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An *in vitro* test of the efficacy of silver-containing wound dressings against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in simulated wound fluid[☆]



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ABSTRACT

An isothermal microcalorimetric assay was used to quantify the efficacy of a silver-containing wound dressing against two common wound pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The growth patterns of the two species were unique and varied depending on the environment in which the organisms were grown. Addition of non-silver-containing dressing altered the growth kinetics while addition of silver (contained either in a dressing or as AgNO₃ solution) was seen to elicit inhibition and/or kill depending on concentration. Tests were conducted in nutrient broth and simulated wound fluid. It was found that minimum inhibitory and minimum bactericidal concentration values were higher in simulated wound fluid and under anaerobic conditions. Bioavailability of silver from the wound dressing was 35% against *S. aureus* in nutrient broth and 68% against both species in simulated wound fluid. The data highlight the importance of developing and conducting *in vitro* assays in biorelevant media.

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1. Introduction

Healing of cutaneous wounds is a complicated process, influenced by a number of factors. Healing may be hampered by underlying conditions or pathology including arterial insufficiency or vascular disease, such as seen with diabetic foot ulcers (Kranke et al., 2012). While endogenous pathophysiological factors, such as chronic inflammation, are recognised to be important features of chronic wounds, it is widely accepted that microorganisms also play an important role (Bowler, 2002). The mere existence of a chronic wound implies bacterial contamination; if progression to permanent colonisation occurs and the bacterial load increases, wound healing may be delayed as microorganisms overcome the host's defences and invade into deeper tissues, resulting in further damage (Landis, 2008). Progression to infection may also be aided by other factors including poor blood supply to the wound and

intrinsic virulence properties of the invading organisms (Siddiqui and Bernstein, 2010; Bowler et al., 2001). Microorganisms involved in wound pathology may originate from either exogenous sources, including from the environment or those introduced by injury, or be endogenous to the surrounding skin and mucous membranes (Siddiqui and Bernstein, 2010). The understanding of the influence of microorganisms in delaying wound healing dates back to the 1960s when Bendy et al. (1964) reported that healing in decubitus ulcers only proceeded when the bacterial load in wound fluid was less than 10⁶ cfu/mL.

It is estimated that 1–2% of the populations in developed countries will at some point suffer from a chronic wound and that global expenditure in treating these conditions is \$13–15 billion annually (Siddiqui and Bernstein, 2010). Measures taken to reduce the bacterial load in chronic wounds may, therefore, be a useful and attractive strategy in reducing this burden. Silver, in particular, has found particular application in medicated wound dressings, as it shows broad antimicrobial (against both Gram-negative and Gram-positive organisms, Mirafteb et al., 2014) and anti-fungal activity (Bowler et al., 2005), although there is debate as to the specific efficacy of silver (Aziz et al., 2012; White and Cutting, 2006) and to its potential toxicity (Hermans, 2006). Understanding the action of silver is complicated by the difficulties inherent in making quantitative measurements *in vivo*, which leaves *in vitro* measurements as

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the best analytical option. As a minimum outcome, development of standard *in vitro* methods will allow quantitative comparison of the efficacy of different products (Ip et al., 2006), while confidence and correlation with *in vivo* performance will develop as the *in vitro* tests become more representative of the chronic wound environment.

A number of standard microbiological tests that can be applied to wound dressings are available, including disc diffusion tests, broth culture tests and viability testing, such as the Live/Dead BacLight™ system (Boonkaew et al., 2013). We argued previously (O'Neill et al., 2003; Gaisford et al., 2009) that the use of isothermal microcalorimetry (IMC) offers many benefits, not least of which is the opportunity to monitor growth of live organisms directly in heterogeneous media containing the actual wound dressing. We demonstrated the efficacy of a commercial product (AQUACEL® Ag, a silver-containing Hydrofiber® dressing) against *Pseudomonas aeruginosa* and quantified the amount of bioavailable silver in the dressing (ca. 25%) by reference to a silver nitrate titration. However, the test was developed in a medium optimised to support growth of *P. aeruginosa* and no other challenge organisms were tested. Here, we use the test to evaluate the efficacy of silver-containing dressings against two species (*P. aeruginosa* and *Staphylococcus aureus*) in simulated wound fluid (SWF), in order to increase the *in vitro*–*in vivo* correlation (IVIVC) of the test. Classical microbiological techniques were employed to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

