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1 **Harnessing Ultrasound-Stimulated Phase Change Contrast Agents to Improve Antibiotic**
2 **Efficacy Against Methicillin-Resistant *Staphylococcus aureus* Biofilms**
3
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18 Bacterial biofilms, often associated with chronic infections, respond poorly to antibiotic
19 therapy and frequently require surgical intervention. Biofilms harbor persister cells, metabolically
20 indolent cells, which are tolerant to most conventional antibiotics. In addition, the biofilm matrix
21 can act as a physical barrier, impeding diffusion of antibiotics. Novel therapeutic approaches
22 frequently improve biofilm killing, but usually fail to achieve eradication. Failure to eradicate the
23 biofilm leads to chronic and relapsing infection, associated with major financial healthcare costs
24 and significant morbidity and mortality. We address this problem with a two-pronged strategy
25 using 1) antibiotics that target persister cells and 2) ultrasound-stimulated phase-change
26 contrast agents (US-PCCA) , which improve antibiotic penetration.
27 We previously demonstrated that rhamnolipids, produced by *Pseudomonas aeruginosa*,
28 could induce aminoglycoside uptake in gram-positive organisms, leading to persister cell death.
29 We have also shown that US-PCCA can transiently disrupt biological barriers to improve
30 penetration of therapeutic macromolecules. We hypothesized that combining antibiotics which
31 target persister cells with US-PCCA to improve drug penetration could eradicate methicillin
32 resistant *S. aureus* (MRSA) biofilms. Aminoglycosides alone or in combination with US-PCCA
33 displayed limited efficacy against MRSA biofilms. In contrast, the anti-persister combination of
34 rhamnolipids and aminoglycosides combined with US-PCCA dramatically reduced biofilm
35 viability, frequently culminating in complete eradication of the biofilm. These data demonstrate
36 that biofilm eradication can be achieved using a combined approach of improving drug
37 penetration of therapeutics that target persister cells.
38
39 **Introduction**
40 *S. aureus* is one of the most important human bacterial pathogens and in 2017 was the
41 cause of 20,000 bacteremia deaths in the US alone[1]. Infections range from minor skin and soft
42 tissue infections (SSTI), implanted device infections to more serious infections such as
43 osteomyelitis, endocarditis and pneumonia [2,3]. In addition to the high degree of mortality, chronic
44 and relapsing *S. aureus* infections are common and associated with significant morbidity. This is

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45 due to frequent treatment failure of *S. aureus* infections. This is best illustrated by SSTIs, with
46 some studies suggesting treatment failure rates as high as 45% and a recurrence rate of 70% [4].
47 Importantly the failure of antibiotic therapy cannot be adequately explained by antibiotic
48 resistance [1]. Failure to clear the infection leads to a need for prolonged antibiotic therapies,
49 increased morbidity and mortality, increased likelihood of antibiotic resistance development as
50 well as an enormous financial healthcare burden.
51 *S. aureus* forms biofilms, bacterial cells embedded in a self-produced extracellular
52 matrix, which act as a protective barrier from the host immune response and other
53 environmental assaults. Biofilms expand up to 1200µm in thickness when attached to indwelling
54 devices such as catheters [5]. Non-surface attached biofilms, in chronic wounds and chronic lung
55 infections, harbor smaller non-surface attached cell aggregates ranging from 2-200µm in
56 diameter [5,6]. These biofilm aggregates are often surrounded by inflammatory immune cells such
57 as neutrophils and embedded in a secondary host produced matrix such as mucus, pus or
58 wound slough [7]. Consequently, biofilm-embedded cells have limited access to nutrients and
59 oxygen and are coerced into a metabolically indolent state [8].
60 It has long been appreciated that biofilms respond poorly to antibiotics [7,9-12]. Most
61 conventional bactericidal antibiotics kill by corrupting ATP-dependent cellular processes;
62 aminoglycosides target translation, fluoroquinolones target DNA synthesis, rifampicin targets
63 transcription and β-lactams and glycopeptides target cell wall synthesis [13,14]. Cells that survive
64 lethal doses of antibiotics in the absence of a classical resistance mechanism are called
65 antibiotic tolerant persister cells [15]. Biofilms are made up of a high proportion of persister cells [15-
66 18]. They are distinct from resistant cells as they cannot grow in the presence of the drug.
67 However, once the drug is removed, persisters grow and repopulate a biofilm and cause a
68 relapse in infection [13]. Anti-persister antibiotics which kill independently of the metabolic state of
69 the cell are more effective against biofilms than conventional antibiotics [19-22]. Tobramycin, an
70 aminoglycoside that requires active proton motive force (PMF) for uptake into the cell is inactive
71 against non-respiring cells, anaerobically growing cells, small colony variants and metabolically
72 inactive cells within a biofilm [20]. We previously reported that rhamnolipids, biosurfactants
73 produced by *P. aeruginosa,* permeabilize the *S. aureus* membrane to allow PMF-independent
74 diffusion of tobramycin into the cell [20,22]. This combination of tobramycin and rhamnolipids
75 (TOB/RL) rapidly sterilized in vitro planktonic cultures as well as non-respiring cells,
76 anaerobically growing cells and small colony variants. However, despite this potent anti-
77 persister activity, TOB/RL reduced biofilm viability by ~3-logs but failed to achieve eradication [20].
78 Notwithstanding the promise of this strategy, eradication of biofilms is arduous, even in vitro,
79 indicating that factors other than the metabolic state of the biofilm-embedded cells are impeding
80 therapy.
81 The biofilm matrix can act as a physical barrier to drug penetration. Penetration of
82 oxacillin, vancomycin, cefotaxime, chloramphenicol and aminoglycoside antibiotics are impeded
83 to some extent into *S. aureus* biofilms [23-25]. Consequently, novel methods of drug delivery into
84 biofilms is a growing area of interest. Ultrasound is a safe, commonplace, portable and relatively
85 inexpensive modality typically used in medical imaging. This imaging capability has been
86 expanded through the use of intravenously administered microbubbles as a contrast agent.
87 These microbubbles are also used in a growing number of therapeutic applications to enhance

