The ability of a colloidal silver gel wound dressing to kill bacteria *in vitro* and *in vivo*

Objective: Inhibiting bacterial biofilms is of major significance for proper wound healing. The choice of the dressing material plays a key role, as bacteria can live in dressings and keep reinfecting the wound. This study examines the effectiveness of a colloidal silver gel (Ag-gel) wound dressing in inhibiting the growth of bacteria in a mouse wound model.

Method: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and two different meticillin-resistant *Staphylococcus aureus* (MRSA) strains were examined. Bacteria were measured *in vitro* on the dressing, and *in vivo* studies were carried out to analyses both the dressing and the infected tissue.

Results: Using colony-forming unit (CFU) assays, over 7 logs of inhibition (100%) were found for *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* for the Ag-gel dressing when compared with the control dressing. *In vivo*, complete inhibition was observered for the three most common bacteria on the Ag-gel dressing and the tissue under that dressing. These results were confirmed by an *in vivo* live imaging system. However, with MRSA strains, only 2–3 logs of inhibition were recorded.

Conclusion: The Ag-gel was effective in preventing biofilm infections caused by both Gram-negative and Gram-positive bacteria.

Declaration of interest: D. Mehta, A. DeSouza, K.W. Moeller and C.D. Moeller all have commercial interests in this product. The other members of the research team have no commercial affiliation or interest in the product.

silver • biofilm • wound • *in vitro* model • *in vivo* model • infection • wound dressing

Silver-containing dressings, such as Silvazine and Acticoat, have shown promise in the control of wound infections in human patients.[1,2] *In vitro* studies have shown that silver can kill Gram-positive and Gram-negative bacteria, in addition to meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE).[3–7] It is thought that silver can destroy bacteria by binding to bacterial proteins, causing structural changes in the bacterial cellular membrane.[5] Silver ions can also bind and denature DNA and RNA, inhibiting bacterial replication.[2–8]

Silver is biologically active when it is soluble, which is the Ag^+ and AgO forms. AgO is the form present in nanocrystalline silver.[9,10] Wright et al.[4] compared silver ions with nanocrystalline silver and found that the nanocrystalline form was more efficacious than the ionic one. The use of nanocrystalline silver dressing has also been reviewed by Fong et al.[9,10]

The present study was carried out to evaluate the ability of a colloidal silver gel (Ag-gel) dressing to kill bacteria both *in vitro* and *in vivo*.

P.L. Tran,[1] PhD; **E. Huynh,**[1] BS; **A.N. Hamood,**[2] PhD; **A. de Souza,**[3] PhD; **D. Mehta,**[3] PhD; **K.W. Moeller,**[4] ND; **C.D. Moeller,**[4] ND; M. Morgan[5], MD; ***T. W. Reid,**[1,2*]

***Corresponding author e-mail:** ted.reid@ttuhsc.edu

1 Departments of Ophthalmology and Visual Sciences, Texas Tech University Health Sciences Center, School of Medicine, Lubbock, Texas, US. **2** Departments of Molecular Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, Texas, US. **3** Viridis BioPharma Pvt. Ltd. Mumbai, India. **4** American Biotech Labs LLC, Alpine, Utah, US. **5** Department of Ophthalmology, Texas Tech University Health Sciences Center, US.

Materials and methods

For *in vitro* studies, control and treated groups of discs were inoculated with test bacterial strains. The biofilms were developed for 24 hours. After incubation, biofilms formed on the discs were examined by confocal laser scanning microscopy (CLSM) and colony-forming unit (CFU) assay.

For *in vivo* studies, control and treated groups of mice were inoculated with the test bacterial strains. Biofilms were allowed to develop for three days and quantified by CLSM, *in vivo* live imaging system (IVIS) and CFU assay.

This study focused on three of the most common wound infecting bacteria: *Staphylococcus aureus* (laboratory as well as two MRSA strains), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The dressing evaluated contained colloidal silver in a gel form (Viridis Biopharma Pvt., Ltd., Mumbai, India). It was tested by evenly spreading 0.5 g on a 1 cm^2 of gauze (hospital-grade rolled gauze bandage (LOT1783A, Johnson and Johnson, New Brunswick, US)). We assessed the bacteria remaining on the wound dressing, as well as the number of bacteria in the wound.

