Research Article

High resolution and rapid separation of bacteria from blood using elasto-inertial microfluidics

Improved sample preparation has the potential to address unmet needs for fast turnaround sepsis tests. In this work, we report elasto-inertial based rapid bacteria separation from diluted blood at high separation efficiency. In viscoelastic flows, we demonstrate novel findings where blood cells prepositioned at the outer wall entering a spiral device remain fully focused throughout the channel length while smaller bacteria migrate to the opposite wall. Initially, using microparticles, we show that particles above a certain size cut-off remain fully focused at the outer wall while smaller particles differentially migrate toward the inner wall. We demonstrate particle separation at 1 μm resolution at a total throughput of 1 mL/min. For blood-based experiments, a minimum of 1:2 dilution was necessary to fully focus blood cells at the outer wall. Finally, *Escherichia coli* spiked in diluted blood were continuously separated at a total flow rate of 1 mL/min, with efficiencies between 82 and 90% depending on the blood dilution. Using a single spiral, it takes 40 min to process 1 mL of blood at a separation efficiency of 82%. The label-free, passive, and rapid bacteria isolation method has a great potential for speeding up downstream phenotypic and genotypic analysis.

**Keywords:**
Bacteria separation / Bloodstream infection / Elasto-inertial microfluidics / Infectious diseases / Non-Newtonian fluid / Sepsis / Viscoelastic flow

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Sepsis is an acute inflammatory response to pathogens by an immune-compromised host body. It may be defined as the body’s systemic inflammatory response syndrome (SIRS) to an infection caused by pathogens. The development of sepsis starts from a site of local infection where microorganisms enter into the bloodstream and spread to different parts of the body. If left untreated, it may lead to shock, multiorgan failure, and death, especially if not recognized early and treated promptly, with an associated rise in mortality of 7% for every hour delay in the administration of appropriate antibiotics [1]. Notably, sepsis is also the final common pathway to death from the current pandemic worldwide such as SARS-CoV-2 / COVID-19 [2]. In the majority of sepsis cases, the causative pathogen is bacteria [3]. The most common bacteria causing sepsis include *Escherichia coli* and *Staphylococcus* among others such as *Pseudomonas*, *Klebsiella*, and *Acinetobacter* [3]. The detailed etiology of organisms causing sepsis highlighting the frequency of infection and their corresponding mortality rate has been studied and reported extensively [3]. Currently, the “gold standard” for the diagnosis of bloodstream infections is by blood cultures. It can take 24–72 h before a complete answer can be reached including the antibiotic resistance profile [4–6]. In addition, detection of fastidious bacteria, which often require a longer time to grow, is challenging [7]. This is mainly due to the low concentration of bacteria in blood in the early stages of sepsis which is about 1–100 CFU/mL [8–11]. For a faster turnaround time, nucleic acid-based techniques are promising, but they suffer from low sensitivity and...
specificity, mainly due to a lack of effective sample preparation methods [7]. Although tremendous improvements have been made over the past few years, including the introduction of next-generation sequencing technologies, sample preparation remains the bottleneck for further expansion of molecular diagnostics into clinical settings. Centrifugation and filtration are two common methods used for separating bacteria from the blood. However, the similar density of bacteria such as E. coli (1.08–1.10 g/cm³) and RBCs (1.086–1.122 g/cm³), makes centrifugation a stand-alone method very challenging for bacterial recovery from blood [11–13]. On the other hand, the main drawback of filtration-based separation is blood clogging on the filter paper from a high population of blood cells, affecting bacterial recovery [11].

Among emerging technologies, microfluidics is a very promising tool and has the potential to enrich the pathogens from the blood sample. The ability to precisely manipulate blood cells has attracted considerable research in the field and several attempts have been made to address sample preparation for sepsis diagnostics. These methods either use the surface markers to isolate bacteria using affinity separation [14], or other properties such as their shape, size, deformability, density, electric or magnetic susceptibility, and hydrodynamic properties [15–20]. To this end, inertial microfluidics is attractive since the active and passive, size-based, technology exploits inherent surface property and hydrodynamic forces that scale with increased flow rate and among them spiral channel designs have been shown to operate at extremely high volumetric flow rates (∼1 mL/min) [15–33]. However, microfluidic technology has been utilized extensively to precisely focus and separate mammalian cells, including circulating tumor cells [34–46], but the separation of small-sized bacteria from blood has been challenging. The main challenge in inertial microfluidics-based bacteria separation is due to the narrow size difference between bacteria (∼1–2 μm) and blood cells (∼3–15 μm). The size of blood cells is approximately 6 to 8 μm for red blood cells (RBCs) [12], approximately 7 to 30 μm for white blood cells (WBCs) [13,47], and approximately 2 to 4 μm for platelets [29]. Initial work in inertial microfluidics was mostly focused on the separation of bacteria from diluted RBCs [48]. Studies performed by M.R. Condina et al. [22,49] used a trapezoidal spiral chip and coupled it with MALDI-TOF to demonstrate the inertial microfluidic-based separation of yeast from a low concentration of beer spoilage bacteria at a high throughput of 1.5 mL/min. It was observed that yeasts and bacteria were separated by cleverly designing the width of the channel outlets. However, higher efficiency of more than 90% was obtained only after three times of recirculating the collected outlet through the spiral channel. Bashir et al. [50], developed a point-of-care microfluidic biochip to quantify nCD64 immune cells, in 30 min at a flow rate of 30 μL/min to diagnose sepsis. In addition, the same group also studied the spontaneous motility of neutrophils in a microfluidic channel from diluted blood and combined it with machine learning to provide the final answer in 6 h with high accuracy [51]. However, sepsis is a very complex disease and the expression of immune cells specific to sepsis is still challenging and not understood well, as these immune cells can also be activated due to other parameters [52] and the information on the presence of viable bacteria to prescribe a specific antibiotic cannot be obtained with this approach. Using a spiral device, Hou et al. recently used a sheath flow to performed size-based differential migration of blood cells to separate bacteria [53]. Here, the bacteria and blood cells both migrate but at different rates and bacteria could be extracted at an efficiency of more than 65%. Although the authors achieved high throughput (with 1:3 diluted blood), the influence of Dean on both big and small-sized particles could have led to a compromise in separation efficiency.

