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ORIGINAL ARTICLE

Inhibition of biofilms by glucose oxidase, lactoperoxidase and guaiacol: the active antibacterial component in an enzyme alginogel

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Key words
Flaminal; Glucose oxidase; Guaiacol; Lactoperoxidase; Wounds

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Abstract

The association of biofilms with wound chronicity has prompted a search for antimicrobial interventions that are effective against biofilms. A patented preparation of glucose oxidase, lactoperoxidase and guaiacol (GLG), which is the antibacterial component of Flaminal®, has been shown to inhibit a wide range of bacteria, but it has not yet been tested on biofilms. This study aims to determine the effect of GLG on biofilms of *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa*. Static biofilms were grown in microtitre plates and on coverslips and treated with a range of concentrations of GLG. Effects were monitored by estimating biofilm biomass by staining with crystal violet, biofilm activity by staining with either resazurin or fluorescein diacetate and biofilm viability by staining with LIVE/DEAD® BacLight™ Bacterial Viability Kit. GLG was able to prevent the formation of biofilms at concentration ≤0·5% (w/v) and higher concentrations were required to inhibit established biofilms. GLG did not disrupt biofilm biomass. Staphylococci were more susceptible to GLG than *P. aeruginosa*. These in vitro findings must be verified by in vivo studies.

Introduction

The first member of a new class of antimicrobial dressings known as enzyme alginogels is Flaminal® (Flen Pharma, Kontich, Belgium). It is essentially a hydrogel that contains a patented GLG enzyme system comprised of two enzymes (glucose oxidase and lactoperoxidase) stabilised by an aromatic oil (guaiacol). In the presence of glucose and iodine, these enzymes produce free radicals via hydrogen peroxide, thiocyanite and hypoiodite, which exert widespread oxidative damage to bacterial cells. Clinical evidence has demonstrated that the enzyme alginogel facilitated healing in chronic leg ulcers (1), burns (2) and chronic and acute wounds of diverse aetiologies (3) and indicated that it maintained a moist wound environment in which continuous debridement was achieved without deleterious effects on wound edges or epithelial cells. Its antimicrobial effect on bacteria capable of causing wound infections has been demonstrated with a range of planktonic cultures in vitro (4,5).

With the discovery that many wounds fail to heal because of the presence of a biofilm (6,7) and the demonstration that microorganisms within biofilms are recalcitrant to antimicrobial agents (8), it has become imperative to determine whether antimicrobial wound care devices are able to inhibit

Key Messages

- concentration of GLG ≤ 0·5% (w/v) prevented the formation of biofilms of *Staphylococcus aureus*, MRSA and *Pseudomonas aeruginosa* in vitro
- *S. aureus* and MRSA were more susceptible to GLG than *P. aeruginosa*
- GLG did not remove biofilm biomass from plastic or glass surfaces
- in vitro tests to evaluate the effects of antimicrobial agents on biofilms should monitor biofilm activity and viability as well as levels of biofilm biomass

biofilms. This study was designed to investigate the ability of GLG enzyme system to prevent the formation of biofilms of representative wound pathogens, as well as to inhibit established biofilms in vitro.

Methods

Test organisms

Cultures of methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus* and *Pseudomonas aeruginosa* were used throughout this study. These bacteria had been isolated from chronic leg ulcers of outpatients attending a local tertiary referral wound care clinic at University Hospital of Wales and stored at −80°C until used. For each experiment, an overnight culture of test organism was cultivated in tryptone soya broth (TSB; Oxoid, Cambridge, UK) at 37°C. Immediately before use each culture was diluted 1:5 in fresh TSB (equivalent to $1{\cdot}6 \times 10^7$ cfu/ml).

Enzyme preparation

An enzyme solution contained 1033 U glucose oxidase and 2680 U lactoperoxidase. Stock substrate solution was prepared by dissolving 50 g glucose and 1·4 g potassium iodide in 48·6 ml deionised water, filter sterilising through 0·22-μm disposable filters into sterile Eppendorf tubes and storing at room temperature. Stock guaiacol solution was prepared by dissolving 124·13 mg guaiacol in 10 ml deionised water, filter sterilising as above into glass bottles and storing for no longer than 4 weeks at 4°C in the dark. Immediately before each experiment, a 4% (w/v) GLG solution was prepared by adding 200 μl stock substrate solution, 100 μl stock guaiacol solution and 10 μl enzyme solution to either 4690 μl 1:5 diluted inoculum or 4690 μl sterile growth medium depending on whether the prevention of biofilm formation or inhibition of established biofilm was being tested, respectively. The working solution was immediately used to prepare doubling dilutions ranging from 4 to 0·0044% (w/v) with either sterile growth medium or 1:5 diluted inoculum depending on whether the prevention of biofilm formation or inhibition of established biofilm was being tested, respectively.

