Interaction of Cationic Liposomes with the Skin-Associated Bacteria *Staphylococcus epidermidis* for the Delivery of Antibacterial Agents

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by

Neil Michael Sanderson

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Division of Biochemistry

School of Biological Sciences

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abstract

The increased use, over the last couple of decades, of implanted biomedical devices such as catheters, prosthetic heart valves and artificial hip joints has led to a greater susceptibility to bacterial infection, most frequently with the Gram-positive microorganism *Staphylococcus epidermidis*. This is normally a non-pathogenic bacteria that inhabits the human skin, but it can become disease-causing when it persists in sites inaccessible to host defences or if these mechanisms are defective. The foreign body biomaterials of implanted devices interfere with these defence mechanisms, and the bacteria can become established in a biofilm on the device surface. Such an infection can have serious consequences for the host, such as septicaemia. Often, complete replacement of the infected implant is the only solution, but this is a complex and expensive procedure. The skin around the insertion site for a device is considered the most common source of entry for the bacteria and so it is essential that this area is kept free of bacteria. Although the skin can be washed and treated with antibacterial agents, such cleaning will be relatively inefficient, leaving a significant number of bacteria still in the skin. Penetration of antimicrobial drugs into the skin could be enhanced by their encapsulation into microscopic phospholipid vesicles - liposomes.

Positively-charged liposomes have been made by incorporation of cationic molecules into the liposome bilayer membranes. These vesicles have a strong affinity for biofilms of the *S.epidermidis* bacteria which carry a negative charge. The interaction has been characterised in terms of incubation time and liposome concentration and examined thermodynamically. The adsoprtion of the liposomes to the bacteria has been shown to be dependent on the ionic strength of the medium, the temperature of incubation and the relative hydrophobicity of the bacterial cells.

The antibacterial agents vancomycin and gentamicin have been encapsulated into such cationic liposomes and their ability to inhibit bacterial growth was examined. With vancomycin, entrapment in the cationic vesicles could enhance the activity of the drug and cause inhibition at low concentrations of vancomycin at which the free drug was ineffective - an important consideration due to the toxicity of vancomycin. The effectiveness of the liposomes was primarily determined by the level of drug entrapment within. Such liposomes could be made part of a cleaning gel for application to the skin or potentially administered intravenously to target to established bacterial infections.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification in this or any other university or other institute of learning.

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Abbreviations

AmB	amphotericin B
BHI	brain-heart infusion
BSA	bovine serum albumin
BZC	benzalkonium chloride
CAPD	continuous ambulatory peritoneal dialysis
CFTR	cystic fibrosis transmembrane regulator
Chol	cholesterol
CMI	cell-mediated immunity
CONS	coagulase-negative staphylococci
CSF	cerebrospinal fluid
CT	computed tomography
CuEDTA	ethylenediaminetetraacetic acid (cupric disodium salt)
DC-Chol	3β[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl] cholesterol
DDAB	dimethyldioactadecylammonium bromide
DOPE	dioleoylphosphatidylethanolamine
DOTAP	*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammoniummethylsulphate
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-triethylammonium
DOX	doxorubicin
dpm	disintegrations per minute
DPPC	dipalmitoylphosphatidylcholine
DPPG	dipalmitoylphosphatidylglycerol
DSC	differential scanning calorimetry
DSPG	distearoylphosphatidylglycerol
DTPA	diethylenetriaminepentaacetic acid
d_w	weight-average diameter
FATMLVs	frozen and thawed multilamellar vesicles

GI	gastrointestinal
HBsAg	hepatitis B surface antigen
HDLs	high-density lipoproteins
HI	humoral immunity
I	ionic strength
i.p.	intraperitoneal
L-AmB	liposomal amphotericin B
LUVs	large unilamellar vesicles
MAC	*Mycobacterium avium* complex
MDP	muramyl dipeptide
MDR	multidrug resistance
MLVs	multilamellar vesicles
MPL	monophosphoryl lipid
MPS	mononuclear phagocyte system
MR	magnetic resonance
MTP-PE	muramyl tripeptide-phosphatidylethanolamine
MTX	methotrexate
N_w	weight-average number of moles of lipid per liposome
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCS	photon correlation spectroscopy
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
%amc	percentage apparent monolayer coverage
$(\%amc)_{max}$	maximum theoretical percentage apparent monolayer coverage
psi	pounds per square inch
RES	reticuloendothelial system
SA	stearylamine
SATA	succinimidyl-*S*-acetylthioacetate

SDS	sodium-*n*-dodecyl sulphate
S-liposomes	sterically-stabilized liposomes
SSLs	sterically-stabilized liposomes
SUVs	small unilamellar vesicles
T_c	chain-melting temperature
TMAG	*N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride
UDP	uridine diphosphate
US	ultrasound
VETs	vesicles produced by extrusion techniques
VRE	vancomycin-resistant enterococci

CHAPTER ONE

INTRODUCTION

Introduction

In March 1995, The Times carried a report on the Liposome 30th Birthday Conference that was held at the Babraham Institute, near Cambridge, celebrating the anniversary of the first publication of a paper giving a description of liposomes, in 1965 [Bangham et al, 1965; Bangham, 1993]. It is a testament to the persistence of workers in the field that the meeting attracted such attention and was held in a celebratory fashion, as it was not so long ago that the initial optimism about the ability of these microscopic lipid vesicles to act as 'magic bullet'-style targeted drug carriers had eroded as it became clear that there were several obstacles to overcome if liposomes were to have any real therapeutic value.

Liposomes are only one type of particulate carrier drug delivery system [Gregoriadis and Florence, 1993b]. Others include polymer-based microspheres made from molecules such as lactic and glycolic acids [Morris et al, 1994; Langer, 1993] or amino acids [Li et al, 1993], colloidal nanoparticles such as those made from poly(methyl methacrylate) [Kreuter, 1994], reconstituted and loaded erythrocytes [Tonetti and De Flora, 1993], niosomes (non-ionic surfactant vesicles) [Uchegbu et al, 1994], emulsion-based lipid microspheres [Yamaguchi and Mizushima, 1994] and lipid-based particles with nonconcentric aqueous compartments [Bonetti and Kim, 1993]. However, liposomes remain the most widely-studied, commonly used and best understood of these systems.

This work involves the delivery of antibacterial agents encapsulated within liposomes to the skin-associated bacteria *Staphylococcus epidermidis*. The introduction is split into two main parts. The first (Section 1.1) looks at the formation and the structure of liposomes, and at some of the uses for these microscopic vesicles, including examples involving their use with bacteria. The second part (Section 1.2) examines

the nature of *Staphylococcus epidermidis*, the infections it can cause and the ways it can be treated.

1.1 LIPOSOMES

1.1.1 Lipids and Bilayers

Liposomes are generally composed primarily of phospholipids. Phospholipids can be divided into two classes, phosphoglycerides and sphingolipids. Phosphoglycerides are most commonly used and they consist of a pair of hydrophobic acyl hydrocarbon chains ('tails') ester-linked to a glycerol backbone with a hydrophilic polar headgroup (a phosphorylated alcohol). The most frequently used phosphoglycerides are phosphatidylcholines (PCs, also known as lecithin), where the head group is phosphocholine. The number of carbon atoms in the acyl chains usually varies from between 14 and 24, and the nomenclature used to describe these hydrocarbons reflects the number of these atoms and the degree of unsaturation. For example, a saturated chain with 16 carbon atoms is known as palmitate and a phosphatidylcholine where both acyl chains are of this type is known as dipalmitoylphosphatidylcholine (DPPC). The structure of DPPC, a synthetic phospholipid, is shown in Figure 1.1.1.

Lipids such as DPPC are amphipathic - they have both hydrophobic (the acyl chains) and hydrophilic (the polar head) parts - and so in aqueous media, they need to take up conformations that minimize the unfavourable interactions between the nonpolar tails and the hydrophilic solvent. How the molecules do this is determined by the relative sizes of the polar and the nonpolar parts. Amphipathic molecules such as surfactants and lysolecithin have just a single acyl chain connected to the polar head and so have a conical shape that, in aqueous media, ensures that the molecules take up a micellar organisation (Fig. 1.1.2, top), with the hydrophobic tails sequestered inside the structure, shielded from the environment by the hydrophilic head groups, satisfying the constraints of the hydrophobic effect. DPPC, on the other hand, has two fatty acyl chains and the overall shape of the molecule is tubular. The acyl chains are too bulky to fit into the interior of a micelle and so instead of forming such

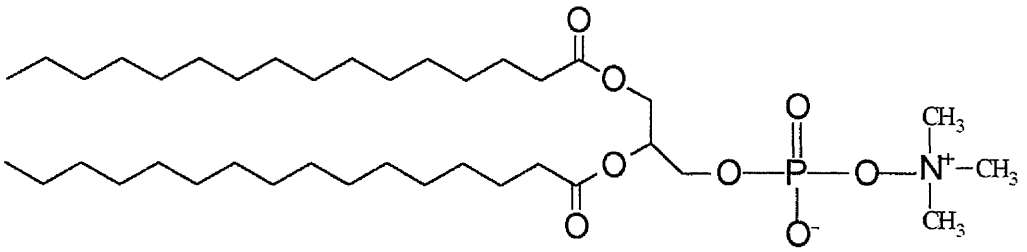


Fig. 1.1.1 Structure of dipalmitoylphosphatidylcholine (DPPC)

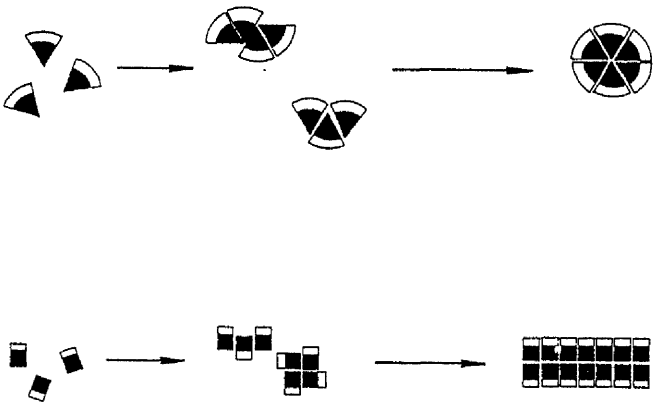


Fig. 1.1.2 Assembly of amphipathic molecules into (top) micellar and (bottom) bilayer structures [New, 1990].

structures, the geometry that actually occurs with such lipids is that of a bilayer (Fig. 1.1.2, bottom) where the lipids are aligned in two layers, with the hydrophobic tails protected from unfavourable interactions by being in the centre of the structure, with the heads exposed to the aqueous environment, an arrangement favoured by the charge density being spread over two acyl chains. This planar arrangement still leaves acyl chains exposed at the edges, a situation that is eliminated when the bilayers fold in on themselves, or when part of the bilayer buds off if forced to curve [Lasic, 1992], to form sealed vesicles - liposomes (Fig. 1.1.3).

1.1.2 Phase transitions

Lipids in a membrane can exist in different phases depending on the temperature. At low temperatures, molecules such as DPPC are aligned in the bilayer tilted relative to the plane of the membrane (by 58°) to compensate for the fact that the headgroup occupies a greater area of the membrane than the two fatty acyl chains (42Å^2 cf. ~39Å^2). This geometry minimizes the space between neighbouring acyl chains caused by the relatively bulky headgroups, bringing adjacent chains closer together to maximize Van der Waals and other non-covalent interactions [New, 1990]. However, as the temperature is increased, the fatty acyl chains are able to take up conformations other than their usual straight-chain configuration. Rotation about carbon-carbon bonds along the chain leads to a *trans*-to-*gauche* isomerism, introducing kinks into the previously fully extended chains and expanding the area they take up in the membranes, eliminating the need for the tilt. As the acyl chains are no longer in their extended straight-chain state, there is a corresponding decrease in the thickness of the bilayer (from 47Å to 39Å). A phase transition occurs, from the tightly-ordered, tilted solid gel phase ($L_{\beta'}$) at low temperatures to a more fluid liquid-crystal phase (L_α) at higher temperatures. The temperature at which this 'chain-melting' occurs (T_c) is determined by the length and degree of unsaturation of the hydrocarbon chains. In general, the longer, and/or more saturated, the chain, the greater the chain-melting temperature.

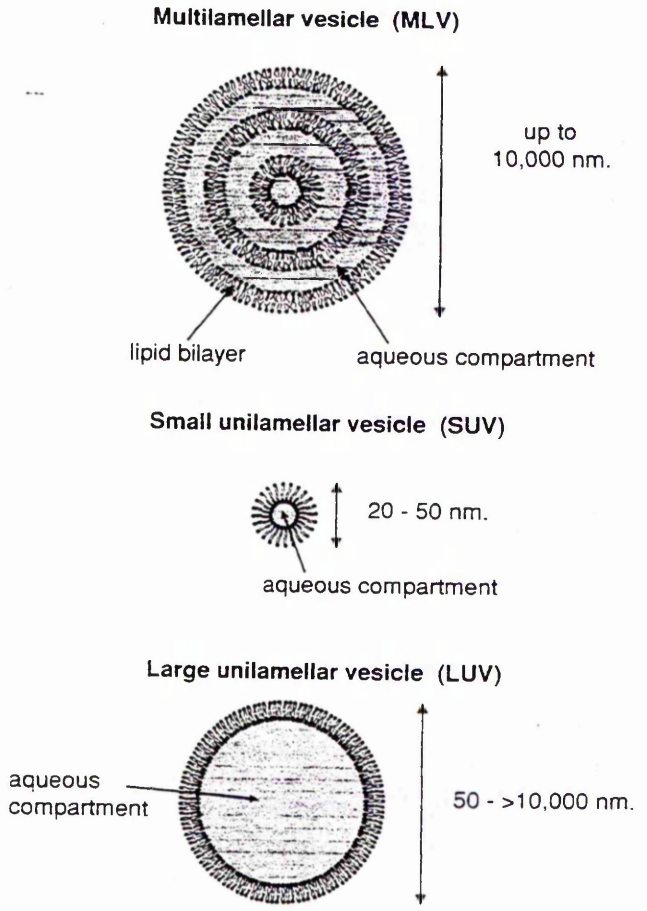


Fig. 1.1.3 Structure of multilamellar and unilamellar vesicles [Jones and Chapman, 1995].

The phase transition is endothermic and can be detected using differential scanning calorimetry (DSC) as there is a peak in the rate of heat absorption at T_c (Fig. 1.1.4). A pre-transition is observed about 5°C below the main transition, corresponding to the transient formation of a 'rippled' arrangement, midway between the two phases.

Membranes composed of a mixture of two lipids of very different chain lengths can show separate transitions. However, mixtures of two more similar lipids will give a T_c that is intermediate between those of the two individual components [New, 1990].

1.1.3 Permeability and Cholesterol

The fluidity of liposomal membranes at ambient temperature is dependent on the chain-melting temperature of the lipids. Generally, saturated chains with more than 14 carbon atoms give rigid gel-like properties to the membrane at body temperatures [Lasic, 1992], having T_c greater than 37°C, and such liposomes are less likely to leak their contents. Thus, permeability can be decreased by extending the chain length - increasing T_c and the thickness of the bilayer. Permeability is at a maximum at T_c itself [Papahadjopoulos et al, 1973].

The incorporation of cholesterol in the liposomal bilayer can have an effect on permeability. Cholesterol (Chol) is an amphipathic molecule and is oriented in the membrane with its hydroxyl group positioned towards the aqueous surface and the hydrophobic chain parallel to those of the lipids (Fig. 1.1.5). Above a certain concentration of cholesterol, the area of the membrane that is taken up by the acyl chains and the steroid cores combined is greater than the area taken up by the headgroups and so the chain tilt does not occur. This is comparable to what happens in lipid membranes without cholesterol above T_c (see Section 1.1.2) and so effectively, with these levels of cholesterol, the phase transition is eliminated altogether.

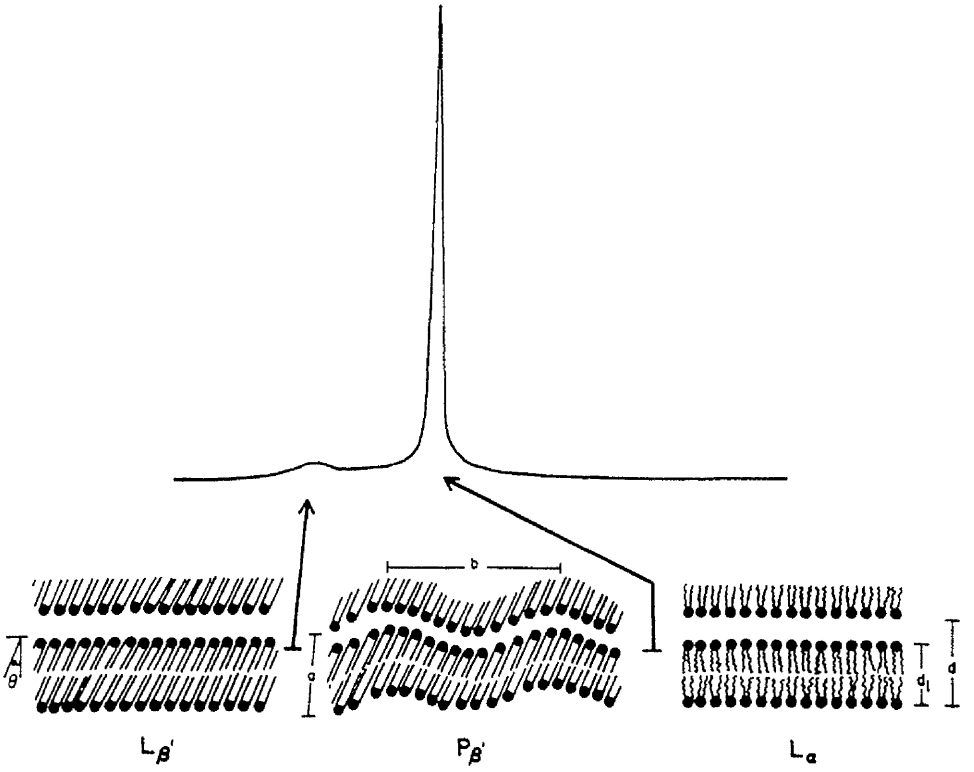


Fig. 1.1.4 Alignment of bilayer membrane lipids and (top) rate of heat absorption during a gel ($L_{\beta'}$) to liquid (L_α) phase transition, passing through a transient 'rippled' phase ($P_{\beta'}$) [Janiak et al, 1976].

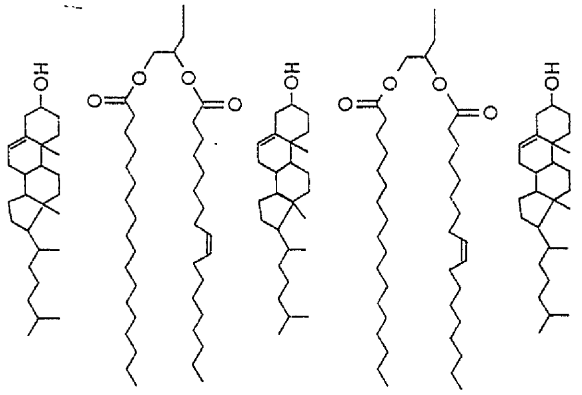


Fig. 1.1.5 Position of cholesterol (far left, centre, far right) in a bilayer [New, 1990]

With high levels of cholesterol the fluidity of the liposome is altered both above and below the chain-melting temperature. Above T_c, the reduced freedom of movement of the acyl chains means that the membrane is more tightly packed, decreasing its fluidity. Below T_c, the fluidity is increased as the phospholipids are pushed apart, weakening the packing of the headgroups. The permeability of liposomes can also be decreased by incorporation of molecules such as cardiolipin [Shibata et al, 1994] or collagen [Pajean and Herbage, 1993].

1.1.4 Formation and Structure of Liposomes

The simplest way of producing liposomes involves dissolving the required lipids in an organic solvent (typically a mixture of chloroform and methanol) and transferring the solution to a round-bottomed flask connected to a rotary evaporator. The organic solvent is then removed on the evaporator under a vacuum (at a temperature above the T_c of the lipids), leaving a thin film of dried lipid evenly distributed on the inside of the flask. The lipids are swollen and hydrated by the addition of the aqueous solvent, and vigorous mixing of the contents of the flask leads to the spontaneous formation of a cloudy suspension of lipid vesicles, each enclosing a volume of the aqueous buffer. Liposomes formed in such a way will be multilamellar vesicles (MLVs) - that is, they will have multiple lipid bilayer shells each enclosing an aqueous compartment in an 'onion skin' arrangement (Fig. 1.1.3, top). This representation of an MLV with concentric layers is a rather simplified description of the actual arrangement [Ostro, 1987].

Although such a population of vesicles is easy to produce, they are not practical for many uses. Firstly, there is no way to control the size of the MLVs made, leading to a very heterogeneous distribution of liposome diameters (200-10,000 nm). This can lead to problems with both *in vitro* experiments, where reproducibility of size is important for comparison between different sets of vesicles, and *in vivo*, where the size of the liposomes can affect their behaviour in the body. Secondly, MLVs have a

very low entrapment volume and encapsulation efficiency - that is, where a solute (e.g. a drug) is included in the buffer used to make the liposomes, with a view to it being incorporated into the aqueous compartments of the subsequently formed vesicles, only a relatively small amount of this solute is actually found within the liposomes due to the high lipid/solvent ratio with MLVs. The encapsulation efficiency can be increased by subjecting the MLVs to a series of cycles of freezing and thawing [Mayer et al, 1985]. These FATMLVs (frozen and thawed MLVs) have increased interlamellar spaces leading to a better distribution of the solute between the liposome and the exterior solvent.

These two problems can be circumvented by using various methods to produce unilamellar vesicles. Unilamellar vesicles have just a single lipid bilayer membrane enclosing one aqueous compartment as its core. Small unilamellar vesicles (SUVs) can be produced by sonicating a population of MLVs with either a probe or, preferably, a bath sonicator [Kodama et al, 1993]. The MLVs are bombarded with high frequency sound waves, causing them to break up into smaller fragments which reseal to form the SUVs, with diameters of around 20-50nm (Fig. 1.1.3, centre). The minimum size of SUVs is determined by the maximum possible crowding that the phospholipid headgroups of the inner part of the bilayer can tolerate as the curvature increases with decreasing diameter. Although SUVs have a more homogenous size distribution than MLVs, there is still the problem of low trapping efficiency/encapsulation. Encapsulation can be increased by the production of large unilamellar vesicles (LUVs). These liposomes, diameter 50-10,000 nm (Fig. 1.1.3, bottom), can be produced in various ways. Reverse-phase evaporation involves removal of an organic phase, by evaporation, from an oil/water emulsion [Chen et al, 1992; Szoka and Papahadjopoulos, 1978]. Ether injection requires that the lipids, in an immiscible mixture of ether and water where they form a monolayer interface, are injected through a needle at a temperature that will cause slow evaporation of the organic phase, leading to the formation of bilayer sheets and subsequently vesicles

[Deamer and Bangham, 1976]. Ethanol injection involves dissolving the phospholipids in the organic solvent and using a fine needle to inject the solution into an excess of aqueous medium [Baztri and Korn, 1973]. The phospholipid molecules are evenly dispersed and the ethanol is instantaneously diluted and can be removed by dialysis [Pons et al, 1993]. Encapsulation efficiency is low, but where this is not an important factor this is a very quick and simple preparation [Campbell, 1995].

Perhaps the most reliable method of producing LUVs, and the method used exclusively in this work, is extrusion [Hope et al, 1985; Olson et al, 1979; Mayer et al, 1986]. The process is detailed in Section 2.2.1 but, briefly, involves transferring MLVs to a small heated chamber where they are subjected to moderate pressure, forcing the vesicles towards the outlet of the device where they encounter a polycarbonate filter. The MLVs are too large to pass through the pores of the filter, but parts of their outer bilayer can be forced through, forming transient cylindrical structures [Clerc and Thompson, 1994] before budding off and resealing to form LUVs. The pores of the filter are of a defined size and the vesicles produced have diameters that approach the filter size. The vesicles are passed through the extruder up to ten times to ensure homogeneity of the population. Such liposomes are referred to as VETs (vesicles produced by extrusion techniques).

Liposomal preparations are affected by age; the phospholipids can hydrolyse, leading to the formation of free fatty acids and lysophospholipids. This hydrolysis can lead to destabilization of the vesicles, limiting the shelf life of any therapeutic preparation [Grit and Crommelin, 1992]. To improve the stability, lyophilizing (freeze-drying) of the vesicles is a good method [Gogineni et al, 1993], especially if a cryoprotectant such as a carbohydrate is included to prevent vesicle fusion and other types of dehydration damage [Engel et al, 1994]. Pharmaceutical formulations need to be sterile, but the use of heat or ionizing radiation can lead to the formation of the

degradation products [Zuidam et al, 1993]. For this reason, sterile filtration seems to be the best method [Goldbach et al, 1995].

1.1.5 Liposomes as Drug Delivery Agents

Liposomes make ideal vehicles for drug delivery [Gregoriadis, 1977]. Firstly, their constituents are similar to those found in cell membranes, and so are non-toxic. Secondly, they have the ability to protect their loads from being diluted or degraded in the blood. Thirdly, and most importantly, they can be specifically targeted to the tissues where the action of the drug is required (See section 1.1.7). In this way, the liposome is increasing the therapeutic index of the drug: delivering the required dose to diseased tissues but by-passing other, healthy tissues, so reducing any toxicity. The ratio of effectiveness to toxicity is increased.

Liposomes can include both water-soluble and lipid-soluble drugs (Fig. 1.1.6). Water-soluble drugs can be encapsulated in the aqueous compartment(s) of the vesicles by including them in the aqueous buffer used to hydrate the lipid film during the formation of the vesicles (see Section 1.1.4). Separation methods such as dialysis, gel filtration or centrifugation [Vemuri and Rhodes, 1995] are used to remove the unencapsulated agent. The amount of drug that is encapsulated is directly proportional to the lipid concentration [Karlowsky and Zhanel, 1992]. As mentioned in Section 1.1.4, the encapsulation efficiency is dependent on the type of liposome used, with LUVs being the most efficient. However, there are various methods of increasing the amount of drug captured; as well as the freeze-thawing method mentioned earlier, there is a technique known as 'remote-loading', where a pH gradient is established across the liposome membrane to load an ionized drug [Kulkarni et al, 1995; Lasic et al, 1992; Praet et al, 1993; Vemuri and Rhodes, 1994].

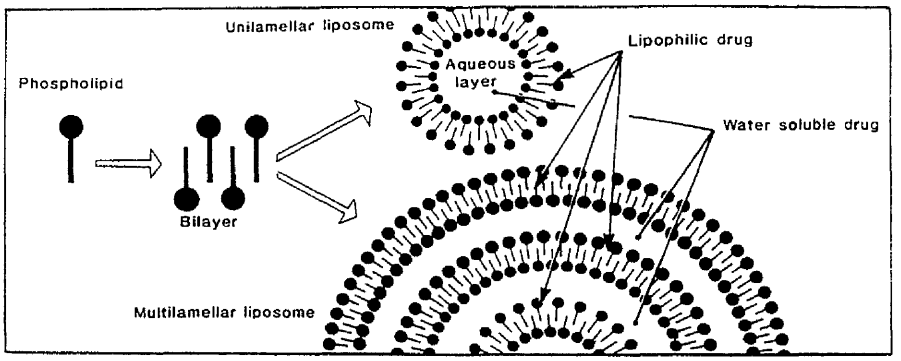


Fig. 1.1.6 Incorporation of water-soluble and lipid-soluble drugs in the lipid bilayers of liposomes [Dean, 1993].

Lipid-soluble drugs become part of the liposome membrane itself when included with the lipids during preparation of the vesicles. The amount of drug incorporated is directly proportional to the total mass of lipid present and virtually complete incorporation can be achieved by selecting a drug concentration below saturation level in the liposome bilayers. Another advantage is the fact that it is not necessary to perform a purification step to remove unencapsulated drug. One example is the incorporation of lipophilic antitumour prodrugs [Mori et al, 1993].

Lipophilic drugs can also be incorporated into the aqueous compartment of liposomes if they are first included within cyclodextrins, a family of hydrophobic, cavity-forming, water-soluble oligosaccharides. These cyclodextrin 'inclusion complexes' are then encapsulated within liposomes [McCormack and Gregoriadis, 1994a]. This system has the advantage of increasing the range of insoluble drugs that can be encapsulated; for example, membrane-destabilizing agents could be included [McCormack and Gregoriadis, 1994b].

1.1.6 Interaction of Liposomes with Cells

Liposomes can interact with cells by several mechanisms [Ostro, 1987; Ostro and Cullis, 1989]. A conventional liposome would not be involved in all these situations, but they are not mutually exclusive (Fig. 1.1.7).

(i) ADSORPTION onto the cell surface. Liposomes will adsorb to the surface of most types of cell, where they will provide a slow and sustained release of an encapsulated drug into adjacent extracellular fluid, from which the drug may pass across the cell membrane due to the concentration gradient, leading to considerable cellular uptake [Nässander et al, 1995].

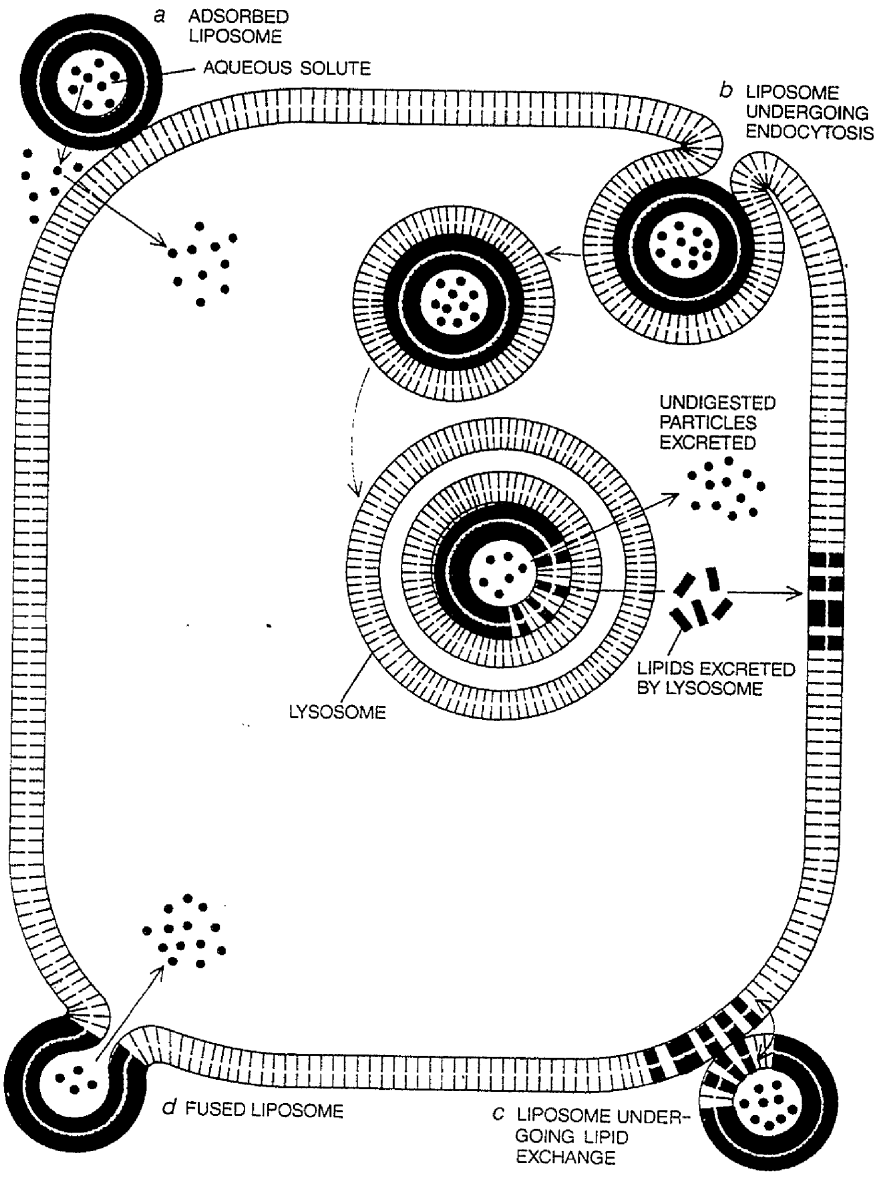


Fig. 1.1.7 The four methods of interaction between a liposome and a cell [Ostro, 1987]

(ii) ENDOCYTOSIS. This occurs only in those cells that are phagocytic e.g. monocytes and macrophages. The liposomal membranes are degraded in lysosomes and the contents of the vesicle, as long as they too are not degraded, are released into the cell.

(iii) LIPID EXCHANGE between liposomal and plasma membranes. The liposomal lipids must be similar to those of the target cell. The aqueous contents of the vesicle do not enter the cell, so lipid exchange is only used to facilitate the transfer of lipid-soluble drugs that have been intercalated into the liposomal membrane.

(iv) FUSION. The outer bilayer of the liposome can interact with, and become part of the cell membrane, leading to direct release of the contents of the vesicle into the cytoplasm of the cell. The extent to which fusion occurs is still debatable [Cullis and Ostro, 1989].

1.1.7 Targeting of Liposomes

There are various ways of targeting liposomes to the sites where the action of the encapsulated/incorporated substance is required.

(a) Passive Targeting

Passive targeting is a bulk recognition mechanism [Sato and Sunamoto, 1992]. The targeting is achieved by altering the structural characteristics of the vesicle, e.g. the hydrophobicity, charge or size. The liposomes will generally be taken up by phagocytic cells (see Section 1.1.9), e.g. macrophages [Banerjee et al, 1994; Brandl et al, 1994; Jung et al, 1993; Mönkkönen and Heath, 1993; Mönkkönen et al, 1994; Weiss et al, 1993].

(b) Active Targeting

Active targeting involves incorporating into the liposomal membrane a specific recognition molecule (e.g. a lectin or an antibody) that interacts with a receptor on the membrane of the target cell (e.g. a carbohydrate or antigen). Antibodies tend to be the type of molecule most commonly linked to liposomes for active targeting and such vesicles are known as immunoliposomes. A wide range of cancer cells can have antibodies raised to them for incorporation into immunoliposomes [Nässander et al, 1995]. The benefits of encapsulating anti-cancer drugs within immunoliposomes are that the number of drug molecules that can be bound to the antibody is increased [Betageri et al, 1993] and no direct chemical binding of the drug is involved which often causes inactivation of the agents [Hashimoto and Suzuki, 1992].

Various methods exist for covalently coupling molecules to the surface of liposomes; for example, succinimidyl-*S*-acetylthioacetate (SATA) can be used to introduce reactive thiol groups at the site of primary amino groups. These thiols can be coupled with thiol-reactive maleimide groups of liposomes. This method has been used to couple glu-plasminogen (which has an affinity for fibrin clots) [Heeremans et al, 1992] and lectins such as succinyl conavalin A and wheat germ agglutinin [Kaszuba et al, 1995] to the surface of vesicles.