Higher bacterial separation efficiency is significant for sepsis diagnostics, as the concentration of bacteria in the blood is very low. Compromising separation efficiency leads to false-positive or false-negative results. In an ideal scenario, both high separation efficiency and throughput are preferred for effective sepsis diagnostics. One way of improving the size resolution is to use the rheological properties of the viscoelastic fluid to manipulate the cells, as blood is a non-Newtonian fluid. In elasto-inertial microfluidics, the combination of elasticity and inertia of viscoelastic fluid, enables size-based 3D focusing of particles [29,54–57]. Using a straight rectangular channel, we have previously reported the separation of bacteria from whole blood using viscoelastic fluid by selectively migrating blood cells away from the walls toward the centerline of the channel while bacteria remain in the streamline and could be separated [58]. Very recently, Lu et al. [59] used curved channels and parallelization strategies to demonstrate the separation of bacteria from diluted blood. In addition, Lee et al. used ten-turn spiral channels to study the multiplex focusing behavior of particles in viscoelastic flows [56]. While all the above methods offer capabilities for precise cell manipulation, the relatively low separation efficiency and limited throughput are hampering practical implementation. The relatively low volumetric flow rate is an inherent limitation of elasto-inertial microfluidics since the synergistic effect of the elastic forces and inertial forces are ideal at moderate flow rates. In our recent work, we extensively studied the behavior of particles in a viscoelastic fluid (polyethylene oxide [PEO]) using different spiral channels and reported focusing of 10 and 15 μm particles at extremely high flow rates (∼2 mL/min) [60]. We studied how particles migrate from the inner wall toward the outer wall and reported stable particles focusing at the outer wall at dynamically high Reynolds numbers.

In this work, we utilize the knowledge of stable focusing of particles to preposition and separate blood cells and bacteria based on size for sepsis applications. Using a two inlet and two outlet spiral device, the diluted blood sample is pinched toward the outer wall by the PEO buffer. The blood cells remained fully focused throughout the channel length, while smaller bacteria follow the Dean vortices and are effectively separated and collected at the inner outlet. To the best of our knowledge, the phenomenon of blood cells remaining fully focused at the outer wall throughout the channel length has
not been shown before. First, the prepositioning, focusing, and separation phenomena were studied using microparticles. Using 1 µm particles (as a model for bacteria), it was possible to separate them from 3 µm particles at an extremely high total flow rate (sheath + sample = 1 mL/min). Thereafter, the optimized spiral geometry was utilized to study and separate bacteria from diluted blood. Using E. coli (~1000 CFU/mL) spiked into a diluted blood sample, bacteria separation is demonstrated at an efficiency of 82 to 90% depending on the blood dilution.

2 Theoretical background

A Poiseuille flow of an incompressible Newtonian fluid through a straight channel will exhibit a parabolic velocity flow profile with a maximum velocity at the center and zero velocity at the walls of the channel, that is, a no-slip boundary condition. The flow of a fluid in the channel is characterized using a dimensionless number Reynolds number ($Re$), defined as $Re = \frac{\rho U D}{\mu}$, where $D = \frac{2w}{h}$ is the hydraulic diameter with “w” and “h” being the width and height of the channel respectively, “$U$” is the bulk velocity of the fluid, “$\rho$” is the fluid density, and “$\mu$” is the dynamic viscosity. The equilibrium position of the particle suspended in the fluid is mainly due to the combined effect of shear-induced lift force ($F_{LS}$) and wall-induced lift force ($F_{W}$). The parabolic flow profile of the fluid induces $F_{LS}$, pushing the particle away from the center of the channel while $F_{W}$ pushes the particle away from the wall. The combination of these two forces, $F_{x}$, is defined as $F_{x} = f_{x}(Re, x) \rho U^2 \frac{a}{h^2}$, will result in an equilibrium focusing position of the particle. Here, $f_{x}(Re, x)$ is the lift coefficient, which is a function of Re, “x” is the position of the particle, and “$a$” is the size of the particle [15]. The number of equilibrium positions of the particles depends upon the geometry of the channel. Segre and Silberberg in 1961, showed that particles attain their equilibrium positions along the annulus of a circular channel at 0.6 radius [61]. In 2007, Di Carlo et al. observed that in a square channel, particles occupy equilibrium positions at the faces of each wall of the channel [15], while Bhagat et al. demonstrated equilibrium positions along the longer sides of a rectangular channel [62]. In the case of a fluid flowing through a curved channel, the curvature of the channel creates a radial pressure gradient creating secondary fluid flows leading to the formation of two counter-rotating Dean vortices, normal to the bulk flow. The drag force, $F_{D}$, caused due to Dean vortices is defined as $F_{D} \sim \rho U a D h \frac{a}{R}$ [15], where “$R$” is the radius of curvature of the channel. The strength of the Dean vortices is determined by a dimensionless Dean number defined as $De = Re \sqrt{\frac{a}{R}}$ [63]. In contrast to a Newtonian fluid, when a viscoelastic (non-Newtonian) fluid flows through a channel, an additional elastic force ($F_{E}$) arises due to the first normal stress difference of the fluid which pushes the particle toward the center of the channel. This force is defined as $F_{E} = a^2 \nabla N_1$, where “$N_1$” is the first normal stress difference [54,64,65]. The elastic property of the fluid is defined by the dimensionless number called the Weissenberg number, defined as $Wi = \frac{\lambda \sqrt{Q}}{\rho U^2}$, where “$Q$” is the volumetric flow rate of the fluid and “$\lambda$” is the relaxation time of the fluid [66]. Here $\lambda = 1.976 \text{ ms}$, which was calculated using the formula: $\lambda = 18 \lambda_2 \left( \frac{C}{C^0} \right)^{0.67}$, where $C^0$ is the polymer overlap concentration = 858 ppm, C is the polymer concentration used = 150 ppm and calculated $\lambda$, is the Zimm relaxation time $= 0.341 \text{ ms}$ [67].

