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Rapid additive-free bacteria lysis using traveling surface acoustic waves in microfluidic channels

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We report an additive-free method to lyse bacteria and extract nucleic acids and protein using a traveling surface acoustic wave (TSAW) coupled to a microfluidic device. We characterize the effects of the TSAW on *E. coli* by measuring the viability of cells exposed to the sound waves and find that about 90% are dead. In addition, we measure the protein and nucleic acids released from the cells and show that we recover about 20% of the total material. The lysis method should work for all types of bacteria. These results demonstrate the feasibility of using TSAW to lyse bacteria in a manner that is independent of the type of bacteria.

Introduction

Studying bacteria at the molecular level is important for the investigation of diseases, detection of pathogens and drug development.[1–4] Lysing the bacteria to extract the intracellular contents, including DNA, RNA and proteins, is an indispensable first step in the analysis of bacteria. Unlike eukaryotic cells, bacterial species have a multi-layered cell wall that is very difficult to lyse.[5] While many techniques, including chemical, thermal and mechanical, can lyse bacteria, a method that is general and effective for all types of bacteria is still lacking. This is particularly important for studies of populations of bacteria, where many different types must be independently lysed with the same technique. For example, while a single chemical can effectively lyse some types of bacteria, it does not work well on all types.[6,7] Moreover, ionic surfactants such as sodium dodecyl sulfate, commonly used for chemical lysis, can dramatically inhibit the activity of polymerases required for downstream genetic analyses.[8] Thermal lysis methods are also not universally applicable and can be time consuming;[9,10] further, heating may denature proteins and may accelerate degradation of nucleic acids, in particular RNA, reducing the accuracy of downstream genetic analyses.[11] Electroporation can lyse bacteria rapidly but will cause partial degradation of the electrodes, limiting the device lifetime.[12] Mechanical lysis is more generic and avoids the problems with thermal and enzymatic techniques. Mechanical lysis methods include bead beating, in which cells are vortexed vigorously with small beads, resulting in physical lysis through collisions;[13–15] sonication, in which sound waves are used to lyse cells through cavitation;[16–18] and the French Press, which forces cells through a small orifice, resulting in cell lysis through a combination of shear forces and a rapid change in pressure.[13] These methods can all efficiently lyse bacteria without substantial damage to proteins and nucleic acids. However, they can only be applied to bulk suspensions of bacteria; lysis of individual cells, which is becoming more important as new single-cell analysis methods are developed, is impossible. Microfluidic devices allow precise control of small volumes and provide a convenient means to handle individual bacteria.[19,20] Lysing of individual mammalian cells in microfluidic devices is readily achieved with chemical or physical means,[21–24] but lysis of bacterial cells in microfluidic devices is mainly restricted to chemical or enzymatic means,[25–27] or to methods that introduce gas bubbles to induce cavitation.[28] However, none of these methods can perform direct lysis of bacterial cells traveling through a microfluidic device, which is essential for single cell analysis of populations of bacteria.

In this paper, we report an additive- and detergent-free method to lyse bacteria in a microfluidic device using acoustic energy to rapidly extract nucleic acids and proteins in a manner that is independent of bacteria type. We use a traveling surface acoustic wave (TSAW) to create sufficient acoustic energy to lyse bacteria. We expose bacteria flowing through a microchannel to a TSAW and extract the nucleic acids and proteins for downstream analyses. This method should work

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for any kind of bacteria and should be particularly suited to populations of multiple species.

Results

We use a hybrid microfluidic device composed of a molded microchannel plasma bonded to a piezoelectric $LiNbO_3$ substrate. The microchannel is formed from polydimethylsiloxane (PDMS) using soft lithography.[29] The dimensions of the PDMS microchannel are length, l = 11 mm, width, w = 400 μm and height, h = 65 μm. Interdigitated transducers (IDTs) are patterned on the piezoelectric substrate to excite the traveling surface acoustic waves. The IDT is deposited onto a 128° Y–X $LiNbO_3$ substrate and is composed of 25 parallel electrodes with an aperture of 7.93 mm. Bacteria cells flow through a straight channel to the section where they are exposed to the TSAW beam while they pass through the aperture of the IDT, as shown in Fig. 1. The average flow velocity of the cells is approximately 10.7 mm per second, determined by dividing the flow rate, 1 ml per hour, by the cross-sectional area of the channel. Since the aperture of the IDT is 7.93 mm, we estimate the residence time for each bacterium in the acoustic field to be about 0.7 seconds. After the cells pass this active region, they flow to the outlet and are collected for subsequent analysis.

