

# The specificity and affinity of immunoliposome targeting to oral bacteria

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## Abstract

Immunoliposomes have been prepared using antibodies raised to an antigenic determinant on the cell surface of the oral bacterium *Streptococcus oralis* (*S. oralis*) in an investigation of their potential to reduce dental plaque. The *N*-succinimidyl-*S*-acetylthioacetate (SATA) derivative of the antibodies were conjugated through the reactive *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoyl-phosphatidylethanolamine (DPPE) incorporated into liposomes. The degree of antibody conjugation to the liposomes was controlled by the percentage of DPPEMBS incorporated into the liposomes. The chemical modification of the antibodies did not affect the ability of the antibodies to bind to a *S. oralis* biofilm. However, the affinity of the immunoliposomes for *S. oralis* was much lower than that of the free antibody. The anti-*oralis* antibodies were highly specific for *S. oralis*. The anti-*oralis* immunoliposomes showed the greatest affinity for *S. oralis*, when targeted to a range of different oral bacterial biofilms. The immunoliposome targeting affinity for *S. oralis* was largely unaffected by the number of antibodies conjugated to the liposomal surface or by the net charge of the liposomal lipid bilayer. The immunoliposomes showed a greater affinity for *S. oralis* than 'naked' (bearing no antibody) liposomes. However, positively charged liposomes, incorporating stearylamine, adsorbed to *S. oralis* with greater affinities than the immunoliposomes. The immunoliposomes appeared to be physically stable over a period of 18 months, as judged by particle-size measurements. © 1998 Elsevier Science B.V.

**Keywords:** Immunoliposome; Liposome targeting; Targeting to bacteria; Bacterial biofilm; Oral bacterium

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## 1. Introduction

Liposomes are versatile drug delivery systems that have great potential in the treatment of infectious diseases [1]. The use of liposomes in the biological milieu enables large quantities of drug to be protected and carried to target specific sites. As phagocytic cells of the reticuloendothelial system (RES) are the natural targets of liposomes injected intravenously [2], antibiotics entrapped in liposomes have been

investigated mainly in infections due to intracellular microbes (including parasites) such as *Salmonella*, *Mycobacterium*, and *Leishmania* [3]. In vivo use of liposomes in the delivery of antimicrobial agents outside the RES can be achieved by manipulation of the liposome size and composition [4]. For example, antibiotic has been successfully targeted, using liposomes, to lung tissue infected with *Klebsiella pneumoniae* [5]. Liposome targeting to strains of skin-associated and oral bacteria has been investigated using a number of different approaches. They include using liposomes with surface bound lectins [6–8], lipo-

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somes prepared from lipid mixtures incorporating negatively charged phosphatidylinositol [9,10], positively charged liposomes incorporating stearylamine [11] and liposomes with surface bound antibodies [12]. Monoclonal antibodies have potential as highly specific targeting vehicles [13] and can be easily produced against determinants of bacterial cell surfaces. Dental plaque is a key factor in dental caries and periodontal disease [14]. Both diseases may be prevented by effectively controlling plaque development. As specific oral bacteria are known to be initial colonisers of the tooth surface, a logical way to control plaque development bacteria is to use immunoliposomes for specific delivery of non-specific antimicrobial agents to early tooth colonisers.

In this study, we have prepared and characterized immunoliposomes with two different surface-bound monoclonal antibodies raised to antigenic determinants on the surface of *Streptococcus oralis* (formally *Streptococcus sanguis* [15]), a predominant initial colonizer of the tooth surface [14]. The *N*-succinimidyl-*S*-acetylthioacetate (SATA) derivatives of the antibodies were conjugated through the reactive *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoylphosphatidylethanolamine (DPPE) incorporated into liposomes prepared by the vesicle extrusion technique [16]. The targeting specificity and affinity of the anti-*oralis* immunoliposomes to films of oral bacteria adsorbed on solid surfaces has been investigated with a view to using immunoliposomes as a potential means of delivering antimicrobial agents to the oral environment. A preliminary abstract of this work has been published [12].

