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Received July 10, 2008
Revised October 15, 2008
Accepted November 10, 2008

Research Article

Red blood cell quantification microfluidic chip using polyelectrolytic gel electrodes

This paper reports on a novel microfluidic chip with polyelectrolytic gel electrodes (PGEs) used to rapidly count the number of red blood cells (RBCs) in diluted whole blood. The proposed microdevice is based on the principle that the impedance across a microchannel between two PGEs varies sensitively as RBCs pass through it. The number and amplitude of impedance peaks provide the information about the number and size of RBCs, respectively. This system features a low-voltage dc detection method and non-contact condition between cells and metal electrodes. Major advantages include stable detection under varying cellular flow rate and position in the microchannel, little chance of cell damage due to high electric field gradient and no surface fouling of the metal electrodes. The performance of this PGEs-based system was evaluated in three steps. First, in order to observe the size-only dependence of the impedance signal, three different sizes of fluorescent microbeads (7.2, 10.0, and 15.0 μm; Bangs laboratories, USA) were used in the experiment. Second, the cell counting performance was evaluated by using 7.2 μm fluorescent microbeads, similar in size to RBCs, in various concentrations and comparing the results with an animal hematoanalyzer (MS 9-5; Melet schloesing laboratories, France). Finally, in human blood sample tests, intravenously collected whole blood was just diluted in a PBS without centrifuge or other pretreatments. The PGE-based system produced almost identical number of RBCs in over 800-fold diluted samples to the results from a commercialized human hematoanalyzer (HST-N402XE; Sysmex, Japan).

Keywords:
Hematoanalyzer / Microfluidic chip / Polyelectrolytic gel electrode / Red blood cells
DOI 10.1002/elps.200800448

1 Introduction

Attention is increasingly being paid to live cells in biological research as well as the clinical diagnosis of diseases. The quantity, shape, and size of the cells in human blood are very important factors in the sustained metabolism of a human body. That is why a large part of cell research and clinical diagnosis involves the quantification of blood cells [1–3]. In particular, the red blood cell (RBC) count (the number of RBCs *per* volume of blood) is a key indication of clinical problems such as anemia, so that the demand for handy, fast, and cost-effective RBC counters is high and continues to grow. For this purpose, a number of conventional methods of cell counting, including manual, optical, and Coulter counter methods, have been developed and are being used [4]. The manual method is most widely used in biological laboratories. In this method, operators count the number of cells in a certain volume visually, through a microscope [5, 6]. However, this method is very time-consuming and may incur significant error. The optical methods, frequently used in fluorescent-activated cell sorters, generally employ lasers of different wavelengths to extract information about the size, shape, and/or internal structure from each cell's fluorescence, scattered light, and transmitted light. The disadvantages of this method include the relatively high cost and difficulty in miniaturization of the optical equipment. Most commercialized automatic blood cell counters adopt the Coulter counter method. It was developed initially by Wallace H. Coulter [7] but is also referred to as the Aperture-Impedance Method [8]. In this method, a small aperture between two electrodes is under an electric field and the impedance change at this aperture is monitored as micro-particles, including cells, pass through [9, 10]. The Coulter

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Abbreviations: EDTA, ethylenediamine tetraammonium salt; **HMDS,** hexa methyl disilazane; **PGE,** polyelectrolytic gel electrode; **PR,** photoresist; **RBC,** red blood cell

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counter is more cost-effective, less time-consuming, and easier to miniaturize than other methods, even though it is not as informative or accurate as the optical method.

With recent advances in micromachining technology, many researches on the development of microfluidic chip-based Coulter counters are currently being carried out [11–16]. In these chip-based systems, the impedance change is monitored in the same way as for macro-scale systems, using a pair of metal electrodes across an aperture fabricated inside a microfluidic chip [17]. Miniaturized flow cytometers are also being attempted based on the fact that the scattered laser light [18–21] and fluorescent emissions provide valuable information about the objects, such as size, number, protein expression, *etc*. [22]. Recently, we have been seeing an acceleration in the development of microfluidic chip flow cytometry [23–25].

