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An in vitro method for the quantitative determination of the antimicrobial efficacy of silver-containing wound dressings

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ABSTRACT

Treatment with silver-containing wound dressings is becoming an increasingly popular strategy to eliminate growth of opportunistic wound pathogens during the healing process. However, there are concerns over the possible side-effects of silver to the patient; coupled to the cost of silver as an ingredient there is a desire to ensure that wound dressings contain the least quantity of active ingredient to ensure the minimum bactericidal concentration (MBC) of silver is maintained in the wound environment. This requires the ability to determine the efficacy of silver directly within the wound environment; an extremely complicated task that is difficult using classical (plate counting) microbiological assays because these cannot be conducted in situ. Here, we report a quantitative method for determining the efficacy of silver in wound dressings using an isothermal calorimetric method. The growth curves of *P. aeruginosa* (NCIMB 8628) were recorded in growth medium and in growth medium containing AQUACEL® Ag Hydrofiber® dressing. It was found that 10 mg of dressing was sufficient to ensure no detectable growth of organism in 2.5 mL of medium inoculated to 10^6 cfu/mL. This corresponded to a silver load of 1.1×10^{-6} moles (equivalent to 4.4×10^{-4} M, in the volume of medium used in the experiment). Experiments conducted with silver nitrate rather than dressing indicated the MBC of silver against *P. aeruginosa* was 1×10^{-4} M. The results suggested that not all of the silver in the dressing was bioavailable, at least over the lifetime of the experiment. One advantage of this effect would be the lack of excess availability of the silver, which allays fears of potential toxicity to the patient and may provide an extended period of time over which the dressing is bactericidal.

1. Introduction

The skin is an extremely effective biological barrier but if breached by trauma the body becomes highly susceptible to microbial attack. Management or prevention of infection in wounds by opportunistic pathogenic organisms is therefore vital if rapid recovery from trauma is to be assured. If a wound remains unhealed for more than 6 weeks, it becomes chronic and in these cases complications become more frequent because of bacterial colonisation (Bowler et al., 2001). Wound management can be facilitated with dressings, which are designed both to act as a temporary barrier and/or to promote wound healing, and a large range of dressings is available on the market. Some of these dressings are medicated, containing active substances to manage the microbial load in the area of the trauma. Typical actives include antibiotics (such as neomycin, bacitracin or polymyxin combinations) and broad-spectrum germicidal agents (for instance silver, iodine or chlorhexidine).

Silver, in particular, has found particular application in medicated wound dressings as it shows broad antimicrobial (against both Gram-negative and Gram-positive organisms) and anti-fungal activity (Bowler et al., 2005). The use of 1% silver sulfadiazine (SSD) with 0.2% chlorhexidine digluconate in a topical cream (Silvazine™,[1]) has been shown to be particularly effective (George et al., 1997) and is the primary treatment for wound care in Australasia (Fraser et al., 2004). Several silver-containing wound dressings are commercially available, including AQUACEL® Ag Hydrofiber®,[1], Acticoat®[2] Absorbent, Silvercel®[3], Urgocell® Silver[4], and PolyMem® Silver[5].

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However, despite its obvious benefits, debate exists as to the specific efficacy of silver in these dressings (White and Cutting, 2006) and to its potential toxicity (Hermans, 2006). For instance, a solution of silver nitrate (0.5%, w/v) in water provides ca. 3200 ppm Ag^+ (Demling and DeSanti, 2001) while Acticoat dressing and Aquacel Ag Hydrofiber dressing provide ca. 55 and 1 ppm Ag^+ respectively (Walker et al., 2006). In saline or wound fluid the concentrations of ionic silver drop to 1 ppm in all cases (Demling and DeSanti, 2001; Walker et al., 2006). Understanding, and ultimately controlling, these issues is difficult with an *in vivo* study because of the complex nature of the wound environment and the difficulties inherent in counting the number of viable organisms therein. An alternative strategy is to develop a quantitative *in vitro* method of analysis; such a method would allow meaningful comparisons between the efficacy of different wound dressings. Indeed, Ip et al. (2006) note that '*A standardised methodology should be established in order to examine and compare the efficacies of the antimicrobial effects of the commercially available 'antimicrobial' coated medical products*'. Once quantified, the quantity of silver in the formulation can be adjusted to minimise potential toxicity by ensuring that the least amount of silver is added that assures the minimum bactericidal concentration (MBC) is maintained in the wound exudate during treatment. Developing such a quantitative assay and applying it to determine the efficacy of a silver-containing wound dressing is the main focus of this work.