## 2. Materials and methods

L- $\alpha$ -dipalmitoylphosphatidylethanolamine (DPPE, product No. P-0890), L- $\alpha$ -dipalmitoylphosphatidylcholine (DPPC, product No. P-0763), cholesterol (Chol, product No. C-8667) and stearylamine (SA, product No. S-9273) were from Sigma (Poole, Dorset, UK). Phosphatidylinositol (PI) grade 1 was from Lipid Products (South Nutfield, Surrey, UK). [ $^3\text{H}$ ]-DPPC (TRK.673) was from Amersham International (Aylesbury, Buckinghamshire, UK). The liposome

extruder was from Lipex Biomembranes (Vancouver, British Columbia). Poretic filters (pore size 100 nm) were from Poretics (Livermore, CA). *N*-succinimidyl-*S*-acetylthioacetate (SATA) was obtained from Calbiochem (Cambridge, UK). *M*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS, product No. 22310) and sulpho-succinidyl 4-(*p*-maleimidophenyl) butyrate (sulpho-SMPB, product No. 22318) were from Pierce and Warriner (Chester, UK). Triethylamine (product No. 23,962-3) was obtained from Aldrich (Gillingham, Dorset, UK). Hydroxylamine (product No. H 9876) was obtained from Sigma (Poole, Dorset, UK).

Two different antibodies 4718 and 4715 (batch No. PL772 and PL779, respectively), raised in mouse to *S. oralis* were gifts from Unilever Research (Colworth Laboratory, Bedford, UK). Alkaline phosphatase rabbit anti-mouse IgG (product No. A-1902) and Sigma 104 phosphatase substrate tablets were obtained from Sigma (Poole, Dorset, UK). *Streptococcus oralis* (*Streptococcus sanguis* strain 209) was a gift from Unilever Research (Port Sunlight Laboratories, Wirral, Merseyside, UK). *Streptococcus mutans* strain D282, *Streptococcus sanguis* strain C104, *Streptococcus gordonii* (*Streptococcus sanguis* strain Cr2B) and *Streptococcus salivarius* strains DBD and NCTC 8618 were from the Manchester University collection. Brain heart infusion (code CM255), yeast extract powder (code L21) and phosphate buffered saline (PBS) tablets were from Oxoid (Basingstoke, Hampshire, UK). Blood agar plates were obtained from Manchester Royal Infirmary (Clinical Sciences Building, Manchester). All other reagents and solvents were of analytical grade and made up in double distilled water.

### 2.1. Preparation of reactive lipid (DPPEMBS) and the SATA derivatives of anti-*oralis* antibodies 4718 and 4715

The MBS derivative of DPPE (DPPEMBS) was prepared as previously described [17]. The SATA-derivatised anti-*oralis* antibodies were prepared by a modification of the method of Duncan et al. [18]. SATA (2.5  $\mu\text{l}$  of 18.8% SATA in dimethylformamide) was added to antibody (10 mg in PBS). After 15 min, the derivatised antibody was separated from

unreacted SATA by gel filtration on a Sephadex G50 column (15 × 2 cm). The quantity of antibody collected was assayed by the protein assay method of Lowry [19].

## 2.2. Preparation of vesicles by extrusion (VETs)

Lipid mixtures (total mass 30–38 mg) plus 5  $\mu$ Ci [ $^3$ H]-DPPC were dissolved in dry chloroform/methanol (4:1 by volume) in a round-bottomed flask. The solvent was removed by rotary evaporation at 60°C to leave a uniform layer of dry lipid. PBS (3 ml, 60°C) was added to form multilamellar vesicles (MLVs). The vesicles were then extruded 5–10 times, under pressurised N<sub>2</sub> gas, through 100 nm filters using an extruder, producing VETs (vesicles produced by extrusion techniques). Samples of the VETs were taken for scintillation counting and for sizing by photon-correlation spectroscopy using a Malvern RR146 autosizer.

## 2.3. Preparation and characterisation of immunoliposomes

Deacetylation of antibody bound SATA was carried out using hydroxylamine solution (0.5 M NH<sub>2</sub>OH, 25 mM EDTA plus solid Na<sub>2</sub>HPO<sub>4</sub> to pH 7.4) to give a final concentration of 0.1 M hydroxylamine in the SATA-derivatised antibody solution for 1 h. The sulphhydryl content of the deacetylated derivative was determined by the method of Ellman [20]. SATA derivatised and deacetylated antibody (1 mg) was added to VETs containing DPPEMBS. The mixture was left overnight at 4°C, then applied to a Sepharose 4B gel filtration column (25 × 2 cm). Fractions (2.2 ml) were collected and assayed for lipid concentration (by scintillation counting) and total protein concentration (bound, and unbound antibody) by the method of Wang and Smith [21]. The weight average number of antibody molecules per immunoliposome ( $\bar{P}_w$ ), described previously [7], was calculated from the protein concentration and the liposome size distribution fitted to a normal weight distribution.