We use a suspension of *Escherichia coli* bacteria, a Gram-negative species, at a concentration of 2×10^9 per milliliter. The throughput in the experiments is 1 ml or 2×10^9 cells per hour. We compare the behavior of two different IDTs, having resonance frequencies of 13 MHz and 160 MHz. We pulse-modulate the output of each IDT at 333 Hz with a duty cycle of 33% using custom-made software and a signal generator. We collect the TSAW-treated bacteria in an Eppendorf tube for further analyses.

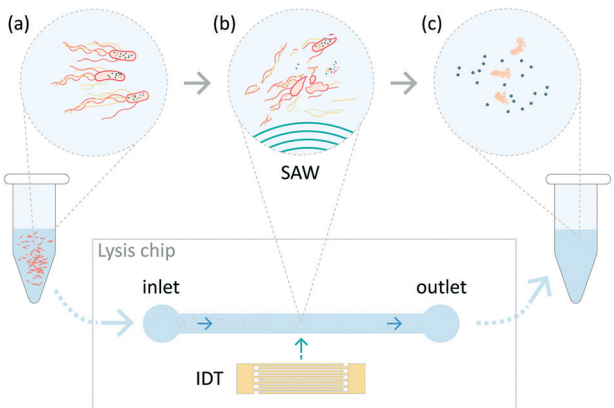

Fig. 1 Overview of the TSAW lysis device (top view). The lysis chip consists of a PDMS microchannel plasma-bonded to a lithium niobate (LiNbO3) substrate with an interdigitated transducer (IDT) adjacent to the microchannel to generate traveling surface acoustic waves. We flow the bacteria suspension through the microchannel (a) at a constant flow rate, expose the bacteria suspension to a TSAW field at 33.3% duty cycle (b) and collect the lysate for further analysis (c).

To characterize the effect of the TSAW treatment on bacterial cells, we determine cell viability and analyze the protein and nucleic acids released. We apply power levels from 3 W to 16 W and 0.5 W to 2.5 W for the 13 MHz and the 160 MHz devices, respectively. In both cases, the maximum power level is limited by the integrity of the device; higher power levels cause cracking of the piezoelectric substrate. After TSAW treatment we remove a fraction of the collected sample and perform viability measurements. We then centrifuge (10 000 rcf, 2 minutes) the remaining suspension to pellet the cells and collect the supernatant for protein measurement and gel electrophoresis of the nucleic acids.

To confirm that TSAW-treated cells were killed, rather than lost during treatment, we simultaneously visualize live and dead bacteria using the SYTO 9 green-fluorescent nucleic acid stain, which stains both live and dead bacteria green, and propidium iodide, which stains bacteria with damaged membranes red. The excitation and emission maxima for SYTO 9 are approximately 480 and 500 nm while for the propidium iodide they are 490 and 635 nm respectively; thus, when both dyes are present, propidium iodide will cause a reduction in the SYTO 9 green-fluorescence.

After treatment with these dyes, we image the bacteria using confocal microscopy. In the TSAW-OFF samples, almost all bacteria have intact membranes (Fig. 2a). When a TSAW of frequency 13 MHz and power level of 15.8 W is applied, the vast majority of cells stain as dead (Fig. 2b). The total number of bacteria in the sample treated with a 15.8 W