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88 biological effects, which include transdermal drug delivery [26] and transient permeabilization of
89 the blood brain barrier [27].
90 When exposed to an ultrasound wave, gas-filled microbubbles in solution will oscillate,
91 with the positive pressure cycle resulting in compression and the negative pressure cycle
92 causing the bubble to expand. In an ultrasound field, microbubbles experience stable cavitation
93 (continuous expansion and contraction) at lower pressures or inertial cavitation (violent collapse
94 of the bubble) at higher pressures [28]. Stable cavitation results in microstreaming; fluid
95 movement around the bubble which induces shear stress to nearby structures (such as
96 biofilms). At higher pressures, inertial cavitation can result in a shockwave, producing high
97 temperatures at a small focus, and create microjets from the directional collapse of the bubble
98 which can puncture host cells and disrupt physical barriers [29]. Both of these pressure regimes
99 have potential for therapeutic applications of ultrasound-mediated microbubble cavitation.
100 Despite the potential of microbubbles to enhance drug delivery, their size (typically 1-4 micron in
101 diameter) and short half-life once injected into solution may limit penetration and subsequent
102 disruption of biofilms.
103 We hypothesized that phase change contrast agents (PCCA), submicron liquid particles
104 (typically 100-400 nanometers in diameter) may be better equipped to penetrate a biofilm.
105 PCCAs generally consist of a liquid perfluorocarbon droplet stabilized by a phospholipid shell.
106 With appropriate ultrasound stimulation, PCCA can convert from the liquid phase to gas,
107 generating a microbubble in their place (Fig. 1a). This process of “acoustic droplet vaporization”
108 (ADV) may enhance drug penetration into biofilms as microbubbles over-expand before
109 reaching their final diameter. Prior to activation, these particles are significantly more stable than
110 microbubbles, with the potential to diffuse into biofilms due to their small size (Fig. 1b).
111 Additionally, with continued ultrasound application, the resulting microbubbles can generate
112 microstreaming, shear stress and microjets as they undergo cavitation (Fig. 1b). We
113 hypothesized that PCCA, in combination with ultrasound (US-PCCA) and antibiotics that target
114 persister cells is a novel biofilm eradication strategy.
115
116 **Results**
117 We first identified drugs with efficacy against biofilms. Antibiotics were chosen based on
118 clinical relevance or previously reported anti-biofilm efficacy in vitro. Mature MRSA biofilms
119 (USA300 LAC) were cultured for 24h in tissue culture treated plates before the addition of
120 antibiotics. Following 24h of drug treatment, biofilms were washed and survivors were
121 enumerated by plating. Tobramycin, mupirocin, vancomycin, and linezolid all caused a
122 significant reduction in surviving biofilm cells (Fig. 2a). In contrast, levofloxacin and gentamicin
123 showed no efficacy against biofilms at clinically achievable concentrations found in serum (C_{max})
124 [24,25] (Fig. 2a).
125 Next, we tested the ability of 30 second (s) US-PCCA treatment to potentiate tobramycin
126 efficacy. Previous studies have indicated that negatively charged components of the biofilm
127 matrix such as extracellular DNA and certain components of polysaccharides impede
128 penetration of positively charged aminoglycosides such as tobramycin [25,30,31]. We hypothesized
129 that US-PCCA might improve tobramycin penetration into biofilms and increase its efficacy.
130 Mature biofilms were washed and transferred to a custom-built temperature-controlled 37°C
131 water bath alignment setup. Tobramycin and PCCAs were added and ultrasound applied at a

