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Research Article

Sound Waves Effectively Assist Tobramycin in Elimination of *Pseudomonas aeruginosa* Biofilms *In vitro*

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Abstract. Microbial biofilms are highly refractory to antimicrobials. The aim of this study was to investigate the use of low-frequency vibration therapy (20–20 kHz) on antibiotic-mediated *Pseudomonas aeruginosa* biofilm eradication. In screening studies, low-frequency vibrations were applied on model biofilm compositions to identify conditions in which surface standing waves were observed. Alginate surface tension and viscosity were also measured. The effect of vibration on *P. aeruginosa* biofilms was studied using a standard biofilm assay. Subminimal inhibitory concentrations (sub-MIC) of tobramycin (5 μg/ml) were added to biofilms 3 h prior, during, and immediately after vibration and quantitatively assessed by (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay (XTT) and, qualitatively, by confocal laser scanning microscopy (CLSM). The standing waves occurred at frequencies <1,000 Hz. Biofilms vibrated without sub-MIC tobramycin showed a significantly reduced metabolism compared to untreated controls ($p<0.05$). Biofilms treated with tobramycin and vibrated simultaneously (450, 530, 610, and 650 Hz), or vibrated (450 and 650 Hz) then treated with tobramycin subsequently, or vibrated (610Hz, 650Hz) after 3 h of tobramycin treatment showed significantly lower metabolism compared to *P. aeruginosa* biofilm treated with tobramycin alone ($p<0.05$). CLSM imaging further confirmed these findings. Low frequency vibrations assisted tobramycin in killing *P. aeruginosa* biofilms at sub-MIC. Thus, sound waves together with antibiotics are a promising approach in eliminating pathogenic biofilms.

KEY WORDS: alginate; biofilm; *Pseudomonas*; tobramycin; vibration.

INTRODUCTION

Formation of biofilms by *Pseudomonas aeruginosa* is well-known to cause serious and clinically significant sequelae in a number of diseases such as wound infections, cystic fibrosis (CF), and implant-related infections, among others (1). Biofilms are complex, spatially oriented, functional communities of one or more species of microorganisms that are encased in an extracellular matrix (EPS) and are attached to a surface and/or to each other (2). These hierarchical and three dimensionally organized microbial communities are well acclimatized to the diverse and dynamic environment of the host (3). The microorganisms in biofilms exhibit phenotypic alterations based on their growth rate and gene expression (4). These characteristic phenotypic variations may result in differential expression of virulence factors, surface molecules, deviations in nutrient utilization, and antimicrobial resistance (5–7).

The slow development and persistent nature of biofilms is rarely resolved by the host immune response (8,9). Persistence of chronicity of wounds mainly associated with the influx of polymorphonuclear leukocytes (PMNLs), imbalance of several inflammatory cytokines, and elevated matrix metalloproteinases (MMPs) (10). Growth of *P. aeruginosa* biofilms in chronic wounds intensifies the inflammation by producing rhamnolipids (11–14). The latter assists the pathogen to escape from phagocytosis. Thus, *P. aeruginosa* elicits further damage to the wound by causing continuous infiltration of PMNLs and triggering the production of MMPs (15).

Thus, eradication of *P. aeruginosa* biofilm infections in chronic wounds and CF patients is considered nearly impossible due to high antibiotic resistance (15,16). Alarmingly, management of chronic wound infections in USA drains on the resources of the health service, costing over $25 billion USD per annum (17–19). Once matured (i.e., approximately 48 h), *P. aeruginosa* biofilms become increasingly resistant to antibiotics (20). Thus, combined therapies of physical debridement of both the biofilm and devitalized tissues of the wound, systemic, and topical antibiotic therapy, antiseptics are often employed in the management of chronic wound infections (21). Though several treatment options have been investigated and tested, antibiotics remain as the first choice for biofilm infections. In CF, for example, aggressive antibiotic therapy is targeted at acute exacerbations of infections and chronic status is treated with long-term antibiotics (22–24).

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As a result of refractory nature of the biofilms, various novel approaches have been sought in eliminating biofilms. Due to promising results obtained in water and food disinfection, ultrasound therapy has been investigated over recent years to eliminate biofilms from the sites of infections while maintaining the biological health of human cells (25–29). However, several undesirable effects such as tissue cavitation, heat generation, and formation of stronger biofilms make the therapeutic usage and clinical translation of ultrasound therapy unclear (4,26,30,31).

Our focus has been to investigate lower frequency vibrations for potential eradication of *P. aeruginosa* biofilms *in vitro*. Lower frequency vibrations less than 20 kHz have yet to be reported in the literature as a possible adjunct treatment in biofilm infections despite their potential to avoid tissue cavitation and heat generation that limits higher frequency treatments. Low frequency vibrations are hypothesized to induce structural and physical changes in biofilms as a result of rheological alterations of the extracellular matrix. These physical changes may influence antimicrobial therapy and/or the phenotype of the bacteria encased within the extracellular matrix. Hence, the aim of this study was to investigate the use of vibration therapy in the frequency range of sound waves (20 Hz–20 kHz) as an innovative approach to control *in vitro P. aeruginosa* biofilms and to assess the effects of vibrations on antibiotic mediated *P. aeruginosa* biofilm eradication.

MATERIALS AND METHOD

Screening Studies: Effect of Vibration on Alginate Gels

Alginate, the major component of *P. aeruginosa* biofilm matrix, was used in screening studies as a model biofilm matrix to simulate biofilms in a mass scale. Alginate (alginic acid sodium salt, from brown algae, viscosity of 2% solution at 250C~250cps, Sigma Aldrich, USA) powder was dissolved in sterile distilled water and shaken 150 rpm at 37°C 24 h to prepare 4% (*w*/*v*) stock suspension. Working suspensions of 1.5, 1, and 0.5% alginate (*w*/*v*) was freshly prepared by dispensing in distilled water (32).

