Weak acids as an alternative anti-microbial therapy

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ABSTRACT

Weak acids such as acetic acid and N-acetyl cysteine (NAC) at pH less than their pKa can effectively eradicate biofilms due to their ability to penetrate the biofilm matrix and the cell membrane. However, the optimum conditions for their activity against drug resistant strains, and safety, need to be understood for their application to treat infections or to inactivate biofilms on hard surfaces. Here, we investigate the efficacy and optimum conditions at which weak acids can eradicate biofilms. We compared the efficacy of various mono and triprotic weak acids such as N-acetyl cysteine (NAC), acetic acid, formic acid and citric acid, in eradicating biofilms. We found that monoprotic weak acids/acid drugs can kill mucoid *P. aeruginosa mucA* biofilm bacteria provided the pH is less than their pKa, demonstrating that the extracellular biofilm matrix does not protect the bacteria from the activity of the weak acids. Triprotic acids, such as citric acid, kill biofilm bacteria at pH < pKa1. However, at a pH between pKa1 and pKa2, citric acid is effective in killing the bacteria at the core of biofilm microcolonies but does not kill the bacteria on the periphery. The efficacy of a monoprotic weak acid (NAC) and triprotic weak acid (citric acid) were tested on biofilms formed by *Klebsiella pneumoniae* KP1, *Pseudomonas putida* OUS82, *Staphylococcus aureus* 15981, *P. aeruginosa* DK1-NH57388A, a mucoid cystic fibrosis isolate and *P. aeruginosa* PA_D25, an antibiotic resistant strain. We showed that weak acids have a broad spectrum of activity against a wide range of bacteria, including antibiotic resistant bacteria. Further, we showed that a weak acid drug, NAC, can kill bacteria without being toxic to human cells, if its pH is maintained close to its pKa. Thus weak acids/weak acid drugs target antibiotic resistant bacteria and eradicate the persister cells in biofilms which are tolerant to other conventional methods of biofilm eradication.

Introduction

Bacterial infection is a serious clinical challenge that is increasingly difficult to treat with the growing prevalence of drug-resistant pathogens. While antimicrobial resistance is a natural phenomenon, the proliferation of resistance has been accelerated by the widespread use of antibiotics in humans, animals and agriculture [1,2]. This is further exacerbated by the observation that biofilm formation is generally associated with increased antimicrobial tolerance for most pathogens [3, 4]. When growing as a biofilm, bacteria are protected by a matrix composed of a cross-linked network of polysaccharides, nucleic acids, proteins and other macromolecules. The biofilm matrix forms a robust and elastic material [5–7], which the drugs that treat biofilm-related infections need to penetrate to kill the bacteria embedded within. Although various hypotheses have been proposed for the mechanism of antibiotic tolerance, many studies indicate that antibiotic tolerance is

caused by limited penetration and deactivation of antibiotics by the biofilm matrix [8]. The penetration of drugs is hindered by the charges on the different components of the matrix, with neutral molecules able to pass through while charged molecules are prevented from penetrating the biofilm [9–14]. For example, positively charged drugs such as ampicillin and ciprofloxacin bind to the negatively charged matrix of *Klebsiella pneumoniae*, which reduces the amount of drug that reaches the bacteria within the biofilm, and may also allow for the pathogen to express adaptive responses [11]. Apart from reduced antibiotic penetration, other biofilm-associated drug tolerance mechanisms include slow growth, adaptive stress responses and the induction of biofilm specific genes [15,16].

The antimicrobial tolerance of biofilms has also been attributed in part to the presence of persister cells within the nutrient-deprived biofilm interior [17–19]. For example, some antibiotics act only on bacteria that are metabolically active and thus the metabolically inactive bacteria in biofilms, such as persister cells, are naturally insensitive. Only colistin and ofloxacin have been shown to be active against non-growing Gram-negative bacteria and to date no antibiotics have been demonstrated as active against non-growing *S. aureus* [20,21]. Furthermore, bacteria throughout the biofilm have different growth kinetics. For example, in vitro studies have shown that the bacteria in the inner-most part of the biofilm microcolonies can be in stationary phase, which limits antibiotic efficacy due to their slow or non-growth phenotype [18,22]. The persister cells in vivo have also been shown to have implications in diseases. For example, *Salmonella* formed non-replicating persister populations upon uptake by macrophages, and *P. aeruginosa* isolates from cystic fibrosis patients produced high levels of drug tolerant persister cells which was linked to the recalcitrance of cystic fibrosis infection [23, 24].

P. aeruginosa is an opportunistic pathogen responsible for infections in a variety of conditions, including burn wounds and indwelling medical devices [25] and has limited susceptibility to many antimicrobial agents. Exposure to various antimicrobial drugs has resulted in the selection for, and rise in prevalence of, multi-drug resistant strains of *P. aeruginosa* [25, 26]. The extracellular matrix of *P. aeruginosa* is comprised mainly of polysaccharides such as alginate, Psl, Pel and extracellular DNA (eDNA) [27]. *K. pneumoniae*, a Gram-negative opportunistic pathogen, is commonly associated with hospital-acquired infections due to its multi-drug resistance [28]. The resistance of *K. pneumoniae* to ampicillin and ciprofloxacin has been partly attributed to slow growth phenotypes when the pathogen forms biofilms [11,29]. The presence of capsular polysaccharides in the matrix of *K. pneumoniae* also contributes to its tolerance [30]. *Pseudomonas putida* is a Gram-negative bacterium and 75% of its matrix is comprised of proteins, with other major components including polysaccharides and eDNA [31]. One of the more prevalent drug-resistant pathogens is the Gram-positive *Staphylococcus aureus*, whose extracellular matrix is largely comprised of cytoplasmic proteins that reversibly associate with the cell surface at low pH [32,33].