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132 range of rarefactional pressures (300-1200kPa). We found that tobramycin efficacy was
133 significantly enhanced at pressures of 300, 600 and 1200 but not 900KPa in the presence of
134 PCCAs (Fig. 2b-c). We confirmed that the addition of PCCA in the absence of ultrasound had
135 no impact on biofilm viability. Similarly, we anticipated that ultrasound alone, in the absence of
136 PCCA would be ineffective, however 1200 kPa did cause a small but significant reduction in
137 surviving cells in the absence of PCCA (Fig. 2b), indicating that potentiation seen at the highest
138 pressure (1200kPa) may not be entirely attributable to PCCA activity. In order to investigate the
139 potentiation effects of PCCA specifically in the regime below ultrasound-alone effects, the
140 higher pressures (900 and 1200kPa) were not evaluated further and the duty cycle lowered to
141 10% for subsequent experiments. The lower pressures, 300 and 600kPa, in combination with
142 PCCA were determined to be most effective at potentiating tobramycin efficacy. This is
143 consistent with our previous findings where lower pressures (above the ADV threshold) resulted
144 in more persistent cavitation activity during a 30s ultrasound exposure and was consistently
145 greatest at macromolecule drug delivery across colorectal adenocarcinoma monolayers [32].
146 Next, we tested the ability of US-PCCA to potentiate mupirocin, vancomycin and
147 linezolid/rifampicin. Mupirocin is a carboxylic acid topical antibiotic commonly used to treat *S.*
148 *aureus* infections that binds to the isoleucyl-tRNA and prevents isoleucine incorporation into
149 proteins [33]. US-PCCA caused a very slight increase in mupirocin killing (41% increase in killing)
150 that was statistically significant but of questionable biological significance (Fig. 2a-b).
151 Vancomycin is a glycopeptide that is the frontline antibiotic to treat MRSA infections.
152 This antibiotic acts by binding to the D-Ala-D-ala residues of the membrane bound cell wall
153 precursor, lipid II, preventing its incorporation and stalling active peptidoglycan synthesis [34].
154 Importantly, some studies have indicated that vancomycin penetration is impeded into biofilms
155 [24]. US-PCCA potentiated vancomycin killing of biofilm-associated cells by 93% (Fig. 3a-b), likely
156 by improving penetration. Notably, potentiation of vancomycin was seen with the C_{max} [35]
157 indicating that at a clinically relevant concentration, US-PCCA has the capacity to improve
158 biofilm killing of the front-line antibiotic used to treat MRSA infections.
159 Linezolid is an oxazolidinone protein synthesis inhibitor that is sometimes combined with
160 the transcriptional inhibitor, rifampicin, for the treatment of *S. aureus* infections [36,37].
161 Linezolid/rifampicin reduced viable cells within the biofilm by almost 3-logs but was not
162 significantly potentiated by US-PCCA. This suggests that US-PCCA has the ability to potentiate
163 some conventional antibiotics but not others. It is possible that US-PCCA does not potentiate
164 the killing of mupirocin and linezolid/rifampicin because the penetration of these drugs is not
165 impeded into biofilms.
166 Although the increased killing of biofilm-associated cells with conventional antibiotics
167 shows promise, we hypothesized that regardless of penetration, antibiotic tolerant persister cells
168 in the biofilm are surviving and thus impeding biofilm eradication. We predicted that utilizing US-
169 PCCA to increase penetration of drugs active against antibiotic tolerant persister cells could
170 achieve eradication of a biofilm.
171 Daptomycin is a lipopeptide antibiotic which inserts into the cell membrane and disrupts
172 fluid membrane microdomains [38]. Daptomycin has potent activity against recalcitrant
173 populations of *S. aureus,* including biofilms [39,40]. Daptomycin in combination with linezolid
174 (DAP/LIN) is the treatment recommended for persistent MRSA bacteremia or vancomycin
175 failure in the Infectious Diseases Society of America 2011 MRSA treatment guidelines [41]. We

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176 found that US-PCCA increased DAP/LIN killing of MRSA biofilms by 83% and 90% at 300kPa
177 and 600kPa, respectively (Fig. 4a-b).
178 Next, we wanted to investigate if US-PCCA could improve efficacy of other drugs with
179 anti-persister activity. Acyldepsipeptides (ADEPs) are activators of the ClpP protease. We
180 previously reported that ADEPs sterilize persisters by activating the ClpP protease and causing
181 the cell to self-digest in an ATP-independent manner [19]. ADEP in combination with rifampicin
182 reduced biofilm cells by >4-logs in 24h. US-PCCA significantly potentiated efficacy of ADEP/RIF
183 at 300kPa but not 600kPa (Fig. 4a-b).
184 Tobramycin combined with rhamnolipids (TOB/RL), has potent anti-persister activity and
185 has eradiated several recalcitrant populations including non-respiring cells, anaerobically
186 growing cells and small colony variants [20]. Despite this potent anti-persister activity, TOB/RL
187 only reduced biofilm viability by ~3-logs and failed to achieve biofilm eradication [20]. We
188 reasoned that drug penetration might be inhibited into the biofilms and hypothesized that
189 improving penetration could lead to biofilm eradication. Applying US-PCCA in combination with
190 TOB/RL increased killing of biofilm cells by 96% and 85% at 300kPa and 600kPa, respectively
191 (Fig. 4a-b). Importantly, half of the biological replicates were eradicated to the limit of detection.
192 Together this data indicates that anti-persister drugs have potent anti-biofilm activity and this
193 can be potentiated further by improving penetration using US-PCCA.
194
195 **Discussion**
196 *S. aureus* biofilms rarely resolve with antibiotic treatment alone and usually require
197 surgical intervention (debridement, drainage, incision) [42]. Many antibiotics reduce bacterial
198 burdens within biofilms but eradication represents an arduous challenge even in vitro [5,15]. In this
199 study, we combine two anti-biofilm strategies to achieve eradication (Fig. 5). Biofilm killing by
200 conventional antibiotics with impeded penetration is improved by US-PCCA (Fig.2b-c, Fig.3a-b,
201 Fig. 5), highlighting the therapeutic potential despite falling short of eradication. Targeting
202 biofilms with anti-persister drugs increases efficacy compared to conventional antibiotics but
203 fails to eradicate (Fig. 4a). Combining these approaches greatly improves biofilm killing, and in
204 some cases resulted in eradication of the biofilm (Fig. 4a, Fig. 5).
205 Antibiotic treatment failure is a complex issue that imposes a heavy burden on global
206 public health. The last new class of antibiotics to be approved by the FDA was in 2003 [43]. Unlike
207 drugs for chronic illnesses that are administered for life (e.g. heart disease, diabetes), antibiotic
208 regimens are comparatively short, rendering the profitability of antibiotic development low [44].
209 The void in the drug discovery pipeline makes sensitizing recalcitrant bacterial populations to
210 already approved therapeutics a promising approach. The use of ultrasound and cavitation-
211 enhancing agents for antibacterial applications, recently termed "sonobactericide", was first
212 published in 2011 [45]. While the field is still developing, a significant prospect of therapeutic
213 ultrasound as a mechanical approach to enhance drug efficacy is its compatibility with any
214 molecular therapeutic.
215 Microbubble oscillation has been shown to cause discrete morphologic changes in a *P.*
216 *aeruginosa* biofilm [46]. Disruption of the physical structure of the biofilm may increase penetration
217 depth of molecules which would otherwise be impeded. Disruption of the biofilm may have other
218 indirect effects on drug efficacy. For example, bacterial biofilms are often hypoxic due to the
219 diffusional distance limit of oxygen. Creating holes in the biofilm may allow oxygen penetration

