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The targeting of phospholipid liposomes to bacteria

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Abstract

Phospholipid liposomes have been prepared from phospholipid mixtures including dipalmitoylphosphatidylcholine/phosphatidylinositol (DPPC/PI) and DPPC/dipalmitoylphosphatidylglycerol (DPPC/DPPG) mixtures and targeted to adsorbed biofilms of the skin-associated bacteria *Staphylococcus epidermidis* and *Proteus vulgaris* and the oral bacterium *Streptococcus sanguis*. The effects of time, liposome concentration and density of bacteria in the biofilm have been studied in detail for *Staphylococcus epidermidis*. The targeting (as assessed by the apparent monolayer coverage of the biofilms by liposomes) to the biofilms was found to be sensitive to the mol% of PI and DPPG in the liposomes and optimum levels of PI were found for targeting to each bacterium. The use of PI and DPPG-containing liposomes for the delivery of the bactericide, Triclosan, to biofilms of *Staphylococcus epidermidis* was studied as a function of the amount of Triclosan carried by the liposomes. All the liposome systems tested inhibited the growth of bacteria from the biofilms after brief (2 min) exposure to Triclosan-carrying liposomes. At low Triclosan levels bacterial growth inhibition by Triclosan-carrying liposomes exceeded that by an equivalent level of free Triclosan. After short periods (min) of exposure of biofilms to Triclosan-carrying liposomes the bactericide was shown to preferentially concentrate in the biofilms relative to its liposomal lipid carrier. The results suggest that phospholipid liposomes with appropriately chosen lipid composition have potential for the targeting and delivery of bactericide to bacteria.

Keywords: Phosphatidylinositol-containing liposome; Liposome; Drug targeting; Bactericide delivery; Triclosan

1. Introduction

The targeting of liposomes to mammalian cells can be achieved by use of site-directing proteins such as lectins [1–4] or antibodies raised to cell surface antigens [5–11]. Liposomes with covalently linked protein (proteoliposomes) have potential for the delivery of drugs although their preparation requires chemical modification of both the liposomal surface and the site-directing protein prior to conjugation [12]. The topical delivery of bactericides to the surface of the skin and the surfaces in the oral cavity is important in the development of personal products such as toothpastes, mouthwashes and shampoos. To be most effective the bactericide should not only be targeted to bacteria but ideally be retained after the initial application. By using liposomes as a means of delivery of bactericide both targeting and retention might be achieved. Before such possibilities can become a reality the nature of the interactions between liposomes and bacteria needs to be investigated. In a recent study of the targeting of liposomes to the oral bacterium *Streptococcus mutans* it was observed that phospholipid liposomes prepared by sonication (SUV) incorporating phosphatidylinositol (PI) would target to *S. mutans* provided the PI level was not too high, i.e., not greater than 10 mol% [13]. Targeting of liposomes by use of selected phospholipids such as PI is of potential value in view of its simplicity, although the mechanism of the process is ill-defined. In order to investigate the potential value of PI and other phospholipids as targeting molecules we have investigated the adsorption of PI-containing and phosphatidylglycerol (PG)-containing liposomes to biofilms of a number of bacteria including the oral bacterium *Streptococcus sanguis* and the skin associated bacteria *Proteus vulgaris* and *Staphylococcus epidermidis*. The use of the liposomes as a means of delivery of

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the bactericide, Triclosan, to *Staphylococcus epidermidis* biofilms and their effect on subsequent growth of the bacterium has been investigated. The results suggest that PI and PG-containing liposomes have considerable potential as a means of targeting liposomes to bacteria and for the delivery of bactericide to adsorbed biofilms.

2. Materials and methods

L-α-Dipalmitoylphosphatidylcholine (DPPC, product No. P 0763) L-α-dipalmitoylphosphatidylglycerol (DPPG, product No. P 9789) and L-α-dipalmitoylphosphatidic acid (DPPA, product No. P 4013) were obtained from Sigma, Poole, Dorset, UK. Phosphatidylinositol (PI, from wheat germ), phosphatidylserine (PS, from bovine spinal cord) and cerebroside (C, from bovine spinal cord) were from Lipid Products, South Nutfield, UK. [^{3}H]DPPC (specific activity 55 Ci/mmol) and [^{14}C]DPPC (specific activity 112 mCi/mmol) were from Amersham International, Amersham, UK. [^{3}H]Triclosan and Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) were from Unilever Research, Port Sunlight Laboratory. Bacteriological agar No. 1 (code L11), brain heart infusion (BHI, code CM 255), yeast extract powder (code L21) and phosphate-buffered saline (PBS) tablets (code BR 14a) were from Oxoid, Basingstoke, Hants, UK. All other reagents were of analytical grade and aqueous solutions were made up with double distilled water.