2. Material and methods

The challenge organisms (*P. aeruginosa*, NCIMB 8628 and *S. aureus*, NCIMB 9518) were grown overnight in nutrient broth (NB; Oxoid Ltd.) for 16 h at 37 °C. Cells were then harvested, washed in phosphate buffered saline (PBS), resuspended in 15% (v/v) glycerol at an organism density of 10^8 cfu/ml and frozen in aliquots (1 mL) over liquid nitrogen (Beezer et al., 1976; Cosgrove, 1979). Aliquots were stored under liquid nitrogen until required. Previous experience (data not shown) has indicated that organisms can be stored for over 6 years in this frozen state and remain viable post thawing with less than 1% decrease in viability. Wound dressings, AQUACEL® (AH) or AQUACEL® Ag (AAGH), were supplied by ConvaTec Ltd. The wound dressings, both comprised of sodium carboxymethylcellulose fibres, differ in that the former has no antimicrobial agent while the latter contains ionic silver. Silver nitrate solution (AgNO_3 , 0.01 M standard solution) was purchased from Riedel-de Haën. Simulated wound fluid was prepared by mixing maximum recovery diluent (MRD; Oxoid Ltd.) with foetal bovine serum (FBS; Invitrogen Ltd.) in equal volumes.

An aliquot of frozen organism was thawed by immersion in a water bath (40 °C) for 3 min, followed by a period of vortexing (1 min). SWF (2.97 mL), pre-warmed to 37 °C was pipetted into the calorimetric ampoule (glass, 3 mL volume). Thawed aliquots of bacteria, used in each experiment, were subcultured to eliminate the possibility of them having been contaminated and to demonstrate stock uniformity. An appropriate mass (see Section 3) of wound dressing or volume of silver nitrate solution was added to the ampoule and the medium was inoculated with bacterial suspension (0.03 mL), giving a final organism density of 10^6 cfu/mL (selected because this is the minimum concentration at which growth is detectable in our instrument; it would, for instance, have been possible to inoculate to a lower concentration, but this would simply have added a time-lag to the data while the culture multiplied to a concentration of 10^6 cfu/mL). Ampoules were sealed with a crimped metal lid (an air-tight seal being ensured with a rubber disc), vortexed for 10 s, transferred to the calorimeter and allowed to reach thermal equilibrium. Data were recorded with

a 2277 Thermal Activity Monitor (TAM; TA Instruments Ltd., UK) operated at 37 °C. Data capture was initiated exactly 30 min post-inoculation with the dedicated software package Digitam 4.1 (1 data point every 10 s, amplifier setting 300 μW). The instrument was calibrated periodically by the electrical substitution method. Data were analysed using Origin 8.1 (Microcal Software Inc.).

MICs of silver nitrate were determined in SWF or NB by a microdilution method in 96-well plates with AgNO_3 concentrations ranging from 1×10^{-3} M to 9.8×10^{-7} M, an inoculum of 1×10^6 cfu/mL and in a total volume of 200 μL . MIC values were evaluated after 16 h incubation at 37 °C.

The minimum mass of wound dressing required to inhibit growth was also determined in SWF with an inoculum of 1×10^6 cfu/mL. Masses of AAGH tested were 1, 2.5, 5, 10, 15, 20 and 25 mg. Minimum bactericidal masses were also recorded as the lowest concentration or mass required, respectively, to give rise to 99.9% kill after plating out 10 μL of sample onto iso-sensitest Agar (Oxoid) and incubation at 37 °C overnight (16 h).

Growth curves for comparison with calorimetric data were obtained by inoculating NB (50 mL) with *S. aureus* to a final population of 1×10^6 cfu/mL. The suspension was dispensed (3 mL) into calorimetric ampoules (11). The ampoules were hermetically sealed as described above. One ampoule was used for TAM analysis and the remaining ten were incubated at 37 °C. At two-hour intervals, an ampoule was vortexed before the seal was broken. Optical density readings were recorded with a spectrophotometer (600 nm, Helios α , Thermo Scientific) and cfu counts were determined by serially diluting and spread plating onto iso-sensitest agar. Colonies were counted following 16 h incubation at 37 °C.

