Published in final edited form as:
J Infect Chemother. 2004 August ; 10(4): 193–199.

Ultrasonic-Enhanced Gentamicin Transport through Colony Biofilms of *Pseudomonas aeruginosa* and *Escherichia coli*

J. C. Carmen[1,2], **J. L. Nelson**[3,4], **B. L. Beckstead**[3,5], **C. M. Runyan**[1,6], **R. A. Robison**[1], **G. B. Schaalje**[7], and **W. G. Pitt**[3,*]

1 *Brigham Young University, Department of Microbiology*

3 *Brigham Young University, Department of Chemical Engineering*

7 *Brigham Young University, Department of Statistics*

Abstract

The hypothesis that ultrasound increases antibiotic transport through biofilms of *Escherichia coli* and *Pseudomonas aeruginosa* was investigated using colony biofilms. Biofilms grown on membrane filters were transferred to nutrient agar containing 50 μg/mL gentamicin. A smaller filter was placed on top of the biofilm and a blank concentration disk was situated atop the filter. Diffusion of antibiotic through the biofilms was allowed for 15, 30, or 45 min at 37°C. Some of these biofilms were exposed to 70 kHz ultrasound and others were not. Each concentration disk was then placed on a nutrient agar plate spread with a lawn of *E. coli*. The resulting zone of inhibition was used to calculate the amount of gentamicin that was transported through the biofilm into the disk. The *E. coli* and *P. aeruginosa* biofilms grown for 13 and 24 h were exposed to two different ultrasonic power densities. Ultrasonication significantly increased the transport of gentamicin through the biofilm. Insonation of biofilms of *E. coli* for 45 minutes more than doubled the amount of gentamicin compared to their non-insonated counterparts. For *P. aeruginosa* biofilms, no detectable gentamicin penetrated the biofilm within 45 min without ultrasound; however, when insonated (1.5 W/cm^2) for 45 min, the disks collected more than 0.45 μg of antibiotic. Ultrasonication significantly increased transport of gentamicin across biofilms that normally blocked or slowed gentamicin transport when not exposed to ultrasound. This enhanced transport may be partially responsible for the increased killing of biofilm bacteria exposed to combinations of antibiotic and ultrasound.

Keywords

biofilm; gentamicin; antibiotic transport; ultrasound; *P. aeruginosa*; *E. coli*

INTRODUCTION

Bacteria within biofilms characteristically exhibit increased resistance to a wide range of antimicrobial agents compared to their planktonic counterparts. Costerton et al. speculated that this increased resistance to antimicrobials is due to changes in bacterial metabolism and genetic expression associated with sessile growth [1]. In addition to phenotypic changes, biofilms may bind or slow the transport of antibiotics, protecting the enclosed bacteria from exposure to lethal levels of antimicrobials [2, 3]. Furthermore, biofilms contain metabolically inactive cells

*Corresponding Author: Dr. William G. Pitt, Chemical Engineering, 350 N Clyde Building, Brigham Young University, Provo UT 84602 USA, email:pitt@byu.edu, Telephone: (801) 422-2589, FAX: (801) 422-0151.
[2]Current Address: University of Kentucky, Department of Microbiology
[4]Current Address: Pfizer Pharmaceutical, Ann Arbor, MI
[5]Current Address: University of Washington, Department of Bioengineering
[6]Current Address: University of Cincinnati, School of Medicine

and "persister" cells that are not responsive to conventional antibiotics [4]. Whatever the cause, the decreased antimicrobial susceptibility renders normal antimicrobial chemotherapy ineffective in the treatment of biofilm-related implant infections. We are investigating a non-invasive means of enhancing antimicrobial agents in order to more easily treat clinical biofilm infections. Previously, our lab has reported that low frequency ultrasound effectively enhanced the action of certain antibiotics in killing bacterial biofilms *in vitro* and *in vivo* [5–11]. The results of these previous experiments are promising; however, we have yet to identify the molecular mechanism of this phenomenon. Drawing upon previous research, we hypothesized that ultrasound increases the transport of certain antibiotics through biofilms of *Escherichia coli* and *Pseudomonas aeruginosa* [9, 12]. Herein we report the results of experiments designed to test this hypothesis.

Previous research investigating the effect of biofilms on antimicrobial transport has shown that biofilms sometimes slow but seldom completely block the transport of antibiotics [4]. Dunne *et al.* examined the diffusion of vancomycin and rifampin across a biofilm of *Staphylococcus epidermidis* using an equilibrium dialysis chamber [13]. After 24 h, the concentration of both antimicrobials exceeded their respective minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in the receiving side of the dialysis chamber. Similarly, Darouiche *et al.* found high concentrations of vancomycin in biofilms of *S. epidermidis* after exposure to the antimicrobial for 24 h [14]. Yasuda *et al.* studied the effect of clarithromycin on the structure of biofilms formed by *P. aeruginosa* and on the transport of ofloxacin and gentamicin across the biofilms [15, 16]. The biofilms that were formed by incubating filters with *P. aeruginosa* for 10 days exhibited reduced permeability of both ofloxacin and gentamicin. In addition, Anderl *et al.* found that ciprofloxacin reached the MIC throughout biofilms of wild-type *Klebsiella pneumoniae* in 20 min, but antibiotically active ampicillin failed to diffuse through the biofilm [17]. However, ampicillin readily diffused through biofilms of mutant *K. pneumoniae* unable to express β-lactamase. Walters reported that both tobramycin and ciprofloxacin diffused across *P. aeruginosa* biofilms given enough time [18]. Ciprofloxacin readily crossed the biofilm, but tobramycin took much longer. Nevertheless, it did reach a concentration five times the MIC after 36 h. Generally, it seems that antibiotics penetrate and cross biofilms. However, enzymatic degradation or ionic bonding may be responsible for inhibited transport across biofilms of *K. pneumoniae* and *P. aeruginosa* biofilms in the case of ampicillin and tobramycin respectively.

