



# Impact of a novel, antimicrobial dressing on in vivo, *Pseudomonas aeruginosa* wound biofilm: Quantitative comparative analysis using a rabbit ear model

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## ABSTRACT

The importance of bacterial biofilms to chronic wound pathogenesis is well established. Different treatment modalities, including topical dressings, have yet to show consistent efficacy against wound biofilm. This study evaluates the impact of a novel, antimicrobial Test Dressing on *Pseudomonas aeruginosa* biofilm-infected wounds. Six-mm dermal punch wounds in rabbit ears were inoculated with  $10^6$  colony-forming units of *P. aeruginosa*. Biofilm was established in vivo using our published model. Dressing changes were performed every other day with either Active Control or Test Dressings. Treated and untreated wounds were harvested for several quantitative endpoints. Confirmatory studies were performed to measure treatment impact on in vitro *P. aeruginosa* and in vivo polybacterial wounds containing *P. aeruginosa* and *Staphylococcus aureus*. The Test Dressing consistently decreased *P. aeruginosa* bacterial counts, and improved wound healing relative to Inactive Vehicle and Active Control wounds ( $p < 0.05$ ). In vitro bacterial counts were also significantly reduced following Test Dressing therapy ( $p < 0.05$ ). Similarly, improvements in bacterial burden and wound healing were also achieved in polybacterial wounds ( $p < 0.05$ ). This study represents the first quantifiable and consistent in vivo evidence of a topical antimicrobial dressing's impact against established wound biofilm. The development of clinically applicable therapies against biofilm such as this is critical to improving chronic wound care.

The effective care of chronic wounds continues to be a difficult, and expensive, problem for clinical practitioners.<sup>1-3</sup> Although several disease processes can contribute to chronic wound pathogenesis, including diabetes mellitus, obesity, and peripheral vascular disease,<sup>4-8</sup> the importance of bacterial biofilm is now being recognized within the scientific community.<sup>9-12</sup> As the predominant state of bacteria within the human body,<sup>13</sup> biofilm structure provides bacteria with a number of mechanisms for defense and survival against their host's innate immune defenses (e.g., inflammatory response), distinguishing biofilm bacteria from their free-floating, "planktonic" counterparts. The self-secreted extracellular polymeric substance, or EPS, that surrounds bacteria within a biofilm provides a physical barrier to host-derived phagocytosis and complement activation, while also preventing the penetration of systemic antibiotics or topically applied antimicrobials.<sup>14,15</sup> Biofilms are also dynamic in their ability to utilize protective cell-cell communication, termed quorum-sensing, and shed planktonic bacteria in an effort to establish new biofilm populations.<sup>9,10,16</sup> The ultimate outcome is an impairment of wound healing, now shown in several in vitro, in vivo, and clinical models.<sup>11,17-21</sup>

The durability of biofilm, and its significance to chronic wound healing, underscores the need for an evolution in current wound care therapy. Wound-bed preparation and treatment have traditionally centered around therapies such as debridement, lavage, and antimicrobials, but with little evidence that they improve chronic wound healing in a quantitative and consistent manner.<sup>22,23</sup> Although we have recently demonstrated that frequent and aggressive, multimodal therapies may be effective in reducing wound biofilm,<sup>20</sup> treatment regimens specifically aimed at biofilm development and maintenance are limited and unproven. Molecular therapies, such as the introduction of D-amino acids<sup>24,25</sup> and RNA-inhibiting peptides,<sup>18</sup> have shown some efficacy both in vitro and in vivo, but the translation of these modalities to the clinical setting remains difficult. Meanwhile, protocols involving the use of specialized dressings have been tested in several different settings but with mixed efficacy.<sup>26,27</sup> Despite a growing understanding of the mechanisms underlying biofilm virulence, the application of this knowledge toward developing effective, anti-biofilm therapies, has not progressed as rapidly.

We have previously established an in vivo model of single-species wound biofilm in the rabbit ear,<sup>19</sup> from which we have

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been able to demonstrate the consistent formation of biofilm among different bacterial species,<sup>28</sup> the impact of comorbidities such as ischemia on biofilm formation,<sup>21</sup> and the combined impact of multiple biofilm pathogens within the same wound.<sup>29</sup> However, beyond an exploration of common wound treatment modalities,<sup>20</sup> we have yet to perform dedicated testing of different novel therapies against the in vivo biofilm produced in our model. Therefore, the goal of this study was to evaluate the impact of a novel, antimicrobial dressing on in vivo *Pseudomonas aeruginosa* biofilm. This impact was measured against both untreated, infected-control wounds and wounds treated with the inert base dressing, with further validation using an in vitro biofilm, dressing-treatment model. We also performed confirmatory tests in polybacterial biofilm wounds containing both *P. aeruginosa* and *Staphylococcus aureus* to provide further relevance for the dressing in the clinical setting.

