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Harnessing ultrasound-stimulated phase change contrast agents to improve antibiotic efficacy against methicillin-resistant *Staphylococcus aureus* biofilms

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ABSTRACT

Bacterial biofilms, often associated with chronic infections, respond poorly to antibiotic therapy and frequently require surgical intervention. Biofilms harbor persister cells, metabolically indolent cells, which are tolerant to most conventional antibiotics. In addition, the biofilm matrix can act as a physical barrier, impeding diffusion of antibiotics. Novel therapeutic approaches frequently improve biofilm killing, but usually fail to achieve eradication. Failure to eradicate the biofilm leads to chronic and relapsing infection, is associated with major financial healthcare costs and significant morbidity and mortality. We address this problem with a two-pronged strategy using 1) antibiotics that target persister cells and 2) ultrasound-stimulated phase-change contrast agents (US-PCCA), which improve antibiotic penetration.

We previously demonstrated that rhamnolipids, produced by *Pseudomonas aeruginosa*, could induce aminoglycoside uptake in gram-positive organisms, leading to persister cell death. We have also shown that US-PCCA can transiently disrupt biological barriers to improve penetration of therapeutic macromolecules. We hypothesized that combining antibiotics which target persister cells with US-PCCA to improve drug penetration could improve treatment of methicillin resistant *S. aureus* (MRSA) biofilms. Aminoglycosides alone or in combination with US-PCCA displayed limited efficacy against MRSA biofilms. In contrast, the anti-persister combination of rhamnolipids and aminoglycosides combined with US-PCCA dramatically improved biofilm killing. This novel treatment strategy has the potential for rapid clinical translation as the PCCA formulation is a variant of FDA-approved ultrasound contrast agents that are already in clinical practice and the low-pressure ultrasound settings used in our study can be achieved with existing ultrasound hardware at pressures below the FDA set limits for diagnostic imaging.

Introduction

S. aureus is one of the most important human bacterial pathogens and in 2017 was the cause of 20,000 bacteremia deaths in the US alone [1]. Infections range from minor skin and soft tissue infections (SSTI), implanted device infections to more serious infections such as osteomyelitis, endocarditis and pneumonia [2,3]. In addition to the high degree of mortality, chronic and relapsing *S. aureus* infections are common and associated with significant morbidity. This is due to frequent treatment failure of *S. aureus* infections. This is best illustrated by SSTIs, with some studies suggesting treatment failure rates as high as 45% and a recurrence rate of 70% [4]. Importantly the failure of

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antibiotic therapy cannot be adequately explained by antibiotic resistance [1]. Failure to clear the infection leads to a need for prolonged antibiotic therapies, increased morbidity and mortality, increased likelihood of antibiotic resistance development as well as an enormous financial healthcare burden.

S. aureus forms biofilms, bacterial cells embedded in a self-produced extracellular matrix, which act as a protective barrier from the host immune response and other environmental assaults. Biofilms expand up to 1200 μm in thickness when attached to indwelling devices such as catheters [5]. Non-surface attached biofilms, in chronic wounds and chronic lung infections, harbor smaller cell aggregates ranging from 2 to 200 μm in diameter [5,6]. These biofilm aggregates are often surrounded by inflammatory immune cells such as neutrophils and embedded in a secondary host produced matrix such as mucus, pus or wound slough [7]. Consequently, biofilm-embedded cells have limited access to nutrients and oxygen and are coerced into a metabolically indolent state [8].

It has long been appreciated that biofilms respond poorly to antibiotics [7,9–12]. Most conventional bactericidal antibiotics kill by corrupting ATP-dependent cellular processes; aminoglycosides target translation, fluoroquinolones target DNA synthesis, rifampicin targets transcription and β-lactams and glycopeptides target cell wall synthesis [13,14]. Cells that survive lethal doses of antibiotics in the absence of a classical resistance mechanism are called antibiotic tolerant persister cells [15]. Biofilms are made up of a high proportion of persister cells [15–18]. They are distinct from resistant cells as they cannot grow in the presence of the drug. However, once the drug is removed, persisters grow and repopulate a biofilm and cause a relapse in infection [13]. Anti-persister antibiotics which kill independently of the metabolic state of the cell are more effective against biofilms than conventional antibiotics [19–22]. Tobramycin, an aminoglycoside that requires active proton motive force (PMF) for uptake into the cell is inactive against non-respiring cells, anaerobically growing cells, small colony variants and metabolically inactive cells within a biofilm [20]. We previously reported that rhamnolipids, biosurfactants produced by *P. aeruginosa,* permeabilize the *S. aureus* membrane to allow PMF-independent diffusion of tobramycin into the cell [20,22]. This combination of tobramycin and rhamnolipids (TOB/RL) rapidly sterilized in vitro planktonic cultures as well as non-respiring cells, anaerobically growing cells and small colony variants. However, despite this potent anti-persister activity, TOB/RL reduced biofilm viability by ~3-logs but failed to achieve eradication [20]. Notwithstanding the promise of this strategy, eradication of biofilms is arduous, even in vitro, indicating that factors other than the metabolic state of the biofilm-embedded cells are impeding therapy.

The biofilm matrix can act as a physical barrier to drug penetration. Penetration of vancomycin, *β*-lactams, phenicols and aminoglycoside antibiotics are impeded to some extent into *S. aureus* biofilms [23–26]. Consequently, novel methods of drug delivery into biofilms is a growing area of interest. Ultrasound is a safe, commonplace, portable and relatively inexpensive modality typically used in medical imaging. This imaging capability has been expanded through the use of intravenously administered microbubbles as a contrast agent. These microbubbles are also used in a growing number of therapeutic applications to enhance biological effects, which include transdermal drug delivery [27] and transient permeabilization of the blood brain barrier [28].

When exposed to an ultrasound wave, gas-filled microbubbles in solution will oscillate, with the positive pressure cycle resulting in compression and the negative pressure cycle causing the bubble to expand. In an ultrasound field, microbubbles experience stable cavitation (continuous expansion and contraction) at lower pressures or inertial cavitation (violent collapse of the bubble) at higher pressures [29]. Stable cavitation results in microstreaming; fluid movement around the bubble which induces shear stress to nearby structures (such as biofilms). At higher pressures, inertial cavitation can result in a shockwave, producing high temperatures at a small focus, and create microjets from the directional collapse of the bubble which can puncture host cells and disrupt physical barriers [30]. Both of these pressure regimes have potential for therapeutic applications of ultrasound-mediated microbubble cavitation. Despite the potential of microbubbles to enhance drug delivery, their size (typically 1–4 μm in diameter) and short half-life once injected into solution may limit penetration and subsequent disruption of biofilms.

