Selectively Concentrating Cervical Carcinoma Cells from Red Blood Cells Utilizing Dielectrophoresis with Circular ITO Electrodes in Stepping Electric Fields

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Abstract

The detection of rare cells, such as circulating tumor cells, circulating fetal cells, and stem cells, is important for medical diagnostics and characterization. The present study develops a handheld electric module which provides stepping electric fields for dielectrophoresis (DEP) to selectively concentrate cervical carcinoma cells (HeLa) from red blood cells, making it low-cost and automated. To observe the experiments, transparent electrodes were fabricated by patterning indium-tin-oxide-coated glass. Positive dielectrophoretic cells were guided toward the center of the microchamber due to the movement of the high-electric-field region. The magnitude of the DEP force acting on HeLa cells is about seven-fold that acting on red blood cells under a given electric field distribution, making it possible to separate HeLa cells from normal blood cells. HeLa cells were successfully concentrated in 160 seconds with an applied peak-to-peak voltage of 16 V at a frequency of 1 MHz.

Keywords: Isolation; Dielectrophoresis (DEP); Stepping electric field; Rare cells

1. Introduction

The manipulation of biological cells is essential to many biomedical applications, such as the isolation and detection of rare cancer cells, the concentration of cells from dilute suspensions, the separation of cells according to specific properties, and the trapping or positioning of individual cells for characterization. Among these applications, concentrating rare cells, such as circulating tumor cells (CTCs), circulating fetal cells, and stem cells, is important in biological and clinical studies [1-3]. Numerous methods for concentrating biological cells have been proposed [1,4], such as immuno-affinity, filtration (ISET, isolation by size of epithelial tumor cells) [5], fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS) [6], cell surface markers, and dielectrophoresis (DEP). Fu et al. [7] developed a microfabricated fluorescence-activated cell sorting (\textmu FACS) device for \textit{Escherichia coli} HB101 cells. The cells are manipulated electrokinetically at a T-shaped fluidic junction on their chip, which is mounted on an inverted optical microscope, and fluorescence is readout with a photomultiplier tube. The application of biotinylated heat-shock protein 60 as a capture molecule for viable \textit{Listeria monocytogenes} in a microfluidic channel was reported [8] and combined with a dielectrophoretic force generated by interdigitated electrodes to enhance the capture rate. A highly sensitive and specific identification of CTCs could prove helpful in the early diagnosis of invasive cancers [9]. Methods for CTC detection are generally divided into cytometric- and nucleic-acid-based techniques, both of which require an enrichment and detection procedure [1,10]. However, most of the aforementioned approaches require the use of antibodies or other molecular recognition elements to target cell populations of interest. Therefore, alternative methods which do not rely on molecular recognition elements and can retain cell function and viability following isolation are desirable.

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DEP is achieved under a non-uniform electric field generated by various electrode patterns. The sign of the DEP force, positive or negative, is controlled by the dielectric properties of the particles and medium, which are functions of frequency. Microfabrication technology has been employed to create microelectrode patterns [11,12], which allow sufficiently large DEP forces to be generated to manipulate particles with small applied voltages. For instance, bacterial cells were trapped using DEP generated by a set of interdigitated electrodes in a continuous flow [13]. By closing the microvalves after trapping the bacterial cells in the sample, the volume is reduced and therefore the locally detectable concentration of the genetic material is enhanced. Moon et al. [14] proposed a microfluidic device that comprises serially integrated multi-orifice flow fractionation (MOFF) and dielectrophoretic cell separators, which are capable of high-speed continuous separation, and separated human breast cancer cells from a blood cell sample. Cells were separated based first on their size in the MOFF channel and then on their dielectrophoretic properties in the DEP channel.