Bacterial strains

All strains were grown in lysogeny broth (LB) or on LB agar plates at 37°C. The strains used were:

- *Staphylococcus aureus* AH133 GFP, a lab strain that constitutively expresses green fluorescent protein (GFP) from a plasmid (pCM11) in the presence of 1 µg/ml erythromycin, obtained from Malone et al.[11]
- *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP, a strain

Fig 1. Effect of Ag-gel dressing on a clinical isolate of *Acinetobacter baumannii* (clinical isolate) **(a)**, MRSA-1 **(b)**, and MRSA-2 **(c)**

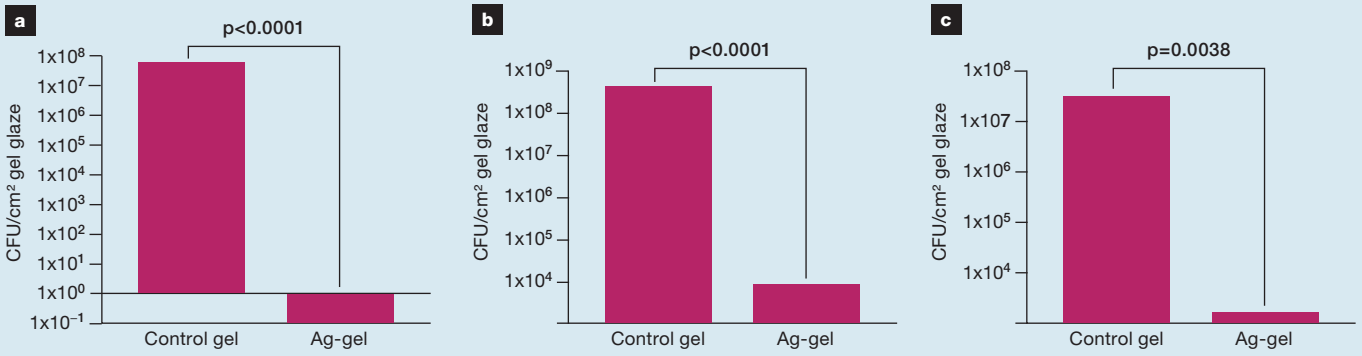


Fig 2. Effect of Ag-gel dressing on *Staphylococcus aureus* AH133 GFP *in vitro* (**a**). Confocal laser scanning microscopy images in the absence **(b)** and presence of Ag-gel **(c)**

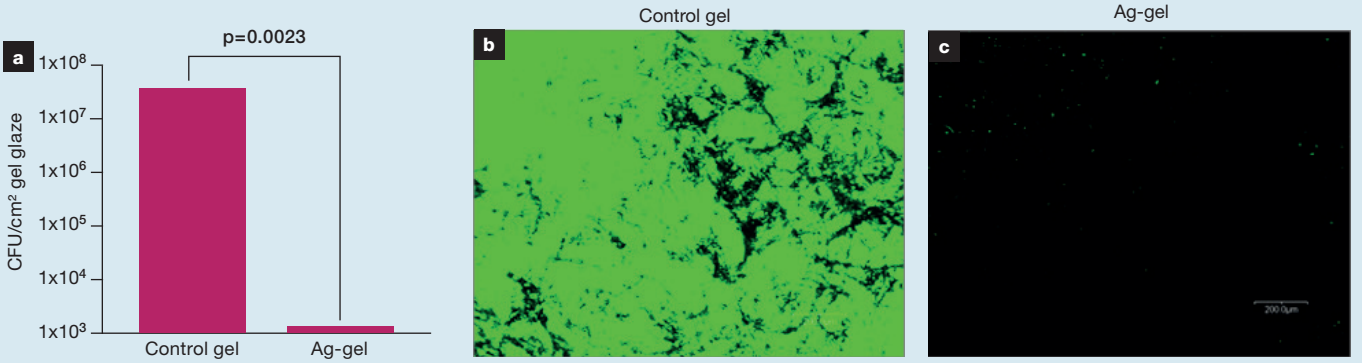
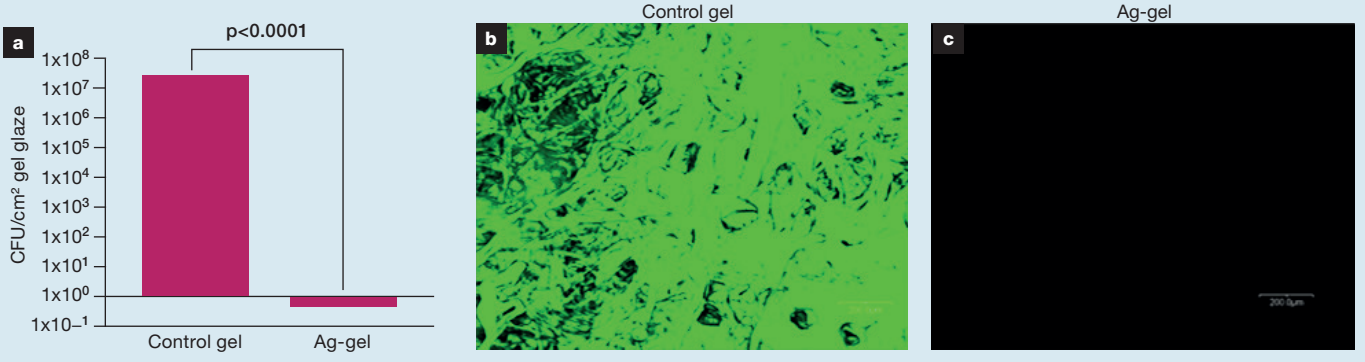


