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Abstract
Medical implants are prone to colonization by bacterial biofilms, which are highly resistant to antibiotics. Normally, surgery is required to replace the infected implant. One promising non-invasive treatment option is to destroy the biofilm with high-intensity focused ultrasound (HIFU) exposure. In our study, *Pseudomonas aeruginosa* bacterial biofilms were grown on graphite disks in a flow chamber for three days prior to exposing them to ultrasound pulses of varying duration or burst period. The pulses were 20 cycles in duration at a frequency of 1.1 MHz from a spherically focused transducer (f/1, 63 mm focal length), creating peak compressional and rarefactional pressures at the disk surface of 30 and 13 MPa, respectively. *P. aeruginosa* were tagged with GFP and cells killed by HIFU were visualized using propidium iodide, which permeates membranes of dead cells, to aid determining the extent of biofilm destruction and whether cells are alive or dead. Our results indicate that a 30-s exposure and 6-ms pulse period or those combinations with the same number of pulses, were sufficient to destroy the biofilm and to kill the remaining cells. Reducing the number of pulses decreased biofilm destruction, leaving more dead and live bacteria on the surface.

Keywords
Biofilms, cell membranes, ultrasonography, bacteria, cell membrane transport

Comments
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Mechanical Destruction of *Pseudomonas aeruginosa* Biofilms by Ultrasound Exposure

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Abstract. Medical implants are prone to colonization by bacterial biofilms, which are highly resistant to antibiotics. Normally, surgery is required to replace the infected implant. One promising non-invasive treatment option is to destroy the biofilm with high-intensity focused ultrasound (HIFU) exposure. In our study, *Pseudomonas aeruginosa* bacterial biofilms were grown on graphite disks in a flow chamber for three days prior to exposing them to ultrasound pulses of varying duration or burst period. The pulses were 20 cycles in duration at a frequency of 1.1 MHz from a spherically focused transducer (f/1, 63 mm focal length), creating peak compressional and rarefactional pressures at the disk surface of 30 and 13 MPa, respectively. *P. aeruginosa* were tagged with GFP and cells killed by HIFU were visualized using propidium iodide, which permeates membranes of dead cells, to aid determining the extent of biofilm destruction and whether cells are alive or dead. Our results indicate that a 30-s exposure and 6-ms pulse period or those combinations with the same number of pulses, were sufficient to destroy the biofilm and to kill the remaining cells. Reducing the number of pulses decreased biofilm destruction, leaving more dead and live bacteria on the surface.

Keywords: Biofilm; destruction; HIFU; medical implant
PACS: 87.18.Fx, *43.35.Wa, *43.80.Qf, 87.50.yt, 87.63.D-

INTRODUCTION

Nowadays, prostheses are widely used in health care, but one major concern with implantation is that even under the most sterile conditions in hospitals, bacterial infections of medical implants may happen. These bacteria, if not planktonic, tend to be sequestered in a layer of exopolymers, and the colonized biofilms formed around implants usually carry along severe resistance to antibiotics. Normally, surgery is required to replace the infected implant before antibiotics is administered. This poses a serious expenditure and discomfort to patients who often are still in the recovery period. Therefore, minimally-invasive therapeutic alternatives to the traditional procedure are desired. Researchers have utilized low-frequency ultrasound (~ 10 kHz) to enhance antibiotics efficacy of treating biofilm infections [1-3]. However, one major disadvantage with this drug delivery approach is the long insonation time, not to mention merely modest success with the treatment of *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilms that have high tolerance to antibiotic dosage [1, 2].

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However, there appears to be promise in using high intensity ultrasound to treat the biofilms non-invasively. Bigelow et al. [4] verified the ability of high intensity focused ultrasound (HIFU) to mechanically destroy *Escherichia coli* (*E. coli*) biofilms. In their study, peak rarefactional pressure was varied to test the effect of power level on bacteria viability. This technique, termed histotripsy, excites HIFU pulses at a low duty cycle to create microbubbles undergoing cavitation that can remove tissue. Histotripsy was originally developed to treat hypoplastic left heart syndrome in neonates through mechanical destruction of bubble activity [5]. Due to the advantages of non-invasive nature, precision and sharp border, histotripsy was also extensively studied to produce lesions *ex vivo* within tissues such as livers, kidneys, prostates, etc. [6].

