

Synergistic Effect and Antibiofilm Activity of a Skin and Wound Cleanser

Ann Beal Salamone, MA¹; Joseph C. Salamone, PhD¹; Rebecca Erin McMahon, PhD¹; Suprena Poleon, BS²; Nina Bionda, PhD³; and Peter D'Arpa, PhD⁴

ABSTRACT

Introduction. Biofilm in chronic wounds impedes the wound healing process. Each biofilm has differing characteristics requiring a multifaceted approach for removal while maintaining a surrounding environment conducive to wound healing. **Objective.** In this study, 3 of the components in a wound cleanser are tested to determine synergy in eradicating biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* in vitro. **Materials and Methods.** The 3 components assessed for synergy were ethylenediamine tetraacetic acid sodium salts (EDTA), vicinal diols (VD; ethylhexylglycerin and octane-1,2-diol), and polyhexamethylene biguanide (PHMB). Each component was assessed individually and in combination while dissolved in a base solution. The Calgary assay method was used for biofilm growth and treatment. Kull Equation analysis for synergy was conducted using viable count results. **Results.** Synergy is defined as the interaction of components to produce a combined effect greater than the sum of their separate effects. The base solution containing all 3 components (EDTA, VD, and PHMB) reduced biofilm viability by more than 5 logs, demonstrating statistically significant synergy. The 3 components tested individually in the base solution resulted in the following: EDTA did not reduce bacteria viability; VD reduced viability by about 1 log; and PHMB reduced *P aeruginosa* viability by about 2.5 logs and MRSA viability by about 4 logs. Of importance, the MRSA biofilm failed to regrow in the recovery plates after combined treatment, indicating complete elimination of the biofilm bacteria. **Conclusions.** The experimental and calculated results indicate the 3 components (VD, EDTA, and PHMB) when used together act synergistically to eradicate MRSA and *P aeruginosa* biofilms in vitro.

KEY WORDS

wound cleanser, biofilm, MRSA, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, synergy, EDTA, vicinal diols, polyhexamethylene biguanide

INDEX

Wounds 2020;32(8):208–216. Epub 2020 May 7

In most natural environments, free-floating bacteria exist transiently and only as a minor population, while the predominant form is biofilm,¹ aggregates of microorganisms within a self-created polymeric matrix wherein they are resistant to host defenses and antimicrobial agents. Biofilm has been reported to be involved in 78% to 90% of human chronic wounds^{2,3} and is associated with delayed wound healing⁴ and other negative wound healing outcomes.⁵⁻⁷

Solutions for the elimination of biofilm have demonstrated significant challenges, in part due to the complexity of the biofilm structure. Biofilm is the construct (microbial community) created through the attachment of microorganisms to substrata within an extracellular polymeric substance (EPS).^{8,9} This construct is stabilized by electrostatic interactions, hydrogen bonds,

and London dispersion forces.^{8,9} The EPS comprises about 50% to 90% of the total biofilm organic matter and varies depending on the microorganisms, environment, and biofilm age.^{8,10,11} The EPS of biofilm in wounds is comprised of dead host tissues, in addition to the substances the microorganisms secrete, as well as proteins, nucleic acids, lipids, polysaccharides, and humic substances.^{8,10,11} These substances and their interactions are targets for biofilm elimination from wounds.

Control of the wound bioburden and biofilm involves multiple treatment modalities and components that impact microbial activity and the integrity and attachment of EPS.^{12,13} Optimally, components are synergistic not just additive. In other words, with synergy, 1 plus 1 is greater than 2 (1 + 1 > 2).

Components of the wound cleanser product studied herein were selected based on the authors' experiences in eye care and water treatment as well as taking into consideration the wound milieu to target a breadth of biofilms. These components comprise: (1) polyhexamethylene biguanide (PHMB), a broad-spectrum polycationic wound care antimicrobial¹⁴⁻¹⁶ that also is used in multipurpose contact lens solutions,¹⁷ water treatment, and numerous consumer products; (2) ethylenediamine tetraacetic acid sodium salts (EDTA), a chelator of divalent metal ions used in wound care, contact lens cleaners, and numerous personal care products¹⁸; and (3) vicinal diols (VD), ethylhexylglycerin and octane-1,2-diol, which are amphiphilic surfactants with moisturizing, antimicrobial, and odor-reducing functions used in underarm deodorants.^{19,20}

Table 1. Wound cleanser components and their properties

INGREDIENT	PROPERTIES	
Antibiofilm components	EPS disruption	Microbial kill
PHMB	In vitro: Cationic polymer that forms polyelectrolyte complexes with polyanions ⁴⁶ Flocculant ⁴⁵	Clinical: Antimicrobial ^{44,50} In vitro: Disrupts microbe cell wall ^{47,48} In vitro: Enters bacterial cells ⁴⁹
EDTA (di- and tri-Na)	Clinical/in vitro: Broad pH range chelator ¹⁸ In vitro: Chelates Ca ²⁺ ⁵⁷ In vitro: Destabilizes matrix integrity ¹⁸	Clinical: Antimicrobial ¹⁸ In vitro: Disrupts microbe cell wall ⁵⁶
VD	Clinical: Reduces odor ^{62,63} In vitro: Dissolves and swells lipids ^{63,64} Amphiphilic surfactants ^{63,64} HLB 7–7.5 ^{63,64}	Clinical: Antimicrobial ^{61,62} In vitro: Antimicrobial ^{61,62} In vitro: Disrupts microbe cell wall ¹⁹ In vitro: Enters microbe cells ¹⁹
BASE SOLUTION IN WATER	PROPERTIES	
P-407	Clinical: Detergent ⁷⁰ Triblock copolymer surfactant ⁶⁹ M _w 9,840–14,600 Daltons ⁶⁹ HLB 18–21.5 ⁶⁹	
HPMC	Clinical: Mucoadhesive ⁷³ Neutral charge ⁷³	
NaCl	Clinical: Osmolality balance ⁷⁴	

EPS: extracellular polymeric substance; PHMB: polyhexamethylene biguanide; EDTA: ethylenediamine tetraacetic acid sodium salts; VD: vicinal diols; HLB: hydrophilic-lipophilic balance; P-407: Poloxamer 407; HPMC: hydroxypropylmethylcellulose; NaCl: sodium chloride

The 3 components (PHMB, EDTA, and VD) were theorized to synergistically disrupt EPS, providing access to the microbes, and then to synergistically permeabilize cell membranes and impair processes needed for viability.