In an attempt to model the diffusion of antibiotics through the alginate produced by *P. aeruginosa* in patients with cystic fibrosis, Williams et al. [19] used a diffusion assay test with alginate that had been exposed to 50-kHz ultrasound at fairly high intensities. Increasing the duration and the intensity of insonation increased the diffusion of tobramycin, piperacillin and ciprofloxacin through the alginate. The increase in diffusion was attributed to a decrease in the viscosity of the alginate as the alginate was fragmented by the intense insonation.

Interaction of gentamicin with the outer membrane of distal cells in the biofilm, as well as uptake of the antibiotic by the cells or binding by exopolysaccharides in the matrix, could significantly increase the amount of time required for cells in the center of biofilm microcolonies to be exposed to inhibitory or bactericidal levels of gentamicin. This is significant because *P. aeruginosa* and several other aerobic and facultative Gram-negative bacilli exhibit transient aminoglycoside resistance in response to exposure to extremely low concentrations of aminoglycosides [20, 21]. It is possible that transient antibiotic resistance exhibited by sessile bacteria could result from early exposure to sub-MIC concentrations of the antibiotic that subsequently upregulates resistance mechanisms. Ultrasound is known to enhance the transport of small molecules across polymer membranes and gels [22, 23]. If similar transport enhancements occur in biofilms, then increased transport might saturate available

binding sites more rapidly, increase the concentration of gentamicin in proximity to sequestered bacteria, and thus reduce exposure of bacteria to sub-MIC concentrations.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics

Cultures of *E. coli* (ATCC 10798) and *P. aeruginosa* (ATCC 27853) were grown on nutrient agar (NA). Planktonic cultures were grown in tryptic soy broth without dextrose (TSB, Difco, Detroit, MI). Gentamicin sulfate (Sigma, St. Louis, MO) was reconstituted in distilled water, filter-sterilized, and added to molten agar that had been cooled to 49°C. Physiological saline solution (PSS) was made by dissolving 9 g of NaCl in 1 L of distilled water followed by autoclave sterilization. The gentamicin MIC values for planktonic suspensions of the *E. coli* and *P. aeruginosa* are 6 and 3 μg/mL respectively. These values are very similar to values reported elsewhere [10, 24–29].

Standard concentration disks

Standard concentration disks were made by loading blank concentration disks (Becton Dickinson, Sparks, MD) with 0.5, 1, 2, 4, 6, 8, 10, and 12 μg of gentamicin as follows. Gentamicin was diluted in sterile distilled water to the desired concentration. Blank concentration disks were placed in sterile polystyrene petri dishes and wetted by pipette with 20 μL of the prepared gentamicin solution. The petri dishes were refrigerated for 48 h to dry the disks, afterwhich the disks were placed in sterile vials and refrigerated until use.

Diffusion experiments

These experiments employed an adaptation of the experimental setup described by Anderl *et al.* [17]. GN-6 Metricel membrane filters of 25 mm diameter (Pall Gelman, Ann Arbor, MI) were placed on NA and inoculated in the center with 10 μL of inoculation culture. This culture was created by diluting 10 μL of overnight culture in 990 μL of TSB. The filters were then incubated for 13 or 24 h at 37°C.

After incubation, the filters and their accompanying biofilm were transferred to different NA plates containing 50 μg/mL gentamicin. A 13-mm GN-6 Metricel filter was placed over the biofilm and a blank concentration disk was positioned on top of the upper filter of the sandwiched biofilm. The concentration disk was wetted with 40 μL of TSB and the plates were exposed to ultrasound or sham (no ultrasound) at 37°C for 15, 30, and 45 min. To apply ultrasound, the gentamicin plates were floated on the surface of water in an ultrasonic bath (SC-100, Sonicor, Copiaque, NY) in which a fixture was constructed to maintain a constant position throughout the experiment. *E. coli* biofilms were insonated at 0 W/cm^2 (sham) and approximately 1.9 W/cm^2 and 2.9 W/cm^2; *P. aeruginosa* biofilms were insonated at 0 W/cm^2 and approximately 1.5 W/cm^2 and 2.5 W/cm^2. After the designated time, the concentration disks were removed from the upper filter, placed in sterile polystyrene petri dishes, and refrigerated. Exposure of the biofilm to ultrasound produced no visible change in the biofilm.

The power densities were measured in separate experiments by placing a calibrated hydrophone (#8103, Bruel & Kjaer, Naerum, DK) in a Petri dish placed in the same position as the experimental Petri dishes. The Petri dish was filled with water to the same height as the agar to produce a similar acoustic situation.

