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A novel approach to antibiofilm susceptibility testing using a thermo-reversible matrix

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Abstract

Biofilm microorganisms are known to have a much higher tolerance to antimicrobials compared to their planktonic equivalents. As a result, traditional antimicrobial susceptibility testing may not extrapolate to clinical treatment of infections of biofilm origin. As a result, there is a need to not only develop antimicrobials with antibiofilm activity, but also suitable *in vitro* testing methods for their evaluation. In this study, we report on a novel method of antibiofilm testing using a thermo-reversible matrix (Poloxamer 407), coupled with live/dead staining of bacteria cultured from the matrix. *Pseudomonas aeruginosa* (NCIMB 8626) was cultured in medium containing Poloxamer 407 at 37°C for 24 h to generate biofilms. The preparation was cooled to liquefy the poloxamer and allow recovery of the biofilm cells, which were then stained with Syto9 to determine viability following exposure to four antimicrobials (polyhexanide, octenadine dihydrochloride, providone-iodine and silver carbonate). Over an 8 min time period, fluorescence levels were spectrophotometrically measured and compared with bacterial controls, cultured in the absence of poloxamer and without antimicrobial. Untreated cells showed no reduction in viability over this period. Importantly, planktonic cells were more susceptible to test agents compared with those of a 'biofilm' phenotype cultured in Poloxamer. Antibiofilm activity was evident for all of the test agents, with highest relative activity seen with octenadine dihydrochloride. In summary, a novel and relatively rapid approach to screen compounds for antibiofilm activity has been described. The method uses standard laboratory equipment and can be readily adapted to test a wide range of microorganisms and other antibiofilm compounds.

Introduction

Biofilms are defined as microbial communities, often attached to a solid surface, where the microbial cells are embedded within a self-generated extracellular polymeric matrix.¹ Such biofilm cells have properties that are distinct from their 'free-living' or planktonic counterparts that typically grow as individual organisms in a liquid environment. A defining feature of biofilm cells is their increased tolerance to many antimicrobial agents² and host defence molecules.

In recent decades, it has become apparent that a significant number of human infections have a biofilm origin. Importantly, in the case of hospital-acquired infections, reports have indicated that over 65% of these are caused by biofilms.¹ In addition, biofilms on medical devices frequently serve as a reservoir of infectious organisms, which are often protected from eradication by host defences or antimicrobials because of their location away from vascularised tissue.

Biofilm cells are known to be more tolerant of antimicrobial agents compared with planktonic equivalents. The reasons for this are complex, multifactorial and in part, relate to the surrounding EPS matrix, which may serve to sequester or neutralise antimicrobial agents, or promote the adaptation of cells to a phenotype that renders them more tolerant. Cells embedded in the biofilm also demonstrated variable growth rates and metabolic activity at different locations within the biofilm, or exhibit differential expression of resistance related genes when in a biofilm.²³

In recent years, there has been significant interest in the involvement of biofilms in impairing wound healing.^{3,4} Chronic wounds are defined as those that do not progress through an expected and orderly reparative process over a 3 month period.⁵ The presence of a recalcitrant biofilm could impede normal wound healing by actively producing hydrolytic enzymes that induce tissue damage, or promote a continuous state of inflammation leading to damage and an inappropriate healing responses.^{6,7} As a consequence, there is urgent need to develop novel antibiofilm compounds with the capacity to be incorporated into wound dressing technology to disrupt wound biofilms and reduce healing time for patients.⁸ With this focus, there is an associated need to produce simple and rapid methods, which can be used to assess the effectiveness of novel compounds against biofilms.⁹

Currently there is a range of broad-spectrum antimicrobial agents which can be incorporated into wound dressings, the most common being various analogues of silver, such as nanocrystalline silver, silver sulfadiazine and ionic silver. Other broad-spectrum wound care antimicrobials include iodine, chlorhexidine, polyhexamethyl-biguanide (PHMB), honey, acetic acid and potassium permanganate, which can be impregnated into the wound dressing itself or in some cases, used as a solution for irrigation prior to dressing application.

