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Targeting and delivery of bactericide to adsorbed oral bacteria by use of proteoliposomes

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Proteoliposomes having surface-bound succinylated concanavalin A (s-conA) have been prepared from a range of phospholipid mixtures by sonication (SUV) and reverse phase evaporation (REV) covering a range of size (weight-average diameter ($\bar{d}_w$)) from approx. 35 to 310 nm and weight-average number of protein molecules per liposomes ($\bar{P}_w$) from approx. 50 to 3000. The targeting of the proteoliposomes to adsorbed biofilms of the bacteria *Streptococcus sanguis* and *Streptococcus mutans* has been assessed from the extent of inhibition of an enzyme-linked immunosorbent assay (ELISA) for bacterial cell surface antigens. The surface-bound lectin enhances targeting relative to 'naked' liposomes of comparable concentration by factors of 2–50 depending on the liposomal lipid composition and $\bar{P}_w$. The effect of the bactericide Triclosan® on the thermal properties and permeability characteristics of liposomes has been studied. At and above a molar ratio of Triclosan® to lipid of 0.6, Triclosan® eliminates the gel to liquid-crystalline phase transition in dipalmitoylphosphatidylcholine (DPPC) containing liposomes and increases the bilayer permeability of both liposomes and proteoliposomes to D-glucose. The proteoliposomes have been used to deliver Triclosan® to *S. sanguis* biofilms and the inhibition of growth of the bacteria after treatment with liposomally delivered Triclosan® has been determined using a microtitre plate re-growth assay and compared with growth inhibition by 'free' Triclosan®. It is shown that for short exposure times (1 to 2 min) proteoliposomally delivered Triclosan® is a more effective growth inhibitor than free Triclosan®. The results are discussed in terms of the targeting, retention and subsequent release of Triclosan® into the bacterial biofilms.

Introduction

Protein-conjugated liposomes (proteoliposomes) are currently of considerable interest as a potential means of targeting of drugs and other therapeutic agents to biosurfaces [1–3]. Antibodies raised to specific cell surface antigenic determinants and plant lectins are the most commonly used proteins for site-directing liposomes [4–9]. The preparation of proteoliposomes having surface bound wheat germ agglutinin (WGA) [4–6,8,9] and concanavalin A (conA) [4,7,10–12] have been described. Proteoliposomes with these lectins bind to surface carbohydrates of mammalian cells [4–6], however, they should also be of value for targeting to other organisms such as bacteria. The bacteria of the oral cavity are responsible for the formation of dental caries and periodontal disease [13]. The microflora of the oral cavity is extensive, however, *Streptococci* comprise a significant proportion of the bacteria present [14] and species such as *S. sanguis* and *S. mutans* have been shown to bind conA [17,18] hence this lectin should make a suitable site-directing macromolecule for targeting proteoliposomes to the bacteria.

In this study proteoliposomes with surface-bound conA have been prepared by conjugation of succinylated conA (s-conA) through the reactive *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoylphosphatidylethanolamine (DPPE) incorporated into liposomes prepared by reverse phase evaporation (REV) or sonication (SUV). The targeting of the proteoliposomes to films of oral bacteria adsorbed on solid surfaces (microtitre plates) has been investigated using a specifically developed enzyme-linked immunosorbent assay (ELISA) and the effectiveness of the proteoliposomes to deliver a bactericide (Triclosan) to the films and inhibit subsequent growth has been investigated. Studies have also been made on the effects of

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Triclosan® on the fluidity and permeability of liposomal bilayers.

Materials and Methods

L-α-Dipalmitoylphosphatidylethanolamine (DPPE) product No. P-0890, L-α-dipalmitoylphosphatidylcholine (DPPC) product No. P-0763, L-α-dipalmitoylphosphatidylglycerol (DPPG) product No. P-9789, sphingomyelin product No. S-1131, cholesterol product No. C-8253 and succinyl-concanavalin A product No. L-3885 were from Sigma (Poole, Dorset, UK). Phosphatidylinositol (PI) grade 1 from wheat germ, molecular weight 846 g mol^{-1} [19] was from Lipid Products (South Nutfield, Surrey, UK). Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was from Pierce and Warriner (Chester, UK) and *N*-succinimidyl-*S*-acetylthioacetate (SATA) was from Calbiochem (Cambridge, UK). [^{3}H]DPPC and [^{14}C]glucose were from Amersham International (Amersham, UK). Triclosan®, [^{3}H]Triclosan® and ganglioside GM_1 were gifts from Unilever Research Port Sunlight Laboratory.

Streptococcus mutans strain D282 and *Streptococcus sanguis* strain CR2b were from the Manchester University Collection. Bacteriological agar No. 1 (code L11), defibrinated horse blood (code SR50), brain heart infusion (code CM255), yeast extract powder (code L21) and PBS tablets were from Oxoid, Basingstoke, Hants, UK. Poly(L-lysine), product No. P1274, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate product No. A-7778, diethanolamine product No. D-888S and Sigma 104 phosphatase substrate tablets were from Sigma. Antibody predominantly to antigen B in the cell wall of *S. mutans* was an IgG fraction of rabbit antiserum to *S. mutans* serotype-c wall protein was a kind gift from Dr. R.R.B. Russell, Hunterian Dental Research Unit, London Hospital Medical College, London. Antigen B is also found in *S. sanguis* which cross-reacts with the antibody [20,21].

Preparation of proteoliposomes

Liposomes were prepared from the required mixture of phospholipids by the sonication technique (SUV) or reverse-phase evaporation (REV) by standard methods [8,9] and conjugated with the SATA derivative of s-conA as previously described [10–12]. The unreacted s-conA was removed by gel filtration on a Sepharose 4B column. The proteoliposomes were physically characterised in terms of size distribution at stages during their preparation 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 diameter ($\bar{d}_w$) and the standard deviation (σ_w) of the distribution. This information together with the protein to lipid molar ratio was used to compute the weight-average number of protein molecules per proteoliposome ($\bar{P}_w$) defined by the relation.

$$\bar{P}_w = \sum_i P_i w_i / \sum_i w_i = \sum_i P_i W(d_i) / \sum_i W(d_i) \quad (1)$$

where P_i and w_i are the number of protein molecules per proteoliposome and weight of proteoliposome of species i, respectively, as previously described [8]. The protein to lipid ratio was determined from a Lowry assay for protein [22] and scintillation counting of [^{3}H]DPPC incorporated in the proteoliposomes with reference to appropriate standards.

Incorporation of Triclosan® in proteoliposomes

To prepare liposomes incorporating Triclosan® in their lipid bilayer the Triclosan® was added to the lipid mixture in the formation of the lipid film prior to hydration in the case of SUV preparations, and to the phospholipid mixture in the organic phase prior to emulsion formation in the case of REV. Above a concentration of 16% by weight of Triclosan® in the lipid mixture, liposome formation was inhibited. Although the solubility of Triclosan® in aqueous media is very low (approx. $9.4 \cdot 10^{-3}$ g l^{-1}), on gel filtration of proteoliposomes incorporating Triclosan® the Triclosan® partitioned out of the proteoliposomes. To avoid this, prior to gel filtration of the proteoliposomes to remove unreacted protein, the samples were 'spiked' with Triclosan® which reduced loss during elution. 'Spiking' involved the addition of 0.3 μCi of [^{3}H]Triclosan to samples prior to gel filtration. In practice because of the low solubility of Triclosan® in aqueous media, for assessing the effects of proteoliposomal delivery of Triclosan® to bacterial films relative to free Triclosan® these experiments were carried out in dilute ethanolic-PBS solutions containing 10% v/v ethanol. The proteoliposomes were stable in these solutions and had the same size within the experimental error as in PBS. This level of ethanol did not inhibit the growth of the bacteria although inhibition by ethanol does occur in 20% (v/v) ethanolic solutions and above. Apart from the Triclosan® delivery experiments all other experiments (targeting, differential scanning calorimetry and permeability studies) the liposomes were in PBS. Triclosan® also gives a positive Lowry assay, hence in order to correct for this in the assay of the protein content of proteoliposomes, a standard curve was prepared for Triclosan® and used to correct the measured absorbances from the known Triclosan® concentrations as measured from the [^{3}H]Triclosan® counts.