2.1. Preparation and characterisation of liposomes

Liposomes were prepared from the required phospholipid mixtures by the vesicle extrusion technique [14]. Routinely the phospholipid mixture (total mass 28–33 mg plus either 5 μCi [^{3}H]DPPC or 1 μCi [^{14}C]DPPC) was dissolved in dry chloroform-methanol (4:1 by volume) in a 50 ml round bottomed flask. The solvent was removed by rotary evaporation at 60°C to leave a uniform lipid layer to which was added 3 ml of PBS at 60°C. The suspension was vigorously mixed on a vortex mixer to form multilamellar liposomes (MLVs) which were extruded 10 times at 60°C through two stacked Poretic polycarbonate filters, pore size 100 nm, at a pressure of 200 psi using a Lipex Biomembranes extruder. The size distributions of the VETs were measured by photon correlation spectroscopy using a Malvern autosizer model RR 146. The scattering data were fitted to an equivalent normal weight distribution $W(d_i)$ to give the weight-average diameters ($\bar{d}_w$) [15].

2.2. Growth of bacteria

Bacteria were obtained from the University of Manchester collection. They were used to inoculate agar plates containing 15–20 ml of agar prepared from 3.7 g of BHI in 100 ml of double distilled water to which was added 1.5 g of bacteriological agar. The agar suspension was boiled to dissolve the agar and sterilized using an autoclave at 15 lbs pressure for 15 min. The plates were inoculated by streaking under aseptic conditions and the inverted streaked plates were incubated at 37°C for 18 h. The resulting colonies were used to inoculate nutrient broth prepared by mixing 3.7 g BHI and 0.3 g yeast extract powder in 100 ml of double distilled water. For growth of *Streptococcus sanguis* (Strain CR2b) the agar plates contained 5% (v/v) defibrinated horse blood and the bacteria were grown anaerobically in broth containing 2% (w/v) sucrose.

The nutrient broth for the bacteria was divided into 10 ml aliquots in glass screwcap bottles and sterilized (15 lbs pressure for 15 min) prior to inoculation. The capped bottles were incubated at 37°C for 18 h after which the bacterial suspensions were centrifuged at 2000 rpm for 15 min, the supernatant discarded and the pellet resuspended in sterile PBS. The centrifugation and resuspension was repeated a further three times and the bacterial concentration adjusted to the required absorbance.

2.3. Adsorption of liposomes (VETs) to bacterial biofilms (Targeting assay)

Assays were carried out in the wells of microtitre plates (Dynatech M129B). Aliquots of bacterial suspension (200 μl of the required absorbance (routinely 0.5 at 550 nm)) were incubated overnight at room temperature to form an adsorbed biofilm. Preliminary experiments established that adsorption of the liposomes to the biofilm reached limiting values when the biofilm was prepared from a bacterial suspension of absorbance in excess of 0.4. After incubation the bacterial suspension was removed and the biofilm washed twice with sterile PBS. Vacant potential binding sites on the biofilms were blocked by incubation with 300 μl of 0.02% (w/v) casein in PBS for 1 h. After removal of the casein solution, wells were exposed to liposome suspensions for the required test time (routinely 2 h). After incubation with liposome suspension the wells were washed three times with PBS and the biofilm was dispersed by addition of 200 μl of 1% (w/v) sodium *n*-dodecylsulfate followed by sonication and a 1 h incubation. Experiments were carried out to establish the effect of the number of washes on the extent of adsorption. It was found that weakly held liposomes were removed in the first and second wash and adsorption remained constant thereafter. Aliquots of the dispersed biofilm (180 μl) were taken for scintillation counting. Control wells containing only bacteria, only PBS and only liposomes were used to assess background levels of activity.

The results of the targeting assays are expressed in terms of the percentage apparent monolayer coverage (%amc) given by

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

Where N_{obs} is the observed number of mol 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 layer of liposomes. L_a was calculated from the equation.

$$L_a = \frac{A_{bf}}{\pi(\bar{d}_w/2)^2}\bar{N}_w$$

Where $\bar{d}_w$ is the weight average diameter of the liposomes having a weight average number of lipid molecules per liposome of $\bar{N}_w$ and A_{bf} is the geometric area of the biofilm. For unilamellar liposomes $\bar{N}_w$ can be calculated from $\bar{d}_w$ assuming an area per lipid molecule in the liposomal bilayer (taken as 0.50 nm^2) and a bilayer thickness (taken as 7.5 nm) as previously described [15]. The area of the biofilm was taken as $2.202 \cdot 10^{-4}$ m^2 which was measured in a previous study for the surface of microtitre plates wells exposed to 200 μl of solution [16].