Organic weak acids have been used to treat infections for thousands of years [34], and there has been a recent resurgence of interest in the use of weak acids to treat bacterial infections. Various modes of action of weak acids on bacteria have been described [35], such as lipophilic properties that allow the uncharged form of weak acids to diffuse freely across the bacterial cell membrane into the cytoplasm until reaching an equilibrium. The decrease in intracellular pH has been implicated in causing growth inhibition, however, bacteria can survive at low intracellular pH [34]. Another model suggests that perturbation of membrane function may be the primary cause for the antimicrobial effect [36]. There is also evidence indicating that weak acids result in anions accumulating inside the cytoplasm, which may have an osmotic effect and alter metabolic processes within the cell [34].

Acetic acid has been shown to kill *P. aeruginosa* and *S. aureus* bacteria and to eradicate wound infections [35,37,38]. N-acetylcysteine (NAC) is a mucolytic agent that is an active ingredient in many 'over-the-counter' drugs used widely for its antioxidant and free radical scavenging property

that is considered safe and has FDA approval [39,40]. Complete eradication of a mucoid strain of *P. aeruginosa* biofilms with NAC was demonstrated at pH of the drug less than its pKa [5]. The mechanism of action for NAC was attributed to its weak acid nature, eliciting a similar response as that of acetic acid, which penetrates the bacterial cell wall in its undissociated state. Citric acid is a triprotic acid with three pKa values and 40% citric acid has been shown to prevent recolonization of oral biofilm on titanium surfaces [41] and reduced survival rate of bacteria in *Pseudomonas* biofilms [42]. Monoprotic acids such as acetic acid and NAC is known to kill bacteria at pH < pKa. However it is not clear if this behaviour can be generalized to all weak acids. The efficacy of triprotic acids such as citric acid across its three pKa values has not been investigated. The optimum concentrations and pH of weak acids/weak acid drugs to treat infections and to achieve complete eradication of biofilm, the breadth of activity as well as the cytotoxicity on human cells due to low pH of drugs are also not well established.

Here we investigate the commonality of weak acids in eradicating biofilms and how the efficacy varies with proticity. We employed various monoprotic acids, such as formic acid, NAC and acetic acid, and the triprotic acid, citric acid, across different pKa values. We also compared the bactericidal activity of monoprotic NAC and triprotic citric acid on biofilms with different morphology and matrix composition as well as biofilms of medically relevant bacteria, including a mucoid cystic fibrosis isolate and an antibiotic resistant strain. Finally, the effect of weak acids on mammalian cells to determine the suitability of weak acids as effective antimicrobials in conjunction with mammalian tissues was explored.

Results and discussion

Weak acids kill biofilm bacteria at pH < pKa

The efficacy of various monoprotic weak acids such as NAC, acetic acid and formic acid on bacterial biofilms is investigated to understand their behavioural similarities. *P. aeruginosa mucA* was grown in a flow cell with a continuous flow (flow rate of 100 μL/min) of 10% Luria-Bertani broth (LB) at pH 6.7 as previously described [6,7]. The flow of medium was stopped to administer NAC, acetic acid or formic acid at pH 3.0 (<pKa) and at pH 5 (>pKa), at a rate of 200 μL/min, using a syringe pump for 30 min. The biofilms were treated with propidium iodide (PI) before and after treatment to check for the presence of dead bacteria. Experiments were also performed without staining with PI before treatment to rule out any effect of the PI stain on the response of the bacteria to weak acids. At pH > pKa, the percentages of live bacteria when treated with NAC, acetic acid and formic acid were 86 ± 4%, 94 ± 3% and 99 ± 0.2%, respectively (Fig. 1b, c and d), which corresponds to the percentage of live bacteria intrinsically present in a biofilm before treatment with any compounds. This suggests that the bacteria are not killed under these conditions. In contrast, at pH < pKa, no live cells were detected, even within the first few minutes of completing the treatment (Fig. 1e, f and g). For all three compounds at pH < pKa, the bacteria in biofilms were completely killed as determined by PI staining and the absence of any green signal from GFP.