Assay of targeting of proteoliposomes to S. sanguis biofilms

The bacteria were stored at −70°C in glycerol and were checked for contamination by streaking a blood

agar plate; 10 ml broth brain heart infusion (BHI, 0.3% (w/v) yeast extract plus 1% (w/v) sucrose) was subsequently inoculated. After 18 h static incubation at 37°C in a candle jar, the bacteria were harvested and washed in sterile PBS (five times). The bacteria were then stored in suspension in sterile PBS at 4°C.

Microtitre plate wells (Dynatech M129B) were loaded with 100 μl bacteria ($OD_{550} = 0.5$) plus 100 μl PBS and incubated overnight at 37°C. The plate was then washed with PBS and blocked with casein solution (0.02% (w/v)). Aliquots of liposomes (100 μl) were added and incubated at 37°C for 2 h. The plate was then washed once with PBS and antibody (to antigen B) (diluted 1:10000 with 0.02% w/v casein in PBS) added and incubated for a further 2 h at 37°C. After this incubation the plate was again washed three times with PBS. Alkaline phosphatase enzyme conjugate (100 μl of 1:500 dilution in 0.02% (w/v) casein in PBS) was added and the plate incubated for a further 2 h at 37°C. After this stage the plate was washed with PBS and substrate (Sigma 104) added. The plate was incubated in the dark at 37°C for various time periods up to 1 h and read at 405 nm using a plate reader (Dynatech MR610) coupled to an Apple IIe microcomputer. Controls included were substrate-biofilm, conjugate-biofilm and antigen-free wells. To determine numbers of cells adhering to the microtitre wells for a given dispension concentration (absorbance), cells were labelled during culture with [^{3}H]thymidine. The numbers of cells adhering were determined by treating the microtitre wells with sodium n-dodecylsulphate (100 μl of 1% (w/v)) and counting 50-μl aliquots.

Assay of targeting of proteoliposomes to S. mutans biofilms

S. mutans grown in sucrose does not possess surface fibrils so the microtitre plate must be chemically treated so that the cells adhere. The plate was pre-treated with 0.1% poly(L-lysine) for 2 h at 37°C. After washing the plate 0.1% (v/v) glutaraldehyde in PBS was added to each well for 5 min at 4°C. The plate was then washed once with PBS before adding 100 μl bacterial suspension ($OD_{550} = 0.5$) in PBS. The plate was incubated overnight at 37°C.

The assay was then continued as for *S. sanguis* except that the optimum alkaline phosphate conjugate concentration differed (100 μl of 1:1000 dilution in 0.02% (w/v) casein in PBS).

In some cases it was necessary to concentrate the liposome suspension prior to assay of targeting. This was done by dialysing the suspension against 20% (w/v) poly(ethylene glycol) (PEG 6000, BDH, Poole, Dorset) in PBS. In experiments in which human saliva was added, saliva was pooled from 10 donors, stored on ice and clarified by centrifugation (10000 $\times g$ for 5 min). It was sterilized either by heat treatment (30 min at 60°C) or by irradiation using a Cobalt-60 source (2.5 rad) for 12 h. After treatment the saliva was divided into aliquots and stored frozen until required. It was used diluted 1:1 by volume with PBS for suspension of the liposomes.

The effectiveness of delivery of liposomal Triclosan® to *S. sanguis* was assessed using a regrowth assay. In control and test experiments, varying amounts of bacterial suspension ($OD_{550} = 0.5$) and sterile PBS were incubated at 37°C overnight in a microtitre well so that the bacteria adhered to the surface of the microtitre plate (100 μl *S. sanguis* contained approx. $6.7 \cdot 10^5$ viable cells as determined by viable cell counting).

After incubation the plate was washed and non-specific binding blocked with 0.02% (w/v) casein in PBS. Varying amounts of liposomes with and without Triclosan® were added to the plate and allowed to adsorb for different time periods (1 min, 2 min or 2 h) at 37°C. The plate was then washed twice with sterile PBS and 100 μl growth medium added (brain heart infusion plus 1% sucrose). The plate was sealed with a sterile plate sealer (Titertek catalogue No. 77-400-05) and incubated in a candle jar for 18 h at 37°C before being read. The effect of 'free' bactericide on the bacteria was also assessed and the minimum inhibitory concentration (MIC) for these conditions determined.

Permeability of proteoliposomes to [^{14}C]glucose in the presence of Triclosan®

The permeability of proteolipsomes was determined from release of encapsulated [^{14}C]glucose by a method similar to that of Johnson and Bangham [23] as previously discussed [11]. Briefly, proteoliposomes encapsulating [^{14}C]glucose and incorporating [^{3}H]Triclosan® were placed in a dialysis bag (Spectrapor 1, molecular weight cut-off 6000–8000) and incubated in 10 ml of stirred PBS at the required temperature. Aliquots (100 μl) were removed from the external medium at various time intervals and permeability and permeability coefficients for glucose determined from the ^{14}C counts and the size of the proteoliposomes as previously described [11].

Differential scanning calorimetry (DSC)

The effect of Triclosan® on the gel to liquid-crystalline phase transition in DPPC/PI REV was studied using a Perkin-Elmer DSC-4 coupled to a thermal analysis data station and calibrated with indium. After preparation and size characterisation of DPPC/PI REV incorporating Triclosan®, they were centrifuged for 1 h at 40000 $\times g$ at 20°C using an MSE 65 ultracentrifuge. The supernatant was immediately removed and 5–10 mg of the pellet accurately weighed into aluminium DSC pans and sealed. Using a heating rate of 5 C°/min^{-1} the samples were thermally scanned. The

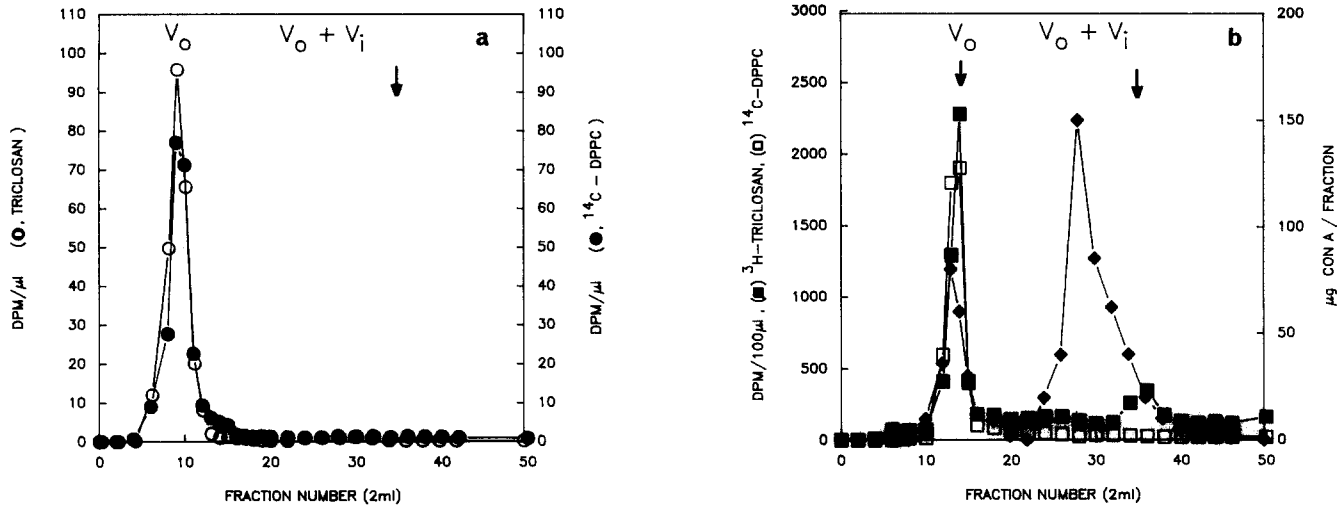

Fig. 1. (a) Elution profile of REV (composition DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5) incorporating Triclosan® from a Sephadex G-200 column. ○, Triclosan®; ● lipid (DPPC). (b) Elution profile of TREV (composition DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5, conjugated with s-conA, $\bar{P}_w = 814$, diameter ($\bar{d}_w$) = 213 nm) incorporating Triclosan® from a Sephadex G-200 column. ■, Triclosan®; □, lipid (DPPC); ◆, s-con-A.

melting point of pure Triclosan® was also measured and found to be 59.4°C, literature values are in the range 54–57°C [24].

