



Isolation of exosomes from whole blood by integrating acoustics and microfluidics

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Contributed by Subra Suresh, August 17, 2017 (sent for review June 5, 2017; reviewed by Gang Bao and M. Taher A. Saif)

Exosomes are nanoscale extracellular vesicles that play an important role in many biological processes, including intercellular communications, antigen presentation, and the transport of proteins, RNA, and other molecules. Recently there has been significant interest in exosome-related fundamental research, seeking new exosome-based biomarkers for health monitoring and disease diagnoses. Here, we report a separation method based on acoustofluidics (i.e., the integration of acoustics and microfluidics) to isolate exosomes directly from whole blood in a label-free and contact-free manner. This acoustofluidic platform consists of two modules: a microscale cell-removal module that first removes larger blood components, followed by extracellular vesicle subgroup separation in the exosome-isolation module. In the cell-removal module, we demonstrate the isolation of 110-nm particles from a mixture of micro- and nanosized particles with a yield greater than 99%. In the exosome-isolation module, we isolate exosomes from an extracellular vesicle mixture with a purity of 98.4%. Integrating the two acoustofluidic modules onto a single chip, we isolated exosomes from whole blood with a blood cell removal rate of over 99.999%. With its ability to perform rapid, biocompatible, label-free, contact-free, and continuous-flow exosome isolation, the integrated acoustofluidic device offers a unique approach to investigate the role of exosomes in the onset and progression of human diseases with potential applications in health monitoring, medical diagnosis, targeted drug delivery, and personalized medicine.

extracellular vesicles | exosomes | blood-borne vesicles | surface acoustic waves | acoustic tweezers

Exosomes are cell-derived nanovesicles (1), ≈30–150 nm in diameter, that carry nucleic acids, proteins, lipids, and other molecules from their cells of origin (2, 3). Exosomes transfer RNA and proteins to the cells they fuse with and play important roles in cell-to-cell communication. Recent research into the characteristics and mechanisms involving exosomes has introduced the potential development of biomarkers for health monitoring and diagnosis of a number of human diseases, including cancer (4), neurodegenerative disease (5), and diseases of the kidney (6), liver (7), and placenta (8). Exosomes represent a unique research opportunity because they are found in nearly all biological fluids (9–11), including blood, saliva, urine, semen, sputum, breast milk, and cerebrospinal fluid. Unlike tissue samples, they can be collected noninvasively over a long period, allowing for continuous monitoring of disease progression and response to therapy. Exosomes also have several advantages over other circulating biomarkers. They are abundant (thousands to billions per microliter of biofluid), and their durability suggests that their internal integrity can be preserved through several freeze-and-thaw cycles.

Currently, differential centrifugation (including gradient ultracentrifugation), which relies on multiple centrifugation steps to sequentially remove whole cells, cellular debris, and subgroups

of extracellular vesicles (EVs) based on their different sizes and densities, is a standard technology for isolating exosomes (12, 13). While differential centrifugation achieves high purity, it is time-consuming (several hours to days), expensive, and inefficient (in that the exosome isolation yields from whole blood are typically low, 5–40% of pre-separation exosome population) (12, 14–17). It also requires trained personnel to operate. Moreover, the high centrifugal force used in ultracentrifugation (100,000–200,000 × *g*) has been shown to cause exosome fusion, promote coagulation, and alter their structures, properties, and functions, which may impact downstream analysis (12, 13, 18). Other methods, including immunoaffinity capture (19, 20), precipitation kits such as ExoQuick (System Biosciences) and Total Exosome Isolation (Invitrogen) (12, 21), microfluidics (17, 22, 23), nanoscale lateral displacement arrays (24), nanostructure-based filtration (25), nanoplasmonic chip (26), magnetochemical sensor (27), and dialysis membrane filtration (28), have been implemented. However, these methods frequently suffer from drawbacks such as the need for additional reagents/labels, long processing time, low reproducibility, low exosome integrity, low exosome purity, and/or low exosome yield.

