In vitro method to assess the antimicrobial activity and potential efficacy of novel types of wound dressings.

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Running title: In vitro method to test wound dressings
SUMMARY

Aim: To develop a simple, reproducible in vitro static diffusion method using cellulose disks and defined species to test antimicrobial efficacy of wound dressings.

Methods and Results: Cellulose disks were inoculated by immersion in cell suspensions of target species *Staphylococcus epidermidis*, *Candida albicans* and *Fusobacterium nucleatum*. Test and control wound dressings were cut into equal sized squares (25 x 25 mm) and applied to the surface of 10 mm thick TYE agar on test beds. Following a 2h equilibration period, inoculated cellulose disks were inserted (one per dressing) at the interface between dressing and agar surface and a small weight applied over each square. At various sampling times, disks were removed and surviving cells enumerated by viable counts. Disk to disk variation for microbial loading was assessed using *S. epidermidis* for both initial (n=16) and standard treatment (n=16) conditions. The coefficient of variation was low (<5%) indicating good reproducibility for cell loading and treatment position on the test bed. Replicate assays (n=6) using *S. epidermidis* and Oxyzyme gels produced similar kill rates with low scatter (R² values >0.9) indicating good reproducibility between assays. Significant differences (P<0.05) in kill rates were observed for different target species, types of dressing and test bed conditions (± blood and nutrients).

Conclusions: The method is reproducible and useful in tracking the death kinetics of test species, enabling the comparison of different types of dressing.

Significance and impact of study: The reported method has significant advantages over established test procedures; it can be applied equally across a wide range of target species (including anaerobes and yeasts), a wide range of conditions, and different types of surface dressings, including those relying upon oxygen diffusion.
INTRODUCTION

A multitude of skin wounds occur in everyday life as a result of accidents, illnesses and after operations. Under various circumstances many become chronic (i.e. remaining unhealed for more than six weeks) and these are frequently complicated by infection (Bowler et al. 2001). Chronic wounds are very common and a great burden for patients, while their treatment is costly for society. Wound dressings of many types and formulations are widely used in the care of chronic wounds, and some are medicated in an attempt to control wound microflora and to limit or reduce infection (Bowler, 2002). Several types of chemical agent are used as germicidal compounds, for example silver ions, iodine, chlorhexidine or (for extreme cases) antibiotics, which diffuse from the dressing onto, or into, the wound bed, thereby inhibiting microbial growth. Recently a new type of dressing technology has been developed in the form of antimicrobial systems that incorporate biological oxidative mechanisms. For example the OXYZYME™ (oxyzyme) system uses the enzyme glucose oxidase to drive the active transport of oxygen through a polymeric hydrogel dressing, utilising hydrogen peroxide as a water soluble carrier. This biochemical process is also used as a means to generate low, controlled doses of anti-microbial iodine in situ, from iodide incorporated in the hydrogel. The levels of iodine produced in the oxyzyme version are very low (compared with the levels used in conventional iodine antiseptic products such as iodine:povidone complexes) but in the IODOZYME™ version the iodine levels are enhanced (though still not to the levels of other iodine products).

Although in vivo test methods on humans or animal models may be used to test the efficacy of wound dressings, they are very expensive and are typically only used in the last resort as a means to confirm physiological or organismal aspects of dressing performance, or to satisfy regulatory acceptance requirements prior to
approval, licensing and market. *In vivo* tests are impractical for testing the effects of every small-step change in the formulation of dressings in their early stages of design and development. For these purposes, *in vitro* tests have been developed and are widely used to measure the antimicrobial potential of dressings against named target species of microbes.

