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ORIGINAL ARTICLE

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A proof-of-concept study of the removal of early and late phase biofilm from skin wound models using a liquid acoustic stream

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Abstract

Chronic wounds fail to progress through the normal stages of healing, with the largest remediable cause of chronicity being presence of a multi-species biofilm. Removal of biofilm from the wound environment is central to wound care. A device for mechanically removing biofilms from wounds has been devised. The removal is caused by small-scale liquid currents and shear, generated by acoustically activated microscopic air bubbles. These bubbles and acoustic waves are delivered onto the wound by a gentle liquid stream, allowing cleaning in situ and removal of debris in the run-off liquid. We have investigated if this liquid acoustic wound stream (LAWS) can remove bacterial biofilm from soft biological wound models and studied the effect of LAWS on the cellular tissues of the substrate. LAWS will efficiently remove early *Pseudomonas aeruginosa* biofilm from an artificial wound in a pig's trotter, 24 hours-mature biofilm of *P. aeruginosa* from a pre-wounded human full thickness skin model (EpiDerm FT), and 3-day mature biofilm of *P. aeruginosa* or *Staphylococcus aureus* from a porcine skin explant. Histological examinations of uninfected EpiDerm models that had been treated by LAWS and then stained with Haematoxylin and Eosin, demonstrated no damage to the human tissue, and wound diameter was smaller in the treated skin models compared with untreated samples. Immunofluorescence staining for cytokeratin 14 showed that keratinocytes had migrated further across the wound in the uninfected samples treated by LAWS. We discuss the implications for wound healing and propose further laboratory and clinical studies to demonstrate the removal of biofilm from patients with chronic leg ulcers and the impact on healing.

KEYWORDS

biofilm, cleaning, debridement, healing, stream, ultrasound, wound

Key Messages

- chronic wounds fail to progress through the normal stages of healing, commonly due to the presence of a multi-species biofilm. Removal of the biofilm infection is central to wound care
- we aim to demonstrate the efficacy of a novel acoustic device, which removes biofilm from wounds by small-scale liquid currents and shear from microscopic air bubbles. We tested this device against three different wound models, infected with *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilms
- this novel device efficiently removed the biofilms from all three wound models, and histological analysis also determined an increase in healing response of uninfected wound models, following treatment with the device

1 | INTRODUCTION

Chronic wounds—wounds that do not progress through the stages of healing in a timely manner—remain a major cause of morbidity and mortality. They form part of a group of difficult-to-treat biofilm phenotypic infections such as cystic fibrosis, implanted device infections and periodontitis.

A systematic review and meta-analysis of chronic wounds in 2019[1] showed a pooled prevalence of 2.21 cases per 1000, of which chronic venous leg ulcers comprised 68%. In this international study, the mean age of patients was >70 years in age. In the United Kingdom, Guest et al[2,3] showed in 2012/13, the NHS had treated 2.2 million wounds of all types, of which 731 000 were leg ulcers. A recent update[4] suggests that the prevalence of all wounds increased by 71% in the 5 years since the original study, and for venous leg ulcers, the figure was 101%. The estimated cost of health care services in the United Kingdom for chronic wounds alone was £5.6 billion[4] in 2017/18, a 48% increase in 5 years. Financial costs do not capture the harm to patients, and a review in 2018[5] noted that pain and loss of mobility were the predominant features contributing to reduced quality of life (QoL). The negative impact on QoL was noted[5] to be similar to that seen in chronic obstructive pulmonary disease and ischaemic heart disease.

Wounds that fail to heal are stuck in the inflammatory phase of healing, and the presence of biofilm is thought to be a major cause of the failure of the wound healing process.[6] Malone et al[7] reviewed the evidence of biofilm in human chronic wounds and concluded that it was "ubiquitous" in wound beds. Costerton et al[8] suggested at the end of the 20th century that bacterial biofilms are a common cause of persistent infections. In his seminal paper 10 years later, Bjarnsholt proposed,[9] with convincing evidence, that biofilms were a significant cause of failure to heal in chronic wounds. This proposition is now universally accepted with the World Union of Wound Healing Societies Position Document[10] setting out the centrality of biofilm management in wound care. More recently, many papers have reported improved healing after the removal of biofilms.[11,12]

The biofilm phenotype gives bacteria an increased adhesion to substrates, protection against host defences and a relative resistance to antibiotics[13] when compared with the planktonic phenotype. This tolerance to antibiotics led to the NICE guidance which recommends antibiotic use[14] only where there is evidence of an acute infection. The difficulty of distinguishing the difference between acute inflammation caused by infection or the stalling of the chronic wound healing process is the probable cause of the high rates of antibiotic use showed by Guest.[4]

Where a biofilm exists on a hard surface, abrasive or erosive techniques to remove the biofilm may be used. However, on soft surfaces the potential for damage to underlying structures must be considered in a harm/benefit analysis. A mainstay for the treatment and management of chronic wounds has long been sharp or vigorous debridement, in which dead tissue and other contaminants are removed from the wound bed,[15] which is endorsed in professional guidelines.[10] However, effective debridement techniques result in pain for the patient, cost for health services, and requirement for skilled practitioners.[15] Active debridement necessarily removes some viable tissue and, in some cases, it is advocated to leave a bleeding wound bed as evidence of a successful procedure.[16] Alternative forms of less abrasive debridement including chemical, biological, and autolytic techniques are available but are also time-consuming and expensive.

This paper proposes a new liquid acoustic wound stream (LAWS) device that gently cleans and removes biofilms from wounds using a rinsing stream of water or saline, unheated and without bio-active chemicals. The pressure (20-40 kPa) exerted by the stream itself[17] alone has negligible effect at removing contaminants, being

1000-1 000 000 less than the pressure in a water jet wound debridement tool.[16] However, the LAWS stream carries sound and microscopic air bubbles onto the wound where they remove biofilm without damaging the underlying tissue. Therefore, this proposed technology is not a debridement tool, but rather a gentle, but efficient, biofilm removal process.