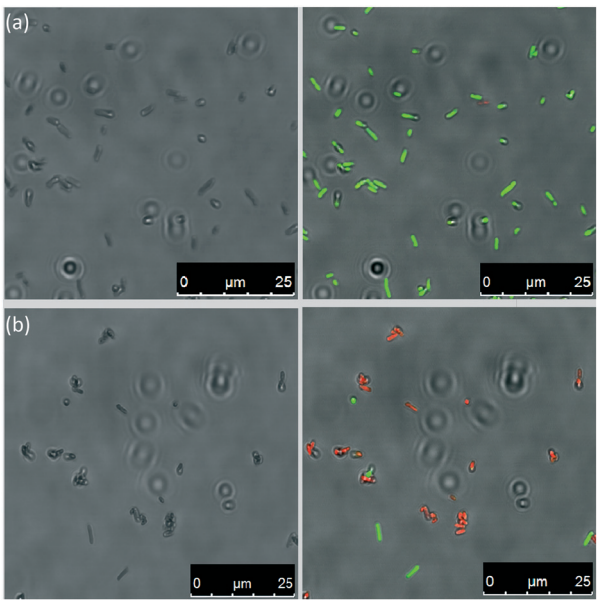

Fig. 2 Confocal fluorescence microscopy images of bacteria viability analysis using a LIVE/DEAD Bacterial Viability kit. (a) Bacteria lysates when the TSAW is OFF. (b) Bacteria lysates when applying traveling surface acoustic waves at a frequency of 13 MHz with a power level of 15.8 W. Left panels are bright field; right panels are merged brightfield and fluorescence. Green fluorescent dyes indicate live bacteria with intact cell membranes, and red fluorescent dyes indicate dead bacteria with damaged cell membranes.

field is less than in the TSAW-OFF sample; this is likely due to some bacteria being completely lysed. Similar results are obtained using the 160 MHz TSAW device.

To quantify the number of viable bacteria after exposure to the TSAW, we collect the treated bacteria suspension, dilute it and plate it on LB Agar. After incubation for 12 hours, we count the number of colonies and determine the concentration of the input and output samples. For all experiments, we use untreated bacteria kept on ice as a control group. A small fraction of the bacteria is lost or killed during passage through the device without TSAW activation. Activation with the TSAW results in a dramatic decrease in the number of viable cells. When the cells are passed through the 13 MHz and 160 MHz resonance frequency devices at power of 3.2 W and 0.7 W, respectively, ~50% of bacteria cells are killed. At the highest applied powers, treatment with either 13 MHz and 160 MHz TSAWs results in a loss of ~90% of viable bacteria, as shown in Fig. 3a and b.

The ultimate goal of lysing the bacteria is to release the proteins and nucleic acids from the cells. To determine if the TSAW treatment has accomplished this, we quantify the protein and nucleic acids released from the cells. We collect sample from the TSAW device output and centrifuge to remove the whole cells and cell debris. Cell debris and particulate matter will be in the pellet and not detected as released. We then determine the concentration of the proteins in the supernatant using an absorbance assay based on bicinchoninic acid (Pierce™ BCA Protein Assay kit, ThermoFisher Scientific). We mix 100 μl of the supernatant with 2 ML BCA working reagent following the enhanced BCA kit protocol. After incubation at 60 °C for 30 minutes, a spectrophotometer (Agilent Technologies Cary 60 UV-vis) is used to measure the absorbance, which provides a relative measure of the protein concentration. For comparison, we create a reference sample by treating an equivalent number of cells with a Protein Extraction kit (Sigma-Aldrich, BugBuster®) to completely lyse the cells. For this reference sample, we determine the protein concentration of the supernatant using the same absorbance test. Protein extraction increases as the power level increases for both TSAW frequencies. At the highest power levels, the concentration of the protein recovered is about 20% of the amount recovered from the completely lysed sample, as shown in Fig. 4.

We also measure the amount of nucleic acids released from the lysed bacteria. We run the supernatant on a 1.2% agarose gel and stain with Gel Red (Biotium, GelRed® Nucleic Acid Gel Stain). For comparison, we create a reference sample by extracting nucleic acids from an equivalent number of *E. coli* using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich). Nucleic acid extraction efficiency increases with increased TSAW power for both frequencies; however, even at the highest powers, the amount extracted remains significantly less than that extracted from the reference sample, as shown in the images of the gels in Fig. 5. We observe some RNA in several TSAW samples as indicated by the faint bands at ~0.1 kb weights in Fig. 5; however, we did not add RNase inhibitor during sample prep and therefore cannot accurately compare RNA extraction efficiencies. For comparison, RNA is not seen in the sample generated with the Bacterial Genomic DNA extraction kit, which includes an RNase step.