Acoustic waves are well-recognized for their high precision and biocompatibility in manipulating cells and other bioparticles

Significance

We have developed a unique, integrated, on-chip technology that is capable of isolating exosomes or other types of extracellular vesicles, directly from undiluted whole-blood samples in an automated fashion. Automated exosome isolation enables biohazard containment, short processing time, reproducible results with little human intervention, and convenient integration with downstream exosome analysis units. Our method of integrating acoustics and microfluidics leads to the isolation of exosomes with high purity and yield. With its label-free, contact-free, and biocompatible nature, it offers the potential to preserve the structures, characteristics, and functions of isolated exosomes. This automated, point-of-care device can further help in advancing exosome-related biomedical research with potential applications in health monitoring, disease diagnostics, and therapeutics.

Author contributions: M.W., Y.O., S.S., Y.S., and T.J.H. designed research; M.W., Y.O., Z.W., R.Z., C.C., and H.L. performed research; M.W., Y.O., Y.S., and T.J.H. contributed new reagents/analytic tools; M.W., Y.O., P.-H.H., P.L., D.Q., M.D., S.S., Y.S., and T.J.H. analyzed data; and M.W., Y.O., P.-H.H., P.L., D.Q., M.D., S.S., Y.S., and T.J.H. wrote the paper.

Reviewers: G.B., Rice University; and M.T.A.S., University of Illinois at Urbana–Champaign. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1709210114/-DCSupplemental.

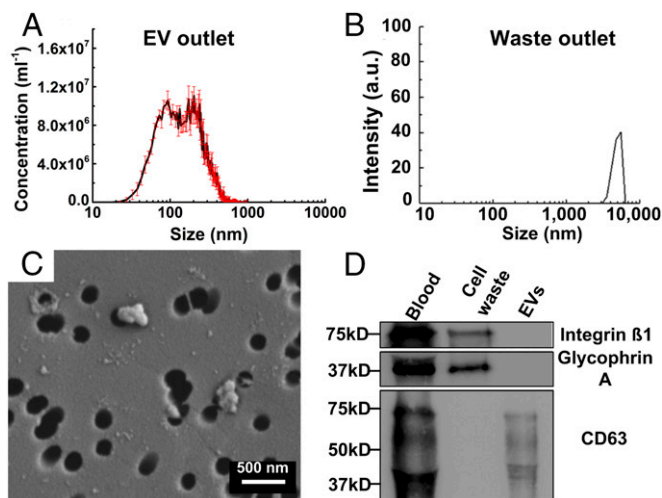


Fig. 3. Characterization of the cell-removal module. (A) Separation of EVs from RBCs and other blood components. NTA was used to characterize the isolated EVs from the collection outlet. (B) RBCs and other blood components collected from waste outlet were characterized by DLS. The ordinate is the relative intensity of signals measured. (C) SEM image of isolated EVs sample loaded on a filter membrane. The EV sample contained vesicles of diameters from ~ 50 to 300 nm. (D) Western blot with expression of RBC marker (GYPA), PLT marker (integrin $\beta 1$), and EV markers (CD63). The proteins from blood, cell waste sample, and isolated EVs were extracted and prepared for electrophoresis.

this acoustofluidic approach to isolate nanoparticles from a mixture of nanoparticles and microparticles.

Based on the conditions optimized by our particle-separation experiments, we proceeded to test our cell-removal module using undiluted whole-blood samples, which contained the anticoagulant EDTA. Because blood cells have a lower acoustic contrast than polystyrene particles, we increased the applied voltage to 40 Vpp. To match the acoustic impedance of whole blood, a 5% dextrose solution in PBS was used as sheath fluid. When the taSSAW field was off, the whole-blood sample flowed into the top outlet. Once the taSSAW was activated, blood components such as RBCs, WBCs, and PLTs changed their flow route and were delivered to the waste outlet (Fig. S2 A and B) and the smaller EV-containing sample was collected.

Samples collected from the two outlets were measured using nanoparticle tracking analysis (NTA) device and dynamic light scattering (DLS). The sample collected at the waste outlet had a visible peak at $\sim 5 \mu\text{m}$, which contained primarily RBCs, while the sample collected at the collection outlet, the isolated EVs sample, contained no particles larger than $1 \mu\text{m}$ (Fig. 3 A and B), thus suggesting that submicrometer particles, such as EVs, were isolated. We used a scanning electron microscope (SEM) and Western blotting to further characterize isolated EVs. The SEM showed that the diameter of isolated EVs ranged between 50 and 300 nm (Fig. 3C). The Western immunoblotting showed that samples from the waste outlet were positive for Integrin $\beta 1$ (PLT marker) and Glycophrin A (a representative marker of RBCs). In contrast, our isolated EVs were immune-positive for CD63, a tetraspanin characteristic of exosomal marker, and negative for PLT and RBC markers (Fig. 3D). Collectively, these results demonstrate that the acoustofluidic cell-removal module is capable of separating EVs directly from undiluted, anticoagulated human blood samples.

