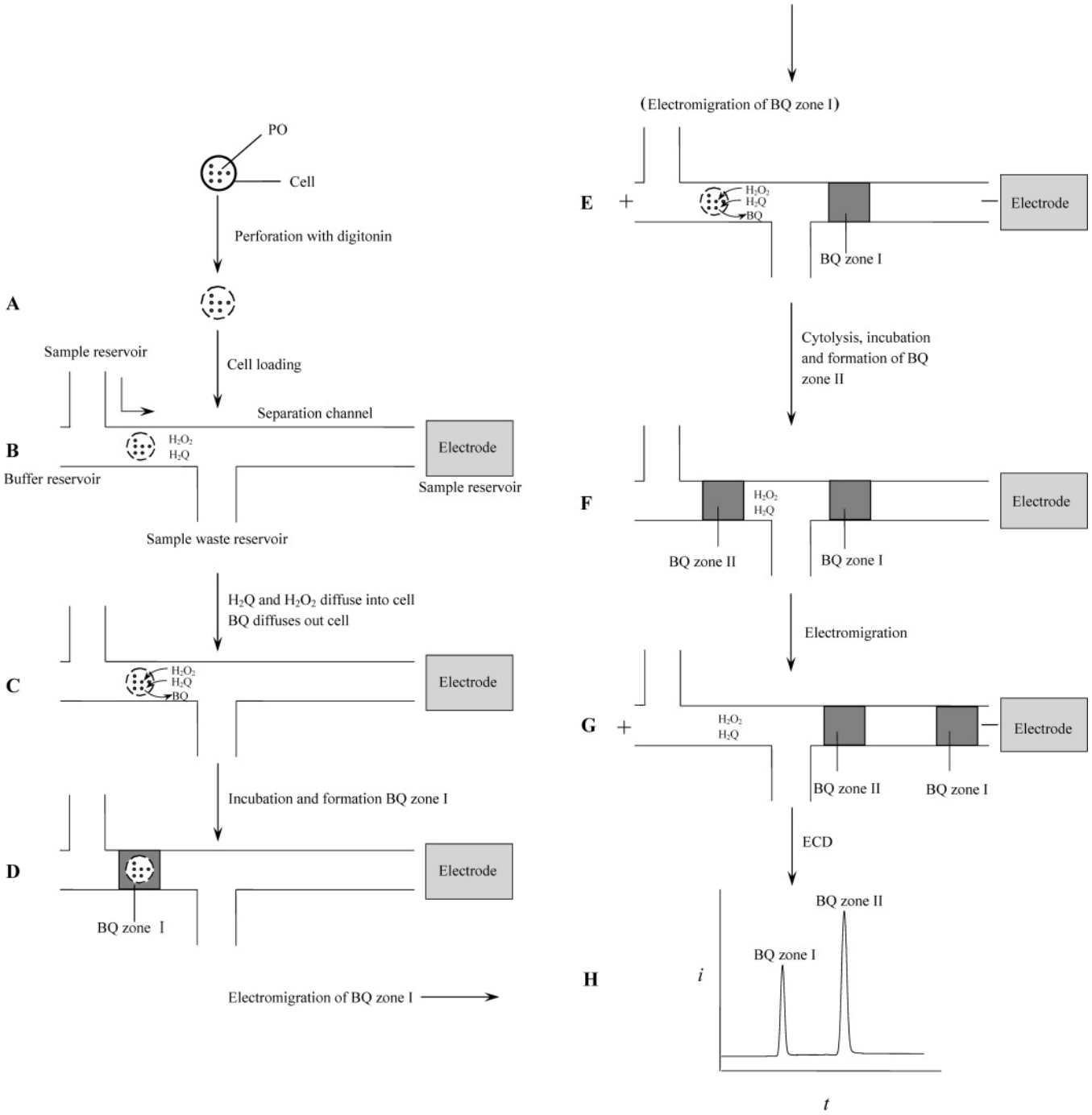

Figure 3. Schematic diagram showing the process of microfluidic chip-ECD of PO activity in single cells without and with cytolysis.

the digitonin-permeabilized cells. To know whether micropores were formed on the cell membrane for neutrophils after incubation with digitonin, trypan blue staining was used. Since cells without micropores are able to pump out this blue dye, no blue appears for the cells in the solution containing trypan blue. However, trypan blue can enter perforated cells through the micropores on the cell membrane. Accordingly, the perforated cells should be blue when they mix with trypan blue. It was found that in the presence of 0.05% trypan blue and 5.0×10^{-6} mol/L digitonin, neutrophils were blue in 5 min, implying formation of the micropores.