## METHODS

### Animals

Under an approved protocol by the Animal Care and Use Committee at the Northwestern University, adult New Zealand white rabbits (3–6 months, ~3 kg) (Covance, Inc., Princeton, NJ) were acclimated to standard housing and fed ad libitum. All animals were housed in individual cages under constant temperature and humidity with a 12-hour light-dark cycle. A total of 12 animals were used for this study.

### Bacterial strains and culture

Wild-type strains of *P. aeruginosa* (obtained from the laboratory of Dr. Barbara H. Iglewski, University of Rochester Medical Center) and *S. aureus* (obtained from the laboratory of Dr. Mark Smeltzer, University of Arkansas Medical Sciences) were utilized for wound infection. *S. aureus* and *P. aeruginosa* strains were grown overnight at 37 °C on *Staphylococcus* and *Pseudomonas* Isolation Agar (Hardy Diagnostics, Santa Maria, CA), and cultured in tryptic soy (TSB) and Luria (LB) broth, respectively, at 37 °C until log-phase was achieved. Bacteria were harvested and washed in phosphate-buffered saline (PBS) three times by centrifugation at 2991 g for 5-minutes at 25 °C. An optical density at the 600-nm wavelength (OD<sub>600</sub>) was measured. An OD<sub>600</sub> equivalent to 10<sup>6</sup> colony-forming units (CFUs)/μL was predetermined empirically for each strain of bacteria used.

### Wound protocol and infection model

Wounding, bacterial infection, and biofilm formation were adapted from principles established in our previously published in vivo, wound biofilm model.<sup>19</sup> Rabbits were anesthetized with intramuscular injection of a ketamine (22.5 mg/kg) and xylazine (3.5 mg/kg) mixture prior to surgery. Ears were shaved, sterilized with 70% ethanol, and injected intradermally with a 1% lidocaine / 1 : 100,000 epinephrine solution at the planned wound sites. Six, 6-mm diameter, full-thickness dermal wounds were created on the ventral ear down to perichondrium and dressed with Tegaderm (3M Health Care, St. Paul, MN), a semi-occlusive transparent film. Individual

biofilm wounds were inoculated with *P. aeruginosa* on post-operative day (POD) 3. Bacterial solutions were diluted such that each wound was inoculated with a total of 10<sup>6</sup> CFU of bacteria in a volume of 10 μL. Bacteria were allowed to proliferate in vivo under the Tegaderm dressing. Topical antibiotics (Ciloxan ointment [Ciprofloxacin 0.3%, Alcon, Fort Worth, TX]) were applied POD4 to eliminate free-floating, planktonic-phase bacteria, leaving a predominately biofilm-phase phenotype. To prevent seroma formation and regrowth of planktonic bacteria, thus maintaining a biofilm-dominant infection, an antimicrobial, absorbent dressing containing polyhexamethylene biguanide (Telfa AMD, Covidien, Mansfield, MA) was applied to biofilm wounds on PODs 5, 6, and then, for control wounds only, every other day until harvest. All dressings were checked daily throughout the protocol. For polybacterial wounds, this protocol was modified by the application of bacterial solutions of 10<sup>6</sup> CFU at a volume of 5 μL for each bacteria (*S. aureus* and *P. aeruginosa*), followed by “mixing” of the two solutions with a pipette tip. An additional antibiotic, Mupirocin (2%) ointment (Teva Pharmaceuticals, Sellersville, PA), was applied to counteract planktonic *S. aureus* within the wounds.

### Dressing materials

Wounds were subject to one of three dressings during the study (Table 1). Telfa AMD was designated as the Active Control dressing, which has been used as the standard control dressing in previous iterations of the rabbit ear wound biofilm model.<sup>19–21,28,29</sup> This dressing allows for consistent formation of intact and viable *P. aeruginosa* wound biofilm, thus representing an appropriate control. The product AQUACEL Hydrofiber was utilized as the Inactive Vehicle dressing, representing a nonwoven gauze pad often used for dressing changes in the clinical setting. The Inactive Vehicle dressing also serves as the base dressing for the novel, antimicrobial dressing, AQUACEL Ag+ Technology Hydrofiber or “Test Dressing,” used in this study. This dressing is impregnated with three different compounds, ionic silver, ethylenediamine

