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Microemulsions are membrane-active, antimicrobial, self-preserving systems

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Microemulsions are physically stable oil/water systems that have potential use as delivery systems for many pharmaceuticals which are normally of limited use due to their hydrophobicity, toxicity or inability to access the site of action. It has been suggested that microemulsions are self-preserving antimicrobials in their own right, although there is little evidence to support this. In this experiment, microemulsions of various compositions were formulated and tested for their stability and antimicrobial action. The physical stability of the different microemulsions was assessed by centrifugation at 4000 *g* and by storage in a water bath at 37 °C for one month, during which no phase separation was observed. The antimicrobial activity of the microemulsions was tested using the compendial method, observation of the kinetics of killing, and transmission electron microscopy (TEM) of microemulsion-exposed cultures of *Pseudomonas aeruginosa* PA01. These latter experiments on *Ps. aeruginosa* indicated distinct signs of membrane disruption. The results indicated that the microemulsions are self-preserved, and that their killing of microbial cultures is very rapid and may be the result of membrane activity.

INTRODUCTION

Interest has rapidly grown in the use of microemulsions as 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).

It has been suggested that microemulsions are self-preserving antimicrobials in their own right (Grindsted Products, Technical Memorandum, TM 5-le; Friberg 1984), but little can be found in the literature to support this theory. The suggestion is made on the basis that bacteria cannot survive in pure fat or oil and that water is necessary for their growth and reproduction. In microemulsions, the water (majority component, v/v) present is effectively bound by the structure of the microemulsion and therefore, access to the water by micro-organisms is limited. Hence, these systems are potentially antimicrobial. It is also suggested that the very structure of microemulsions is harmful to the bacterial or microbial cell and, in particular, that they adversely affect the structure and function of the bacterial membranes (Friberg 1984; Jones *et al.* 1997; Bortoleto *et al.* 1998). Literature survey has revealed only two references investigating the inherent antimicrobial properties of microemulsions (Wright 1996; Hamouda *et al.* 1998) and, while both are valid papers, neither has undertaken a definitive test to prove this activity. It is suggested that such a phenomenon should be demonstrable by application of an internationally accepted challenge test (United States Pharmacopoeia 1995) for the examination of antimicrobial effectiveness in pharmaceutical preparations. In addition, it would be reasonable to expect any successful antimicrobial preparation to be stable in various physical conditions over extended periods of

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time. The objectives of this study were to examine the stability and self-preserving antimicrobial properties of various microemulsion systems.

MATERIALS AND METHODS

Chemical reagents

Brij 35 and Tween 80 were obtained from Merck. Isopropylmyristate (content > 98%) and *n*-pentanol (GC assay, 99%) were obtained from Merck-Schunardt. Oleic acid (chemically pure) and cyclohexanol were obtained from Riedel-Dehaen. Ethyl oleate (GPR) was obtained from BDH Chemicals. Liquid paraffin was obtained from Fluka. The following reagents were required for the calibration of the pycnometer and were purified by fractional distillation before use: cyclohexane (GPR, 99%; Merck) and benzene (APR, 99·5%; Peking Chemicals).

Preparation of microemulsions

Mixtures were prepared by mass (Sartorius analytical balance with a precision of ±0·00001 g) in stoppered flasks 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/co-surfactant 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:

$$V^e = w(total)/d(total) - [w(mix)/d(mix) + w(oil)/d(oil)] \quad \text{(Equation 1)}$$

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. The pycnometers were calibrated by the measurements of the densities of the pure solvents. Results are given in Table 1 and are compared with published values. Table 2 gives the composition of the microemulsions prepared.