It is not a simple matter to measure the efficacy of silver in wound dressings because of the heterogeneity of both the silver speciation (Parsons et al., 2005) and the system (comprising dressing, aqueous medium, silver and organism). Thorn et al. (2005) note that '*An accurate determination of kill rates is the key to comparing formulations, different environmental effects on killing and the susceptibility of different species*'. Conventional microbiological assay techniques, such as plating and counting, are time consuming, do not provide information on real-time microbial growth nor *kill rates* in the actual wound environment, do not compensate for the fraction of the microbial load that is dead or not-viable and exclude any contribution to product efficacy caused by physical effects such as gelling of the dressing matrix. In addition, a proportion of the bacteria will become entrapped in the dressing matrix and will not be available for plate counting. In spite of these limitations, the use of this classical approach to study the efficacy of silver-containing wound dressings has been reported by Ip et al. (2006), where it is noted that at least qualitative comparisons can be drawn between dressings.

Some authors have attempted to develop assays for counting organisms directly within the dressing under *in vitro* conditions. Thorn et al. (2005) proposed a static diffusion method by placing cellulose disks inoculated with various species between an agar base and a wound dressing and, more recently, the same authors proposed a method using bioluminescent *P. aeruginosa* and monitoring light production (Thorn et al., 2007). Both methods were problematic; in the former case the data were not recorded in real-time, required overnight culturing on agar media and the method was species dependent, while in the latter case the correlation between photon and viable counts was not consistent between wound dressings. Gallant-Behm et al. (2005) compared the use of in vitro disc diffusion and time kill-kinetic assays for the evaluation of efficacy of silver-containing wound dressings, concluding that the methods were not comparable and that disc diffusion methods are of little value to these materials.

Here, we propose a calorimetric method for quantitatively monitoring the efficacy of silver in wound dressings, following earlier qualitative proof-of-concept work (O'Neill et al., 2003). Briefly, isothermal calorimetry (IC) monitors the rate of heat production (power) in a sample; the power signal is quantitatively proportional to the number of viable organisms in the sample. Organism growth (or inhibition/death) can thus be measured in media with and without wound dressing because the technique is not dependent on the physical form of the sample and does not impose a requirement for optical clarity. While recognising that the approach is not perfect, calorimetry addresses many of the concerns listed above in relation to plate-counting, allowing real-time measurement of organism growth in more realistic (although still not equivalent to the in vivo case) media. The organisms do not need to be sampled nor removed from their environment and calorimetric data are not affected by non-viable cells. There is the drawback that heat, being ubiquitous, is produced or absorbed by every event occurring within the sample, which means that the power signal obtained is (potentially) a composite of many processes. However, careful experimental design can ameliorate many of these issues and we have discussed several approaches for dealing with complexity in calorimetric data (Gaisford, 2005; O'Neill, 2005). We report here data for the efficacy of AQUACEL® Ag Hydrofiber® dressing against *P. aeruginosa*, a common wound pathogen.

2. Materials and methods

The challenge organism (*P. aeruginosa*, NCIMB 8628) was grown overnight in a bacterial culture medium (Oxoid Nutrient Broth, ONB). Late exponential growth phase cells were then harvested, washed in phosphate buffered saline (PBS), resuspended in 15% (v/v) glycerol to 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 for experimentation. Previous experience (data not shown) has indicated that *P. aeruginosa* 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® Hydrofiber (AH) or AQUACEL® Ag Hydrofiber® (AAgH), were supplied by ConvaTec Ltd. The wound dressings, both comprising sodium carboxymethyl cellulose, differ in that the former has no antimicrobial agent while the latter contains ionic silver. Silver nitrate solution (0.01 M standard solution) was purchased from Riedel-de Haën. All experiments were prepared under aseptic conditions.

