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EXPERIMENTAL THERAPEUTICS

In Vitro Antibacterial Activity of Hydrogen Peroxide and Hypochlorous Acid, Including That Generated by Electrochemical Scaffolds

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ABSTRACT Hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are biocides used for cleaning and debriding chronic wound infections, which often harbor drug-resistant bacteria. Here, we evaluated the *in vitro* activity of H_2O_2 and HOCl against 27 isolates of eight bacterial species involved in wound infections. Minimum inhibitory concentrations (MICs) and minimum biofilm bactericidal concentrations (MBBCs) were measured. Compared to their respective MICs, MBBCs of isolates exposed to H_2O_2 were 16- to 1,024-fold higher, and those exposed to HOCl were 2- to 4-fold higher. We evaluated the selection of resistance after exposure of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms to 10 iterations of electrochemically generated HOCl or H_2O_2 delivered using electrochemical scaffolds (e-scaffolds), observing no decrease in antibiofilm effects with serial exposure to e-scaffold-generated H_2O_2 or HOCl. Twenty-four-hour exposure to H_2O_2-generating e-scaffolds consistently decreased the number of CFU of *S. aureus* and *P. aeruginosa* biofilms by ~5.0 $\log_{10}$ and ~4.78 $\log_{10}$ through 10 iterations of exposure, respectively. Four-hour exposure to HOCl-generating e-scaffolds consistently decreased the number of CFU of *S. aureus* biofilms by ~4.9 $\log_{10}$, and 1-h exposure to HOCl-generating e-scaffolds consistently decreased the number of CFU of *P. aeruginosa* biofilms by ~1.57 $\log_{10}$. These results suggest that HOCl has similar activity against planktonic and biofilm bacteria whereas the activity of H_2O_2 is less against biofilm than planktonic bacteria, and that repeat exposure to either biocide, generated electrochemically under the experimental conditions studied, does not lessen antibiofilm effects.

KEYWORDS H_2O_2, HOCl, biofilm, e-scaffold, MIC, MBIC, MBBC, resistance

Chronic wound infections caused by antibiotic-resistant bacteria are a challenge in clinical practice. In the United States, costs associated with treating chronic wound infections exceed $10 billion yearly (1). Such infections are commonly associated with the presence of biofilms in wound beds, making them especially difficult to treat, since many antimicrobial agents are poorly active against bacterial biofilms (2). Biofilms in wound beds can hamper wound healing by impairing movement of keratinocytes/fibroblasts or decreasing angiogenesis, for example (3–5). Antiseptics and topical disinfectants used for cleaning and debriding chronic wound infections include chlorhexidine, povidone-iodine, sodium hypochlorite, hypochlorous acid (HOCl), peracetic acid, quaternary ammonium compounds, and hydrogen peroxide (H_2O_2), to name a few (6, 7). Biofilms in wound beds may hinder the optimal efficacy of these biocides. Among the various biocides, H_2O_2 and HOCl are generated as part of natural cellular inflammatory responses and are noteworthy for their inherent potential properties in eliminating biofilms in wound beds and stimulating wound healing (8–11). Increased migration

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and differentiation of keratinocytes and fibroblasts have been reported in the presence of H_2O_2 and HOCl (12, 13). A limitation in the application of H_2O_2 and HOCl to wounds is, however, that they are rapidly oxidized/reduced in wound environments, losing activity over time. Therefore, the continuous generation and delivery of H_2O_2 and HOCl to wound beds to reduce biofilms could be considered for ideal antibacterial effects.

Few studies have assessed the antibiofilm activity of H_2O_2 and HOCl in terms of minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm bactericidal concentrations (MBBCs) against biofilms formed by numerous/broad-spectrum clinically important pathogens. Since wound infections often involve biofilms in wound beds, antibiofilm activity is an important consideration. Previously, we described H_2O_2- and HOCl-generating electrochemical scaffolds (e-scaffolds) as prototypes of devices being developed to treat wound infections (13, 14).

Here, we studied the susceptibility of selected clinically relevant Gram-positive and -negative bacteria by determining MICs, MBICs, and MBBCs of H_2O_2 and HOCl. The literature has contrasting reports regarding the selection of H_2O_2 and HOCl resistance (15–17), so we also assessed whether there would be a decrease in antibiofilm activity with serial application of H_2O_2 or HOCl. Specifically, we tested *S. aureus* and *P. aeruginosa* biofilms with multiple exposures to H_2O_2- and HOCl-generating e-scaffolds.

