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An EPR investigation of surfactant action on bacterial membranes

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Abstract

The effects of the surfactants, alcohol ethoxylate, amine ethoxylate, amine oxide and SDS on cell membranes were investigated using the lipid soluble spin label 5-doxyl stearic acid (5-DS). Electron paramagnetic resonance (EPR) spectroscopy revealed that the action of the surfactants was to significantly increase membrane fluidity of *Proteus mirabilis*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*. The action of these surfactants as biocides was investigated and found to be dependent on the type of organism tested. There was, however, no direct correlation between enhanced membrane fluidity observed due to the action of the surfactants and biocidal activity. Data presented suggest that perturbing the fluidity of the cytoplasmic membrane is not immediately responsible for cell death.

Keywords: Biocide; Spin labelling; Surfactant

1. Introduction

Surface active agents (surfactants) are commonly added to cleaning products because of their ability to wet surfaces, penetrate soil and solubilise fatty materials. Many of these products are also required to deliver a hygiene benefit and the surfactants contribute to a varying extent to the microbiocidal effect. Surfactants are amphiphiles, containing both polar and non-polar regions on the same molecule and are broadly classified as cationic, anionic, non-ionic or amphoteric depending on the nature of the hydrophile yielded in aqueous solution [1].

Cationic surfactants, of which the quaternary ammonium compounds have been best studied, possess strong bactericidal properties [2–4]. These surfactants alter the permeability of the cell membranes leading to loss of function and cell death [5]. Amphoteric agents have bactericidal properties similar to quaternary ammonium salts however their activity is maintained over a wider pH range [6] and they are claimed to be less readily inactivated by proteins [7]. The mechanisms of biocidal action of other surfactants has received little attention but is also likely to involve interaction with the cytoplasmic membrane and increases in permeability. Anionic surfactants

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are used in hygienic cleaners especially at low pH and at high concentrations anionics such as sodium dodecyl sulphate can induce bacterial lysis. Anionic fatty acids are in general more active against Gram-positive bacteria than against Gram-negative bacteria [8]. Non-ionics are generally assumed to be inactive.

In this report, ranges of surfactants representative of non-ionic, anionic and amphoteric types are compared for their ability to cause fluidisation of the bacterial and yeast cell membranes and for their biocidal properties. The use of lipid soluble nitroxide spin labels in conjunction with electron paramagnetic resonance (EPR) spectroscopy, offers a sensitive method for the investigation of membrane fluidity changes without unduly perturbing the cells under investigation. The data presented here suggests that there is no direct correlation between enhanced membrane fluidity due to the action of the surfactant and biocidal activity.

2. Materials and methods

2.1. Organisms used

Proteus mirabilis (strain ATCC 14153), *Staphylococcus aureus* (strain NCTC 10788) and *Saccharomyces cerevisiae* (strain ATCC 9763).

2.2. Peptone solution

European suspension test (EST) peptone solution was prepared by dissolving 1 g of bacteriological peptone and 8.5 g sodium chloride in 1 l of distilled water. The pH was adjusted to 7.0 (± 0.1) using a 0.1 M solution of NaOH or HCl. The EST peptone solution was then steam sterilised (autoclaved) prior to use.

2.3. Water of standard hardness (WSH)

WSH was prepared from two solutions; solution A containing 31.74 g anhydrous $MgCl_2$ and 73.99 g anhydrous $CaCl_2$ and solution B containing 56.03 g $NaHCO_3$. 1.5 ml of solution A and 2 ml of solution B were filtered using a 0.45 μm filter and added to 494 ml of distilled water.

2.4. Quench solution

Two buffer solutions containing 0.4 M $NaH_2PO_4 \cdot 2H_20$ (Fisher Scientific) (buffer A) and 0.4 M $Na_2HPO_4 \cdot 12H_20$ (Fisher Scientific) (buffer B) were prepared initially. A mixed buffer solution containing 117 ml of buffer A and 183 ml of buffer B was then prepared. To 250 ml of the mixed buffer solution 0.75 g Lecithin (about 90% egg) (Fisher Scientific), 0.75 g bacteriological peptone (Oxoid), 7.5 g Tween 80 (Sigma) and 1.25 g sodium thiosulphate 5-hydrate (Hopkins and Williams) was added. The mixture was then stirred until all the solid had dissolved. The quench solution was then sterilised by autoclaving before use.

