The interaction of cationic liposomes with the skin-associated bacterium *Staphylococcus epidermidis*: effects of ionic strength and temperature

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Abstract

Cationic liposomes have been prepared from dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol) and stearylamine (SA). These phospholipid vesicles were exposed to adsorbed biofilms of the skin-associated bacteria *Staphylococcus epidermidis*, to which they showed a strong affinity. The interaction (as assessed by the apparent monolayer coverage of the biofilms by liposomes) was described in terms of a Langmuir adsorption isotherm which enabled determination of the maximum theoretical coverage of the bacterial surface and association/dissociation constants. The interaction was shown to be dependent on the ionic strength of the surrounding medium; on increasing the ionic strength the biofilm-vesicle dissociation constant decreased. This suggested that the adsorption was mediated by electrostatic effects. The adsorption of the vesicles was examined at various temperatures, enabling determination of thermodynamic parameters for the interaction. The adsorbed state of the liposomes was energetically favoured and the interaction was enthalpy driven. The Gibbs energies of adsorption were in a range from -15 to -19 kJ mol^{-1} and the enthalpies of adsorption from -26 to -22 kJ mol^{-1}. Studies using cell populations of different hydrophobicity showed that the hydrophobic character of the bacterial cells also had an effect on the adsorption of the vesicles to the biofilm.

1. Introduction

Coagulase-negative staphylococci (CNS) such as *Staphylococcus epidermidis* are the bacteria most frequently associated with infection of indwelling medical devices such as catheters, prosthetic heart valves and artificial joints [1]. These Gram-positive microorganisms are normally harmless colonizers of the human skin, but they can become pathogenic. Implantation of the foreign body biomaterials leads to a local environment which hampers the defence mechanisms that the host normally uses to deal with bacterial infection [2]. Most *S. epidermidis* infections are of a nosocomial origin; they originate and spread within hospitals themselves [3]. The skin around the insertion site for the implant is the most common source of the infecting bacteria.

The first stage in the development of the infection involves adhesion of the bacteria to the device via various non-covalent interactions and also ligand-mediated interactions involving receptor-like structures (adhesins) on the bacterial surface [4,5]. Once attached, the bacteria multiply and colonize the surface of the device. The bacteria then secrete a highly adhesive, negatively-charged polysaccharide substance (slime) which they become embedded in to form a biofilm that provides protection against antibiotics [6].

Such infections can prove very painful to the patient and are potentially fatal. Often, total replacement of the device is the only option, a complicated and expensive procedure [7]. One method of preventing such device-associated infections is to treat the patient with an antibiotic such as vancomycin [8]. However, vancomycin is potentially toxic and cannot be given orally. Intravenous administration of the drug is required over several weeks, with frequent monitoring of the blood necessary. Vancomycin can be applied topically prior to implantation, but penetration of this water-soluble drug into the skin is low. Cutaneous penetration can be enhanced by encapsulation within phospholipid vesicles (liposomes) [9]. Liposomes have been

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shown to interact with bacteria [10–12] and can be used as vehicles for delivery of antibacterial agents.

Cationic liposomes, incorporating positively-charged stearylamine (SA), have previously been shown to interact with biofilms of *S. epidermidis* [13,14]. We examine here this interaction and how it is affected by temperature, ionic strength and bacterial cell hydrophobicity. The thermodynamic parameters involved in the adsorption of the liposomes are determined, giving insights into the nature of the interaction.

2. Materials and methods

L-α-Dipalmitoylphosphatidylcholine (DPPC, product No. P0763), cholesterol (Chol; product No. C8667) and stearylamine (SA; product No. S9273) were from Sigma, Poole, Dorset, UK. [³H]DPPC (specific activity 55 Ci mmol⁻¹) and [³H]thymidine (specific activity 82 Ci mmol⁻¹) were from Amersham, Amersham, UK. Bacteriological agar No. 1 (code L11), brain heart infusion (BHI, code CM255), yeast extract powder (code L21), tryptone soya broth (TSB, code CM129) and phosphate-buffered saline (PBS) tablets (code BR14a) 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 using the vesicle extrusion technique (VETs) [15,16]. DPPC (27 mg), cholesterol (7 mg) and stearylamine (4 mg), together with 5 μCi [³H]DPPC, were dissolved in *tert*-butyl alcohol (20 ml) in a 100 ml round-bottomed flask. The solvent was removed by rotary evaporation at 60°C to leave a thin, even lipid film to which was added PBS buffer (3 ml, pH 7.4) at 60°C. For the preparation of VETs the suspension was vigorously mixed on a vortex mixer to form multilamellar liposomes (MLVs) followed by extrusion 10 times at 60°C through two stacked polycarbonate filters (Poretics, Livermore, CA; 100 nm pore size) under a pressure of 200–500 psi.