Sound and liquid have been used to clean for many decades in ultrasonic cleaning baths.[18] However, the use of inertial cavitation in a cleaning bath is a violent process that may damage tissue and requires the item to be cleaned to be placed in a bath, immersed in a "soup" containing removed contaminants.[19] Moreover, the ultrasonic bath cannot "clean in place," cannot accommodate objects larger than itself, and the act of immersing a target within the bath can degrade the sound field and generate spots of little activity where no cleaning occurs.[20]

The occurrence of inertial cavitation in an ultrasonic cleaning bath, or the operation of an ultrasonic debridement tool, is characterised by the presence of high amplitude ultrasonic pressure oscillations, which cause gas bubbles to pulsate, expanding to many times their original volumes, and then violently collapse. On collapse, the bubble might involute to form a liquid jet, which passes through the bubble and impacts the liquid at the far bubble wall, generating a blast wave.[21] These effects cause damage and erosion to solids near the bubble.

The LAWS introduced in this paper utilises a different form of bubble wall motion. Acoustic fields of much lower amplitudes cause small bubble pulsations. It was discovered that, if tuned correctly, the sound field stimulates ripples on the bubble wall that can be detected by the scattering of a second sound field, of much higher frequency, from the bubble.[22] Such bubble wall ripples excited by this device as they sit on a tissue surface can be visualised microscopically (Figure 1). These ripples cause liquid microcirculatory currents close to the bubble wall[23] and shear forces on solid surfaces near the bubble, and these can be delivered onto a target down a water stream if the sound can be made to propagate down the stream[19] and if, at the target, it can generate the conditions needed to excite these waves.[24,25]

Earlier studies found that by delivering shear and liquid microcirculatory currents at the end of a liquid stream, they could remove a range of contaminants from solid surfaces, including brain tissue from surgical steel,[26] bacteria from hay,[26,27] and marine biofilms from hull material.[28] It was noted that, if the surface is structured, contoured or contains crevices from which contaminant is difficult to remove and hence difficult to clean, acoustic forces will drive the rippling bubbles into pores to enhance cleaning.[29] This was demonstrated, for example, by the removal of dental biofilms[30] from model teeth.

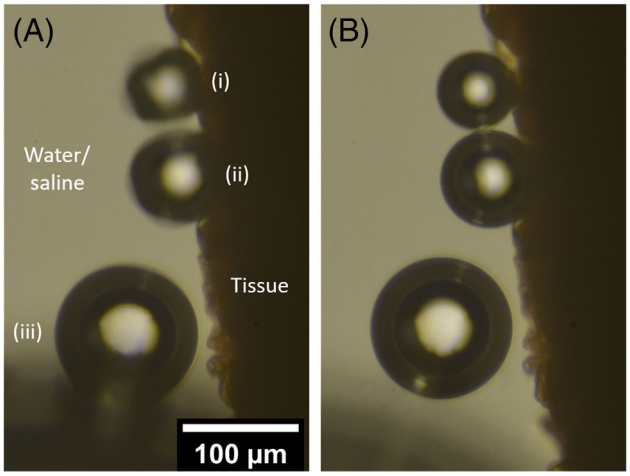

FIGURE 1 Ultrasonically induced bubble pulsation. Example brightfield micrographs demonstrating bubbles (A) excited by the 132 kHz field to a pulsation motion that is too small to discern on this scale, three bubbles oscillate in a sound field next to a tissue surface. Bubbles (i) and (ii) exhibit very different forms of surface waves: bubble (i) has waves that are larger wavelength and larger amplitude than the surface waves on bubble (ii). This is because the size of the bubble is a determining factor for what waves are excited on a given bubble (as predicted by theory; Maksimov and Leighton[24]). The surface motion on bubble (iii) is barely discernible on this scale. The sound field is switched off 125 ms later (B), and the consequent cessation of surface waves shows a clear difference—that is, the loss of the surface waves seen in (A) makes the now-stationary bubble wall clear and free of the motion seen in (A). In each frame the exposure time was 0.25 ms intergrating over approximately 16 frames of the surface wave motion, causing the bubble wall in (A) to blur.

The ability of the LAWS device to clean soft substrates safely was illustrated by its use to clean salad leaves without damaging the leaves.[31]

Removal of the biofilm without damage to the underlying wound bed might improve chronic wound healing and offer advantages for health care. This paper describes a proof-of-concept study to investigate whether a LAWS, based on the principles set out above, could safely remove a bacterial biofilm from a soft, complex biological surface in a variety of wound models.

2 | METHODS

Full details of the methods used are given in Supplementary Methods and Materials.

2.1 | Wound cleaning device

The details regarding the operation of the LAWS device have been published previously[20,26,31] and are detailed in