The effect of GLG in the prevention of biofilm formation

To determine the lowest concentration of GLG to prevent biofilm formation, the working solution prepared above using 1:5 inoculum was immediately used to prepare a doubling dilution series ranging from 4 to 0·0044% (w/v) with 1:5 diluted inoculum. Into each of two 96-well microtitre plates, 50 μl aliquots were dispensed into wells as follows: eight wells in column 1 received 2% (w/v) GLG, eight wells in column 2 received 1% (w/v) GLG, eight wells in column 3 received 0·5% (w/v) GLG, eight wells in column 4 received 0·25% (w/v) GLG, eight wells in column 5 received 0·125% (w/v) GLG, eight wells in column 6 received 0·0625% (w/v) GLG, eight wells in column 7 received 0·0312% (w/v) GLG, eight wells in column 8 received 0·0156% (w/v) GLG, eight wells in column 9 received 0·0088% (w/v) GLG and eight wells in column 10 received 0·0044% (w/v) GLG. Into wells in column 11 was dispensed 50 μl 1:5 inoculum (positive control) and into wells in column 12 was dispensed 50 μl sterile TSB (negative control). Plates were incubated aerobically at 37°C for 24 hours, and one plate was used to estimate biofilm biomass using crystal violet staining and the other was used to estimate biofilm activity using fluorescent staining.

Cultivation of established biofilms

Overnight cultures of test bacteria incubated at 37°C in TSB (were diluted 1:5 in fresh TSB and 50 μl (equivalent to $1{\cdot}6 \times 10^7$ cfu/ml) was inoculated into wells of 96-well, flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) and incubated aerobically without shaking at 37°C for 24 hours. Eight wells in each plate contained 50 μl TSB as a negative control. When 48-hour established biofilms were required, the liquid phase of wells was carefully removed from 24-hour established biofilms and gently replaced with 50 μl TSB to replenish nutrients. Plates were then incubated at 37°C for 24 hours (total incubation period was 48 hours). Similarly, for 72-hour established biofilms, TSB was replenished in 24-hour established biofilms as above and repeated again after 48 hours, and plates were incubated at 37°C for another 24 hours (72 hours in total). For microscopic evaluation, 24-, 48- and 72-hour biofilms were also cultivated as described above using glass coverslips placed into the wells of a 24-well microtitre plate (Nunc). The initial inoculum was 300 μl overnight culture diluted 1:5 in TSB and biofilms were fed at 24-hour intervals with fresh TSB. All experiments were performed on at least two occasions.

The effect of GLG on established biofilm

The effect of GLG in inhibiting established biofilms was investigated on biofilms that had been established in wells for 24, 48 or 72 hours at 37°C. From the 4% (w/v) stock solution of GLG, a serial doubling dilution range between 0·004 and 2% (w/v) was prepared in sterile TSB immediately before use. Then the liquid phase of each well in the microtitre plates containing established biofilm was carefully removed without disturbing adherent cells and discarded. Remaining biofilm was gently washed with 50 μl sterile phosphate-buffered saline (PBS; Oxoid), replaced with 50 μl aliquots of a range of concentrations of GLG diluted in TSB and incubated at 37°C for 24 hours. In each plate, eight wells were used with TSB alone (negative control) and eight wells with inoculated TSB but not GLG (positive control). In some plates, wells containing GLG without guaiacol and test organism were also set-up to investigate the effect of guaiacol. Each GLG dilution was tested with either four or eight replicates per plate and all experiments were performed on at least two occasions.

Evaluation of biofilm biomass

To estimate biofilm biomass, the liquid phase was carefully removed from each well and discarded, and adherent biofilm

was gently washed with 100 µl PBS. For *S. aureus* and MRSA, but not for *P. aeruginosa*, cells were fixed by adding 100 µl 99% methanol to each well for 15 minutes. Adherent biofilm was then stained with 50 µl 0·25% (w/v) crystal violet for 15 minutes. Soluble crystal violet was removed and biofilm was washed twice more with PBS; biofilm-bound crystal violet was solubilised using 100 µl acetic acid [7% (w/v)] and absorbance was measured at 570 nm using a Spectrostar nano plate reader (BMG Labtech, Buckinghamshire, UK) (9). Minimum inhibitory concentration (MIC_{90}) was determined as the concentration of GLG that reduced biofilm biomass by at least 90% compared with untreated controls and MIC_{50} the concentration that reduced biomass by 50%.