Zones of inhibition and quantitation of gentamicin

The concentration disks were placed on NA plates spread with a lawn of *E. coli* (ATCC 10798). The calibration disks containing standard amounts of gentamicin were also placed on NA plates spread with *E. coli*. A lawn was assured by spreading the plate with 100 μL of planktonic *E.*

coli culture created by diluting 10 μL of overnight culture in 990 μL of TSB. The plates were incubated for 24 h at 37°C. The zones of inhibition created by the gentamicin in the disks were measured with a 150 mm stainless dial caliper with 0.02 mm graduations (Cole Palmer, Vernon Hills, IL). The sizes of the zones of inhibition caused by the calibration concentration disks were correlated with the amount of gentamicin within the disk that created it. A plot of average zones of inhibition versus mass of gentamicin was created using the calibration disks (Fig. 1). The following correlation was used to convert the measured experimental zones of inhibition to micrograms of gentamicin in the disk,

$$G = 0.53\mu g\,(exp((ZI - 6.68mm)/2.67mm) - 1) \quad (1)$$

in which G is the mass of calibration gentamicin applied to the disk, and ZI is the diameter of the zone of inhibition, and 6.68 mm is the diameter of the concentration disk.

Biofilm thickness

Biofilms grown on the 25 mm membranes were stained with SYTO 9 and propidium iodide (LIVE/DEAD BacLight, Molecular Probes, Eugene, OR) [30, 31] and examined under a laser scanning confocal microscope (Zeiss LSM10, Oberkochen, Germany). The vertical thickness was measured in several places on each biofilm and ranged from 55 to 133 microns, depending on the species of bacteria.

Statistical analysis and graphic presentation

The data were analyzed using a heterogeneous variance ANOVA procedure in which the variances as well as the means are modeled as functions of the factors. The Kenward-Roger method [32] was used to approximate the degrees of freedom for the tests. Statistical tests were performed for treatment length, biofilm age, and presence and intensity of ultrasound. Additional tests to identify any interactions of these effects were performed. The data is presented in the form of box plots, which depict the interquartile range (25^{th} and 75^{th} percentile), the median, adjacent values, and outliers in the data, represented respectively by the top and bottom of the box, the central line, the error bars, and the dots.

RESULTS

The present experiments were designed to test the hypothesis that ultrasound increases the rate of transport of antibiotic through the biofilms into concentrations disks. Parallel experiments were constructed to directly compare the amount of gentamicin transport with and without application of ultrasound. Other factors that were studied included the species of biofilm and the age of the biofilm.

The results in general showed the presence of any biofilm significantly decreased the transport of antibiotic into the disk and the amount of accumulated gentamicin increased with time (see Tables 1 and 2 and Figures 2 and 3). Most importantly, the disks atop ultrasonicated biofilms contained more gentamicin than the non-insonated disks.

For *E. coli*, 45 minutes of ultrasound more than doubled the amount of gentamicin transported through the biofilm; shorter lengths of insonation time also increased the amount of transport, but not to the same extent. The ANOVA on the complete data set showed that the amount of transport into the concentration disk increased with intensity of ultrasound ($p < 0.0001$), increased with length of treatment time ($p < 0.0001$) and decreased with the age of biofilm ($p < 0.0001$).

For *P. aeruginosa* it is noteworthy that colony biofilms of both age effectively blocked the transport of gentamicin within the time frame studied; however, significant transport through

the same biofilm was observed upon application of ultrasound (Figure 3). The ANOVA on the complete data set showed that transport increased with intensity of ultrasound ($p < 0.0001$), with length of treatment time ($p < 0.0001$), and decreased with biofilm age ($p < 0.0001$).

DISCUSSION

Factors affecting transport

These results show that several factors influence the transport of antibiotic within or through a biofilm. In addition to ultrasound (which will be discussed below), the amount of transport is a strong function of the species of biofilm bacteria, the age of the biofilm, and the time allowed for transport. It is not surprising that the amount of antibiotic crossing the biofilm increased with time, since more time was allowed for the gentamicin to accumulate in the disk. However, it is noteworthy that the amount crossing the biofilm significantly decreased with the age of the biofilm, indicating that a 13-h biofilm is physically, chemically or biologically different than a 24-h biofilm. Measurements with the confocal microscope showed no significant difference in thicknesses of the 13-h 24-h biofilms ($F > 0.3$). With respect to biological differences, Sauer et al. have shown that there are several stages of biofilm development in which genetic expression changes during the course of biofilm development [33]. Such transient changes in the biofilm genetic expression and resulting phenotype appear to impact the transport of antibiotics as the biofilm matures.

Stewart et al. have shown that many factors can retard the diffusion of antibacterial agents through biofilms [3, 34, 35]. Such factors include the volume fraction of solids in the biofilm, reversible binding (adsorption/desorption) of agent to the biofilm components, irreversible binding to a fixed number of sites, and inactivation or enzymatic degradation of agent within the biofilm. Our observations that gentamicin transport through *E. coli* and *P. aeruginosa* biofilms is significantly influenced by biofilm age suggest that one or more of Stewart's "retarding" factors (cited above) increase as the biofilm matures. Often older biofilms are observed to be more recalcitrant towards antimicrobial agents [36], and we posit that decreased transport due to binding, inactivation, or enzymatic degradation in older biofilms could contribute to their recalcitrance.

With respect to the influence of the biofilm species, in the absence of ultrasound, gentamicin penetrated the *E. coli* biofilm in 30 min. However, no detectable amount of gentamicin traversed the colony biofilms of *P. aeruginosa* in 45 min. Subsequent experiments revealed that gentamicin penetrated both our 13-h and 24-h biofilm within 180 min (data not shown). Anderl et al., using a wild type and a β-lactamase deficient mutant of *K. pneumoniae* showed that detectable amounts of ampicillin could not penetrate wild-type biofilms in 240 minutes, whereas measurable amounts of antibiotic penetrated the mutant biofilm in only 10 minutes [17]. They attributed the lack of measurable penetration to the enzymatic degradation of ampicillin by the wild-type biofilm. In our experiments neither of the two species is considered to have any specific gentamicin-degrading enzymes expressed, at least in the planktonic phenotype. Walters et al. [18] showed that a similar aminoglycoside, tobramycin, penetrated a *P. aeruginosa* colony biofilm after about 13 hrs, much longer than the 45 minutes examined herein.