Acoustic Vibration of Alginate Gels

The front of an electroacoustic transducer (i.e., a loudspeaker cone, 14 cm diameter, X3057, Coral, Japan) was covered with a polyethylene film and carefully retained around the circumference of the cone in order to achieve a stretched and wrinkle-free surface. The speaker cone was connected to an amplified signal generator (Model 142 HF VCG generator, Wavetek, CA, USA). A culture dish (tissue culture dish, 60 x 15mm, Sarstedt Inc, NC, USA) was firmly attached to the center of the polyethylene film using double-sided tape (3 M, St. Paul, MN, USA).

Ten milliliters of alginate solutions were added to the culture dish and exposed to acoustic vibrations across the range of 20 Hz–20 kHz. The response of the alginate solutions to the acoustic vibrations was observed by tracking the surface oscillations in the liquid. These surface movements were visually observed, and specific events corresponding to sound frequencies were recorded. During vertical oscillation of the solution by the electroacoustic transducer, the solution can form standing waves (Faraday instabilities) on the surface (33,34). The frequencies at which the solutions developed these surface patterns were recorded. Specifically, the frequency of onset (frequency of onset), the frequency associated with the greatest amplitude of the standing wave (frequency of max), and the frequency at which the standing wave disappeared (frequency of end).

To simulate the mechanical stability of the biofilm matrix provided by cross-linked multivalent cations (35) and the calcium ion content in found in the sputum of CF patients, predetermined concentrations of $CaCl_2$ were added to the preparations of alginate (estimated concentration of Ca^{2+} in the CF sputum range from 0.5 to 1.8 mM) (36,37). Specifically, 0.5, 1.2, and 1.8 mM of $CaCl_2$ were added to 1.5% alginate solution, and 0.5 mM $CaCl_2$ was added to 1.5, 1, and 0.5% alginate solutions. The acoustic vibration of these alginate-calcium mixtures was then performed as described above. Each experiment was performed at room temperature, three different times by two independent researchers due to the visual determination of the onset, greatest amplitude, and the disappearance of standing waves during vibration.

Measurement of Viscosity of Alginate

The viscosity of alginate under three different vibration conditions was measured using the Cannon-Fenske Routine Viscometer (size 300) at room temperature (25°C). First, 10 ml of 1.5% alginate solution with 0.5 mM $CaCl_2$ was vibrated in a culture dish for 2 min, poured in to the viscometer and the flux time in the viscometer was determined. Second, alginate solution was vibrated for 2 min and left still for 8 min, poured to viscometer and flux time was determined. Third, the alginate solution added to the viscometer was vibrated while alginate was fluxing and time was recorded. Each experiment was conducted at 400, 480, 520, 530, and 600 Hz frequencies at room temperature at three different occasions.

Measurement of Surface Tension of Alginate

The changes in the surface tension of the alginate under two different vibration conditions were measured using the Fisher Surface Tensiometer (Fisher Scientific, Model 20). First, 10 ml of 1.5% alginate with 0.5 mM $CaCl_2$ was vibrated in a culture dish for 2 min, and surface tension of alginate was measured immediately at 25°C. Second, alginate with similar concentration was vibrated for 2 min and left aside for 8 min for resting. At the end of 8 min, the surface tension was measured.

The culture dish with alginate was placed in the sample table, and sample table was raised until the platinum-iridium ring was completely wetted and immersed in alginate. Then, the torsion arm was released and the instrument adjusted to zero reading. The sample table was lowered while maintaining the index at the reference. Surface of the alginate distended and the sample table lowered until the alginate breaks. The reading at the scale was considered as the apparent surface tension. Each experiment was conducted at 400, 480, 520, 530, and 600 Hz frequencies in three different occasions.

Biofilm Studies: Effect of Vibration on *P. aeruginosa* Biofilm Models

Microorganisms

P. aeruginosa PAO1 was used throughout the study. The identity of the bacteria was confirmed with commercially available API 20 E kit (Biomérieux, Mercy I'Etoile, France). All isolates were stored in multiple aliquots at −70°C, after confirming their purity.

Growth Media

Blood Agar and Brain Heart Infusion (BHI) solution (Sigma Aldrich, USA) were used for culturing *P. aeruginosa*.

Microbial Inocula

Prior to each experiment, *P. aeruginosa* was subcultured on blood agar for 18 h at 37°C. A loopful of the overnight bacterial growth was inoculated into BHI medium, and, incubated for 18 h in an orbital shaker (80 rpm) at 37°C. The resultant growth was harvested, washed twice in phosphate buffered saline (PBS, pH 7.4) and resuspended in BHI. The concentration of *P. aeruginosa* was adjusted 1×10^7 cells/ml by spectrophotometry (Infinite M200 microplate reader, TECAN US Inc, NC, USA) and confirmed by hemocytometric counting.

Biofilm Formation

P. aeruginosa biofilms were developed as described by Bandara *et al.* (38) with some modifications. Commercially available sterile, polystyrene, flat bottom tissue culture treated culture dishes (tissue culture dish, 35×10 mm, Corning Glass Works, NY, USA) were used to develop *P. aeruginosa* biofilms. First, 1 ml of a standard cell suspension of bacteria (10^7organisms/ml) was prepared and transferred into culture dishes and incubated for 1.5 h (37°C, 75 rpm, VWR incubating orbital shaker, VWR International, PA, USA) to promote microbial adherence to surface of the dishes. After the initial adhesion phase, the cell suspensions were aspirated, and culture dishes were washed twice with PBS to remove loosely adherent cells. A total of 3 ml of BHI was transferred and the dishes were reincubated for 24 h (37°C, 75 rpm), and dishes washed twice with PBS to eliminate traces of the medium. Thus, the effect of vibrations was studied on preformed biofilms in a period of 24 h.

Biofilm Vibration

The effect of vibration on *P. aeruginosa* biofilms were tested in three different ways. First, 24 h *P. aeruginosa* biofilms were vibrated for 2 min and treated with tobramycin (5 μg/ml, ~1/13 of biofilm MIC). Second, 5 μg/ml of tobramycin was added to *P. aeruginosa* 24-h biofilms immediately before vibration for 2 min. Third, 24 h *P. aeruginosa* biofilms were treated with 5 μg/ml tobramycin and incubated for 3 h at 37°C 75 rpm. The biofilm was vibrated for 2 min thereafter. Frequencies of 450, 530, 610, 650, or 700 Hz were used to treat the bacterial biofilms. Thus, vibration-/drug- (negative control), 610 Hz/drug- (vibration positive control), vibration-/drug+ (drug positive control), and test treatments (450 Hz/drug+, 530 Hz/drug+, 610 Hz/drug+, 650 Hz/drug+, and 700 Hz/drug+) were studied. After treatment with antibiotic and vibration, all samples were incubated for additional 24 h at 37°C and 75 rpm. At the end, biofilms were washed with PBS twice, and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay was performed. Each experiment was performed quadruplicates at three different occasions at room temperature.

