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Synergistic effects of heat and antibiotics on *Pseudomonas aeruginosa* biofilms

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Abstract

Upon formation of a biofilm, bacteria undergo several changes that prevent eradication with antimicrobials alone. Due to this resistance, the standard of care for infected medical implants is explantation of the infected implant and surrounding tissue, followed by eventual reimplantation of a replacement device. Recent studies have demonstrated the efficacy of heat shock for biofilm eradication. To minimize the heat required for *in situ* biofilm eradication, this study investigated the hypothesis that antibiotics, while ineffective by themselves, may substantially increase heat shock efficacy. The combined effect of heat and antibiotics on *Pseudomonas aeruginosa* biofilms was quantified *via* heat shock in combination with ciprofloxacin, tobramycin, or erythromycin at multiple concentrations. Combined treatments had synergistic effects for all antibiotics for heat shock conditions of 60 °C for 5 min to 70 °C for 1 min, indicating an alternative to surgical explantation.

Keywords

biofilm; heat shock; antibiotics; infection; *Pseudomonas aeruginosa*

Introduction

Biofilms are densely packed colonies of bacteria immobilized on surfaces ranging from water pipes to implanted devices. The behavior of these sessile bacteria is significantly different than their planktonic, or free swimming, counterparts, making them much more robust and difficult to eradicate (Anwar et al. 1992; Costerton et al. 1999; Piddock 2006). Biofilms foul industrial surfaces (Abdallah et al. 2014) and in medical settings they cause a wide range of infections and sterility issues in hospitals (Rohde et al. 2007; Francolini & Donelli 2010; Gbejuade et al. 2015). Biofilms are a growing concern for both the medical and industrial fields since they form resistant infections by encasing themselves in a protective layer called extracellular polymeric substance (EPS). Each year in the US >100,000 implanted medical devices become infected with a biofilm, resulting in long painful recovery processes and occasional deaths. These infections typically require explantation of the infected implant and surrounding tissue, followed by eventual reimplantation of a replacement device (Montanaro et al. 2011; Tran & Tran 2012;

Fernandes & Dias 2013). The second implant has twice the likelihood of infection as the first implant (Rohde et al. 2007). These procedures greatly decrease patient quality of life and cost the US billions of dollars annually (Darouiche 2004). These problems have persisted for decades, prompting calls for new approaches to mitigate biofilms.

Biofilms have been shown to increase the resistance of bacteria to antibiotics *via* several factors including transport limitations, persister cells, and gene regulation changes. Due to these changes in bacterial behavior, biofilms require up to 128 times greater concentrations of antibiotics than their planktonic counterparts (Ceri et al. 1999; Abdi-Ali et al. 2006). Using antibiotic concentrations sufficient for biofilm treatment is usually not feasible due to the toxic effects to the patient at these concentrations (Hengzhuang et al. 2011). The transport limitations mentioned above are due largely to the EPS surrounding the bacteria and limiting the transport of antibiotics to the bacterial cells (Anderl et al. 2003; Walters III et al. 2003). This slower transport of antibiotics can result in concentrations below the minimum inhibition concentration, which not only fail to eradicate the biofilm, but have also been shown to increase the likelihood of evolutionary resistance to that antibiotic in the biofilm (Gbejuade et al. 2015; Howlin et al. 2015). Another consequence of EPS transport limitation is the nutrient gradient in the biofilm and the resulting variation in metabolic activity among the bacteria. This gradient creates pockets of bacteria, called persister cells, that have low metabolic rates and low division rates, further decreasing the efficacy of the antibiotics that typically target either a metabolic pathway or a replication process (Anderl et al. 2003; Walters III et al. 2003; Nguyen et al. 2011). Additionally, the proximity of bacteria to one another allows for constant communication between the cells, called quorum sensing, which can cause upregulation of genes that increase antibiotic resistance (Phelan et al. 2013), such as the upregulation of efflux pumps. Efflux pumps regulate some transport across the cell membrane and when upregulated can increase the rate at which the antibiotics are pumped out of the bacteria (de Kievit et al. 2001; Aeschlimann 2003). Due to the increased resistance of a biofilm to antibiotics, other approaches to eradication must be investigated.