## 2.4. Preparation of bacterial biofilms

Bacteria were stored at –70°C in glycerol or semi-skimmed milk. Streaked blood agar plates were

incubated upside down at 37°C for 18 h in a candle jar, then stored for one month at 4°C. Bacteria were grown for 18 h at 37°C in brain heart infusion (BHI) broth (3.7% BHI, 0.3% yeast extract plus 1% sucrose), then harvested by centrifugation and washed four times in sterile PBS. The final bacterial suspension (O.D.<sub>550</sub> = 0.5) was loaded into microtitre plate wells (200  $\mu$ l each well) and left overnight at room temperature for the cells to adhere. Before use, the plates were washed (3 × 300  $\mu$ l) with PBS.

## 2.5. Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates of bacterial biofilms were incubated with BSA (1% in PBS) for 15 min at room temperature. The plates were then washed (3 × 300  $\mu$ l PBS) and incubated with free anti-*oralis* antibody, derivatised free anti-*oralis* antibody, anti-*oralis* immunoliposomes or a PBS control (200  $\mu$ l). After 2 h at 37°C, the plates were washed (3 × 300  $\mu$ l PBS) and incubated for 1 h at 37°C with a second antibody conjugate (200  $\mu$ l, diluted 1:1000 in PBS). The plates were then washed again (3 × 300  $\mu$ l PBS) and incubated with substrate (100  $\mu$ l, two tablets in 10 ml 9.7% diethanolamine, 0.01% MgCl<sub>2</sub>·6H<sub>2</sub>O buffer, pH 9.8). The plate was then read at 410 nm using a plate reader (Dynatech MR610 coupled to an Apple IIe microcomputer).

## 2.6. Targeting assay

To measure the targeting of the liposomes to the biofilms, microtitre plates of bacterial biofilms were first blocked with BSA (1% in PBS) for 15 min at room temperature. In the experiments with cationic liposomes, blocking with BSA was omitted. The plates were then washed (3 × 300  $\mu$ l PBS) and incubated with liposomes (200  $\mu$ l) at various concentrations for 2 h at 37°C. The plates were washed again (3 × 300  $\mu$ l PBS) and the targeted vesicles, adsorbed to the biofilms, were solubilised by SDS (5%, 200  $\mu$ l). After 30 min the plates were sonicated in a water bath for 5 min and 180  $\mu$ l of the SDS solution was taken for scintillation counting. The results of the targeting assay are expressed in terms of percentage monolayer coverage (%amc) given by:

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

where  $N_{\text{obs}}$  is the measured number of moles of lipid

adsorbed to the biofilm and  $L_a$  the number of moles that would be adsorbed if the biofilm was covered with a close-packed monolayer of liposomes.  $L_a$  was calculated from the following equation:

$$L_a = \left( A_{\text{bf}} / \pi (\bar{d}_w / 2)^2 \right) \times \bar{N}_w \quad (2)$$

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  $A_{\text{bf}}$  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 of  $50 \text{ nm}^2$  and a bilayer thickness of  $7.5 \text{ nm}$  as previously described [23]. The area of the biofilm was taken as  $2.202 \times 10^{-4} \text{ m}^2$  which was measured in a previous study for the surface of the microtitre plate wells exposed to  $200 \mu\text{l}$  of solution [24].

### 3. Results

#### 3.1. Characterisation of immunoliposomes

SATA was found to introduce an average of 1.75 and 1.03 sulphhydryl groups into the anti-*oralis* antibodies 4718 and 4715 respectively. Separation of unbound SATA-derivatised anti-*oralis* antibody from VETs with covalently conjugated anti-*oralis* antibody was achieved by elution through a Sepharose 4B column; typically  $\approx 40\%$  of the total antibody was associated with the VETs. The extent of antibody conjugation is controlled by the percentage of reactive lipid DPPEMBS in the liposome mixture. The weight average number of proteins per liposome,  $\bar{P}_w$  [7], increases with increasing percentage of DPPEMBS in the VETs as shown in Fig. 1(A). Both, anti-*oralis* antibodies 4718 and 4715 show the same extent of conjugation with increasing DPPEMBS. While these results were reproducible with freshly prepared materials, the extent of conjugation decreased on storage of the derivatised antibodies which accounts for some of the lower values of  $\bar{P}_w$  for a given mol% DPPEMBS shown in Figs. 4 and 5.