To date, increasing numbers of investigations have used different *in vitro* and *in vivo* models to examine the efficacy of common (and novel) wound antimicrobials to disrupt and kill wound biofilms.

The 'gold standard' type of wound biofilm model could be seen as an animal model, or human patient with a chronic, non-healing wound. These models would naturally replicate the exact environmental conditions and surface sub-strata that would promote biofilm development. However, the use of *in vivo* modelling inherently contains uncontrollable elements of the host *i.e.*, immune function, stress levels and mobility rates. In addition, supporting an *in vivo* model, such as a mouse, rat or pig can be very costly in terms of maintaining the minimum standards expected by animal welfare regulations; likewise, a human patient with a chronic wound will require regular visits and commitment from a wound health specialist for treatment and monitoring. In either case, *in vivo* modelling brings with it a range of added complications, costs and time constraints associated with ethical research.

There are a variety of *in vitro* models which have been employed to assess the efficacy of wound care technologies at disrupting infective biofilms. Thorn *et al.*,¹⁰ developed a model that utilised a flow-cell that allowed perfusion of simulated wound fluid through a porous matrix on which the biofilm cells had attached, thereby allowing the researcher to measure the antibiofilm effects of novel wound dressings. This type of system however, requires peristaltic pumps, bespoke containers and incubators for analysis, which significantly increase the initial setup costs.

Other methods to assess the properties of antibiofilm compounds have used 'off the shelf' devices such as the Calgary Biofilm Device (CBD) or similar microtitre plate-based systems.^{11,12,13} With the CBD, biofilms of a particular microbial species are cultured on a range of pegs inserted into a microtitre plate with growth media and once developed, the pegs (and biofilm) can be removed and subjected to a range of antimicrobial susceptibility testing. A key benefit of using this type of device is the number of simultaneous replicates that can be performed due to the device developed around a standard 96-well plate model.

To date, there has been accumulating interest in the use of poloxamer polymers for biofilm research. Poloxamers are inert, non-ionic, co-polymers, which can be dissolved in standard bacteriological culture media such as Muller-Hinton Broth or Tryptone Soya Broth. A key feature of poloxamers is their thermo-reversible properties. When dissolved in a medium at a temperature below 15°C (approximately), poloxamers are liquid; however, increasing the temperature above 15°C, causes the poloxamer to transform to a semi-solid gel, which can then be re-liquefied by lowering the temperature below 15°C. Importantly, it has already been reported that microorganisms cultured in a semi-solid poloxamer matrix form micro-colonies exhibiting a biofilm phenotype with increased tolerance to disinfectants, antimicrobials and silver containing wound dressings.^{14,15,16} The thermo-reversible nature of poloxamers, coupled with their ability to induce a biofilm phenotype,

makes it an attractive material to investigate the efficacy of antibiofilm compounds. In this present investigation, we have evaluated the use of poloxamer within a 96-well plate model as a novel screening approach to assess antibiofilm activity of 4 different compounds previously used in the management of chronic wounds.

Materials and methods

All microbiological culture media and reagents were supplied by Oxoid Ltd (Basingstoke, Hampshire, UK) unless otherwise stated and were prepared as recommended by the manufacturer. Poloxamer 407 (Lutrol F127; Sigma, Poole, UK) for use in biofilm development was prepared at 30% (w/v) in Tryptic Soy Broth (TSB). The medium was sterilised in 250-ml volumes by autoclaving, and then liquefied at 5°C. Sodium Chloride Peptone Broth (SCPb) was also sterilised by autoclaving.

Preparation of microbial biofilms in Poloxamer 407

An overnight culture of *Pseudomonas aeruginosa* (NCIMB 8626) was prepared in TSB at 35°C in a humidity controlled incubator. Dilution of this culture in TSB was performed to give a standardised optical density (OD) of 0.8 at 620_{nm}. A 100-µl volume of the diluted culture was inoculated into 10 ml of liquefied (chilled) poloxamer-TSB medium and vortex mixed to ensure homogenous cell distribution. The viable cell count of *P. aeruginosa* in the poloxamer matrix was approximately 10⁵ colony forming units (cfu)/ml, as determined by quantitative culture on solid agar media. Two hundred and fifty-µl volumes of inoculated poloxamer-TSB medium were added to 1.5 ml microcentrifuge tubes and these were incubated at 35°C in a humidity-controlled incubator for 24 h to generate biofilm phenotypes. Planktonic control cultures of *P. aeruginosa* NCIMB 8626 were similarly prepared in TSB medium devoid of poloxamer.