2.5. Culture of organisms

The bacterial cultures were maintained at 4°C on tryptone soya agar slopes or for the yeast malt extract agar slopes. The slopes were subcultured every 2–3 weeks. To prepare a culture for the bactericidal assay, a loopfull of the bacteria was inoculated into 100 ml of nutrient broth No. 2 (Oxoid) or malt extract broth for yeast. The bacterial suspension was incubated in a shaking water bath at 37°C (28°C for the yeast) for 24 h. After incubation, the bacterial cell culture was separated from the broth solution by centrifugation at 3500 rpm for 5 min. The bacterial cells were then washed in sterile distilled water before centrifugation for a further 5 min. Finally the cells were resuspended into EST peptone solution to give a suspension with an optical density of 0.5 at 600 nm, and stored at 4°C before use.

2.6. Biocidal assay

We employed the European suspension test (EST) methodology with a standard contact time of 5 min. The surfactant, amine oxide, amine ethoxylate, alcohol ethoxylate and SDS were prepared at a stock concentration of 100 mM. The pH of the surfactant solutions was adjusted to 3.5 using HCl. Prior to use, the surfactant solutions were diluted 1:15 in water of standard hardness (WSH), as specified in the European suspension test for bacterial activity, CEN 1276. Tests for bactericidal activity were performed as follows; 5 ml of diluted test solution was added to

4 ml of bovine serum albumin (7.5 mg ml^{-1}), 1 ml of bacterial suspension ($OD_{600} = 0.5$) was added to the test formulations and incubated at room temperature for a contact time of 5 min. The bactericidal assay was terminated by transferring 1 ml of the assay mixture into 9 ml of quench solution and left for a further 5 min. After quenching the bactericidal assay, 1 ml of the solution was diluted further into 9 ml of EST peptone solution. This dilution process was repeated to give a series of six 10-fold diluted bacterial suspensions. The number of viable colony forming units in the bacterial suspensions was then determined. This was achieved by the transfer of 1 ml of the bacterial solution into a petri dish and overlaying with molten agar. The bacterial suspension/molten agar mixture was mixed well before allowing it to set. The agar used was tryptone soya, malt extract and CLED for *S. aureus*, *S. cerevisiae* and *P. mirabilis* respectively. The plates were then inverted prior to incubating at 37°C (or 28°C for yeast) for 48 h. After incubation, the individual colonies on the plates were then counted.

2.7. Spin labelling P. mirabilis

P. mirabilis was grown on blood agar base plates for 24 h at 37°C. The cells were harvested from the plates with plastic spreaders into phosphate buffered saline (PBS, Dulbecco A, pH 7.4). The cell suspension was then centrifuged at 3500 rpm for 20 min and finally resuspended in fresh PBS to give an OD_{600} of 2.5. Spin labelling was achieved by initially drying to a film 100 μl of the spin label 5-doxyl stearic acid (2.5 mg ml^{-1}) (Sigma) in a glass tube under nitrogen. Two ml of the cellular suspension was added to the glass tube and mixed for 5–7 min. The organisms were then incubated at 37°C for 1 h before removing excess unbound spin label by washing 1 ml of the spin labelled cells twice with 1 ml of PBS. The cell pellet was then resuspended in 0.4 ml of PBS before adding 0.5 ml of the surfactant solution for 4 min. After 4 min the cells were centrifuged for 1 min before transferring the cell pellet to a sealed glass capillary. The EPR spectra were obtained immediately.