Organisms and culture maintenance

Candida albicans ATCC 10231, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538 were obtained from the American Type Culture Collection (Maryland, USA). Cultures of *E. coli*, *Ps. aeruginosa* and *Staph. aureus* were maintained and stored on nutrient agar (Oxoid) slopes in a darkened cupboard at room temperature after incubation at 35 °C for 48 h. *Candida albicans* cultures were maintained and stored on Sabouraud Dextrose Agar (Oxoid) slopes in a darkened cupboard at room temperature after incubation at 20 °C for 48 h. All culture media were sterilized by autoclaving at 121 °C, 15 psi for 15 min. An overnight culture of each micro-organism was used to inoculate the surface of an appropriate sterile solid agar medium. These cultures were then incubated at either 35 °C (bacteria) or 20 °C (fungi). Cultures of bacteria and fungi 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

Table 1 Experimental and published densities of pure liquids measured at 25 °C

Solvent	Density (g cm^{-3} at 25 °C)		
	Experimental	Published	Reference
Water	nd	0·997	CRC Solubility Tables
Cyclohexanol	0·9460	0·956	CRC Solubility Tables
n-Pentanol	0·8115	0·811	CRC Solubility Tables
Oleic acid	0·8910	0·883*	CRC Solubility Tables
Ethyloleate	0·8660	na	na
Isopropylmyristate	0·8502	na	na
Liquid paraffin	0·8476	na	na

nd = not done; na = not available.
*The density was calculated from the molar volume (320 cm^3 mol^{-1}) and the molecular weight (282·5 g mol^{-1}).

Table 2 Composition (v/v) of the microemulsions prepared for this study

Formula no.	Tween 80 (%)	Pentanol (%)	Ethyl oleate (%)
1	15	6	1
2	**15**	**6**	**3**
3	20	8	3
4	25	10	3
	Tween 80 (%)	Cyclohexanol (%)	Ethyl oleate (%)
5	15	6	1

The balance of percentage was made up with water in all cases. Emboldened (2) was used for the TEM experiment.

all cases, the final cell concentration was determined by viable counts of cells.

Challenge test procedure

Aliquots (20 ml) of each microemulsion were aseptically transferred into four sterile test tubes. Each tube was inoculated with sufficient washed cells of a different test culture to give a final cell concentration of 10^6 cells ml^{-1} (0·1 ml). Control tubes were prepared containing sterile normal saline instead of a microemulsion preparation. The contents of all tubes were mixed using a rotamixer and incubated at 20 °C for a period of 28 days. At intervals (0 h and 24 h, and 7, 14, 21 and 28 days), aliquots (0·1 ml) were removed from each tube and added to an empty sterile Petri plate. A volume (20 ml) of cool (55 °C) sterile agar (nutrient agar or Sabouraud Dextrose Agar as appropriate) was added to each plate and the contents gently mixed in order to give a pour-plate for each sample. Previous experimentation (results not given here) had shown that this dilution ratio was sufficient to neutralize the effects of the microemulsions present. All plates were incubated at either 35 °C for not less than 3 days, or 20 °C for not less than 5 days, for bacterial cultures and fungal cultures, respectively. This procedure was performed in triplicate for each test tube prepared.

Filtration experiment

It was suggested that the absence of viable cells in the tubes prepared as above was due to adsorption of the microemulsions to the bacterial cells and their subsequent hydrophobic interaction with the walls of the test tubes. In order to investigate this possibility, the tubes were prepared in duplicate and their contents mixed by the use of a rotamixer. The contents of the tubes were then rapidly passed through a sterile bacterial membrane ($0·22 \pm 0·02$ μm; Sartorius, Germany) in order to entrap any bacterial cells present before they could adhere to the walls of the tubes. After filtration, each membrane was washed with sterile saline (250 ml) and aseptically transferred to the surface of a sterile agar plate of the appropriate medium (Nutrient Agar for the bacteria and Sabouraud Dextrose Agar for the yeast). The plates were then incubated at either 35 °C for 24 h or 20 °C for 5 days, as appropriate.