The lipid content was determined by scintillation counting of [³H]DPPC. Size measurements were made by photon correlation spectroscopy (PCS) using a Malvern Autosizer model RR146. The scattering data were fitted to an equivalent normal weight distribution $W(d_i)$ to give the weight-average diameter ($\bar{d}_w$) [17].

2.2. Preparation of bacterial cultures

Staphylococcus epidermidis (NCTC 11047) was the wild type laboratory strain used in this study. It was stored at −70°C and cultured on tryptone soya broth and then inoculated into TSB broth and incubated for 20 h at 37°C

before use. Hydrophobic and hydrophilic subpopulations of cells and adhesion defective mutants were derived from this parent strain for use in this study.

Hydrophobic and hydrophilic subpopulations were isolated using a modification of the hexadecane partition assay [18]. An overnight culture of *S. epidermidis* NCTC 11047 grown in TSB was harvested by centrifugation at 2000 rpm and washed three times before resuspension in sterile phosphate buffer, pH 7.2. To enrich for a hydrophobic subpopulation, 3 ml of the suspension ($OD_{440} = 0.5$) and 200 μl of hexadecane were vortexed together for 10 s under sterile conditions and allowed to partition for 15 min. Bacteria that had partitioned into the hexadecane phase were isolated and purified on agar plates. A few single colonies were then reinoculated into broth and the same enrichment process was repeated, for 34 passages in all.

A hydrophilic subpopulation of cells was enriched for in a similar way, except that the partitioning was preceded by a longer vortexing (60 s) and the bacteria in the buffer phase were the ones plated out. Again, the enrichment cycle was repeated 34 times.

The enriched subpopulations and the wild type strain were then tested to quantify the level of affinity for hexadecane [18]. Washed cell suspensions of overnight cultures of the two populations ($OD_{440} = 0.5$) were distributed in 3 ml volumes into six test tubes and 200 μl of hexadecane layered into each tube. Tubes were vortexed for 60 s and allowed to partition for 15 min. The lower aqueous phase was used to determine the OD_{440} of each sample with the buffer as a blank. The percentage affinity of the bacteria for hexadecane was calculated as:

$$\% \text{ affinity} = \frac{OD_i - OD_p}{OD_i} \times 100\% \tag{1}$$

where OD_i represents the OD_{440} of the initial suspension and OD_p the OD_{440} of the partitioned suspension. In the wild type, 30% of the cells were hydrophobic and 70% were hydrophilic. The hydrophobic bacteria were more adhesive to catheters than the hydrophilic ones [19].

Adherence-defective mutants of *S. epidermidis* were prepared by transposon mutagenesis. A transposon that conferred erythromycin resistance was carried by a temperature-sensitive plasmid vector which was introduced into the bacteria by protoplast transformation, selecting for the plasmid-borne resistance for chloramphenicol. The plasmid replicated at 30°C and during growth of the bacteria at this temperature the transposon inserted randomly into the bacterial chromosome. Subsequent growth of the bacteria on erythromycin agar at 43°C (a temperature at which the plasmid could not replicate) selected a collection of transposon-generated mutant clones. The adherence-defective mutants were isolated from the collection on the basis of their decreased hydrophobicity using the hexadecane adhe-

sion assay [18]. Four different hydrophilic mutants were isolated. The mutant used in this work was designated M3.

Bacterial cell suspensions for use in the liposome adsorption experiments were prepared as follows. Bacterial stocks were used to inoculate agar plates prepared from BHI (3.7 g) in double distilled water (100 ml) to which was added bacteriological agar (1.5 g). The plates were inoculated by streaking and the inverted streaked plates were incubated at 37°C for 18 h. The resulting colonies were used to inoculate aliquots (10 ml) of nutrient broth prepared by mixing BHI (3.7 g) and yeast extract powder (0.3 g) in double distilled water (100 ml). These were incubated in capped bottles at 37°C for 18 h after which the bacterial suspensions were centrifuged (2000 rpm; 15 min), the supernatant discarded and the pellet resuspended in sterile PBS. The centrifugation and resuspension were repeated a further three times and the bacterial concentration adjusted to give an absorbance of 0.5 at 550 nm.