Results

Effect of incorporation of Triclosan® on the preparation and properties of liposomes

The solubility of Triclosan® in the lipid bilayer of liposomes gives rise to a number of effects, particularly associated with the fluidity of the bilayer. In the preparation of REV or targeted REV ('TREV') with surface bound s-conA, the emulsion which is formed prior to reverse phase evaporation is more stable when Triclosan® is present but can be 'broken' by vortexing prior to evaporation of the organic solvent. Liposomes (REV and TREV) incorporating Triclosan® behave in a similar way to Triclosan®-free liposomes on gel filtration. Fig. 1a shows a typical elution profile for REV incorporating Triclosan® from a Sephadex G-200 column and Fig. 1b shows separation of unreacted s-conA from conjugated liposomes (TREV) by elution from a Sepharose 4B column. In both cases the liposomally

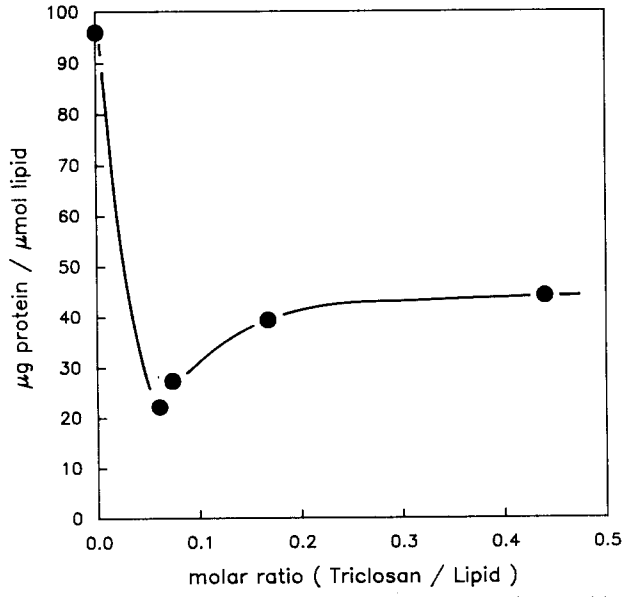

Fig. 2. Dependence of s-conA conjugation to REV (composition DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5) on Triclosan® to total lipid molar ratio. The REV had diameters ($\bar{d}_w$) in the range 200–340 nm.

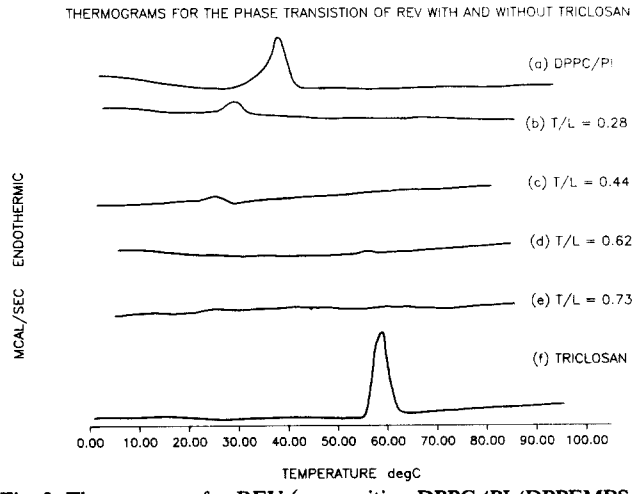

Fig. 3. Thermograms for REV (composition DPPC/PI/DPPEMBS, molar ratio, 84.4:8.1:7.5) incorporating increasing amounts of Triclosan® and for pure Triclosan®. (a) REV, diameter ($\bar{d}_w$) = 240 nm. (b) REV+Triclosan®, molar ratio Triclosan® to total lipid (T/L = 0.28, diameter ($\bar{d}_w$) = 262 nm. (c) REV+Triclosan®, T/L = 0.44, diameter ($\bar{d}_w$) = 254 nm. (d) REV+Triclosan®, T/L = 0.62, diameter ($\bar{d}_w$) = 538 nm. (e) REV+Triclosan®, T/L = 0.73. (f) Pure Triclosan®.

TABLE I

The effect of Triclosan® on the permeability coefficients of liposomes and proteoliposomes to [^{14}C]glucose

Liposome type [a]	Permeability coefficient (cm h^{-1}) (×10^7)		P_{37}/P_{20}
	20°C	37°C	
REV	0.24	8.87	36.9
REV + Triclosan®	2.29	6.69	2.9
TREV ($\bar{P}_w$ = 1150)	0.30	15.0	50.0
TREV ($\bar{P}_w$ = 490) + Triclosan®	4.22	9.99	2.4

[a] DPPC/PI/DPPEMBS (mol ratio 10.4:1:0.91) liposomes. Triclosan® was present at a molar ratio to lipid of 0.34:1.

associated Triclosan® elutes with the lipid. A free Triclosan® peak can also be separated (Fig. 1b) and elutes in the inner volume of the column. This arises from 'spiking' the liposomes with [^{3}H]Triclosan® just prior to gel filtration to avoid loss of Triclosan® from the bilayer.

Triclosan® has a detectable effect on the conjugation of liposomes with s-conA as shown in Fig. 2. The extent of conjugation in the absence of Triclosan® can be controlled by the mol% of reactive lipid DPPEMBS (the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide derivative of dipalmitoylphosphatidylethanolamine) [8,12]. However, in the presence of increasing levels of Triclosan®, the extent of conjugation (μg protein/μmol lipid) is reduced for both TREV and targeted multilamellar vesicles (MLV).

TABLE III

Targeting of DPPC/PI/DPPEMBS liposomes to adsorbed biofilms of Streptococcus mutans (strain D282) in PBS as a function of PI content

Liposome composition (DPPC/PI/DPPEMBS, mol%)	$\bar{d}_w$ (nm)	[lipid] (μmol ml^{-1})	% inhibition of ELISA
82:8:10 (SUV)	53.9	1.40	88
82:8:10 (REV)	143	1.10	76
74:16:10 (SUV)	63.7	1.64	46
70:20:10 (SUV)	82.8	2.16	16
65:25:10 (SUV)	78.3	1.84	18
56:34:10 (SUV)	101	1.62	9.5

The incorporation of Triclosan® into the bilayer of liposomes prepared from DPPC/PI/DPPEMBS eliminates the gel to liquid-crystalline phase transition of DPPC which can be seen by DSC measurements provided the PI level is not greater than approx. 50 mol% [25]. Fig. 3 shows thermograms for REV with increasing molar ratios of Triclosan® to lipid. The chain-melting temperature of DPPC was 40.6°C and the enthalpy of the transition was 33.6 kJ mol^{-1}, in good agreement with literature values [25,26]. At and above a molar ratio of 0.6, no DPPC phase transition occurs, suggesting that the Triclosan® fluidizes the bilayer. As a consequence of this the temperature dependence of the permeability of the bilayer to [^{14}C]glucose is markedly changed (Table I) on incorporation of Triclosan®. The ratio of the permeability coefficients at 37°C and 20°C decrease both in REV and TREV from approx. 37 and 50, respectively, to 2–3 on incorporat-

TABLE II

Targeting of liposomes and proteoliposomes with surface-bound s-conA to adsorbed biofilms of Streptococcus sanguis (strain CR2$_b$) in PBS/saliva