At present we have no explanation as to why gentamicin easily penetrates biofilms of *E. coli*, but not *P. aeruginosa*. Both biofilms contain negatively charged polysaccharides that can bind the positively charged aminoglycoside. However, it is possible that there are more binding sites or tighter binding sites in the *P. aeruginosa* biofilm.

Another factor that influences the rate of antibiotic transport is the chemistry of the antibiotic itself. Anderl et al. have shown that in wild-type *K. pneumoniae* biofilm, the rates of ampicillin

and ciprofloxacin penetration were vastly different, particularly in the wild-type biofilm wherein ampicillin failed to penetrate, but ciprofloxacin penetrated easily [17]. However, in the mutant biofilm (in which β-lactamase activity would not be present to interfere with ampicillin transport) the initial rate of ampicillin transport was much more rapid than that of ciprofloxacin.

Effect of Ultrasound

Ultrasound is known to enhance the transport of small molecules across porous membranes [22, 37] and increase transport of larger molecules such as DNA through agarose [23] or insulin through skin [38]. In general, the precise mechanisms of such increased transport have not been identified and may be different in each situation; but the enhancement is often speculatively attributed to increased microconvection from ultrasonic heating [37], to ultrasonic vibrational interactions with bubbles (cavitation events) [39], to reduction in boundary layer thickness due to of turbulence or microconvection [37], or to "oscillatory-enhanced dispersion" caused by oscillatory flow in channels [6]. In fact, the data of Tables 1 and 2 show that there is a large amount of gentamicin flux across the porous filter membranes in the absence of the biofilm, and that the flux is increased with application of ultrasound. Because the amount of gentamicin transported into the disk without biofilm is much larger than with biofilm, the biofilm (with or without ultrasound) is much less permeable to gentamicin than the micro-porous polymer membrane, and thus the biofilm is controlling the overall rate of transport.

A large amount of data was collected concerning the effect of ultrasound on the transport of gentamicin through biofilms, most of which could not be presented in this paper [40]. When all of the data was considered statistically, a relationship between intensity and transport emerges. Compared to when using non-insonated biofilms, the amount of gentamicin contained in the disks increased significantly when the biofilms were exposed to ultrasound for 30 to 45 min. For *E. coli* biofilms, exposure to 2.9 W/cm^2 resulted in the transport of larger amounts of gentamicin than exposure to 1.9 W/cm^2. Similarly for *P. aeruginosa* biofilms, higher insonation intensity increased the amount of transport. Thus there appears to be a dose-response relationship between the intensity of ultrasound and the amount of trans-biofilm transport. Such an intensity-dependent transport is consistent with transport mechanisms based upon cavitation events, microconvection, and oscillatory-enhanced diffusion.

A very interesting set of data is shown in Figure 3 in which insonation at 1.5 W/cm^2 rendered a previously impenetrable *P. aeruginosa* biofilm permeable to gentamicin transport. As discussed previously, we do not attribute the lack of penetration in the absence of ultrasound to inactivation of gentamicin by the biofilm. This data indicates that gentamicin can be successfully transported through the biofilm within 15 minutes given the additional "boost" by the ultrasound.

Even in the presence of ultrasound the age of the biofilm has an effect of upon the transport. More antibiotic was transported across the younger biofilms than the older ones (compare Figs 2A and 3A to 2B and 3B). Thus physiologic or phenotypic differences within a single species of biofilm (as well as differences due to species) still contribute to the transport in the presence of ultrasound.

Our data does not allow us to differentiate or identify which of the possible mechanisms for ultrasonic-enhanced transport are operational within the biofilm. We tend to discount the possible role of cavitation because micrographs of *P. aeruginosa* biofilms [18] do not show many voids that could serve as nuclei for cavitation bubbles. Rather, we posit that vibrations in the biofilm may increase convection in pores and channels by oscillatory-enhanced dispersion, and thus increase the overall rate of transport beyond that due to diffusion alone. The enhanced transport does not negate the normal adsorption nor the binding interactions between the antibiotic and the components of the biofilm; these probably occur as usual. But we postulate

that binding sites are filled more quickly as ultrasound enhances the transport, and thus the breakthrough time (when antibiotic first enters the disk) decreases, and a larger amount of antibiotic eventually enters the disk in the allotted time.

Recent observations by many investigators indicate that the recalcitrance of biofilms cannot be attributed entirely to the lack of eventual penetration of antibiotic into the biofilm [4, 17, 18, 35, 41]. The lack of oxygen penetration may also be involved [18]. We propose that just as ultrasound can increase the transport of gentamicin within biofilms, it can also increase the transport of oxygen and other small molecules that may increase the metabolic state and render the cells more susceptible to the antibiotic. Ultrasound does increase the effectiveness of gentamicin in killing cells in *E. coli* and *P. aeruginosa* biofilms [5–8]. Although we have yet to identify the exact mechanism(s), the observations reported herein support the role of two possible processes. First, ultrasound may enhance diffusion of oxygen into the biofilm and render metabolically inactive bacteria sufficiently active so that the antibiotics are effective. Secondly, the enhanced rate of transport of antibiotic into the biofilm may kill the cells before they can respond genetically to resist the antimicrobial attack. More work remains to be done to identify and take advantage of the role of ultrasound in enhancing the action of antibiotics against biofilms.