Taking dimensions of antibody fragments from the Brookhaven Protein Data Bank, the Y-shaped structure of an IgG has overall dimensions of  $18.8 \text{ nm} \times 18.8 \text{ nm} \times 4.1 \text{ nm}$ , irrespective of whether the

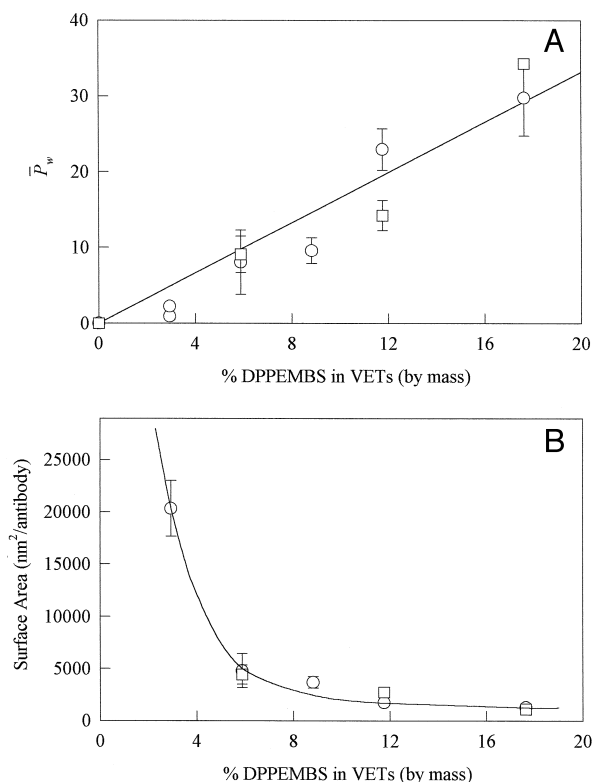


Fig. 1. Dependence of weight-average number of (A) – anti-*oralis* antibody molecules per liposome ( $\bar{P}_w$ ) and (B) – surface area per anti-*oralis* antibody molecules on the surface of immunoliposomes as a function of the percentage of reactive lipid (DPPEMBS), by mass, in DPPC/PI/DPPEMBS VETs. (○) – Anti-*oralis* antibody 4715, and (□) – anti-*oralis* antibody 4718. The error bars are from three experiments.

molecule lies flat or with the *Fc* or *Fab* regions uppermost. Assuming rotational motion, the excluded surface area ( $\pi(18.8)^2$ ) is  $1110 \text{ nm}^2$  [22]. Increasing the DPPEMBS content of the liposomes increases conjugation of anti-*oralis* antibody until a minimum area per antibody molecule of  $\sim 1000 \text{ nm}^2$  is achieved. This is shown in Fig. 1(B). The results suggest that a close-packed monolayer of antibody molecules on the liposomal surface can be achieved with a minimum DPPEMBS concentration of 17.5%.

Fig. 2 shows the adsorption of free anti-*oralis* antibody, derivatised anti-*oralis* antibody and anti-*oralis* immunoliposomes to a *Streptococcus oralis* bacterial biofilm measured by an ELISA. A characteristic sigmoidal antibody dilution curve of antibody adsorbing to *S. oralis* is seen for free antibody, SATA derivatised antibody, deacetylated antibody

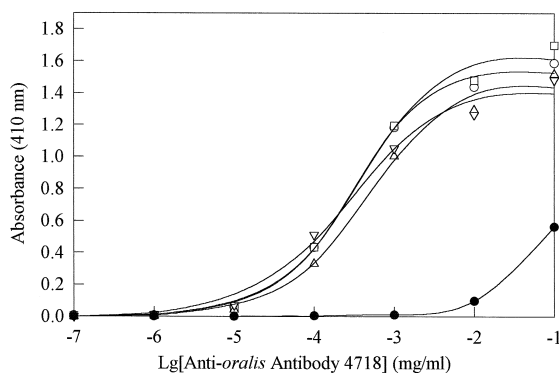


Fig. 2. Adsorption of free anti-oralis antibody 4718, chemically modified anti-oralis antibody 4718 and anti-oralis 4718 immunoliposomes to *S. oralis*, measured by an ELISA. Antibody targeting 2 h at 37°C.  $\bar{P}_w$  for immunoliposomes = 47. (○) – Free antibody, (□) – SATA-derivatised antibody, (△) SATA-derivatised antibody plus hydroxylamine, (▽) – SATA-derivatised antibody plus hydroxylamine plus sulpho-SMPB, and (●) – antibody conjugated to DPPC/PI/DPPMBs VETs.

and SATA derivatised antibody conjugated to sulpho-SMPB through a thioether cross-linkage. For antibody conjugated to liposomes incorporating DPPMBs, the sigmoidal antibody adsorption curve is displaced to the right by approximately three orders of magnitude in terms of antibody concentration. Thus, the antibody concentration required for immunoliposome adsorption is approximately 1000 times greater than that required for an equivalent ELISA signal measuring free antibody adsorption.