Recovery of biofilm cells

After 24 h incubation, the microcentrifuge tubes were 'flash cooled' at -70°C for 2-3 min to liquefy the poloxamer. A 500-µl volume of chilled (5-6°C) SCPb was added to each poloxamer biofilm and mixed by repeated pipetting (this step prevented the poloxamer from re-solidifying when returned to room temperature). Bacteria were then harvested by centrifugation (3000 g for 5 min).

Susceptibility testing

Test antibiofilm compounds were prepared in distilled water and were 0.1% (v/v) polyhexanide (Arch UK Biocides Ltd, Castleford, UK), 0.1% (v/v) octenadine dihydrochloride (Shulke, Sheffield, UK), 1% (w/v) providone-iodine (Vetasept, Animal Care Ltd, York, UK) and 1% (w/v) silver carbonate (Fisher Scientific Ltd, Loughborough, UK).

A 100- μ l volume of test antibiofilm was added to the recovered bacterial cell pellets and incubated at 18°C for 1 min periods to a maximum of 8 min. Negative controls used 100 μ l of sterile deionised water in place of the antibiofilm agent. After incubation, 1 ml of neutralising solution (lecithin NaCL buffered peptone; Heipha GmbH, Eppelheim, Germany) was added for polyhexanide and octenadine dihydrochloride treated samples, whilst sodium thioglycolate was used as a neutraliser for both silver carbonate and iodine.

The treated cells were centrifuged and washed with SCPB to remove any remaining neutraliser and re-suspended in 100 μ l of SCPB together with 100 μ l of Syto9 live stain (Baclight, Life Technologies, Paisley, UK) prepared according to manufacturer's instructions. Incubation with Syto9 was for 15 min in the dark and at room temperature. Two 100- μ l volumes were then transferred to separate wells of a black-clear bottom 96 well plate. The plate was scanned using an optical plate reader (Varioskan; Thermo-scientific, Loughborough, UK) measuring excitation/emission at 480/500 nm at 7 separate points in each well and the data captured using dedicated software.

Statistical analysis

The raw data from the Syto9 fluorescence emission (at 500 nm) was exported to excel (Microsoft). The data was normalised against the mean time zero readings for the negative controls and the percentage reduction in fluorescence over the 8 min period of analysis calculated. Statistical analysis performed using GraphPad Prism (GraphPad Prism 5.01, California, USA).

Results

Figure 1 illustrates the results of reduction in fluorescence (reduced viability) of *P. aeruginosa* following exposure to 0.1 % polyhexanide. It was evident that there was no reduction in viability of *P. aeruginosa* for either planktonic or biofilm control preparations in the absence of the antimicrobial. Both planktonic and biofilm preparations treated with 0.1% polyhexanide showed increasing reduction in viability over the 8 min exposure period and the extent of this antimicrobial effect was equivalent for both planktonic and biofilm preparations.