Microfluidic devices with vertical interdigitated electrodes embedded in the channel sidewalls have been designed for cellular separation by dual-frequency coupled dielectrophoretic forces [15]. DEP generated by three-dimensional carbon electrodes has been integrated with a compact disk (CD)-based centrifugal microfluidic platform to enhance filtering efficiency [16]. The isolation of HeLa cells from normal peripheral blood cells has been achieved on a silicon chip containing a five-by-five array of microlocations due to the differences in their dielectric properties [17]. Gascoyne et al. [18] developed an approach for isolating malaria-parasitised cells by dielectrophoretic manipulation in spatially inhomogeneous, travelling electrical fields. Applying four-phase signals to a spiral array of four parallel microelectrodes caused normal erythrocytes to be trapped at the electrode edges, whereas parasitised cells were levitated and carried toward the center of the spiral by the travelling field. DEP field-flow fractionation (DEP-FFF) has been applied to the isolation of TICs from clinical blood specimens using simulated cell mixtures of cultured tumor cells with peripheral blood [19]. MDA-435, MDA-468, and MDA-231 cells were successfully isolated from blood samples by exploiting cell morphological characteristics without labeling procedures.

Insulating structures (obstacles) have been used to produce non-uniform electric fields, termed insulator-based DEP (iDEP) [20]. Geometrical constrictions formed by insulating structures have been employed to squeeze the electric field in a conductive medium, hence producing a local maximum of the electric field gradient. A microfluidic device composed of a series of microchannels constructed with insulating microstructures for the concentration of micro/nanoparticles using direct-current DEP in a continuous fluid flow was proposed [21]. All the incoming particles are trapped when a sufficiently high voltage (> 400 V) is applied. The distance between metal electrodes in iDEP devices can be on the order of centimeters; therefore, high voltages are usually required. High voltage may result in Joule heating and could damage cells. A pore-type iDEP device with microfabricated SU-8 membranes positioned between two indium tin oxide (ITO) electrodes, which reduce the required applied voltage, has been developed for particle trapping [22].

An alternative technique, termed contactless DEP (cDEP), has been proposed to provide the non-uniform electric fields in microfluidic channels required for the dielectrophoretic manipulation of cells without direct contact between the electrodes and the sample [23]. The electric field is created in the sample microchannel using electrodes inserted into two conductive microchambers which are separated from the sample channel by thin insulating barriers. Microfluidic devices capable of selectively isolating live human leukemia cells from dead cells utilizing their electrical signatures have been developed [24]. The frequency response of breast cancer, leukemia, macrophages, and red blood cells (RBCs) has been investigated using a cDEP device [25]. Hence, conditions (such as the applied electric frequency and the medium conductivity) to detect and enrich CTCs from a peripheral blood sample can be determined to develop methods to isolate these rare cells. Additionally, the dielectrophoretic response of prostate tumor initiating cells (TICs) has been observed to be distinctly different from that of non-TICs in a microfluidic system employing cDEP [26]. Therefore, marker-free TIC separation from non-TICs utilizing their electrical signatures through DEP has been achieved. The method of cDEP relies on the application of a high-frequency alternating-current (AC) electric signal to electrodes which are capacitively coupled to a microfluidic channel, which limits its applications [27].

Many existing dielectrophoretic approaches for the isolation and characterization of CTCs require large equipment; therefore, the development of portable point-of-care devices for medical diagnostics and analysis is needed. In our previous study [28], interdigitated curvy electrodes were designed to generate stepping electric fields by switching the electric field to an adjacent electrode pair via relays, which were controlled by an 8-bit microcontroller. Our previous results demonstrated that positive dielectrophoretic cells could be conveyed along the direction of the stepping electric field powered by a function/arbitrary waveform generator. Opaque circular gold electrodes were employed to collect HeLa cells from RBCs in our previous studies [29,30]. RBC to HeLa cell ratios of 13 and 130 were used to demonstrate the feasibility of the method. A microfabricated module for DEP with circular microelectrodes for the rapid isolation of HeLa cells from RBCs is proposed in the present study. Circular microelectrodes were designed to generate a stepping electric field by switching the electric field to an adjacent electrode pair via relays. Two different designs of the central electrode are proposed and their performance is evaluated. To observe the experiments, transparent electrodes were fabricated by patterning ITO-coated glass. Experiments were conducted to demonstrate the capability of the proposed device to selectively concentrate cancer cells.