To quantify the extraction efficiency, we collect the supernatants derived from 3 million cells treated with TSAWs using 13 MHz at 15.8 W and 160 MHz at 2.5 W and load these samples onto agarose gels. For comparison, we create an "extraction standard curve" consisting of genomic DNA extracted from 0.3 million, 0.6 million, 1 million, 1.5 million, 3 million bacteria using the Bacterial Genomic DNA kit. By comparing the relative band intensities of the TSAW and the standard-curve samples, we find that the amount of DNA extracted by treating 3 million cells with either 13 MHz at 15.8 W or 160 MHz at 2.5 W is comparable to the amount of DNA obtained by complete lysis of between 0.3 M and 0.6 M cells, as shown in Fig. 6. Thus, the extraction efficiency is between 10% and 20%.

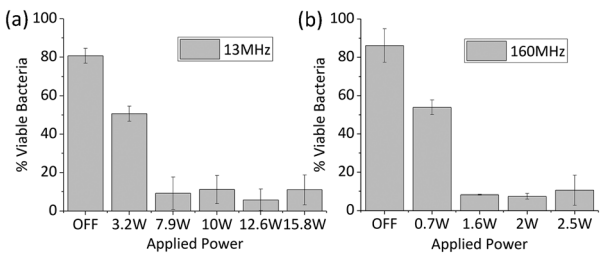

Fig. 3 Relative viability of bacteria treated with various applied power levels. (a) Traveling surface acoustic waves at a frequency of 13 MHz. (b) Traveling surface acoustic waves at a frequency of 160 MHz. A control sample using bacteria on ice was prepared and used to determine the 100% viability value. "OFF" indicates passage of bacteria through the channel with TSAW OFF. The error bars indicate standard deviations based on four independent tests for 13 MHz and three independent tests for 160 MHz.

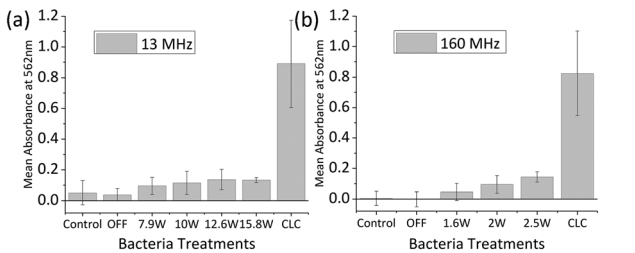

Fig. 4 Quantitative analysis of protein released from bacteria after TSAW treatment. We treat bacteria at the indicated power level, centrifuge the suspension, determine the protein concentration in the supernatant using a BCA Protein Assay kit. (a) Traveling surface acoustic waves at frequency of 13 MHz. The error bars indicate standard deviations based on five independent tests. (b) Traveling surface acoustic waves at frequency of 160 MHz. The error bars indicate standard deviations based on three independent tests. "Control" indicates bacteria on ice. For "CLC" complete lysis control, we analyse the supernatant from bacteria after complete lysis using a Protein Extraction kit.

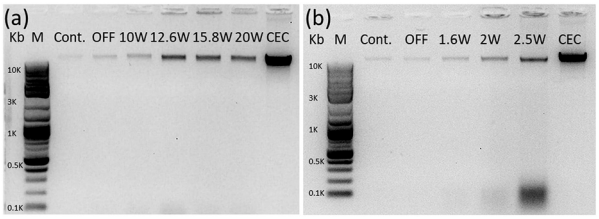

Fig. 5 Agarose gel electrophoresis analysis of nucleic acids extracted at indicated TSAW power level. (a) Traveling surface acoustic waves at frequency of 13 MHz. (b) Traveling surface acoustic waves at frequency of 160 MHz. The no-lysis control ("Cont.") is generated from bacteria kept on ice. The complete extraction control ("CEC") is prepared using the Bacterial Genomic DNA kit. "M" is 100 bp DNA ladder; "kb" is kilobases.