Fig 3. Effect of Ag-gel dressing on *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP *in vitro* (**a**). Confocal laser scanning microscopy images in the absence **(b)** and presence of Ag-gel **(c)**



with a plasmid (pMRP9-1) containing a gene that codes for GFP, obtained from Davies et al.[12] To maintain the plasmid, the strain was grown in the presence of 300 µg/ml carbenicillin

- *Staphylococcus aureus* Lux Xen29, a strain with a plasmid containing a gene that codes for the luciferase protein. To maintain the plasmid, the strain was grown in the presence of 40 µg/ml kanamycin. This strain was also available at our lab at Texas Tech University Health Sciences Center
- *Pseudomonas aeruginosa* PAO1 Lux Xen5, available at our lab
- A clinical isolate of *Acinetobacter baumannii* obtained from infected wounds of patients, available at our lab
- MRSA strains of *Staphylococcus aureus*, obtained from a leg wound (MRSA-1) and from the blood culture of a septicaemia patient (MRSA-2) collected by our lab.

These MRSA strains and the *Acinetobacter baumannii* isolate were acquired under an approved Institutional

Review Board protocol, Texas Tech University Medical Center, Lubbock, Texas.

Colony-forming unit assays: *in vitro*

Segments of wound dressing material or gauze were cut in 1 cm^2 pieces and used in test and control groups. Approximately $1x10^2$ to $1x10^3$ CFU of the test bacteria were inoculated on a blank sterile 6 mm cellulose disc (BD Diagnostic System, Sparks, US). The discs were transferred onto LB agar plates with appropriate antibiotics. Gauze pieces containing either Ag-gel or control gel were placed over the discs inoculated with bacteria. The gauzes were gently pressed down using a sterile forceps. The plates were then incubated at 37°C for 24 hours. Following incubation, each cellulose disc inoculated with *Staphylococcus aureus* AH133 GFP or *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP was examined by CLSM using a Nikon A1+/AIR+ Confocal Microscope (Nikon Inc., Melville, US). The discs were also examined by the CFU assay.[13,14] Bacterial counts were expressed as CFU per disc. Thus, since 1 ml of solution was obtained from each sample, and 10 µl was used for the dilutions, the equation for back-calculating the bacterial concentration on the original material was CFU x dilution factor x 100. All experiments were performed in triplicate.

Mouse wound infection model

Tests were conducted on adult female Swiss Webster mice (n=6) weighing 20–24 g. The mice were anaesthetised using a mixture of isoflurane and oxygen, and their backs were shaved. Shaved areas were completely cleansed with 95% ethanol and a 0.5 cm^2 area of skin was removed centrally in the shaved area. A layer of 0.5 g of either hydrogel (control) or Ag-gel was added to 1 cm^2 of gauze. The Ag-gel gauze was placed on the wounds of all the test mice. The gauze was secured using transparent, semipermeable, polyurethane OpSite dressing (Smith & Nephew, Andover, US) over the back of the mouse. Infection of the wound was initiated by injecting approximately $1x10^2$ to $1x10^3$ CFU of the test strain into the wound bed. The mice were monitored twice a day for signs of infection or distress. After three days of observation, mice treated with *Staphylococcus aureus* Lux or *Pseudomonas aeruginosa* Lux were imaged using IVIS, and were then euthanised.

The dressings were removed and the connective tissue around the wound was then dissected, removed and weighed. The extracted dressings and tissues were gently rinsed and homogenised. The suspensions were then analysed by a CFU assay. For mice treated with *Staphylococcus aureus* AH133 GFP or *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP, the dressings and the connective tissue around the wound were recovered and examined under CLSM, and followed by a CFU assay. For mice treated with *Acinetobacter baumannii* and MRSA clinical isolates (CI), the dressings and the connective tissue around the wound were recovered and examined by the CFU assay only. Animals were treated in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center.

Biofilm detection by assay using the dressing

Bacteria were grown overnight, washed once with sterile 1X phosphate-buffered saline (PBS) (pH 7.4), resuspended in sterile 1X PBS (pH 7.4) to an optical density (OD600) of 0.5 (~108 CFU/ml), and serially diluted [10-fold]. Aliquots containing $1x10^2$ to $1x10^3$ CFU of the tested strain were inoculated into the wound bed between either an Ag-free (control) dressing or test dressing (Ag-gel) and the wound. These were allowed to grow for three days. Biofilms were quantified by determining the CFU per cm^2 of dressings. After removal from the mouse, each piece of dressing was gently washed twice with sterile 1X PBS to remove any planktonic bacteria. Excess PBS was drained from the dressing by using sterile filter paper and the dressing was then transferred to a sterile homogenised tube containing 2 ml of sterile PBS to count the bacteria. The dressings were homogenised 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 sterile 1X PBS, and 10 µl aliquots of each dilution were spotted onto LB agar plates. Bacterial counts were determined as described above. The results were reported as attached CFU per cm^2 of dressing. All experiments were done in triplicate.