2.4. Delivery of Triclosan to biofilms (Re-growth assay)

For *Staphylococcus epidermidis* the effectiveness of the liposomes incorporating Triclosan in inhibiting bacterial growth relative to an equivalent concentration of free Triclosan was assessed using a regrowth assay. For these experiments the liposomes were prepared in PBS containing 10% (v/v) of ethanol. Triclosan is oil-soluble and has a very low solubility in water; it is more soluble in 10% (v/v) ethanol solutions and measurable free concentrations can be obtained. In 10% (v/v) ethanol the lowest concentration at which 100% inhibition of growth is obtained was found to be approx. 1 μg/100 μl although 90% inhibition of growth occurs at 0.5 μg/100 μl and 25% growth inhibition is observed as low as 0.01 μg/100 μl. For the delivery experiments [^{3}H]Triclosan was added to the lipid mixture during the preparation of the lipid film prior to formation of the VETs. [^{3}H]Triclosan containing liposomes were labelled with [^{14}C]DPPC. Prior to use, the Triclosan loaded liposomes were passed through a Sepharose 4B column to remove any free Triclosan. The liposomes were targeted to the *Staph. epidermidis* biofilms, prepared as described above, for the required time period (routinely 2 min) together with controls of equivalent levels of free Triclosan. Control wells with only bacteria and only PBS were also included. After targeting, the well contents were removed, the biofilms were washed with PBS and 200 μl of growth media were added to each well. The plate was incubated for 18 h after which the biofilms were dispersed and the absorbance of each well was measured at 630 nm using a Dynatech MR 610 plate reader coupled to an Apple IIe Microcomputer. The increase in absorbance over the 18 h period was taken as a measure of bacterial growth.

3. Results

3.1. Kinetics of adsorption to Staphylococcus epidermidis biofilms

The kinetics of adsorption of DPPC/PI liposomes to *Staph. epidermidis* were studied as a function of liposome

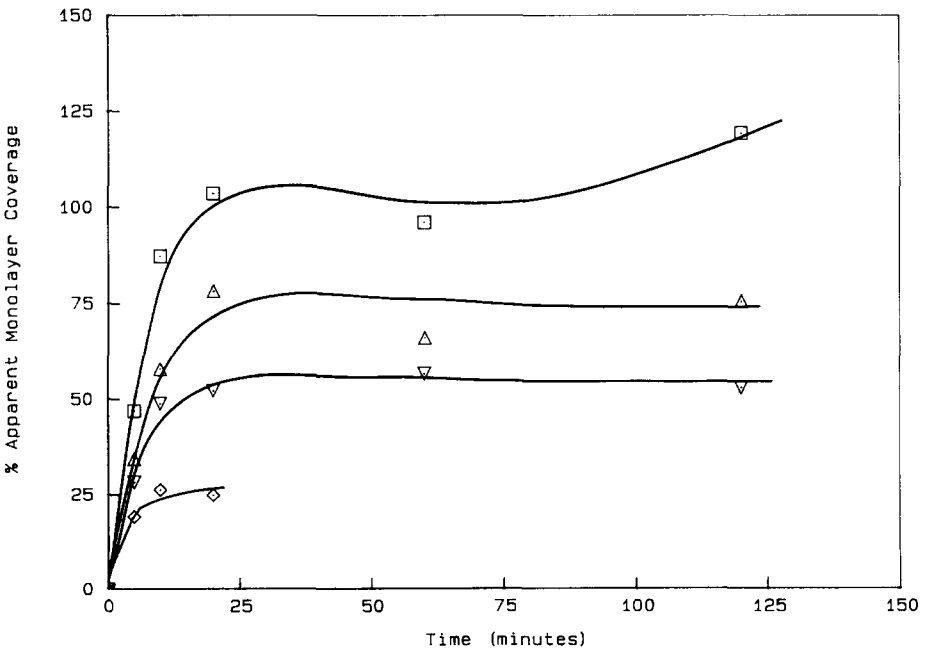

Fig. 1. The kinetics of adsorption of DPPC/PI (molar ratio 1:0.129) liposomes ($\bar{d}_w$ = 156 nm) to *Staphylococcus epidermidis* biofilms. The liposomal lipid concentrations were □, 13.8 mM; △, 6.90 mM; ▽, 3.45 mM; ◇, 0.863 mM. Casein was used as blocking agent and the biofilms were washed three times with PBS prior to assay.