Heat has been used to kill bacteria for hundreds of years; for planktonic bacteria this approach was formalized by Pasteur in 1864. Where feasible, biofilms are sterilized using pressurized autoclaves at temperatures > 120 °C. Many biofilm-covered surfaces cannot fit in an autoclave, however, or withstand such temperatures and pressures (Chmielewski & Frank 2006). Medical implants, obviously, cannot be subjected to those temperatures and pressures *in vivo*. While pasteurization has been studied extensively for mitigating planktonic bacterial populations using milder temperatures and shorter exposure times, comparatively little is known about the effects of such heat shocks on biofilms. Recent studies have shown biofilm population reductions of up to six orders of magnitude when subjected to temperatures of 70 °C to 80 °C (Chmielewski & Frank 2006; O'Toole et al. 2015; Wahlen et al. 2016). These temperatures can be wirelessly applied directly to the implant surface using a magnetic nanoparticle and polymer composite coating (Coffel & Nuxoll 2015) and the current standard of care sets a low bar for consequent damage to adjacent tissue. Nonetheless, strategies that enhance efficacy at a milder heat shock would reduce both the heating power requirement and the tissue damage for eradicating the infection. The objective of this study was to determine whether antibiotics increase the

efficacy of the heat shock, and if so, to quantify the breadth and magnitude of their synergistic effect.

The application of a mild temperature increase (a 5 °C increase or less) has enhanced antibiotic efficacy against biofilms in some cases (Allan et al. 1988; Hassani et al. 2007), although the decrease in population density was still modest. With the ability to wirelessly deliver localized heat directly at an implant surface *in situ*, more aggressive temperature increases can be achieved (Coffel & Nuxoll 2015). This prompts the need to investigate the combined effect of these two orthogonal mitigation strategies, antibiotics and heat, together. This study investigated the combined effect of antibiotics with heat using *Pseudomonas aeruginosa* biofilms. *P. aeruginosa* is a common model organism and is the third most common biofilm former on joint implants (Montanaro et al. 2011). The antibiotics ciprofloxacin, tobramycin, and erythromycin were investigated because of their different mechanisms of action, different molecular sizes, stability at higher temperatures, and frequency of use in patients. Ciprofloxacin is the smallest of these antibiotics (331 g mol⁻¹), has been shown to dehydrate at 120 °C, but remains stable below 100 °C (Turel & Bukovec 1996; Zupan et al. 2001; El-Gamel et al. 2012). It inhibits DNA gyrase and topoisomerase IV which in turn hinders the cell's ability to replicate (American Society of Health-System Pharmacists 2008). Tobramycin is slightly larger than ciprofloxacin at 468 g mol⁻¹ and has no appreciable decomposition until 164 °C (Dash & Suryanarayanan 1991). Tobramycin binds to the 30S ribosomal subunit in the 16S rRNA A-site preventing protein synthesis (Davis 1987; Yang et al. 2006; Bulitta et al. 2015). Erythromycin is the largest of these antibiotics (734 g mol⁻¹) and does not experience dehydration until 105.6 °C (Marian et al. 2013). Erythromycin hinders protein synthesis by binding to the 50S ribosomal subunit in the exit tunnel and causes addition defects (Siibak et al. 2009; Wilson 2014; Shishkina et al. 2015). The effect of each antibiotic on both planktonic and biofilm bacteria was determined across a concentration range of 0.25 µg ml⁻¹ to 32 µg ml⁻¹ for each antibiotic, with further concentrations investigated as needed. Selected concentrations were then added to heat shock trials ranging from 37 °C to 80 °C and 1 min to 30 min exposure time. Bacterial cell count reductions for these combined treatments were then compared with reductions by corresponding mono-treatments to identify and quantify synergistic activity.

Materials and methods

Biofilm growth

Cryogenically preserved *Pseudomonas aeruginosa* PAO1 (15692, American Type Culture Collection, Manassas, VA) was thawed and streaked on an agar plate (Difco Nutrient Agar, Sparks, MD, USA). The agar plates were inverted and incubated at 37 °C for 24 h. An inoculum was made by suspending two colonies from the agar plate in 5 ml of sterile tryptic soy broth (TSB, BD Bacto, Sparks, MD, USA) made as directed and incubated at 37 °C for 24 h, achieving an average concentration of $2.12 \times 10^9 \pm 0.07 \times 10^9$ colony forming units per milliliter (CFU ml⁻¹). One milliliter of inoculum was then diluted into 15 ml TSB and mixed gently. One hundred fifty microliters of the diluted mixture were then placed into each well of the 96-well plate, except for the negative control wells which received 150 µl of TSB. The MBEC™ assay system was used (Innovotech, Edmonton, AB, Canada) in which a

corresponding array of 96 pegs protrudes from the plate lid into the wells, providing a convenient array of substrata for biofilm growth which is then readily transferred to new wells (Harrison et al. 2010). The peg lid was placed into the 96-well plate then sealed using Parafilm and placed on an orbital shaker table (VWR 1000, 15 mm orbit, Thorofare, NJ, USA) at 160 rpm and incubated at 37 °C for 24 h.