RESULTS

Susceptibility of planktonic bacteria to hydrogen peroxide and hypochlorous acid. The 27 bacterial isolates studied had mean H_2O_2 MICs ranging from 0.20 to 3.19 mM (Table 1). Gram-positive and -negative bacteria showed wide H_2O_2 MIC ranges. *P. aeruginosa* PA14 had a mean H_2O_2 MIC of 3.19 mM, whereas its isogenic mutant strain lacking the catalase genes *katA* and *katB*, *P. aeruginosa* PA14 Δ*katAB*, had a mean H_2O_2 MIC of 0.20 mM, ~16-fold lower than the parent strain.

The 27 bacterial isolates studied had mean HOCl MIC values ranging from 0.5 to 1.99 mM (Table 1), a tighter range than H_2O_2 and below concentrations considered toxic to mammalian cells (~15.12 mM) (10). As with H_2O_2, both Gram-positive and -negative bacteria showed similar HOCl MIC ranges.

Susceptibility of bacterial biofilms to hydrogen peroxide and hypochlorous acid. (i) Biofilm inhibitory concentrations. As shown from Table 1, mean H_2O_2 MBICs ranged from 0.40 to 170 mM. For most tested Gram-positive bacteria, H_2O_2 MBICs were similar or slightly higher than corresponding MIC values. For Gram-negative bacteria, except for *P. aeruginosa*, H_2O_2 MBICs were also similar/slightly higher than their MICs. Almost all *P. aeruginosa* isolates, except *P. aeruginosa* PA14 Δ*katAB*, had 128- to 256-fold MBICs compared to MICs.

All bacteria studied had mean HOCl MBICs similar to or slightly higher than their respective MICs; an exception was *P. aeruginosa* IDRL-7543, which had a mean HOCl MBIC of ≥3.97 mM, markedly higher than its MIC (0.99 mM).

(ii) Biofilm bactericidal concentrations. Mean H_2O_2 MBBC values ranged from 51 to 680 mM, 32- to 512-fold higher than their MIC or MBIC. *P. aeruginosa* isolates had the highest H_2O_2 MBBC values of the bacteria studied. Almost all *P. aeruginosa* isolates had an overall 256- to 512-fold higher H_2O_2 MBBC than MIC and MBIC.

Mean HOCl MBBCs ranged from 0.66 to ≥3.97 mM, the same or slightly higher than the respective MICs and MBICs. HOCl MBBCs for the Gram-positive bacteria studied were generally similar to their MICs and MBICs, whereas for Gram-negative bacteria, HOCl MBBCs were generally higher than their MICs and MBICs.

Measurement of susceptibility following repeated exposure of *S. aureus* and *P. aeruginosa* biofilms to H_2O_2 and HOCl generated by e-scaffolds. Figure 1 shows the results of repeated e-scaffold treatment of *S. aureus* USA100 and *P. aeruginosa* IDRL-11442 biofilms. Based on our prior e-scaffold studies, ~45 mM H_2O_2 is typically generated over a 24-h treatment period and ~22 mM HOCl over a 4-h treatment period (13, 14). When *S. aureus* USA100 biofilms were exposed to an H_2O_2-producing e-scaffold for 24 h, a mean reduction of ~5.00 $\log_{10}$ CFU/cm^2 was observed compared to controls over 10 iterations (Fig. 1A). When the same biofilms were exposed to an HOCl-generating e-

TABLE 1 Susceptibility of bacterial isolates (planktonic and biofilm forms) to H_2O_2 and HOCl