Each component was evaluated individually and compared with the 3 components combined in the cleanser product for synergistic activity in disrupting and killing *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms. Each test solution was dissolved in a base solution containing a non-ionic surfactant poloxamer 407 (P-407), a mucoadhesive hydroxypropylmethylcellulose (HPMC), and sodium chloride (NaCl). A statistically significant synergy index of less than 1 was determined, which proved synergy.

MATERIALS AND METHODS

Wound cleanser materials and compositions

Aqueous wound cleanser compositions were created by solvating PHMB, EDTA

di- and tri-sodium salts, ethylhexyl-glycerin and octane-1,2-diol (ie, VD, monoalkyl glycerol, and monoalkyl glycol, respectively) (Sensiva SC 50 and Sensiva SC 10; Schülke & Mayr GmbH), P-407, hydroxypropylmethylcellulose (HPMC), and sodium chloride (NaCl) in water at physiological osmolality and pH 5.5.

Bacterial strains

Methicillin-resistant *S aureus* strain (MRSA) USA-300 is a predominant community-associated methicillin-resistant strain that causes significant morbidity and mortality.²¹ *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC 27312) was originally isolated from an infected wound.²²

Experimental design

Correlational research was conducted to determine the synergy between 3 components of a wound cleanser based on anti-biofilm effectiveness. Biofilm was grown using the Calgary Biofilm Device (Innovotech Inc). The biofilm was treated

under specified conditions with the test solutions. The log survival of the biofilm microbes (the data) was mathematically assessed for synergy using the Kull equation.

Calgary assay for survival of *S aureus* or *P aeruginosa* grown in biofilm

The minimum biofilm eradication (MBEC) assay with the biofilm device was utilized.²³ An MBEC 96-peg lid (Innovotech Inc) was placed into a 96-well plate filled with 150 µL of an overnight culture of *S aureus* or *P aeruginosa* diluted to 0.1 OD₆₀₀. During method development, 2 inoculation volumes were investigated (100 µL and 150 µL). There was no significant difference in the overall number of biofilm-associated bacteria for either species with respect to different inoculation volumes. The higher inoculation volume of 150 µL was selected for this synergy study.

The MBEC plate was covered with parafilm to prevent evaporation and was incubated with shaking (75 rpm) for 48 hours. The biofilm-coated pegs were removed and rinsed with 200 µL/well of PBS twice

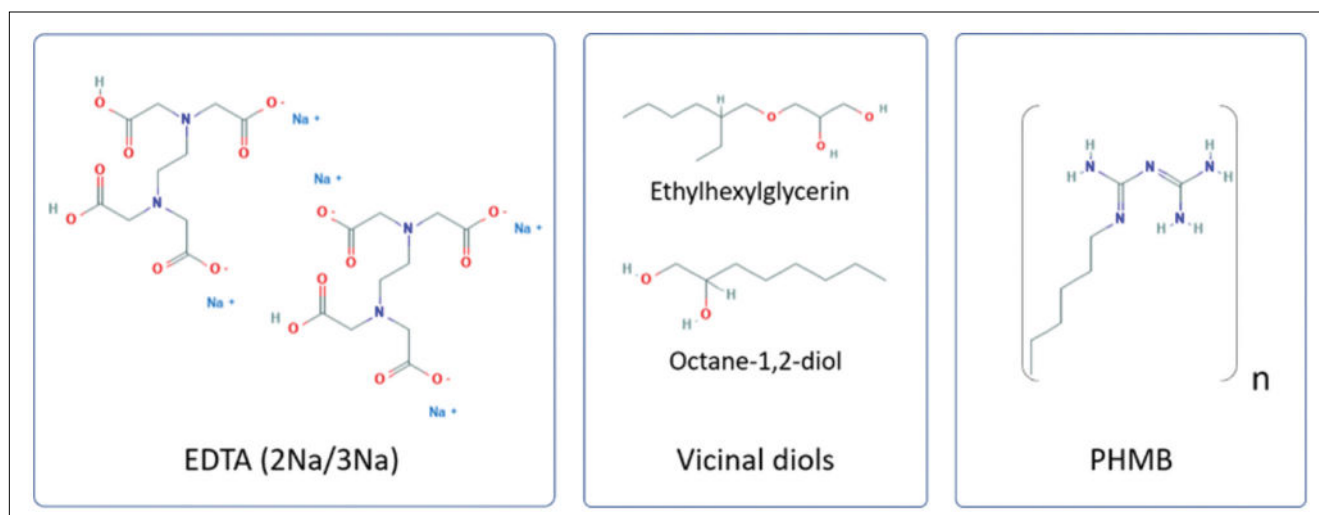


Figure 1. Wound cleanser components tested for synergism in eradicating biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Structures obtained from PubChem.³⁰

EDTA: ethylenediamine tetraacetic acid sodium salts; PHMB: polyhexamethylene biguanide

to remove loosely adhered bacteria. The volume of 200 μL /well of PBS, test solution, or neutralizer was selected to provide for complete submersion of the biofilm-coated pegs. Submersion of the pegs in the same volume of each solution eliminates potential data variations due to a portion of the biofilm surface not being exposed to the solutions. In clinical practice, washing/soaking of a wound's complete biofilm surface with a cleanser is desired. The pegs then were transferred into a 96-well plate containing the test or control solutions (200 μL /well) for a 15-minute incubation at room temperature with shaking (75 rpm). After treatment, the pegs were transferred into the neutralization plate containing 200 μL /well of Dey-Engley broth (Becton Dickinson) for 5 minutes before being transferred into the recovery plate (200 μL /well of tryptic soy broth; Becton, Dickinson and Company), which then was sonicated for 20 minutes to disintegrate the biofilms. From each well of the recovery plate, 100 μL was removed, serially diluted, and plated to enumerate colony forming units (CFUs). The remaining volume of the recovery cultures was incubated overnight to assess regrowth.