In this article, we show that by prepositioning the particle at the outer wall using sheath fluid (PEO), the forces $F_{D}$ and $F_{W}$ balance the effect of $F_{D}$ and $F_{W}$ and keep the larger particles at a stable focusing position at the outer wall throughout the channel length. On the other hand, smaller particles are trapped in the Dean vortices and migrate toward the opposite wall. This phenomenon is utilized to demonstrate the focusing and differential migration of bacteria from blood cells to achieve size-based rapid separation.

3 Materials and methods

3.1 Fabrication of the microchannels

The master mold was obtained using standard photolithography techniques. AutoCAD 2017, (AutoDesk, USA) software was used to design the two-turn spiral channel. It was printed on a Mylar mask. A negative photore sist SU-8 was spin-coated onto a 4-inch silicon wafer. It was exposed to a UV light source through the Mylar mask and was developed using SU-8 developer (Micro resist technology, Germany) to obtain the channel design onto the silicon wafer. A PDMS replica was produced using the standard soft lithography technique. Sylgard 184 silicon elastomer and curing agent was mixed in a 10:1 ratio and the silicon elastomer was poured onto the SU-8 master and was cured for a minimum of 6 h at 65°C. The PDMS mold was peeled, cut and holes were punched using a 0.75 mm diameter Harris UniCore puncher to obtain inlets and outlets. Clean glass and the PDMS mold were bonded using an oxygen plasma treatment (Femto Science Cute Plasma System, South Korea) and were incubated at 110°C for 10 min to ensure robust bonding.

3.2 Chip characterization

A two-turn spiral channel with a rectangular cross-sectional geometry design (by Bhagat et al. [68]) was used to study the behavior of particles of different sizes using elasto-inertial microfluidics in our work. The design is shown in Fig. 1 in the center. The microchannel had a width of $w = 500 \mu$m and a height of $h = 50 \mu$m. The width of the channel at outlet 2 (O2) is 350 µm and at outlet 1 (O1) is 150 µm. The aspect ratio, defined as width/height ($w/h$) was 10. The blockage ratio is defined as $a/h$, where “$a$” is the diameter of the particle and “$h$” is the channel height, ranged from 0.02 to 0.2.
3.3 Particle experiments

The spiral chip was flushed with 0.1% BSA (prepared from 10% BSA blocker in PBS, Thermo Fischer Scientific) and was incubated overnight at room temperature before starting the experiments. BSA is used to prevent the adsorption of particles/cells to the channel walls and tubing. For particle experiments, 10 μL of different sizes of fluorescently labeled polystyrene microparticles (FluoSpheres, Thermo Fischer Scientific) were spiked into 10 mL of 150 ppm concentration (0.015%) PEO, molecular weight = 2000 kDa and was used as a sample fluid. This gives approximately a final concentration of 10⁷ particles per milliliters. A total of 150 ppm PEO was used as sheath fluid. Different sized particles (1, 3, 5, 7, and 10 μm) were used for particle experiments. A syringe pump (Pump 33 DDS, Harvard Apparatus) was used to pump the sample and sheath. The inlets and outlets of the chip were connected using Cole-Parmer Tygon tubing with inner diameters of 0.254 mm and an outer diameter of 0.762 mm. The focusing of particles in the microchannels was observed using a fluorescence microscope. The sample flow rate for all experiments was kept constant at 50 μL/min, while the sheath flow rate was varied. Fluorescent image analysis was performed using ImageJ software (https://imagej.nih.gov/ij/).

For quantification, a hemocytometer was used to count particles collected from the two outlets. In this study, separation efficiency is defined as the number of target particles or cells collected at the specified outlet divided by the total number of target particles or cells in a sample mixture at the inlet.

Yield is defined the same as separation efficiency but it is used when there is only one type of particle or cell in the sample. For obtaining side view images of the particles, the experimental setup is shown in Supporting information Fig. S3. The scattering effect by thick PDMS layer was reduced by adding a drop of nondrying immersion oil used for fluorescent microscopy (Cargille immersion oil; type FF; code:159) in between the support glass and the PDMS-glass chip.