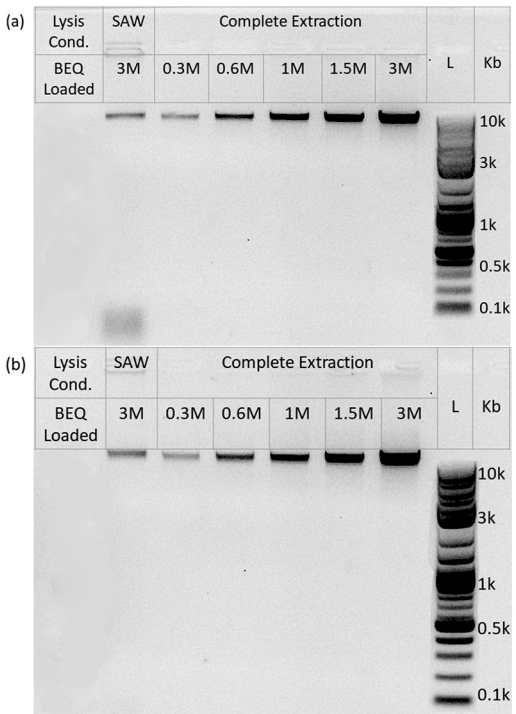

Fig. 6 TSAW extraction efficiency relative to detergent-based method. Agarose gel electrophoresis analysis of nucleic acids released from bacteria treated with TSAW at (a) frequency of 13 MHz at power level of 15.8 W and (b) frequency of 160 MHz at power level of 2.5 W. We loaded lanes with supernatant from indicated number of bacteria cells ("BEQ" indicates Bacteria equivalents). We loaded "Complete Extraction" lanes with supernatant obtained by treating the indicated number of cells with the GenElute™ Bacterial Genomic DNA kit. Band sizes of DNA ladder ("L") are indicated in kilobases ("kb").

For both the proteins and the nucleic acids, the amount of material recovered from the TSAW device is only 20%, even at the highest power levels. However, the viability assay suggests that only 10% of the bacteria remain viable after exposure to the TSAW. Moreover, the number of bacteria collected is visually reduced upon passage through the TSAW device, suggesting some are completely destroyed by exposure to the TSAW. This suggests that the actual extraction efficiency is significantly higher, and that some DNA from lysed cells is lost. This microfluidic device was not designed to optimize collection of the lysate, and thus performance will be improved with an optimized device design.

Materials and methods

Device design

Our device consists of a PDMS microchannel plasma bonded onto a 128° Y–X lithium niobate ($LiNbO_3$) onto which a single IDT has been deposited. We design the microchannels and IDTs using AutoCAD (Autodesk, Inc., San Rafael, CA) and print them onto a photomask. The photomask is purchased from CAD/Art Services, Inc. (Bandon, OR) and imaged with a resolution of 15 400 dpi for lithography. The PDMS microchannel features an air gap above the IDT to prevent damping of the SAW by direct contact with PDMS.[30–32] The microchannel is 11 mm in length and 400 μm in width. The thickness of the PDMS wall between the air gap and fluid-filled channel is 250 μm and provides sufficient stability while minimizing sound absorption.

We design straight interdigitated transducers (IDTs) with twenty-five connected and parallel finger pairs with overlapping width (aperture) of 7.93 mm. The IDTs are designed at operating frequencies of 13 MHz and 160 MHz, with finger spacings of 75 μm and 6.2 μm respectively, which can produce SAW wavelengths of 300 μm and 24.86 μm respectively. The metallization coverage ratio is $a/p = 1$ throughout the transducers, where the electrode width is a and the distance between fingers is p. The interdigitated electrodes on either side are interconnected by bus bars that merge into square contact pads to which we apply external voltages.

IDT fabrication

We use a 4″ diameter black 128° Y–X lithium niobate with thickness of 500 μm and double-side polished wafers as the piezoelectric substrate. We use acetone and isopropanol to clean each wafer and then bake on a heating plate at 180 °C for at least 3 minutes to remove any residual moisture. We create a 300 nm thick sacrificial layer on the top of cleaned wafer by spin coating with LOR 3A (MicroChem Corp., Westborough, MA) at 3000 rpm, then baking the wafer at 180 °C for 7 minutes. Subsequently, we form a 500 nm layer of photoresist on top of the sacrificial layer by spin coating with Shipley S1805 (MicroChem Corp., Westborough, MA) at 4000 rpm and baking for 1 minute at 115 °C. After these processes, we use a mask-less alignment tool (MLA150, Heidelberg Instruments, Germany) to transfer the designed IDT patterns to the wafer. We expose the wafer to UV-light using 40 mJ cm^{-2} and a wavelength of 405 nm. We use CD-26 developer (Microposit MF, Dow Electronic Materials, Marlborough, MA) for wafer development for 75 seconds and clean it by a rinse with deionized water, and finally dry it with nitrogen. Before metal deposition, we clean the wafer in an oxygen plasma for 5 minutes at 150 W and 40 sccm gas flow (Anatech SCE-106 plasma barrel etcher, Anatech USA, Union City, CA) to remove organic residues from the wafer surface that could