Agar diffusion methods can be used to measure zones of inhibition around test dressings placed onto lawns of indicator bacteria, grown on appropriate media (Thomas and Russel 1976; Nathan *et al.* 1978), and this method has been further developed using agar plugs to simulate the effect of a large microbiological challenge to a non-infected wound (Thomas *et al.* 1983). However it has since been argued that results from such diffusion methods are dependent on the nature of the active compounds and their formulation. Some types of compound are immobilised within the dressing matrix so may not diffuse far into the test agar and consequently show little in the way of zones of inhibition. Furthermore, both diffusion rate of active compounds and growth rate of lawn target species are highly sensitive to physicochemical effects of temperature, pH, water activity (and for diffusion) agar gel concentration and depth. The method does not distinguish between bacteriocidal or bacteriostatic effects and it is not unsurprising that the agar diffusion/zone of inhibition method can produce variable results at odds with those observed *in vivo* for potentially effective wound dressings (Andrews *et al.* 1982). Some recently developed dressings carry antimicrobial agents in a matrix that swells as it absorbs water – an effect that is of great value in treating an exuding wound. However, this causes a severe problem in the agar diffusion/zone of inhibition test, because the dressing absorbs water from the agar gel (causing shrinkage and distortion) and swells
during the time period of the experiment to obscure much (or all) of the developing clear zone.

Holland and Davis (1985) developed a suspension method whereby test dressings are held on a glass rod applied to the surface of a continually mixed suspension of indicator micro-organism. The suspension method has clear advantages over those measuring zones of inhibition since samples can be removed over time to determine accurate kill curves against a wide range of defined target species over a wide range of experimental conditions (for example in dirty conditions with the addition of blood or serum). An accurate determination of kill rates is the key to comparing different formulations, different environmental effects on killing and the susceptibility of different species. However, the method of Holland and Davis relies on aqueous diffusion to draw out the active components from the test dressing and (using a stirred vessel) is too dynamic to represent the situation in vivo. Moreover, the method would not be appropriate for gel dressings which rely upon oxygen diffusion and the integrity of the in-built biochemical mechanisms for generating the active killing agents in situ. For these, as well as conventional release dressings, an alternative testing method is proposed.

The aim of this investigation was to develop a simple, reproducible in vitro static diffusion method, using cellulose disks as a matrix in which to hold defined indicator species in close but indirect contact with the dressing under test. Organisms held in the disks are exposed to any anti-microbial effects for defined periods of time and under controlled physicochemical conditions. The organisms can be readily recovered from the disk for the quantification of survivors, thus revealing the strength of any antimicrobial effects. To offer advantages over existing techniques, the method must allow quantifiable measures of kill rate to be accurately determined within a
convenient and reproducible laboratory environment and must be applicable to a wide range of test species (including anaerobes and yeasts) and a wide range of wound dressings and conditions.

**MATERIALS AND METHODS**

**Growth and maintenance of micro-organisms**

The following species were used in the experiments: *Staphylococcus epidermidis* (laboratory typed strain isolated from human skin), *Candida albicans* NCTC 10288 and *Fusobacterium nucleatum* (subspecies *nucleatum*) ATCC 10953, all obtained from laboratory stocks. *S. epidermidis* was maintained on Nutrient Agar (Oxoid Ltd, Basingstoke, UK), *C. albicans* on Potato Dextrose Agar (Oxoid Ltd, Basingstoke, UK) and *F. nucleatum* on Fastidious Anaerobe Agar (Oxoid Ltd, Basingstoke, UK) supplemented with 5% defibrinated horse blood (TCS Biosciences, Buckingham, UK). Broth cultures of *S. epidermidis* and *C. albicans* were grown in 1% tryptone-0.5% yeast extract and *F. nucleatum*, in brain-heart infusion broth. *S. epidermidis* and *C. albicans* were incubated at 37°C aerobically (Genlab M1005L incubator, Cheshire, UK), and *F. nucleatum* was incubated at 37°C anaerobically (MK3 Anaerobic work station, Don Whitley Scientific, Shipley, UK). All cultures were sub-cultured weekly.

