Dielectrophoretic Characterization of Red Blood Cells

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Abstract

Medical microdevices have been touted as the doctor's 'toolkit of the future' for their potential ability to perform a variety of diagnostic tasks while utilizing only a single drop of blood. This work describes a unique lab-on-a-chip device that performs blood sample preparation via cell rupture to facilitate access to intracellular biomarkers. Traditional rupturing is accomplished via chemical lysis, which can leave unwanted chemicals in microdevice channels and interfere with subsequent separations, purifications, and analysis of desired proteins or other biomarkers. A recent alternative advanced by our lab group is rupturing the blood cells via dielectrophoresis; it is chemical free and entirely field dependent, so the intracellular contents of the red blood could be subsequently analyzed on the same chip. The purpose of this work is to quantify the rate at which red blood cells rupture when exposed to a 1kHz alternating current electric field. The two variables tested herein are the blood type and the age of the blood after donation has occurred. Rupturing behaviors at a 1 kHz dielectrophoretic field were quantified as a function of age for blood types A+, B+, AB+, O+ and O-. Age dependency was tested by conducting rupture experiments of the cells every three days after blood donation. The work presented herein focuses on the rupturing rate of five different blood types on the day of donation. Erythrocyte rupturing rates were quantified by examining the still frames of video of ruptured red blood cells to determine the rupture rate. Rupturing rate varied based on age and blood type from no rupturing to approximately 90% of the original number of red blood cells in 14 minutes. This work would advance and simplify medical microdevices by enabling one step lysis in line with separation, purification, and analysis for fully integrated diagnostic blood tests.

Introduction

Microdevices, also known as lab-on-a-chip devices or micro total analysis systems, emerged in the 1990's for the use of cell identification and analysis. These devices are seen as one of the key growth industries for the 21st century [1]. The accessibility of these devices make them very attractive for use as point-of-care devices in situations such as roadside emergencies, natural disasters, or for use in remote field locations where access to traditional bench scale equipment is difficult. These miniature devices require dedicated techniques for cell separation and subsequent cell analysis. One such technique explored herein is the use of dielectrophoretic electric fields for red blood cell hemolysis.

Dielectrophoresis (DEP) is the movement of particles in a non-uniform alternating current (AC) field. When a charged particle is subjected to a non-uniform AC field, the particles become polarized and move in accordance with their dielectrophoretic force. The particle will experience a force towards the high or low electric field density regions based on its polarizability [2]. If the particle is more polarizable than the medium the particle will move

towards the region of high field density. If the particle is less polarizable than the medium, it will move towards the low field density regions. Bioparticles and cells are more complex than homogenous colloidal particles and therefore demonstrate more complex behaviors in dielectrophoretic fields [3]. Dielectrophoresis has been explored as a way to quickly separate and identify living cells [4]. Previous red blood cell dielectrophoretic studies have focused on frequencies nearing 1MHz and have recorded the movement of the red blood cells as function of age and blood type [3,5]. While these tools have focused on intact cell manipulations, our work has revealed what 1 kHz dielectrophoresis can rupture cells such that access to sub cellular analytes is possible.

Therefore dielectrophoresis may be useful for cellular preparation for subsequent proteomic and genomic analysis. Common methods of cell destruction include chemical lysis or electroporation. The most common chemicals used for red blood cell destruction are hypochlorous and hypobromous acid [6]. The use of such chemicals in microdevices can present complex design challenges. These challenges include overcoming problems with on chip storage of reagents, flowing and contacting the chemicals with the cells, mixing to insure adequate contact time, the addition of a washing step after separation and the possibility of unwanted effects in the final analysis [7]. Chemical lysis can be caustic and cause harmful or unwanted interactions in later analyses.

Through the fields of electroporation and pulsed field electrophoresis, it is known that cells can also be irreversibly damaged either accidentally or as a means of cell rupturing using electric fields much higher than 10kHz [8]. It has been demonstrated that the membranes of both free flowing cells and those adhered to the bottom of a channel can be permeabilized by electroporation using a mechanical valve to create direct current pulses. This technique eliminates the cost of microfabricated electrodes that are often found in cell lysis devices [9]. Cell destruction by dielectrophoresis however, uses much weaker fields than by electroporation and is usually due to a breakdown of the membrane surrounding the cell [8]. Lysis by dielectrophoresis has many advantages such as the lack of chemicals allowing for integration of lysis and analysis on a single microchip and the low power needed for a dielectrophoretic approach would enable device portablilty. One application of dilectrophoretic cell lysis is in medical diagnostics. Blood is a convenient diagnostic fluid because it is easy to attain uncontaminated samples and provides chemical information about systemic physiology. Blood is composed of erythrocytes (red blood cells = 45%), leukocytes (1%) and plasma (55%) [10]. Due to erythrocyte prevalence in blood, this work focuses on red blood cells.

