Microsystems for Whole Blood Purification and Electrophysiological Analysis

Arum Han, Ki-Ho Han, Swomitra K. Mohanty, and A. Bruno Frazier

Abstract—**This paper presents the development of a microsystem for whole blood purification and electrophysiological analysis of the purified cells. Magnetophoresis using continuous diamagnetic capture (DMC) was utilized for whole cell purification and electrical impedance spectroscopy (EIS) was utilized for electrophysiological analysis of the purified cells. The system was developed on silicon and plastic substrates utilizing conventional microfabrication technologies and plastic microfabrication technologies. Using the magnetophoretic microseparator, white blood cells were purified from a sample of whole blood. The experimental results of the DMC microseparator show that 89.7% of the red blood cells (RBCs) and 72.7% of the white blood cells (WBCs) could be continuously separated out from a whole blood using an external magnetic flux of 0.2 T. EIS was used as a downstream whole cell analysis tool to study the electrophysiological characteristics of purified cells. In this work, primary cultured bovine chromaffin cells and human red blood cells were characterized using EIS. Further analysis capabilities of the EIS were demonstrated by successfully obtaining unique impedance signatures for chromaffin cells based on the whole cell ion channel activity.**

Index Terms—**Blood Cell Separator, Diamagnetic Capture (DMC), Magnetophoresis, Electrical Impedance Spectroscopy (EIS), Electrophysiology**

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I. INTRODUCTION

There has been great interest in microsystems that accept whole blood as the input sample, separate the different components of the blood including the cells, and analyze the components to extract the genetic or pathological information [1, 2]. One goal of such systems is to accept whole blood as the input sample and to extract/study the rare cells within the blood, such as white blood cells or cancerous cells. Cell separation systems developed to date use either samples that are purified off-chip or tagged cells in which the tag provides a means of collecting a given sub-population of cells from a complex media. To analyze these cells, fluorescent or radioactive molecules tagging has been widely used. Detection methods that can differentiate cells by their physiological status or cell types without the requirement of tagging can eliminate these additional sample preparation processes. Furthermore, for microsystems intended to be deployed in the field, integrated on-chip purification systems that enable the use of whole blood as an input sample are essential.

Extraction and concentration of rare cells in whole blood requires sample purification. For example, dielectrophoresis (DEP) has been used to manipulate cells and separate different types of cells, but with marginal success for samples containing a large number of cells with very low concentrations of the target cells (e.g., whole blood) [3]. Conventional high gradient magnetic separation (HGMS) methods have been used to separate paramagnetic and diamagnetic particles from water, soil, or air [4-6]. This method has advantages, such as the capacity to produce a large separation force with simple

device structures, low cost, ease of use, and the non-hydrolytic nature of magnetic fields. Based on the inherent magnetic properties of blood cells, studies of conventional macro scale magnetophoretic separators [7-15] have focused on developing cell separators that use the HGMS method. However, these macroseparators are limited in applications since the magnetic force that can be attained is low due to the system-level geometric constraints limiting the magnetic field gradient and due to the weak susceptibilities of native biological cells. To overcome the low magnetic force on biological cells and to take advantage of the geometrical scaling effects of miniaturization, our group has previously proposed a high gradient magnetophoretic microseparator fabricated by microfabrication technologies [16]. In our previous work, the theoretical model for calculating the magnetic forces produced by the magnetophoretic microseparator was derived and verified by comparison with experimental results.

Electrical impedance spectroscopy is a technique mainly used to characterize tissues based on the knowledge of their electrical properties in the frequency spectrum [17]. In biological tissues, electric current influences components that have a net electric charge and/or dipolar electrical moment, such as ions, polar molecules of tissue water, and protein and lipid structures of membranes or cell interfaces [18]. The induced conduction and dielectric relaxation phenomenon are used to study the electrical properties of tissues, defined by conductivity and permittivity. These properties of tissues depend on frequency.

From the various applications of impedance spectroscopy, distinguishing different types of tissues and studying pathological tissues are of great interest [19]. Among the studies relative to pathological tissues, one of the more promising areas seems to be the characterization of cancerous tissues. In cancerous tissues, the permittivity and conductivity will be higher than that of healthy tissues. Several macrosystem-based studies have been conducted to characterize breast cancer tissues using impedance spectroscopy [17].