**Experimental wound dressing**

The wound dressings tested were oxyzyme and iodozyme hydrogels (Insense, Bedford, UK), and Elastoplast™ plasters (Beiersdorf AG, Hamburg, Germany). The oxyzyme hydrogels comprised a 100 x 100 x 1.5 mm first polymeric hydrogel (the wound contact layer containing glucose and potassium iodide) and a second polymeric hydrogel measuring 60 x 60 x 1.4 mm (containing entrapped glucose
oxidase), placed on top of the wound contact layer. Once the two layers are brought into contact with each other, glucose diffuses into the entrapped enzyme layer where it is oxidised to form gluconate and hydrogen peroxide, which further reacts with iodide to form antimicrobial iodine. The iodozyme hydrogels also comprised two layers of similar construction, but these were supplied in two formulations with different, elevated iodide concentrations (identified as Test Hydrogels 401 & 402) to give enhanced antimicrobial iodine levels. Both types of iodozyme comprised of a 100 x 100mm glucose and iodide wound contact layer and a 100 x 100mm enzyme surface layer. Iodozyme Test Hydrogel 402 had a higher iodide concentration than Test Hydrogel 401. The Elastoplast™ plasters comprised an adhesive surface (which was removed) surrounding a pad (size 100 x 25 mm) impregnated with the antiseptic chlorhexidine digluconate. All wound dressings were supplied in their standard packaging, and cut into square sections (25 x 25 mm) immediately prior to use.

**Disks and disk inoculation**

Disks (11.2 mm diameter to give an area of 1 cm²) were cut from sheets of cellulose filter paper (Size 1, Whatman International Ltd., Maidstone, UK) using dissection scissors. Disks were sterilised by UV irradiation (G15 T8 UV lamp, Toki Corporation, Japan) for 20 minutes at a distance of 50 cm from lamp to disk. Test cell suspensions were prepared from neat 24h broth cultures of *S. epidermidis*, 48h broth cultures of *C. albicans* and 72h broth cultures of *F. nucleatum*, diluted if necessary with sterile broth to give optical density readings between 0.5 and 0.7 using a 1cm light path CE-373 linear readout grating spectrophotometer (Cecil Instruments, Cambridge, UK) at wavelength 540nm. UV-sterilised 1cm² cellulose disks (n=32) were then immersed in vortex mixed test cell suspensions for 5 minutes, removed into
a sterile plastic petri-dish and excess fluid removed from each disk by sterile blotting. All disks then contained approximately the same numbers of viable cells at about $10^7$ cfu per disk.

**Test Method**

Test and control wound dressings (n=16, 25 x 25 mm) were evenly distributed on to the surface of 10mm thick nutrient agar made from tryptone (1.0% w/v), yeast extract (0.5% w/v) (TYE) and pure agar (Oxoid Ltd, Basingstoke, UK) at a concentration of 1.0% (w/v). Media was sterilised, poured and set within large square assay plates (235mm x 235mm). For some experiments the nutrient components were replaced by PBS in the same strength agar. Test hydrogel wound dressing samples comprised the iodide + glucose wound contact layer and the enzyme surface layer placed on top, whereas the control hydrogel wound dressing samples (control 1) comprised only the iodide + glucose wound contact layer without the surface enzyme layer (thus preventing generation of hydrogen peroxide and iodine). Sample positions were determined by reference to a grid matrix (intersection of rows and columns) and the sequence of samples determined according to latin square principles. The test plates loaded with dressing samples were pre-incubated for two hours to allow for initial gel swelling of about 20 % (w/w) and the establishment of reaction-diffusion equilibration of ROS flux prior to the introduction of the test disks containing equivalent numbers of target species. These microbe-laden test disks were placed between the nutrient agar layer and topical wound dressings of both test and control, the components being manipulated with sterile forceps. Uncovered disks (n=16) were also placed onto the test plates containing equivalent numbers of target species as an additional uncovered control (control 2). Small metal weights (12 g) on nylon mesh
pads were then applied onto the top surface of each hydrogel square in order that contact between gel, cellulose disk and agar surface was not disturbed with further gel swelling (of approximately 20% over 12 h), following the initial equilibrium stage. The large plate assay with disks and gels was incubated at 25°C for the duration of the experiments.

