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ORIGINAL ARTICLE

The ability of quaternary ammonium groups attached to a urethane bandage to inhibit bacterial attachment and biofilm formation in a mouse wound model

Phat L Tran[1], Eric Huynh[1], Abdul N Hamood[2], Anselm de Souza[3], Gregory Schultz[4], Bernd Liesenfeld[5], Dilip Mehta[3], Daniel Webster[6] & Ted W Reid[1,2]

1 Departments of Ophthalmology and Visual Sciences, Texas Tech University Health Sciences Center, School of Medicine, Lubbock, TX, USA
2 Departments of Molecular Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, TX, USA
3 Viridis BioPharma Pvt. Ltd, Mumbai, India
4 Department of Obstetrics and Gynecology, University of Florida, College of Medicine, Gainesville, FL, USA
5 Quick Med Technologies, Inc., Gainesville, FL, USA
6 Departments of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, School of Medicine, Lubbock, TX, USA

Key words
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Correspondence to
TW Reid, PhD
Departments of Ophthalmology and Visual Sciences
Texas Tech University Health Sciences Center
School of Medicine
3601 4th Street STOP 7217
Lubbock, TX 79430
E-mail: ted.reid@ttuhsc.edu

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Abstract

For proper wound healing, control of bacteria or bacterial infections is of major importance. While caring for a wound, dressing material plays a key role as bacteria can live in the bandage and keep re-infecting the wound. They do this by forming biofilms in the bandage, which slough off planktonic bacteria and overwhelm the host defense. It is thus necessary to develop a wound dressing that will inhibit bacterial growth. This study examines the effectiveness of a polyurethane foam wound dressing bound with polydiallyl-dimethylammonium chloride (pDADMAC) to inhibit the growth of bacteria in a wound on the back of a mouse. This technology does not allow pDADMAC to leach away from the dressing into the wound, thereby preventing cytotoxic effects. *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were chosen for the study to infect the wounds. *S. aureus* and *P. aeruginosa* are important pathogens in wound infections, while *A. baumannii* was selected because of its ability to acquire or upregulate antibiotic drug resistance determinants. In addition, two different isolates of methicillin-resistant *S. aureus* (MRSA) were tested. All the bacteria were measured in the wound dressing and in the wound tissue under the dressing. Using colony-forming unit (CFU) assays, over six logs of inhibition (100%) were found for all the bacterial strains using pDADMAC-treated wound dressing when compared with control-untreated dressing. The CFU assay results obtained with the tissues were significant as there were 4–5 logs of reduction (100%) of the test organism in the tissue of the pDADMAC-covered wound versus that of the control dressing-covered wound. As the pDADMAC cannot leave the dressing (like other antimicrobials), this would imply that the dressing acts as a reservoir for free bacteria from a biofilm and plays a significant role in the development of a wound infection.

Introduction

Bacterial infections in wounds can result from burns (1), diabetic ulcers (2), trauma (3) and surgery (4,5). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the main bacteria that are known to contribute to serious complications in wound infection (1–5). Statistical

Key Messages

- the pDADMAC foam wound dressing was capable of over six logs of inhibition of both Gram-negative and Gram-positive bacteria under a dressing used on an open

wound on the back of a mouse compared to a control foam bandage without pDADMAC
- the pDADMAC foam dressing showed a total inhibition of biofilm formation for all the test organisms
- the fact that the pDADMAC foam was able to totally block biofilm formation in the tissue of the wound under the bandage is highly significant
- this result implies that a dressing can act as a reservoir for bacteria, leading to infection, and that pDADMAC blocks the reservoir effect, which lets the tissue deal with the residual free bacteria present in the wound

data of infections by *S. aureus* have shown a mortality rate of 19–38% (6–8) while that of *P. aeruginosa* range from 26% to 55% (9, 10). This high mortality rate is because of bacteraemia of the wound. While these two bacteria are of importance because of their toxigenicity, *Acinetobacter baumannii* is a strain of antimicrobial-resistant Gram-negative bacteria thought to be one of the most difficult to treat (11). It is a frequent cause of infections because of its ability to persist for extended periods of time on surfaces under a wide range of environmental conditions. Hence, *S. aureus*, *P. aeruginosa* and *A. baumannii* were chosen for the evaluation of their biofilm-forming ability on dressing materials used on an open wound.