3.4 Blood and bacteria experiments

Fresh blood samples were collected from healthy donors at the blood center GeBlod, Stockholm, Sweden in EDTA tubes and were stored at 4°C. All blood-based experiments were performed on the same day of blood collection. Freshly prepared PEO solution was used to dilute blood in 1:10 (1 mL of whole blood in 9 mL of PEO solution), 1:5, and 1:2 dilutions. *Escherichia coli* ATCC 25 922 strain (*E. coli*) and *Staphylococcus Capitis* (*S. Capitis*) were used in this work. Bacteria were grown on a Muller Hilton (MH) agar (Sigma Aldrich, Germany) for 17 h, incubated at 37°C. To spike bacteria into blood, a bacterial colony from the overnight grown MH agar plate was inoculated into 5 mL of MH broth and optical density (OD) was adjusted to 0.2 using an Ultrospec 10 cell density meter (Amersham Biosciences, UK) measured at 600 nm. Serial dilution was performed using MH broth and 100 μL.
of each dilution was plated \((n = 3)\) on blood agar plates. The agar plating count for each dilution was deduced after overnight culture at a 37°C incubator. A suitable dilution was selected based on the agar plate count and bacteria were fluorescently labeled using the Backlight viability kit (Thermo Fischer, USA) protocol for visualization and to check their viability. The emission of green fluorescence light depicts viable bacteria while red fluorescence light indicates dead ones.

For blood-based experiments, 100 µL of fluorescently labeled bacteria was spiked into diluted blood. After processing the sample through the spiral chip, inlets and outlets fractions were quantified. To achieve this, the sample inlet and outlets were centrifuged at 4000 g for 10 min and the pellet was resuspended into equal volumes of PEO solution. To quantify the bacterial growth at the outlet fractions, 100 µL of the sample inlet and the outlets were plated overnight and the final colony forming units per milliliters of the sample (CFU/mL) was calculated \((n = 3)\). By comparing the counts at the different outlets and the inlets, the separation efficiency was calculated.

### 3.5 White blood cells Ficoll separation

Standard Ficoll density gradient centrifugation was performed on 1 mL of whole blood to separate blood cells components. Whole blood was diluted in 1:5 v/v using 1X PBS and was added to 7.5 mL of Ficoll solution. The mixture was centrifuged for 30 min at 4000 g without break. The WBCs layer was carefully removed and resuspended in 1X PBS. This was washed by centrifuging for 10 min at 3500 g. The supernatant was discarded and the pellet containing WBCs was resuspended in 150 ppm PEO and used for spiral experiments.

### 3.6 Eukaryotic cell staining

For staining WBCs, Cell trace Calcein green AM dye ordered from Thermo Fischer scientific was used. Isolated WBCs were stained with calcein green AM dye by adding 1 µL of dye to 1 mL of the sample and incubated at room temperature for 15 min in dark, to avoid photobleaching of the fluorescent dye. To visualize platelets, 1 mL of the inlet and outlets solutions was treated with RBC lysis buffer (1:10 ratio), which consists of 135 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA in 10 mL of Milli-Q water and was incubated for 10 min at room temperature. The mixture was centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the pellet was resuspended with 2 mL of 1X PBS. The mixture was centrifuged again (washing step) at 1200 rpm for 5 min and the pellet was resuspended in 100 µL of 1X PBS. To stain platelets, the final mixture was mixed with 10 µg/mL concentration of Alexa Fluor 647 anti-human CD61 antibody and was incubated in dark for 25 min at 4°C. The solution was washed by adding 1 mL of 1X PBS and centrifuging at 1200 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 70 µL of 1X PBS and then visualized using a fluorescent microscope.

### 4 Results and discussion

#### 4.1 High-resolution particle focusing

In viscoelastic flows through curving microchannels, the following dominant forces affect particle focusing: lift forces \((F_L, F_D)\), elastic force \((F_E)\), and a curvature-induced Dean's drag force \((F_D)\). The combined interaction of the forces results in particles migration and focusing. Previous work from our group and others has mainly focused on particle migration toward an equilibrium position using either inertial and elastoinertial microfluidics [34,48,53,58,60,68,69]. In this work, we show that it is possible to preposition particles at the inlet using a viscoelastic sheath buffer (PEO) and keep the particles fully focused throughout the channel length above a certain size cut-off. This behavior is independent of length in flow through spiral microchannels. Smaller particles are affected by the Dean force and entrapped into the Dean vortices and continuously migrate away from the equilibrium position. Figure 1 shows the working principle of the method, indicating schematic illustration (cross-sectional view) and the corresponding experimental results (top view) of 1 and 7 µm particles suspended in PEO solution. The sample was introduced at the outer wall, and images were taken at four regions of the spiral channel (region 1–4, as highlighted on the spiral). A sheath fluid (PEO) pinches the sample containing large (7 µm in red) and small (1 µm in green) particles to occupy a narrow stream at the outer wall of the inlet (region 1). As the hydrodynamic forces are developed, the larger particles \((7 \mu m)\) remain focused due to the balance between the three main forces while the smaller particles \((1 \mu m)\) are trapped in the Dean vortices and differentially migrate away from the outer wall \((\text{regions 2 and 3})\). Finally, the smaller particles reach the inner wall and can then be separated \((\text{region 4})\).