**Table 1.** Study dressing groups

Dressing	Product
Active Control	Telfa AMD antimicrobial nonadherent dressing, Covidien, Mansfield, MA (a cotton gauze pad impregnated with polyhexamethylene biguanide [PHMB])
Inactive Vehicle	AQUACEL Hydrofiber Dressing, ConvaTec Ltd, Deeside, United Kingdom (a nonwoven pad constructed from a proprietary gelling fiber)
Test Dressing	AQUACEL Ag+ Hydrofiber Dressing, ConvaTec Ltd, Deeside, United Kingdom (a nonwoven pad constructed from a proprietary gelling fiber containing ionic silver, ethylenediamine tetra-acetic acid [EDTA], and benzethonium chloride)

tetra-acetic acid (EDTA), and benzethonium chloride, which in combination function to clear wound biofilm. Ionic silver is known as an effective antimicrobial that functions to kill viable bacteria. However, its efficacy alone against biofilm-infected wounds remains limited and unclear.<sup>20</sup> EDTA, as it is formulated in Europe, or sodium edetate, as it is available in the United States, is a pharmaceutical grade excipient that is thought to enhance the activity of ionic silver by preventing ionic silver consumption within wound exudate while also weakening biofilm structure by removing divalent metal cross-links between bacteria.<sup>30</sup> Benzethonium chloride is another excipient that is also thought to aid in the disruption of biofilm structure.<sup>31</sup> Together, the two excipients expose more bacteria to the antimicrobial activity of the silver, effectively reducing biofilm burden.

### Study design and treatment protocol

Animals were designated to one of two experimental study arms: Inactive Vehicle dressing vs. Active Control dressing ( $n = 6$  animals) or Test Dressing vs. Active Control dressing ( $n = 6$  animals). For each rabbit, ears were then designated as either Active Control or Study (Test Dressing or Inactive Vehicle dressing) ear, with each of the six wounds on that ear following the same protocol. This allowed for each wound to have its own internal control on the contralateral ear for improved statistical validity. Dressing changes were performed on *P. aeruginosa* biofilm-infected wounds every other day (QOD) starting on POD6, the time at which a steady-state, predominantly biofilm infection is present.<sup>19</sup> After each primary dressing change, Tegaderm (used to cover the dressing) was reapplied over all wounds. From each study arm, one animal was designated for harvest on POD8 for viable bacterial count measurement. Another animal from each study group was designated for harvest on POD10, also for viable count measurement. On POD12, the remaining four animals from each group were euthanized and their wounds harvested for histological analysis ( $n = 18$  wounds per study or control group) or bacterial counts ( $n = 6$  wounds per study or control group). All wounds were excised using a 10-mm biopsy punch (Acuderm Inc., Fort Lauderdale, FL).

Confirmatory experiments in polybacterial wound animals were performed by once again comparing treated, Test Dressing wounds, and Active Control wounds ( $n = 7$  animals). Two animals each were designated for viable bacteria count measurement on POD6 and POD10, and three animals were designated for histological analysis on POD18.

### Viable bacterial count measurements

The dorsal sides of wounds used for bacterial counts were removed to eliminate the inclusion of bacteria outside of the infected wound surface. Any overlying dressings were also removed from the wounds prior to preparation for bacterial count measurement. To recover bacteria, biofilm-infected wound samples were placed in tubes prefilled with homogenizer beads (Roche Diagnostics, Indianapolis, IN). One mL of PBS was added to the tube and was homogenized for 90 seconds at 2991 g in a MagNA Lyser homogenizer (Roche Diagnostics), followed by sonication (Microson Ultrasonic Cell Disrupter, Heat Systems-Ultrasonics, Inc, Farmingdale, NY) for 2 minutes at 6–8 W to disrupt any biofilm present.

The resulting solutions were serially diluted and plated on *Pseudomonas* Isolation Agar plates and incubated overnight at 37 °C. Total viable counts (TVCs) were determined by standard colony counting method. For polybacterial wounds, solutions were serially diluted and plated on both *Pseudomonas* and *Staphylococcus* Isolation Agar plates.

### Histological analysis

Wounds excised for histological analysis were bisected at their largest diameter for hematoxylin and eosin (H&E) staining. Tissues were fixed in formalin, embedded in paraffin, and cut into 4- $\mu$ m sections. Paraffin was removed with a xylene wash, followed by a standard H&E staining protocol to prepare samples for analysis under a light microscope. Slides were examined for quantification of epithelial and granulation gaps, and total epithelial and granulation areas, using a digital analysis system (NIS-Elements Basic Research, Nikon Instech Co., Kanagawa, Japan), as previously described.<sup>19</sup>

### In vitro biofilm-gauze dressing protocol

A stock suspension of planktonic *P. aeruginosa* was prepared in maximal recovery diluent, adjusted to approximately  $10^8$  CFU/mL by optical density measurement, and the count was confirmed by standard microbiological counting techniques. A simulated wound fluid (SWF) medium (consisting of tryptone soy broth and fetal calf serum at a ratio of 1 : 1 v/v) was prepared. Finally, a biofilm culturing solution was prepared by mixing 9.9 mL of SWF with 0.1 mL of the bacterial stock suspension in a sterile 100 mL Duran bottle.