Antibiotic preparation

A stock of 5 mg ml⁻¹ of ciprofloxacin in sterile, de-ionized water was prepared with ciprofloxacin hydrochloride (MP Biomedicals, Santa Ana, CA, USA) and mixed thoroughly. Tobramycin stock was made in a similar fashion by mixing 5 mg ml⁻¹ of tobramycin sulfate salt (Sigma-Aldrich, St Louis, MO, USA) in sterile, de-ionized water. Erythromycin was obtained from MP Biomedicals and the stock was prepared by mixing 5 mg ml⁻¹ erythromycin into ethanol as directed. Each antibiotic mixture was then filter sterilized through a 0.22 µl PES membrane sterile filter (Millex®GP filter unit) and stored at 2 °C. Each antibiotic was then diluted from the stock solution into sterile TSB at an array of concentrations in a 96-well challenge plate used that day.

Antibiotic exposure

After growth for 24h, the array of biofilm-covered pegs was transferred into a 96-well rinse plate (Costar® 96 well flat bottom cell culture, Corning Incorporated, NY, USA) containing 200 µl well⁻¹ of de-ionized, sterile water at ambient temperature for 2 min to remove bacteria not incorporated in the biofilm. The peg lid was then transferred to a 96-well challenge plate of wells each containing 200 µl of various concentrations of ciprofloxacin, tobramycin, or erythromycin diluted in TSB. For the antibiotic experiments without heat shock the peg lid was exposed to a single challenge plate for 24 h in an incubator at 37 °C before rinsing again for 2 min in a new rinse plate. The peg lid was then transferred to a recovery plate containing 200 µl well⁻¹ of fresh, sterile TSB for resuspension and enumeration.

Antibiotic and heat exposure

To investigate the combined effect of antibiotics and heat shock, biofilms cultured and exposed to antibiotics as discussed above were removed from their antibiotic challenge plate after only 4 h. They were quickly transferred to a challenge plate with the same array of antibiotic concentrations, preheated to the target temperature by a thermostatted water bath. Temperatures of 37 °C, 50 °C, 60 °C, 70 °C, and 80 °C were studied at exposure times of 1, 5, and 30 min. The peg array was left in the heated challenge plate for the desired exposure time, then transferred to a new challenge plate with the same antibiotic concentrations at 37 °C and incubated for the remainder of the total 24 h antibiotic exposure time. Following a total antibiotic exposure time of 24 h, the peg lid was rinsed once again for two minutes in a new rinse plate and placed into a recovery plate for resuspension and enumeration. Each growth and challenge plate step is shown in Figure 1.

Enumeration of bacteria in biofilms

To disrupt the biofilms and re-suspend the bacteria in a homogenous solution for serial dilution, each recovery plate with biofilm-covered pegs was sonicated for 10 min at 45 kHz in a VWR Symphony 9.5 L sonicator (Radnor, PA, USA). The sonicated recovery plate suspensions were serially diluted tenfold in a 96-well flat-bottom culture plate. Twenty microliters of each dilution were spot plated on agar plates and allowed to absorb for ~ 20 min before the agar plates were inverted and incubated at 37 °C for 20 to 24 h. The resulting CFUs were then counted and recorded. The logarithmic population density, $\log\left(\frac{CFU}{peg}\right)$, was calculated using Equation 1:

$$\log\left(\frac{CFU}{peg}\right) = \log\left[\frac{(plate\ count) * 10^{dilution\ factor} * \left(\frac{200\ \mu L}{20\ \mu L}\right)}{peg} + 1\right] \quad (1)$$

where the dilution factor is the number of tenfold dilutions corresponding to the sample counted and the plate count is the number of CFUs counted in that sample. The $(200\ \mu L / 20\ \mu L)$ is the ratio of the total recovery suspension to the amount that was sampled. Dilutions showing 3 to 50 CFUs were used for analysis; when two dilutions fit this range, the less dilute sample was used. The upper range (50) prevents counting error due to overlapping CFUs and the lower range (3) prevents a single CFU from altering the $\log\left(\frac{CFU}{peg}\right)$ value by > 0.125 by chance. By this rubric, population densities below $\log\left(\frac{CFU}{peg}\right) = 1.49$ cannot be quantified. In cases where the undiluted recovery well sample yielded < 3 CFUs, lower $\log\left(\frac{CFU}{peg}\right)$ were calculated but should be considered below the quantification limit. For samples with no CFU evident, the “+1” in Equation 1 ensured that the $\log\left(\frac{CFU}{peg}\right)$ value was 0 rather than mathematically undefined; its effect on values above the quantification limit is negligible.