Kinetics of killing

Overnight cultures of *Ps. aeruginosa* and *Staph. aureus* were washed twice (with sterile normal saline; centrifugation at 4000 *g*) and used to prepare test cultures in fresh medium with a known inoculum size (*Ps. aeruginosa*, $5·9 \times 10^5$ cfu ml^{-1}; *Staph. aureus*, $1·6 \times 10^6$ cfu ml^{-1}). These cultures were then challenged with concentrations of one of the microemulsion preparations (Formula number 2; 15% Tween 80, 6% pentanol, 3% ethyl oleate and 76% water) and incubated at 35 °C for 6 min. Samples (0·1 ml) were taken from each tube at 10, 20, 30 and 40 s, and 1, 2 , 3, 4 , 5 and 6 min, and prepared by dilution series for viable counts on sterile nutrient agar plates. All plates were incubated at 35 °C for 24 h.

Transmission electron microscopy

An overnight culture of *Ps. aeruginosa* PA01 (grown in 25 ml chemically-defined medium (CDM; Dinning *et al.* 1998) and incubated at 37 °C in an orbital incubator at 200 oscillations min^{-1}) was divided into two equal parts. The aliquots (12·5 ml) were centrifuged (4000 *g* for 15 min) and washed once in sterile distilled water (10 ml). One pellet was resuspended in sterile distilled water (5 ml; control) and the other was resuspended in Microemulsion 2 (5 ml; Tween 80, 15%; pentanol, 6%; ethyl oleate, 3%; and water, 76% v/v) for 5 min. The suspensions were then centrifuged at 4000 *g* and washed twice with sterile distilled water (10 ml) to remove the presence of the test materials. The final pellets were resuspended in glutaraldehyde (5 ml; 2·5% v/v solution in water) and incubated at room temperature (20 °C) for 1 h prior to a further centrifugation (4000 *g* for 15 min) and a further two washes in sterile distilled water (10 ml). The fixed pellets were then resuspended in osmium tetraoxide solution (0·2% OsO_4 in 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·2) and left to fix/stain overnight. The suspensions were then centrifuged (4000 *g* for 15 min) and rinsed twice with distilled water.

Uranyl acetate solution (2% aq.) was then added to the pellets and left overnight. The pellets were then washed twice with distilled water. Cells were dehydrated through a graded ethanol series with two changes in absolute ethanol. The pellets were then washed twice with propylene oxide (100%) and infiltrated with a 1:1 (v/v) mixture of propylene oxide and Durcapan resin (Sigma) on a rotary wheel (4 rev min^{-1}) for 2 h at room temperature. A fresh 1:1 (v/v) mixture of propylene oxide and Durcapan resin was added and allowed to infiltrate the pellets for a further 2 h as above. The pellets were then infiltrated with 100% Durcapan resin as above overnight. Pellets were transferred to flat embedding moulds with fresh resin and placed in a 60 °C oven for 24 h to polymerize the resin. Sections were cut from the resin-fixed pellets using a Reichert OMU-3 ultramicrotome fitted with a diamond knife and mounted/collected on 50 mesh pioloform coated copper grids. After staining with uranyl acetate and lead citrate, sections were examined using a JEOL-1200 EX transmission electron microscope.

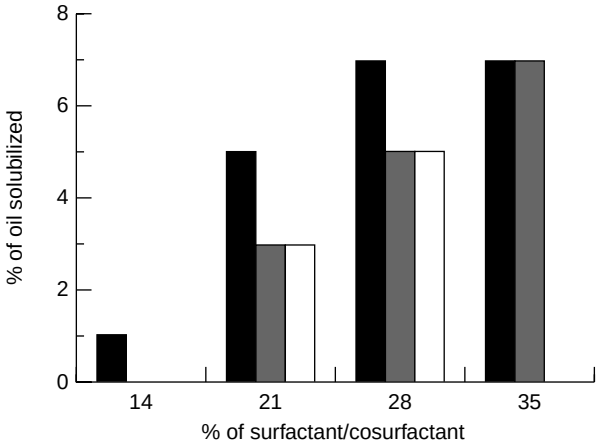

Fig. 1 Percentage of solubilized oil at different surfactant/co-surfactant concentrations. (■), Tween 80/pentanol/ethyl oleate; (▩), Tween 80/cyclohexanol/ethyl oleate; (□), Brij 35/pentanol/oleic acid

