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Separation of Sub-micron Particles from Micron Particles using Acoustic Fluid Relocation Combined with Acoustophoresis

Gayatri P. Gautam¹, Rubi Gurung¹, Frank A. Fencel², and Menake E. Piyasena^{1,*}

¹Department of Chemistry, New Mexico Institute of Mining and Technology, Socorro, NM 87801, USA

²Center for Biomedical Engineering & Department of Chemical and Biological Engineering, University of New Mexico, Albuquerque, NM 87131, USA

Abstract

Acoustophoresis has gained increasing attention as a gentle, non-contact, and high throughput cell and particle separation technique. It is conveniently used to isolate and enrich particles that are greater than 2 μm ; however, its use in manipulating particles smaller than 2 μm is limited. In this work, we present an alternative way of using acoustic forces to manipulate sub-micrometer particles in continuous flow fashion. It has been shown that acoustic forces can be employed to relocate parallel laminar flow streams of two impedance mis-matched fluids. We demonstrate the separation of sub-micron particles from micron particles by the combination of acoustophoresis and acoustic fluid relocation. The micron particles are focused into the middle of the flow channel via primary acoustic forces while sub-micron particles are moved to the side via drag forces created by the relocating fluid. We demonstrate the proof of the concept using binary mixtures of particles comprised of sub-micron/micron particles, micron/micron particles, and bovine red blood cells with *E. coli*. The efficiency of the particle enrichment is determined via flow cytometry analysis of the collected streams. This study demonstrates that by combining acoustic fluid relocation with acoustophoresis, sub-micron particles can be effectively separated from micron particles at high flow rates and it can be further implemented to separate binary mixtures of micron particles if the volumetric ratio of two particles is greater than 10 and the larger particle diameter is about 10 μm . The combined method is more appropriate to use than acoustophoresis in situations where acoustic streaming and differences in acoustic impedance of fluids can be concerns.

In the presence of a resonance acoustic field, the clean high-density fluid (dark grey) and the low-density sample fluid are relocated. During this process, *E. coli* are separated from the red blood cells (RBC).

Graphical Abstract

*Corresponding Author, sesathura.piyasena@nmt.edu.

Compliance with ethical standards

Conflict of interest Gayatri P. Gautam and Menake E. Piyasena declare that they have filed patent application on this technology that uses principles discussed here. Rubi Gurung and Frank A. Fencel declare no competing interests.

Keywords

Acoustophoresis; Acoustic fluid relocation; Particle separation; Microfluidics

Introduction

Separation and enrichment of viruses, bacteria, exosomes, DNA, and other similar nano and sub-micrometer components in the presence of micrometer components in biological fluids is necessary for many clinical, and biological sample preparation and analyses [1–4]. Centrifugation has been the most common mode of isolation and enrichment of such nano and sub-micrometer particles [5–7]. However, the use of strong centrifugal forces can adversely affect morphological and functional properties of these components [2, 8]. Most often, time-consuming extended centrifugation methods including, differential and density gradient centrifugation are necessary to enrich smaller particles [9]. Centrifugation needs larger volumes of samples and often results in aggregation of particles and a significant sample loss. Thus mild yet efficient and fast methods for nano and sub-micrometer particle separation and enrichment are warranted.

Several microfluidic flow through techniques have been developed to isolate and concentrate particles [10–15], and among them, acoustophoresis has gained tremendous interest as a non-contact, gentle, label-free, and high throughput particle separation and enrichment technique. In acoustophoresis, primary acoustic force generated by a resonance standing acoustic wave field is applied to manipulate particles and cells suspended in the microfluidic flow [12, 16]. The magnitude of the primary acoustic forces (F), which is responsible for the particle manipulation, is proportional to the particle volume, thus larger particles experience greater acoustic forces than smaller particles as described by the following equation [17, 18].

$$F = -\frac{(\pi p_0^2 V_p \beta_m)}{2\lambda} \phi(\beta, \rho) \sin 2kx \quad (1)$$

Where p , V_p , β , λ , p , ϕ , k and x represent the pressure amplitude of the standing wave, volume of the particle, compressibility, wavelength of the acoustic wave, density, acoustic contrast factor, wave number (defined as $2\pi/\lambda$) and distance of the particle from the acoustic pressure node, respectively. Letters p and m in subscript refer to the particle and the

surrounding medium. The acoustic contrast factor is determined by the density and the compressibility of the particle and the surrounding medium as shown by the equation 2.