OD measurements of planktonic bacteria

The planktonic bacteria that had escaped the biofilm *via* dispersion after the heat treatment were optically observed to better understand biofilm dispersion after heat treatment while antibiotics were still present. To estimate the free-swimming bacteria following the antibiotic treatment, the OD (BioTek Gen5 Microplate Reader, Winooski, Vermont, USA) at 600 nm was measured for each well in the second challenge plate. The negative controls in the challenge plate contained only TSB and were used to calculate an average background.

Confocal laser scanning microscopy (CLSM)

To visually demonstrate the effect of heat on biofilm viability, high population density ($\sim 10^9$ CFU cm^{-2}) biofilms grown on flat microscope slides in a drip flow reactor were imaged using CLSM. Immediately prior to imaging, the biofilms were stained using the FilmTracer LIVE/DEAD Biofilm Viability Kit (Molecular Probes, Inc. Eugene, OR, USA). The assay consists of SYTO9 which is a membrane permeable dye that binds to nucleic acid. The

propidium iodide displaces the SYTO9 in cells with a damaged membrane due to a higher affinity for nucleic acid than SYTO9. The biofilm was then visualized using a 40x dip lens and an upright Bio-Rad Radiance 2100 multiphoton/confocal microscope (Hemel-Hempstead, UK). The SYTO9 dye excites at a wavelength of 488 nm by using a helium-neon laser with an emission wavelength of 500 nm while the propidium iodide excites at a wavelength of 568 nm by using an argon laser with an emission wavelength at 635 nm. The lack of overlapping peaks allows distinction between the live and dead bacteria in the biofilm. Biofilm images were obtained as 1024×1024 pixel arrays by horizontally rastered scans, repeated in 1 μm vertical increments. The images were processed using the open-source ImageJ processor.

Statistical analysis

The statistical analyses of both the planktonic OD results and the logarithmic CFU results were reported by their arithmetic mean and standard deviation (SD). The reported OD numbers had a measured average background of 0.8 subtracted from their original raw values. Statistical analysis was performed in GraphPad Prism 7 using the two-way ANOVA with p-values of 0.05 to determine statistical differences. Each data point had three replicates per MBEC™ plate and 2 replicate plates. A total of 8 dilutions were used for the enumeration and two replicate plates for each dilution set, resulting in 12 replicates total for each trial.

Results

Antibiotic biofilm trials

None of the antibiotics reduced the biofilm below the quantifiable population density at physiologically relevant concentrations, as indicated in Figure 2. Figure 2 shows biofilm population densities after exposure to antibiotic concentrations ranging from $0.032 \mu\text{g ml}^{-1}$ to $128 \mu\text{g ml}^{-1}$ in twofold dilutions for 24h at 37°C . Ciprofloxacin and tobramycin did show a power-law decrease in biofilm population density with increasing antibiotic concentration up to $32 \mu\text{g ml}^{-1}$, with ciprofloxacin prompting consistently lower populations. Erythromycin showed little effect at any concentration.

These results were used to select the concentrations for the combined antibiotic and heat shock trials. Intravenously, ciprofloxacin, tobramycin, and erythromycin are administered at concentrations of 4, 4, and $2 \mu\text{g ml}^{-1}$, respectively (American Society of Health-System Pharmacists 2008). At these concentrations ciprofloxacin and tobramycin had significant population reduction effects on their own. Therefore, lower concentrations, 0.125 and $1.0 \mu\text{g ml}^{-1}$ for ciprofloxacin and 1.0 and $2.0 \mu\text{g ml}^{-1}$ for tobramycin, were also used for the combined treatment trials so that increased bacterial death from combined activity could be better quantified. Moreover, the local concentration of unbound antibiotic at the biofilm *in vivo* is typically less than the targeted plasma concentrations (Brunner et al. 1999). The lower concentrations had little impact on biofilm population density by themselves (discernable for ciprofloxacin) while the intermediate concentrations had a clear power-law effect. Larger concentrations (64 and $128 \mu\text{g ml}^{-1}$) of erythromycin were used to increase the likelihood of observing any antibiotic effect at all.

Effect of antibiotics and heat on biofilms

Biofilms were more resistant to both heat and antibiotics than their planktonic counterparts. Without antibiotics, the heat treatment alone resulted in a binary effect, killing all the biofilm bacteria at 60 °C for 30 min, 70 °C for 5 min, and 80 °C for 1 min, while milder treatments had no statistically significant effect, as seen in Figures 3 through 5. The effect of heat shock on biofilms is demonstrated visually in Figure 6, which compares a biofilm shocked at 80 °C for 1 min to a control biofilm “shocked” at the incubation temperature of 37 °C. In the CLSM image the live cells are dyed green and the dead cells are dyed red. Without heat shock, the biofilm reductions agree with the results shown in Figure 2, showing that the transfer of the biofilms to fresh 37 °C wells had little impact on biofilm viability. These results reconfirm that while ciprofloxacin and tobramycin at higher concentrations significantly reduce biofilm populations, they cannot, on their own, reliably eliminate them as seen with the more aggressive heat shocks. Even at low concentrations, however, these antibiotics have a significant synergistic impact within a key window of heat shock conditions.