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220 and stimulate the metabolic state of the residing persister cells, rendering them sensitive to
221 antibiotics. In support of this, ultrasound in combination with microbubbles has previously been
222 reported to alter the metabolic state of bacterial biofilms [46,47].
223 We hypothesized that PCCAs may be more efficient than microbubbles at penetrating
224 biofilms due to their relatively small size and increased stability. The use of US-PCCAs has
225 previously shown to increase vancomycin killing of MRSA biofilms [48]. In contrast to the current
226 study, Hu et al. used perfluoropentane as the perfluorocarbon core, which requires higher
227 pressures than octofluoropropane to vaporize. Even in the absence of an antibiotic, US-PCCA
228 caused a significant reduction in biofilm matrix and metabolic activity measured by three-
229 dimensional fluorescence imaging and resazurin [47]. The difference in quantification method
230 makes comparison with the previous study difficult (we enumerated bacterial survivors),
231 however our results demonstrate a significant improvement in efficacy using shorter treatment
232 times (30 s vs 5 minutes)[49]. In addition, the low boiling point PCCAs used in the current study
233 present the advantage that the same low-pressure ultrasound settings can be used for both
234 ADV and subsequent microbubble cavitation. Indeed, this can be achieved with clinically
235 available ultrasound hardware at pressures below the FDA set limits for diagnostic imaging.
236 Additionally, PCCA formulation is a variant of FDA-approved ultrasound contrast microbubbles
237 that have been clinically used for over 25 years in Europe, Asia and USA. This approach may
238 improve the efficacy of existing approved drugs without the additional need for the extensive
239 regulatory approval which accompanies a new molecule. Likewise, as it uses ultrasound
240 parameters that are achievable with clinically available equipment, this has the potential for
241 rapid translation to clinical practice without the need for further technological development.
242 The ultrasound parameters used in our study mostly varied acoustic pressure and have
243 not yet been optimized for in vivo application. While acoustic pressure is a large contributor to
244 PCCA activation and stimulation, other parameters of frequency, duty cycle, treatment time and
245 PCCA concentration could be further evaluated. Ultrasound is also used clinically for
246 debridement of wounds to disperse biofilms [50]. Evaluation of PCCA drug potentiation using
247 lower frequencies and higher intensities typical for this application could give further insight into
248 clinical integration strategies. Future experiments will evaluate the potentiation of antibiotics in a
249 *S. aureus* mouse skin and soft tissue infection (SSTI). For topical applications such as soft
250 tissue infections, we believe maintaining cavitation activity for the duration of the treatment will
251 be crucial for efficacy, as no new cavitation nuclei will be introduced as would be the case in
252 intravenously administered PCCA (replenished by blood flow).
253
254 **Methods**
255 **Biofilm assays**
256 Biofilm assays were performed using the USA300 MRSA strain LAC. It is a highly characterized
257 community-acquired MRSA (CA-MRSA) strain isolated in 2002 from an abscess of an inmate in
258 Los Angeles County jail in California [51]. LAC was cultured overnight (18h) in brain heart infusion
259 (BHI) media (Oxoid) in biological triplicates. Each culture was diluted 1:150 in fresh media and
260 2-3ml was added to the wells of 24-well or 12-well tissue culture treated plates (Costar),
261 respectively. Biofilms were covered with Breathe-Easier sealing strips (Sigma) and incubated at
262 37ºC for 24h. Biofilms were carefully washed twice with PBS and fresh BHI media containing
263 antibiotics was added. Biofilms were covered and incubated at 37ºC for 24h. Biofilms were