RESULTS

Physical stability studies

A fixed surfactant/co-surfactant ratio of 2·5 was used in all the microemulsions. The maximum oil (ethyl oleate) solubilized by each surfactant/co-surfactant concentration was determined by preparing microemulsions with oil concentrations of 1, 3 and 5%. The components were mixed manually by shaking and then visually observed for any phase separation. No phase separation was observed at the 5% oil concentration and therefore, microemulsions containing 7 and 9% oil were prepared and observed. Ethyl oleate was soluble in the Tween 80/*n*-pentanol combination up to 7% at a surfactant/co-surfactant concentration of 28% and 35%. However, liquid paraffin and isopropyl-myristate were not soluble in the Tween 80/cyclohexanol systems. Therefore, these microemulsions were only formulated with ethyl oleate. Due to the high viscosity of the solutions containing Brij 35, only 14 and 21% solutions were prepared, and oleic acid was soluble up to 5% at 21% surfactant/co-surfactant concentrations (Fig. 1).

Further examination of the physical stability of the microemulsions was performed by placing them in a centrifuge at 4000 *g* for 15 min at room temperature (20 °C). No phase separation was observed in the samples after centrifugation. The same samples were then stored for 1 month at 37 °C and no phase separation was observed. These results indicate that the prepared emulsions are stable under conditions of centrifugation and incubation for extended periods.

The viscosity (using a calibrated viscometer) and volume change upon microemulsion formation was measured for Tween 80/*n*-pentanol/ethyl oleate microemulsions. The results are given in Figs 2 and 3, respectively. These results exhibit the maximum values of the measured property at a surfactant/co-surfactant concentration of 28%. The microemulsion droplet interface is formed from surfactant and co-surfactant molecules and will be penetrated by some of the oil molecules. The occurrence of oil solubility maxima at the same surfactant/co-surfactant concentration of the

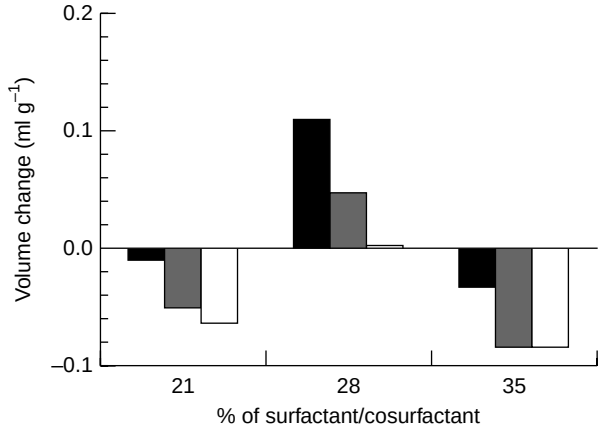

Fig. 2 Volume change (ml g^{-1}) of Tween 80/pentanol/ethyl oleate microemulsion formation at different oil percentages. (■), 1% oil; (▩), 3% oil; (□), 5% oil

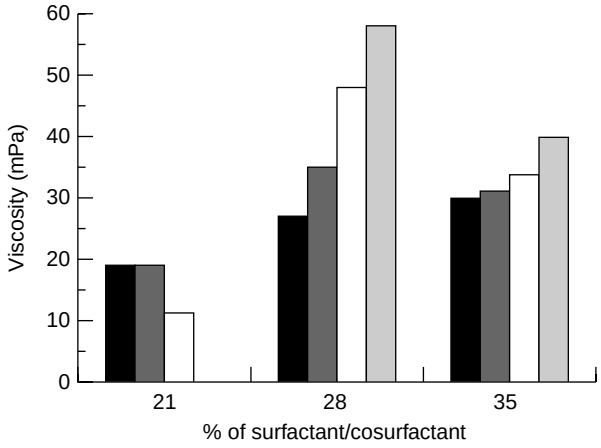

Fig. 3 Viscosity (mPa) of Tween 80/pentanol/ethyl oleate microemulsions at different oil percentages. (■), 1% oil; (▩), 3% oil; (□), 5% oil; (▤) 7% oil.