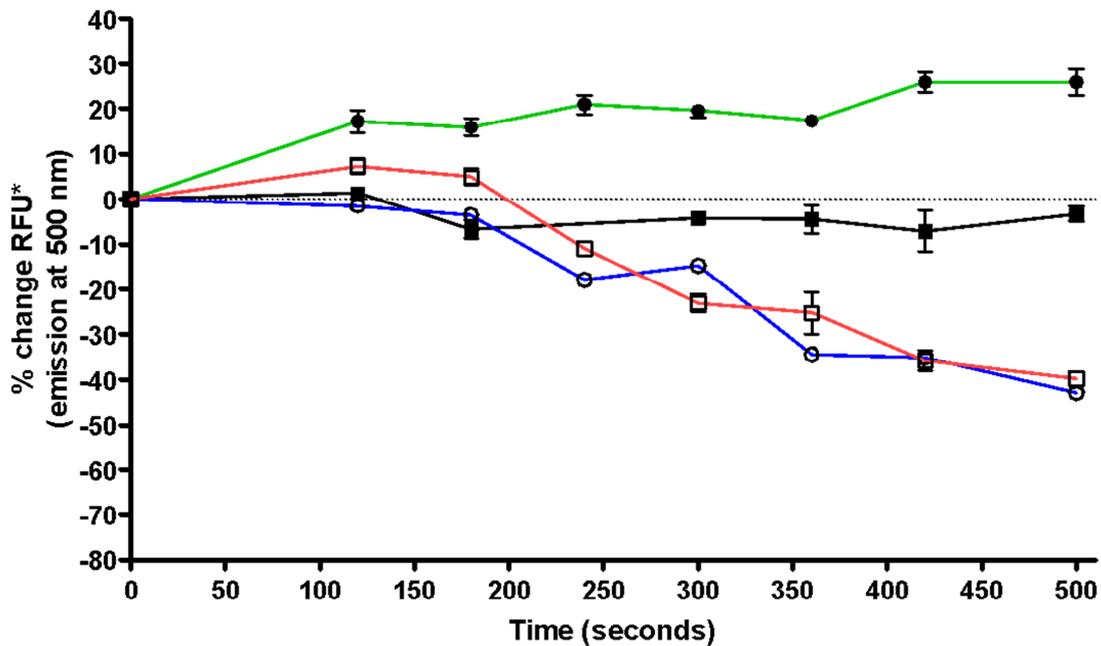


Figure 1. Reduction in fluorescence caused by 0.1% polyhexanide from viable *P. aeruginosa* cells stained with Syto9. Solid black line (▪) = biofilm control (TSB + 30% poloxamer + Sterile H₂O). Blue line (○) = biofilm test (TSB + 30% poloxamer + polyhexanide). Green line (●) = planktonic control (TSB + Sterile H₂O). Red line (□) = planktonic test (TSB + polyhexanide). Error bars show ± SEM (n=14; 2 biofilm/planktonic samples read at 7 different spatial points). *Relative Fluorescence Units.

Interestingly, in the case of treatment with both 0.1% octenadine dihydrochloride (figure 2) and 1% silver carbonate (figure 3), whilst both biofilms and planktonic preparations were killed by the antimicrobials, there was a notable delay in antimicrobial effect against the biofilms preparations. Treatment using 1% providone-iodine (Figure 4) again showed reduced antimicrobial activity against biofilm derived cells, however this was not seen as a delayed effect, but was evident by a greater reduction in planktonic cell viability at all time points.