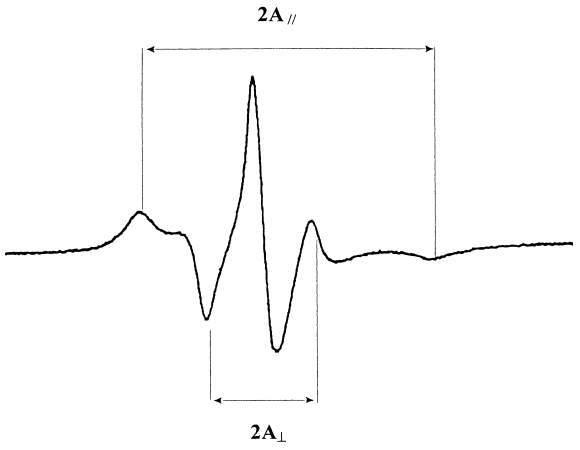

Fig. 1. Typical EPR spectrum of the 5-doxyl stearic acid spin label in a biological membrane. $2A_{\parallel}$ and $2A_{\perp}$ represent easily measurable spectral parameters that can be incorporated into the order parameter equation to give a measure of membrane fluidity.

2.8. Spin labelling S. aureus and S. cerevisiae

Spin labelling was carried out using a modified procedure of Stewart et al. [9]. The Gram-positive bacteria and the yeast were initially grown on tryptone soya agar and malt extract agar plates respectively for 24 h at 37°C (or 28°C for the yeast). Spin labelling was carried out as for *P. mirabilis* except that the spin label mixture was vortexed for 30 s prior to incubation at room temperature for 5 min. Treatment with the surfactants was carried out as already described.

2.9. EPR spectroscopy

The technique of spin labelling EPR spectroscopy relies on the spectral anisotropy produced when a stable free radical, such as a nitroxide is bound either to a biological molecular assembly or to an intercalated probe molecule. Instead of the characteristic three line (isotropic) solution spectrum obtained for a free nitroxide radical species, an anisotropic (powder-like) spectrum is obtained, see Fig. 1. This arises because when the spin probe is intercalated into the membrane bilayer it can align itself along an axis parallel to the normal of the membrane, hence the largest interaction will be the hyperfine coupling constant in this plane, i.e. $A_{\parallel}$ and the minimum will be in the perpendicular direction, i.e. $A_{\perp}$. Comparison of

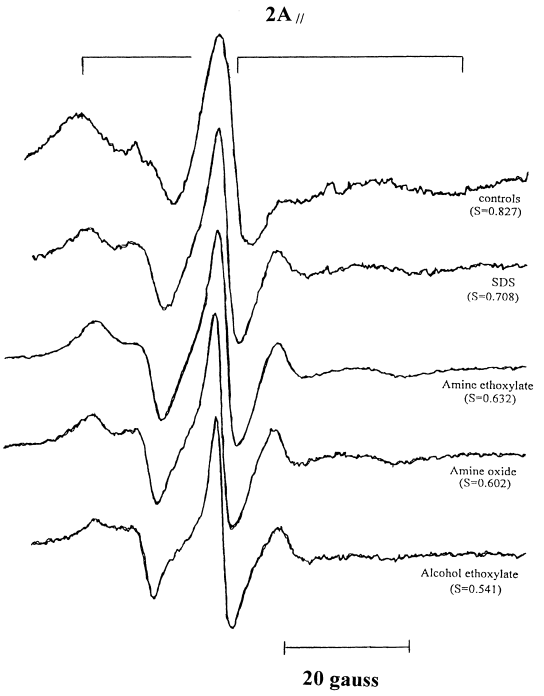

Fig. 2. EPR spectra showing the fluidising effects of the surfactants solutions on *P. mirabilis*. Cells were labelled with 5-DS spin label and treated with the surfactant solutions according to Section 2.

these values with the theoretical principal values A_{zz} and A_{xx} allows one to calculate a measure of membrane fluidity. Such a measure is the order parameter (S), where the membrane fluidity can be derived from the spectral line splitting. S is the ratio of the observed hyperfine anisotropy ($A_{\parallel}-A_{\perp}$) to the 25 Gauss theoretical maximum ($A_{zz}-A_{xx}=25$ gauss) if the spin label was rigidly immobilised (Fig. 1).