Data analysis

For this study, *t* tests with unequal variances were performed on the ranked data using the R function pairwise *t* test (with pooled.

sd = FALSE and Benjamini-Hochberg correction for multiple comparisons).²⁴ This test performs better than the Mann-Whitney *U* test in controlling Type I errors when variances are unequal.²⁵⁻²⁷

Synergy among the test solutions was determined using the Kull equation,²⁸ which calculates a Synergy Index (SI), equaling 1 for simple additivity, greater than 1 for antagonism, and less than 1 for synergy. The SI is the sum of the terms that are the ratios of the viable counts (vc) resulting from treatment with the complete cleanser (CC) — multiplied by the weight fraction (wf) of the ingredient evaluated in that term — to the vc resulting from the single ingredient (either EDTA, VD, or PHMB) (**Formula**).

RESULTS

Polymeric biguanides (PHMB), VD (ethylhexyl glycerin and octane-1,2-diol), and EDTA have demonstrated qualitative synergistic biocidal activity in antibiofilm research studies.²⁹ Here, quantitative studies with statistical analysis are presented, which verify statistically significant synergy.

Together, the 3 components with the base components of the complete wound cleanser are listed in **Table 1**, and the structures of the 3 components under study for synergistic antibiofilm activity are shown in **Figure 1**.³⁰

For assessing the synergistic activity of EDTA, VD, and PHMB in eliminating single-species biofilms of *P aeruginosa*

FORMULA

$$SI = \frac{(CC_{vc} * EDTA_{wf})}{EDTA_{vc}} + \frac{(CC_{vc} * VD_{wf})}{VD_{vc}} + \frac{CC_{vc} * PHMB_{wf}}{PHMB_{vc}}$$

Where:

SI: Synergy Index (*Synergy*: SI<1; *Additivity*: SI=1; *Antagonism*: SI>1); EDTA_{vc}: vc after EDTA treatment; VD_{vc}: vc after VD treatment; PHMB_{vc}: vc after PHMB treatment; CC_{vc}: vc after treatment with CC (ie, EDTA, VD, and PHMB); EDTA_{wf}: wf of EDTA=0.115; VD_{wf}: wf of VD=0.708; PHMB_{wf}: wf of PHMB=0.177

The confidence interval of the SI was constructed using bootstrap resampling (R package boot, bootstrap replicates = 10 000, set.seed = 1) of the vc data (replacing 0 CFU with 0.1 CFU).

and *S aureus*, the solutions listed in **Table 2** were prepared. These solutions all contain the base solution components: the neutral-charged, amphiphilic surfactant P-407 and the neutral-charged,

Table 2. Test solutions used to assess synergistic antibiofilm activity of EDTA, VD, and PHMB

SOLUTION	EDTA-2Na (wt %)	EDTA-3Na (wt %)	VD ^a 1 (wt %)	VD ^b 2 (wt %)	PHMB (ppm/wt %)	P-407 (wt %)	HPMC (wt %)	OSMOLALITY (mOsm/kg)	pH
A	0.05	0.015	–	–	–	2	0.2	334	5.5
B	–	–	0.3	0.1	–	2	0.2	347	6.2
C	–	–	–	–	1,000/0.1	2	0.2	335	6.3
D ^c	0.05	0.015	0.3	0.1	1,000/0.1	2	0.2	352	5.7

^a VD-1: Sensiva SC-50

^b VD-2: Sensiva SC-10

^c Composition of BIAKÖS™ Antimicrobial Skin and Wound Cleanser (Sanara MedTech Inc)

EDTA: ethylenediamine tetraacetic acid sodium salts; VD: vicinal diol; PHMB: polyhexamethylene biguanide; P-407: Poloxamer 407; HPMC: hydroxypropylmethylcellulose

mucoadhesive HPMC. In addition, solutions A through C contain 1 of the biofilm-disrupting components: EDTA, VD, or PHMB, respectively. Solution D is the CC comprised of all 3 biofilm-disrupting components in the base solution.

Biocidal activity of the test solutions was evaluated on biofilms grown using the biofilm device (ie, on pegs attached to the lids of 96-well culture plates).^{23,31} The biofilms were treated in blinded fashion with test solutions A–D (Table 2). After the treatments, the viable bacteria remaining adherent to the pegs were enumerated to determine viable bacterial counts (CFUs). As shown in Figure 2, solution A, containing EDTA and without VD or PHMB, was not effective at reducing the viability of either species' biofilm. Slightly effective was solution B, containing VD, which reduced the viability of both species by about 1 log. Solution C, containing PHMB, was the most effective of the single components, reducing viability by about 2.5 logs or about 4 logs. However, the complete wound cleanser, solution D, reduced the viability of the *S aureus* and *P aeruginosa* monospecies biofilms by more than 5 logs (Figure 2 and Tables 3, 4). Of importance, *S aureus* biofilms that yielded 0 CFU after treatment also failed to regrow after incubating the recovery plates overnight, demonstrating complete biofilm elimination from the pegs.

To assess synergy among EDTA, VD, and PHMB, the SI was calculated using the Kull equation.²⁸ The SI for these 3