Data were recorded with a 2277 Thermal Activity Monitor (TA Instruments Ltd., UK) at 37 °C. An aliquot of *P. aeruginosa* was thawed by immersion in a water bath (40 °C) for 3 min, followed by a period of vortexing (1 min). Nutrient broth (ONB, 2.475 mL), prewarmed to 37 °C was pipetted into the calorimetric ampoule (glass, 3 mL volume). 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 *P. aeruginosa* (0.025 mL), giving a final organism density 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 capture was initiated exactly 30 min post inoculation with the dedicated software package Digitam 4.1 (1 data point every 10 s, amplifier range 300 μW). The instrument was calibrated periodically by the electrical substitution method. Data were analysed using Origin (Microcal Software Inc.).

3. Results and discussion

It is important to make some comments on the nature of the assay we propose prior to discussing results. Any *in vitro* method will differ from the *in vivo* case; the relevancy of any differences depends on the use to which the data are put. Here the *in vivo* situation is an extremely difficult environment to reproduce; one

primary concern is that in a wound environment any organisms will grow as biofilms or microcolonies rather than as planktonic cultures (James et al., 2008), which can affect the susceptibility of an organism to an antimicrobial agent. Recent results (Bjarnsholt et al., 2007) suggested that bactericidal concentration of silver required to eradicate *P. aeruginosa* biofilms is 10–100 fold higher than that required to eradicate planktonic bacteria and the authors suggest this may mean the silver concentration in some wound dressings may be too low. Percival et al. (2008) showed that AAgH is effective against biofilms as well as planktonic cultures but it is important to note that in the experiments reported here the organism is growing in planktonic culture. The aim of this initial study is to demonstrate that calorimetry has the potential for quantifying the efficacy of silver-containing wound dressings; further increases in the complexity of the study environment, both by using simulated wound fluids and growing biofilms would increase confidence in any *in vitro–in vivo* correlation (IVIVC) but do not diminish the usefulness of the present data.

To make quantitative measurements of the efficacy of the wound dressing it is necessary to have control over the repeatability of the growth curves of the *P. aeruginosa* aliquots. This is because in these experiments it is the organism that is used as an analytical standard with which to probe the efficacy of the dressing. It is not easy to accomplish this by growing fresh cultures for each experiment, because of natural batch-to-batch variability, and so we developed an alternative strategy: A batch of *P. aeruginosa* (NCIMB 8628) was grown, harvested and resuspended to a density of 10^8 cfu/mL before being frozen over liquid nitrogen in aliquots while in the late exponential growth phase. Aliquots were thawed prior to each experiment which means that the entire experimental series reported herein was performed with the same batch of organism. Samples used for study in the calorimeter were inoculated to a cell density of 10^6 cfu/mL 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). Since the calorimeter records power as a function of time, growth of microorganisms will result in an exponentially increasing signal until the nutrients in the growth medium are exhausted or the environment in the cell is no longer conducive to cell growth (which may be caused by a build-up of toxic metabolites and/or a gradual decrease in pH). Post this point, the organisms will die and the power signal will return to baseline (which is, in a properly set-up instrument, zero).

The data in Fig. 1 reflect this and show the growth curves of six *P. aeruginosa* samples in the calorimeter. Taking the total area under the growth curve (total heat output) as an indicator of organism numbers shows reproducibility to 6.3% (3.51 ± 0.22 J). The growth curve is complex and characteristic of organism growth in an undefined medium with restricted oxygen (the ampoule is sealed and the oxygen level is limited to that dissolved in the medium and present in the headspace). Briefly, the initial exponential phase represents aerobic metabolism, which is then followed (at ca. 5 h) by a switch to anaerobic metabolism and a second exponential growth phase. Subsequent peaks and troughs represent sequential utilisation of the major carbohydrate sources typically found in a complex growth medium. A more detailed description of microbial calorimetry can be found in Beezer (1980) and James (1987).