$$\phi(\beta, \rho) = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m} \quad (2)$$

In a typical acoustophoretic particle isolation setting, a microfluidic channel with branched inlets and outlets is utilized to transfer particles from the sample fluid stream to a clean fluid stream, and the sample fluid is infused through lateral inlets while the clean fluid, often a biological buffer is introduced through a central inlet. Upon applying an acoustic standing wave field, larger particles in the mixture are transferred to the central fluid stream while smaller particles remain in the lateral fluid streams [12, 19]. This method works reasonably well as long as the acoustic impedance of the central fluid is equal to or greater than that of the lateral fluid. However, if the acoustic impedance of the lateral fluid is higher than that of the central fluid, the high impedance fluid will relocate to the center of the channel while the low impedance fluid relocates to the lateral position on applying acoustic forces resulting a phenomenon termed as acoustic fluid relocation [20]. In this situation, the lateral stream that moves to the center may carry the suspended particles along with it, hence affecting the purity [20, 21]. Often, bio-fluids are relatively denser, and while extracting components in bio-fluids into clean buffers streams, sample spillover along with the bio-fluid can exist. Furthermore, the primary acoustic force is proportional to the cubed radius of the particle, hence the direct isolation of particles that are smaller than 2 μm diameter becomes difficult in most experimental conditions. Besides, acoustic streaming induced drag forces also impede effective acoustophoresis when particles are smaller [22–24].

Recently, acoustophoretic manipulation of sub-micrometer particles has been demonstrated with two-dimensional acoustophoresis [25], traveling and standing surface acoustic wave techniques [26–29], acoustic trapping [30, 31], and acoustic seed particle trapping [32]. Most of these methods are promising, yet, to realize more viable and high-throughput methods for sub-micrometer or smaller particle isolation, it is essential to develop simple and continuous flow based acoustic separation methods. For example, surface acoustic wave based methods, as well as traveling wave methods require precise control in fabricating interdigitated electrodes and acoustic trapping, and seed particle-based approach is a non-continuous, batch separation process. In the current work, we explore the feasibility of utilizing acoustic fluid relocation to separate sub-micrometer particles from micrometer particles in simple, rapid, and efficient manner.

To generate acoustic fluid relocation, initially, the high impedance fluid must be in the lateral stream, and the low impedance fluid must be in the central stream. The acoustic force density generated in high impedance fluid will act on the low impedance fluid, causing the fluid relocation [27]. Fluid streams will still switch along their axial positions even if the impedance of two fluids is significantly not different [20]. Albeit, the impedance-mismatched fluids result in poor performance in direct acoustophoresis, same conditions can be creatively adapted for effective separation of nano and sub-micrometer particles from

micrometer particles. When a mixture of sub-micrometer (diameter $<1\ \mu\text{m}$) particles and micrometer (diameter $>1\ \mu\text{m}$) particles suspended in the low-impedance fluid is used as the central stream, micrometer particles will experience the strong acoustic forces, thus they will remain in the center (acoustophoresis) while sub-micrometer particles will be dragged to the two lateral sides along with the low impedance fluid (acoustic fluid relocation). Separation of sub-micrometer particles from micrometer particles can be achieved by combining the two processes mentioned above.

The overall goal of the current work is to demonstrate the use of acoustic fluid relocation combined with acoustophoresis to purify binary mixtures of particles as an alternative approach to direct acoustophoresis. We propose our approach to be used where direct acoustophoresis is challenging due to mismatched fluid properties and acoustic streaming of nano and sub-micron particles. The method described here utilizes similar experimental conditions as in typical acoustophoresis, thus no need of change in device preparation. However, our approach eliminates the use of fluids with a similar density which is a critical requirement in typical acoustophoresis of particle mixtures, especially with biological fluid like blood. The method we explain here is more suitable to separate nano and sub-micron particles from microparticles; nonetheless, it can be implemented to separate binary mixtures of microparticles of some specific sizes. In the current work, first, we demonstrate the isolation of sub-micrometer particles from micrometer particles by fluid relocation, and then extend the work to investigate the limit of separation of two different sized micron particles. Finally, we demonstrate the separation of a biological sample comprised of a mixture of *Escherichia coli* (*E. coli*) and bovine red blood cells (B-RBCs). The separation of two different sized micron particles can be extended to separate certain types of cancer cells (e.g. MCF-7) that are larger than healthy cells. The enriched sample streams can be collected separately and analyzed via flow cytometry [18, 33, 34]. Even though the difference in impedance of two media is the characteristic parameter that mainly governs the acoustic fluid relocation [20], we worked in a regime that difference in fluid density can merely be utilized for relocating the fluid streams.