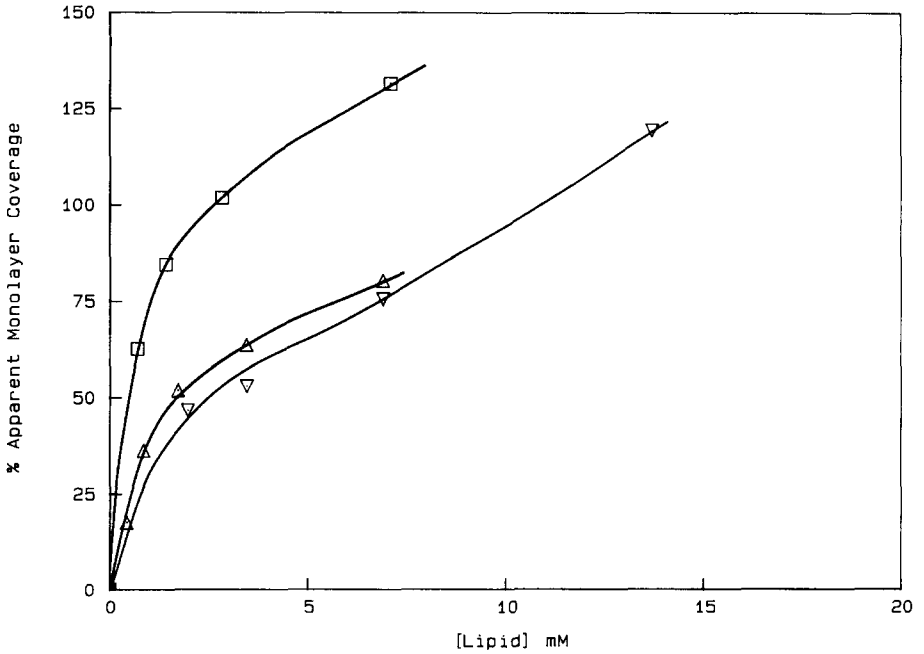

Fig. 2. The dependence of adsorption of DPPC/PI liposomes to bacterial biofilms on liposomal lipid concentration □, *Staphylococcus epidermidis* biofilm, DPPC/PI molar ratio (1:0.129), $\bar{d}_w$ = 87.7 nm; ▽, *Streptococcus sanguis* biofilm, DPPC/PI molar ratio (1:0.129), $\bar{d}_w$ = 92.0 nm; △, *Proteus vulgaris* biofilm, DPPC/PI molar ratio (1:0.161), $\bar{d}_w$ = 92.2 nm. The time of incubation of the liposomes with the biofilm was 2 h, casein was used as the blocking agent and the biofilms were washed three times with PBS prior to assay.

concentration (Fig. 1). It was found that the adsorption reached limiting values after 30–60 min at all the concentrations studied in a range up to 14 mM liposomal lipid. Routinely an incubation time of 120 min was adopted in subsequent experiments. The dependence of %amc after 120 min incubation on the liposomal lipid concentration was also studied and the data are shown for *Staph. epidermidis* and other bacteria in Fig. 2. Adsorption rises steeply between a lipid concentration of 0 and 5 mM and more slowly thereafter. Liposomal lipid concentrations in the region of 1–2 mM are sufficiently high to give adsorption levels which were easily measurable. The levels of adsorption for the different bacteria may reflect subtle differences in the bacteria glycocalyx.

3.2. Dependence of targeting on liposomal lipid composition

Previous studies on *Streptococcus mutans* showed that liposomes containing low levels of PI effectively targeted to this bacterium [13]. The dependence of liposomal composition on targeting to *Staphylococcus epidermidis, Proteus vulgaris* and *Streptococcus sanguis* was investigated. For each system an attempt was made to use batches of liposomes of approximately the same size and to keep the liposomal lipid concentration constant. Figs. 3 to 7 show the results of these experiments. For *Staph. epidermidis* (Fig. 3) the %amc was very sensitive to the PI content of DPPC/PI liposomes, the optimum PI level being approx. 11 mol%. It is also shown in Fig. 3 that on addition of Triclosan at relatively high concentrations, [Triclosan]/[lipid] molar ratio of the order of 0.03, although the extent of adsorption at optimum PI levels was reduced the profile of adsorption as a function of the PI content of the liposomes was not affected. DPPG was also effective in targeting to *Staph. epidermidis* and here also an optimum level (6.78 mol%) of DPPG in DPPC liposomes was found (Fig. 4). Pure DPPG liposomes (the control) were less effective but addition of PI to DPPG

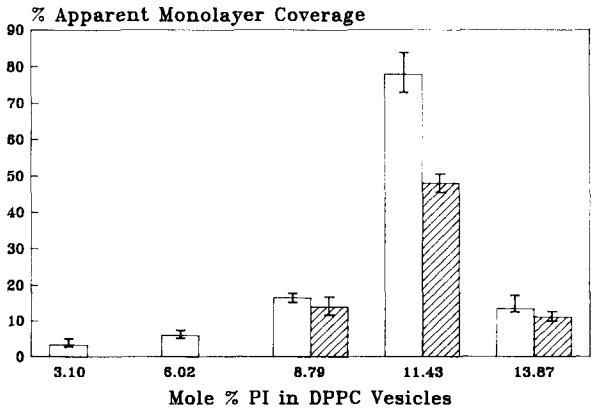

Fig. 3. Dependence of adsorption of DPPC/PI liposomes to *Staphylococcus epidermidis* biofilms on mol% PI content. The liposomes were incubated with the biofilm for 2 h, casein was used as the blocking agent and the biofilms were washed three times with PBS prior to assay. The liposomal lipid concentration was 1.34 ± 0.06 mM, $\bar{d}_w$ = 79.8 ± 6.7 nm. The shaded blocks refer to liposomes incorporating Triclosan at a molar ratio of Triclosan to lipid of 0.03.