Biofilm detection by assay using the tissue

At the end of the experiment (three days), the mice were euthanised and the tissue of the wounds was removed. The tissues were weighed. They were then homogenised and the CFU assay was performed in a manner similar to the assay used for the dressing above. The results were reported as attached CFU per gram of tissue. All experiments were done in at least triplicate.

In vivo live imaging studies

Mice infected with either *Staphylococcus aureus* Lux Xen29 or *Pseudomonas aeruginosa* Lux Xen5 were observed at day 3 after treatment. The mice were lightly anaesthetised, and the infected wounds were visualised using an IVIS Lumina XR system with Living Image software (Perkin Elmer, US). This instrument allows us to directly detect pathogenic infections in living animals by producing images through bioluminescence. Bioluminescence offers a method for monitoring infections *in vivo* over a period of several days. It is sensitive and non-invasive, and requires fewer animals than conventional methodologies. After imaging, the mice were euthanised, and the gauze dressings as well as the wound tissue were recovered. The samples were then analysed by the CFU assay as described above. Imaging experiments were conducted at the Texas Tech University Health Sciences Center Image Analysis Core Facility (Lubbock, TX). All experiments were done in triplicate.

Fig 4. Effect of Ag-gel on *Staphylococcus aureus* Lux Xen29 in the dressing and the wound tissue. Number of bacteria (colony forming units CFU) in the gauze and tissue from treated and untreated samples **(a)**. *In vivo* live imaging of *Staphylococcus aureus* Lux **(b** and **c)**

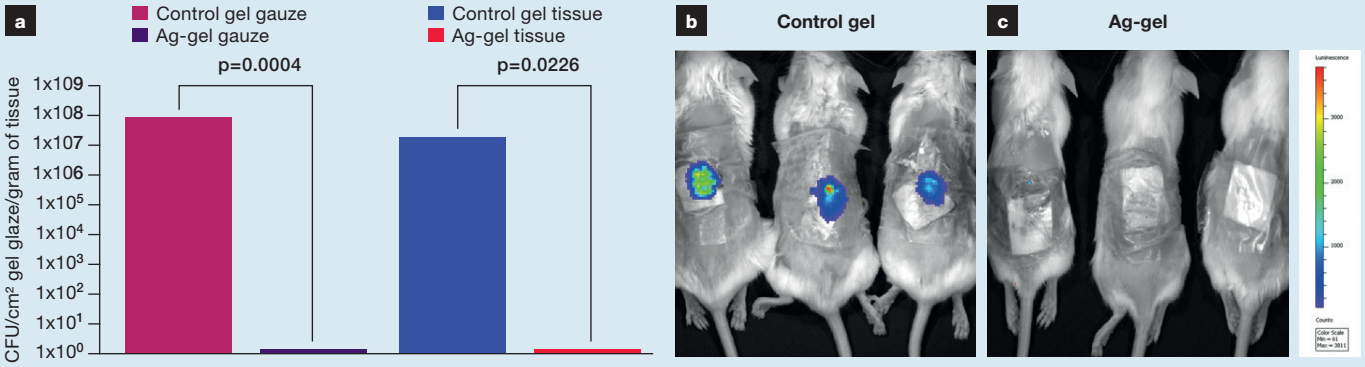
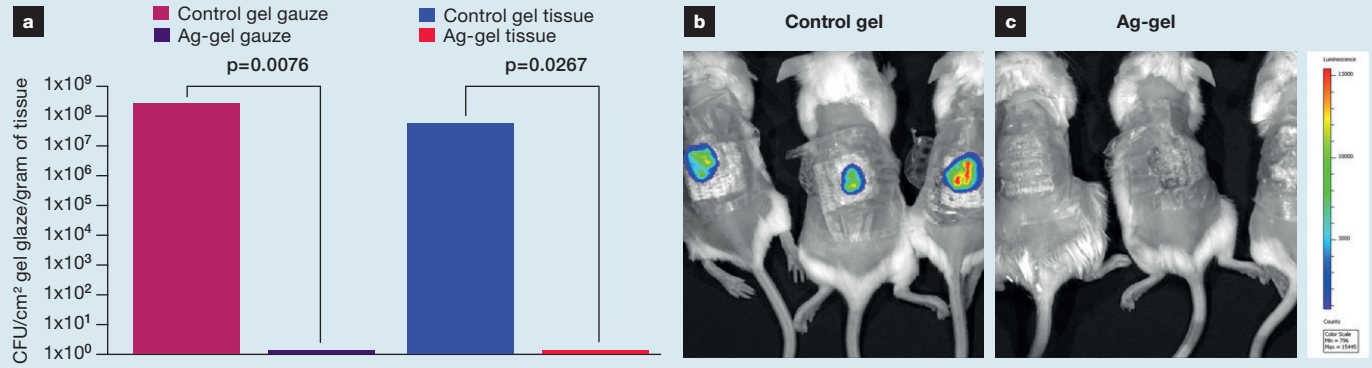