Bacteria	Isolate designation	Isolate characteristics	Value (means ± SD, in mM) for[a]:					
			H_2O_2			HOCl		
			MIC	MBIC	MBBC	MIC	MBIC	MBBC
S. aureus	USA100	Clinical isolate, resistant to methicillin	0.40 ± 0.00	0.40 ± 0.00	85 ± 29	1.65 ± 0.57	1.32 ± 0.57	1.32 ± 0.57
S. aureus	USA200	Clinical isolate, resistant to methicillin	0.27 ± 0.11	0.40 ± 0.00	85 ± 29	1.65 ± 0.57	0.99 ± 0.00	1.32 ± 0.57
S. aureus	USA300	Clinical isolate, resistant to methicillin	0.40 ± 0.00	0.66 ± 0.23	68 ± 29	1.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
S. aureus	IDRL-6169	Prosthetic hip isolate; resistant to methicillin and mupirocin	0.40 ± 0.00	0.66 ± 0.23	51 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
S. aureus	Xen 30	Clinical isolate; resistant to methicillin	0.66 ± 0.23	0.53 ± 0.23	119 ± 78	1.32 ± 0.57	1.32 ± 0.57	1.32 ± 0.57
S. aureus	IDRL-4284	Clinical isolate; resistant to methicillin	0.66 ± 0.23	0.66 ± 0.23	170 ± 59	1.99 ± 0.00	1.32 ± 0.57	0.99 ± 0.00
S. epidermidis	ATCC 35984	Catheter sepsis isolate; resistant to methicillin	0.53 ± 0.23	0.53 ± 0.23	170 ± 59	1.65 ± 0.57	1.32 ± 0.58	1.65 ± 0.57
S. epidermidis	IDRL-6461	Prosthetic knee infection isolate; susceptible to methicillin	0.53 ± 0.23	0.66 ± 0.23	136 ± 59	1.32 ± 0.57	0.99 ± 0.00	0.99 ± 0.00
S. epidermidis	Xen 43	Catheter isolate; susceptible to methicillin	0.40 ± 0.00	1.06 ± 0.46	102 ± 0.00	1.32 ± 0.57	1.32 ± 0.58	1.32 ± 0.57
E. faecalis	ATCC 29212	Urine isolate	3.19 ± 0.00	1.86 ± 1.22	136 ± 59	0.66 ± 0.29	1.32 ± 0.57	1.65 ± 0.57
E. faecalis	IDRL-8618	Prosthetic hip infection isolate	0.53 ± 0.23	1.33 ± 0.46	102 ± 0.00	0.50 ± 0.00	1.32 ± 0.57	1.99 ± 0.00
E. faecalis	IDRL-7107	Prosthetic knee infection isolate	3.19 ± 0.00	4.25 ± 1.84	170 ± 59	0.50 ± 0.00	1.99 ± 0.00	2.32 ± 1.52
E. faecium	IDRL-11790	Abscess isolate; resistant to vancomycin and penicillin, and susceptible to linezolid	0.80 ± 0.00	0.80 ± 0.69	55 ± 45	0.99 ± 0.00	0.82 ± 0.28	1.32 ± 0.57
E. coli	IDRL-10366	bla_{KPC}-positive isolate; resistant to ceftolozane-tazobactam, imipenem, meropenem, ertapenem, ceftriaxone, and cefepime	1.33 ± 0.46	0.66 ± 0.23	170 ± 59	0.99 ± 0.00	1.32 ± 0.57	1.32 ± 0.57
E. coli	IDRL-7029	Prosthetic hip infection isolate	1.59 ± 0.00	1.86 ± 1.22	340 ± 118	0.99 ± 0.00	1.32 ± 0.57	3.31 ± 1.15
E. coli	IDRL-6199	Prosthetic knee infection isolate	2.13 ± 0.92	1.59 ± 0.00	408 ± 0.00	0.99 ± 0.00	1.65 ± 0.57	3.31 ± 1.15
E. coli	IDRL-8110	Blood isolate	2.66 ± 0.92	3.72 ± 2.44	340 ± 118	0.99 ± 0.00	1.65 ± 0.57	3.97 ± 0.00
P. aeruginosa	IDRL-7262	Prosthetic hip infection isolate	0.66 ± 0.23	170 ± 58.89	408 ± 0.00	0.99 ± 0.00	1.65 ± 0.57	1.65 ± 0.57
P. aeruginosa	Xen 5	Blood isolate	2.13 ± 0.92	170 ± 58.89	612 ± 353	0.99 ± 0.00	>3.97	>3.97
P. aeruginosa	PAO1, ATCC 47085	Wound isolate; type strain	2.66 ± 0.92	153 ± 88.33	680 ± 236	0.99 ± 0.00	1.65 ± 0.57	1.99 ± 0.00
P. aeruginosa	PA14	Wild-type laboratory strain	3.19 ± 0.00	85 ± 29.44	408 ± 0.00	0.99 ± 0.00	1.65 ± 0.57	1.65 ± 0.57
P. aeruginosa	PA14 Δ*katAB*	*katA* and *katB* double knockout of PA14	0.20 ± 0.00	3.72 ± 2.43	51 ± 0.00	0.99 ± 0.00	1.32 ± 0.57	1.65 ± 0.57
P. aeruginosa	IDRL-11442	Groin isolate; resistant to piperacillin-tazobactam, cefepime, ceftazidime, meropenem, aztreonam, ciprofloxacin, levofloxacin; susceptible to colistin	0.60 ± 0.34	51 ± 0.00	170 ± 59	0.99 ± 0.00	1.65 ± 0.57	1.32 ± 0.57
A. baumannii	ATCC 17978	Meningitis isolate	0.80 ± 0.00	2.13 ± 0.92	85 ± 29	0.83 ± 0.29	0.99 ± 0.00	1.32 ± 0.57
A. baumannii	ATCC BAA-1605	Sputum isolate; resistant to ceftazidime, gentamicin, ticarcillin, piperacillin, aztreonam, cefepime, ciprofloxacin, imipenem and meropenem	0.80 ± 0.00	2.12 ± 0.92	68 ± 29	0.83 ± 0.29	1.32 ± 0.57	0.83 ± 0.29
A. baumannii	ARLG-1268	Wound isolate; resistant to amikacin, ampicillin, cefepime, ceftazidime, ciprofloxacin and tobramycin	1.06 ± 0.46	2.66 ± 0.92	102 ± 0.00	0.66 ± 0.29	0.66 ± 0.29	0.66 ± 0.29
K. pneumoniae	IDRL-10377	bla_{KPC}-positive isolate; resistant to ceftolozane-tazobactam, imipenem, meropenem, ertapenem, ceftriaxone and cefepime	0.40 ± 0.00	2.12 ± 0.92	102 ± 0.00	0.99 ± 0.00	0.66 ± 0.29	0.99 ± 0.00