(c) Physical Targeting

Circumstances leading to a change in the stability of the liposomal membrane can be exploited as a means of physical targeting. For example, vesicles are most permeable at the chain-melting temperature and so can be made heat-sensitive. Liposomes can be formulated to be stable at 37°C but to be less stable when the temperature is raised to 40°C [Block et al, 1975], for example by externally heating (hyperthermia) a region known to contain a tumour [Ono et al, 1992; Weinstein et al, 1979]. The release of the contents can be enhanced in the presence of high-density lipoproteins (HDLs). Temperature-sensitive vesicles can also be made by coating the liposomes

with a thermosensitive polymer such as poly(*N*-isopropylacrylamide) [Kono et al, 1994]. The polymer forms aggregates at temperatures higher than 31°C, inducing membrane defects that will ultimately lead to rupture of the liposome.

Alternatively, the vesicles can be made to be sensitive to the pH of the surroundings. If the liposomes are primarily composed of lipids which form unstable membranes, such as unsaturated phosphatidylethanolamine (PE) which normally adopts an inverted hexagonal micelle form instead of a bilayer structure [Cullis and de Kruijff, 1979], then they can be stabilized by materials that are charged at neutral pH but become uncharged in more acidic environments (e.g. at sites of infection, inflammation and tumours). When the charge is removed, the stabilizing effect is lost and the encapsulated contents released. Such stabilizing molecules include fatty acids, palmitoyl homocysteine, cholesterol hemisuccinate and *N*-succinyl PE [Connor et al, 1984; Ellens et al, 1984; Hazemoto et al, 1993; Yatvin et al, 1980].

1.1.8 Alternative Routes of Administration

Oral delivery of vesicles is not the most feasible route of administration, due to the action of bile salts and lipases. However, rigid liposomes (e.g. those incorporating cholesterol) are less susceptible to these effects [Rowland and Woodley, 1981] and some work has been done using this route, including the encapsulation of insulin to give some protection of the contents against degradation [Patel and Ryman, 1976] and of BSA to induce an antigenic responses in rats [Fujii et al, 1993].

Many therapeutic agents are applied to the surface of the skin in the form of gels or creams, but one of the major disadvantages of such transdermal drug delivery is the low penetration rate of substances, especially when hydrophilic drugs are used [du Plessis et al, 1994]. The use of liposomes has been investigated as an alternative to these standard topical applications, and they have been shown to act as penetration enhancers [Yarosh, 1990]. The vesicle serves as a solubilization matrix, providing a

lipophilic environment for water-soluble compounds that is more compatible with the environment of the skin. The significant increase in concentration of the drug in the skin is accompanied by a reduction in the systemic adsorption of the drug [Mezei and Gulasekharam, 1980].

The barrier function of the skin is almost entirely due to the outermost layer of the epidermis, the dead cells of the stratum corneum. Penetration through this layer is passive and relies on diffusion [Dean, 1993]. On application to the surface of the skin, the liposomes can enter the epidermis and are seen as deformed spherical structures between the skin cells [Gregoriadis, 1994a] where the liposome lipid bilayers can fuse with the lipid layers of the stratum corneum [A. Blume et al, 1993]. The phospholipids of the vesicles do not penetrate into the deeper skin layers, but act as depots for sustained release of the encapsulated contents. The amount of time that the drug is in contact with the skin is increased. Such vesicles can be used for the topical administration of anti-inflammatory drugs and antibiotics. For example, a liposome gel of the antifungal agent econazole has recently been introduced [Schrier and Bouwstra, 1994].

The ability of liposomes to enhance penetration into the skin has attracted the attention of the cosmetics industry [Memoli et al, 1993]. 'Empty' liposomes with no encapsulated contents can be used as part of a moisturiser, delivering moisture and supplying lipid molecules. Water molecules can also be delivered to the deeper layers of the skin, either bound to encapsulated sugars or amino acids, or as hydrated lipids [Lasic, 1992]. As well as these moisturising substances, liposomes can incorporate other active ingredients including sunscreens [Gilchrest et al, 1993; Wolf et al, 1993; 1995; Yarosh et al, 1994], vitamins [Kirilenko and Gregoriadis, 1993] proteins and antibiotics. The ability of such topical liposomes to deliver their contents could be increased by receptor-mediated targeting, for example by incorporating rhamnose in the liposomes, making them specific for the rhamnose receptor of keratinocytes

[Gregoriadis, 1994a]. It should be noted that such treatments are not universally recognized as being effective [Komatsu et al, 1986].

The use of topical preparations of liposomes has extended beyond the skin to other tissues, including the eyes [Gregoriadis and Florence, 1993a]. The benefits of topical therapy are also seen with pulmonary surfaces [Schreier et al, 1993]. Vesicles can be formulated from materials that are similar to the components of the lung surfactant; a complex mixture of around 85 % phospholipid, mainly DPPC with some DPPG and cholesterol [Taylor and Newton, 1992]. Local delivery is achieved by administration as an aerosol (e.g. with a nebulizer) or via intratracheal administration. The use of vesicles prolongs the residence time of drugs within the lungs and enables targeting to alveolar macrophages [Berg et al, 1993; Perry and Martin, 1995; Tschaikowsky, 1994], which is particularly useful with diseases such as cystic fibrosis (see Section 1.1.10) [Dean, 1993]. Examples of liposomal pulmonary treatments include encapsulation of detirelix (involved in fertility treatments) [Bennett et al, 1994] and delivery of desferrioxamine [Tabak and Hoffer, 1994; Tabak et al, 1994] or antioxidant enzymes [Baker et al, 1992; Senga et al, 1990] as treatment against oxidative lung damage. Topical treatment with liposomes is also possible with the oral mucosa [Ponchel, 1994], where there are problems with standard treatments due to the high permeability of the mucosa. Encapsulation helps to localize the drug within the epithelium. Such administration has been used with the corticosteroid hormone triamcinolone [Mezei and Gulasekharam, 1980; Sveinsson and Holbrook, 1993].

The effectiveness of actively-targeted liposomes can be increased if they are injected directly into the compartment where the target cells are located. For example, ovarian cancers usually spread over the serous membranes of the peritoneal cavity and so intraperitoneal (i.p.) administration may help to control the disease. Immunoliposomes designed to interact with the carcinoma cells were shown to bind

to human ovarian carcinoma cells growing in the peritoneal cavity of mice [Nässander et al, 1995]. Encapsulation reduces reabsorption of the drug through the serous membrane and out of the cavity [Genne et al, 1994].

Submucosal or subcutaneous administration leads to accumulation of liposomes in the lymph nodes as well as in the blood [Akamo et al, 1994]. These routes are often used when drugs cannot be injected intravenously, for example when the drug has low aqueous solubility [Zuidema et al, 1994]. Administration is faster after intramuscular injection compared with the subcutaneous route due to the abundance of capillaries in the muscle tissue [Kadir, 1993].

1.1.9 Circulation Lifetime of Liposomes

When intravenously administered, the circulation lifetime of liposomes can be as short as a few minutes due to the fact that they are rapidly removed from the bloodstream by the cells of the reticuloendothelial system (RES, also known as the mononuclear phagocyte system, MPS). Liposomes accumulate in the major sites of the RES, primarily Kupffer cells of the liver and fixed macrophages of the spleen, due to the scavenging action of these cells, which recognize the liposomes as being foreign. This removal from circulation reduces the ability to target to non-RES tissues and the short lifetime hampers their ability to act as sustained release depots [Harwood, 1992]. Problems in the circulation are due to destabilization of liposomes by plasma proteins such as high-density lipoproteins (HDLs) [Mui et al, 1994], which can result in loss of encapsulated or bound molecules and/or liposome degradation due to phospholipid exchange and transfer, and the action of opsonins, which coat the surface of circulating liposomes resulting in increased interaction with phagocytic cells [Chonn et al, 1992; Harashima et al, 1993b]. Generally, large liposomes are cleared from circulation more rapidly than small ones and negatively charged vesicles have a shorter half-life than neutral and positively charged ones [Hernández-Caselles

et al, 1993; Lee et al, 1992]. The half-life is also increased with increasing rigidity of the membrane [Brandl et al, 1994].

This uptake of liposomes by the RES can be useful when it is the reticuloendothelial system itself that is infected. For example, the disease leishmaniasis is caused by infection of macrophages by protozoa of the genus *Leishmania*. Visceral leishmaniasis (also known as kala-azar), a fatal disease prevalent in many tropical parts of the world, is caused by the pathogen *Leishmania donovani*. It can be treated with antimonial compounds, but these are toxic. Encapsulation of the drugs inside vesicles leads to the agent interacting almost exclusively with the macrophages, reducing the toxicity and greatly increasing the therapeutic efficacy. However, recent clinical reports seem to indicate that the pathogen is becoming resistant to antimonial therapy, and polyene antibiotics such as amphotericin B and hamycin are being examined for their potential as alternatives. Hamycin has been encapsulated in liposomes that have been coated with mannose to stimulate receptor-mediated uptake and this system has been shown to be extremely effective in treating a murine model of the disease [Banerjee et al, 1994]. Attempts to target the broad spectrum antibiotic ribavirin to cat macrophages for treatment of infectious peritonitis virus in a similar fashion have not been successful [Weiss et al, 1993].

However, normally this uptake needs to be avoided and several methods have been used to minimize the interaction of vesicles with the RES. Early ideas including trying to 'blockade' the system by administration of 'empty' drug-free liposomes or dextran sulphate [Patel et al, 1983; Goto and Ibuki, 1994]. The use of the empty vesicles as a blockade showed that liposomes cleared by the RES accumulate primarily in the liver and so this method has potential for targeting subsequently administered doses of drug-laden vesicles specifically to the spleen [Hamori et al, 1993]. Dextran sulphate is toxic to hepatic macrophages and so was used to alter the distribution of injected liposomes, but the effects seemed to be only temporary (less

than 48 hours) [Patel et al, 1983]. Unfortunately, the use of both these methods meant that the RES was compromised which was potentially dangerous to the patient. Attention soon switched to altering the liposomes themselves, so as to avoid their recognition and uptake. The initial idea behind RES-avoiding liposomes was to try and mimic properties of red blood cells, which are not removed from circulation so rapidly. The liposomes were disguised as 'self' in this way by incorporation of ganglioside GM_1. These liposomes were found to have increased lifetimes and were termed 'Stealth' liposomes [Allen, 1994] for their ability to avoid detection and uptake. The inclusion of GM_1 reduces the degradation of the liposomes [Allen and Chonn, 1987]. The ganglioside decreases the total amount of blood protein bound to the surface of the vesicles [Chonn et al, 1992], reducing the rate of uptake though not the mechanism [Parr et al, 1993]. The sialic acid of GM_1 was essential as was its position relative to the carbohydrates of the ganglioside and relative to the membrane surface. The effect of the ganglioside is species-specific to a certain extent, as in rats it is a signal for clearance by the RES and so shortens the circulation time [Liu et al, 1995].

Although these liposomes had obvious therapeutic benefits, the difficulty in obtaining large quantities of GM_1 [Yamauchi et al, 1995] meant that their large scale use was somewhat impractical. A different approach was taken by another group who postulated that conjugation of poly(ethylene glycol) (PEG) to liposomes might extend their circulation lifetimes in the same way that PEG-conjugation increased the half-life of proteins [Klibanov et al, 1990]. Incorporation of PEG (conjugated to phosphatidylethanolamine) into LUVs significantly increased the half-life, and to a greater extent that GM_1 did. PEG is an amphipathic, linear polymer which exhibits considerable conformational flexibility. Its action is thought to be due to (i) decreased hydrophobicity and/or steric barrier activity preventing adsorption of opsonins onto the surface of the liposomes [Parr et al, 1994; Torchilin et al, 1994a], and (ii) reduction of lipid exchange with HDLs. This barrier function of PEG led to such

Stealth liposomes being also referred to as sterically stabilized liposomes (SSLs) [Oku and Namba, 1994; Woodle and Lasic, 1992] or S-liposomes [Allen, 1994] (cf. 'conventional' C-liposomes). As well as the advantages of increased circulation, S-liposomes have pharmacokinetics that are independent of liposome dose [Allen and Hansen, 1991], unlike normal liposomes where saturation of the RES occurs at higher doses. The rate of removal of S-liposomes is related to the rate of removal of the stabilizing component (PEG) from the liposomes.

The activity of PEG is more effective for rigid liposomes (e.g. those incorporating cholesterol, which are resistant to HDL destabilization [Zakharova et al, 1993]) where inclusion of low Mwt PEG had a greater effect. Conversely, with more fluid vesicles high Mwt PEG (5000 or 12000) polymers are required, indicating that the steric barrier activity is directly correlated to the polymer chain length [Maruyama et al, 1992a]. The more rigid liposomes need only a small polymer for increased stability.

Such sterically stabilized liposomes are useful for sustained release and targeted delivery. Their increased circulation time means that they can accumulate efficiently in a solid tumour model system by penetrating the leaky vasculature. Incorporation of anti-cancer agents such as doxorubicin into S-liposomes has been shown to significantly increase survival times when injected into mice with tumours [Maruyama et al, 1992b; Yuan et al, 1994]. The length of the PEG chains can 'mask' any targeting molecules incorporated into the liposome; this problem can be overcome by attaching the molecule instead to the end of the PEG chains [G. Blume et al, 1993].

Other molecules used to create longer-circulating liposomes include a lipophilic derivative of glucuronic acid [Doi et al, 1994], *N*-acylphosphatidylethanolamine (a negatively-charged phospholipid with a third acyl chain) [Mercadal et al, 1995], the

block co-polymer poloxamine-908 [Moghimi et al, 1993a; 1993b], polyacrylamide and poly(vinyl pyrrolidone) [Torchilin et al, 1994b]. Polymers of sialic acid have been suggested as an alternative to PEG [Gregoriadis et al, 1993a; McCormack and Gregoriadis, 1994].

1.1.10 Liposomal Amphotericin B

The first clinically proven and licensed application of liposome-based therapy was in the field of invasive fungal infections. Opportunistic fungal infections are a major problem in immunocompromised patients, e.g. those with AIDS [Coker et al, 1991] and neutropenia (decreased neutrophil count) [Chopra et al, 1992; Mills et al, 1994]. The mortality rate of such infections is very high. Amphotericin B (AmB) is normally the drug used to treat such infections; however, problems are caused by the chronic toxicity of the drug [Lopez-Berestein, 1987]. Adverse side effects include acute fever, electrolyte imbalances and renal toxicity. This dose-limiting toxicity is due to the method of action; the drug binds to ergosterol, a sterol in the fungal cell membrane, leading to formation of AmB/sterol aggregates which associate into transmembrane barrels with aqueous pores, through which ions and other solutes can pass, leading to cell death [Lambing et al, 1993]. The problems come because AmB also binds to cholesterol in the mammalian cell membrane.

The toxicity of the agent can be reduced by its incorporation into liposomes e.g. SUVs composed of soya PC and cholesterol [Gokhale et al, 1993a, 1993b]. The highly lipophilic nature of AmB means that it can be tightly bound into the bilayer. The drug has a higher affinity for the lipids of the carrier than for the mammalian cell membrane. The agent is selectively transferred to the pathogen [Bakker-Woudenberg et al, 1993]. Incorporation of AmB into liposomes retains the antifungal activity but reduces the toxicity [Anaissie et al, 1991]. The mode of action is not fully understood but seems to involve enhanced immunity and higher tissue levels, as the liver, spleen and bone marrow (all RES organs) are the main sites of systemic fungal infections

[Gray and Morgan, 1991; Ng and Denning, 1995]. Its uptake by organs such as the kidney, brain and heart is lower, which explains the reduction in toxicity. The liposomal AmB is also taken up by circulating macrophages which transport the agent to the site of infection [Mehta et al, 1994].

Liposomal AmB (L-AmB) has been shown to be well tolerated [Emminger et al, 1994; Ralph et al, 1993] with minimal adverse effects [Mills et al, 1994], especially when compared with the acute toxicity seen with the free drug [Ringdén et al, 1994]. It was originally used to treat leishmaniasis, as both the infective agent and liposomes are taken up by phagocytes [Schmitt, 1993]. It is now used to treat infections such as histoplasmosis, cryptococcosis [Coker et al, 1991], candidiasis [Gokhale et al, 1993b; Karyotakis and Anaissie, 1994] and aspergillosis. Immunotargeting of L-AmB [Schmitt, 1993] and incorporation of the macrophage activator tuftsin [Owais et al, 1993] may offer more effective therapy. The response rate amongst infected patients treated with L-AmB seems to be higher where the patient has had no prior treatment with the free drug [Ng and Denning, 1995].

Liposomal AmB is commercially available as AmBisome [de Marie et al, 1994; Ng and Denning, 1995; Zoubek et al, 1992] which is licensed in several countries including the U.K. The drug is supplied as lyophilized SUVs of composition PC:Chol:DSPG:AmB (ratio 2 : 1 : 0.8 : 0.4). It has been suggested that its use might be limited due to the expense of a course of the treatment [Mills et al, 1994]; liposomal AmB is estimated to cost around $ 800 for each patient [Seaman et al, 1995].

1.1.11 Liposomes in Cancer Treatment

Doxorubicin (DOX, commercial name Adriamycin) is an anticancer drug, an anthracycline antibiotic that is frequently used to treat solid tumours [Daoud et al, 1992]. Its clinical usage is limited by its dose-limiting toxicity and so it is a good

candidate for encapsulation into liposomes, especially into the long-circulating S-liposomes detailed in Section 1.1.8 [Allen, 1994; Sadzuka et al, 1995]. The liposomes direct DOX away from sites which have tight capillary junctions (e.g. heart muscle and GI tract) and towards sites with loose capillary junctions (e.g. bone marrow, tumour) and to organs rich in the RES (liver, spleen, lymph nodes) [Cowens et al, 1993; Lee et al, 1995], significantly reducing the side effects of the drug [Huang et al, 1992]. Solid tumours are usually resistant to free doxorubicin, but encapsulation leads to significant extravascular accumulation [Yuan et al, 1994]. Other factors that contribute to the enhanced therapeutic effects of liposomal DOX include the altered pharmacokinetics [Kim, 1993], enhanced endocytosis by tumour cells or accelerated membrane turnover [Guaglianone et al, 1994], the slow release of drug into the tumour [Lilley et al, 1994] and decreased systemic elimination [Vaage et al, 1994]. DOX-liposomes can also be targeted more specifically to tumour cells, for example by incorporating antibodies to tumour markers [Ahmad et al, 1993; Ohta et al, 1993] or attaching folate to the liposome surface via a PEG spacer; the folate-binding protein has been shown to be greatly overexpressed in a large number of tumours [Lee and Low, 1995]. Liposomal DOX can be administered to the gastric mucosa for delivery to regional lymph nodes in treatment of gastric carcinomas [Akamo et al, 1994].

The effectiveness of the DOX liposomes can be increased if the synthetic monocyte activator muramyl dipeptide (MDP) is incorporated into the liposomal membrane (in the form of the lipophilic derivative muramyl tripeptide-phosphatidylethanolamine, MTP-PE) [Creaven et al, 1990; Hoedemakers et al, 1993; Tanguay et al, 1994]. The MDP activates monocytes and macrophages, by way of morphological and biochemical changes and the production of enzymes and monokines, to kill tumour targets [Melissen et al, 1992; Roerdink et al, 1987]. Incorporating the lipid form of this activator into the liposomal membrane makes it more effective than when just encapsulating MDP inside the vesicles [Daemen et al, 1993; Kleinerman et al, 1995].

It is thought that the doxorubicin could have an additive or synergistic effect on the activator, preventing metastasis and prolonging survival [Shi et al, 1993].

Liposomal DOX has been used in cases of Kaposi's sarcoma - a cancer of connective tissue that affects one-third of patients with AIDS [Harrison et al, 1995]. These malignant tumours arise from blood vessels in the skin and appears as purple or brown patches on the skin. The chemotherapy is toxic and since its effects are only palliative (i.e. providing temporary relief from the symptoms but not acting as a cure) the treatment needs to be as mild as possible. The sarcoma lesions consist of proliferating spindle-like cells that form many slit-like spaces through which vesicles may extravasate, making liposomal DOX a highly active form of treatment with neutropenia as the only major toxicity.

Encapsulated doxorubicin can also be used to get around the problem of multidrug resistance (MDR), a major obstacle in cancer treatment [Rahman et al, 1992]. The resistance of cultured tumour cells to cytotoxic drugs is frequently due to the expression of a plasma membrane P-glycoprotein, encoded by the MDR genes, that functions as a drug-efflux pump. It seems that liposome-encapsulated doxorubicin is more toxic to resistant cells than the free drug, with modulation of MDR found in Chinese hamster LZ cells [Thierry et al, 1989], human colon cancer cells [Rahman et al, 1992], ovarian carcinoma cells, renal cell carcinomas [Law et al, 1994] and breast cancer lines [Thierry et al, 1993] that have been treated. The liposomes appear to interact with the P-glycoprotein, thus reducing the amount of drug the glycoprotein can bind.

There are, at present several human phase I and phase II trials of liposome-encapsulated DOX in progress [Gabizon et al, 1994; Law et al, 1994]. Formulations include TLC D-99 [Cowens et al, 1993; Embree et al, 1993], Lipodox [Law et al, 1994] and Doxil [Harrison et al, 1995; Vaage et al, 1993]. Other anthracycline

antibiotics could be encapsulated and used in this way. Annamycin has been shown to be more effective than DOX when used intravenously against several tumour models [Zou et al, 1994]. This drug has a marked affinity for lipid membranes with enhanced uptake and retention. Daunorubicin has been encapsulated and has shown similar results to liposomal DOX [Guaglianone et al, 1994]. It is undergoing clinical trials as DaunoXome.

One problem with liposomally administered DOX is that the altered biodistribution may cause toxicity in those organs where the vesicles do accumulate [Daemen et al, 1995]. In particular, the Kupffer cells of liver have important roles in homeostasis and host defence. For example, defects in the clearance mechanism of these phagocytic cells can lead to severe conditions such as disseminated intravascular coagulation (caused by uncleared circulating immune complexes triggering activation of platelets and neutrophils), septicaemia (due to circulating microorganisms), metastatic spread of tumours and spillover of uncleared endotoxins from the gut [Brandl et al, 1994; Lutz et al, 1995]. Greatly decreased phagocytic capacity has been demonstrated in animals treated with liposomal DOX [Daemen et al, 1995].

Other types of anticancer agent have been used in liposomes. For example, methotrexate (MTX) has been entrapped with positively charged vesicles and been shown to enhance antitumour activity against solid rodent tumours and resistant human leukaemic cell lines when compared to the free drug [Kim et al, 1994].

1.1.12 Immunological Uses of Liposomes

Liposomes can be used as adjuvants; inert and non-specific agents that boost the immune response to weak antigens [Dean, 1993; Gregoriadis, 1985; Gregoriadis, 1994a; 1994b; Stevens, 1993]. The criteria for a good adjuvant are that the agents should be inexpensive, non-toxic, non-immunogenic, stable, biodegradable and should preferably induce both humoral immunity (HI, involving circulating

antibodies) and cell-mediated immunity (CMI, resulting from the action of antibodies bound to the surface of lymphocytes). The lack of toxicity with liposomes gives them an advantage over more conventional adjuvants such as alum (aluminium hydroxide). The antigens can either be encapsulated within the liposome or, especially if it is a small peptide, the antigen can be covalently linked to the outer membrane of the vesicle, although the coupling agent used must be non-immunogenic [Friede et al, 1993]. With HI, the liposomes act as depots, supplying antigen-presenting cells such as macrophages with released and/or entrapped antigen, leading to increased processing and presentation by the cells to promote antibody production [Alving, 1987]. CMI is induced because the vesicles present the antigens in a hydrophobic environment similar to that seen with proteins conjugated to lipids [Alving, 1992]. The adjuvanticity effect does not seem to be related to any characteristic of the vesicles although its action can be improved by increasing the uptake of the liposomes by macrophages (e.g. by coating the liposome with mannose for receptor-mediated targeting) or by using the liposomes in combination with co-adjuvants such as avridine, a synthetic lipoidal amine that is an immunopotentiating agent [Fatunmbi et al, 1992a; 1992b]. An example is the strong immune response to diphtheria toxoid obtained in mice with no side effects such as hypersensitivity or the development of granulomas [Gregoriadis, 1994a]. Hepatitis B surface antigen (HBsAg) proteins - secreted as lipoprotein particles by liver cells during infection - have been reconstituted into vesicles and shown to induce a larger amount of antibodies than with the free antigen [Gómez-Gutiérrez et al, 1995] as have reconstituted influenza virus envelopes and surface antigens [Gregoriadis et al, 1992] and tetanus toxoid molecules [Alpar et al, 1992]. Liposomally encapsulated *Vibrio cholerae* antigens have been encapsulated and delivered intraintestinally to produce a highly immunogenic vaccine where none previously existed [Chandrasekhar et al, 1994]. Immunity against malaria involves both humoral and cellular mechanisms directed against various epitopes expressed at different times during the complex life-cycle of the parasites involved, protozoa of the genera *Plasmodium* and *laverania*. For

effective immunization, these numerous antigens need to be expressed as potently as possible, preferably in a multi-stage, multi-component, vaccine preparation. Encapsulation into liposomes can provide such a system [Gordon, 1993].

Attenuated (i.e. live but with reduced virulence) or killed bacteria and viruses can be used as very effective vaccines and encapsulation of such microorganisms within 'giant' vesicles (up to several thousand nanometres in diameter) may have some advantages [Antimisiaris et al, 1993]. Vesicles could be used where multiple vaccines (soluble antigens such as proteins and particulate antigens like the attenuated viruses) or vaccine formulations containing cytokines are needed. In these cases, simultaneous presentation of all the elements may improve the immune response.

Adjuvancy can also be conferred by use of lipid A, isolated from Gram-negative bacterial lipopolysaccharide. This immunomodulating lipid is involved in macrophage activation and so incorporation into liposomes will promote increased delivery of the adjuvant to these phagocytic cells, with a reduction in its toxicity [Alving, 1993]. Lipid A can also augment antibody responses to membrane-bound antigens such as gangliosides if attached to the liposomes in the form of monophosphoryl lipid A (MPL) [Gordon, 1993; Ravindranath et al, 1994].

Liposomes are very attractive as carriers of allergens, and vesicle-mediated immune enhancement can substantially reduce hypersensitivity reactions at the site of injection [Dean, 1993], lowering the levels of the IgE antibodies that trigger the release of inflammatory mediators from mast cells and basophils, a process known as desensitization. Allergens such as pollen, mites and cat hair have been made to interact with negatively charged liposomes by increasing the pH of their solution [Genin et al, 1994]. Food is a potent cause of allergies and a major allergen from mustard seeds (*Sin a* I) has been successfully used in vesicles [Oñaderra et al, 1994].

Both B-cell and T-cell epitopes can be carried by liposomes which reduces the need to covalently link them to carrier proteins which have the problems of antigenic competition and epitope masking [Gregoriadis et al, 1993b].

1.1.13 Liposomes and the HIV Virus

The preferential uptake of liposomes by cells of the RES could be exploited to improve the effectiveness of antiviral agents administered against HIV infection. Macrophages are crucial to the pathogenesis of the virus because they act as reservoirs of the pathogen, propagating the agent throughout the immune system. Encapsulation of the antiviral drug within liposomes should lead to its concentration within these cells after administration, improving efficacy and reducing toxicity [Désormeaux and Bergeron, 1995]. Vesicles have been used in such a way for delivery of dideoxynucleoside drugs against HIV-1 [Mirchandani and Chien, 1993]. A further advantage to using liposomes against this infection is that they can be administered subcutaneously, leading to their accumulation at high levels in the lymph nodes. This could help to preserve the microenvironment of follicular dendritic cells in the lymphoid tissue and so protect the infected host from developing the characteristic immunodeficient state [Désormeaux and Bergeron, 1995]. It has also been suggested that anionic cardiolipin-based vesicles can fuse with HIV-1 and impair the ability of the virus to fuse with the plasma membrane of cells [Konopka et al, 1993].

1.1.14 Liposomes in Diagnostic Imaging

Encapsulation of contrast media within liposomes can be used to enhance diagnostic imaging. Radionuclides such as In-111 and Tc-99m have been enclosed within vesicles and used to generate images of liver metastases, myocardial infarcts, abscesses, lymph nodes, tumours and synovial images in arthritic joints [Gray and Morgan, 1991]. For example, Tc-99m, linked to diethylenetriaminepentaacetic acid (DTPA), has been encapsulated into RES-avoiding palmityl glucuronide vesicles

which are passively targeted to tumour tissues after intravenous administration. The vesicles are imaged by use of a γ-camera, identifying the tumour location [Oku et al, 1993]. Iodinated contrast agents such as the nonionic monomer iopromide can be encapsulated for use in computed tomography (CT). Through RES uptake, they can produce selective enhancement of the liver and the spleen [Krause et al, 1993]. Since phagocytic activity is not exhibited by tumour tissues, the contrast agent will be concentrated in the healthy tissue, leaving 'blank' patches corresponding to the tumour location [Sachse et al, 1993]. Paramagnetic agents such as gadolinium-DTPA can be incorporated into the liposomal membrane to improve imaging seen with magnetic resonance (MR). This contrast agent has been bound to stearylamine and included within liposomes that have been coated with mannan and polysaccharide, leading to accumulation primarily in the liver and the lungs [Kunimasa et al, 1992]. Continuous extrusion methods have recently been developed to produce liposomal contrast agents for MR and CT with high encapsulation efficiencies and excellent long-term storage stability in relatively large quantities [Schneider et al, 1995]. Finally, nitrogen gas has been entrapped within liposomes and used as ultrasound (US) contrast agents called Aerosomes [Unger et al, 1992]. Bubbles of gas reflect sound efficiently; however, their use as contrast agents is limited by the fact that they are cleared by the lungs and do not normally pass through the pulmonary microcirculation into the left side of the heart. Aerosomes were used to image myocardial infarcts in rabbits and showed considerable improvement in echocardiographic contrast compared to free gas bubbles [Unger et al, 1993].

1.1.15 Cationic Liposomes and Gene Transfer

Many methods exist to facilitate transfer of polynucleotides into cells, including electroporation [Simon et al, 1993] and calcium phosphate precipitation. Liposomes can also be used and have the advantages of convenience, reproducibility and efficiency [Felgner and Ringold, 1989]. One commercially available liposome-based system is Lipofectin, which consists of sonicated vesicles composed of the positively

charged lipid DOTMA (N[1-(2,3-dioleyloxy)propyl]-*N,N,N*-triethylammonium) and neutral DOPE (dioleoylphosphatidylethanolamine) [Kamata et al, 1994; Takehara et al, 1995]. DOPE is used because it is considered to fuse with cell membranes (i.e. it is 'fusogenic') and seems to reduce the toxicity of the cationic compound [Gustafsson et al, 1995]. When mixed with DNA, these polycationic liposomes spontaneously form complexes of around four liposomes per plasmid. The DNA is not encapsulated within the liposomes but is trapped inside the complex. The net charge on the DNA/liposome complex should be positive and so interacts with negative charges on the target cell surfaces. This 'lipofection' was shown to be up to 100 times more effective than either calcium phosphate or DEAE-dextran transfection techniques, depending on the cell line used [Felgner et al, 1987]. However, synthesis of the DOTMA is complex and the commercial reagent is expensive, so another cationic molecule, the cholesterol derivative DC-Chol (3β[*N*-(*N,N*-dimethylaminoethane)-carbamoyl] cholesterol) has been used to prepare sonicated liposomes with DOPE [Gao and Huang, 1991].

The events that occur during the interaction of liposome complex with cell are not fully understood, but the transfection appears to be mediated by fusion and endocytosis through lipid-associating receptors [Jääskeläinen et al, 1994; Watanabe et al, 1994], and inhibition of the endocytic pathway (by incubation in hypertonic media or by depletion of cellular ATP with sodium azide and 2-deoxyglucose) in various cell types has shown that binding to the cell surface is not in itself sufficient and that the uptake is dependent on endocytosis [Wrobel and Collins, 1995].

These DC-Chol/DOPE liposomes have been shown to provide effective transfection with a variety of cell lines and are being used for treatment of cystic fibrosis which is caused by a genetic mutation. Transfection of the normal cystic fibrosis transmembrane conductance regulator (CFTR) gene into mouse models can lead to correction of the defect [Middleton et al, 1994]. The mutation of the CFTR gene

leads to a disturbance of the transport of ions and water across the luminal surface of the secretory epithelia of the gut, pancreas, lungs, biliary ducts and sperm ducts. The result of this defect is a characteristic dry mucus. With liposomal delivery of the gene, the vesicles fuse with the cell membrane and are degraded, delivering the complexed DNA to the cell. Correction of the defect is detected using electrophysiological measurements. Intratracheal instillation and nebulisation have been successfully used to introduce the liposome/CFTR plasmid complexes into the airways of mice [Coutelle, 1995] and trials are now being done to examine the efficacy and safety of this transfer into the nasal cavity of patients in the U.K. [Johnson, 1995]. Phase I trials into treatment of pulmonary cystic fibrosis by liposomes are now underway [Coutelle, 1995]. DNA/liposome complexes have also been injected directly into the central nervous system [Imaoka et al, 1995].

As well as correcting defects, the liposomal transfection of genetic material can be used to create animal models of human diseases. Hepatitis C virus is a major cause of liver disease, but the only feasible animal model involves the inoculation of chimpanzees, a very expensive procedure. Using the Lipofectin reagent, the full length virus genome has been expressed in rat hepatocytes [Takehara et al, 1995].

Other positively-charged molecules used to make cationic liposomes include SA (stearylamine) [Kim et al, 1994], DOTAP (*N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethyl-ammoniummethylsulphate) [Jääskeläinen et al, 1994; Zhang et al, 1993], DDAB (dimethyldioctadecylammonium bromide) [Rose et al, 1991], TMAG (*N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride) [Yagi et al, 1993] and BZC (benzalkonium chloride) [Nakamori et al, 1993].

Although cationic liposomes are efficient vehicles for gene transfer, there are problems of toxicity and increased clearance from the blood. These problems can be circumvented by the use of sterically stabilized liposomes (see Section 1.1.9). Such

S-liposomes can be given a positive charge if the PEG used to confer the extended circulation lifetime is amino functionalized [Zalipsky et al, 1994]. This gives better blood circulation kinetics and tissue distributions, increasing the chances of utilizing DNA/liposome complexes for *in vivo* applications.

The methods detailed above involve formation of DNA/liposome complexes; however, attempts have been made to encapsulate the genetic material within positively charged vesicles [Yagi et al, 1993] and it has been claimed that the cytotoxicity was less than with the lipofection methods. Negatively charged vesicles (incorporating phosphatidylserine) have also been used to encapsulate genetic material and have been used to passively target the human α1-antitrypsin gene to mouse hepatocytes [Aliño et al, 1994]. The small liposomes are able to penetrate sinusoidal pores of the liver.