Liposome composition (mol%)	$\bar{d}_w$ (nm)	$\bar{P}_w$	[lipid] (μmol ml^{-1})	% inhibition of ELISA	Lectin target enhancement
REV (DPPC/PI/DPPEMBS, 56:34:10)	106	0	1.98	14	–
TREV (DPPC/PI/DPPEMBS, 56:34:10)	117	54	0.350	21	8.5
REV (DPPC/PI/DPPEMBS, 82:8:10)	198	0	2.84	13	–
TREV (DPPC/PI/DPPEMBS, 82:8:10)	191	471	1.13	42	8.1
REV (DPPC/PI/DPPEMBS, 74:16:10)	190	0	1.77	8	–
TREV (DPPC/PI/DPPEMBS, 74:16:10)	180	609	0.282	12	9.4
REV (DPPC/PI/DPPEMBS, 82:8:10)	259	0	3.08	14	–
TREV (DPPC/PI/DPPEMBS, 82:8:10)	240	855	0.965	27	6.2
REV (DPPC/PI/DPPEMBS, 82:8:10)	201	0	1.59	13	–
TREV (DPPC/PI/DPPEMBS, 82:8:10)	244	3188	0.368	25	8.3
REV (DPPG/DPPEMBS, 90:10)	110	0	2.00	10	–
TREV (DPPG/DPPEMBS, 90:10)	106	82	0.25	25	20
REV (DPPC/DPPG/DPPEMBS, 50:40:10)	121	0	2.32	15	–
TREV (DPPC/DPPG/DPPEMBS, 50:40:10)	113	109	0.243	25	16
REV (DPPG/PI/DPPEMBS, 83.6:10:6.4)	109	0	0.550	30	–
TREV (DPPG/PI/DPPEMBS, 83.6:10:6.4)	114	68	0.253	35	2.5
REV (DPPC/Sp M/GM1/DPPEMBS/Stealth^m)	279	0	1.13	13	–
TREV (DPPC/Sp M/GM1/DPPEMBS/Stealth^m)	310	3377	0.430	10	2.0
REV (DPPC/Stearylamine/DPPEMBS, 85:5:10)	198	0	1.06	7	–
TREV (DPPC/Stearylamine/DPPEMBS, 85:5:10)	197	422	0.342	10	4.43

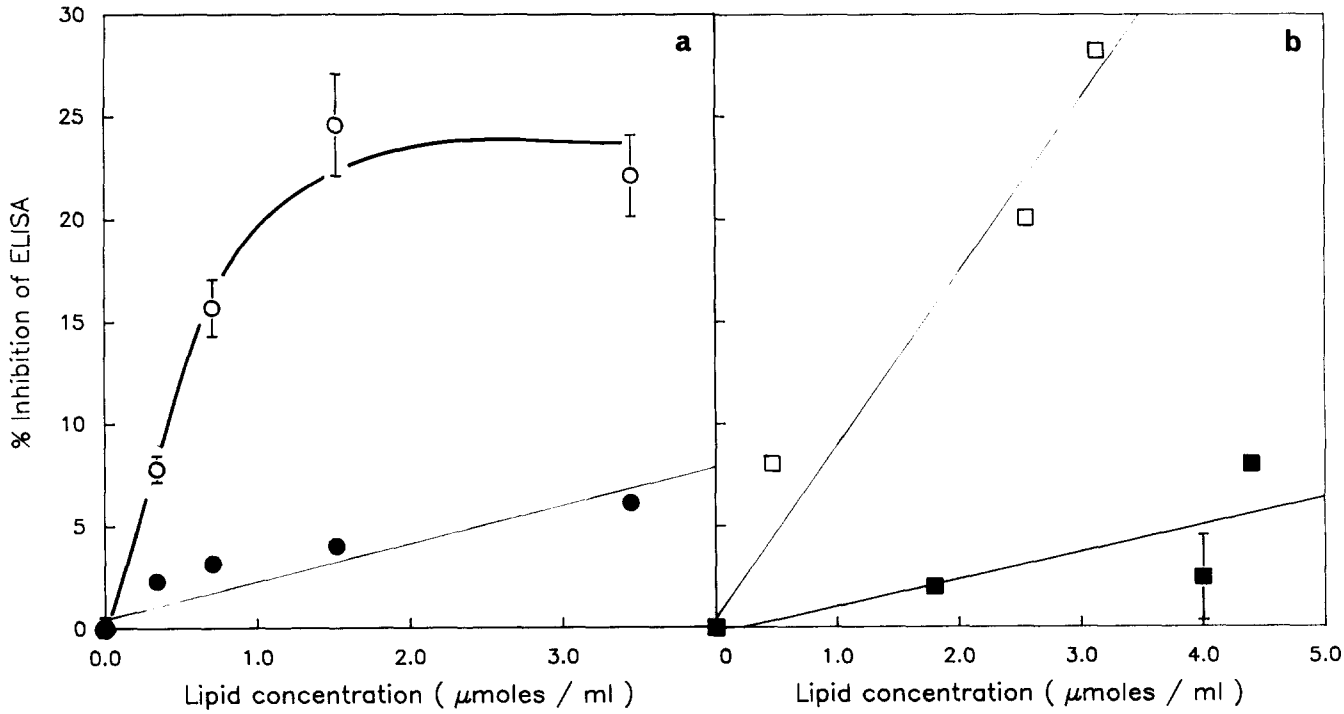

Fig. 4. Dependence of inhibition of ELISA for bacterial cell surface antigens on liposomal lipid concentration (a) *S. sanguis* biofilm; ●, REV (composition, DPPC/PI/DPPEMBS, molar ratio, 71.7:20.7:7.6, diameter ($\bar{d}_w$) = 140 nm); ○, TREV (composition, DPPC/PI/DPPEMBS, molar ratio 71.7:20.7:7.6, diameter ($\bar{d}_w$) = 170 nm, $\bar{P}_w$ = 616) (b) *S. mutans* biofilm; ■, SUV (composition DPPC/PI/DPPEMBS, 71.7:20.7:7.6 diameter ($\bar{d}_w$) = 71 nm); □, TSUV (composition, 71.7:20.7:7.6, diameter ($\bar{d}_w$) = 87 nm, $\bar{P}_w$ = 95).

ing Triclosan® at a molar ratio to lipid of 0.34:1, as a consequence of the increase in permeability induced by Triclosan® at 20°C.

Proteoliposome targeting

The results of targeting proteoliposomes, having a range of lipid composition and protein surface density, to adsorbed biofilms of *S. sanguis* and *S. mutans* was assessed on the basis of the extent of inhibition of an ELISA assay of bacterial cell surface antigens relative to the inhibition observed with 'protein-free' liposomes. The principle of the ELISA is that the targeting and adsorption of the proteoliposomes to the bacterial biofilms sterically blocks antigenic sites on the film

TABLE IV

Targeting of liposomes and proteoliposomes with surface-bound s-conA to adsorbed biofilms of Streptococcus mutans (strain D282) in PBS