[a]Susceptibility data values (i.e., MIC, MBIC, and MBBC) are represented as means ± SD ($n = 3$). All experiments were performed in triplicates. *S. aureus* USA100, USA200, and USA300 strains were provided by Henry Chambers III (University of California, San Francisco). Xen 30, Xen 43, and Xen 5 strains were provided by Caliper Life Sciences. *P. aeruginosa* PAO1, PA14, and PA14 Δ*katAB* strains were provided by Daniel Hassett (University of Cincinnati). *A. baumannii* ARLG-1268 was provided by the Antibacterial Resistance Leadership Group of the National Institutes of Health.

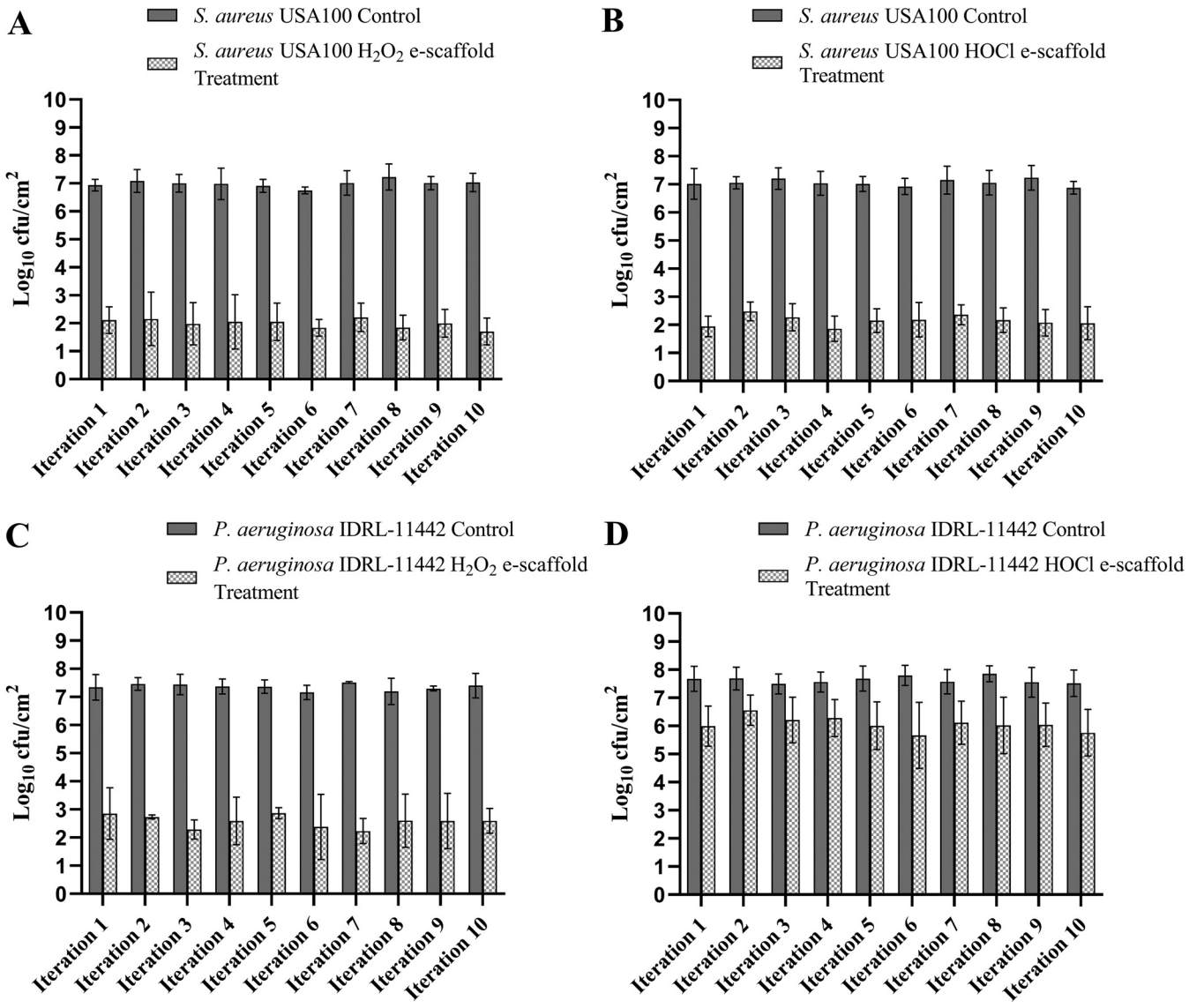

FIG 1 Repeated treatment with e-scaffolds demonstrating no decrease in effect over 10 sequential iterations. (A) *Staphylococcus aureus* USA100 biofilms exposed to an H_2O_2 generating e-scaffold. (B) *S. aureus* USA100 biofilms exposed to an HOCl-generating e-scaffold. (C) *Pseudomonas aeruginosa* IDRL-11442 biofilms exposed to an H_2O_2 generating e-scaffold. (D) *P. aeruginosa* IDRL-11442 biofilms exposed to an HOCl-generating e-scaffold. Data are expressed as means ± SD (n = 3). All the experiments were performed in triplicate.

scaffold treatment for 4 h, a mean reduction of ~4.90 $\log_{10}$ CFU/cm^2 compared to controls was observed over 10 iterations (Fig. 1B). The degree of effect was maintained with repeated e-scaffold treatment, with reductions in CFU counts remaining consistent between iterations for both e-scaffold types.

When *P. aeruginosa* biofilms were exposed to an H_2O_2-producing e-scaffold for 24 h, a mean reduction of ~4.78 $\log_{10}$ CFU/cm^2 was observed compared to controls over 10 iterations (Fig. 1C). When the same *P. aeruginosa* biofilms were exposed to an HOCl-generating e-scaffold treatment for 1 h, a mean reduction of ~1.57 $\log_{10}$ CFU/cm^2 was observed compared to controls over 10 iterations (Fig. 1D). *P. aeruginosa* biofilms were exposed to the HOCl-producing e-scaffold for only 1 h, since longer exposure times resulted in biofilm eradication (data not shown). Reductions in CFU counts of *P. aeruginosa* biofilm were consistent over 10 iterations.