Ciprofloxacin at the bloodstream concentration of 4 $\mu\text{g ml}^{-1}$, and at 1 $\mu\text{g ml}^{-1}$ substantially reduced biofilms with at least a five-order magnitude decrease in viable bacteria regardless of the heating, as seen in Figure 3. Combined with 1 min heat shocks at 70 °C, these ciprofloxacin concentrations left no viable bacteria however, despite the fact that the 1 min heat shock at 70 °C by itself had no discernable effect. Even the ciprofloxacin at a concentration of 0.125 $\mu\text{g ml}^{-1}$, which had little effect on biofilms by itself, reduced biofilm populations by five orders of magnitude when combined with otherwise ineffective heat shocks at 60 °C for 5 min and 70 °C for 1 min.

Tobramycin was the second most effective antibiotic on its own and demonstrated this synergy with heat more clearly than either of the other two antibiotics. Figure 4 shows the trend of prolonged heat exposure time increasing the overall efficacy of biofilm mitigation at all non-zero tobramycin concentrations, regardless of temperature. No trend is seen at the control temperature (37 °C) nor is this trend observed without tobramycin, except for complete elimination at 60 °C for 30 min, 70 °C for 5 min, and 80 °C for 1 min, as mentioned earlier. Within each of the concentration sets for tobramycin exposure at 50 °C for 30 min and 60 °C for 5 min resulted in similar amounts of mitigation. At 50 °C for 30 min, 60 °C for 5 min, and 70 °C for 1 min the effect of the combined heat and antibiotics was larger than either treatment by itself.

Erythromycin showed no effect at any concentration for the control temperature (37 °C) or in combination with 50 °C heat treatments as seen in Figure 5. However, biofilms heated at 60 °C for 5 min and treated with 64 $\mu\text{g ml}^{-1}$ or 128 $\mu\text{g ml}^{-1}$ erythromycin decreased viable bacteria by an average of 1.5 log(CFU peg^{-1}) and 2.7 log(CFU peg^{-1}), respectively. At 70 °C for 1 min the biofilms exposed to 64 $\mu\text{g ml}^{-1}$ decreased by 2.3 log(CFU peg^{-1}) and when exposed to 128 $\mu\text{g ml}^{-1}$ the number of viable bacteria decreased by 6.0 log(CFU peg^{-1}). Similar to the other antibiotics, the combined antibiotics and heat shock approach showed the greatest increase in efficacy over either individual treatment in the heat shock window of 60 °C for 5 min to 70 °C for 1 min.

Effect of antibiotics on dispersed planktonic bacteria

Further confirming the biofilm measurements, the media in which the biofilms were incubated after heat shock showed virtually no bacteria for treatments in which the corresponding biofilm was destroyed. Moreover, in antibiotic free trials the heat shock had no apparent effect on the ability of the bacteria to disperse from the biofilm and repopulate a fresh well. However, the presence of antibiotics strongly inhibited planktonic bacterial growth regardless of the presence of heat shock. All three concentrations of ciprofloxacin, 0.125, 1.0, and 4.0 $\mu\text{g ml}^{-1}$ seen in Figure 7, and tobramycin, 1.0, 2.0, and 4.0 $\mu\text{g ml}^{-1}$ seen in Figure 8, were effective against the planktonic bacteria with the higher two of the three concentrations killing off almost all the free-swimming bacteria regardless of heat shock. Erythromycin had no discernable effect at 2 $\mu\text{g ml}^{-1}$ as seen in Figure 9. However, at 64 $\mu\text{g ml}^{-1}$ there appeared to be a significant reduction in population even without heat shock, and a further decrease in viable planktonic bacterial cells at 128 $\mu\text{g ml}^{-1}$. Notably, while a 60 °C heat shock for 5 min had no effect by itself, in the presence of erythromycin the bacterial population was reduced from the non-heat-shocked values, this effect was even more pronounced and significant with a heat shock at 70 °C for 1 min. The synergistic effect of antibiotics with heat shocks at 60 °C for 5 min and 70 °C for 1 min were observed with all the antibiotics, but most prominently with erythromycin.