Liposome composition (mol%)	$\bar{d}_w$ (nm)	$\bar{P}_w$	[lipid] (μmol ml^{-1})	% inhibition of ELISA	Lectin target enhancement
SUV (DPPC/PI/DPPEMBS, 82:8:10)	53.9	0	1.40	88	–
TSUV (DPPC/PI/DPPEMBS, 82:8:10)	70.0	270	0.21	88	6.7
SUV (DPPC/PI/DPPEMBS, 82:8:10)	55.6	0	1.79	56	–
TSUV (DPPC/PI/DPPEMBS, 82:8:10)	84.3	357	0.10	58	18.5
SUV (DPPC/PI/DPPEMBS, 72:18:10)	49.8	0	1.91	16	–
TSUV (DPPC/PI/DPPEMBS, 72:18:10)	34.0	46	0.15	26	20.6
SUV (DPPC/PI/DPPMBS, 72:20:8)	72	0	4.0	2.4	–
TSUV (DPPC/PI/DPPMBS, 72:20:8)	87	82	2.55	20	13.1
SUV (DPPC/PI/DPPMBS, 72:20:8)	72	0	4.0	2.4	–
TSUV (DPPC/PI/DPPMBS, 72:20:8)	87	100	3.13	28	14.9
SUV (DPPC/PI/DPPMBS, 72:20:8)	72	0	4.0	2.4	–
TSUV (DPPC/PI/DPPMBS, 72:20:8)	87	102	0.47	8.0	28.4
SUV (DPPC/PI/DPPMBS, 72:20:8)	72	0	4.0	2.4	–
TSUV (DPPC/PI/DPPMBS, 72:20:8)	87	110	7.02	19	4.5
SUV (DPPC/PI/DPPMBS, 72:20:8)	72	0	4.0	2.4	–
TSUV (DPPC/PI/DPPMBS, 72:20:8)	87	153	3.9	32	13.7
SUV (DPPC/PI/DPPMBS, 72:20:8)	72	0	4.0	2.4	–
TSUV (DPPC/PI/DPPMBS, 72:20:8)	87	250	0.46	13	47.1

surface reducing adsorption of the antibody, antibody conjugate and hence the signal. Because of dilution during gel filtration of the proteoliposomes the final concentrations were lower than those of the liposomes prior to conjugation. When possible the proteoliposomes were concentrated by dialysis against poly(ethylene glycol). However, to allow for liposome concentration differences between liposomes and proteoliposomes, the enhancement of targeting due to surface-bound protein was assessed from the percentage inhibition of the ELISA for targeting from the equation

$$\text{Lectin target enhancement (LTE)} = \frac{(\%\ \text{inhibition}/[\text{lipid}])_{+\text{lectin}}}{(\%\ \text{inhibition}/[\text{lipid}])_{-\text{lectin}}} \quad (2)$$

Table II shows the LTE values for a range of proteoliposomes with surface-bound s-conA targeted to *S. sanguis* biofilms. In every system the proteoliposomes (TREV) targeted effectively relative to liposomes (REV) of the same lipid composition.

Eqn. 2 assumes that there is a linear relationship between % inhibition and lipid concentration for both proteoliposomes (TREV, TSUV) and liposomes (REV, SUV). Fig. 4 shows that for low lipid concentrations (< 0.5 μmol ml^{-1} (TREV), < 3 μmol ml^{-1} (TSUV)) this is a reasonable approximation.

For biofilms of *S. mutans* it was found that liposomes with a low PI content would effectively target relative to liposomes with a high PI content. Table III shows the results of experiments to determine the dependence of targeting on the PI content of liposomes, from which it is seen that the % inhibition of the ELISA decreases markedly on increasing the mol% PI to 20% and above with a corresponding decrease in DPPC content.

Table IV shows the LTE values for a range of proteoliposomes with surface-bound conA targeted to *S. mutans* biofilms. As for *S. sanguis* (Table II) the proteoliposomes (TSUV) targeted effectively relative to liposomes (SUV) of the same lipid composition. The % inhibition of the ELISA was however greater for TSUV formed from lipid mixtures with less than 20 mol% PI.

Effect of Triclosan® on the growth of S. sanguis

The very low solubility of Triclosan® in water ($9.4 \cdot 10^{-3}$ g l^{-1}) has led to its use in dilute aqueous ethanolic solutions of the order of 10% (v/v) ethanol. Ethanol has an inhibitory effect on bacterial growth and hence it is important to be able to distinguish between the effects of ethanol and Triclosan®. Fig. 5a shows that the growth of *S. sanguis* over a period of 2 h is not markedly inhibited by 10% (v/v) ethanol. The critical region for growth inhibition is between 10% and 20% (v/v) ethanol. In 10% (v/v) ethanol the minimum inhibitory concentration of Triclosan® is in the range 0.005%–0.008% (w/v) in 10% (v/v) ethanolic media (Fig. 5b). Subsequent delivery experiments were carried out using 10% (v/v) ethanolic solutions, which had no significant effects on the size or stability of the proteoliposomes.

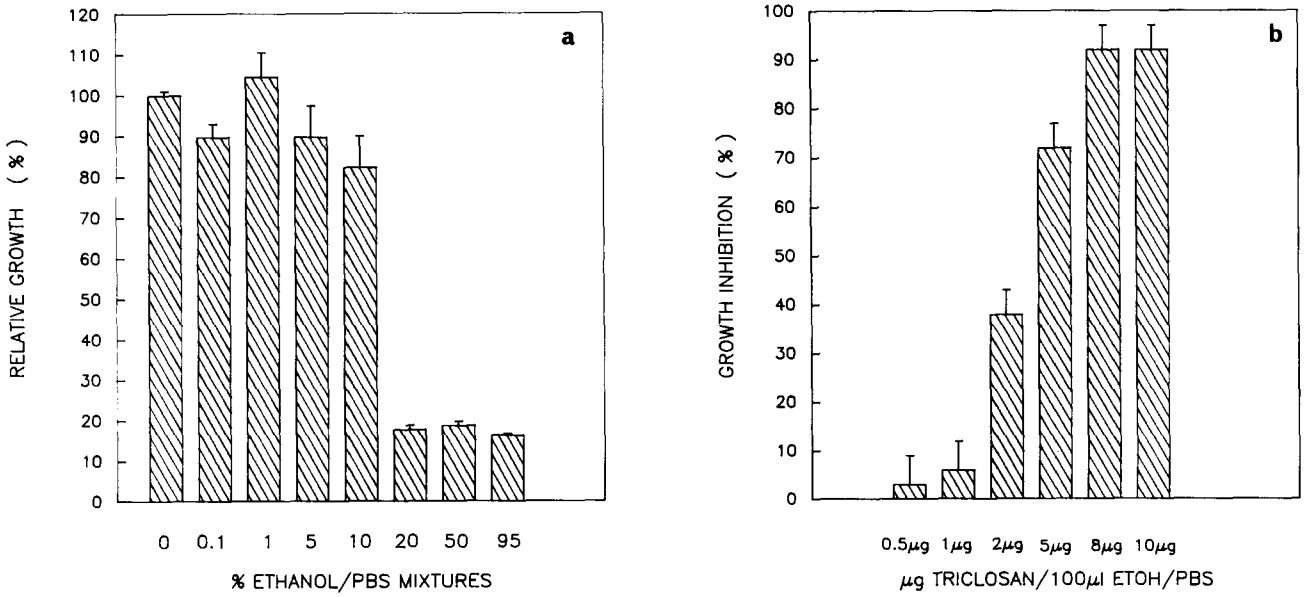

Fig. 5. (a) The effect of ethanol on the growth of *S. sanguis*. Cells ($1.42 \cdot 10^5$) were incubated for 2 h with ethanol/PBS (100 μl) followed by 18 h with growth medium (200 μl) at 37°C. (b) The effect of Triclosan® in 10% (v/v) ethanol/PBS on the growth of *S. sanguis*. Cells ($1.42 \cdot 10^5$) were incubated for 2 h with Triclosan® solution at the levels indicated followed by 18 h with growth medium (200 μl). The error bars are the standard errors ($n = 18$).

