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Microemulsions are highly effective anti-biofilm agents

I.S.I. Al-Adham[1], N.D. Al-Hmoud[2], E. Khalil[3], M. Kierans[4] and P.J. Collier[2]

[1]School of Pharmacy & Medical Technology, University of Petra, Amman, Jordan, [2]School of Contemporary Sciences, University of Abertay Dundee, Dundee, Scotland, UK, [3]Faculty of Pharmacy, University of Jordan, Amman, Jordan, and [4]Department of Biological Sciences, University of Dundee, Dundee, Scotland, UK

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ABSTRACT

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Aims: The demonstration of the antibiofilm effects of pharmaceutical microemulsions.

Methods and Results: Microemulsions were prepared as physically stable oil/water systems. Previous work by this group has shown that microemulsions are highly effective antimembrane agents that result in rapid losses of viability in planktonic populations of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In this experiment a microemulsion preparation was used upon established biofilm cultures of *Ps. aeruginosa* PA01 for a period of 4 h. The planktonic MIC of sodium pyrithione and the planktonic and biofilm MICs of cetrimide were used as positive controls and a biofilm was exposed to a volume of normal sterile saline as a treatment (negative) control. Results indicate three log-cycle reductions in viability within the microemulsion treated biofilm, as compared to those observed in control treatments of similar biofilms (one log-cycle reduction in viabilities).

Conclusions: The results indicate that the microemulsions are highly effective antibiofilm agents.

Significance and Impact of the Study: This study suggests that microemulsions may have a role in the treatment of industrial and environmental biofilms.

Keywords: antimicrobial, biofilm, membrane-active, microemulsions, self-preservation.

INTRODUCTION

Previous work by this group (Al-Adham *et al.* 2000) clearly indicated that formulated microemulsions have significant antimicrobial action against bacterial cells within very short time-scales. The results of this work exhibited losses in viability of greater than 6 log-cycles over a period as short as 60 s, in the case of planktonic populations of both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Subsequent transmission electron microscopy indicated significant losses in outer membrane structural integrity as a result of short exposures (1 min) to the microemulsion preparation (Al-Adham *et al.* 2000). Control experiments involving the testing of the components of the microemulsions yielded no significant antimicrobial activity. These results suggest that microemulsions are highly effective antimembrane agents that result in cell death within very short time periods.

Microemulsions have potential use as cosmetic agents and pharmaceutical drug delivery systems. This is mainly due to their relatively high oil content, which improves the bioavailability of hydrophobic drugs (Kovarik *et al.* 1994; Trull *et al.* 1994). Evidence exists to suggest that microemulsions can also be used to control the rate of drug release within a therapeutic situation (Trotta *et al.* 1989; Pattarino *et al.* 1993). Microemulsions are also used in the development of novel cosmetic preparations, which must exhibit acceptable levels of product preservation (Friberg 1984).

The proven ability of microemulsions to act as effective antimicrobial agents against planktonic cultures of *Ps. aeruginosa* (Al-Adham *et al.* 2000) has implications in the potential use of microemulsions as antimicrobial agents against this normally intransigent microorganism. It has been suggested that this observed level of efficiency may

Correspondence to: Dr P.J. Collier, School of Contemporary Sciences, University of Abertay Dundee, Dundee, DD1 1HG, Scotland (e-mail: mltpjc@abertay.ac.uk).

indicate significant antibiofilm activity by the same process. The objectives of this study are to examine the ability of a chosen microemulsion formulation to reduce the viability of an established biofilm population of *Ps. aeruginosa*.

MATERIALS AND METHODS

Chemical reagents

Brij 35 and Tween 80 were obtained from Merk, Whitehouse Station, NJ, USA. Isopropylmyristate (content > 98%) and *n*-Pentanol (gas chromatography assay, 99%) were obtained from Merk-Schunardt, Haar, Germany. Oleic acid (chemically pure) and cyclohexanol were obtained from Riedel-DeHaën, Seelze, Germany. Ethyl oleate (general purpose reagent, GPR) was obtained from BDH Chemicals, Poole, England. Liquid paraffin was obtained from Fluka, Buchs, Switzerland. The following reagents were required for the calibration of the pycnometer and were purified by fractional distillation before use: cyclohexane (GPR, 99%; Merk, Germany) and benzene (APR, 99·5%; Peking Chemicals, Beijing, China).