3. Results and discussion

The use of IMC for monitoring bacterial growth has been long established and discussed (Beezer, 1980; Braissant et al., 2010). We have chosen to plot the data as cumulative heat curves, since these most closely resemble the growth curves produced by classical techniques (Von Ah et al., 2009), so we provide first some interpretation of the data. The output from the calorimeter is a plot of power (μW , or $\mu\text{J s}^{-1}$) as a function of time (t). It follows that the area under the curve is equal to the heat released (μJ). Fig. 1[D] shows the typical power–time trace for *S. aureus* inoculated into NB. It is apparent that the calorimetric trace is complex, comprising a series of peaks and troughs that correspond to different phases of microbial growth. Assignment of those phases can be made by reference to growth curves determined by optical density (OD) or cell number measurements (Fig. 1[A] and [B]). Biphasic exponential growth occurs initially (0–6 h), followed by a stationary phase (6–14 h) before commencement of cell death. Because experiments are conducted in hermetically sealed ampoules, with a small head-space, it is assumed that the initial exponential phase (ca. 1 h) represents aerobic metabolism, after which the oxygen in the ampoule is exhausted and the bacteria switch to anaerobic metabolism, resulting in the second exponential phase (ca. 5 h). These growth phases are seen in the calorimeter as exothermic peaks over the same time period. Interestingly, while the OD and cell numbers remain constant during the stationary phase, the calorimeter records a broad, increasing exotherm over the same period. Since cell numbers do not increase during the stationary phase, this power must be associated with the bacteria utilising an increasingly diverse range of nutrients. If cumulative heat is plotted with time, Fig. 1[C], biphasic exponential growth is clearly evident prior to the stationary phase (although the gradient of the stationary phase is not zero because of the effect of the exothermic heat measured during this phase).

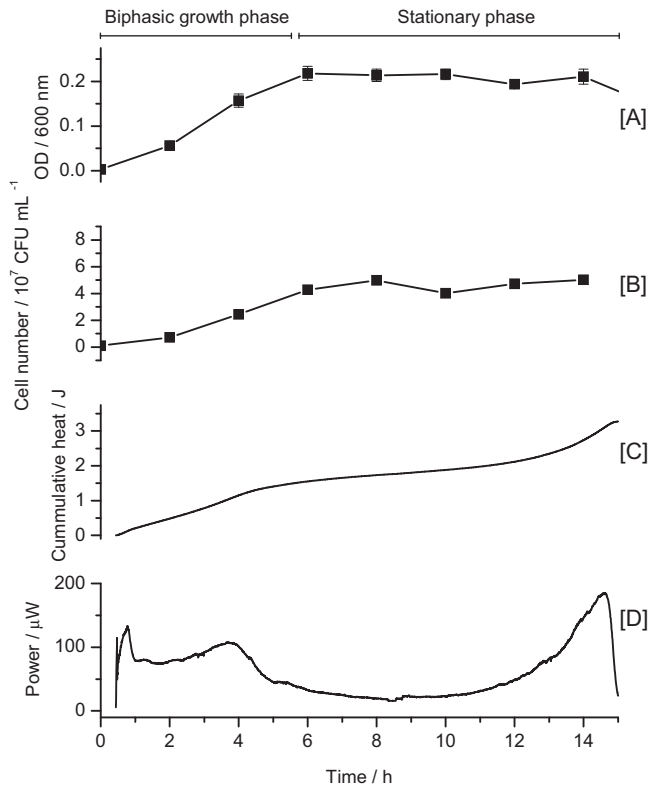


Fig. 1. Comparison of power–time data [D] with plots of cumulative heat [C], cell number [B] and optical density [A] for *S. aureus* grown in NB. The growth and stationary phases are indicated.

Two further points are of note. Firstly, because the calorimeter requires a minimum cell density of 1×10^6 cfu/mL in order to measure a detectable power, it is not possible to differentiate between MIC and MBC by calorimetric analysis alone (the use of isothermal calorimetry for MIC determination has recently been discussed, Von Ah et al., 2009), since experiments conducted with antibacterial agents at both inhibitory and bactericidal concentrations would result in a zero power signal. For this reason, ampoules were opened after each experiment and viability counts performed. Secondly, the use of frozen inocula removes natural generational variability in the bacterial species, but the main aim of the work is the development of an assay that is of practicable application to assessment of wound dressing design and performance. However, the reproducibility of the growth curves we obtain (typically to ca. 6%) means that we can use the organisms to make quantitative comparisons when varying dose and media. We are also focused on the use of IMC as an *in vivo* test for product efficacy but note that it may be possible to extend its use to the clinic by sampling wound exudate.

Fig. 2 shows the normal growth curves for *S. aureus* in NB and SWF in the absence of any dressing. For comparison, the corresponding power–time data are also shown. It is apparent that substrate availability (and so utilisation) is substantially different in the two media, commensurate with their different compositions. In particular, anaerobic growth commences sooner, and lasts longer, in SWF. Plate counts on samples removed from the calorimeter once the power signal had returned to zero showed 1×10^7 cfu/mL viable cells in NB and 1.5×10^8 cfu/mL viable cells in SWF, confirming that the cells switch to a survival stasis mechanism once the nutrient supply is exhausted and suggesting that SWF is a better medium for supporting *S. aureus* growth. This outcome immediately highlights the importance of medium selection and the need to match the *in vivo* case.