### 3.2. Specificity of anti-oralis immunoliposomes

The affinity of free anti-oralis antibodies 4718 and 4715 for various bacterial biofilms was assessed using an ELISA method. The results are shown in Fig. 3. A characteristic sigmoidal antibody dilution curve is found for each antibody adsorbing to *Streptococcus oralis*. About half of *S. oralis*'s antigenic sites are occupied when antibody concentrations in the order of  $0.1 \mu\text{g ml}^{-1}$  are used and the antigenic sites are completely saturated using antibody concentrations in the order of  $1 \mu\text{g ml}^{-1}$ . For anti-oralis antibody clone 4718 (Fig. 3A), there is a slight cross reaction with the bacterium *S. sanguis* C104, and slightly more so with *S. gordonii*. The anti-oralis antibody 4718 does not bind significantly to *S. salivarius* DBD, *S. salivarius* 8618 or *S. mutans* D282.

For anti-oralis antibody clone 4715 (Fig. 3B), there is no cross reaction with other bacteria, but at high antibody concentrations ( $0.1 \text{ mg ml}^{-1}$ ) the antibody begins to bind non-specifically to all the bacterial biofilms.

The affinity of anti-oralis immunoliposomes for various bacterial biofilms was measured using the targeting assay. The results of targeting anti-oralis 4718 immunoliposomes and anti-oralis 4715 immunoliposomes to five different oral bacteria are shown in Fig. 4(A) and (B), respectively. Anti-oralis 4718 immunoliposomes adsorb with twice the affinity to *S. oralis* than to other bacteria. Anti-oralis 4715 immunoliposomes adsorb with more than three times the affinity to *S. oralis* than to other bacteria. Non-specific adsorption of anti-oralis immunoliposomes to other bacteria results in  $\approx 8\%$  monolayer coverages of the bacterial biofilms after 2 h at 37°C.

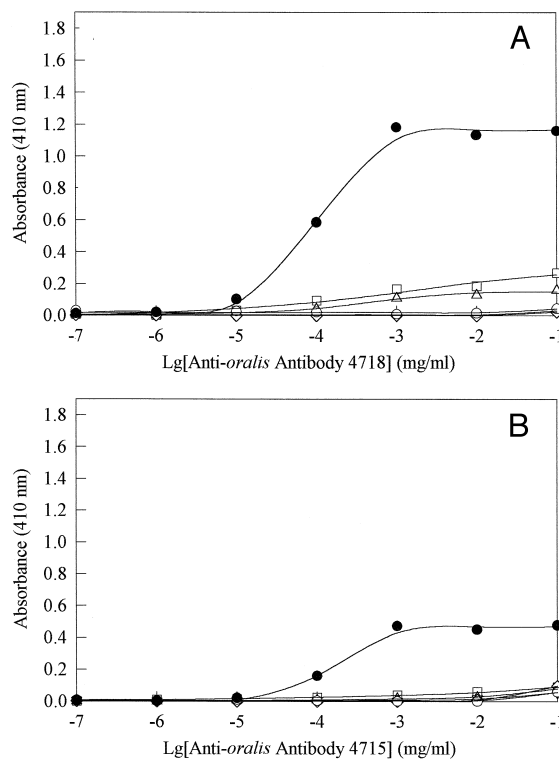


Fig. 3. Affinity of anti-oralis antibodies (A) 4718 and (B) 4715 for various oral bacteria. Anti-oralis antibody incubation 2 h at 37°C, anti-mouse alkaline phosphatase antibody incubation 1 h at 37°C. (●) – *S. oralis*, (□) – *S. gordonii*, (△) – *S. sanguis* C104, (▽) – *S. salivarius* DBD, (◇) – *S. salivarius* 8618, and (○) – *S. mutans* D282.