Test bed sampling
At various sampling times, wound-dressing squares were lifted in order to remove test or control disks (using sterile forceps). One of the uncovered control disks was also removed at the same sampling time. Each sample disk was added to a 10 ml volume of sterile PBS containing 0.05% (w/v) Na$_2$S$_2$O$_3$ as I$_2$-neutralizer and vortex mixed for 1 minute to release surviving cells in suspension. Neat suspensions were serially diluted in a 1:10 dilution series down to 10$^{-3}$ in PBS. Viable counts were then performed of neat and diluted suspensions using a spiral plater (Don Whitley Scientific, Shipley, UK) on to appropriate recovery medium to determine the numbers of survivors at different time points. Plates were incubated under appropriate conditions (37°C aerobically or anaerobically) and the number of survivors determined as colony forming units per sample (ie. cfu cm$^{-2}$ since the disks have a surface area of 1cm$^2$).

Effect of nutrients on Oxyzyme activity
To test the effect of the presence of nutrients and blood on kill rates of S. epidermidis within the test bed environment, four different types of assay plate agar were used: 1% w/v agar in phosphate buffered saline (PBS; the control for this experiment), 1% w/v agar in TYE, TYE + 1% defibrinated horse blood, and TYE + 10% defibrinated
horse blood. This experiment was conducted only on the very low iodine version, oxyzyme.

**Analysis of results**

For each test species and each condition, plots were made of log numbers of survivors over reaction time. Kill rates were determined by linear regression, enabling direct comparison of the results over time for the test and two controls for each target species. Graph construction, statistical analyses, and modelling were conducted with the use of GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA). An Analysis of Covariance (ANCOVA) was used to ascertain whether there was a significant difference between rates. A P value of <0.05 was regarded as significant. In order to take into account any change in cfu cm$^{-2}$ within control samples and to allow direct comparison of all test organisms and conditions, log plots were also constructed using the following formula (Cove and Holland, 1983): 

$$Y = (\log \frac{N_t}{N_0})_{\text{test}} - (\log \frac{N_t}{N_0})_{\text{control}}$$

Where: $N_0 =$ cfu per cm$^{-2}$ at zero time and $N_t =$ cfu cm$^{-2}$ at later time (t)

As for the initial plots, linear regression of the log number of survivors over time was used to determine kill rates and an Analysis of Covariance performed.

**RESULTS**

The oxyzyme wound dressings exhibited a significant antimicrobial effect against all three of the microorganisms tested. To analyse the reproducibility of the disk loading, disk to disk variation was calculated using *Staphylococcus epidermidis* before and after treatment exposure (Table 1). The log mean cfu cm$^{-2}$ following treatment was lower, as is expected if the treatment is effective, but the SD was higher. This increase
in variability following treatment indicates a lack of uniformity in terms of position on the test bed and, therefore, in, equivalence of treatment. The increase in variance is, however, still well within acceptable levels (< 3% variance) showing the method to have achieved good disk to disk reproducibility. With regard to inter-assay reproducibility, the data in table 2 and figure 1 shows the mean kill rate for *Staphylococcus epidermidis* from six independent repeat assays, conducted on different days. The kill rate, K, was calculated to be -0.349 and the goodness of fit (R² = 0.903) shows low scatter, indicating close reproducibility between assays. When log numbers of survivors were plotted (fig 1), *Staphylococcus epidermidis* exhibited an accelerating kill rate (fig 1). However, for the purposes of statistical comparison with other conditions and test species, simple linear regression of all data points was used to calculate the mean kill rate shown in table 2.

The death kinetics for *C. albicans* are shown in fig 2. The kill rate (K= -0.224) corresponded to a D-value of 4.466 hours and was significantly different (P<0.0001) from either no change or that observed for either of the two control conditions. The death kinetics for *F. nucleatum* are shown in fig 3. All three sample conditions (test and both controls) caused kill rates, but all kill rates were significantly different from each other (p<0.0001), with the test conditions giving the highest kill rate (K= -3.657) corresponding to a D-value of 0.273 hours.

All test data were compiled to create a comparative graph of the kill rates for all three test organisms (fig 4) using the formula stated in the method, which accounts for any population changes occurring within control samples. All kill rates were significantly different (P<0.0001) and hence the order of sensitivity to the oxyzyme test dressings was *F. nucleatum, S. epidermidis, C. albicans*. 
The effect of nutrients and blood added to the test bed environment (to emulate contaminated conditions, as in a wound) on the antimicrobial activity of the very low iodine version, oxyzyme, was to reduce the kill rate to below that obtained in the control medium (PBS agar). The reduction compared to control was 56.16% by TYE, 89.34% with the addition of 1% blood and 99.22% with the addition of 10% blood (fig 5).

