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Colloids and Surfaces
A: Physicochemical and Engineering Aspects 186 (2001) 43–53

COLLOIDS AND SURFACES A

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# The interaction of phospholipid liposomes with mixed bacterial biofilms and their use in the delivery of bactericide

Anne M. Robinson [a], Mark Bannister [a], Jonathan E. Creeth [b], Malcolm N. Jones [a,*]

[a] *School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK*
[b] *Unilever Research, Port Sunlight Laboratory, Bebington, Wirral, Merseyside L63 3JW, UK*

**Abstract**

The interaction of cationic liposomes, prepared by the extrusion technique (VETs) from dimyristoylphosphatidylcholine (DMPC), cholesterol and dimethyldioctadecylammonium bromide (DDAB), covering a range of compositions from 0–18.5 mole% DDAB, with a range of biofilms of oral bacteria has been studied. It has been found that the adsorption of the liposomes to the biofilms showed specificities with respect to bacterial species and strains, adsorption was greatest for strains of *Streptococcus salivarius*. Using this information, the adsorption of cationic liposomes to mixed biofilms of two bacteria, differing in adsorption characteristics, (*S. salivarius* DBD and *Streptococcus sanguis* C104) was investigated as a function of biofilm composition. A linear relationship between liposome adsorption and biofilm composition was found, which showed that each bacterium in the biofilm adsorbed independently. Similar results were obtained for the adsorption of anionic liposomes (composition DMPC-phosphatidylinositol), although the extent of adsorption was smaller, and for cationic liposomes (composition dipalmitoylphosphatidylcholine (DPPC)-cholesterol-stearylamine) adsorbed to mixed biofilms of skin-associated bacteria (*Staphylococcus epidermidis* and *Proteus vulgaris*). The cationic and anionic liposomes were used to deliver the bactericide Triclosan to *S. salivarius* DBD and *S. sanguis* C104 biofilms and their mixed biofilms. Anionic liposomes were most effective in inhibiting growth of *S. sanguis* C104 biofilms whereas, growth of *S. salivarius* DBD could not be effectively inhibited by liposomal Triclosan. Growth inhibition of mixed biofilms by liposomal Triclosan, reflected the effects found on the single species biofilms, anionic liposomes showing growth inhibitions in biofilms with a high content of *S. sanguis* C104. 

*Keywords:* Phospholipid liposomes; Bacterial biofilms; Extrusion technique (VETs); Dimethyldioctadecylammonium (DDAB)

## 1. Introduction

There have been numerous studies on the use of liposomes to deliver antibacterial agents, both to bacterial infections of the mononuclear phagocyte system (MPS) [1–3], to the lung [4] and to clinically important bacteria [5–10]. The use of lipo-

* Corresponding author. Tel.: +44-161-2755093; fax: +44-161-2755082.
*E-mail address:* mjones@fsl.scg.man.ac.uk (M.N. Jones).

0927-7757/01/$ - see front matter 
PII: S0927-7757(01)00481-2

somes as delivery systems in the treatment of infectious diseases has been reviewed [11]. Studies have also been reported on the delivery of bactericides such as Triclosan and chlorhexidine [12–15] and antibiotics [16] to bacterial biofilms. All the above mentioned studies have been concerned with the specific adsorption (targeting), of liposomes to a single species of bacteria either in suspension culture or as an immobilised biofilm. In practice, this is seldom the situation since both the skin and the oral cavity are hosts to a range of bacteria. For example, in the human oral cavity, the streptococci constitute the most numerous group of bacteria and are the most commonly occurring microorganisms in oral infections. Streptococci are the primary colonisers of the salivary pellicle that coats the tooth surface leading to dental plaque that consists of a range of bacteria [17]. *Streptococcus salivarius* is the most prevalent salivary streptococcal species but constitutes only a minor portion of the plaque flora indicating that, there is selectivity involved in early plaque formation [18]. The question of competition between species for adsorption of liposomes appears not to have been addressed. In this paper, we have taken the first steps in investigating the adsorption of liposomes to immobilised biofilms containing binary mixtures of the oral bacteria (*Streptococcus sanguis* plus *S. salivarius*) and the skin-associated bacteria (*Staphylococcus epidermidis* plus *Proteus vulgaris*). *Staphylococcus epidermidis* is normally a harmless Gram-positive bacterium, but can be associated with infections affecting prosthetic devices such as catheters [19], heart valves [20] and artificial joints [21], where the local environment of the implants can become acidic and ischaemic, impairing host defences. The inhibition of growth of the mixed biofilms of the oral bacteria by liposomally delivered Triclosan was also investigated.

## 2. Materials and methods

L-α-dipalmitoylphosphatidylcholine (DPPC, product No. P-0763), L-α-dimyristoylphosphatidylcholine (DMPC, product No. P-63 92), cholesterol (product No. C-8667) dimethyldioctadecylammonium bromide (DDAB, product No. D-2779) and stearylamine (SA, product No. S-9273) were from Sigma Chemical Company, Poole, Dorset, UK. Phosphatidylinositol (PI) grade 1 (from wheat germ) was from Lipid Products, South Nutfield, Redhill, UK. [$^3$H]DPPC (product No. TRK 673, specific activity 55 Ci mmole$^{-1}$), [$^{14}$C] DPPC (product No. CFA 604, specific activity 100 mCi mole$^{-1}$) and [$^3$H]-thymidine (product No. TRK 758) were from Amersham International, Amersham, UK. [$^3$H]-Triclosan (Irgasan) was from Unilever Research, Port Sunlight Laboratory, Wirral, UK. Bacteriological agar No. 1, brain heart infusion (BHI), yeast extract powder, defibrinated horse blood and phosphate buffered saline (PBS) tablets were from Oxoid Ltd., Basingstoke, Hants, UK.