We hypothesized that phase change contrast agents (PCCA), submicron liquid particles (typically 100–400 nm in diameter) may be better equipped to penetrate a biofilm. Liposome encapsulated drugs (which are similar in size to PCCAs) have previously been shown to penetrate *P. aeruginosa* biofilms [31,32]. In addition, unlike microbubbles, PCCA have been shown to penetrate blood clots and generate substantial internal erosion during sonothrombolysis [33]. PCCAs generally consist of a liquid perfluorocarbon droplet stabilized by a phospholipid shell. With appropriate ultrasound stimulation, PCCA can convert from the liquid phase to gas, generating a microbubble in their place (Fig. 1a). This process of "acoustic droplet vaporization" (ADV) may enhance drug penetration into biofilms as microbubbles over-expand before reaching their final diameter. Prior to activation, these particles are significantly more stable in circulation than microbubbles, with pharmacokinetic half-lives on the order of 45 min compared to approximately 4 min for microbubbles [34,35], with the potential to diffuse into biofilms due to their small size (Fig. 1b). Additionally, with continued ultrasound application, the resulting microbubbles can generate microstreaming, shear stress and microjets as they undergo cavitation (Fig. 1b and c). Typical PCCA formulations use perfluorocarbons with bulk boiling points near body temperature (e.g. dodecafluoropentane, 29 °C boiling point) and may induce undesired bioeffects as they require acoustic pressures above 3–6 MPa for ADV [36,37]. Conversely, low boiling-point PCCA filled with octofluoropropane (−36.7 °C boiling point) can be vaporized with peak negative pressures as low as 300 kPa at 1.0 MHz frequency [38]. These low boiling-point PCCA have been shown safe to use in vivo at moderate mechanical indices (MIs) and can be activated with clinically available hardware [39,40]. We hypothesized that low boiling-point PCCA, in combination with ultrasound (US-PCCA) and antibiotics that target persister cells would be a novel biofilm eradication strategy.

Results and discussion

Antibiotic efficacy against biofilm cells

We first identified drugs with efficacy against biofilms. Antibiotics were chosen based on clinical relevance or previously reported antibiofilm efficacy in vitro. Mature MRSA biofilms (USA300 LAC) were cultured for 24h in tissue culture treated plates before the addition of antibiotics. Following 24h of drug treatment, biofilms were washed and survivors were enumerated by plating. Tobramycin, mupirocin, vancomycin, and linezolid all caused a significant reduction in surviving biofilm cells (Fig. 2a). In contrast, levofloxacin and gentamicin showed no efficacy against biofilms at clinically achievable concentrations found in serum (C_{max}) [24,25] (Fig. 2a).

Efficacy of combined US-PCCA and tobramycin therapy

Next, we tested the ability of 30 s (s) US-PCCA treatment to potentiate tobramycin efficacy. Previous studies have indicated that negatively charged components of the biofilm matrix such as extracellular DNA and certain components of polysaccharides impede penetration of positively charged aminoglycosides such as tobramycin [25,26,41]. We hypothesized that US-PCCA might improve tobramycin penetration into biofilms and increase its efficacy. Mature biofilms were washed and transferred to a custom-built temperature-controlled 37 °C water bath alignment setup (Fig. 1b). Tobramycin and PCCAs were added and ultrasound applied at a range of rarefactional pressures (300–1200 kPa).

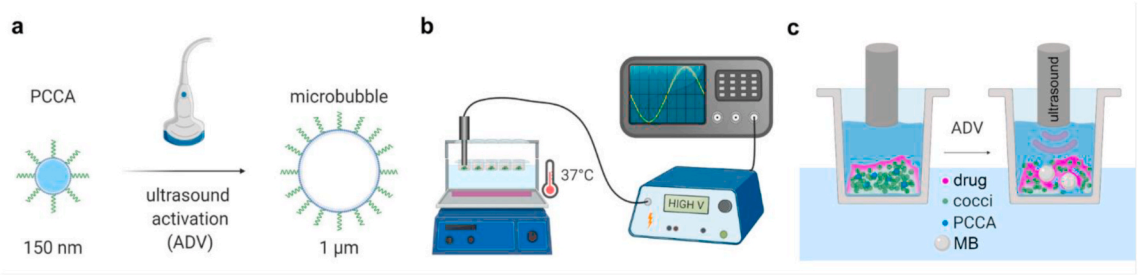

Fig. 1. PCCA and ultrasound disrupts biofilm and increases drug penetration. (a) Nanoscale PCCA are in a stable liquid phase. When exposed to ultrasound, the lipid shell containing superheated liquid perfluorocarbon is destabilized, causing the liquid to vaporize (acoustic droplet vaporization, ADV) to the gas phase and expand into a microbubble. (b) Experimental schematic for in vitro ultrasound exposure. An arbitrary waveform generator is used to generate a 1 MHz sine wave which is amplified and transmitted to an ultrasound transducer which is positioned over a bacterial biofilm in a well plate. The well plate is positioned in a custom fabricated water bath and coupled to water maintained at 37 °C. The bottom of the water bath is lined with ultrasound absorber material to reduce acoustic reflections. A lid with circular holes is used to center the ultrasound transducer within each well at a consistent height. (c) The stability and small size of PCCA makes them ideal to diffuse into biofilms prior to ultrasound application. Ultrasound stimulation can vaporize PCCA to microbubbles that can physically disrupt biofilms and enhance drug penetration.