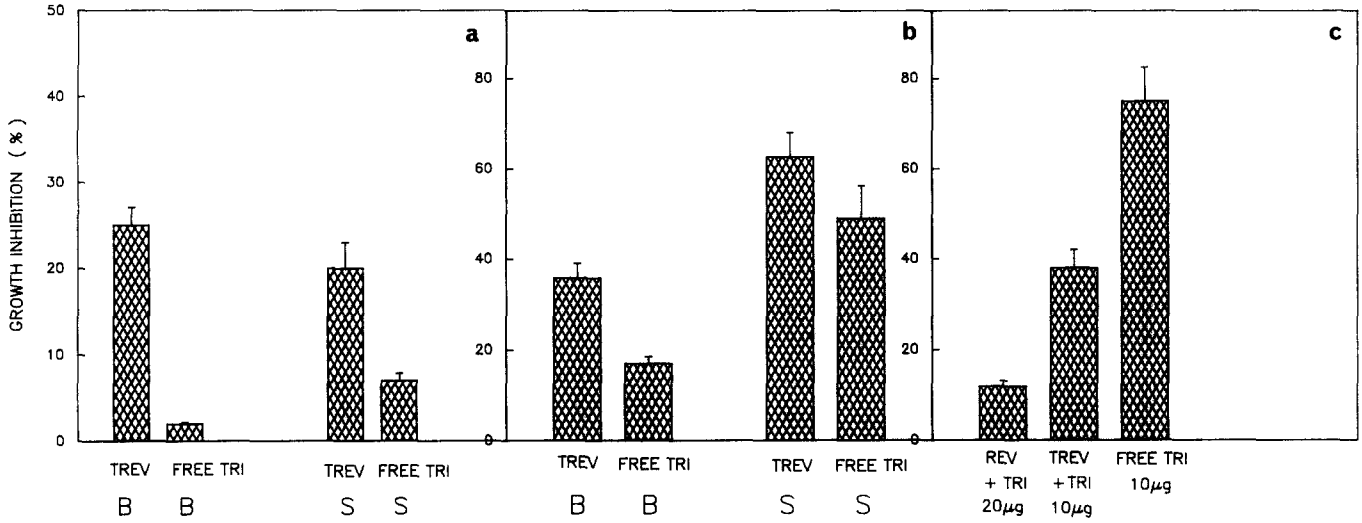

Fig. 6. Inhibition of growth of *S. sanguis* from adsorbed biofilms by liposomally carried and free Triclosan® (a) TREV (composition, DPPC/PI/DPPEMBS, molar ratio, 84.4:8.1:7.5, diameter ($\bar{d}_w$) = 339 nm, $\bar{P}_w$ = 1467, [lipid] = 3.61 μmol ml^{-1}, LTE = 11.3), containing 5 μg/100 μl Triclosan® in PBS or PBS-saliva (1:1, by volume) and free Triclosan® (5 μg/100 μl). The biofilm ($5.9 \cdot 10^4$ cells) was exposed for 1 min to the proteoliposomes or free Triclosan® followed by growth medium. (b) TREV (composition, DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5, diameter ($\bar{d}_w$) = 255 nm, $\bar{P}_w$ = 46, [lipid] = 3.71 μmol ml^{-1}, LTE 5.6) containing 2.05 μg/100 μl Triclosan® in PBS or PBS-saliva (1:1, by volume) and free Triclosan® (2.05 μg/100 μl). The biofilm ($1.42 \cdot 10^5$ cells) was exposed for 2 min to the proteoliposomes or free Triclosan® followed by growth medium. (c) REV and TREV (composition, DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5, diameter ($\bar{d}_w$) = 251 nm (REV), 213 nm (TREV), $\bar{P}_w$ = 814, [Lipid] = 1.096 μmol ml^{-1} (REV), 0.961 μmol ml^{-1} (TREV)) containing the indicated levels of Triclosan® (per 100 μl). The biofilm ($1.42 \cdot 10^5$ cells) was exposed for 2 h to the liposomes or free Triclosan® followed by growth medium.

Delivery of Triclosan® to S. sanguis biofilms by proteoliposomes

The effectiveness of proteoliposomes targeted to *S. sanguis* biofilms adsorbed on microtitre plates was determined using the 're-growth' assay in which the ability of the bacteria to grow after exposure to proteoliposomes incorporating Triclosan® was determined and compared with re-growth after exposure to identi-

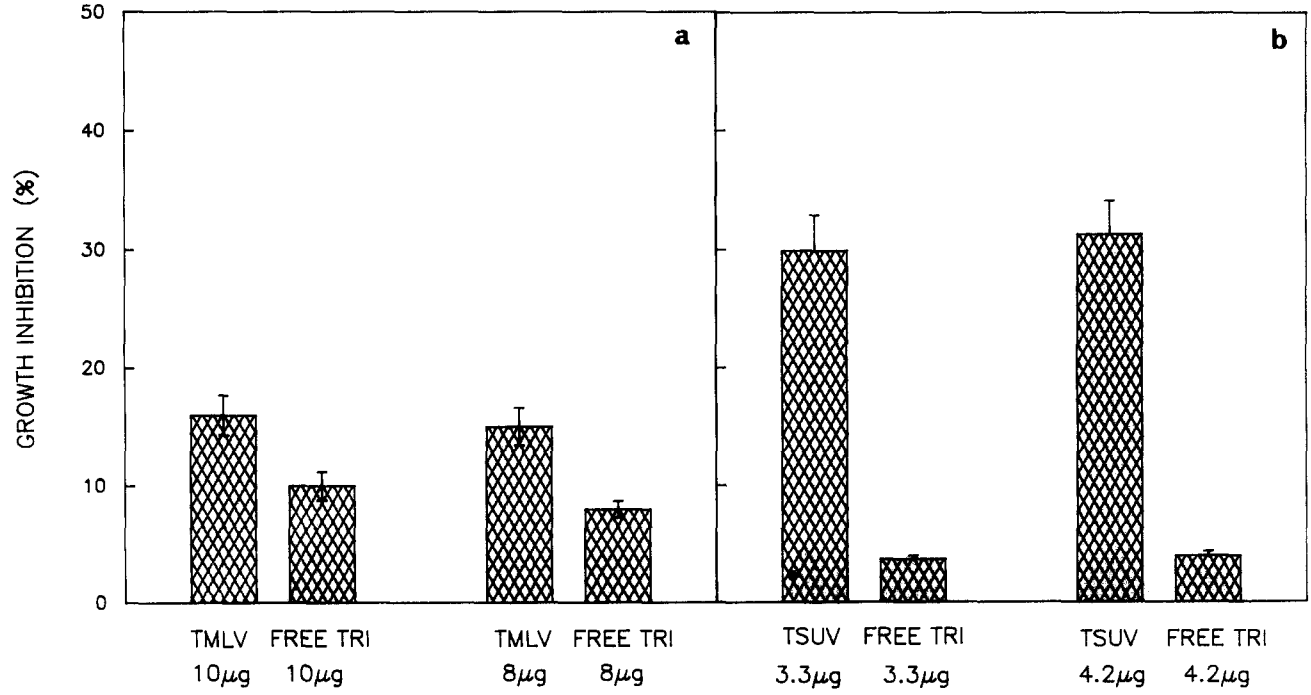

Fig. 7. Inhibition of growth of *S. sanguis* from adsorbed biofilms by liposomally carried and free Triclosan®. (a) TMLV (composition, DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5, diameter ($\bar{d}_w$) = 703 nm, $\bar{P}_w$ = 3898, [Lipid] = 2.6 μmol ml^{-1}) containing the indicated levels of Triclosan® (per 100 μl) in PBS-saliva (1:1, by volume). (b) TSUV (composition, DPPC/PI/DPPEMBS molar ratio 84.4:8.1:7.5, diameter ($\bar{d}_w$) = 37 nm, $\bar{P}_w$ = 66, [Lipid] = 0.54 μmol ml^{-1}) containing the indicated levels of Triclosan® (per 100 μl) in PBS-saliva (1:1, by volume). The biofilm ($1.18 \cdot 10^5$ cells) was exposed for 1 min to the proteoliposomes or free Triclosan® followed by growth medium.