Previous studies on the antimicrobial properties of a non-leaching urethane wound-dressing material that has long chain polymers with high densities of quaternary amines (pDADMAC) attached to its surface showed that they were effective in controlling bacterial biofilms when studied in vitro (12). The present study was carried out to see whether the same wound-dressing material can play an active role in the treatment process of a wound in vivo using a mouse wound model.

Materials and methods

Foam dressing

The polyurethane foam dressing used for evaluation had a high molecular weight (~250 k Daltons) polymer of quaternary amines [polydiallyl-dimethylammonium chloride (polyDADMAC, 0·3%), Quick-Med, Boca Raton, FL] permanently bonded onto it. The pDADMAC-containing foams were obtained from Viridis Biopharma, Mumbai, India under licensing from Quick-Med Technologies, Inc.. The control (pDADMAC-free) foams were obtained from ABL Medical. They were tested as 1 cm^2 swatches.

Test organisms

The strains used were (i) *S. aureus* AH133, a lab strain that constitutively expresses green fluorescent protein from a plasmid (pCM11) in the presence of 1 μg/ml erythromycin (13); (ii) *P. aeruginosa* strain PAO1 Lux; (iii) a clinical isolate of *A. baumannii* (obtained from patients with infected wounds); (iv) *S. aureus* Lux; and (v) MRSA strains of *S. aureus*, obtained from a leg wound (MRSA 1) and from the blood culture of a septicaemia patient (MRSA 2). The *S. aureus* strain AH133 was obtained from C.L. Malone (13). The *P. aeruginosa* strain PAO1 Lux and *S. aureus* Lux are strains available in our lab at Texas Tech University Health Sciences Center. The *A. baumannii* clinical isolate and both the MRSA isolates were obtained from the clinical lab at Texas Tech University Health Sciences Center under the approved Institutional Review Board protocol, Texas Tech University Medical Center, Lubbock, TX. All strains were grown in Luria Bertani (LB) Broth, Mueller Hinton Broth or on LB Agar plates at 37°C.

Mouse wound infection model

The tests were conducted on adult female Swiss Webster mice ($n = 4$), weighing 20–24 g. A similar control group was also maintained. The mice were anaesthetized using a mixture of isoflurane and oxygen, and their backs were shaved. Shaved areas were completely cleansed with 95% ethanol, and 0·5 cm^2 of skin was removed centrally in the shaved area. Either control or pDADMAC foams (1 cm^2) were placed on the wounds. The foams were secured in place by applying a clear OPSITE wound dressing (Smith & Nephew, Mississauga, Ontario, Canada) over the back of the mouse. The dressing was then lifted with a pair of forceps, and an aliquot containing 10^3–10^4 colony-forming unit (CFU) of bacteria in 50 μl of phosphate buffered saline (PBS) (pH 7) was injected in the area between the bandage and the wound. The mice were monitored twice a day for signs of infection or distress. After 48 hours of observation, the mice were euthanized. The foam dressings were removed, and the connective tissues of the wounds were then dissected and removed. The extracted foams and tissues were gently rinsed in PBS, and the biofilms were analysed by CFU assay. Animals were treated in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center in Lubbock, TX.

Colony-forming unit assays

The biofilm formation on the wound dressings was determined by the colony-forming unit assay previously described (14) with some modifications. Bacteria were grown overnight, washed once with PBS (pH 7·4), re-suspended in PBS (pH 7·4) to an optical density (OD_{600}) of 0·5 (10^8 CFU/ml) and serially diluted (10-fold). Fifty microlitres of aliquots containing 10^3–10^4 CFU were injected under either an untreated (control) foam or test foam coated with pDADMAC-PU on the back of the mice. These were allowed to grow for 24–48 hours. Biofilms were quantitated by determining the CFU per square centimeter of foam. Each foam piece was gently washed twice with sterile PBS to remove any planktonic bacteria. Excess PBS was drained from the foam using sterile filter paper, and the foam was then transferred to a sterile 15-ml conical tube containing 5 ml of PBS for enumeration of bacteria. The tubes were placed in a water bath sonicator for 10 minutes to loosen the cells within the biofilm and then vigorously vortexed three times for 1 minute to detach the cells. Suspended cells were serially diluted (10-fold) in PBS, and 10-μl aliquots of each dilution were spotted onto LB Agar plates. All experiments were performed in triplicates.