Experimental

Materials

Silicon wafers (100 mm diameter) were purchased from University Wafer (Boston, MA). AZ 9260 positive photoresist and its developer were purchased from AZ EM (Branchburg, NJ). Hexa-methyldisilazane (HMDS) was purchased from Ultra Pure Solutions (Castroville, CA). SCHOTT BOROFLOAT®-33 glass slides ($75 \times 50 \times 1\ \text{mm}^3$) were purchased from Applied Microarrays Inc. (Tempe, AZ). Poly(dimethylsiloxane) (PDMS, Sylgard 184) was purchased from Ellsworth Adhesives (Germantown, WI). Silicone tubing (0.64 mm ID) were purchased from Cole Parmer (Vernon Hills, IL). PZT ceramic transducers were purchased from APC International Ltd (Mackeyville, PA). Acetone, PBS buffer tablets, NaCl, and fluorescein sodium salt were purchased from Sigma Aldrich (St. Louis, MO). B-RBCs were purchased from Innovative Research (Novi, MI). *E. coli* was purchased from Carolina Biological Supply (Burlington, NC). Nile-red polystyrene particles (NR-ps) were purchased from

Spherotech (Lake Forest, IL) and CountBright™ absolute counting beads were purchased from Life Technologies (Carlsbad, CA).

Fabrication of microfluidic devices

A silicon-glass microfluidic device consisting of one main channel with trifurcated inlets and outlets was constructed using standard silicon microfabrication methods reported elsewhere [18]. Briefly, a 100 mm silicon wafer was photo-patterned with the positive photoresist and etched via deep reactive ion etching. The width of the channel was chosen based on the frequency of the acoustic transducers available in the lab. A profilometer was used to measure the width and the depth of the fabricated channel. The final channel was 5 cm long, 250 μm wide, and 80 μm deep. The width and length of the each inlet and outlet were 84 μm and 1 cm respectively. A borosilicate glass slide was anodically bonded to the top surface of the etched wafer. Silicone tubing ($I = 2$ cm) were attached for liquid connection (Fig. 1a). To generate standing acoustic waves, a piezoelectric transducer ($I = 3$ cm, $w = 0.5$ cm) with the resonance frequency of 2.91 MHz was superglued to the underneath of the channel (Fig. 1b) in the longitudinal direction and the assembled device was mounted on a glass slide using PDMS slabs as supports and double-sided tapes as the adhesive (Fig. 1b).

Preparation of samples

We used following fluids to carry out relocation experiments; DI water (without further purification), 1X phosphate buffered saline (1X PBS, containing 137 mmol/L of NaCl, 2.7 mmol/L of KCl, 10 mmol/L of Na_2HPO_4 and 1.8 mmol/L of KH_2PO_4), 0.1X phosphate buffered saline (0.1X PBS) prepared by 10 fold dilution of the 1X PBS, and the high density 1X PBS (containing 200 mmol/L of NaCl, in addition to the rest of 1X buffer components). To demonstrate the proof of the concept, fluorescent and non-fluorescent solutions with different mass densities, solutions containing mixtures of suspended micron particles and sub-micron particles, *E. coli*, and B-RBCs were utilized. 2.5 μM fluorescein solutions were prepared by dissolving fluorescein sodium salt in D.I. water and 0.1X PBS. For the particle mixtures, fluorescent NR-ps with different diameters were suspended in D.I. water. B-RBCs and *E. coli* were diluted in 1X PBS buffer.

Optimization of the device for fluid relocation

First, acoustic fluid relocation was established in the microfluidic device using two fluids with different mass densities. The 1X PBS (137 mM NaCl, 2.7 mM KCl) and 2.5 μM fluorescein solution prepared in D.I. water were introduced to the microfluidic device via the two lateral and the central inlets respectively (Fig. 2a) using syringe pumps (KD Scientific, MA) at flow rates of 25 and 75 $\mu\text{L}/\text{min}$ respectively. Acoustic standing wave field was applied after the three flow streams were stabilized and the optimum fluid relocation was established by fine-tuning the resonance frequency.