Acknowledgements

Funding for this research was provided by NIH grant HL59923 and by Brigham Young University. Brian Daniels did the confocal microscopy and Angela Dunford measured the MIC values.

References

1. Costerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A Common Cause of Persistent Infections. Science 1999;284:1318–1322. [PubMed: 10334980]
2. Stewart PS. Biofilm Accumulation Model that Predicts Antibiotic Resistance of *Pseudomonas aeruginosa* Biofilms. Antimicrob Agents Chemother 1994;38:1052–1058. [PubMed: 8067737]
3. Stewart PS. Theoretical Aspects of Antibiotic Diffusion into Microbial Biofilms. Antimicrob Agents Chem 1996;40:2517–2522.
4. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother 2001;45:999–1007. [PubMed: 11257008]
5. Johnson LL, Peterson RV, Pitt WG. Treatment of bacterial biofilms on polymeric implants using antibiotics and ultrasound. J Biomat Sci Polymer Ed 1998;9:1177–1185.
6. Peterson RV, Pitt WG. The effect of frequency and power density on the ultrasonically-enhanced killing of biofilm-sequestered *Escherichia coli.* Colloids and Surfaces B: Biointerfaces 2000;17:219–227.
7. Qian Z, Sagers RD, Pitt WG. The Effect of Ultrasonic Frequency upon Enhanced Killing of P. aeruginosa Biofilms. Annals Biomed Eng 1997;25:69–76.
8. Qian Z, Sagers RD, Pitt WG. The role of insonation intensity in acoustic-enhanced antibiotic treatment of bacterial biofilms. Colloids and Surfaces B: Biointerfaces 1997;9:239–245.
9. Qian Z, Sagers RD, Pitt WG. Investigation of the mechanism of the bioacoustic effect. J Biomed Mater Res 1999;44:198–205. [PubMed: 10397921]
10. Rediske AM, Roeder BL, Brown MK, Nelson JL, Robison RL, Draper DO, et al. Ultrasonic enhancement of antibiotic action on *Escherichia coli* biofilms: an *in vivo* model. Antimicrob Agents Chemother 1999;43:1211–1214. [PubMed: 10223938]
11. Rediske AM, Roeder BL, Nelson JL, Robison RL, Schaalje GB, Robison RA, et al. Pulsed ultrasound enhances the killing of *E. coli* biofilms by aminoglycoside antibiotics in vivo. Antimicrob Agents Chemother 2000;44:771–772. [PubMed: 10681355]
12. Pitt WG, Ross SA. Ultrasound increases the rate of bacterial cell growth. Biotechnol Prog 2003;19:1038–1044. [PubMed: 12790676]

13. Dunne WM Jr, Mason EO Jr, Kaplan S. Diffusion of rifampin and vancomycin through a *Staphylococcus epidermidis* biofilm. Antimicrobial Agents and Chemotherapy 1993;37:2522–2526. [PubMed: 8109913]

14. Darouiche RO, Dhir A, Miller AJ, Landon GC, Raad II, Musher DM. Vancomycin Penetration into Biofilm Covering Infected Prostheses and Effect on Bacteria. J Infect Diseases 1994;170:720–723. [PubMed: 7915751]

15. Yasuda H, Ajiki Y, Koga T, Kawada H, Yokota T. Interactions between biofilms formed by *Pseudomonas aeruginosa* and clarithromycin. Antimicrobial Agents and Chemotherapy 1993;37:1749–1755. [PubMed: 8239580]

16. Yasuda H, Ajiki Y, Koga T, Yokota T. Interaction betwee clarithromycin and biofilms formed by *Staphylococcus epidermidis*. Antimicrobial Agents and Chemotherapy 1994;38:138–141. [PubMed: 8141568]

17. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial Agents and Chemotherapy 2000;44:1818–1824. [PubMed: 10858336]

18. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of Antibiotic Penetration, Oxygen Limitation, and Low Metabolic Activity to Tolerance of Pseudomonas aeruginosa Biofilms to Ciprofloxacin and Tobramycin. Antimicrob Agents Chemother 2003;47:317–323. [PubMed: 12499208]

19. Williams KA, Clark HA, Allison DG. Use of ultrasound to facilitate antibiotic diffusion through Pseudomonas aeruginosa alginate. J Antimicrob Chemother 1995;36:463–473. [PubMed: 8830010]

20. Karlowsky JA, Hoban DJ, Zelenitsky SA, Zhanel GG. Altered *denA* and *anr* gene expression in aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. Journal of Antimicrobial Chemotherapy 1997;40:371–376. [PubMed: 9338489]

21. Karlowsky JA, Saunders MH, Harding GAJ, Hoban DJ, Zhanel GG. In vitro characterization of aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 1996;40:1387–1393. [PubMed: 8726006]

22. Li H, Ohdaira E, Ide M. Enhancement in Diffusion of Electrolyte through Membrane Using Ultrasonic Dialysis Equipment with Plane Membrane. Jpn J Appl Phys 1995;34:2725–2729.