Determination of HRP Activity and PO Activity in Neutrophil Extract with the On-Capillary Enzyme-Catalyzed Reaction by CE-ECD. Both PO and HRP are peroxidases and can catalyze the same substrates by the same mechanism. Since

(79) Gonçalves, C. A.; Hall, A.; Sim, A. T. R.; Bunn, S. J.; Marley, P. D.; Cheah, T. B.; Dunkley, P. R. *J. Neurochem.* **1997**, *69*, 2387−2396.
(80) Cheah, T. B.; Bobrovskaya, L.; Gonçalves, C. A.; Hall, A.; Elliot, R.; Lengyel, I.; Bunn, S. J.; Marley, P. D.; Dunkley., P. R. *J. Neurosci. Methods* **1999**, *87*, 167−174.
(81) Adam, S. A.; Sterne-Marr, R.; Gerace, L. *J. Cell Biol.* **1990**, *11*, 807−816.
(82) Moroianu, J.; Blobel, G.; Radu, A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9699−9704.
(83) Nakielny, S.; Dreyfuss, G. *Curr. Biol.* **1997**, *8*, 889−95.
(84) Love, D. C.; Sweitzer, T. D.; Hanover, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10608−10613.
(85) Roehrig, S.; Tabbert, A.; Ferrando-May, E. *Anal. Biochem.* **2003**, *318*, 244−253.

the principle of the high-throughput single-cell analysis described here is similar to CE, CE-ECD of HRP was used to obtain the optimal conditions for detection of PO activity. HRP can be strongly adsorbed on the wall of an uncoated capillary. Usually, the capillary has to be modified to reduce adsorption.[86] In this work, spermine was added to the running buffer to overcome the influence of HRP adsorption on the electropherograms. It was found that PBS containing 5.0×10^{-4} mol/L H_2Q, 8.0×10^{-4} mol/L H_2O_2, and 5.00×10^{-5} mol/L spermine (pH 7.4) for the CE running buffer; 20 kV for the separation voltage; and -0.20 V versus SCE for the detection potential were suitable for the detection of HRP. The electrophoretic peak area, q, could be used to quantify PO activity. The linear range of HRP activity concentration (i.e., PO activity concentration) was 2.00×10^{-3} to 2.00×10^{-1} unit/mL, with a correlation coefficient of 0.997. The activity concentration LOD calculated from q obtained for the activity concentration at the low end of its linear range was 1×10^{-3} unit/mL, when the signal-to-noise ratio was 3. Its activity linear range and activity LOD were 2.2×10^{-9} to 2.2×10^{-7} unit and 2×10^{-9} unit, respectively, calculated according to the injection volume of 1.1 nL, The relative standard deviations (RSDs) of the method were 1.2% for t_m and 2.2% for q ($n = 3$).

Only one peak appeared on the electropherogram of the neutrophil extract. The peak could be identified as the peak corresponding to PO on the basis of the migration time, as compared with the electropherogram of HRP. It was noted that no other peaks from other electroactive compounds in the cell extract were detected. The possible reasons were that they had escaped from the cells through the micropores on the cell membrane during chemical cell perforation and washing of the perforated cells, or their concentrations were too low, or the detection potential was unsuitable for these components. The mean activity concentration of PO in the extract obtained by the standard calibration method was 6.78×10^{-3} unit/mL. To prove the reliability of the method, a certain amount of standard HRP was added to the extracts, and then the extracts were measured. From the detected activity concentrations in the extracts with and without the standard HRP, the recoveries calculated were between 96 and 108%. According to the mean activity concentration determined and the cell concentration of 7.9×10^5 cells/mL, the calculated mean PO activity in a single neutrophil was 8.6×10^{-9} unit with an RSD of 2.5% for the extract sample (sample I).