Although the chip-based cell counter remarkably contributed to improve the performance and miniaturization of cell counting devices, there are still a few problems that should be addressed. The conventional microfluidic Coulter counters measure the impedance changes between the coplanar metal electrodes in the respective chambers or two facing electrodes on opposite walls of the channel [13, 19]. When two electrodes are in the chambers that are connected *via* a small hole along which the electric field is applied, multiple cells may happen to be in the pore at the same time passing in a row with a short interval and thus give rise to apparent one single signal, the so-called coincident effect. Considering the size of RBCs and the pore, it is not negligible. This may lead to erroneous counting, which takes place more frequently at higher flow rate or higher concentration of the cells. One of the attempts to figure out this problem was to use a pair of electrodes facing each other on the pore wall. However, as shown by many reports, it is difficult to fabricate the well-defined solid-state electrodes in such an arrangement. Even if such electrodes can be made, only ac current with high frequency is allowed to obtain the data for impedance analysis because the largest part of electric dc or low-frequency potential drops across the electrode surface and thus the presence of a cell between the electrodes does not bring about significant change in impedometry.

The present study proposes a simple dc impedance analysis system to rapidly count the number of RBCs, by incorporating polyelectrolytic gel electrodes (PGEs) in the microchannel wall on a microfluidic chip. When an electric field is applied across a pair of PGEs spaced the width of the microchannel apart, the counter ions of the polyelectrolytes carry the charge. This unique system has been utilized to implement cytometry and velocimetry in a microfluidic chip [14], and an effective micro-mixer has been constructed based on the ion depletion between PGEs [26]. The PGEs located between the microchannel and the electrode chamber prohibit not only direct contact between metal electrodes and diluted blood, but also aggregation of the blood cells or bubbles at the electrode surface. As a result, cells passing through the microchannel between the PGEs produce significant impedance changes very sensitively for their size [13, 27]. Furthermore, this method is expected to require a far smaller blood volume (~2 μL) and to work with a lower dilution factor than the Aperture-Impedance Method, where a dilution factor of 20 000 is necessarily required to reduce the coincident effect [28].

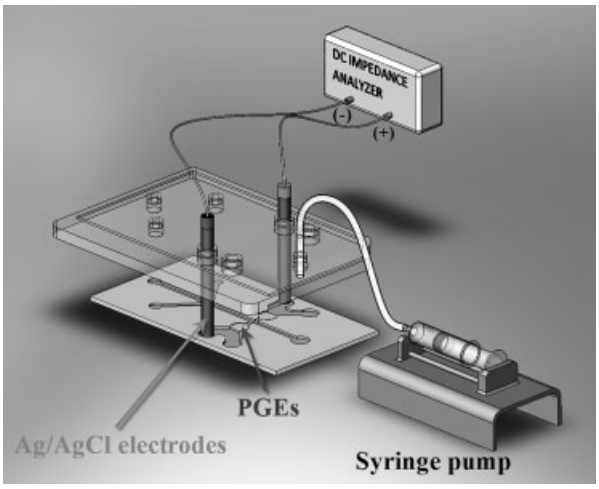

Figure 1. Schematic of the proposed microfluidic chip: the microchannels etched on a glass substrate are 70 μm wide and 30 μm deep.

Figure 1 shows a schematic diagram of the developed PGE-based system. Anions, Cl^- ions in the present system, flow through the PEGs upon 0.4 V dc biased between two Ag/AgCl electrodes in 1 M KCl internal filling solution. When blood cells pass through the microchannel between the PGEs, the impedance across them changes abruptly to generate sharp negative peaks in the current waveform. The number of these impedance peaks corresponds exactly to that of the blood cells passing through. This study shows a possibility of a reliable chip-based RBC counter using whole blood without any separation or pretreatment, except dilution, by comparing the results with a commercialized hematoanalyzer.

