Microfluidic Chip based Hematoanalyzer Using Polyelectrolytic Gel Electrodes

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ABSTRACT

We reports on a novel microfluidic chip with polyelectrolytic gel electrodes (PGEs) used to rapidly count the number of red blood cells in diluted whole blood. The number and amplitude of dc impedance peaks provide the information about the number and size of red blood cells, respectively. This system features a low-voltage dc detection method and non-contact condition between cells and 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 were used in the experiment. Second, the cell counting performance was evaluated by using 7.2 μm fluorescent microbeads, similar in size to red blood cells, in various concentrations and comparing the results with an animal hematoanalyzer. Finally, in human blood sample tests, intravenously collected whole blood was just diluted in a phosphate buffered saline without centrifuge or other pretreatments. The PGEs-based system produced almost identical numbers of red blood cells in over 800-fold diluted samples to the results from a commercialized human hematoanalyzer.

Keywords: microfluidic chip, polyelectrolytic gel electrode, red blood cells, hematoanalyzer

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 RBC count (the number of red blood cells 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 the 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 (FACS), 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 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, a lot of research on the development of microfluidic chip-based Coulter counters [11-15] is currently being carried out. 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 [16]. Miniaturized flow cytometers are also being attempted [17-20] based on the fact that the scattered laser light and fluorescent emissions provide valuable information about the objects, such as size, number, protein expression etc. [21]. Recently, we have been seeing an acceleration in the development of microfluidic chip flow cytometry [22-24].

The present study proposes a simple system to rapidly count the number of red blood cells, by incorporating polyelectrolytic gel electrodes (PGEs) 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 [25]. The PGEs located between the microchannel and the electrodes 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, 26]. 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 imperative to overcome the coincidence effect. [27].

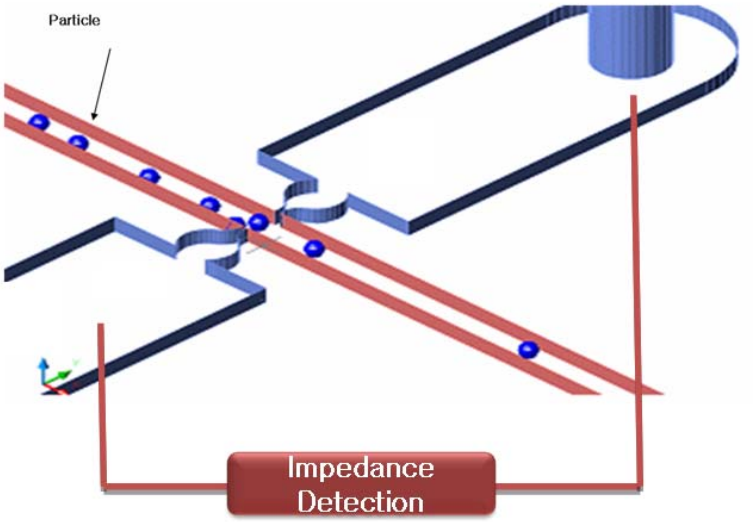

Fig. 1. Schematic of the proposed microfluidic chip: the microchannels etched on a glass substrate are 70 μm wide and 30 μm deep.

Fig. 1 shows a schematic diagram of the developed PGEs-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. Since the impedance is measured from the current upon a given DC bias that is applied perpendicularly to the cellular flow direction, unlike the Aperture-Impedance method, it is expected that the coincidence effect and the required minimum dilution ratio are subtantially reduced. Further improvement can be made if a sheath flow is used for hydrodynamic focussing. This study shows a possibility of a reliable chip-based RBCs counter using whole blood without any separation or pretreatment, except for dilution, by providing preliminary results in good agreement with a human hematoanalyzer.