Discussion

Biofilms are present in many locations, from the plaque on teeth to the bottom of ships' hulls. On implanted medical devices, a biofilm infection can be life threatening and often requires surgical explantation followed by intensive antibiotic treatment before surgical implantation of a replacement device (Darouiche 2004). This stems from much higher resistance to antibiotics of bacteria in biofilms compared to their planktonic counterparts (Anwar et al. 1992; Costerton et al. 1999; Piddock 2006), a phenomenon demonstrated again in this study, with all three antibiotics showing more efficacy against free-swimming bacteria than biofilm bacteria. In the most extreme case, erythromycin at 37 °C had no effect against the biofilm at any concentration, but decreased the OD of the planktonic bacteria by 90% at 128 $\mu\text{g ml}^{-1}$. Tobramycin and ciprofloxacin had some efficacy against the biofilms, but in each case reached an asymptote where they could not further reduce the bacterial population below about 3 log(CFU peg^{-1}) or 1 log(CFU peg^{-1}), respectively, while virtually eliminating planktonic bacteria. Heat shock, on the other hand, appears to effectively mitigate biofilms, although conduction of heat to the tissue may cause significant damage which must be minimized (Hengzhuang et al. 2011; O'Toole et al. 2015; Wahlen et al. 2016) and the combination of antibiotics with the heat could lower the required temperatures.

As antibiotics are typically administered to infected patients as a first line of defense, their effect on the efficacy of heat shock is of prime interest. It was hypothesized that synergistic effects would be observed from improved transport of the antibiotics through the EPS or increased bacterial metabolism increasing the antibiotic susceptibility of the bacteria. The heat shock window for such observations is limited on one end by shocks effective enough to eradicate the bacterial biofilms on their own (60 °C for 30 min, 70 °C for 5 min, and 80 °C for 1 min) and on the other end by shocks so weak that even amplified effects are not

discernable. The bimodal distribution of biofilm response to heat was previously shown not to be affected by the maturity of the biofilm (Ricker et al. 2017). This synergistic window was observed at 70 °C for 1 min and at 60 °C for 5 min for all the antibiotics and additionally at 50 °C for 30 min for tobramycin. At these temperatures and exposure times the biofilms were not affected by either heat shock or antibiotics alone, but when combined these treatments had a substantial effect on the biofilm population.

One proposed mechanism for the synergism is the increased transport of the antibiotics through the EPS at higher temperatures. Some antibiotics such as aminoglycosides like tobramycin have been shown to have slow transport across the EPS, while other antibiotics such as ciprofloxacin appear to have little transport limitation across the EPS (Kumon et al. 1994; Walters III et al. 2003). This aligns with data from this study showing that ciprofloxacin was more effective against biofilms than tobramycin, with little to no effect seen from erythromycin. This trend also follows the molecular masses of the antibiotics closely which may influence the diffusivity of the molecules based on their sizes. The limited duration of the elevated temperature (1 to 30 min) compared to the overall exposure time (24 h) suggests that this contribution was limited however, as the diffusivity should only increase about 50% according to the Wilke-Chang diffusivity model (Wilke & Chang 1955). Chemical reactions or physical adsorption of the antibiotics by the EPS would also effectively limit their transport (Stewart 1996). However, in this case the change of antibiotics at the 4 h point should increase their efficacy regardless of temperature and that was not observed either.

Many researchers have suggested that the limiting factor for antibiotic efficacy in biofilms is not transport, but rather decreased metabolism of many of the bacteria in the biofilm (Walters III et al. 2003). The decreased metabolism of bacteria based on their locations in the biofilm is well documented (Walters III et al. 2003; Nguyen et al. 2011; Stewart et al. 2015) and likely contributes to the observed synergism in this study. With little baseline metabolic activity, inhibition of replication or protein synthesis has little consequence on bacterial viability, severely limiting antibiotic efficacy in biofilms. Thermal stimulation of metabolic activity would increase this efficacy substantially. This study supports this hypothesis and identifies temperature and exposure time thresholds at which the metabolism may increase. All antibiotics showed synergistic efficacy at 70 °C for 1 min and at 60 °C for 5 min, with tobramycin also synergistically effective at 50 °C for 30 min. Tobramycin can also cause bacterial wall damage (Kadurugamuwa, Lam, et al. 1993; Kadurugamuwa, Clarke, et al. 1993; Bulitta et al. 2015) which may increase its synergy with heat shock. A further implication of this hypothesis is that heat shocks at those conditions without antibiotics may increase the activity of the bacteria in the biofilm.