Particle manipulation and separation

To study the manipulation of sub-micron particles via fluid relocation, experiments were conducted with aqueous suspensions comprised of 0.530, and 0.840 μm diameter NR-ps

particles separately. Each sub-micron particle suspension was introduced to the channel via the central inlet at a flow rate of 50 $\mu\text{L}/\text{min}$ while particle-free 1X PBS was introduced via the two lateral inlets at a flow rate of 75 $\mu\text{L}/\text{min}$. These flow rates were chosen after a series of flow rate tests to obtain optimum widths for three fluid streams during the fluid relocation. To investigate the separation of sub-micron particles from micron particles, a binary mixture comprised of microspheres and sub-microspheres suspended in D.I. water was introduced from the central inlet and 1X PBS buffer was introduced from two lateral inlets. The composition of both particle types was maintained to be as equal as possible. For biological samples, a mixture of B-RBCs and *E. coli* in 1X PBS (low-density buffer) was introduced via the central inlet and high density 1X PBS was introduced via two lateral inlets. The particle concentration of each suspension was maintained at $\sim 1 \times 10^5$ particles/mL for NR-ps and 1×10^6 cells/mL for biological samples. A comparative study was carried out to compare the efficacy of our method and the acoustophoresis in separating a binary mixture of micron particles by using a mixture comprised of 5.1 and 11.0 μm diameter NR-ps particles. Acoustophoresis experiment was performed using the standard method reported elsewhere [18, 33, 35]. Briefly, a sample containing particle mixture in 1X PBS was flowed through lateral inlets and particle free 1X PBS was flowed through the central inlet at the flow rates of 75 $\mu\text{L}/\text{min}$ and 150 $\mu\text{L}/\text{min}$, respectively. A minimum resonance acoustic power was used to selectively focus 11.0 μm at the center of the microchannel. For the combined method, same flow rates and voltage were used, however sample containing mixture of particles was passed through central inlets and 1X PBS solution was passed through the lateral inlets. The resonance standing acoustic waves were generated using a waveform generator (RIGOL DG 1022, RIGOL Technologies Inc., OR) and amplified via an RF amplifier (E&I 350L, Electronics & Innovation Ltd., NY). The acoustic performance parameters (frequency, applied voltage, amplitude) were monitored via an oscilloscope (Tektronix TBS 1052B, Tektronix Inc., OR).

Fluorescence imaging and flow cytometry analysis

To evaluate the fluid flow relocation and particle separation, fluorescence images and video clips of fluid and particle streams in the microfluidic channel were captured above the PZT or near the trifurcated outlet using an epi-fluorescence microscope equipped with a sCMOS camera (Orca-FLASH 4.0, Hamamatsu, Japan). The flow profile was obtained by line scanning the image across the channel (ImageJ software, NIH). A BD FACSCalibur flow cytometer was used to analyze samples collected from each outlet before and after the fluid relocation. The composition of NR-ps particles and cells in a particular sample was determined via flow cytometry dot plots. Pure samples of NR-ps particles and cells were first measured to define the regions of interest in each dot plot and set the gates accordingly for each particle or cell type. These regions were later used to identify and calculate the percentage composition of particles or cells present in a particular mixture. NR-ps particle populations were gated based on their fluorescence intensity via FL2 fluorescence channel of the flow cytometer that detects fluorescence at 585 ± 21 nm and side scattering and the data were presented in the form of dot plots of side scatter (SSC) vs. fluorescence intensity (FL2). Mixtures of non-labeled cells (*E. coli* and B-RBCs) were analyzed using their size difference and hence, presented as dot plots of SSC vs. forward scatter (FSC). All data collection and analysis were performed using BD CellQuestTM Pro (BD Bioscience, San

Jose, CA) and FCS Express 6 (De Novo Software, Glendale, CA) software respectively. Total of 10,000 events were collected during each measurement, and the instrument threshold was set at FL2 and/or SSC to remove unwanted events resulting from the non-fluorescent particles and/or debris. To determine the particle concentration in samples, an internal calibration method was employed using flow cytometry standard beads [36]. Briefly, one milliliter of bead sample was mixed with 50 μL of standard CountBright™ absolute counting beads. The concentration of particles was calculated using the following equation [37].

$$\text{Concentration (per mL)} = \left(\frac{\# \text{ of particle events}}{\# \text{ of standard bead events}} \right) \left(\frac{\text{Standard beads in total volume}}{\text{Total volume (mL)}} \right) \quad (3)$$