2. Theory and design

The DEP force ($F_{DEP}$) acting on a spherical particle of radius $R$ suspended in a fluid with permittivity $\varepsilon_r$ is given as:
where $E_{rms} = 2\pi R^2 \varepsilon_0 \text{Re}(f_{CM}) \sqrt{\varepsilon_r}$

\[ F_{CM} = 2\pi R^2 \varepsilon_0 \text{Re}(f_{CM}) \sqrt{\varepsilon_r} \]

(1)

where $\text{Re}(f_{CM})$ is the real part of the Clausius-Mossotti factor and $E_{rms}$ is the root-mean-square of the external electric field in an AC field. The Clausius-Mossotti factor ($f_{CM}$) is a parameter of the effective polarizability of a particle. It varies with the complex dielectric properties of the particle and the surrounding medium, which are functions of the frequency of the applied field ($f$). The Clausius-Mossotti factor for a spherical particle is represented as:

\[ f_{CM} = \frac{\varepsilon_p - \varepsilon_m}{\varepsilon_p^2 + 2\varepsilon_m} \]

(2)

where $\varepsilon_p$ and $\varepsilon_m$ are the complex permittivities of the particle and the medium, respectively. The complex permittivity is related to the conductivity $\sigma$ and angular frequency $\omega = 2\pi f$ as:

\[ \varepsilon' = \varepsilon - j\frac{\sigma}{\omega} \]

(3)

where $j$ equals $\sqrt{-1}$. Therefore, the DEP force depends not only on the difference between the dielectric properties of the particles and those of the suspending medium solution, but also on the size of the particles. The DEP force can be either positive, pulling particles toward the region with a high electric field gradient, or negative, repelling particles away from the region with a high electric field gradient.

A cell should be modeled taking its heterogeneous structure into account with the multi-shell model [31] when evaluating the dielectric properties of cells. The effective dielectric property of a multi-shell sphere can be simplified to a single-shell dielectric model with a cytoplasm surrounded by a shell of lipid membrane [32,33]. The dielectric properties of viable mammalian cells can be formulated using the single-shell model (protoplast model), which is based on a spherical particle consisting of a cytoplasm and a lossless capacitive membrane [34,35]. The membrane of cells mainly consists of a very thin lipid bilayer and is highly insulating with a conductivity of about 10$^{-7}$ S/m. The conductivity of cytoplasm (interior part of a cell) can be as high as 1 S/m, since cells contain many ions and charged particulates.

Therefore, the effective permittivity can be derived by neglecting the conductance of the membrane in the protoplast model; thus, the Clausius-Mossotti factor for viable cells can be rewritten as:

\[ f_{CM}(\omega) = \frac{\omega^2 (r_n - r_e)^2 + j\omega (r_n - r_e) - 1}{\omega^2 (2r_n + r_e)^2 - j\omega (2r_n + r_e) - 2} \]

(4)

where $r_n = e_n R/\sigma_n$ and $r_e = e_e/\sigma_e$ are time constants, and $\sigma_n$ and $\sigma_e$ are the electrical conductivity and permittivity of the cytoplasm, respectively. The parameters $C_m$ and $R$ represent the effective capacitance of the membrane and the radius of the cell, respectively. Moreover, the constants $r_n$ and $r_e$ can be defined as $r_n = e_n/\sigma_n$ and $r_e = e_e/\sigma_e$, respectively, where $\varepsilon_n$ and $\varepsilon_m$ are the electrical conductivity and permittivity, respectively, of the suspension medium.

Based on the protoplast model, viable HeLa cells suspended in a sucrose medium ($\varepsilon_c = 78; \sigma = 1.76 \times 10^{-3}$ S/m) exhibit a strongly positive dielectrophoretic response; i.e., the Clausius-Mossotti factor is 1.0 at a frequency of 1 MHz [35]. RBCs, which are the main part of a blood sample, are biconcave discs with a diameter of 7 to 8 µm and a width of 2 µm. It is assumed that RBCs are spheres with a diameter of 4.8 to 6 µm based on a volume of 60 to 120 fL [36]. For RBCs, the membrane capacitance ($C_m$) is 0.9 µF/cm², and the internal dielectric permittivity ($\varepsilon_c$) and conductivity ($\sigma_c$) are 57 $\varepsilon_0$ and 0.52 S/m, respectively [37].