Evaluation of biofilm activity

Metabolic activity in biofilms of *S. aureus* and MRSA was evaluated by the reduction of blue-coloured resazurin to pink-coloured fluorescent resorufin, and the esterase activity in biofilms of *P. aeruginosa* was estimated by the conversion of non fluorescent fluorescein diacetate (FDA) to yellow fluorescent fluorescein (10). For staphylococci, wells were washed once with 100 µl PBS, and then 100 µl PBS and 5 µl resazurin (Promega, Southampton, UK) were added to each well and the plates were incubated in the dark at 37°C for 60 minutes. Fluorescence was measured at excitation wavelength of 560 nm and emission wavelength of 590 nm by Tecan Infinite (Reading, UK). For *P. aeruginosa*, a stock solution of 10 mg/ml FDA was dissolved in acetone, dispensed into Eppendorf tubes and stored at −20°C until required; at the time of requirement it was diluted 1:50 in MOPS buffer pH 7·00 freshly before each assay (10). Liquid phase was gently removed from wells and adherent cells were washed with 100 µl MOPS. Then, 100 µl FDA working solution was added to each well, the plate was incubated at 37°C in the dark for 1 hour and fluorescence was measured at an excitation wavelength of 494 nm and emission wavelength of 518 nm by Tecan Infinite. MIC_{90} was determined as the concentration of GLG that reduced biofilm activity by at least 90% compared with untreated controls.

Evaluation of biofilm viability

Viability of biofilms was assessed using LIVE/DEAD BacLight™ Bacterial Viability Kits (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Essentially, coverslips were washed with 100 µl sterile deionised water, stained for at least 20 minutes with dye and washed with deionised water. Then, they were mounted onto glass slides and visualised by a Nikon Eclipse 80i fluorescent microscope with oil immersion and 100× lens. For detection of SYTO 9 (green channel), a 488-nm excitation and 520-nm emission filter was used. For propidium iodide detection (red channel), a 543-nm excitation and 572-nm emission filter was used. Uptake of SYTO 9 stained the cells green; propidium iodide was excluded from living cells and gained entry only into dead cells that stained red. Images were analysed using Volocity software (Perkin Elmer, Cambridge, UK).

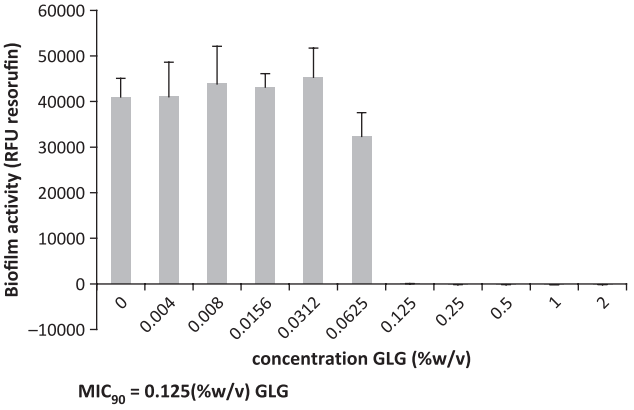

Figure 1 The effect of GLG (glucose oxidase, lactoperoxidase and guaiacol) in preventing the formation of *Staphylococcus aureus* biofilm determined by estimating metabolic activity with resazurin staining. Minimum inhibitory concentration (MIC_{90}) = 0·125% (w/v) GLG.

Table 1 Minimum concentrations of GLG that prevented the formation of biofilms

	Biofilm biomass		Biofilm activity
Bacterium tested	MIC_{90} (% w/v) GLG*	MIC_{50} (% w/v) GLG*	MIC_{90} (% w/v) GLG*
MRSA	ND	0·3	0·125
Staphylococcus aureus	ND	0·125	0·125
Pseudomonas aeruginosa	0·5	ND	0·5

GLG, glucose oxidase, lactoperoxidase and guaiacol; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; ND, not determined.
*Mean MICs were calculated from two experiments.

Results

The effect of GLG in preventing the formation of biofilms in microtitre plates

The concentrations of GLG needed to prevent the formation of biofilm of each of MRSA, *S. aureus* and *P. aeruginosa* in microtitre plates were determined using crystal violet to estimate biofilm biomass and resazurin or FDA to monitor biofilm metabolic activity. MIC_{90} for metabolic activity was readily detected visually and by measuring fluorescence (e.g. *S. aureus* in Figure 1) for all test organisms (Table 1). However, although staining the biomass of biofilms with crystal violet gave detectable endpoints for *P. aeruginosa* biofilms (Figure 2), clearly defined endpoints for MRSA and *S. aureus* (Figure 3) were not found. Hence, MIC_{50} rather than MIC_{90} was reported for these bacteria (Table 1). In many experiments where the concentration of GLG needed to prevent biofilm was investigated, reduced levels of biomass were detected at GLG concentrations above the MIC (Figure 3). This suggested that biofilm had started to accumulate before an inhibitory effect was achieved, and although the biofilm showed no detectable metabolic activity (Figure 1), it had been neither disrupted nor dispersed by GLG.