Results and discussion

Acoustic fluid relocation

Acoustic fluid relocation has been demonstrated in microfluidic devices to enhance the laminar flow mixing [38] and to study its impact on acoustophoresis [20]. In the current work, we explored its potential as a new approach for rapid and efficient separation of binary mixtures of particles. First, we established the optimum fluid relocation parameters by using the three streams of two density mis-matched solutions as described in the experimental section. The laminar flow condition in the device makes these three streams flowing parallel to each other with a negligible diffusive mixing occurring at each interface (Fig. 2a). The acoustic frequency was scanned from 2.5 MHz to 3.5 MHz while keeping the applied voltage (V_{pp}) constant at 20.0 V_{pp} and found that the maximum fluid relocation occurs at 3.01 MHz. Once the effective resonance frequency was established, the applied voltage was scanned from 2 V_{pp} to 20 V_{pp} to find the lowest possible applied voltage that can generate fluid relocation and it was found that stable fluid relocation occurs at 10.8 V_{pp} . The width of each fluid stream was maintained by controlling the relative fluid flow rates using syringe pumps, and the width of each 1X PBS stream and the D.I. water stream was kept to approximately one-third of the channel width (Fig. 2b). During fluid relocation (see Electronic Supplementary Material (ESM) Movie S1), two 1x PBS streams in lateral positions were relocated and combined at the center of the channel (Fig. 2c) while the fluorescein stained water stream in the center was split and relocated to the two lateral positions of the channel (Fig. 2c). The line scan of flow profile (Fig. 2d) suggests that each of these lateral streams was about 65 μm wide while the width of the new central stream was about 130 μm (Fig. 2d). The expansion of the newly formed central stream was expected as it comprised two combined streams of initial lateral streams. The epi-fluorescence images (Fig. 2a, 2c) show the absence of sharp fluid boundaries between 1X PBS and water streams due to the diffusion of fluorescein at each interface [21, 39, 40]. When 1X PBS solution was replaced with a ten-fold diluted 1X PBS (0.1X PBS), the stream relocation occurred as before (ESM Fig. S1) and the ten-fold dilution of the buffer had no visible impact on the

extent and the speed of relocation. Our observation further confirms that denser solution initially must be at the lateral position for relocation to occur as reported earlier by Deshmukh et al. [20]. In these experiments, fluorescein was just used to visualize flow paths. When the experiment was repeated with 2.5 μM fluorescein in water as two lateral streams and pure D.I. water as the central stream, fluid relocation was not observed (ESM Fig S1), implying that there is no effect from 2.5 μM fluorescein on fluid relocation. However, we can anticipate that fluid relocation can occur at higher fluorescein content since the mass density of the medium increases. Further, we tested total flow rate of up to 500 $\mu\text{L}/\text{min}$ without the loss of fluid relocation, thus high- throughput flow manipulation is achievable.

Relocation of sub-micron particles

To demonstrate the separation of sub-micron particles from micron particles via acoustophoresis combined fluid relocation, we first investigated the relocation of individual submicron particle type using NR-ps particles with the diameters of; 0.53, and 0.84 μm . Sub-micron particles of a given size suspended in D.I. water were introduced into the device as explained in experimental section and observed a complete relocation of sub-micron particles to the lateral stream at an acoustic standing wave field of 3.01 MHz (± 10 kHz) and applied voltage of 12 V_{pp} , as shown in Figure 3a (initial) and 3b (relocated). The small dots in the middle of the channel are a small fraction of particles that are adhered to the surface of the channel, and there was no influence from them for the fluid relocation. The extent of the relocation of sub-micron particles was determined by flow cytometry measurements of particle streams collected from each outlet before and during the fluid relocation. Here we report the relocation of 0.53 and 0.84 μm particles only. The fluorescence intensity of particles smaller than 0.53 μm was not high enough for us to capture visible epi-fluorescence images. Further, the smaller particle population lies near to the lower threshold region of flow cytometry dot plots where sample debris can also present; consequently, concentration calculations can be inaccurate. However, we can anticipate that smaller particles will have weaker acoustic focusing forces, thus enhanced relocation, and therefore should perform better. Flow cytometry analysis shows that two lateral streams contain a small number of particles before the fluid relocation (Fig. 3c) and this can be due to the diffusion and random movement of some particles. Due to this, the average sample loss was 988 ± 125 particles/mL in three replicates for 0.53 μm particles, and this is negligible comparing to the total composition. The total average concentration of particles in two lateral streams has increased to 83977 ± 1813 particles/mL during the fluid relocation. This 84-fold increase in particle concentration proves that sub-micron particles can be effectively manipulated via drag forces of relocating fluid. The concentration of particles in the original particle stream (center) has decreased by $\sim 80\%$ (Fig. 3c) during the process. For 0.84 μm particles, the average concentration of particles in two lateral streams was 788 ± 136 particles/mL (Fig. 3c), and the average concentration of particles in two lateral streams has increased to 79416 ± 80 particles/mL during fluid relocation. The concentration of 0.84 μm particle is enhanced by 100 fold and concentration of particles in the original particle stream (center) has decreased by $\sim 88\%$ (Fig. 3c) during fluid relocation.