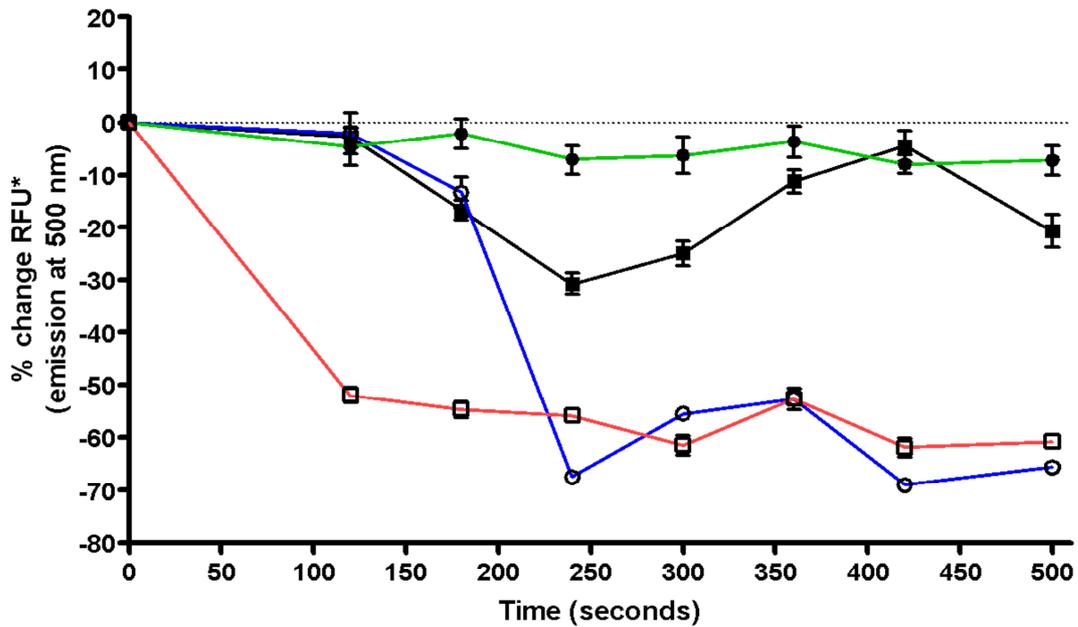


Figure 2. Reduction in fluorescence by 0.1% octenadine dihydrochloride from viable *P. aeruginosa* cells stained with SYTO9. Solid black line (▪) = biofilm control (TSB + 30% poloxamer + Sterile H₂O). Blue line (○) = biofilm test (TSB + 30% poloxamer + octenadine dihydrochloride). Green line (●) = planktonic control (TSB + Sterile H₂O). Red line (□) = planktonic test (TSB + octenadine dihydrochloride). Error bars show ± SEM (n=14; 2 biofilm/planktonic samples read at 7 different spatial points). *Relative Fluorescence Units.

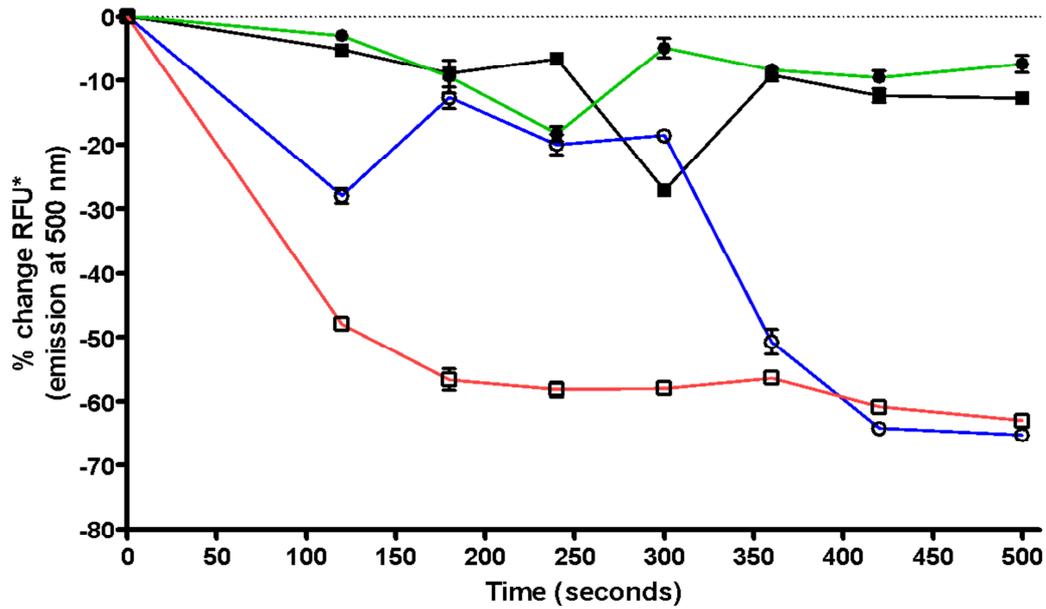


Figure 3. Reduction in fluorescence by 1% silver carbonate from viable *P. aeruginosa* cells stained with SYTO9. Solid black line (▪) = biofilm control (TSB + 30% poloxamer + Sterile H₂O). Blue line (○) = biofilm test (TSB + 30% poloxamer + silver carbonate). Green line (●) = planktonic control (TSB + Sterile H₂O). Red line (□) = planktonic test (TSB + silver carbonate). Error bars show ± SEM (n=14; 2 biofilm/planktonic samples read at 7 different spatial points). *Relative Fluorescence Units.