23. Ma Y, Yeung ES. Effect of Ultrasound on the Separation of DNA Fragments in Agarose Gel Electrophoresis. Anal Chem 1990;62:1194–1196. [PubMed: 2363513]

24. Staneck JL, Glenn S, Dipersio JR, Leist PA. Wide Variability in Pseudomonas-Aeruginosa Aminoglycoside Results among 7 Susceptibility Testing Procedures. Journal of Clinical Microbiology 1989;27:2277–2285. [PubMed: 2511223]

25. Pitt WG, McBride MO, Lunceford JK, Roper RJ, Sagers RD. Ultrasonic Enhancement of Antibiotic Action on Gram-Negative Bacteria. Antimicrob Agents and Chemother 1994;38:2577–2582. [PubMed: 7872751]

26. Andrews J, Walker R, King A. Evaluation of media available for testing the susceptibility of Pseudomonas aeruginosa by BSAC methodology. Journal of Antimicrobial Chemotherapy 2002;50:479–486. [PubMed: 12356791]

27. Ioannides-Demos LL, Liolios L, Wood P, Spicer WJ, McLean AJ. Changes in MIC alter responses of Pseudomonas aeruginosa to tobramycin exposure. Antimicrobial Agents and Chemotherapy 1998;42:1365–1369. [PubMed: 9624477]

28. Kronvall G. Analysis of a Single Reference Strain for Determination of Gentamicin Regression Line Constants and Inhibition Zone Diameter Breakpoints in Quality Control of Disk Diffusion Antibiotic Susceptibility Testing. J Clin Microbiol 1982;16:784–793. [PubMed: 6818249]

29. Landrygan J, James PA, Brooks D, Kubiak EM. Reproducibility of control organism zone diameters for batches of IsoSensitest agar manufactured from 1996 to 2000 using the BSAC disc susceptibility test method. Journal of Antimicrobial Chemotherapy 2002;49:391–394. [PubMed: 11815586]

30. Takenaka S, Iwaku M, Hoshino E. Artificial Pseudomonas aeruginosa biofilms and confocal laser scanning microscopic analysis. J Infect Chemother 2001;7:87–93. [PubMed: 11455498]

31. Heersink J, Costerton WJ, Stoodley P. Influence of the Sonicare toothbrush on the structure and thickness of laboratory grown Streptococcus mutans biofilms. Am J Dent 2003;16:79–83. [PubMed: 12797563]

32. Kenward MG, Roger JH. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 1997;53:983–997. [PubMed: 9333350]
33. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J Bacteriol 2002;184:1140–1154. [PubMed: 11807075]
34. Stewart PS, Grab L, Diemer JA. Analysis of biocide transport limitation in an artificial biofilm system. J Appl Microbiol 1998;85:495–500. [PubMed: 9750280]
35. Stewart PS, Raquepas JB. Implications of reaction-diffusion theory for the disinfection of microbial biofilms by reactive antimicrobial agents. Chem Engr Sci 1995;50:3099–3104.
36. Anwar H, Strap JL, Costerton JW. Establishment of Aging Biofilms: Possible Mechanism of Bacterial Resistance to Antimicrobial Therapy. Antimicrob Agents and Chemother 1992;36:1347–1351. [PubMed: 1510427]
37. Floros JD, Liang H. Acoustically Assisted Diffusion Through Membranes and Biomaterials. Food Tech 1994;48:79–84.
38. Mitragotri S, Blankschtein D, Langer R. Ultrasound-Mediated Transdermal Protein Delivery. Science 1995;269:850–853. [PubMed: 7638603]
39. Nyborg WL. Biological Effects of Ultrasound: Development of Safety Guidelines. Part II: General Review. Ultrasound Med Biol 2001;27:301–333. [PubMed: 11369117]
40. Carmen J. An Investigation of the Mechanism of the Action of Ultrasound and Antibiotics on *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*M.S. Thesis;Brigham Young University. 2001; pp. 78.
41. Dodds MG, Grobe KJ, Stewart PS. Modeling Biofilm Antimicrobial Resistance. Biotech Bioeng 2000;68:456–465.