2.3. Adsorption of liposomes (VETs) to bacterial biofilms

Adsorption assays were carried out in wells of microtitre plates (Dynatech M129B). Aliquots of bacterial suspension (either wild-type, subpopulation or adherence-defective mutants) were incubated overnight (18 h) at room temperature to form an adsorbed biofilm. After adsorption, the bacterial suspension was removed and the biofilm washed three times with sterile PBS. After washing, the adsorbed biofilms in the wells were incubated with a liposome suspension for 2 h (at temperatures ranging from 4°C to 37°C), after which the wells were washed three times with PBS and the biofilm dispersed by addition of sodium n-dodecylsulphate (SDS, 1% w/v; 200 μl) followed by incubation (30 min) at room temperature and a brief sonication (2 min). Aliquots of the dispersed biofilm (180 μl) were taken for scintillation counting. Control wells containing only bacteria, only PBS or only liposomes were used to assess background levels of activity.

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

$$\% \text{ amc} = \frac{N_{obs}}{L_a} \times 100 \qquad (2)$$

Where N_{obs} is the observed number of moles of lipid adsorbed to the biofilm and L_a the number of moles of lipid which would be adsorbed if the biofilm was covered with a close-packed layer of liposomes. L_a was calculated from the equation

$$L_a = \frac{A_{bf} \, \overline{N}_w}{\pi \left(\overline{d}_w / 2 \right)^2} \qquad (3)$$

Where $\overline{d}_w$ is the weight average diameter of the liposomes having a weight average number of moles of lipid per liposome of $\overline{N}_w$ and A_{bf} is the geometric area of the

biofilm. $\overline{N}_w$ was calculated from $\overline{d}_w$ assuming an area per lipid molecule in the liposomal bilayer (taken as 0.5 nm^2) and a bilayer thickness (taken as 7.5 nm) as described previously [17]. 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 plate wells exposed to 200 μl of solution [20].

Adsorption assays performed at ionic strengths higher than that of the stock PBS solution were done by diluting the liposomes in high ionic strength solutions made by using more than one PBS tablet per 100 ml distilled water used normally. The ionic strength of PBS was 188 mM, and so 2 × concentrated PBS had ionic strength 376 mM, 3 × PBS 564 mM and 4 × PBS 752 mM. The final ionic strengths of the liposome suspensions used (taking into account that the liposomes were initially made up in 1 × PBS) were 357 mM, 526 mM and 696 mM. Increasing the PBS concentration did not alter the pH.

2.4. Counting of cells attached to microtitre plates

To assess the number of bacterial cells that attached to each well of the microtitre plate, bacteria were inoculated in liquid nutrient media as described above, using 5 ml aliquots of broth, each containing 50 μl of [³H]thymidine (= 50 μCi). The bacteria were allowed to grow in the nutrient media for 18 h and were then washed several times with PBS (to ensure removal of any labelled thymidine not associated with the bacteria) and the OD_{550} adjusted to 0.5. Aliquots (10 μl) of the bacteria at this dilution were taken for scintillation counting and the number of cells in the bacterial suspension was determined by haemocytometry. From the cell counts and the data obtained from the scintillation counting, the specific activity of the bacterial cells could be calculated (dpm/cell). Aliquots (200 μl) of the labelled bacteria were then incubated overnight in the wells of a microtitre plate to enable biofilm formation. After washing, SDS (10% w/v; 200 μl) was added to the wells. The plate was incubated at room temperature for 1 h and then briefly sonicated (2 mins) to ensure dispersion of the biofilm by the detergent. Aliquots (180 μl) were taken from these wells for scintillation counting. From these counts, and from the specific activity of the cells, the number of cells attached as a biofilm to each well was calculated.

3. Results

3.1. Hydrophobicity of Staphylococcus epidermidis strains

Table 1 shows the hydrophobicity values of S. epidermidis strains as measured using the affinity for hexadecane. Statistical analysis (non-parametric Mann–Whitney test) showed that the hydrophobic bacteria had a significantly greater affinity for hexadecane than the wild type

Table 1

Hydrophobicity values of *S. epidermidis* strains determined by affinity for hexadecane

S. epidermidis population	Mean % hydrophobicity
Wild type	88.7% ($\pm 4.2\%$)
Hydrophobic subpopulation	92.8% ($\pm 1.3\%$)
Hydrophilic subpopulation	61.2% ($\pm 4.8\%$)
M3 mutant	2.1% ($\pm 0.6\%$)

Fig. 2. Dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) to *S. epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ; ▲, 526 mM; ▼, 696 mM. Incubation time 2 h, temperature 25°C.

($P = <0.01$), whereas the hydrophilic cells had significantly lower affinity than the wild type. The mutant M3 had a very low hydrophobicity and clearly has a very hydrophilic surface.