In an effort to seek optimal exposure conditions for bacteria killing, our histotripsy work varied exposure time and burst period of ultrasound pulses during the treatment of *P. aeruginosa* biofilms in view of weaker resistance of *E. coli* to antibiotics. This work is motivated by the prospect of shortening therapy time with this modality. In our study, biofilms were grown on graphite disks in a fluid flow environment, and the effectiveness of ultrasound exposure was assessed with the indicator of colonization percentage of killed and live cells remaining on the disks. This article introduces biofilm preparation and ultrasound exposure, and provides preliminary results.

METHODS

Preparation of *P. Aeruginosa* Biofilms

Preliminary studies were conducted to check the influence of agitation on biofilm growth. Sterilized graphite disks (12.7 mm × 6.35 mm × 3.175 mm) were placed into test tubes filled with medium containing *P. aeruginosa* strain culture, tryptone yeast extract and trace elements. Graphite was chosen because of its common use in medical implants such as heart valves. Then biofilms were incubated with and without a shaker. Microscopic inspection showed that biofilms were grown denser on the stationary disks. Additionally, even though the preliminary studies also showed that 48 h was sufficient to develop robust *P. aeruginosa* biofilms, 72 h duration was always strictly implemented throughout the study.

The *P. aeruginosa* strain PAO1 (pP*nptII-gfp*Gm) was taken from -70 ^{o}C ultra-low freezer room, and the bacteria contained green fluorescent protein (GFP) from jelly fish for easy identification of cells. Then the culture was streaked onto tryptic soy agar (TSA) petri dishes prior to overnight incubation at 37 ^{o}C. A flow system as in Fig. 1 was used to grow biofilms on the top surface of graphite disks. The basic components include a 6-L flask as medium reservoir, a pump (77120-70, Masterflex, Vernon Hills, Illinois), a bubble trap, a growth chamber and a waster container. The customized bubble trap [7], mainly consisting of a vertical syringe and a polycarbonate base with a circular channel drilled through, was made to prevent the air bubble from passing to the downstream segment. The growth chamber held 3 graphite disks into the 3 wells located in its bottom. Based on flow simulation, a flow rate at ~ 1.2 mL/min corresponding to an interior size of 61.0 mm × 30.5 mm × 7.6 mm was adequate to ensure a laminar and uniform flow in the vicinity of biofilms. This low rate is fairly

attractive given the 72 h biofilm growth time, corresponding to a total of 5.2 L of $1/10^{th}$ strength tryptic soy broth (TSB) medium for each batch of biofilm preparation. Besides a growth chamber, another chamber was created to hold only one graphite disk at a time during ultrasound exposure. This chamber was so designed to ensure no blockage of ultrasound beam. An interior size of 90.2 mm × 67.3 mm × 6.4 mm was finalized after simulation indicated a similar flow environment to that in the growth chamber.

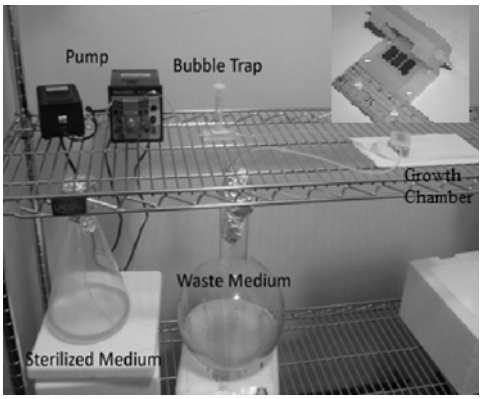

FIGURE 1. Set-up of the flow system for biofilm growth

Prior to biofilm growth initiation, autoclaved 1% bleach and sterile nano-purified water was run to sterilize the flow system and to dilute the bleach, respectively. Then the graphite disks in the wells of growth chamber were inoculated with 5 mL of autoclaved TSB medium with an optical density of .05. Once the growth was finished, the biofilms attached on 3 graphite disks were taken microscopic pictures of in order to ensure the occurrence of healthy biofilms before subjecting them to ultrasound exposure.