Using aseptic techniques throughout, substrates for biofilm growth were prepared using a biopsy punch to cut 35-mm discs of NA gauze (Systagenix, Gatwick, United Kingdom). Discs were placed in the biofilm culturing solution and incubated for 6 hours at 35 °C ( $\pm 3$  °C) in a shaking incubator to encourage bacterial attachment. Following incubation, each gauze sample (with attached bacteria) was removed and transferred onto separate sterile 140 mm Petri dishes where they were covered with an Active Control dressing, mimicking to the in vivo protocol. Each Active Control dressing was then hydrated with 5 mL of SWF, and a sterile L-shaped spreader was used to ensure contact between the dressing and gauze-biofilm substrate. Petri dishes were incubated for a further 48 hours at 35 °C ( $\pm 3$  °C) to allow the biofilm to develop while suppressing any planktonic growth. A number of disc samples were analyzed by scanning electron microscopy (SEM) to confirm the presence of biofilm.

Prepared gauze-biofilm samples were placed onto fresh Petri dishes and covered with either the Test Dressing, an Inactive Vehicle dressing, or an Active Control dressing (5 cm  $\times$  5 cm). Each dressing sample was hydrated with 5 mL of SWF, the Petri dish lids were replaced, the dishes were sealed with Parafilm M (Bemis Flexible Packaging, Neenah, WI) to prevent dehydration and then incubated at 35 °C ( $\pm 3$  °C) for 24, 48, 72, and 96 hours ( $n = 3$  for each dressing and each time point).

Following incubation, dressings were removed and gauze-biofilm substrates were separately transferred into a sterile stomacher bags containing 30 mL of DE neutralizing broth (DENB). The biofilm was removed and disrupted by vigor-

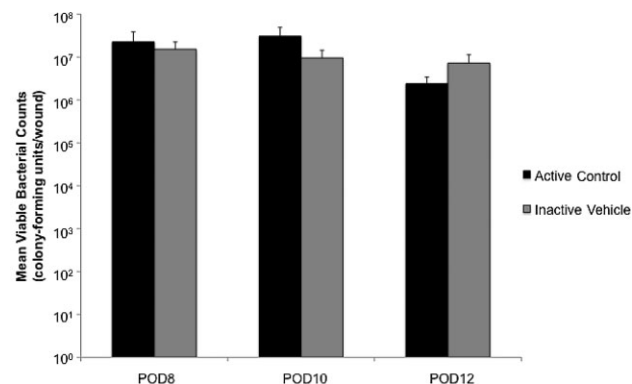
ously homogenizing in a stomacher blender for 4 minutes. Duplicate TVCs were performed on each homogenized suspension by performing 10-fold serial dilutions in DENB, and then inoculating appropriate dilutions onto predried tryptic soy agar plates, spreading and incubating at 35 °C (±3 °C) for at least 48 hours before enumeration. TVCs of untreated gauze-biofilm at controls at time zero were determined in the same way to establish T<sub>0</sub> counts.

**Statistical analysis**

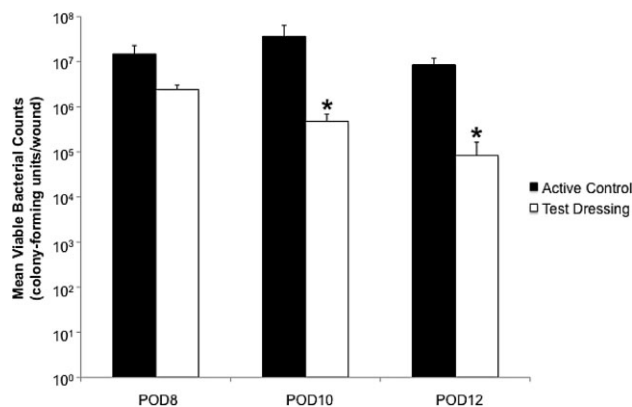
Data are presented in graphical form as mean ± standard errors when applicable. Statistical analyses were performed for all in vivo experiments using a paired, two-tailed Student’s *t*-test with the comparison of each treated wound with its paired control. The level of significance was set at *p* < 0.05. All analyses were performed at the Northwestern University.

**RESULTS**

To understand the impact of the tested dressings on biofilm burden, total viable bacterial count measurements (TVCs) were performed over time, and the results are displayed in Figures 1–3. When comparing wounds treated with the Inactive Vehicle vs. the Active Control dressings, there were no significant differences in TVCs between the dressings or over time (Figure 1). From POD8 to POD12, the amount of *P. aeruginosa* present in wounds remained relatively stable in both groups, with no reduction in bacterial burden despite multiple treatments. In contrast, wounds treated with the Test Dressing showed reduction of biofilm burden within the wounds over time (Figure 2). There was a trend toward lower bacteria at POD8 (after one treatment), and at POD10 and POD12 there was a significant reduction in the number of viable bacteria present in wounds (*p* < 0.05), indicating in vivo efficacy of the Test Dressing. Similarly, when comparing the Inactive Vehicle and Test Dressing treated wounds, there was a significant difference between the two dressings at both POD10 and POD12 (Figure 3).