Fig. 2 shows the growth of *P. aeruginosa* in the presence of AH (i.e. the dressing with no antimicrobial ingredient). Several differences can be observed between these data and those of Fig. 1. Firstly, the initial aerobic phase occurs much sooner (the exponential phase is still present, but the data are lost because of the 30 min equilibration time of the experiment). Secondly the shape of the

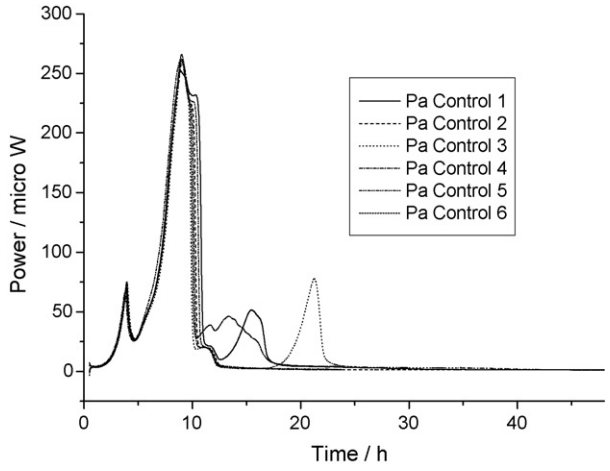

Fig. 1. Power–time data showing the growth curves for 6 repeats of *P. aeruginosa*.

growth curve is different (growth occurs for a greater period of time, although the maximum power attained is lower) and thirdly the total heat output in the presence of AH is greater than in the control. It is difficult definitively to explain these phenomena, for many of the reasons noted earlier regarding interpretation of calorimetric data in the absence of additional data, but several factors may be important. Physical phenomena, such as gelling of the dressing will inevitably lead to entrapment of organisms and a concomitant reduction in the diffusion rates of the substrates required for microbial growth; it is also possible that organisms may grow on the surface of the gelled dressing, further affecting the kinetics of the growth profile. Furthermore, it is entirely plausible that the cellulosic structure of the dressing behaves as a pH 'buffer', acting to maintain a different pH in the environment of the swelled dressing compared with the bulk growth medium, prolonging the time period over which growth is viable.

It should be noted that in this experiment the volume of solvent to dressing is vastly higher than would be the case when used clinically, which would result in the micro-environment of the organism being substantially different. Typically, a $10 \times 10\,cm^2$ piece of AH would take up ca. 18 mL of 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

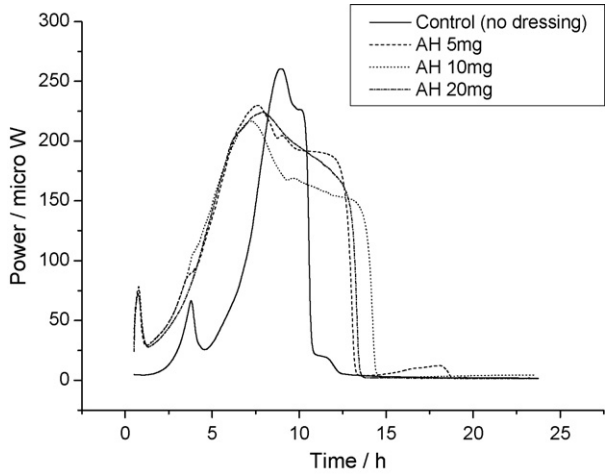

Fig. 2. Power–time data showing the growth curves for *P. aeruginosa* in a growth medium in the presence of AH.