Ultrasound Exposure

The graphite disk with biofilm was embedded into the single well of the exposure chamber. Subsequently, the chamber was filled with sterile water, sealed up, flipped upside down and submerged in a bath of degassed water at room temperature (20^{o} C). Then a pulser/receiver (Panametrics 5900 PR, Waltham, MA) was excited to locate the graphite disk with a 3-axis computer-controlled positioning system (BiSlide Assemblies, VELMEX Inc., Bloomfield, NY). After the alignment process, the transducer was driven by a high-power amplifier (1140LA-CI, Electronics & Innovation Ltd., Rochester, NY) to begin HIFU exposure. During exposures, a rectangular region of 12.75 mm by 6.75 mm was scanned across the entire surface of biofilm in a raster pattern with a step size of 0.75 mm. Meanwhile, a pump was run at its minimum rate so that the resultant flow inside chamber would make the possibly dislodged bacteria drift away and flow downstream directly to the degassed water bath. A piece of saran wrap was used to separate the graphite disk in the exposure chamber from the rest of the water bath.

The ultrasound exposure consisted of 20-cycle *sine* wave bursts. The source was a 1.1MHz single-element spherically focused transducer (H-101, Sonic Concepts, Inc., Bothell, MA) with a focal length of 63.4 mm and an active diameter of 63.6 mm. The

acoustic field at 127.2 V peak-to-peak excitation corresponding to our exposures has a peak compressional pressure of 17.8 MPa and a peak rarefactional pressure of 7.86 MPa at focus, which yielded a peak rarefactional pressure of ~ 13 MPa at the disk surface due to the reflection of graphite disk. The coefficient of reflection for graphite was measured to be .65 in our preliminary study. The pressure of 13 MPa guarantees the ability of histotripsy to kill bacteria as indicated by Bigelow et al [4].

There were 8 exposure conditions plus a sham exposure and an autoclaving case scenario. The pause between scan steps, namely exposure time for each treatment location, and burst period, were varied. For the sham exposures, the associated disks stayed in the exposure chamber with purified water running through for 90 minutes. 6 repetitions were performed for each condition bringing the total number of biofilms used in this study to 60. And the sequence of implementing exposure conditions was randomized to remove bias throughout the procedure.

Determination of Cell Colonization Percentage

After each ultrasound exposure, the disk was gently dipped into sterile water 3 times to get rid of the non-firmly-attached bacteria debris. Then the disk was stained with a live/dead stain containing propidium iodide that can penetrate through the membrane of only damaged or dead cells. Consequently, fluorescent microscopy could visualize both killed and live cells. This concerned the use of two types of illumination filters that can bring out the GFP in green and broken cell membranes in red, respectively. 3 sub-domains were used for each disk to cover one entire surface in microscopy. Each sub-domain has exactly the same field of view when depicting the extent of biofilm destruction by assessing colonization percentages. Hence, for each experimental condition, there were 18 samples for both live and dead cells. The colonization percentages were evaluated based on the filtration of the intensity of picture pixel, using MetaVue software. Lastly, one-tailed Student's *t* test was performed for statistical analysis in all combined experimental groups.

RESULTS

Shown in Fig. 2 are the colonization percentages of bacteria cells both live and dead, attached on disks after each ultrasound exposure. The y-coordinate is in log10 scale, and the standard deviation is also included above each bar for each experimental condition. The results for autoclaved (sterile) biofilms are used as a reference for comparison. Therefore, the 10 experimental groups along the x-coordinate can be regarded as gradually increasing exposure level while maintaining acoustic pressures constant. More specifically, the exposure times per treatment location are 5 s, 5 s, 5 s, 30 s, 15 s, 5 s, 15 s and 30 s for the middle 8 exposure conditions, respectively, yielding the total exposure time as 15 min, 15 min, 15 min, 90 min, 45 min, 15 min, 45 min and 90 min in turn. Additionally, the burst periods are 12 ms, 6 ms, 3 ms, 6 ms, 3 ms, 1 ms, 1 ms and 1 ms, leaving the duty cycle as 0.15%, 0.3%, 0.61%, 0.3%, 0.61%, 1.82%, 1.82% and 1.82% in turn. Also, the corresponding numbers of pulses at each treatment location are 417, 833, 1667, 5000, 5000, 5000, 15000 and 30000, respectively.