impair metal adhesion. We create a 10 nm thick titanium adhesion layer and a 50 nm thick gold layer by using electron beam physical vapor deposition (Denton Explorer 14, Denton Vacuum LLC, Moorestown, NJ). We use a Remover-PG bath (MicroChem Corp, Westborough, MA) at 80 °C for the lift-off process and clean it with isopropanol. Prior to dicing, we add a protective layer by spinning Shipley S1813 at 3000 rpm and baking the wafer at 115 °C for 2 minutes. We cut the wafer into square pieces of 17.4 mm by 17.4 mm using an automated dicing saw (DAD321, DISCO Corp., Tokyo, Japan). The protection layer is then removed by soaking individual IDTs in acetone for ~5 minutes. The wafer is cleaned with isopropanol prior to use.

PDMS fabrication

We fabricate the PDMS microchannel following the process recommended in the manufacturer's data sheet for SU-8 3000 series photoresists (MicroChem Corp., Westborough, MA). First, we dispense a small amount of SU-8 3050 resist onto the silicon wafer. We spin coat at 2000 rpm to create a layer of about 65 μm in height. We then pre-bake the wafer for 20 minutes at 95 °C on a hot plate. We place the photomask (CAD/Art Services Inc., Bandon, OR) on the wafer photoresist and pattern with UV light on a contact mask aligner (ABM, Scotts Valley, CA). The wafer is post-exposure baked for 5 minutes at 95 °C and then developed by immersion in polyethylene glycol monomethyl ether acetate (484431, Sigma-Aldrich Co. LLC, St. Louis, MO) and mixing for 12 minutes using an orbital shaker (Roto Mix 8 × 8, Thermo Fisher, Waltham, MA). After developing, we rinse the wafer with isopropanol and blow dry it with compressed nitrogen. We create PDMS replicas by placing the wafer in a petri dish and covering with PDMS prepared by mixing Sylgard 184 base and cross-linker (Dow-Corning, Midland, MI) in a 10 : 1 weight ratio using a Thinky mixer (AR-100, Thinky Corp., Tokyo, Japan) and then degassed for 20 minutes. We cure the poured PDMS overnight in a 65 °C oven. We cut the PDMS replica into individual lysis devices prior to use. We make inlet and outlet holes using a 1.2 mm diameter biopsy punch (Uni-Core, GE Healthcare Life Sciences, Pittsburgh, PA). We bond each individual PDMS microchannel onto a square piece of lithium niobate substrate by treating both pieces for 10 s using an oxygen plasma stripper (PE-50, Plasma Etch, Carson City, NV) and then using a microscope for alignment.

Lysis apparatus

The assembled microchannel with the piezoelectric substrate is mounted onto a printed circuit board (PCB). The PCB is connected to the bus bars of the IDTs by two pogo pins and to an amplifier (LZY-22+, Mini-Circuits, Brooklyn, NY) by standard RF adaptors (SMA to MMCX male). The amplifier is driven by the signal from an RF waveform generator (SMB100A, Rhode & Schwarz, Munich, Germany) controlled by a computer. During the lysis experiments, we use the RF waveform generator in modulation mode to send pulsed, high-frequency signal to the IDT to generate pulsed traveling surface acoustic waves. We use two excitation frequencies, 13 MHz and 160 MHz. The pulsed signals have a frequency of 333 Hz and a duty cycle of 33%.