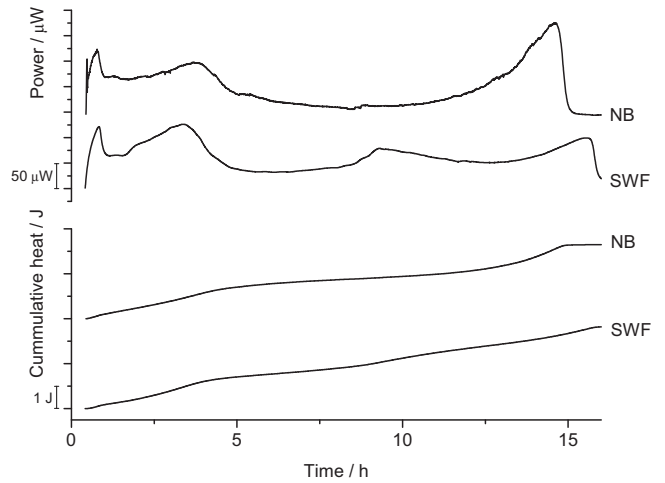


Fig. 2. Comparison of the calorimetric growth curves of *S. aureus* in NB and SWF (top) with the corresponding plots of cumulative heat (bottom).

Fig. 3 shows the growth curves for *S. aureus* in NB in the presence of increasing masses of AH (i.e. the dressing with no antimicrobial ingredient). The initial aerobic phase is present in all cases, and finishes slightly later in the presence of the dressing, but the main changes are seen during the anaerobic growth phase. Anaerobic growth is fastest in the presence of a small amount of dressing and reduces as the mass of dressing increases. Plate counts showed ca. 1×10^7 cfu/mL viable cells present after all experiments.

As we discussed previously (Gaisford et al., 2009) absolute interpretation of the factors causing such changes, without ancillary data is difficult, but it is likely that physical phenomena, such as gelling of the dressing, will result in entrapment of organisms and a concomitant reduction in the diffusion rates of the substrates required for microbial growth. *S. aureus* is a non-motile species and cells tend to aggregate which means it is more prone to settling than *P. aeruginosa*. In the presence of gelled dressing entrapment of organisms would prevent settling but may also affect the kinetics of substrate diffusion and utilisation. It is also plausible that the weakly acidic carboxylate salt structure of the dressing behaves as a pH ‘buffer’. If so, then the pH in the environment of the swelled dressing would differ from the bulk growth medium, potentially prolonging the time period over which growth is viable.

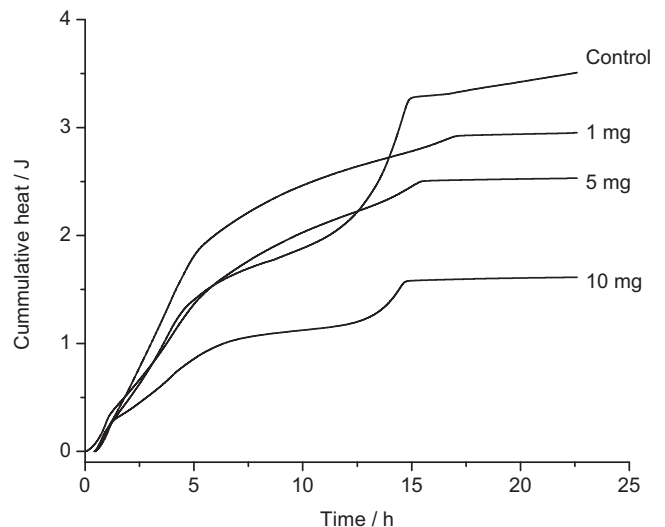


Fig. 3. Calorimetric growth curves of *S. aureus* in NB in the presence of increasing masses of AH.