The local unbound antibiotic concentration experienced by the biofilm will usually be lower than the plasma antibiotic concentration. It is particularly encouraging that the strongest demonstrations for synergistic action by ciprofloxacin or tobramycin came at the lowest (non-zero) concentrations investigated, and indicates enhanced biofilm destruction at concentrations below typical systemic dosing. It is important to note, however, that this system is not necessarily constrained to systemically tolerable concentrations, as the antibiotic could be delivered locally by the coating itself, exposing the biofilm to

concentrations far higher than would be tolerated systemically. Moreover, with a self-heating coating this release could be thermally triggered, concentrating it in a specific time window coordinated to the heat shock. This raises the possibility that even poorly matched antibiotics such as erythromycin, which showed no effect by itself and a synergistic effect only at concentrations 32–64 times the standard bloodstream concentration, may still be released locally at the biofilm surface at a concentration high enough to be effective against even poorly matched bacteria. This poor match is most likely due to one of the efflux pumps found in *P. aeruginosa* which is particularly effective against erythromycin (Zhao et al. 1998), with minimum inhibitory concentrations of $\sim 512 \mu\text{g ml}^{-1}$ (Morita et al. 2014), higher than the concentrations used in these experiments. However, it is unclear whether antibiotic and heat shock synergy would be observed at any concentration against a species with complete genetic resistance to the given antibiotic.

Thermal ablation at temperatures up to 100 °C is already a common medical procedure where the goal is destruction of unwanted tissue (Diederich 2005). This is typically done endoscopically to minimize the destruction of surrounding tissue, though the process is still invasive and not feasible for complete coverage of a medical implant. Recent advances in the transmission of heat to otherwise inaccessible surfaces raises the prospect of a non-surgical alternative to implant infection control. By equipping the implant with a magnetically susceptible coating, its surface temperature can be raised on demand by exposing it to an alternating magnetic field (Coffel & Nuxoll 2015). The use of a coating to heat the implant surface would result in some tissue damage, but may significantly reduce tissue loss compared to the current method of treatment for biofilm infected implants (Coffel & Nuxoll 2016). The Cumulative Equivalent Minutes at 43 °C (CEM43) (Dewhirst et al. 2003; Yarmolenko et al. 2011) varies strongly with the surrounding heat sink (for example, perfusive blood carrying away the heat). However, even with the heat sink 5 mm away and no closer perfusion, a 1 min heat shock at 70 °C would expose only the nearest 2.5 mm to 200 or more CEM43, shown to significantly damage muscle tissue, with the exposure dropping off rapidly in the next two millimeters. Underscoring the importance of the antibiotic synergism observed here, heat shocks which eliminated biofilms without antibiotics (5 min at 70 °C and 1 min at 80 °C) would expose 3.3 or 3.1 mm, respectively, to more than 200 CEM43. The extensive tissue damage from the removal of the implant (including the removal of the surrounding tissue) in addition to the subsequent surgeries to re-implant a device, together with the increased risk of infection, sets a low bar regarding tolerable tissue damage, although direct comparison of the two treatment procedures will require *in vivo* trials.

In practice, it is unlikely that any biofilm infection therapy would be implemented without concurrent systemic antibiotics. This study indicates that with these antibiotics in combination with a heat shock treatment, biofilms may be eliminated at lower temperatures and exposure times than previously observed, significantly reducing the damage to surrounding tissue. Reduced heat shocks also require less heating power. With a decreased demand for heating power a coating wirelessly heated *via* induction from an alternating magnetic field would require a lower magnetic field strength and would improve the ability to localize the field.

Conclusions

An elegant solution to biofilm infection and biofouling is the use of heat. This study showed that the use of antibiotics in conjunction with heat can have a synergistic mitigation effect against *P. aeruginosa* biofilms. Heat shocks for 1 min at 70 °C and 5 min at 60 °C, which had no mitigation effect on their own, prompted a sharp decrease in biofilm population density when combined with any of the three antibiotics of different classes, even at concentrations that have no effect on their own. While heat shock likely does increase antibiotic transport through the EPS, the results of this study suggest transport is not the limiting factor in antibiotic efficacy in these biofilms. More consistent with these results is the theory that metabolic activity is severely limited in a fraction of biofilm bacteria, and that heat shocks for 1 min 70 °C and 5 min at 60 °C, or in some cases, 30 min at 50 °C, will stimulate activity for antibiotic efficacy. This synergism significantly reduces the required thermal load and the negative impacts of this load on the surrounding tissue and materials.

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Figure 1: Biofilm growth and combined heat shock and antibiotic treatment.

Stock *P. aeruginosa* PA01 cells were prepared to produce biofilms which were grown in an MBEC™ assay on an orbital shaker table. The biofilms were then transferred *via* the peg lid to a rinse plate to rinse off any loosely adhered bacteria then moved over to the challenge plate containing antibiotics and controls in different wells. After 4 h of the initial challenge plate the biofilms were heat shocked in a heated water bath and then swiftly transferred to a new challenge plate for the remainder of the total 24 h antibiotic exposure. The biofilms were rinsed once again and then placed in a recovery plate for sonication, dilution, and enumeration.