1.1.16 Other Uses of Liposomes

When liposomes were originally discovered, the first use envisaged for them was the reconstitution of proteins extracted from cell membranes, enabling the proteins to be studied in a cell-free system but in an environment similar to the one they were taken from, allowing functional studies to be done [Eytan, 1982]. Examples include the reconstitution of proton pumps [Perotti and Chanson, 1993] and of epithelial proteins [Nakamura et al, 1994].

Encapsulation of substances other than drugs within the aqueous core of phospholipid vesicles can provide various advantages. For example, encapsulation of the enzyme tyrosinase will, on administration, increase the plasma levels of L-DOPA. This can be used to treat Parkinson's disease where levels of dopamine, which has L-DOPA as a precursor, are low [Miranda et al, 1993]. Haemoglobin can be encapsulated within liposomes (haemosomes) as a non-antigenic substitute for red blood cells [Brandl and Gregoriadis, 1994; Langdale et al, 1992]. The kinetics of oxygen binding are very

similar to those seen with erythrocytes. The main use for such a substance would be for resuscitation of trauma victims from severe shock and it would be especially useful when no suitable blood donor could be immediately found. Obviously, the smaller the liposomes were, the longer would be the half-life of the transfused vesicles. Problems can occur if the complement pathway is activated by the liposomes, leading to phagocytosis [Harashima et al, 1993a; Szebeni et al, 1994].

Photosensitive drugs are susceptible to degradation in light and so it is advantageous if they can be encapsulated in liposomes along with chemical light absorbers and antioxidants [Loukas et al, 1995].

One useful application of liposomes is in administration of drugs to pregnant mothers. Most drugs are able to cross the placenta and so can come into contact with the unborn child, leading to developmental abnormalities in the foetus. Liposomes do not cross the placenta so readily and so encapsulation should significantly reduce this transfer and hence the risk of deformation [Onur et al, 1992].

1.1.17 Interaction of Liposomes with Bacteria

As well as human and animal cells, liposomes can also interact with microorganisms such as bacteria. Due to the fact that injected liposomes tend to be taken out of circulation by phagocytic cells (See section 1.1.8) they were considered suitable vehicles for delivery of antibacterial agents to organisms that were resident within these cells. For example, *Escherichia coli* can survive inside macrophages, reducing the efficacy of many antibiotics. However, encapsulation of chloramphenicol or streptomycin within neutral or negatively-charged LUVs led to a 10-fold increase in the intracellular antibacterial activity on administration, due to uptake of the liposomes [Stevenson et al, 1983]. *Mycobacterium avium* complex (MAC), an opportunistic infection in immunosuppressed patients, invades and replicates within monocytes and macrophages and so has been shown to be susceptible to streptomycin

[Gangadharam et al, 1995], gentamicin [Klemens et al, 1990], azithromycin and rifabutin [Onyeji et al, 1994b] when these agents are encapsulated [Janoff, 1992]. Antibacterial liposomes can also be used when an extracellular infectious agent has been engulfed by the phagocytic cell; administration of liposomal dihydrostreptomycin sulphate has been shown to enhance that antibacterial action of peritoneal macrophages that have attacked *Staphylococcus aureus* [Bonventre and Gregoriadis, 1978] and these infections have also been treated with vancomycin and teicoplanin in vesicles [Onyeji et al, 1994a]. Treatment can also be given where infection is in the fixed phagocytic cells of the RES; for example *Brucella sp.* infections have been treated with liposomes encapsulating aminoglycoside antibiotics [Fountain et al, 1985] and salmonellosis in the spleen and liver has been eliminated with large doses of liposomal streptomycin [Tadakuma et al, 1985].

Away from phagocytic cells, levels of *Pseudomonas aeruginosa*, a bacterial contaminant of surgical wounds, have been reduced when treated with a local application of encapsulated tobramycin [Price et al, 1994] and with neutral and cationic liposomes containing entrapped ciprofloxacin [Nicholov et al, 1993]. Liposomes with the lipophilic bactericide Triclosan incorporated have been used to treat various extracellular pathogens including various oral bacteria such as *Streptococcus mutans* and *Streptococcus gordonii*, as well as *S.epidermidis* [Jones et al, 1994a; 1994b].

1.2 STAPHYLOCOCCUS EPIDERMIDIS

1.2.1 Foreign Body Infections

One unfortunate consequence of recent advances in therapeutic procedures is that the increasing use of indwelling medical devices such as catheters has led to a greater susceptibility to bacterial infection at the site of implantation [for review, see Jansen and Peters, 1993]. As well as being a major medical problem, such foreign body associated infections can prove to be very expensive. For example, replacement of a prosthetic hip joint is estimated to increase the total cost of the operation by 400-600%. A variety of microorganisms are associated with these infections, but staphylococci are by far the most frequently isolated bacteria, particularly coagulase-negative staphylococci (CONS) such as *Staphylococcus epidermidis*.

1.2.2 Classification

Staphylococci are members of the family Micrococcaceae. They are non-motile, non-spore-forming, facultatively anaerobic, clustering cocci that produce catalase [Moreira and Daum, 1995; Pfaller and Herwaldt, 1988]. They are Gram-positive bacteria and have a relatively thick and complex cell wall (Fig. 1.2.1). Their name derives from the fact that they occur in grape-like clusters. The staphylococci are mainly associated with skin, skin glands and mucous membranes of warm-blooded animals. The human skin represents a diverse environment, and a number of staphylococcus species have evolved to inhabit the many varied microenvironments found on this complex organ [Kleeman et al, 1993]. There are around 31 species of staphylococcus, and around half of these are found in human skin. The coagulase-negative staphylococci are distinguished from the more virulent *Staphylococcus aureus* by their inability to form clots in mammalian plasma, as they lack the extracellular coagulase protein [Moreira and Daum, 1995].

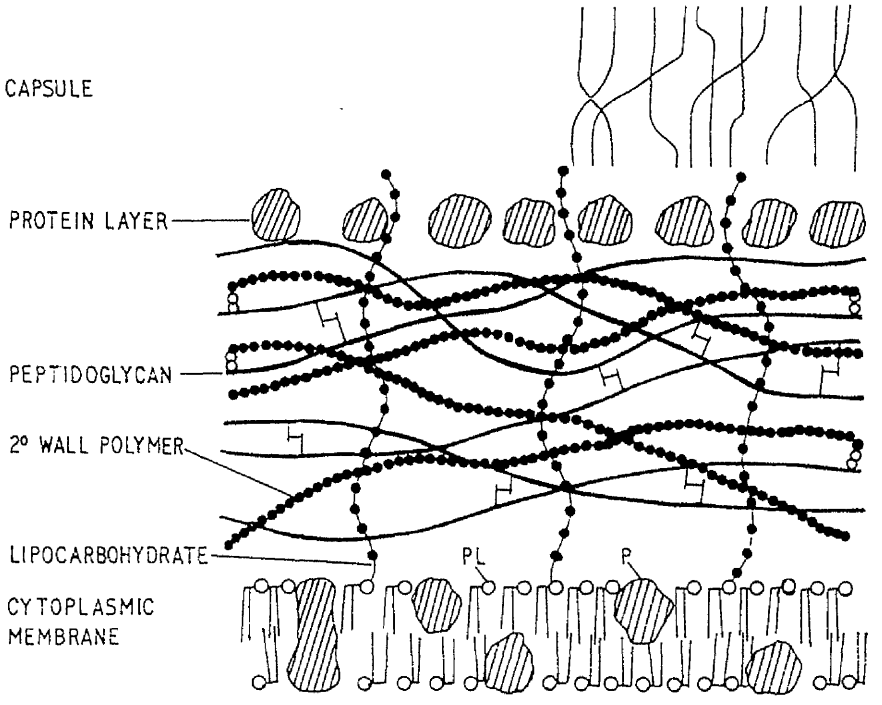


Fig. 1.2.1 Structure of a typical Gram-positive cell wall. P = protein, PL = phospholipid [Hancock and Poxton, 1988].

1.2.3 Onset of Infection

Normally, *Staphylococcus epidermidis* is a non-pathogenic organism associated with human skin. Indeed, infants are colonized by CONS soon after birth. Because of this, and because of their large numbers on the skin, they were originally considered clinically insignificant. However, such low-virulence organisms can become opportunistic pathogens, harmless to a normal individual but disease-causing when they persist in sites inaccessible to host defences or if these mechanisms are defective. The seriousness of these infections is in many cases attributable to the presence of foreign body biomaterials which provide a relatively ischaemic and acidic environment that hampers the normal methods the body uses to deal with bacterial infection such as leukocyte phagocytosis. Also, on implantation the devices become coated with fibrinogen and fibronectin which cause neutrophils to become 'lazy' and inefficient at phagocytosis [Eng et al, 1995]. *S.epidermidis* is the principal cause of infections of; peripheral and central venous catheters [Raad et al, 1993], prosthetic heart valves [Etienne et al, 1988], artificial joints [Verheyen et al, 1993], CSF shunts, Spitz-Holtzer valves (used to drain CSF in patients with hydrocephalus) [Hussain et al, 1991], genitourinary prostheses [Teichman et al, 1993] and other prosthetic devices. Various methods exist to characterize the different strains of *S.epidermidis* [Bétrémieux et al, 1995; Etienne et al, 1988; Hu et al, 1995; Jones 1993; Lebeau et al, 1993; Lina et al, 1993], which is important when identifying the source of infection, for example to differentiate between an outbreak of *S.epidermidis* and a series of unconnected infections.

Certain other conditions can predispose a patient to the infection. These include; malignancy, neutropenia (low neutrophil count), defective neutrophil function [Baker et al, 1992] and the need for recurring corticosteroid treatment. Infection is also a problem in low-birth-weight premature infants (less than four weeks old) [Huebner et al, 1994; Nataro et al, 1994].

1.2.4 Spread of Infection

Most of the *S.epidermidis* infections are of a nosocomial origin; that is, they originate and spread within the hospitals themselves. CONS such as *S.epidermidis* account for around 25% of all nosocomial infections in the U.S. [Perl et al, 1994]. The vast majority of nosocomial bacterial infections are now device-associated [McDermid et al, 1993a; 1993b]. The skin around the insertion site for the device is considered the most common source of microorganisms that cause these infections, although infection by oral routes has been identified [Lina et al, 1994] and even library books have been singled out as possible vectors for the spread of *S.epidermidis* [Brook and Brook, 1994]. The bacteria are also the organisms most commonly found on contact lenses [Farber et al, 1995].

1.2.5 Pathogenesis

The development of the disease can be divided into three major stages [Jansen and Peters, 1993]:

(a) Primary adhesion

This is the critical event in the infection. The bacteria initially adhere to the surface of the biomaterial via hydrophobic interactions, electrostatic attraction and van der Waals forces. There is also ligand-mediated interaction between receptor-like structures on the bacterial surface (adhesins) and the device itself and/or host-derived factors (serum and tissue proteins) that coat the surface of the device shortly after implantation [Muller et al, 1993; Patrick et al, 1990; Rupp and Archer 1992]. Most biomaterials commonly used are hydrophobic, providing a strong driving force for the irreversible adsorption of these host factors, increasing the probability of bacterial adhesion. One team [Wang et al, 1993a, 1993b] has suggested that it is platelets that mediate this adhesion, not the surface-adsorbed proteins. Divalent cations, especially Mg^{2+}, may also be involved [Dunne Jr. and Burd, 1992].

(b) Colonization

S.epidermidis has a limited capacity for tissue invasion and requires the presence of a foreign body for continued growth. The bacteria multiply and accumulate to cover the surface of the biomaterial. Electron microscopy (EM) has shown that colonization is necessary, but not sufficient alone, to lead to infection [Raad et al, 1993].

(c) Biofilm formation

A matrix of extracellular slime (glycocalyx) is formed. This is the major factor in establishing the foreign body infection. The bacteria secrete this highly adhesive, negatively-charged polysaccharide and become embedded in a biofilm consisting of the fibrous glycocalyx and various host substances (e.g. fibronectin). The end result is a thick matrix composed of many layers of *S.epidermidis* and copious amounts of slime. The glycocalyx has been visualized in detail using transmission EM and image analysis software [Lambe Jr. et al, 1994] and has been found to be approximately 99% water. Production of slime is enhanced under conditions of iron limitation [Deighton and Borland, 1993]. The exact composition of slime has not yet been determined [Hussain et al, 1991], but the polysaccharide seems to be rich in glucosamine and neutral sugars with small amounts of sulphates and glucuronic acid [Arvaniti et al, 1994; Hussain et al, 1993; Karamanos et al, 1995].

In biofilms, the bacteria have a lower metabolic activity. They can be sustained indefinitely and are less vulnerable to antibiotics. Slowly growing bacteria are difficult to kill using antibiotics, as has been demonstrated by comparing the degree of resistance shown by bacteria growing in liquid nutrient to that shown by the same bacteria on a non-nutrient surface such as cotton fibres [Eng et al, 1995]. EM has shown films up to 160μm thick, and these are thought to act as penetration barriers against antimicrobial agents although the effectiveness of this supposed shielding effect has recently been questioned [Eng et al, 1995]. The extracellular slime also interferes with the host defence mechanisms. It has been shown to intercede in the

production of specific antibodies and to shield the bacteria from phagocytosis and opsonophagocytosis, including bacterial uptake and subsequent intracellular killing.

1.2.6 Effects of Infection

As mentioned above, most nosocomial bacteraemias (bacteria in the blood) are due to device-associated infection. This can lead to serious consequences, most frequently septicaemia: potential widespread destruction of tissues by the bacteria. For example, *S.epidermidis* commonly causes central venous catheter-related bacteraemia in patients with depleted granulocytes (granulocytopenia). In such cases, lethal secondary complications can occur, including thrombotic emboli (blood clots), endocarditis (inflammation of the endocardium and heart valves) and meningitis [Guiot et al, 1994]. *S.epidermidis* is the most common cause of sepsis in premature infants [Mattsson et al, 1994], where immunity may be hampered by several factors including lowered neutrophil function and decreased levels of immunoglobulin G and complement. Immunity in such babies may be further impaired by the use of lipid emulsions in parenteral (non-oral) nutrition; such emulsions can affect neutrophil or RES function [Fischer et al, 1994].

These infections can also affect patients suffering from renal diseases who are undergoing continuous ambulatory peritoneal dialysis (CAPD), which involves the use of a catheter [Rowe and Miller, 1993]. *S.epidermidis* is the most common organism causing peritonitis in CAPD patients, leading to inflammation of the serous membrane of the abdominal cavity, and is a common cause of dialysis failure [Betjes et al, 1993; Calame et al, 1995].

1.2.7 Treatment of Infection

Often, removal and replacement of the infected device is the only option [Parsons et al, 1993]. However, various strategies can be adopted to try and avoid this drastic treatment.

(a) Preventative

The difficulties met trying to deal with device-associated *S.epidermidis* infections means that prevention is of the upmost importance. Needless to say, strict aseptic procedures are essential during operation and insertion of the prosthetic device. Frequent handwashing by all staff involved in surgery is vital. Bacteria on the skin may be reduced by removing them physically with a detergent and water or by killing them with bactericidal agents. A reduction in the risk of infection can be achieved by minimizing the length of the operation and by special measures such as the use of a laminar air flow [Jansen and Peters, 1993].

(b) Antibiotics

Resistance to methicillin, a semisynthetic form of penicillin used to treat infections caused by bacteria that destroy natural penicillin, is very widespread in *S.epidermidis* isolated from hospitals. Such resistance can be detected in a variety of ways [Hedin and Löfdahl, 1993] and is due to the production by the bacteria of a low affinity penicillin-binding protein, PBP 2a, that bestows cross-resistance to all ß-lactam antibiotics. One team are currently trying to target drugs to the PBP protein itself [Entenza et al, 1994].

The only non-experimental antibiotic to which staphylococci are considered uniformly sensitive to is vancomycin which is effective against the majority of gram-positive organisms. Vancomycin, alone or in combination with rifampin, is ideally given to the patient several days before surgery, especially if an infected device is being replaced [Bandyk and Esses, 1994]. The rifampin appears to facilitate the diffusion of vancomycin across the staphylococcal biofilm, possibly due to the creation or exposure of additional binding sites for the antibiotic [Dunne Jr. et al, 1993]. Similarly, vancomycin can be used in tandem with protamine sulphate, an agent that interacts with the bacterial surface and disrupts the protective barrier formed by the biofilm [Teichman et al, 1993, 1994]. There are various problems

associated with using vancomycin, not least the fact that it is potentially toxic and cannot be given orally. Another similar antimicrobial agent, teicoplanin, has been shown to reduce the number of *S.epidermidis* adhering to catheters to a significantly greater extent than vancomycin [Kropec et al, 1993]. Teicoplanin has a novel pharmacokinetic profile that allows easier administration than with vancomycin and this has led to its release for clinical use in Europe; however, usage may soon be limited by the finding that vancomycin can select for methicilin-resistant *S.aureus* with decreased teicoplanin susceptibilty [Mainardi et al, 1995]. Synthetically modified derivatives of teicoplanin could be used to get around this problem [Goldstein et al, 1995].

(c) Treated devices

One approach to impeding infection is to make the implanted device itself resistant to infection. For example, precoating polyethylene with *S.epidermidis*-derived slime has been shown in some cases to block bacterial adherence and elicit production of antibodies that also inhibit adhesion [Giridhar et al, 1994]. Another strategy is to incorporate an antibacterial agent into the device, for example by use of an antibiotic-impregnated network fixed to the implant [Goëau-Brissonnière, 1995]. Gelatin-sealed grafts have been shown to bind rifampin *in vivo* for at least seven days [Goëau-Brissonnière et al, 1994] and were resistant to infection when used for the *in situ* replacement of an infected graft. Liberation of high doses of antibiotic over a period of weeks prevents the bacterial colonization of the polymeric surface [Schierholz et al, 1994]. Extracellular slime formation can be reduced by treatment with anti-inflammatory drugs such as sodium salicylate leading to an inhibition in attachment to the biomedical devices [Farber et al, 1995].

1.3 AIMS OF THE PROJECT

The aims of this project were two-fold:

(i) To examine the nature of the interaction of cationic liposomes with biofilms of *Staphylococcus epidermidis* bacteria. Factors that were to be studied included the effects of the ionic strength of the medium, the temperature of incubation of liposomes with the bacteria, the hydrophobicity of the bacterial cells and the reversibility of the interaction.

(ii) To encapsulate antibacterial agents (vancomycin and gentamicin) into vesicles and to target these liposomes to the bacteria, comparing their effectiveness in inhibiting bacterial growth to that found with equivalent concentrations of the free drug.

CHAPTER TWO

MATERIALS AND METHODS

Materials and Methods

2.1 MATERIALS

The materials that were used in this work were obtained from the following companies.

From *Sigma-Aldrich* Company Ltd., Poole, England: Cholesterol (Chol), dimethyldioctadecylammonium bromide (DDAB), ethylenediaminetetraacetic acid (cupric disodium salt, CuEDTA), gentamicin, Sepharose 4B, stearylamine (SA), and vancomycin (hydrochloride).

From *Oxoid* Ltd., Basingstoke, England: Bacteriological agar, brain-heart infusion (BHI), phosphate buffered saline (Dulbecco 'A' tablets, PBS) and yeast extract powder.

From *BDH* Laboratory Supplies, Poole, England: Folin & Ciocalteau's phenol reagent, sodium-*n*-dodecyl sulphate (SDS).

From *Amersham International* plc, Aylesbury, England: [^{3}H]-dipalmitoyl-phosphatidylcholine (DPPC), [^{3}H]-thymidine.

From *Sygena* Ltd., Switzerland: Dipalmitoylphosphatidylcholine (DPPC).

From *Fisons* Scientific Equipment, Loughborough, England: *tert*-Butyl alcohol.

From *Poretics* Corporation, Livermore, USA: Polycarbonate membrane filters (25mm diameter filters, 100 nm pores).

From *Dynatech* Laboratories Inc., Virginia, USA: Immulon 2 flat bottom microtitre plates (protein-binding polystyrene).

From *Philip Harris* Scientific, Manchester, England: Sterile circular polystyrene Petri dishes (9 cm).

From *Sarstedt* Ltd., Leicester, England: Acryl-Cuvettes (for use with the spectrophotometer) and four-sided cuvettes (for use with the autosizer).

2.2. METHODS

2.2.1 Preparation of Liposomes

Liposomes were prepared using the vesicle extrusion technique [Hope et al, 1985; Olson et al, 1979; Mayer et al, 1986]. All liposomes made were primarily dipalmitoylphosphatidylcholine (DPPC), with or without cholesterol. Cationic vesicles were made by including stearylamine or DDAB (Fig. 2.2.1) in the lipid mixture

The required lipids (25-50 mg), together with 5μCi [^{3}H]-DPPC, were dissolved in *tert*-butyl alcohol (20ml) in a 100ml round bottomed flask. This organic solvent was used instead of the standard chloroform/methanol solutions normally utilized, because the cholesterol in the mixtures used could have precipitated out prior to deposition of the phospholipids, leading to blockage of the extruder filters by cholesterol microcrystals [as reported in "The Extruder Manual", Lipex Biomembranes Inc.]. The solvent was removed by rotary evaporation at 60^{o}C to leave a thin lipid film, evenly distributed over the inside of the flask. PBS buffer (3ml, pH 7.4) was added to the flask at 60^{o}C and vigorously mixed on a vortex mixer to hydrate the lipid film and remove it from the surface of the flask, forming a cloudy white suspension of multilamellar liposomes (MLVs). The vesicles were extruded using the Extruder (Lipex Biomembranes Inc, Vancouver, Canada). The suspensions of MLVs were pipetted into the barrel of the extruder which was kept at 60^{o}C by attaching it to a thermostatted circulating water bath. This ensured that the liposomes were kept above the phase transition temperature of the constituent lipids throughout the procedure. The extruder was pressurized by the release of nitrogen gas from an attached cylinder (200-500 psi). This pressure forced the liposomes through a pair of stacked membrane filters with pores sizes of 100 nm. The extruded vesicles were collected from the outlet tube and reapplied to the extruder, generally for five passes

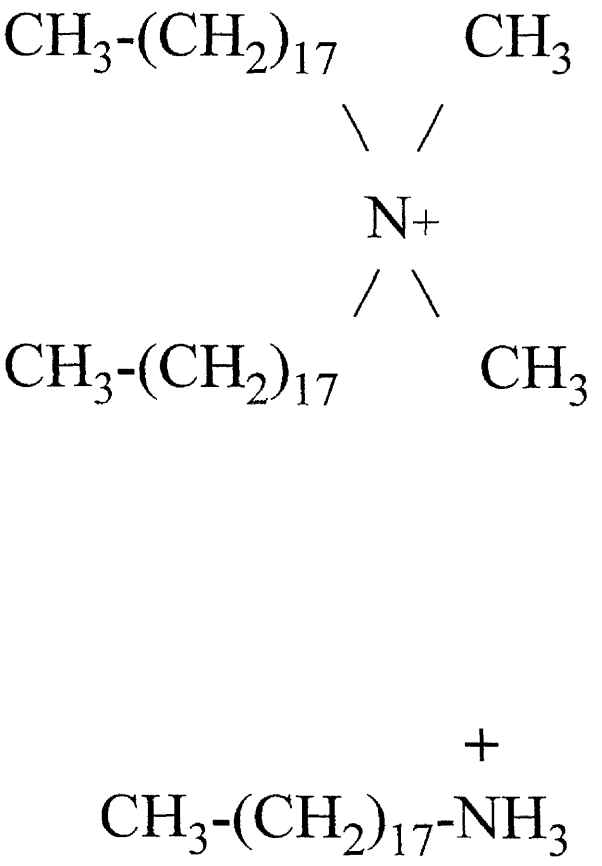


Fig. 2.2.1 Chemical structures of (top) dimethyldioctadecylammonium bromide (DDAB) and (bottom) stearylamine (SA).

in total. Successful formation of extruded vesicles (VETs) with diameters of around 100 nm was characterized by a distinctive orange-red tinge seen when the liposome suspension was held up to the light.

The lipid content was determined by scintillation counting of [^{3}H]-DPPC. Liposome samples were taken for counting both before and after extrusion, enabling calculation of the percentage of lipid that passed through the extruder, which was always in excess of 93 %.

2.2.2 Encapsulation of Vancomycin or Gentamicin in Liposomes

Where entrapment of the antibacterial agents in the aqueous compartment of liposomes was required, the procedure was as above except that water (3 ml) was used as the hydrating aqueous solvent instead of PBS. A quantity of the drug was dissolved in this water prior to it being added to the dried lipid film to form the vesicles.

2.2.3 Freeze-Thawing

Repeated cycles of freezing and thawing of multilamellar liposomes has been reported to increase the encapsulation efficiency of the vesicles [Mayer et al, 1985]. MLVs with encapsulated vancomycin were transferred from the round-bottomed flask into a Pyrex tube. The vesicles were rapidly frozen by suspending the tube in liquid nitrogen for a few minutes. The liposomes were then thawed by transferring the tube into a water bath (at 60^{o}C). This procedure was performed five times and then the MLVs were extruded in the usual manner.

2.2.4 Characterization of Liposome Size

The liposomes were sized using a Malvern Autosizer (model RR146, Malvern Instruments, Malvern, England). For sizing, a sample of the liposomes (50 µl) was added to 2 ml of PBS in a four-sided cuvette. The cuvette was placed in the

temperature-controlled cell of the Autosizer, which was set at 25°C. Values for temperature, viscosity and optical density of the solution were inputted into the associated microcomputer. Analysis of the sample by the Autosizer gave values for mean diameter, peak size and a standard deviation, giving an indication of the polydispersity of the population of vesicles.

2.2.5 Gel Filtration of Liposomes

Liposomes with vancomycin or gentamicin encapsulated within were separated from free drug in the surrounding solution by gel filtration. The vesicles were applied to a Sepharose B column (internal dimensions 24 cm x 1.8 cm) and fractions (2-3 ml) were collected using a fraction collector. Blue dextran and potassium chromate were used to identify, respectively, the void volume (V_o) and the inner volume (V_i). Aliquots (10 µl) were removed from each sample and taken for scintillation counting to identify the peak liposome fraction. A further 100 µl was taken from each fraction to assay for the amount of encapsulated drug.

2.2.6 Assay for Concentration of Drug Encapsulated

The amount of vancomycin or gentamicin present within the filtered liposomes was determined by using the modification by Wang and Smith [1975] of the method used for the measurement of proteins devised by Lowry *et al* [1951]. A calibration curve for the assay was established using drug standards made up in double distilled water (0-1 mg ml^{-1}). To 100 µl of each standard or sample was added 0.5 ml of Wang and Smith reagent, made up from 250 mg CuEDTA, 100 ml sodium carbonate (20 %, w/v) and 10 ml of 10M sodium hydroxide in one litre of distilled water. The samples were mixed on a vortex mixer and left for 15 minutes at room temperature. To each tube was then added 0.5 ml of SDS (10 %, w/v). The samples were mixed to solubilize the liposomes and then 50 µl of cold Folin-Ciocalteau's reagent (50 %, v/v) was added to each tube. The samples were mixed again and incubated at 30°C for 30 minutes. After incubation, the absorbance of the tubes was measured at 700 nm. The

This was the density of cells required to give a confluent monolayer of bacteria in the wells of the microtitre plate.

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

Table showing the relative hydrophobicity values of the various populations of *Staphylococcus epidermidis* used in this work.

standards were used to construct a calibration curve which enabled determination of the concentration of drug within the vesicles. Curves were constructed for vancomycin (Fig. 2.2.2) and gentamicin (Fig. 2.2.3).

2.2.7 Preparation of Bacterial Cultures

Staphylococcus epidermidis bacteria (NCTC 11047) were obtained from the University of Manchester collection. They 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^{o}C for 18 hours. 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^{o}C for 18h after which the bacterial suspensions were centrifuged (2000rpm; 15 min), the supernatant discarded and the pellet resuspended in sterile PBS. The centrifugation and resuspension were repeated a further three times and the bacterial concentration adjusted by dilution with PBS to give an absorbance of 0.5 at 550 nm.

2.2.8 Hydrophobic and Hydrophilic Subpopulations

The hydrophobic and hydrophilic subpopulations of *Staphylococcus epidermidis* NCTC 11047 were a kind gift from Dr. Pauline Handley, University of Manchester. The total population of the bacteria was subdivided into two subpopulations on the basis of their hydrophobicity. They were separated on the basis of their adsorption to hydrophobic droplets of hexadecane in a partitioning assay [Geertsema-Doornbusch et al, 1993]. The total population divided into a relatively hydrophobic subpopulation (30 % of the cells) and a relatively hydrophilic subpopulation (70 % of the cells). The hydrophobic bacteria were more adhesive to catheters than the hydrophilic ones.

Vancomycin is a large glycopeptide containing an amino sugar called vancosamine linked to three aromatic rings; a bis-resorcinol system and several amino acid residues that are detected by the protein assay.

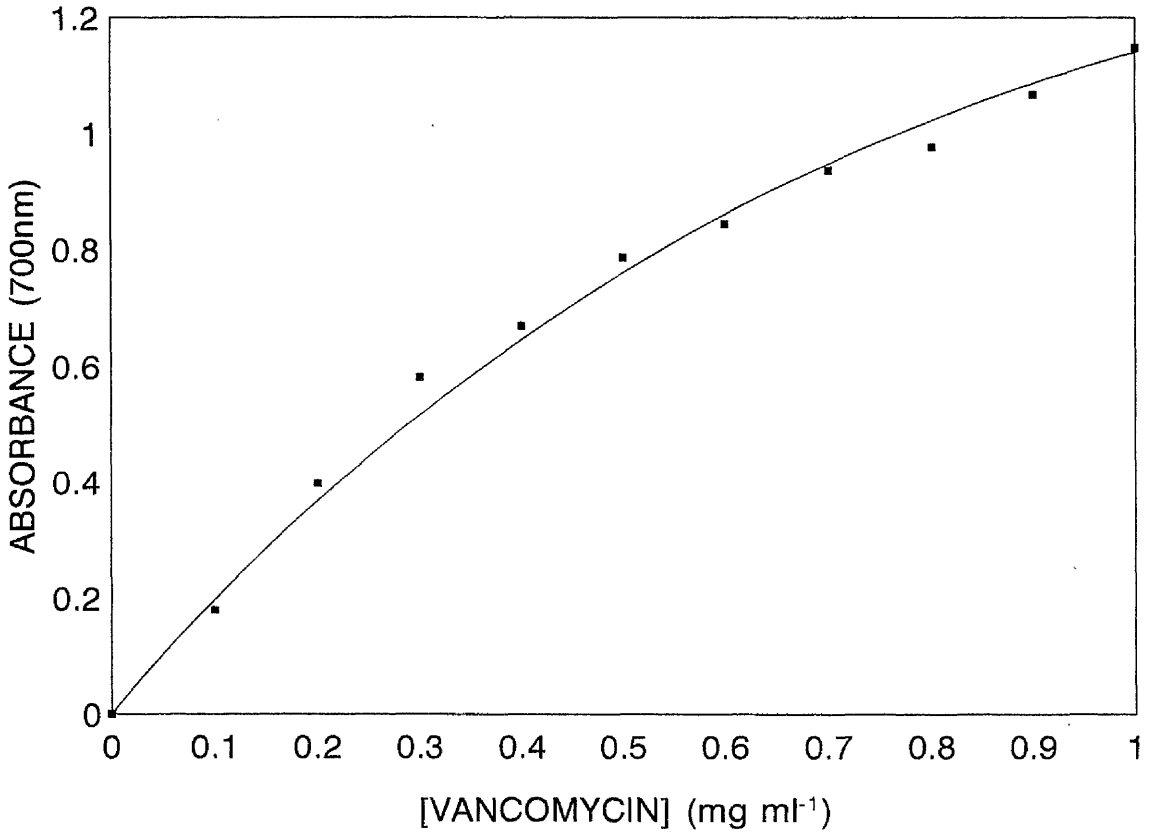


Fig. 2.2.2 Standard curve of vancomycin concentrations obtained from the Wang and Smith/Lowry protein assay.

Gentamicin consists of two amino sugars connected to a central L-deoxystreptamine residues. The amino sugars are probably responsible for the response of gentamicin to the protein assay.

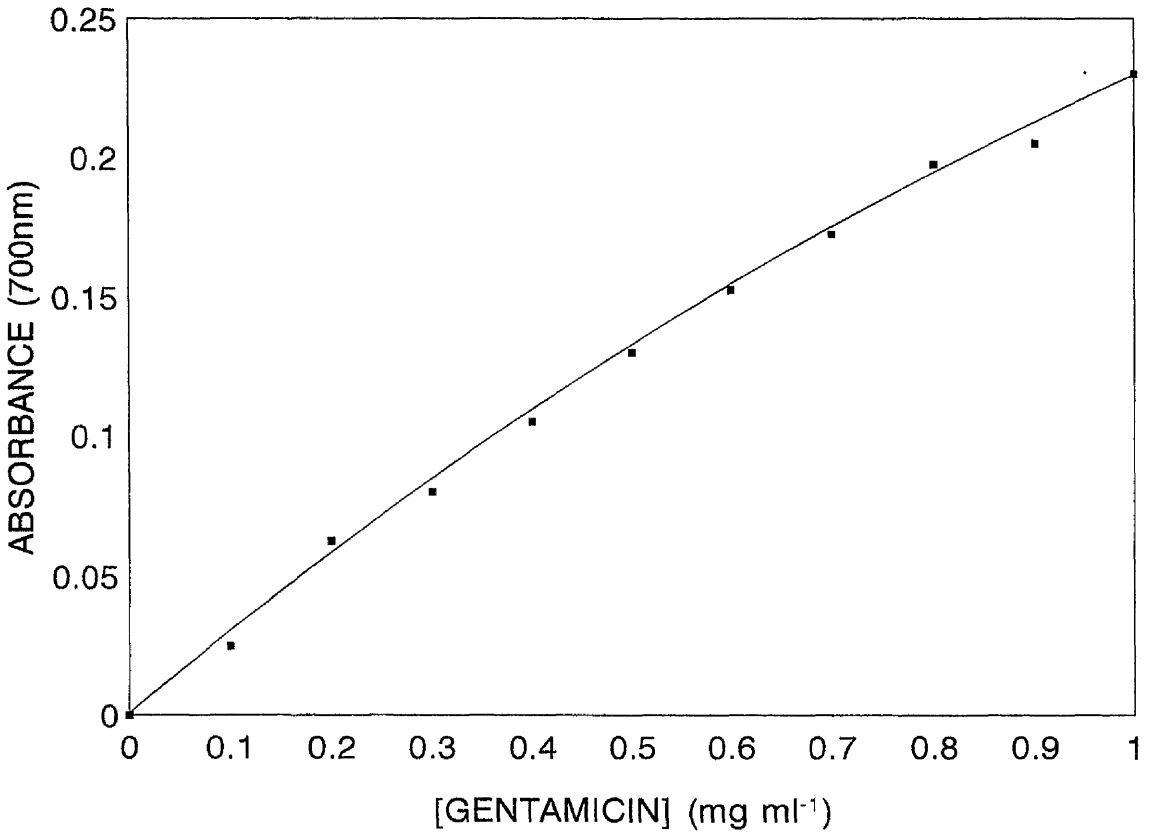


Fig. 2.2.3 Standard curve of gentamicin concentrations obtained from the Wang and Smith/Lowry protein assay.