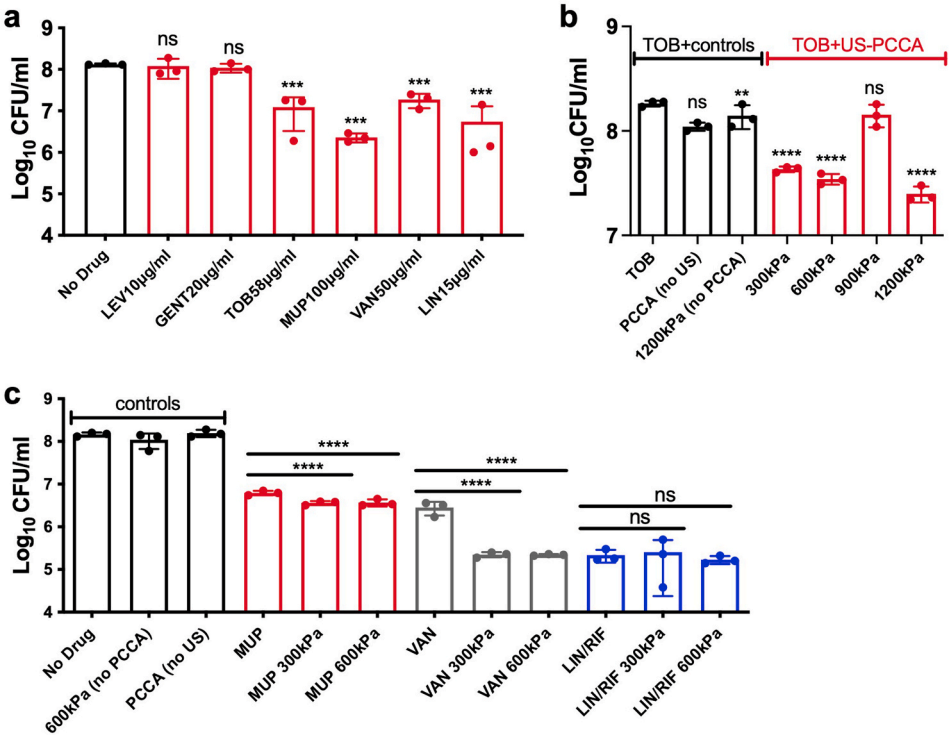

Fig. 2. US-PCCA improves antibiotic killing of MRSA biofilms. MRSA strain LAC biofilms were cultured overnight in brain-heart infusion (BHI) media in 12-well (a–b) or 24-well (c) tissue culture treated plates. Biofilms were washed and treated with antibiotics. Where indicated, plates were transferred to a custom-built temperature-controlled 37 °C water bath alignment setup. PCCA were added and 30 s ultrasound exposure was applied at indicated pressures and 20% duty cycle (b) or 10% duty cycle (c). After 24 h, biofilms were washed, sonicated for disruption and surviving cells were enumerated by serial dilution plating. Survivors were presented as $\log_{10}$ CFU/ml. The averages of $n = 3$ biologically independent samples are shown. The error bars represent the standard deviation. Statistical significance was determined using a one-way analysis of variance (ANOVA) with Dunnett's (a) or Sidak's multiple comparison test (b–c). **, ***, **** denotes $P < 0.005$, $P < 0.0005$, $P < 0.0001$, respectively. LEV, levofloxacin; GENT, gentamicin; TOB, 58 μg/ml tobramycin; MUP, mupirocin; VAN, vancomycin; LIN, linezolid, RIF, 10 μg/ml rifampicin; ns, not significant; US-PCCA, ultrasound-stimulated phase change contrast agents.

We found that tobramycin efficacy was significantly enhanced at pressures of 300, 600 and 1200 but not 900 kPa in the presence of PCCAs (Fig. 2b and c). We confirmed that the addition of PCCA in the absence of ultrasound had no impact on biofilm viability. Similarly, we anticipated that ultrasound alone, in the absence of PCCA would be ineffective, however 1200 kPa did cause a small but significant reduction in surviving cells in the absence of PCCA (Fig. 2b), indicating that potentiation seen at the highest pressure (1200 kPa) may not be entirely attributable to PCCA activity, and that mechanisms other than cavitation (e.g. acoustic radiation force) may impact potentiation at this pressure. It has been previously determined that low-intensity ultrasound could potentiate gentamicin killing in *P. aeruginosa* biofilms without evidence of physical disruption [42]. Additionally, studies in mammalian cells show non-lethal metabolic changes and cytoskeletal rearrangement in response to low-frequency ultrasound [43,44]. In order to investigate the potentiation effects of PCCA specifically in the regime below ultrasound-alone effects, the higher pressures (900 and 1200 kPa) were not evaluated further and the duty cycle lowered to 10% for subsequent experiments. The lower pressures, 300 and 600 kPa, in combination with PCCA were determined to be most effective at potentiating tobramycin efficacy. This is consistent with our previous findings where lower pressures (above the ADV threshold) resulted in more persistent cavitation activity during a 30s ultrasound exposure and was consistently greatest at macromolecule drug delivery across colorectal adenocarcinoma monolayers [45].

Efficacy of combined US-PCCA with clinically relevant antibiotic therapy

Next, we tested the ability of US-PCCA to potentiate mupirocin, vancomycin and linezolid/rifampicin. Mupirocin is a carboxylic acid topical antibiotic commonly used to treat *S. aureus* infections that binds to the isoleucyl-tRNA and prevents isoleucine incorporation into proteins [46]. US-PCCA caused a very slight increase in mupirocin killing (41% increase in killing) that was statistically significant but of

questionable biological significance (Fig. 2c).

Vancomycin is a glycopeptide that is the frontline antibiotic to treat MRSA infections. This antibiotic acts by binding to the D-Ala-D-ala residues of the membrane bound cell wall precursor, lipid II, preventing its incorporation and stalling active peptidoglycan synthesis [47]. Importantly, some studies have indicated that vancomycin penetration is impeded into biofilms [24]. US-PCCA potentiated vancomycin killing of biofilm-associated cells by 93% (Fig. 2c), likely by improving penetration. Notably, potentiation of vancomycin was seen with the C_{max} [48] indicating that at a clinically relevant concentration, US-PCCA has the capacity to improve biofilm killing of the front-line antibiotic used to treat MRSA infections.

Linezolid is an oxazolidinone protein synthesis inhibitor that is sometimes combined with the transcriptional inhibitor rifampicin for the treatment of *S. aureus* infections [49,50]. Linezolid/rifampicin reduced viable cells within the biofilm by almost 3-logs but was not significantly potentiated by US-PCCA (Fig. 2c). This suggests that US-PCCA has the ability to potentiate some conventional antibiotics but not others. It is possible that US-PCCA does not potentiate the killing of mupirocin and linezolid/rifampicin because the penetration of these drugs is not impeded into biofilms.