Exosome-Isolation Module. To examine whether our exosome-isolation module could separate EV subgroups, namely microvesicles from exosomes, we input a mixture of purified exosomes

and microvesicles derived from primary human trophoblasts (PHTs) to a standalone exosome-isolation module. The isolation and culture of PHT cells from human placentas and the purification of PHT-derived microvesicles and exosomes from PHT-conditioned medium were described elsewhere (16). We identified an optimized driving frequency of 39.4 MHz based on pilot experiments using a nanoparticle mixture of 110 and 340 nm (Fig. S3). Then, we set the sample flow rate and sheath flow rate as 4 and $8 \mu\text{L}/\text{min}$, respectively. With the standing SAW field switched on, and under an input voltage of 45 Vpp, larger bioparticles were deflected and directed to the waste outlet. We then conducted NTA of the isolated samples from both outlets as well as of the original mixture of the same volume. The original mixture of purified microvesicles and exosomes exhibited a broad size distribution from ~ 50 to 600 nm (Fig. 4A); specifically, there was a single peak at 122 nm corresponding to exosomes, whereas other peaks appeared between 170 and 300 nm, representing the broader distribution of microvesicles rather than exosomes. Additionally, the concentration distribution curve reached a valley at 140 nm, which was therefore chosen as the separation cutoff size. The sample at the collection outlet exhibited two peaks, at ~ 81 and 99 nm, which represented slight shifts from the inlet peak corresponding to a size of 122 nm. This difference may be attributed to the resolution limits of NTA when testing highly heterogeneous samples. When we examined the morphology of the isolated exosomes (Fig. 4C) using transmission electron microscopy (TEM), the mean size of isolated vesicles was ~ 100 nm, which is consistent with the NTA results and the predicted size of exosomes. In contrast, the sample collected from the waste outlet exhibited several peaks larger than 170 nm, along with very few components that were less than 100 nm. These results demonstrated that our acoustofluidic device was able to separate two distinct EVs from each other (i.e., PHT-derived microvesicles from exosomes).

We further used NTA to quantify the concentrations of the mixture of trophoblastic microvesicles and exosomes, isolated microvesicles, and isolated exosomes. Given that the final

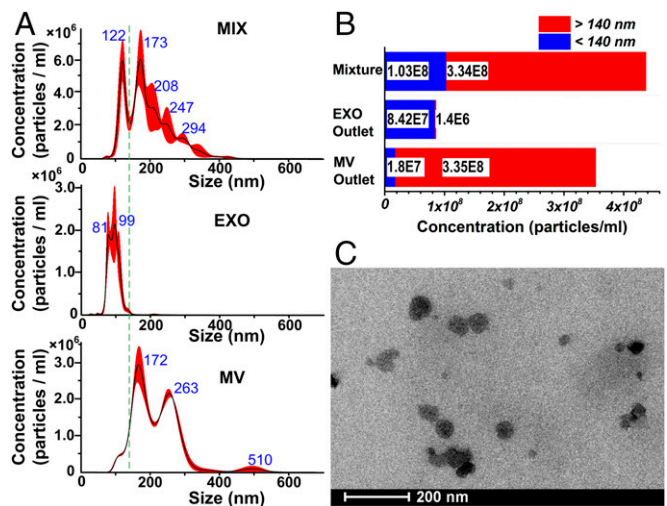


Fig. 4. Separation of exosomes from microvesicles using the exosome-isolation module. (A) Size distribution of original mixture (MIX), isolated EXO, and MV samples. The data were obtained from at least three NTA assays. The black line and the red area represent the fitting curve and the error bar, respectively. The y axis is the concentration of particles. The peak positions are marked. The green dashed line is located at 140 nm, which is set as the cutoff size. (B) Quantitative characterization of exosome/microvesicle separation, showing the concentrations of vesicle subgroups (cutoff size at 140 nm) in the mixture and processed samples. The concentration is expressed as the number of particles per microliter. (C) TEM image of isolated exosome samples.