The percentages of live and dead bacteria were quantified using fluorescent intensity of PI stain and GFP. After treatment with weak acids at pH < pKa, there were no detectable live bacteria (Fig. 1i). Furthermore, no biofilm regrowth was observed after 24 h. Although PI staining is used widely to determine the viability of bacteria, a recent study has shown that extracellular DNA (eDNA) may interfere with the viability estimate leading to the overestimation of the dead cell count [43]. Hence, we also checked the number of colony forming units (CFU) of the bacteria in the biofilms after treatment with different acids and found that the CFU/mL was below the detection limit when treated with weak acids at pH < pKa (Fig. 1h). This confirmed that NAC, acetic acid and formic acid exhibited a common behaviour in which the undissociated form of weak acids (pH < pKa) penetrate the *P. aeruginosa mucA* biofilm matrix and kill all the bacteria embedded within. This also suggests that the viability
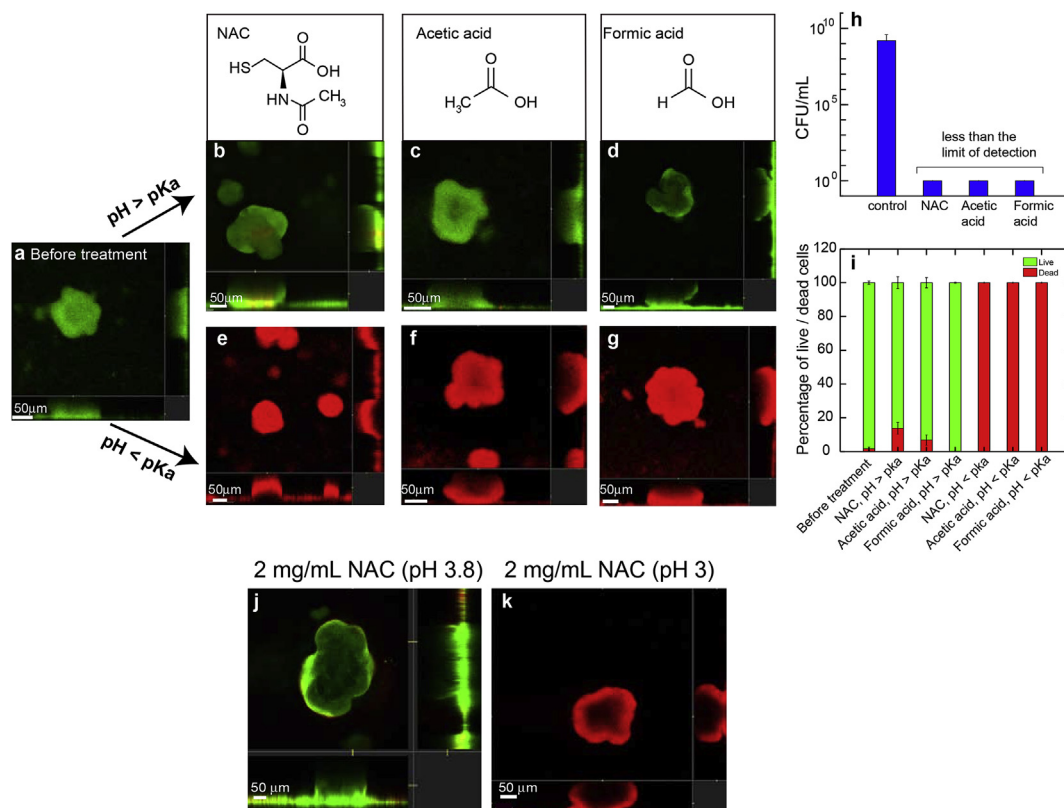
Fig. 1. *P. aeruginosa mucA* biofilms treated with monoprotic weak acids at pH below their pKa values kill all of the bacteria in the biofilm. (a) GFP tagged biofilm treated with PI stain before treatment with drugs shows only a few dead bacteria. No significant killing was observed when biofilms were treated with (b) NAC (c) acetic acid and (d) formic acid at pH > pKa. All of the bacteria in the biofilm were killed when treated with (e) NAC (f) acetic acid and (g) formic acid at pH < pKa. (h) CFU per mL of bacteria in biofilms after treatment with weak acids at pH 3 showed no detectable colonies. (i) The percentage of live and dead bacteria when treated with NAC, acetic acid and formic acid at pH 5 (>pKa) and pH 3 (<pKa). (j, k) Biofilms treated with 2 mg/mL NAC at pH 3.8 and 3 kills bacteria in the biofilms only at pH 3.

staining is a good proxy for the ability of cells to grow on plates under the conditions used here.

In all of the above experiments, the pH of the drug was altered by changing the concentration of the drug. Hence to show that the killing of bacteria is not just due to the changes in concentrations, experiments were performed with the same concentration of NAC at different pH. Here, the pH was changed by preparing NAC in different solvents. For example, 2 mg/mL NAC prepared using PBS buffer and 10% LB had a pH of 3.8 and 3 respectively. It should be noted that only 0.5 mg/mL NAC is required to prepare a solution of pH 3.8 with 10% LB. Higher concentration of NAC was required with PBS due to the buffering action of PBS. The bacteria in biofilms were killed only when the pH of the drug was maintained at pH 3 (Fig. 1k). Hence pH of the drug relative to its pKa is the key factor in killing the biofilm bacteria.

The biofilm killing efficacy of citric acid varies between its three pKa values

Given that the monoprotic weak acids outlined above killed biofilm bacteria when the pH < pKa, we then tested the biofilm killing efficacy of different concentrations of citric acid, a triprotic acid (pKa1 = 3.13, pKa2 = 4.76 and pKa3 = 6.41) on *P. aeruginosa mucA*. We observed that GFP tagged *P. aeruginosa mucA* biofilms treated with citric acid at pH 3 (<pKa1) displayed no detectable live bacteria (Fig. 2a), but that citric acid was ineffective at pH 5 (>pKa2), with 95 ± 4% of biofilm cells being viable (Fig. 2e). At pH levels between pKa1 and pKa2, 88 ± 5%, 70 ± 10% and 10 ± 4% of bacteria in biofilms were killed at pH 3.6, 3.8 and 4, respectively (Fig. 2f). The CFU/mL also showed a similar trend with no detectable CFU at pH 3, a decrease in CFU from $2.5 \times 10^9 \pm 2 \times 10^9$ CFU/mL to $1.8 \times 10^7 \pm 1 \times 10^7$ CFU/mL at pH 3.7 and no change in CFU at pH

5.6 (Fig. 2g). It should be noted that at concentrations corresponding to pH between pKa1 and pKa2, the bacteria in the interior of the microcolonies were killed, while the cells on the periphery appeared to be alive, although the percentage of these viable cells varied (Fig. 2b, c and d).

Since the biofilm microcolonies have a metabolically active subpopulation of bacteria on the periphery and dormant bacteria within the microcolony [44], we hypothesised that citric acid at pH between pKa1 and pKa2 kills the metabolically inactive population of bacteria in biofilms. This behaviour is similar to many of the membrane-targeting drugs such as colistin, EDTA, SDS and chlorhexidine that specifically kill the inactive subpopulation and do not affect the active subpopulation. The metabolically active subpopulation of *P. aeruginosa* adapts to these membrane targeting drugs through different genetic mediated determinants [45,46]. The mechanism by which these drugs kill the metabolically inactive, but not the active bacteria in biofilms is not clearly understood.