### 2.1. Bacteriology

*Streptococcus mutants* strain D282, *S. sanguis* strain C104, *S. gordonii*, *S. salivarius* strains DBD and NCTC 8618, *S. epidermidis* and *P. vulgaris* were kindly supplied from the University of Manchester collection, by Dr Pauline S. Handley. The bacteria were cultured on brain heart infusion agar by overnight incubation at 37°C. For the growth of streptococci and *P. vulgaris* the agar plates also contained 5% v/v defibrinated horse blood. The plates were incubated in aerobic or 5% $CO_2$ environments. The 5% $CO_2$ environment was produced in a container in which the $CO_2$ concentration was enhanced by removal of oxygen using combustion of a candle. A nutrient broth composed of BHI plus 0.3% w/v yeast extract was used for broth culture. Cultures were grown at 37°C for 18 h, after which they were centrifuged (2000 rpm; 15 min), the supernatant discarded and the pellet resuspended in sterile PBS. The centrifugation and resuspension were repeated a further three times and the bacterial concentration adjusted by dilution with PBS to give an absorbance of 0.5 at 550 nm. The same procedure was used to grow [$^3$H]-labelled bacteria except that [$^3$H]-thymidine (100 μl of 1 mCi ml$^{-1}$) was added per 10 ml of broth just following the innoculation and the bacterial pellet was washed with sterile PBS until the radioactive counts in the

supernatant became negligible (five washes). The cell dispersions (absorbance 0.5) were used to prepare immobilised biofilms by addition of 200 μl aliquots to the wells of microtitre plates (Immulon 2 flat bottomed 96-well plates, Dynatech Laboratories Inc., Virginia, USA), followed by overnight incubation at room temperature. This procedure results in a biofilm of close-packed cells as earlier demonstrated by electron microscopy [22]. In the preparation of binary bacterial biofilms, the two bacterial suspensions were added to the microtitre plate wells in the proportions required, by volume, to give a total volume of 200 μl. The bacteria were incubated overnight at room temperature, after which the supernatant was removed from the biofilm and dispersed in 200 μl of 10% w/v sodium *n*-dodecyl sulphate and left for 1 h. Aliquots (180 μl) were taken and added to 3.5 ml of scintillation fluid for counting.

### 2.2. Preparation and characterisation of liposomes

Liposomes were prepared using the vesicle extrusion method with a Lipex extruder (Lipex Biomembranes, Inc. Vancouver BC Canada) [15]. All the liposomes contained DPPC as the major component. The required lipids (total mass approximately 30 mg) together with either 5 μCi [$^3$H]-DPPC or [$^{14}$C]-DPPC when contained [$^3$H]-Triclosan were dissolved in either 3 ml chloroform–methanol (4:1 by volume) or 20 ml of tert-butyl alcohol, for cholesterol containing liposomes in a 100 ml round-bottomed flask. The solvent was removed by rotary evaporation at 60°C to leave a thin lipid film. The film was hydrated by the addition of 3 ml of phosphate buffered saline (PBS), pH 7.4 at 60°C with vigorous mixing, using a vortex mixer, to give a suspension of multilamellar vesicles (MLVs). The MLVs were then extruded at 60°C through two stacked polycarbonate filters (pore size 100 nm) under a nitrogen pressure of 200–500 psi. The extrusion was repeated five to ten times to produce a uniform suspension of largely unilamellar liposomes of diameter approximately 100 nm.

The extruded liposomes were characterised in terms of lipid concentration by scintillation counting of the [$^3$H]-DPPC and in terms of size by photon correlation spectroscopy (PCS) using a Malvern Autosizer model RR146. Unencapsulated Triclosan was separated from the liposomes by gel filtration as earlier described [15].

### 2.3. Adsorption of liposomes to bacterial biofilms (targeting assays)

Liposome adsorption measurements on the biofilms were carried out in the wells of microtitre plates as earlier described [15]. Microtitre plates of bacterial biofilms were blocked with BSA (1% in PBS) for 15 min at room temperature. The plates were then washed ($3 \times 300$ μl PBS) and incubated with liposomes (200 μl) at various concentrations and for the required time interval at 37°C. The plates were washed again ($3 \times 300$ μl PBS) and the targeted vesicles, adsorbed to the biofilms, were solubilised by SDS (5%, 200 μl). After 30 min the plates were sonicated in a water bath for 5 min and 180 μl of the SDS solution was taken for scintillation counting. Liposome adsorption was expressed as the percentage apparent monolayer coverage of the biofilm by liposomes, calculated from the projected diameter of the liposomes (using $d_w$, the liposome weight average diameter) and the area of the biofilm (calculated as $2.202 \times 10^{-4}$ m$^2$, for a 200 μl solution [15]).

The results were expressed in terms of the percentage apparent monolayer coverage (% amc) calculated from the equation

$$\%\text{amc} = \frac{N_{obs}}{L_a} \times 100 \quad (1)$$

where $N_{obs}$, is the observed number of moles of lipid adsorbed to the biofilm and $L_a$ the number of moles of lipid which would be adsorbed if the biofilm was covered with a close-packed monolayer of liposomes. $L_a$ was calculated from the biofilm and the liposome dimensions, as earlier described [15].