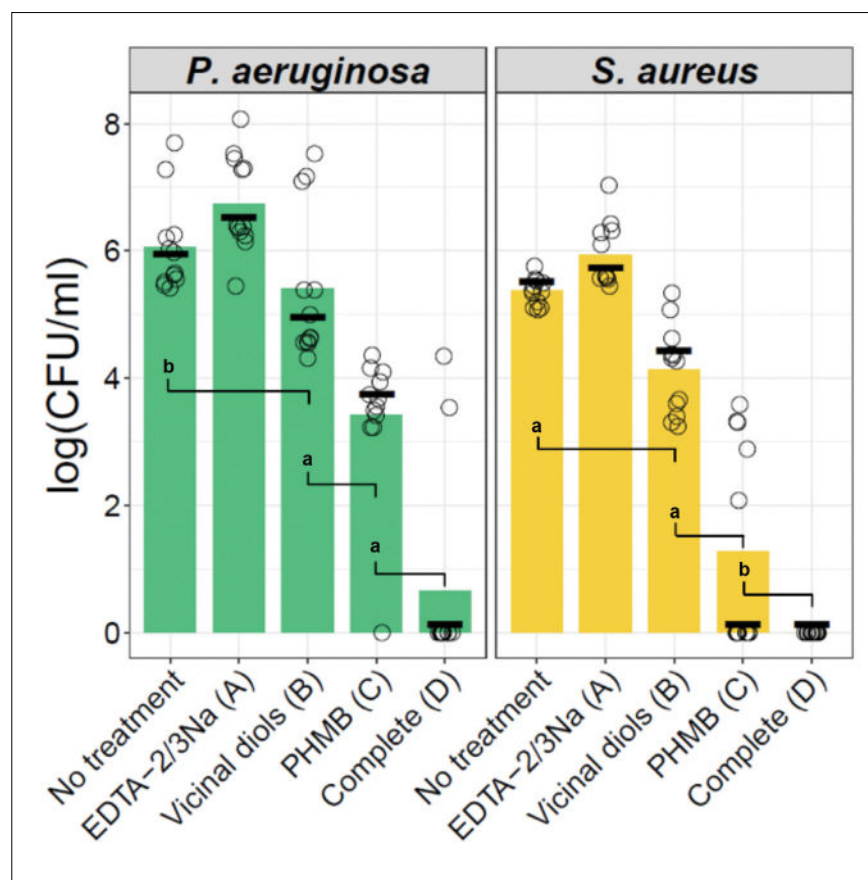


Figure 2. Viable cell counts after treatment of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms with the base solution containing either EDTA, VD, or PHMB; or the complete wound cleanser solution containing EDTA, VD, and PHMB.

Individual viable cell count determinations (CFU) are shown as open circles (n=12). Letters in parentheses correspond to the solutions listed in Table 2. The height of the green and yellow bars represents mean CFUs. The median is marked by thick horizontal lines.

Significant differences between the groups are shown for a one-sided t test for unequal variances on ranked data: ^aP<.001 and ^bP<.05.

EDTA: ethylenediamine tetraacetic acid sodium salts; VD: vicinal diol; PHMB: polyhexamethylene biguanide; CFU: colony forming unit

components in eradicating *P aeruginosa* and *S aureus* biofilms was 0.148 (95% CI, 0.019–0.340) and 0.032 (95% CI, 0.027–0.050), respectively. These SI values demonstrate evidence of highly synergistic biocidal activity by the components. Similar values of SI were calculated using median instead of mean log (CFUs) or when only VD and PHMB solutions were included in the analysis (ie, omitting solution A from the equation).

DISCUSSION

Using the Kull equation to analyze the biofilm viability experimental results, the

combination of PHMB, EDTA salts, and VD had been found to provide synergistic, not simply additive, antibiofilm effectiveness in this report. The Kull equation was created to determine synergy of anti-fungal mixtures that were tested against planktonic fungi. Subsequently, Schmaus et al³² used the Kull equation for synergy determination of mixtures of 1,2 alkane diols as antimicrobial agents when tested on planktonic microorganisms. To the authors’ knowledge, the use of the Kull equation to prove antibiofilm synergy for any application, including wound cleansers, has not previously been published.

Biofilm infections persist in wounds as a result of biofilm EPS blocking access of antimicrobial agents to their sites of action as well as microbes in biofilm having depressed metabolism and activated protective stress responses.³³⁻³⁶ Thus, the removal of biofilm from wounds is important to promote wound healing,³⁷ and an aggressive multimodal therapy that includes debridement, frequent lavage, and antimicrobial treatment is supported by clinical evidence.^{35,37} Such repeated attacks on biofilm forces it to reattach and reform, temporarily driving it into an immature state more susceptible to host defenses and antimicrobials.^{12,38} However within hours posttreatment, biofilm can reform³⁹ and spread into tissues where it adheres firmly,² thus repeatedly applied debridement⁴⁰ and topical biocides have been used to deter biofilm from reforming and entrenching into tissue.^{2,35} Of note, microbes do not proliferate unchecked in healthy tissue.⁴¹ This suggests that a multifaceted approach is required in vivo to remove all causes of tissue damage, such as compromised circulation, edema, or repeated trauma, while protecting injured tissue from microbial invasion and/or biofilm formation.⁴²

As listed in **Table 1** and, discussed further on, PHMB, EDTA, and the VD each individually are known to disrupt EPS and permeabilize microbial cell membranes and impair processes needed for viability.

Polyhexamethylene biguanide is a

Table 3. Mean CFU differences between treatments

STRAIN	TREATMENT	MEAN LOG ₁₀ CFU (% OF CONTROL)	SD
<i>Pseudomonas aeruginosa</i>	No treatment	6.06 (100)	0.73
	EDTA-2/3Na (A)	6.75 (111)	0.76
	VD (B)	5.41 (89)	1.17
	PHMB (C)	3.41 (56)	1.13
	Complete (D)	0.66 (11)	1.55
<i>Staphylococcus aureus</i>	No treatment	5.37 (100)	0.21
	EDTA-2/3Na (A)	5.93 (110)	0.5
	VD (B)	4.14 (77)	0.69
	PHMB (C)	1.27 (24)	1.61
	Complete (D)	0 (0)	0

CFU: colony forming unit; SD: standard deviation; EDTA: ethylenediamine tetraacetic acid sodium salts; VD: vicinal diol; PHMB: polyhexamethylene biguanide

Table 4. P values for differences between treatments

		NO Tx	EDTA-2/3Na (A)	VD (B)	PHMB (C)
<i>Pseudomonas aeruginosa</i>	EDTA-2/3Na (A)	0.99	-	-	-
	VD (B)	0.39	7.2e-04	-	-
	PHMB (C)	6.2e-09	5.8e-11	1.3e-04	-
	Complete (D)	5.8e-11	1.7e-12	2.1e-06	6.7e-04
<i>Staphylococcus aureus</i>	EDTA-2/3Na (A)	0.99	-	-	-
	VD (B)	3.1e-05	4.6e-07	-	-
	PHMB (C)	1.8e-06	3.4e-07	3.1e-05	-
	Complete (D)	2.3e-16	5.1e-13	5.9e-10	0.011