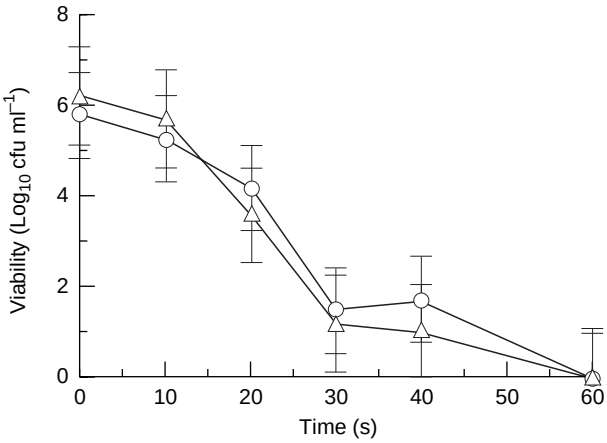

Fig. 4 Time exposure viability curves for the addition of microemulsion 2 (15% Tween 80, 6% pentanol and 3% ethyl oleate) to cultures of *Pseudomonas aeruginosa* ATCC 9027 (○) and *Staphylococcus aureus* ATCC 6538 (△). Error bars are calculated from the standard error of the dataset

maximum expansion in volume and viscosity of the microemulsion might indicate the limits of interfacial packing of microemulsion components. This series of values also indicates the unique surfactant concentration for any surfactant/co-surfactant combination which will yield the optimum oil solubility. Microemulsion formation accompanied by maximal changes in observed physical properties has also been reported by Fubini *et al.* (1988).

Challenge test procedure and filtration experiment

No growth was observed on any plate prepared during the USP challenge test (United States Pharmacopoeia 1995). This result indicates either that all the prepared microemulsions had a 100% killing rate towards all the test micro-organisms, or that the viable cells were adhering to the walls of the test tubes as a result of adsorption to the hydrophobic microemulsions. This latter hypothesis was disproved by the membrane filtration experiment. This experiment clearly indicated that bacterial and fungal cells, which could not have passed through the membranes, should, if still viable, have exhibited growth on agar plates. No such growth was observed on any plate, indicating that the cells were non-viable. The results of these two experiments suggest that the microemulsions, as prepared containing no free antimicrobial compounds, are capable of inhibiting microbial growth and viability.

Kinetics of killing

An experiment was designed to observe the kinetics of killing of one of the microemulsion preparations. In this experiment, the changes in viability of two cultures of bacterial cells (*Ps. aeruginosa* and *Staph. aureus*) were observed over a short period of time after exposure to a known concentration of the microemulsion. Figure 4 shows the rate of killing observed for these cultures and gives clear evidence of a true biocidal dynamic. Both cultures started with a viable count of approximately 10^6 cells $ml^{-1.}$ This viability decreased rapidly over a period of 60 s until no viable cells were observed. This indicates that a 5 log reduction in bacterial titre was obtained in approximately 45 min. The $LT_{90\%}$ value (time at which 90% of the original population has been killed by the antimicrobial) for this experiment was approximately 15 s for both micro-organisms. $LT_{90\%}$ values of this order suggest that the antimicrobial compound involved is highly and effectively biocidal.

Transmission electron microscopy

Comparison of control cells (Fig. 5a) and microemulsion-treated cells (Fig. 6a) of *Ps. aeruginosa* at 20 000× magnification immediately suggests some extra- and intracellular effect upon the test cells by the microemulsion. These images are general fields of bacterial cells after 1 min of exposure to the microemulsion (Fig. 6a) or water (Fig. 5a), followed by identical preparation treatments for electron microscopy. Figure 5(a) shows a normal cellular morphol-