volume of each outlet is $1.5\times$ the input sample volume, reflecting the PBS dilution effect during the course of separation, we corrected the particle concentrations measured from NTA by dividing by this dilution factor (1.5). We calculated that the original mixture contained 1.03×10^8 particles per microliter that were smaller than 140 nm and 3.34×10^8 particles per microliter that were larger than 140 nm. The concentration of particles collected from the exosome outlet was 8.42×10^7 per microliter (<140 nm) and 1.4×10^6 per microliter (>140 nm). At the microvesicle outlet, the particle concentration was 1.8×10^7 per microliter (<140 nm) and 3.35×10^8 per microliter (>140 nm). The total numbers of vesicles before and after separation were 4.37×10^8 per microliter and 4.386×10^8 per microliter, respectively, and the percentages of small (<140 nm) particles were comparable before (23.6%) and after (23.3%) separation. These values suggest that the acoustofluidics-based separation technique had a high sample yield with minimal loss during the separation process. We defined the recovery rate as the fraction of particles recovered below 140 nm among the particles of that size in the inlet solution. Similarly, we define the purity of particle isolation as the fraction of isolated particles below 140 nm among the collected particles of all sizes. Overall, the present exosome-isolation device showed a recovery rate and purity of 82.4% and 98.4%, respectively, for particles smaller than 140 nm in diameter. Despite the demonstrated recovery rate and purity, it should be noted that the particles smaller than 140 nm may contain nonexosomal particles and protein aggregates, which could be considered contaminants for downstream analysis of exosome.

Isolation of Exosomes from Undiluted Blood Using the Integrated Device. Following testing and optimizing the individual modules, we integrated the cell-removal module and exosome-isolation module into a single acoustofluidic chip. On this integrated chip, the distance between the two modules was set sufficiently apart to avoid interference between the acoustic fields of the two modules, allowing the integrated device to operate as efficiently as the optimized individual modules using the same parameters and designs. We used undiluted human blood from healthy donors for EV isolation (Fig. 5 and Fig. S4). The flow rates of each inlet were set to 4 $\mu\text{L}/\text{min}$ for the blood sample, 4 and 12 $\mu\text{L}/\text{min}$ for sheath

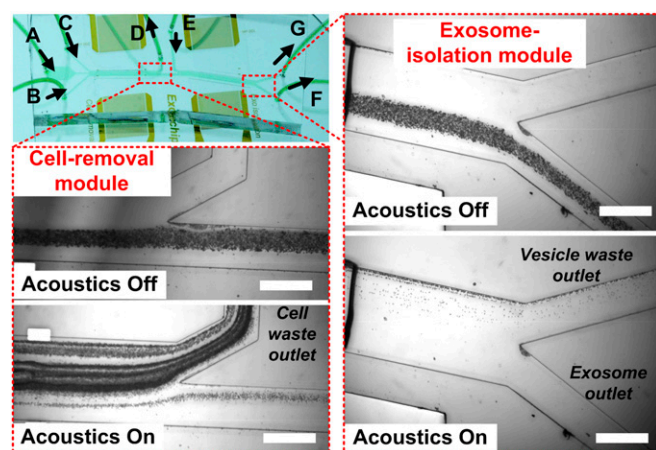


Fig. 5. Isolation of exosomes from whole blood using the integrated device using acoustofluidics. In our experiments, inlet A is for whole blood; inlets B, C, and E are for sheath flows. Outlet D is cell waste. Outlets F and G are for isolated exosomes and vesicle waste, respectively. Images were taken under the microscope at the corresponding areas of the device. Blood components were directed to each corresponding outlet when the acoustic wave was on. White stripe in the four grayscale panels indicates the centerline location of the CCD image sensor. (Scale bar: 500 μm .)

flows in the cell-removal module, and 10 $\mu\text{L}/\text{min}$ for sheath flow in the exosome-isolation module. The driving frequency and voltage of the input rf signal for the integrated device were the same as those used for individual modules described above. When the acoustic field was off, the blood stream was focused in the middle of channel and directed into the device outlet F in Fig. 5 (Top Left). When the rf signal was on for both modules, blood components were separated into different outlets after passing through the cell-removal module. The vast majority of blood cells and PLTs were deflected to a cell waste outlet (outlet D in Fig. 5, Top Left) and the remaining components continued to flow downstream to the exosome-isolation module where the apoptotic bodies, microvesicles, and the remaining part of cells are deflected to the vesicle waste outlet (G in Fig. 5, Top Left), thereby isolating exosomes from whole-blood samples in the device outlet (F in Fig. 5, Top Left), which we subsequently refer to as the “exosome outlet.”