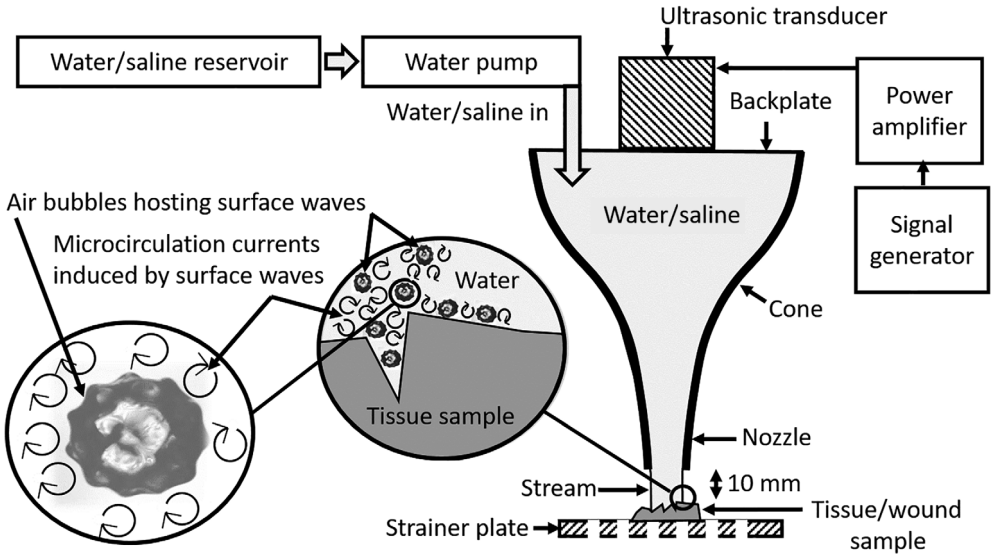

FIGURE 2 Schematic of liquid acoustic wound stream (LAWS) system. Schematic diagram of the experimental set up for the LAWS system cleaning a tissue or wound sample. The two inserts demonstrate the ultrasonically induced activity of the air bubbles that is associated with the cleaning effects of the LAWS. Diagram is adapted from Malakoutikhah et al 2020[20] and Chong et al 2021[31]

Figure 2. Full details can be found in Supplementary Methods and Materials. In summary, the device is a hand-held nozzle through which liquid passes at 2 L/min and a wave generator that supplies a signal at 132 kHz to the ultrasonic transducer. Acoustic pressure amplitudes below the Blake Threshold (the minimum condition that must be exceeded to generate inertial cavitation) were generated in the water stream at the location of the tissue. In these experiments, either 0.9% saline or plain water was passed through the device at a rate of 2 L/min at room temperature and delivered with the nozzle tip held at 1 cm from the wound.

2.2 | Bacterial cultures

Pseudomonas aeruginosa PA01 (pMF230) and a community-associated methicillin-resistant *Staphylococcus aureus* (USA 300) from laboratory stock, both expressing green fluorescent protein (GFP), were used to infect the wound models. Full details are given in Supplementary Materials.

2.3 | Wound models

For the first model, whole pig's trotters were sourced from the local butcher and rough wounds, about 20 mm in diameter, were made through the epidermal and dermal layer with a sterile scalpel.

Pre-wounded reconstituted human epithelial cultures (EpiDerm Full Thickness, MatTek Corp., Ashland, Massachusetts) were used in the second series of experiments. The cultures are derived from human neonatal foreskin tissue to form a multi-layered highly differentiated model of human skin and contains both keratinocytes and fibroblasts.

Finally, an ex vivo pig skin explant wound model was prepared using the method of Yang et al.[32] The preparation was modified and full details of the modification, together with details of the other wound models, are given in Supplementary Material.

2.4 | Wound treatments

Wound models were either washed with plain water or 0.9% saline through the LAWS device either with the sound turned on or off at a rate of 2 L/min as described in the results. Control wound models were untreated.

2.5 | Analysis

Photomicrographs of the wound bed and surrounding tissues before and after treatment were obtained in situ using episcopic differential interference contrast (EDIC) microscopy coupled with epifluorescence. The images obtained were analysed using ImageJ software (National Institutes for Health, USA) to detect the percentage coverage of residual GFP positive bacteria/biofilm.

Sections of the EpiDerm FT models were processed, sectioned, and stained with Haematoxylin & Eosin (H&E) to examine the anatomical structure. Further sections were stained for the presence cytokeratin 14.