3.2. Dependence of adsorption of liposomes on lipid concentration

The adsorption of the DPPC/Chol/SA liposomes (in PBS buffer, ionic strength 188 mM) to *S. epidermidis* biofilms was studied as a function of liposome concentration at three different temperatures (4°C, 25°C, 37°C) as shown in Fig. 1. The level of stearylamine used in the liposomes (21.3 mol%) had been identified previously as that which gave optimum targeting to *S. epidermidis* [21]. Over the range of liposome concentrations studied, adsorption was greater when the incubation temperature was higher. However, as the thermodynamic parameters show (see Table 3 below), the Gibbs energies of adsorption are greater at the lower temperature so that although adsorption (%amc) is less at the lower temperature the liposomes are more strongly adsorbed, consistent with an electrostatic interaction. At all three temperatures, the %amc rises steeply between a liposomal lipid concentration of 0 and 1 mM and more slowly thereafter. Neutral liposomes com-

posed of DPPC/Chol with no SA did not adsorb to the bacteria significantly, giving %amc of less than 5% at all the liposomal concentrations studied.

3.3. Dependence of adsorption on ionic strength of media

The concentration dependence of liposomal adsorption was studied at three higher ionic strengths (357 mM, 526 mM, 696 mM), as well as in the standard PBS buffer, at the three different temperatures. Fig. 2 shows the effect of ionic strength on the adsorption at one of the temperatures studied (25°C). Over the range of liposomal concentrations examined, the %amc decreased when the ionic strength
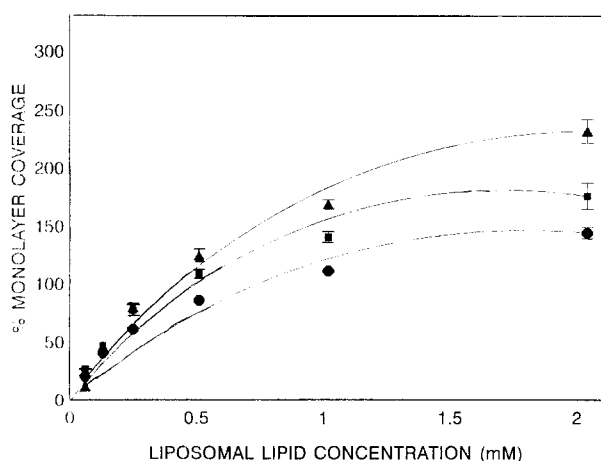

Fig. 1. Dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) to *S. epidermidis* biofilms on the temperature of incubation. ●, 4°C; ■, 25°C; ▲, 37°C. Incubation time 2 h, ionic strength 188 mM.
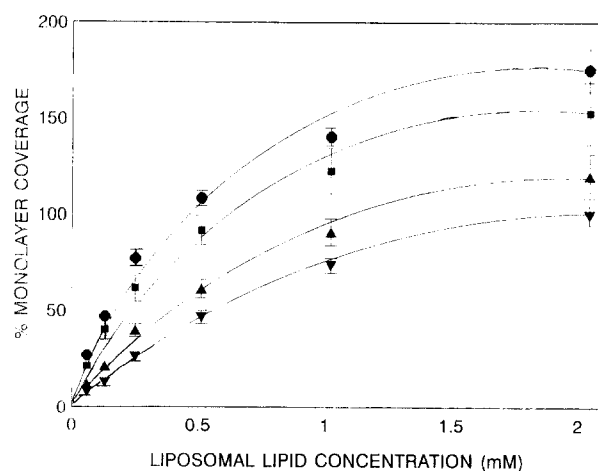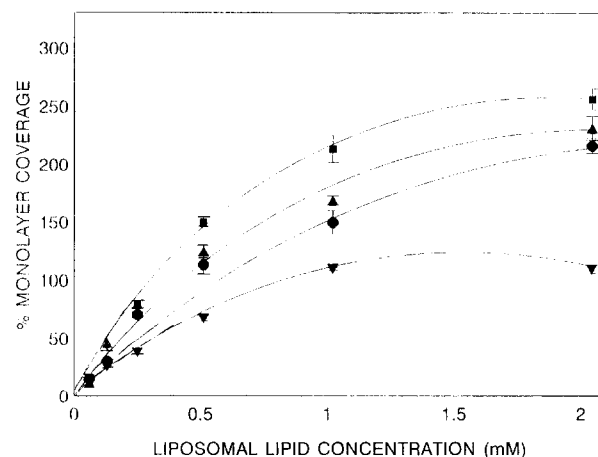

Fig. 3. Adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) to biofilms of hydrophobic and hydrophilic subpopulations of *S. epidermidis*. ▲, total population; ■, hydrophobic subpopulation; ●, hydrophilic subpopulation; ▼, adherence-defective mutants. Incubation time 2 h, ionic strength 188 mM, temperature 37°C.