The conductance of the membrane is ignored in the present study. The Clausius-Mossotti factor for RBCs determined from Eq. (4) is 0.91 at a frequency of 1 MHz. Therefore, the magnitude of the DEP force acting on HeLa cells with a diameter of approximately 10 µm is about seven-fold that acting on RBCs under a given electric field distribution in a sucrose medium, making it possible to separate HeLa cells and RBCs.

The operational concept of separating HeLa cells is illustrated in Fig. 1a. When an electric field is applied to the two adjacent microelectrodes, a high-electric-field region is generated between the electrode pair. The applied electric field is subsequently switched to the adjacent electrode pair via relays (from the outermost to the center pair of microelectrodes) to generate a stepping electric field. Positive dielectrophoretic cells are guided along the direction of the stepping electric field due to the movement of the high-electric-field region to the center of the circular electrode. The high-electric-field region between adjacent electrodes gradually shrinks towards the center. The movement of HeLa cells toward the center electrodes is faster than that of RBCs. As a result, the HeLa cells are concentrated onto the central microelectrode and isolated from RBCs. The pattern of central electrodes affects cell concentration, since HeLa cells are eventually collected on the central electrode. Schematic diagrams of the two proposed central electrodes are shown in Fig. 1b. A 30-µm-wide straight electrode was employed to collect HeLa cells at the center of the chamber. A lollipop-shaped electrode with a circle (90 µm in diameter) was also designed as the central electrode. Numerical simulations were performed to examine the differences between these two designs.

3. Experimental design

3.1 Chip fabrication

A biocompatible material, polydimethylsiloxane (PDMS), was adopted for the microchamber in the microchip for cell concentration. A schematic of the microchip with circular microelectrodes is shown in Fig. 2a. The cells were introduced into the microchamber using a pipette. The microchip was fabricated in-house using standard soft-lithography techniques. The electrodes were patterned by etching ITO glass substrates using an HCl solution. A PDMS prepolymer mixture (Sylgard-184 Silicone Elastomer Kit, Dow Corning, Midland, MI, USA) was diluted with hexane, with a PDMS to hexane weight ratio of 1:5. The 3-µm-thick hexane-diluted PDMS prepolymer was spin-coated onto the electrodes to prevent electrolysis and cell adherence to the electrodes. The mold
A handheld electric module with a voltage-to-frequency converter and an operational amplifier was developed in our previous study [29]. The microfabricated chip was mounted on the electric module, which generated the stepping electric field for cellular concentration, as shown in Fig. 2c. The cells were observed and recorded using an inverted fluorescence microscope (Model CKX41, Olympus, Tokyo, Japan), a mounted CCD camera (DP71, Olympus, Tokyo, Japan), and a computer with Olympus DP controller image software.