Recent advance in microfabrication and lab-on-a-chip concept enabled electrical impedance spectroscopy devices to measure small sizes of samples quickly and accurately and to be integrated with other analyses methods. These systems have been expanded to detect and characterize single cells. Measuring electrical impedance of human polymorphoneuclear leukocytes and teleost fish red blood cells [20], erythrocytes, leukocytes, and embryonic renal cells [21, 22], human prostate carcinoma cell lines (DU-145) [23], and bovine chromaffin cells [24] using microfabricated devices have been shown. These studies show that detecting different types of cells, cell viability, and cell reaction to toxins are possible. Most of these systems utilize fluidic channels through which samples can be flown and opposing electrodes positioned inside these channels to measure the electrical impedance. Positioning a single cell between these electrodes can be accomplished by controlling fluid pressure and channel geometry. By measuring the impedance of a single cell, high-resolution cellular assays can be performed with minimum interferences. Although these systems show promising results in characterizing single cells, inaccuracy in aligning single cells between opposing electrodes typically results in higher deviation. A system with accurate cell positioning capabilities and with multiple pairs of electrodes at different vertical levels to be used in tomography-type analysis will enhance the understanding of correlations between electrical impedances and physiological characteristics of cells.

Additionally, there have been several studies to use microfabricated electrical impedance spectroscopy devices for interrogating the electromagnetic behavior of biological or chemical samples. These devices have been used to sense variations in solution temperatures [25], ionic concentrations [26], and bacterial metabolism [27, 28]. These systems typically analyze target solutions using arrays of metal electrodes in a fluidic chamber or channel.

This paper presents the design, fabrication, and characterization of a continuous diamagnetic capture (DMC) mode magnetophoretic microseparator for separating blood cells based on their native magnetic properties and a micro electrophysiological analysis system (μ-EPAS) for single cell manipulation and electrical impedance spectroscopy, as shown in Figure 1. Using our previously developed theoretical model of the magnetophoretic microseparator, the present DMC microseparator is designed. Quantitative measurements of the relative separation percentages of RBCs and WBCs are reported and compared with theoretically estimated results.

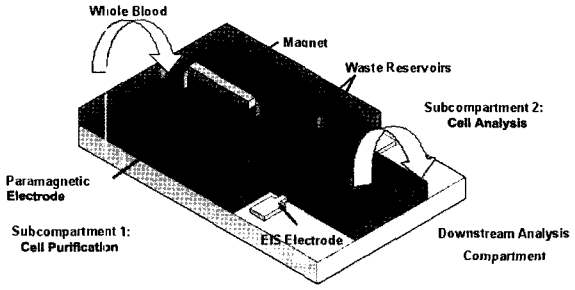


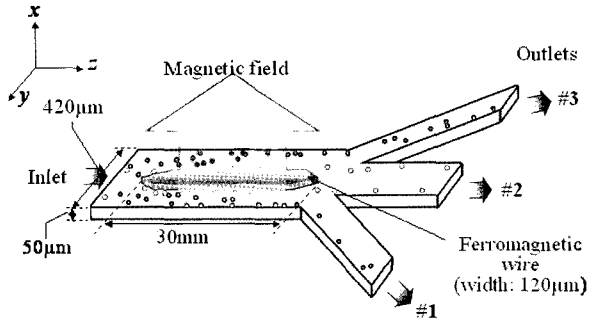
Fig. 1. Illustration of a microsystem for blood sample analysis with sample purification and cell analysis

The μ-EPAS has the capability to perform impedance spectroscopy, general extracellular stimulation/recording using integrated multi-electrode array, and patch clamp recording on a single cell. Electrical impedance measurement on two different sub-populations of bovine chromaffin cells and red blood cells are reported. The ion channels of chromaffin cells were blocked and the electrical impedance was measured to study the electrophysiological activities of the cells.

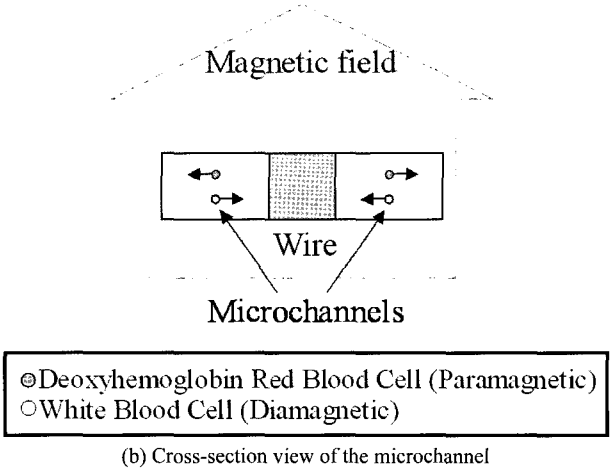
II. METHODOLOGY

1. Whole Cell Purification System

Figure 2(a) shows a schematic of the magnetophoretic microseparator. The magnetophoretic microseparator consists of one inlet and three outlets, which were numbered #1 to #3 from left to right. When an external magnetic field is applied normal to the microchannel (Figure 2(a)), the RBCs as paramagnetic particles are forced away from the ferromagnetic wire and the WBCs as diamagnetic particles are drawn closer, as shown in Figure 2(b). Therefore, the RBCs are forced into outlets #1 and #3, and the WBCs are forced into outlet #2. Blood cells can be separated continuously as the whole blood passes through the microchannel of the DMC microseparator. This geometric configuration of the magnetophoretic macroseparator has been termed the diamagnetic capture (DMC) mode [15]. The DMC microseparator consists of a microchannel, defined by glass-to-glass thermal bonding between two Borofloat™ glass slides. A ferromagnetic nickel wire was electroplated along the length of the microchannel, as shown in Figure 2(a). For the first