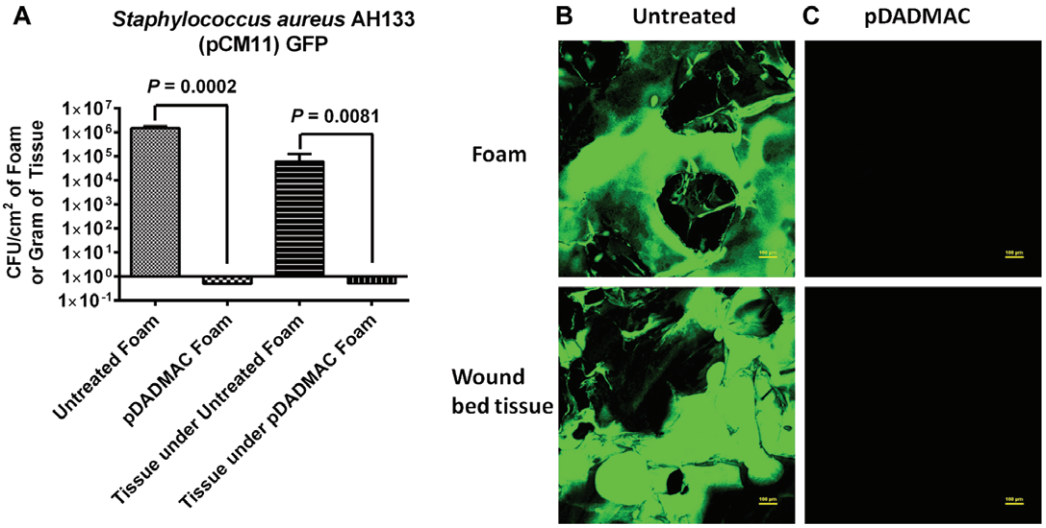

Figure 1 Effect of pDADMAC foam on *Staphylococcus aureus* AH133 (pCM11) GFP: (A), colony-forming unit (CFU) study; (B), confocal laser scanning microscope (CLSM) images of untreated control dressing; and (C) pDADMAC dressing.

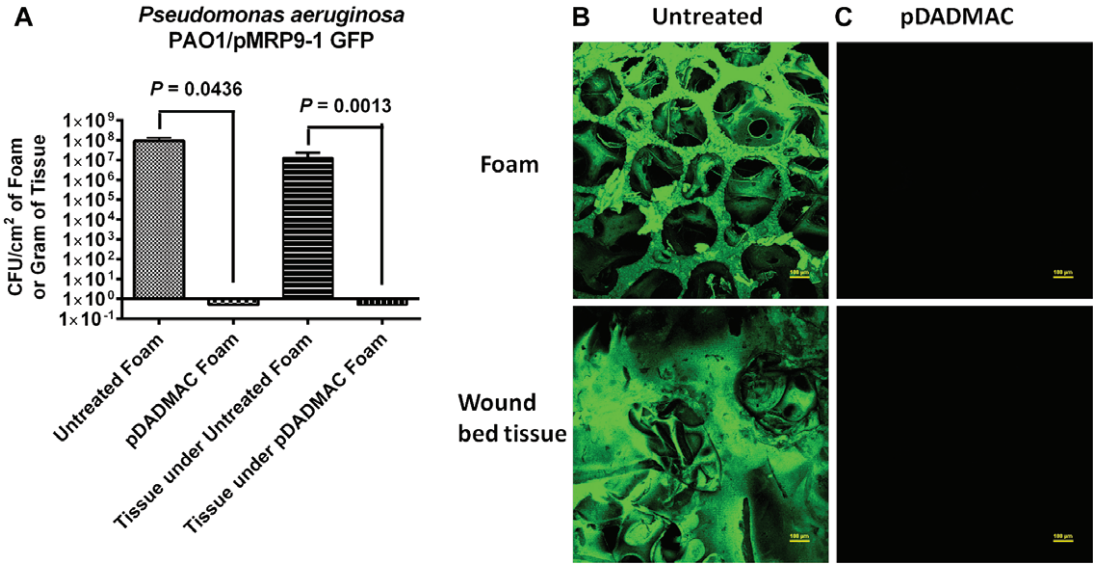

Figure 2 Effect of pDADMAC foam on *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP in the foam bandage dressing and the wound tissue: (A), colony-forming unit (CFU) results; (B) and (C), confocal laser scanning microscope (CLSM) images of untreated control dressing and pDADMAC dressing, respectively.