We did not observe the emergence of resistance with exposure of *S. aureus* or *P. aeruginosa* biofilms to H_2O_2- or HOCl-producing e-scaffolds over 10 iterations. We measured the susceptibilities of planktonic and biofilm forms of *S. aureus* USA100 and *P. aeruginosa*

TABLE 2 Planktonic and biofilm susceptibilities of *Staphylococcus aureus* USA100 and *Pseudomonas aeruginosa* IDRL-11442 before and after repeated e-scaffold exposure for 10 sequential iterations

Bacteria	Value (means ± SD, in mM) for[a]:					
	H_2O_2			HOCl		
	MIC (planktonic)	MBIC (biofilm)	MBBC (biofilm)	MIC (planktonic)	MBIC (biofilm)	MBBC (biofilm)
S. aureus USA100 before e-scaffold exposure	0.40 ± 0.00	0.40 ± 0.00	85 ± 29	1.65 ± 0.57	1.32 ± 0.57	1.32 ± 0.57
S. aureus USA100 after e-scaffold exposure for 10 sequential iterations	0.66 ± 0.23	0.66 ± 0.23	68 ± 29	1.99 ± 0.00	1.65 ± 0.57	1.65 ± 0.57
P. aeruginosa IDRL-11442 before e-scaffold exposure	0.60 ± 0.34	51 ± 0.00	170 ± 59	0.99 ± 0.00	1.32 ± 0.57	0.99 ± 0.00
P. aeruginosa IDRL-11442 after e-scaffold exposure for 10 sequential iterations	0.66 ± 0.23	85 ± 29	204 ± 0.00	1.32 ± 0.57	0.99 ± 0.00	1.65 ± 0.57

[a]Susceptibility data values (i.e., MIC, MBIC, and MBBC) are represented as means ± SD ($n = 3$). All experiments were performed in triplicate.

IDRL-11442 after 10 iterations of exposure to both H_2O_2- and HOCl-producing e-scaffolds. As evident from Table 2, there was no significant difference in the mean MIC, MBIC, or MBBC before and after e-scaffold exposure, suggesting that repeated exposure to H_2O_2 or HOCl as delivered herein does not affect susceptibility to H_2O_2 or HOCl.