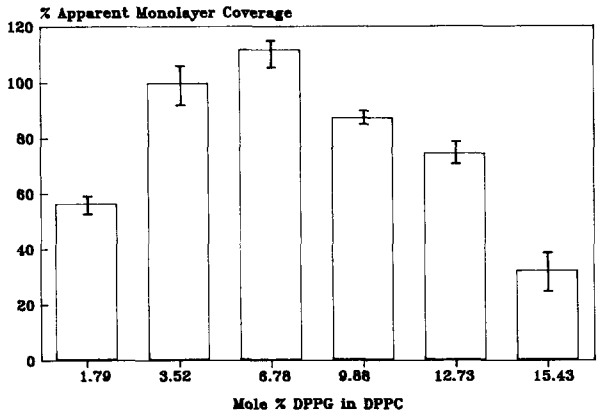

Fig. 4. Dependence of adsorption of DPPC/DPPG liposomes to *Staphylococcus epidermidis* biofilms on mol% DPPG content. The liposomes were incubated with the biofilm for 2 h, casein was used as the blocking agent and the biofilms were washed three times with PBS prior to assay. The liposomal lipid concentration was 1.34 ± 0.08 mM, $\bar{d}_w = 86.6 \pm 3.0$ nm.

improved targeting (Fig. 5). These results suggested that the existence of optimum compositions for targeting for liposomes containing the phospholipids PI and DPPG related to the hydroxy content of the head groups. This observation prompted us to assess liposomes prepared from the ternary mixture DPPC/phosphatidic acid (PA)/cerebroside, however these gave low %amc (Fig. 5).

The composition profile for targeting DPPC/PI liposomes to *Proteus vulgaris* consistently ($n = 4$) gave two peaks at 8.8 mol% and 13.9 mol% PI (Fig. 6). The importance of PI head groups in targeting is also apparent here; on replacing PI with PS it was found that DPPC/PS liposomes did not target significantly. The optimum level

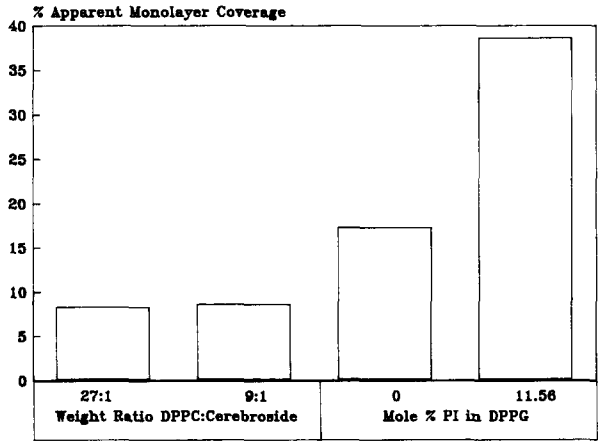

Fig. 5. Dependence of adsorption of DPPG, DPPG/PI and DPPC/PA/C liposomes to *Staphylococcus epidermidis* biofilms on lipid composition. The liposomes were incubated with the biofilm for 2 h, casein was used as the blocking agent and the biofilms were washed three times with PBS prior to assay. The compositions, concentrations and sizes of the liposomes from left to right were as follows: DPPC/PA/C, weight ratio, 1:0.037:0.111, 1.36 mM, $\bar{d}_w = 95.1$ nm; DPPC/PA/C, weight ratio 1:0.037:0.037, 1.27 mM, $\bar{d}_w = 97.9$ nm; DPPG, 1.34 mM, $\bar{d}_w = 81.1$ nm; DPPG/PI, molar ratio 1:0.146, 1.36 mM, $\bar{d}_w = 91.7$.

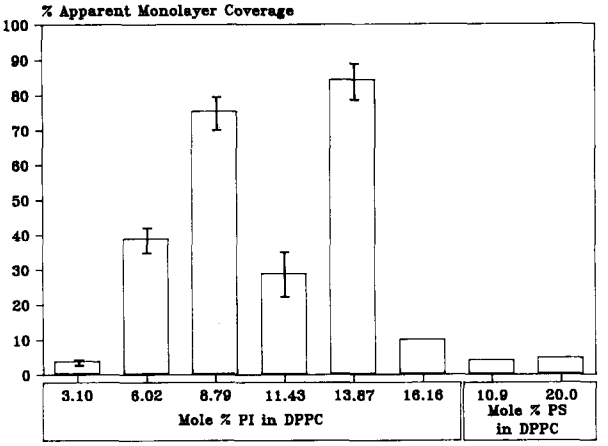

Fig. 6. Dependence of adsorption of DPPC/PI and DPPC/PS liposomes to *Proteus vulgaris* biofilms on lipid composition. The liposomes were incubated with the biofilms for 2 h, casein was used as the blocking agent and the biofilms were washed three times with PBS prior to assay. For the DPPC/PI liposomes the liposomal lipid concentration was 1.36 ± 0.07 mM, $\bar{d}_w = 85.0 \pm 9.6$ nm. For the DPPC/PS liposome the liposomal lipid concentration was 1.41 ± 0.09 mM, $\bar{d}_w$ 102.6 ± 0.7 nm.

of PI in DPPC for targeting to *S. sanguis* was 17.1 mol% PI (Fig. 7).