Note, the total flow rate \((\text{sample + sheath})\) is at 1 mL/min. For the particle focusing, while scaling differently, all the forces \((F_L, F_D, F_E, F_D)\) affect the particles. The elastic force, \(F_E\) from the viscoelastic nature of PEO aids \(F_E\) to balance the effect of \(F_D, F_L\), to maintain an equilibrium position at the outer wall for large particles throughout the channel length. For smaller particles, a significant decrease in the magnitude of \(F_D\) and \(F_L\) due to their small size will perturb their equilibrium position, resulting in smaller particles getting trapped into Dean vortices and migrating toward the inner wall. Notably, introducing the sample closer to the particle equilibrium position allows particles above a certain cut-off to be fully focused throughout the entire channel length.

Initially, we used microparticles to examine the focusing phenomena before evaluating the spiral chip for blood and bacteria separation. We first characterized the spiral chip to understand the focusing and separation behavior using 1 and 7 µm particles, keeping in mind the average size of RBCs and WBCs are ≥7 µm while bacteria are approximately 1 µm.
Further, we compared the behavior of 1 and 7 μm particles suspended in Newtonian (1X PBS) and non-Newtonian (PEO) fluid at different starting positions at the inlet wall and observed that prepositioning the particles at the outer wall of the spiral using PEO enabled improved size-based particle separation at extremely high total volumetric flow rates (Sample + sheath = 1 mL/min) as shown in Supporting information Fig. S1. We evaluated several parameters that influence the particle focusing behavior such as preposition of the particle at the inlet, flow rate, particle size, and PEO concentration. The effect of PEO concentration on the particle behavior is shown in Supporting information Fig. S2. PEO concentration of 150 ppm was found to be optimal as it provided the best trade-off between high separation efficiency without compromising with throughput. Following, we describe and discuss the detailed analysis performed to better understand particle migration and focusing on a PEO-based non-Newtonian fluid.

4.2 Influence of Re on particle behavior

To investigate the influence of Re, 1 and 7 μm particles were pushed at different flow rates, and the particle distribution was examined at the outlet (region 4 in Fig. 1). The sample flow rate was kept constant at 50 μL/min, while the sheath flow rate was increased systematically from 100 μL/min to 1 mL/min (Re = 5 to 35). In Fig. 2A, the fluorescent intensity distribution of 1 and 7 μm particles along the lateral position are shown using a heat map. At a low flow rate (Re = 5–8), both 1 and 7 μm particles are partly spread out due to insufficient inertial, Dean, and elastic forces. In addition, at low total flow rates, there is also an insufficient pinching effect of the PEO buffer (1:2 ratio), which results in the spreading of the particles. As the flow rate increases, a gradual spreading of 1 μm was observed, and at a flow rate of 500 μL/min (Re = 18), the particles spread completely and reach the inner wall. As the flow rate was increased further to 700 μL/min (Re = 25), it was observed that 1 μm particles start to focus toward the inner wall. The particles gradually move close to the inner wall as the flow rate reaches up to 900 μL/min (Re = 32). With further increased flow rate (Re = 35), the smaller 1 μm particles start to spread again. In contrast, 7 μm particles stay focused toward the outer wall, and at higher flow rates, they are stable and tightly focused. The particles, initially show a broader distribution of positioning at the outer wall, at flow rates of 100 and 200 μL/min (Re = 5 to 8). High-resolution focusing was achieved as the Re was increased (from Re = 12 to 35). Note that while the Dean forces quickly move the smaller particles laterally toward the inner wall, the migration toward the outer wall in the second round is significantly slowed due to dominant shear-induced lift force countering the influence of Dean and elastic forces maintaining the particles toward the inner wall. This phenomenon eventually enables high-resolution size-based particle separation.

To further understand the focusing of particles in PEO along with the channel height (50 μm), the spiral chip was turned sideways and images were captured close to the outer wall of the outlet region, (region 4 in Fig. 1) at a total flow rate of 1 mL/min. The experimental setup for this experiment is shown in Supporting information Fig. S3. We used different-sized particles (1, 7, and 10 μm) for comparison. It was observed that smaller 1 μm (green) particles (positioned at the inner wall), spread all along with the height of the channel while larger particles, 7 μm (red) and 10 μm (blue) show 3D focusing at the channel center (Fig. 2B). Normalized particle intensity distribution along the channel height was quantified using imageJ as shown in the graph of Fig. 2B. It can be observed that the larger the particle, the better is the focusing along with the channel height, as observed by a sharp Gaussian curve for 10 μm particles. The table in Fig. 2C shows the calculated values of different dimensionless numbers, De and Wi for a corresponding change in Re.