Upon collecting samples from the exosome and vesicle waste outlets, we characterized the cell-removal efficiency. The original blood sample, separated vesicle waste, and isolated exosome sample were each collected into 1.5-mL centrifuge tubes and spun at 3,000 rpm for 10 min. As shown in Fig. 6A, the volume of cells in the whole-blood sample was nearly half of the total volume, which is typical for human blood. In contrast, there were few ($<0.1\%$) blood cells remaining in the isolated exosome sample and the vesicle waste (Fig. 6A). We further quantified the number of blood cells in the exosome sample, using a hemocytometer. The concentration of cells was 2.08×10^4 per milliliters in the sample collected from the exosome outlet, while the RBC count reference ranged from 4.7 to 6.1×10^{10} per milliliter, yielding a cell-removal rate greater than 99.999%. We then measured the size distribution of isolated exosome samples through NTA. This was compared with NTA of plasma that was separated from the whole-blood sample using standard centrifugation. The sample collected from the exosome outlet showed a clear, narrow peak at around 100 nm, which corresponded to exosomes, while the plasma control displayed a flat, disperse curve covering a broad range from ~ 50 nm to 1 μm (Fig. 6B). As control, we isolated human plasma exosomes using OptiPrep gradient ultracentrifugation, and compared the size distribution of exosomes isolated by two different approaches. The peak of exosomes using gradient ultracentrifugation was slightly larger than that of exosomes using the acoustofluidic device (Fig. S5). This difference could be explained by the effect of ultracentrifugation on exosomes, causing some aggregation of exosomes and/or even fusion of small, “contaminating” particles (18, 35). Collectively, the NTA results demonstrated that the acoustofluidic device differentiated subgroups of EVs based on size, and thereby isolated exosomes from the mixture.

Having demonstrated the removal of blood components, including RBCs, WBCs, PLTs, and microvesicles from undiluted whole-blood samples, we sought to verify that the sample isolated from blood is indeed composed of exosomes. We used Western blot analysis to examine the expression of exosomal protein markers in the samples collected from all three outlets and a diluted blood sample. We analyzed the expression of EV membrane tetraspanin CD63, membrane-binding protein TSG101, endoplasmic reticulum protein HSP90, and heat shock cognate protein 70 (HSC70). Among the four samples examined, the sample collected from the exosome outlet showed a high expression of HSP90, HSC70, CD63, and TSG101 (Fig. 6C), confirming the presence of exosomes in the samples. These proteins were also present in original blood samples, as expected. The other two outlets, referred to as vesicle waste and cell waste, showed very low levels of exosomal markers.

We further investigated whether exosomes isolated by our integrated acoustofluidic chip were contaminated by RBC's RNA transcripts. It has been demonstrated (36) that four mRNA genes encoding Ferritin light chain (FTL), Glycophorin A (GYPA),