The death rate constant for *S. epidermidis* treated with the three test hydrogel wound dressing formulations (oxyzyme, iodozyme 401 & iodozyme 402) and the test dressing impregnated with chlorhexidine digluconate are shown in table 3. All wound dressings gave kill rates significantly different (p<0.001) to either of the two controlled conditions, and all kill rates were significantly different from each other (p<0.001). The order of efficacy of the dressings was; iodozyme 402, iodozyme 401, chlorhexidine dressing and oxyzyme.

**DISCUSSION**

The *in vitro* static diffusion method described was capable of measuring the killing effect of hydrogel wound dressings against all test organisms. Variance of the disk population of survivors appears to increase with treatment time but the level of variance was still low, showing good disk to disk reproducibility. The assay to assay consistency was also high as indicated by the high $R^2$ values which measure the goodness-of-fit (or scatter) of all the points from repeated runs. The ability to follow the death kinetics of the eukaryotic yeast *C. albicans* indicates the usefulness of the method for testing the efficacy of wound dressings against yeast infection. *C. albicans* has a cell size 20 to 30 times larger than bacterial cells and it was killed at a slower rate throughout the assay period. The slower rate of for *C. albicans* compared with *S.
*epidermidis* has been observed previously during photodynamic treatment with the same test strains (Zeina *et al.* 2001). The anaerobe *F. nucleatum* was killed at the highest rate. Although aerobic conditions alone (control 1 and 2) produced a killing effect, this background rate was less than that induced by the active test dressing, so the difference between control and test rates can be ascribed to the antimicrobial efficacy of the dressing. It was apparent that control 1 (iodide + glucose wound contact layer without the surface enzyme layer) produced a higher kill rate than control 2, possibly due to oxygen and the inactive iodide in the test dressing being slowly oxidised to form trace levels of iodine, perhaps by the test microbial cells. With the majority of wounds being poly-microbial, involving aerobes and anaerobes it is important to encompass anaerobes in the determination of the antimicrobial activity and efficacy of any novel type of wound dressings.

The kill rate from oxyzyme (which is the very low iodine version) was extremely dependent on the organic composition of the killing environment. Antimicrobial activity was reduced in the presence of nutrients (TYE) and blood. Nutrient-rich conditions are likely to reduce the bactericidal activity by acting directly against the antimicrobial agents or indirectly by affecting the physiological state of the test organism (Leeming *et al.* 1986). PBS would provide the least interference with any antimicrobial effects and would, therefore, be the most sensitive and most useful for testing minor differences between dressing formulations. Media rich in organic solutes such as TYE, blood and serum would be useful for testing the ability of dressings (such as the higher iodine versions iodozyme 401 and 402) designed to work on wounds with a high degree of blood or serous discharge.

To show that the method could be applied to different types of skin dressing, three different hydrogel formulations (oxyzyme, iodozyme 401 & 402) and a
conventional wound dressing impregnated with chlorhexidine digluconate were
tested. Kill rates were clearly discriminated in each case. As expected, kill rates
increased with increases in the concentration of iodide in the hydrogel wound
dressing. These results illustrate how the method can be used to compare different
types and/or formulations of wound dressings and which in principle could be applied
to any active test surface. This opens up numerous applications, for example
monitoring the loss of antimicrobial efficacy on storage under different conditions.

*In vitro* methods that depend upon zones of inhibition from seeded lawn
growth of test organisms only give semi-quantitative data (no kill rates) and conflate
both static and killing properties of the surface dressings. The later development of
the aqueous diffusion model (Holland *et al.* 1985) was advantageous since it enabled
the determination of comparable kill rates within a controlled system. However both
systems are limited in their application in testing surface dressings, for example being
totally inappropriate for those which are reliant on oxygen diffusion. A method using
a polyurethane sponge within a static diffusion model has been described
(Grzybowski *et al.* 1996). However there is insufficient information from this report
to show variability, kill rates or effectiveness of the test method using anaerobes
and/or dressings requiring oxygen diffusion.