Table 2

Attachment of hydrophobic and hydrophilic subpopulations of *S. epidermidis* and adherence-defective *S. epidermidis* mutants to the wells of microtitre plates (Immulon 2), as determined by labelling of bacteria with [^{3}H]-thymidine

S. epidermidis subpopulation	Cell density (cells m^{-2})
Wild type	$2.34 \cdot 10^{10} \pm 0.11 \cdot 10^{10}$
Hydrophobic subpopulation	$2.34 \cdot 10^{10} \pm 0.15 \cdot 10^{10}$
Hydrophilic subpopulation	$1.76 \cdot 10^{10} \pm 0.08 \cdot 10^{10}$
M3 mutant	$2.15 \cdot 10^{9} \pm 0.07 \cdot 10^{9}$

Incubation time 18 h, room temperature.

was increased. Similar results were obtained at 4°C and 37°C (not shown).

3.4. Effect of hydrophobicity of bacteria on adsorption

The attachment of cationic liposomes to biofilms of the hydrophobic and hydrophilic subpopulations of *S. epidermidis* was compared to that seen with the total population of the bacteria and with the adherence-defective mutant (Fig. 3). Over the range of liposomal concentrations studied, there was a greater extent of adsorption to biofilms of the hydrophobic subpopulations than to biofilms of the total population of the bacteria. Conversely, significantly lower attachment was seen with the hydrophilic subpopulation when compared to the total population. Adsorption to biofilms of the M3 mutant of *S. epidermidis* was substantially lower than for the wild-type or the subpopulations, with a levelling off of the monolayer coverage at around 1 mM lipid concentration suggesting that a saturation level of liposome attachment had been reached.

3.5. Number of cells attached to microtitre plate wells

The level of attachment of the various subpopulations of *S. epidermidis* to the wells of the microtitre plates was determined by labelling the bacteria with [^{3}H]thymidine and by cell counting using a haemocytometer. With the specific activity of a single cell known, the number of bacteria attached to a well was calculated by dispersing the biofilms with detergent and taking a sample for scintillation counting. Table 2 shows the relative levels of the various bacterial cells attached to wells of a microtitre plate after 200 μl of a bacterial suspension had been incubated in the plate for 18 h.

4. Discussion

Adsorption of SA-containing liposomes to biofilms of *S. epidermidis* was found to be dependent on liposomal lipid concentration (Fig. 1). The %amc increased with liposome concentration, giving values of well over 100%. The biofilms were formed by adsorption of bacteria from

suspensions which had an absorbance 0.5 at 550nm, leading to a close packed multilayer of cells that can be visualized by electron microscopy [22]. It should be noted that the values of %amc over 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 smooth hemispheres on the surface of the microtitre plate then their surface area would be doubled and hence the %amc halved. Hemispheres with a rough surface would have a larger surface area and the %amc would be reduced further. However, should the liposomes collapse on the bacterial surface the projected area occupied would be larger, reducing L_a in Eq. (3) and hence increasing the %amc.

At all three temperatures studied, the plot of %amc against liposomal concentration was hyperbolic. At higher levels of lipid, saturation of the biofilm is approached. The extent of adsorption as a function of liposomal concentration can be described in terms of a Langmuir adsorption isotherm [23], which assumes that there is an equilibrium between adsorbed and free liposomes. If θ is the fraction of the surface area that is covered by adsorbed liposomes in equilibrium with liposomes, concentration C, in the bulk phase then the Langmuir adsorption isotherm may be written as:

$$\theta = \frac{C}{K_d + C} \tag{4}$$

where K_d is the dissociation constant and θ is given by $(\%amc)/(\%amc)_{max}$, where $(\%amc)_{max}$ is the maximum apparent monolayer coverage. Substituting for θ in Eq. (4) and reciprocating gives the linear form of the isotherm:

$$\frac{1}{\%amc} = \frac{1}{C} \cdot \frac{K_d}{(\%amc)_{max}} + \frac{1}{(\%amc)_{max}} \tag{5}$$

from which K_d and $(\%amc)_{max}$ can be obtained, from the slope and intercept of the plot of $1/\%amc$ against $1/C$. Fig. 4 shows such a double-reciprocal plot of the data in Fig. 2. Increasing the ionic strength of the incubation medium did not alter $(\%amc)_{max}$ but K_d increased.