Tissue: At the end of the experiment (48 hours), the mouse was euthanized, and the tissue of the wound was removed. This was then used for a CFU assay in a manner similar to that used for the foam wound dressing above.

Biofilm analysis by fluorescence microscopy

The pieces of foam placed on the wounds on the mice were inoculated with bacteria as described above and were studied at the end of the 48-hour incubation. Three control and three pDADMAC foam segments were then examined for the presence of biofilm using a fluorescence microscope. As some fluorescence persisted in the wound even though all of the bacteria were dead, as determined by the CFU assay, the foams were treated with 0·05% protease to eliminate the fluorescence of the green fluorescent protein (GFP) protein that was released by the dead bacteria. This was followed by washing with 1 mg/ml bovine serum albumin (BSA) to quench the protease. In this way, it was possible to compare the fluorescence of the control bandage with the pDADMAC bandage.

Statistical analysis

Results of the CFU assays were analysed with Prism® version 4.03 (GraphPad Software, San Diego, CA) with 95% confidence intervals (CIs) of the difference. Comparisons of the in vivo biofilms formed on pDADMAC-free and pDADMAC

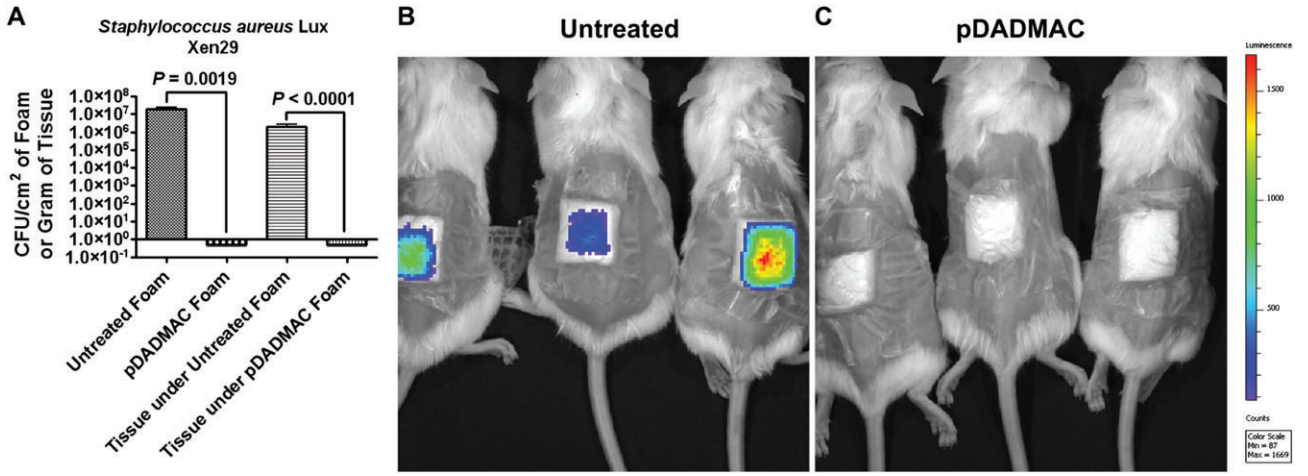

Figure 3 Effect of pDADMAC foam on *Staphylococcus aureus* Lux Xen29 in the foam bandage dressing and the wound tissue: (A), colony-forming unit (CFU) results; (B) and (C), in vivo imaging system (IVIS) of *S. aureus* Lux Xen29.