(a) Perspective view of the microchannel that has one inlet and three outlets



(b) Cross-section view of the microchannel

Fig. 2. Illustrations of the DMC microseparator

fabrication step, the bottom glass substrate (Borofloat™ glass of 0.7 mm thick, Howard Glass Co., USA) was etched 50 μm in depth using 25% HF solution. Next, a Ti/Cu/Cr seed layer for nickel electroplating was evaporated onto the bottom glass substrate, as shown in Figure 3(a). The ferromagnetic wire was fabricated by nickel electroplating, as shown in Figure 3(b) followed by glass-to-glass thermal bonding at 685°C for 3.5 hours (Figure 3(c)).

The integrated microfluidic interface (IMI) fabricated by stereolithography was used to realize the microfluidic interconnect [29]. Nitrile rubber o-rings (Size 001-1/2, McMASTER-CARR, USA) were used for sealing the microfluidic interconnect. An ultraviolet (UV) adhesive (1187-M, DYMAX Co., USA) was used for adhesive bonding on the IMI, and capillary forces pulled the adhesive into the gaps between the IMI and the glass chip. The UV adhesive was then cured by placing it under a UV light for 30 minutes, completing the fabrication of the DMC microseparator, as shown in Figure 3(d). Finally, to reduce the adhering of blood cells to the microchannel wall, polyethylene glycol surfactant was coated onto the

surface of the microchannel. Figure 4(a) and 4(b) show the fabricated DMC microseparator and a close-up of the separation point with the three outlets, respectively.

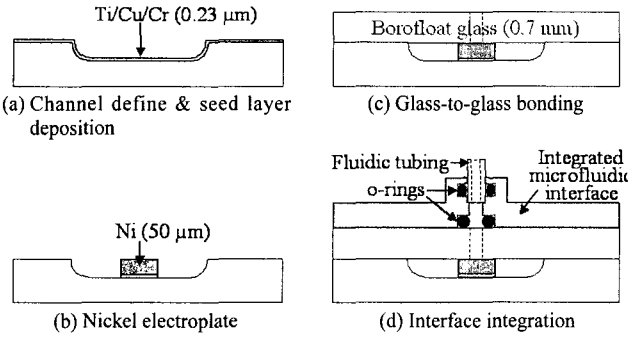


Fig. 3. Microfabrication process for the DMC microseparator

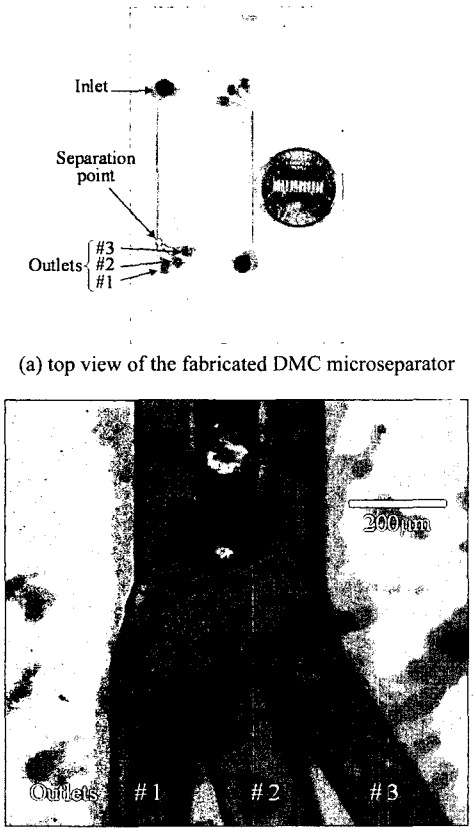


Fig. 4. Micrographs of fabricated DMC microseparator

2. Electrical Impedance Spectroscopy System

The system is composed of an array of analysis cavities, each capable of analyzing a single cell at a time, a vacuum via to hold the cell in position during the impedance analysis operation, and multiple electrodes surrounding the circumference of the cell to measure the