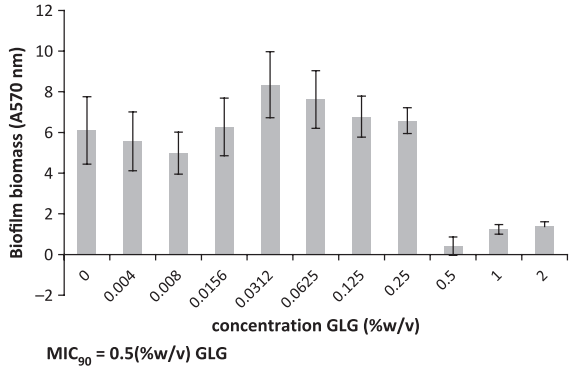

Figure 2 The effect of GLG (glucose oxidase, lactoperoxidase and guaiacol) in preventing the formation of *Pseudomonas aeruginosa* biofilm determined by estimating biomass by staining with crystal violet. Minimum inhibitory concentration (MIC_{90}) = 0·5% (w/v) GLG.

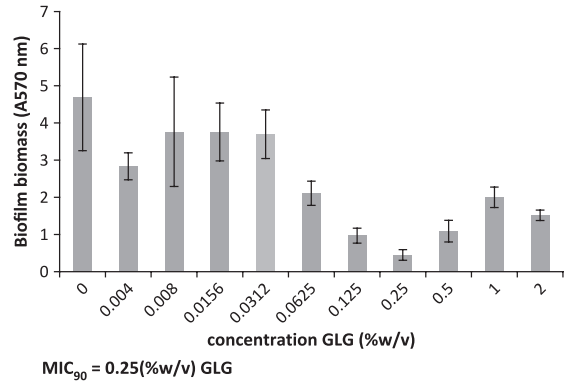

Figure 3 The effect of GLG (glucose oxidase, lactoperoxidase and guaiacol) in preventing the formation of *Staphylococcus aureus* biofilm determined by estimating biomass by crystal violet staining. Minimum inhibitory concentration (MIC_{90}) = 0·25% (w/v) GLG.

Table 2 MIC_{90} of GLG against established biofilms estimated by biofilm activity

	Biofilm biomass		
Bacterium tested	24-hour biofilm*	48-hour biofilm*	72-hour biofilm*
MRSA	0·8 ± 0·27 (5)	0·88 ± 0·25 (4)	1 (3)
Staphylococcus aureus	0·8 ± 0·27 (5)	0·88 ± 0·25 (4)	1·3 ± 0·6 (3)
Pseudomonas aeruginosa	1 (4)	1·3 ± 0·6 (3)	1·67 ± 0·6 (3)

GLG, glucose oxidase, lactoperoxidase and guaiacol; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*.
*Mean MIC_{90} ± standard deviation (number of assays).

The effect of GLG in inhibiting biofilms established in microtitre plates

A similar observation was made in investigating the effect of GLG on established biofilms. MIC_{90} values were obtained for all test organisms by measuring the biofilm activity (Table 2), but definitive endpoints were not found by staining with crystal violet for biomass. With *P. aeruginosa* decreased

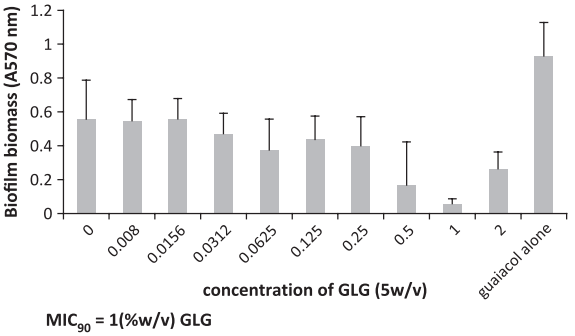

Figure 4 The effect of GLG (glucose oxidase, lactoperoxidase and guaiacol) on the biomass of 24-hour established *Pseudomonas aeruginosa* biofilm determined by crustal violet staining. Minimum inhibitory concentration (MIC_{90}) = 1% (w/v) GLG.

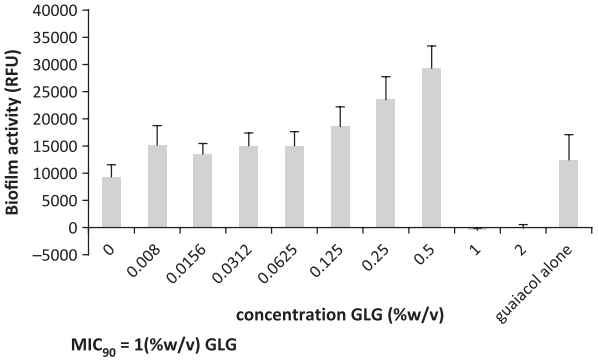

Figure 5 The effect of GLG (glucose oxidase, lactoperoxidase and guaiacol) on the metabolic activity of 24-hour established *Pseudomonas aeruginosa* biofilm determined by staining with fluorescein acetate. Minimum inhibitory concentration (MIC_{90}) = 1% (w/v) GLG.