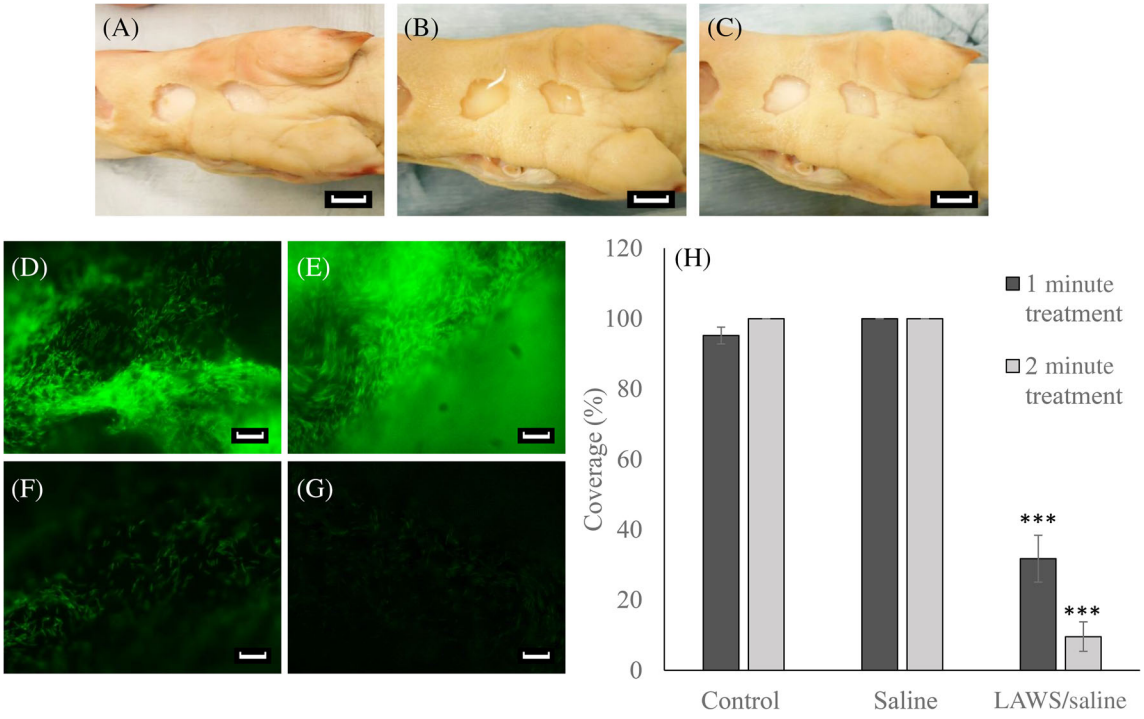

FIGURE 3 Pig trotter wound model. Example images of (A) ~2 cm diameter wounds produced within frozen/thawed pig trotters before inoculation, (B) post inoculation of *Pseudomonas aeruginosa* pMF230 incubated at 37°C for 5 hours, and (C) post 2 min liquid acoustic wound stream (LAWS) treatment. Scale bars represent 2 cm. Representative episcopic differential interference contrast/EpiFluorescence (EDIC/EF) micrographs of green fluorescent protein (GFP)-tagged *P. aeruginosa* biofilms in (D) the control (untreated wounds), (E) after a 1 minute saline wash at a flow rate of 2 L/min, (F) after a 1 minute LAWS treatment at a flow rate of 2 L/min, and (G) after a 2 minutes LAWS treatment at a flow rate of 2 L/min. Scale bars represent 10 μm. Image analysis (H) of the EDIC/EF micrographs demonstrating the residual percentage coverage of GFP tagged *P. aeruginosa* pMF230 within the pig trotter wounds after 5 hour incubation at 37°C: control (untreated wounds), after a 1 or 2 minutes saline wash at a flow rate of 2 L/min (saline) and after a 1 or 2 minutes LAWS treatment at a flow rate of 2 L/min (LAWS/saline). Error bars represent the SEM (n = 3), One-Way analysis of variance/Tukey post-hoc test demonstrated ***$P \leq .001$ when compared with the untreated controls

3 | RESULTS

3.1 | Cleaning

3.1.1 | Pig's trotter wound model

A sample of *P. aeruginosa* was cultured on the wound bed for 5 hours. Compared with the unwashed control wound beds, washing with saline alone had no significant effect on the residual coverage of GFP tagged bacteria in the model. Washing with LAWS for 1 minute reduced the coverage by 73% and washing for 2 minutes resulted in a 90% reduction as shown in Figure 3.

3.1.2 | EpiDerm FT wound model

A 100 μL aliquot of *P. aeruginosa* was inoculated into the wound bed and the model cultured for 24 hours at 37°C and 5% CO_2 to produce an early-stage biofilm. The micrographs in Figure 4 show the effect of washing the Epiderm FT model with either saline or a LAWS. Control sections were not washed. The wash with saline alone appears to have spread the bacteria more evenly across the wound model but without removing a significant quantity of bacteria. The reduction achieved after 2 minutes of washing with a LAWS is statistically significant with $P \leq .001$.

3.1.3 | Pig skin explant wound model

Explants were either inoculated with *P. aeruginosa* or *S. aureus* and cultured for 3 days at 37°C and 5% CO_2 with daily change of media and filter. After 3 days, each wound sample was imaged, washed for 1 minute with LAWS/water and re-imaged. The results are shown in Figure 5. The reduction in coverage with *P. aeruginosa*

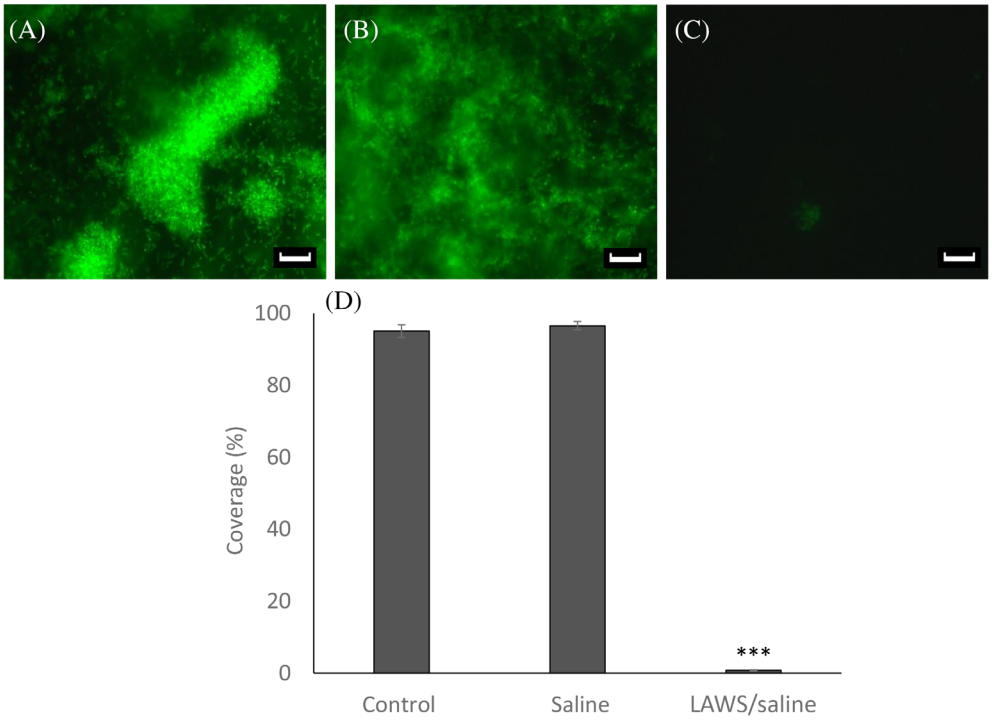

FIGURE 4 Epiderm full thickness (EFT) wound models. Representative episcopic differential interference contrast (EDIC)/EF micrographs of green fluorescent protein (GFP)-tagged *Pseudomonas aeruginosa* pMF230 biofilm within the EFT wound models: (A) with no treatment, (B) after a 2 minutes saline wash at a flow rate of 2 L/min, and (C) after a 2 minutes liquid acoustic wound stream (LAWS)/saline treatment at a flow rate of 2 L/min. Scale bars represent 10 μm. Image analysis (D) of the EDIC/EF micrographs demonstrating the percentage coverage of GFP tagged *P. aeruginosa* pMF230 biofilm within the EFT wound models after 24-hour incubation at 37°C. Error bars represent the SEM (n = 3), One-Way analysis of variance/Tukey post-hoc test demonstrated ***$P \leq .001$ when compared with the non-treated controls