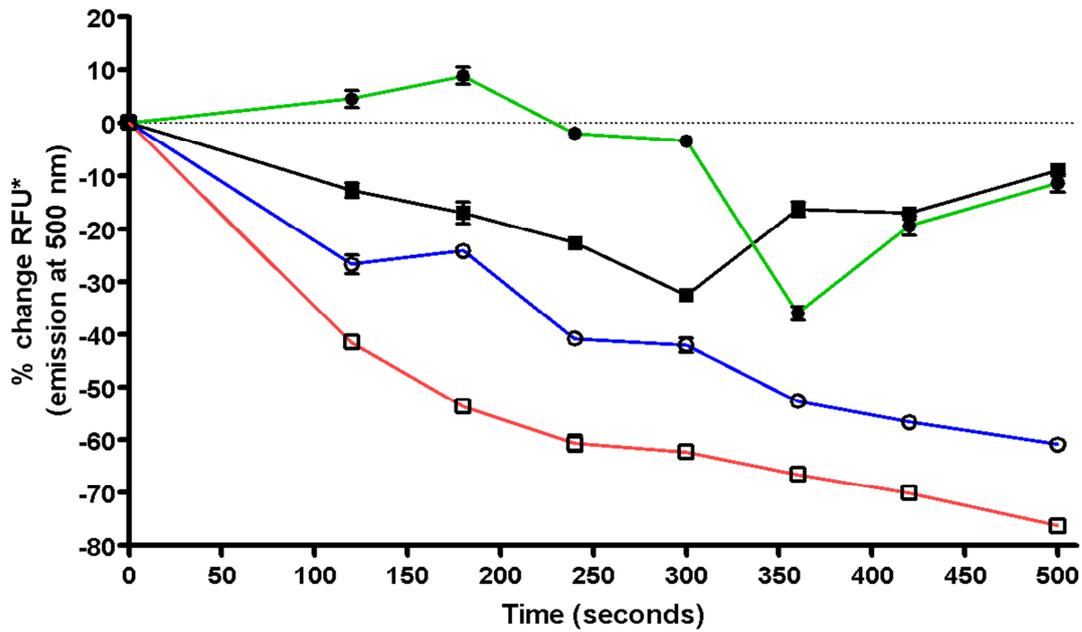


Figure 4. Reduction in fluorescence by 1% providone-iodine from viable *P. aeruginosa* cells stained with SYTO9. Solid black line (▪) = biofilm control (TSB + 30% poloxamer + Sterile H₂O). Blue line (○) = biofilm test (TSB + 30% poloxamer + Providone-iodine). Green line (●) = planktonic control (TSB + Sterile H₂O). Red line (□) = planktonic test (TSB + providone-iodine). Error bars show \pm SEM (n=14; 2 biofilm/planktonic samples read at 7 different spatial points). *Relative Fluorescence Units.

Discussion

In recent years, the treatment of microbial infections has become increasingly problematic due to the emergence of microbial species with an acquired resistance to over-prescribed antibiotics. This antimicrobial resistance has primarily arisen due to selective pressures of excessive and non-targeted use of antibiotics as part of clinical therapy.¹⁷ In addition, with increasing use of implanted medical devices in modern medicine, such as prosthetics, intravenous and urinary catheters, the prevalence of biofilm-originating infections has also increased.

The reason why biofilms are more tolerant to antimicrobials is multifactorial and most likely relates to the poor penetration of the agent through the biofilm matrix (due to diffusion limitation and sequestration) or inactivation of the agent by the EPS of the biofilm. Even if the agent can access the biofilm cells, it is thought that low metabolic activity of members of the biofilm population or those actually expressing an altered and more resistant phenotype (*i.e.* persister cells) are able to survive. To counter these problems, there is clearly an urgent need to develop novel antimicrobial therapies that also exhibit activity against biofilms. Of course, methodology that allows the efficacy of antimicrobial function to be assessed against biofilms is a necessary component of this antimicrobial development.