### 3.3 Cell sample preparation

Studies have shown that tumor cells are likely to exist in the bone marrow or in the peripheral blood of patients with various carcinomas [38], such as cervical cancer [39]. A human cervical carcinoma cell line (HeLa cells) was cultured [29] for an experimental demonstration of isolating HeLa cells using the proposed microfabricated module. The cells were stained using a standard fluorescence assay with calcein AM (Molecular Probes, Eugene, OR, USA) prior to the experiment.
Calcein AM is a green fluorescent dye which is able to penetrate the cell membrane into the cytosol and transform into a fluorescent form when it is hydrolyzed by esterases located inside cells. The cell samples were then suspended in an 8.62 wt% sucrose solution with a measured conductivity of 1.76 × 10⁻³ S/m. The sucrose solution was employed to increase the osmolarity to normal physiological levels. This isotonic solution was used for the dielectrophoretic separation experiments. A physiological fluid, for example phosphate-buffered saline (PBS), should be employed to increase clinical relevance. However, it is challenging to use DEP with a highly conductive medium due to Joule heating and bubble formation [23]. Furthermore, the whole blood is a suspension of mostly elastic particles (i.e., cells) in a Newtonian fluid of plasma, which results in non-Newtonian characteristics. The viscosity and viscoelasticity of whole blood depend on the shear rate the blood experiences. Plasma and blood cells are the two major components of whole human blood. In terms of cellular fraction, there are approximately 5 × 10⁹ cells per milliliter of whole blood, including erythrocytes, leukocytes, platelets, and phagocyte cells [40]. Whole blood is usually treated by adding some anticoagulant, such as EDTA, or diluted by physiological sodium chloride irrigation solution, whose fluidic properties are similar to those of water. For simplifying the experimental procedures, the peripheral blood collected from volunteers was diluted in sucrose solution with a concentration of 3.25 × 10⁵ cells/mL. Most of the cells in a blood sample are RBCs and the concentration of white blood cells (WBCs) is about a thousandth of the total cell concentration in the blood [40]. The concentration of WBCs in the diluted solution was approximately 3.25 × 10³ cells/mL. Few WBCs were observed in the microchamber. Hence, the influence of WBCs is ignored in the present study. It should be noted that the size and shape of WBCs are similar to those of cancer cells; therefore, isolating cancer cells from the WBCs is an important issue in clinical applications.

4. Results and discussion

High-strength electric fields, a long exposure time, or both could result in rupture of the cell membrane and cell lysis [41,42]. The field strength required for the lysis of mammalian cells is on the order of magnitude of 10⁶ V/m and cell lysis can be achieved in less than 33 ns using a 1 ms pulse length [43]. A peak-to-peak voltage of 16 V at a frequency of 1 MHz was thus applied here to ensure the viability of cells. Numerical simulations of electric fields using the commercial software package CFD-ACE⁺ (ESI Group, France) [29] were performed to investigate the differences between the two proposed designs. The simulation results of the magnitude of the gradient of the electric field squared (|\( \nabla E^2 \)|) with a peak-to-peak voltage of 16 V applied to the two most inner electrodes for the straight and lollipop-shaped central electrodes are shown in Fig. 3. The numerical results indicate that the high-electric-field-gradient region was distributed along the edge of the central electrode for the straight electrode. However, for the lollipop-shaped central electrode, it was found that |\( \nabla E^2 \)| at the circular part of the central electrode (90 µm in diameter) was much higher than that along the 30-µm-wide straight part of the electrode connected to the circular part. The high-electric-field-gradient region was focused at the center for the lollipop-shaped central electrode. This design is beneficial for making the high-electric-field-gradient at the center of the chamber. The simulated |\( \nabla E^2 \)| with an applied peak-to-peak voltage of 16 V from the outermost microelectrode pair to the central microelectrode pair via switching relays is shown in Fig. 4. The numerical results indicate that the high-electric-field-gradient region moves along the direction of the stepping electric field. Moreover, the size of the high-electric-field-gradient region changed with the pattern of the microelectrodes. Particles with a positive dielectrophoretic response could be guided along the direction of the stepping electric field toward the center of the microchamber, and concentrated at the central pair of electrodes.

![Figure 3. Simulation results of the magnitude of the gradient of the electric field squared (|\( \nabla E^2 \)|) for two designs of center microelectrode pair. The voltage applied is 16 V.](image)

![Figure 4. Simulation results of the magnitude of the gradient of the electric field squared (|\( \nabla E^2 \)|) with the electric field applied from the outermost microelectrode pair to the center microelectrode pair via switching relays. The voltage applied for the stepping electric field is 16 V.](image)
pair of electrodes was turned on to aggregate the cells. The electric field was held for about 20 seconds and then switched to the next adjacent pair of electrodes. The duration of cellular concentration from the outermost to the central pair of microelectrodes was about 160 seconds. The concentrations of HeLa cells and RBCs introduced into the microchamber were $2.5 \times 10^5$ cells/mL and $3.25 \times 10^6$ cells/mL (the ratio of RBCs to HeLa cells was 13), respectively. Initially, both the HeLa cells and RBCs were distributed randomly on the substrate. However, the magnitude of the DEP force acting on HeLa cells is about seven-fold that acting on RBCs under a given electric field distribution in a sucrose medium. HeLa cells were thus more attracted to the central electrodes than were RBCs when the stepping electric field was applied.