The average adult has 4.2 million to 6.2 million red blood cells per microliter. For males the average is 5.5 million red blood cells per microliter as opposed to 4.5 million red blood cells per microliter for females [10]. Red blood cells are biconcave disks of diameter 7 microns and thickness 2 microns; this shape maximizes the surface through which the cells can transport oxygen and carbon dioxide. The life span of a red blood cell in circulation is approximately 120 days and undergoes physiological changes during that time such as increased membrane fragility which can make red blood cells more susceptible to hemolysis [10].

An incredibly important factor when working with blood is the blood type due the varying polysaccharide molecules, or complex carbohydrate antigens found on the cell membrane. The ABO blood typing system was discovered by Landsteiner in 1900 [11]. This blood typing system is defined by the presence or absence of two main antigens. Type A blood is classified by the presence of the A antigen, whereas type B blood is classified by the presence of

the B antigen. Type AB blood has both A and B antigens and type O blood has neither antigen [10]. Another major factor in the ABO system is the presence or absence of powerful antibodies in the surrounding cell plasma. Normally the body does not produce antibodies unless exposed to the antigen; an exception is for the antibody against the antigen that the body does not naturally produce. Antigen A and antigen B only vary by a subsitution on the terminal end of the antigen. Antigen A has N-acetyl-D-galactosamine, whereas B has N-acetyl-D-glucosamine. However because the number of antigens per cell can be as high as 1.5 million this small change makes a large cellular difference [7]. *In vivo* hemolysis, the natural demise of red blood cells in the body, is largely controlled by a protein binding to the red blood cell membrane, specifically to the antibody that has bound to the surface antigen to form an antigen-antibody complex. This complement protein then binds and induces hemolysis [10]. Due to the *in vivo* role of antigens in red blood cell destruction it is hypothesized that blood type will influence *in vitro* red blood cell destruction. In addition to the A and B antigens there is a third antigen that is important in matching blood types during transfusions [10]. This antigen is known as the Rhesus factor. If the Rh antigen is present, the blood type is classified as positive and if it is absent the blood type is positive [12]. Therefore, the eight resulting blood types are A+,B+,O+,AB+,A-,B-,O-,AB-. Due to the fact that the antigens that define blood type are found on the cell membrane and the cell membrane plays a major role in the ability of the cell to rupture, it is hypothesized that the rupturing of the red blood cell will be dependent on the blood type based on previously presented initial results [13].

The purpose of this work is to quantify the rate at which red blood cells rupture when exposed to a 1kHz alternating current electric field. The two variables tested herein are the blood type and the age of the blood after donation has occurred. Blood type is an important factor because it is an easily known chemical variant in the population that can be used to assess antigen role in membrane stability. Also testing the same blood type from various donors can assess the reproducibility of the experimental technique. Age of the blood after donation is important because often field tests will need to be later verified in a laboratory setting. Also blood banks could use this tool (i.e. rupture times) as an indicator of the stability and health of the stored samples. Rupturing behaviors at a 1 kHz dielectrophoretic field were quantified as a function of age for blood types A+, B+, AB+, O+ and O-. The was to assess the impact of the A and B antigens as well as the presence or absence of the Rhesus factor. Age dependency was tested by conducting rupturing experiments of the cells approximately every three days after blood donation.

Materials and Methods

Blood Sample Preparation

Whole blood samples representing blood types A+, B+, AB+, O+ and O- were obtained via venipuncture from anonymous donors at the Longest Student health center. This blood was then drawn into a vacutainer with 1.8 mg K_2 EDTA anticoagulant per mL of blood. After donation the blood vial was stored at 5^{O}C in a Biosafety Refrigerator. Just prior to experiments, whole blood was mixed with an isotonically matched phosphate buffer saline (0.140molar NaCl, 0.02489 molar KH_2PO_4, 0.00907molar K_2HPO_4) before being injected in a 600:1 V/V mixture into a custom fabricated microdevice for rupture.