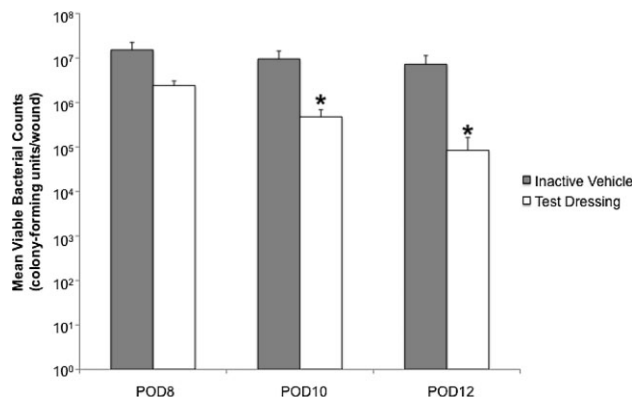
**Figure 1.** Mean viable bacterial counts in Active Control and Inactive Vehicle dressing wounds. Wounds with the Inactive Vehicle dressing showed no difference in viable bacteria when compared with Active Control wounds over time. POD, postoperative day.



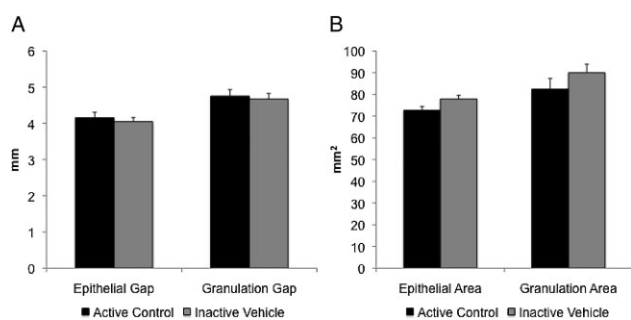
**Figure 2.** Mean viable bacterial counts in Active Control and Test Dressing wounds. Wounds treated with the Test Dressing showed significantly lower levels of viable bacteria when compared with Active Control wounds on both POD10 and POD12. POD, postoperative day. (\**p* < 0.05).

To correlate with viable bacterial count measurements, histological analyses of wound healing parameters were performed at POD12 for all dressings. When measuring both epithelial and granulation tissue in-growth, there were no measurable differences between Inactive Vehicle and Active Control wounds at POD12 (Figure 4). In contrast, similar to the bacterial count findings, the Test Dressing resulted in significant improvements (vs. Active Control) in all measured histological parameters, indicating an end-effect of the dressing on both bacterial burden and overall wound healing (Figure 5). The superiority of the Test Dressing over the Inactive Vehicle dressing is further emphasized when comparing them histologically, with again significant improvements seen following Test Dressing treatment (Figure 6).

In vitro examination of the Active Control, Inactive Vehicle, and Test Dressings was performed to further validate the aforementioned in vivo findings. SEM of control gauzes



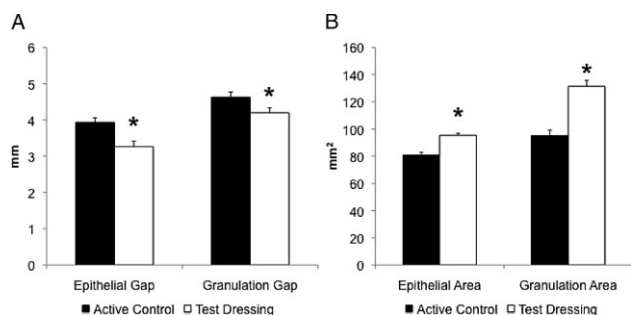
**Figure 3.** Mean viable bacterial counts in Inactive Vehicle and Test Dressing wounds. Wounds treated with the Test Dressing showed significantly lower levels of viable bacteria when compared with Inactive Vehicle wounds on both POD10 and POD12. POD, postoperative day (\**p* < 0.05).