2 Materials and methods

2.1 Microfluidic chip fabrication

Figure 2 shows the developed microfluidic chip, with 70 μm wide and 30 μm deep microchannels fabricated using only standard photolithograpic techniques. A slide glass (no. 2947; Corning) was soaked in a piranha solution (H_2O_2:H_2SO_4 = 1:3) and cleaned for 45 min. After cleaning with the piranha solution, it was washed with deionized (DI) water (NANOpure Diamond, Barnstead, USA) and then with acetone (CMOS grade; J.T.Baker, USA) and methanol (CMOS grade; J.T.Baker). This process was repeated three times. Any moisture was completely removed from the glass with clean air, and the glass was placed on a hot plate at

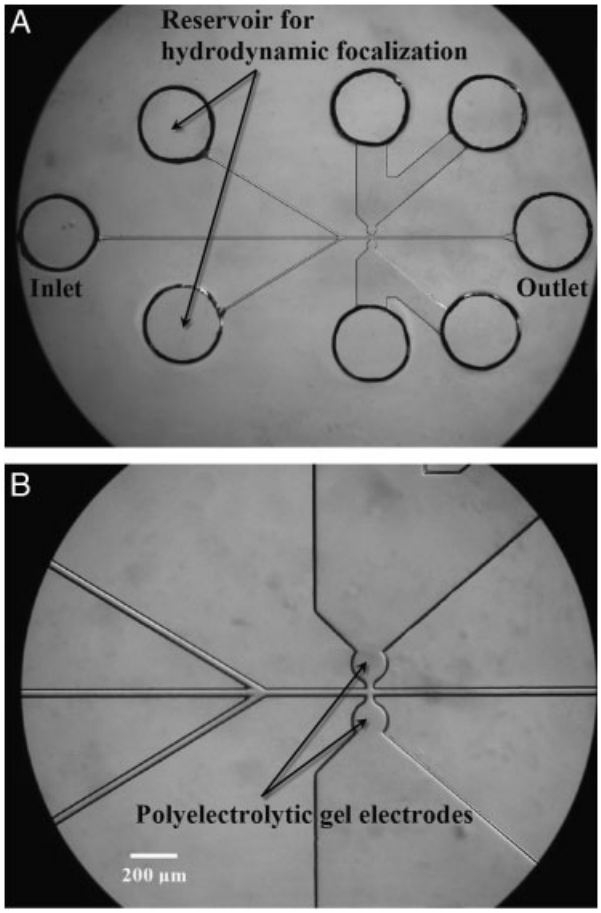

Figure 2. (A) The fabricated microfluidic glass chip. The sample flow is hydrodynamically focused between two sheath flows to reduce coincident effect and thereby to operate with a lower dilution factor. (B) A pair of PGEs are faced each other on the microchannel wall perpendicular to the flow direction.