Fig 5. Effect of Ag-gel on *Pseudomonas aeruginosa* Lux Xen5 in the dressing and the wound tissue. Number of bacteria (colony forming units CFU) in the gauze and tissue from treated and untreated samples **(a)**. *In vivo* live imaging of *Pseudomonas aeruginosa* Lux **(b** *and* **c)**



Statistical analysis

The results of the CFU assays were analysed with Prism version 4.03 (GraphPad Software, San Diego, US) with 95% confidence intervals (CIs) of the difference. Comparisons of the *in vitro* and *in vivo* biofilms formed on Ag-gel dressings and Ag-free ones were analysed by a two-tailed unpaired t-test to determine significant differences. All experiments were done in triplicate.

Results

Effect of Ag-gel on bacterial biofilm formation for *in vitro* studies

The results for the *in vitro* studies using *Acinetobacter baumannii* isolate, MRSA-1, and MRSA-2 are illustrated in Fig 1 (a, b and c, respectively). The Ag-gel showed over 4 log of reduction in both cases of MRSA strains when compared with the control as showed in Fig 1 (b, c). The Ag-gel treated dressing was able to cause 7 log of killing (100% inhibition) for the clinical isolate of *Acinetobacter baumannii* (Fig 1a).

The effect of the colloidal silver on *Staphylococcus aureus* AH133 GFP is shown in Fig 2. Fig 2a also shows a similar reduction of 4 logs, as compared with the control. The CLSM seen in Fig 2 (b, c) confirmed the CFU results seen in Fig 2a. Ag-gel from Fig 2c significantly reduced the biofilm formation of *Staphylococcus aureus* AH133 GFP (Fig 2c) as compared with the control gel from Fig 2b.

The CFU assay results for *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP presented in Fig 3a show complete killing by the Ag-gel (over 7 log). The CLSM images of the dressings without and with the Ag-gel in Fig 3 (b, c) respectively confirm the results of the CFU assay.

Effect of Ag-gel dressing on bacterial biofilm formation on a mouse wound

The results of these studies are presented in Fig 4–8. After the three-day incubation period, over 7 log (100%) inhibition was seen with the CFU assay for *Staphylococcus aureus* Lux Xen29 and *Pseudomonas aeruginosa* Lux Xen5 with the Ag-gel dressing as compared with the control gel dressing (Fig 4a and Fig 5a). The CFU assay results also showed complete inhibition of bacterial growth in the wound tissue. At the end of 3 days, the mice were imaged using IVIS. The images obtained with *Staphylococcus aureus* Lux Xen29 as test organism are shown in Fig 4 (b, c) and with *Pseudomonas aeruginosa* Lux Xen5 in Fig 5 (b, c). The images show an absence of viable cells on the Ag-gel treated mice but a well-established biofilm was observed on the control mice. These results are in agreement with the results obtained with the CFU assays.

Similar results were obtained for *Acinetobacter baumannii* clinical isolate *in vivo* (Fig 6a), where complete inhibition of bacterial growth was observed both on the gauze and wound tissue treated with Ag-gel. However, since no Lux strain of *Acinetobacter baumannii* was available, only CFU results are presented. When tested in wounds, the two strains of MRSA did not show complete inhibition. A reduction of approximately 2–3 log was seen with the Ag-gel dressing versus the control gel dressing, both on the gauze and on the wound tissue as seen in Fig 6 (b, c). Values are the means of n=6 replicate ± SD, and the bacterial counts are presented as CFU per cm^2 of dressing or per gram of tissue.

In order to check if the Lux results obtained with *Staphylococcus aureus* and *Pseudomonas aeruginosa* were due to any characteristic of the Lux construct, the *in vivo* experiments were repeated with the GFP constructs of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results are presented in Fig 7 and 8. The CFU assay results show a significant reduction of *Staphylococcus aureus* AH133 GFP on the gauze; while complete inhibition was seen in the wound tissue as seen in Fig 7a. Fig 8a shows that there was complete inhibition of *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP both on the gauze and wound tissue. The CLSM results confirm the CFU assay results as shown in Fig 7 (b, c) and 8 (b, c). The confocal images of the dressing and tissue using the GFP constructs were similar to the IVIS images using Lux constructs.

