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Lysing Bacterial Spores by Sonication through a Flexible Interface in a Microfluidic System

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Cell disruptions using ultrasonic energy transmitted through a flexible interface into a liquid region has limitations because the motion of the vibrating tip is not completely transferred into the liquid. To ensure that some degree of contact will be maintained between the ultrasonic horn tip and the flexible interface, the liquid must be pressurized. The pressure conditions that yield consistent coupling between the ultrasonic horn tip and the liquid region were explored in this study by using an analytical model of the system and test fixture experiments. The nature of the interaction between the horn tip and the flexible interface creates pulses of positive pressure rises, increase in temperature, streaming flow, and almost no cavitation in the liquid. There was sufficient energy to create a cloud of microspheres, or beads, that maintain a consistent pattern of ballistic motion in the liquid. The sonication was found to be repeatable by studying video recordings of bead motion and was shown to be statistically consistent using measurements of temperature rise. Sonication of bacterial spores to obtain measurements of released nucleic acid and SEM images of damaged spores were used to verify the effects of liquid pressure on the horn−interface−liquid coupling.

Rapid, automated systems based on the polymerase chain reaction (PCR) for detecting and identifying small quantities of microbes in real-world samples requires the integration of one or more sample processing steps. We have been working toward combining in a cartridge format[1] steps that would reduce the complexity of the sample, concentrate the microbial targets, and disrupt the cells or spores to liberate the nucleic acids for subsequent PCR analysis without any user involvement once the process is initiated. Spores present the most challenging target to prepare due to a protective cortex that is highly resistant to harsh physical and chemical treatments. Therefore, a desirable lysis method would destroy the integrity of the spore cortex, be amenable to the cartridge format, and provide an extract that does not inhibit PCR.

Conventional sonication devices[2−6] typically require the manual placement of an ultrasonic probe directly into the liquid, but cross-contamination, cavitation-induced foaming, and potential aerosolization present serious complications. This report describes a fluidic module that rapidly captures bacterial spores on a filter, washes the spores with water, and disrupts the spores in the presence of glass beads by applying ultrasonic energy through a thin-film flexible interface. The feasibility of rapidly disrupting spores by sonicating through a flexible interface has been previously demonstrated,[7] but there was still a need to add a target-capturing filter to the module and to determine the conditions that yield consistent results. The efficiency of spore lysis using this approach is dependent on physical parameters that affect the transfer of ultrasonic vibration through the interface. Data are presented that correlate these parameters with an analytical model to elucidate the principles in the horn−interface−liquid coupling mechanism and to establish criteria to rapidly lyse spores reproducibly in a microfluidic system.

EXPERIMENTAL SECTION

A sonication test stand (Figure 1A) was designed to hold a 40 kHz ultrasonic horn (Sonics & Materials, Inc., Newtown, CT) against a flow-through cartridge. The cartridge was designed to hold a filter to capture the target material for sonication and PCR analysis. Alternately, the sonication process was studied by capturing visual images of bead motion and measurements of temperature rise. In this way, the mechanical nature of the sonication event was studied separately from the chemical analysis of cell disruptions.

Each cartridge was assembled with a fresh piece of thin-film interface material (Flexicon) and mounted into the nest in the sonication tester so that the interface was supported by the horn. For visual study, the cartridge was then primed with a solution containing approximately 10 000 of 106-μm blue polystyrene beads (IMT).

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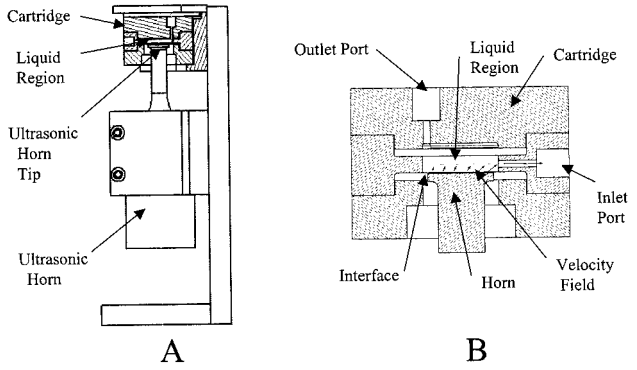

Figure 1. (A) The cartridge is shown mounted in the sonication test stand with the horn tip below the flexible interface and the liquid region to be sonicated, above. (B) Cut-away view showing the velocity field of liquid motion, induced by horn motion, toward the inlet port. The mass of liquid induced into motion can be approximated using one-half of the volume of the entire liquid region.