3.3. Delivery of bactericide (Triclosan) and growth inhibition in Staphylococcus epidermidis biofilms

The use of liposomes for the delivery of Triclosan to *Staph. epidermidis* biofilms was investigated by determining the extent of inhibition of growth of the bacterium from biofilms. Fig. 8 shows the extent of inhibition of growth after an 18 h incubation period with growth medium after exposure of the biofilms to liposomes carrying Triclosan for a brief time period (2 min). Also shown are the effects of free Triclosan at the same level as that in the liposomal solutions. The liposomal phospholipid composi-

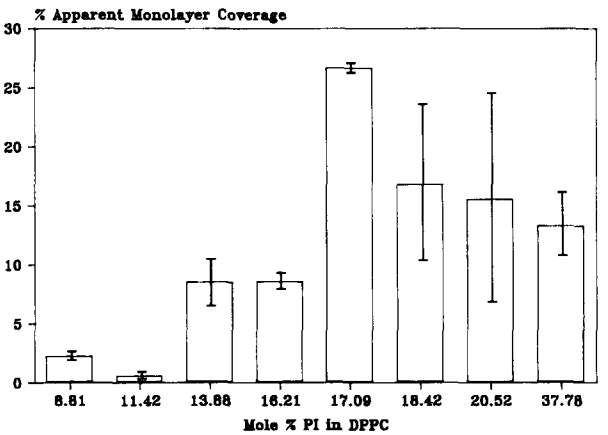

Fig. 7. Dependence of adsorption of DPPC/PI liposomes to *Streptococcus sanguis* biofilms on lipid composition. The liposomes were incubated with the biofilms for 2 h, casein was used as the blocking agent and the biofilms were washed three times prior to assay. The liposomal lipid concentration was 0.3 mM, $\bar{d}_w = 191 \pm 56$ nm.

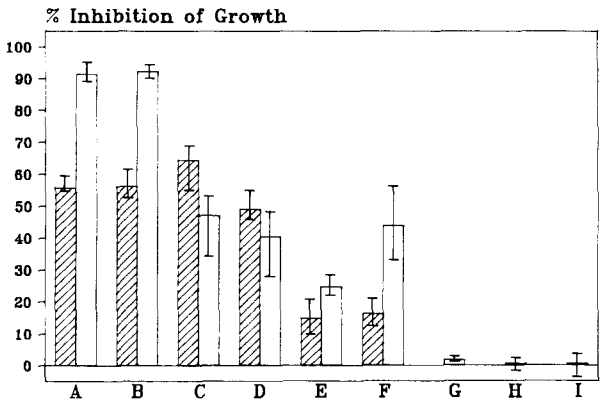

Fig. 8. The effect of liposomally-delivered (shaded bars) and free (open bars) Triclosan on the inhibition of growth of *Staphylococcus epidermidis* from biofilms, as a function of Triclosan concentration. The biofilms were exposed to liposomes for 2 min and bacteria were grown for 18 h at 37°C. The compositions (molar ratios), lipid concentrations, Triclosan levels ([T] and [T]/[lipid]) and sizes of the liposomes from A to I were as follows: A, DPPC/PI (1:0.129), 8.97 mM, [T] = 0.514 mM, [T]/[lipid] = 0.0573, $\bar{d}_w$ = 86.2 nm; B, DPPC/PI (1:0.129), 5.31 mM, [T] = 0.262 mM, [T]/[lipid] = 0.0493, $\bar{d}_w$ = 106.9 nm; C, DPPG/PI(1:0.146), 2.86 mM, [T] = 2.33 μM, [T]/[lipid] = $8.15 \cdot 10^{-4}$, $\bar{d}_w$ = 101 nm; D, DPPG/PI (1:0.146), 9.68 mM, [T] = 0.747 μM, [T]/[lipid] = $0.77 \cdot 10^{-4}$, $\bar{d}_w$ = 94.1 nm; E, DPPC, 2.22 mM, [T] = 0.219 μM, [T]/[lipid] = $0.99 \cdot 10^{-4}$, $\bar{d}_w$ = 112.0 nm; F, DPPC, 3.61 mM, [T] = 0.886 μM, [T]/[lipid] = $2.45 \cdot 10^{-4}$, $\bar{d}_w$ = 106.3 mM; G, DPPC/PI (1:0.129), 1.38 mM, [T] = 0, $\bar{d}_w$ = 125.9 nm; H, DPPC/PI (1:0.129), 13.8 mM, [T] = 0, $\bar{d}_w$ = 125.9 nm; I, DPPC/PI (1:0.096), 13.5 mM, [T] = 0, $\bar{d}_w$ = 114.1 mM. The error bars represent the standard deviation of at least 6 measurements.