Preparation of microemulsions

Microemulsion 2 (15% Tween 80, 6% Pentanol, 3% Ethyl Oleate, in water) was prepared by the use of a Sartorius analytical balance (precision of ± 0·00001 g) in a stoppered flask in order to minimize evaporation and contamination. The densities of the pure and mixed components were measured using a capillary pycnometer of 25 and 50 ml volume at a constant temperature of 25 ± 0·01 °C. The measurements were taken in triplicate and were reproducible in the range of ± 0·001 mg cm^{-3}. The density of the surfactant/cosurfactant mixture, d(mix), of the required concentration was measured. The volume change (V_e) of the microemulsion formation was calculated by application of Equation 1:

$$V_e = w(\text{total})d(\text{total}) \ (1) \ [w(\text{mix})d(\text{mix}) + w(\text{oil})d(\text{oil})] \quad (1)$$

where w = weight, d = density and the volume change is calculated as $cm^3\ g^{-1}$ of the microemulsion, due to the difficulty of obtaining the exact molecular weights of the surfactants.

Organisms and culture maintenance

Pseudomonas aeruginosa ATCC 9027 was obtained from the American Type Culture Collection (Maryland, USA). Cultures were maintained and stored on Nutrient Agar (Oxoid CM3; Oxoid Ltd, Basingstoke, England) slopes in a darkened cupboard at room temperature after incubation at 35 °C for 48 h. All culture media were sterilized by autoclaving at 121 °C, 15 psi for 15 min. An overnight culture was used to inoculate the surface of nutrient agar (Oxoid CM3) plates. Cultures were then incubated at 35 °C. Cultures of bacteria were harvested by washing the surfaces of incubated agar plates with sterile saline TS solution (0·05% w/v polysorbate 80, 0·9% w/v NaCl) to give a final cell concentration of 10^8 cells ml^{-1}. In all cases the final cell concentration was determined by viable counts of cells. Planktonic mid-logarithmic phase cultures were prepared by inoculation of sterile 100 ml Erlenmeyer flasks containing 25 ml of CDM (Dinning *et al.* 1998), which were then incubated at 37 °C in an orbital incubator (Gallenkamp, Basingstoke, UK). Inocula were taken from these cultures at times and O.D. values which indicated that the cells were in the mid-logarithmic phase of growth (data not shown here).

Establishment of test biofilms

Biofilm cultures of *Ps. aeruginosa* PAO1 were grown within Sorbarod™ filter plugs (Ilacon Ltd, Kent, UK), which were perfused with culture media (chemically defined medium, CDM; Dinning *et al.* 1998) at 37 °C according to the protocol of Hodgson *et al.* (1995). Lengths (40 mm) of clear PVC tubing (10 mm i.d.) were prepared to contain single Sorbarod™ filters. The Sorbarod™ filter consists of a cylindrical paper sleeve, 10 mm in diameter and 20 mm in length, encasing a compacted concertina of cellulose fibres. Each mounted Sorbarod™ was prewetted with the addition of an aliquot (5 ml) of sterile normal saline (0·9% w/v) and then inoculated in a drop-wise fashion, with *Ps. aeruginosa* PAO1 (10^8 cfu ml^{-1}; 10 ml) from a sterile disposable syringe. A new sterile syringe was introduced into the deep end of the PVC tubing containing the Sorbarod™. The plunger of the syringe was removed and the rubber seal left in place. A sterile disposable needle (0·8 × 40 mm) was inserted through the rubber seal to act as a media feed. The media inlet tubing, from a peristaltic pump and reservoir, was then attached to the proximal end of the mounted needle. Multiples of these units were established within an incubator set at 37 °C.

The Sorbarod™ biofilms were then perfused with CDM at a known rate (0·5 ml min^{-1}) over extended periods of time and the stability of the biofilm population was established by performing regular viable counts on the eluate cells, in order to determine the rate with which these cells were being lost from the surface of the biofilm. Pseudo-steady states were established at which the growth rate of the biofilm was reproducible, measurable and significantly slower than in corresponding broth cultures. Biofilms of

Ps. aeruginosa could be maintained in pseudo-steady state for periods of up to 100 h.

Microemulsion and biocide challenge test

Biofilms established as above were allowed to enter pseudo-steady state for a period of 72 h and were subsequently challenged by the addition of an aliquot (2 ml) of either Microemulsion 2 or an aliquot (2 ml) of the planktonic minimal inhibitory concentration (MIC; 64 μg ml^{-1}) of sodium pyrithione (NaPT), the planktonic (20 μg ml^{-1}) and biofilm (32 μg ml^{-1}) MICs of cetrimide. A control biofilm was challenged by the addition of an aliquot (2 ml) of sterile normal saline. Normal media flow was suspended for 4 min (this period is equivalent to a flow rate of 0·5 ml min^{-1}) during the addition of these aliquots. Viable counts of eluate cells produced by these biofilms were determined by the tube dilution method at hourly intervals from 1 h prior to the addition of microemulsion or biocide up to 4 h after addition. The results were plotted as logarithm of the viable count *vs.* time.