150°C for 100 min. The glass was then cooled at room temperature for 90 s. Next, the glass was coated with HMDS (Hexa methyl disilazane; Clariant, Switzerland) using a spin coater (Won, Korea) at 6000 rpm for 30 s. HMDS helps stronger adherence of photoresist (PR) to the glass. The glass coated with HMDS was placed on a hot plate at 120°C for 3 min and cooled at room temperature for 90 s. PR AZ4620 (Clariant) was spread on the glass using a spin coater. The spin coater was activated at 500 rpm for 7.5 s and at 6000 rpm for 30 s. Following this, the PR-coated glass was soft baked on a hot plate of 100°C for 90 s and cooled at room temperature for 90 s. Then the designed pattern was written on a mask film (accumax photoplotter film; Kodak, USA) using a high-resolution mask printer, silverwriter (Mania barco, Germany). The mask film and the glass were aligned using a UV aligner (MDA-400M; Midas, Korea) and exposed to UV light for 10 s. The exposed PR was developed by immersing it into the AZ400K developer (Clariant), which was replaced by a new one every 90 s to prevent the PR from being overdeveloped. Before being soaked in a new developer solution, the glass surface was cleaned with DI water and dried with clean air, and this process was repeated three times. The degree of development was checked through a microscope, and then the glass was hard baked on a hot plate of 150°C for 15 min. After sufficient cooling at room temperature, the glass was etched for 45 min using 6:1 buffered oxide etch solution (J.T.Baker) and was washed with DI water and an ultrasonic cleaner (351OE-DTH; Brasonic, USA) for 15 min to remove impurities remaining on the pattern. Then the electrode reservoir and channel inlet/outlet holes were drilled where necessary. For bonding, a cover glass (no. 2947; Corning, USA) of the same size as the patterned glass was prepared and washed in a piranha solution for 45 min. Washing sequentially with DI water, acetone, and methanol allowed the complete removal of moisture. The cover glass placed on the patterned glass was kept in a 600°C furnace (CRF-M15; CEBER, Korea) for 5 h. The bonded glass chip was stored in a desiccator at constant temperature and humidity.

2.2 PGE fabrication

The PGEs were fabricated using photopolymerization technique, which was based on our previous research [14]. Before polymerization, for covalent linkage between the polymer and the substrate, the microchannels were coated with 3-(trimethoxysilyl)propyl methacrylate under dark conditions at room temperature for 1 h. The 3-(trimethoxysilyl)propyl methacrylate-coated microchannels surface was then washed with methanol (J.T.Baker). Diallyldimethylammonium chloride (Sigma-Aldrich, USA) was used as the monomers, which was prepared with a photoinitiator (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone; Sigma-Aldrich) and a cross-linker (*N*,*N*′-methylene-bisacrylamide; Sigma-Aldrich) making up 2% of the monomer solution. The aqueous diallyldimethylammonium chloride solution was injected into the microchannels through the reservoirs of the microfluidic chip. The mask and microchip were aligned using a UV aligner (MDA-400M; Midas) to determine the desired location of PGE formation and then were exposed to UV light (365 nm) of 20 mW/cm^2 intensity for 4.0 s. Finally the PGEs and microchannels were washed with 1 M KCl electrolyte solution and stored in 1 M KCl before use.

2.3 Instrumentation

A 0.4 V dc bias that was generated by external self-developed dc impedance analyzer produced approximately 13 μA dc current and applied between two Ag/AgCl electrodes immersed into isotonic 1 M KCl solution in the PGEs on the microfluidic chip. The amount of AgCl on the Ag/AgCl electrode was sufficient to maintain the ionic current between the PGEs at least 1 h. The dc impedance analyzer

converted the impedance change induced by the microbeads or cells passing along the microchannel between the PGEs into voltage pulses. An ac coupling circuit removed the dc component and then the impedance signal was amplified 2000 times by operational amplifier parts; the voltage pulses were digitized with a 50 kHz sampling rate. The number of microbeads or blood cells were counted using a self-developed Labview (National Instrument, Labview 8.2) program.

2.4 Sample preparation

To evaluate the performance of the PGE-based system, we used fluorescent microbeads that were dispersed in PBS at pH 7.4 and well recognized individually by an inverted fluorescence microscope (TE2000-U; Nikon, Japan). The average diameters of the beads (Bangs laboratories, USA) were 7.2, 10.0, and 15.0 μm whose coefficients of variation were less then 10% in order to calibrate the impedance signal amplitude as a function of the bead size. Performances of the PGE-based system and animal hematoanalyzer (MS 9-5; Melet schloesing laboratories, France) were compared by employing 7.2 μm fluorescent microbeads similar in size to RBCs [29]. The whole blood samples were collected from healthy subjects, which were voluntarily donated by the authors. A solution of ethylenediamine tetraammonium salt (EDTA) was added to a sample and mixed to prevent coagulation. The mixture was diluted with PBS according to the given dilution factor and directly injected into the microfluidic chip.