Citric acid at pH between pKa1 and pKa2 is ineffective in killing planktonic bacteria in log phase

The above response elicited by citric acid was intriguing and we subsequently tested the hypothesis that citric acid at pH between pKa1 and pKa2 would be effective in killing the dormant but not the metabolically active bacteria. This hypothesis was tested using *P. aeruginosa mucA* planktonic cultures. The number of colony forming units (CFU/mL) were recorded after 0, 1 and 3 h of growth, post exposure to varying concentrations of NAC and citric acid ranging from pH 3.0 to pH 3.7. In the case of both NAC and citric acid at pH < pKa or pKa1, no viable cells
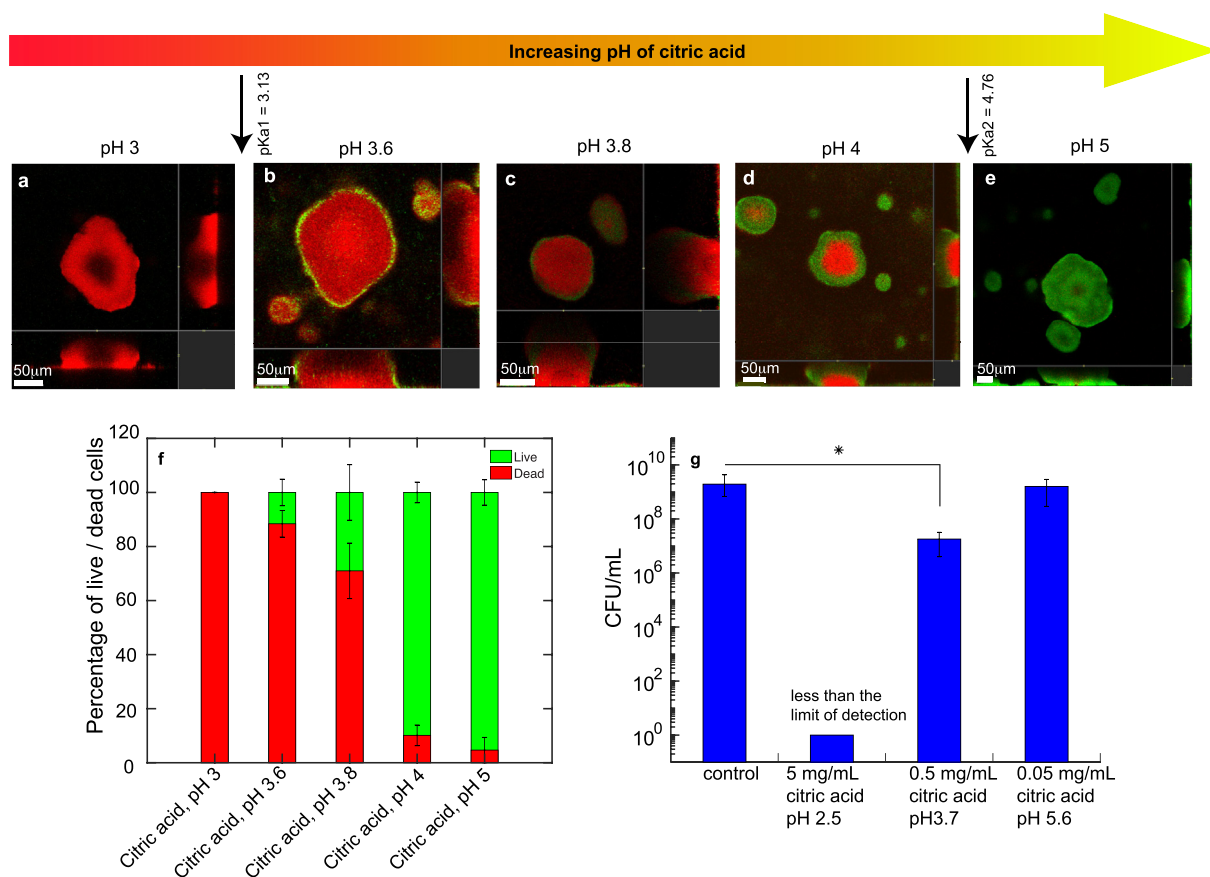
Increasing pH of citric acid

pKa1 = 3.13

pKa2 = 4.76

pH 3 | pH 3.6 | pH 3.8 | pH 4 | pH 5

Fig. 2. The killing efficacy of citric acid differs at concentrations between its three pKa values when *P. aeruginosa mucA* biofilms are treated with different concentrations of citric acid. (a) Citric acid at pH 3 kills the bacteria in biofilms effectively. (b, c, d) Citric acid at pH 3.6, 3.8 and 4 kills bacteria within biofilm colonies but does not kill cells at the periphery of the microcolonies. (e) Citric acid at pH 5 is ineffective in killing the biofilm bacteria. (f) The percentage of live and dead bacteria when treated with citric acid at pH 3, 3.6, 3.8, 4 and 5. (g) CFU per mL of *P. aeruginosa mucA* biofilms treated with citric acid at pH 2.5, 3.7 and pH 5.6. Asterisks indicate statistically significant differences between pairs of values (*p < 0.05). Limit of detection is 10 CFU/mL.

were detected after 1 h of incubation (Fig. 3a). This suggests that NAC and citric acid at pH < pKa or pKa1 kill cells within 60 min of incubation. However, at a concentration of 3.25 mg/mL citric acid (pH 3.7; between pKa1 and pKa2), the number of planktonic bacteria that remained viable after 3 h was the same as that of the control, albeit with an initial decrease after 1 h.