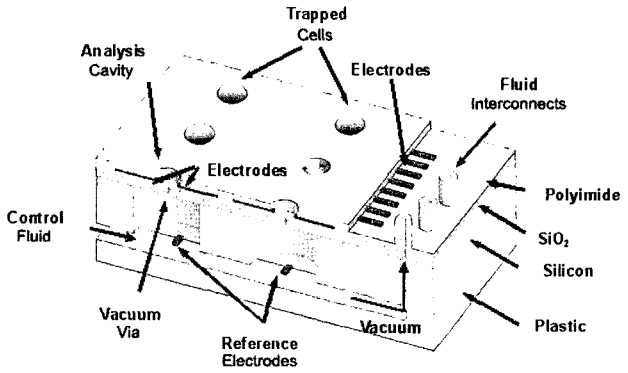


Fig. 5. Illustration of the micro electrophysiological analysis system (μ-EPAS)

electrical characteristics of the captured cell. Additionally, the μ-EIS system has microchannels to apply negative pressure through the vacuum via and to control the extracellular biochemical environment, and fluid interfaces to connect the microsystem with macro scale fluid handling. Figure 5 shows a schematic cross-section of the system.

The system was fabricated on a 3-inch diameter silicon wafer starting with 1.8 μm thick SiO_2 film deposition on the surface using plasma enhanced chemical vapor deposition. A 3.0 μm diameter vacuum via was etched through the SiO_2 film using inductive coupled plasma (ICP, Plasma-Therm Inc., USA). A 3.5 μm thick polyimide layer (PI2566, HD Microsystems L.L.C., USA) was spin-coated, followed by baking at 200°C hotplate for 2 hours. A gold film was deposited to a thickness of 3500 Å and patterned to create one or two pairs of opposing electrodes positioned around the vacuum via and inside the analysis cavities. A second layer of 3.5 μm thick polyimide was spin-coated over the patterned electrodes. To pattern the polyimide, aluminum was chosen as an etch mask. Aluminum was sputtered on the polyimide and 10.0 μm and 16.0 μm diameter analysis cavities were patterned. The polyimide was etched using reactive ion etching (RIE) in a 90% oxygen, 10% CHF3 environment, followed by removal of the aluminum layer in an aluminum etchant (Transene Company, Inc., USA). Circles with 200 μm in diameter were patterned on the backside of the Si wafer using NR9-8000 photoresist (Futurrex Inc., USA). The Si wafer was etched all the way through from the backside using ICP Bosch etching process. The SiO_2 film in the front of the wafer served as the etch stop. On a 3-inch diameter silicon wafer, arrays of 16 analysis cavities were

positioned on each quarter of the wafer, resulting in 64 analysis sites overall.

Microchannels and fluid interfaces were created using stereolithography. Stereolithography is a technique where a laser is used to cure photosensitive polymer structures layer-by-layer [30]. The design incorporates arrays of fluid channels where each channel covers two analysis cavities. These channels and ports are used to apply negative pressure through the via to capture a single cell and to control the extracellular biochemical environment. Each channel has an inlet and outlet port, into which standard 1/16 inch outer diameter fluorinated ethylene propylene (FEP) tubing (Upchurch Scientific Inc., USA) can be attached. Two-part epoxy was used to seal the tubing to the ports. This stereolithographically defined parts were aligned to the overlying silicon wafer and bonded using two-part epoxy. Using this design, cells can be trapped in two analysis cavities at the same time and analyzed in parallel. Additional designs capable of trapping up to 16 cavities in parallel were designed and fabricated.

The system was used to compare the impedance of two sub-populations of bovine chromaffin cells and human red blood cells. Bovine chromaffin cells were prepared by Dr. Engisch at Emory University (Department of Physiology, Atlanta, GA). The cultured cells were transferred to a centrifuge tube and centrifuged for 10 minutes (Fisher Scientific Centrifuge Model 225, Fisher Scientific Co., USA). After centrifugation, the culture media were disposed of and 3 ml of D-PBS (Dulbecco's PBS 1x, 1.00mg/L, D-glucose with 36 mg/L sodium pyruvate, calcium, Gibco, USA) was added. Red blood cells were taken from a human sample, centrifuged, and diluted in a D-PBS solution. Five micro liter of cell suspension was dropped on top of the analysis cavity. Negative pressure was applied from the backside using a syringe to pull the cell into the analysis cavity. A microscope was used to visualize positioning of the cells. Once a cell was captured, the valve at the outlet tubing was closed to maintain the cell in position and the electrical impedance was measured. The impedance was measured between opposing electrodes using a precision impedance analyzer (4294A, Agilent Technologies Inc., USA). The electrodes were in direct contact with the outer surface of the cell membrane with the cell snugly fitted into the analysis cavity. For the electrophysiological studies of chromaffin cells, calcium ion channels were blocked by adding 1.0 M CdCl (Sigma-Aldrich Co., USA) to the cell suspension. The measured impedance was compared to that of normal cells.