High-Throughput ECD of PO Activity inside Single Cells. To know whether PO stays in perforated cells, peroxidase staining was adopted. Peroxidases can catalyze the oxidation of the substrate 3,3,5,5-tetramethylbenzidine (TMB_{red}) to its oxidized form TMB_{ox} in the presence of H_2O_2. The reaction product TMB_{Ox} appears blue. This reaction is very sensitive and is widely used for detection of peroxidases in ELISA.[87] Here, we used the method to monitor PO. After neutrophils were perforated for 15 min in PBS containing 5.0×10^{-6} mol/L digitonin, the perforated neutrophils were removed. No blue color appeared in the supernatant that mixed with 2.0×10^{-4} mol/L TMB_{red} and 1.00×10^{-3} mol/L H_2O_2, indicating that there was no PO in the supernatant. However, when TMB_{red} and H_2O_2 were added into the cell suspension containing the perforated cells, blue color was clearly observed. In this case, TMB_{red} and H_2O_2 entered the cells through the micropores on the cell membrane, and TMB_{red} was oxidized to blue TMB_{ox} by PO within cells. These results indicated that PO remained in the perforated cells and did not escape from perforated cells.

For the high-throughput single-cell analysis, the characteristics of the peak of the BQ zone on the elution curve depended on the delivery time of a single cell, t_d, in the capillary. A long t_d could result in a high peak due to a long reaction time, which could enhance the detection sensitivity, and result in a wide peak due to the diffusion of BQ along the longitudinal axis of the capillary, which could reduce the peak resolution. In addition, a long t_d decreased the analysis throughput. In the present high-throughput experiments, a t_d of 5 min was suitable for a sufficiently high and narrow peak. The high-throughput detection of PO activity within single neutrophils was according to the process illustrated in Figure 1. The perforated neutrophils were mixed with PBS containing enzyme substrates H_2Q and H_2O_2 in the cell injector shown in Figure 2. Then they were continuously introduced through the capillary by nitrogen pressure as the microsampler and microreactor. When perforated cells moved in the capillary, H_2Q and H_2O_2 diffused into the cells, and BQ produced by the intracellular enzyme-catalyzed reaction diffused out from the cells through the micropores on the cell membrane. The BQ zones were formed around every cell and synchronously moved with the cells toward the capillary outlet. The BQ zones were continuously detected at the working electrode positioned at the capillary outlet. In these experiments, a 50-μm-i.d. capillary was used to reduce the solution resistance in the capillary. It could be noted that the lower the cell concentration was, the larger the interval between peaks was (see Figure 4), which would reduce the analysis throughput. However, higher cell concentrations would lead to the peaks' overlapping each other. In this case, the peak would be 2-fold wider than that of a single cell, which could be obtained from the elution curve of lower cell concentrations. A cell concentration of 4.0×10^3 cells/mL was found be appropriate for the high-throughput detection of PO activity in single cells. In this case, the peaks could be well-separated with almost the same peak width. When the temperature of the whole detection system was 37 °C, the blank current from BQ produced in the system made the baseline increase with increasing run time (see Figure 4). This was because of the direct oxidation of H_2Q to BQ by oxygen, peroxide itself, and trace metals, as well as PO inside the perforated cells in the system, especially in the cell injector. To decrease the BQ amount in the cell injector via decreasing the enzyme-catalyzed reaction rate, both cell suspension and PBS containing H_2Q and H_2O_2 at 4 °C were used, and the cell injector was placed in an ice bath. To increase the rate of the intracellular enzyme-catalyzed reaction in the capillary, the capillary as the microreactor was passed through a warm water bath of 37 °C, as shown in Figure 2. When PBS containing H_2Q and H_2O_2 in the cell injector was continuously propelled through the capillary and was detected using the detection system, the blank baseline did not increase with time, implying that BQ produced by direct oxidation of oxygen, peroxide itself and trace metals in the system could be neglected. Figure 5 shows a segment of 32 min on the elution curve of intact neutrophils, which were continuously introduced through the capillary by nitrogen pressure. The first

(86) He, Z.; Wang, H.; Sun, X.; Jin, W. *Electroanalysis* **2002**, *14*, 1674−1678.
(87) Frey, A.; Meckelein, B.; Externest, D.; Schmidt, M. A. *J. Immunol. Methods* **2000**, *233*, 47−56.