2.2.9 Preparation of Adherence-Defective Mutants

The adherence-defective mutants were a kind gift from Dr. Alan Jacob and Mr. Baoqing Guo, University of Manchester. They were prepared by transposon mutagenesis of *S.epidermidis* NCTC 11047, the same strain that was used as the wild-type for the other parts of this work. A transposon that conferred erythromycin resistance was included on 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 into the bacterial DNA. Subsequent growth of the bacteria on erythromycin agar at 43°C (a temperature at which the plasmid would not replicate) selected a collection of transposon-generated mutant clones. The adherence-defective mutants were isolated on the basis of their decreased hydrophobicity using the hexadecane adhesion assay [Geertsema-Doornbusch et al, 1993]. Four different hydrophobic mutants were isolated and were found to demonstrate reduced adherence to catheter polyurethane and microtitre plate polystyrene. The mutant used in this work was designated M3.

2.2.10 Adsorption of VETs to Bacterial Biofilms (Targeting Assays)

Targeting assays were carried out in wells of microtitre plates. Aliquots (200 µl) of the bacterial suspension were incubated overnight at room temperature to form an adsorbed biofilm. After adsorption, the bacterial suspension was removed and the biofilm washed three times with sterile PBS using a plate washer. After washing, the adsorbed biofilms in the wells were incubated with a liposome suspension for up to two hours (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; 200µl) followed by incubation (30 min) at room temperature and a brief sonication (2 min). Aliquots of the dispersed biofilm (180µl) were taken for scintillation counting. Control wells containing only bacteria, only PBS or only liposomes were used to assess background levels of activity. The extent

of targeting was assessed by calculating the percentage of the biofilm that was covered by attached liposomes, from the known surface area of the biofilm and the area taken by up by each of the adsorbed vesicles, with the number of vesicles calculated by the scintillation count of the dispersed biofilm (see Section 3.1.2).

The composition of PBS was as follows; (pH 7.4)

$[NaCl] = 160$ mM

$[KCl] = 3$ mM

$[Na_2HPO_4] = 8$ mM

$[KH_2PO_4] = 1$ mM

The ionic strength of PBS was 188 mM. Targeting 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 100ml distilled water used normally. So, 2x concentrated PBS had ionic strength 376 mM, 3x PBS 564 mM, and 4x PBS 752 mM. Increasing the PBS concentration did not alter the pH. The final ionic strengths of the liposome dilutions used (taking into account the fact that the liposomes were made up in PBS, ionic strength 188 mM) were 357 mM, 526 mM and 696 mM (Table 2.2.1).

2.2.11 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 in Section 2.2.7, but using 5 ml aliquots of broth (instead of 10 ml), each containing 50 µl of [^{3}H]-thymidine (=50 µCi). As above, the bacteria were allowed to grow in the nutrient media for 18 hours 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

Total vol.	Lipid dilution	PBS conc	Lipid vol.	PBS vol.	Water vol.	I (mM)
1400	10	1	140	1260	0	188
1400	20	1	70	1330	0	188
1400	40	1	35	1365	0	188
1400	80	1	18	1383	0	188
1400	160	1	9	1391	0	188
1400	320	1	4	1396	0	188

Total vol.	Lipid dilution	PBS conc	Lipid vol.	PBS vol.	Water vol.	I (mM)
1400	10	2	140	1260	0	357
1400	20	2	70	1294	36	357
1400	40	2	35	1312	53	357
1400	80	2	18	1321	62	357
1400	160	2	9	1325	66	357
1400	320	2	4	1327	69	357

Total vol.	Lipid dilution	PBS conc	Lipid vol.	PBS vol.	Water vol.	I (mM)
1400	10	3	140	1260	0	526
1400	20	3	70	1282	48	526
1400	40	3	35	1294	71	526
1400	80	3	18	1300	83	526
1400	160	3	9	1303	88	526
1400	320	3	4	1304	91	526

Total vol.	Lipid dilution	PBS conc	Lipid vol.	PBS vol.	Water vol.	I (mM)
1400	10	4	140	1260	0	696
1400	20	4	70	1278	52	696
1400	40	4	35	1287	78	696
1400	80	4	18	1291	91	696
1400	160	4	9	1294	98	696
1400	320	4	4	1295	101	696

Table 2.2.1 Composition of liposome dilutions used in ionic strength experiments. Liposomes were made in PBS (188 mM) and diluted with solutions of higher ionic strength (2 x PBS, 3 x PBS, 4 x PBS). All volumes are in microlitres.

Scintillation counting was done using a Beckman LS 7800 counter. For each sample, 2.5 ml of Ecoscint A scintillation fluid was added. Samples were counted for 5 minutes each (dpm).

0.5. Aliquots (10 μl) of the bacteria at this dilution were taken for scintillation counting. A drop of the bacterial suspension was placed on a haemocytometer slide and examined under a light microscope. For each sample, the number of cells were counted in three sets of 16 small squares, giving a mean number of cells per set. The volume of the set of squares was 4×10^{-6} cm^3 and from this count could be derived the number of bacteria in 1 ml. From this figure, and with 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 one hour 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 could be calculated.

2.2.12 Delivery of Antibacterial Agents to Biofilms

The effectiveness of liposomally-encapsulated vancomycin or gentamicin in inhibiting bacterial growth relative to the free drug was determined by performing a regrowth assay. Using techniques to prevent contamination by indigenous bacteria, aliquots (250 μl) of the liposomes were incubated with bacterial biofilms for the required time periods. Controls were done with equivalent concentrations of the free vancomycin, and with wells containing only bacteria and wells with only PBS. After targeting, the well contents were removed and the biofilms washed three times with sterile PBS. After washing, sterile nutrient broth (200 μl) was added as growth medium to each well. The plate was incubated at 37°C for 18 hours and then the biofilms were dispersed. The absorbance of each well was measured at 630 nm using a Dynatech MR 60 plate reader (coupled to an Apple IIe computer). The absorbance after the 18 hour period was taken as a measure of bacterial growth.

CHAPTER THREE

RESULTS

Results

3.1 INTERACTION OF LIPOSOMES WITH BACTERIA

3.1.1 Characterization of Liposomes

The vesicles were sized using an Autosizer (Fig. 3.1.1), an instrument designed to measure particle sizes in the range 10-3000 nm using photon correlation spectroscopy (PCS), also known as dynamic light scattering. The samples of liposomes were illuminated with a monochromatic laser light beam. The beam interacts with the many thousands of "scattering centres" (vesicles in suspension), which scatter (re-radiate) the light. The scattered light is detected at 90^{o} by a photomultiplier and these signals are analyzed by digital autocorrelation. Whilst in suspension, the liposomes are undergoing Brownian motion, random in direction but at a rate determined by the diffusion coefficient (D) of the suspended vesicles. The smaller a vesicle is, the faster it will move and the larger D will be; D is inversely proportional to the size of the liposome. The Autosizer effectively calculates D by analyzing the scattered intensity at two different times; the larger D is (i.e. the smaller the liposome is) the more the intensity differs between the two times. The radii of the liposomes is related to their diffusion coefficients by the Stokes-Einstein equation;

$$D = \frac{k\ T}{6\ \pi\ \eta\ R_h} \qquad (1)$$

R_h = hydrodynamic radius of the scattering molecule

η = viscosity of the medium

k = Boltzmann's constant

T = absolute temperature

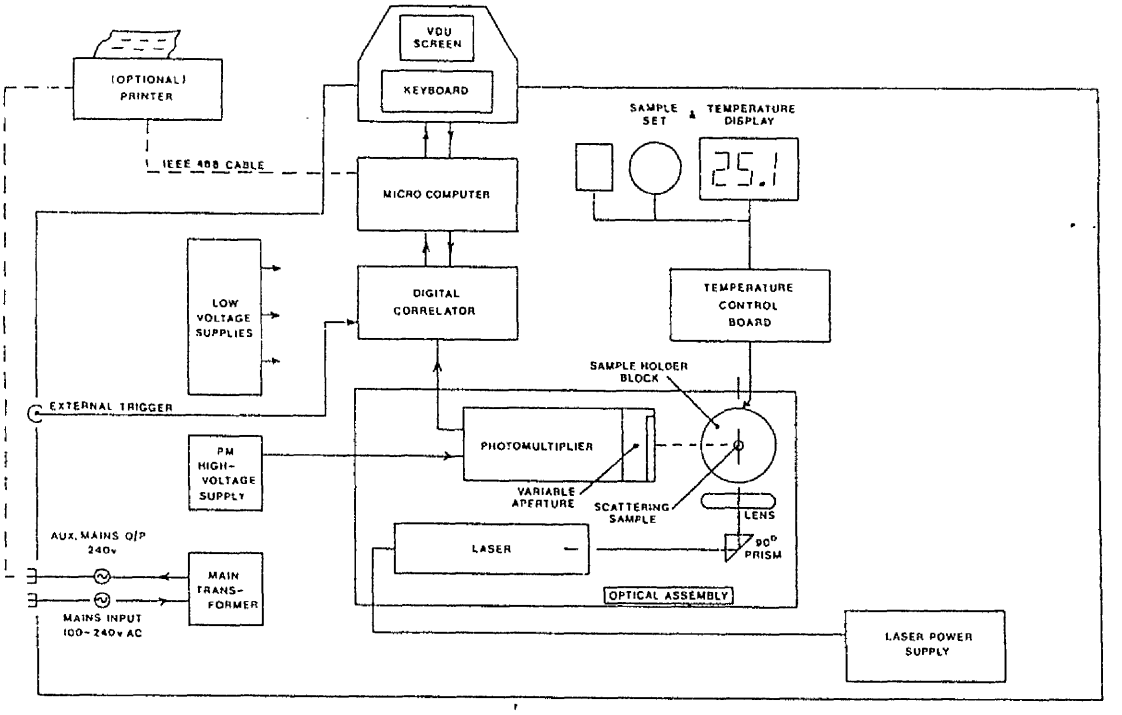


Fig. 3.1.1 Schematic diagram of the Autosizer.

The parameters of viscosity and refractive index used were those for water at a temperature of 25°C. The viscosity was 0.8904 N m^{-2} s^{-1} and the refractive index was 1.33. The diffusion coefficient was measured by the autosizer.

The viscosity, refractive index and temperature of the suspending medium are known and from these, and the measured diffusion coefficient, the mean size of the particles is determined. The Autosizer output gives the diffusion coefficient, mean size and polydispersity index (Pusey's Q factor) as well as the peak size and a standard deviation of the distribution. The values for the peak size and standard deviation of the distribution are shown in Table 3.1 for a wide range of liposome formulations (designated compositions A to L).

With a polydisperse system such as a population of VETs, there will be a range of diffusion coefficients. The autocorrelation techniques used in PCS produce a single correlation function which needs to be transformed into this distribution. The correlation function can be related to the z-average diffusion coefficient (D_z);

$$D_z = \frac{\sum_i n_i M_i^2 D_i}{\sum_i n_i M_i^2} \qquad (2)$$

n_i = number of species *i*

M_i = mass of species *i*

D_i = diffusion coefficient of species *i*

Because the liposomes are roughly spherical, they obey Stokes law and the diffusion coefficient is related to the particle diameter. With the Autosizer, the scattering seen is fitted to the normal weight distribution of particle sizes that would give the equivalent scattering. It is assumed that the distribution of particle (vesicle) sizes follows a normal weight distribution, so that the scattering of data is fitted to this distribution. The parameters calculated (diffusion coefficients and size) are those which correspond to the equivalent normal weight distribution irrespective of what

MOLE%	DPPC	Chol	SA	DDAB	Peak Size (nm)	S.D.
A	67.02	32.98	-	-	122.0 (± 1.0)	36.5 (± 0.5)
B	52.75	25.96	21.29	-	127.6 (± 1.2)	36.24 (± 1.0)
C	65.14	32.05	-	2.81	115.5 (± 1.5)	37.5 (± 3.5)
D	63.36	31.18	-	5.46	114.5 (± 1.5)	33.0 (± 2.0)
E	61.68	30.35	-	7.97	122.0 (± 3.0)	30.0 (± 9.0)
F	60.68	29.57	-	10.35	124.0 (± 8.0)	35.0 (± 5.0)
G	58.56	28.82	-	12.62	127.8 (± 5.0)	29.0 (± 4.7)
H	43.75	43.06	-	13.19	126	22
I	66.82	18.79	-	14.39	132.3 (± 1.1)	27.7 (± 1.3)
J	57.12	28.11	-	14.77	122	29
K	42.94	42.26	-	14.8	123.5 (± 3.5)	28.5 ± 1.5
L	85.39	-	-	14.61	96.25 (± 1.1)	26.3 (± 1.5)

Table 3.1.1 Peak size and standard deviation of the size distribution of liposomes of various compositions, as measured by photon correlation spectroscopy.

the actual distribution is. However, for vesicles it is very likely that they do not vary significantly from a normal weight distribution. This normal distribution W(d) gives a measure of the degree of polydispersity of the particle diameters (d) and can be written as

$$W(d) = \frac{1}{\sigma_w\sqrt{2\pi}} \exp\left(\frac{-(d-\bar{d}_w)2}{2\sigma_w}\right) \quad (3)$$

$\bar{d}_w$ = median value of the distribution

σ_w = standard deviation

The median value of the distribution, also known as the weight-average diameter, is calculated from the output of the autosizer; the peak size, mean diameter and standard deviation, and the upper and lower limits of the distribution. A BBC Basic program has been written to perform these calculations and the listing for this program is given in Appendix I.

Fig 3.1.2 compares the size distribution of a sample of cationic liposomes before and after extrusion. Fig. 3.1.3 demonstrates the effect the inclusion of cholesterol has on the size of cationic liposomes.

3.1.2 Targeting Assays

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

$$\%amc = \frac{N_{obs}}{L_a} X\ 100 \quad (4)$$

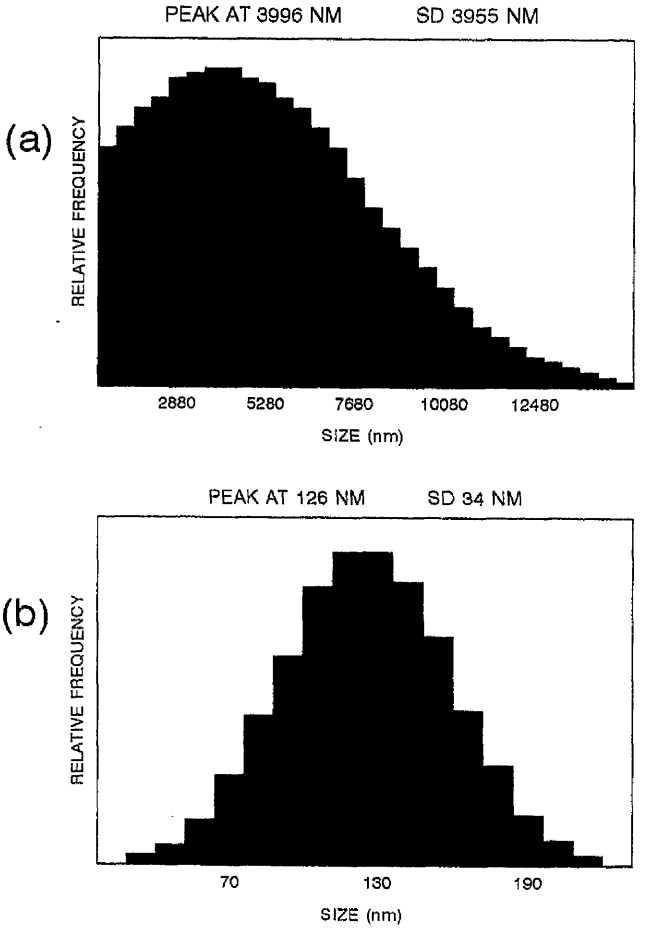


Fig 3.1.2 Size distribution of cationic liposomes, both before (a) and after (b) extrusion through filters with 100 nm pores. Composition DPPC/Chol/SA (molar ratio 1:0.49:0.4)

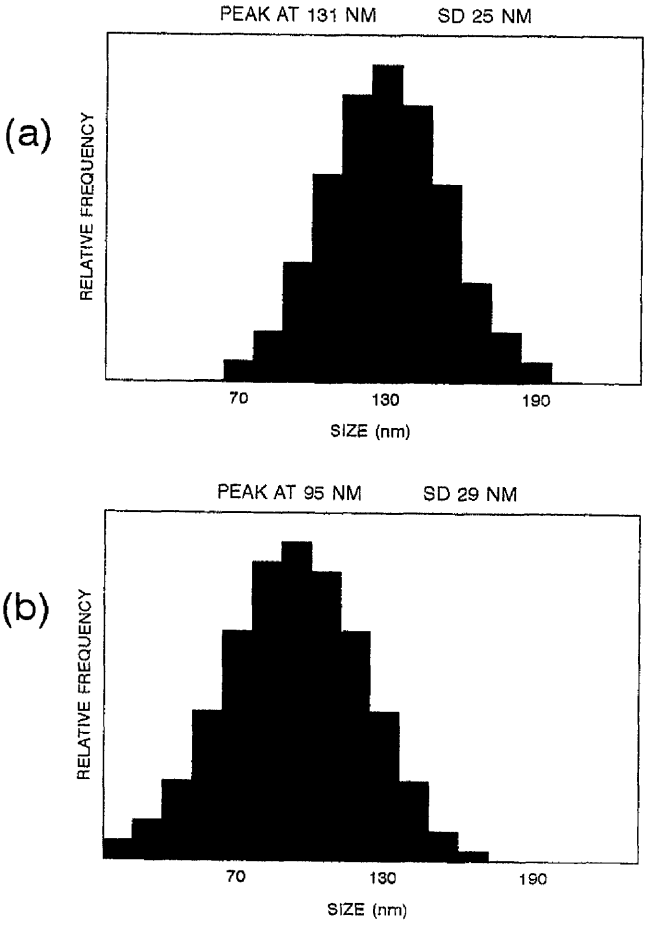


Fig 3.1.3 Size distribution of extruded cationic vesicles incorporating DDAB both (a) with cholesterol (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22), and (b) without cholesterol (DPPC/DDAB, molar ratio 1:0.17) in the liposomal membrane.

Control wells containing only bacteria or only PBS always gave background levels of radioactivity (< 40 dpm when counted for [^{3}H]). For each well containing a bacterial biofilm that liposomes were added to, an equivalent suspension of liposomes was added to a well that did not contain a biofilm under equivalent conditions. Any resulting radioactivity in these control wells, corresponding to vesicles adsorbed to the microtitre plate material , was subtracted from the corresponding result for the wells with bacteria. This 'control' attachment to 'empty' wells was always less than 5% amc.

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

$$L_a = \frac{A_{bf}\ \bar{N}_w}{\pi\ (d_w/2)^2} \qquad (5)$$

$\bar{d}_w$ = weight-average diameter of the liposomes

$\bar{N}_w$ = weight-average number of moles of lipid per liposome

A_{bf} = geometric area of the biofilm

The factor $\pi(\bar{d}_w/2)^2$ is the projected area that each vesicle will occupy on the biofilm. $\bar{N}_w$ was calculated from $\bar{d}_w$ assuming an area per lipid molecule in the liposomal bilayer (taken as $0.5nm^2$) and a bilayer thickness (taken as 7.5nm) as described previously [Hutchinson et al, 1989; Tahara and Fujiyoshi, 1994]. The area of the biofilm was assumed to be that of the area of the well that was exposed to the bacterial suspension. This area was taken as $2.202 \times 10^{-4}m^2$ which was measured in a previous study for the surface of microtitre plate wells exposed to 200µl of the suspension [Chapman et al, 1990]. An example of the calculations used to determine the monolayer coverage of the bacteria is given in Appendix II.

3.1.3 Time Dependency of Adsorption

The adsorption of the DPPC/Chol/SA liposomes to *S.epidermidis* biofilms was dependent on the length of time that the liposomal suspension was exposed to the bacteria. The plot of incubation time against monolayer coverage (Fig. 3.1.4) could be divided into three stages. Between 0 and 30 minutes, the adsorption of the liposomes was very rapid, increasing from 0 to 56 % during this time period. This

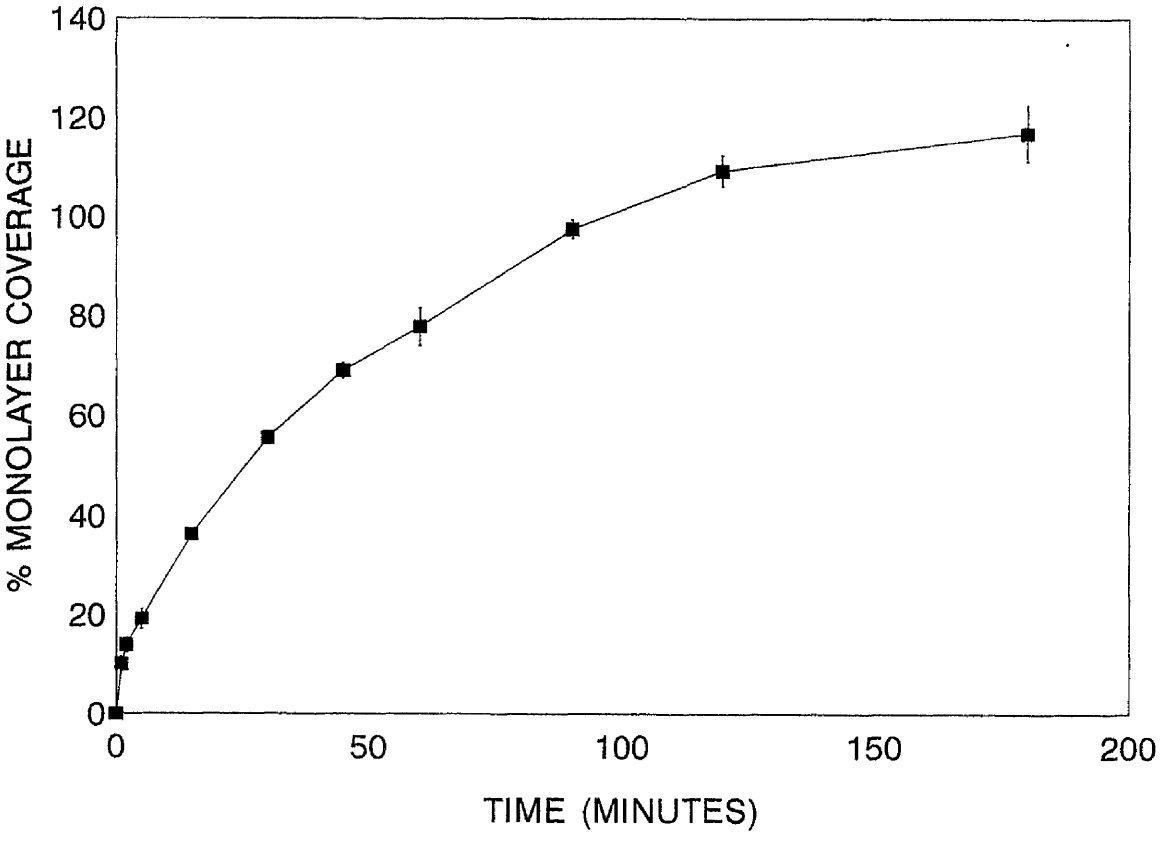


Fig. 3.1.4 The kinetics of adsorption of DPPC/Chol/SA VETs (molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms. Liposomal lipid concentration 0.51 mM, ionic strength 188 mM, temperature 37^{o}C.

reflected attachment of liposomes to a surface initially uncovered by vesicles. A significant level of attachment (14 %) was seen after only two minutes. Between 30 and 90 minutes, the attachment was less rapid, increasing from 56 % to 98%. The attachment levelled off between 90 and 180 minutes, as the surface was becoming covered in a large number of vesicles, when the monolayer coverage only increased from 98 % to 117 %.

From the results of this experiment, it was decided that two hours would be chosen as the exposure time in all subsequent experiments involving the stearylamine liposomes.

3.1.4 Effect of Liposome Concentration

The adsorption of the DPPC/Chol/SA liposomes to *S.epidermidis* biofilms was studied as a function of liposome concentration at 37°C (Fig. 3.1.5). The level of stearylamine used in the liposomes (21.3 mole%) had been identified previously as one which gave optimum targeting to *S.epidermidis* [Song and Jones, 1994]. The %amc rose steeply between a liposomal lipid concentration of 0 and 1 mM and more slowly thereafter. Concentrations in the region of 0.2-0.3 mM were sufficiently high to give adsorption levels in the region of 50-80 %. Neutral liposomes composed 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. Therefore, it was the presence of the cationic stearylamine that caused the interaction of the liposomes with the bacterial biofilm.

3.1.5 Effect of Ionic Strength of Media

The concentration dependence of adsorption at 37°C was studied at three higher ionic strengths (357 mM, 526 mM, 696 mM), as well as in the standard PBS buffer (188 mM). Over the range of liposomal concentrations studied, the general pattern was that the %amc decreased when the ionic strength was increased (Fig. 3.1.6). The

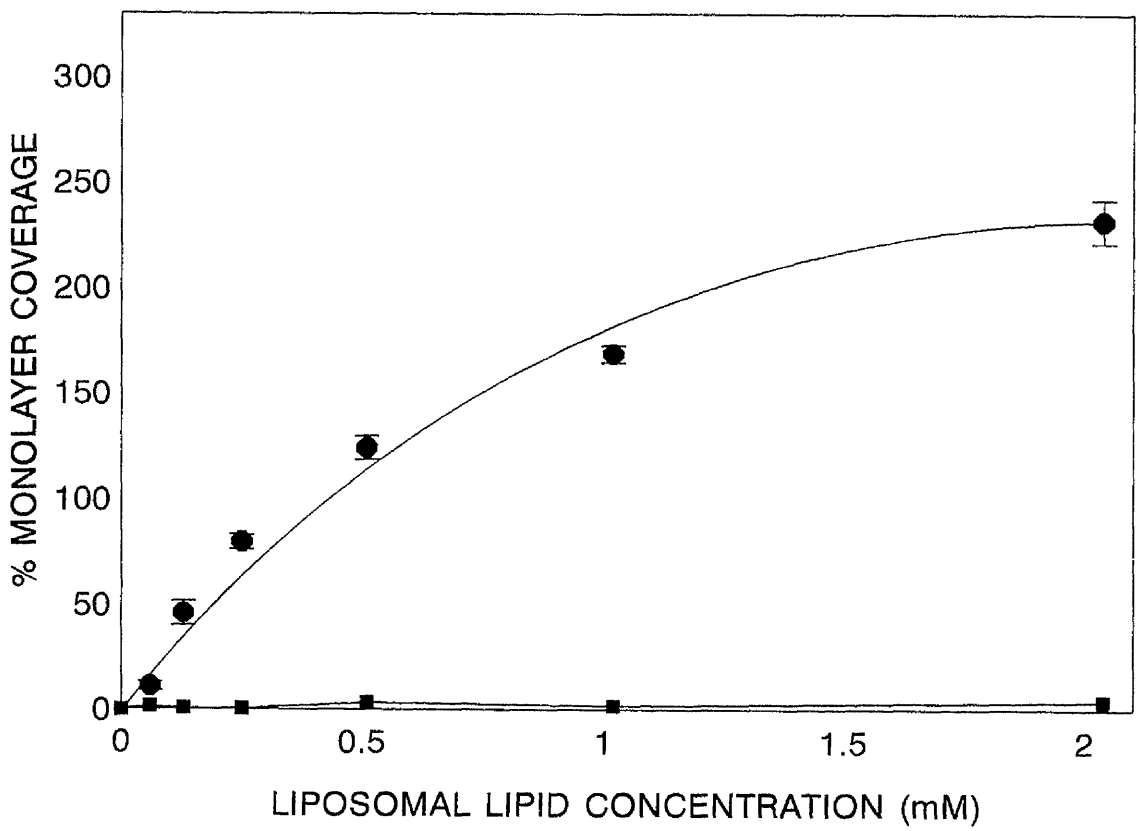


Fig. 3.1.5 The dependence of adsorption of cationic liposomes to *S.epidermidis* biofilms on liposomal lipid concentration. ●, cationic DPPC/Chol/SA VETs (molar ratio 1:0.49:0.4); ■ neutral DPPC/Chol VETs (molar ratio 1:0.82). Incubation time of the liposomes with the biofilm was 2 hours, ionic strength 188 mM, temperature 37°C.

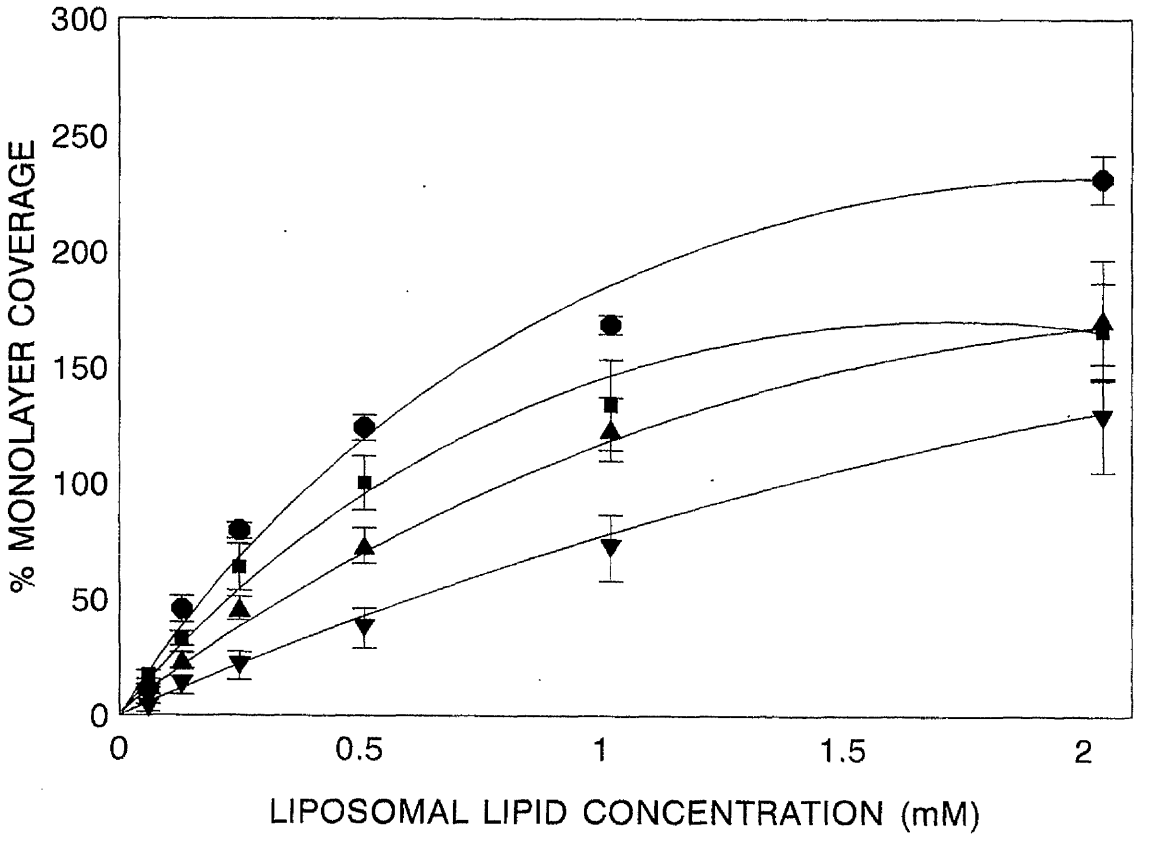


Fig. 3.1.6 Dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours, temperature 37^{o}C.

highest levels of adsorption were obtained with PBS at 188 mM, and the lowest levels at the highest ionic strength studied (696 mM). Although the %amc rose more steeply between 0 and 1 mM at the lower ionic strengths, over the higher range of concentrations studied (1 to 2 mM) the level of adsorption tended to level off at the lower ionic strengths (188 mM and 357 mM) whereas with the higher ionic strengths (526 mM and 696 mM) the level increased steadily over this range, indicating that the plateau seen with the lower strength media had not been reached at the lipid concentrations studied. Indeed, the plot at 696 mM is almost linear. This indicates that at lipid concentrations greater than those studied (2 mM), the difference in %amc seen with ionic strength will not be as great (see Discussion, Section 4.1.5).

To assess if there was any aggregation of the cationic vesicles in the higher ionic strength solutions, the liposomes were added to samples of these solutions (giving a final liposome concentration of 0.51 mM) and mixed, with the diameter monitored throughout a two hour period (Fig. 3.1.7). An increase in the diameter measured would reflect the formation of vesicle aggregates.

No aggregation was observed at 188 mM and 357 mM during the two hours. However, some aggregation was seen at 526 mM and 696 mM, reflected in a threefold increase in the measured diameter. This corresponded to the formation of small aggregates of around ten liposomes.

Some osmotic shrinkage of the vesicles may have occurred concurrently with the aggregation when the vesicles were mixed with the high ionic strength solutions [Lerebours et al, 1993]; however, this was not very likely as the experiment was performed at a temperature lower than the chain-melting temperature of the liposomal lipids. The shrinkage only occurs above this temperature [Mosharraf et al, 1995].