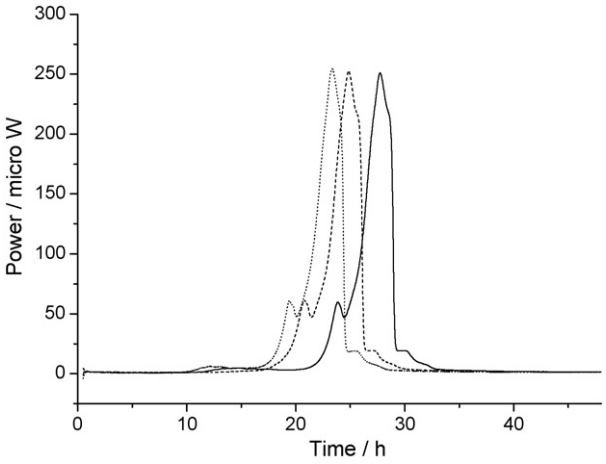

Fig. 3. Power–time data showing the growth curves for *P. aeruginosa* in growth medium with a small quantity of AAgH (1 mg).

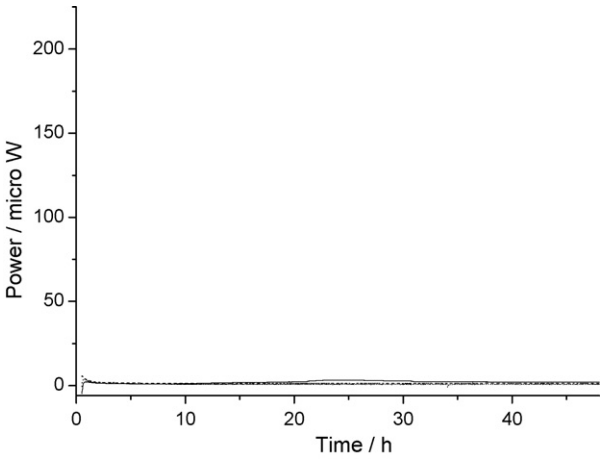

Fig. 5. Power–time data showing the growth curves for *P. aeruginosa* in growth medium with a small quantity of AAgH (10 mg).

used during treatment. Growth in wound fluid will also differ significantly from growth in an ideal medium. The net effect of these factors impacts upon IVIVC but does not preclude quantitative comparison between experiments, and we will return to the effect of the dressing in simulated wound fluid in a future publication.

Completely different behaviour was noted when AAgH was present in the growth medium, Figs. 3–5. Most obviously, the shape of the growth curve conforms to that seen for growth in ONB alone and not to that seen for growth in the presence of AH. Since it is the ionic silver that is the antimicrobial agent, rather than the cellulosic polymer of the dressing, it is likely that in this case organisms cannot grow on the surface of, and in the gelled vicinity of, the AAgH and, hence, growth is consistent with that in nutrient broth. This finding acts to support the hypothesis proposed above for the different growth profile seen in the presence of AH. With a very small amount of dressing (1 mg, Fig. 3), growth is delayed by 15–20 h (i.e. AH is bacteriostatic, Newman et al., 2006), but is then consistent with the control sample (3.25 ± 0.12 J, compared with 3.51 ± 0.22 J for the control). This suggests that the silver is present in sufficient quantity to kill the majority of the initial microbial load, but that the survivors then continue to multiply. This pattern of behaviour would be observed upon addition of virtually any antibacterial agent to a wound environment, which confirms that the system used here is a good *in vitro* model. The calorimeter subsequently detects growth as the number of organisms increases above 10^6 cfu/mL.

As the mass of AAgH dressing is increased (5 mg, Fig. 4), a significantly enhanced bactericidal effect is observed. Of the three repeats, one showed no growth within the experimental observation period, one showed retarded growth and one showed growth consistent with the control. These differences in behaviour are most likely a result of variations in the exposure of the organisms to the gelled dressing. With a larger mass of dressing (10 mg, Fig. 5) no growth was observed, over a 50 h observation period.