The method described here, using cellulose filter disks inoculated with
indicator species placed under the test dressings, offers significant advantages over
those already in existence. It allows accurate, quantifiable and comparable kill rates to
be determined within a convenient and reproducible *in vitro* static diffusion test-bed
system. This method can be applied equally across a wide range of target species
(including anaerobes and yeasts), a wide range of conditions, and against different
types of surface dressings, including those relying upon oxygen diffusion.
REFERENCES:


**Table 1.** Disk to disk reproducibility prior to and following equal 5 hour treatment exposure by oxyzyme hydrogel wound dressing against *Staphylococcus epidermidis*.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Number of samples (n)</th>
<th>Log mean cfu per cm$^2$ ± Standard Deviation (SD)</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment (Zero Time)</td>
<td>16</td>
<td>7.064 ± 0.061</td>
<td>0.867</td>
</tr>
<tr>
<td>Equal Treatment (5h)</td>
<td>16</td>
<td>6.230 ± 0.170</td>
<td>2.731</td>
</tr>
<tr>
<td>Test Organism</td>
<td>Kill Rate* (K = slope) (log cfu reduction h^{-1}) (mean ±SD)</td>
<td>N</td>
<td>R^2</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------------------------------------------</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>-0.349 ± 0.100</td>
<td>6</td>
<td>0.903</td>
</tr>
<tr>
<td>C. albicans</td>
<td>-0.224 ± 0.017</td>
<td>3</td>
<td>0.976</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>-3.657 ± 0.517</td>
<td>3</td>
<td>0.943</td>
</tr>
</tbody>
</table>

* All slopes are significantly non-zero (P < 0.0001) and significantly different from controls and each other (P < 0.001).
Table 3. The death rate constant for *S. epidermidis* treated with the three test hydrogel wound dressing formulations and a conventional test dressing impregnated with chlorhexidine digluconate.

<table>
<thead>
<tr>
<th>Test Dressings</th>
<th>Kill Rate* ($K = \text{slope}$) (mean ± SD)</th>
<th>$R^2$</th>
<th>D Value (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxyzyme</td>
<td>-0.342 ± 0.038</td>
<td>0.954</td>
<td>2.924</td>
</tr>
<tr>
<td>Iodozyme 401</td>
<td>-1.333 ± 0.228</td>
<td>0.919</td>
<td>0.750</td>
</tr>
<tr>
<td>Iodozyme 402</td>
<td>-2.524 ± 0.310</td>
<td>0.971</td>
<td>0.396</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>-0.491 ± 0.064</td>
<td>0.894</td>
<td>2.037</td>
</tr>
</tbody>
</table>

* All slopes are significantly non-zero ($P < 0.0001$) and significantly different from controls and each other ($P < 0.001$).
FIGURE LABELS

Figure 1. Death kinetics of *S. epidermidis* treated with an oxyzyme hydrogel. Test treated with active hydrogel (▲), control with inactive hydrogel (□), untreated control (Δ), hatched line shows minimum level of detection. Lines of best fit on the test data relate to a linear regression of data points and error bars show ± SD (n=6).

Figure 2. Death kinetics of *Candida albicans* treated with an oxyzyme hydrogel. Test treated with active hydrogel (▲), control with inactive hydrogel (□), untreated control (Δ), hatched line shows minimum level of detection.

Figure 3. Death kinetics of *Fusobacteium nucleatum* treated with an oxyzyme hydrogel. Test treated with active hydrogel (▲), control with inactive hydrogel (□), untreated control (Δ), hatched line shows minimum level of detection.

Figure 4. Comparative graph of the death kinetics of *S. epidermidis* (■), *C. albicans* (▲) and *F. nucleatum* (●) treated with an oxyzyme hydrogel dressing.

Figure 5. The effect of nutrients on the survival of *S. epidermidis* treated with an oxyzyme hydrogel dressing over a 24 hour period. PBS control (■), TYE (♦), TYE + 1% Blood (▼), TYE + 10% Blood (▲).