Continuous Separation of White Blood Cells From Whole Blood Using Viscoelastic Effects

Justin K. S. Tan, Sung-Yong Park, Hwa Liang Leo, and Sangho Kim

Abstract—White blood cells (WBCs) are the only cellular constituent containing genetic materials, and, hence, are candidate biomarkers for a host of diseases. However, conventional methods for WBC separation tend to have low sample purity and separation efficiency, which will have adverse implications on downstream polymerase chain reaction (PCR) analyses. In this study, we introduce a two-stage microfluidic device which harnesses the elastic property of a non-Newtonian fluid for size-based separation of WBCs from whole blood. The device displayed high resolution and efficiency in separating polystyrene particles and blood cells of different sizes up to a flow rate of 150 µL/min in polyvinylpyrrolidone solutions. We performed a separate parametric study to evaluate the effects of the fluid elasticity and flow rate on the separation performance. The hematocrit of the blood sample was varied from 0.01% to 20% to investigate the effect of increased intercellular interactions on the separation efficiency. An optimized set of parameters was selected to demonstrate the applicability of the device to the separation of WBCs from diluted whole blood, with excellent efficiency and purity (>90%). This microfluidic device will be especially useful for blood fractionation applications requiring high sample purity and speedy processing. Additionally, the apparent flow-rate insensitivity of the separation allows for its potential use in point-of-care applications.

Index Terms—Biomedical microelectromechanical systems, medical diagnosis, microfluidics, white blood cells.

I. INTRODUCTION

EUKOCYTES, white blood cells (WBCs), are the second most populous cellular constituent in our blood. They are responsible for a host of physiological functions, with their primary function being the regulation of the immune system. Unlike other blood cells, WBCs contain genetic materials, hence making them candidate biomarkers for blood-based diagnostics of various pathological conditions, such as heart disease [1]–[4], cancer [5], pneumonia [6] and HIV [7] and other pathological disorders [8]–[11]. However, such clinical applications require the extraction and purification of WBCs from a whole blood sample, as other blood cells may jeopardize the accuracy of cell counts [12], [13] and compromise the efficiency of polymerase chain reaction (PCR) for DNA analysis [14]. As such, significant efforts have been invested in developing strategies to isolate WBCs from a blood sample. However, conventional efforts such as density gradient centrifugation leukapheresis are hampered by challenges in handling WBCs due to their limited distribution in blood as well as marginal success in achieving sample purity.

Microfluidics is a platform that offers significant advantages for WBC manipulation, owing to its ability to manipulate particles through unique flow phenomena at the micron scale while simultaneously maintaining precise control over shear conditions in the microchannel. Previous studies have demonstrated the use of microfluidics for WBC isolation or enrichment through the use of passive methods such as hydrodynamic focusing [15]–[21] and affinity flow fractionation [22]–[25], as well as active methods such as acoustophoresis [26]–[29] and magnetophoresis [30]–[35]. While active methods boast higher separation efficiencies and purities, they often require complicated setup configurations in addition to sample pre-preparation steps prior to separation. These hamper the practical usage of these devices, especially for point-of-care applications. In one study, using a series of pillar microstructures to induce inertial flow deformations, Sollier et al. [16] achieved a separation recovery of up to 80%, with limited contamination by other cellular components. Similarly, Jung and Han [34] employed a multi-layer filtration chip to achieve high purities of up to 93% although this was undermined by lower WBC recovery rates. A summary of other recent work on passive separation of WBCs using microfluidics is provided in Table I. We utilized two parameters to assess the performance of the separation techniques: (i) Separation efficiency referring to the total amount of cells recovered out of the amount of cells present in the original sample and (ii) Separation purity referring to the amount of target cells collected as a fraction of the total number of cells collected.

The common observation among these reported WBC separation techniques was that a high separation efficiency was often accompanied by a low separation purity and vice versa. However, for better clinical applicability, WBCs separation should be performed with both high efficiency and purity.