Efficacy of combined US-PCCA with anti-persister antibiotic therapy

Although the increased killing of biofilm-associated cells with conventional antibiotics shows promise, we hypothesized that regardless of penetration, antibiotic tolerant persister cells in the biofilm are surviving and thus impeding biofilm eradication. We predicted that utilizing US-PCCA to increase penetration of drugs active against antibiotic tolerant persister cells could further improve antibiotic therapy against biofilms.

Daptomycin is a lipopeptide antibiotic which inserts into the cell membrane and disrupts fluid membrane microdomains [51]. Daptomycin has potent activity against recalcitrant populations of *S. aureus,* including biofilms [52,53]. Daptomycin in combination with linezolid (DAP/LIN) is the treatment recommended for persistent MRSA bacteremia or vancomycin failure in the Infectious Diseases Society of America 2011 MRSA treatment guidelines [54]. We found that US-PCCA increased DAP/LIN killing of MRSA biofilms by 87% and 90% at 300 kPa and 600 kPa, respectively (Fig. 3a).

Next, we wanted to investigate if US-PCCA could improve efficacy of other drugs with anti-persister activity. Acyldepsipeptides (ADEPs) are activators of the ClpP protease. We previously reported that ADEPs sterilize persisters by activating the ClpP protease and causing the cell to self-digest in an ATP-independent manner [19]. ADEP in combination with rifampicin reduced biofilm cells by > 4-logs in 24h. US-PCCA significantly potentiated efficacy of ADEP/RIF at 300 kPa but not 600 kPa (Fig. 3a).

Tobramycin combined with rhamnolipids (TOB/RL), has potent anti-persister activity and has eradicated several recalcitrant populations

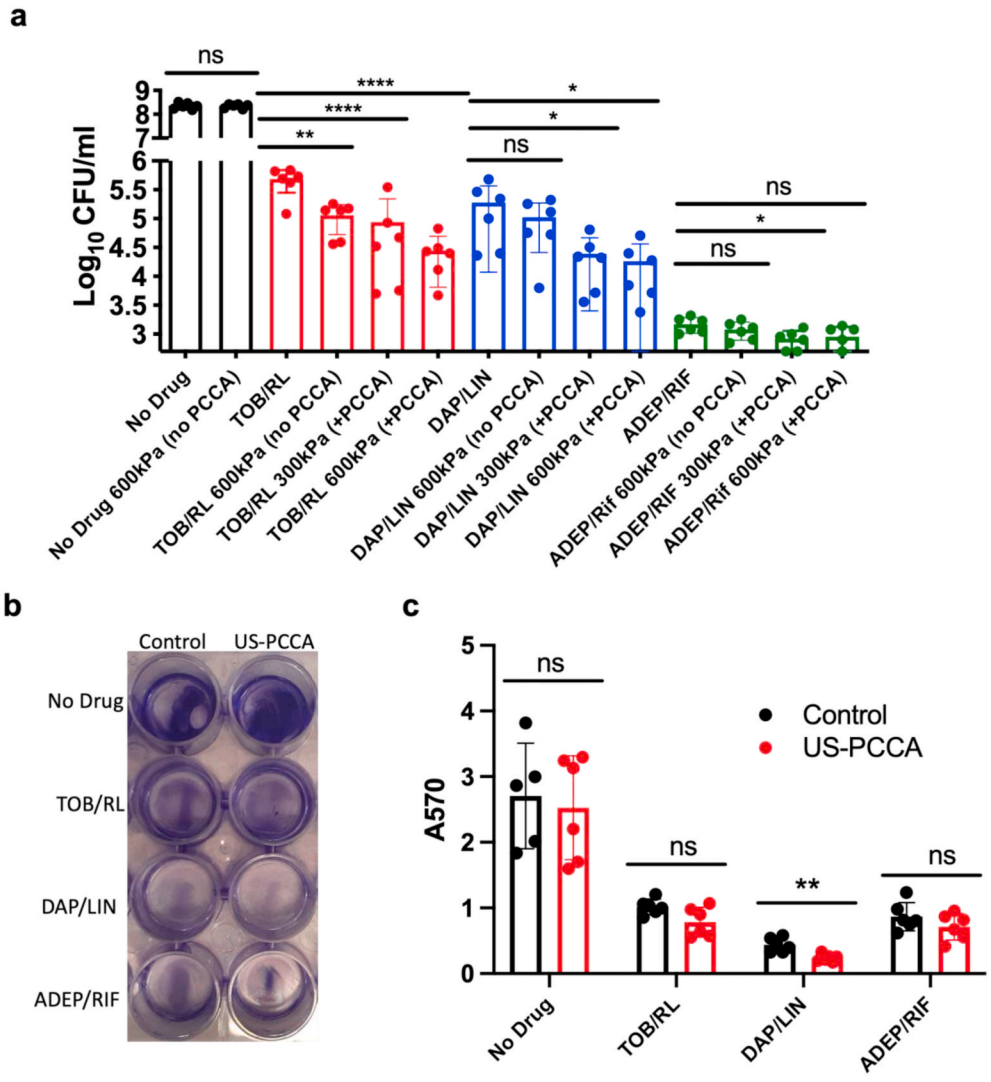

Fig. 3. US-PCCA improves anti-persister antibiotic therapy against MRSA biofilms. MRSA strain LAC biofilms were cultured overnight in brain-heart infusion (BHI) media in 24-well tissue culture treated plates. Biofilms were washed and treated with antibiotics and transferred to a custom-built temperature-controlled 37 °C water bath alignment setup. PCCAs were added and 30s ultrasound exposure was applied at 300 kPa or 600 kPa (b–c) and 10% duty cycle. After 24h, biofilms were washed, sonicated for disruption and surviving cells were enumerated by serial dilution plating (a) or stained with crystal violet (b). The averages of $n = 6$ biologically independent samples are shown. The error bars represent the standard deviation. Statistical significance was determined using a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (a) or multiple unpaired *t*-test (2-tailed) (c). *, **, **** denotes $P < 0.05$, $P < 0.005$, $P < 0.0001$, respectively. TOB, 58 μg/ml tobramycin; RL, 30 μg/ml rhamnolipids; DAP, 100 μg/ml daptomycin; LIN, 15 μg/ml linezolid; RIF, 10 μg/ml rifampicin; ADEP, 5 μg/ml acyldepsipeptide; ns, not significant; US-PCCA, ultrasound-stimulated phase change contrast agents. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

including non-respiring cells, anaerobically growing cells and small colony variants [20]. Despite this potent anti-persister activity, TOB/RL only reduced biofilm viability by ~3-logs [20]. We reasoned that drug penetration might be inhibited into the biofilms and hypothesized that improving penetration could further improve efficacy against biofilms. Applying US-PCCA in combination with TOB/RL increased killing of biofilm cells by 82% and 94% at 300 kPa and 600 kPa, respectively (Fig. 4a). Reduction in viable cfu was also associated with a decrease in biofilm biomass, as measured by crystal violet staining (Fig. 3b and c).