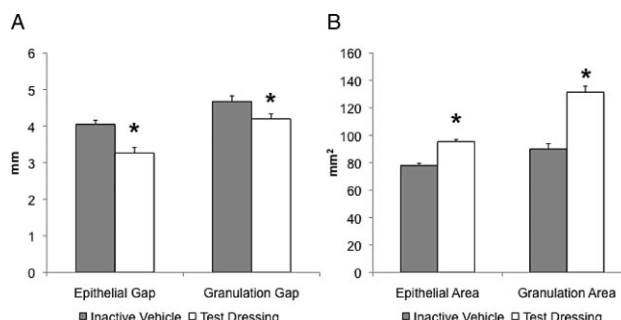
**Figure 4.** Comparison of quantitative histological parameters for Active Control and Inactive Vehicle dressing wounds. Wounds with the Inactive Vehicle dressing showed no differences in epithelial and granulation tissue gaps (A) and epithelial and granulation tissue areas (B) when compared with Active Control wounds.

following 48 hours of incubation with *P. aeruginosa* verified the formation of biofilm on the gauze substrate, prior to treatment with either the control or experimental dressings (Figure 7). Bacterial counts from each dressing group, measured over time, revealed a significant decrease in biofilm burden at 24 hours of Test Dressing application when compared with the other two groups (Figure 8). This trend continued up to 96 hours, during which the level of *P. aeruginosa* found on the gauze substrate in the Test Dressing group was consistently below 30 CFU/gauze, the limit of detection.

Confirmation of the in vivo efficacy of the Test Dressing against biofilm wounds was performed in polybacterial wounds containing both *P. aeruginosa* and *S. aureus*. On gross examination of wound size over time, Test Dressing wounds visually showed significant improvements in wound healing relative to Active Control dressed wounds through POD18 (Figure 9). Correlating with these findings, histological analysis of polybacterial wounds treated with Test Dressing showed significant improvements in epithelial gap and granulation area measurements ( $p < 0.05$ ) (Figure 10A and B). Measurement of viable bacteria within the wounds at



**Figure 5.** Comparison of quantitative histological parameters for Active Control and Test Dressing wounds. Wounds with the Test Dressing showed significantly smaller epithelial and granulation tissue gaps (A) and larger epithelial and granulation tissue areas (B) when compared with Active Control wounds, indicating overall improved wound healing ( $*p < 0.05$ ).

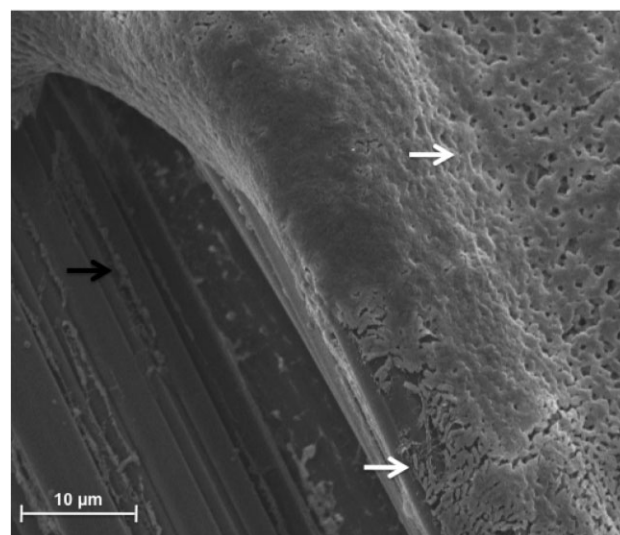


**Figure 6.** Comparison of quantitative histological parameters for Inactive Vehicle and Test Dressing wounds. Wounds with the Test Dressing showed significantly smaller epithelial and granulation tissue gaps (A) and larger epithelial and granulation tissue areas (B) when compared with Inactive Vehicle wounds indicating overall improved wound healing ( $*p < 0.05$ ).

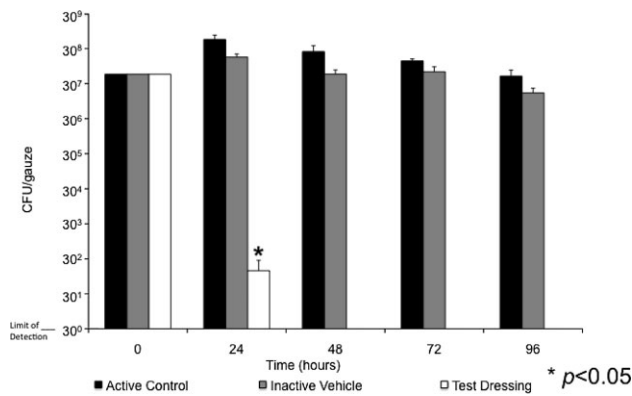
POD10 also confirmed a reduction in the burden of *P. aeruginosa* within polybacterial wounds as a result of treatment with the Test Dressing ( $p < 0.05$ ) (Figure 10C). However, there were no significant differences in the viable amount of *S. aureus* within these same wounds.