Separation of binary mixtures of particles:

a) Separation of micron sized particles and sub-micron sized particles—After establishing the manipulation of sub-micron particles via fluid relocation, next, we investigated its feasibility for isolation of sub-micron particles from a binary mixture that also contained micron particles. For this, a mixture of 0.25 and 2.07 μm (diameter) NR-ps particles suspended in the low-density fluid (water) was introduced from the central inlet as mentioned in the experimental section (Fig. 4a). On applying resonance acoustic standing wave field, 2.07 μm particles were focused and remained in the central stream while 0.25 μm particles were dragged along with the low-density fluid and relocated as lateral streams as shown in the fluorescence streak image (Fig. 4b) and ESM Movie S2. The dot plots of side scatter vs fluorescence emission were used to measure the purity of NR-ps population in each sample (Fig. 4c-d). The initial mixture in the central stream (Fig. 4c) contained $52\pm 1\%$ of 0.250 μm and $40\pm 1\%$ of 2.07 μm particles. During the fluid relocation, the central stream was enriched with 2.07 μm particles and its composition was increased to $81\pm 2\%$ while the composition of 0.250 μm particles was reduced to $9\pm 0.4\%$. The remaining population (about 10%) was considered as debris in the sample. The two combined lateral streams had $92\pm 2\%$ 0.250 μm particles and 0.1% 2.07 μm particles. We also tested few other combinations of binary mixtures; 0.53 μm and 2.07 μm , 0.84 μm and 2.07 μm , 0.84 μm and 4.24 μm , and 0.84 μm and 11.0 μm (ESM Fig. S2). Overall, data suggest that the lateral stream is efficiently enriched with the sub-micron particles, with the percentage of 90% or above. The larger microspheres in the mixture will experience higher primary acoustic forces such that they will be focused and remained in the center stream with minimum influence from drag forces arising from relocating fluid while the smaller particles in the mixture are efficiently migrated to the two lateral positions of the channel. The presence of a small fraction of smaller particles in the central stream (Fig. 4d) during fluid relocation is associated with the diffusion and spillover of particles to the central stream as mentioned before. We found that, if the collected fraction of the central stream is recycled once more through the device, the central stream can be enriched entirely with micron particles.

b) Separation of micron-sized particles—The ability to separate a binary mixture of micron particles via our approach was investigated with two binary mixtures containing particles of 5.1 and 11.0 μm (ESM Movie S3) and 2.07 and 11.0 μm diameter respectively. For 5.1 and 11.0 μm , the initial mixture contained $64\pm 1\%$ 5.1 μm particles and $34\pm 1\%$ 11 μm particles (Fig. 5a). In the presence of acoustic focusing and fluid relocation, the central stream was enriched with 11.0 μm microspheres ($83\pm 1\%$). However, it had $17\pm 0.4\%$ of 5.1 μm particles as well (Fig. 5b) while the lateral stream was fully enriched with 5.1 μm particles ($99\pm 1\%$) and 11.0 μm particles were not present (Fig. 5c). These data from flow cytometry analysis of collected streams confirms a good separation of micron particles of two sizes, especially the lateral stream with pure 5.1 μm particles. On recycling the central stream once more, the central stream was enriched with 11.0 μm particles ($95\pm 2\%$) (Fig. 5e) by relocating the remaining 5.1 μm particles to the lateral streams (Fig. 5f). We observed a similar outcome when a mixture of 2.07 and 11.0 μm particles was used (ESM Fig. S3). This experiment validates that fluid relocation combined with acoustophoresis can be utilized to separate mixtures of micrometer particles of specific size ranges. When the size increases, the primary acoustic forces on micron-sized particles become dominant and the drag forces

of the relocating fluid will not be effective anymore. Further, we tested the binary mixtures containing micron particles of 6.43/11, 3.42/6.43, 4.24/6.43, and 2.07/5.0 μm ; however, the separation was poor, thus we conclude that the volume difference of two particles in the binary mixture is too close obtaining an adequate separation. To estimate the limits of particle size and their combination in binary mixtures of micron particles that can effectively be separated, we calculated the volumetric ratios of micron particle mixtures tested, as in equation 4.