Previous studies have reported that bacteria embedded in biofilms can be coerced into a viable but non-culturable (VBNC) state in response to antibiotic pressure [55,56]. To determine if antibiotic/ultrasound caused cell death rather than inducing a VBNC state, we examined the viability of cells within residual biofilms following anti-biotic/ultrasound treatment. Biofilms were stained with LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit and imaged with confocal laser scanning microscopy (CLSM). The viability of the biofilm was defined as a ratio between the total fluorescent signal above the threshold level

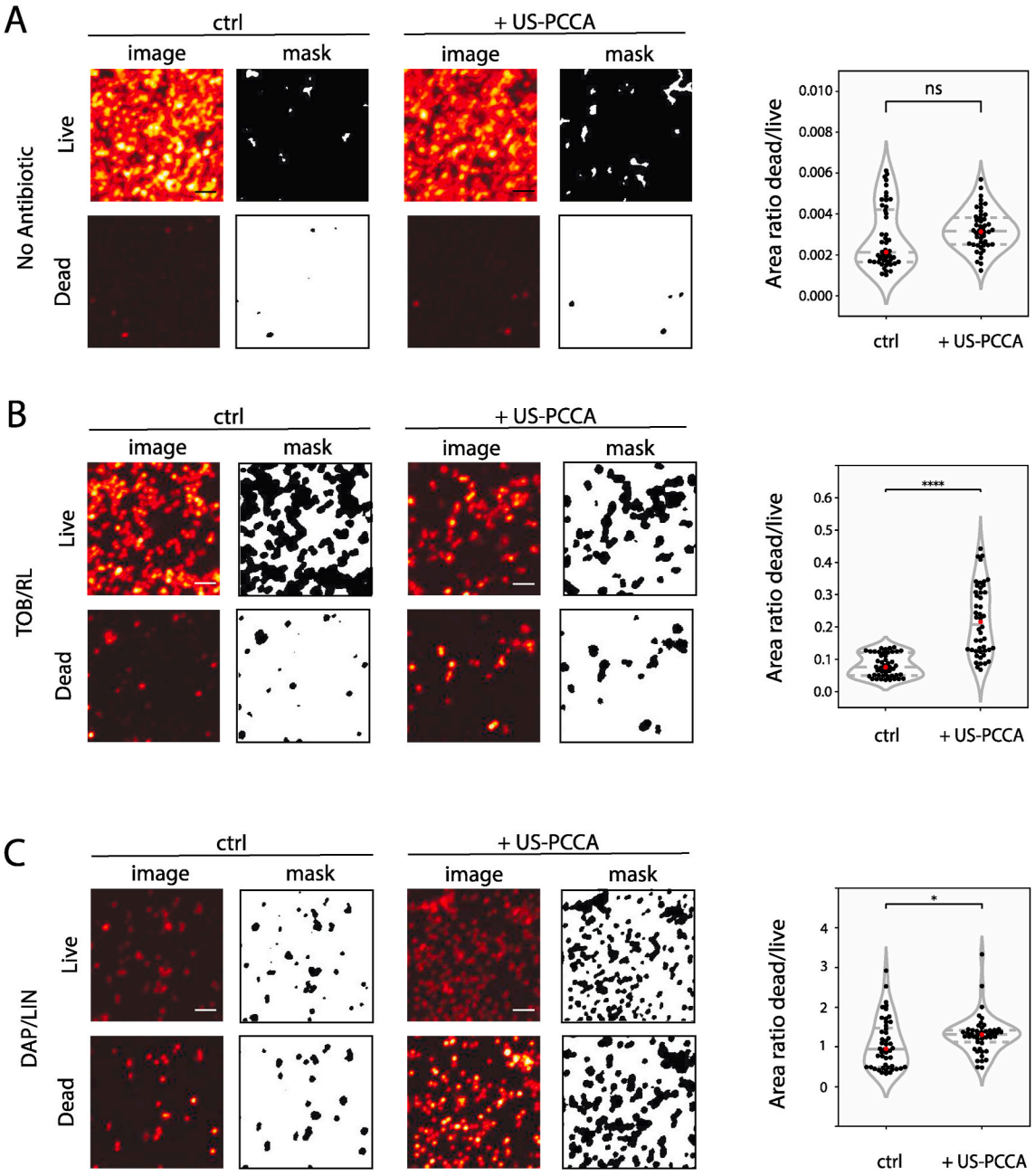

Fig. 4. US-PCCA in combination with anti-persister drugs reduce viability of MRSA biofilms. Biofilm viability assay in no antibiotic condition (a) or treated with TOB/RL (b) or DAP/LIN (c) with and without the exposure to ultrasound at 600 kPa. Upper rows show the biofilms stained with SYTO 9 representing live (total) bacteria present and their corresponding segmentation masks (black: areas covered by bacteria), while lower rows show dead bacteria within the biofilms and their segmentation masks. Scale bars indicate 5 μm. Violin and swarm plots represent the distribution of areas occupied by dead/live bacteria in independent fields of view within the biofilms (n = 16 fields for each condition from 3 biological replicates each). Statistical significance of the difference between pairs was evaluated using a Student's two-sided *t*-test. *, **** denotes $P < 0.05$, $P < 0.0001$, respectively. TOB, 58 μg/ml tobramycin; RL, 30 μg/ml rhamnolipids; DAP, 100 μg/ml daptomycin; LIN, 15 μg/ml linezolid; ns, not significant; US-PCCA, ultrasound-stimulated phase change contrast agents; ctrl, control. Representative images (~4% of the area in the center) of the fields of view with values closest to the condition medians were chosen for presentation and are indicated in the swarm plots by a red point. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

covered by dead (propidium iodide positive) and total bacteria (SYTO9-positive). US-PCCA had no impact on the viability of an untreated biofilm but significantly decreased viability of the cells within biofilms treated with the anti-persister therapies tobramycin combined with rhamnolipids (TOB/RL) and daptomycin combined with linezolid (DAP/LIN) (Fig. 4). Together this data indicates that anti-persister drugs have potent anti-biofilm activity and this can be potentiated further by improving penetration using US-PCCA.