### 2.4. Permeability measurements

The rate of release of Triclosan from liposomes was measured and used to determine apparent permeability coefficients. Triclosan is oil soluble and carried by the liposome bilayer. The rate of

release from the bilayer is thus equivalent to an apparent permeability of the bilayer to Triclosan. Liposomes were prepared (total lipid concentration 10 mg $ml^{-1}$) containing Triclosan and a [$^3$H]-Triclosan tracer (10 μCi). The liposomes were separated from extraliposomal Triclosan by gel filtration, using a Sepharose 4B column. The liposome peak fraction (2 ml) was placed in a dialysis bag (Spectra/por® dialysis membrane, molecular weight cut-off 3500). The bag was placed in a vial containing PBS (10 ml) plus 10% ethanol at 37°C and the rate of release of Triclosan from the liposomes followed by taking 300 μl aliquots for scintillation counting as a function of time. A control experiment was carried out using a bag containing only free Triclosan to ensure that the apparent release of Triclosan from the liposomes was not affected by the permeability of the dialysis membrane tubing. The permeabilities (p) were calculated from the equations

$$\ln(1 - \text{fraction released}) = -\frac{pt}{V_c} \quad (2)$$

where $V_c$ is the internal volume of the liposomes. The permeability coefficients ($P$) were calculated from the slopes of the plots of ln (1-fraction released) vs. $t$ and the relationship,

$$P = \frac{p(R-h)^3}{V_c 3R^2} \quad (3)$$

where $R$ is the radius of the liposome and $h$ the bilayer thickness taken as $75 \times 10^{-8}$ cm [23].

## 3. Results

The adsorption of cationic liposomes incorporating DDAB (13.0 mole%) to a range of streptococci was studied as a function of liposomal lipid concentration (Fig. 1). These bacteria show a relatively wide variation in adsorption characteristics even within this genus, and there are significant differences between strains. *S. salivarius* DBD gave the highest adsorption and *S. sanguis* C104 the lowest. It should be noted that values of apparent monolayer coverage above 100% do not necessarily imply multilayer formation on the biofilms, since the calculations are based on the geometric surface area of the film and do not allow for surface roughness. The effect of the amount of DDAB in the liposomes on apparent monolayer coverage at a constant liposomal lipid

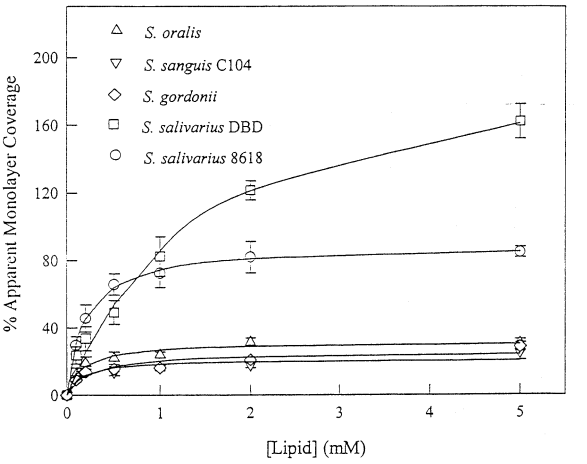


Fig. 1. Adsorption of cationic liposomes (composition DMPC/cholesterol/DDAB, molar ratio 64:23:13, i.e. 13 mole% DDAB, $d_w = 1\ 26$ nm) as a function of liposomal lipid concentration after 30 min incubation with bacterial biofilms at 21°C.

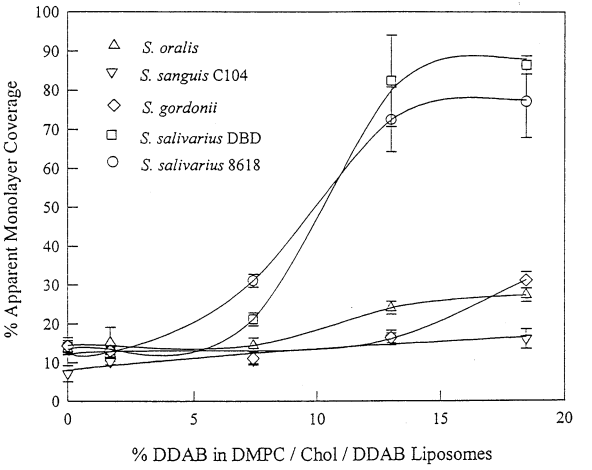


Fig. 2. Adsorption of cationic liposomes (composition DMPC/cholesterol/DDAB, molar ratio 76.8:23.2:0, i.e. 0 mole% DDAB, $d_w = 120$ nm; 75.1:23.1:1.7 i.e. 1.7 mole% DDAB, $d_w = 121$ nm; 69.5:23.1:7.4, i.e. 7.4 mole% DDAB, $d_w = 131$ nm; 64:23:13 i.e. 13 mole% DDAB, $d_w = 126$ nm; 58.5:23.0:18.5, i.e. 18.5 mole% DDAB, $d_w = 117$ nm) as a function of mole% DDAB after 30 min incubation with bacterial biofilms at 21°C.

concentration (1 mM) was also studied. Fig. 2 shows that adsorptions follows a sigmoidal curve as a function of mole% DDAB for this range of streptococci, for each strain, adsorption increases with DDAB mole% and for the *S. salivarius* the data reach plateaux above which further increases in mDDAB mole% do not increase adsorption.