Discussion

This study presents quantitative data of the

Fig 6. Effect of Ag-gel on the number of colony forming units (CFU) in gauze or the tissue in the presence of a clinical isolate of *Acinetobacter baumannii* **(a)**, *Staphylococcus aureus* MRSA-1 **(b)** and *Staphylococcus aureus* MRSA-2 **(c)**

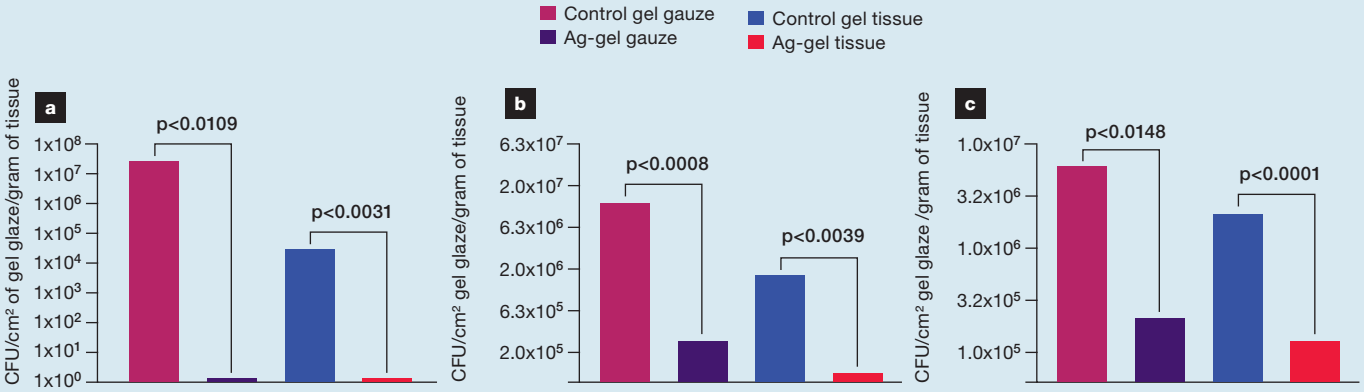
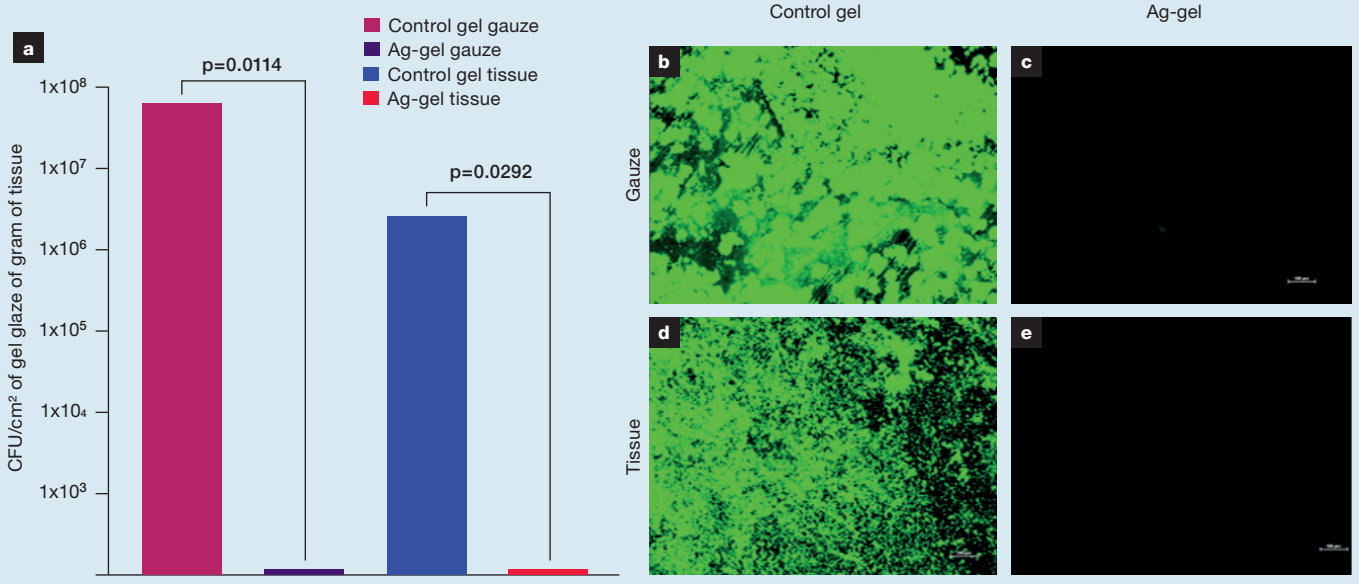