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264 carefully washed twice with PBS before dispersal in a sonicating water bath (5min) and vigorous
265 pipetting. Surviving cells were enumerated by serial dilution and plating. Antibiotics were added
266 at concentrations similar to the C_{max} in humans; 10μg/ml levofloxacin[52] (Alfa Aesar), 20μg/ml
267 gentamicin[53] (Fisher BioReagents), 58μg/ml tobramycin[54] (Sigma), 50μg/ml vancomycin
268 hydrochloride[35] (MP Biomedicals), 15μg/ml linezolid[55] (Cayman Chemical), 10μg/ml rifampicin[56]
269 (Fisher BioReagents), 100μg/ml daptomycin[57] (Arcos Organics), with the exception of the topical
270 antibiotic mupirocin (Sigma) (administered at 100μg/ml) and acyldepsipeptide antibiotic
271 (ADEP4) which was added at 10x MIC (10μg/ml) which previously showed efficacy against *S.*
272 *aureus* biofilms[19]. For daptomycin activity, the media was supplemented with 50mg/L of Ca^{2+}
273 ions. Where indicated tobramycin was supplemented with 30μg/ml rhamnolipids[22] (50/50 mix of
274 mono- and di-rhamnolipids, Sigma). Where indicated biofilms were treated with PCCA and
275 ultrasound.
276
277 **PCCA Generation**
278 Phase change contrast agents were generated as previously reported [58] [Sheeran et al. 2012].
279 Briefly, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-
280 phosphoethanolamine-N-methoxy(polyethylene-glycol)-2000 (DSPE-PEG2000) (Avanti Polar
281 Lipids, Alabaster, AL, USA) were dissolved in 5% glycerol, 15% propylene glycol (both from
282 Fisher Chemical, Waltham, MA, USA) in PBS (v/v) at a 1:9 ratio, to a total lipid concentration of
283 1 mg/ml. Lipid solution (1.5ml) was dispensed into 3ml crimp-top vials and degassed under
284 vacuum for 30 minutes and then backfilled with octofluoropropane (OFP) gas (Fluoro Med,
285 Round Rock, TX, USA). The vials were activated by mechanical agitation (VialMix, Bristol-
286 Myers-Squibb, New York, NY, USA) to generate micron scale OFP bubbles with a lipid coat.
287 The vials containing bubbles were cooled in an ethanol bath to -11C. Pressurized nitrogen (45
288 PSI) was introduced by piercing the septa with a needle and used to condense the gaseous
289 octofluoropropane into a liquid, creating lipid-shelled perfluorocarbon submicron droplets
290 (PCCA). Particle size and concentration was characterized the Accusizer Nano FX (Entegris,
291 Billerica, MA, USA).
292
293 **Ultrasound Experiments**
294 Ultrasound experiments were conducted in 12 or 24 well tissue culture plates using a custom
295 fabricated water bath ultrasound alignment setup to maintain 37C during the experiment, similar
296 to a design used previously with cell monolayers [32]. Briefly, alignment guides were positioned
297 above the wells to ensure reproducible transducer placement to the center of each well on top
298 of the biofilm and 10mm from their bottom. To limit acoustic reflections and standing waves from
299 the bottom of the well plate, ultrasound gel was applied on the outside bottom of each well
300 before the plate was positioned in the water bath, the bottom of which was lined with acoustic
301 absorber material. The water temperature was maintained at 37C throughout the experiment by
302 placing the water bath setup on a heated plate and monitored by thermocouple. A 1.0MHz
303 unfocused transducer (IP0102HP, Valpey Fisher Corp) was characterized via needle
304 hydrophone and driven with an amplified 20- or 40-cycle sinusoidal signal defined on an
305 arbitrary function generator (AFG3021C, Tektronix, Inc.; 3100LA Power Amplifier, ENI) at a
306 pulse-repetition frequency of 5000 Hz (10% or 20% duty cycle). Peak negative pressures of
307 300, 600, 900 and 1200kPa were used in the experiments. To avoid ultrasound-alone effects on

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308 the biofilm, we focused on the lower pressures, 300 and 600kPa, determined most effective at
309 potentiating tobramycin efficacy with PCCA and lowered the duty cycle from 20% in Fig. 1 to
310 10% in Fig. 2-3 as this was shown to have a more modest effect in our prior work and resulted
311 in significant drug delivery [32]. Where indicated, 10µl of PCCA was added to each well ((1.17 ±
312 0.4) x10^{11} particles / mL, 0.18 µm diameter) and mixed gently by pipetting. The transducer was
313 positioned in the well in the media above the biofilm and ultrasound treatment was applied for
314 30 seconds. Following treatment, each plate was incubated at 37C for 24h before enumerating
315 survivors (described in detail above).
316

317 **Statistical information**

318 The averages of n = 3 biologically independent samples are shown. The error bars represent
319 the standard deviation of the mean. Statistical analysis was performed using Prism 8
320 (GraphPad) software. One-way ANOVA with Sidak's or Dunnett's multiple comparison test (as
321 indicated in the figure legends). Statistical significance was defined as $P < 0.05$.
322

323 **Acknowledgements**

324 This work was supported in part by NIH grants R01AI137273 and Cystic Fibrosis Research
325 Grant to B.P.C and R33CA206939 to P.A.D. We are grateful to Nikki J. Wagner, Lauren C.
326 Radlinski and Matthew Wolfgang for technical advice, as well as Brian Velasco for assistance
327 with PCCA preparation.
328

329 **Competing interests**

330 P.A.D declares that he is a co-inventor on a patent describing the formulation of low boiling-
331 point perfluorocarbon agents and a cofounder of Triangle Biotechnology, a company that has
332 licensed this patent. Additionally, P.G.D, P.A.D., B.P.C., V.P. and S.E.R. are all co-inventors on
333 a provisional patent describing the use of low boiling-point phase change contrast agents for
334 enhancing the delivery of therapeutics agents to biofilms. Additionally, B.P.C and S.E.R are co-
335 inventors on a provisional patent describing the use of rhamnolipids for potentiating antibiotic
336 efficacy. A.E.S declares that she has no competing interests.
337

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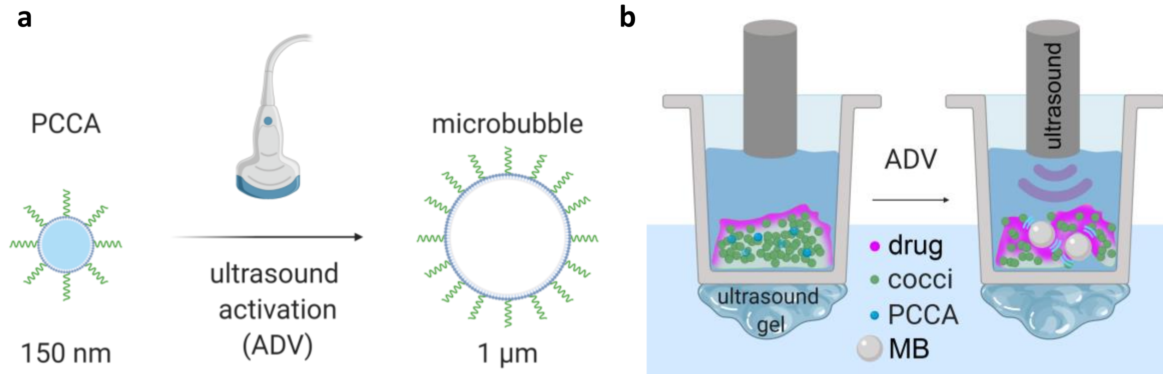
504 