III. Results and Discussion

1. Whole Cell Purification Systems

1) Separation of Red Blood Cells

To prepare the blood sample, bovine whole blood was diluted to a ratio of 1:10 using a 3 mM isotonic sodium hydrosulfite (Fisher Scientific, Pittsburgh, PA, USA) solution. Figures 6(a) shows an image of RBCs passing through the microchannel at a flow velocity of 0.2 mm/sec with an external magnetic flux of 0.2 T using a permanent magnet. Figure 6(b) shows an image of RBCs flowing at an average velocity of 0.2 mm/sec without the external magnetic flux. The images prove that RBCs are forced away from the ferromagnetic wire within the magnetic

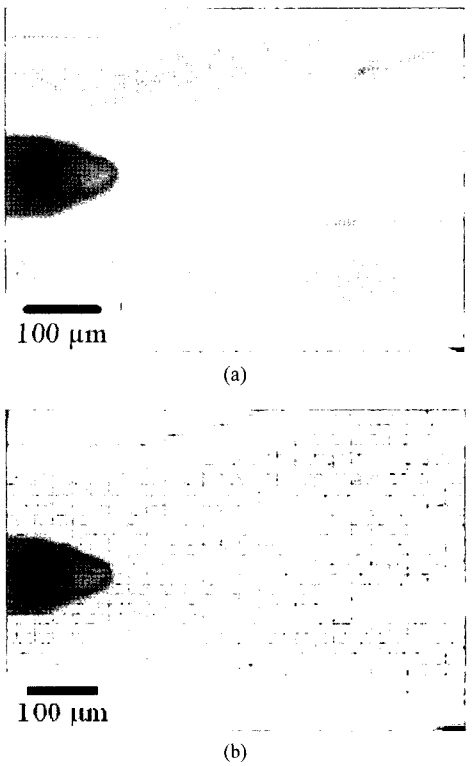


Fig. 6. RBCs passing through the microchannel of the DMC microseparator at (a) an average flow velocity of 0.2 mm/sec with an external magnetic flux, and (b) an average flow velocity of 0.2 mm/sec without an external magnetic flux

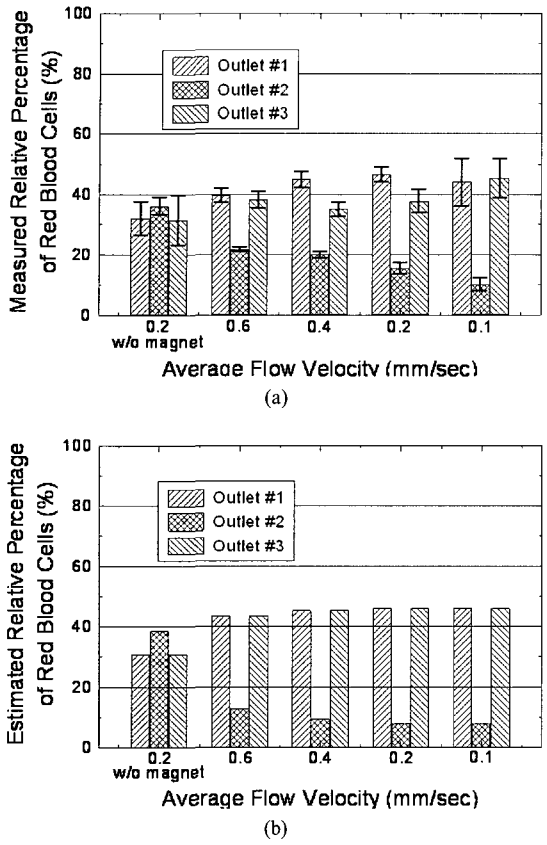


Fig. 7. The relative separation percentage of RBCs at each outlet of the DMC microseparator for various average flow velocities: (a) measured results; (b) estimated results

field. These experimental results also demonstrate that the DMC microseparator can move RBCs from the edge of the wire which is 50 μm away within 5 minutes. The measured relative separation percentage of RBCs at each outlet (Figure 7(a)), measured three times with a Coulter counter (Beckman Coulter, Inc., Fullerton, CA, USA), shows that the DMC microseparator separates out 89.7 % of the RBCs from outlets #1 and #3 at 0.1 mm/sec flow velocity. Figure 7(b) shows the estimated relative separation percentage of RBCs while varying the flowing velocity. The difference between the estimated and measured relative separation percentage of RBCs from the outlet #2 was 2.4 % to 8.9 %. The main reason of this may be the friction effect between the RBCs and the glass surface on the bottom of the microchannel. The friction effect explains why the measured relative separation percentage of RBCs was generally lower than the estimated result, as shown in Fig. 7(a) and 7(b).