![Figure 5](image1.png)

**Figure 5.** Experimental results of the isolation of HeLa cells from a blood sample. The concentrations of RBCs and HeLa cells in the microchamber were $3.25 \times 10^6$ cells/mL and $2.5 \times 10^5$ cells/mL, respectively. The voltage applied and frequency were 16 $V_p$ and 1 MHz, respectively. The time interval of relay switching was 20 seconds.

The experimental results indicate that the HeLa cells were selectively concentrated at the center pair of microelectrodes from RBCs with a recovery rate of around 64.5% (in which the ratio of RBCs to HeLa cells was 13), when the time interval of relay switching was set to 20 seconds. The recovery rate, which is defined as the number of cells collected at the central electrode divided by the number of total cells initially distributed in the microchamber, was evaluated by manually counting the number of cells. Experiments on samples with lower cellular density were also performed. A sample of rare cells is that containing less than 1,000 target cells per milliliter [4]. The volume of the microchamber (113 nL) was too small to perform experiments with samples with such a low concentration. However, the main purpose of this investigation is to demonstrate the working principle of inverting the stepping electric fields. The experimental results for the cell concentration of $10^4$ cells/mL are shown in Fig. 6, in which the ratio of RBCs to HeLa cells is 325. Several experiments with a RBC to HeLa cell ratio of 325 were conducted. The HeLa cells were selectively concentrated onto the central microelectrode and isolated from the blood sample even with only a single cell in the microchamber. RBCs and WBCs occupying the central electrode initially may exclude HeLa cells. However, the concentration of CTCs increases at the central electrode due to the stepping electric fields. The immunoassay could be further employed after collecting CTCs to overcome the interference of WBCs and RBCs to improve the sensitivity of detection.

![Figure 6](image2.png)

**Figure 6.** Experimental results of the isolation of low-concentration HeLa cells from a blood sample. The concentrations of RBCs and HeLa cells in the microchamber were $3.25 \times 10^6$ cells/mL and $10^4$ cells/mL, respectively. The voltage applied and frequency were 16 $V_p$ and 1 MHz, respectively. The time interval of relay switching was 20 seconds.

The proposed microdevice for the isolation of cancerous cells is capable of enhancing sensitivity for subsequent CTC detection. However, the influences of undiluted blood samples, the existence of WBCs, and the usage of physiological fluid should be further investigated in the future to make this technique more clinically relevant. The most important challenge of CTC detection is the extreme low concentration of various types of CTCs. Increasing the volume of the proposed device or integrating it with a microfluidic platform in the future might solve this issue.

5. Conclusion

A microfabricated module for DEP with circular microelectrodes for selectively concentrating HeLa cells from...
RBCs was proposed and its feasibility was demonstrated. The applied electric field was switched to the adjacent electrode pair via relays to generate a stepping electric field. Transparent electrodes were patterned by etching ITO glass substrates. Positive dielectrophoretic cells were guided by the movement of the high-electric-field-gradient region. The magnitude of the DEP force acting on HeLa cells is about seven-fold that acting on RBCs under a given electric field distribution in a sucrose medium, making it possible to separate HeLa cells and normal blood cells. Initially, both the HeLa cells and RBCs were distributed randomly on the substrate. When the stepping electric field was applied, the high-electric-field region between adjacent electrodes gradually shrunk toward the center. The movement of HeLa cells toward the central microelectrode and isolated from the blood sample. The proposed microdevice for the selective concentration of cancerous cells is capable of enhancing sensitivity of subsequent CTC detection. Moreover, the proposed microfabricated module is relatively simple and can be applied in point-of-care applications. The proposed device can be applied in the fields of clinical diagnostics, biological assays, and biomedicine.

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