We also compared the effect of citric acid at pH 3.7 on planktonic bacteria in the log phase as well as in the stationary phase of growth (Fig. 3b). The number of viable cells in the log phase and stationary phase

of growth was $1.5 \times 10^8 \pm 5 \times 10^7$ CFU/mL and $1.5 \times 10^7 \pm 5.8 \times 10^6$ CFU/mL respectively after 1 h and $3.9 \times 10^8 \pm 3.3 \times 10^8$ CFU/mL and $1.6 \times 10^7 \pm 1 \times 10^7$ CFU/mL after 3 h of treatment. In both cases, there were no viable cells detected after 24 h of treatment. Hence, there is a faster decrease in the number of cells in the stationary phase compared to the log phase culture. This confirmed that citric acid at pH between pKa1 and pKa2 kills the slow growing cells at a faster rate compared to the metabolically active bacteria. However, further experiments need to be performed to assess if the bacteria in the viable outer layer of biofilms in
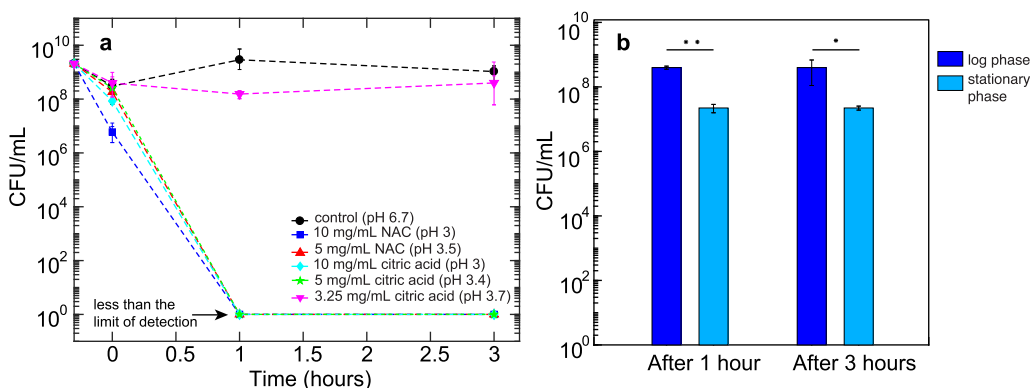

Fig. 3. (a) CFU per mL of *P. aeruginosa mucA* planktonic cultures in log phase treated with NAC at pH 3 (blue squares), NAC at pH 3.5 (red triangles), citric acid at pH 3 (cyan diamonds) and citric acid at pH 3.4 (green stars). When treated with citric acid at pH 3.7 (magenta triangles), which is between pKa1 and pKa2, the planktonic bacteria were not killed. (b) Comparison of the effect of citric acid at pH 3.7 on planktonic bacteria in the log phase and in the stationary phase of growth. Asterisks indicate statistically significant differences between pairs of values (*p < 0.05, **p < 0.001). The limit of detection is10 CFU/mL.

Fig. 2b, c and d are metabolically active.

NAC is broadly effective in killing biofilm bacteria

Although most bacteria form biofilms, their biofilm morphology and matrix composition vary considerably [47,48]. To investigate whether weak acid drugs has broad spectrum activity against a wide range of bacteria, we tested the effect of a monoprotic NAC and triprotic citric acid on *K. pneumoniae, S. aureus* and *P. putida.* These species were grown in flow cells for 2 d to establish biofilms of more than 50 μm thickness, which were considered to be mature biofilms in this system, based on previous observations [49]. The biofilms were then treated with 10 mg/mL NAC or citric acid (pH 2.5) for 30 min and their viability was assessed using the PI stain.

Before treatment with NAC or citric acid, $99 \pm 0.2\%$, $95 \pm 3\%$ and $60 \pm 31\%$ of *K. pneumoniae, P. putida* and *S. aureus,* respectively, were considered alive or viable, as determined by the GFP signal (Fig. 4a–c). After treatment with NAC or citric acid, no viable *K. pneumoniae* or *P. putida* cells could be detected in the biofilm (Fig. 4d, e, g and h). In the case of *S. aureus,* $94 \pm 6\%$ of bacteria were dead after NAC treatment (Fig. 4f and j), while $50 \pm 43\%$ of bacteria appeared viable after citric acid treatment (Fig. 4i and j).

The CFU/mL obtained from biofilms before and after treatment showed no detectable colonies in the case of *K. pneumonia* and *P. putida* after treatment with NAC and citric acid. *S. aureus* on the other hand showed a decrease from $2 \times 10^9 \pm 1.8 \times 10^9$ CFU/mL to $8.6 \times 10^5 \pm 7.2 \times 10^5$ CFU/mL and $5.4 \times 10^5 \pm 2.9 \times 10^5$ CFU/mL when treated with NAC and citric acid respectively (Fig. 4k). This suggests that weak acids show broad activity against biofilms formed by the bacterial species tested here. It should be noted that regrowth of biofilms was observed when *S. aureus* treated with NAC and citric acid was left to grow for 24 h after treatment, whereas the other species of bacteria did not show any sign of regrowth (Supplementary Fig. 1).

NAC effectively kills antibiotic-resistant biofilm bacteria

Having demonstrated that weak acids are broadly effective in killing a range of biofilm bacteria, we propose that these compounds can be used as an alternative drug to treat infections caused by antibiotic resistant strains of bacteria. To test this hypothesis, we investigated the efficacy of NAC on two clinical isolates: (i) *P. aeruginosa* DK1-NH57388A, a mucoid cystic fibrosis isolate [50], and (ii) *P. aeruginosa* PA_D25, which is a multidrug resistant strain [51]. It should be noted that these strains were not GFP tagged and hence the biofilms were stained with a live-dead reagent (SYTO9-PI) for visualisation and quantification.

For untreated *P. aeruginosa* biofilms (Fig. 5a and d), ~99% and 92% of the bacteria in microcolonies were alive. When treated with 10 mg/mL NAC (pH 2.5) for 30 min and again stained with PI, bacteria within these colonies displayed a red signal. Fig. 5b and e show an overlay of the live and dead stains. After treatment with NAC, the bacteria initially stained with SYTO9 were killed in biofilms formed by both *P. aeruginosa* strains (Fig. 5g). Since staining of bacteria with SYTO9 and PI before treatment may alter the bacterial cells, experiments were also performed without the initial staining (Supplementary Fig. 2).