2. MATERIALS AND METHODS

2.1 Microfluidic chip fabrication

Fig. 2 shows the developed microfluidic chip, with 70 μm wide and 30 μm deep microchannels fabricated using only standard photolithograpic techniques. A Corning 2947(7.5 x 2.5 x 0.1 cm^3) glass 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, USA). This process was repeated three times. Any moisture was completely removed from the glass with clean air, and the glass was put on a hot plate at 150 ^oC for 100 min. The glass was then cooled at room temperature for 90 s. Next, the glass was coated with HMDS (Hexa methyl di silazane, Clariant, Switzerland) using a spin coater (Won Corp., 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 ^oC for 3 min and cooled at room temperature for 90 s. Photoresist (PR) AZ4620 (Clariant, Switzerland) 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 ^oC for 90 s and cooled at room temperature for 90 s. Then the pattern designed using Silverwriter (Mania barco, Germany) was drawn on a mask film (accumax photoplotter film, Kodak, USA). 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 AZ400K developer (Clariant, Switzerland) which was replaced it by new one every 90 s. 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 ^oC 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, USA), and was washed with DI-water and a ultrasonic cleaner (351OE-DTH, Brasonic, USA) for 15 min to remove impurities remaining on the pattern. Then electrode reservoir and channel inlet/outlet holes were drilled where necessary. For bonding, a Corning 2947 cover glass 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 ^oC furnace (CRF-M15, CEBER, Korea) for 5 h. The bonded glass chip was stored in a desiccator at constant temperature and humidity.

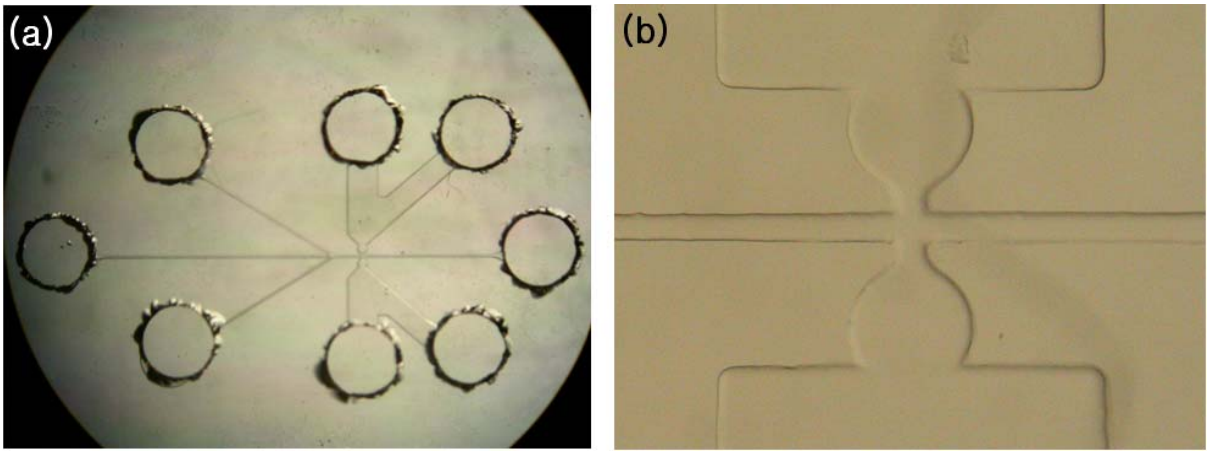

Fig. 2. (a) The fabricated microfluidic glass chip. The sample flow is hydrodynamically focused between two sheath flows to reduce coincident effect and thereby to operate with a lower dilution factor. (b) A pair of PGEs are faced each other on the microchannel wall perpendicularly to the flow direction.

2.2 PGE fabrication

The PGEs were fabricated using photopolymerization of the monomer solution by UV irradiation on specific parts of the glass chip[28]. Before polymerization, for solid adherence of the polymer to the microchannel wall, the microchannels were coated with TMSMA(3-(Trimethoxysilyl)propyl methacrylate) under dark conditions at room temperature for 1 h. The TMSMA-coated microchannel surface was then washed with methanol (J.T.Baker, USA). An aqueous DADMAC(65 wt%, Sigma-Aldrich, USA) solution was mixed with a photoinitiator(2-hydroxy-4'-(2-hydroxyethoxy)-2-