Fig 7. Effect of Ag-gel on *Staphylococcus aureus* AH133 GFP in the dressing and the wound tissue number of colony forming units (CFU) in gauze or the tissue **(a)**. Confocal laser scanning microscopy images of *Staphylococcus aureus* AH133 GFP in the presence **(c** and **e)** of or absence of Ag-gel **(b** and **d)**



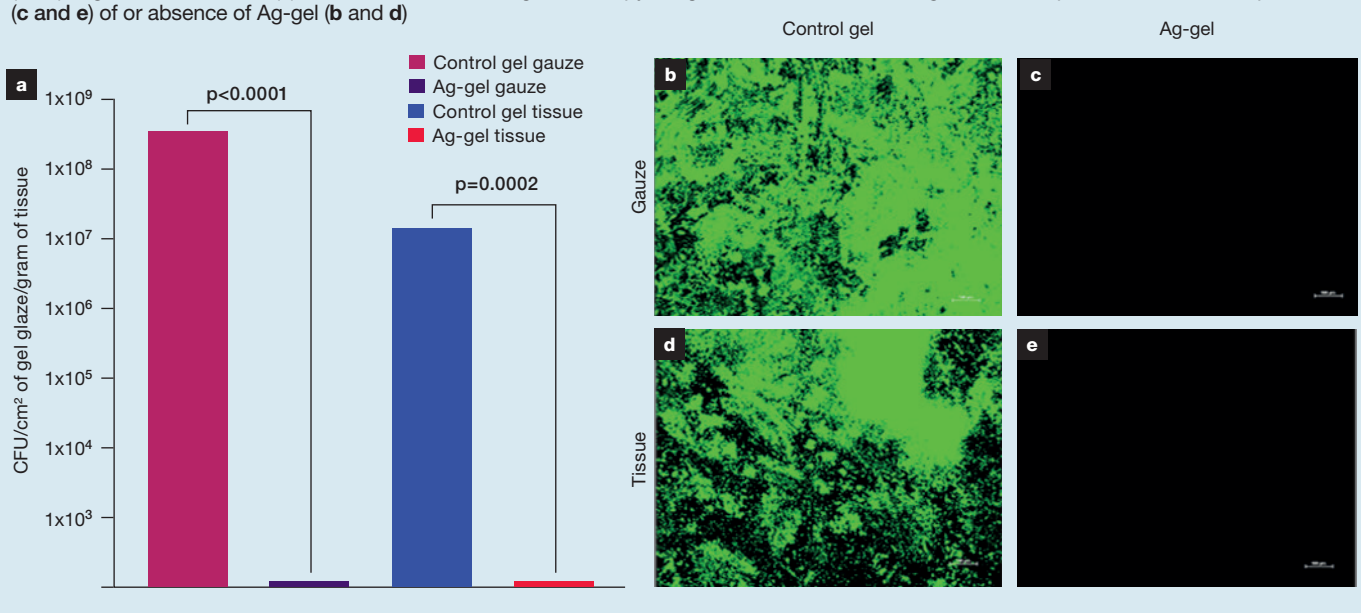


Fig 8. Effect of Ag-gel on *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP in the dressing and the wound tissue, number of colony forming units (CFU) in gauze or the tissue (**a**). Confocal laser scanning microscopy images of *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP in the presence (**c and e**) of or absence of Ag-gel (**b** and **d**)

antimicrobial effect on both Gram-positive and Gram-negative bacteria of Ag-gel, layered on a gauze and used as a dressing material.

The CFU assay results of *in vitro* studies using Ag-gel treated dressings showed over 7 log of killing (100%) for *Pseudomonas aeruginosa* GFP, *Staphylococcus aureus* GFP and *Acinetobacter baumannii* as compared with a control gel dressing containing no Ag-gel. These results were confirmed by CLSM studies with *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP and *Staphylococcus aureus* AH133 GFP biofilm on the Ag-gel and control gel dressings. However, both MRSA strains of *Staphylococcus aureus* showed only 2–4 log of reduction with Ag-gel as compared with the control dressing by the CFU assay.