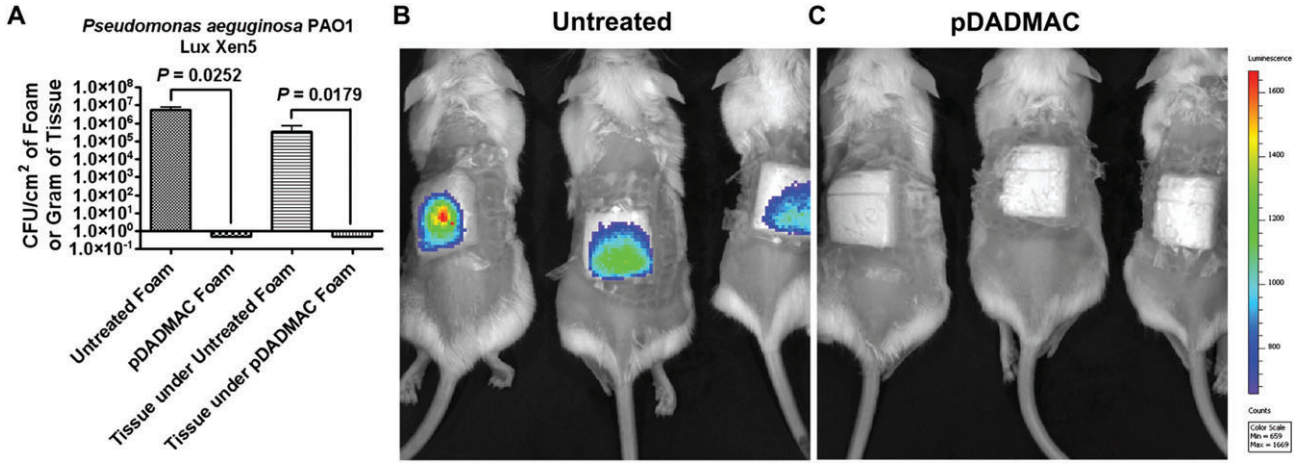

Figure 4 Effect of pDADMAC on *Pseudomonas aeruginosa* Lux Xen5 in the foam bandage dressing and the wound tissue: (A), colony-forming unit (CFU) results; (B) and (C), in vivo imaging system (IVIS) of *P. aeruginosa* Lux Xen5.

foams were analysed by a two-tailed unpaired *t*-test to determine significant differences. All experiments were performed in triplicates.

Results

Bacterial biofilm formation on polyurethane foam

Different strains of bacteria were inoculated under the foam wound dressing with and without pDADMAC on the back of the mouse for 48 hours. At the end of the incubation period, the CFU assay was performed, and the results are presented in Figures 1–5. The pDADMAC foam completely blocked biofilm development for the bacteria tested when compared with the control foam. After 3 days of growth, over six logs of inhibition were seen for *P. aeruginosa, S. aureus, and A. baumannii* in the pDADMAC wound dressing as compared to the control dressing. Visualisation of the CFU results from Figures 1–5 with the confocal laser scanning microscope (CLSM) and the in vivo imaging system (IVIS) confirm these

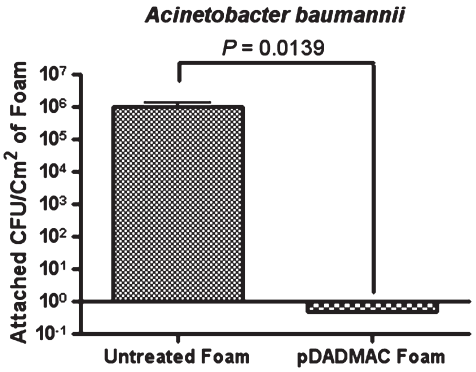

Figure 5 Effect of pDADMAC foam on *Acinetobacter baumannii*.

results. In addition, two different strains of MRSA that were tested over seven logs of inhibition were found with the pDADMAC foam versus the control bandage (Figure 6). In each case, the pDADMAC wound dressing resulted in the complete