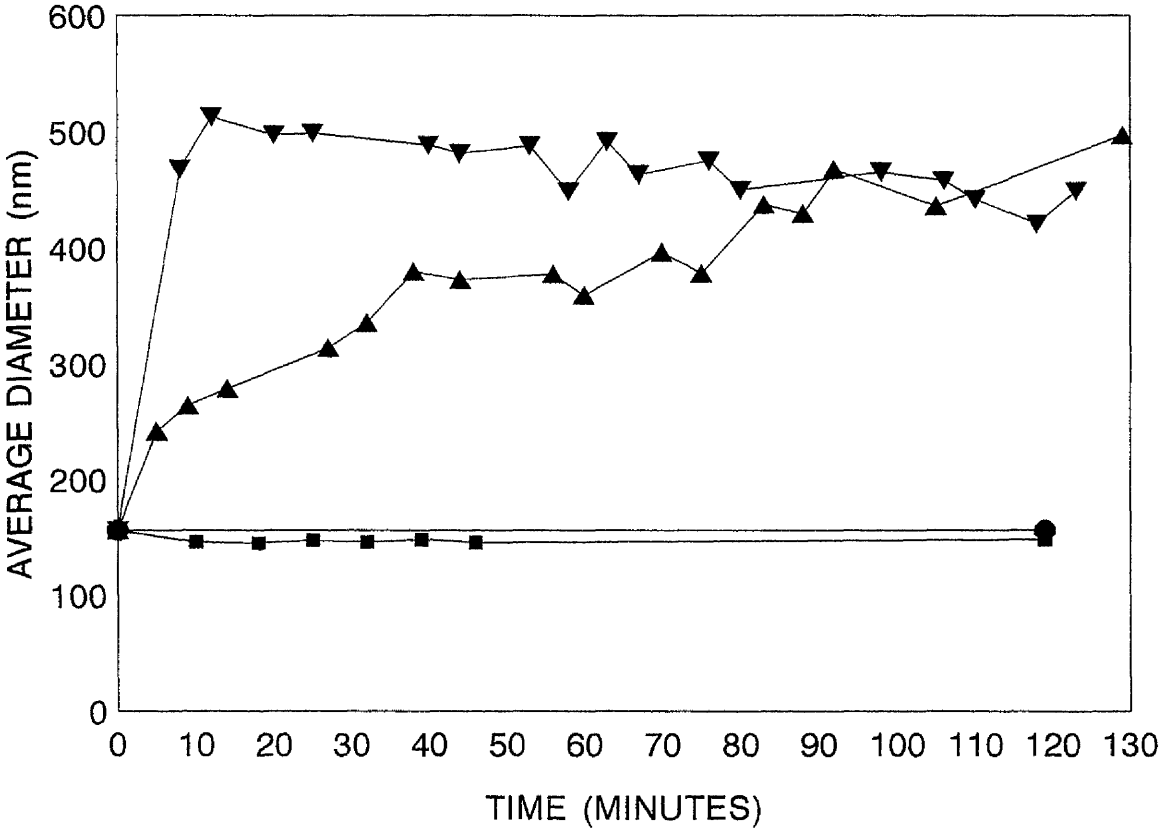


Fig. 3.1.7 Average diameter of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) on addition to media of higher ionic strength. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Liposomal lipid concentration 0.51 mM, temperature 25°C.

3.1.6 Reversibility of the Interaction

The adsorption of the stearylamine-VETs was examined to see if it was reversible; in other words to see if increasing the ionic strength would have the effect of lowering the level of attachment of liposomes to a biofilm where they had already been allowed to attach under the optimal conditions (incubation for two hours at 37^{o}C in PBS buffer). If increasing ionic strength removed attached vesicles then their adsorption to the biofilm was reversible.

The concentration of the liposomes exposed to the biofilm in PBS was 0.51 mM, which gave a monolayer coverage of 124.0 % (± 12.4 %). When this biofilm was exposed to a subsequent medium of higher ionic strength (not containing liposomes) for a further two hours at 37^{o}C, this level fell indicating a removal of attached liposomes induced by the increased level of free ions. The greatest liberation of adsorbed vesicles was seen when the biofilm was exposed to the highest strength solution (696 mM) when the level of monolayer coverage fell by more than half to 53.0 % (± 5.6 %). Within experimental error, the levels of attachment seen after the incubation of the biofilm and adsorbed vesicles with medium of higher ionic strength were comparable to those seen when the liposomes were initially allowed to adsorb in the equivalent high strength medium (Fig. 3.1.8). These results indicate that the adsorption of cationic vesicles to *S.epidermidis* is reversible. Pretreatment of the biofilms with the high ionic strength solutions had no effects on adsorption of subsequently administered liposomes (results not shown).

3.1.7 Effect of Temperature of Incubation

The adsorption of the vesicles was examined at 4^{o}C, 25^{o}C and 37^{o}C (Fig. 3.1.9). Over the range of liposome concentrations studied, the greatest level of adsorption was seen at the highest temperature (37^{o}C) and the least adsorption at the lowest temperature (4^{o}C). Similar hyperbolic plots were seen at each temperature, with a rapid increase in the %amc observed between 0 and 1 mM and a levelling off of the

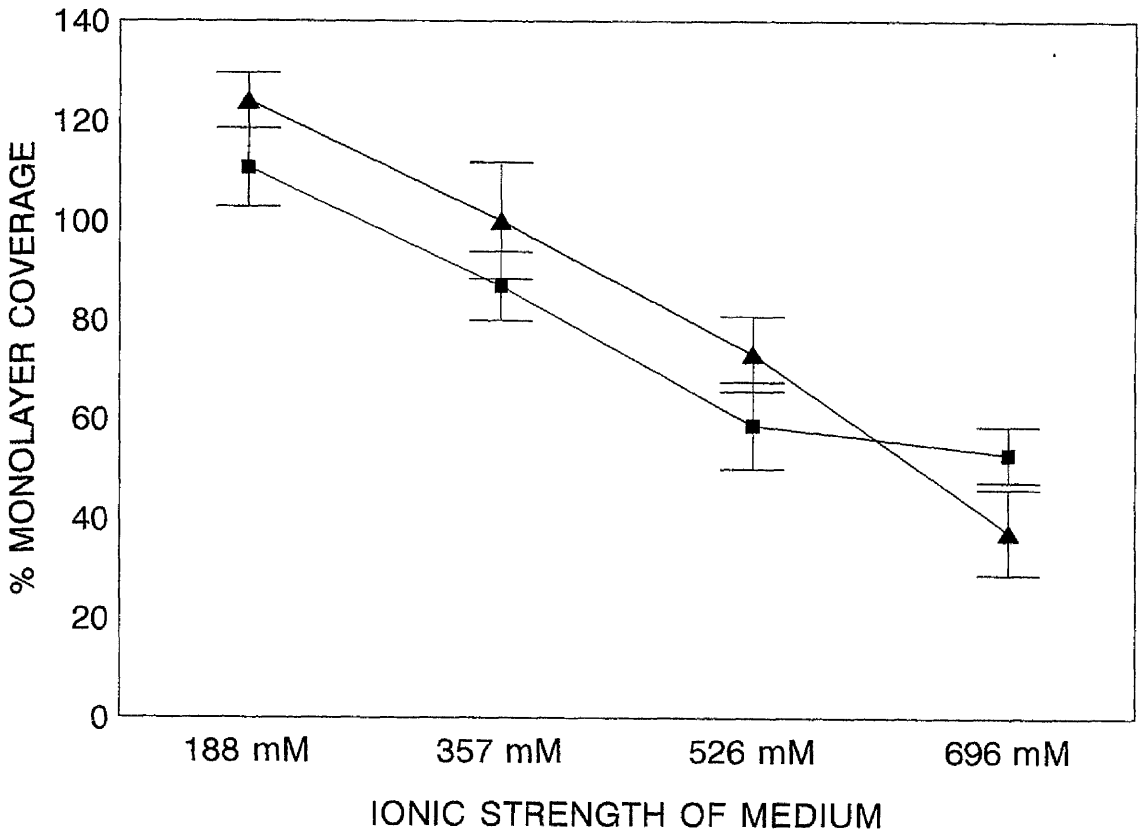


Fig. 3.1.8 Reversibility of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms. ▲, adsorption of liposomes as a function of ionic strength of the medium; ■, adsorption of liposomes after initial adsorption from PBS (188 mM) followed by a further incubation in medium of higher ionic strength to induce detachment of adsorbed vesicles. Liposomal lipid concentration 0.51 mM, incubation times 2 hours each, temperature 37^{o}C.

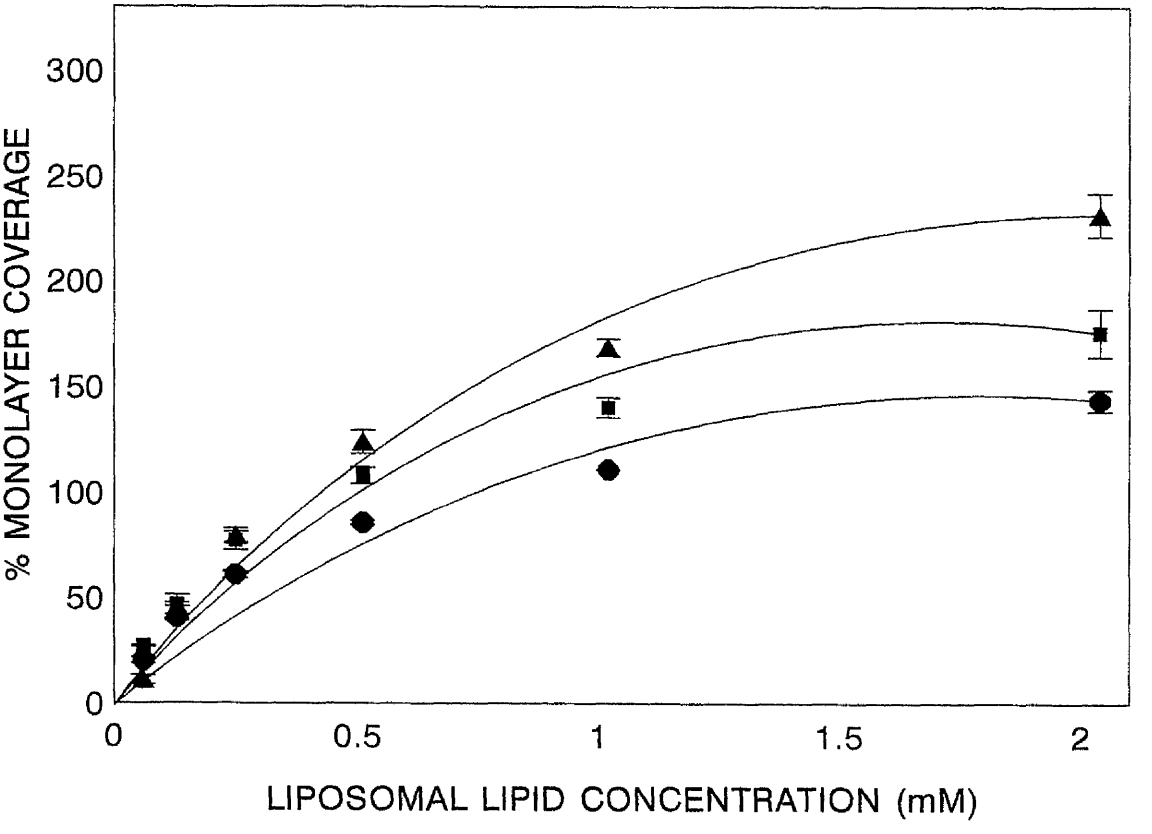


Fig. 3.1.9 Dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the temperature of incubation. ●, 4°C; ■, 25°C; ▲, 37°C. Incubation time 2 hours, ionic strength 188 mM.

level of coverage between 1 and 2 mM.

3.1.8 Effect of Ionic Strength at Different Temperatures

The effect of ionic strength on the adsorption of cationic liposomes to the bacterial biofilms was examined at 4^{o}C (Fig 3.1.10) and 25^{o}C (Fig 3.1.11). At both these temperatures, ionic strength had the same effect as observed at 37^{o}C (see above); over the range of lipid concentrations studied, increasing the ionic strength lowered the extent of liposomal adsorption. Also, at each of the ionic strengths, the %amc increased with increasing temperature.

3.1.9 Adsorption to Hydrophobic and Hydrophilic Subpopulations

The strain of *S.epidermidis* used in this work (NCTC 11047) had previously been subdivided into two subpopulations on the basis of their hydrophobicity as determined by a hexadecane partitioning assay. Both subpopulations formed biofilms in the wells of microtitre plates and the level of adsorption of cationic liposomes to these bacteria was compared to that observed with biofilms of the total population (Fig. 3.1.12). Over the range of vesicle concentrations examined, there was greater adsorption to films of the hydrophobic subpopulation than to films of the total population of bacteria. Conversely, significantly lower attachment was seen to the hydrophilic subpopulation compared to the total population.

3.1.10 Adsorption to Adherence-Defective Mutants

Attachment of the cationic liposomes to biofilms of the M3 mutant of *S.epidermidis* was substantially lower than that seen with the wild-type bacteria over the range of liposomal concentrations studied (Fig. 3.1.13). With the mutants, a saturating level of liposomal adsorption was reached at a relatively low concentration of liposomes, reflected in a levelling off of the monolayer coverage at around 1 mM lipid.

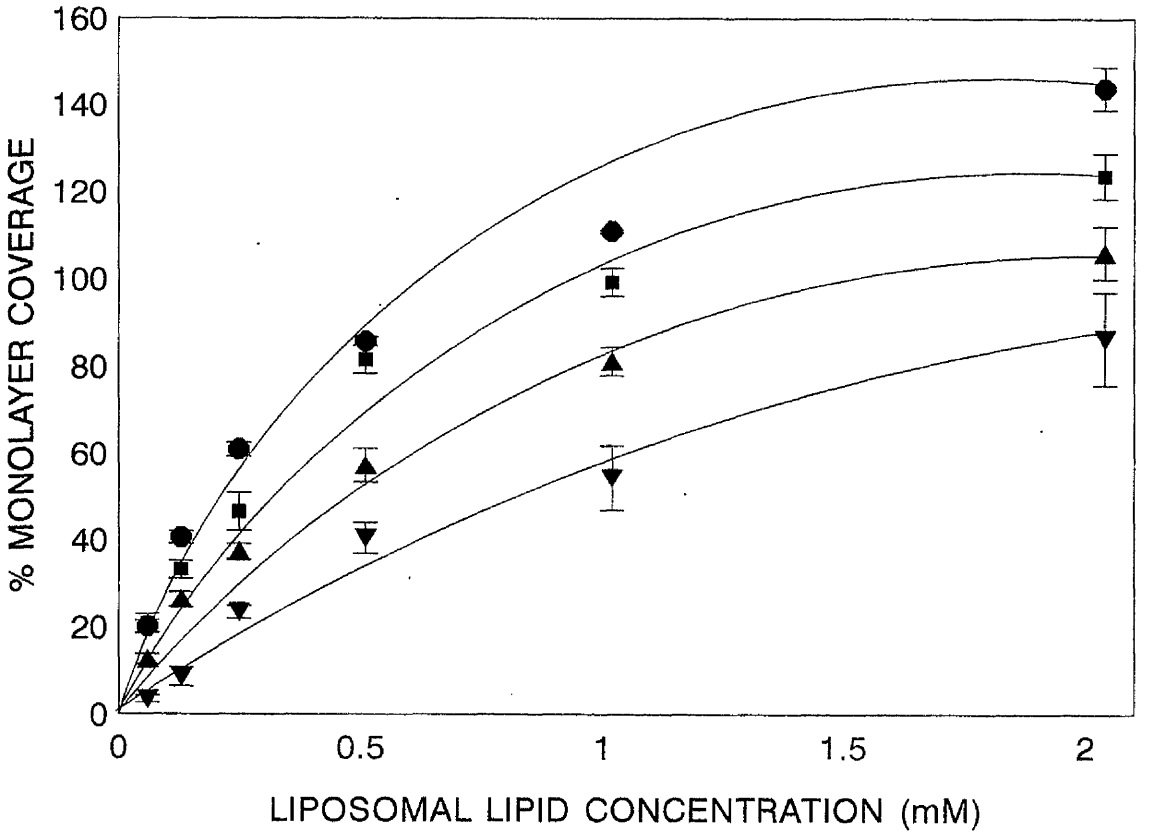


Fig. 3.1.10 Dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours, temperature $4^{o}C$.

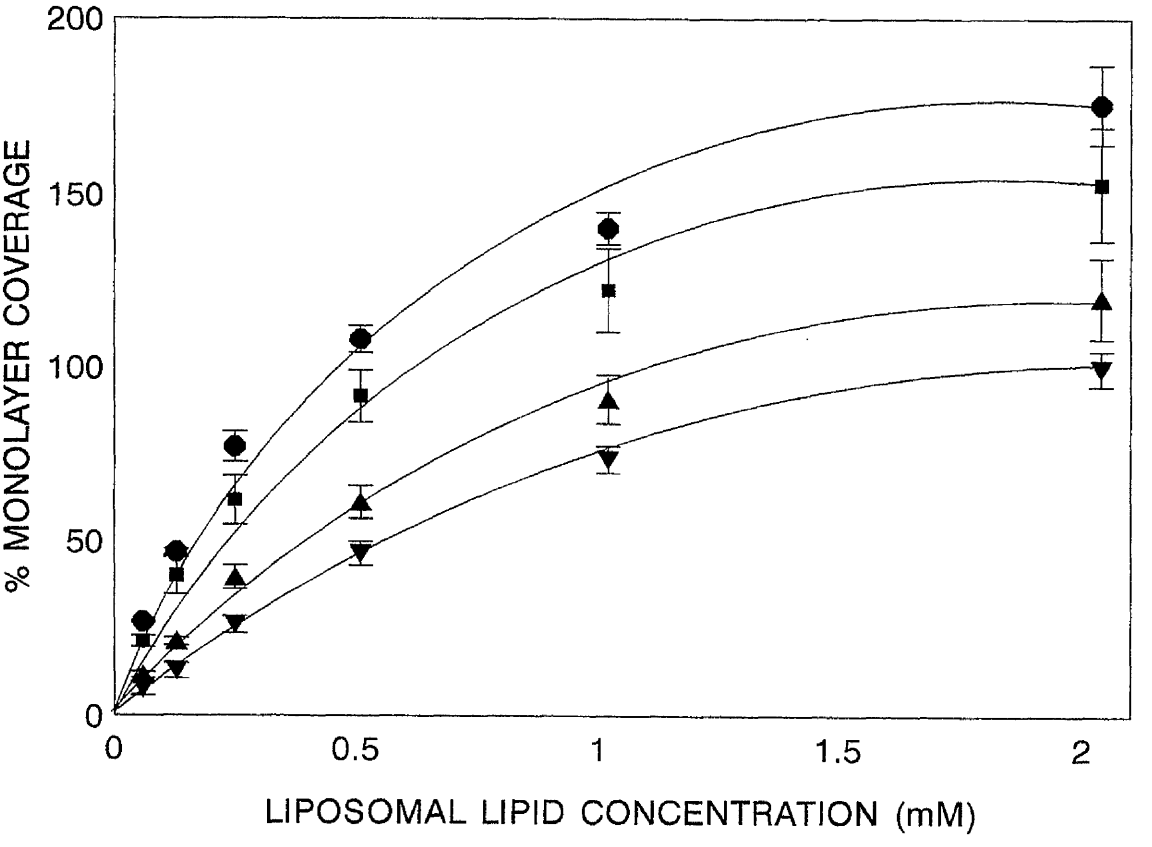


Fig. 3.1.11 Dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours, temperature 25°C.

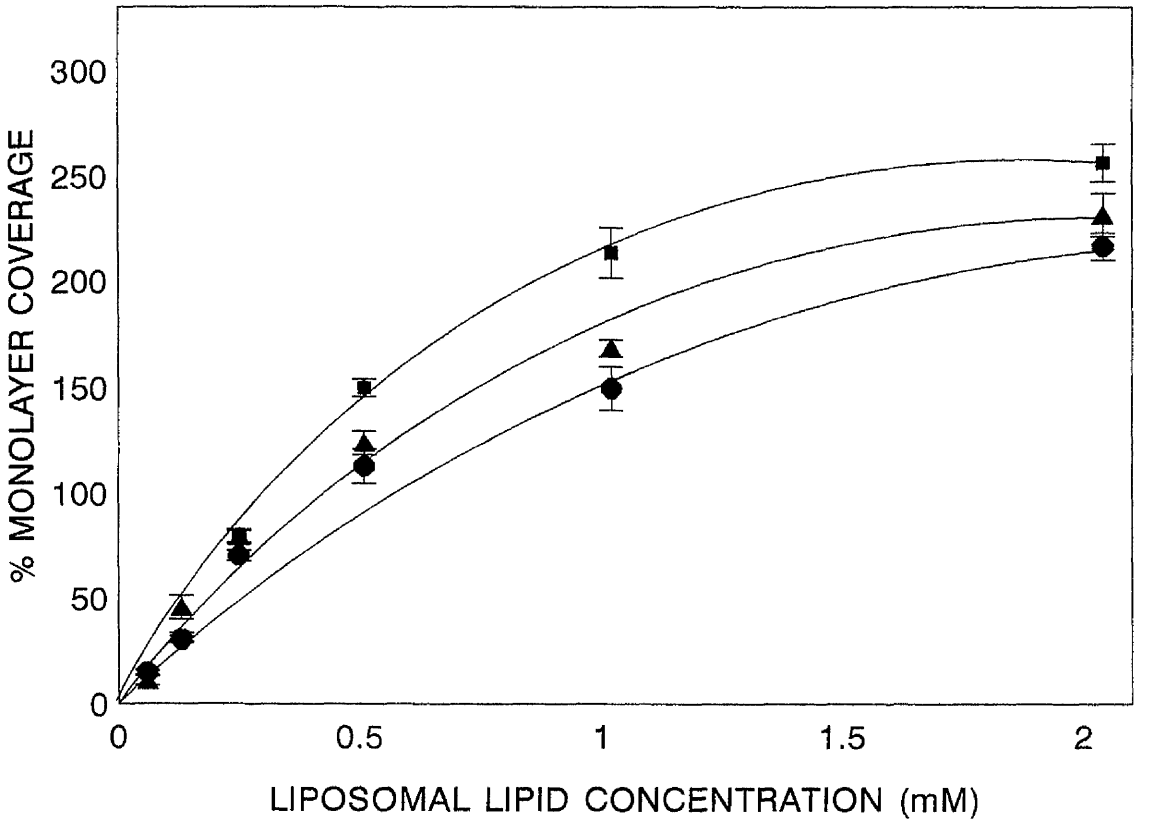


Fig 3.1.12 Adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to biofilms of hydrophobic and hydrophilic subpopulations of *S.epidermidis*. ▲, total population; ■, hydrophobic subpopulation; ●, hydrophilic subpopulation. Incubation time 2 hours, ionic strength 188 mM, temperature 37^{o}C.

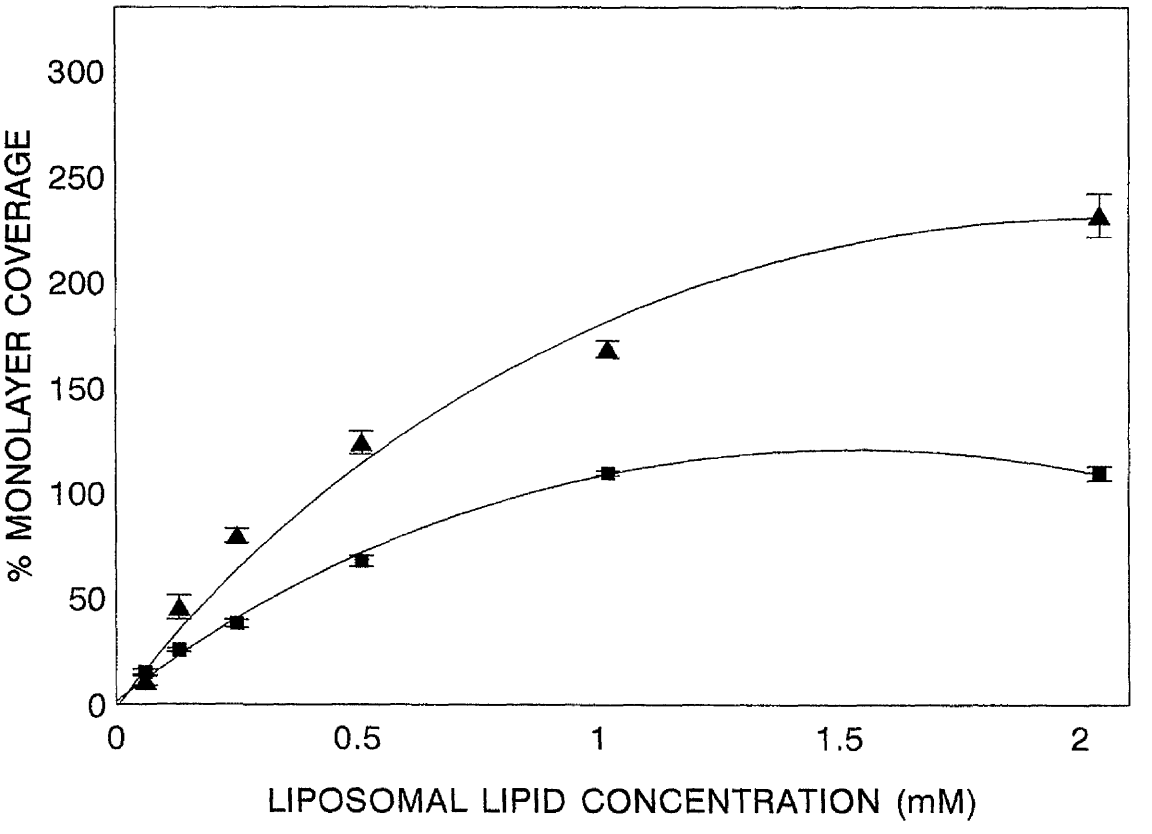


Fig. 3.1.13 Adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to biofilms of adherence-defective mutants *S.epidermidis*. ▲, *S.epidermidis* NCTC 11047 wild-type; ■, *S.epidermidis* M3 mutant. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

3.1.11 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. After inoculation in nutrient broth containing the radiolabelled thymidine, the harvested bacterial cells were washed in buffer to ensure that the only radioactivity present was that incorporated by the bacteria; four washes were sufficient to ensure the removal of any free thymidine (Fig 3.1.14).

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. For example, with the total population of NCTC 11047 bacteria;

Cells in 1 ml of suspension ($OD_{550} = 0.5$)	=	9.03×10^7
dpm (^{3}H) in 1 ml of suspension	=	174,646
specific activity	=	1.935×10^{-3} dpm/cell
dpm of cells attached per well	=	9980.8
number of cells per well	=	$9980.8/1.935 \times 10^{-3}$
	=	5,157,671 cells

Fig. 3.1.15 shows the results for the attachment of these bacteria, plus the hydrophobic and hydrophilic subpopulations and the adherence-defective mutants. The number of cells of the hydrophobic subpopulation attached per well was similar to the number seen with the total population, but there was significantly less attachment of the hydrophilic cells. The mutants had lost much of their adhesiveness for the plates; the attachment of the wild-type cells was around ten times greater.

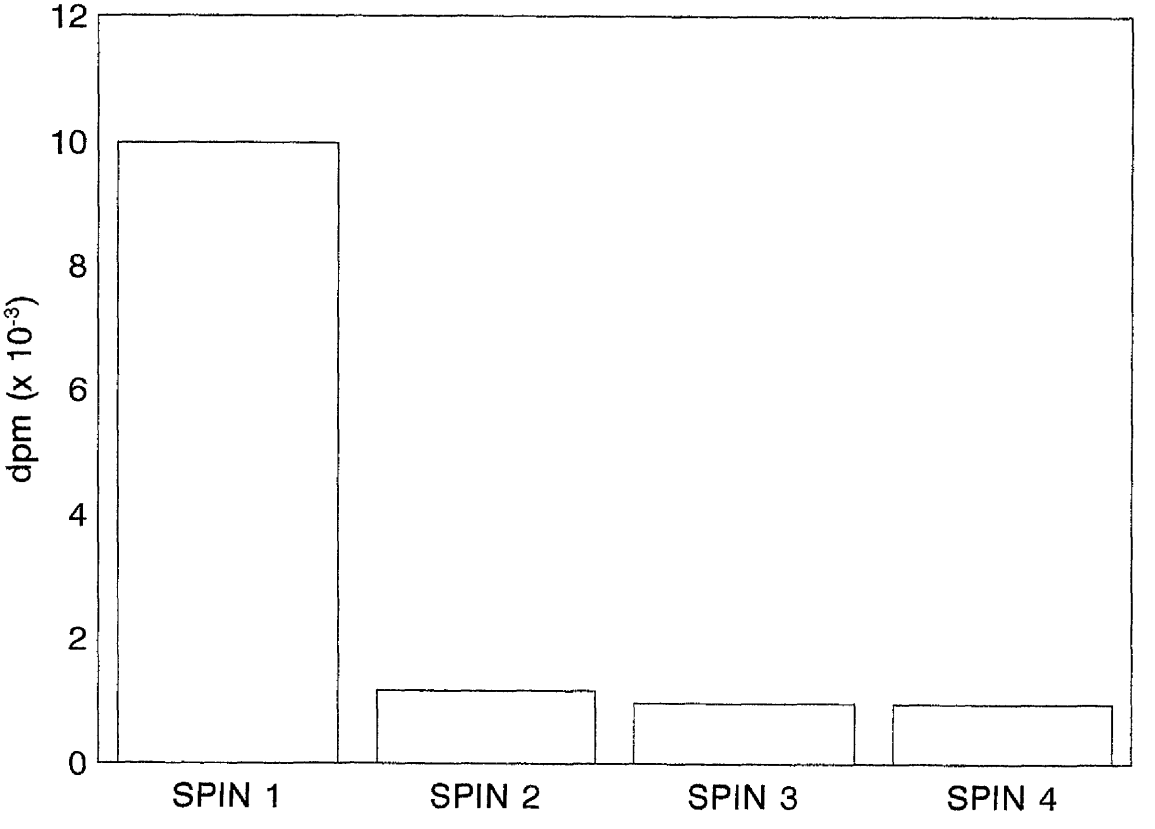


Fig. 3.1.14 Scintillation counts (dpm) of samples (10 μl) taken from resuspended volumes (10 ml) of [3H]-thymidine labelled *S.epidermidis* after successive centrifugations (2000 rpm, 10 mins each).

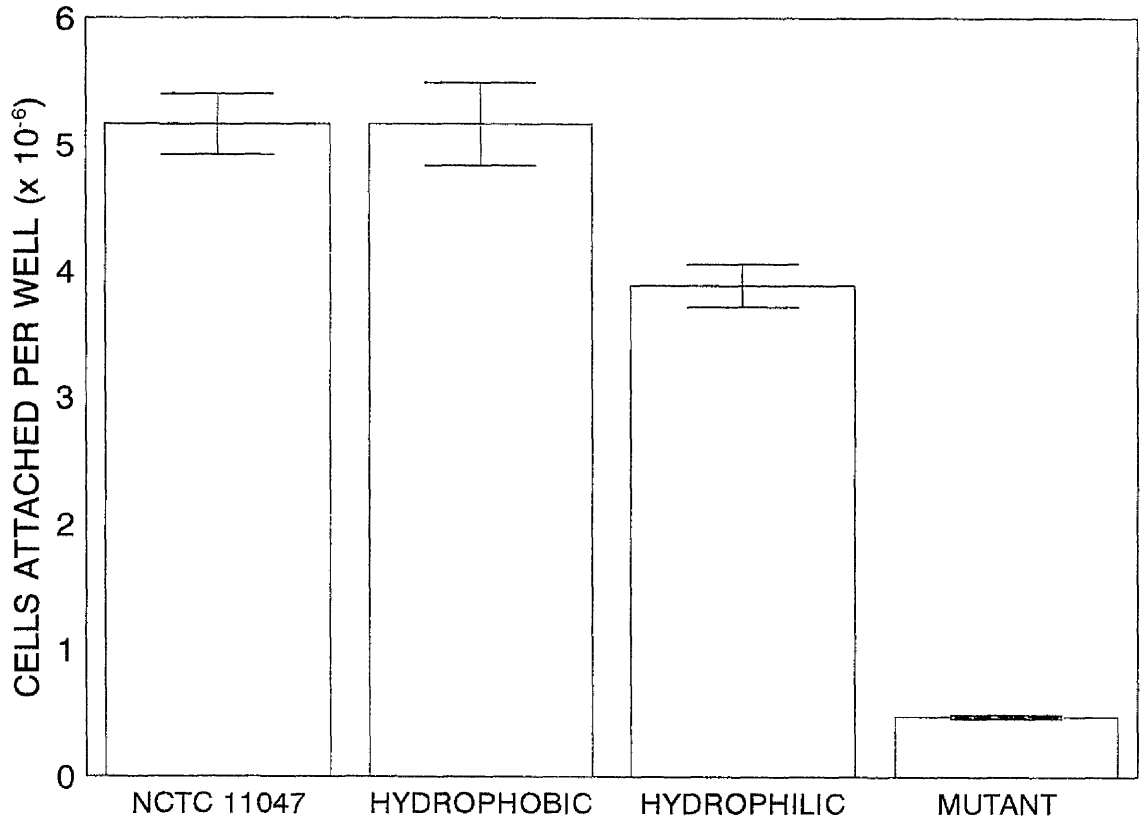


Fig. 3.1.15 Attachment of hydrophobic and hydrophilic subpopulations of *S.epidermidis*, and adherence-defective *S.epidermidis* mutant to the wells of microtitre plates (Immulon 2), as determined by labelling of bacteria with [^{3}H]-thymidine. Incubation time 18 hours, room temperature.

3.2 EFFICIENCY OF ENCAPSULATED AGENTS

3.2.1 Adsorption of Cationic DDAB-VETs to *S.epidermidis*

For targeting of liposome-encapsulated antibacterial agents (vancomycin and gentamicin) to *S.epidermidis*, stearylamine was not used as the cationic component in the vesicles due to its reported toxicity [Klang et al, 1994; Oku and Namba, 1994; Swenson et al, 1988]. The liposomes were instead given a positive charge by incorporation of dimethyldioctadecylammonium bromide (DDAB) into the bilayer (see Fig. 2.2.1). Adsorption of such liposomes was examined as a function of the percentage of DDAB in the membrane of DPPC/Chol liposomes (see Fig. 3.2.1). A negligible level of attachment was seen with liposomes having a DDAB content of 2.81 mole%. With levels of between 5.46 and 10.35 mole% DDAB, hyperbolic plots were seen when the monolayer coverage (%amc) was examined as a function of liposomal lipid concentration. With levels of DDAB greater than 12 mole%, a much larger degree of adsorption was observed over the range of liposome concentrations studied. Analysis of these curves (see Discussion, Section 4.2.1) showed that the adsorption seemed to reach a peak with an optimum level of DDAB of around 14.77 mole% and so this proportion of the positively charged component was the one used in all subsequent experiments.