The CFU data of *in vivo* tests indicate over 7 log of inhibition (100%) with the Ag-gel for *Staphylococcus aureus* Lux Xen29, *Pseudomonas aeruginosa* Lux Xen5, and *Acinetobacter baumannii* clinical isolate as compared with the control gel dressing. The quantification of bacteria left behind in the wound after removal of the gel dressing was of significant importance. It was found that the tissue covered with the Ag-gel dressing showed complete killing (100%) of *Staphylococcus aureus* Lux Xen29 (>7 log), *Pseudomonas aeruginosa* Lux Xen5 (7 log), and *Acinetobacter baumannii* clinical isolate (>4 log), when compared with tissue covered with control dressing. This could possibly be due to the non-Ag-gel dressing acting as a reservoir for the bacteria, allowing them to overcome the host defences and reinfect the wound. These results were confirmed by the IVIS, which showed the absence of both *Staphylococcus aureus* Lux Xen29 and *Pseudomonas aeruginosa* Lux Xen5 in Ag- gel treated wounds. However, the MRSA strains were inhibited to a lower extent in the *in vivo* studies. The results obtained by CLSM with GFP constructs for both *Staphylococcus aureus* AH133 GFP and *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP were similar to the Lux data. In both cases, there was total inhibition of the biofilm in both the dressing and the wound.

Limitations

The bacteria on both the bandage and the wound tissue were quantified by the CFU assay. In experiments where no bacteria were detected, from the diluted samples, a 100 µl sample of undiluted solution from the 1 ml obtained from the bandage or the wound tissue was then spotted on a plate. The bacteria in the sample were then calculated as CFU x 10. This would mean that the smallest amount of bacteria that we could detect would statistically be approximately 10 bacteria. However, since all experiments were performed in triplicate the smallest amount of bacteria that we could statistically detect would be 3–4 bacteria. This was displayed as zero on the graphs.

Conclusion

The Ag-gel was found to be capable of over 7 log (100%) inhibition of both Gram-negative and Gram-positive bacteria when used on a dressing in a mouse wound model compared with control gel dressing. The Ag-gel dressing showed total inhibition of biofilm formation on the dressing and in the wound tissue for all the three test organisms. The results were confirmed by IVIS for *Pseudomonas aeruginosa* Lux Xen5 and *Staphylococcus aureus* Lux Xen29 and with CLSM studies for *Pseudomonas aeruginosa* PAO1/pMRP9-1 GFP and *Staphylococcus aureus* AH133 GFP on the dressing and the wound tissue. However, MRSA strains of *Staphylococcus aureus* were not inhibited to a great extent both *in vivo* and *in vitro*. JWC

References

1 Church, D., Elsayed, S., Reid, O. et al. Burn wound infections. Clin Microbiol Rev 2006; 19: 2, 403–434.
2 Lansdown, A.B. Silver. I: Its antibacterial properties and mechanism of action. J Wound Care 2002; 11: 4, 125–130.
3 Tredget, E.E., Shankowsky, H.A., Groeneveld, A., Burrell, R. A matched-pair, randomized study evaluating the efficacy and safety of Acticoat silver-coated dressing for the treatment of burn wounds. J Burn Care Rehabil 1998; 19: 6, 531–537.
4 Wright, J.B., Lam, K., Burrell, R.E. Wound management in an era of increasing bacterial antibiotic resistance: a role for topical silver treatment. Am J Infect Control 1998; 26: 6, 572–577.
5 Yin, H.Q., Langford, R., Burrell, R.E. Comparative evaluation of the antimicrobial activity of ACTICOAT antimicrobial barrier dressing. J Burn Care Rehabil 1999; 20: 3, 195–200.
6 Thomas, S., McCubbin, P. A comparison of the antimicrobial effects of four silver-containing dressings on three organisms. J Wound Care 2003; 12: 3, 101–107.
7 Thomas, S., McCubbin, P. An in vitro analysis of the antimicrobial properties of 10 silver-containing dressings. J Wound Care 2003; 12: 8, 305–308.
8 Dunn, K., Edwards-Jones, V. The role of Acticoat with nanocrystalline silver in the management of burns. Burns 2004; 30 Suppl 1: S1–9.
9 Fong, J., Wood, F. Nanocrystalline silver dressings in wound management: a review. Int J Nanomedicine 2006; 1: 4, 441–449.
10 Fong, J., Wood, F., Fowler, B. A silver coated dressing reduces the incidence of early burn wound cellulitis and associated costs of inpatient treatment: comparative patient care audits. Burns 2005; 31: 5, 562–567.
11 Malone, C.L., Boles, B.R., Lauderdale, K.J. et al. Fluorescent reporters for Staphylococcus aureus. J Microbiol Methods 2009; 77: 3, 251–260.
12 Davies, D.G., Parsek, M.R., Pearson, J.P. et al. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 1998; 280: 5361, 295–298.
13 Nidadavolu, P., Amor, W., Tran, P.L. et al. Garlic ointment inhibits biofilm formation by bacterial pathogens from burn wounds. J Med Microbiol 2012; 61: 662–671.
14 Tran, P.L., Hamood, A.N., de Souza, A. et al. A study on the ability of quaternary ammonium groups attached to a polyurethane foam wound dressing to inhibit bacterial attachment and biofilm formation. Wound Repair Regen 2015; 23: 1, 74–81.