The CFU/mL obtained from the two bacterial strains are shown in Fig. 5c and f. After exposure of both strains of bacteria to 10 mg/mL NAC (pH 2.5; pH < pKa), no viable cells could be detected by CFU determination. When treated with 3 mg/mL NAC (pH 3.3; pH = pKa), the number of viable cells decreased from $8.2 \times 10^8 \pm 3.2 \times 10^8$ CFU/mL to 74 ± 43 CFU/mL for *P. aeruginosa* DK1-NH57388A and from $3.1 \times 10^9 \pm 3 \times 10^9$ CFU/mL to 390 ± 142 CFU/mL for *P. aeruginosa* PA_D25. Hence, we showed that NAC is efficient in eradicating biofilms formed by cystic fibrosis isolates as well as *P. aeruginosa* PA_D25, which is multidrug resistant. While this suggests that weak acids are effective irrespective of drug resistance or sensitivity, further work would be needed to verify this with a range of different species.

Low pH of drugs is non-toxic to a human cell-line

To treat biofilm-related infections, the weak acid concentration has to be such that the pH is less than the pKa. For example, the effective concentration of NAC has to be less than 3.3. To ascertain whether this pH range is toxic to human cells or tissue, we assayed HeLa cells grown in 96 well plates to a confluence of approximately 80% and exposed to NAC
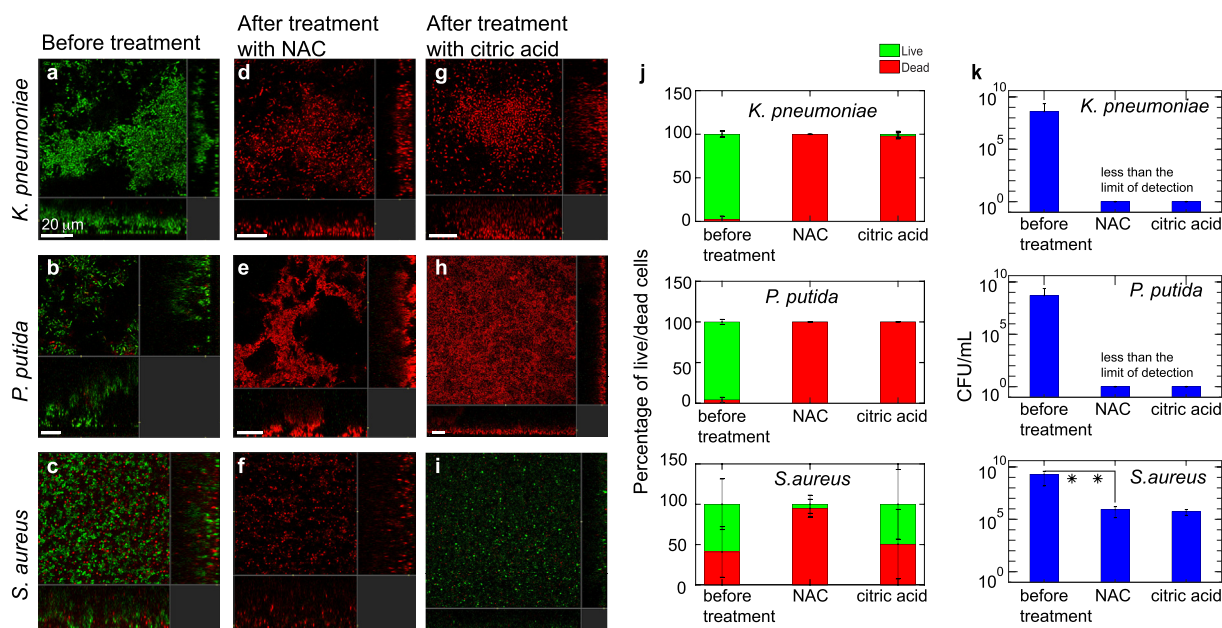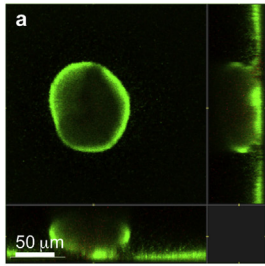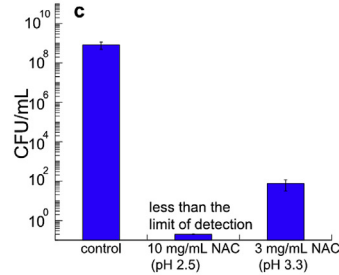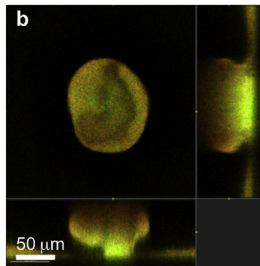

Fig. 4. (a–c) Representative confocal images of biofilms formed by *K. pneumoniae, P. putida* and *S. aureus* bacteria expressing GFP. (d–f) Green and red channels after the biofilms were treated with 10 mg/mL NAC (pH 2.5). (g–i) Green and red channels after the biofilms were treated with 10 mg/mL citric acid (pH 2.5). (j) The percentage of dead and live bacteria in biofilms before and after treatment with NAC and citric acid. (k) CFU per mL of the bacteria in biofilms before and after treatment with NAC and citric acid. Asterisks indicate statistically significant differences between pairs of values (**p < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
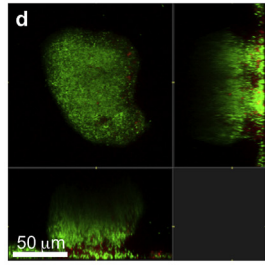
P. aeruginosa DK1-NH57388A