$$S = (A_{\parallel}-A_{>\perp})/(A_{zz}-A_{xx})$$

$S=0$ when the probe moves free in a homogenous solution and $S=1$ when the probe is held in a rigid environment. Intermediate values of S between 1 and 0 represent intermediate motion of the probe [10]. The above expression is only an approximation of the order parameter because the solvent terms, such as polarity are not included. (Within this study relative changes were measured for the three systems and hence the absolute values for the order parameters are not essential.)

Prior to EPR spectroscopy 50 μl of $K_3Fe(CN)_6$ was added at a concentration of 0.1 M to reoxidise any spin label reduced by the cells. Time averaged (4×1 min) scans were obtained with spectral settings of 100 G scan range, 2 G field modulation, 10 mW microwave power and 0.064 s time constant. All measurements were obtained at room temperature using a Varian E 104 EPR spectrometer with computerised data acquisition.

3. Results

The effects of the surfactants on the cell wall/membrane were investigated using the lipid soluble spin label 5-doxyl stearic acid (5-DS). Spin labelling of *P. mirabilis*, *S. aureus* and *S. cerevisiae* revealed that the action of the surfactant was to significantly increase fluidity (shown by a decrease in order parameter, S) of the cell membrane (Table 2). This can be visualised as a decrease in $2A_{\parallel}$ of the resulting immobilised EPR spectra (Fig. 2).

Measurement of the order parameter (S) showed for *P. mirabilis* and *S. aureus* that the increase in membrane fluidity was of the following order: alcohol ethoxylate > amine oxide > amine ethoxylate > SDS (i.e. alcohol ethoxylate had the greatest fluidising effect on the membranes) (Table 1). However

Table 1
Summary of the effect of surfactant solutions on order parameter (S)

Organism	Control	Amine oxide	Amine ethoxylate	Alcohol ethoxylate	SDS
P. mirabilis	0.827 ± 0.003	0.602 ± 0.011*	0.632 ± 0.001*	0.541 ± 0.001*	0.708 ± 0.005*
S. aureus	0.811 ± 0.09	0.653 ± 0.017*	0.691 ± 0.006*	0.645 ± 0.017*	0.737 ± 0.005*
S. cerevisiae	0.681 ± 0.001	0.600 ± 0.002*	0.644 ± 0.004*	0.509 ± 0.008*	0.593 ± 0.008*

Data represent the mean ± standard error of four separate measurements. Changes in fluidity are significantly different from controls at *$P<0.005$.

Table 2
Biocidal activity of surfactants expressed as log reduction in viable CFUs (see Section 2 for concentrations used)

Organism	Amine oxide	Amine ethoxylate	Alcohol ethoxylate	SDS
P. mirabilis	1.031 ± 0.021	2.419 ± 0.184	0.324 ± 0.145	0.023 ± 0.005
S. aureus	6.484 ± 0.157	6.231 ± 0.061	0.059 ± 0.021	0.447 ± 0.011
S. cerevisiae	4.475 ± 0.003	4.776 ± 0.003	0.924 ± 0.067	0.095 ± 0.071

Data represent the mean log reduction ± standard error from duplicate determinations of two separate experiments.

for *S. cerevisiae* the effect on membrane fluidity was of the following order: alcohol ethoxylate > SDS > amine oxide > amine ethoxylate (Table 1). Alcohol ethoxylate induced the greatest increase in membrane fluidity in the three organisms tested.

The changes in membrane fluidity caused by the surfactants clearly represent gross perturbations. We therefore sought to correlate the changes in membrane fluidity due to the action of the surfactants with biocidal activity. It was found that the biocidal efficiency of the surfactants was dependent on the type of organism tested.