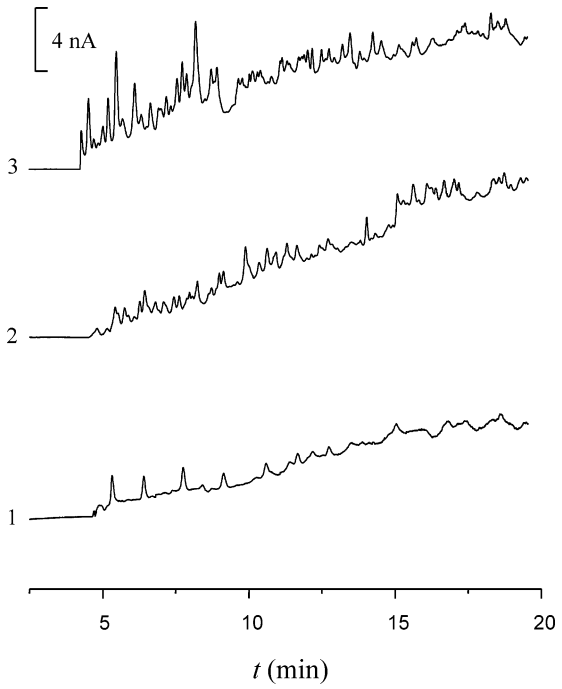

Figure 4. Continuously recorded elution curves of perforated neutrophils at different cell concentrations. Cell concentration (cells/mL): (1) 4.0×10^3, sample I; (2) 5.0×10^3, sample II; (3) 10×10^3, sample IV. Perforation for 15 min with 5.0×10^{-6} mol/L digitonin. Capillary: 45 cm in length, 50-μm i.d.; running buffer, PBS containing 5.0×10^{-4} mol/L H_2Q and 8.0×10^{-4} mol/L H_2O_2 (pH 7.4); detection potential, −0.20 V versus SCE; temperature of cell injector, ~4 °C; temperature of capillary, 37 °C.

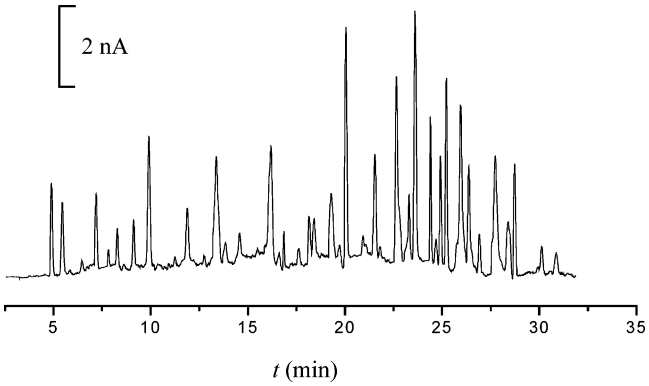

Figure 5. Continuously recorded elution curve of human perforated neutrophils (sample I). Cell concentration, 4.0×10^3 cells/mL; other conditions as in Figure 4.

peak appeared at ~5 min. Resolved peak envelopes from 39 cells were analyzed in this run for an average detection rate of >1 cell/min. The baseline was very flat, and all peaks could be separated well. Since the mean PO activity in a cell determined using the high-throughput analysis was the same as that determined for the cell extract, the PO activity in single cells, A_s, could be calculated according to eq 3 from the mean peak area, q_m, and each peak area, q_c, as well as the mean PO activity in a cell, A_m, determined for the cell extract. For the neutrophil sample (sample I) shown in Figure 5, q_m and A_m were 1.5×10^{-8} C and 8.6×10^{-9} unit, respectively. The A_s values in the 39 neutrophils were $(1.1-28) \times 10^{-9}$ unit, which had the same order of magnitude compared

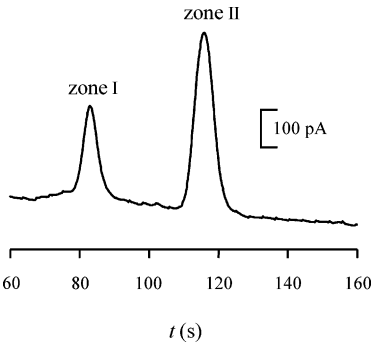

Figure 6. Electropherograms of a single perforated human neutrophil without and with cytolysis by using microfluidid chip-ECD. The intracellular enzyme-catalyzed reaction for 5 min for a perforated neutrophil and then the in-channel enzyme-catalyzed reaction for 5 min for the "released" PO from the lyzed neutrophil (sample V). Other experimental conditions: CE running buffer, PBS containing 5.0×10^{-4} mol/L H_2Q and 8.0×10^{-4} mol/L H_2O_2 (pH 7.4); separation electric field strength, 100 V/cm; detection potential, −0.20 V versus SCE.