tions were chosen to give relatively high levels of targeting. For high levels of Triclosan (drug to lipid molar ratio of the order of 0.05) DPPC/PI liposomes inhibit bacterial growth but not as effectively as free Triclosan. At low levels of Triclosan (drug to lipid molar ratio of the order of 10^{-4}) inhibition was greater for liposomally delivered Triclosan than for free drug. Liposomes without PI inhibited significantly less than PI-containing liposomes. Liposomes without Triclosan did not inhibit growth even at very high lipid concentrations. The extent of growth inhibition by Triclosan delivered by PI-containing liposomes was similar in the drug to lipid molar ratio range 0.05 to 10^{-4} suggesting that the drug:lipid ratio was not the limiting factor. As Fig. 3 shows targeting was not as great in the presence of high drug/lipid ratios of the order of 0.03 which in part might account for the fact that growth inhibition was not greater the higher the drug/lipid ratio.

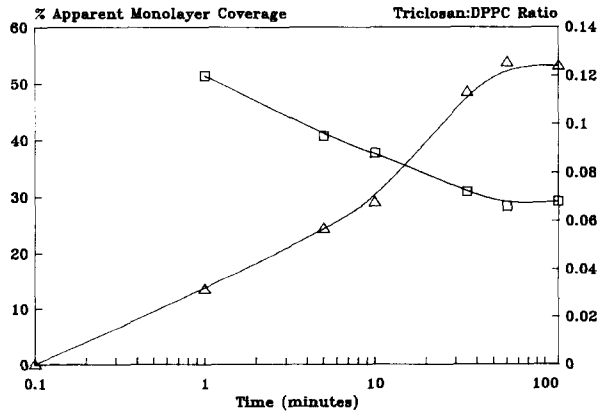

Fig. 9. The relative adsorption of Triclosan and lipid to *Staphylococcus epidermidis* biofilms as a function of time. The liposomes contained [^{14}C]DPPC and [^{3}H]Triclosan and the initial count ratio [^{3}H]/[^{14}C] was 0.055. The liposomes (DPPC/PI, molar ratio 1:0.129) were at a lipid concentration of 1.38 mM, $\bar{d}_w$ = 95 nm. Right hand axis [^{3}H]/[^{14}C] ratio (□), left hand axis %amc (△).

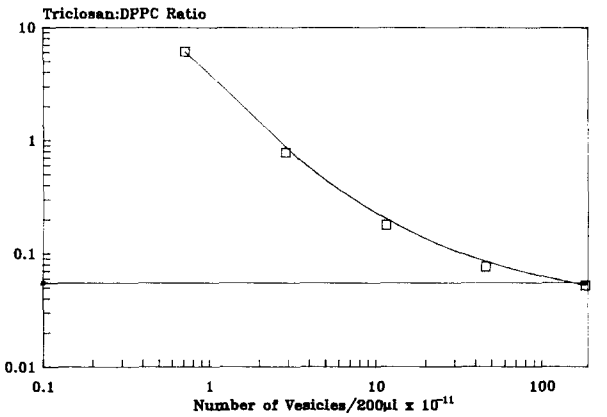

Fig. 10. The effect of liposome concentration on the relative adsorption of Triclosan and lipid to *Staphylococcus epidermidis* biofilms. The [^{3}H]/[^{14}C] ratio in the biofilm was measured after targeting for 2 h at 37°C. The liposomal composition was DPPC/PI, molar ratio 1:0.129, $\bar{d}_w$ = 95 nm. The [^{3}H]/[^{14}C] ratio in the liposomes was 0.055. The *x*-axis covers a lipid concentration for 8.04 μM to 8.04 mM.

To investigate the transfer of the bactericide into the biofilm, liposomes incorporating [^{14}C]DPPC and [^{3}H]Triclosan were prepared and targeted and the [^{3}H]/[^{14}C] ratio in the biofilm was measured as a function of time (Fig. 9). The [^{3}H]/[^{14}C] ratio in the biofilm after exposure to liposomes for 1 min was 0.12 and fell to 0.067 after 2 h. The initial [^{3}H]/[^{14}C] ratio in the liposomal suspension was 0.055. These results demonstrate that initially Triclosan is preferentially taken up by the biofilm in short time intervals, but after long times of exposure to liposomes the [^{3}H]/[^{14}C] ratio in the biofilm approaches that in the liposomal suspension.

The effect of the concentration of liposomes on the uptake of Triclosan by the biofilms was also studied for a targeting time of 2 h (Fig. 10). At low liposome concentrations the relative Triclosan content of the biofilms was found to exceed that of the adsorbed lipid, i.e., the [^{3}H]/[^{14}C] ratio in the biofilms exceeded that in the bulk phase (0.055) at low liposome concentrations but approached the bulk phase ratio at high concentrations.