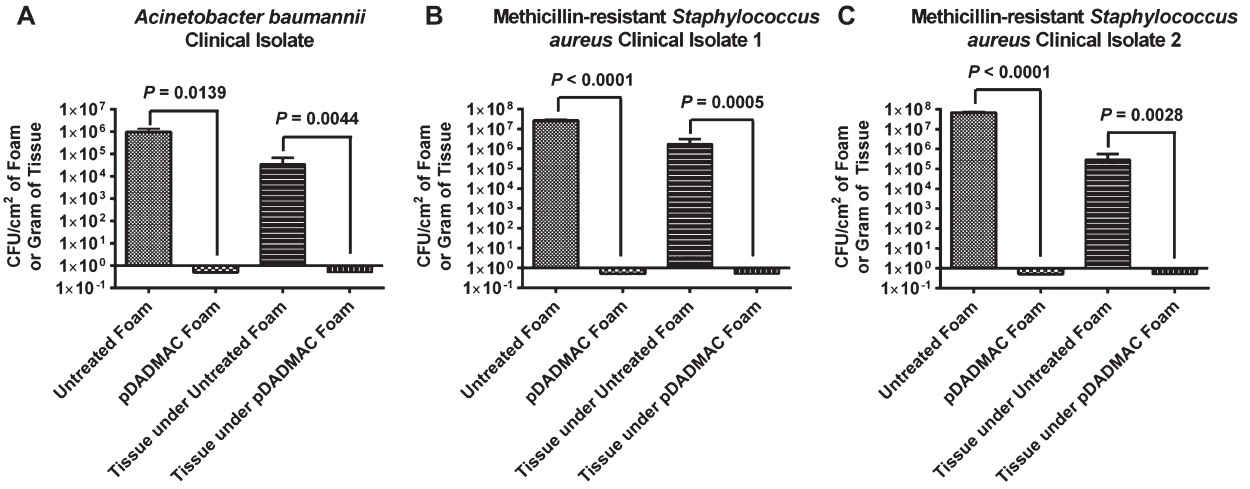

Figure 6 Effect of pDADMAC in the foam bandage dressing and in the wound tissue treated with (A) *Acinetobacter baumannii* clinical isolate, (B) *Staphylococcus aureus* MRSA (leg wound isolate) and (C) *S. aureus* MRSA (blood isolate).

inhibition of biofilm formation. Values are the mean of three or more replicates ± standard deviation (SD), and the CFU determined are per 1 cm^2 of foam.

Similar results were obtained with *S. aureus* GFP AH133 as presented in Figure 4.

Bacterial biofilm formation in the tissue under the bandages

After treatment with the different strains of bacteria for 3 days and the removal of the foam bandages as described above, the tissue under the foam bandage was removed, and CFU assays were performed. As can be seen in Figures 1–5, over four logs of inhibition were seen for the growth of *P. aeruginosa, S. aureus, and A. baumannii* in the tissue with the pDADMAC foam bandage as compared to the control foam bandage. In each case, the pDADMAC foam bandage resulted in the complete inhibition of biofilm formation. In addition to the CFU assay, the in vivo results for the *S. aureus* AH1333 GFP and *P. aeruginosa* PAO1 GFP/pMRP9-1 studies were also examined by CLSM (Figure 1(B) and (C) and 2(B) and (C)), and they were found to agree with the results presented in Figures 1(A) and 2(A). Moreover, visualisation of the CFU results from wound tissue in Figures 3, 4 with the IVIS confirm these results. Similar results were obtained for an *A. baumanni* clinical isolate in vivo where the complete inhibition of bacterial growth was observed with pDADMAC foam bandage. These results can be seen in Figure 5. However, as no Lux or GFP strain was available, only CFU results are presented. In addition, when two different strains of MRSA were tested in wounds, complete inhibition of over approximately five logs was seen for the pDADMAC versus the control on the wound tissue as seen in Figure 6.

Fluorescence images of the *S. aureus* GFP protein on treated and untreated foam wound dressing

The images of *S. aureus* GFP fluorescence are seen in Figure 7. The treatment with protease completely destroyed any fluorescence from the GFP on the pDADMAC foam but not on the control foam, showing that the bacteria on the control foam were still alive, but the bacteria on the pDADMAC foam were dead.

Discussion

In a previous study (12), it was shown that a non-leaching urethane wound dressing material that had a high molecular weight (~250 k Daltons) polymer of quaternary amines [polydiallyl-dimethylammonium chloride (polyDADMAC)] attached to its surface was able to inhibit biofilm formation in vitro. Because of the difference in growth conditions of bacteria in a wound, we felt that it was necessary to test this same material in a wound. In addition, the effect of pDADMAC-treated dressing material on the growth of bacteria in the wound as well as bacterial growth on the foam was determined. The results of this study are significant as it yields information regarding the ability of the wound dressing to act as a reservoir for bacteria that would subsequently get released and grow in the wound.