RESULTS AND DISCUSSION

Figure 1 shows the comparative data for viable counts of eluate cells of separate biofilms treated with Microemulsion 2, NaPT, Cetrimide and no treatment. This figure clearly shows that all five biofilms exhibited a small reduction in eluate viability between T = −1 h and T = 0 h. The most likely reason for this observation is the addition of the agents as a single aliquot (2 ml) at T = 0 h followed by an immediate sampling for viability, whilst normal flow of media was suspended. This implies a 'wash-out' of bacterial cells from the surface of the biofilm as the result of a 'slug' of non-nutrient liquid passing over its surface. However, as the result of this addition was similar in all cases, it is suggested that the subsequent data would not be adversely affected by this phenomenon.

−1	1·18E+08	5·90E+08	1·31E+08	8·35E+06	9·00E+06
0	7·85E+07	3·50E+07	6·80E+07	1·10E+07	2·27E+06
1	3·70E+07	9·20E+06	4·95E+07	1·30E+08	9·75E+05
2	3·00E+07	4·75E+04	2·49E+07	1·65E+08	4·30E+05
3	6·10E+07	2·16E+04	1·11E+06	7·05E+06	2·00E+07
4	5·40E+06	1·93E+04	9·80E+06	1·23E+06	1·69E+06

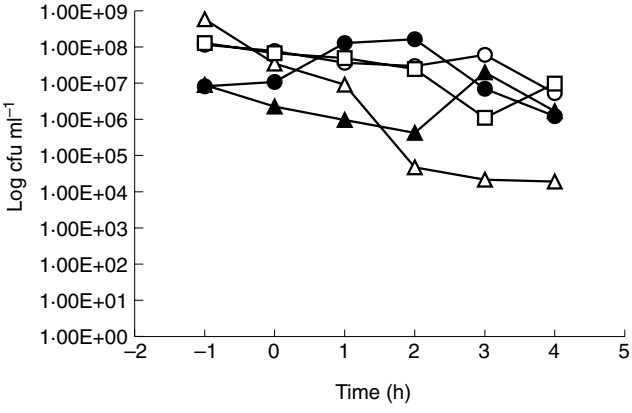

Fig. 1 Graph of the logarithm of the viable count of eluate cells from the control biofilm (2 ml sterile normal saline) (○), sodium pyrithione treated biofilm (2 ml of 64 μg ml^{-1}) (△), Microemulsion 2 treated biofilm (2 ml) (□), cetrimide (2 ml of 20 μg ml^{-1}) (●) and cetrimide (2 ml of 32 μg ml^{-1}) (▲) against time

Subsequent to the addition of sterile normal saline (2 ml) at T = 0 h, the control cells exhibited a steady decline in viability for the 4 h of the experiment. The original count approximated 10^8 cfu ml^{-1} and fell to 7×10^6 cfu ml^{-1} after 4 h, a fall of just over one log-cycle. A similar loss in viability was observed with the eluate cells from the biofilm exposed to the planktonic MIC value of NaPT (64 μg ml^{-1}). This count started at approximately 10^8 cfu ml^{-1} and fell to 10^7 cfu ml^{-1} over the same period. Again this represents a loss in viability of just one log-cycle. Comparison of these two results suggests that the NaPT had no observable antibiofilm effect at the concentration applied here, as its associated loss in viability of eluate cells was similar to that observed in the control (saline) experiment. Cetrimide was used as a possible biofilm-active positive control at both its planktonic (20 μg ml^{-1}) and biofilm (32 μg ml^{-1}) MICs (Abdel Malek *et al.* 2002 and unpublished results from this group). In both cases a reduction of approximately one log-cycle in viable counts was observed (1×10^{-7} to $1{\cdot}1 \times 10^{-6}$ cfu ml^{-1}). This result indicates that cetrimide has similar antibiofilm properties to NaPT at both its planktonic and biofilm MICs.

The eluate viability at T = 0 h for the biofilm challenged with Microemulsion 2 was 5×10^7 cfu ml^{-1}. This value fell to 2×10^4 cfu ml^{-1} after 4 h, upon addition of the active agent. This represents a loss in viability of over three log-cycles in a 4-h period, or greater than three times the rate of kill observed in both the control biofilm and those treated with MICs of NaPT and cetrimide. This result indicates that Microemulsion 2 has significant antibiofilm activity, as assessed by reductions in the viability of eluate cells. This approach is valid, as such cells are the direct product of binary fission amongst the population of adherent cells within the biofilm and, therefore, indicate a loss of viability within that structure.

This result supports our previous observations regarding the antimicrobial nature of microemulsions against planktonic bacterial cells (Al-Adham *et al.* 2000). In addition to their obvious antiplanktonic nature, it is now possible to suggest that microemulsions are highly effective antibiofilm

agents as well. This may also suggest that a future role for this interesting group of pharmaceutical products may be found in their application in the treatment of normally intransigent biofilms in industrial and environmental situations. However, further studies are required if they are to have potential in the treatment of medically associated biofilms.

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