Tx: treatment; EDTA: ethylenediamine tetraacetic acid sodium salts; VD: vicinal diol; PHMB: polyhexamethylene biguanide

broad-spectrum antimicrobial, biocompatible^{43,44} cationic polymer that has a low average molecular weight range of 2000 to 4500 Daltons. Polyhexamethylene biguanide can penetrate EPS and form polyelectrolyte complexes with polyanions, such as DNA and polysaccharides, causing flocculation and large aggregates that may be removed more easily.⁴⁵ When PHMB is present, aggregation of EPS polyanions (alginates) in *P aeruginosa* biofilm is visually observable as “clumps.” Bueno and Moraes⁴⁶ used this effect to bind PHMB to chitosan-alginate wound dressings for sustained release of PHMB. Once in contact with microbes, the polycation PHMB interacts with microbial membrane anions to disrupt the cell membrane with resultant leakage of cytoplasmic components and inhibition of membrane-bound enzymes^{47,48}; additionally, PHMB enters bacterial cells, condenses chromosomal DNA, and arrests cell division.⁴⁹ Also, PHMB has been recommended as an antimicrobial compound of choice for chronic wounds and burns.⁵⁰

Ethylenediamine tetraacetic acid sodium salts (di- and tri-) functions over a wide pH range (2–12), which is a requirement as both the wound milieu pH range can be from acidic to basic depending on native biochemical processes for wound healing⁵¹ and multispecies biofilms (aerobic, facultative, and anaerobic) can have pH gradient ranges from acidic to basic.⁵² The EDTA is known to chelate divalent metal cations essential for bacterial growth⁵³ and destabilize bacterial membranes and matrix integrity.^{18,53–55} As an example, EDTA is reported to potentiate the antimicrobial effects of quaternary ammonium compounds by extraction of lipopolysaccharide from *P aeruginosa* cell walls.⁵⁶ Cationic quaternary ammonium compounds absorb on negatively charged cell walls concurrently with EDTA chelation of cell wall metal cations with a resulting loss of lipopolysaccharides and increased cell permeability.⁵⁶ Also, EDTA is reported to inhibit excess matrix metalloproteases by chelating zinc and calcium,⁵⁷ thereby facilitate wound healing.⁵⁸ Consistent with healthy human tissue, the wound cleanser’s EDTA (di- and tri-sodi-

um) provides a slightly acidic pH, which, together with physiologic osmolarity to prevent cell dehydration from high osmolarity or cell swelling from low osmolarity, contribute to mitigating pain while being non-cytotoxic to human tissue.^{59,60}

The VD, ethylhexylglycerin and octane-1,2-diol, are multifunctional personal care ingredients commonly used in underarm deodorants, with moisturizing and antimicrobial activities resulting in odor reduction due to inhibition of odor-causing Gram-positive bacteria (eg, *Corynebacterium* spp, *Leifsonia aquaticum*, *Ochrobactrum anthropi*, *Kocuria rhizophila*).^{61–64} They are known to disrupt microbial membranes and synergistically boost the effectiveness of preservatives such as parabens or phenoxyethanol¹⁹ — eg, ethylhexylglycerin potentiated the lethality of phenoxyethanol against *P aeruginosa* and *Aspergillus niger*.⁶⁵ With 8 carbon atoms and 2 hydroxyl groups on adjacent carbons, these amphiphilic surfactants, with an hydrophilic-lipophilic balance (HLB) of 7 to 7.5, provide humectance (hydration) and emollience (occlusivity, softening, lubrication, spreading, and delivery of actives), solvate out lipids and humic components (ie, from biofilm), and have antimicrobial activities differing from PHMB.^{63,64} While PHMB has broad-spectrum activity against bacteria, fungi, protozoa, and viruses, the VD are particularly effective against Gram-positive bacteria and yeasts.^{65,66}

By incorporating the combination of PHMB, EDTA, and VD, synergistic antibiofilm effectiveness was found, not just additive effectiveness. The SI values determined were 0.148 with *P aeruginosa* and 0.032 with MRSA. The SI values are notably lower than 1, thus indicating high synergy.

The base solution for these studies and used in the wound cleanser was developed to complement the synergistic antibiofilm efficacy of the 3 components (PHMB, EDTA, and VD). The base solution comprises water, a salt, a mucoadhesive, and a surfactant.

Surfactants lower the interfacial tension between substances and can loosen and remove dirt, debris, slough and loosen

biofilm from the wound. Very hydrophilic surfactants, such as poloxamer 188 with an HLB of 29, have been used to aid in the removal of biofilm from wound surfaces.^{12,67,68} However, the nonionic surfactant, P-407 in the base solution, not only lowers interfacial tension to aid in removal of debris but also is a detergent (HLB 18–21.5)⁶⁹ that incorporates (ie, emulsifies) hydrophobic materials into water.⁷⁰ These hydrophobic materials may be present as lipids, proteins, and polysaccharides, for instance. By incorporating these organic substances into water, they are easier to remove by irrigation. In addition to serving as a detergent and surfactant, P-407 aids in the solubility of the VD due to its amphiphilic competency.⁷¹ Poloxamer 407 (Pluronic 127; Sigma-Aldrich) is a triblock copolymer consisting of a central hydrophobic block of about 101 repeats of polypropylene glycol flanked by 2 hydrophilic blocks of about 56 repeats of polyethylene glycol. Of note, P-407 helps to maintain the activity of PHMB⁷ and VD.

Mucoadhesives are used to increase residence time of a composition on a mucosal membrane such as found in the gastrointestinal tract, lungs, and eyes. Mucosal membranes contain up to 95% water, with the remaining components comprising glycoproteins, lipids, and other hydrophilic organic matter.⁷² In general, a wound bed has similarities to mucosal tissue, such as higher water content combined with the presence of hydrophilic organic matter, as found in wound exudate as well as biofilm. Therefore, the mucoadhesive, water-soluble, neutral-charged HPMC⁷³ forms a hydrated film on the wound surface and, hence, increases the cleanser’s residence time.