DISCUSSION

We assessed planktonic and biofilm susceptibilities to H_2O_2 and HOCl of 27 bacterial isolates. Mean H_2O_2 MICs ranged from 0.20 to 3.19 mM. Low concentrations of H_2O_2 disrupt cell membranes, oxidize DNA, and destabilize enzymes and proteins (16). Moreover, H_2O_2 is rapidly oxidized to a hydroxyl radical (·OH), which promotes oxidative stress (17). In other studies, H_2O_2 MIC values have been reported to range between 0.40 and 14 mM, similar to those observed here (18, 19). In prior studies, variable susceptibility to H_2O_2 has been reported and tolerance to H_2O_2 described as more strain than species specific (19). The exact mechanism of action of HOCl is not fully understood. HOCl is a highly active oxidizing agent that disrupts cellular activities of proteins and oxidative phosphorylation and inhibits DNA synthesis (16). In one study, the HOCl MIC of bacterial isolates was >0.025% (~3.78 mM), similar to what was found with *P. aeruginosa* IDRL-7543 (20). Mazzola et al. described a MIC range of HOCl against various bacterial species of 0.02 to 0.06% (3 to 9 mM) (21) and that these values were dependent on the pH of the HOCl solution. Thus, HOCl MICs may depend on factors such as pH; at pH 4.0 to 7.0, HOCl was most active (21). At pH >7.5, HOCl is no longer the active moiety in solution, as free chlorine speciation becomes dominated by OCl^-.

Prior work has shown that biofilms have reduced susceptibilities to H_2O_2 and HOCl compared to the same isolates grown planktonically (22, 23). Compared to their planktonic forms, biofilms are more evolved and complex. Bacteria in biofilms grow slowly and have overall reduced metabolic activity. They are also encased in an extracellular polymeric substance matrix comprised of DNA, proteins, and polysaccharides, which protects them from adverse environmental conditions and confers mechanical and biochemical protection against biocides and antibiotics (24, 25). Additionally, the interior of biofilms has a lower pH than the surface, alongside less oxygen and water availability, which may render biocides ineffective (26). The MBICs of H_2O_2 were not higher than MICs for most bacterial isolates used in this study. The exception was *P. aeruginosa*, which showed 128- to 256-fold higher H_2O_2 MBIC than MIC values. Overall, Gram-negative bacteria had relatively higher MBBCs for H_2O_2 than Gram-positive bacteria. *Escherichia coli* and *P. aeruginosa* isolates studied were found to have the most tolerance to H_2O_2 when grown as biofilms. Perumal et al. found similar results (18); they performed MIC and MBBC assays to evaluate the activity of various disinfectants against Gram-negative bacteria, observing that bacterial biofilms were 266-fold less susceptible to H_2O_2 than bacteria in the planktonic state. The addition of another acidic agent (e.g., peracetic acid or 2-furoic acid) in combination with H_2O_2 improved the susceptibility of bacteria to these agents when exposed for short time intervals. In several

other studies, bacterial biofilms were exposed to H_2O_2 for short durations as part of surface contact treatments, with varying results (15, 27). For example, in one study, among different disinfectants used, only H_2O_2 and sodium hypochlorite removed both *S. aureus* and *P. aeruginosa* biofilm matrix and bacterial viable mass (28). It is expected that over a 24-h period, H_2O_2 will be oxidized into other reactive oxygen species (ROS), including hydroxyl radical and singlet oxygen species, and undergo autocatalytic degradation to oxygen and water. Bacterial cells embedded in outer layers of biofilms can produce free radical scavenger molecules that destroy some ROS during early stages of interactions, which occur when biofilm cells are presented with H_2O_2 (29). Prior studies have revealed that H_2O_2 cannot effectively penetrate mature biofilms with outer surface biofilm layers decomposing H_2O_2 and abrogating its effective diffusion into interior layers (30). The effective diffusion coefficients of solute molecules like H_2O_2 and HOCl are reduced in biofilm environments compared to water (31). Expression of new genes and their resulting products has been hypothesized to play a prominent role in reduced susceptibility of biofilms toward biocides (32). In addition, bacterial cells present in biofilm layers can produce a plethora of enzymes, including catalases, peroxidases, glutathione reductase, and superoxide dismutase (16), which can break down H_2O_2, HOCl, and antibiotics. A common enzyme produced by bacteria to destroy H_2O_2 is catalase. The degradation of H_2O_2 due to catalase production could be a reason we observed high MBBC values with H_2O_2 exposure. Among the isolates studied here, *E. coli* and *P. aeruginosa* are known to have strong SOS response signaling pathways when challenged with sublethal concentrations of H_2O_2. Work done by Elkin et al. demonstrates a protective role of catalase genes *katA* and *katB* in *P. aeruginosa* mutant strains (in planktonic and biofilm forms) when exposed to sublethal concentrations of H_2O_2 (33). The authors conclude that KatA catalase is important for conferring resistance to H_2O_2, especially at high concentrations, whereas KatB catalase helps confer resistance when initial levels of H_2O_2 are sublethal. In our study, *P. aeruginosa* PA14 Δ*katAB* had a 16-fold lower MBIC value than its parent wild-type isolate, *P. aeruginosa* PA14. This supports the idea that catalase produced by *P. aeruginosa* has a protective role against H_2O_2 by degrading it. *E. coli* isolates have distinct stress response elements when exposed to H_2O_2; induction of SoxR and OxyR regulons is mainly responsible for providing resistance to H_2O_2 (34). The high MBBC values observed here may be attributed, at least in part, to the activation of enzymes connected to oxidative stress response signaling. H_2O_2 has a higher probability of being degraded in the presence of bacterial enzymes than other biocides. Therefore, it is our view that to ideally use H_2O_2 as an antibiofilm agent, a high working concentration of H_2O_2 along with a long surface contact time are likely to be needed.