Sample preparation

We select *E. coli* BL21 (T7 Express Competent sold by New England Biolabs), Gram-negative bacteria, as a model in our study. These cells have an outer membrane, a cell wall, and a cytoplasmic membrane. We culture the bacteria in lysogeny broth (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) overnight at 37 °C in a shaking incubator. We measure the concentration of the bacteria using a NanoDrop spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific), adjust the concentration to 2×10^9 per milliliter and keep it constant during each experiment. To avoid LB medium interfering with assays, we wash the bacteria suspensions 3 times using PBS (centrifugation performed at 10 000 rcf for 2 minutes). To generate complete-lysis reference samples, we completely lyse cells using the Protein Extraction kit (Sigma-Aldrich, BugBuster). We generate DNA extraction reference samples using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Inc.).

Discussion and conclusion

We have demonstrated a microfluidic device for bacteria lysis that uses traveling surface acoustic waves and operates continuously in a flow-through manner. The goal of these experiments is to demonstrate the feasibility of this method for lysing bacteria in a generic, species-independent manner. We show that the application of high-energy ultrasound waves generated with a TSAW device are indeed capable of lysing bacteria. We test TSAWs at two frequencies: the lower frequency allows the application of higher power levels to the device before the substrate cracks. However, the ability to focus and manipulate the beam is correspondingly reduced given the larger wavelength. The higher frequency provides a more limited accessible power range before the substrate fails; however, the shorter wavelength offers greater potential control of the acoustic field.

For both frequencies, the TSAW is capable of killing the vast majority of the bacteria that pass through the sound wave; at the highest power levels, more than 90% of the bacteria are no longer viable. This is the first example of the use of TSAWs for efficient, rapid, lysis of bacteria flowing through a microfluidic device. While the properties of surface acoustic waves are understood,[33–37] the exact physical mechanism of the interaction that leads to the cell lysis is not clear. Although the use of acoustic energy to lyse mammalian cells, has been reported, mammalian cells are much easier to lyse than bacteria, which possess a thick, multi-layered cell wall. In addition, these methods all incorporated extra features to facilitate lysis. For example, devices were fabricated to include micro-pillars, or polystyrene beads were added to the cell sample to increase lysis through collisions.[21–24]

The viability of cells treated with SAW radiation varies with different conditions, such as the SAW frequency, the applied power, the exposure time, and the volume and density of the cell suspension,[38] and SAW devices have been used to sort individual living cells. For example, Franke *et al.* and Ma *et al.* both describe the use of narrow TSAW beams with a low power to sort living cells by acoustic streaming, enabling highly accurate sorting of individual cells with high viability.[39,40] Our chip is designed for cell lysis and is operated using different parameters. We tested several IDT patterns and locations within the chip to define a TSAW path on the piezoelectric substrate that is appropriate for cell lysis. We also used various excitation power and exposure times and found that pulsed TSAW excitation at 333 Hz with a duty cycle of 33% is effective for cell lysis while allowing for prolonged operation of the device.

It is possible that heating effects contribute to cell lysis in our devices.[41–44] The device exhibits some heating at the highest TSAW powers, becoming warm to the touch. However, this is not the case at lower powers, even when ~50% of the cells are killed. Because the residence time of the cells in the acoustic energy is well below 1 s, and because the fluid is constantly flowing, the degree of heating of the cells themselves is likely to be minimized. While the exact mechanism of the lysis remains to be established, it is controlled by the TSAW and is independent of cell type. Thus, this is highly useful in any study of populations of bacteria, where a wide variety of species, with different characteristics, are present, and where a generic form of lysis is essential.

These experiments provide the important proof of concept that TSAW can lyse bacteria flowing through a microfluidic device. In the present study, we were able to recover ~20% of total cell content after TSAW cell lysis, and we believe that our planned work to systematically optimize device design and operation will greatly improve this rate. In addition, one of our ultimate goals with this work is to develop methods to lyse individual bacteria and collect the full contents of each cell for further analysis. For example, if the RNA and DNA of individual bacteria can be collected into single droplets, bead-based barcoding methods and next generation sequencing will enable highly parallelized analysis of the transcripts and/or the genomic DNA of individual cells.

Thus, future work will encompass new microfluidic chip designs that will enable the bacteria to be lysed individually, and the contents of each cell to be collected separately for further analysis on a single-cell basis. For example, TSAW excitation could be triggered when individual bacterial cells are detected in the device channel. Upon TSAW lysis, the intracellular contents of that cell could be encapsulated into a droplet. This method, with further improvements, will greatly enable single cell analysis of populations of bacteria.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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