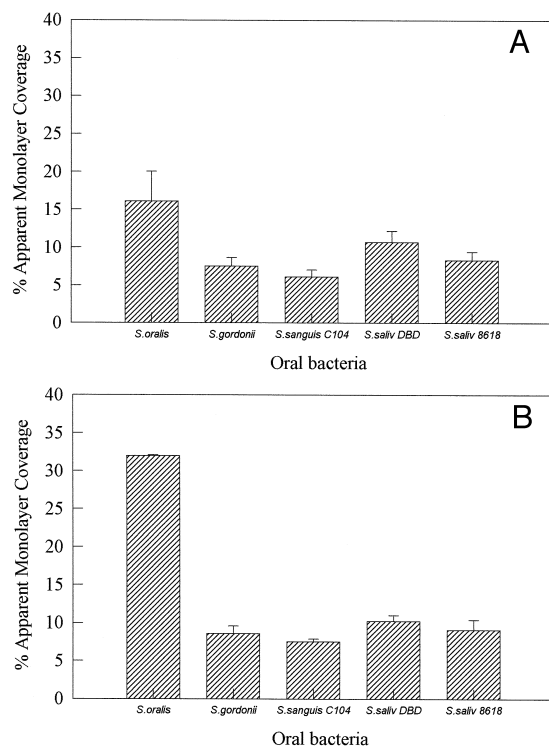


Fig. 4. Adsorption of anti-oralis immunoliposomes to various oral bacteria. Targeting 2 h at 37°C, lipid concentration 2 mM. (A) – Anti-oralis 4718 VETs, liposome composition DPPC/PI/DPPEMBS (82.8: 2.6: 14.6, molar ratio),  $\bar{d}_w = 120$  nm,  $\bar{P}_w = 38$ . (B) – Anti-oralis 4715 VETs, liposome composition DPPC/PI/DPPEMBS (82.8: 2.6: 14.6, molar ratio),  $\bar{d}_w = 117$  nm,  $\bar{P}_w = 17$ . The error bars are from four experiments.

This adsorption is likely to be a result of lipid, rather than antibody, interactions as the free antibody adsorption to these bacteria is almost negligible (Fig. 3(A) and (B)).

### 3.3. Affinity of anti-oralis immunoliposomes

Fig. 5 shows how immunoliposome targeting is dependent on liposome concentration, measured by lipid content. Anti-oralis immunoliposome adsorption increases with increasing lipid concentration and is saturable. Anti-oralis 4718 immunoliposomes adsorb to *S. oralis* with approximately twice the affinity of ‘naked’ liposomes (Fig. 5A). Anti-oralis 4715 immunoliposomes adsorb to *S. oralis* with approximately five times the affinity of ‘naked’ liposomes. Cationic liposomes adsorb to *S. oralis* with a greater affinity than anti-oralis immunoliposomes and the

adsorption of cationic liposomes is dependent on the percentage of cationic lipid incorporated into the VETs preparation (Fig. 5B). However, the targeting affinity of anti-oralis immunoliposomes is not significantly influenced by the charge on the liposomal lipid bilayer (Fig. 6). The affinity of anti-oralis immunoliposomes for *S. oralis* is not significantly different depending on the weight average number of antibody molecules ( $\bar{P}_w$ ) conjugated, within the range examined (Fig. 6B). The weight average number of