2) Separation of White Blood Cells

To improve the reliability of the experiments related to the WBCs, fresh bovine blood was used within 12 hours after being drawn. This precaution was taken due to concern about change in the magnetic susceptibility [14], and the surface properties of WBCs over time. To prepare WBC-rich blood sample, 5 ml bovine whole blood was centrifuged and mixed with 10 ml of 3 mM isotonic sodium hydrosulfite solution. The WBC-rich blood sample was probed by incubation at 37°C for 40 minutes with a cell permeable nucleic acid fluorescent dye (S-7575, Molecular Probes, Inc., Eugene, OR).

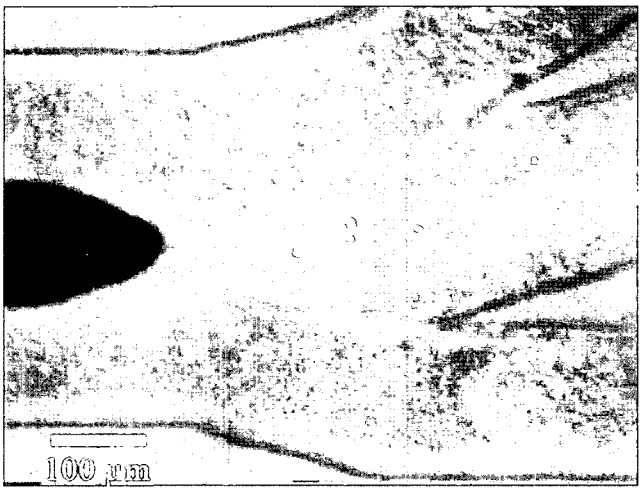
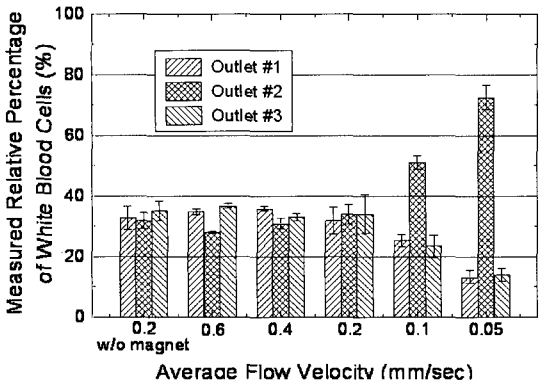
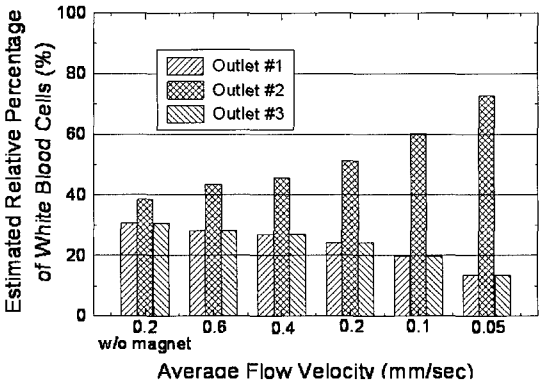


Fig. 8. Fluorescent probed WBCs passing through the microchannel of the DMC microseparator at an average flow velocity of 0.05 mm/sec

Figure 8 shows an image of the WBCs, probed with the fluorescent dye, flowing at a velocity of 0.05 mm/sec through the microchannel with an external magnetic flux of 0.2 T. By counting WBCs through a microscope (ME600, Nikon Instruments, Inc., Melville, NY) with a fluorescence detector (Y-FL, Nikon Instruments, Inc.), the relative separation percentage of WBCs at each outlet was measured for varying flow velocities of 0.05 mm/sec to 0.6 mm/sec, as shown in Figure 9. The average and standard deviation were calculated from three measured data sets. The experimental results show that the DMC microseparator separates out 72.7 % of the WBCs from the outlet #2 at 0.05 mm/sec flow velocity. To estimate the relative separation percentage of WBCs, the viscosity of the WBCs and average radius of the WBCs were assumed to be 0.96×10^{-3} N·s/m^2 and 5 μm [31], respectively. Under the assumption, the relative magnetic susceptibility of the WBC was fitted to -0.234×10^{-6}(SI), which makes an



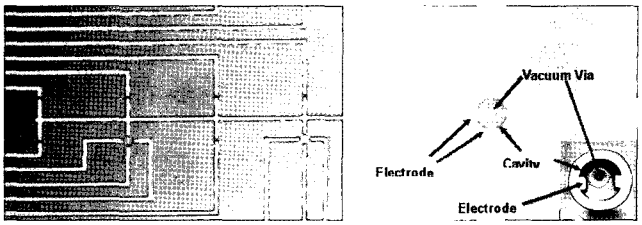
(a)