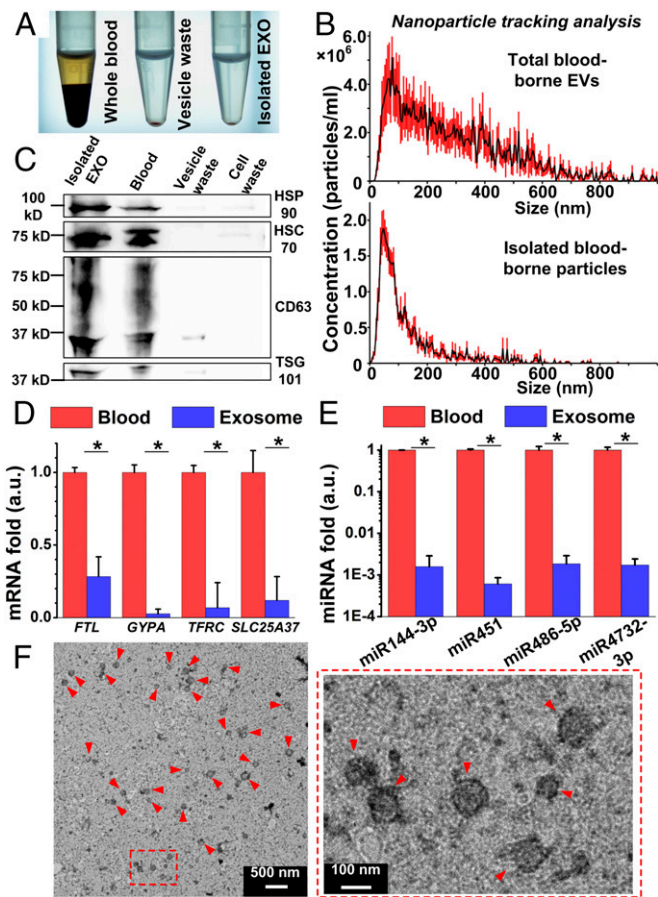


Fig. 6. Characterization of exosome isolation from whole blood using the integrated acoustofluidic chip. (A) Removal of blood cells and PLTs. In the original sample (undiluted whole blood), RBCs occupied approximately half of the volume. The isolated exosome sample and vesicle waste sample contain a minimal amount of blood cells. (B) EVs in blood plasma showed a dispersed size distribution that ranged between 30 nm and 1 μ m. The size distribution of collected exosome sample exhibited a major peak at <100 nm. (C) Western blot of exosome markers, showing a prominent expression in the isolated exosome and blood samples, while the other samples (vesicle waste and cell waste) exhibited low expression level of exosomal proteins. (D and E) The expression (expressed as relative fold difference) of individual mRNAs (D) and miRNAs (E) in human blood and isolated exosomes. The data represent three independent experiments. * $P < 0.05$ (ANOVA) (F) TEM images of isolated exosomes. The exosomes (red arrows) have a characteristic round shape and a cup-like structure.

Transferrin receptor (TFRC), and Solute carrier family 25 member 37 (SLC25A37) are predominantly expressed in human RBCs. We compared the relative levels of these transcripts in samples of human blood input and isolated exosomes. We found that all four transcripts expressed in RBCs were decreased by 75~90% between the input to the first module and the output from the second module in our acoustofluidic device (Fig. 6D). Similarly, we examined relative levels of RBC-dominant miRNAs in whole blood and isolated exosomes. miRNAs are known to be packaged in exosomes and other extracellular vesicles, and RBCs strongly express four miRNAs including miR-144-3p, miR-451, miR-486-5p, and miR-4732-3p (37–40). Consistent with the mRNA results (Fig. 6D), our miRNA results (Fig. 6E) indicated that isolated exosomes barely, if any, expressed these four RBC miRNAs. We observed a similar pattern of mRNA and miRNA expression using samples derived from the gradient-based ultracentrifugation (Fig. S6). Together, the mRNA and miRNA results suggest that the exosomes isolated by our acoustofluidic devices have little contamination by RBCs. Finally, we examined the morphology of isolated exosomes

using TEM. A large number of vesicles were, as marked by arrows, of diameter \sim 100 nm with cup-like concavity (Fig. 6F), consistent with the established morphology of exosomes (41). These results support the ability of our acoustofluidic platform to isolate morphologically intact exosomes.

Discussion

We have demonstrated an acoustofluidic platform that is capable of isolating exosomes directly from undiluted human blood. The integrated device is based on acoustofluidics and contains two separation modules, which provide the flexibility to handle multiple subpopulations of a complex sample. By tuning the input power of the rf signal and fluid flow rates, the cutoff size for each of the two separation modules can be adjusted to ensure the selection of specific subgroups. This feature enables the flexibility to adjust for a range of particle sizes and applications.

Blood is one of the most complex biological fluids, with components and properties that vary greatly among individuals or within an individual at different time points. These factors challenge existing separation techniques. Consider, for example, the experimental hurdles arising from the blood lipid level. The lipid particles have a negative acoustic contrast, in that they are pushed to antinodes in the standing acoustic field. As such, lipid particles concentrate at antinodes and tend to aggregate (36). Aggregation of lipids disturbs laminar flow and the acoustic field pattern, which in turn reduces separation efficiency. Therefore, for blood samples with high lipid levels, the sheath/sample flow rate needs to be appropriately adjusted with an increased buffer flow rate to suppress lipid aggregation. Another solution might be the addition of a third acoustofluidic module designed to remove lipids from undiluted blood.