methylpropiophenone) and a cross-linker(N,N'-Methylene-bisacrylamide) making up [together] 2% of the DADMAC content. The aqueous DADMAC solution was injected into the microchannel through the reservoirs of the microfluidic chip. The mask and chip were aligned using a UV aligner (MDA-400M, Midas, Korea) to determine the desired location of PGEs formation, UV light exposure of 20 mW cm^{-2} intensity for 4 s. Then the PGEs and microchannel 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 applied between two Ag/AgCl electrodes immersed into an internal filling solution in the reservoirs on the microfluidic chip produced around 13 μA dc current. A DC impedance analyzer converted the impedance change due to the passage of microbeads or cells into voltage pulses. After amplification by 2000 times, the voltage pulses were digitized with a 5MHz 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 PGEs-based system, we used fluorescent microbeads that were dispersed in PBS at pH 7.4 and well recognized individually by an optical microscope. The average diameters of the beads were 7.2, 10.0 and 15.0 μm and 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 PGEs-based system and animal hematoanalyzer were compared by employing 7.2 μm fluorescent microbeads similar in size to RBCs. The whole blood samples, which were generously donated by the volunteers, were mixed with a solution of ethylenediamine tetraammonium salt (EDTA) to prevent coagulation. They were 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's inlet and PBS was introduced into the two side channels' inlets for the sheath flow. The samples and PBS drawn from the outlet by a syringe pump(KDS100, KD Scientific) with a flow rate of 10 μl/h resulted in a hydrodynamic focusing to reduce the coincidence 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 PGEs-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 fleorescent 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 PGEs-based system and a clinical human hematoanalyzer for comparison.

3. RESULTS AND DISCUSSION

3.1 Impedance Amplitude of three different sized microbeads

The correlation between the amplitude of impedance change and the size of fluorescent microbeads was examined using three different sized fluorescent microbeads(7.2, 10.0 and 15.0 μm in diameter). 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 which 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 region between the two PGEs, a pulse-shaped signal appears due to the impedance change, as shown in Figure 3 (a). Because the impedance change between PGEs is directly related to the microbead size, larger microbeads bring about greater pulse amplitudes, as shown in Figure 3 (b). Since we can perform

a low-voltage DC impedance analysis thanks to the PGEs, the resultant impedance change is dependent not on the bead position across the microchannel but only on the bead size.

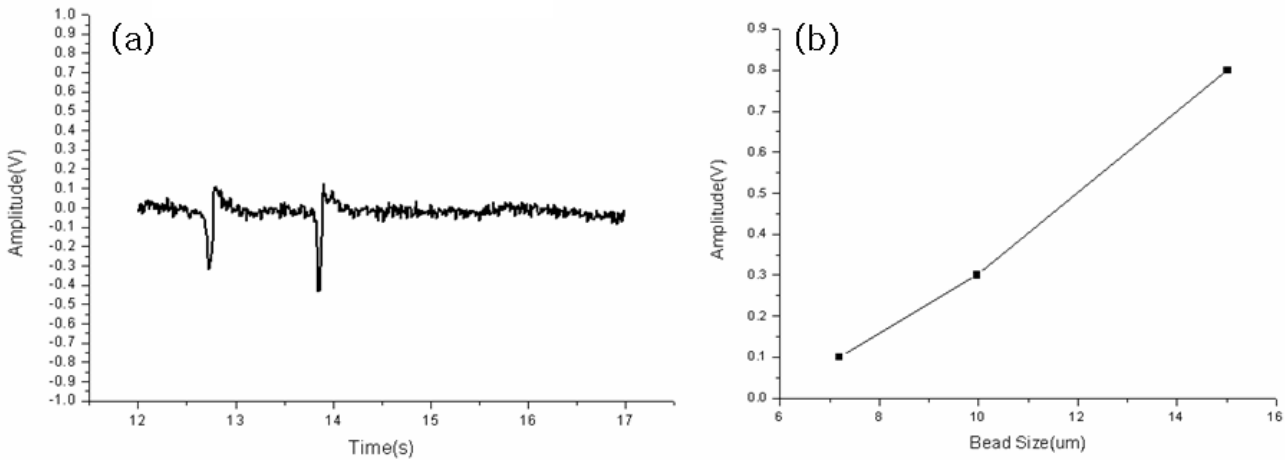

Fig. 3. (a) Measured Impedance signal between a pair of PGEs induced by a flow of three 10.0 μm fluorescent microbeads. (b) The amplitude of the impedance change resulted from the bead size independently of the flow rate and the bead location across the microchannel. Each point was tested eight times.