In order to adjust osmolality to a physiologically normal range (290–320 mOsm/kg), sodium chloride is added as a component of the base solution. When products used on wounds are hypertonic, water is pulled out of surrounding tissue through osmosis, which causes dehydration and cell size shrinkage.⁷⁴ The opposite effect occurs when osmolality is hypotonic; the surrounding tissue pulls in water causing cell size

enlargement.⁷⁴ Pain is created with either too high or too low osmolality, and neurological damage is suffered.⁷⁴ Therefore, a physiologically balanced osmolality is preferred for protection of healthy tissue in and surrounding the wound.

In summary, the targeted points of the wound cleanser are (1) synergistic antibiofilm components (PHMB, EDTA, and VD) complemented by (2) P-407 to remove loose debris from the wound surface through interfacial tension reduction as well as to incorporate hydrophobic materials (ie, emulsification) into water and (3) the mucoadhesive HPMC to increase residence time on wound surfaces. Additionally, physiologically balanced pH and osmolality are gentle to human tissue.^{59,60,74}

Clinical validation of the antimicrobial cleanser is in progress with bacterial fluorescence and DNA sequencing. This also includes targeted data points of wound healing progression and economic evaluation. The algorithm for use of this wound cleanser is provided under the guidelines from the International Wound Infection Institute International Consensus Update 2016/Wound Infection in Clinical Practice for “Effective Wound Infection Management,”⁷⁵ which recommends regular wound evaluation for signs of infection and to “cleanse the wound with each dressing change.” The cleanser effectiveness may be impacted by enzymes, ointments, or oils in the wound bed. Irrigation of the wound bed may be performed with the cleanser to thoroughly rinse the wound bed from these agents.

LIMITATIONS

Biofilm in wounds treated in vivo is expected to be susceptible to the same synergistic biocidal activities that have been observed for biofilm treated in vitro due to the multitargeted chemical approach. However, biofilm in vivo is affected by systemic environmental factors, such as host immune response, cardiovascular sufficiency, age, nutrition, and local environmental factors, such as repeated trauma. Therefore, the biofilm composi-

tion and density distribution (both EPS and microbial cells) will be influenced by these factors in each biofilm-containing wound. The synergistic biocidal activity likely extends to biofilms of other microbial species because of the gross similarity (proteins, polysaccharides, lipids, humic substances) of EPS across species; however, the current findings are limited to monospecies biofilms of *P aeruginosa* and *S aureus*, common species that infect wounds. Translational research is needed to verify these results in clinical wounds.

CONCLUSIONS

Highly synergistic antibiofilm activity was observed for the wound cleanser components — VD, EDTA, and PHMB, in an aqueous base solution of P-407 and HPMC where osmolality and pH were at physiological levels — against *P aeruginosa* and MRSA monospecies biofilms in vitro. Clinical studies to compare these in vitro synergy results with clinical outcomes is the next research step. **W**

ACKNOWLEDGMENTS

Affiliations: ¹Rochal Industries LLC, San Antonio, TX; ²University of North Texas Health Science Center, Fort Worth, TX; ³Fyber, Ithaca, NY; and ⁴BioMedWrite, North Potomac, MD

Correspondence: Ann Beal Salamone, MA, Chairman of the Board, Rochal Industries LLC, Research & Development, 12000 Network Blvd, B-200, San Antonio, TX 78249; absalamone@rochalindustries.com

Disclosure: This study was funded by Rochal Industries LLC (San Antonio, TX). Dr. Joseph C. Salamone, Ann Beal Salamone, Dr. Rebecca E. McMahon and Suprena Poleon are/were employees of Rochal Industries LLC. iFyber was paid to design and conduct the blinded biofilm studies. BioMedWrite was paid to co-write this manuscript and to independently conduct statistical analyses.

REFERENCES

1. Wolcott RD, Ehrlich GD. Biofilms and chronic infections. *JAMA*. 2008;299(22):2682–2684. doi:10.1001/jama.299.22.2682
2. Attinger C, Wolcott R. Clinically addressing biofilm in chronic wounds. *Adv Wound Care (New Rochelle)*. 2012;1(3):127–132.
3. Malone M, Bjarnsholt T, McBain AJ, et al. The

prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. *J Wound Care*. 2017;26(1):20–25. doi:10.12968/jowc.2017.26.1.20

4. Metcalf DG, Bowler PG. Biofilm delays wound healing: a review of the evidence. *Burns Trauma*. 2013;1(1):5–12. doi:10.4103/2321-3868.113329
5. Bloemsma GC, Dokter J, Boxma H, Oen IM. Mortality and causes of death in a burn centre. *Burns*. 2008;34(8):1103–1107. doi:10.1016/j.burns.2008.02.010
6. Omar A, Wright JB, Schultz G, Burrell R, Nadworny P. Microbial biofilms and chronic wounds. *Microorganisms*. 2017;5(1). doi:10.3390/microorganisms5010009
7. Withycombe C, Purdy KJ, Maddocks SE. Micro-management: curbing chronic wound infection. *Mol Oral Microbiol*. 2017;32(4):263–274. doi:10.1111/omi.12174
8. Flemming HC, Wingender J. Relevance of microbial extracellular polymeric substances (EPSs)—part I: Structural and ecological aspects. *Water Sci Technol*. 2001;43(6):1–8.
9. Mayer C, Moritz R, Kirschner C, et al. The role of intermolecular interactions: studies on model systems for bacterial biofilms. *Int J Biol Macromol*. 1999;26(1):3–16.
10. Vu B, Chen M, Crawford RJ, Ivanova EP. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules*. 2009;14(7):2535–2554. doi:10.3390/molecules14072535
11. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis*. 2002;8(9):881–890.
12. Das Ghatak P, Mathew-Steiner SS, Pandey P, Roy S, Sen CK. A surfactant polymer dressing potentiates antimicrobial efficacy in biofilm disruption. *Sci Rep*. 2018;8(1):873. doi:10.1038/s41598-018-19175-7
13. Kostakioti M, Hadjifrangiskou M, Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med*. 2013;3(4):a010306. doi:10.1101/cshperspect.a010306
14. Moore K, Gray D. Using PHMB antimicrobial to prevent wound infection. *Wounds UK*. 2007;3(2):96–102.
15. Wiegand C, Abel M, Ruth P, Hipler UC, HaCaT keratinocytes in co-culture with *Staphylococcus aureus* can be protected from bacterial damage by polihexanide. *Wound Repair Regen*. 2009;17(5):730–738. doi:10.1111/j.1524-475X.2009.00536.x