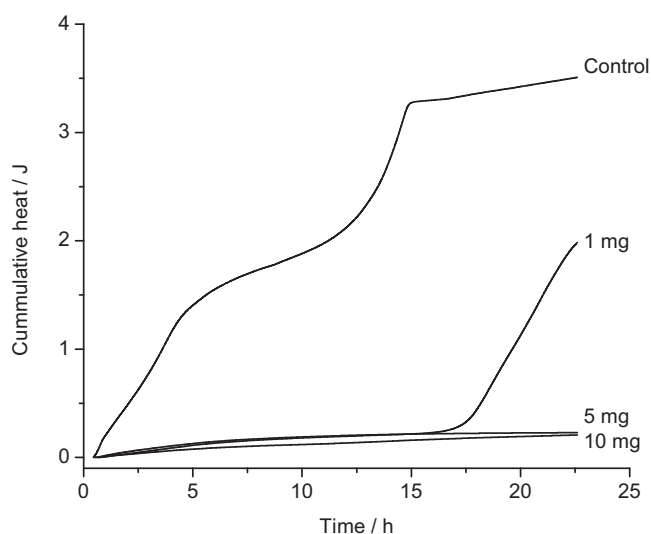


Fig. 4. Calorimetric growth curves of *S. aureus* in NB in the presence of increasing masses of AAgH.

As a general point, it should be noted that in this experiment the volume of solvent to dressing is higher than would be the case when used clinically, which would result in the micro-environment of the organism being different. Typically, a $10 \times 10 \text{ cm}^2$ piece of AH would adsorb ca. 18 mL of isotonic fluid (Parsons et al., 2005) and would be changed periodically (usually every 3–4 days or when it becomes saturated) during treatment. In the experiments reported here, the ratio of dressing to medium is roughly 1/125 of that which would be used during treatment. Nevertheless, it is clear both that the presence of dressing changes the growth kinetics of the organism and that the calorimeter is sensitive to such change, although the total increase in cell numbers is constant with or without the dressing present.

The growth of *S. aureus* is also significantly altered in the presence of AAgH (i.e. the silver-containing dressing), Fig. 4. Growth is significantly delayed when just 1 mg of dressing is present and no growth is seen when larger masses are used. Viable counts at the end of each experiment showed ca. 1×10^8 cfu/mL for 1 mg of dressing and 1×10^6 cfu/mL for dressing masses up to 10 mg. Since growth did not commence until ca. 16 h after inoculation when in the presence of 1 mg of AAgH the bacteria were in a growth phase when the experiment was stopped, which may account for the relatively high cell count. Stasis occurs with larger masses of dressing.

The silver in AAgH is present as a counter ion to the carboxymethylcellulose which forms the basis of the gellable but water insoluble fibrous structure of the dressing. The silver must dissociate from the fibre by an ion exchange process and then diffuse through the dressing matrix in order to exert a bactericidal effect. This raises the question as to the fraction of silver that is bioavailable. This can be determined by titration with silver nitrate solution (Fig. 5). It is apparent that the growth patterns are significantly disrupted by the presence of AgNO_3 . Growth is delayed in the presence of $1 \times 10^{-5} \text{ M}$ AgNO_3 , reduced almost to baseline in the presence of $5 \times 10^{-5} \text{ M}$ AgNO_3 and is zero in the presence of $1 \times 10^{-4} \text{ M}$ AgNO_3 . Plate counts were 1×10^8 , 5×10^6 and zero respectively, confirming the trend seen for the AAgH experiments above. One possible explanation for this effect is that because there is no dressing, entrapment of the organisms in the gelled dressing matrix (where presumably the silver concentration is saturated) cannot occur, and so the organisms are exposed to what is effectively a lower silver concentration in bulk solution. Another is that small amounts of silver may reduce the integrity of the cell

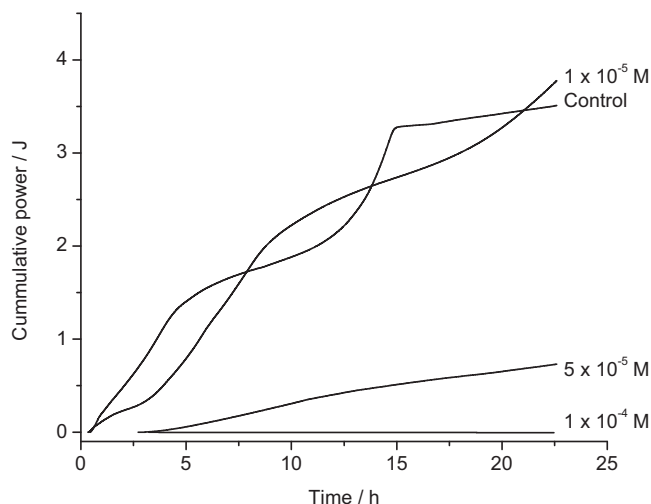


Fig. 5. Calorimetric growth curves of *S. aureus* in NB in the presence of increasing concentrations of AgNO_3 solution.

membrane, enhancing uptake of nutrients. Hence, an assay based on silver nitrate solution alone would underestimate the potency of AAgH.