Of late, viscoelastic focusing has been garnering attention in the field of particle separation. This is due to the intrinsic flows properties of viscoelastic fluids which allow for precise manipulation of particles without the need for complicated channel geometries. Elastic forces arising from non-uniform distribution of the first normal stress difference \(N_1\) in viscoelastic fluids...
TABLE I
OVERVIEW OF MICROFLUIDIC WBC SEPARATION TECHNIQUES

<table>
<thead>
<tr>
<th>Reference</th>
<th>Principle</th>
<th>Efficiency</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[15]</td>
<td>Modified pinched flow fractionation</td>
<td>100%</td>
<td>87%</td>
</tr>
<tr>
<td>[16]</td>
<td>Inertial migration induced by channel structure</td>
<td>80%</td>
<td>80%</td>
</tr>
<tr>
<td>[17]</td>
<td>Inertial migration in spiral microchannel</td>
<td>96.2%</td>
<td>86.8%</td>
</tr>
<tr>
<td>[18]</td>
<td>Inertial migration in spiral microchannel</td>
<td>90%</td>
<td>90%</td>
</tr>
<tr>
<td>[19]</td>
<td>Filtration using micropores</td>
<td>93.4%</td>
<td>14.8%</td>
</tr>
<tr>
<td>[20]</td>
<td>RBC lysis and WBC trapping using microstructures</td>
<td>60%</td>
<td>100%</td>
</tr>
<tr>
<td>[21]</td>
<td>Inertial migration in a curved channel</td>
<td>84%</td>
<td>70%</td>
</tr>
<tr>
<td>[22]</td>
<td>Filtration using micropillars</td>
<td>25%</td>
<td>89%</td>
</tr>
</tbody>
</table>

account for cross-streamline lateral migration of suspended particles. Accordingly, these forces can be modulated by varying the channel geometry, flow rate, and fluid property. This phenomenon, aided by the biocompatibility of many biopolymer-based viscoelastic fluid media, has been capitalized on for biological particle manipulation. Recently, Ahn et al. [36] demonstrated particle separation by employing the centerline focusing capabilities of elastic fluids when the flow approaches the inertial regime. This, however, suffered from the low separation efficiency of the collected particles. Our group has more recently demonstrated that 15-μm particles could effectively be separated from red blood cells (RBCs) and platelets using a viscoelastic fluid [37].

In this study, we exploit the cross-streamline properties of viscoelastic fluids for highly efficient separation of WBCs from whole blood. Blood cells display stark contrasts in their physical properties, the most obvious being their distinct size differences. Consequently, whole blood diluted in a biocompatible viscoelastic polymer solution exhibits a differential cell fractionation behavior due to the size dependence of the elastic force. In our microfluidic design [38], all the blood cells were focused and initialized in the first stage, before being separated in the second stage by size-dependent elastic force effects. This allowed for highly efficient separation of WBCs from a sample of whole blood by selecting the appropriate fluid and flow properties. A parametric study was performed to evaluate the effect of medium elasticity, flow rate, and hematocrit on the separation performance, with the goal of achieving sheathless separation of WBCs while preserving the recovery and purity of the separated sample.

II. MATERIALS AND METHODS

A. Sample Preparation

An elastic-dominant aqueous solution with a negligible shear thinning characteristic (Polyvinylpyrrolidone, PVP) was used as the suspending medium. PVP is biocompatible and has previously been used as a plasma expander for blood [39]. The solution was prepared by dissolving PVP powder (Mₘ = 360,000, Sigma Aldrich, USA) in PBS at three different concentrations (0.5, 1, and 3 wt%). The viscosities of the fluid samples were measured by a commercial rheometer (HAAKE MARS III, Thermo Scientific, USA) to be 3.39, 4.87, and 13.25 mPa·s, respectively at 25 °C.