For the experiments examining delivery of antibacterial agents, four types of vesicle were used;

1. Neutral liposomes
   DPPC/Chol, molar ratio 1:0.21
   (17.42 mole% Chol)

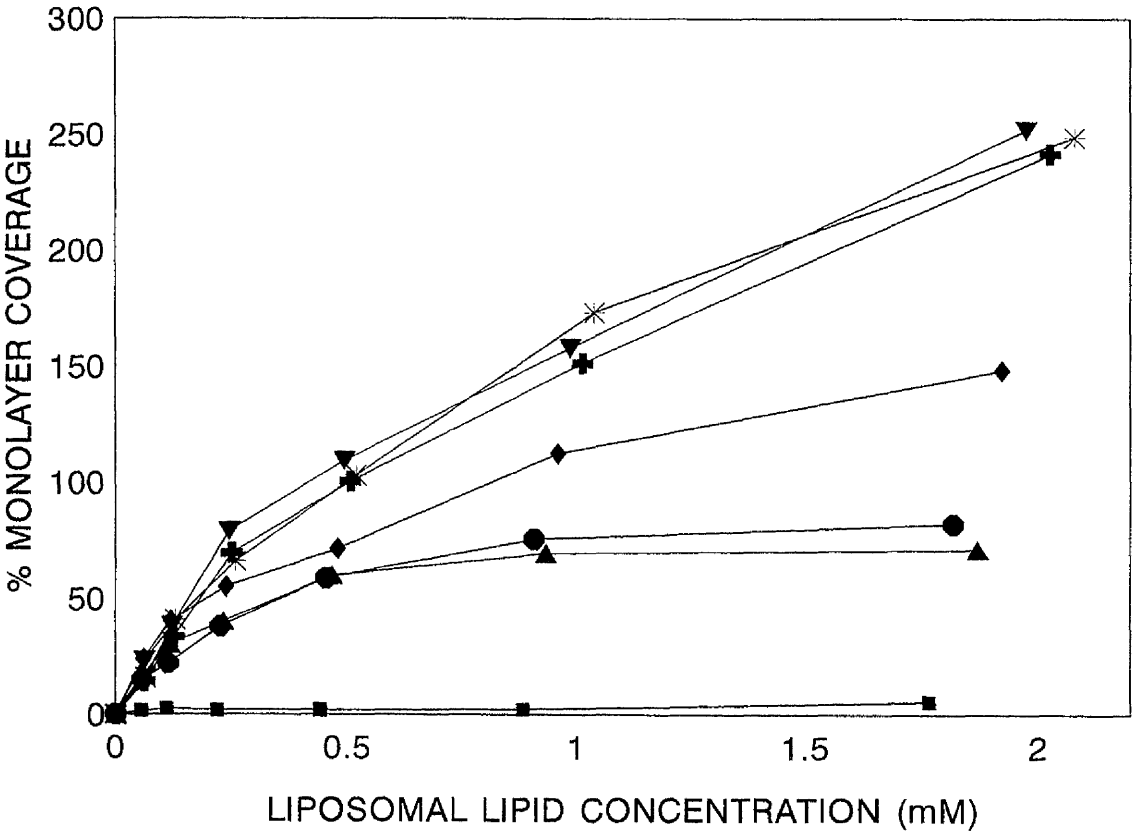


Fig. 3.2.1 Liposome concentration-dependent adsorption of DPPC/Chol/DDAB VETs to *S.epidermidis* biofilms as a function of mole% DDAB in the liposome membrane. ■, 2.81 mole% DDAB; ●, 5.46 mole% DDAB; ▲, 7.97 mole% DDAB; ◆, 10.35 mole% DDAB; ▼, 12.62 mole% DDAB; ✚, 14.77 mole% DDAB; ✳, 16.81 mole% DDAB. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

2. Cationic liposomes - no cholesterol

   DPPC/DDAB, molar ratio 1:0.17

   14.61 mole% DDAB

3. Cationic liposomes - with cholesterol

   DPPC/Chol/DDAB, molar ratio 1:0.28:0.22

   14.39 mole% DDAB (18.79 mole% Chol)

4. Cationic liposomes - high cholesterol content

   DPPC/Chol/DDAB, molar ratio 1:0.98:0.34

   14.80 mole% DDAB (42.26 mole% Chol)

The aqueous solvent used in making these liposomes was double distilled water. PBS buffer was not used because it appeared to interact with vancomycin when the drug was dissolved in it, leading to the blockage of the membrane filter of the extruder when production of such vesicles was attempted.

The adsorption of these vesicles to the biofilms was examined as a function of liposomal lipid concentration (Figs. 3.2.2 - 3.2.4). The adsorption assays were performed at room temperature and the exposure times were 2 mins, 15 mins and 30 mins, reflecting the conditions that were subsequently used in the regrowth assays (see Section 3.2.3).

With the neutral liposomes, there was no adsorption to bacteria at any concentration of liposomes. Using the cationic vesicles, adsorption was seen and a longer period of incubation led to the attachment of a greater number of liposomes, as would be expected. With increasing lipid concentration, the difference in levels of adsorption seen with the three incubation time periods became more pronounced. Generally, the levels of adsorption seen after 15 mins incubation were closer to those seen after 30,

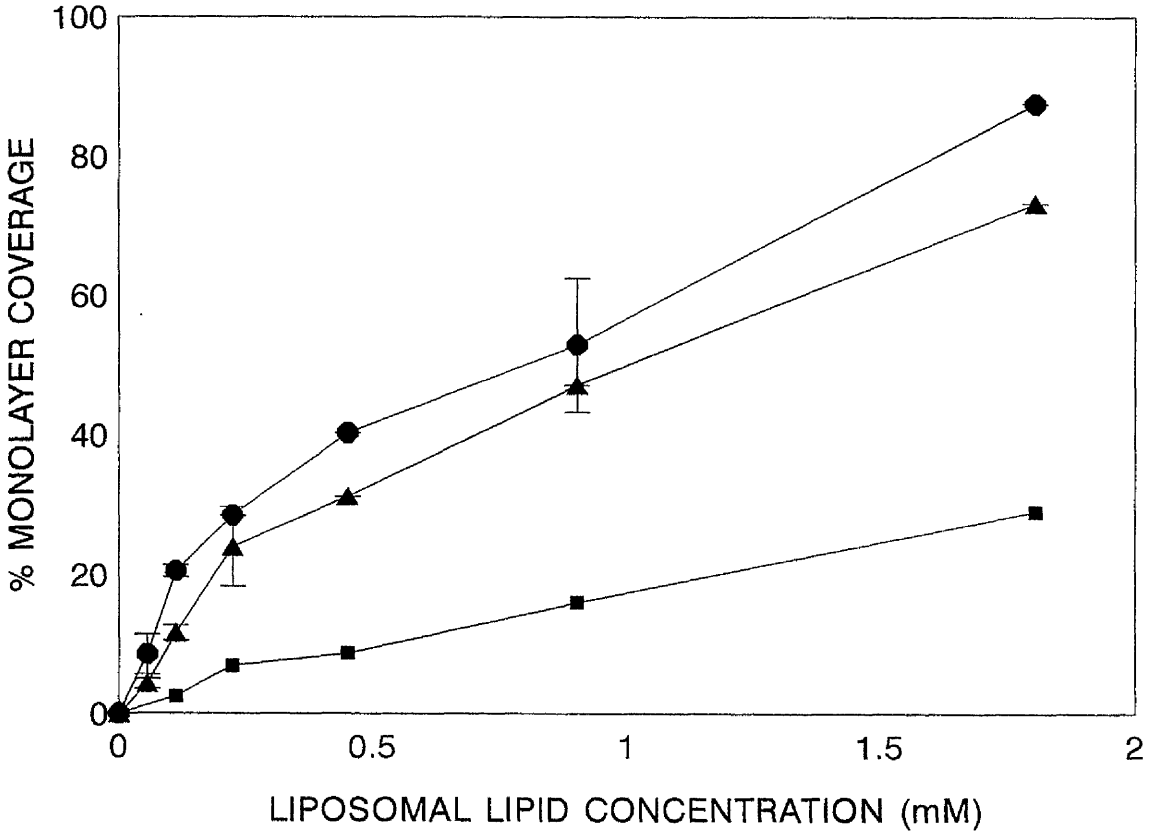


Fig. 3.2.2 The dependence of adsorption of cationic liposomes with no cholesterol (DPPC/DDAB, molar ratio 1:0.17) to *S.epidermidis* biofilms on liposomal lipid concentration and the time of incubation. ■, 2 minute incubation; ▲, 15 minute incubation; ●, 30 minute incubation.

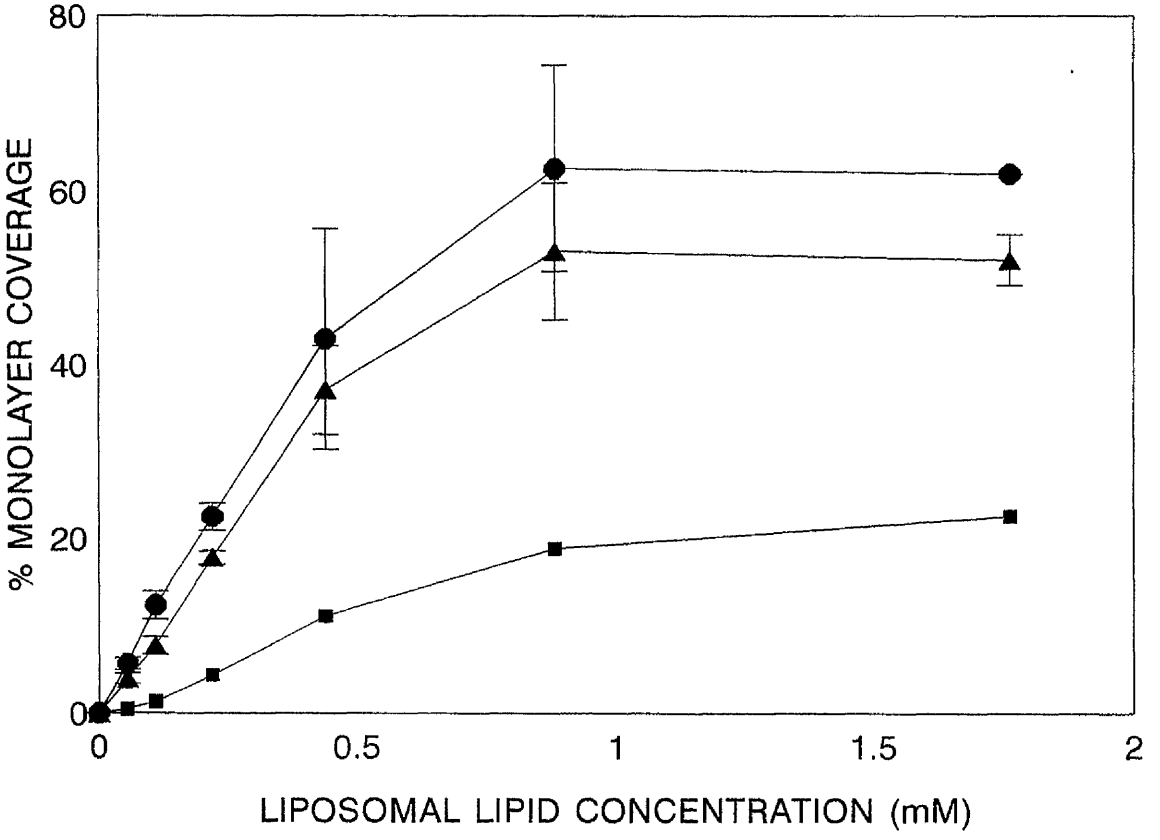


Fig. 3.2.3 The dependence of adsorption of cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) to *S.epidermidis* biofilms on liposomal lipid concentration and the time of incubation. ■, 2 minute incubation; ▲, 15 minute incubation; ●, 30 minute incubation.

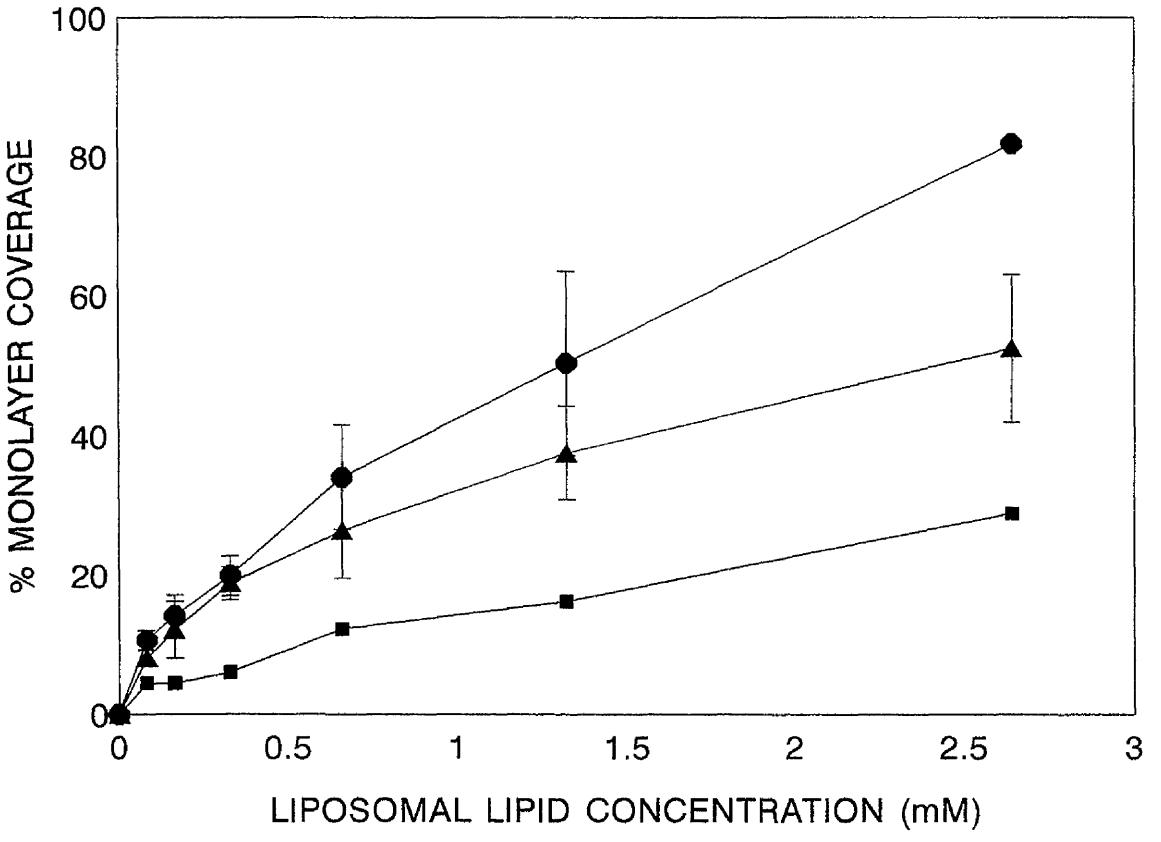


Fig. 3.2.4 The dependence of adsorption of cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34) to *S.epidermidis* biofilms on liposomal lipid concentration and the time of incubation. ■, 2 minute incubation; ▲, 15 minute incubation; ●, 30 minute incubation.

mins than the levels seen after 2 mins, reflecting the time-dependent kinetics of adsorption (c.f. the similar results obtained with stearylamine-VETs, see Fig. 3.1.4). There was little difference seen in adsorption between the three different compositions of cationic liposome; each gave a coverage of about 60-80 % at a lipid concentration of 1.5 mM after 15 mins.

3.2.2. Encapsulation of Antibacterial Agents

Free vancomycin was separated from liposomes encapsulating the drug using gel filtration chromatography (Fig. 3.2.5). The liposomes eluted in the void volume of the column. Scintillation counting showed a peak in the level of [^{3}H]-DPPC in fractions 6 and 7, corresponding to a volume of around 16-19 ml. A Wang and Smith/Lowry protein assay indicated the presence of vancomycin in these fractions, corresponding to drug encapsulated within the vesicles. The assay also revealed a much larger peak in the elution profile around peaks 19 and 20 (50-53 ml), corresponding to elution of unencapsulated vancomycin that had penetrated to the inner volume of the pores of the Sepharose beads.

Calibration curves for the assay were used to determine the amount of encapsulated vancomycin (see Fig. 2.2.2). Depending on the level of vancomycin included in the aqueous solution used in the production, the concentration of encapsulated vancomycin within the aqueous compartments of the liposomes ranged from 45 μg ml^{-1} to 830 μg ml^{-1}. These figures corresponded to encapsulation efficiencies of 2-4 %, where

$$\text{encapsulation efficiency} = \frac{\text{[vancomycin] in initial solution}}{\text{[vancomycin] encapsulated}} \times 100\ \% \qquad (6)$$

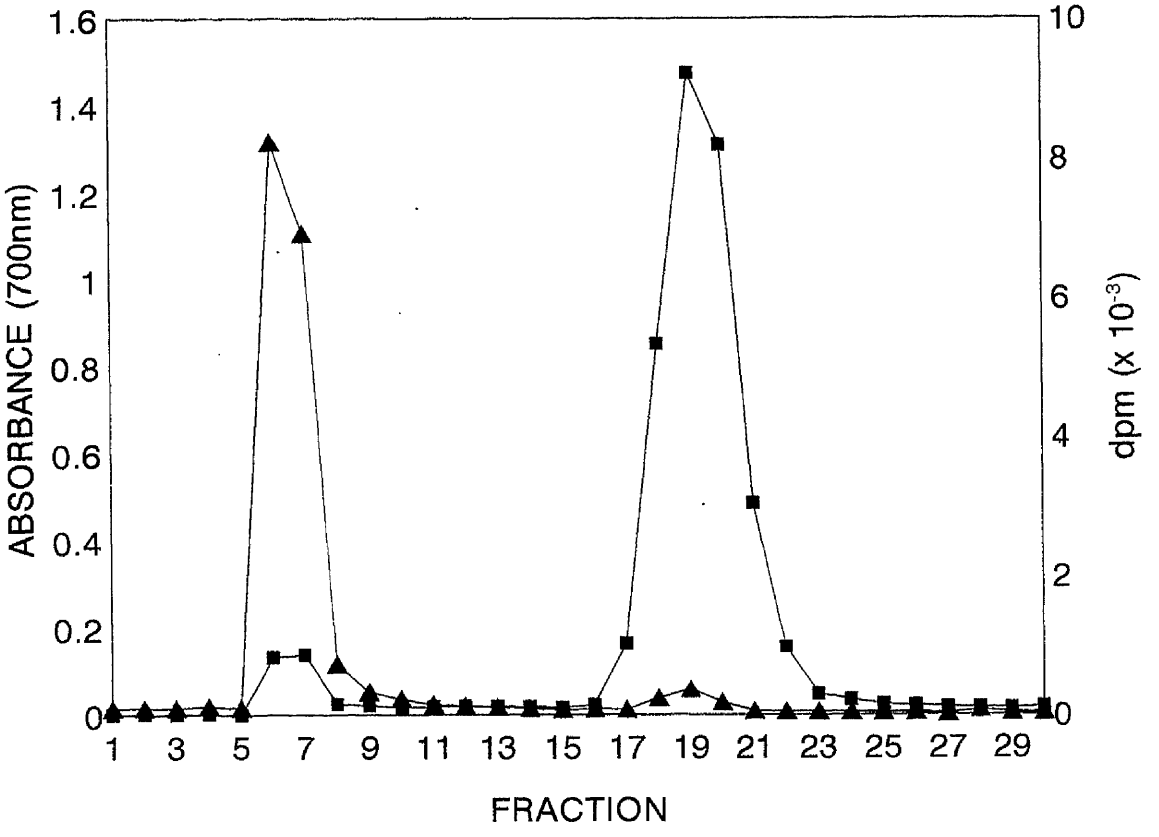


Fig. 3.2.5 Elution profile of cationic VETs (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) after gel filtration on a Sepharose 4B column. ■, absorbance (700 nm) of a 100 µl sample of the fraction measured for protein concentration using a Wang and Smith/Lowry assay; ▲, scintillation count (^3H) of a 10 µl sample of the fraction. Fraction size approximately 2.65 ml.

These values were low, but were typical of encapsulation seen when a water-soluble substance is passively encapsulated within vesicles. Freeze-thawing of the vesicles prior to extrusion did not increase this efficiency.

The encapsulation efficiency was found to be independent of the amount of drug included during the production of the liposomes. However, the entrapment did seem to differ with liposomal composition. For the cationic liposomes with no cholesterol, the average encapsulation efficiency was 0.6 % compared with 1.8 % when cholesterol was present in the membrane.

3.2.3 Regrowth Assays

The effectiveness of the various liposomal preparations of the antibacterial agents vancomycin and gentamicin were assessed using a regrowth assay. Various concentrations of the drugs (free or encapsulated within liposomes) were exposed to biofilms of *S.epidermidis* adsorbed to microtitre plate wells and incubated at room temperature for 2, 15 or 30 mins. Nutrient broth was subsequently added to the biofilms under conditions that would allow for optimum growth of bacteria. The effectiveness of the antibacterial treatment was measured by comparing bacterial growth after 18 hours to that seen in wells with bacteria that had not been treated by the drug (representing 0 % inhibition of growth) and to growth seen in wells that did not contain a bacterial biofilm (defined as 100 % inhibition of growth). The growth of bacteria in each well was measured using a plate reader (wavelength 630 nm). The inhibition of growth was defined as;

$$\text{Inhibition} = \frac{A_{630\ nm}(\text{bacteria}) - A_{630\ nm}(\text{treated bacteria})}{A_{630\ nm}(\text{bacteria}) - A_{630\ nm}(\text{no bacteria})} \times 100\% \qquad (7)$$

'Empty' liposomes (i.e. those not encapsulating any antibacterial agent) did not cause inhibition of growth at any concentration. Therefore, the inhibition observed with encapsulated drug could only have been caused by the agent itself.

3.2.4 Vancomycin - Effect of Incubation Time

The effectiveness of free and liposome-encapsulated vancomycin was assessed after incubation for periods of 2, 15 and 30 mins. In this work, the highest concentration of liposomal vancomycin used was 50 μg ml^{-1}. There were two reasons for this. Firstly, at concentrations higher than this, inhibition with the free drug was very high (greater than 75 %) and so there would be little scope for enhancement of antibacterial action using liposomes. For example, preliminary work showed that at a vancomycin concentration of 200 μg ml^{-1}, cationic liposomes (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22, liposomal lipid concentration 3.4 mM) caused 72.1 % inhibition of growth after 15 mins, compared with 77.0 % inhibition with the free drug. Secondly, due to the low encapsulation of vancomycin within liposomes, using higher concentrations would have used up a large volume of the liposomal preparations, restricting the number of experiments that could have been performed at the more 'sensitive' concentrations of drug below 50 μg ml^{-1}.

The inhibition of growth of *S.epidermidis* caused by free vancomycin was dependent on the length of time that the drug was incubated with the biofilm before being washed off (Fig. 3.2.6). At all concentrations of the drug examined, there was a greater extent of inhibition with longer incubation times. With only 2 mins incubation, the inhibition fell steadily between 50 and 10 μg ml^{-1}. With 15 and 30 mins incubation, the retardation of growth was high and relatively constant between 50 and 20 μg ml^{-1}. The critical region was between 20 and 10 μg ml^{-1}, where there was a significant decrease in inhibition after both 15 and 30 mins. At the lower

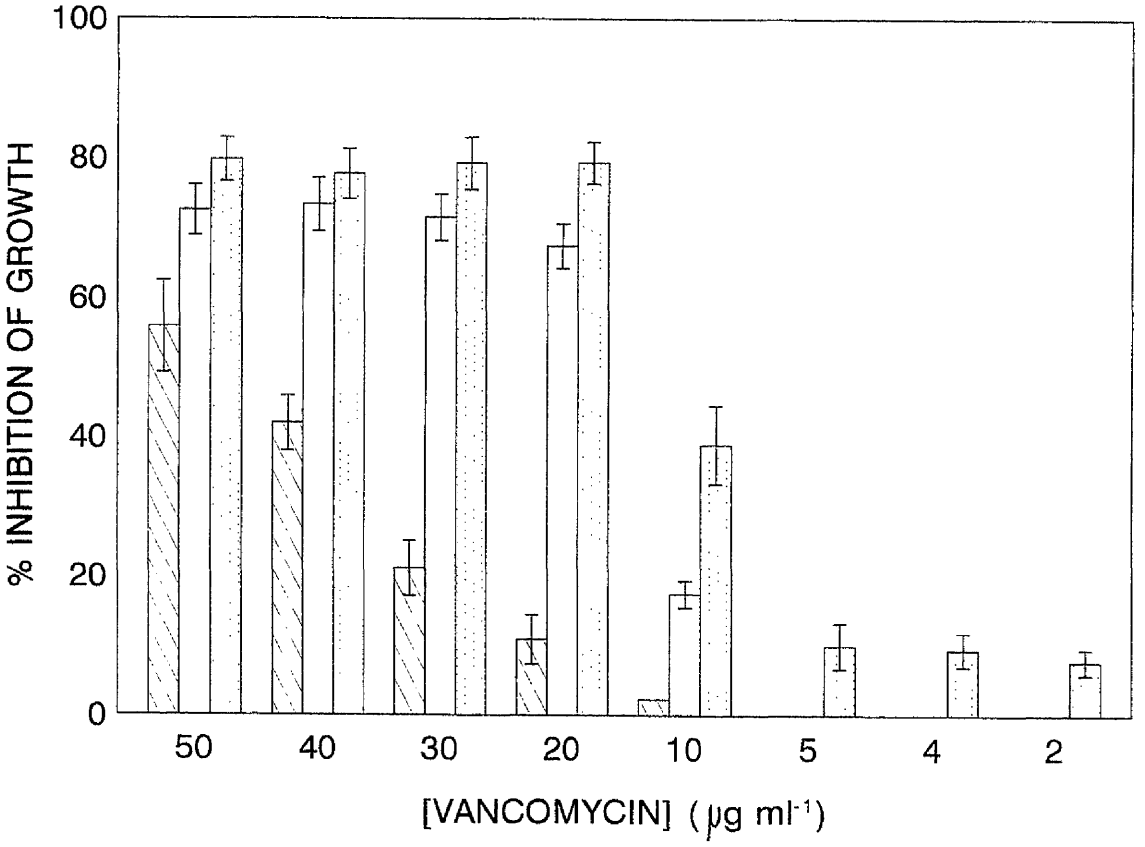


Fig. 3.2.6 Effect of incubation time on the inhibition of bacterial growth by free vancomycin. Shaded bar, 2 mins incubation; empty bar, 15 mins incubation; dotted bar, 30 mins incubation.

concentrations of the free drug examined (2-5 μg ml^{-1}), inhibition was only seen after 30 mins.

Various levels of entrapment of vancomycin were seen when preparing the vesicles (see Section 3.2.2). The effect of incubation time was studied using liposomes that had relatively high and relatively low encapsulation of the drug, where the concentration of the drug within liposomes was calculated in terms of the amount of vancomycin per mg of liposomal lipid - the higher this number, the greater the encapsulation.

(a) Neutral liposomes

The uncharged vesicles with relatively low entrapment of drug (Fig. 3.2.7) showed little time dependence, although no inhibition was seen after 2 mins incubation at any concentration used. With higher entrapment (Fig. 3.2.8), a greater dependence on incubation time was seen, with the incubation after 2 mins and after 15 mins falling off gradually between 50 and 30 μg ml^{-1} and between 50 and 20 μg ml^{-1} respectively. With 30 mins incubation, significant (around 20 %) inhibition was still observed at 20 μg ml^{-1}.

(b) Cationic liposomes - no cholesterol

With these vesicles, there was no significant inhibition seen after 2 mins incubation. There was little difference between the inhibition observed after 15 mins and 30 mins, either with relatively low (Fig. 3.2.9) or relatively high (Fig. 3.2.10) encapsulation of drug.

(c) Cationic liposomes - with cholesterol

With both relatively low (Fig. 3.2.11) and relatively high (Fig. 3.2.12) vancomycin entrapment within these liposomes, the inhibition after 2 mins incubation fell

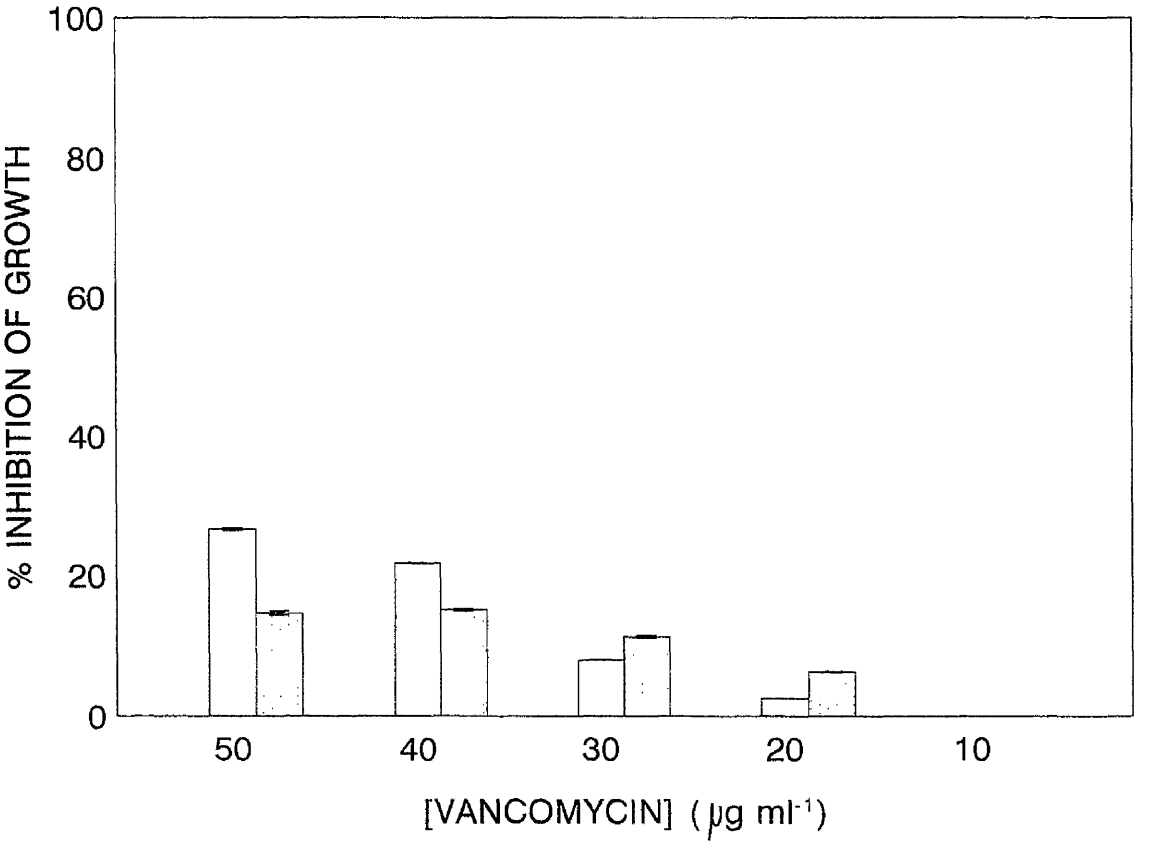


Fig. 3.2.7 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in neutral liposomes (DPPC/Chol, molar ratio 1:0.21, 0.081 mg vancomycin/mg liposomal lipid). Empty bar, 15 mins incubation; dotted bar, 30 mins incubation. No inhibition seen with 2 mins incubation over the range of drug concentrations studied.

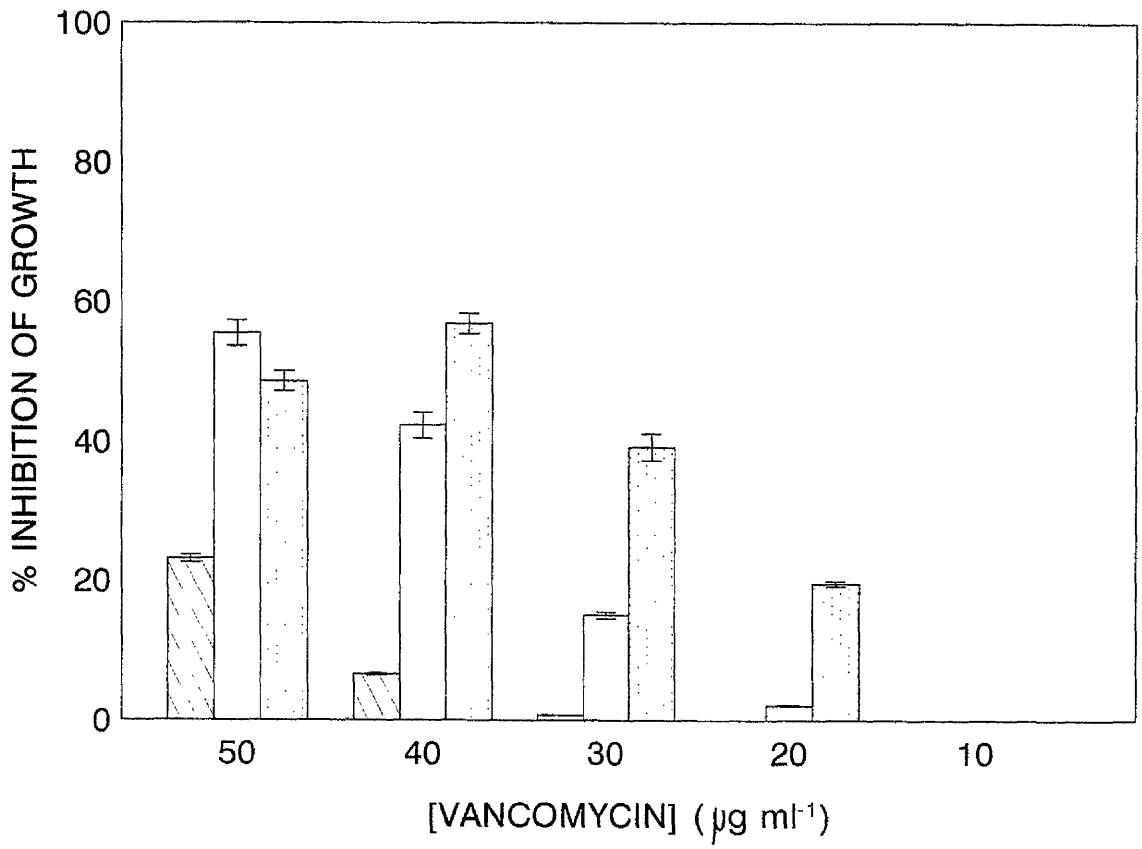


Fig. 3.2.8 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in neutral liposomes (DPPC/Chol, molar ratio 1:0.21, 0.132 mg vancomycin/mg liposomal lipid). Shaded bar, 2 mins incubation; empty bar, 15 mins incubation; dotted bar, 30 mins incubation.