Conclusions

S. aureus biofilms rarely resolve with antibiotic treatment alone and usually require surgical intervention (debridement, drainage, incision) [57]. Many antibiotics reduce bacterial burdens within biofilms but eradication represents an arduous challenge even in vitro [5,15]. In this study, we combine two anti-biofilm strategies to improve therapy against biofilms (Fig. 5). Targeting biofilms with anti-persister drugs increases efficacy compared to conventional antibiotics (Fig. 4a). Biofilm killing by conventional antibiotics with impeded penetration is improved by US-PCCA (Fig. 2b and c, Fig. 5), highlighting the therapeutic potential. US-PCCA combined with anti-persister therapies further improves biofilm killing in vitro (Figs. 3–5). Although the clinical relevance of this strategy is not yet known, targeting two of the main drivers of biofilm antibiotic tolerance concurrently (metabolically indolent persister cells and poor drug penetration) leads to a biofilm with drastically reduced biomass and viable cells, which may facilitate subsequent immune clearance in vivo.

Antibiotic treatment failure is a complex issue that imposes a heavy burden on global public health. The last new class of antibiotics to be approved by the FDA was in 2003 [58]. Unlike drugs for chronic illnesses that are administered for life (e.g. heart disease, diabetes), antibiotic regimens are comparatively short, rendering the profitability of antibiotic development low [59]. The void in the drug discovery pipeline makes sensitizing recalcitrant bacterial populations to already approved therapeutics a promising approach. The use of ultrasound and cavitation-enhancing agents for antibacterial applications, recently termed “sonobactericide”, was first published in 2011 [60]. While the field is still developing, a significant prospect of therapeutic ultrasound as a mechanical approach to enhance drug efficacy is its compatibility with any molecular therapeutic.

Microbubble oscillation has been shown to cause discrete morphologic changes in a *P. aeruginosa* biofilm [61]. Disruption of the physical structure of the biofilm may increase penetration depth of molecules which would otherwise be impeded. Disruption of the biofilm may have other indirect effects on drug efficacy. For example, bacterial biofilms are often hypoxic due to the diffusional distance limit of oxygen. Creating holes in the biofilm may allow oxygen penetration and stimulate the metabolic state of the residing persister cells, rendering them sensitive to antibiotics. In support of this, ultrasound in combination with microbubbles has previously been reported to alter the metabolic state of bacterial biofilms [61,62].

We hypothesized that PCCAs may be more efficient than microbubbles at penetrating biofilms due to their relatively small size and increased stability. PCCA have been shown to enhance cavitation erosion of blood clots for example, as they are able to penetrate and cause internal erosion in the middle of bovine clot samples from nanodroplet-mediated sonothrombolysis, whereas microbubble-mediated ultrasound generated only surface erosion [33]. PCCA enhanced penetration into the biofilm matrix may therefore enhance the disruption of the biofilm matrix under ultrasound cavitation. The use of US-PCCAs has previously shown to increase vancomycin killing of MRSA biofilms [63]. In contrast to the current study, Hu et al. used perfluoropentane as the perfluorocarbon core, which requires higher pressures than octofluoropropane to vaporize. Even in the absence of an antibiotic, US-PCCA caused a significant reduction in biofilm matrix and metabolic activity measured by three-dimensional fluorescence imaging and resazurin [62]. The difference in quantification method makes comparison with the previous study difficult (we enumerated bacterial survivors), however our results demonstrate a significant improvement in efficacy using shorter treatment times (30 s vs 5 min). In addition, the low boiling point PCCAs used in the current study present the advantage that the same low-pressure ultrasound settings can be used for both ADV and subsequent microbubble cavitation. Indeed, this can be achieved with clinically available ultrasound hardware at pressures below the FDA set limits for diagnostic imaging. Additionally, PCCA formulation is a variant of FDA-approved ultrasound contrast microbubbles that have

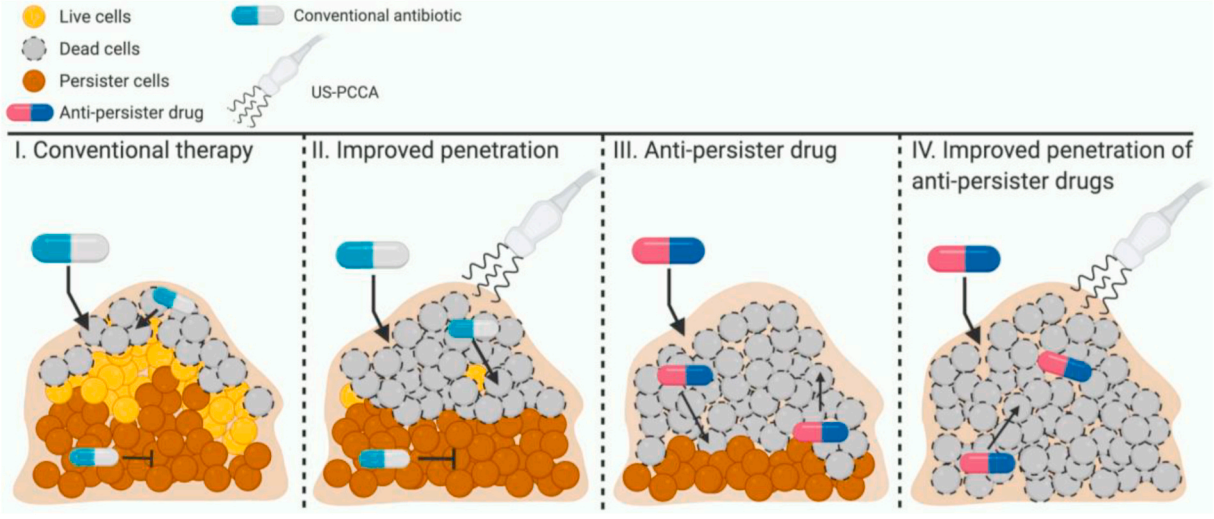

Fig. 5. Schematic representing a dual approach to improving antibiotic therapy against *S. aureus* biofilms. (I) Biofilms display remarkable tolerance to antibiotics. Susceptible cells at the biofilm periphery die (dead cells) while less metabolically active cells within the biofilm are tolerant to conventional antibiotics (persister cells). Failure to eradicate the biofilm leads to relapse in infection following removal of the antibiotic. (II) Improving penetration of conventional antibiotics using US-PCCA will improve efficacy of some conventional antibiotics that do not penetrate well through the biofilm matrix. This strategy is futile as it does not improve killing of persister cells. (III) Targeting biofilms with antibiotics which kill persister cells (anti-persister drug) improves efficacy but if drug penetration is impeded into the biofilm, some persister cells will remain following drug treatment and could contribute to relapsing infections. (IV) Improving penetration of anti-persister drugs into the biofilm could enhance biofilm killing and reduce relapse of infection following removal of the antibiotic. Schematic created with BioRender.com.