With the current device configuration, we have successfully separated and isolated bioparticles larger than 150 nm from exosomes. Notably, this isolated exosome sample may contain non-exosomal particles and protein aggregates that have a size similar to exosomes or smaller particles. To obtain exosomes with the highest purity, we plan to integrate additional acoustofluidic-based separation modules into the current device setup. As indicated in Table S1, Fig. S7, and SI Simulation Assays for Isolating Nonexosomal Particles and Soluble Proteins from Exosomes, these additional acoustofluidic modules will allow us to further isolate exosomes from (i) particles that have a similar size to exosomes (30–150 nm) but different acoustic contract factors, and (ii) particles that are smaller (i.e., <30 nm) than exosomes.

Our technology, predicated upon acoustofluidics, offers the following distinct advantages over other available means to separate exosomes from biological fluids:

- i) Automation, high reproducibility, and biohazard containment: In conventional exosome-isolation assays, samples need to be subjected to a multistep protocol using several instruments. Throughout this process, a trained technician must manually interact with the samples. In contrast, the acoustofluidic approach can isolate exosomes (or other subgroups of EVs) directly from biological fluids (e.g., undiluted blood) with a single device in an automated manner. Thus, it offers a simpler approach with enhanced biosafety and a higher likelihood of consistent and reliable results. Furthermore, after determining the optimal acoustic field settings, routine operation of the acoustofluidic system requires less training compared with conventional approaches.
- ii) Exosome-separation speed: While differential centrifugation approaches take hours to days for exosome isolation from whole blood, the entire process to isolate exosomes from 100 μ L undiluted human blood can be achieved within \sim 25 min using acoustofluidics.
- iii) Exosome yield and purity: We have demonstrated an exosome purity of \sim 98% and a yield of \sim 82% by using a mixture

of purified exosomes and microvesicles derived from PHT cells in our experiments.

- iv) Continuous flow configuration: Many exosome-separation platforms must be operated in batch mode. Acoustofluidics is capable of separating exosomes in continuous flow. Such devices involving continuous flow can be conveniently integrated into existing microfluidic-based exosome analysis device to enable an all-in-one, on-chip exosome processing and analysis system.
- v) Potential to isolate structurally intact and biologically active exosomes: Many existing exosome-isolation technologies have difficulties in isolating biologically active and structurally intact exosomes; the isolation process often alters the morphology, content, and functions of the exosomes (14, 18, 42). The present strategy offers a label-free, contact-free, and potentially gentle method that has the potential to minimize disruption of the captured exosomes. The acoustic power intensity and frequency we used in our experiments are in a similar range to those in ultrasonic imaging, which has been proven to be a safe technique. Using our device, exosomes are exposed to a low-power-intensity acoustic field for several seconds. This may compare favorably to differential centrifugation, which subjects exosomes to hours of exposure to forces as high as $200,000 \times g$. This combination

of factors yields a higher likelihood of preserving the biological, biophysical, and structural integrity of the isolated exosomes for further investigation.

Methods

Device Fabrication and Experimental Setup. The device is fabricated by standard soft-lithography and lift-off process. More details are in *SI Device Fabrication* and *SI Experimental Setup*.

Isolation of Exosomes Using Gradient Ultracentrifugation. Exosomes were isolated from whole-blood specimens using an OptiPrep gradient ultracentrifugation as previously described (16). The collection of placentas used for cell isolation and culture was reviewed and approved by the Institutional Review Board (IRB) at the University of Pittsburgh.

Characterization of Exosomes. The isolated exosomes are characterized by NTA, Western blot, electron microscopy, and quantitative polymerase chain reaction (qPCR). More details can be found in *SI Characterization of Exosomes*.

ACKNOWLEDGMENTS. The authors are grateful to Hunter Bachman for editing the manuscript. We acknowledge support from the National Institutes of Health (Grant R01 HD086325) and National Science Foundation (Grant IIP-1534645). M.D. acknowledges partial support from Singapore - Massachusetts Institute of Technology Alliance for Research and Technology (SMART).

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