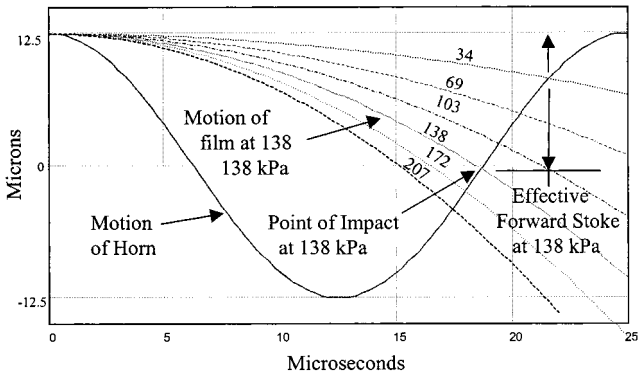

Figure 2. Horn displacement (25 μm P−P) and film displacement (at pressures of 34, 69, 103, 138, 172, and 207 kPa) against time in microseconds. The film under any of the pressures shown cannot keep up with the retreat of the horn. The point of impact between the horn and interface and the effective forward stroke of the interface at 138 kPa is shown.

Table 1. [a]

appl. static press. (kPa)	est. forward stroke (μm)	est. dyn. press. (kPa)	meas. temp rise (°C)	std. dev. (°C)	no. of pts.	CV (%)
25-μm horn amplitude						
34	5	4.1	3.8	1.0	13	26.6
69	8	7.6	13.4	1.1	14	8.4
103	11	9.6	15.4	1.2	13	7.7
138	14	11.0	26.5	3.0	19	11.5
38-μm horn amplitude						
app. static press. (kPa)	est. forward stroke (μm)	est. dyn. press. (kPa)	meas. temp rise (°C)	std. dev. (°C)	no. of pts.	CV (%)
34	6	7.6	20.7	4.0	12	19.6
69	10	13.1	32.3	4.0	12	12.3
103	13	17.2	43.1	3.3	12	7.7
138	16	20.7	53.9	6.1	18	11.3

[a] The estimated dynamic pressure, effective forward stroke, and measurements of temperature rise increase as the static pressure is increased for the same horn amplitude. Measurements of temperature rise were taken after 10 s of sonication; the results indicate reasonable repeatability at applied pressures from 69 to 138 kPa.

The amplitude of the horn was adjusted to either 25 or 38 μm peak-to-peak (using a microscope), the regulator controlling the air pressure that pressurizes the liquid in the cartridge was adjusted to 34, 69, 103, or 138 kPa, and the ultrasonic horn was energized for 10 or 20 s. A video camera was used to capture still images or moving videos of beads in motion. Measurements of temperature rise were taken (without beads) using a thermocouple that was inserted into the liquid region through the outlet port of the cartridge.

The sonication and disruption of *Bacillus subtilis* spores was performed using cartridges assembled with 0.22-μm filters (Millipore) and approximately 60 mg of 106-μm glass beads (Sigma). Samples of purified 10^5 spores/mL were passed through the liquid fixture, capturing the spores on the filter, and followed by a 3-mL wash solution of DI water. The spores were sonicated for 20 s using a horn amplitude of 25 μm and pressures of 34, 69, 103, and 138 kPa. PCR analysis of resulting DNA was obtained using the Smart Cycler (Cepheid). For SEM/EDX analysis, the filters were dried to capture spore debris on the surface and sputter-coated with a thin layer of gold and palladium. The filters were observed using a JEOL 6400F microscope with 10 keV of beam energy (Surface Science Laboratories, Mountain View, CA).

RESULTS AND DISCUSSION

The hypothesis was that a constant applied static pressure in the liquid would result in a stable interaction between the horn tip and the flexible interface−liquid region, leading to consistent sonication of the target spores. The pressure was applied to maintain a consistent degree of coupling to the horn tip.

Model of Horn-Interface-Liquid Interaction. A static pressure is applied at the inlet port of the cartridge (Figure 1b), located at the midpoint of the liquid volume; therefore, approximately one-half of the liquid mass must be induced into motion along with the interface. Treating the liquid as incompressible for the purpose of this motion analysis is a reasonable approximation, because the pressures involved are relatively low.[8] The pressure in the liquid creates a force on the surface area of the interface that acts on this liquid mass (M = 0.13 g). The motion of the interface under pressure is determined using Newtonian principles.[9] The sinusoidal motion of the horn tip at 25 μm peak-to-peak amplitude and frequency of 40 kHz is graphed (Figure 2), along with the motion of the film interface at applied static pressures from 34 to 207 kPa.