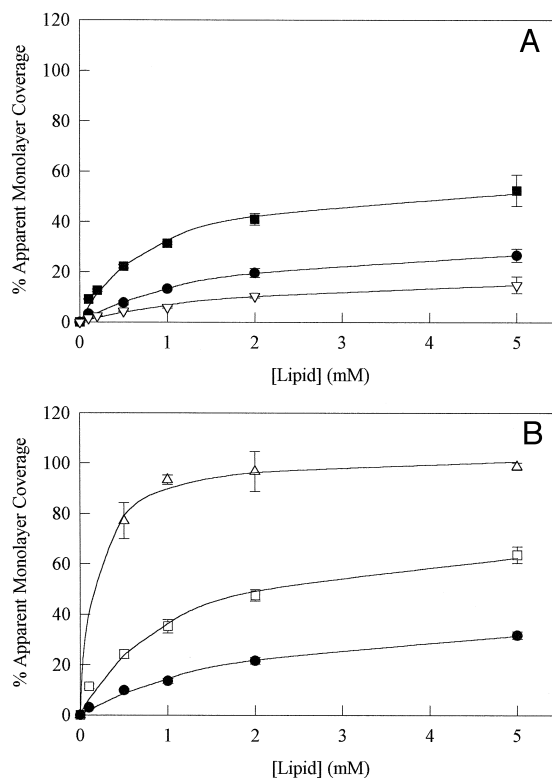


Fig. 5. (A) Adsorption of anti-oralis antibody immunoliposomes, compared with ‘naked’ liposomes, to a *S. oralis* biofilm as a function of lipid concentration. Liposome composition DPPC/PI/DPPEMBS (82.8: 2.6: 14.6, molar ratio). Targeting 2 h at 37°C. (■) – Anti-oralis 4715 VETs,  $\bar{d}_w = 107$  nm,  $\bar{P}_w = 5.3$ . (●) – Anti-oralis 4718 VETs,  $\bar{d}_w = 115$  nm,  $\bar{P}_w = 6.8$ . (▽) ‘Naked’ VETs,  $\bar{d}_w = 114$  nm. (B) – Adsorption of cationic vesicles, compared to anti-oralis immunoliposomes, to a *S. oralis* biofilm as a function of liposome lipid concentration. Targeting 2 h at 37°C. (△) – Cationic VETs DPPC/Chol/SA (61.4: 20.8: 17.8 molar ratio),  $\bar{d}_w = 132$  nm, (□) Cationic VETs DPPC/Chol/SA (72.3: 24.1: 3.6 molar ratio)  $\bar{d}_w = 137$  nm, (●) – Anti-oralis 4718 immunoliposomes DPPC/PI/DPPEMBS (82.8: 2.6: 14.6, molar ratio)  $\bar{d}_w = 112$  nm,  $\bar{P}_w = 30.0$ . The error bars are from four experiments.

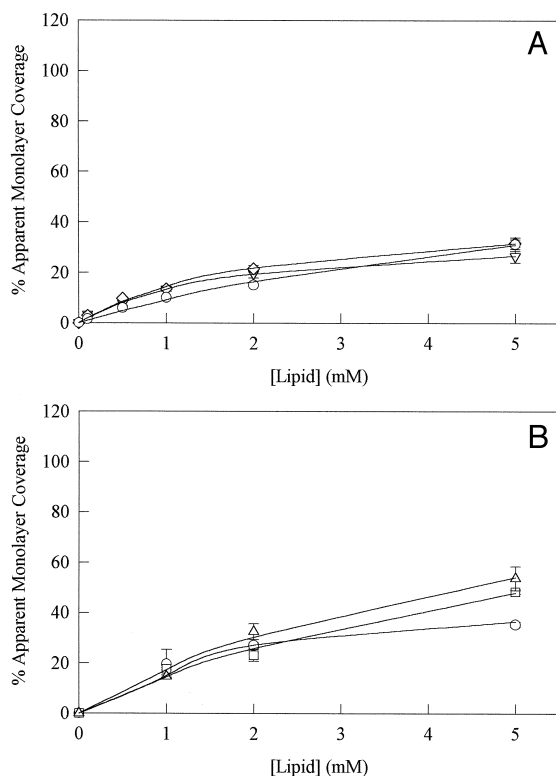


Fig. 6. (A) – Adsorption of anti-oralis 4718 immunoliposomes, with various lipid compositions, to *S. oralis* as a function of liposomal lipid concentration. Targeting 2 h at 37°C. (V) – Negatively charged VETs {2.6 mol% PI} liposome composition DPPC/PI/DPPEMBS (82.8:2.6:14.6, molar ratio),  $\bar{d}_w = 120$  nm,  $\bar{P}_w = 38$ , (◇) – positively charged VETs {3.6 mol% SA} liposome composition DPPC/Chol/SA/DPPEMBS (59.0:24.9:3.6:12.5, molar ratio),  $\bar{d}_w = 109$  nm,  $\bar{P}_w = 44$ , (○) – neutral VETs liposome composition DPPC/DPPEMBS (85.0:15.0, molar ratio)  $\bar{d}_w = 111$  nm,  $\bar{P}_w = 19$ . (B) – Adsorption of anti-oralis antibody 4715 immunoliposomes, bearing various numbers of antibody molecules per liposome to a *S. oralis* biofilm, as a function of lipid concentration. (○) – Liposome composition DPPC/PI/DPPEMBS (92.6:2.6:4.8 molar ratio),  $\bar{d}_w = 113$  nm,  $\bar{P}_w = 9.7 \pm 2.7$ , (□) – liposome composition DPPC/PI/DPPEMBS (87.8:2.6:9.6 molar ratio),  $\bar{d}_w = 115$  nm,  $\bar{P}_w = 23.6 \pm 2.3$ , (△) – liposome composition DPPC/PI/DPPEMBS (82.8:2.6:14.6, molar ratio),  $\bar{d}_w = 112$  nm,  $\bar{P}_w = 30.0 \pm 5.9$ . The error bars are from four experiments.

antibodies per liposome is controlled by the percentage of reactive lipid (DPPEMBS) in the VETs preparation. The targeting curves for immunoliposomes with  $\bar{P}_w$ 's of 9.7, 23.6 and 30.0 are all similar, only at high lipid concentrations (5 mM) does there seem to be a trend for targeting to be limited by the number of antibody molecules on the liposomal surface. The

size of the anti-oralis immunoliposomes was monitored over an 18 month period. They were found to be physically stable, with no significant changes in the mean size or size distribution width.