505 **Fig 1. PCCA and ultrasound disrupts biofilm and increases drug penetration.** Nanoscale
506 PCCA are in a stable liquid phase. When exposed to ultrasound, the lipid shell containing
507 superheated liquid perfluorocarbon is destabilized, causing the liquid to vaporize (acoustic
508 droplet vaporization, ADV) to the gas phase and expand into a microbubble (a). The stability
509 and small size of PCCA makes them ideal to diffuse into biofilms prior to ultrasound application.
510 Ultrasound stimulation can vaporize PCCA to microbubbles that can physically disrupt biofilms
511 and enhance drug penetration (b).
512
513

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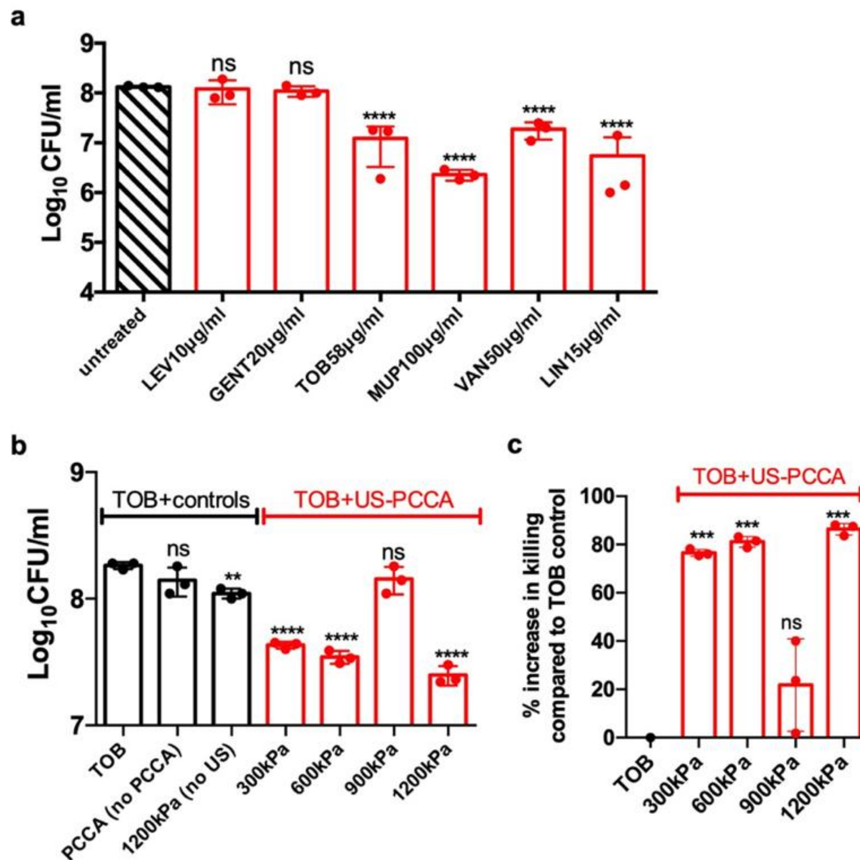

514
515 **Fig. 2: MRSA biofilms display high tolerance to clinically relevant antibiotics.** MRSA strain
516 LAC biofilms were cultured overnight in brain-heart infusion (BHI) media in 12-well tissue culture
517 treated plates. Biofilms were washed and treated with antibiotics (a-c). Where indicated, 12-well
518 plates were transferred to a custom-built temperature-controlled 37°C water bath alignment
519 setup. PCCA were added and 30s ultrasound exposure was applied at indicated pressures and
520 20% duty cycle (b-c). After 24h, biofilms were washed, sonicated for disruption and surviving
521 cells were enumerated by serial dilution plating. Survivors were presented as $\log_{10}$CFU/ml (a-b)
522 or as % increase in killing compared to tobramycin control (c) (extrapolated from a). The
523 averages of $n = 3$ biologically independent samples are shown. The error bars represent the
524 standard deviation. Statistical significance was determined using a one-way analysis of variance
525 (ANOVA) with Dunnett's (a, c) or Sidak's multiple comparison test (b). **, ***, **** denotes
526 P<0.005, P<0.0005, P<0.0001, respectively. LEV, levofloxacin; GENT, gentamicin; TOB,
527 tobramycin; MUP, mupirocin; VAN, vancomycin; LIN, linezolid, ns, not significant; US-PCCA,
528 ultrasound-stimulated phase change contrast agents.
529
530