The results indicate that the attractive interaction between the cationic vesicles and negatively-charged sites on the bacterial surface or the extracellular slime (e.g., teichoic acids) is mediated by electrostatic effects, in that compression of the diffuse double layers surrounding these oppositely-charged surfaces and the increased ionic screening at higher ionic strength weakens the attraction between bacterium and liposome. The change in ionic strength does not affect the $(\%amc)_{max}$ because on extrapolation of the liposomal concentration to infinity, the ionic strength effect is abolished.

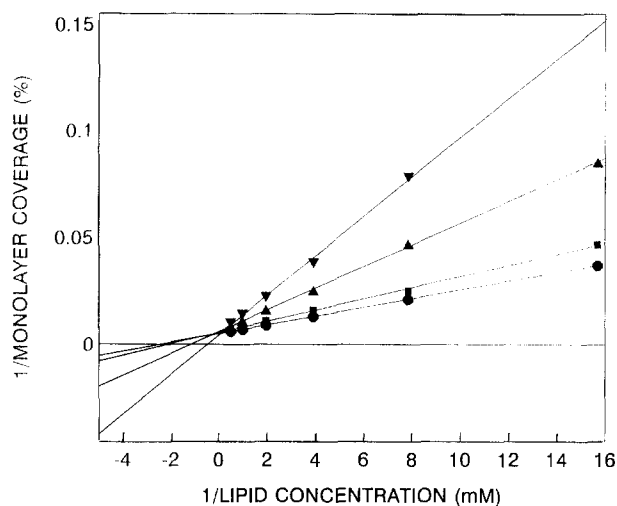The effect of ionic strength on aggregation of the

Fig. 4. Double-reciprocal plot of the data in Fig. 2, showing the dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) to *S. epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 h, temperature 25°C.
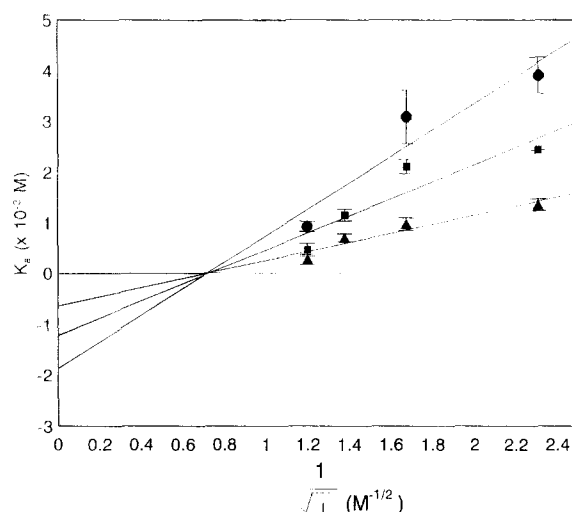

Fig. 5. Dependence of the association constant (K_a) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) and *S. epidermidis* biofilms on the reciprocal of the square root of the ionic strength of the medium and the temperature of incubation. ●, 4°C; ■, 25°C; ▲, 37°C. Incubation time 2 h.

cationic liposomes was examined over a 2-h period using photon correlation spectroscopy (results not shown). No aggregation was observed at 188 mM or 357 mM during this time. However, some aggregation was seen at 526 mM and 696 mM reflected by, at most, a threefold increase in the apparent diameter of the liposomes, corresponding to the formation of small aggregates of around ten liposomes.

The screening of ionic interactions is related to the thickness of the ionic atmosphere associated with the bacterium and liposome surfaces. The reciprocal of the Debye–Hückle parameter, κ, is generally taken as a measure of the thickness of the ionic atmosphere and is related

to ionic strength (I) for a symmetrical electrolyte by the equation [24]:

$$\frac{1}{\kappa} = \left(\frac{\varepsilon_0 \varepsilon_r kT}{2N10^3 e^2}\right)^{1/2}\left(\frac{1}{\sqrt{I}}\right) \tag{6}$$

where N, e, ε_0, ε_r, k and T are Avogadro's constant, electronic charge, permittivity of vacuum, relative permittivity of the medium, Boltzmann constant and temperature, respectively. It follows that for an electrostatic attractive interaction, adsorption would be expected to decrease as the ionic strength increases and the ionic interactions are increasingly screened. Thus the association constant K_a ($= 1/K_d$) might be expected to be related to the thickness

Table 3

Calculated values of the maximum theoretical monolayer coverage ($(\%amc)_{max}$), the association constant (K_a) and associated energy changes for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) and *S. epidermidis* biofilms in media of various ionic strengths and at three different temperatures