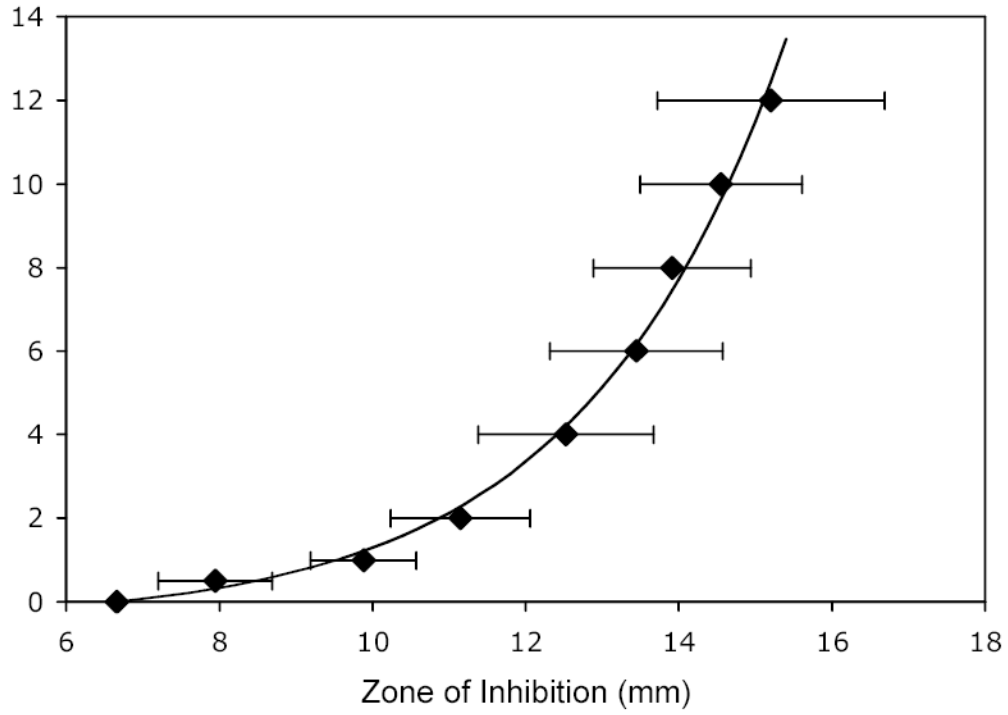

Figure 1.
The correlation between the amount of gentamicin contained in a concentration disk and the resulting size of the zone of inhibition of *E. coli*. The mean and standard deviation (n=8) are represented by the diamonds and error bars; the line is the correlation given in Equation 1.

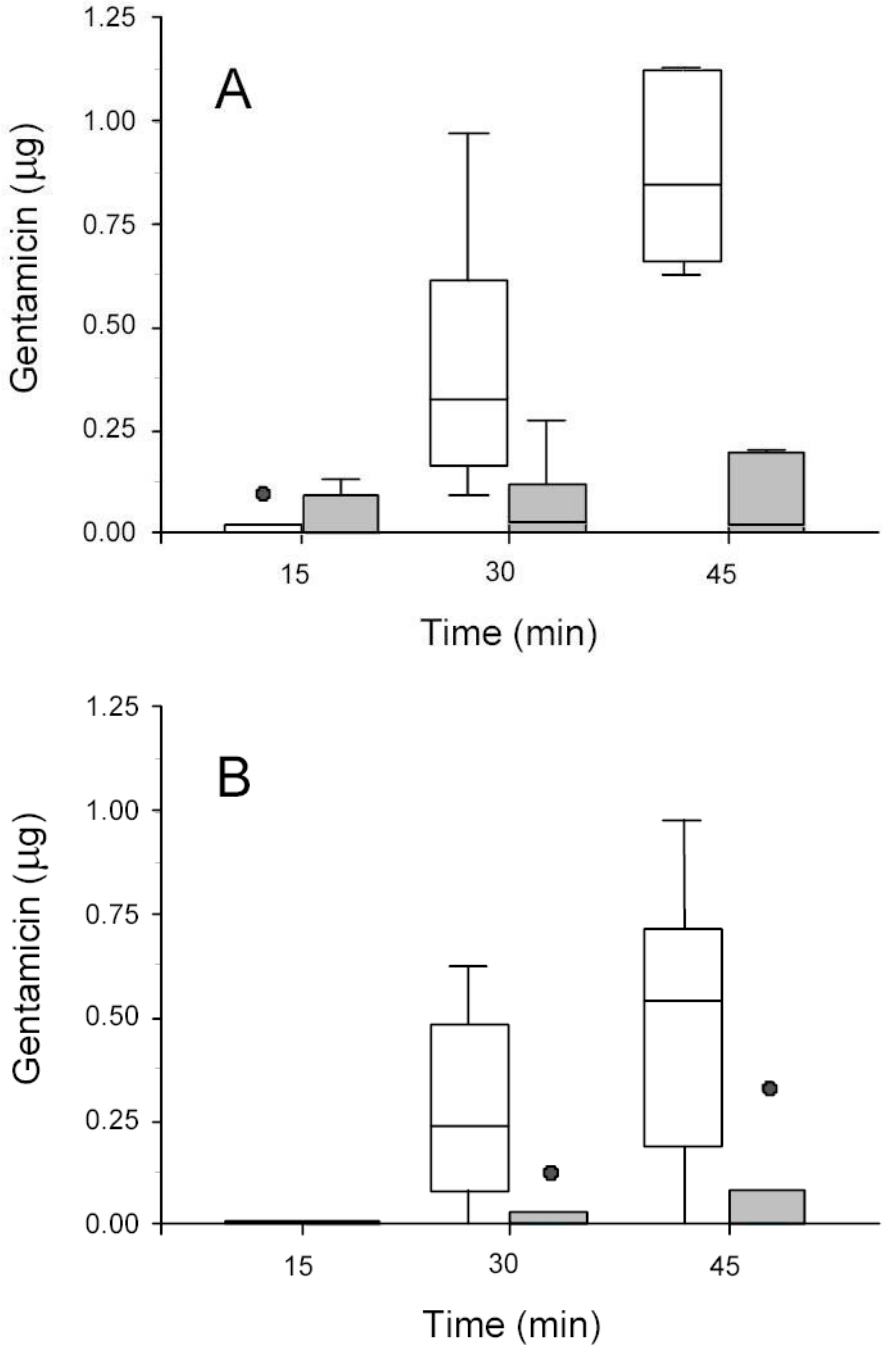

Figure 2.
Gentamicin in disks recovered from ultrasonicated (1.9 W/cm^2) (open box) and sham (non-insonated) (gray box) *E. coli* biofilms. A: 13-hr-old biofilms. B: 24-hr-old biofilms.