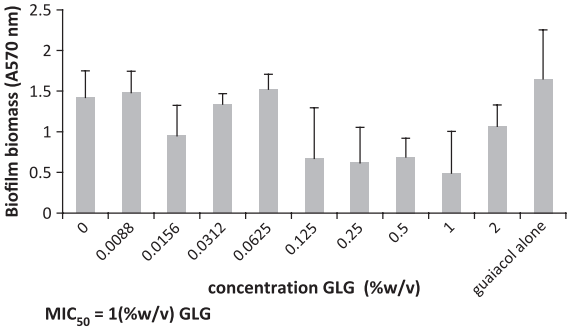

Figure 6 The effect of GLG (glucose oxidase, lactoperoxidase and guaiacol) on the biomass of 24-hour established *Staphylococcus aureus* biofilm determined by crystal violet staining. Minimum inhibitory concentration (MIC_{50}) = 1% (w/v) GLG.

levels of biomass (Figure 4) largely coincided with MICs deduced by FDA assays for biofilm activity (Figure 5), but for MRSA and *S. aureus* biofilm biomass persisted after exposure to even the highest concentrations of GLG tested here (Figure 6). Control wells containing guaiacol without glucose oxidase or lactoperoxidase showed no inhibitory

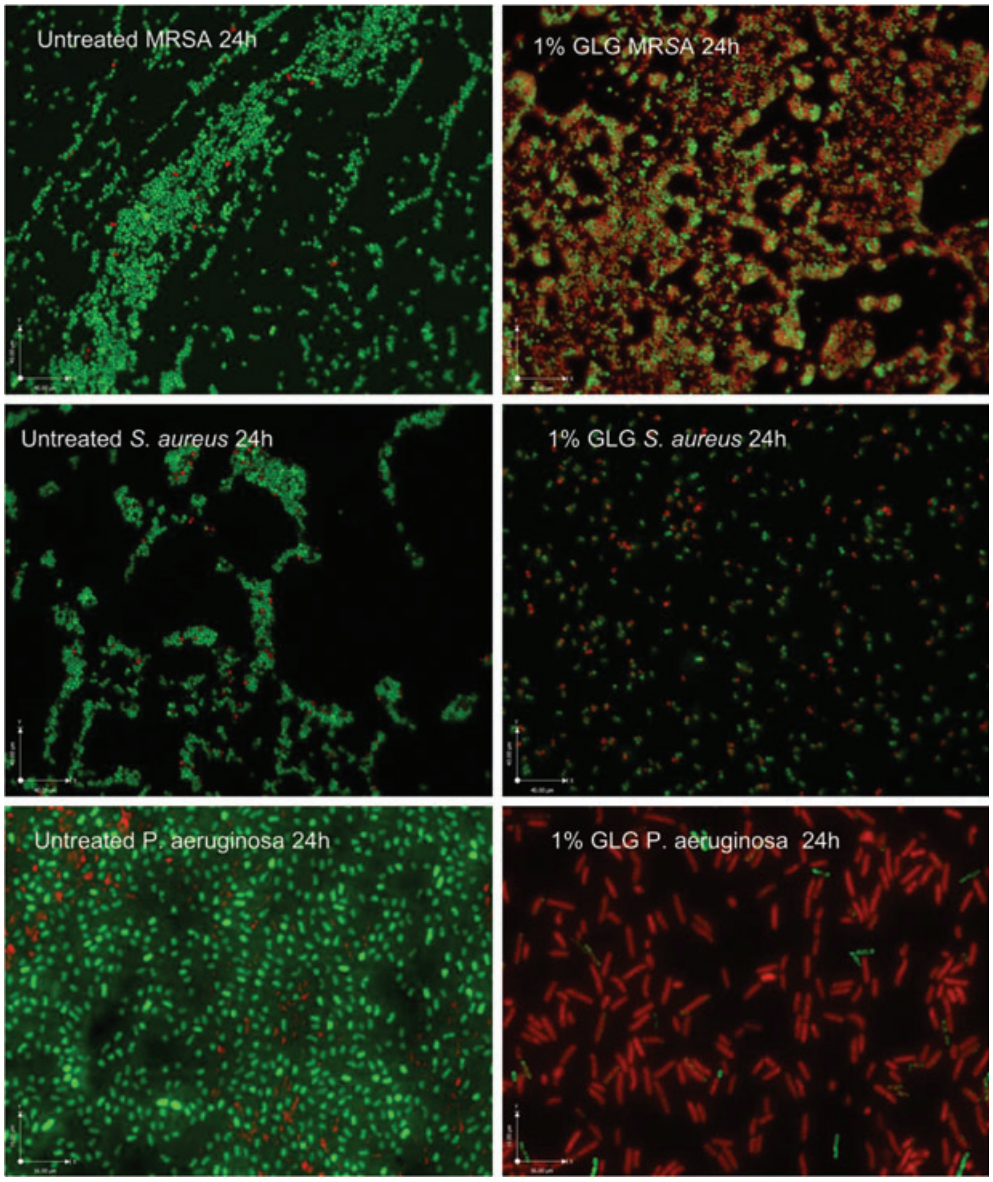

Figure 7 The effect of 1% (w/v) GLG (glucose oxidase, lactoperoxidase and guaiacol) for 24 hours at 37°C in preventing biofilm formation monitored with LIVE/DEAD® BacLight™.

effects on either biofilm activity or biomass (Figures 4–6). Biofilms established for 24, 48 and 72 hours demonstrated similar sensitivity to GLG (Table 2).