$$\text{Volumetric Ratio} = \frac{\text{Volume of the large particle}}{\text{Volume of the small particle}} \quad (4)$$

The table 1 suggests that if the volumetric ratio of particles in the binary micron particle mixture is about 10 or greater, an effective separation can be expected. However, when the diameters of both particles are greater than 11 μm , regardless of the volumetric ratio, separation is difficult, either via typical acoustophoresis or our combined approach. These larger particles experience very high acoustic forces which prevents discrimination of two sizes. Therefore, we compared only the microspheres of 11 μm or smaller. While assuming the minimum volumetric ratio of two sizes needed for separation as 10, particle diameters of different combinations in binary mixtures were plotted (Fig. 6). The straight line in the Fig. 6 shows the theoretical boundary line of two diameters of micron particles which corresponding the volumetric ratio of 10. Any combination that lies on or above this line will be separated. For example, if the particle 1 diameter is 2 μm , then the particle 2 diameter needs to be 4.31 μm for the volumetric ratio to be 10. Thus, 2 μm particles can be separated from particles of 4.31 μm or greater. The filled circles represent experimentally observed microsphere combinations that were separated whereas the open circles represent microsphere combinations that were not separated via fluid relocation. It could have been more useful if wider combinations of particle sizes were tested; however, the limited availability of various sizes prevented us from performing a broader analysis.

c) Separation of *E. coli* and bovine red blood cells—To demonstrate the capability and the efficiency of separation and enrichment of small pathogenic microorganisms in blood via our method, we used a sample of *E. coli* and B-RBCs. These cells were non-stained, thus they were identified by their size difference via flow cytometry forward scatter plots. The relative positions of pure *E. coli* and B-RBCs in cytometry scatter plots were first assigned by analyzing unmixed samples. The percentages of two populations in the initial mixture were $56 \pm 1\%$ of *E. coli* and $41 \pm 1\%$ of B-RBCs (Fig. 7a). During the fluid relocation, *E. coli* relocated to the lateral stream, and its composition was found to be $98 \pm 2\%$ (Fig. 7b). The composition of the new central stream was $80 \pm 1\%$ RBCs and $19 \pm 0.4\%$ *E. coli* (Fig. 7c). We could further enrich the middle stream with B-RBCs by recycling the central stream once more (data not shown) as we reported before. The separation of *E. coli* from B- RBCs demonstrates the efficacy of our approach for the separation of micro-organisms in blood.

Acoustophoresis vs acoustophoresis combined acoustic fluid relocation for micrometer particle separation

By scanning the applied voltage at the resonant frequency of the device, 3.01 MHz, we found that a minimum voltage of 14.5 V_{pp} is required to focus 11 µm particles via acoustophoresis. Flow cytometry analysis of samples separated via acoustophoresis alone suggests that at this minimum voltage, the composition of 11.0 µm particles in the central stream was only 53±1% (ESM Fig. S4), whereas, for the combined method this was 83±1%. Petersson *et al.* had demonstrated efficient separation of microparticles of close range sizes via acoustophoresis by precisely controlled flow rates, power, microchannel dimensions, and medium composition [12]; however, our approach is much efficient, simpler and easier to setup and control, thus it will be more convenient to use for the separation of binary mixtures of micron particles with the sizes < ~11 µm and the volumetric ratio of >10.