Microdevice Fabrication

Custom microdevices were fabricated using the soft photolithography technique developed by Whitesides [14]. A high resolution AutoCAD mask was printed at 32,512 dpi by Fineline Imaging (Colorado Springs, CO). The autocad design can be seen below in Figure 1. Silica wafers were washed with various organic solvents (ethanol, isopropanol and acetone) and surface functionalized with sodium hydroxide. After drying, negative photoresist was spin coated onto the wafer at an even thickness of 200 microns. After baking the photoresist the AutoCAD mask was placed over the coated wafer and exposed to a UV light to imprint the mask design on the photoresist layer. After the masked photoresist was baked, the wafer was placed in the photoresist developer such that the features of the mask remained on the wafer and any excess photoresist was washed off with isopropanol and acetone. Polydimethylsiloxane (PDMS) elastomer (Dow Corning) and a curing agent were then cast over the design on the wafer and baked overnight at 65°C. The PDMS casting was gently peeled off the wafer, and sample ports were punched out with a biopsy punch. After electrodes were embedded in the PDSM the device was treated in a UV Ozone Generator for 10 minutes and thus irreversibly bound to a glass slide.

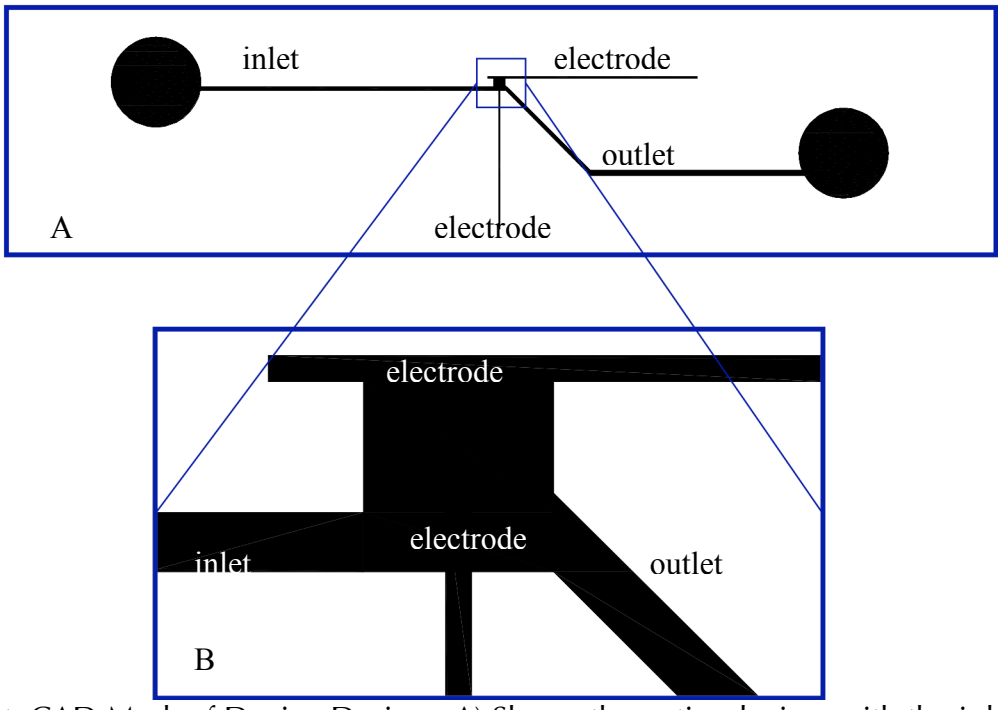

Figure 1. AutoCAD Mask of Device Design. A) Shows the entire device, with the inlet and outlet ports at the ends and the perpendicular lines for electrodes. B) Shows the experimental chamber of dimensions 800x800 microns with inlet and outlet channels, as well as channels for the two electrodes.

Experimental Procedure and Image Capturing

The two main variables tested for their impact on erythrocyte rupturing were age of the blood and blood type. It was important to hold the field density constant at .03 Vpp/micron for all experiments. This was done by measuring the gap distance between the electrodes and calculating the peak-to-peak voltage by:

$$E = V/d$$

where E is the constant field density of .03 volts/micron. The peak-to-peak voltage V was adjusted given the measured distance between the electrodes, d. Age experiments were determined from day 0 (the day the blood was drawn) and every two days thereafter.