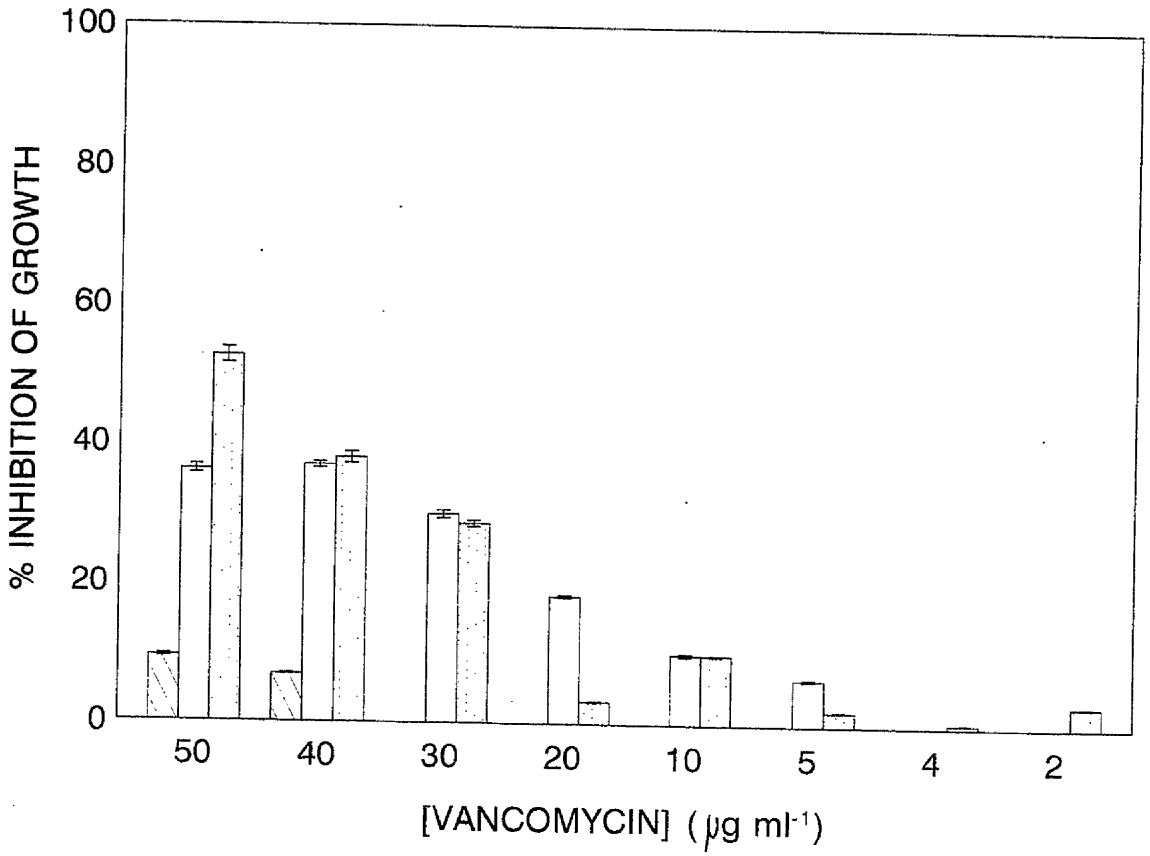


Fig. 3.2.9 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in cationic liposomes with no cholesterol (DPPC/DDAB, molar ratio 1:0.17, 0.066 mg vancomycin/mg liposomal lipid). Shaded bar, 2 mins incubation; empty bar, 15 mins incubation; dotted bar, 30 mins incubation.

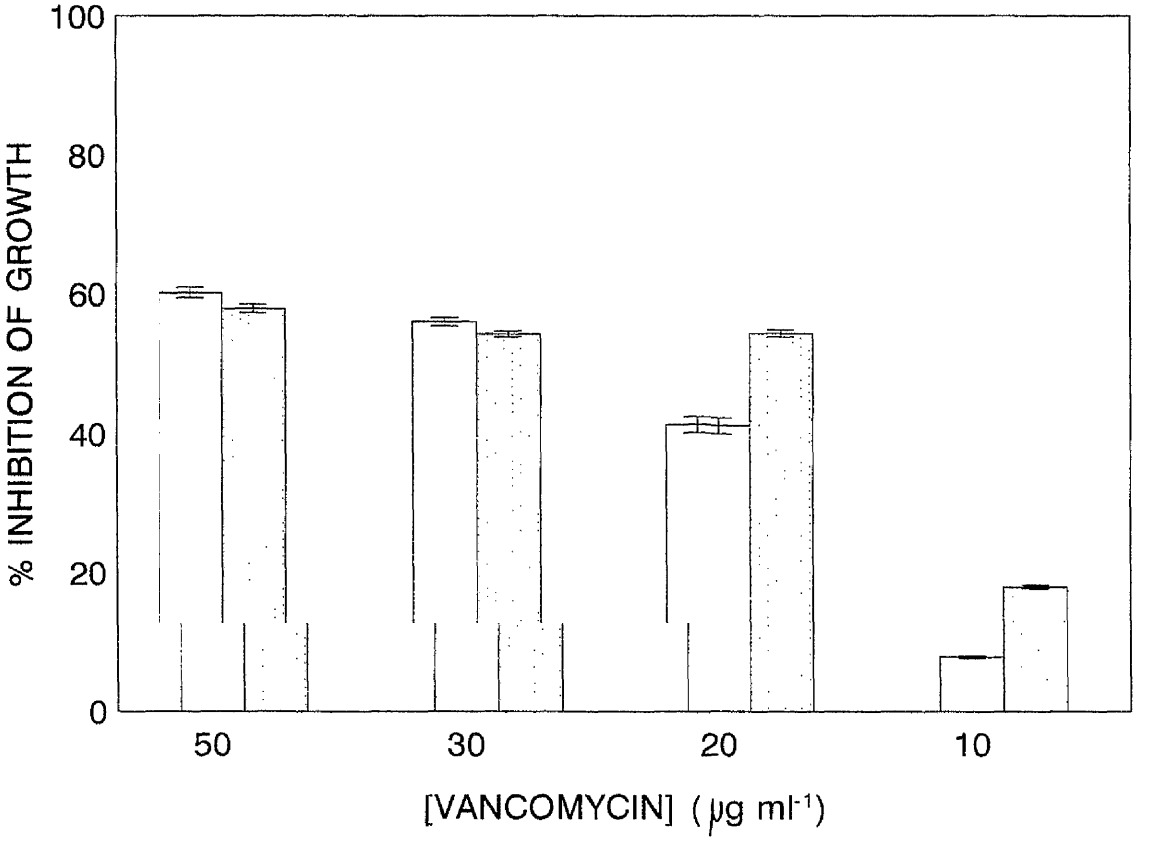


Fig. 3.2.10 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in cationic liposomes with no cholesterol (DPPC/DDAB, molar ratio 1:0.17, 0.173 mg vancomycin/mg liposomal lipid). Empty bar, 15 mins incubation; dotted bar, 30 mins incubation. 2 mins incubation not done.

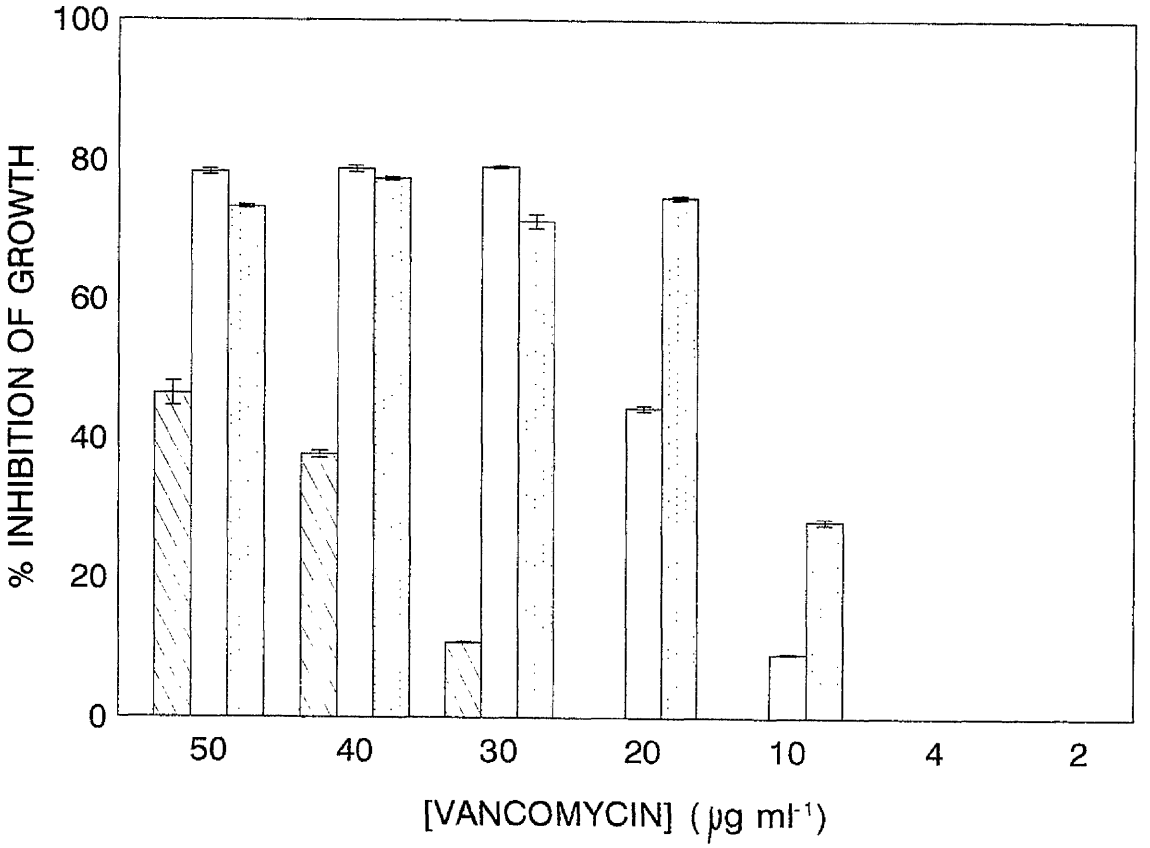


Fig. 3.2.11 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22, 0.080 mg vancomycin/mg liposomal lipid). Shaded bar, 2 mins incubation; empty bar, 15 mins incubation; dotted bar, 30 mins incubation.

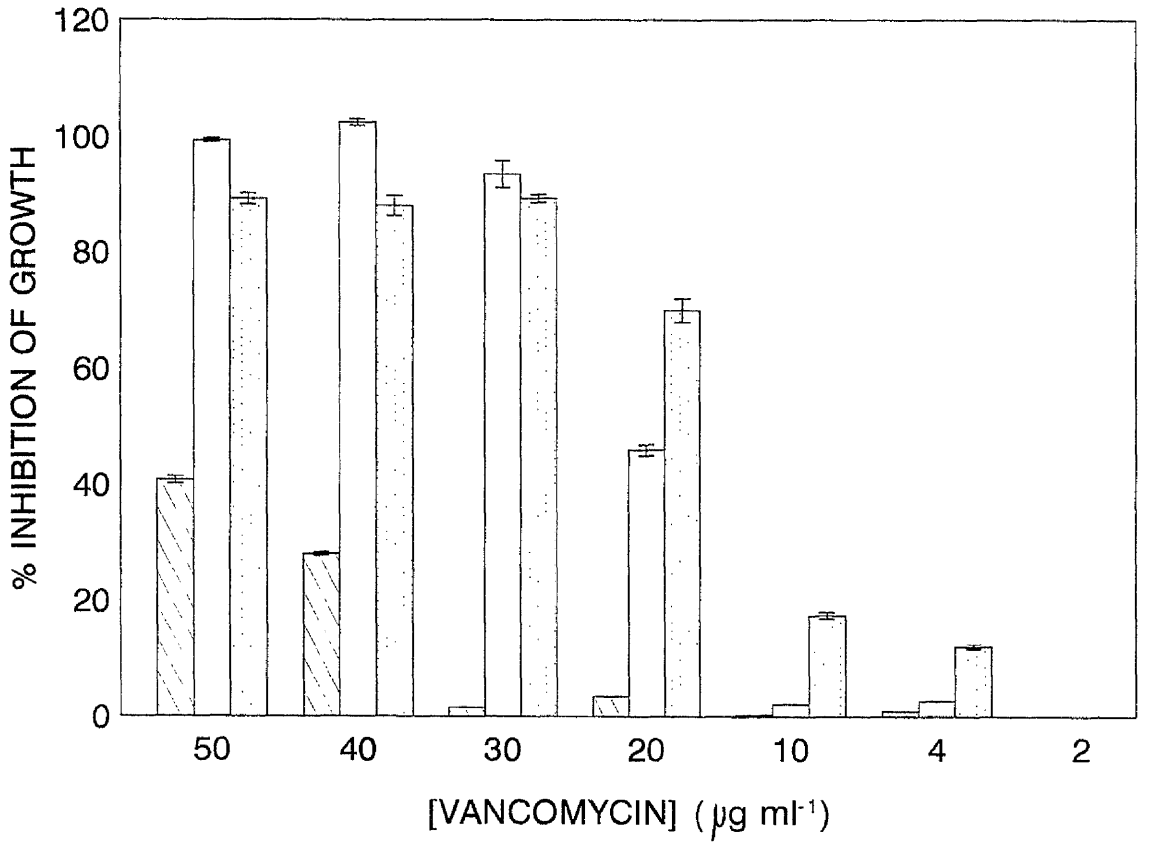


Fig. 3.2.12 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22, 0.147 mg vancomycin/mg liposomal lipid). Shaded bar, 2 mins incubation; empty bar, 15 mins incubation; dotted bar, 30 mins incubation.

between 50 and 20 μg ml^{-1}. With both sets of liposomes, there was little difference seen with 15 mins and 30 mins incubation between 50 and 30 μg ml^{-1}, but at 20 μg ml^{-1} and below there was significantly more inhibition after 30 mins than after 15 mins.

(d) Cationic liposomes - high cholesterol content

At relatively low levels of encapsulation with these liposomes (Fig. 3.2.13), a significantly larger degree of inhibition was seen after 30 mins incubation compared with 15 mins at vancomycin concentrations of 20 μg ml^{-1} and lower. With relatively high levels of encapsulation (Fig. 3.2.14), there was little difference in inhibition after 15 mins and 30 mins incubation at concentrations of vancomycin of 10 μg ml^{-1} and above, but below this the 30 mins incubation was more effective.

3.2.5 Vancomycin - Effect of Level of Encapsulation in Liposomes

As indicated above, the inhibition of bacterial growth by liposomes was dependent on the concentration of vancomycin within the liposomes. In other words, at a fixed concentration of vancomycin, the inhibition varies depending on the liposomal lipid concentration needed to achieve that concentration. The effectiveness of various concentrations of vancomycin was examined using several preparations of liposomal vancomycin with different degrees of drug entrapment. Their relative effectiveness was examined after incubation periods of 2, 15 and 30 mins.

(a) 2 mins incubation

With this short incubation period, there is little dependence of inhibition on entrapment. The free drug seemed more effective at inhibiting growth than the equivalent concentration of vancomycin in any of the liposomal formulations with a relatively large amount of drug encapsulated (Fig. 3.2.15). With free vancomycin, significant inhibition (10-20%) was seen with concentrations as low as 20-30 μg ml^{-1}.

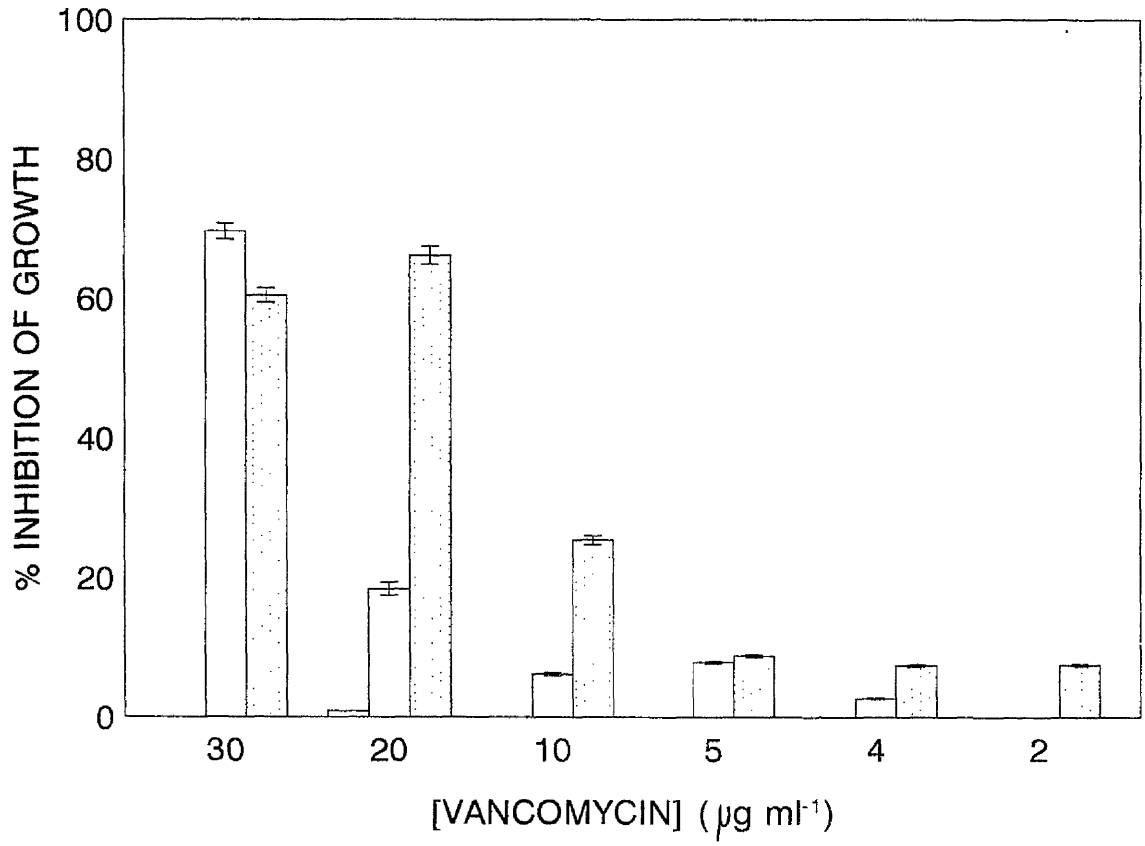


Fig. 3.2.13 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34, 0.019 mg vancomycin/mg liposomal lipid). Shaded bar, 2 mins incubation; empty bar, 15 mins incubation; dotted bar, 30 mins incubation.

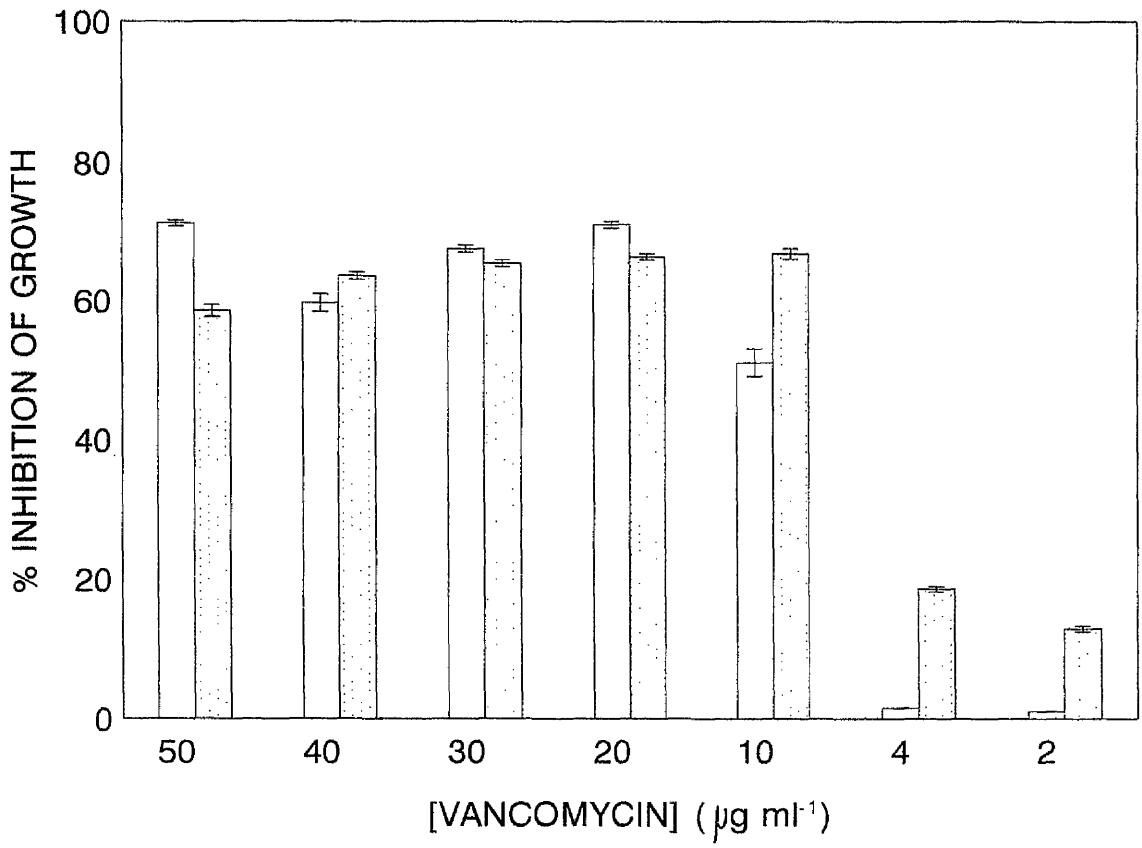


Fig. 3.2.14 Effect of incubation time on the inhibition of bacterial growth by vancomycin encapsulated in cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34, 0.315 mg vancomycin/mg liposomal lipid). Empty bar, 15 mins incubation; dotted bar, 30 mins incubation. 2 mins incubation not done.

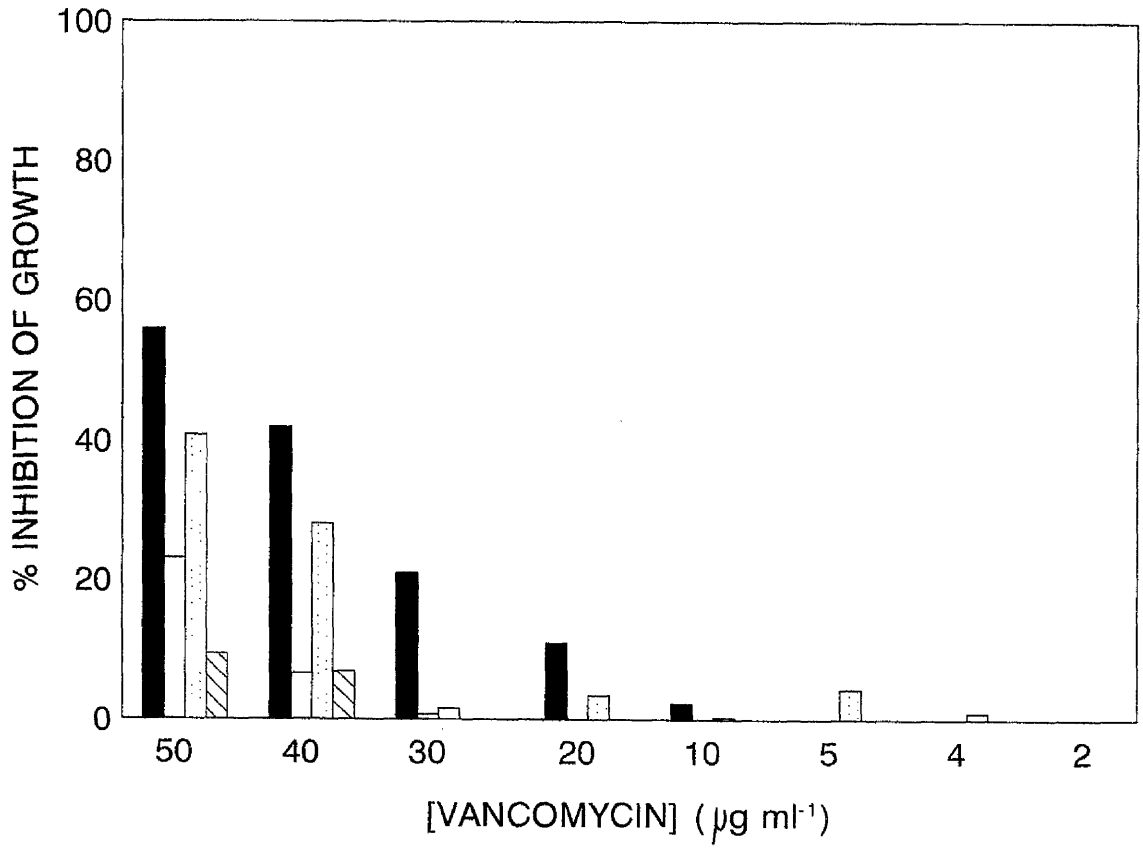


Fig. 3.2.15 Effectiveness of vancomycin encapsulated in neutral and cationic liposomes in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, vancomycin encapsulated in neutral liposomes (DPPC/Chol, molar ratio 1:0.21, 0.132 mg vancomycin/mg liposomal lipid); dotted bar, vancomycin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22, 0.147 mg/mg lipid); shaded bar, vancomycin encapsulated in cationic liposomes without cholesterol (DPPC/DDAB, molar ratio 1:0.17, 0.066 mg/mg lipid). Incubation time 2 mins.

With liposomes, no significant inhibition was seen with any of the formulations at concentrations lower than 40 μg ml^{-1}.

The cationic liposomes containing cholesterol were the most effective vesicles for inhibiting growth, and at the higher drug concentrations examined (40-50 μg ml^{-1}) showed levels of incubation that were only some 10-15 % lower than the levels seen with the free drug. They were more effective than the neutral liposomes, perhaps reflecting the fact that even after 2 mins there was substantial attachment of these cationic liposomes (10-20 % monolayer coverage, see Fig. 3.2.3) while there was no attachment of the neutral ones.

The cationic liposomes that did not contain cholesterol in the membrane were no more effective than the neutral liposomes. This could be due to the much lower degree of drug encapsulation seen with these vesicles: 0.066 mg of vancomycin per mg of liposomal lipid, c.f. 0.132 mg and 0.147 mg for the neutral and cationic-cholesterol liposomes, respectively.

(b) 15 mins incubation

After 15 mins incubation (Fig. 3.2.16), the free drug caused inhibition at concentrations of 10 μg ml^{-1} and above. The critical concentration was between 20 and 10 μg ml^{-1}, where the inhibition fell from 67.4 % (± 3.2 %) to 17.4 % (± 1.9 %).

With the neutral liposomes (Fig. 3.2.16), the encapsulation seemed to decrease the efficiency of the drug, as neither set of liposomes used caused as much inhibition as the free drug did at any concentration of vancomycin. The level of entrapment did seem to have an effect; the vesicles with a relatively high vancomycin content were about twice as effective at inhibiting growth than the liposomes with a lower internal drug concentration.

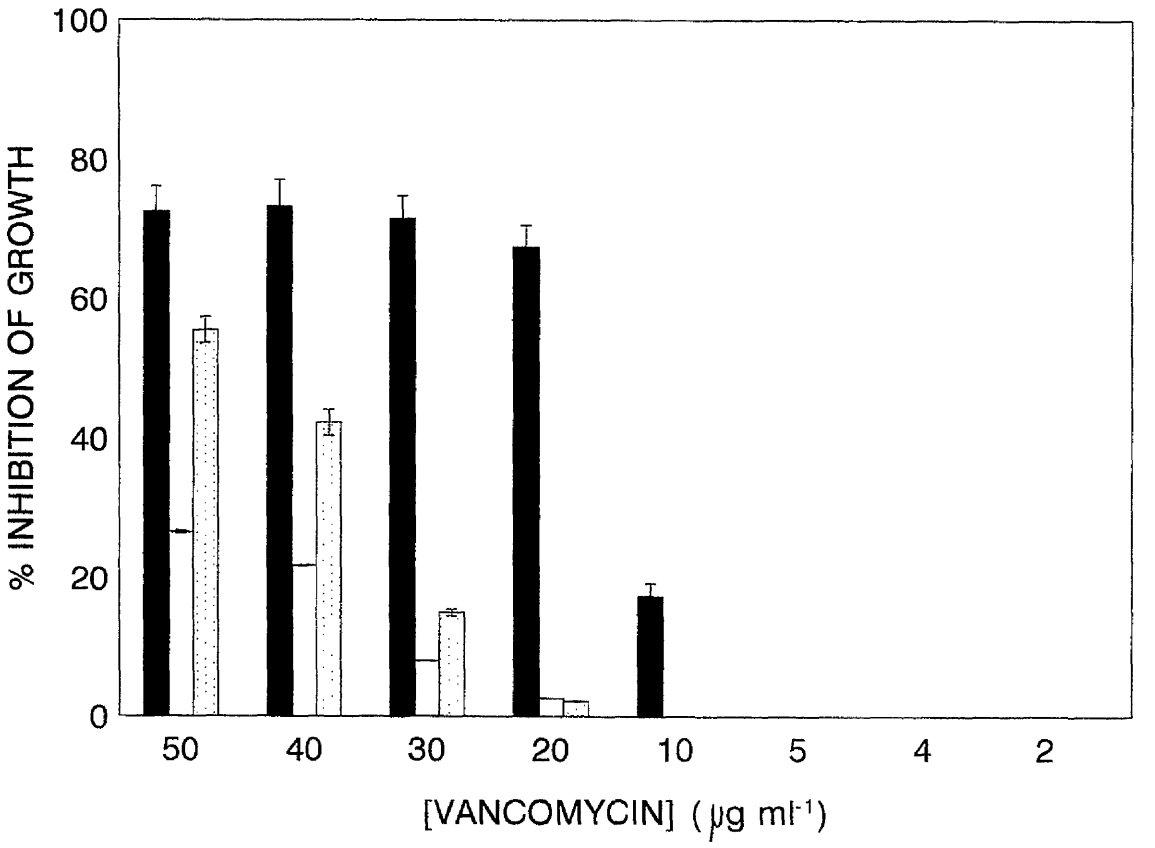


Fig. 3.2.16 Effectiveness of vancomycin encapsulated in neutral liposomes (DPPC/Chol, molar ratio 1:0.21) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.081 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.132 mg/mg lipid). Incubation time 15 mins.

When the drug was encapsulated in cationic liposomes that did not have cholesterol in the membrane (Fig. 3.2.17), a similar effect was seen, where increasing the amount of vancomycin within the vesicles enhanced the antibacterial action at a fixed drug concentration. Between 50 and 20 μg ml^{-1}, the two sets of vesicles with the highest levels of entrapment (0.092 and 0.173 mg/mg lipid) showed inhibition to a slightly smaller extent to that seen with the free drug. However, at a vancomycin concentration of 10 μg ml^{-1} the liposomes with the highest entrapment (0.173 mg/mg lipid) showed a slightly higher degree of inhibition than the free drug did, and all three sets of vesicles showed some significant inhibition (around 10 %) at a vancomycin concentration of 5 μg ml^{-1}, a level at which the free drug did not inhibit growth of *S.epidermidis*.

The cationic liposomes incorporating cholesterol were extremely effective compared to the free drug (Fig. 3.2.18). With the highest degree of encapsulation (0.187 mg/mg lipid) the liposomes caused significantly more inhibition (about 15 % higher) than the free drug at vancomycin concentrations between 50 and 30 μg ml^{-1}. At concentrations of 10 μg ml^{-1} and below, the two sets of vesicles with highest drug entrapment (0.129 and 0.187 mg/mg lipid) were much more effective than the free drug; at 5 μg ml^{-1} and below, the liposomes had considerable antibacterial action (20-30 % inhibition) whereas the free drug had none. The dependence of inhibition on the level of encapsulation was most obvious at vancomycin concentrations of 20 μg $ml^{-1}$and below.

The cationic liposomes with a high cholesterol content similarly showed a dependence of inhibition on the entrapment level, most obviously at concentrations of drug of 20 μg ml^{-1} and below (Fig. 3.2.19). Again, the liposomes caused inhibition at vancomycin concentrations of 5 μg ml^{-1} and below, concentrations at which the free drug was ineffective.

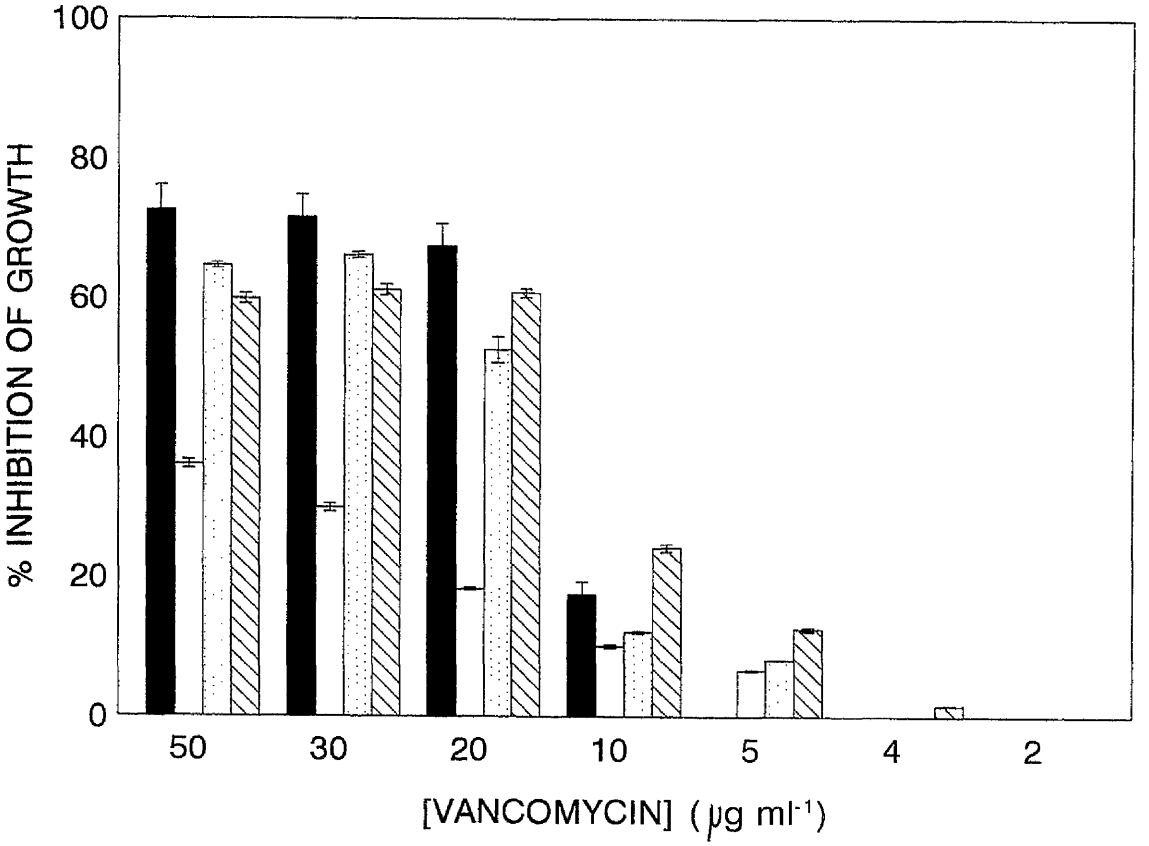


Fig. 3.2.17 Effectiveness of vancomycin encapsulated in cationic liposomes with no cholesterol (DPPC/DDAB, molar ratio 1:0.17) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.066 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.092 mg/mg lipid); shaded bar, liposomes (0.173 mg/mg lipid). Incubation time 15 mins.