2.5 Experimental procedure

In the impedance signal calibration experiment, fluorescent microbeads of difference sizes (7.2, 10.0, and 15.0 μm in diameter) were injected into the main microchannel inlet and PBS was introduced into the two side channel's inlets for the sheath flow. The samples and PBS drawn from the outlet by a syringe pump (KDS100; KD Scientific, USA) with a flow rate of 10 μL/h resulted in a hydrodynamic focusing to reduce the coincident effect. The Cl^- ions in the 1 M KCl electrolyte solution carry the charge through the PGEs to maintain a current driven by a dc bias. Fluorescent microbeads traveling along the microchannel interfere with the current flow between PGEs to cause a change in impedance whose amplitude is proportional to the bead size.

The RBC counting capability of the PGE-based system was validated in two steps; first using four solutions of different concentrations of 7.2 μm fluorescent microbeads and then using human blood samples in different dilution ratios. The same fluorescent bead solution was also counted with an animal hematoanalyzer and the results from the two systems were compared. Human whole blood samples were diluted in PBS at various ratios and simultaneously counted with the PGE-based system and a clinical human hematoanalyzer (HST-N402XE; Sysmex, Japan) for comparison.

3 Results and discussion

3.1 Amplitude calibration of the impedance change

The correlation between the amplitude of impedance change and the size of fluorescent microbeads was examined using three different-sized fluorescent microbeads. As shown in Fig. 2, there is a main microchannel between a pair of PGEs facing each other perpendicular to the flow direction. The PGEs separate the internal filling solution of 1 M KCl in the electrode chamber from the sample solution flowing in the main microchannel so that the microbeads to be measured do not come into direct contact with the Ag/AgCl electrodes. This is an advantage because cells very close to the surface of the metal electrode could get damaged due to the high electric potential gradient, a situation that is often encountered in conventional particle or cell counters based on the Coulter principle. With a dc bias applied between two Ag/AgCl electrodes, Cl^- ions carry the charge and produce a dc current through the PGEs. When a bead or a blood cell passes the

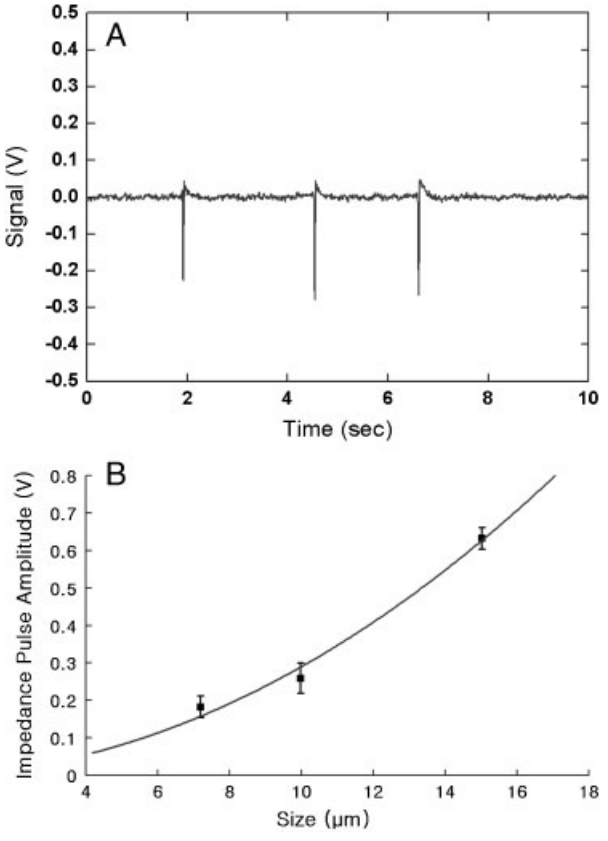

Figure 3. (A) Measured impedance signal between a pair of PGEs induced by a flow of three 10.0 μm fluorescent microbeads. (B) The amplitude of the impedance change resulted from the bead size independently of the flow rate and the bead location across the microchannel. Each point was tested eight times.

region between the two PGEs, a pulse-shaped signal appears due to the impedance change, as shown in Fig. 3A. Because the impedance change between PGEs is directly related to the microbead size, larger microbeads bring about greater pulse amplitudes, as shown in Fig. 3b. Since we can perform a low-voltage dc impedance analysis with the PGEs, the resultant impedance change is dependent not on the bead position across the microchannel but only on the bead size.