Determination of Minimum Inhibitory Concentration of Tobramycin on P. aeruginosa Biofilms (MIC)

P. aeruginosa biofilms were developed in sterile 96 well plates (BD biosciences, USA) as described above. Biofilms were washed twice with PBS, and tobramycin was administered in a concentration gradient. The plates were incubated for 24 h at 37°C and 75 rpm.

At the end of incubation period, XTT reduction assay was performed to quantify the viability of biofilms. The lowest concentration of the antibiotic at which the bacteria demonstrate 80% of loss of viability compared to the solvent control is considered as the MIC_{80} (MIC) of the antibiotic against *P. aeruginosa* biofilms. Due to extremely high antimicrobial resistance of biofilms (more than 5,000 times of MIC of planktonic counterpart), difficulty in achieving such a higher drug concentrations (MIC90 or MIC100) in most experimental set ups as a result of the chemical and physical properties of the drug, unavailability of established standards such as CLSI guidelines in measuring MIC in biofilms and reporting MIC80 in recent studies as an accepted parameter for biofilm studies, we used MIC80 as a standard parameter throughout the study (39,40). The assay was performed quadruplicates at three different occasions.

XTT Reduction Assay

At the end of incubation of both test and control biofilms, a standard XTT reduction assay was performed as described by Bandara *et al.* (41). XTT reduction assay measures the viability of biofilms by means of bacterial cell metabolic activity. In brief, commercially available XTT powder (Sigma, MO, USA) was dissolved in PBS to a final concentration of 1 mg/ml. Then, the solution was filter-sterilized (0.22 μm pore size filter) and stored at −70°C. Freshly prepared 0.4 mM menadione (Sigma, MO, USA) solution was used for XTT reduction assay. XTT solution was thawed and mixed with menadione solution at 20:1 (*v*/*v*) immediately before the assay. Thereafter, PBS:XTT:menadione in 79:20:1 proportion were added into each culture dish containing biofilms and incubated in the dark for 5 h at 37°C. The color changes were measured with a microtiter plate reader (Infinite M200 microplate reader, TECAN US Inc, NC, USA) at 492 nm. All assays were carried out in triplicate on three different occasions.

Confocal Laser Scanning Microscopy

Biofilms were prepared on sterile cover slips attached to sterile culture dishes (Tissue culture dish, 60×15mm, Sarstedt Inc, NC, USA) as described above. Preformed 24-h biofilms

were treated with tobramycin and vibrated as mentioned above and incubated for another 24 h at 37°C at 75 rpm. At the end of incubation, the prewashed coverslips were stained with Live/Dead stain (LIVE/DEAD BacLight Bacterial Viability kit, Invitrogen, Eugene, USA) (38). The biofilm was then analyzed by fluorescent microscopy (using confocal laser scanning microscope, Leica TCS SP5 II Fixed Stage Upright Microscope, Leica Microsystems, IL, USA).

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS software (version 16.0). The Mann-Whitney *U* test was performed to compare significant differences between the control and each test sample of alginates and *P. aeruginosa* biofilm. A P value <0.05 was considered statistically significant.

RESULTS

Vibration Screening Study on Alginate Gels

The purpose of the vibration screening study was to assess macroscopic changes in models of biofilm gels in response to acoustic vibrations. These studies were not intended to exactly mimic biofilm structure and composition. Rather, they were intended to screen the effects of alginate, calcium ion, and vibration on the model gel systems. The different concentrations of alginate used in this study exhibited different responses to vibration frequencies as determined by our experimental setup. Despite the wide range of frequencies tested, the standing waves observed on the surfaces of the samples occurred at frequencies less than 1,000 Hz. When the concentration of alginate increased from 0.5 to 1.5%, the vibration frequencies range relating to standing wave observation increased (Fig. 1a). However, when $CaCl_2$ was added to aforementioned solutions of alginate, vibration frequencies associated with the maximal wave formation decreased (Fig. 1b). In addition, the range of the frequencies in which the gels exhibited standing waves narrowed (Fig. 1b). With increasing calcium ions, the 1.5% alginate gel (Fig. 1c) the frequencies associated with the standing waves tended to decrease. The effects of gel volume were assessed and it was demonstrated that this had no significant effects on the observed data.