after washing was statistically significant with $P \leq .001$. The reduction in coverage with *S. aureus* after washing was also statistically significant with $P \leq .01$.

3.2 | Healing

3.2.1 | EpiDerm FT wound model

Examination of the H&E-stained sections of uninfected wound models allows measurement of the length of the tongue of reepithelialisation (Figure 6). The EpiDerm FT wound model is known to heal with the addition of human serum, and measurement of the length of the tongue is a method of quantifying healing in this model. There was no significant difference in tongue length between the control (no wash) and saline wash samples but the difference between the LAWS saline treated models and the controls is significant ($P \leq .05$).

Immunofluorescent staining for cytokeratin 14 (Figure 7) further confirmed the significant increase in the rate of reepithelisation observed in the LAWS treated wound models when compared with the control and saline treated samples.

4 | DISCUSSION

The fundamentals of the LAWS device have previously been shown to remove bacterial biofilm from hard surfaces.[20,27,28,30] However, soft, biological surfaces offer very different and inconstant acoustic parameters. Variable surface transmission and reflection combined with varying tissue impedance inevitably alter the acoustic environment. These proof-of-concept studies were undertaken to investigate the potential for LAWS treatment to remove biofilm from wound models.

The centrality of the presence of biofilm in chronic wounds as the cause of failure to heal was formally recognised in 2016[10] and is supported by continuing research. Removal of the biofilm is a primary objective of chronic wound management. Although substances such as honey[33] and EDTA[34] have been reported to impair biofilm maintenance, debridement or vigorous cleaning is

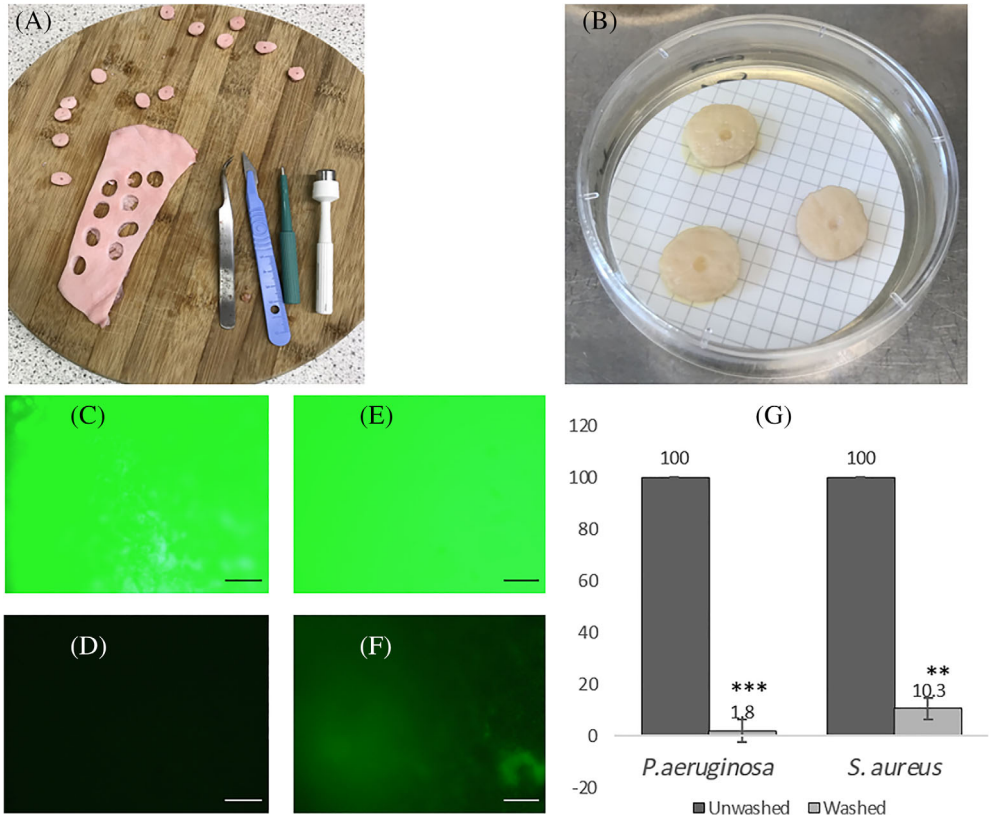

FIGURE 5 Porcine skin explant wound models. Example images of (A) creating pig skin explant wound model and (B) three wound models prepared for culture. Representative episcopic differential interference contrast (EDIC)/EF micrographs of 3-day old green fluorescent protein (GFP)-tagged *Pseudomonas aeruginosa* pMF230 (C and D) and s-GFP tagged *Staphylococcus aureus* (E and F) wound models before and after washing for 1 minute with plain water through the liquid acoustic wound stream (LAWS) device at a flow rate of 2 L/min. Scale bars represent 10 μm. Chart (G) shows percentage coverage of the biofilm, and the error bars represent the SEM (n = 3). One-Way analysis of variance test demonstrated $^{**}P \leq .01$ and $^{***}P \leq .001$ reduction after treatment

the current gold standard in the management of chronic wounds,[10] even with the limitations regarding healthy tissue damage and cost as discussed previously. Surgical or sharp debridement is an effective way to remove biofilm, but there is limited evidence for other types of biofilm removal techniques.[35] However, physical debridement does not remove all the biofilm and regrowth of biofilm occurs readily, requiring repeat treatment. The concept of continuing management of wound biofilm as an individual component of wound care is gaining traction.