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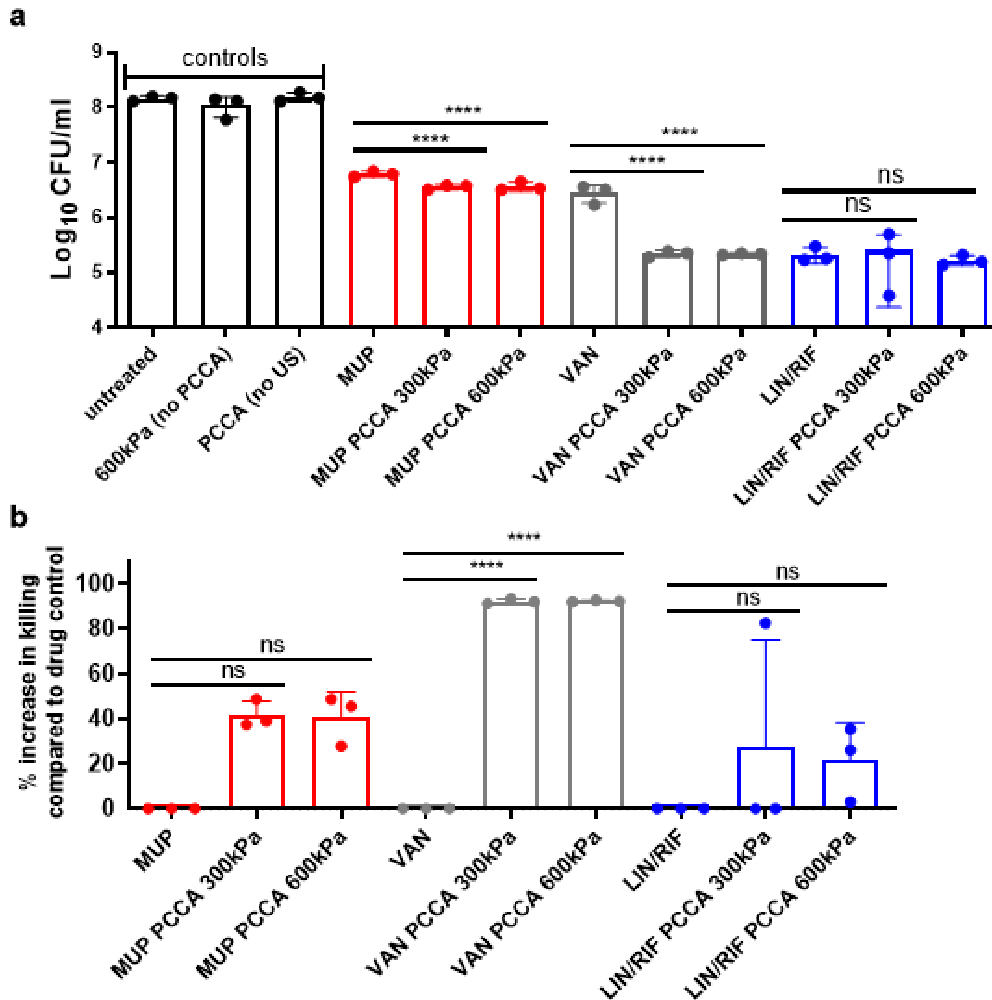

531
532 **Fig. 3: US-PCCA improve vancomycin killing of MRSA biofilms.** MRSA strain LAC biofilms
533 were cultured overnight in brain-heart infusion (BHI) media in 24-well tissue culture treated
534 plates. Biofilms were washed and treated with antibiotics and transferred to a custom-built
535 temperature-controlled 37°C water bath alignment setup. PCCA were added and 30s ultrasound
536 exposure was applied at indicated pressures and 10% duty cycle. After 24h, biofilms were
537 washed, sonicated for disruption and surviving cells were enumerated by serial dilution plating.
538 Survivors were presented as log_{10}CFU/ml (a) or as % increase in killing compared to antibiotic
539 control (b) (extrapolated from a). The averages of $n = 3$ biologically independent samples are
540 shown. The error bars represent the standard deviation. Statistical significance was determined
541 using a one-way analysis of variance (ANOVA) with Sidak's multiple comparison test. ****
542 denotes P<0.0001, respectively. MUP, 100µg/ml mupirocin; VAN, 50µg/ml vancomycin; LIN,
543 15µg/ml linezolid; RIF, 10µg/ml rifampicin; ns, not significant; US-PCCA, ultrasound-stimulated
544 phase change contrast agents.
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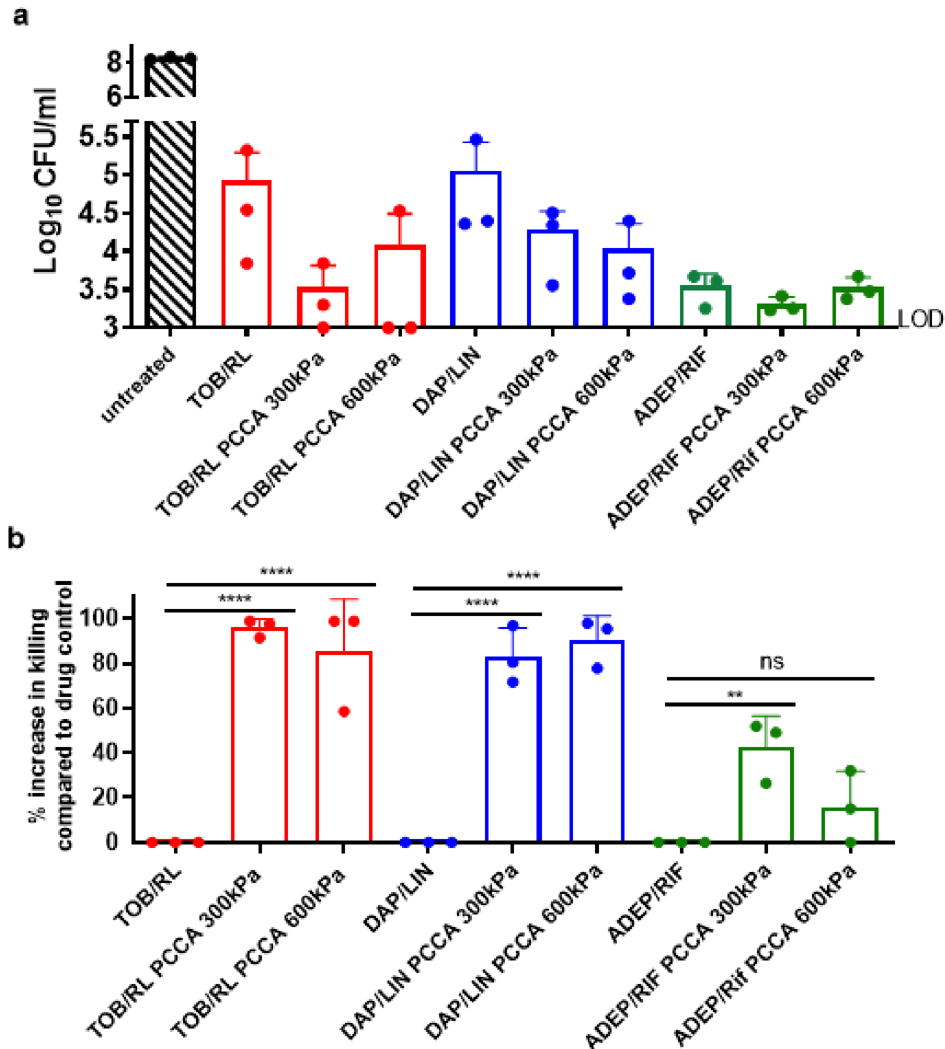