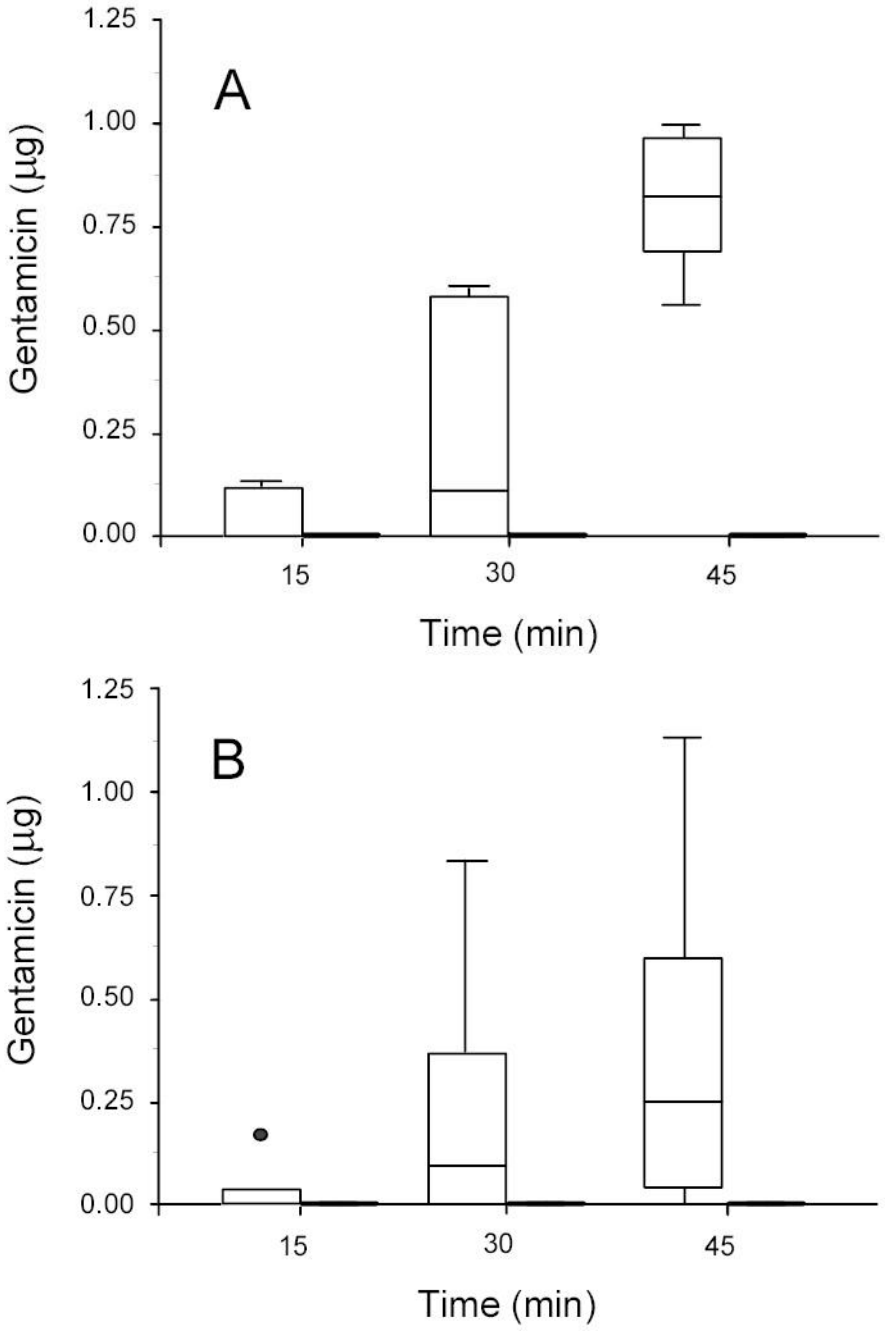

Figure 3.
Gentamicin in concentration disks recovered from ultrasonicated (1.5 W/cm^2) (open box) and sham (non-insonated) (gray box) *P. aeruginosa* biofilms. A: 13-hr-old biofilms. B: 24-hr-old biofilms.

Table 1

Average amount of gentamicin (μg) accumulated in disks (n=6) atop biofilms of *E. coli*.

Power density		**0 W/cm^2**			**1.9 W/cm^2**			**2.9 W/cm^2**	
Exposure time	**15 min**	**30 min**	**45 min**	**15 min**	**30 min**	**45 min**	**15 min**	**30 min**	**45 min**
No Biofilm*	1.38†	1.95	1.79	1.76	3.24	3.48	1.30	2.76	3.08
13-hr biofilm	0.03	0.06	0.07	0.01	0.38	0.84	0.08	0.25	0.60
24-hr biofilm	0.00	0.02	0.05	0.00	0.26	0.47	0.04	0.25	0.46

* The disk was place atop 2 filter membranes on the gentamicin plate with no biofilm present.

† The concentrations were determined by zones of inhibition.

Table 2

Average amount of gentamicin (μg) accumulated in disks (n=6) atop biofilms of *P. aeruginosa*.

Power density	**0 W/cm^2**			**1.5 W/cm^2**			**2.5 W/cm^2**		
Exposure time	**15 min**	**30 min**	**45 min**	**15 min**	**30 min**	**45 min**	**15 min**	**30 min**	**45 min**
No Biofilm*	0.94†	1.70	1.38	1.35	2.38	2.37	1.74	2.81	3.25
13-hr biofilm	0.00	0.00	0.00	0.04	0.23	0.79	0.09	0.76	1.45
24-hr biofilm	0.00	0.00	0.00	0.03	0.20	0.34	0.00	0.22	0.80

* The disk was place atop 2 filter membranes on the gentamicin plate with no biofilm present.

† The concentrations were determined by zones of inhibition.