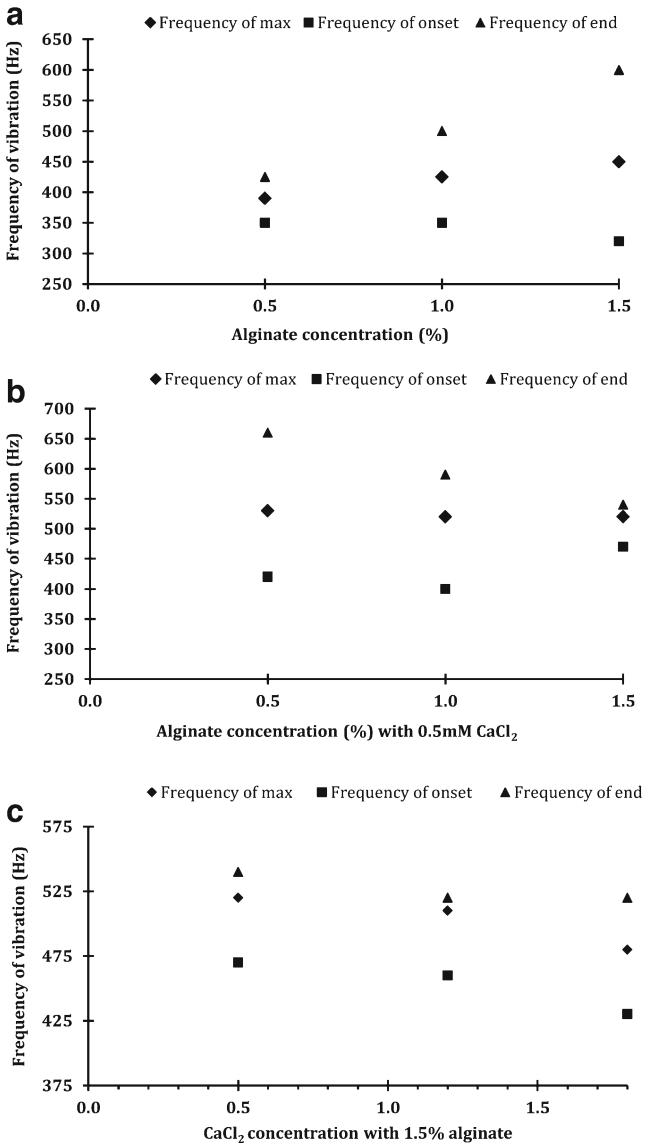

Fig. 1. Effects of the concentration of alginate and $CaCl_2$ content on alginate surface movements/standing waves. **a** Different alginate concentrations (%); note the widening of the range of frequency when increasing the concentration of alginate. **b** Different alginate concentrations with 0.5 mM $CaCl_2$; note the narrowing of the range of frequency when increasing the concentration of alginate in the presence of $CaCl_2$. **c** Different $CaCl_2$ concentrations with 1.5% alginate; note the widening of the range of frequency when increasing the concentration of $CaCl_2$. However, frequency max was slightly affected in different conditions tested in all three occasions

Vibration Affects the Surface Tension of the Alginate

The mean static apparent surface tension of alginate was 43.17±1.04 dynes/cm. In both studies, (surface tension measured immediately, and surface tension measured after 8 min rest), there were no significant differences in the surface tension of alginate compared to control. Despite the lack of statistical significance, there was a reduction trend in the surface tension of alginate vibrated at 400 Hz immediately following vibration compared to its control. Similarly, after 8 min of resting a trend to lower surface tension was observed for alginate samples that were vibrated at 400, 520, and 530 Hz frequencies compared to nonvibrated samples.

Sound Waves Do Not Affect the Viscosity of Alginate

The viscosity of 1.5% alginate with 0.5 mM CaCl2 was determined to be 56.64±1.54 cSt. There were no significant changes in the viscosity of alginate observed when vibrated and measured immediately, after a rest period, or during vibration (Fig. 2). However, some interesting trends were noted. Although not statistically significant, there were a trend of an increase in viscosity for vibration treatments between 480 and 600 Hz measured immediately after vibration (Fig. 2a). When alginate was left to rest 8 min after vibration,

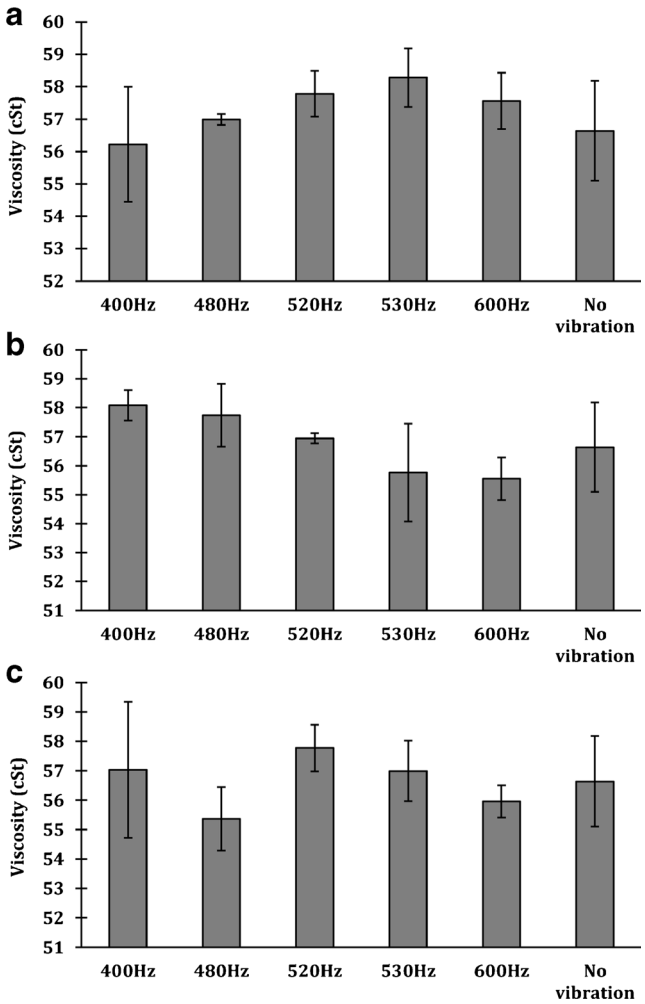

Fig. 2. A composite histogram of viscosity data of alginate exposed to different vibration strategies. **a** Viscosity of alginate immediately after vibration. **b** Viscosity of vibrated alginate after 8 min of resting. **c** Viscosity of alginate when vibrating. There were no significant differences of the viscosity of vibrated alginate compared to nonvibrated control. However, note the trend of reducing the viscosity when rested for 8 min after vibration compared to the increase in the viscosity when measured while vibration and immediately after

some lowering of viscosity was observed in some cases (e.g., for the 600 Hz treatment). Measurement of viscosity taken during flux also showed nonsignificant results (Fig. 2c).

Minimum Inhibitory Concentration of *P. aeruginosa* Biofilms

Minimum inhibitory concentration (MIC80) of tobramycin for *P. aeruginosa* planktonic cells was 1 μg/ml and 64 μg/ml for mature biofilms.

Sound Waves Affect the Metabolic Activity of Biofilms

There were significant differences of the metabolic activity of *P. aeruginosa* biofilms when exposed to a variety of combinations of vibration and tobramycin treatment as indicated by XTT reduction assay findings.