The figure illustrates that the horn loses contact with the film as it retreats, but again makes contact in a head-on collision as the two speed toward each other. The resulting effective forward stroke of the film at 138 kPa is illustrated in Figure 2, for which the calculated relative velocity between the horn and interface at the time of impact is 4.59 m/s. The periodic effective forward stroke, which is always less than the peak-to-peak amplitude of the horn, and the relative velocity at impact are the parameters that determine the level of sonication that is imparted to the liquid.

The impact of the horn creates a disturbance in the liquid, which results in a dynamic pressure pulse. Using theory for bulk and waterhammer flow,[8] the pressure disturbance can be estimated from the Bernoulli Principle (11 kPa at 138 kPa applied pressure and 25-μm horn displacement). The mathematical model

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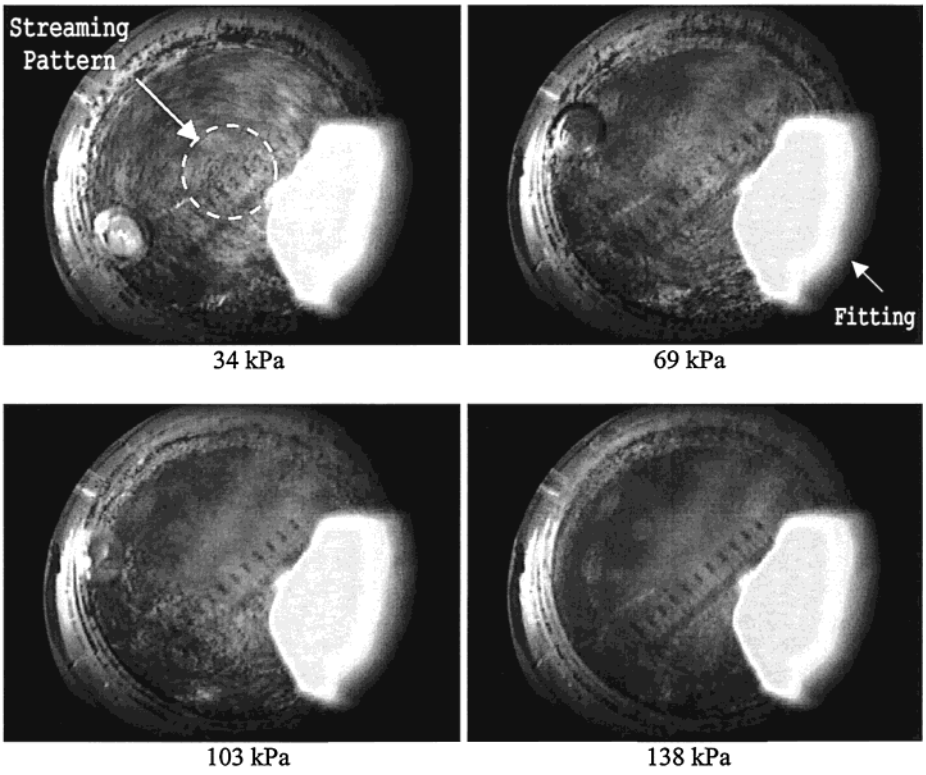

Figure 3. Bead motion under sonication at applied static pressures of 34, 69, 103, and 138 kPa (horn displacement fixed at 25 μm P−P). The bead motion becomes more vigorous as the pressure is increased, moving more rapidly and becoming more blurred in the still frame. The presence of an occasional air bubble did not affect the results.

indicates that the dynamic pressure and effective forward stroke increase as the static pressure is increased (Table 1) for the same horn amplitude, which suggests that the intensity of sonication depends on the applied static pressure.

Comments on Cavitation. The nature of the horn−flexible interface−liquid interaction is such that large pressure drops are not produced, because the film separates from the retreating horn; hence, cavitation bubbles do not form. In contrast, when an ultrasonic horn is placed directly into a liquid at atmospheric pressure, cavitation bubbles formed due to pressure drops near the radiating surface are carried away in streaming flows.

To confirm this, a microscope was used during sonication. Low levels of fine cavitation bubbles would sometimes form at very low static pressures, well below 5 psi, depending on the way the interface settled against the horn tip. If the static pressure was increased during observation, then the formation of bubbles would cease. The lack of cavitation bubbles supports the expectation that large pressure drops would not occur, because the film interface cannot keep up with the retreating horn.