16. Kamaruzzaman NF, Tan LP, Mat Yazid KA, et al. Targeting the bacterial protective armour; challenges and novel strategies in the treatment of microbial biofilm. *Materials (Basel)*. 2018;11(9). doi:10.3390/ma11091705
17. Yanai R, Ueda K, Nishida T, Toyohara M, Mori O. Effects of ionic and surfactant agents on the antimicrobial activity of polyhexamethylene biguanide. *Eye Contact Lens*. 2011;37(2):85–89. doi:10.1097/ICL.0b013e31820cebc3
18. Finnegan S, Percival SL. EDTA: an antimicrobial and antibiofilm agent for use in wound care. *Adv Wound Care (New Rochelle)*. 2015;4(7):415–421.
19. Langsrud S, Steinhauer K, Luthje S, Weber K, Goroncy-Bermes P, Holck AL. Ethylhexylglycerin impairs membrane integrity and enhances the lethal effect of phenoxyethanol. *PLoS One*. 2016;11(10):e0165228. doi:10.1371/journal.pone.0165228
20. Okukawa M, Watanabe T, Miura M, Konno H, Yano S, Nonomura Y. Antibacterial activity of 1,2-alkanediol against *Staphylococcus aureus* and *Staphylococcus epidermidis*. *J Oleo Sci*. 2019;68(8):759–763. doi:10.5650/jos.ess19074
21. McClure JA, Zhang K. Complete genome sequence of a community-associated methicillin-resistant *Staphylococcus aureus* hypervirulent strain, USA300-C2406, isolated from a patient with a lethal case of necrotizing pneumonia. *Genome Announc*. 2017;5(22):e00461–17. doi:10.1128/genomeA.00461-17
22. Schroeter J. Über einige durch Bakterien gebildete Pigmente. In: Cohn F, ed. *Beiträge zur Biologie der Pflanzen*. J. U. Kern's Verlag. Breslau, 1875:109–126.
23. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol*. 1999;37(6):1771–1776.
24. R Core Team. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, 2016.
25. Zimmerman DW, Zumbo BD. Rank transformations and the power of the Student *t*-test and Welch *t*-test for non-normal populations with unequal variances. *Can J Exp Psychol*. 1993;47(3):523–539. doi:10.1037/h0078850
26. Ruxton G. The unequal variance *t*-test is an underused alternative to Student's *t*-test and the Mann-Whitney U test. *Behavioral Ecology*. 2006;17(4):688–690.
27. Cribbie RA, Bewell C, Wilcox RR, Keselman HJ. Tests for treatment group equality when data are nonnormal and heteroscedastic. *J Modern Applied Statistical Methods*. 2007;6(1):117–132. doi:10.22237/jmasm/1177992660
28. Kull FC, Eisman PC, Sylwestrowicz HD, Mayer RL. Mixtures of quaternary ammonium compounds and long-chain fatty acids as antifungal agents. *Appl Microbiol*. 1961;9(6):538–541.
29. Salamone J, Salamone AB, inventors; Rochal Industries LLC, assignee. Biocidal compositions and methods of using the same. US patent 8,829,053 B2. September 9, 2014.
30. Kim S, Chen J, Cheng T, et al. PubChem 2019 update: improved access to chemical data. *Nucleic Acids Res*. 2019;47(D1):D1102–D1109. doi:10.1093/nar/gky1033
31. Harrison JJ, Stremick CA, Turner RJ, Allan ND, Olson ME, Ceri H. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nat Protoc*. 2010;5(7):1236–1254. doi:10.1038/nprot.2010.71
32. Schmaus G, Lange S, Joppe H. Synergistic mixtures of 1,2 -alkane diols. US7582681. 2009.
33. Costerton J, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284(5418):1318–22.
34. Stewart PS, Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol*. 2002;292(2):107–113.
35. Seth AK, Geringer MR, Gurjala AN, et al. Treatment of *Pseudomonas aeruginosa* biofilm-infected wounds with clinical wound care strategies: a quantitative study using an in vivo rabbit ear model. *Plast Reconstr Surg*. 2012;129(2):262e–274e. doi:10.1097/PRS.0b013e31823aeb3b
36. Rani SA, Pitts B, Beyenal H, et al. Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol*. 2007;189(11):4223–4233.
37. Snyder RJ, Bohn G, Hanft J, et al. Wound biofilm: current perspectives and strategies on biofilm disruption and treatments. *Wounds*. 2017;29(6):S1–S17.
38. Stewart PS, Grab L, Diemer JA. Analysis of biocide transport limitation in an artificial biofilm system. *J Appl Microbiol*. 1998;85(3):495–500.
39. Wolcott RD, Rumbaugh KP, James G, et al. Biofilm maturity studies indicate sharp debridement opens a time-dependent therapeutic window. *J Wound Care*. 2010;19(8):320–328.
40. Madhok BM, Vowden K, Vowden P. New techniques for wound debridement. *Int Wound J*. 2013;10(3):247–251. doi:10.1111/iwj.12045
41. Nakatsuji T, Chiang H-I, Jiang SB, Nagarajan H, Zengler K, Gallo RL. The microbiome extends to subepidermal compartments of normal skin. *Nat Commun*. 2013;4:1431–1447. doi:10.1038/ncomms2441
42. Hurlow J, Couch K, Laforet K, Bolton L, Metcalf D, Bowler P. Clinical biofilms: a challenging frontier in wound care. *Adv Wound Care (New Rochelle)*. 2015;4(5):295–301.
43. Müller G, Kramer A. Biocompatibility index of antiseptic agents by parallel assessment of antimicrobial activity and cellular cytotoxicity. *J Antimicrob Chemother*. 2008;61(6):1281–1287. doi:10.1093/jac/dkn125
44. Daeschlein G, Assadian O, Bruck JC, Meinel C, Kramer A, Koch S. Feasibility and clinical applicability of polyhexanide for treatment of second-degree burn wounds. *Skin Pharmacol Physiol*. 2007;20(6):292–296.
45. Lopez-Maldonado EA, Oropeza-Guzman MT, Ochoa-Teran A. Improving the efficiency of a coagulation-flocculation wastewater treatment of the semiconductor industry through zeta potential measurements. *J Chem*. 2014. doi:10.1155/2014/969720
46. Bueno CZ, Moraes AM. Influence of the incorporation of the antimicrobial agent polyhexamethylene biguanide on the properties of dense and porous chitosan-alginate membranes. *Mater Sci Eng C Mater Biol Appl*. 2018;93:671–678. doi:10.1016/j.msec.2018.07.076
47. Ikeda T, Ledwith A, Bamford CH, Hann RA. Interaction of a polymeric biguanide biocide with phospholipid membranes. *Biochim Biophys Acta*. 1984;769(1):57–66.
48. Broxton P, Woodcock PM, Gilbert P. Binding of some polyhexamethylene biguanides to the cell envelope of *Escherichia coli* ATCC 8739. *Microbios*. 1984;41(163):15–22.
49. Chindera K, Mahato M, Sharma AK, et al. The antimicrobial polymer PHMB enters cells and selectively condenses bacterial chromosomes. *Sci Rep*. 2016;6:23121. doi:10.1038/srep23121
50. Kramer A, Dissemmond J, Kim S, et al. Consensus on wound antiseptics: update 2018. *Skin Pharmacol Physiol*. 2018;31(1):28–58. doi:10.1159/000481545
51. Schneider LA, Korber A, Grabbe S, Dissemmond J. Influence of pH on wound-healing: a new perspective for wound-therapy? *Arch Dermatol Res*. 2007;298(9):413–420.