The biofilms vibrated without antibiotic treatment ("610Hz/drug-") showed a significant reduction of their metabolism compared to undisturbed controls ("vibration-/drug-", $p<0.05$, Fig. 3). Subminimal inhibitory concentrations of tobramycin ("vibration-/drug+") significantly reduced the metabolic activity of *P. aeruginosa* biofilms compared to both undisturbed controls ("vibration-/drug-") and biofilms vibrated at 610 Hz without tobramycin ("610 Hz/drug-", $P<0.05$). These statistical differences were observed in all three treatment regimens as shown in Fig. 3 (Fig. 3a–c: tobramycin treatment immediately after vibration; simultaneous vibration and tobramycin treatment; and tobramycin treatment at the end of 3-h resting period after vibration, respectively).

In contrast, biofilms treated with tobramycin similar concentrations and vibrated simultaneously at 450, 530, 610, and 650 Hz ("450 Hz/drug+, 530 Hz/drug+, 610 Hz/drug+, and 650 Hz/drug+") demonstrated significantly greater reduction in the biofilm metabolism ($P<0.05$) compared to biofilms treated with tobramycin alone ("vibration-/drug+", Fig. 3b). Biofilms treated with tobramycin and simultaneously vibrated at 700 Hz ("700 Hz/drug+") did not show any significant changes of XTT readings compared to *P. aeruginosa* biofilm treated with tobramycin alone ("vibration-/drug+", Fig. 3b).

Biofilms that were treated with tobramycin immediately after vibration at 450 and 650 Hz ("450 Hz/drug+ and 650 Hz/drug+") demonstrated a significant reduction of their XTT readings compared to biofilms treated with antibiotic alone ("vibration-/drug+", $P<0.05$, Fig. 3a). In contrast, biofilms that were treated with tobramycin immediately after vibration at 530, 610, and 700 Hz ("530 Hz/drug+, 610 Hz/drug+, and 700 Hz/drug+") did not elicit significant difference in XTT readings compared to *P. aeruginosa* biofilm treated with tobramycin alone ("vibration-/drug+", Fig. 3a).

P. aeruginosa biofilms vibrated at the end of 3-h incubation period of tobramycin treatment at 610 and 650 Hz ("610 Hz/drug+ and 650 Hz/drug+") showed a significant reduction in biofilm metabolic activity compared to *P. aeruginosa* biofilm treated with tobramycin alone ("vibration-/drug+", $P<0.05$, Fig. 3c). However, *P. aeruginosa* biofilms treated similarly and vibrated at 450, 530,, and 700 Hz ("450 Hz/drug+, 530 Hz/drug+, and 700 Hz/drug+") did not show significant effect in their metabolism compared to control biofilm treated with antibiotic alone ("vibration-/drug+", Fig. 3c).

Biofilm vibration at the 450 Hz frequency showed different responses depending on when the vibration treatment was applied. Specifically, there were significantly lower metabolic activities of the biofilms vibrated before and during antibiotic treatment compared to biofilms vibrated after 3 h of antibiotic treatment ($p<0.05$). Similar findings were observed when biofilms were vibrated with 610 Hz. However, in this case, the metabolism of the biofilms vibrated during antibiotic treatment was significantly lower than the biofilms vibrated before tobramycin treatment at 610 Hz ($p<0.05$). At 650 Hz, the biofilms treated with tobramycin and vibrated simultaneously demonstrated a significantly lower activity than that of biofilms vibrated before or 3 h after tobramycin treatment ($p<0.05$). However, there were no significant changes between the metabolism of biofilms vibrated at 650 Hz before and after tobramycin treatment ($p<0.05$). There were no significant changes noted among any treatment groups vibrated at 530 or 700 Hz.