As noted earlier, MIC and MBC values need to be confirmed with a classical microbiological assay, the values being reported in Table 1. Since the amount of silver in AAgH is 1.2% (w/w) (Parsons et al., 2005), it is easy to calculate the total silver load administered if the mass of dressing is known. The results showed the MIC and MBC values to be equivalent and equal to 3.7×10^{-4} for AAgH and $1.3 \times 10^{-4} \text{ M}$ respectively for AgNO_3 . Expressed another way, assuming all of the silver in AgNO_3 is bioavailable, 35% of the silver in AAgH is bioavailable against *S. aureus*, a slightly higher fraction than was noted previously (25%, Gaisford et al., 2009) when the tests were performed against *P. aeruginosa*.

Figs. 6–8 show corresponding growth curves for *S. aureus* in the presence of increasing masses of AH, AAgH and AgNO_3 respectively in SWF. Dealing with the AH data first, several differences from the control are apparent. Firstly, the initial aerobic phase occurs much sooner (the exponential phase is still present, but the data are not recorded because of the 30 min equilibration time). Secondly, while similar gross patterns are seen in the presence of dressing, growth appears to occur more slowly. Plate counts showed ca. 1×10^7 cfu/mL viable cells after all experiments, showing that growth rates were altered but not inhibited, as was observed in the NB data discussed above.

In the presence of AAgH detectable growth was seen to reduce and this correlated with viable count data; 1×10^8 , 1×10^8 and 1×10^6 cfu/mL with 1, 5 and 10 mg samples respectively. The AgNO_3 titration data were interesting, significant growth being

Table 1
MIC and MBC values of wound dressing (AAGH) and silver nitrate for *P. aeruginosa* and *S. aureus* determined under aerobic conditions in SWF and NB.

System	MIC/M	MBC/M
<i>P. aeruginosa</i> in SWF		
AAGH	9.3×10^{-5}	3.7×10^{-4}
AgNO_3	6.3×10^{-5}	2.5×10^{-4}
<i>S. aureus</i> in SWF		
AAGH	1.9×10^{-4}	3.7×10^{-4}
AgNO_3	1.3×10^{-4}	2.5×10^{-4}
<i>S. aureus</i> in NB		
AAGH	3.7×10^{-4}	3.7×10^{-4}
AgNO_3	1.3×10^{-4}	1.3×10^{-4}

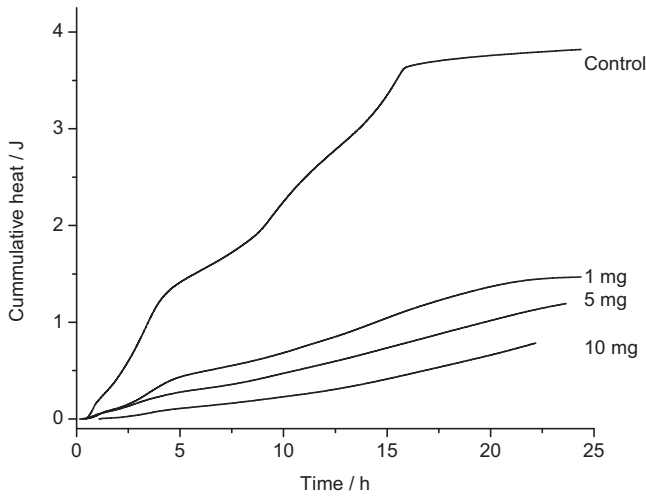


Fig. 6. Calorimetric growth curves of *S. aureus* in SWF in the presence of increasing masses of AH.

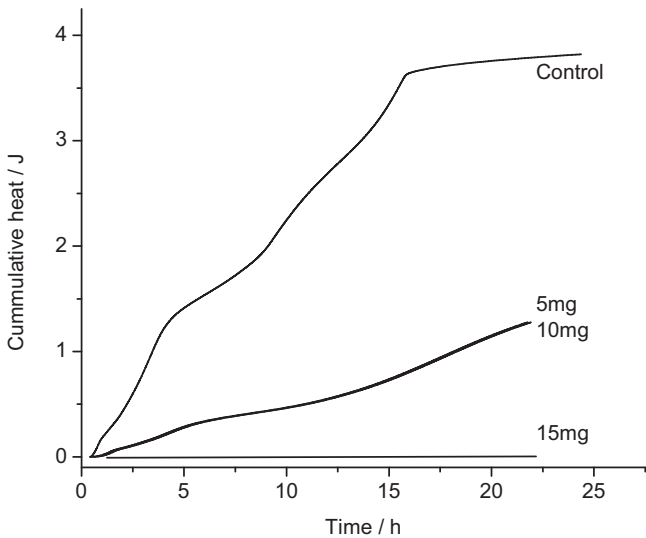


Fig. 7. Calorimetric growth curves of *S. aureus* in SWF in the presence of increasing masses of AAgH.