Before treatment | **After treatment with NAC**

P. aeruginosa PA_D25
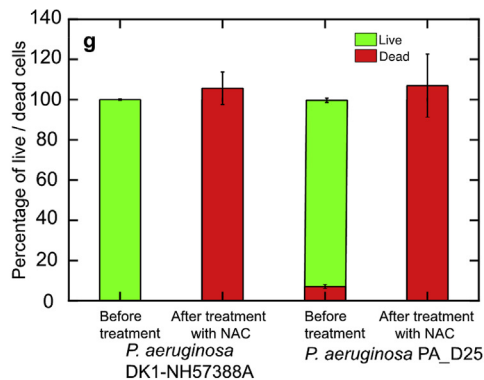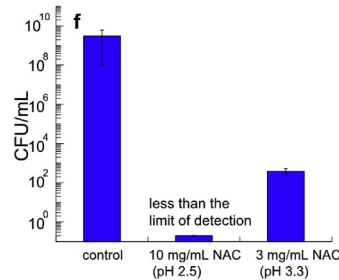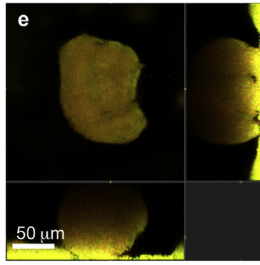
Before treatment | **After treatment with NAC**

Fig. 5. Representative confocal images of micro-colonies formed by (a) *P. aeruginosa* strain DK1-NH57388A, a mucoid cystic fibrosis isolate and (d) *P. aeruginosa* PA_D25, an antibiotic resistant strain, before treatment with NAC. The biofilms were stained with the live-dead reagents. (b, e) After treatment with 10 mg/mL NAC (pH 2.5), live-dead staining showed that majority of the bacterial cells in the colony were killed. (c, f) The CFU per mL of the bacteria treated with NAC at pH 2.5 and 3.3. The limit of detection is 10 CFU/mL. (g) The percentage of live and dead cells before and after treatment with NAC.

in phosphate buffered saline (PBS) at concentrations from 2 to 5 mg/mL (pH 3.8 to 3, ≈ pKa value) for 15 min. A significant percentage of HeLa cells remained viable when treated with NAC at concentrations less than 3 mg/mL (pH 3.3), as measured using Presto Blue (Fig. 6a). NAC treatment from 2.4 mg/mL (pH 3.5) to 3 mg/mL (pH 3.3) was effective in killing *P. aeruginosa* biofilm cells (Fig. 6b), while remaining non-toxic to human cells. Hence, we propose that weak acids can be safely used to treat bacterial infections in humans/animals provided the pH is maintained between 3.5 and 3.3.

Conclusions

Here we investigated the efficacy of weak acids in eradicating biofilms, especially those formed by antibiotic resistant bacteria, as well as how the efficacy varies with proticity of the weak acids. We found that the undissociated form of monoprotic weak acids can completely kill bacteria in biofilms. Triprotic acid behaved differently between its three pKa values, with complete eradication of biofilm at pH < pKa1. At pH between pKa1 and pKa2, citric acid was effective in killing bacteria at the core of the biofilm colonies, but was ineffective in killing the cells at the biofilm periphery. We also showed that weak acids have a broad spectrum of activity and killed bacteria in biofilms formed by *K. pneumoniae*, *P. putida*, *S. aureus*, as well as antibiotic-resistant and cystic fibrosis isolates. As low pH of the acid was shown to be non-toxic to a human cell line, weak acids could represent an alternative therapeutic agent against antibiotic resistant biofilm infections.

Materials and methods

Flow chamber

Poly(dimethyl siloxane) (PDMS) flow cells were fabricated from a 3D printed stamp, using a Sylgard 184 kit (Dow Corning, UK). The flow cell had a straight channel with dimensions of 0.2 cm × 0.5 cm × 3 cm (height × width × length) [6]. PDMS monomer and the curing agent were mixed in a 10:1 ratio (w/w) and this mixture was placed in a vacuum chamber for 1 h to remove air bubbles trapped during mixing. The mixture was then slowly poured into the mold and left at room temperature for 24 h after which it was incubated at 70 °C for 1 h. Once the PDMS cooled, it was removed from the mold, oxygen-plasma treated and bonded to a glass coverslip. Inlet and outlet holes of 1 mm diameter were created using Uni-CoreTM punchers (Sigma-Aldrich, St. Louis, MO, USA) before bonding.

Flow system

Continuous flow of nutrients was provided by a simple, gravity fed system, which comprised of an inverted conical flask (1 L), with a one-
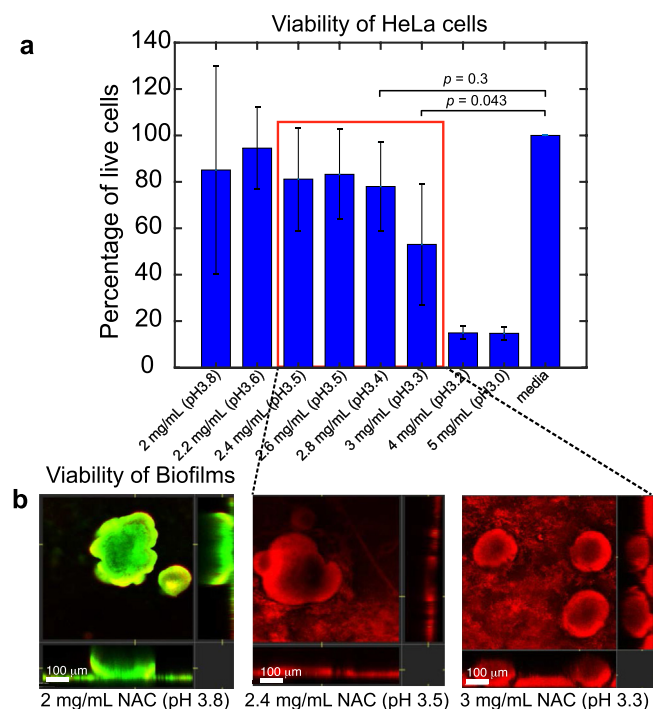
a