The amphoteric and non-ionic surfactants, amine oxide and amine ethoxylate respectively had the strongest biocidal action on *S. aureus* (log reduction > 6) and *S. cerevisiae* (log reduction > 4) (Table 2). *P. mirabilis* was more resistant to the biocidal activity of amine oxide and amine ethoxylate (log reduction of 1.031 ± 0.021 and 2.419 ± 0.184 respectively) when compared to the Gram-negative and the yeast. The anionic surfactants SDS and alcohol ethoxylate had poor biocidal action on all the organisms tested (log reduction < 1). The anionic surfactants studied were less effective in cell killing than the amine oxide and amine ethoxylate.

No correlation could be made between biocidal activity and the extent of membrane fluidisation by the surfactants. The non-ionic surfactant alcohol ethoxylate induced the greatest membrane fluidisation in all the organisms tested, but had only moderate to poor biocidal activity. The second non-ionic surfactant, amine ethoxylate which was a good membrane fluidiser for all the organisms tested, displayed good biocidal activity on the Gram-positive species and yeast, but only moderate killing of the Gram-negative organisms. Clearly other factors other than changes in membrane fluidity play a role in the biocidal action of surfactants.

4. Discussion

Fatty acid spin labels are known to probe the phospholipid domains of Gram-negative, Gram-positive and yeast cells. They enter almost exclusively into the phospholipids domains [11] in Gram-negative cells. Nettleton and co-workers [12] have suggested that the nitroxide probe can redistribute between the plasma membrane and membranes of other organelles in eukaryotic cells. It would seem reasonable to assume that both the phospholipid domains in the outer and cytoplasmic membranes are probed in *P. mirabilis* and that the resultant spectra are indicative of the overall fluidity in both regions. Rapport et al. [13] have shown the fatty acid spin label to be able to partition into the fluid outer and inner membranes of *P. aeruginosa*. However, the exact partitioning of the spin probe between the outer membrane and the cytoplasmic membrane remains to be fully determined.

In this study, spin labelling of *P. mirabilis*, *S. aureus* and *S. cerevisiae* has revealed that the surfactants have a strong fluidising effect on their membrane structures. Its extent was dependent on the surfactant used and the organism tested. The non-ionic surfactant, alcohol ethoxylate, induced the greatest increases in fluidity (as determined by a decrease in order parameter) on all three organisms studied. It is generally thought that increases in cytoplasmic membrane permeability lead (directly or indirectly) to cell death. However, the best membrane fluidiser alcohol ethoxylate, was not the most potent biocide. The exact correlation between the extent of membrane fluidisation by the surfactants and their biocidal activity remains to be fully elucidated. Our results suggest that perturbing the fluidity of the cytoplasmic membrane is not immediately responsible for cell death.

The gross changes in membrane fluidity observed in this study represent interference in the tight packing of the phospholipid hydrocarbons induced by the action of the surfactants. The actions of the surfactants may be similar to those of chaotropic anions (i.e. agents that are able to break up the water structure near the polar head groups and with the capability of perturbing the lipid bilayer) [14]. Some chaotropic agents are able to influence the head group structure without disrupting the long range organisation of the lipid bilayer. Similarly, if the surfactants used only perturbed the surface as opposed to compromising the whole lipid bilayer, this may account for the discrepancy that some good membrane fluidisers, as determined by measurement using 5-doxyl stearic acid, are not good biocides. Further work is required to determine the effects of the surfactants used in this study deep within the membrane.

Takasakia et al. [15] found that the action of the quaternary ammonium disinfectant, didecylimethyl ammonium chloride, enhanced membrane fluidity of liposomes prepared from cell phospholipids of *S. aureus*. This led them to propose that the action of the cationic surfactant was first to cause damage to the cell membrane which results in the loss of function of the cell membrane. Our findings are in agreement with their results which suggest that enhanced membrane fluidity due to the action of the surfactants alone is not sufficient for enhanced biocidal action, but has to be coupled to a loss of membrane function. In this respect the amphoteric and non-ionic surfactants, amine oxide and amine ethoxylate may represent important reagents to potentiate the action of other biocides.

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