4. Discussion

Adsorption of PI-containing liposomes to bacterial biofilms was found to be dependent on time (Fig. 1) and

liposomal lipid concentration (Fig. 2). Since the liposome sizes were very similar for the three systems shown in Fig. 2 it follows that the %amc increases with liposome concentration. In all the experiments the biofilm is formed by adsorption of bacteria from suspensions of absorbance 0.5. Under these conditions electron microscopy showed that the bacteria form a close-packed multilayer [17]. It should be noted that values of the % apparent monolayer coverage (amc) above 100% do not necessarily imply that the liposomes are forming a multilayer on the biofilm. The calculation of %amc is made with reference to the geometric surface area of the biofilm (microtitre plate well) and depending on the surface roughness of the biofilm, will to some degree overestimate the coverage. For example if the bacteria were represented as hemispheres on the surface of the microtitre plate then their surface area would be doubled and hence the %amc halved.

The extent of adsorption for liposomes of approximately the same size and concentration is very sensitive to lipid composition. For biofilms of *Staphylococcus epidermidis*, DPPC liposomes incorporating PI or DPPG (Figs. 3 and 4) show optimum targeting at 11.4 mol% and 6.8 mol%, respectively. Pure DPPG liposomes target less effectively than DPPG/PI liposomes while DPPC/PA/cerebroside liposomes target poorly (Fig. 5). DPPC/PI liposomes also target effectively to *Proteus vulgaris* and *Streptococcus sanguis* biofilms (Figs. 6 and 7). The dependence of targeting to *Proteus vulgaris* on PI content is complex, while DPPC/PS liposomes target poorly to this bacterium (Fig. 6). Taken overall the data suggest that it is the hydroxy content of the inositol and glycerol head groups which must play a significant role in determining the extent of adsorption. The existence of optimum levels of the hydroxy-containing head groups in the liposomal surface suggests a balance between opposing factors, specifically hydroxy content, which confers attractive interactions, most likely hydrogen bonding, and in classical terms a negative charge. Both PI and PG are negatively charged phospholipids and will confer a negative charge on the liposomal surface which will lead to an electrostatic repulsion between the liposomes and the negatively charged bacteria. For all the bacteria studied here and for *Streptococcus mutans* [13] high levels of PI inhibit targeting. The existence of optimum levels of PI for adsorption has been considered theoretically on the basis of a lattice model to represent the interaction between surface polymer of the bacterial glycocalyx and the PI head groups protruding from the liposomal surface [18]. Using such a model it was shown that optimum levels for adsorption to bacteria of hydroxy-bearing liposomes could arise as a consequence of a balance between the 'self interactions' between the surface polymers and PI head groups in the bacterium and liposome surfaces and the 'cross interactions' between them. Only a relatively low energy of interaction, less than a single hydrogen bond, is required between the PI head groups and the surface polymer residues to produce an interaction profile in which the cross interactions predominate at specific PI head group surface densities giving rise to optimum PI levels for adsorption.

Liposomes of optimum lipid composition are effective for the delivery of Triclosan to biofilms over a wide range of bactericide concentration (0.5 mM to 0.2 μM of liposomally-carried Triclosan) in that all the systems studied inhibited bacterial growth to some degree (Fig. 8). However, relative to inhibition by equivalent levels of 'free' Triclosan, liposome delivery only has an advantage at low drug/lipid molar ratios, although in vivo the ability to target specifically to bacteria would be advantageous at all concentrations.

The data in Figs. 9 and 10 suggest that Triclosan is preferentially transferred from the liposomal bilayer to the bacterial biofilm. After brief exposure (min) to liposomes at relatively high concentrations (Fig. 9) or longer term exposure (2 h) to low concentrations of liposomes (Fig. 10), the Triclosan content (relative to phospholipid) of the biofilm with an adsorbed liposomal layer exceeds that in the initial bulk phase (liposomal suspension). These observations can be explained in terms of either transfer of bactericide from adsorbed or bulk phase liposomes via the aqueous phase or by assuming that a proportion of the liposomes participate in a mobile equilibrium between the adsorbed and bulk phases and that while in the adsorbed state Triclosan diffuses into the biofilm; the resulting Triclosan-poor or Triclosan-free liposomes exchanging in the adsorbed phase with Triclosan-rich liposomes in the bulk. The rate of Triclosan transfer into the biofilm and its very low solubility in the aqueous phase would suggest that the latter mechanism is perhaps more likely together with our observation that Triclosan remains incorporated in VETs at high levels for a long period (months).

In conclusion the results suggest that by careful control of lipid composition it is possible to target liposomes to bacteria and that liposomes have potential as a means of delivery of oil-soluble bactericides such as Triclosan to adsorbed biofilms, particularly at low drug/lipid molar ratios.

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