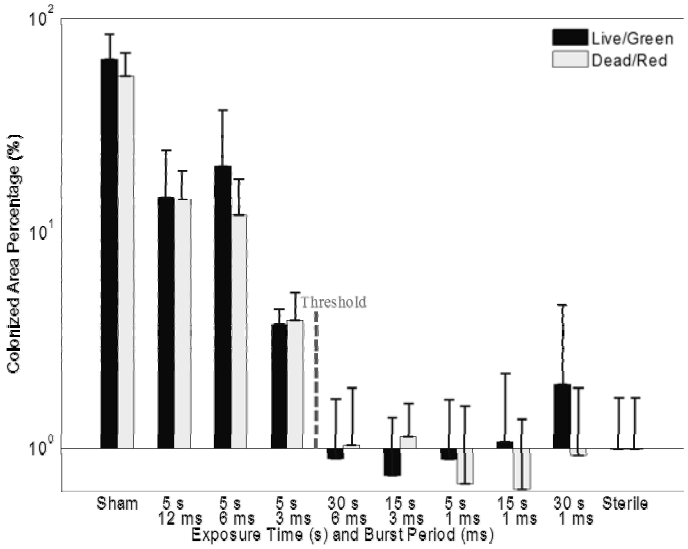

FIGURE 2. Colonization percentage of both live and dead bacteria

The results for the sham exposures suggested that *P. aeruginosa* bacteria in sterile water might die from the limitation of nutrients' supply with time. However, the decrease (by 36%) in the area percentage of live cells for shame exposures is not as dramatic as for those exposure conditions sitting right to the threshold mark in Fig. 2. Those exposures had led to nearly 99% decrease in the live cell percentage. Moreover, there is a difference of 2 orders of magnitude in the colonization percentages of both killed and live bacteria among groups. Also, the untreated group (sham) was statistically significantly different from all the others ($p < .00001$; $n = 18$). All this testified to a pronounced success in killing bacteria with histotripsy. Also, all of the five exposure groups on the right to the threshold are not statistically significantly different from the sterile ($p > .13$; $n = 18$).

DISCUSSION AND CONCLUSIONS

In our study, consistent *P. aeruginosa* biofilms were grown on graphite disks in a fluid flow environment, and then those biofilms were exposed to HIFU under 8 different exposure conditions plus a sham exposure and a sterile condition. The goal of this work is to study the effect of exposure conditions on biofilm destruction. Our experiments confirmed the capability of this HIFU modality for treating biofilms. The results also indicated that more bacteria either live or dead, remained on disks after the ultrasound exposure of fewer pulses. For those exposures with more pulses, the dead cells "disappeared" or only few damaged cells remained on disks, perhaps because stronger exposures tended to dislodge those dead cells and scatter them away with water flow during ultrasound exposure or the dipping process before staining. Additionally, one plausible explanation for the non-unity-sum phenomenon with the two percentages in each group is that ultrasound beam distorted and moved the biofilm bacteria all over before truly killing bacteria so that the bacteria distribution was not bearing a life-or-death situation.

Beginning from the 30s-6ms group through the 30s-1ms one, all of the five groups are not statistically significantly different from the sterile ($p > .13$; $n = 18$). This observation justifies the existence of a threshold (marked as a vertical dashed line in Fig. 2) for exposure condition in the sense of destroying *P. aeruginosa* biofilm. Stronger exposures than this threshold would reach equivalent efficacy to the sterilization in the sense of destroying *P. aeruginosa* biofilms.

This preliminary research demonstrates that exposure time at each treatment location and burst period can be optimized to shorten the biofilm treatment time. However, other parameters such as the step size in the raster scan can be used as a study variable in future experiments. Consequently, the therapy time can be shortened so that HIFU can be used to treat biofilm infection on artificial implants in an efficient way.

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