52. Vroom JM, De Grauw KJ, Gerritsen HC, et al. Depth penetration and detection of pH gradients in biofilms by two-photon excitation microscopy. *Appl Environ Microbiol*. 1999;65(8):3502–3511.
53. Chew BP, Tjoelker LW, Tanaka TS. In vitro growth inhibition of mastitis causing bacteria by phenolics and metal chelators. *J Dairy Sci*. 1985;68(11):3037–3046.
54. Metcalf D, Parsons D, Bowler PG. Development of a next-generation antimicrobial wound dressing. *Acta Med Croatica*. 2016;70(1):49–56.
55. Percival SL, Salisbury AM. The efficacy of tetrasodium EDTA on biofilms. *Adv Exp Med Biol*. 2018;1057:101–110. doi:10.1007/5584_2017_134
56. Adair FW, Geftic SG, Gelzer J. Resistance of *Pseudomonas* to quaternary ammonium compounds. Resistance of *Pseudomonas* to quaternary ammonium compounds. *Appl Microbiol*. 1971;21(6):1058–1063.
57. Tezvergil-Mutluay A, Agee KA, Hoshika T, et al. The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices. *Dent Mater*. 2010;26(11):1059–1067. doi:10.1016/j.dental.2010.07.006
58. Rodrigues M, Bonham CA, Minniti CP, Gupta K, Longaker MT, Gurtner GC. Iron chelation with transdermal deferoxamine accelerates healing of murine sickle cell ulcers. *Adv Wound Care (New Rochelle)*. 2018;7(10):323–332. doi:10.1089/wound.2018.0789
59. Percival SL, McCarty S, Hunt JA, Woods EJ. The effects of pH on wound healing, biofilms, and antimicrobial efficacy. *Wound Repair Regen*. 2014;22(2):174–186. doi:10.1111/wrr.12125
60. Jones EM, Cochrane CA, Percival SL. The effect of pH on the extracellular matrix and biofilms. *Adv Wound Care (New Rochelle)*. 2015;4(7):431–439.
61. Lawan K, Kanlayavattanukul M, Lourith N. Antimicrobial efficacy of caprylyl glycol and ethylhexylglycerine in emulsion. *J Health Res*. 2009;23(1):1–3.
62. KaB M, Lüthje S, Herweg S, Stepan S. Systematic investigations in the antimicrobial efficacy of glycerine esters with fatty acids of different chain length. *SOFW J*. 2013;139(12).
63. The Derm Review. Ethylhexylglycerin. Published Sept 28, 2018. <https://thederreview.com/ethylhexylglycerin/>
64. schülke inc. Smooth – safe – sensiva®. www.schulke-us.com/schulke_sensiva_SC10.html. 2020.
65. Beilfuss W, Leschke M, Weber K. A new concept to boost the preservative efficacy of phenoxyethanol. *SOFW J*. 2005;131(11):2–7.
66. Beilfuss W. A multifunctional ingredient for deodorants. *SOFW J*. 1998;124(6):360–366.
67. Anglen JO, Gainor BJ, Simpson WA, Christensen G. The use of detergent irrigation for musculoskeletal wounds. *Int Orthop*. 2003;27(1):40–46.
68. Yang Q, Larose C, Della Porta AC, Schultz GS, Gibson DJ. A surfactant-based wound dressing can reduce bacterial biofilms in a porcine skin explant model. *Int Wound J*. 2017;14(2):408–413. doi:10.1111/iwj.12619
69. BASF Pluracare L/F Grades, Poloxamer Technical Information. January 2008. EMM 070801e-00:1–10.
70. Percival SL, Chen R, Mayer D, Salisbury AM. Mode of action of poloxamer-based surfactants in wound care and efficacy on biofilms. *Int Wound J*. 2018;15(5):749–755. doi:10.1111/iwj.12922
71. Bodratti AM, Alexandridis P. Formulation of poloxamers for drug delivery. *J Funct Biomater*. 2018;9(1):11–35. doi:10.3390/jfb9010011
72. Smart JD. The basics and underlying mechanisms of mucoadhesion. *Adv Drug Deliv Rev*. 2005;57(11):1556–1568.
73. Agarwal S, Murthy RS. Effect of different polymer concentration on drug release rate and physicochemical properties of mucadhesive gastroretentive tablets. *Indian J Pharm Sci*. 2015;77(6):705–714.
74. Danziger J, Zeidel ML. Osmotic homeostasis. *Clin J Am Soc Nephrol*. 2015;10(5):852–862. Published correction appears in *Clin J Am Soc Nephrol*. 2015;10(9):1703.
75. International Wound Infection Institute (IWII). Wound infection in clinical practice: principles of best practice. *Wounds Int*. 2016;3–30.