Figure 2: Antibiotic effect on biofilms.

The ciprofloxacin and tobramycin followed a power-law decrease for a discrete concentration range before reaching an asymptote, with ciprofloxacin having a larger effect on the biofilms than tobramycin. Erythromycin showed no statistical differences for each concentration tested.

Figure 3: Effect of heat and ciprofloxacin on the biofilm.

Biofilms were incubated at 37 °C with the indicated ciprofloxacin concentration for 24 h before enumeration. 4 h into the incubation they were heat shocked for the indicated time and temperature (in some cases at 37 °C as controls). Error bars indicate SD (n = 12).

Asterisks indicate trials that were statistically different from the corresponding controls (the 37 °C within that concentration group) as determined by two-way ANOVA (p < 0.05). Heat treatments of 60 °C for 30 min, 70 °C for 5 min, and 80 °C for 1 min eradicated the biofilm independently of the ciprofloxacin concentration, indicated by an “NG” on the graph.

Figure 4: Effect of heat and tobramycin on the biofilm.

Biofilms were incubated at 37 °C with the indicated tobramycin concentrations for 24 h before enumeration. 4 h into this incubation the biofilms were heat shocked for the indicated time and temperature (in some cases at 37 °C as controls). Error bars indicate the SD ($n = 12$). Asterisks indicate trials that were statistically different from their corresponding controls (the 37 °C within that concentration group) as determined by two-way ANOVA ($p < 0.05$). Heat treatments of 60 °C for 30 min, 70 °C for 5 min, and 80 °C for 1 min eradicated the biofilm independently of the tobramycin concentration, indicated by an “NG” on the graph.

Figure 5: Effect of heat and erythromycin on the biofilm.

Biofilms were incubated at 37 °C with the indicated erythromycin concentrations for 24 h before enumeration. 4 h into this incubation the biofilms were heat shocked for the indicated time and temperature (in some cases at 37 °C as controls). Error bars indicate the SD ($n = 12$). Asterisks indicate trials that were statistically different from their corresponding controls (the 37 °C within that concentration group) as determined by two-way ANOVA ($p < 0.05$). Heat treatments of 60 °C for 30 min, 70 °C for 5 min, and 80 °C for 1 min eradicated the biofilm independently of the erythromycin concentration, indicated by an “NG” on the graph.

Figure 6: Confocal visual effect of heat on *P. aeruginosa* biofilms.

P. aeruginosa biofilms grown in a drip flow reactor were heat shocked and visualized with Syto9 and propidium iodide dyes under a CLSM. (A) The control heat shock at 37 °C for 2 min had more green fluorescence (live cells dyed with Syto9) than (B) the biofilm heat shocked at 80 °C for 1 min which had more red fluorescence (dead cells dyed with propidium iodide).

Figure 7: Effect of ciprofloxacin on the planktonic bacteria dispersed from the biofilm.

Antibiotic efficacy against the planktonic bacteria dispersed from the heat shocked biofilms was measured by optical density. Error bars indicate the SD ($n = 12$). A baseline correction of 0.8 based on negative controls was applied. Biofilms which died before enumeration also showed no surviving planktonic bacteria. However, planktonic bacteria from surviving biofilms were also eliminated at ciprofloxacin concentrations of $1 \mu\text{g ml}^{-1}$ and above. Asterisks indicate trials that were statistically different from their corresponding controls (the 37°C within that concentration group) as determined by two-way ANOVA ($p < 0.05$).

Figure 8: Effect of tobramycin on the planktonic bacteria dispersed from the biofilm.

Antibiotic efficacy against the planktonic bacteria dispersed from the heat shocked biofilms was measured by optical density. Error bars indicate the SD ($n = 12$). A baseline correction of 0.8 based on negative controls was applied. Biofilms which died before enumeration also showed no surviving planktonic bacteria. However, planktonic bacteria from surviving biofilms were also eliminated at tobramycin concentrations of 2 µg mL⁻¹ and above. Asterisks indicated trials that were statistically different from their corresponding controls (the 37 °C within that concentration group) as determined by two-way ANOVA ($p < 0.05$).

Figure 9: Effect of erythromycin on the planktonic bacteria dispersed from the biofilm.

Antibiotic efficacy against the planktonic bacteria dispersed from the heat shocked biofilms was measured by optical density. Error bars indicate the SD (n = 12). A baseline correction of 0.8 based on negative controls was applied. Biofilms which died out before enumeration also showed no surviving planktonic bacteria. However, planktonic bacteria from surviving biofilms were also reduced at erythromycin concentrations of 64 µg ml and above. Asterisks indicated trials that were statistically different from their corresponding controls (the 37 °C within that concentration group) as determined by two-way ANOVA (p < 0.05).