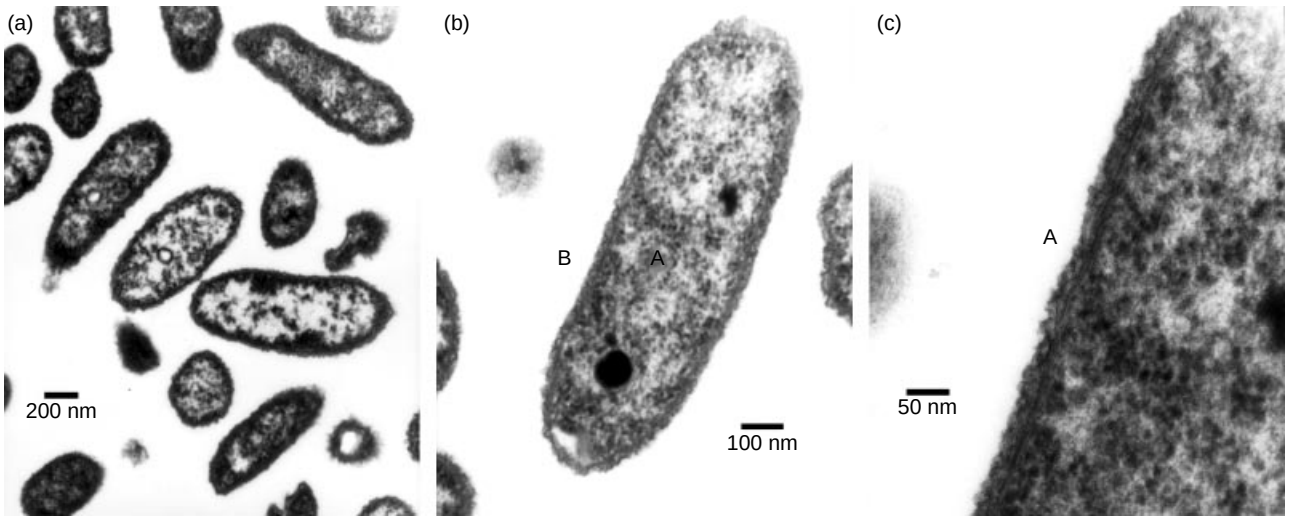

Fig. 5 Transmission electron micrographs of *Pseudomonas aeruginosa* ATCC 9027 exposed to water for 60 s and then prepared for TEM by the method above and observed using a JEOL-1200 EX transmission electron microscope at 20 000× magnification (a), 50 000× magnification (b) and 100 000× magnification (c). Figure 5(a) shows normal cytosolic components (A) and an intact cell envelope (B). Figure 5(c) shows fine detail (A) of the cell envelope structure