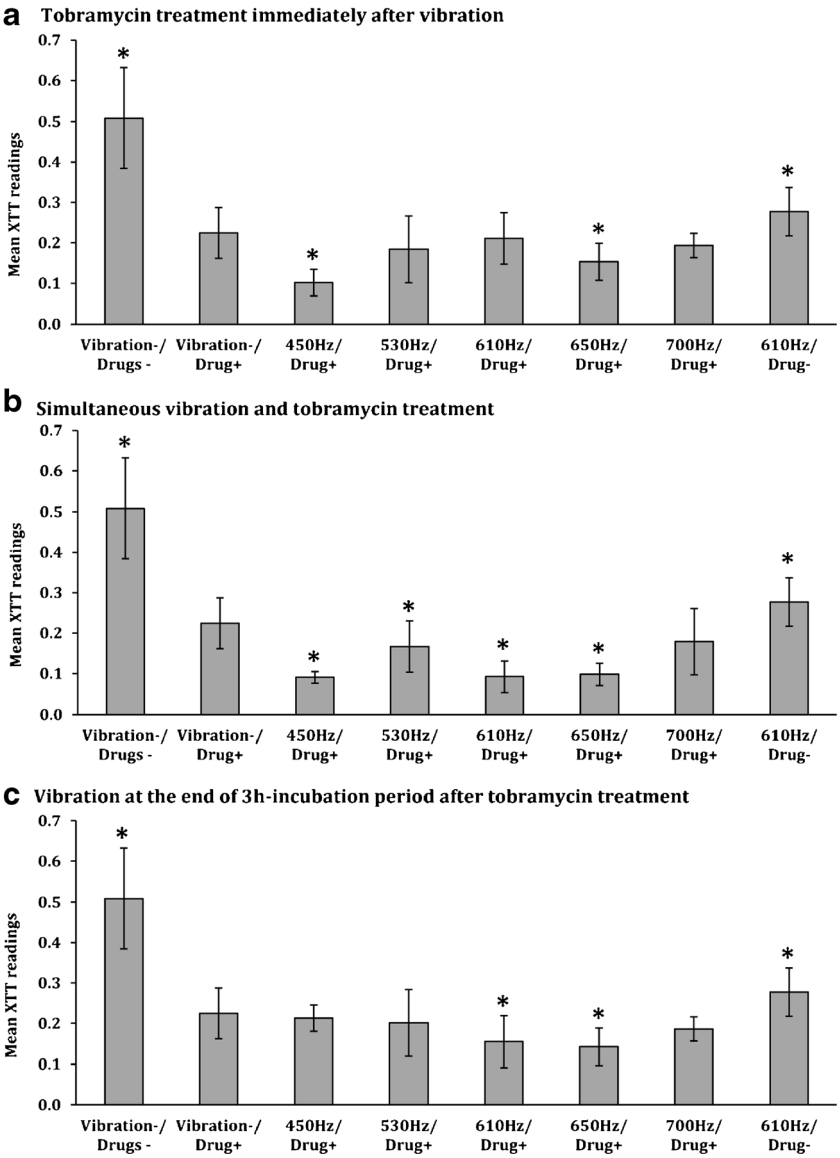

Fig. 3. A composite figure showing the effect of the various frequencies of vibration and tobramycin (5 μg/ml) treatment on the metabolic activity of *P. aeruginosa* biofilms. **a** Tobramycin treatment immediately after vibration. **b** Simultaneous vibration and tobramycin treatment. **c** Vibration at the end of 3-h incubation period after tobramycin treatment. Note the significant reduction of the metabolic activity of *P. aeruginosa* biofilms treated only with tobramycin (vibration-/drug+) compared to undisturbed controls (vibration-/drug-) and biofilm only treated with 610 Hz vibration (610 Hz/drug-, $P<0.05$). When treated with tobramycin and vibration, significant reductions of metabolic activity were noted in 450- and 650-Hz-treated biofilms in *(A)*, 450-, 530-, 610-, and 650-Hz-treated biofilms in *(B)*, and 610- and 650-Hz-treated biofilms in *(C)* compared to tobramycin alone-treated biofilm ($P<0.05$). *Asterisk* indicates significant changes in metabolic activity compared to biofilms treated only with tobramycin (vibration-/drug+) and $p<0.05$ considered statistically significant

Sound Waves Affect the Morphology of *P. aeruginosa* Biofilms

Live/Dead staining of *P. aeruginosa* biofilms with CYTO9 and propidium iodide demonstrated significant changes in matrix architecture due to vibration treatment.

The biofilm control which was not exposed to antibiotics or vibration showed a dense and spatially oriented hierarchical growth with some extracellular materials visible under CLSM. These biofilms showed typical cellular distribution as both live and dead cells were proportionally distributed ("vibration-/drug-", Fig. 4a). When the biofilm was treated with subminimum inhibitory concentration of tobramycin (5 μg/ml), the cellular content of the biofilm was considerably reduced while more dead cells appeared in the CLSM image. However, some structural organization of the biofilm was still preserved in the sample

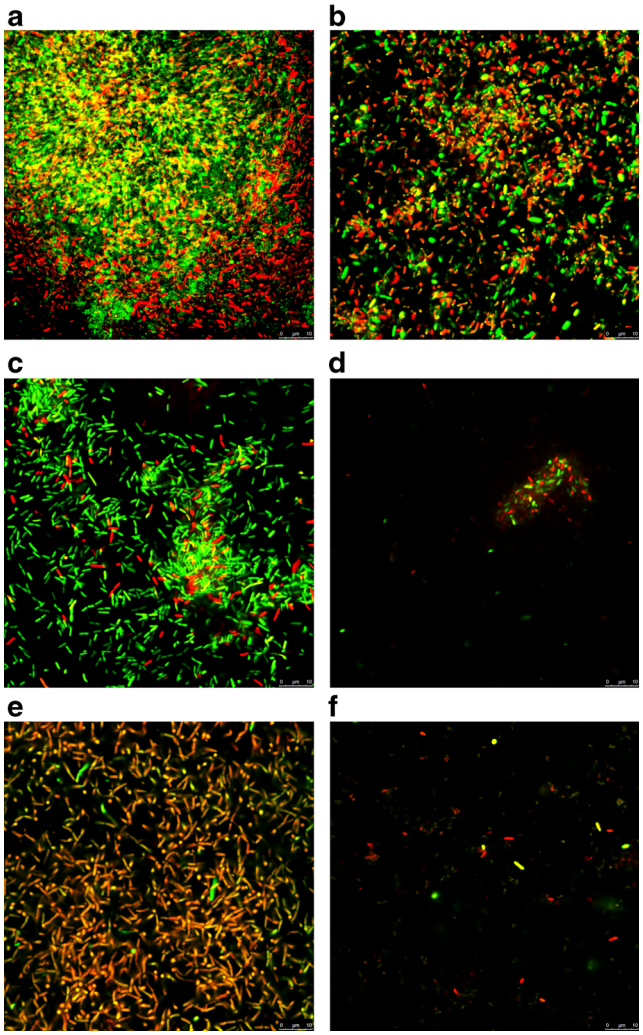

Fig. 4. CLSM images of *P. aeruginosa biofilms* treated with vibration (450 Hz) and tobramycin (5 μg/ml) (magnification ×40) (stained using a LIVE/DEAD BacLight bacterial viability kit; Invitrogen); live cells are stained in green and dead cells in red. **a** Undisturbed control. **b** Treated only with tobramycin (5 μg/ml); note the reduction of the cellular content in (**b**) and the increment of the dead cell proportion compared to (**a**). **c** Exposed only to 450 Hz vibration; note the considerable reduction of the biomass and increase in dead cell ratio compared to (**a**) and (**b**). **d** Simultaneous exposure to tobramycin and vibration; note the significant reduction of biomass and scattered debris compared to (**a**), (**b**), and (**c**). **e** Tobramycin treatment immediately after vibration; note the higher proportion of dead and dying cells and appearance of "hyphae-like" cells. **f** Vibration after 3 h of incubation period with tobramycin treatment. Note the significant destruction of the biofilm architecture compared to (**a**), (**b**), and (**c**)

("vibration-/drug+", Fig. 4b). When *P. aeruginosa* biofilms were exposed to 450 Hz vibration for 2 min and incubated without antibiotics ("450Hz/drug-"), there was a considerable reduction of biofilm biomass; however, the proportion of live:dead cells have been increased compared to Fig. 4a, b. Under these vibration treatment conditions, CLSM imaging also showed some retention of structural organization of the biofilm (Fig. 4c).