These experiments indicate the minimum bactericidal concentration of ionic silver needed in the dressing (we did not plate out samples post inoculation because in earlier work (O'Neill et al., 2003) we demonstrated that a zero signal in the calorimeter corresponded to complete cell death). The data do not quantify the *fraction* of the silver load that is bioavailable. In order to quantify this, experiments were repeated without dressing but with added silver nitrate (AgN, which is assumed to be completely bioavailable, as it is in solution). At a concentration of 1×10^{-5} M Ag, growth was seen in all samples, Fig. 6, although there was a time delay consistent with that seen for the AAgH samples. The peak areas were consistent with the control data (3.10 ± 0.1 J compared with 3.51 ± 0.22 J for the control). At a concentration of 5×10^{-5} M AgN growth was delayed and only seen in two of the three samples, Fig. 7

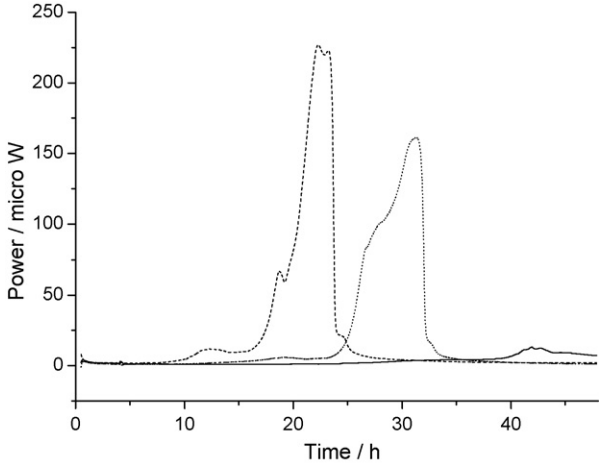

Fig. 4. Power–time data showing the growth curves for *P. aeruginosa* in growth medium with a small quantity of AAgH (5 mg).

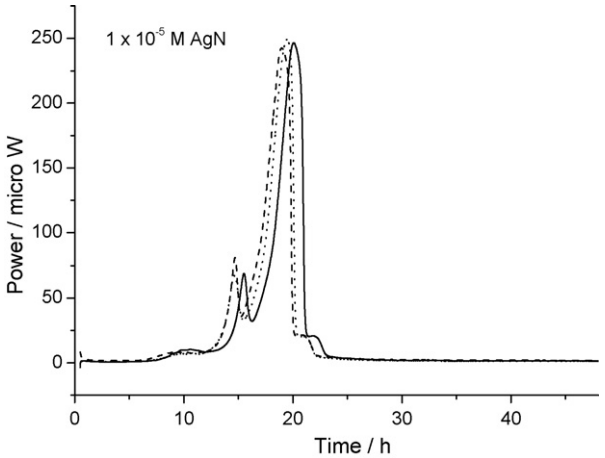

Fig. 6. Power–time data showing the growth curves for *P. aeruginosa* in growth medium containing silver nitrate (1×10^{-5} M).

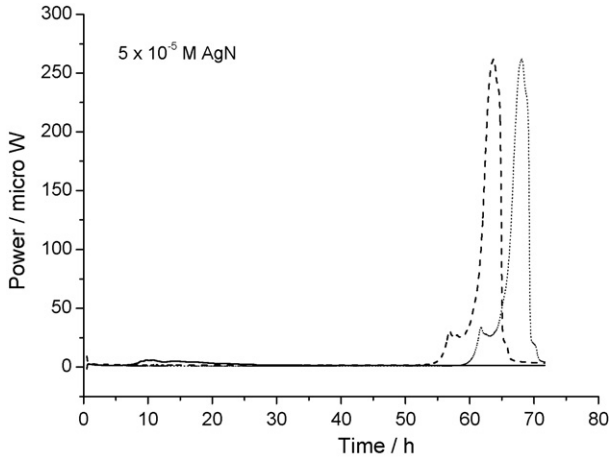

Fig. 7. Power–time data showing the growth curves for *P. aeruginosa* in growth medium containing silver nitrate (5×10^{-5} M).