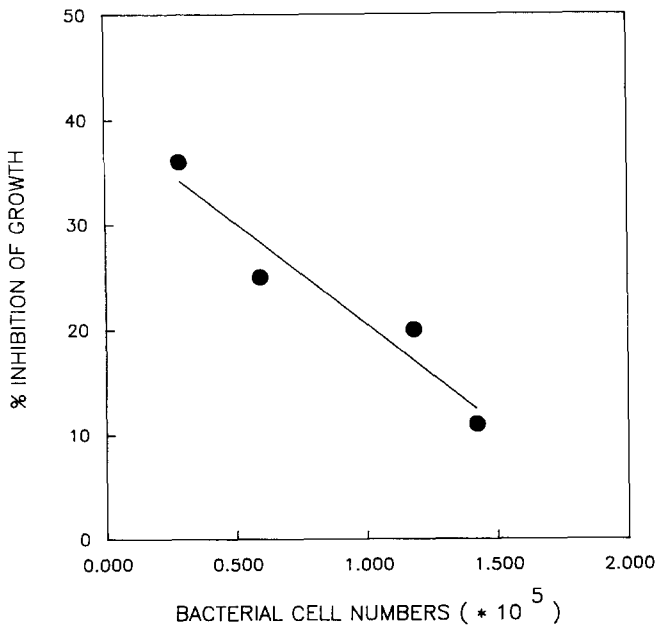

Fig. 8. The effect of cell number in biofilms of *S. sanguis* on the inhibition of growth by liposomally delivered Triclosan®. The biofilm was exposed to TREV (composition DPPC/PI/DPPEMBS, molar ratio 84.4:8.1:7.5, diameter ($\bar{d}_w$) = 257–325 nm, $\bar{P}_w$ = 698–767, [Lipid] = 0.6–1 μmol ml^{-1}) in PBS-saliva (1:1, by volume) containing Triclosan® (5–6 μg/100 μl) for 1 min followed by growth medium.

cal amounts of 'free' Triclosan® in solution. Two brief exposure times were used (1 and 2 min) and a long exposure period (2 h). Figs. 6a and b show the data for the brief exposure times and also the effects of saliva on the regrowth process. For the four systems, growth inhibition was greater for proteoliposomally (TREV) delivered Triclosan® than for free Triclosan®, particularly when a one minute exposure time was used. In contrast (Fig. 6c) there was no advantage in the use of proteoliposome delivery for a long time of exposure; although the TREV were superior to REV, neither liposomal system was as effective as free Triclosan®. Fig. 7 compares the effect of targeted SUV (TSUV) and MLV (TMLV) on growth inhibition for a 1-min exposure time. As for TREV, for this exposure time TSUV were effective delivery systems but TMLV, although more effective than free Triclosan®, were not as effective in inhibiting growth as TSUV.

The extent of regrowth of bacteria from an adsorbed biofilm was found to depend on the number of cells per microtitre well (Fig. 8). From the dimensions of the microtitre wells the geometric surface area of the adsorbed biofilm was determined. For a well containing a 100 μl volume of cell dispersion the surface area exposed to cells is $1.37 \cdot 10^{-4}$ m^2. The dimensions (diameter) of *S. sanguis* are of the order of 1 μm, which gives a projected area of the order of 10^{-12} m^2, so that 10^5 cells per microtitre well would cover approximately 10^{-7} m^2, which is much less than the available well surface area. The cells could in principle spread as a monolayer for which the % inhibition of growth would be largely independent of coverage. However, in the presence of saliva the cells change [27] so that multilayer patches form and for these the % inhibition of growth will depend on the patch thickness and hence the number of cells in the microtitre well (Fig. 8).

Discussion

Effects of Triclosan® on bilayer properties

The incorporation of Triclosan® into liposomes affects both the extent of protein coupling and the properties of the proteoliposome bilayer. The elimination of the gel to lipid crystalline phase transition of DPPC in DPPC PI liposomes (Fig. 3) as the molar ratio of Triclosan® to lipid is increased shows that Triclosan® has a fluidizing effect on the liposomal bilayer. The temperature of coupling of s-conA to DPPC/PI/DPPEMBS is below the phase transition of DPPC so that in the absence of Triclosan® there will be some degree of lateral phase separation and the reactive lipid (DPPEMBS) will be inhomogeneously distributed (i.e., 'patched'). The data suggests that this patching facilitates coupling, possibly because the effective concentration of reactive lipid is high in the patches. Addition of Triclosan® will eliminate the patches and hence reduce the extent of coupling, however the effect appears greatest at lower Triclosan® to lipid ratios. It is not clear why this is so.

Consistent with the elimination of the DPPC phase transition by Triclosan®, the bilayer becomes more permeable to glucose at lower temperatures (e.g., 20°C) and the temperature coefficient (P_{37}/P_{20}) of the permeability is markedly reduced (Table I) as a consequence of an increased permeability at 20°C and a decreased permeability at 37°C relative to liposomes and proteoliposomes in the absence of Triclosan®.

Targeting and delivery of Triclosan® to biofilms

The data in Tables II and IV show that proteoliposomes with surface-bound s-conA effectively target to adsorbed biofilms of both *S. sanguis* and *S. mutans*. ConA is believed to bind to the hydroxyl groups of C-3, C-4 and C-6 of the α-D-glucosepyranosyl and α-D-mannosepyranosyl rings of the extracellular polysaccharide produced by streptococci in the presence of sucrose [28]. The lectin target enhancements range from approximately 2 to 50 depending on the phospholipid composition and the surface density of s-conA. For a given proteoliposome the extent of inhibition of the ELISA for bacterial cell surface antigens increases linearly with lipid at low liposomal lipid concentrations (Fig. 4). It might be expected that the efficiency of

targeting (extent of ELISA inhibition) of proteoliposomes would depend on the concentration of proteoliposomes and on $\bar{P}_w$. The concentration of proteoliposomes is directly proportional to lipid concentration and inversely proportional to their surface area ($4\pi(\bar{d}_w/2)^2$). Thus the parameter $(c/\bar{d}_w^2)\bar{P}_w$, which is proportional to the proteoliposome concentration times the weight-average number of surface-bound s-conA molecules, should relate to the extent of inhibition of the ELISA of surface antigens by a given type of proteoliposome. Fig. 9 shows the % inhibition of the ELISA plotted as a function of $(c/\bar{d}_w^2)\bar{P}_w$ for TREV targeted to *S. sanguis* and TSUV targeted to *S. mutans*. The data were selected from Tables II and IV on the basis of lipid composition and are for the DPPC/PI/DPPEMBS s-conA proteoliposomes apart from where indicated. For this type of TREV and TSUV (provided the mol% PI is > 20) inhibition increases monotonically with $(c/\bar{d}_w^2)\bar{P}_w$. It is clear that for TSUV inhibition reaches a saturation point (Fig. 9b).

The lipid composition of the proteoliposomes has a significant effect on targeting; the TREV proteoliposomes containing DPPG do not fit on the curve of Fig. 9a and TSUV containing less than 20 mol% PI do not fit on the curve of Fig. 9b. However, for proteoliposomes with a given lipid composition, targeting to particular bacteria is directly related to $(c/\bar{d}_w^2)\bar{P}_w$.

Triclosan® effectively inhibits the growth of *S. sanguis* in 10% (v/v) ethanolic solutions at concentrations at and above 0.05 g l^{-1} when biofilms of *S. sanguis* are exposed to Triclosan® for 2 h or more (Fig. 5b). However, brief exposure times result in more limited inhibition of growth (Fig. 6). In contrast, when Triclosan® is incorporated into proteoliposomes and a biofilm is briefly exposed (1 or 2 min) to the proteoliposomes, significantly larger extents of growth inhibition are observed for both TREV (Figs. 6a and b) and TSUV (Fig. 7b). In the case of TREV, addition of human saliva increases inhibition by free Triclosan®, but targeted proteoliposome delivery remains greater than for free Triclosan®. Delivery of Triclosan® by multilamellar proteoliposomes is also more effective than free Triclosan®, although the differences are not as large as for TREV or TSUV (Fig. 7a). It is clear that proteoliposomes are effective delivery systems for the bactericide when only short exposure times are possible, as for example in delivery to the oral cavity by a mouthwash. The success of bactericide delivery to biofilms on brief exposure to targeted proteoliposomes lies in the retention of the proteoliposomes incorporating bactericide by the bacterial film and subsequent release of bactericide. In contrast, free Triclosan® is easily washed from the biofilm and is not retained to any great extent in a 1–2 min period. The process of transfer of Triclosan® from the proteoliposomal bilayer to the cells in the biofilm is probably one of simple diffusion, since uptake of Triclosan® by cells is thought to be a passive process [28].