In order to investigate the adsorption to biofilms containing binary mixtures of cells, the pair *S. salivarius* DBD plus *S. sanguis* C104 were chosen for study since, these show the largest difference in affinities for the cationic liposomes incorporating DDAB. Studies were also carried out on a pair of skin-associated bacteria, *S. epidermidis* plus *P. vulgaris* that adsorb cationic liposomes incorporating stearylamine.

It was important to investigate the composition of the biofilms formed on the microtitre plate wells. The binary biofilms of varying compositions were made by incubation of mixtures of the separate bacteria of varying volume % in the plate wells. To determine the composition of the biofilms, experiments were made using mixtures of bacteria, in which one species was radiolabelled with [$^3$H]-thymidine. Fig. 3 shows the results for the streptcocci combinations. The radioactivity of the biofilm was found to increase linearly with increase in % by volume of the radio labelled species in the initial suspension. The radioactivity decreased linearly with increase in the % by volume of the unlabelled species. For $^3$H-*S. salivarius* DBD the regression coefficient was 0.986 and for $^3$H-*S. sanguis* C104 0.984.

Fig. 4 shows similar data for the skin-associated bacterial combination. The radioactivity of the biofilm was again found to be linearly related to the radioactive species in the original bacterial suspension. For $^3$H-*S. epidermidis* the regression coefficient was 0.977 and for $^3$H-*P. vulgaris* 0.948.

The adsorption of cationic liposomes incorporating DDAB, at two liposomal lipid concentrations, to the streptococcus binary biofilms, is shown in Fig. 5(a) as a function of the % *S. salivarius* DBD by volume in the suspension used to prepare the biofilm. The apparent monolayer coverage is found to increase linearly with the amount of the more strongly adsorbing bacterium in the biofilm. Similar data were also obtained

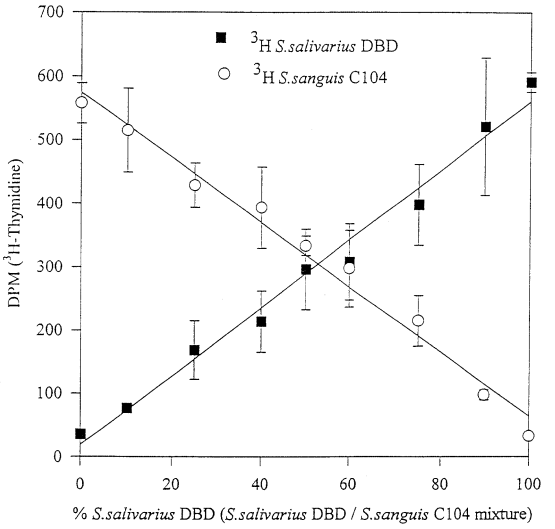


Fig. 3. Adsorption of $^3$H-labelled bacteria *S. salivarius* DBD (■) and *S. sanguis* C104 (O) to microlitre plate wells from mixtures of the bacterial suspensions (absorbance 0.5 at 550 nm, only one bacterium being $^3$H-labelled at a time) as a function of vol%. The bacterial suspensions were incubated for 24 h at 21°C to produce the mixed biofilm which was then washed to remove free bacteria.

when anionic liposomes incorporating phosphatidylinositol were studied (Fig. 5(b)) However, the extent of adsorption of these liposomes is only small and there is only a small variation between adsorption to the biofilms of the pure species.

Fig. 6 shows the apparent monolayer coverage with cationic liposomes incorporating SA as a function of the % *S. epidermidis* in the biofilms of the skin-associated bacteria binary combination. In this case, there is a large difference between adsorption to biofilms of the pure species ($\sim$ 170%) but linearity between adsorption and the amount of the most strongly adsorbing species in the biofilm is still found.

The effect of the free Triclosan on the inhibition of growth of bacteria from biofilms of the two strains of streptococci was determined after a 30 min exposure time followed by a 4 h growth period in nutrient broth (Fig. 7(a) and (b)). *S. sanguis* C104 is more sensitive to Triclosan than *S. salivarius* DBD. At a free Triclosan concentration of approximately 4 μg ml$^{-1}$, the inhibition of growth was 92% for *S. sanguis* C104 but only 16%

for *S. salivarius* DBD. The sensitivity to Triclosan was also reflected in the growth inhibition by liposomes carrying Triclosan. Growth inhibition of *S. sanguis* by cationic liposomes incorporating Triclosan, was less than by free Triclosan (Fig. 8(a)) but significantly greater than for *S. salvarius* DBD whose growth was not inhibited. Surprisingly, anionic liposomes incorporating PI, although adsorbing only weakly to pure *S. sanguis* C104 biofilms (Fig. 5) delivered Triclosan effectively, but the cationic liposomes did not inhibit growth of *S. salivarius* DBD (Fig. 8(b)).

The delivery of Triclosan by both cationic and anionic liposomes to binary bacterial biofilms, reflected both the greater effectiveness of anionic liposome delivery to the pure *S. sanguis* C104 biofilms and the greater efficacy of liposomal Triclosan. Growth inhibition was greater with biofilms containing a smaller proportion of *S. salivarius* DBD and correspondingly larger proportion of *S. sanguis* C104 (Fig. 9). As for the single species biofilms, Triclosan in cationic liposomes failed to inhibit growth above a few percent from mixed biofilms.