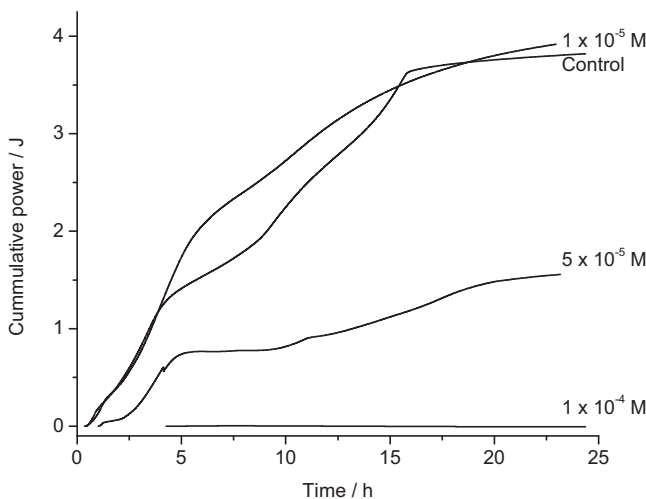


Fig. 8. Calorimetric growth curves of *S. aureus* in SWF in the presence of increasing concentrations of AgNO_3 .

Table 2

MIC and MBC values of AgNO_3 for *S. aureus* determined under anaerobic conditions in NB and SWF.

	MIC/M	MBC/M
NB	1.3×10^{-4}	1.3×10^{-4}
SWF	2.5×10^{-4}	n/d

detected at concentrations of 1×10^{-5} and 5×10^{-5} M, although zero power was seen at a concentration of 1×10^{-4} M. Plate counts were 1×10^8 , 6×10^7 and 1×10^6 cfu/mL respectively (in other words, no MBC was reached). Clearly, the effect of a small amount of silver is greater in SWF than NB. In addition to the possible mechanisms discussed above, SWF contains more proteins than NB. Since it has been shown that small quantities of silver are able to denature DNA fibrils (Hobot et al., 2008) one additional explanation for the growth seen in the presence of 1×10^{-5} M AgNO_3 may be that the silver denatures some of the proteins in the medium, creating additional, and more easily utilised, nutrient sources.

MIC and MBC data are given in Table 1. The results showed MIC and MBC values of 1.9×10^{-4} and 3.7×10^{-4} M respectively for AAgH and 1.3×10^{-4} and 2.5×10^{-4} M respectively for AgNO_3 . This means 68% of the silver in AAgH is bioavailable in SWF, almost double that seen in NB. The greater bioavailability may reflect a higher dissociation rate of the silver from the carboxymethylcellulose when in SWF compared with NB because of differences in pH and/or ionic strength.

One consideration with classical determination of MIC and MBC values is that the system is typically aerobic, and yet our interpretation of the calorimetric data is that following an aerobic phase, growth progresses anaerobically. Hence, MIC and MBC values for *S. aureus* were determined anaerobically for comparison, Table 2. For growth in NB the values were the same, whether determined aerobically or anaerobically, but in SWF the MIC increased to 2.5×10^{-4} M while no bactericidal effect was seen, over the concentration range used. This implies that inhibition and kill are harder to achieve in SWF under anaerobic conditions, which confirms the findings from the calorimeter. This also implies that the conditions in the ampoule are indeed anaerobic, as postulated earlier, because if they were not, a bactericidal effect should have been observed.

The final set of data show the growth of *P. aeruginosa* in SWF, an extension of the previous study that reported data for this organism in NB (Gaisford et al., 2009). Fig. 9 shows the

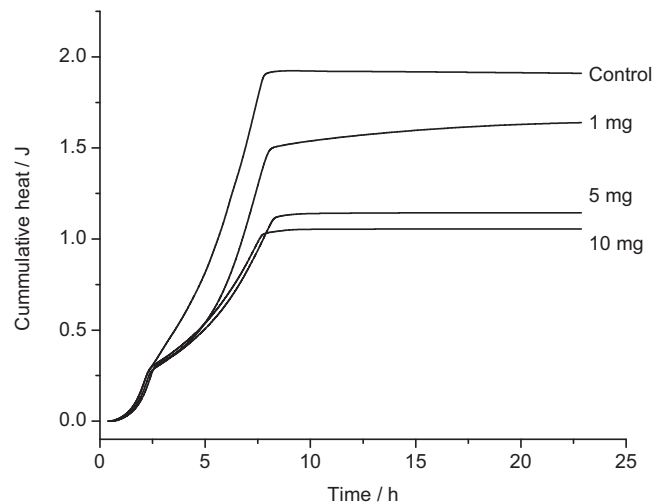


Fig. 9. Calorimetric growth curves of *P. aeruginosa* in SWF in the presence of increasing masses of AH.