In this study, we have shown that a gentle stream of aqueous liquid containing bubbles activated by sound can safely remove laboratory grown biofilm of between 5 hours and 3 days maturity from three types of wound models. No biocidal substances were added to the water stream and room temperature liquid was used in the device. This gives the device low running costs and permits use in remote locations requiring only power and a supply of water.

The evolution of a biofilm is well described. In a review of wound biofilm management,[36] it was noted that bacteria attach to surfaces within a matter of minutes and microcolonies are formed within 2-4 hours. Exocellular polymeric substance is secreted by 6 hours, providing enhanced protection to the bacteria. Continuing development, including increasing tolerance to biocides, leads to fully mature biofilm in 2-4 days. For these proof-of-concept experiments, biofilm of a few hours, 1 day and 3 days maturity were used to cover a range of biofilm maturities with these wound models.

P. aeruginosa biofilm was efficiently removed from the pig's trotter model (Figure 3) but the substrate is dead tissue. The pig's trotters were initially used to determine the efficacy of the LAWS device at removing biofilm from a wound model with all the soft tissue and bone in place, as the composition of the substrate requiring cleaning might affect the acoustical propagation of the LAWS devices. Although this was not an established wound model, the novelty of the LAWS device to acoustically

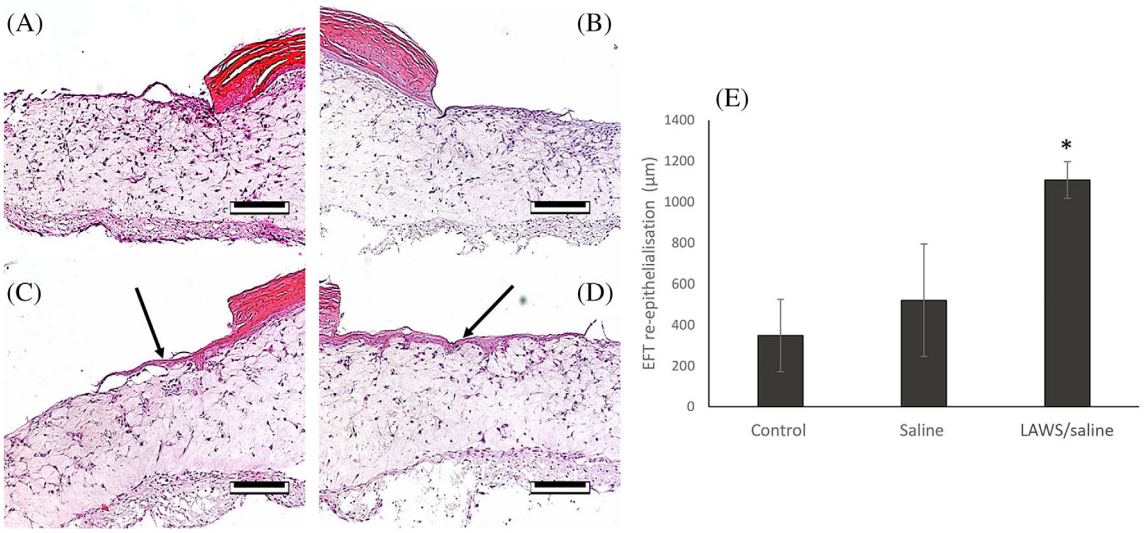

FIGURE 6 Effects of UAS on uninfected wounds. Representative bright-field micrographs of Haematoxylin and Eosin stained sections of epiderm full thickness skin tissue, 7 days after: (A) no treatment (control), (B) a 2 minutes saline wash at 2 L/min (saline), and (C and D) two examples post 2 minutes liquid acoustic wound stream (LAWS) treatment (LAWS/saline). The black arrows in micrographs C and D highlight the re-epithelialisation tongue observed in these sections. Scale bars represent 500 µm. Image analysis measurements (E) of the extent of reepithelialisation 7 days post treatment are shown. Error bars represent the SEM (n = 3), One-Way analysis of variance/Tukey post hoc test demonstrated *$P \leq .05$ when compared with the non-treated controls