The release of Triclosan from cationic and anionic liposomes was determined using a dialysis method. Fig. 10 shows the linear plots based on Eq. (2) for Triclosan release from liposomes, together with results from a control experiment for the transport of free Triclosan across the dialysis membrane. The control experiment clearly demonstrates that transport of Triclosan through the dialysis membrane is very much faster than through the liposomal bilayers. The permeability coefficients of the liposomal bilayers calculated from Eq. (3) were $9.26 \times 10^{-8}$ cm $h^{-1}$ for the anionic liposomes and $2.57 \times 10^{-8}$ cm $h^{-1}$ for the cationic liposomes. After a period of 6 h, these permeabilities correspond to the release of 32% of the encapsulated Triclosan from the anionic liposomes and 9.7% from the cationic liposomes. The difference in permeability coefficients for Triclosan between anionic and cationic liposomes possibly relates to stabilisation of the Triclosan in cationic bilayers due to lone pair hydroxyl-cation interactions in the bilayer interface.

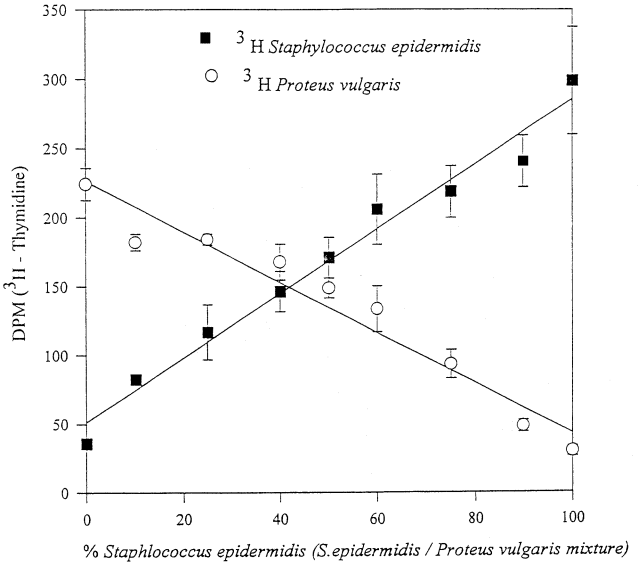


Fig. 4. Adsorption of $^3$H-labelled bacteria *S. epidermidis* (■) and *P. vulgaris* (O) to microtitre plate wells from mixtures of the bacterial suspensions (absorbance 0.5 at 550 nm, only one bacterium being $^3$H-labelled at a time) as a function of vol%. The bacterial suspensions were incubated for 24 h at 21°C to produce the mixed biofilm which was then washed to remove free bacteria.

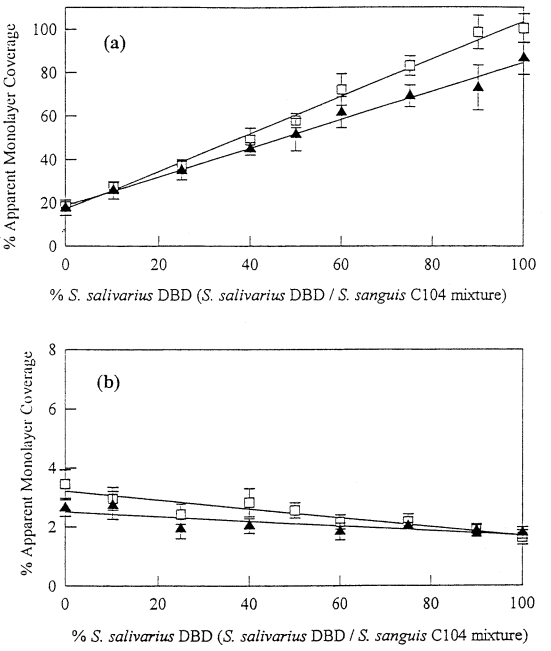


Fig. 5. Adsorption of liposomes to mixed bacterial bioflims (*S. salivarus* DBD and *S. sanguis* C104) (a) Adsorption of cationic liposomes (composition DMPC/cholestero1/DDAB molar ratio 58.5:23.0:18.5; $d_w$ 120 nm), (b) adsorption of anionic liposomes (composition DMPC/PI, molar ratio 82.5:17.5; $d_w$ 108 nm). The liposomes were incubated with the biofilms for 30 min at 37°C, liposomal lipid concentration 2 mM (▲) and 5 mM (□).

## 4. Discussion

Cationic liposomes display a range of adsorption specificities with respect to species and strains of oral bacteria which is most marked when the liposomes contain greater than 15 mole% DDAB (Figs. 1 and 2). These specificities were exploited in the choice of pairs of bacteria for the study of adsorption to biofilms containing a mixed population of species.

Interactions between cationic liposomes and the surface of bacteria are possibly dominated by ionic forces between the positive liposome surface and negative polyolphosphates of teichoic acid [24] on the bacterial surface. Negative zeta potentials indicating negative surface charge have been reported for a number of oral bacteria such as *Streptococcus sanguis*, *mutans* [25] and *salivarius* [26].