Bead Motion, Streaming Flow, and Temperature Rise Study. Images of bead motion in Figure 3 (captured using a video camera coupled to a microscope) indicated that at the lower static pressures the bead motion is not as active as at the higher pressures (at constant horn amplitude). These observations were in agreement with the expectation that the sonication of the liquid would be a function of applied static pressure for a given amplitude of the horn.

The absorption of the pressure pulse due to the viscosity of the liquid creates energy gradients, which yield the conditions for creation of stable streaming flows.[10,11] Moving images of streaming flow and particular flow patterns that remained stable were observed. It was also noted that the streaming flow increased as the applied pressure was increased for constant horn amplitude.

The consistency of sonication can be accessed using measurements of temperature rise caused by the energy absorbed.[10−12] Measurements of temperature rise indicated a repeatable temperature rise at the same pressure and horn amplitude (Table 1), which suggests that sonication is consistent.

Sonication of Spores for PCR Analysis and SEM Images. Quantitative, real-time PCR has been demonstrated to be an effective method for monitoring spore lysis by measuring the amount of free spore DNA in the liquid. As the number of lysed bacterial spores in the reaction increases, the threshold cycle (C_T, the cycle in which a significant positive signal is first detected) obtained from the real-time PCR detection curve decreases.[7] The real-time PCR results in Figure 4 demonstrate an inverse correlation of C_T and the applied pressure during sonication, with the lowest C_T obtained at the highest test pressure of 138 kPa. This indicates that as the pressure was increased, the level of sonication apparently was increased, because of the increasing degree of spore lysis (with horn amplitude constant at 25 μm).

SEM micrographs (Figure 5) of the filters depicted no damage to unsonicated *B. subtilis* spores, light damage to spores at the

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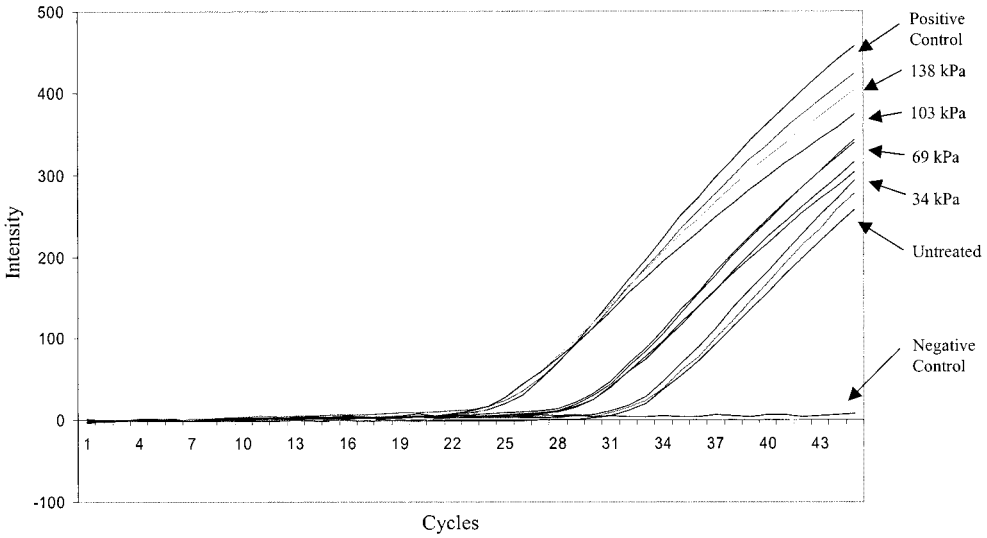

Figure 4. Real-time PCR analysis of sonicated spores. As the pressure was increased, lower C_T values and higher signal amplitudes were observed, which indicated that the level of sonication was increasing. The highest test pressure, 138 kPa, is near the positive control, and the lowest test pressure, 34 kPa, is near the untreated (unsonicated) control.