3.2 Quantification of fluorescent microbeads in differential concentrations

Adult humans have about 4.2–6.3 million RBCs *per* microliter and the diameter of a typical human erythrocyte disk is 6∼8 μm [29]. Fluorescent microbeads of 7.2 μm in diameter whose size was similar to RBCs were counted using both the PGE-based system and a commercialized animal hematoanalyzer at four different concentrations between 1 and 6 thousand *per* μL in considering more than 800 times dilution in real applications. As shown in Fig. 4, the count results match each other well within a reasonable error range. The PGE-based system produced stable count results even at a high concentration, where the coincident effect worsened. It is believed that the narrow sample flow of ∼20 μm induced by 1-D hydrodynamic focusing, as shown in Fig. 5, was effective. It is especially meaningful in that a relatively simple setup having one main and two PBS-filled side microchannels drawn by a single syringe pump at the outlet achieved it. Any sophisticated sheath flow control was not necessary because the impedance pulse amplitude in the PGE-based system is immune to the position of the microbeads within the microchannel. This is another benefit of using low-voltage dc impedance analysis.

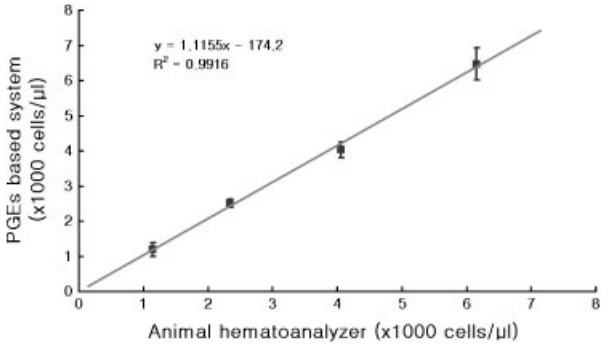

Figure 4. The relationship between results from the PGE-based system and a commercialized animal hematoanalyzer at four different concentrations of the fluorescent microbeads of 7.2 μm in diameter, a similar size to RBCs.

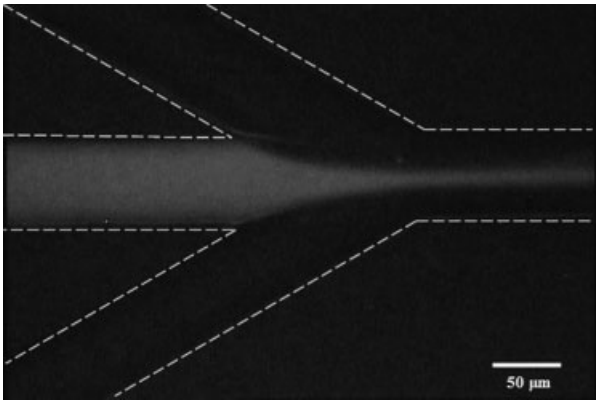

Figure 5. The sample flow width shrunk down by 1-D hydrodynamic focusing mitigated the coincident effect.