## DISCUSSION

The management and treatment of biofilm-infected chronic wounds remains a challenging problem for both patients and practitioners.<sup>1-3</sup> With dynamic and robust defense mechanisms,<sup>4-16</sup> the presence of biofilm requires new



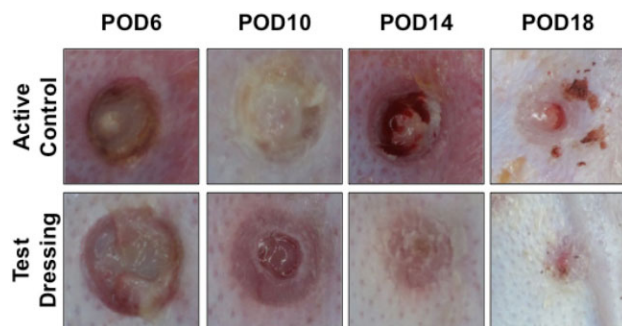
**Figure 7.** Scanning electron microscopy of Active Control gauze following 48 hours of incubation with *Pseudomonas aeruginosa*. Black arrow indicates clean gauze, in contrast to white arrows which show evidence of rod-shaped bacteria, *P. aeruginosa*, forming a lattice-like matrix on the gauze, consistent with biofilm.



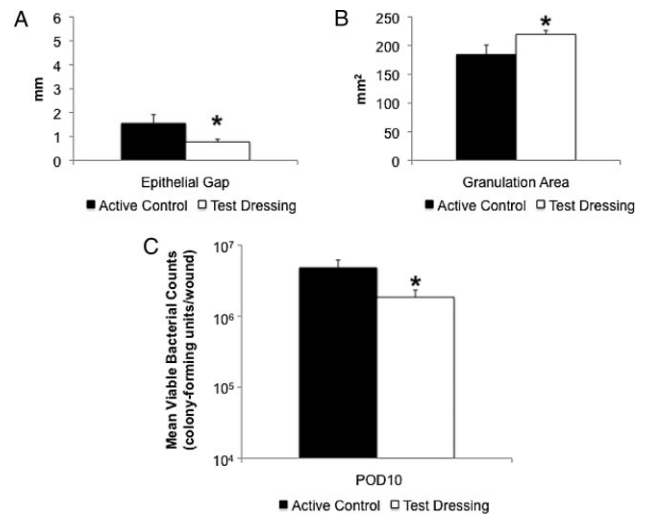
**Figure 8.** Viable bacterial counts measured from Active Control, Inactive Vehicle, and Test Dressing used on in vitro, *Pseudomonas aeruginosa*, biofilm-infected gauze. Over time, bacterial counts significantly decreased in the Test Dressing group relative to the other two groups at 24 hours. This trend continued up to 96 hours with undetectable levels of bacteria in the Test Dressing group from 48 to 96 hours.

innovative therapies to both reduce bacterial burden and allow for wound closure. With only a limited amount of literature addressing the effective treatment of wound biofilm,<sup>11,17–21</sup> we utilized an established in vivo model of wound biofilm, and its in vitro counterpart, to investigate the efficacy of a novel, antimicrobial dressing against *P. aeruginosa* biofilm.

Previous studies examining the impact of different topical therapies for chronic wound biofilm burden have shown only mixed results, relying primarily on anecdotal or limited clinical experience rather than rigorous investigation. Wolcott and Dowd<sup>32</sup> described a patient with a dorsal foot burn with suspected biofilm infection, who underwent topical nanogel and Acticoat treatment combined with oral antibiotic to achieve significant healing after 4 weeks. Meanwhile, Percival et al.<sup>26</sup> demonstrated that silver-containing dressings decreased total bacterial burden when used against an in vitro biofilm, but did not translate these experiments into an in vivo model or examine its impact on wound healing. Davis et al.<sup>27</sup> utilized



**Figure 9.** Gross photographs of polybacterial wounds treated with Active Control or Test Dressing over time. Test Dressing wounds showed visual improvement in healing over time relative to Active Control wounds at each photographed time point.



**Figure 10.** Quantitative histologic parameters and viable bacterial count measurements in polybacterial wounds treated with Active Control and Test Dressings. Test Dressing wounds showed significant improvements in epithelial gap (A) and granulation area (B), indicating an overall improvement in healing. Test Dressing wounds also showed a significant decrease in mean viable *Pseudomonas aeruginosa* within polybacterial wounds (C) (\* $p < 0.05$ ).

different topical, antimicrobial bandages in a partial-thickness porcine wound biofilm model, which showed only mixed efficacy in reducing *S. aureus* biofilm burden. Others have looked at the impact of topical enzymatic debriding agents in infected, granulating rodent wounds, showing a substantial decrease in viable bacteria while accelerating healing rates.<sup>22</sup> However, these results were not tested against an established, in vivo, wound biofilm, limiting their applicability to the chronic, biofilm-infected wound. Therefore, the literature remains limited and inconclusive as to the utilization of topical dressing- or antimicrobial-based therapies on biofilm-infected wounds.