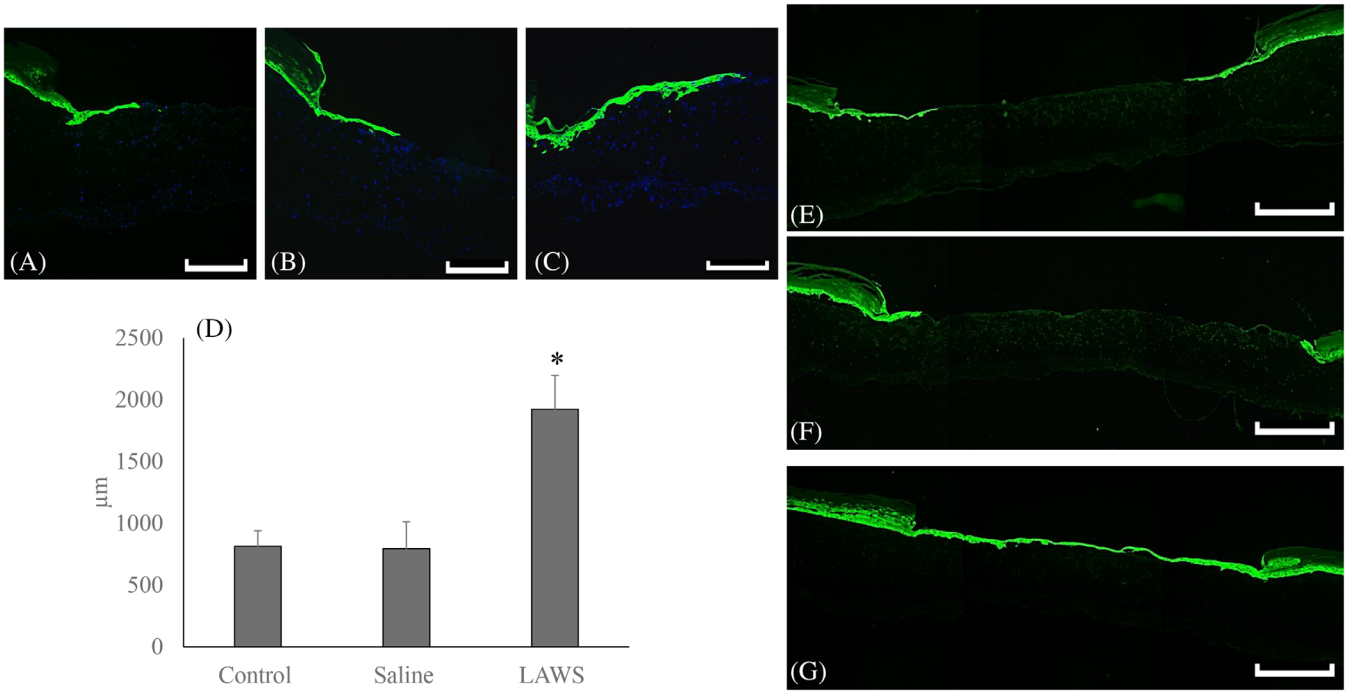

FIGURE 7 Cytokeratin-14 immunofluorescent staining of epiderm full thickness (EFT) sections. Representative epi-fluorescent micrographs of CK-14 immunofluorescent labelled EFT sections taken 7 days after: (A) no treatment (control), (B) 2 minutes saline wash at 2 L/min (saline), and (C) post 2 minutes liquid acoustic wound stream (LAWS) treatment. The green fluorescence shows the CK-14 positive keratinocyte migrations across the wound. The samples were then counterstained with DAPI to highlight the cell nuclei. Micrographs E-G (CK-14 immunofluorescent labelled EFT sections taken 7 days after: no treatment, a 2 minutes saline wash at 2 L/min, and post 2 minutes LAWS treatment, respectively) show x/y scans of the whole wound area, highlighting the complete keratinocyte migration across the LAWS treated wound bed compared with the untreated and saline treated controls. Scale bars represents 500 µm. Image analysis measurements of the mean distance of keratinocyte (CK-14) migration (µm) 7 days post treatment are shown in "graph D." Error bars represent the SEM (n = 3), One-Way analysis of variance/Tukey post hoc test demonstrated *$P \leq .05$ when compared with the non-treated controls

clean a wound through a stream of water, required a novel wound model to determine any detriment to the acoustical cleaning effect, if such a detriment exists, caused by the underlying soft tissue and bone that would not be modelled within the established in vitro wound models used below.

The Epiderm Full Thickness (EpiDerm FT) model is a form of reconstructed human epidermis and consists of human keratinocytes and fibroblasts in a multilevel and differentiated model of human skin. It has had wide use in dermatological research including wound healing work to support the reduction of animal use in research.

The EFT models were cultured at 37°C/5% CO_2 and a 24-hour *P. aeruginosa* biofilm created in the wound bed. The biofilm was removed from the wound bed using LAWS technology whereas washing with simple saline had no significant effect on the bacteria as is shown in Figure 4. This confirms that LAWS was able to remove early-stage bacterial biofilm of a clinically relevant bacteria from a viable human wound model.

Finally, using an established, and published pig skin explant infected wound model,[32] mature biofilms of GFP expressing *P. aeruginosa* or *S. aureus* were grown for 3 days in the wound model. Treatment with LAWS again showed efficient removal of the biofilm confirming a cleaning action against both Gram-negative and Gram-positive mature biofilm bacteria in a published wound model.

We have shown removal of biofilms of two different bacterial species commonly found in chronic wounds and of a maturity of between 5 hours and 3 days in three different wound models. This suggests that a single LAWS treatment could have a beneficial effect on wound management. Future research will determine if there is any re-growth of residual bacteria within these models, and the efficacy of repeat LAWS treatment on wound management.

It has long been known that sound may have a positive effect on cell function[37] and the sound and forces generated could induce cell proliferation and tissue recovery. Keratinocytes do act as mechano-receptors leading to cellular migration and proliferation.[38]

One documented feature of the Epiderm Full Thickness (EpiDerm FT) model is that it will continue to heal after wounding if cultured suggesting functional integrity. Histological analysis of the EpiDermFT through H&E staining and immunofluorescent analysis of CytoKeratin14 has demonstrated equivalent morphology to normal human skin and both techniques allow differentiation and understand of the wound healing process.[39,40] Microscopic examination of sections of the model stained with H&E showed no damage to the structure of the skin and a review of H&E stained sections from Epiderm wound models that had not been infected but washed with either water or LAWS and subsequently cultured for 7 days showed that LAWS treated models continued to heal.

A review of the H&E-stained EFT sections and measurement of the tongue of reepithelialisation seen at the wound edge showed a significantly increased reepithelialisation after washing with LAWS when compared with no washing or washing with saline alone. Serial sections were examined for CytoKeratin14 by immunofluorescent staining as shown in Figure 7. This confirmed that the increased wound healing following LAWS treatment seen with H&E staining, suggesting increased keratinocyte migration/proliferation.