4.3 Size-based differential migration of particles

For sepsis application, the spiral device needs to focus on blood cells of different sizes at the outer wall while allowing the bacteria to migrate away from the wall. To find out the particle size cut-off above which particles remain focused, we evaluated the spiral device using different-sized particles. Experimentally, 1 μm particles were mixed with particles of different sizes (2, 3, 5, and 7 μm) and the migration and focusing behavior was observed (Fig. 3A). Using the current spiral design, keeping bacterial (1 μm) separation from blood (≥3 μm) in mind, we demonstrate the separation of 1 μm from 3 μm particles with high resolution. While the 2 μm particles are not separated from the 1 μm particles at the outlet, it is noteworthy to mention that, while 1 μm particles have made a turn along the inner wall, the 2 μm particles are yet to make the complete turn (Dean cycle). Furthermore, 3 and 5 μm particles migrate toward the inner wall while 7 μm particles are fully focused. As previously described in Fig. 1, the stable position of the larger particles is due to the balance between lift, elastic, and Dean forces. As the particle size is reduced, the effect of these forces is also significantly reduced due to the smaller particle size ($F_L \propto a^4$, $F_D \propto a^4$; and $F_E \propto a^3$). This reduction in the magnitude of $F_L$ (to the power of 4) and $F_D$ (to the power of 3) will affect the stable equilibrium position for the smaller particles and they migrate along with the dean vortices toward the inner wall. For a low-aspect-ratio channel geometry (width >> height), focusing is strongly dependent on the particle size to channel height ratio ($a/h$). Using low-aspect-ratio channel geometry, we previously suggested a minimum $a/h$ ratio more than 0.1 for focusing on inertial microfluidics [70]. Interestingly, our findings in the current work also indicate stable focusing at $a/h$ ratio of more than 0.1. Stable focusing in our case means particles remaining fully focused at the outer wall. This would translate to a particle size above 5 μm in the current spiral design ($h = 50 \mu m$). The graph in Fig. 3B shows the normalized fluorescent intensity distribution of particles along the lateral direction of the channel. The Black dotted vertical line in
Figure 2. Particle focusing behavior at different Re. (A) Heat map of normalized fluorescent intensity distribution along with the lateral position (width: 0 to 500 μm) of the channel for different Re for 1 and 7 μm particles. At low Re, both 1 and 7 μm particles are not well focused with significant spread observed for 1 μm particles. At higher flow rates (Re = 22–35), 1 μm particles migrate toward the inner wall, while 7 μm particles stay focused at the outer wall. (B) Focusing behavior of particles along the height of the channel (side view) imaged near the outer wall of the spiral outlet. The smaller particles (1 μm) spread all along the channel height, while the 7 and 10 μm particles are 3D focused at the center of channel height. Scale bar: 50 μm. The graph on the right shows the normalized fluorescent intensity distribution along with the height of the channel for 1, 7, and 10 μm. (C) The calculated values of De and Wi for the range of Re tested.

4.4 High-resolution particle separation

In comparison to inertial microfluidics, it is possible to manipulate particles with higher resolution using elasto-inertial microfluidics due to the additional viscoelastic forces. In this work, we take advantage of the relatively different scaling of the forces ($F_I \propto a^4$, $F_D \propto a$; and $F_E \propto a^3$) to continuously separate particles. As can be seen in Fig. 3B, the 2 μm particles are separated from the 3 μm particles. To investigate the possibility to separate the particles at 1 μm resolution, we mixed the 2 and 3 μm particles in the PEO and processed the sample. The result is shown in Fig. 4A, indicating 1 μm separation resolution is possible. We performed hemocytometer-based quantification to count the two outlet fractions for experiments with samples involving a mixture
of 2 and 3 μm (Fig. 4B) and 1 and 3 μm (Fig. 4C). A total of 87% of 2 μm particles were collected in O1, while 100% of 3 μm particles were collected in O2 (Fig. 4B). Furthermore, we mixed the 1 and 3 μm particles and processed them. As can be seen in Fig. 4C, the separation efficiency for 1 μm particles was 96% at O1, while 100% for 3 μm particles were collected in O2. To reiterate, the total volumetric flow rate used was 1 mL/min. While outside the scope of this work, redesigning the width of the channel at O2 to around 300 μm (as observed in the graph of Fig. 3B) should enable higher separation efficiency, especially for the 2 μm particles.

4.5 Focusing of blood cells

Blood is a non-Newtonian fluid and the viscosity of the blood is dependent on the hematocrit content, plasma protein concentration, and blood cell count. In flow-through spirals, the blood rheology will affect the Dean and lift forces differently. In addition, the viscoelastic nature of the PEO used will also interact with the blood cells. Initially, we found that whole blood needs to be diluted to keep them focused. Three different dilutions of blood (1:2, 1:5, and 1:10) were tested. Notably, all the diluted blood cells are focused at the outer wall and effectively collected through the outer outlet channel (Fig. 5A). The 1:2 diluted blood (~25% hematocrit) sample is broader, indicating more particle-particle interaction. To investigate the optimal dilution, we mixed 1 μm particles with diluted blood and processed them through the spiral device. Figure 5B shows the normalized intensity distribution graph for 1 μm and blood along the width of the channel for different blood dilutions. For blood, the grayscale in ImageJ was used to calculate the intensity values. The graph clearly shows the spread of 1 μm particles as the hematocrit content increases (from 1:10 to 1:2), with the highest intensity merging toward the outer wall. In addition, a gradual spread of blood cells at the outer wall with increased hematocrit content (from 1:10 to 1:2) can also be observed. Hemocytometer-based quantification of the collected outlet fraction in Fig. 5C shows that, for 1:10 diluted blood (~5% hematocrit), 96% of 1 μm particles were collected at the inner outlet (O1), 93% for 1:5 diluted blood (~10% hematocrit), and 82% for 1:2 diluted blood, respectively. On the other hand, 100% of blood cells were collected in O2 for all three cases. The results indicate that the blood cells are fully focused throughout the channel length and as the concentration of blood cells increases (hematocrit), the separation efficiency of 1 μm particles reduces, indicating cell-particle interaction. Consequently, there is a need to dilute the blood sample, and an increase in dilution will result in improved separation efficiency of 1 μm particles. It is possible that some particles get stuck to blood cells due to high solid content. In addition, high solid content may ultimately
affect how the local forces interact on neighboring particles. Although more work is needed to effectively decipher the effect of solid content, it is evident that high solid content prevents smaller particles from being effectively carried by the Dean vortices toward the inner wall and, thus, reducing the efficiency of separation. The challenges from particle-particle interaction due to high hematocrit content and the requirement of blood dilution are also reported previously by Shen et al. and Zhou et al., respectively [71,72].