3.2 Counting the microbead in differential concentrations

Adult humans have about 4.2 to 6.3 million RBCs per microliter and the diameter of a typical human erythrocyte disk is 6~8 μm. Fluorescent microbeads of 7.2μm in diameter were counted using both the PGEs-based system and a commercialized animal hematoanalyzer at four different concentrations between 1 to 6 thousand/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.

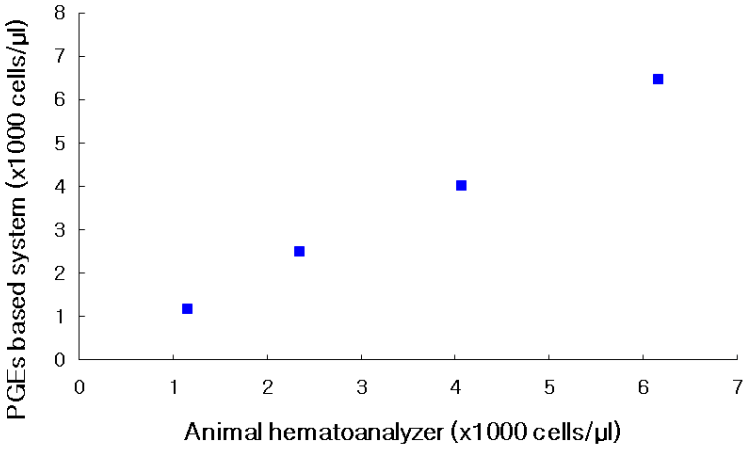

Fig. 4. The relationship between results from the PGEs-based system and a commercialized animal hematoanalyzer at four different concentrations of the fluorescent microbeads of 7.2 μm in diameter, a similar size to RBCs.

The PGEs-based system produced stable count results even at a high concentration, where the coincidence effect worsened. It is believed that the narrow sample flow of ~20 μm induced by 2D hydrodynamic focusing 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 PGEs-based system is immune to the position of the microbeads within the microchannel. This is another benefit of using low-voltage DC impedance analysis.

3.3 Counting the RBC

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 PGEs-based cell counter, 2μl 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. Fig. 6 shows a comparison between the two systems' count results. 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 coincicence effect results in a deterioration of the PGEs-base 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 red blood cells was successfully counted with just a simple dilution of whole blood without other pretreatments such as centrifuging or filtering.

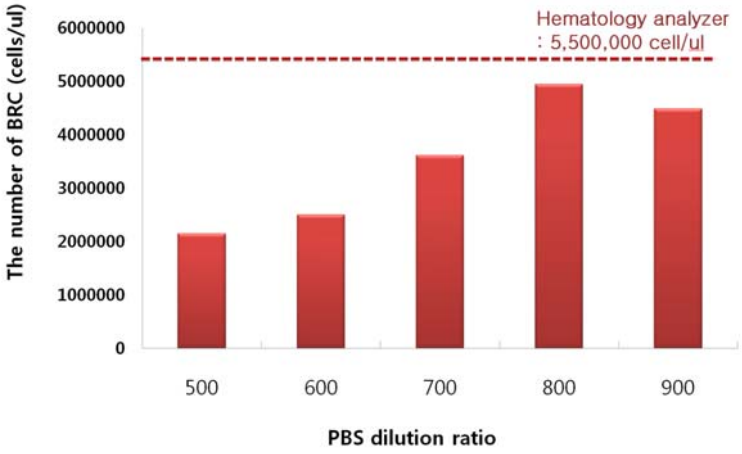

Fig. 5. RBC count results for human whole blood samples diluted with PBS to a total of eight different ratios. For comparison, percentage values of the result from a clinical human hematoanalyzer were marked on each result with a different dilution ratio.

4. CONCLUDING REMARKS

We propose the technique of PGEs-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 an errors of approximately 10% in comparison with a clinical hematoanalyzer. The only required sample was 2μL 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 PGEs-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 hard to find one with practical clinical applications, mainly because most of them are based on relatively large and expensive optical equipments which 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 (POCT) type cell counter.

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