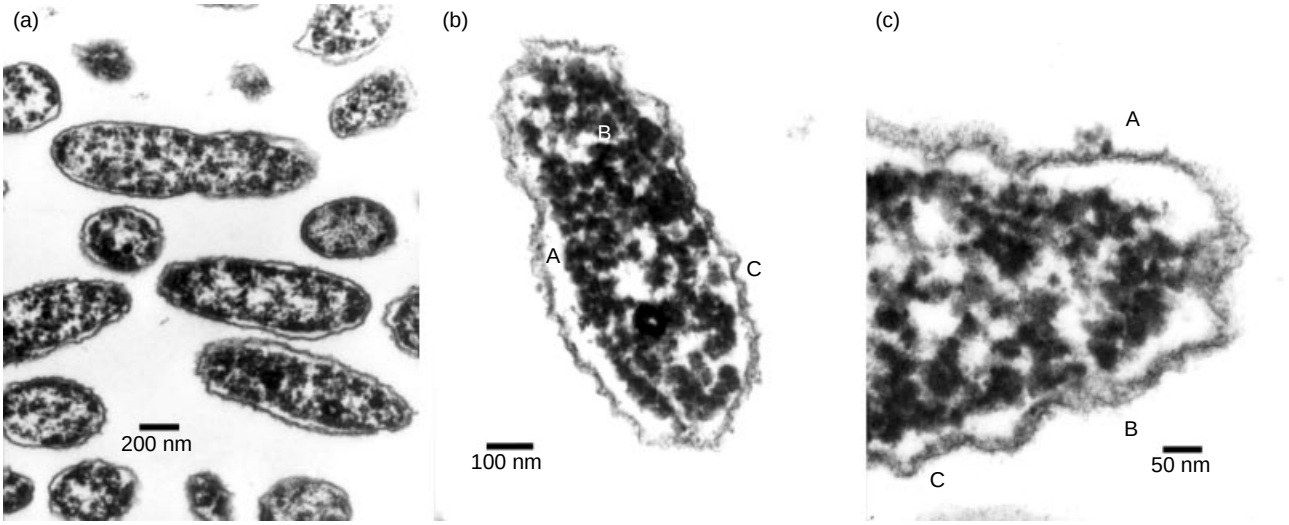

Fig. 6 Transmission electron micrographs of *Pseudomonas aeruginosa* ATCC 9027 exposed to microemulsion 2 for 60 s and then prepared for TEM by the method above and observed using a JEOL-1200 EX transmission electron microscope at 20 000× magnification (a), 50 000× magnification (b) and 100 000× magnification (c). Figure 6(b) shows crenation or separation of the cytoplasmic membrane from the cell envelope (A), coagulation of cytosolic components (B) and a disrupted outer membrane structure (C). Figure 6(c) shows membrane sloughing (A), membrane breaching (B) and 'blebbing' (C)

ogy with distinct internal components in the form of cytosol (dark internal structures) and nucleoid (pale internal structure). Figure 6(a) shows a separation of the cytosol and cytoplasmic membrane from the peptidoglycan layer and some obvious internal coagulation of cytosolic components.

These observations are supported at higher magnification (50 000×) in Fig. 5(b) (control) and 6(b) (test); clear signs of crenation (A) can be seen in Fig. 6(b), along with coagulation of cytosolic components (B). This image (Fig. 6b) also shows some membrane effects (C) which are abnormal when compared with those (B) of the control cells (Fig. 5b).

Figure 5(c) shows a high magnification (100 000×) image of a control cell in which a normal cytosol and cell envelope (A; cytoplasmic membrane, cell wall and outer membrane) can be seen. At the same magnification, the microemulsion-exposed cell exhibits many signs of membrane disfunction, including membrane sloughing (A), membrane breaching (B) and 'blebbing' (C).

DISCUSSION

The results of the stability tests show that the microemulsions are stable over extended periods and through various treatments. Such stability is desirable if these products are to be used as pharmaceutical preparations and cosmetics. They also indicate a low level of potential microbial activity, a major reason for losses in stability in pharmaceutical and cosmetic products. Such observed stability will be a function of both the chemical nature of the microemulsions and their inherent antimicrobial nature.

The high levels of inherent antimicrobial activity observed in these microemulsions, demonstrated, for example, by the results of the USP and kinetics of killing experiments, indicate that these microemulsions are indeed self-preserving systems. The observed 5 log reduction in bacterial titre over 45 s, coupled with $LT_{90\%}$ values of 15 s observed with these microemulsions, suggests excellent biocidal activity. Such rapid biocidal activity is indicative of a direct attack on the structural integrity of the cell rather than a secondary effect through metabolic inhibition (Gilbert 1984); the use of TEM on microemulsion-exposed cells may yield evidence of this. The results of TEM indicate that the microemulsion used (Formula No. 2) is capable of significant antimembrane activity, resulting in the gross disturbance and disfunction of membrane structure. This level of disfunction could potentially result in the death of the cell and may explain the rapid loss of cell viability observed in the kinetics of killing experiment.

CONCLUSIONS

These results clearly indicate that the microemulsions are stable, self-preserving antimicrobial agents, with a highly effective killing rate against *Staphylococcus aureus* and the particularly resistant bacterial species, *Pseudomonas aeruginosa*. These findings suggest that some of the observed levels of antimicrobial activity associated with microemulsion-encapsulated antibiotics may be due to the direct activity of the microemulsions themselves against the bacterial cytoplasmic membrane. Further experimentation to investigate this phenomenon and its implications regarding the use of microemulsions is desirable.

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