The effect of GLG on biofilms grown on coverslips

To determine the effect of GLG on the viability of bacterial cells within biofilms, a viability kit was used to stain live cells with fluorescent green dye and dead cells with red fluorescent dye. As mentioned earlier, the effect of 1% (w/v) GLG in preventing biofilm formation and in disrupting established biofilms after 24-hour incubation at 37°C was investigated. It was observed that GLG caused extensive cell death for all test organisms. Inoculating test bacteria onto coverslips with 1% (w/v) GLG prevented the formation of biofilm by *S. aureus* and *P. aeruginosa*, with single cells predominating. Although MRSA started to form a biofilm, most cells were dead at 24 hours (Figure 7).

When 1% (w/v) GLG was added to established biofilms, *S. aureus* (Figure 8) and *P. aeruginosa* (Figure 9) showed more extensive cell death than MRSA (Figure 10). The age of the *P. aeruginosa* biofilm did not seem to influence susceptibility to GLG, but younger staphylococcal biofilms were more susceptible to GLG than older ones.

Discussion

The role of lactoperoxidase in inhibiting bacteria is well established. This enzyme occurs in milk, tears, intestinal secretions and saliva where it reacts with hydrogen peroxide, iodide and thiocyanate to generate short-lived, weak oxidising

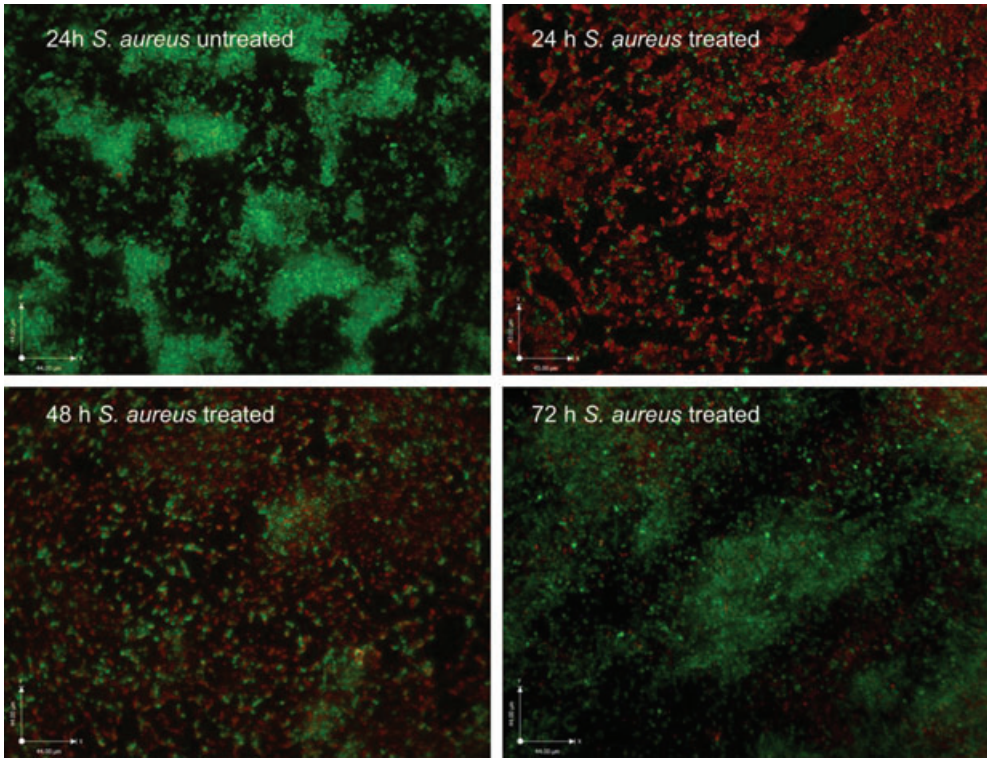

Figure 8 The effect of 1% (w/v) GLG (glucose oxidase, lactoperoxidase and guaiacol) for 24 hours at 37°C on viability of established *Staphylococcus aureus* biofilms monitored with LIVE/DEAD® BacLight™.

agents such as hypothiocyanite and hypoiodite to enhance human defence mechanisms against bacterial invasion across mucosal surfaces by *Escherichia coli*, oral streptococci and *Bacillus cereus* (11–13). These oxidising agents are able to penetrate bacterial cell walls and cause non specific oxidation of membranes and damage to DNA often by reacting with thiol groups. The glucose oxidase in GLG oxidises glucose to generate a ready source of hydrogen peroxide for lactoperoxidase. Guaiacol (otherwise known as 2-methoxy phenol) is an aromatic oil derived from the breakdown of woody plant material, which is thought to potentiate the activity of lactoperoxidase. Although it is not itself inhibitory (Figures 4–6) it acts as a universal substrate for peroxidases (14).