546
547 **Fig. 4: US-PCCA improves anti-persister antibiotic therapy against MRSA biofilms.** MRSA
548 strain LAC biofilms were cultured overnight in brain-heart infusion (BHI) media in 12-well
549 (TOB/RL) or 24-well (all other drugs) tissue culture treated plates. Biofilms were washed and
550 treated with antibiotics and transferred to a custom-built temperature-controlled 37°C water bath
551 alignment setup. PCCAs were added and 30s ultrasound exposure was applied at indicated
552 pressures and 10% duty cycle. After 24h, biofilms were washed, sonicated for disruption and
553 surviving cells were enumerated by serial dilution plating. Survivors were presented as
554 log_{10}CFU/ml (a) or as % increase in killing compared to antibiotic control (b) (extrapolated from
555 a). The averages of $n = 3$ biologically independent samples are shown. The error bars represent
556 the standard deviation. Statistical significance was determined using a one-way analysis of
557 variance (ANOVA) with Sidak's multiple comparison test (b). **, **** denotes P<0.005,
558 P<0.0001, respectively. TOB, 58µg/ml tobramycin; RL, 30µg/ml rhamnolipids; DAP, 100µg/ml
559 daptomycin; LIN, 15µg/ml linezolid; RIF, 10µg/ml rifampicin; ADEP, 5µg/ml acyldepsipeptide;
560 ns, not significant; US-PCCA, ultrasound-stimulated phase change contrast agents, LOD; limit
561 of detection.
562

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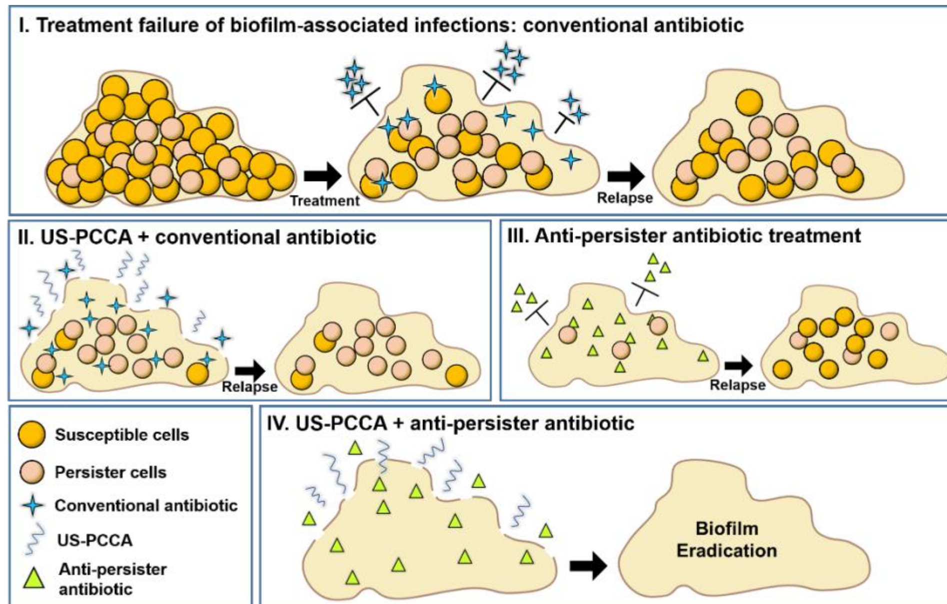

563
564 **Fig. 5: Schematic representing a dual approach to improving antibiotic therapy against *S.*
565 *aureus* biofilms.** (I) Biofilms display remarkable tolerance to antibiotics. Susceptible cells at the
566 biofilm periphery die (susceptible cells) while less metabolically active cells within the biofilm are
567 tolerant to conventional antibiotics (persister cells). Failure to eradicate the biofilm leads to
568 relapse in infection following removal of the antibiotic. (II) Improving penetration of conventional
569 antibiotics using US-PCCA will improve efficacy of some conventional antibiotics that do not
570 penetrate well through the biofilm matrix. This strategy is futile as it does not improve killing of
571 persister cells and leads to relapse. (III) Targeting biofilms with antibiotics which kill persister
572 cells (anti-persister antibiotics) improves efficacy but if drug penetration is impeded into the
573 biofilm, some persister cells will remain following drug treatment and lead to relapse. (IV)
574 Improving penetration of anti-persister antibiotics into the biofilm can lead to the eradication of
575 all cells within a biofilm and prevent relapse of infection following removal of the antibiotic.
576