In contrast, when *P. aeruginosa* biofilms were exposed to tobramycin and vibration simultaneously ("450 Hz/drug+"), there was a significant reduction of the biofilm biomass and biofilm architecture was significantly disturbed showing only a single colony of the bacteria (Fig. 4d). More cellular debris were also scattered around the cells.

Compared to controls, *P. aeruginosa* biofilms treated with tobramycin immediately after vibration 450 Hz showed significantly higher population of dead and dying bacteria ("450 Hz/drug+", Fig. 4e). Though the biofilm architecture was partially preserved, the bacterial cells are elongated and appeared as "hyphae" compared to the control. Biofilm extracellular matrix was not visible in the samples imaged (Fig. 4e).

When *P. aeruginosa* was treated with tobramycin for 3 h and vibrated, a significant destruction of the biofilm architecture was noted compared to controls. There were few scattered bacteria which were visible in the CLSM images. Scattered biofilm debris were also noted ("450 Hz/drug+", Fig. 4f)

DISCUSSION

Microbial biofilms are highly refractory to antibiotics. Mechanisms associated with antimicrobial resistance are the presence of a dense microbial matrix which acts as a barrier for drug penetration throughout the entire biofilm, provides binding sites and deactivating sites (5,42), limited nutritional and oxygen accessibility to the deeper layers of the biofilm cells (43,44), local pH changes in the core of the biofilm (45,46), heterogeneous metabolism (3,47), and physiologically dormant "persister" cells (48,49). Overcoming these barriers and protective mechanisms remain a constant challenge in eliminating biofilms and biofilm-associated infections.

In addition to single and combined antibiotic therapies, various novel approaches have being sought to improve the outcome of biofilm-associated infections in the past decade. The principle behind these approaches is to overcome indigenous protective mechanisms of the biofilms. One promising approach utilized is ultrasound combined with antibiotic treatment (50). Ultrasound approach is based on longitudinal and/or shear waves through the material with linear particle motion in a cavitation range (50). It has been shown that ultrasound treatment reduces the final drug concentration needed to achieve desired microbial inhibition and minimize the generation of antimicrobial resistance among microbes (51–53). Though the exact mechanism by which cavitation improves the antibiotic sensitivity of biofilm is not yet known, it is possible that cavitation may rupture the biofilm matrix, hence, increasing antibiotic penetration and acceleration of bacterial metabolism through active transportation of oxygen and nutrients (53). It is also suggested that mechanical damage, abrogation of bacterial adhesion, and bypassing the obstacle of conditioning-films are also possible effects of ultrasound therapy (54–58). However, the major practical disadvantages of ultrasound therapy are cavitation-induced tissue damage and localized tissue heating (50). In addition, ultrasound requires professionally trained staff, relatively large instruments, and is not yet available for use on indwelling devices. Thus, an approach that can overcome aforementioned limitations needed to be sought.

To address these shortcomings, the study described here has been conducted using low frequency sound waves, essentially in the form of physical vibrations. Sound waves are safe and user-friendly, and upon optimization, these waves could potentially be applied in controlling biofilms in chronic wounds, indwelling devices, and in the airways. Kopel *et al.* (2011) have investigated the usage of a device containing a

piezo-element capable of transmitting relatively low-frequency (100 kHz, 0.3 $mWcm^{-2}$) surface acoustic waves into indwelling catheters (50). Simultaneous application of acoustic waves with antibiotics showed a significant reduction of *Escherichia coli*, *Staphylococcus epidermidis*, and *P. aeruginosa* biofilms in urinary catheters (50). In our study, vibrations that induce standing waves in alginate gels were investigated. In a screening study, very low frequencies (in the range less than 1,000 Hz) were observed to create these instabilities. These standing waves were also observed in the cultured biofilms of *P. aeruginosa*. We hypothesized that vibrations at these frequencies may result in disruption of the biofilm and potentially enhance antimicrobial activity. Initially, we used alginate gels, the major component of biofilm matrix to simulate the biofilm matrix *in vitro* as a screening tool to identify potential interactions of these low frequency vibrations with the biofilm matrix (59). It is known that the concentration of alginate can vary among different patients and various stages of *P. aeruginosa* infections. There are few reports in the literature that indicate the concentration of alginate or its viscosity in biofilms. Pedersen *et al.* (1990) reported that the alginate concentration in CF patients with *P. aeruginosa* infections averaged 35.5 mg/ml (range, 4 to 101 mg/ml) (32) and the viscosity of *P. aeruginosa* biofilms was reported to be $3.6\times10^5\pm2.6\times10^5$ (mean±1 SD) N s/m^2 (60). Thus, using previous data, we have used three concentrations of alginate throughout the study. (59). Due to possible precipitation of Ca^{2+}, 1.5% alginate was used as the highest concentration (37). The wavelengths studied here were between 20 Hz and 20 kHz and well below the cavitation power intensities (for ultrasound frequencies of 100 kHz, cavitation is generated at acoustic intensities in the range of 0.5×10^3–2×10^3 $mWcm^{-2}$ while the intensities generated in this study is 10^{-13}–1 $mWcm^{-2}$). In addition, alginate screening confirmed that vibration did not affect the temperature of the alginate solutions suggesting negligible heat generation (data not shown).

Sound Waves Could Induce Gel Disruption

According to the observations of alginate surface standing waves, the concentration, volume of alginate, and $CaCl_2$ content have minimal effects on low frequency vibration of alginate gels. The frequency needed to vibrate alginate and result in a surface standing wave was relatively insensitive to alginate concentration used. Although a simplified model of a biofilm, these findings from the screening study were encouraging in reference to *P. aeruginosa* biofilms. Little differences in the frequencies required to initiate a maximal standing wave in the gels were observed under a variety of conditions. Thus, the heterogeneity of biofilms present in an uncontrolled clinical setting may not pose technical challenges in selecting vibration frequencies and applying vibration therapy to disrupt biofilms exist in bacterial infections.