been clinically used for over 25 years in Europe, Asia and the USA. This approach may improve the efficacy of existing approved drugs without the additional need for the extensive regulatory approval which accompanies a new molecule. Likewise, as it uses ultrasound parameters that are achievable with clinically available equipment, this has the potential for rapid translation to clinical practice without the need for further technological development.

The ultrasound parameters used in our study mostly varied acoustic pressure and have not yet been optimized for in vivo application. While acoustic pressure is a large contributor to PCCA activation and stimulation, other parameters of frequency, duty cycle, treatment time and PCCA concentration could be further evaluated. The selected frequency of 1 MHz is lower than the predicted resonant frequency of the resulting microbubbles. However, optimal PCCA activation parameters and optimal microbubble oscillation parameters may not be the same and will require further investigation. Ultrasound is used clinically for debridement of wounds to disperse biofilms at frequencies below 1 MHz [64]. Interestingly, the lower frequency of 250 kHz was recently shown to enhance sonoporation due to large radial excursions of microbubbles well below their acoustic resonant frequency [65,66]. Evaluation of PCCA drug potentiation using lower frequencies and higher intensities typical for this application could give further insight into clinical integration strategies. Future experiments will evaluate the potentiation of antibiotics in a *S. aureus* mouse skin and soft tissue infection (SSTI). For topical applications such as soft tissue infections, we believe maintaining cavitation activity for the duration of the treatment will be crucial for efficacy, as no new cavitation nuclei will be introduced as would be the case in intravenously administered PCCA (replenished by blood flow).

Methods

Biofilm assays

Biofilm assays were performed using the USA300 MRSA strain LAC. It is a highly characterized community-acquired MRSA (CA-MRSA) strain isolated in 2002 from an abscess of an inmate in Los Angeles County jail in California [67]. LAC was cultured overnight (18h) in brain heart infusion (BHI) media (Oxoid) in biological triplicates. Each culture was diluted 1:150 in fresh media and 2 or 3 ml was added to the wells of 24-well or 12-well tissue culture treated plates (Costar), respectively. Biofilms were covered with Breathe-Easier sealing strips (Sigma) and incubated at 37 °C for 24h. Biofilms were carefully washed twice with PBS and fresh BHI media containing antibiotics was added. Biofilms were covered and incubated at 37 °C for 24 h. Biofilms were carefully washed twice with PBS before dispersal in a sonicating water bath (5min) and vigorous pipetting. Surviving cells were enumerated by serial dilution and plating. Antibiotics were added at concentrations similar to the C_{max} in humans; 10 μg/ml levofloxacin [68] (Alfa Aesar), 20 μg/ml gentamicin [69] (Fisher BioReagents), 58 μg/ml tobramycin [70] (Sigma), 50 μg/ml vancomycin hydrochloride [48] (MP Biomedicals), 15 μg/ml linezolid [71] (Cayman Chemical), 10 μg/ml rifampicin [72] (Fisher BioReagents), 100 μg/ml daptomycin [73] (Arcos Organics), with the exception of the topical antibiotic mupirocin (Sigma) (administered at 100 μg/ml) and acyldepsipeptide antibiotic (ADEP4) which was added at 10x MIC (10 μg/ml) which previously showed efficacy against *S. aureus* biofilms [19]. For daptomycin activity, the media was supplemented with 50 mg/L of Ca^{2+} ions. Where indicated tobramycin was supplemented with 30 μg/ml rhamnolipids [22] (50/50 mix of mono- and di-rhamnolipids, Sigma). Where indicated biofilms were treated with PCCA and ultrasound. For crystal violet staining, biofilms were carefully washed twice with PBS, and dried in a 65 °C oven for 1 h. Biofilms were stained with 1 ml 0.4% crystal violet for 5min, and washed 3x with PBS. Wells were photographed and stain was solubilized with 2 ml 5% acetic acid and absorbance measured at 570 nm.

PCCA generation

Phase change contrast agents were generated as previously reported [39,74]. Briefly, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene-glycol)-2000 (DSPE-PEG2000) (Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in 5% glycerol, 15% propylene glycol (both from Fisher Chemical, Waltham, MA, USA) in PBS (v/v) at a 1:9 ratio, to a total lipid concentration of 1 mg/ml. Lipid solution (1.5 ml) was dispensed into 3 ml crimp-top vials and degassed under vacuum for 30 min and then backfilled with octofluoropropane gas (Fluoro Med, Round Rock, TX, USA). The vials were activated by mechanical agitation (VialMix, Bristol-Myers-Squibb, New York, NY, USA) to generate micron scale octofluoropropane bubbles with a lipid coat. The vials containing bubbles were cooled in an ethanol bath to −13C. Pressurized nitrogen (45 PSI) was introduced by piercing the septa with a needle and used to condense the gaseous octofluoropropane into a liquid, creating lipid-shelled perfluorocarbon submicron droplets (PCCA). Particle size and concentration was characterized the Accusizer Nano FX (Entegris, Billerica, MA, USA).