4.6 Bacteria separation from diluted blood

Before testing the behavior of bacteria spiked in blood, we tested and optimized how bacteria behave in viscoelastic fluids. For initial optimization, PEO was spiked with high concentration (10⁷ CFU/mL) of fluorescently labeled Gram-negative *E. coli* and Gram-positive *S. capitis* in separate experiments, processed through the spiral device at a total flow rate of 1 mL/min. We observed similar behavior for both Gram-negative and Gram-positive with a yield of 94 and 89% for *E. coli* and *S. capitis*, respectively, measured using agar plating (Supporting information Fig. S4).

Following, to study the separation of blood cells, *E. coli* was spiked at a concentration of 1000 CFU/mL into 1:10, 1:5, and 1:2 diluted blood. As expected, the bacteria migrated to the inner wall and were collected at outlet O1, whereas blood cells stay near the outer wall and collected at outlet O2 (Fig. 6A). Images of the fluorescently labeled *E. coli* (using Backlight viability kit) and blood cells were captured using a hemocytometer at the inlets and outlets that were separated in the spiral chip, at a total flow rate of 1 mL/min. The blood cells (RBCs) were observed only in O2 and a majority of *E. coli* were observed in O1 (Fig. 6C). Quantification of the outlets was performed using blood agar plating. Blood agar plating for each case was plated (n = 3), and a representative image for each plate is shown in Supporting information Fig. S6. A separation efficiency of 82 to 90% of *E. coli* (Fig. 6B) was obtained depending on the blood dilution, while blood was recovered through the outer outlet for all cases. The higher the blood dilution the higher is the separation efficiency for bacteria separation, in agreement with the results obtained for 1 μm particles (see Fig. 5). The viability of blood cells and bacteria in PEO buffer was also tested by comparing it with the cells in 1X PBS as shown in Supporting information Fig. S8. We observed that the viability of blood cells or bacteria was not affected due to PEO. The separation efficiency for *S. capitis* spiked in 1:10 diluted blood was similar to that of *E. coli*, as shown in Supporting information Fig. S5A. In addition, *E. coli* concentrations ranging from 10⁷ to 10³ CFU/mL spiked in 1:10 diluted blood resulted in similar separation efficiency, as shown in the graph of Supporting information Fig. S5B. Furthermore, we investigated the outlets to check the presence of WBCs and platelets in both the outlets, shown in Supporting information Fig. S7. Ficoll separation was performed to isolate pure WBCs and was processed through the spiral device. The outlets were stained using calcein green AM dye and hemocytometer analysis revealed 100% of WBCs collected in O2 (Supporting information Fig. S7A). Further to measure the platelets, both the outlets were collected and treated with RBC lysis buffer and stained using anti-CD61 antibody dye (See “Methods” section for the complete protocol). Hemocytometer analysis showed that 78% of platelets were collected in blood outlet (O2) and the remaining 22% were collected in the bacterial outlet, which is attributed to a different size range of platelets (ranging from 2 to 4 μm) as shown in the graph of Supporting information Fig. S7B. However, it is important to note that majority of the platelets were collected in the blood outlet (O2). Hence, not only does the device separate 100% of the WBCs and RBCs but also the majority (78%) of the platelets. While outside the focus of the current study, further optimization of the spiral design, such
Figure 5. Focusing on blood cells for different blood dilutions. (A) Blood cells remain well focused at the outer wall at higher blood dilutions (1:10 and 1:5). As the hematocrit content increases (~25% in case of 1:2 dilution), blood cells are focused in a broader band due to an increase in particle-particle interaction. Scale bar: 100 μm. (B) Normalized intensity graph depicting the position of blood cells and 1 μm particles for different blood dilutions (1:10, 1:5, and 1:2) along the channel width. (C) Particle separation from diluted blood. The separation efficiency of 96, 93, and 82% was obtained for 1 μm particles from the 1:10, 1:5, and 1:2 diluted blood samples, respectively.

as changing the resistance at the outlets, might further improve the platelet separation from bacteria. Further, to examine the separation performance of this device at clinical relevant bacteria concentrations, we spiked *E.coli* and *S.capitis* into 1:5 and 1:2 diluted blood at different concentrations (from ~1000 to ~40 CFU/mL), in separate experiments and analyzed the outlets (graph and table of Supporting information Fig. S5). The bacterial count was quantified using the blood agar plating technique. A bacterial separation efficiency of more than 70% using 1:5 diluted blood and more than 65% using 1:2 diluted blood was obtained for *E.coli* concentrations of more than 100 CFU/mL collected at O1. For *S. capitis*, a separation efficiency of more than 65% and more than 50% using 1:5 and 1:2 diluted blood was obtained respectively, for concentrations of more than 100 CFU/mL. In general, we observed a decrease in separation efficiency as the bacterial concentration was reduced. One of the reasons for a decrease in separation efficiency at low bacterial concentration can be attributed to cell-cell interaction with the increase in hematocrit content. This has been reported previously by various studies [53,71,72]. To test this, we performed a control experiment with *E.coli* spiked in PEO and quantified the collected outlets using agar plating. Surprisingly, we observed a similar behavior, where the yield decreased from 79 to 60% at O1 for *E.coli* concentrations approximately 200 and approximately 40 CFU/mL, respectively (Supporting information Table. S1). Hence, we speculate the limitation of agar plating-based analysis which is known to give variable results at a low bacterial concentration to be the major factor at play. While it is a topic for future work, there is a need to investigate this more in detail.