Ionic strength	Temp. (K)	$(\%amc)_{max}$ (%)	K_a (M)	ΔG_a (kJ mol^{-1})	ΔH_a (kJ mol^{-1})	$T\Delta S_a$ (kJ mol^{-1})	ΔS_a (kJ K^{-1} mol^{-1})
180 mM	277.15	126 ($\pm$10)	3920 ($\pm$355)	-19.1 ($\pm$0.2)	-21.7 ($\pm$5.4)	-2.590	-0.00933
	298.15	200 ($\pm$0.1)	2453 ($\pm$2)	-19.3 ($\pm$0.0)	-21.7 ($\pm$5.4)	-2.300	-0.00773
	310.15	303 ($\pm$24)	1372($\pm$116)	-18.6 ($\pm$0.2)	-21.7 ($\pm$5.4)	-3.020	-0.00975
342 mM	277.15	123 ($\pm$20)	3098 ($\pm$530)	-18.5 ($\pm$0.4)	-23.0 ($\pm$9.3)	-4.490	-0.01620
	298.15	181 ($\pm$11)	2119 ($\pm$140)	-18.9 ($\pm$0.2)	-23.0 ($\pm$9.3)	-4.030	-0.01350
	310.15	294 ($\pm$37)	985 ($\pm$124)	-17.8 ($\pm$0.3)	-23.0 ($\pm$9.3)	-5.240	-0.01690
504 mM	277.15	115 ($\pm$20)	2212 ($\pm$426)	-17.7($\pm$0.4)	-24.0 ($\pm$2.7)	-6.240	-0.02250
	298.15	170 ($\pm$14)	1160 ($\pm$115)	-17.5 ($\pm$0.2)	-24.0 ($\pm$2.7)	-6.490	-0.02180
	310.15	284 ($\pm$33)	713 ($\pm$82)	-16.9 ($\pm$0.3)	-24.0 ($\pm$2.7)	-7.040	-0.02270
666 mM	277.15	121 ($\pm$12)	935 ($\pm$102)	-15.8 ($\pm$0.3)	-26.0 ($\pm$3.0)	-10.200	-0.03690
	298.15	230 ($\pm$64)	472 ($\pm$133)	-15.3 ($\pm$0.7)	-26.0 ($\pm$3.0)	-10.700	-0.03600
	310.15	320 ($\pm$117)	276 ($\pm$102)	-14.5 ($\pm$1.0)	-26.0 ($\pm$3.0)	-11.500	-0.03710

Incubation time 2 h.

of the ionic atmosphere $(1/\kappa)$ or $1/\sqrt{I}$. Fig. 5 shows the plot of K_a vs. $1/\sqrt{I}$ in which K_a decreases linearly with increasing $\sqrt{I}$. At all three temperatures tested, the intercept on the x-axis when $K_a = 0$ was 0.72, corresponding to an ionic strength of approximately 1.95 M, at which the electrostatic interaction would be swamped by electrolyte and no adsorption would occur.

Table 3 summarizes the values of $(\%amc)_{max}$ and K_a obtained for the interaction under the various conditions of incubation temperature and ionic strength. From the association constants, the Gibbs energy change on association was calculated from $\Delta G_a = -RT \ln K_a$ (Table 2, column five) and by application of the Gibbs–Helmholtz equation, the enthalpy of association (ΔH_a):

$$\left[\frac{\partial(\Delta G_a/T)}{\partial(1/T)} \right]_P = \Delta H_a \qquad (7)$$

The calculations are based on a standard state of 1 M liposomal lipid. Fig. 6 shows the plots of $\Delta G_a/T$ vs. $1/T$ from which values of ΔH_a were determined at each ionic strength. Values for $T\Delta S_a$ and ΔS_a were calculated from the equation:

$$\Delta G_a = \Delta H_a - T\Delta S_a \qquad (8)$$

and are given in Table 2 (columns six–eight).

All the values of ΔH_a are negative; showing that the adsorption of liposomes is an exothermic process as expected for an attractive ionic interaction. Increasing the ionic strength from 188 mM to 696 mM increased the enthalpy of association from -21.7 kJ mol^{-1} to -26.0 kJ mol^{-1}.