The mean MICs of HOCl against the bacteria studied ranged from 0.50 to 1.99 mM. In contrast to H_2O_2, we did not observe large variations in MIC, MBIC, or MBBC ranges. The mechanism of action of HOCl is incompletely defined, and how bacterial molecular stress mechanisms respond to it are also poorly understood. It has been proposed that the transport of free chlorine into biofilms is a significant factor in imparting resistance (35). In work done by Castillo et al., HOCl was used as oral rinses to remove dental plaque (36). HOCl was a more effective antibacterial agent than chlorhexidine and reduced bacterial viability of different periodontopathic bacteria found in biofilms. The authors suggested that HOCl can oxidize taurine, an amino acid, promoting the formation of chlorine-taurine complexes that have antibacterial activity. In another study, 0.018% HOCl (2.72 mM) removed lipopolysaccharides found in *Porphyromonas gingivalis* biofilms. The authors suggested that HOCl forms chlorohydrins, which attack acyl chains in unsaturated fatty acids, causing cell membrane damage along with cytolysis (37). HOCl has been found to interact with sulfur-containing amino acids, aromatic amino acids, nitrogen-containing compounds, and lipids (38). Various ATP-independent HOCl-sensing chaperones, like Hsp33, RidA, CnoX, etc., have been found to be activated as part of the immediate counter-response to HOCl, especially in Gram-negative bacteria.

In previous work, we evaluated e-scaffold antibiofilm activity against biofilms of *S.*

aureus, *P. aeruginosa*, and *A. baumannii* (13, 14, 39). In these studies, we observed time-dependent increases in antibiofilm activity with >4-$\log_{10}$ biofilm reductions after 24 h of treatment for biofilms exposed to H_2O_2-producing e-scaffolds and complete eradication of biofilms when exposed to HOCl-producing e-scaffold for 4 h. We also found that treatments were not toxic to host tissue (13, 14). Given the prolonged exposure to biocides associated with e-scaffolds, there might be concerns about selection for resistance to H_2O_2 or HOCl. Here, we show that H_2O_2- and HOCl-generating e-scaffolds maintain activity against *S. aureus* and *P. aeruginosa* biofilms after 10 iterations of exposure under the conditions studied. Until now, there have been few studies of biocide resistance in planktonic bacteria over several generations of exposure. Ikai et al. evaluated antibacterial activity of hydroxyl radicals generated by the photolysis of H_2O_2 (40), examining repeated biocide exposure over 40 continuous generations in selected bacterial pathogens and reporting no evidence of selection of biocide resistance.

Our e-scaffold system continuously produces small amounts of H_2O_2 or HOCl, below concentrations that are toxic to tissue. We produced ~45 mM H_2O_2 in 24 h of continuous treatment and ~22 mM HOCl in 4 h (13, 14). By continuously producing small amounts of these biocides, we achieved an ~5-$\log_{10}$ reduction in the number of CFU of *S. aureus* USA100 despite a mean H_2O_2 MBBC of 85 mM. The continuous production of H_2O_2 and HOCl likely can overwhelm oxidative stress response systems in bacterial biofilms to the point where that they cannot respond.

A limitation of this study is that we performed the biocide resistance experiments on biofilms formed for short durations. This does not fully represent the chronic wound infection environment, which frequently harbors mature biofilms. Furthermore, susceptibility testing was done on biofilms on pegged lids whose material composition is different from that of biofilms used for resistance iteration testing and also may not represent the actual situation found in wound infections. Additionally, we only tested two bacterial strains commonly found in wounds to evaluate the potential emergence of resistance. Another limitation is that we grew subsequent iterations of biofilms from two/three colonies of bacteria, which were exposed to e-scaffold treatment. This reduces the probability of selecting a mutation in the next iteration. Bacteria also were grown without selective pressure (in broth culture and then used to establish biofilms for 24 h). Finally, selective evolution of biocide resistance depends on the initial number of cells before treatment (41), and we did not study large population sizes.