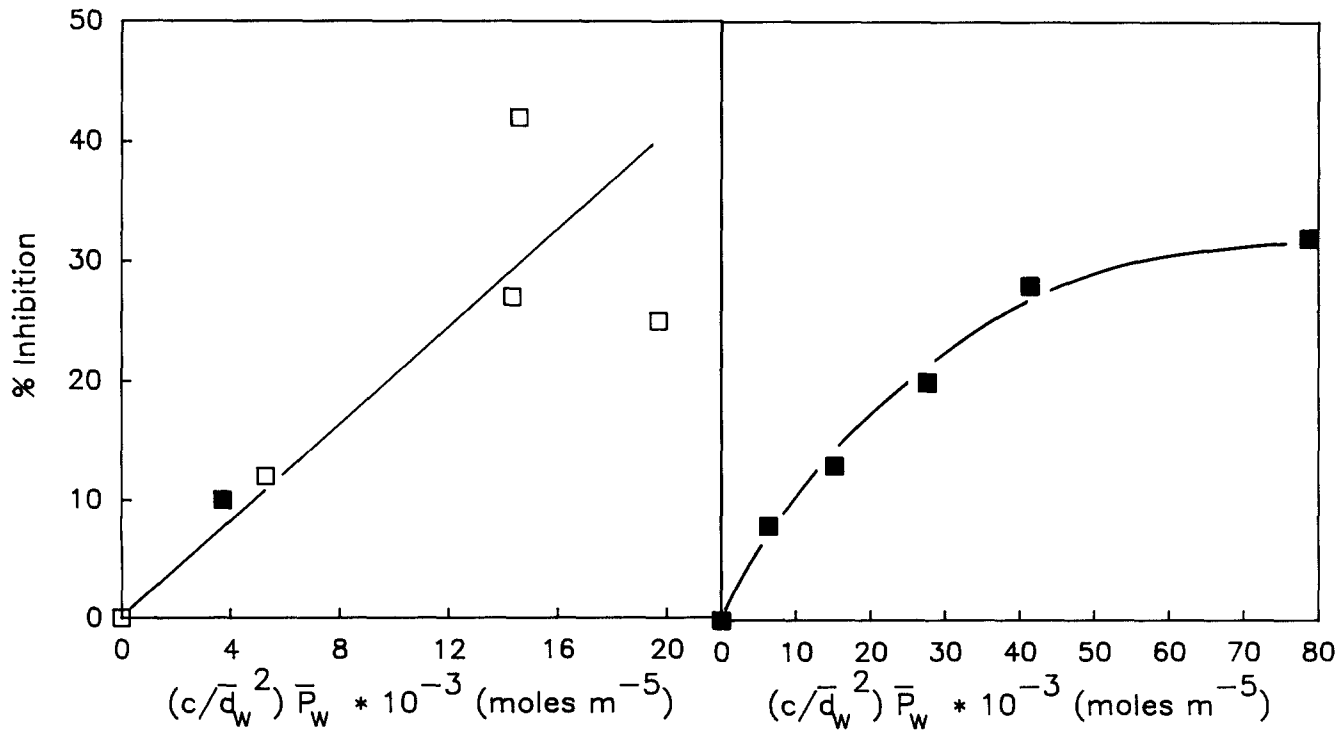

Fig. 9. Dependence of inhibition of ELISA for bacterial cell surface antigens on the parameter $(c/\bar{d}_w^2)\bar{P}_w$. (a) TREV (composition DPPC/PI/DPPEMBS s-conA) targeted to *S. sanguis* biofilm. The solid point is for DPPC stearylamine DPPEMBS s-conA proteoliposomes. (b) TSUV (composition DPPC/PI/DPPEMBS s-conA) targeted to *S. mutans* biofilms.

Targeting to the oral cavity

The studies described demonstrate that proteoliposomes with surface bound lectin can be used to target and deliver bactericide to biofilms of oral bacteria. In vivo oral bacteria are not the only potential binding sites for the proteoliposomes. The glycoprotein in the glycocalyx of the cells of the buccal mucosa may also act as receptors for proteoliposomes. Although the distribution of proteoliposomes between such potential sites and bacterial surfaces is not known, such adsorption could only help to improve retention of proteoliposomes (and bactericide) in the oral cavity.

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References

1 Peters, P.A.M., Storm, G. and Crommelin, D.J.A. (1987) Adv. Drug Deliv. Rev. 1, 249–266.
2 Leserman, L. and Machy, P. (1987) in Liposomes from Biophysics to Therapeutics (Ostro, M.J., ed.), Chapter 5, pp. 157, Marcel Dekker, New York.
3 Wright, S. and Huang, L. (1989) Adv. Drug Deliv. Rev. 3, 343–389.
4 Carpenter-Green, S. and Huang, L. (1983) Anal. Biochem 135, 151–155.
5 Liautard, J.P., Vidal, M. and Philipot, J.R. (1985) Cell Biol. Int. Rep. 9, 1123–1137.
6 Hutchinson, F.J. and Jones, M.N. (1988) FEBS Lett. 234, 493–496.
7 Margolis, L.B., Bogdanov, A.A., Jr., Gordeva, L.V. and Torchilin, V.P. (1988) in Liposomes as Drug Carriers (Gregoriadis, G., ed.), Chapter 52, pp. 727, John Wiley and Sons, New York.
8 Hutchinson, F.J., Francis, S.E., Lyle, I.G. and Jones, M.N. (1989) Biochim. Biophys. Acta 978, 17–24.
9 Hutchinson, F.J., Francis, S.E., Lyle, I.G. and Jones, M.N. (1989) Biochim. Biophys. Acta 987, 212–216.
10 Francis, S.E. and Jones, M.N. (1990) Biochem. Soc. Trans 18, 876–877.
11 Francis, S.E., Lyle, I.G. and Jones, M.N. (1990) Biochim. Biophys. Acta 1062, 117–122.
12 Francis, S.E., Hutchinson, F.J., Lyle, I.G. and Jones, M.N. (1992) Colloids Surfaces 62, 177–184.
13 Loesche, W.J. (1986) Microbiol. Rev. 50, 353–380.
14 Hardie, J.M. and Bowden, G.H. (1974) in The normal microbial flora of man (Skinner, F.A. and Carr, J.G., eds.), Academic Press, New York.
15 McCarthy, C., Snyder, M.L. and Parker, R.B. (1965) Arch. Oral Biol. 10, 61–70.
16 Carlsson, J., Grahnen, H. and Jonsson, G. (1975) Caris Res. 9, 333–339.
17 Doyle, R.J. and Birdsell, D.C. (1972) J. Bacteriol. 109, 652–658.
18 Ellwood, D.C., Hardie, J.M., Browning, P.M. and Bowden, G.H. (1973) J. Dent. Res. 52. 955–956.
19 Ter-Minassian-Saraga, L. and Madelmont, G. (1982) J. Colloid Interface Sci. 85, 373–388.
20 Russell, R.R.B. (1979) J. Gen. Microbiol. 114, 109–115.
21 Russell, R.R.B. (1980) J. Gen. Microbiol. 118, 383–388.
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
23 Johnson, S.M. and Bangham, A.D. (1969) Biochim. Biophys. Acta 193, 82–91.
24 Furia, T.E. and Schenkel, A.G. (1968) Soap Chem. Specialities 44, 47–50.
25 Hammond, K., Lyle, I.G., and Jones, M.N. (1984) J. Coll. Int. Sci. 99, 294–296.
26 Jones, M.N. (1988) in Biochemical Thermodynamics (Jones, M.N., ed.), Chapter 5, pp. 197, Elsevier, Amsterdam.
27 Handley, P.S., Carter, P.L., Wyatt, J.E. and Hesketh, L.M. (1985) Infect. Immun. 47, 217–227.
28 Rolla, G. (1971) Arch. Oral Biol. 6, 967–969.
29 Meincke, B.E., Kranz, R.G. and Lynch, D.L. (1980) Microbiosystems 28, 133–147.