In the current study, we have presented quantitative data on the eradication of both Gram-positive and Gram-negative bacteria by pDADMAC attached to a polyurethane foam, used as a wound-dressing material on an open wound on the back of a mouse. The data on the foam showed over six logs of eradication (100%) against *S. aureus, P. aeruginosa, and A. baumannii* (Figures 1–6) as compared with a control bandage with no quaternary amine attached. The bacteria were quantitated by counting in a colony-forming unit assay. The biofilms formed because of the bacteria on untreated foam were also shown by fluorescence microscopy as seen in Figures 1–6. The results from the CFU analysis and the fluorescence microscopy complement each other.

Of greater importance was the study of the amount of bacteria left behind in the wound after removal of the dressing. It was found that the pDADMAC dressing showed the complete eradication (100%) of *S. aureus, P. aeruginosa, and*

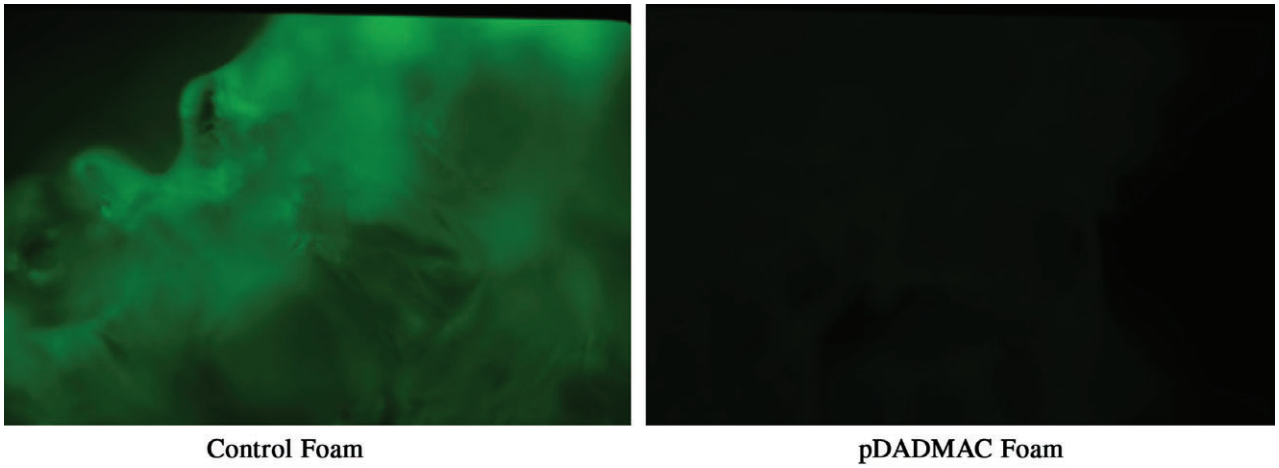

Figure 7 Fluorescent images of foam dressing.

A. baumannii (Figures 1–5) as compared with a control bandage with no quaternary amine attached. It can be concluded that the non-pDADMAC dressing acts as a reservoir for the bacteria and allows them to overcome the host resistance and grow in the wound. It was previously (12) shown that a short time was required for eradication with the pDADMAC foam. It was found that the *S. aureus* was eradicated by the foam in less than 15 minutes. However, Gram-negative bacteria such as *P. aeruginosa* and *A. baumannii* took more time, 240 minutes and 60 minutes, respectively. It would appear that Gram-positive bacteria are eradicated quickly on contact, but Gram-negative bacteria need more time to be eradicated, probably because of the presence of the outer membrane. As no bacteria were found in the tissue infected with both the Gram-positive and Gram-negative bacteria, it would appear that the foam eradicated the bacteria fast enough to prevent them from seeding and infecting the wound. Some of the bacteria must have entered the wound; however, the wound under the pDADMAC dressing showed no bacteria present.

Conclusion

The pDADMAC foam was capable of over six logs of inhibition of both Gram-negative and Gram-positive bacteria under a dressing used on an open wound on the back of a mouse compared to a control foam bandage without pDADMAC. The pDADMAC foam dressing showed a total inhibition of biofilm formation for all the test organisms. The fact that the pDADMAC foam was also able to totally block biofilm formation in the tissue of the wound under the bandage is highly significant. This result implies that a dressing can act as a reservoir for bacteria leading to infection and that pDADMAC blocks the reservoir effect, which lets the tissue deal with the residual free bacteria present in the wound.

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