Ultrasound experiments

Ultrasound experiments were conducted in 12 or 24 well tissue culture plates using a custom fabricated water bath ultrasound alignment setup to maintain 37 °C during the experiment, similar to a design used previously with cell monolayers (and the full design of this water bath can be found in the supplemental materials of that publication) [45]. Briefly, alignment guides were positioned above the wells to ensure reproducible transducer placement to the center of each well on top of the biofilm and 10 mm from their bottom. To limit acoustic reflections and standing waves from the bottom of the well plate, the plate was coupled to a water bath, the bottom of which was lined with acoustic absorber material. The water temperature was maintained at 37 °C throughout the experiment by placing the water bath setup on a heated plate and monitored by thermocouple. A 1.0 MHz unfocused transducer (IP0102HP, Valpey Fisher Corp) was characterized via needle hydrophone and driven with an amplified 20- or 40-cycle sinusoidal signal defined on an arbitrary function generator (AFG3021C, Tektronix, Inc.; 3100LA Power Amplifier, ENI) at a pulse-repetition frequency of 5000 Hz (10% or 20% duty cycle). Peak negative pressures of 300, 600, 900 and 1200 kPa were used in the experiments. Previous experiments using octofluoropropane PCCAs at these pressures demonstrated that higher pressures significantly reduced stable and inertial cavitation persistence over a 30-s exposure whereas lower pressures sustained cavitation activity [45], indicating inertial cavitation at high pressures and a subsequent reduction of cavitation nuclei due to bubble destruction. To avoid ultrasound-alone effects on the biofilm, we focused on the lower pressures, 300 and 600 kPa, determined most effective at potentiating tobramycin efficacy with PCCA and lowered the duty cycle from 20% in Figs. 2b to 10% for subsequent figures, as this was shown to have a more modest effect in our prior work and resulted in significant drug delivery [45]. Where indicated, 10 μl of PCCA was added to each well ($(1.17 \pm 0.4) \times 10^{11}$ particles/mL, 0.18 μm diameter) and mixed gently by pipetting. The transducer was positioned in the well in the media above the biofilm and ultrasound treatment was applied for 30 s. Following treatment, each plate was incubated at 37 °C for 24 h before enumerating survivors (described in detail above).

Microscopy

Biofilms were cultured in 24-well plates and treated with antibiotics and US-PCCA as described above. Following 24 h of antibiotic therapy, biofilms were washed in 0.85% NaCl and stained with LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit, for microscopy & quantitative assays (Invitrogen) for 15 min in the dark. Biofilms were washed gently in PBS

and submerged in 0.5 ml PBS for imaging. Images were acquired on a Zeiss LSM 700 confocal microscope, using an LD Plan Neofluar 40X/0.6 DIC II objective, with the correction collar set to 1.0. The "live" stain was acquired with a 488 nm laser, with a 490–555 nm band pass emission filter. The "dead" stain was acquired with a 555 nm laser, with a 615 nm long pass emission filter. The multiple beam splitter position was set to 615 nm, and the microscope was operated in line-switching mode. A transmitted light image was acquired simultaneously in the 555 nm channel. For each channel, the laser power, conventional PMT master gain and digital offset were adjusted to ensure no pixels had a value of 0, and no pixels were saturated (saturation value 4095). The pinhole was set to 1 AU for the longest wavelength fluorophore (the "dead" stain), and its diameter in um was kept constant in the other channel. Images were taken with zoom set to 1.0X, 1024 × 1024 pixels, for a pixel size of 0.156 μm. Images were averaged 4 times in line mode and unidirectional laser scanning was used. A field of 4 by 4 images was acquired centered roughly in the middle of each well, using tilescan mode without overlap. Because of imperfections in stage movement, some images overlapped slightly with their neighbors; we cropped 3.5% of each image border to avoid measuring any cells twice in our analysis. The Z plane selected for imaging was the one with the maximal number of cells, which was typically the Z plane in the sample closest to the bottom of the well. All images were acquired the same day, with the same settings. Controls with unstained samples showed that with these settings autofluorescence from bacteria or biofilms was undetectable.

Live/dead quantification

Quantification of the bacteria viability from confocal images was performed using Python (3.8.3) with Numpy (1.18.5), Pandas (1.0.5), Skimage (0.16.2) and Seaborn (0.10.1) libraries. Each field within a tiled scan was considered an independent image. For each condition, three biological replicates have been imaged in sixteen fields of view (total 48 images for each condition). Images in both channels were smoothed using a gaussian filter (sigma = 1) and segmented with a global threshold (100 a.u.). The viability of the biofilm was defined as a ratio between the area (or total fluorescent signal above the threshold level) covered by dead (propidium iodide positive) and live/total bacteria (SYTO9-positive). A Student's two-sided *t*-test was performed as implemented in Python Scipy (1.5.0) stats.ttest_ind to compare control and ultrasound conditions. We observed the same results using a range of relevant threshold values as well as comparing the integrated intensity ratios above the threshold in both channels.

Statistical information

The averages of $n = 3$ or $n = 6$ biologically independent samples are shown (as indicated in the figure legends). The error bars represent the standard deviation of the mean. Statistical analysis was performed using Prism 8 (GraphPad) software. One-way ANOVA with Sidak's or Dunnett's multiple comparison test (as indicated in the figure legends). Statistical significance was defined as $P < 0.05$.

Data availability

Additional data that support the findings of this study are available from the corresponding authors, Virginie Papadopoulou (papadopoulou@unc.edu) and Sarah E. Rowe (seconlon@email.unc.edu), upon request.

CRediT authorship contribution statement

Phillip G. Durham: Conceptualization, Methodology, Visualization, Writing – original draft. **Ashelyn E. Sidders:** Visualization, Writing – review & editing. **Jenna E. Beam:** Visualization, Methodology. **Katarzyna M. Kedziora:** Methodology, Visualization, Writing – review & editing. **Paul A. Dayton:** Writing – review & editing. **Brian P. Conlon:** Writing – review & editing. **Virginie Papadopoulou:** Conceptualization, Methodology, Visualization, Writing – original draft. **Sarah E. Rowe:** Conceptualization, Methodology, Visualization, Writing – original draft.

Declaration of competing interest

P.A.D declares that he is a co-inventor on a patent describing the formulation of low boiling-point perfluorocarbon agents and a cofounder of Triangle Biotechnology, a company that has licensed this patent. P.G.D, P.A.D., B.P.C., V.P. and S.E.R. are all co-inventors on a provisional patent describing the use of low boiling-point phase change contrast agents for enhancing the delivery of therapeutics agents to biofilms. B.P.C and S.E.R are co-inventors on a provisional patent describing the use of rhamnolipids for potentiating antibiotic efficacy. A. E.S, J.E.B. and K.M.K declare no competing interests.

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