Polystyrene (PS) microspheres (Fisher Scientific, USA) of 2, 6, and 15 μm diameters were suspended in the viscoelastic fluid at the final particle concentration of 0.01% (v/v). Before each experiment, the particle suspensions were mixed thoroughly using a micropipette to prevent particle aggregation and to achieve homogeneity in the particle suspension. Whole blood samples were obtained from a commercial source (i-DNA, Singapore). The blood samples were prepared with an anticoagulant (EDTA) and stored at 4 °C prior to use. In each experiment, the blood was aliquoted into separate microcentrifuge tubes and left to equilibrate to room temperature. WBCs were labeled with Acidine Orange (AO) (Life Technologies, USA), which stains double-stranded DNA green, hence excluding other blood cells from being labeled. In brief, a 0.5% stock solution was prepared by dissolving 50 mg of AO in 10 mL of distilled water. WBC viability was evaluated before and after separation using Trypan Blue exclusion. For pre-separation viability testing, the dilute blood sample was incubated with 0.4% Trypan Blue (Invitrogen, USA) at the ratio of 1:1 for 3 min. The sample was then loaded on a disposable hemocytometer and WBCs were checked for blue staining. This process was repeated with the sample collected at the target outlet after separation. To evaluate the optimum PVP concentration and flow rate for WBC separation from other cells, RBCs and WBCs were suspended in PVP solutions (0.5%, 1%, and 3% concentrations) at the cell concentration of 0.01% (v/v). For the study on the effect of hematocrit on the separation performance, RBCs were suspended at hematocrit levels of 0.01%, 0.1%, 1%, 10%, and 20%. To exclude the effects of PVP concentration on the separation dynamics, different PVP concentrations were used to dilute each sample to the appropriate hematocrit, so that the final PVP concentration of all the samples could be maintained at 1%.

All devices were flushed with 1% bovine serum albumin (Sigma Aldrich, USA) prior to use to minimize cell adhesion. For the particle separation experiments, flow rate was varied from 1 to 150 μL/min using a syringe pump (Hamilton Company, USA). The PS microspheres were observed using an inverted microscope (Olympus IX71, Olympus, Japan) with a high-speed video camera (Photron, USA). The lateral positions of PS microspheres were measured in the expansion region using custom-built MATLAB codes. The lateral positions (Y) were normalized by the width of the channel and thus Y varied from 0 (inner wall) to 1 (outer wall). The labeled WBCs were visualized with an sCMOS fluorescence camera (PCO, Germany) under epi-illumination with a blue filter (U-MWB2, Olympus, Japan; peak excitation and emission of 490 and 520 nm, respectively). For each blood sample, a sample size of 1000 cells was analyzed. Statistical analyses of the difference in lateral migration were performed using a two-tailed unpaired student’s t-test.
μ° is the characteristic shear rate, Q is the volume flow rate, h is the channel height, and w is the channel width. The elastic effect becomes significant when Wi ≫ 1, where as the inertial effect dominates for Wi ≈ 0. The medium elastic force in polymer solutions drives particles toward low shear regions under inertia-less flows (Wi > 1). This allows for particles to be entrained along characteristic equilibrium positions according to the channel cross section geometry. In a square channel, the regions at the corners as well as at the center have the lowest shear rates while the regions near the walls have the highest shear rates. By modifying the channel cross section to have a high aspect ratio, two-dimensional particle focusing along the vertical center plane can be achieved due to minimized corner-trapping of the particles [41]. In a viscoelastic fluid, the force \( F_E \) acting on the particle and velocity \( V_E \) of the particle are proportional to \( N_1 \) and the shear rate gradient as follows:

\[
F_E \propto a^2 \frac{\partial N_1}{\partial y}
\]

(1)

\[
V_E \propto a^2 W_i \frac{\partial \gamma}{\partial y}
\]

(2)

where \( a \) is the particle radius, \( y \) refers to the lateral position and \( \gamma \) is the local shear rate. Both the resultant elastic force and lateral migration are dependent on the particle size, with larger particles experiencing a larger elastic force and hence a higher migration rate. Both parameters are also directly proportional to the medium viscoelasticity, represented by \( W_i \) and the gradient of \( N_1 \).

A schematic of the proposed device for sheathless particle separation is depicted in Fig. 1. The device can be divided into two stages – initialization (1st stage) and separation (2nd stage). In the 1st stage, all particles are focused to the vertical center plane without the use of sheath flows simply by setting the cross section to have a high aspect ratio in the 1st stage of the device [41]. At the end of the 1st stage, particles focused along the vertical center plane of the channel are initialized at the inner walls of the channel after the 1st bifurcation that marks the progression to the 2nd stage. Following this, particles migrate under the influence of the elastic force exerted by the channel wall to equilibrium positions depending on the particle size. Since the elastic force acting on particles is proportional to the particle size (see Eqs. (1) and (2)), larger particles migrate faster and thus adopt positions nearer to the center of the channel.