A Zeiss Axiovert 200M inverted light microscope with a high resolution Axiocam camera was used to record images of the erythrocytes every fifteen seconds for fourteen minutes. The total number of remaining erythrocytes in the images were manually tabulated. All data was graphed using a percentage as a function of time:

$$X_{cells} = 100 * \frac{\#RBC_{time=t}}{\#RBC_{time=0}}$$

where the number of red blood cells at time zero was the initial amount of red blood cells visible in the field. These experiments were replicated twice a day every two days following donation. This allowed for a calculation of standard deviation for each blood type on Day 0.

Results

The number of ruptured cells were compiled and tabulated according to the formula above for the blood types A+, B+, AB+, O+ and O-. This was completed for Day 0, the day of donation and results compiled for the 14 minute run. A complete rupture profile was established for each blood type, as well as an overall percentage of red blood cells ruptured during the course of the experiment.

The percentage of red blood cells ruptured as a function of time for blood group A for one of the two Day 0 experiments is shown in Figure 2. For the first minute after the electric field is applied, the number of cells in the field of view increases due to cell migration into the imaging field of view. The cells then rapidly begin to rupture such that >30% rupture is achieved in the second minute. It is also interesting to note that after 300 seconds there seems to be no additional rupturing.

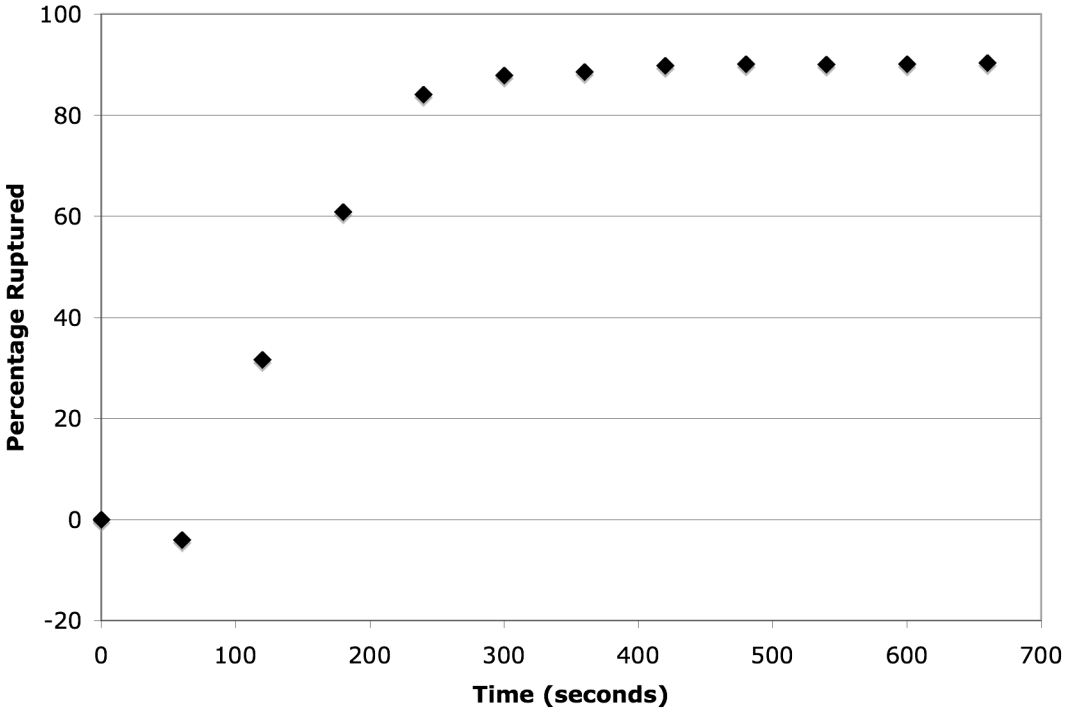

Figure 2: Rupturing of A+ on Day 0. The dip below 0% indicates a slight increase in the number of cells in the viewing area. Approximately 90% rupture is achieved in 660 seconds.

When comparing these profiles for each blood type, it became apparent that the rate of rupture varied greatly based on blood type for all of the Day 0 experiments. The percent of rupturing is compiled as a function of the blood type in Figure 3. For example, B+ red blood cells demonstrate minimal rupture over the course of 14 minutes, whereas approximately 50% of the O- red blood cells are ruptured. Standard deviation is not reported with A+ blood because while one experiment yielded bubbles at the electrodes (usually indicative of transient electrolysis reactions at the Platinum surface) and >90% rupture, the second experiment demonstrated no bubble formation and 0% rupture. The electrical connection was likely not complete in this second experiment and is in the process of being repeated. Also there are noted variations in the B+, O+ and O- results, which is hypothesized to be a result of the device design.