3.3 Quantification of human RBCs

In all commercially available clinical hematoanalyzers, a blood sample is diluted by a factor of 20 000∼40 000 before RBC counting. In order to determine the minimal dilution factor for the developed PGE-based cell counter, 2 μL of human whole blood samples were diluted with PBS and EDTA solution to a total of eight different ratios. The diluted samples were then counted using the PGEs-based system. Figure 6 shows the count results from the two systems. It was found that 800 is the lowest dilution factor able to achieve a comparable RBC count to a commercial system. Below that, it seems the coincident effect results in a deterioration of the PGEs-based system's cell counting performance. The effects of other blood cells of white blood cells and platelets were shown to be negligible. The number of white blood cells in the blood samples is roughly 0.1% of that of RBCs and platelets can be discerned from RBCs due to their noticeably different pulse amplitude.

Consequently, the number of RBCs was successfully counted with just a dilution of 2 μL whole blood (1:800) without any pretreatment such as centrifuging or filtering. The PGEs in the walls of the microchannel ensure good electric connections along the circuit. Simultaneously, the PGEs efficiently prevent the blood cells from aggregation and to suppress the production of bubbles at the electrode surface by mechanically separating the electrodes from the diluted blood. The sample volume

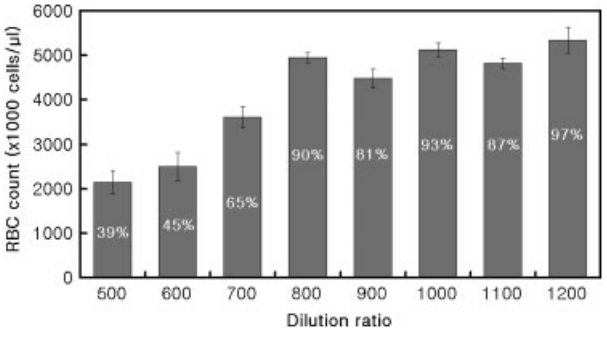

Figure 6. RBC count results for human whole blood samples diluted with PBS to a total of eight different ratios. For comparison, percentage values of the result from a clinical human hematoanalyzer were marked on each result with a different dilution ratio.

required for this new device was small enough to substantially cut the cost for clinical analysis. Moreover, the chip-integrated PGE system in this study was experimentally demonstrated to be on a par with the commercialized clinical human hematoanalyzers in performance. It is believed that the proposed system suggests the way to the handheld chip-based hematoanalyzer for point-of-care testing.

4 Concluding remarks

We propose the technique of PGE-based cell counting using a low-voltage-biased dc impedance analysis as a new strategy for a chip-based cell counter. RBCs in human whole blood samples were counted with error approximately within 10% in comparison with a clinical hematoanalyzer. The required sample was 2 μL of whole blood diluted by a factor of more than 800 with a buffer and EDTA solution, which can be readily implemented in a microfluidic chip. The greatest advantage of the developed PGE-based system is that it requires no preprocessing, neither centrifugation nor filtering, to separate blood cells. Furthermore, it can protect the blood cells from electrical damage and undesirable aggregation on the surface of electrodes because there is no direct contact between them.

Although a number of miniaturized chip-type blood cell counters have been suggested, it is difficult to find one with practical clinical applications, mainly because most of them are based on relatively large and expensive optical equipment that often require sophisticated alignments and fine tunings. Our whole system, including the external electronic circuitry, is simple and compact enough to be a point-of-care testing-type cell counter.

For this reason, the proposed PGEs-based system has sufficient potential for many valuable clinical applications in the near future.

This research was supported by MKE (Ministry of Knowledge Economy), Korea under the ITRC (Information Technology Research Center) support program supervised by the IITA (Institute of Information Technology Advancement) (IITA-2009-(C1090-0902-0002)). The authors also acknowledge the support by the Nano/Bio Science & Technology Program (M10536090001-05N3609-00110) of the Ministry of Education, Science and Technology (MEST) and the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. R11-2007-012-02002-0(2009)).

The authors have declared no conflict of interest.

5 References

[1] Norris, P. J., Rosenberg, E. S., *J. Mol. Med.* 2002, *80*, 397–405.

[2] Hanscheid, T., *Clin. Lab. Haem.* 1999, *21*, 235–245.