(mean area 3.34 J—there is no standard deviation as only two areas were measured). At a concentration of 1×10^{-4} M AgN no growth was seen, even over 140 h, Fig. 8. The MBC for aqueous silver is thus assigned here to be 1×10^{-4} M.

AAgH contains 1.2% (w/w) ionic silver. In 10 mg of dressing, therefore, there are 1.1×10^{-6} moles of silver (equivalent to 4.4×10^{-4} M, in the volume of medium used in the ampoule). This value is greater than the MBC suggested in the silver nitrate experiments and warrants some discussion. The data suggest that not all the silver in the dressing is bioavailable, at least over the lifetime of the experiment. Two factors potentially affect this event. The first is that the silver present is ionic and hence must dissociate from the dressing prior to becoming bioavailable; there will clearly be a rate associated with this process which may be rate limiting over the lifetime of the experiments conducted here. Two immediate advantages of this effect would be the lack of excess availability of the silver, which allays fears of potential toxicity to the patient and an extended period of time over which the dressing is bactericidal. The second is that there will be a series of equilibria in the system between dressing and ionic silver, ionic silver and aqueous silver and aqueous silver and organism. Again, the relative positions of these equilibria will act both to delay dissolution and to make the dressing act as a reservoir of silver, with the same beneficial effects noted above. While the data suggest that a relatively high quantity of silver should be incorporated into the dressing it should be remembered that in clinical application the ratio of dressing to wound fluid would be vastly increased relative to the experiments reported here and that the dressing would be changed every 3–4 days.

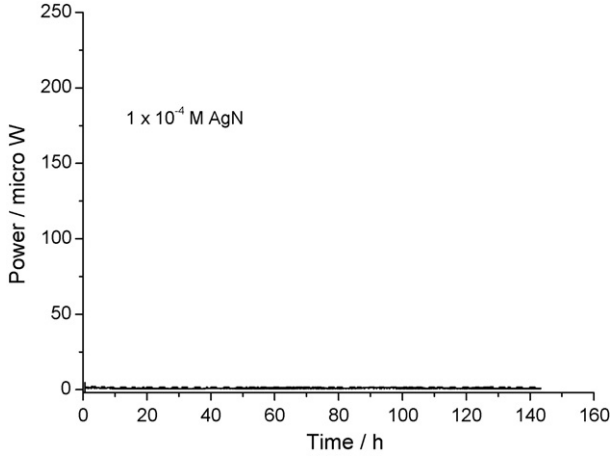

Fig. 8. Power–time data showing the growth curves for *P. aeruginosa* in growth medium containing silver nitrate (1×10^{-4} M).

4. Summary

Assessment of the efficacy of silver-containing wound dressings in vivo is challenging; in the absence of a suitable assay for in vivo studies a suitable in vitro method is desirable. Once an *in vitro* method has been established, its correlation with, and eventual extension to, *ex vivo* and then *in vivo* study can be attempted. Of the currently reported *in vitro* methods all require sampling and concomitant plate counting to determine organism numbers, with the presumption that growth on an agar plate is representative of growth in the experiment. Here, we propose an alternative methodology that progresses measurements closer to the in vivo case by using calorimetry to monitor growth of organisms directly. Although the method has some limitations, including the fact that the organism culture is planktonic, it does (as discussed in the introduction) overcome many of the significant issues associated with plate counting and we see no reason why it could not be extended to studies of organisms growing in biofilms (such as the work on denture surfaces by Morgan and Beezer, 1998). Because all living organisms produce heat, the approach is valid for any organism type. Utilising a standard inoculum of organism is essential if the assay is to be used for quantitative comparison of efficacy, either in a batch-to-batch study of one particular dressing or in a study of different dressings. Again, one effect of this is to distance the assay from the *in vivo* case but it is necessary to reach a compromise in experimental factors in order to achieve a quantitative outcome. We have shown that for one particular silver-containing dressing the calorimeter can quantify the efficacy of silver against *P. aeruginosa*. We would expect the approach to be valid for other dressings, organisms and growth media and present the method here so that it may be used in other cases.

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