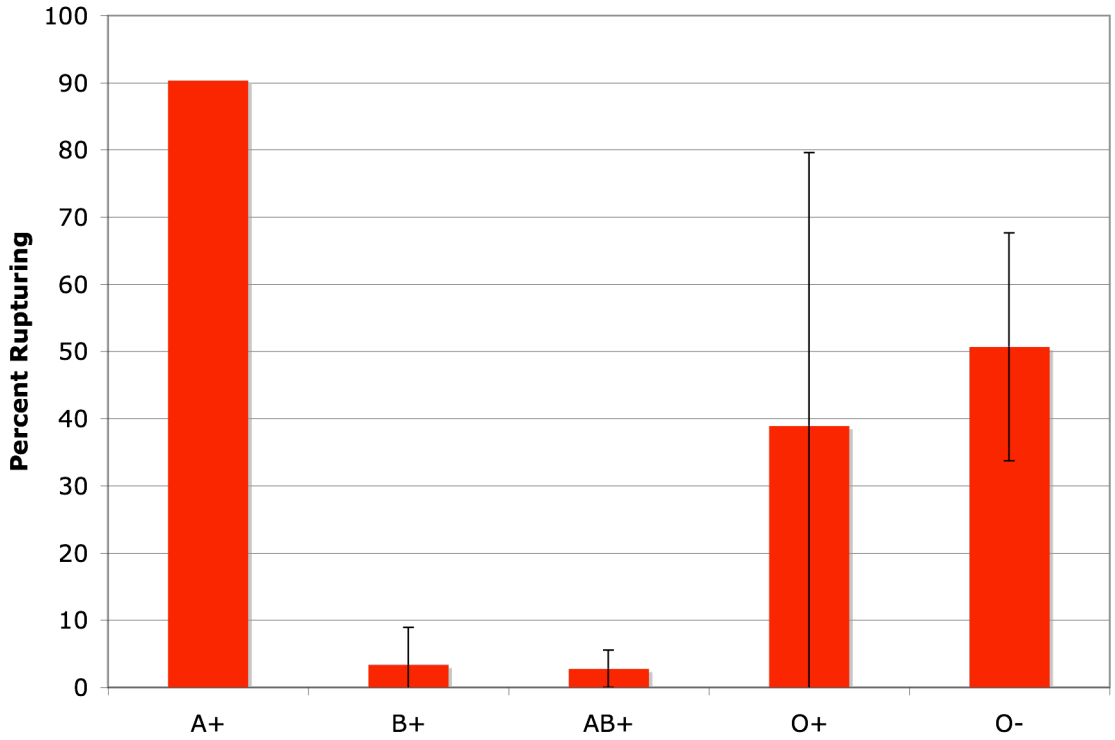

Figure 3: Percentage Rupturing over the Course of the Experiment. A+ ruptures more of its cells over the course of the experiment than any other blood type. Blood types containing B antigen (B+ and AB+) don't appear to rupture at all.

It is interesting to note that the presence or absence of the A and B antigens appear to have more of an effect on rupturing percentage than does the presence of the Rh antigen. This can be seen when comparing the large variation in rupturing percentages between A+, B+, AB+ and O+ but the comparatively similar rupturing percentages between O+ and O-.

Conclusions

Preliminary data from the influence of dielectrophoretic fields on various blood types suggests interesting trends. It was hypothesized that the blood type would have an effect on the rupturing rate due to their influence of antigens on membrane stability. Preliminary results suggest rupturing rate is influenced by blood type. It can be seen that the B+ blood barely ruptures whereas the O- blood ruptures approximately 50% of its cells in 14 minutes. The placement of the electrodes and their shape must be strictly controlled to keep an accurate field density of .03Vpp/micron for all of the experiments. The next step in this project is to quantify the affect of age on the rupturing rate of the red blood cells. This will involve completing analysis of experimental runs completed 2, 4 and 6 days after donation.

These experiments will further understanding the membrane properties of the red blood cells as they age. Understanding membrane properties of red blood cells will allow for the eventual implementation of dielectrophoretic rupturing onto lab-on-a-chip devices. The need to understand how these membrane properties change over time becomes very important when tests done in the field must be repeated later or when blood banks want to test blood in storage

for its viability. These rupturing experiments give us important information about how various blood types respond to electric fields while aging.

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