[3] Lancaster, C., Kokoris, M., Nabavi, M., Clemmens, J., Maloney, P., Capadanno, J., Gerdes, J. *et al.*, *Methods* 2005, *37*, 120–127.

[4] Bertucco, L., Nunnari, G., Randieri, C., *Proc. IEEE* 1998, *1*, 341–345.

[5] Gross, W. B., *J. Am. Vet. Med. A* 1984, *185*, 343–343.

[6] Nielsen, L. K., Smyth, G. K., Greenfield, P. F., *Biotechnol. Progr.* 1991, *7*, 560–563.

[7] Nicholson, J. K., Stein, D., Mui, T., Mack, R.., Hubbard, M., Denny, T., *Clin. Diagn. Lab. Immunol.* 1997, *4*, 309–313.

[8] Zimmermann, I., *Eur. J. Pharm. Biopharm.* 1997, *43*, 101–102.

[9] Larsen, U. D., Blankenstein, G., Branebjerg, J., *Transducers 97* 1997, *2*, 1319–1322.

[10] Saleh, O. A., Sohn, L. L., *Rev. Sci. Instrum.* 2001, *72*, 4449–4451.

[11] Zhe, J., Jagtiani, A., Dutta, P., Hu, J., Carletta, J., *J. Micromech. Microeng.* 2007, *2*, 304–313.

[12] Roberts, K., Parmeswaran, M., Moore, M., Muller, R. S., *Proc. IEEE* 1999, *3*, 1668–1673.

[13] Gawad, S., Schild, L., Renaud, P. H., *Lab Chip* 2001, *1*, 76–82.

[14] Chun, H. G., Chung, T. D., Kim, H. C., *Anal. Chem.* 2005, *77*, 2490–2495.

[15] McClain, M. A., Culbertson, C. T., Jacobson, S. C., Allbritton, N. L., Sims, C. E., Ramsey, J. M., *Anal. Chem.* 2003, *75*, 5646–5655.

[16] Hong, J. I., *Biochip J.* 2008, *2*, 12–26.

[17] Satake, D., Ebi, H., Oku, N., Matsuda, K., Takao, H., Ashiki, M., Ishida, M., *Sens. Actuators B Chem.* 2002, *83*, 77–81.

[18] Ormerod, M. G., *Flow cytometry: A Practical Approach*, Oxford University Press, Oxford 2000.

[19] Gawad, S., Cheung, K., Seger, U., Bertsch, A., Renaud, P., *Lab Chip* 2004, *4*, 241–251.

[20] Tang, H., Gao, Y. F., *IEEE Sens. J.* 2005, *5*, 1346–1352.

[21] Mogensen, K. B., Klank, H. P. K. J., *Electrophoresis* 2004, *25*, 3498–3512.

[22] Kruger, J., Singh, K., O'Neill, A., Jackson, C., Morrison, A., O'Brien, P., *J. Micromech. Microeng.* 2002, *12*, 486–494.

[23] Fu, A. Y., Chou, H. P., Spence, C., Arnold, F. H., Quake, S. R., *Anal. Chem.* 2002, *74*, 2451–2457.

[24] Huh, D., Gu, W., Kamotani, Y., Grotberg, J. B., Takayama, S., *Physiol. Meas.* 2005, *26*, R73–R98.

[25] Chung, T. D., Kim, H. C., *Electrophoresis* 2007, *28*, 4511–4520.

[26] Chun, H. G., Kim, H. C., Chung, T. D., *Lab Chip* 2008, *8*, 764–771.

[27] Ayliffe, H. E., Frazier, A. B., Rabbitt, R. D., *J. Microelectromech. S* 1999, *8*, 50–57.

[28] Andrew, J. d. M., Joshua, B. E., *J. Appl. Phys.* 2007, *101*, 084903.

[29] Henry, J. B., *Clinical and Diagnosis Management by Laboratory Methods*, Harcourt Brace Jovanovich Inc., Philadelphia 1991, pp. 553–757.