#### 4. Discussion

The number of sulphydryl groups introduced into the anti-oralis antibodies compares well with the introduction of sulphydryl groups into other molecules, used for liposome targeting, by the same conjugation technique [22,23,25]. Conjugation of anti-oralis antibodies to DPPEMBS increases as the percentage of reactive lipid (DPPEMBS) in the vesicles increases, in agreement with previous studies [17,25,26].

The chemical modification of anti-oralis antibody 4718, in several ways which relate to the liposome conjugation process, does not affect the ability of the antibody to bind to a *S. oralis* biofilm. However, anti-oralis 4718 immunoliposomes appear to adsorb much less effectively than the chemically altered free antibody. These results imply that it is the presence of the liposome attached to the antibody that is affecting the affinity of the immunoliposomes for the biofilm and not the derivatisation of the antibody. It is possible that this could be attributable to the second ELISA antibody conjugate binding inefficiently to liposomally bound close-packed anti-oralis antibody or to steric effects of the bulky liposomes in the biofilm.

Anti-oralis antibodies 4718 and 4715 are found to be very specific for the bacterium *S. oralis*. Even with other *S. sanguis* bacteria, there is little cross reaction (*S. oralis* and *S. gordonii* were formally classed as *S. sanguis* [15]). Anti-oralis immunoliposomes are relatively less specific for *S. oralis* than the free antibody because of the non-specific interactions of the vesicles with the other bacteria. However, the anti-oralis immunoliposomes are still found to adsorb with the greatest affinity to *S. oralis* so that antibody specificity is retained to a certain degree after conjugation to the liposomes. *S. salivarius* DBD shows the largest non-specific immunoliposome adsorption. This can be attributed to its carrying fimbriae, rather than fibrils (as in the case of *S. salivar-*

ius 8618), on its cell surface [27] which have been associated with hydrophobicity [28], helping liposome targeting.

It is unclear why anti-*oralis* 4715 immunoliposomes target to *S. oralis* with a greater affinity than anti-*oralis* 4718 immunoliposomes (Fig. 5A), since both antibodies bind to antigens of the protein SSP5, the *S. sanguis* salivary agglutinin receptor [29], also found in *S. oralis* (Unilever Research, personal communication). The targeting efficiency of immunoliposomes depends on the numbers of antibodies per liposome only at high liposome concentrations. There are several reports of immunoliposome binding being influenced by the degree of antibody conjugation [30,31].

By comparison with the affinity of cationic vesicles for *S. oralis*, immunoliposomes show relatively low binding affinities. Immunoliposome adsorption to *S. oralis* is presumed to occur as a result of specific interaction between the antibody and a specific cell surface antigen. Cationic liposome adsorption to *S. oralis* is likely to occur as a result of a non-specific electrostatic forces. We have found that cationic liposomes adsorb strongly to several other bacteria including *Staphylococcus epidermidis* [32] and *aureus*. Because most bacteria have a net negative charge on the cell surface [33], cationic vesicle targeting is likely to occur largely through an electrostatic interaction.

When anti-*oralis* immunoliposomes were prepared from more than one type of lipid mixture, it was found that targeting does not depend on the net charge of the liposomal lipids, most likely because antibody molecules conjugated to the liposomal surface mask the net charge on the liposomal lipid surface.

Both, chemical (hydrolysis, oxidation) and physical instability (aggregation, phase separation in the bilayer, fusion, loss of encapsulated drug) of liposomes on storage have been described in detail [34]. Anti-*oralis* immunoliposomes appear physically stable with regards to the size distribution over an 18 month period although chemical stability and retention of antibody specificity and affinity require further study.

In conclusion, liposomes can be specifically targeted to oral bacteria by use of monoclonal antibodies. This may prove to be of importance for specific

delivery of antimicrobial agents to oral bacteria for specific plaque control. By selectively inhibiting bacteria implicated in dental caries and periodontal disease, or bacteria involved in early plaque development, it is hypothesised that the ecological niche of disease-causing bacteria is vacated and filled by other more benign organisms of the oral flora, or that by inhibiting initial tooth colonisers, plaque development is slowed.

It is noteworthy that oral streptococci can be found in other types of infection in immunologically compromised and neutropenic patients, such as septicaemia and the Adult Respiratory Distress Syndrome [35]. With improvements for stabilisation of liposomes in the blood being made with polyethylene glycol-immunoliposomes (for review, see Ref. [36]), drug delivery to specific oral bacteria in systemic infections, by use of specific immunoliposomes, may also be of future importance.

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