For the binary mixtures of bacteria it was important to be able to control the proportion of a particular species in the biofilm. Any preferential adsorption for one particular species on the solid support (microtitre plate well), would lead to a population containing a disproportionate number of that species within the biofilm. Radiolabelling of one species in the binary mixture enables the proportion of that species in the biofilm, to be checked in relation to its proportion in the bacterial suspension, from which the biofilm was formed. The results showed a linear relationship between the amount of the radiolabelled species in the biofilm and the percentage by volume of the species in the bacterial suspension, used to form the biofilm (Figs. 3 and 4). The mixed bacterial suspension were formed from suspensions of the individual species, having the same absorbance (0.5 at 550 nm). Mixing these suspensions in different relative proportions maintained the same absorbance and hence, the same cell concentrations across the composition range. The only assumption in the procedure was that the suspensions of a given species of the same absorbance contained approximately the same num-

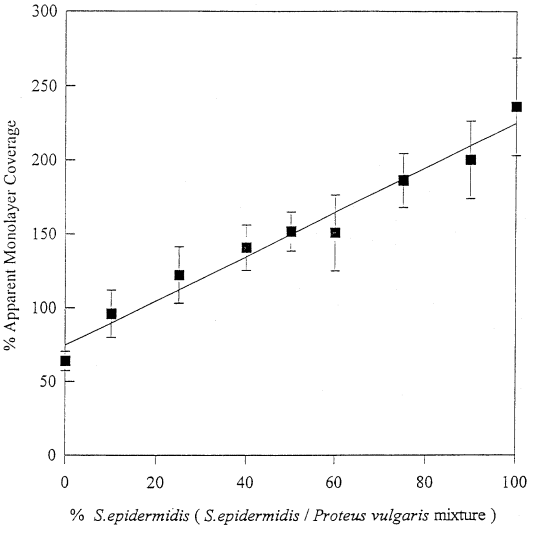


Fig. 6. Adsorption of cationic liposomes to mixed bacterial biofilms (*S. epidermidis* and *P. vulgaris*). Liposome composition DPPC/cholesterol/SA molar ratio 52.8:26.0:21.2, $d_w = 129$ nm, liposomal lipid concentration 2 mM. The liposomes were incubated with the biofilm for 2 h at 37°C.

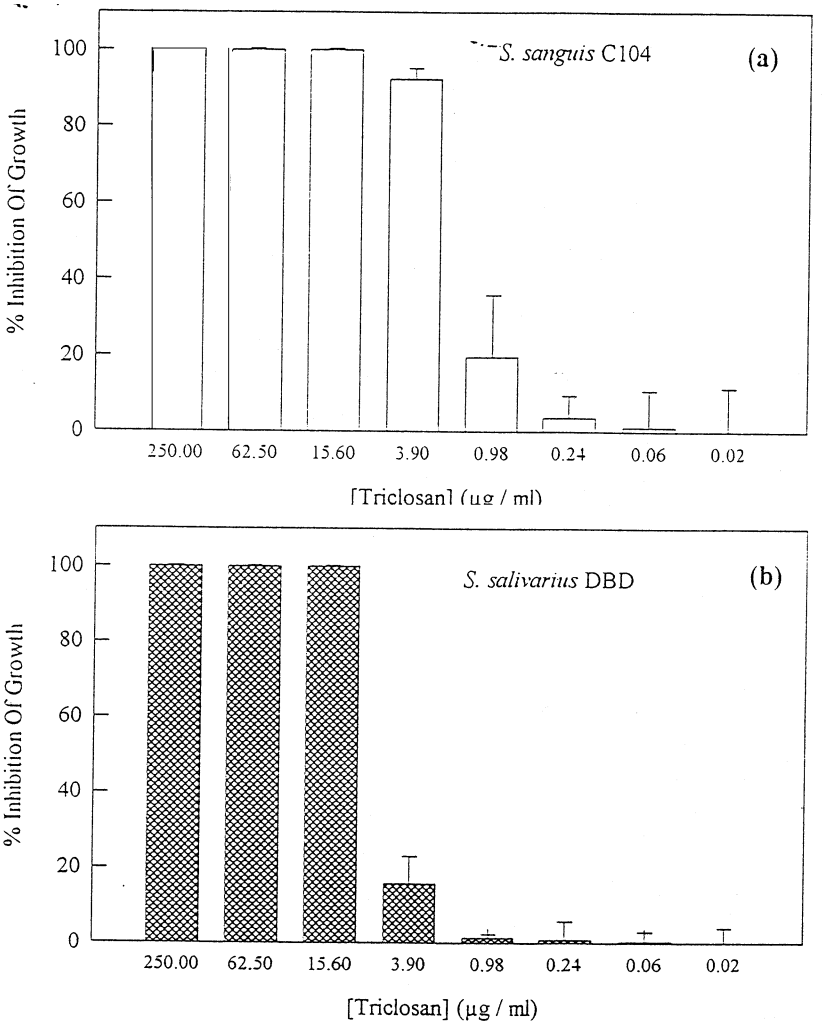


Fig. 7. The effect of Triclosan on the growth of *S. sanguis* C104 (a) and *S. salivarius* DBD (b) biofilms. Triclosan incubation time 30 min at 37°C. The biofilms were incubated with sterile nutrient broth for 4 h at 37°C; mean ± SD ($n = 6$).

ber of cells. The linearity of the plots in Fig. 3 and Fig. 4 indicate that the two species adsorb to the solid support independently and in proportion to their concentrations in the mixed bacterial suspension.

This is further confirmed by the data in Fig. 3, where the labelling of the cells with $^{3}$H-thymidine was approximately the same for the two species and the cross-over point occurs at approximately 50%.