The required channel dimension for each stage was determined based on the channel blockage ratio, \( \beta = \frac{w}{h} \). Previous studies have reported a critical value of 0.1 for the effective focusing of particles to a single equilibrium position at the center of the channel [42], [43], and to a single plane at the center of the channel when using channels of high aspect ratio [42]. In a high aspect ratio channel (in our case, the height to width ratio was ~5), the shear rate becomes high at the longer side walls and diminishes at the center plane. Hence, the elastic force focuses particles towards the center plane, hence allowing for two-dimensional focusing of all particles. In the 1st stage of our device, the channel width was selected such that \( \beta \approx 0.1 \) for the smallest particles. Consequentially, all particles could

B. Microchannel Fabrication

The microchannels were fabricated using Polydimethylsiloxane (PDMS) (Dow Corning, MI, USA) by the standard photolithography and replica molding procedures. The SU-8 2075 negative photoresist (MicroChem, USA) was spin-coated onto a polished silicon wafer to the desired thickness, after which the mold was subjected to subsequent soft baking, UV exposure, and post baking. The SU-8 developer (MicroChem, USA) was then used to develop the features on the mold. To fabricate the microchannels, PDMS prepolymer and curing agent were mixed at a 10:1 ratio (w/w) and poured onto the silicon mold before degassing and baking for 2 hr at 70 °C. The PDMS microchannels were then peeled off from the mold, and inlet and outlet ports were punched using a 1.5 mm biopsy punch. The microchannels were irreversibly bonded to microscope glass slides by oxygen plasma treatment. The device had a height of 125 μm throughout, and widths of 25 μm (1st stage), 50 μm (2nd stage) and 500 μm (expansion region).

III. WORKING PRINCIPLE

Polymer solutions exhibit non-Newtonian behaviors due to the presence of elastic polymer chains. These solutions typically display certain unique flow phenomena due to the anisotropic stress distribution arising from the stretching and tensioning of streamlines along the direction of flow [40]. When confined within the constraints of a microchannel, viscoelastic flows demonstrate useful flow focusing behaviors within specific flow regimes. This elastic effect can be quantified using the non-dimensional Weissenberg number, Wi, which is given by Wi = λpγc = \( \frac{2\lambda_p Q}{b_w} \), where \( \lambda_p \) is the polymer relaxation time,

\[ \beta > 0.1 \text{ for all particles} \]

\[ \beta > 0.1 \text{ only for larger particles} \]

Fig. 1. Schematic diagram of the microchannel design for WBCs separation from RBCs by size. Insets A to D show the schematics of the particle distributions in the cross-sectional view. As illustrated, particles enter the inlet dispersed (A), but attain vertical focusing in the 1st stage (B). At the end of the 1st stage, all particles are focused to the inner wall at the bifurcation (C) followed by size-dependent migrations in the 2nd stage (D), allowing for separation into the two outlets.
be focused in the 1st stage. The expansion region of 500 μm width after the 2nd stage subsequently amplified the separation distance between the different sized particles. The 2nd bifurcation at the end of the expansion region allowed particles to be separated based on their lateral positions in the channel. The 2nd bifurcating point (separation boundary) was set to be 0.35 of the channel width away from the inner side wall. To separate different target samples, this bifurcating point may be shifted, or the resistances of the downstream channels may be adjusted accordingly using negative pressure pumps to avoid redesigning the microchannel.