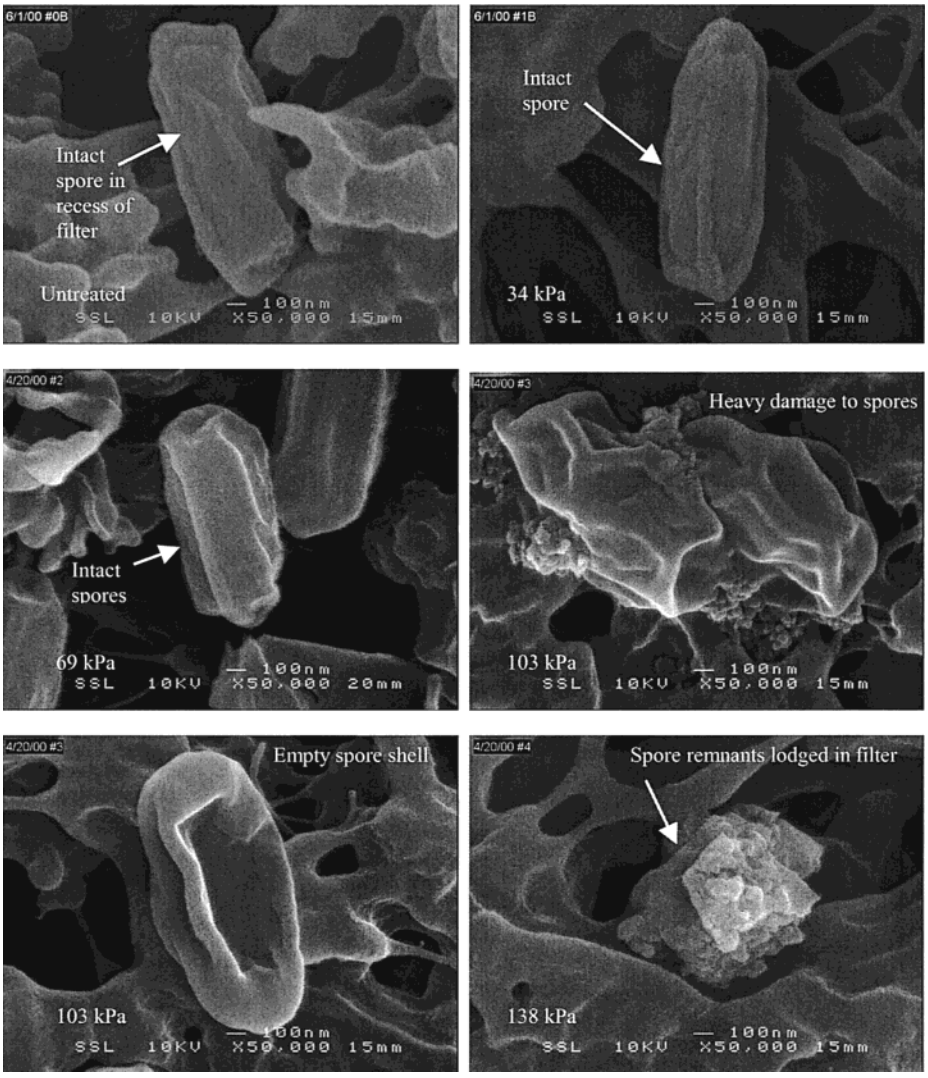

Figure 5. SEM images of sonicated spores. Images shown are for 34, 69, 103, and 138 kPa pressures and a horn amplitude of 25 μm (untreated control also shown). Damage to spores was observed to be more extensive as the pressure was increased, indicating the increased intensity of sonication.

lower pressures, intermediate damage at 103 kPa, and extensive damage at 138 kPa (all tests done using 25 μm horn amplitude). Debris seen near partially damaged spores, particularly at 103 and 138 kPa pressures, appeared to be derived from disrupted spores and was similar to globular material seen in other studies.[13] Spore damage at 138 kPa was so extensive that detecting remnants of material required extensive searching of the filter surface.

Parameters for Consistent Sonication. The study shows there are three parameters that determine the consistency of sonication: (1) static pressure applied to the liquid region, (2) the amplitude of the horn tip, and (3) the mass load (liquid, beads, and interface). Consistent sonication is achieved when these parameters are maintained at defined values.

It is important to note that it is the horn amplitude, not the power, which is held constant, because sonication is an amplitude-driven event.[14] The power drawn fluctuates as the load varies, differs if air bubbles form in the liquid region, drifts as the ultrasonic transducer heats, depends on the design of the particular transducer being used, and is affected by the efficiency of the ultrasonic power supply circuitry.

CONCLUSIONS

The study shows that maintaining a regulated static pressure will yield a consistent, effective forward stroke of the interface and leads to streaming flows and dynamic pressure pulses that act together to get the beads moving in violent motion, producing sufficient sonication to achieve cell disruptions. The results indicate that through an understanding of the physics of ultrasonic wave generation, one can augment sample processing, such as for spore and/or cell disruption for biological analysis. We are continuing the development of this technology, which will lead to a fully portable system for automated lysis and analysis for laboratory and field applications.

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