Viability of HeLa cells

b

Viability of Biofilms

2 mg/mL NAC (pH 3.8)   2.4 mg/mL NAC (pH 3.5)   3 mg/mL NAC (pH 3.3)

Fig. 6. (a) Viability of HeLa cells treated with different concentrations of NAC. The viable cells fluoresced when incubated with Presto Blue for 30 min. The cells not treated with NAC were labelled as media and normalized to 100%. (b) Biofilms treated with 2 mg/mL NAC (pH 3.8) was not killed whereas the ones treated with 2.4 mg/mL (pH 3.5) and 3 mg/mL (pH 3.3) was killed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hole rubber stopper. A clinical IV administration set (B. Braun) was inserted into the rubber stopper to allow for adjustment of the flow rate.

Biofilm formation

An alginate-overproducing *P. aeruginosa mucA* strain [52], *P. putida* OUS82 [53], *S. aureus* 15981 [54], *K. pneumoniae* KP1 [49], *P. aeruginosa* DK1-NH57388A [50] and *P. aeruginosa* PA_D25 [51] were used for the study. Fluorescently-tagged strains were constructed by the insertion of a mini-Tn7-enhanced green fluorescent protein (eGFP)- Gm^r cassette as previously described [55]. Overnight cultures of these strains were grown in Luria-Bertani broth (5 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) at 37 °C under shaking conditions (200 rpm). The overnight cultures were diluted to an optical density at 600 nm (OD$_{600}$) of 0.4, and 350 μL was injected into the flow cell and incubated for 1 h to allow the bacteria to attach to the glass surface. After the initial attachment, 10% LB medium was supplied to the biofilm at a flow rate of 100 μL/min. The biofilms were then allowed to grow for 2 d.

Treatment, staining and imaging

N-acetyl cysteine, acetic acid, citric acid and formic acid were purchased from Sigma-Aldrich. Weak acids were prepared using 10% LB, 100% LB or PBS buffer. The pH of weak acids at a particular concentration can be different depending on the solvent used due to their buffering action. NAC at concentrations 10, 2 or 0.1 mg/mL (pH 2.5, 3 and 5 respectively) and citric acid at 2, 0.6, 0.5, 0.4 or 0.1 mg/mL (pH 3, 3.6, 3.8, 4 and 5) prepared using 10% LB medium was used for the treatment of biofilms. Acetic and formic acids were diluted using 10% LB medium to obtain a pH of 3 or 5. Ten or 5 mg/mL (pH 3 and 3.5) NAC and 10, 5 or 3.25 mg/mL (pH 3, 3.4 and 3.7) citric acid prepared using 100%

LB was used to treat planktonic cultures. Two to 5 mg/mL (pH 3.8 to 3) NAC was prepared using PBS buffer for treatment of HeLa cells.

A live-dead viability kit containing SYTO9 and propidium iodide (PI) (Thermo Fisher Scientific, USA) was used to stain biofilms. The GFP tagged biofilms were stained with 0.3% (v/v) of PI for 20 min to visualize the presence of dead bacteria or eDNA in biofilms before treatment with a drug. The stain was then flushed out and biofilms imaged. The biofilms were treated with the drug for 30 min after which the drug was flushed out with 10% LB. The treated biofilms were again stained with PI stain to visualize the dead bacteria after treatment. The biofilm bacteria that were not GFP tagged were stained with both SYTO9 and PI at concentrations of 0.3% (v/v) before treatment.

Imaging of the biofilms was performed using a Fluoview 1000 confocal microscope (Olympus Japan) with a 20 × or 60 × oil immersion objective. Two image channels were acquired for each stack using GFP 488 and Alexa 594 excitation filters. The number of z-stacks depended on the height of the colonies.

At least three independent repeats of all the experiments were performed.

CFU counts of planktonic culture and biofilms

P. aeruginosa mucA planktonic cultures were grown in LB medium to an optical density at 600 nm (OD$_{600}$) of 0.4. This culture was then diluted 10 times with LB containing 10 and 5 mg/mL NAC (pH 3 and 3.5), 10, 5 and 3.25 mg/mL citric acid (pH 3, 3.4 and 3.7). The number of colony forming units per mL (CFU/mL) were recorded after 0, 1 and 3 h.

To measure the CFUs of biofilm bacteria after treatment with drugs, biofilms were grown in open flow cells as described previously [6], and treated with NAC. The flow cells were then opened, the biofilms scraped out and diluted in 10% LB medium, vortexed to homogenize the bacteria in the solution and plated on LB agar plates after a serial dilution.

HeLa cells culture and viability detection

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermofisher Scientific) in 96 well plates to ≈ 80% confluence. NAC solutions ranging from 2 to 5 mg/mL were prepared using PBS buffer. The corresponding pH ranged from 3.8 to 3, respectively. The HeLa cells were rinsed with PBS and incubated with NAC for 15 min, after which the cells were again rinsed with PBS to remove the NAC and the incubated with Presto Blue for 30 min. The viable cells change the Presto Blue solution into a fluorescent product, which is detected from the absorbance measurement.

CRediT authorship contribution statement

Binu Kundukad: Conceptualization, Investigation, Methodology, Data curation, Writing - original draft, Validation, Writing - review & editing. **Gayathri Udayakumar:** Investigation. **Erin Grela:** Investigation. **Dhamanpreet Kaur:** Investigation. **Scott A. Rice:** Validation, Writing - review & editing. **Staffan Kjelleberg:** Validation, Writing - review & editing. **Patrick S. Doyle:** Conceptualization, Validation, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https ://doi.org/10.1016/j.bioflm.2020.100019.

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