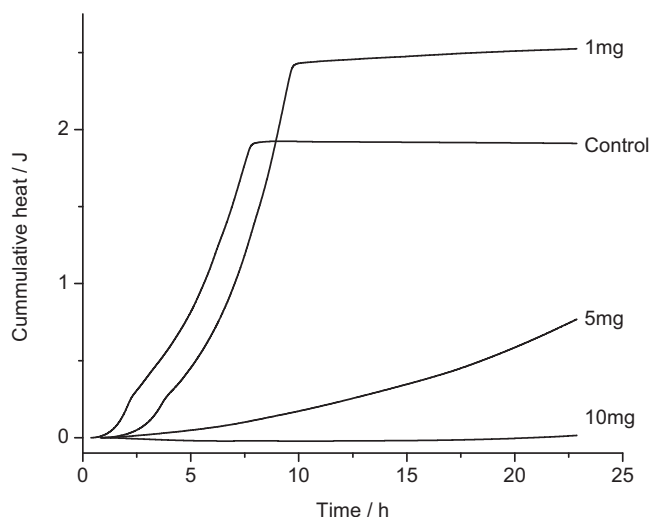


Fig. 10. Calorimetric growth curves of *P. aeruginosa* in SWF in the presence of increasing masses of AAgH.

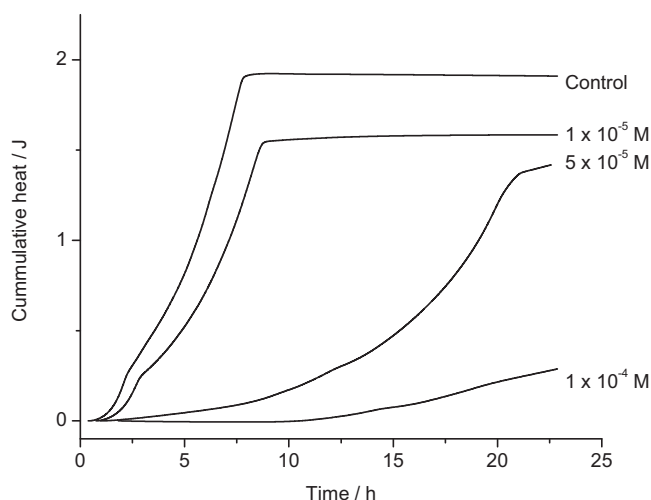


Fig. 11. Calorimetric growth curves of *P. aeruginosa* in SWF in the presence of increasing concentrations of AgNO_3 .

control growth curve and the changes induced with increasing masses of AH. As noted earlier in the case of *S. aureus*, a change in the growth pattern is noted, presumably for the same reasons of entrapment and changes in the rate of supply/availability of nutrients. Fig. 10 shows the effect of increasing masses of AAgH. Growth is delayed in the presence of 1 mg of dressing and inhibited in the presence of larger masses. MIC and MBC data are given in Table 1. The results showed MIC and MBC values of 9.3×10^{-5} and 3.7×10^{-4} M. The MBC value is larger than that reported previously in NB (1×10^{-4} M, Gaisford et al., 2009), again a reflection of the fact that inhibition and kill are harder in SWF. Titration with AgNO_3 solution showed slightly delayed growth at 1×10^{-5} M, significantly delayed growth at 5×10^{-5} M and no growth at 1×10^{-4} M, Fig. 11. The corresponding MIC and MBC values were 6.3×10^{-5} and 2.5×10^{-4} M respectively, Table 1. Thus, the efficacy and bioavailability of silver against both species are equal in SWF (equivalent to 68% of the silver-load in AAgH).

4. Summary

Different organisms are seen to produce different growth curves when studied with IMC. Growth curves also change with respect to medium. Addition of AH is seen to alter the growth kinetics while addition of silver (as either AAgH or AgNO_3) is seen to elicit inhibition and/or kill depending on concentration. MIC and MBC values are seen to be higher in simulated wound fluid and under anaerobic conditions, highlighting the importance of conducting assays in biorelevant media.

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