Sound Waves Do Not Affect the Biofilm Viscosity and Surface Tension

Vibration therapy used in this study did not show any significant results in the resultant viscosity and the surface tension of alginate gels measured at any frequency or different time points during/after vibration treatments. There was, however, an inverse relationship between the viscosity of alginate measured immediately after vibration and after resting period of 8 min following vibration. It seems that when the gels were rested, there was a tendency of increasing viscosity of alginate vibrated with lower frequencies. This could be a factor to take into consideration when designing antibiotic therapy after vibration. For instance, if antibiotics were given immediately after vibration with lower frequencies (relative to the range we tested), the drug penetration through alginate (thus, biofilms) may be improved compared to application of the antibiotic treatment after a period of resting. Nevertheless, ideal frequencies and the treatment sequence are yet to be determined.

Sound Waves are Promising Approach to Reduce *P. aeruginosa* Biofilm Viability and as a Synergistic Therapy with Antibiotics

Low frequency vibration applied on the biofilms of *P. aeruginosa* appeared to be a promising approach to reduce *in vitro* bacterial burden. Biofilms vibrated without any antibiotic treatment showed a significant reduction of the biofilm metabolism, indicating that vibration itself can either disrupt biofilm or kill the bacteria in the biofilm to reduce the bacterial burden. In contrast, ultrasonic waves (continuous ultrasonication at a frequency of 500 kHz and power density of 10 mW/cm^2, *in vitro*), did not affect *P. aeruginosa* biofilm structure or cell organization within the biofilms (57). In fact, low frequency vibration treatments (i.e., vibration treatment alone "vibration+/drug-") resulted in similar reductions in the metabolic activity of biofilms to those that were treated with subminimal concentrations of tobramycin alone ("vibration-/drug+"). Importantly, combined vibration and antibiotic treatments have additive effects compared to their individual effects on *P. aeruginosa* biofilms tested. According to the data reported here, simultaneous treatment of tobramycin and application of vibration yield better elimination of biofilms as measured by their lower metabolic activity compared to vibration of biofilm before antibiotic addition or vibration of the biofilm after 3 h of antibiotic treatment. Tobramycin together with 650 Hz ("650 Hz/drug+") exhibited the best suppression on *P. aeruginosa* biofilms under all three treatment regimens tested. However, it is still not clear whether the effect of vibration on biofilms merely due to mechanical disruption of the biofilm matrix or the possibility of bacteria to sense the vibration waves. Kopel *et al.* 2011 have noted that there were some significant changes in the bacterial transcriptome in response to surface acoustic waves (50). Though the effect of environmental factors on the transcriptome, they identified the switch anaerobic respiration to aerobic respiration by downregulating of *narI-G* operon. This is suggested due to increased aeration as a result of microstreamimg (50). Also, there was a significant change in the expression profile of PQS signaling.

Accordingly, although vibration therapy alone induces significant reduction in biofilm viability, it is likely that it would not be considered an alternative therapy for antibiotics. Rather, it may be considered as an adjunct therapy with antibiotics as the combination of the two treatments together was observed to be better than the individual treatments when used alone. Interestingly, Hazen *et al.* (2006) previously

demonstrated that vibration by surface acoustic waves prevented the biofilm formation in indwelling devices (61).

Biofilm XTT findings further support the assumptions made with viscosity observations. As mentioned above, alginate vibrated at higher frequencies and rested for 8 min showed lower viscosity; thus, we assumed that it would be the best time and frequency for antibiotic administration. Interestingly, biofilms vibrated with 610 and 650 Hz and rested for 3 h before tobramycin treatment elicited significantly lower biofilm viability compared to 540 and 530 Hz, hence, supporting aforementioned alginate results.

Ultrastructural views of *P. aeruginosa* biofilms further confirmed the findings obtained from the XTT assay. Generally, uninterrupted biofilm controls of *P. aeruginosa* were dense, confluent, relatively well defined and uniformly distributed, with some extracellular polymeric materials and healthy distribution of live and dead cell proportions. In contrast, biofilms treated with vibration alone were less dense with cell clumps, higher proportions of dead cells indicating the effect of vibration. More importantly, biofilms treated with both tobramycin and vibration showed severely disrupted biofilms. There were only few colonies visible under CLSM. One of the most distinguished features observed in vibrated and tobramycin-treated biofilms is the elongated "hyphae-like" structures. Wright *et al.*, in 1987, demonstrated that *P. aeruginosa* mucoid strains were capable of forming filamentous cells under certain experimental conditions such as magnesium depletion during exponential growth phase. Stationary phase cells were minimally formed filamentous cells. They suggested that magnesium may be attached to biofilm matrix thus preventing them from reaching the bacterial cells. However, they ruled out the possibility of lack of oxygen and glucose in the media causing the filamentous growth (62). In contrast, *Pseudomonas fluorescens* showed filamentous growth when oxygen was limited (63). Similarly, in a recent study, it was explained that anaerobic respiration is responsible for cell elongation and filament formation during biofilm development of *P. aeruginosa* PA01 (64). The presence of nitrile reductase that reduces nitrite to nitric oxide during anaerobic respiration was found to play a main role in filamentation (64). Therefore, it is tempting to speculate that during vibration, oxygen trapped in biofilm may have been removed creating more anaerobic environment to bacterial cells thus shifting respiration from aerobic to anaerobic. This may have led to formation of filamentous cells. These findings qualitatively confirm the beneficial combined effects of vibration with subminimal doses of tobramycin.

With shear stress, biofilms form more rigid biofilms and more EPS which would negatively affect the clearance of biofilms. Thus, it is an important factor when to determine the vibration frequency (60). According to data reported here and in previous studies, optimal biofilm elimination is highly dependent on different types of acoustic energy, varying in both frequency and intensity. Thus, the levels of vibration energy applied may play crucial roles in the outcomes of treating existing biofilms (58).

SUMMARY

According to alginate and biofilm data, it is proposed that sound waves are a promising approach in assisting antibiotics to kill bacterial biofilms in chronic wounds and in CF lungs. However, preliminary studies conducted by Dror *et al.* 2009 on sheep trachea to evaluate the use of acoustic waves in preventing intratracheal biofilm formation noted the complexity of applying this concept (58). Further investigations on optimization of vibration therapy within the spectrum of sound waves will greatly assist biofilm control in devastating infections in living being.

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