The adsorption of cationic liposomes to the binary biofilms was found to be a linear function of the biofilm composition for both the oral and skin-associated bacteria combinations (Figs. 5 and 6). This suggests that each species in the biofilm is adsorbing liposomes independently. Any preferential adsorption by one species would have given rise to a deviation from linearity giving either a convex adsorption curve as the concentration of the more strongly adsorbing species increased, or a concave curve with increase in the concentration of the more weakly adsorbing species. The observed adsorption is essentially 'ideal' behaviour reminiscent of the total vapour pressure as a

function of mole fraction, for an ideal liquid mixture in which the vapour pressure of each component obeys Roault's law and is directly proportional to its mole fraction. Here, the % amc for each species in the biofilm may be expressed by equations of the form

$$\%\ \mathrm{amc}_1 = (\%\ \mathrm{amc})_1^{\circ}\phi_1 \quad (4)$$

$$\%\ \mathrm{amc}_2 = (\%\ \mathrm{amc})_2^{\circ}(1-\phi_1) \quad (5)$$

where the subscripts 1 and 2 refer to the two bacterial species and the superscript (o) refers to adsorption to the pure biofilms. $\phi_1$ is the volume fraction of species 1 in the suspension used to form the biofilm. The total % amc is thus given by

$$\%\ \mathrm{amc} = \%\ \mathrm{amc}_1 + \%\ \mathrm{amc}_2 \quad (6)$$

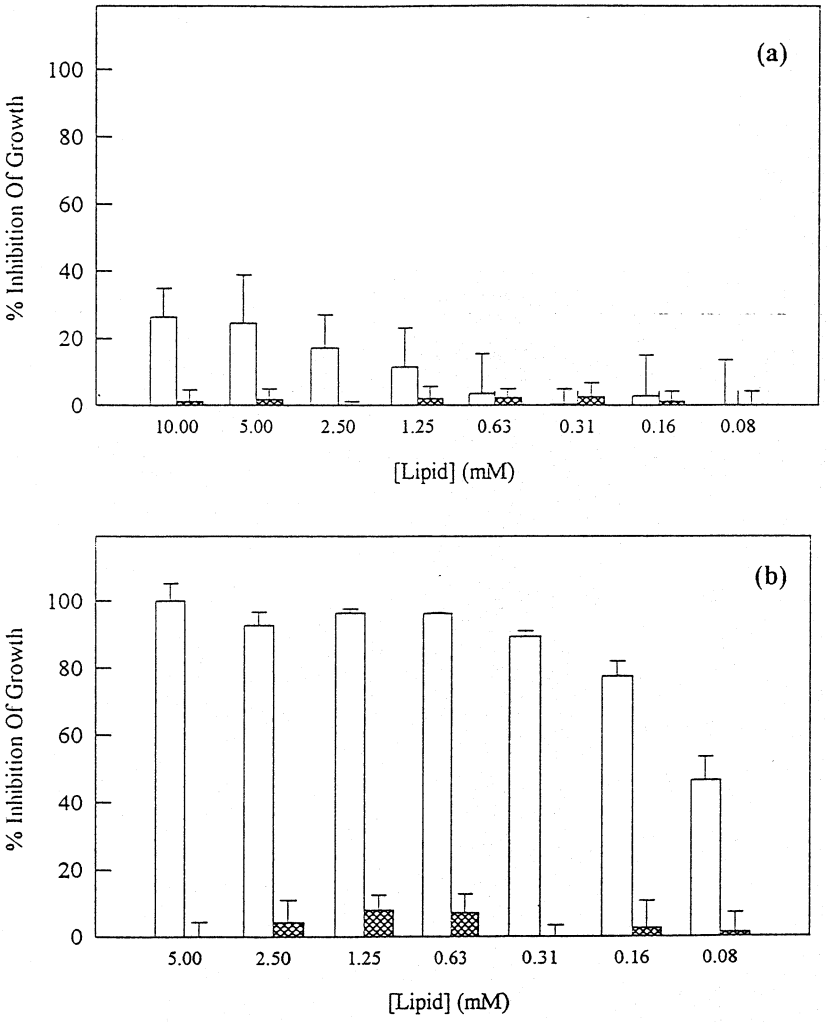


Fig. 8. Growth inhibition by liposomal Triclosan of *S. sanguis* C104 (open blocks) and *S. salivarius* DBD (hatched blocks) by cationic liposomes (a) (composition DMPC/cholestero1/DDAB molar ratio 58.5:23:18.5; $d_w = 1$ 17 nm) and anionic liposomes; (b) (composition DMPC/PI molar ratio 82.5:17.5; $d_w = 118$ nm) as a function of liposomal lipid concentration. The concentrations of liposomal Triclosan were in a range from 498–3.98 μl $ml^{-1}$ with a constant mass per mole of lipid of 49.8 g (mole lipid)$^{-1}$ (a) and in a range 498–7.97 μl $ml^{-1}$ with a constant mass per mole of lipid of 99.7 g (mole lipid)$^{-1}$, (b) Liposome incubation time with biofilm 30 min at 37°C. The biofilms were incubated with sterile nutrient broth for 4 h at 37°C; mean ± SD ($n = 6$).