In conclusion, our data suggest that HOCl has similar activity against planktonic and biofilm bacteria, whereas H_2O_2 is substantially less active against biofilm than planktonic bacteria. We did not observe the emergence of antibiofilm resistance with repeated exposure to either H_2O_2- or HOCl-producing e-scaffolds under the conditions studied.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. The 27 isolates studied are listed in Table 1. Isolates were removed from −80°C freezer stocks and streaked onto sheep blood agar plates.

Susceptibility of planktonic bacterial isolates to hydrogen peroxide or hypochlorous acid. H_2O_2 and HOCl MICs for *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *E. coli*, *P. aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* were determined by a modified broth microdilution protocol described in the Clinical and Laboratory Standards Institute (CLSI) guidelines (42). Overnight-grown bacterial colonies were used to inoculate 5 ml of tryptic soy broth (TSB; catalog no. 211825; BD Company, Sparks, MD) and cultures grown to a 0.5 McFarland standard. A 30% (wt/wt) stock solution of H_2O_2 (H1009; Sigma-Aldrich) was diluted to a 5% working solution in cation-adjusted Mueller-Hinton broth (CAMHB) for susceptibility assays. This was serially diluted to concentrations ranging from 1.632 to 0.19 mM so that each well contained 50 μl of H_2O_2, and then 50 μl of CAMHB (212322; BD Company, Sparks, MD) containing ~5×10^5 CFU of bacteria was added to wells of U-bottom 96-well plates (non-tissue culture treated; 35117; Corning Incorporated, Corning, NY). A stock solution of 0.0525% (~7.94 mM) HOCl (Aquaox, Loxahatchee, FL) was diluted in CAMHB to create testing concentrations ranging from 3.97 to 0.062 mM, and the addition of bacteria was done as described above. Plates were incubated at 37°C for 18 to 20 h and MICs recorded as the wells with the lowest concentration of H_2O_2 or HOCl with no turbidity. All experiments were performed in triplicate, with data represented as means ± standard deviations (SD).

Susceptibility of bacterial biofilms to hydrogen peroxide or hypochlorous acid. Minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm bactericidal concentrations (MBBCs) of H_2O_2

and HOCl against bacteria were determined using a pegged-lid microtiter plate assay (43). Briefly, 150 μl of bacterial suspension in TSB (standardized to a 0.5 McFarland) was added to wells of flat-bottom 96-well plates (243656; Thermo Scientific, Roskilde, Denmark) and covered with 96-pegged lid (445497; Nunc-TSP; Thermo Scientific). Plates were incubated for 6 h at 37°C on an orbital shaker (120 rpm). Pegged lids were rinsed with 1× phosphate-buffered saline (PBS; 10× PBS buffer; AM9625; Invitrogen) and transferred to a microplate containing serial dilutions of H_2O_2 (1.632 to 0.19 mM) or HOCl (3.97 to 0.062 mM) in CAMHB. Plates were incubated for 24 h at 37°C without shaking. MBICs were recorded as the lowest concentration of biocide showing no visible bacterial growth. Next, the pegged lids were washed in PBS and transferred to recovery microtiter plates containing 200 μl of CAMHB per well and incubated at 37°C for an additional 24 h. MBBCs were recorded as the wells with the lowest concentration of H_2O_2 or HOCl with no turbidity. All experiments were performed in triplicate, with data represented as means ± SD.

Repeated exposure of methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* biofilms to H_2O_2 and HOCl generated by e-scaffolds to assess decrease in activity with repetitive exposure. For these experiments, we used H_2O_2- and HOCl-generating e-scaffolds made of carbon fabric designed and assembled as in our previous study (14). e-scaffolds electrochemically reduce dissolved oxygen to H_2O_2 when polarized at -0.6 $V_{Ag/AgCl}$ or produce HOCl when polarized at $+1.5$ $V_{Ag/AgCl}$ (13, 14). We evaluated changes in activity using polarized e-scaffolds against *S. aureus* USA100 and *P. aeruginosa* IDRL-11442. Biofilms were grown *in vitro* in 6-well plates for 24 h at 37°C and then exposed to H_2O_2-generating e-scaffolds for 24 h (for both *S. aureus* and *P. aeruginosa*). For HOCl-generating e-scaffold treatment, *S. aureus* biofilms were exposed for 4 h and *P. aeruginosa* for 1 h at room temperature (initial inoculum, $\sim 1 \times 10^4$ CFU [CFU/well]). Controls were biofilms exposed to nonpolarized e-scaffolds. After e-scaffold treatment, biofilms were removed and quantified and results reported as $\log_{10}$ CFU/cm^2, as previously described (39). After the first treatment, two to three colonies recovered from quantitative culture were used to prepare a new biofilm, which was again exposed to treatment for the same time; this was repeated for 10 iterations. At the end of 10 iterations of exposure, we again determined the MIC, MBIC, and MBBC of the two studied bacterial isolates. All experiments were performed in triplicate, with data represented as means ± SD.

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