Chronic wounds are defined as those that do not heal in an orderly fashion and typically beyond a 3-month time frame.⁵ The impact of chronic wounds is not limited to patient morbidity, but also increases healthcare costs. It was reported that in 1999, annual costs associated with chronic wounds in the USA alone, exceeded \$1 billion.¹⁸ This cost is continually increasing given the changing demographics of societies, where the over 80 year olds represent is the most rapidly expanding age group.

In recent years, the occurrence of biofilms within wounds has been implicated with impaired wound healing.^{19,20} The exact reasons why biofilms delay wound healing remain to be established. It has, however, been proposed that production of microbial virulence factors in the wound environment, such as hydrolytic enzymes and toxins, directly promote tissue damage.²¹ In addition, a continual stimulation of the host's immune response through wound colonisation by a recalcitrant biofilm may also lead to indirect tissue damage from the immune factors and cells.

Pseudomonas aeruginosa is an opportunistic bacterial pathogen and one that is adept at biofilm formation.²² This bacterial species is also renowned for its high resistance to many antimicrobials and the fact that it can also produce biofilms exacerbates this problem. *Pseudomonas aeruginosa* is a frequent coloniser of wounds and also has been implicated with chronic wounds and as such, this species was seemed to be an appropriate model organism to use in the present investigation.

Given the problems outlined above, this present study investigated a novel approach for the screening of antimicrobials that might exhibit activity against *P. aeruginosa* biofilm cells.

These 'biofilm' cells were cultured within a thermo-reversible gel matrix using Poloxamer 407, which has been previously proven to induce a biofilm phenotype. Test antimicrobials were those that have previously been used to treat wound infections. The observation that 3 of the 4 test agents (*i.e.* 0.1% octenadine dihydrochloride, 1% silver carbonate and 1% povidone iodine) exhibited reduced activity for *P. aeruginosa* cultured in the Poloxamer matrix compared to planktonic culture would indeed indicate successful generation of bacteria with a biofilm phenotype.

In the case of both octenadine dihydrochloride and silver carbonate, increased periods of antimicrobial exposure were required to generate equivalent antimicrobial effects as those evident with planktonic cultures. The reason for this is unclear, but could relate to factors such as alterations in cell wall structures; or the presence of EPS surrounding the cell which can either prevent antimicrobial contact with the cell membrane or limit penetration itself into the cell.

A different profile of tolerance was seen for biofilm cells exposed to povidone iodine (figure 4). In these cases, at any given time point a higher proportion of viable cells was evident for the biofilm cells compared with the planktonic cells. It is tempting to speculate here that a subpopulation of cells (*i.e.* persister cells) is present in the biofilm that were able to resist the effects of 1 % povidone iodine. Further investigation, with longer exposure periods would be needed to test this hypothesis. Interestingly, comparable antimicrobial activity for both biofilm and planktonic cells was evident with 0.1 % polyhexanide (figure 1), suggesting that an antimicrobial mechanism could be present that was unaffected by the biofilm phenotype generated by the poloxamer.

There are clear benefits to this novel antimicrobial screening method. Key amongst these are that cells of a biofilm phenotype can be tested and a more relevant antimicrobial efficacy determined against likely wound colonisers. However, during these experiments some potential limitations were noted, such as unknown interactions between the antimicrobial and the Syto9 stain. It is quite possible that certain antimicrobials *i.e.* bleach based disinfectants or surfactants, may have unwanted reactions with fluorescent stains, potentially reducing the data accuracy. Overall, this method was able to screen multiple antimicrobials and can readily be expanded to culture other microbial species.

Standard laboratory materials were used in this research, although there is the need for a spectrophotometer that can detect Syto9 fluorescence. It is envisaged that with the anticipated advent of newer antimicrobial agents for use in wound care, the described methodology will aid manufacturers in identifying agents with antibiofilm properties. Overall this will ultimately benefit the patient and clinicians through a better understanding

of the biofilm/antimicrobial interaction, thus reducing the healing time and costs to the health service.

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