Conclusion

In this work, we introduced a new, rapid and efficient method to isolate sub-micrometer particles from micron particles by combining the acoustophoresis with acoustic fluid relocation. The proof of the concept study was performed for binary mixtures comprised of sub-micron and micron particles, and *E. coli* spiked bovine RBC. We demonstrated that when the primary acoustic force is relatively weak, the fluid drag force generated by the relocating fluid is capable of separating particles or cells of two sizes. We separated sub-micrometer particles into the two lateral streams while micrometer particles into the central stream of a trifurcated microfluidic channel. The two lateral streams comprised almost entirely (97% or more) of smaller particles after the separation. We observed that the central stream may contain a small percentage (10% or less) of smaller particles due to the spillover and diffusion; however, we could improve the separation further by recycling the central stream once more. We also demonstrated separating a binary mixture of micron particles comprised of 11 and 5.1 µm particles. We were interested in separating relative particle sizes in biological applications and, 11 µm was considered as the upper limit. With 11 µm particles, the closest size we could separate was 5.1 µm, and the separation of particles with sizes >5.1 µm was not effective. To estimate the separation limits of particle size and their combination in binary mixtures of micron particles, we calculated the volumetric ratios of micron particle mixtures and, we can propose the ratio has to be 10 or higher. We used a minimum total flow rate of 200 µL/min during the separation of sub-micron particles from micron particles, and we did not observe acoustic streaming of particles, further enhancing the separation. Moreover, the total flow rate can be as high as 500 µL/min, and which offers faster separation. Further, our method eliminates the use of lengthy centrifugation processes to enrich clinically important biological components. We envision that the technique we demonstrated in this work has the potential for rapid and simple enrichment of clinically significant nanometer scale elements like viruses, bacteria, and cellular components like exosomes, lipid particles, and DNA from blood and other biofluids and this will eventually help early detection of pathogenic species present in biological fluids. Further, we propose our approach to be used where direct acoustophoresis is challenging due to mismatch fluid properties and acoustic streaming of nano and sub-micron particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Silicon microfluidic device. Top view of the DRIE device with three silicone tubing inlets and outlets. Microchannel is enclosed with an anodically bonded glass slide and silicone tubing- PDMS slab is plasma sealed to the slide (a). Bottom view of the device with the attached transducer and the PDMS supports (b).

Fig. 2.

Relocation of the fluid streams on applying resonance acoustic wave field. Laminar flow of three streams in the absence of acoustic force. The two lateral streams are 1X PBS and the central stream is 2.5 μ M fluorescein in DI water (a). Corresponding flow profile obtained via ImageJ (b). Relocation of solutions in the presence of acoustic wave field. Fluorescein solution is relocated to the lateral streams while 1X PBS solution is relocated to the central stream (c), and corresponding flow profile (d).

Fig. 3. Relocation of sub-micro particles. Epi-fluorescence micrograph of 0.53 μm particles at the central stream (a). Relocation of the sub-micron particles to the lateral stream (b). Concentration of sub-micron particles in each stream (c).

Fig. 4.

Top: Epi-fluorescence micrograph for the separation of 0.25 μm and 2.07 μm NR-ps particles via the combined method. Mixture of 0.25 μm and 2.07 μm particles flowing in the central stream in the absence of acoustic wave field (a). 0.25 μm particles are dragged to the lateral stream along with the fluid and 2.07 μm particles are focused at the central stream in the presence of acoustic wave field (b). Bottom: Scatter plots for flow cytometry analysis of samples collected from: Central stream, acoustic off (c); central stream, acoustic on (d); and lateral stream, acoustic on (e).

Fig. 5.

Scatter plots of flow cytometry analysis for the separation of a mixture of 5.1 and 11.0 μm NR-PS particles. Analysis shows the percentage of each particle type present in streams. Acoustic off, central stream; 1st run (a). Acoustic on, central stream; 1st run (b). Acoustic on, lateral stream; 1st run (c). Acoustic off, central stream; 2nd run (d). Acoustic on, central stream; 2nd run (e). Acoustic on, lateral stream; 2nd run (f).

Fig. 6. Volumetric ratio curve for the effective separation of binary mixtures of micron particles. The straight line represents where the volumetric ratio of two micron particles is equal to 10. Any micron particle combination (between 1–12 μm diameter) above the line can be separated via the combined method.

Fig. 7.

Flow cytometry scatter plots for the separation of a mixture of *E. coli* and B-RBCs. Central stream, acoustic off (a); lateral stream, acoustic on (b); central stream, acoustic on (c).

Table 1.

Determination of limits of particle dimensions and binary mixtures for micro-particle separation

Particle 1 Diameter (µm)	Particle 2 Diameter (µm)	Volumetric Ratio (particle 1/ particle 2)	Separation
11.0	6.43	5.6	No
11.0	5.10	10	Yes
11.0	2.07	15.1	Yes
11.0	0.34	2245.6	Yes
6.43	4.24	3.5	No
6.43	3.42	6.6	No
5.1	2.07	14.9	Yes
4.24	6.34	126.6	Yes
2.07	0.34	14.9	Yes