Inhibition by GLG of a diverse range of planktonic bacteria has already been demonstrated in vitro (6,7). In this study, the concentrations of GLG required to inhibit bacteria established in biofilms were higher than those required to inhibit planktonic bacteria in laboratory tests (6,7). This was not unusual (8). Several factors are known to contribute to the increased tolerance of biofilm communities to antimicrobial agents, such as diminished growth rates of cells in mature biofilms, restricted diffusion rates through biofilm matrix components and the presence of persister cells. Nevertheless, the formation of biofilms of three important wound pathogens was prevented by clinically achievable concentrations of GLG (Table 1) and concentrations of GLG two to ten times higher were able to inhibit established biofilms (Table 2). Marked differences in the susceptibility to GLG of 24- and 72-hour established biofilms were not evident (Table 2). Consistently, MRSA and *S. aureus* were more susceptible to GLG than *P. aeruginosa*, but all bacteria were inhibited in vitro by the concentrations of GLG used clinically in Flaminal. Monitoring the loss of viability of biofilms by staining with LIVE/DEAD BacLight and the loss of biofilm activity by fluorescent stains suggested a bactericidal mode of action of GLG and, therefore, confirmed the deductions of de Smet (5).

Crystal violet has long been used to quantify the biomass of biofilms that are adherent on the surfaces of plastic culture vessels (15) and a quantitative method was developed for evaluating staphylococcal biofilms (16). Reduction in biofilm biomass has been successfully monitored in biofilms treated with honey, suggesting disruption of established biofilm (17,18). However, in this study, it was found that although MIC values could be determined for each of the test bacteria treated with GLG using crystal violet, endpoints derived from staining biofilm biomass were not as distinct as those derived from estimations of biofilm activity using fluorescent stains. Hence, biofilm biomass was detected at concentrations of GLG above the MIC, indicating that it had not been entirely disrupted even though metabolic activity was at undetectable levels. A similar observation was made when glucose oxidase and lactoperoxidase were tested on biofilms grown on steel and polypropylene surfaces (19). In that study, the use of polysaccharide-hydrolysing enzymes to remove biofilm killed by glucose oxidase and lactoperoxidase was recommended. One inference is that using crystal violet to estimate the extent of a biofilm is limited to the detection of biomass without providing information on either its metabolic activity

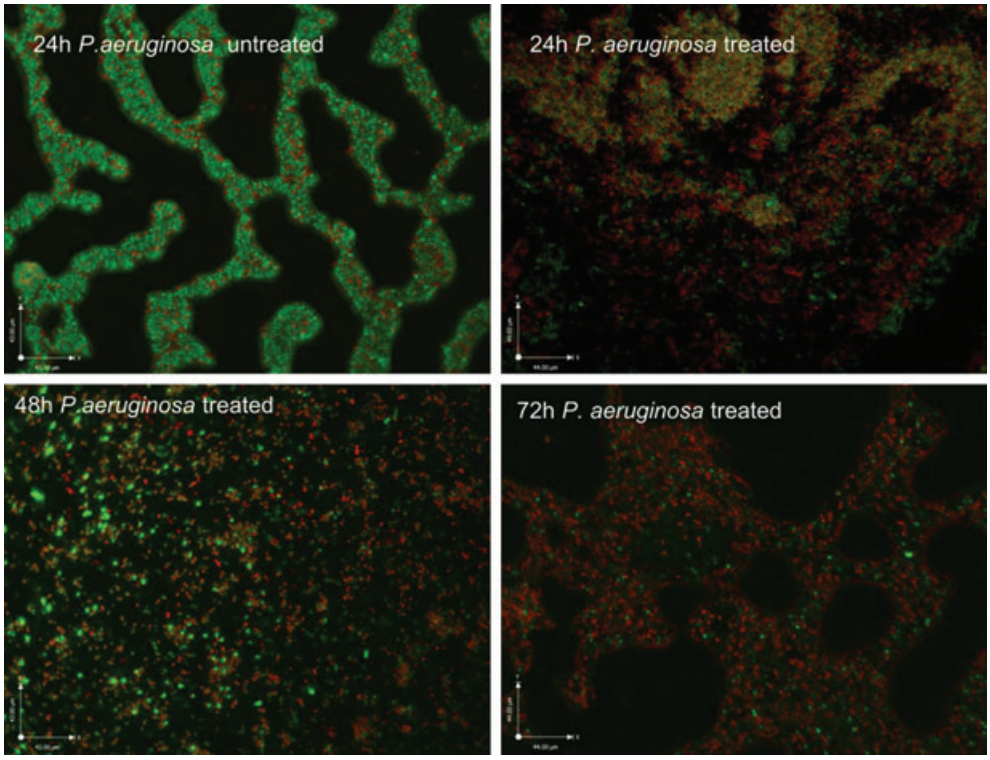

Figure 9 The effect of 1% (w/v) GLG (glucose oxidase, lactoperoxidase and guaiacol) for 24 hours at 37°C on viability of established *Pseudomonas aeruginosa* biofilms monitored with LIVE/DEAD® BacLight™.