Over the range of ionic strengths studied, $|\Delta H_a|$ is greater than $|T\Delta S_a|$ and so the association is primarily enthalpy driven. However, as the ionic strength was increased, the $T\Delta S_a$ term becomes more dominant. At all the temperatures and ionic strengths studied, the negative en-
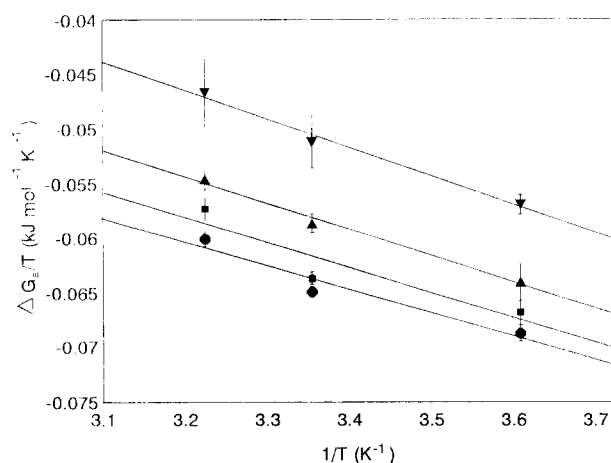

Fig. 6. Plot of $\Delta G_a/T$ against reciprocal temperature for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126$ nm) and S. epidermidis biofilms. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 h.

Table 4
Calculated values of the maximum theoretical monolayer coverage $((\%amc)_{max})$, the dissociation constant (K_d), Gibbs free energy change of dissociation (ΔG_d) and number of liposomes bound per bacterium at saturation for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4, $\bar{d}_w = 126.48$ nm) and biofilms of the total population of S. epidermidis NCTC 11047 and the hydrophobic and hydrophilic subpopulations and adherence-defective mutants

S. epidermidis population	$(\%amc)_{max}$	K_d (mM)	Max. liposomes per bacterium
Hydrophobic	418 ($\pm$74)	1.106 ($\pm$0.211)	14.152
Wild type	303 ($\pm$24)	0.729 ($\pm$0.061)	10.296
Hydrophilic	245 ($\pm$20)	0.621 ($\pm$0.062)	11.015
M3 mutant	119 ($\pm$23)	0.426 ($\pm$0.087)	44.065

Incubation time 2 h, ionic strength 180 mM, temperature 37°C.

tropy change of association (ΔS_a) is consistent with the more ordered adsorbed state of the liposomes.

The data in Fig. 3 were used to derive values of $(\%amc)_{max}$ and K_d for the various types of S. epidermidis. From the numbers of cells making up the biofilms (see Table 2) and the number of liposomes corresponding to 100% monolayer coverage, the maximum number of liposomes attached per bacterium was determined (Table 4). Both the monolayer coverage and the dissociation constant were highest with the hydrophobic subpopulation. The pooled population of S. epidermidis was estimated to be made up of 70% hydrophilic and 30% hydrophobic bacteria. If a mean figure for $(\%amc)_{max}$ is calculated, based on these relative proportions a value of 296.8% is obtained, which compares with the experimental value of 303.3%. This implies that the relative abundance of the two subpopulations attached to the plate reflected their proportion in the bacterial pool. When examining the number of attached liposomes per bacterium, within experimental error, there was little difference in attachment of liposomes between the total population and the hydrophilic subpopulation, reflecting the fact that the majority of the bacteria in the total population are hydrophilic. However, the extent of attachment to the hydrophobic bacteria was slightly higher.

Photon correlation spectroscopy of the bacteria gave a size (diameter) ranging up to a maximum of approximately 4000 nm with a weight-average diameter of 1518 nm [22]. Assuming a smooth sphere model these figures give surface areas of $50.3 \cdot 10^{-12}$ m^2 (maximum) and $7.24 \cdot 10^{-12}$ m^2 (average). Thus for liposomes of 125 nm diameter with a projected area of $\pi(d_w/2)^2$ the number of liposomes required to form a close-packed monolayer on half the bacteria surface (assuming the other half is adsorbed to the biofilm) would be between approximately 2000 (maximum) and 300 (average). The measured values (Table 4) are larger than these estimates, the differences perhaps arising either because of the crude assumptions made in the above calculations and also because the liposomes could adsorb to the extracellular slime excreted by the

bacteria which constitutes part of the adsorbed bacterial biofilm.

With the M3 mutant, the cell density in the biofilm was much lower (see Table 1), reflecting the decreased adherence of this mutant. Therefore, when the maximum theoretical coverage is reached, the number of liposomes attached per bacterium is much higher with the mutant bacteria, suggesting that adsorption to extracellular slime in the biofilm may be particularly important in this case.

These results demonstrate the strong affinity of stearyl-amine-containing liposomes for *S. epidermidis* bacteria. The interaction seems to be of an electrostatic nature and involves negative charges associated with the bacterial biofilm – either on the bacteria or the surrounding biofilm. Such vesicles could potentially be used for targeted delivery of antibacterial agents for treatment of device-associated bacterial infection.

Acknowledgements

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