(b)

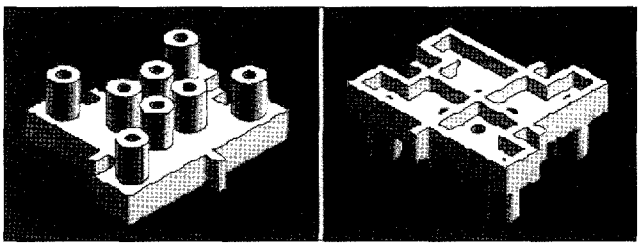
Fig. 9. The relative separation percentage of WBCs at each outlet of the DMC microseparator for various average flow velocities: (a) measured results; (b) estimated results

estimated relative separation percentage of WBCs equal to the measured value of 72.7 % from the outlet #2 at 0.05 mm/sec flow velocity.



(a) (b)

Fig. 10. μ-EPAS system showing (a) array of cavities and (b) enlarged view showing the 16.0 μm diameter polyimide cavity with a 3.0 μm diameter vacuum via at the center and four electrodes coming into the cavity. Inset: A scanning electron micrograph showing the two-electrode design with a vacuum via, cavity, and electrodes



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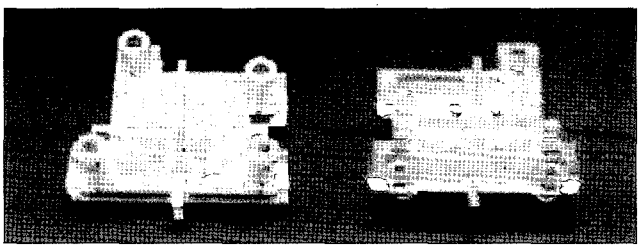

(b)

Fig. 11. Backside fluid channels and fluid interface fabricated using stereolithography: (a) Design and (b) Fabricated part

2. Electrical Impedance Spectroscopy System

Figure 10 shows a micrograph of the fabricated system. Arrays of analysis sites and electrodes connecting each analysis cavity on a three-inch diameter silicon wafer can be seen. Figure 10(a) shows arrays of eight cavities with two and four opposing electrode configurations interfacing with each analysis cavity. Figure 10(b) shows a 16.0 μm diameter polyimide cavity with a 3.0 μm vacuum via in SiO_2 and four 4.0 μm wide opposing gold electrodes. The inset shows a scanning electron micrograph of the analysis cavity where the vacuum via, two opposing electrodes, and the polyimide cavity can be observed. Figure 11 shows the backside fluid channels and fluid interface including inlet/outlet ports created using stereolithography. The inner diameter of the ports was 1700 μm, into which a tubing was fit in tightly.

The impedance measurements from red blood cells and bovine chromaffin cells are plotted in Figure 12. Large differences in cell impedance were observed at lower frequencies of the spectrum. At higher frequencies, the curves leveled out to a characteristic impedance value due to the elimination of the membrane capacitive component. Figure 12(a) shows that the magnitude measurement for red blood cells showed up commonly at two different levels. The first population fell in between 1 kΩ and 10 kΩ, and accounted for most of the cells observed. The other population fell in between the 100 kΩ to 1000 kΩ range. Distinct phase readings were observed for chromaffin cells and red blood cells. This is shown in Figure 12(b) where the chromaffin cells with a lower magnitude had a lower phase, while chromaffin cells with

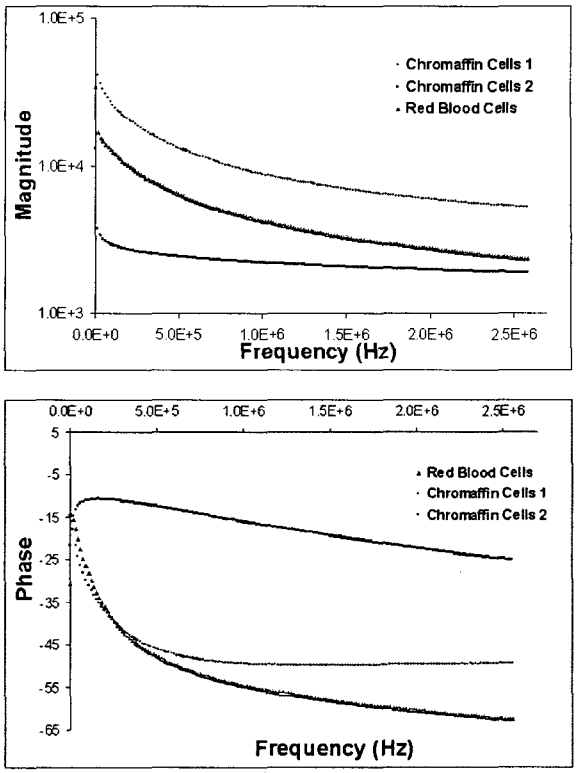

Fig. 12. Electrical impedance spectrum for different sub-populations of bovine chromaffin cells and human red blood cells