IV. RESULTS AND DISCUSSION

A. Particle Focusing in the 1st Stage

In the 1st stage of the device, particles were entrained along the vertical plane of the channel. We examined the effect of flow rate on the particle focusing in the 1st stage. The particles appeared to be randomly dispersed near the inlet (see Fig. 2(a)), whereas they were well focused near the 1st bifurcation (see Fig. 2(b) and (c)), which initializes the particles for separation in the 2nd stage of the device (see Fig. 2(d)). Fig. 2(e) shows the normalized off-center position of 2 μm PS particles in the 1st stage at various flow rates. At the 0.5% PVP concentration, tight particle focusing around the central plane is observed for flow rates above 0.1 μL/min, implying the effective initialization prior to the 2nd stage. The lateral distribution of particles followed a normal distribution, with R² values > 0.9. The central band became progressively narrower as the flow rate increased, hence illustrating a higher degree of focusing at the channel center. At low flow rates (< 0.1 μL/min), the channel length seemed insufficient to achieve compact focusing, due to the low magnitude of the elastic force. For the higher PVP concentrations, particles were focused at all flow rates due to the increased relaxation time, which translated to higher Wi. Within the flow rates used in this study, no inertial destabilizing effects were observed [45]. Our results illustrated that the effective focusing of all the particles used in the present study was achievable within the channel length of 3 cm when the flow rate is greater than 0.1 μL/min.

B. Lateral Migration of Particles in the 2nd Stage

To evaluate the difference in particle migration in the 2nd stage of the device, the viscoelasticity-driven lateral displacement of particles passing through the expansion region at the outlet was measured. An increase in the polymer concentration increases the elasticity of the solution [46]. As the flow rate increases, the elastic forces increase in magnitude as well. However, fluid inertia will also increase simultaneously. If the fluid inertia effects surpass the elastic effects, the inertial destabilizing effects begin to play a dominant role in particle dynamics. This compromises the focusing efficiency, as the particles begin to migrate under the influence of the combined contribution of the wall repulsion and shear gradient lift forces. This requires the selection of an appropriate polymer concentration and flow rate to ensure effective elastic focusing in the 1st stage, and highly efficient separation in the 2nd stage.

We examined the particle separation performance of 6 and 15 μm microspheres at three different polymer concentrations (see Fig. 3). The particles were injected into the inlet at flow rates ranging from 5 to 100 μL/min. As the particles progressed to the 2nd stage, they experienced an elastic force driving them away from the channel wall and towards the centerline. The differences in the particle lateral positions were enhanced in the expansion region. As a result, particles could be effectively separated in the 2nd stage based on their sizes. For the 0.5% PVP solution (see Fig. 3(a)), there was little distinction in the normalized lateral positions (Y) between the 6 and 15 μm particles, regardless of the flow rates used. The 6 μm particles remained widely dispersed over a band ~0.1 of the channel width and some overlap was observed between the two particles at low flow rates. The separation improved as Q increased, and both particle populations seemed to reach an apparent equilibrium lateral position at flow rates > 50 μL/min. Furthermore, these equilibrium positions shifted closer to the channel center as the
PVP concentration increased. Specifically, at 1% PVP concentration, the distance (normalized by the channel width) between the two particles at $Q > 50 \mu\text{L/min}$ (0.11 and 0.13 for 50 and 100 $\mu\text{L/min}$, respectively) appeared to be the largest among the three concentration cases. For the 3% PVP, the elastic force was sufficient to drive even the smaller particles to $0.42 \pm 0.03$ of the channel width, whereas the 15 $\mu\text{m}$ particles were located at $0.50 \pm 0.08$. This reduced the separation resolution between the particles.

C. WBC Separation From Whole Blood Sample

WBCs have diameters in the range of 5 to 24 $\mu\text{m}$ [47], with considerable overlap in diameter with RBCs (~8 $\mu\text{m}$) [48]. However, because of their biconcave morphology, RBCs have an effective diameter of 2.8 $\mu\text{m}$ [49]. As such, there is a mismatch in the elastic forces exerted on the two different cell types which results in their differential migration. We performed parametric studies to determine the optimal parameters for the maximal separation efficiency and purity. While Eqs. (1) and (2) describe the differences in the elastic force and migration rate between different sized particles, they were analytically derived based on rigid spherical particles [36],[43]. Thus, it is of note that these do not consider effects of the particle shape or deformability, which may influence the cross-streamline migration of particles [49].