While high throughput is desirable for any application, a need to process large volumes is extremely very important for sepsis applications, as the bacterial concentration per millilitre of blood in early sepsis is very low. Hence, for sepsis applications, large volumes (2–7 mL whole blood) will be necessary for any sample preparation method. Ideally, this should not be on the cost of separation efficiency. Using our current spiral device, considering only the sample flow rate of 50 μL/min, it takes 40 min to process 1 mL of whole blood (with ~25% hematocrit) at a separation efficiency as high as 82%. On the other hand, for a separation efficiency of 90%, it would take approximately 3 h to process 1 mL of blood (with ~5% hematocrit). In general, there is a trade-off between separation efficiency and throughput for any given technology. To this end, in comparison to the current state of the art, our method is well suited for high separation efficiency without compromising throughput too much. In Fig. 7, we compare
Figure 6. *E. coli* separation from diluted blood. (A) Qualitative analysis showing fluorescently labeled (using bacterial viability kit) *E. coli* migrating to the inner wall and collected at O1 while blood cells remain focused at the outer wall and collected at O2. Scale bar: 100 μm. (B) Quantification of *E. coli* (1000 CFU/mL) spiked into three diluted blood samples (1:10, 1:5, and 1:2). Agar plating-based quantification shows *E. coli* separation efficiency between 90 and 82% depending on blood dilution. (C) Hemocytometer was used to observe fluorescently labeled *E. coli* (using Backlight viability kit) and blood cells, at the inlet and outlets that were separated in the spiral chip, at a total flow rate of 1 mL/min. The blood cells (RBCs) were observed only in O2 and the majority of *E. coli* were observed in O1. Scale bar: 0.1 mm.

previous work published using both active (magnetophoresis [73], acoustophoresis [74,75], and dielectrophoresis [76,77]) and passive (size-based separation [48,53,58,78]) to our current work involving bacteria isolation from blood, after taking the blood dilution and sample throughput per chip for each study into consideration. While a more fair comparison would be to use similar experimental conditions, that is, same bacteria concentration, blood dilution ratio, same design, etc., we found that most of the work (about 80% of the studies) used high bacteria concentration of ≥10⁴ CFU/mL (highlighted in blue) [48,53,74,76–78]. In addition, most of the work that shows good separation efficiency has lower throughput [48,58,59,73,74,76–78] while studies that highlight higher throughput show lower separation efficiency [53,73]. The separation efficiency corresponding to the three different blood dilutions at a bacterial concentration of 1000 CFU/mL in our current work is highlighted in red (Fig. 7). The relatively high separation efficiency in this work is attributed to prepositioning the blood sample at the outer wall and to the addition of viscoelastic enhancer (PEO), which maintains the blood cells to remain focused throughout the length of the spiral channel while bacteria migrate.

5 Concluding remarks

In this article, we report a label-free elasto-inertial microfluidics-based passive method for separating viable bacteria from diluted blood at high efficiency and faster sample processing compared to conventional methods. We demonstrate that by prepositioning particles at the outer wall using viscoelastic buffer (PEO), high-resolution focusing of bigger particles at the outer wall can be achieved, while smaller particles migrate to the inner wall for effective separation. This stable focusing behavior at the outer wall is attributed to the elastic force ($F_e$) that arises due to the viscoelastic buffer (PEO) to counteract the effect of $F_L$ and $F_D$, enabling high-resolution 3D focusing. Prepositioning particles at the outer wall enable continuous and high-resolution particle separation via entrapment into the Dean vortices strictly based on size. We demonstrate 1 μm particle separation resolution (separation between 2 and 3 μm particles) at a total throughput of 1 mL/min (with a sample flow rate of 50 μL/min), a throughput only previously demonstrated using inertial microfluidics mainly for larger particle sizes. For blood-based experiments, we used PEO to sheath the blood sample (using 1:10, 1:5, and 1:2 blood dilutions) to preposition them at the outer wall of a spiral inlet. We show that blood cells remain fully focused at the outer wall throughout the entire spiral length while bacteria continuously migrate for efficient separation. The viable bacteria are recovered free of larger blood cells (RBCs and WBCs) and a majority of the platelets, readily available for downstream analysis. In this work, we demonstrate the separation of bacteria from 1 mL of blood in 40 min at an efficiency of 82% for a bacteria concentration of 1000 CFU/mL. The microfluidic platform opens up possibilities of effectively and rapidly separating viable bacteria from blood and should

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warrant clinical value as a stand-alone sample preparation method in clinical settings.

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The authors have declared no conflict of interest.

Data availability statement

The data that support the finding of this study are available from the corresponding author upon reasonable request.

Author contributions

S. Narayana Iyengar and A. Russom were responsible for conceptualizing the idea. S. Narayana Iyengar performed the experiments and wrote the manuscript. T. Kumar, G. Mårtensson, and A. Russom are responsible for reviewing and editing the manuscript. A. Russom and G. Mårtensson were the principal and cosupervisors for this work, respectively. A. Russom is responsible for funding and resource acquisition. All authors have approved the final version of the manuscript.

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