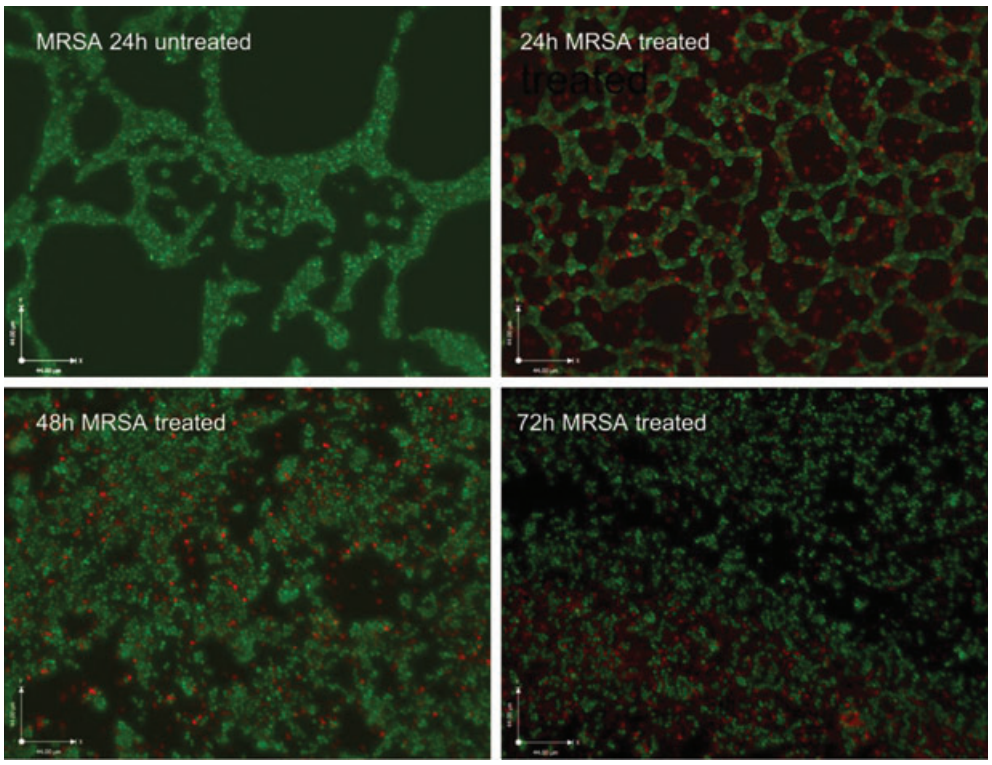

Figure 10 The effect of 1% (w/v) GLG (glucose oxidase, lactoperoxidase and guaiacol) for 24 hours at 37°C on viability of established MRSA biofilms monitored with LIVE/DEAD® BacLight™.

or viability. Hence, monitoring biofilm activity or viability with fluorescent dyes is likely to be a more reliable laboratory method than estimating biofilm biomass determinations alone. Another possibility is that inactive biofilm biomass present in wounds following treatment with GLG may have to be removed by debridement.

The biofilm-based wound management approach developed by Wolcott and Rhoads advocates the use of sharp debridement coupled with exposure to antibiofilm treatments (20). The rationale for this approach being that antimicrobial susceptibility of microbial cells during biofilm formation is greater than that found in microbial cells present in mature biofilms, so that any microorganisms attempting to regenerate biofilm will be more susceptible to antimicrobial agents than those within mature biofilms (21). GLG would seem to be a suitable antimicrobial intervention to use following debridement. Another advantage of introducing GLG into cutaneous wounds is that low concentrations of hydrogen peroxide may stimulate keratinocyte proliferation and migration (22,23) and generate free radicals that act as a signalling molecule in wound healing (24).

At present, the choice of antimicrobial agents available for treating biofilms in wounds is limited. This study demonstrates that the active antibacterial component in Flaminal can prevent the formation of biofilms and inhibit established biofilms in vitro. Clinical evidence of its efficacy in reducing wound bioburden is now required. Its effect on wound biofilm will be more difficult to investigate in vivo until a reliable, near-patient biofilm diagnostic tool becomes available. Then, a comparative study of Flaminal and other topical treatments could be envisaged.

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