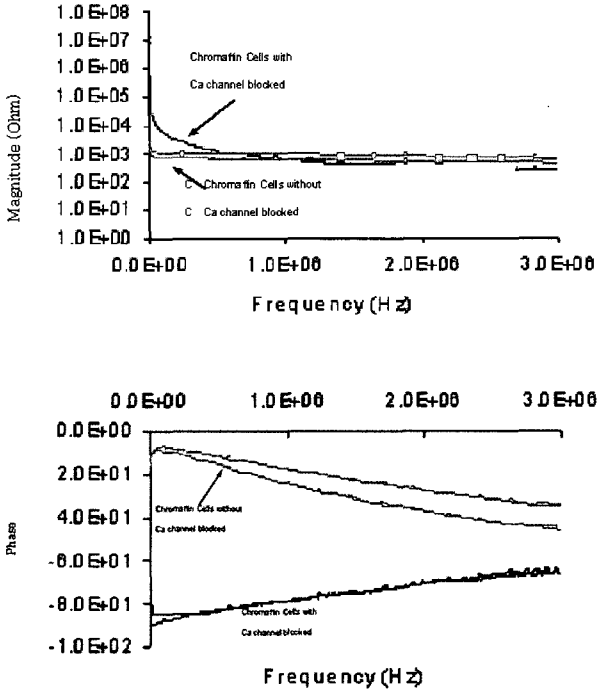

Fig. 13. Electrical impedance spectrum for chromaffin cells with Ca channel blocked compared to normal cells

a higher magnitude exhibited a higher phase. Chromaffin cells averages in sizes of 12 μm and 20 μm in diameters. The magnitude did show some segregation according to size with larger cells having a lower magnitude and the smaller cells having a smaller magnitude. However there was some overlap between the magnitude readings making it difficult to see a significant difference. The phase data showed a much more significant difference between cell types.

Figure 13 shows the impedance measurement of chromaffin cells with calcium channels blocked compared to normal cells of 20 μm in diameter. At lower frequencies, the cells with ion channels blocked showed a higher magnitude than normal chromaffin cells. The initial readings were in the 10 kΩ range and then dropped to the 1 kΩ range at higher frequencies. The phases for cells with calcium channels blocked were significantly different compared to that from normal cells. Larger phases were recorded at lower frequencies and smaller phases were recorded at higher frequencies.

Two different groups of chromaffin cells were observed by the μ-EPAS. Correlating the sizes of the cell to impedance showed that although there was some relation, it did not account for all the differences observed. This may be due to cell viability. The health of chromaffin cells declines soon after sample preparation and can significantly affect the measurement. The recordings from the cells with calcium channels blocked show that the μ-EPAS has some sensitivity to ion channel functions, especially when looking at the phase data. This is significant in that the system may be able to detect how ion channel activity of the cells has been affected due to various chemical stimuli or pathogens that alter ion channel functionalities.

IV. Conclusions

A microsystem for whole blood purification and electrophysiological analysis of the purified cells has been developed. The system was developed on silicon and plastic substrates utilizing conventional microfabrication technologies and plastic microfabrication technologies. Whole blood was purified using magnetophoresis in continuous diamagnetic capture (DMC) mode to increase the concentration of white blood cells. The results show that 89.7 % of the red blood cells (RBCs) could be

continuously separated out from a whole blood sample within 5 minutes using an external magnetic flux of 0.2 T. Electrical impedance spectroscopy (EIS) was utilized for electrophysiological analysis of single cells. Primary cultured bovine chromaffin cells and human red blood cells were manipulated and the electrical impedances were measured. Ion channels of chromaffin cells were blocked by toxins and the effect was monitored using EIS. Good discrimination between different cell types and different electrophysiological status were observed.

ACKNOWLEDGMENTS

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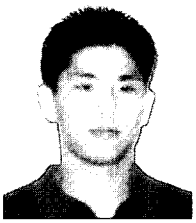

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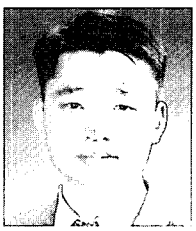

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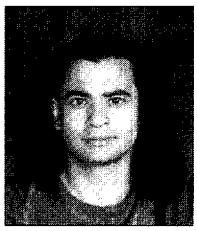

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