This proof-of-concept study has several limitations. First, the biofilms used were single species and a laboratory grown culture, although containing bacterial species commonly found in chronic wounds. Real life biofilms contain various species of bacteria, and other microorganisms, living in a cooperative community, the composition of which may vary over time. Primarily studies to determine efficacy of wound treatments use single species or polymicrobial biofilms on abiotic surfaces using CDC bioreactors or agar culture.[41-43] However, the formation and morphology of biofilms on abiotic surfaces does not replicate those formed on tissue relevant wound-based models. Wound based models such as the porcine explant mode are currently developed only to study single species biofilms.[32,44] Therefore, the use of single species biofilms was deemed acceptable for this proof-of-concept study looking at the simple question of whether a liquid acoustic stream can remove biofilm from a soft, complex surface such as a wound bed.

A further limitation is inherent in the use of wounds models. The first model used, a pig's trotter, was intended to simulate a complex biological surface, inclusive of all the soft tissue and bone as previously described. Most of the previous work using the LAWS had been conducted with hard contaminants on a hard surface. Some work had been undertaken removing marine biofilm on a ship's hull[28] or bacterial biofilm from a tooth model[30] although again these experiments involved biofilm on abiotic hard surfaces. The pig's trotter model consisted of dead tissue and the duration of the culture was limited by disintegration.

The pre-wounded, human reconstituted epithelium (HRE) skin model Epiderm FT and the pig skin explant model, both established wound models, are more representative of a wound as they remained alive. However, both models represent an acute rather than a chronic wound and lacked a functioning circulatory and immune system. As the objective of this study was to investigate the removal of a biofilm from a soft biological surface this limitation is minor.

The use of a liquid acoustic stream may have a number of consequences for the bacteria exposed to the stream. It is believed that bacteria may be killed, some may be rendered viable but not culturable (VBNC) and others may be left alive (Dr C Highmore, University of Southampton, personal communication). The use of a standard method such as serial dilution and determining the residual colony forming units (CFU) post treatment would not therefore be a suitable means of quantifying the removal of biofilm from the wound models without the possibility of under detecting viable bacterial that would not be cultured in the VBNC state.

Fluorescent imaging, a technique used for visualising both in vitro and clinical biofilms,[43,45] was used in this study to detect all bacteria (regardless of VBNC state) within the models used, thus overcoming the aforementioned limitations of CFU counts. The bacteria used for these studies express GFP. Image analysis of an epifluorescent photomicrograph does not provide a precise measure of the number of bacteria remaining on the wound models after cleaning. However, it does provide a measure of the abundance of bacteria in the wound which is likely to be consistent between wounds undergoing different treatments. It is an effective measure of the efficacy of the removal of the GFP-tagged biofilm in a short-term proof-of-concept experiment such as those reported here.

Published studies into the effect of treatments on wound healing tend to rely on animal-based wound models.[42,43] However, in this proof-of-concept study it would not have been feasible to include animal studies within the cost or timeframe. Alternatively, we used the human relevant EFT tissues as non-animal replacement to study wound healing.[39,40]

The use of LAWS treatment could reduce the use of antiseptics or antibiotics required to control the bacteria, as the bacteria are efficiently removed from the wound. Reduction in the use of antibiotics has its own benefits in terms of antibiotic stewardship. This is of particular importance in the management of chronic wounds where over prescription of antibiotics is well recognised.[4] Unlike sharp debridement, there is no requirement for a skilled operator and irrigation with a stream of water may be more acceptable to the patient.

Evidence from the histological examination of treated, uninfected HRE samples shows no evidence of damage to underlying tissues suggesting that the use of a LAWS will be safe for the patients. Use of the device would fit easily into the initial wound cleaning stage of usual wound care and only require a minor change to the current cleaning process.

This study has proven the concept that LAWS will reduce mature biofilm load from soft, biological surfaces, such as wound models. We suggest that this simple technique may improve wound care. The cleaning of wounds by removing biofilm is currently the central plank of wound care and is known to lead to improvements in healing. This device offers an easier method of wound cleaning at lower cost and not requiring a skilled operator. Further studies are currently being undertaken to quantify biofilm removal (both via fluorescent and CFU analysis) of polymicrobial biofilms comparing LAWS to alternative wound treatments designed to gently remove biofilms. Important future work would also include studies of the formation, structure and relevant maturity of the biofilms being tested within the models used. The data reported here and from future studies, combined with human volunteer data not reported here, will be used to demonstrate the removal of "real world" biofilm from clinically significant chronic wounds.

CONFLICT OF INTEREST

T.G.L. is Executive General Director and Inventor-in-Chief of the company (Sloan Water Technology, Ltd.) that holds the patent to this technology and C.C.H. was used part-time by the company but took no salary. T.J.S. was employed by the University of Southampton during this work, but since its completion has joined the staff of Sloan Water Technology, Ltd.

AUTHOR CONTRIBUTIONS

Thomas J. Secker carried out laboratory experimentation, data analysis, interpretation, participated in the design of the study, and drafted the manuscript; Christopher C. Harling carried out laboratory experimentation, data analysis, interpretation, participated in the design of the study, and drafted the manuscript; Chloe Hand carried out laboratory experimentation and data analysis; David Voegeli conceived the study, participated in the design of the study, aided the laboratory experimentation, data analysis and interpretation, and edited the manuscript; Charles W. Keevil oversaw the microbiological components and helped edit the manuscript; Timothy G. Leighton conceived the study, participated in the design of the study, coordinated across disciplines, and helped draft the manuscript. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

The datasets generated and analysed during this study will be openly available from the University of Southampton repository at https://doi.org/10.5258/SOTON/D2193.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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