with the values (6.5−21 nU) of another sample measured by voltammetry.[88]

$$A_s = A_m \, q_c / q_m \tag{3}$$

Error Analysis. In the method, the electrochemical response may depend on membrane features such as the membrane area determined by cell size; the number, size, and thickness of the micropores; and the kinetic behavior of intermembrane transport of the enzyme substrates through micropores, as well as the detection conditions. To determine the effects of these factors on the detected peak area that reflected the PO activity inside perforated cells, we designed an experiment to evaluate the effects using microfluidic chip-ECD. The details of the experiment were described in Experiment Section. After a single perforated cell attached in the separation channel of microfluidic chip was incubated for 5 min with H_2Q and H_2O_2, an electric field strength of 100 V/cm was applied to the separation channel for 30 s to separate the attached cell and the BQ zone (BQ zone I) around the cell (Figure 3E), which was produced by the intracellular enzyme-catalyzed reaction. Then the cell was lysed under a high electric field strength. When the electric field strengths were higher than 400 V/cm, the cell could be lysed easily. The cytolysis of the cell took ~5 s using an electric field strength of 400 V/cm. The "released" PO from the lysed cell was incubated for 5 min with H_2Q and H_2O_2 again. Another BQ zone (BQ zone II) was formed through the in-channel enzyme-catalyzed reaction (Figure 3F). When an electric field strength of 100 V/cm was applied, both BQ zones were migrated to the detection end of separation channel and were detected at the channel outlet (Figure 3G). Two electrophoretic peaks corresponding to both BQ zones were recorded. Figure 6 shows a typical electropherogram of both BQ zones for a single neutrophil. The peak areas of both electrophoretic peaks (q_I for BQ zone I and q_{II} for BQ zone II) corresponded to the same PO activity of the cell. The peak area of zone I was smaller than that of zone II, as expected. One reason

(88) Gao, N.; Zhao, M.; Zhang, X.; Jin, W. *Anal. Chem.* **2006**, *78*, 231−238.

Table 1. Peak Area of BQ Zone I, q_I; Peak Area of BQ Zone II, q_{II}; and Peak Area Ratio, q_{II}/q_I, Using Microfluidic Chip-ECD (SampleV)

cell	q_I (nC)	q_{II} (nC)	q_{II}/q_I
1	0.332	0.810	2.44
2	0.915	2.47	2.70
3	1.06	3.31	3.12
4	3.18	8.38	2.64
5	0.461	1.15	2.49
6	4.35	12.1	2.78
7	0.528	1.60	3.03
8	1.57	4.03	2.57

was because BQ in zone I was from the cell interior via the micropores on the cell surface during the intracellular enzyme-catalyzed reaction, but BQ in zone II was directly generated via the in-solution enzyme-catalyzed reaction. Another reason was the environment difference of the enzyme between the cell and PBS. It was observed that the q_I and q_{II} values of each cell were different from cell to cell, with a maximum difference of 13-fold for q_I or 15-fold for q_{II} (Table 1). The RSD of q_{II}/q_I with a mean value of 2.7 was 8.7% ($n = 8$), which reflected the total RSD from the microfluidic chip-ECD method and the cell difference. We have known that when the microfluidic chip-ECD was used to determine PO in the neutrophil extracts, the RSD of the method was 2.7%, which was equivalent to that (2.5%) obtained by using capillary-ECD described here. Therefore, the RSD from the cell difference was estimated to be ~6%.

CONCLUSION

The enzyme activity in single cells can be determined without cytolysis by a combination of chemical cell perforation and the intracellular enzyme-catalyzed reaction. Cells are chemically perforated with digitonin before detection. A capillary as the microsampler and microreactor is used to perform the high-throughput analysis of PO activity in single neutrophils. An average detection rate of >1 cell/min can be achieved. Our studies demonstrate conclusively the feasibility of quantitative analysis of the enzyme activity in single cells without cytolysis. In this method, the biological macromolecules that can be adsorbed on the capillary wall or on the working electrode do not escape from cells and do not interfere with the determination of enzyme activity. Not only the precision of the results can be improved, but also the high-throughput single-cell analysis for enzyme activity can be realized without microchannel treatment between runs. Although we perform this work using ECD, other detection approaches, such as LIF detection, can also be applied, if suitable enzyme substrates are chosen. It should be noted that the method is limited to the assaying of cytosolic enzymes in cells that do not contain cell walls.

ACKNOWLEDGMENT

This project was supported by the National Natural Science Foundation of China (Grant No. 20235010 and 20475033).

Received for review February 13, 2006. Accepted March 15, 2006.

AC060278V