In the separation experiment, the diluted whole blood samples were fed into the device at flow rates ranging from 50 to 150 $\mu\text{L/min}$. The maximum flow rate for the 3% PVP sample was set to 75 $\mu\text{L/min}$, above which the maximum shear stress exceeded 100 Pa, resulting in loss of WBC viability. PVP concentrations of 0.5%, 1%, and 3% were used in this set of experiments. Fig. 4 shows the normalized lateral positions of RBCs and WBCs for the various flow rates and PVP concentrations. Platelets were excluded from the measurement as they were assumed to migrate significantly less than RBCs, owing to their much smaller size (1-2 $\mu\text{m}$). WBCs demonstrated a similar migration behavior to the 15 $\mu\text{m}$ microspheres, albeit with a larger standard deviation of the cell positions. This may be attributed to the larger size heterogeneity between the WBC subpopulations.

To quantitatively evaluate the separation performance of the device, two parameters, purity and efficiency, were utilized. Purity was calculated based on the number of WBCs collected in the target outlet (Outlet 2) as a fraction of all cellular components collected in the outlet, whereas the separation efficiency ($n$) was defined as the ratio of WBCs collected in the target outlet to the total number of WBCs in both outlets. The throughput of the device was ~100 WBCs/min, which can be further augmented by parallelization of the device. The separation efficiency was calculated to be >95% for all flow rates for the 1% and 3% PVP cases. The calculated purity for the PVP cases was 100% for all flow rates. Furthermore, the wide range of flow rates (50 to 150 $\mu\text{L/min}$) demonstrated the suitability of the device for POC applications stemming from its apparent flow rate insensitivity, which would allow for possible hand operation in place of conventional syringe pumps. The 0.5% PVP case demonstrated low separation efficiencies (<30%) as the WBCs could not migrate sufficiently even at high flow rates. The pseudoshear stress (the mean flow velocity divided by the channel hydraulic diameter) across the range of applied flow rates was less than 100 Pa. Hence, the actual shear stresses acting on the cells at their equilibrium positions were assumed to be within the physiological range to avoid WBC dysfunction (10–60 Pa) [51],[52]. Accordingly, the WBC viability was measured to be ~95% before and after the separation by our device (see Fig. 5). In summary, the optimal set of parameters for WBC separation from the diluted whole blood was 1% PVP at 150 $\mu\text{L/min}$. Under this condition, separation efficiency and purity were both 100% across repeated experiments.

D. Hematocrit Effect on WBC Separation

Timely diagnosis of diseases demands rapid sample processing, requiring a high device throughput. The preceding sections...
illustrated the effects of medium elasticity and flow rate on the separation of WBCs. To augment the separation throughput, we next evaluated the effect of hematocrit on the separation. Based on our findings shown in Fig. 4, an optimum PVP concentration of 1% and flow rate of 150 μL/min were chosen to maximize the separation resolution of both cell types. Fig. 6 shows the probability of the normalized lateral positions of WBCs as well as stacked images of RBCs and fluorescence-labelled WBCs in the expansion region. We observed a distinct separation between WBC and RBC bands at a hematocrit of 0.01%. However, the separation distance was reduced with increasing hematocrit as the RBC band became wider. At 0.1% and 1% hematocrit, there was some overlap between the WBC and RBC bands, as each cell band widened. At higher hematocrits of 10% and 20%, the WBCs were dispersed within the RBC band and were unable to be separated from the RBCs. This was due to the enhanced cell-cell interactions in the 1st and 2nd stages, impairing the initialization and differential displacements of the WBCs and RBCs. This illustrates a limit to the sample concentration for efficient cell separation to maximize the separation efficiency and purity.

V. CONCLUSION

In the present study, a continuous, sheathless size-based particle separation device using a viscoelastic fluid was applied to the separation of WBCs from diluted blood samples. Based on the results for PVP concentrations, hematocrits, and flow rates used in the present study, the optimum separation was achieved under the condition of 1% PVP, 0.01% hematocrit, and 150 μL/min. This method of particle separation requires the dilution of cells in a non-native medium, which needs an additional preparation step prior to the separation and reduces the throughput of the device. However, this pre-dilution may be removed by the use of a parallel buffer flow to achieve the desired dilution on-chip. In addition, the throughput can further be enhanced, if needed, through parallelization of the device. To optimize the proposed device for the separation of biological particles, further improvements might be required to consider the non-uniformity of their physical properties such as size, shape, and deformability. Nonetheless, the proposed device promises significant potential for separating shear-sensitive, biological particles of varying sizes in a viscoelastic fluid.

REFERENCES