Our work suggests that the topical application of this novel Test Dressing to *P. aeruginosa* biofilm-infected wounds may help to significantly reduce bacterial burden over time both in vitro and in vivo, with a subsequent improvement in in vivo wound healing. This study represents the first to report quantifiable and consistent efficacy of a topical antimicrobial dressing against established in vivo biofilm in a validated animal model. The Test Dressing is a silver-containing dressing that is based upon the existing Hydrofiber product AQUACEL Ag, but contains additional excipients that increase the rate of antimicrobial activity and enhance the efficiency of the ionic silver. The increase in efficiency is thought to be due to a disruption of the EPS that encases otherwise free-floating bacteria, increasing their exposure to the antibacterial silver. As previously discussed, the known, potent antimicrobial properties of silver have made silver-based dressings attractive for biofilm management,<sup>26</sup> but to date they have had limited success. However, the Test Dressing utilized in this study may potentially have the additional ability to dissolve and penetrate the protective biofilm EPS, a

barrier for a number of other topical therapies. As seen in our previous work with other conventional, clinical treatment strategies,<sup>20</sup> the combination of topical Silvadene with other treatment modalities that disrupt the EPS (e.g., debridement, lavage) can significantly reduce biofilm burden and improve in vivo wound healing. Similarly, the Test Dressing investigated in this study may provide a combined treatment modality approach that is both antimicrobial and targets the EPS, through the use of a single dressing, further validating its potential efficacy and utility.

The inherent multiple treatment modalities within the Test Dressing underscores the potential for its use both individually in biofilm-infected wounds, but also in conjunction with other therapies. As previously discussed, earlier studies with this model have reinforced the principles of frequent, multimodality therapy as being the most effective against biofilm.<sup>20</sup> Similarly, experimental work with bacteriophages, and debridement prior to phage application, has also revealed promising, synergistic results.<sup>33,34</sup> Mechanical debridement presumably disrupts the EPS, allowing for the antibacterial properties of the bacteriophage to be utilized. Dual-action therapy involving both mechanical debridement, and the inherent chemical debridement and antimicrobial effects of the Test Dressing, may further improve bacterial burden and wound healing outcomes.

Through the use of our validated, polybacterial biofilm model,<sup>29</sup> we were able to confirm the efficacy of the Test Dressing against biofilm-infected wounds. Interestingly, the Test Dressing only showed a significant impact on the level of *P. aeruginosa* within the wounds, not *S. aureus*. Despite this limited impact on bacterial burden, there was a measurable difference in overall wound healing between untreated and treated wounds. In validating our polybacterial biofilm model, we also demonstrated that *P. aeruginosa* appears to be the predominant pathogen within a *P. aeruginosa* and *S. aureus* mixed biofilm wound.<sup>29</sup> Furthermore, *P. aeruginosa* appears to be the more virulent organism within these wounds, and therefore having a greater impact on wound healing.<sup>29</sup> Given this established dynamic, it is conceivable that the impact of Test Dressing on polybacterial biofilm wound healing is primarily due to its effects on *P. aeruginosa* within these wounds. It is unclear why a similar efficacy is not seen against *S. aureus*, indicating that this Test Dressing may require some customization based on the individual bacteria within a given wound. Further work is needed to better understand the potential benefits of the Test Dressing in a polybacterial biofilm setting.

As with all studies, we do acknowledge some limitations with our approach. We chose to primarily investigate only one bacterium, *P. aeruginosa*, as part of this study. Clinically, the majority of chronic wounds infected with biofilm tend to contain multiple bacteria,<sup>9,12,34</sup> which warrants studies involving polybacterial biofilm. As previously discussed, with our recent validation of an in vivo, polybacterial modification of our model,<sup>29</sup> future work will involve a more thorough investigation of this Test Dressing as part of treatment against polybacterial biofilm. In addition, our study showed the efficacy of the Test Dressing in both reducing bacterial burden and improving wound healing, but it does not validate the proposed anti-biofilm mechanism. Future studies focused on delineating the underlying molecular mechanisms will help our understanding of how to modulate the dressing for different biofilm-infected wounds, as well as provide a better understanding for developing future therapeutics.

Wound biofilm research continues to grow exponentially, with validated in vitro and in vivo models essential to this development. However, effective therapeutics against wound biofilm remain limited, requiring new, innovative approaches. Our study has shown success with a novel, antimicrobial dressing that meshes a novel therapeutic regimen with the principles of traditional wound dressing care. We believe that these results establish a foundation for further dressing-based in vivo biofilm research, while providing impetus for translation of this work to the clinical setting.

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