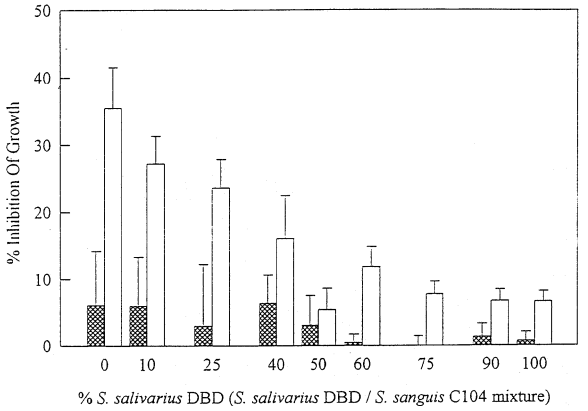


Fig. 9. Growth inhibition of mixed bacterial biofilms (*S. sanguis* C 104 plus *S. salivarius* DBD) by cationic liposomes (hatched bars) (composition DMPC/cholesterol/DDAB molar ratio 58.5:23:18.5; $d_w = 117$ nm, liposome lipid concentration 0.2 mM; Triclosan concentration 1.99 μl ml$^{-1}$ (9.96 g (mole lipid)$^{-1}$)) and anionic liposomes (open bars) (composition DMPC/PI molar ratio 82.5:17.5; $d_w = 118$ nm, liposome lipid concentration 0.2 mM; Triclosan concentration 3.99 μl ml$^{-1}$ (19.94 g (mole lipid)$^{-1}$)) as a function of biofilm composition. Liposome incubation time with biofilm 30 min at 37°C. The biofilms were incubated with sterile nutrient broth for 4 h at 37°C; mean ± SD ($n = 6$).

$$= \{(\%\ \text{amc})_1^\circ - (\%\ \text{amc})_2^\circ\}\phi_1 + (\%\ \text{amc})_2^\circ \qquad (7)$$

so that when $\phi_1 \to 1$, % amc $\to (\%\ \text{amc})_1^\circ$ and when $\phi_1 \to 0$, % amc $\to (\%\ \text{amc})_2^\circ$.

It follows from Eq. (7) that % amc is a linear function of $\phi_1$ as observed in Fig. 5(a) and Fig. 6.

Ideality is also observed for the adsorption of anionic liposomes to the mixed biofilms of oral bacteria (Fig. 5(b)) although as differences between adsorption to the pure biofilms is small deviations from ideal, independent adsorption would hardly be expected.

The delivery of Triclosan by cationic liposomes was effective only in inhibiting the growth from *S. sanguis* C104 biofilms, despite the fact that the adsorption of the liposomes was less than that of *S. salivarius* DBD biofilms. The extent of growth inhibition of *S. sanguis* C104 by cationic liposomes was however, not as effective as inhibition by Triclosan, carried by anionic liposomes containing PI (Fig. 8(b)). This behaviour was also reflected in the delivery of Triclosan to mixed biofilms (Fig. 9). As the proportion of *S. salivarius* DBD increased in the biofilm, the ability of the anionic liposomes to inhibit growth decreased. Cationic liposomes were not effective against the mixed biofilms.

The difference in Triclosan delivery between cationic and anionic liposomes is clearly not simply related to the extents of adsorption of the liposomes, since cationic liposomes adsorb very much more than anionic liposomes. It is only possible to speculate at present about the origin of this difference. The permeability studies clearly show that anionic liposomes are approximately four times more permeable to Triclosan than cationic liposomes, although this is a very significant difference, in a time period of 30 min that was used for targeting, the amounts of Triclosan released by anionic and cationic liposomes are 5 and 3%, respectively. Such a small difference in Triclosan release would perhaps not be expected to lead to such large differences in growth inhibi-

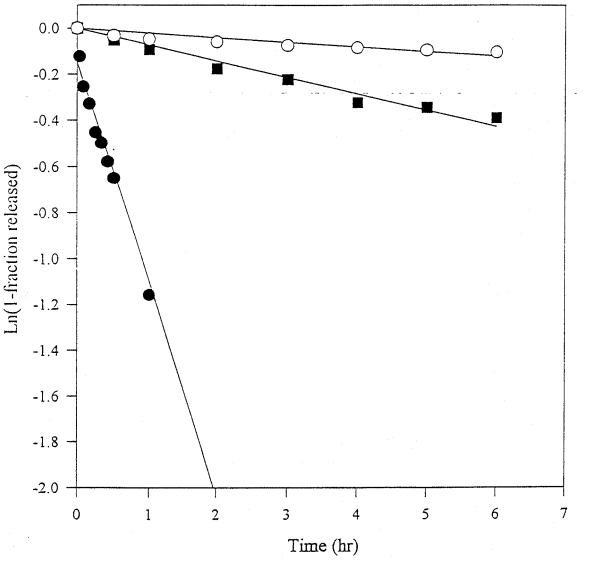


Fig. 10. Rate of release of Triclosan according to Eq. (2) from dialysis bag (●), cationic liposomes (O) and anionic liposomes (■). Cationic liposome composition DMPC/cholesterol/DDAB molar ratio 58.5; 23:18.5; $d_w = 117$ nm;Triclosan concentration 133 μl ml$^{-1}$ (17.47 g (mole lipid)$^{-1}$); liposomal lipid concentration 7.67 mM. Anionic liposome composition DMPC/PI molar ratio 82.5:17.5; $d_w = 118$ nm; Triclosan concentration 766 μl ml$^{-1}$ (112.8 g (mole lipid)$^{-1}$); liposomal lipid concentration 6.88 mM. The free Triclosan concentration was 650 μg ml$^{-1}$

tion. Further studies on the nature of the mechanism of interaction between the liposomes and the bacteria and specifically to what extent the liposomes remain intact on adsorption may be of value in this context.

## Acknowledgements

We thank the BBSRC for a CASE studentship for Anne M. Robinson.

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