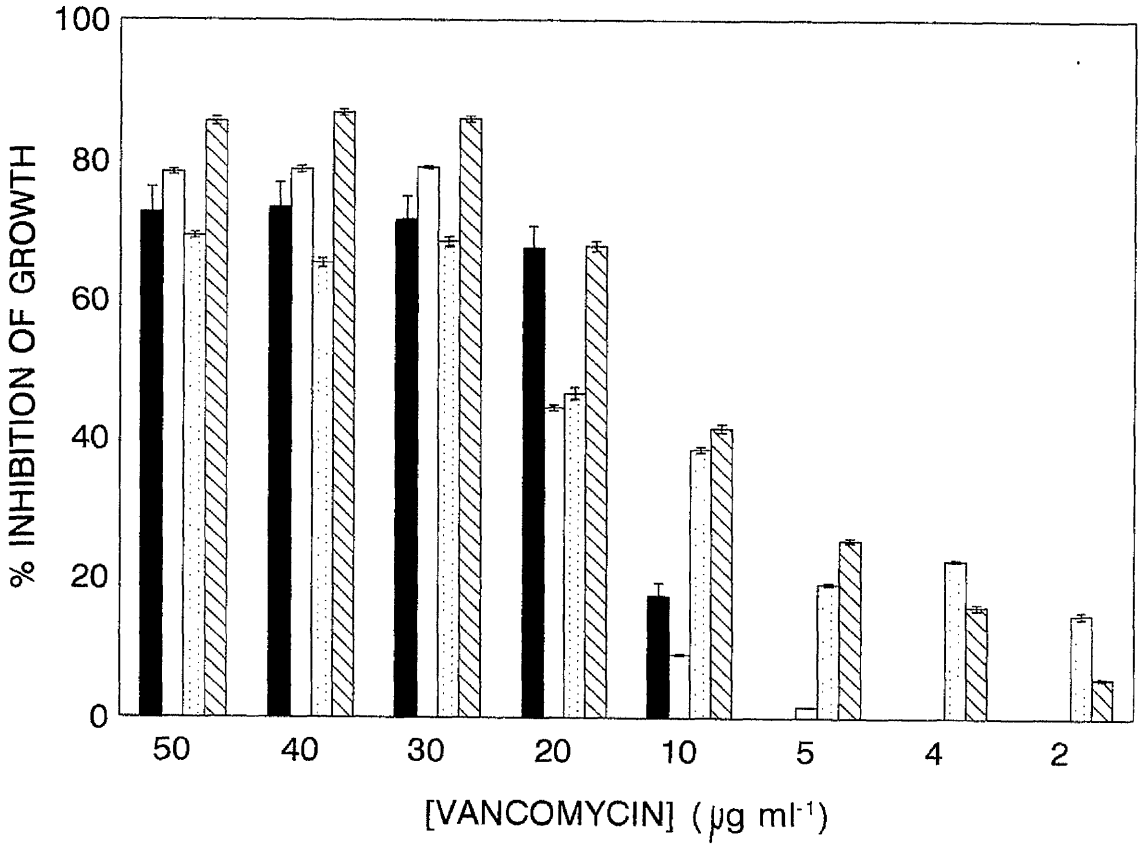


Fig. 3.2.18 Effectiveness of vancomycin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.080 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.129 mg/mg lipid); shaded bar, liposomes (0.187 mg/mg lipid). Incubation time 15 mins.

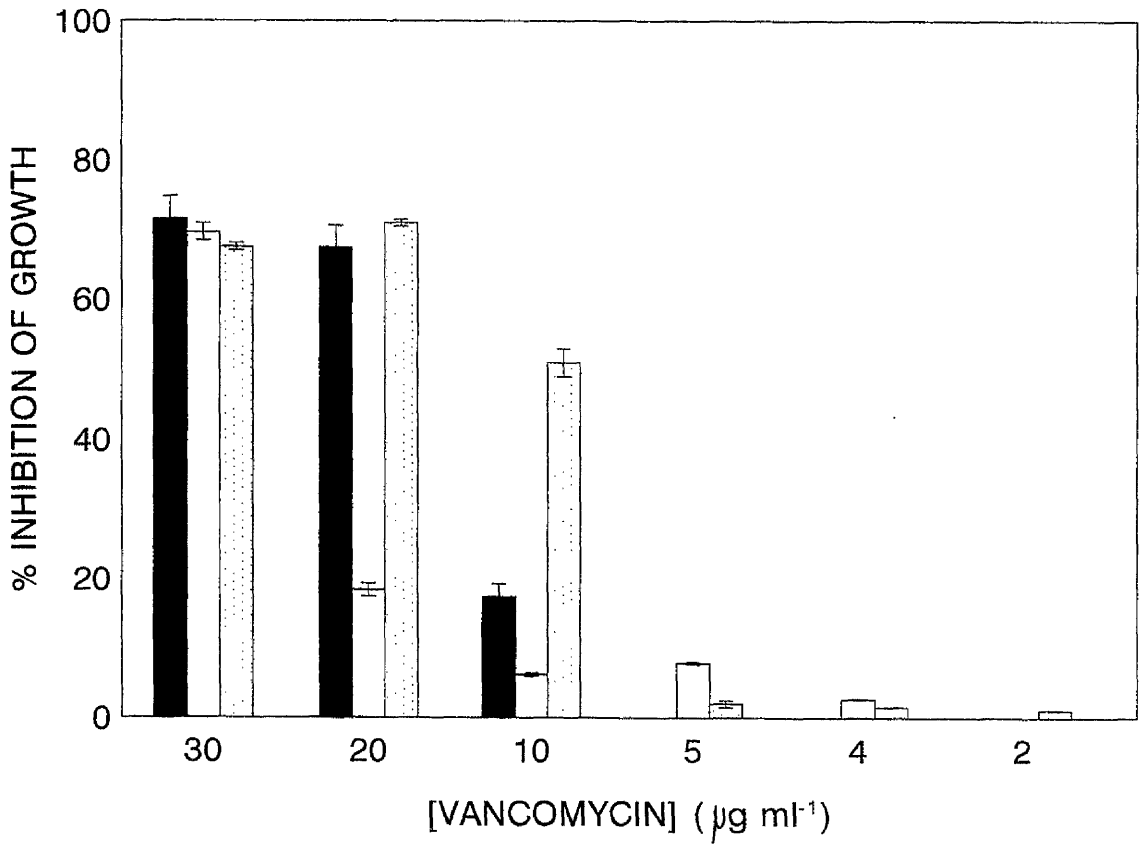


Fig. 3.2.19 Effectiveness of vancomycin encapsulated in cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.019 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.315 mg/mg lipid). Incubation time 15 mins.

(c) 30 mins incubation

After 30 mins (Figs. 3.2.20), the free drug caused a large degree of inhibition of bacterial growth at concentrations between 50 and 20 μg ml^{-1}, with a significant decrease between 20 and 10 μg ml^{-1}. Unlike the 15 mins incubations (see above), there was significant inhibition (around 10 %) at levels of 5 μg ml^{-1} and below.

Encapsulation within neutral liposomes reduced the effectiveness of the drug (Fig. 3.2.20). and no inhibition was seen with the vesicles at drug concentrations of 10 μg ml^{-1} and below. As with the 15 min incubation, the inhibition achieved was dependent on the level of encapsulation.

Similarly, encapsulation in cationic vesicles not incorporating cholesterol reduced the effectiveness of the drug after 30 mins incubation (Fig. 3.2.21). Although the dependence of inhibition on entrapment was again seen, even the vesicles with the highest level of encapsulation (0.173 mg/mg lipid) did not show enhanced antibacterial effects at vancomycin concentrations of 10 μg ml^{-1} and below, unlike after 15 mins incubation with these vesicles where there was enhancement (see above).

As with the 15 mins incubation, the cationic liposomes incorporating cholesterol were very effective (Fig. 3.2.22). With the highest level of entrapment (0.187 mg/mg lipid), the liposomes were either as effective (between 50 and 10 μg ml^{-1}) or more effective (5 μg ml^{-1} and below) than the free drug.

The effect of drug entrapment on inhibition of bacterial growth was also seen with the cationic liposomes with a high cholesterol content (Fig. 3.2.23). This dependence was most pronounced at vancomycin levels of 10 μg ml^{-1} and below, with the liposomes having the highest level of entrapment (0.315 mg/mg lipid) showing enhanced antibacterial effects at this level.

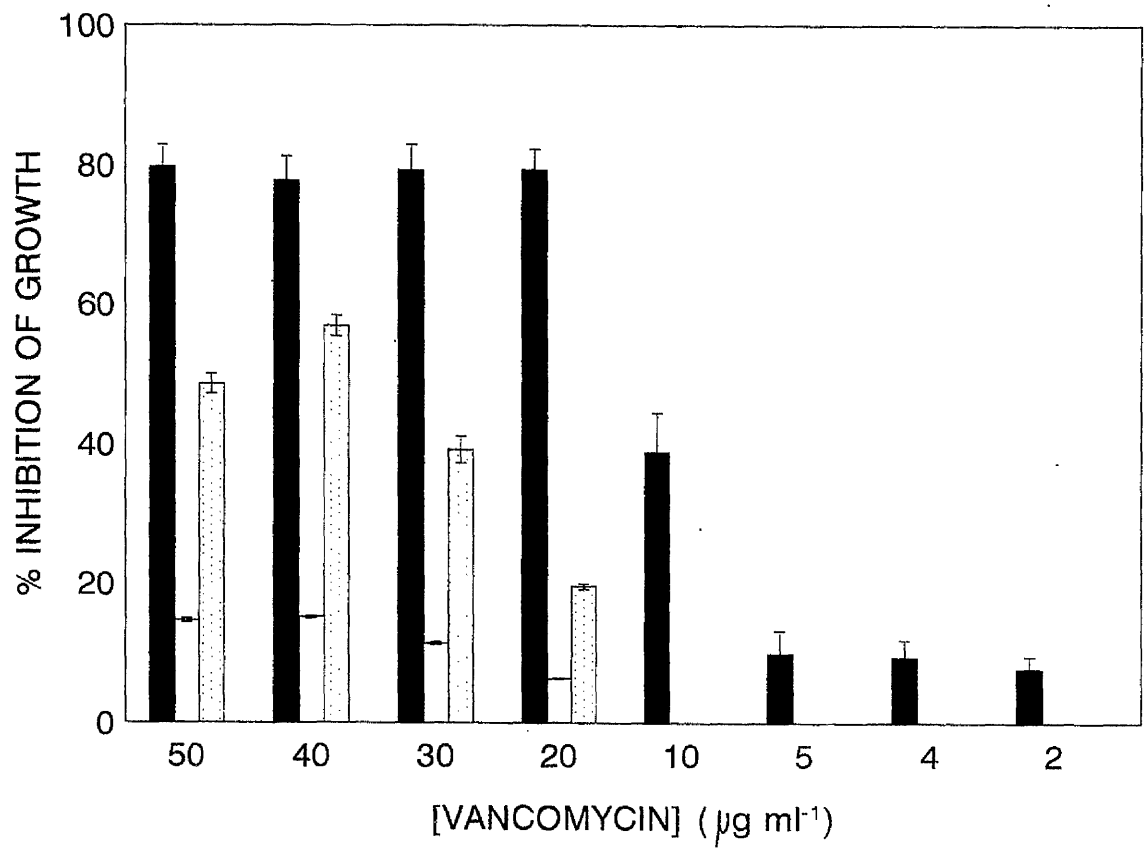


Fig. 3.2.20 Effectiveness of vancomycin encapsulated in neutral liposomes (DPPC/Chol, molar ratio 1:0.21) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.081 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.132 mg/mg lipid). Incubation time 30 mins.

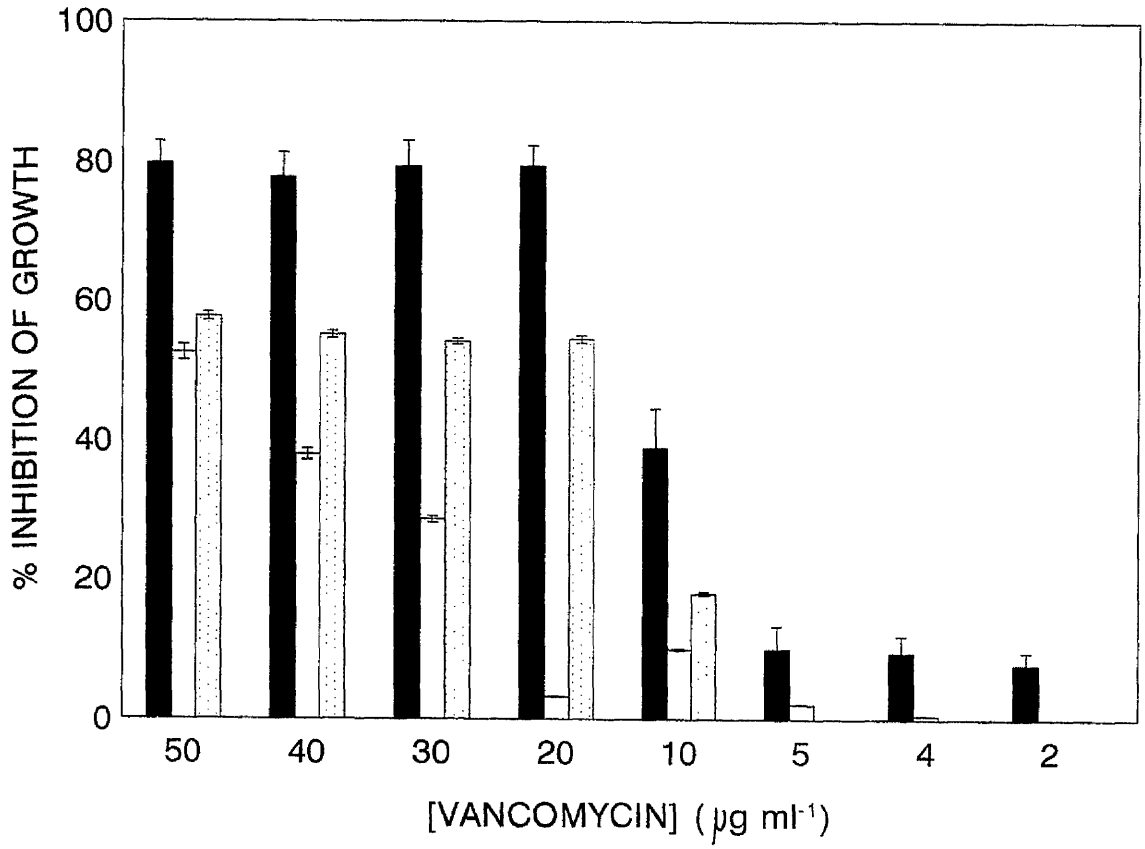


Fig. 3.2.21 Effectiveness of vancomycin encapsulated in cationic liposomes without cholesterol (DPPC/DDAB, molar ratio 1:0.17) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.066 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.173 mg/mg lipid). Incubation time 30 mins.

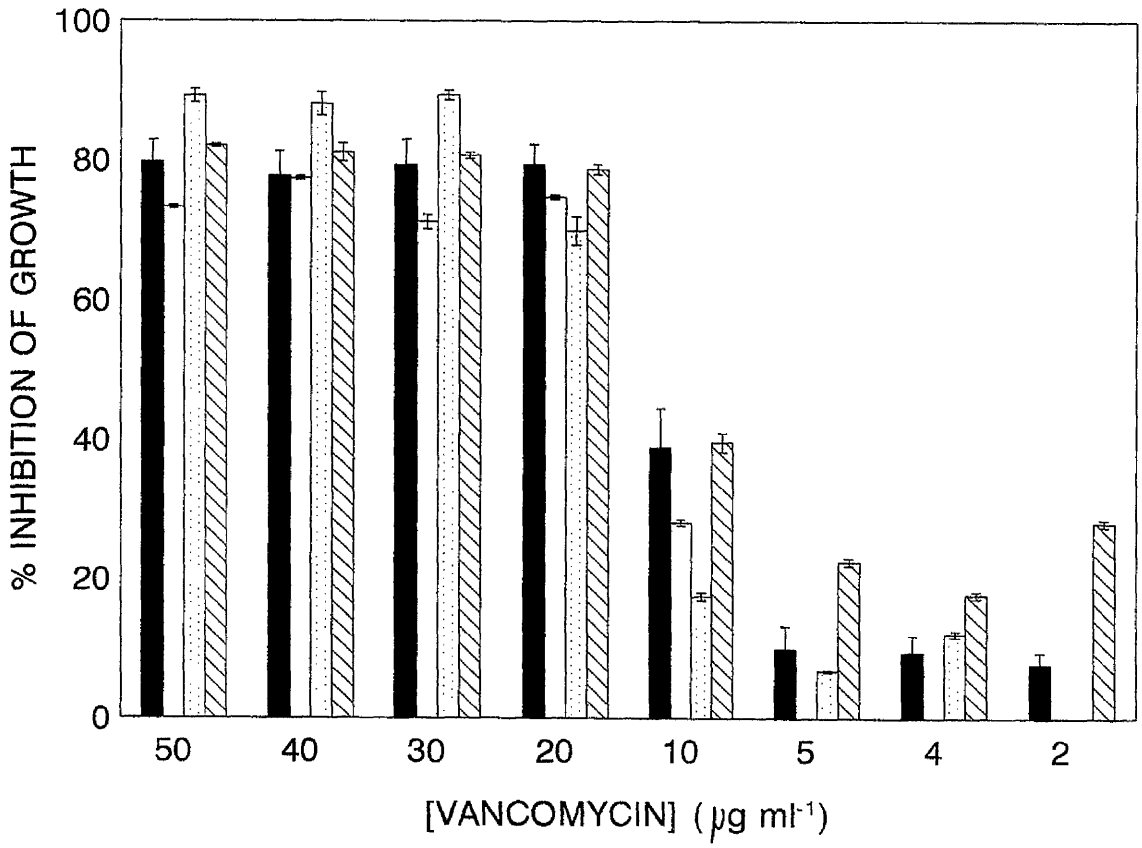


Fig. 3.2.22 Effectiveness of vancomycin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.080 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.147 mg/mg lipid); shaded bar, liposomes (0.187 mg/mg lipid). Incubation time 30 mins.

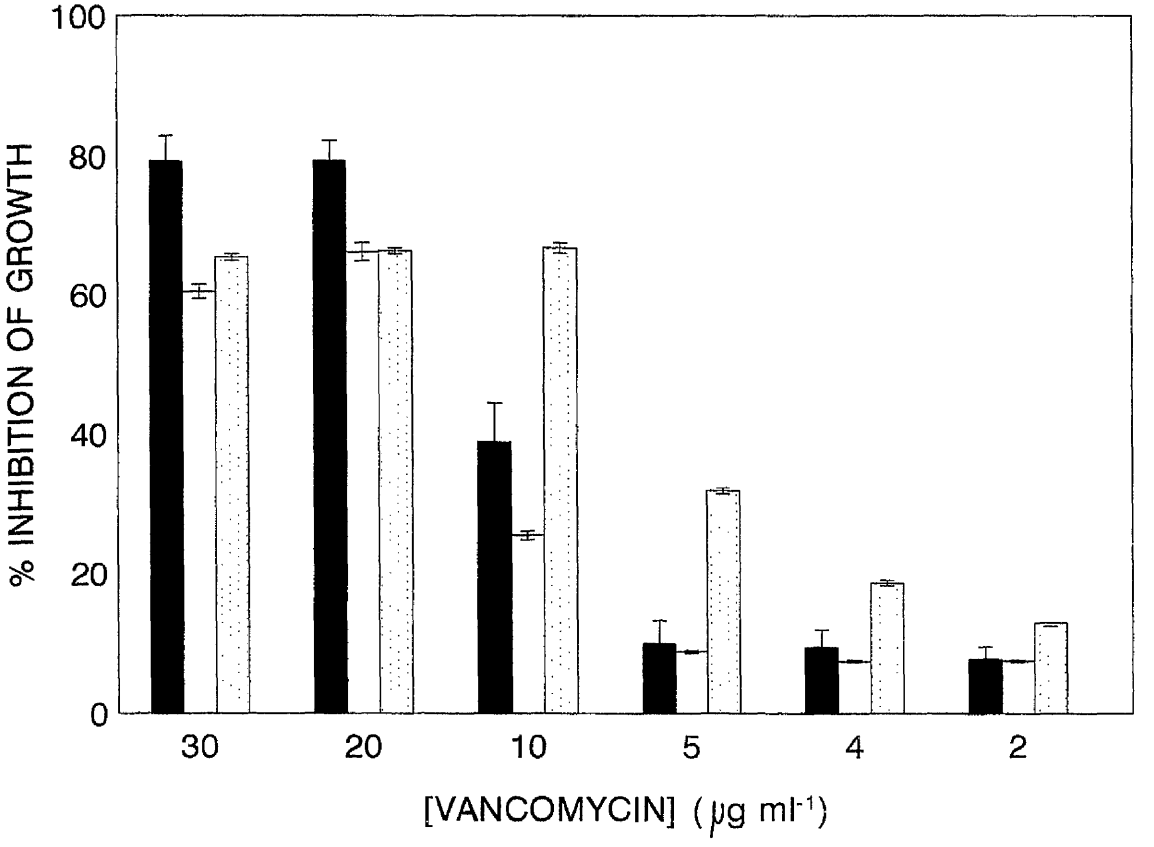


Fig. 3.2.23 Effectiveness of vancomycin encapsulated in cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34) in inhibiting bacterial growth compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.019 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.315 mg/mg lipid). Incubation time 30 mins.

3.2.6 Effectiveness of Liposomal Compositions

As it is not possible to have an exact control over the concentration of a water-soluble substance that is passively encapsulated within liposomes and, as Section 3.2.5 demonstrated, there was a strong relationship between this entrapment level and the effectiveness of liposomally encapsulated vancomycin, it was difficult to compare the different types of liposome used in this work. However, by examining the results obtained from a few sets of the vesicle preparations that did contain similar concentrations of the drug, some differences were observed.

Fig. 3.2.24 compares the effectiveness of neutral and cationic liposomes with encapsulated vancomycin at a level of around 0.080 mg/mg lipid (15 mins incubation). From these results it appeared that the cationic liposomes incorporating cholesterol in the membrane were the most effective at inhibiting growth. The effectiveness of the vancomycin liposomes was in the order

cationic liposomes (low cholesterol)	>	cationic liposomes (no cholesterol)	>	neutral liposomes

For the liposomes with a high cholesterol content (42.26 mole%), there was not a set of vesicles with a similar entrapment level. However, a set with a lower vancomycin concentration (0.019 mg/mg lipid) caused inhibition of growth similar to that seen with the low cholesterol content (18.79 mole%) vesicles, even though those vesicles had a higher level of encapsulation.

Fig. 3.2.25 compares neutral and cationic liposomes with higher levels of entrapment (around 0.130 mg/mg lipid). Again, the cationic liposomes were much more effective at inhibiting growth than the neutral ones. These results were contrasted to those obtained with the high-cholesterol cationic liposomes. Even with a higher entrapment

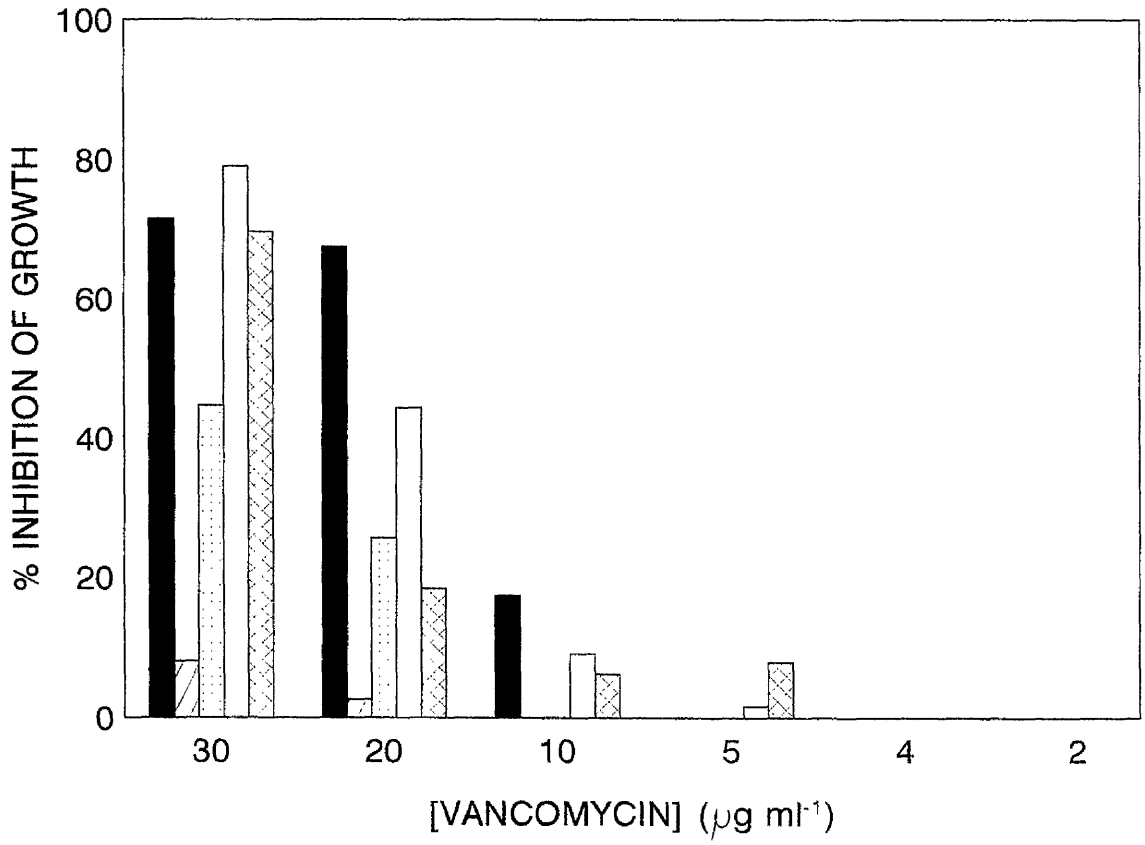


Fig. 3.2.24 Comparison of bacterial growth caused by free vancomycin and by vancomycin encapsulated in cationic and neutral liposomes with similar levels of drug entrapment. Closed bar, free vancomycin; shaded bar, vancomycin in neutral liposomes (0.081 mg vancomycin/mg liposomal lipid); dotted bar, in cationic liposomes without cholesterol (0.077 mg/mg lipid); empty bar, in cationic liposomes with low cholesterol content (0.080 mg/mg lipid); cross-hatched bar, in cationic liposomes with high cholesterol content (0.019 mg/mg lipid). Incubation time 15 mins, room temperature.

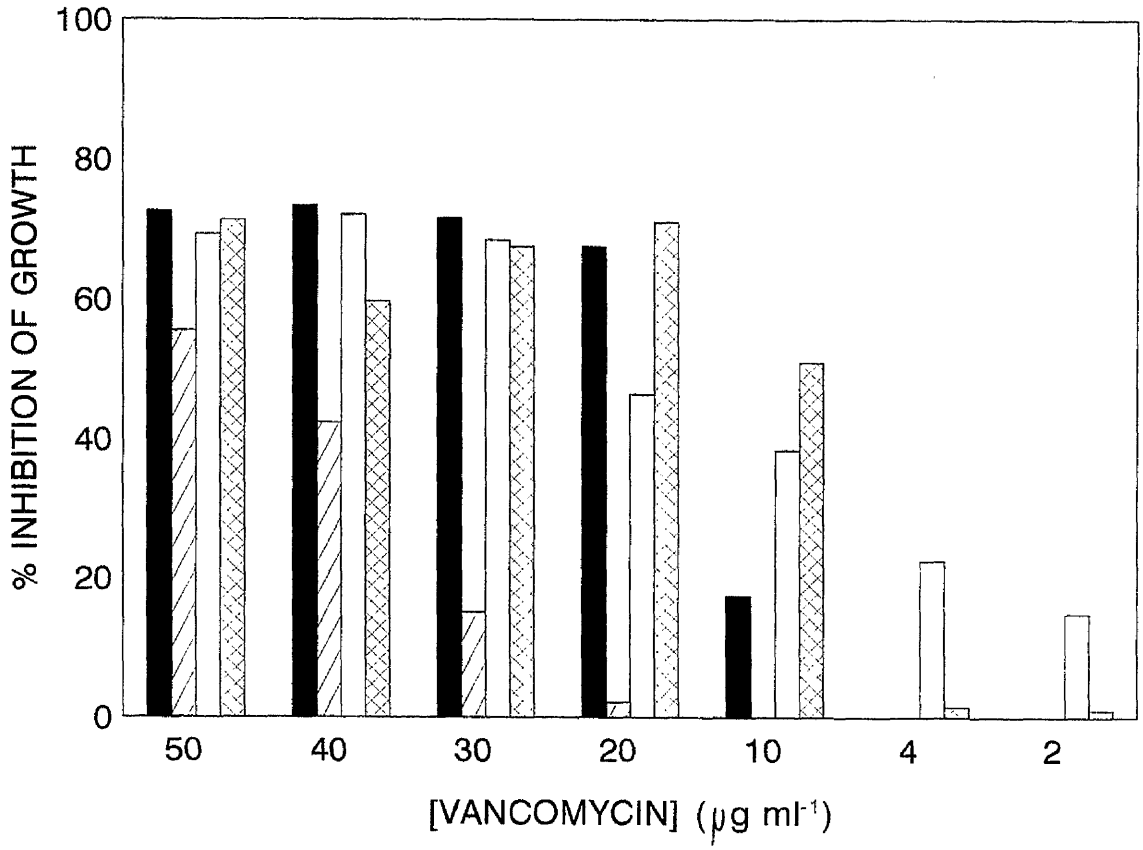


Fig. 3.2.25 Comparison of bacterial growth caused by free vancomycin and by vancomycin encapsulated in cationic and neutral liposomes with similar levels of drug entrapment. Closed bar, free vancomycin; shaded bar, vancomycin in neutral liposomes (0.132 mg vancomycin/mg liposomal lipid); empty bar, in cationic liposomes with low cholesterol content (0.129 mg/mg lipid); cross-hatched bar, in cationic liposomes with high cholesterol content (0.315 mg/mg lipid). Incubation time 15 mins, room temperature.

level (0.315 mg/mg lipid) these cholesterol-rich vesicles were not more effective than the cationic vesicles with the lower cholesterol content, especially at the lower drug concentrations (2-4 μg ml^{-1}). Hence, the final order of effectiveness was established as

cationic liposomes > cationic liposomes > cationic liposomes > neutral liposomes
(low cholesterol) (high cholesterol) (no cholesterol)

3.2.7 Liposomal Encapsulation of Gentamicin

Knowing the conditions that gave the greatest enhancement of bacterial targeting using liposomal vancomycin (cationic liposomes with cholesterol in the membrane, high entrapment of the drug, 15 mins incubation), the effectiveness of liposomally encapsulated gentamicin was examined using similar methods (Fig. 3.2.26).

The inhibition of bacterial growth caused by liposomal gentamicin was comparable to that obtained when the free drug was used at high concentrations of gentamicin (200-800 μg ml^{-1}). However, with lower concentrations of the drug (2-100 μg ml^{-1}), the free gentamicin was much more effective than the liposomal form.

3.2.8 Vancomycin and Gentamicin in Combination

With a view to encapsulating both antibacterial agents together in a liposomal preparation, the activity of vancomycin and gentamicin in combination was compared to the effects of the two agents in isolation (Fig. 3.2.27). None of the combinations of the free drugs tested were more effective at inhibiting bacterial growth than the sum of the two free drugs used in isolation. Indeed, if anything, the gentamicin seemed to inhibit the action of the vancomycin (or vice versa), e.g. 10 μg ml^{-1} vancomycin caused 57.7 % (± 0.8 %) inhibition of growth whereas 10 μg ml^{-1} in combination with 30 μg ml^{-1} gentamicin (25 % vancomycin : 75 % gentamicin)

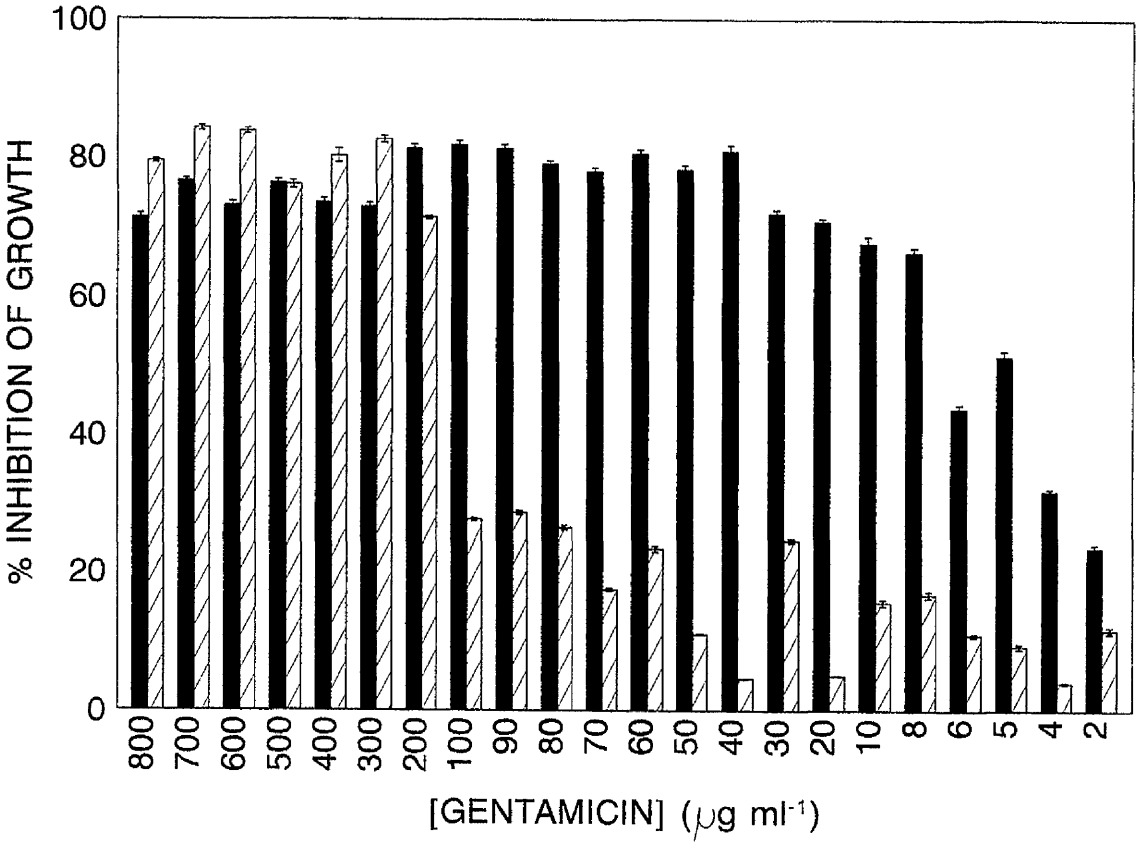


Fig. 3.2.26 Effectiveness of gentamicin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) in inhibiting bacterial growth compared to free gentamicin. Closed bar, free gentamicin; shaded bar, liposomal gentamicin (0.354 mg gentamicin/mg liposomal lipid). Incubation time 15 mins.

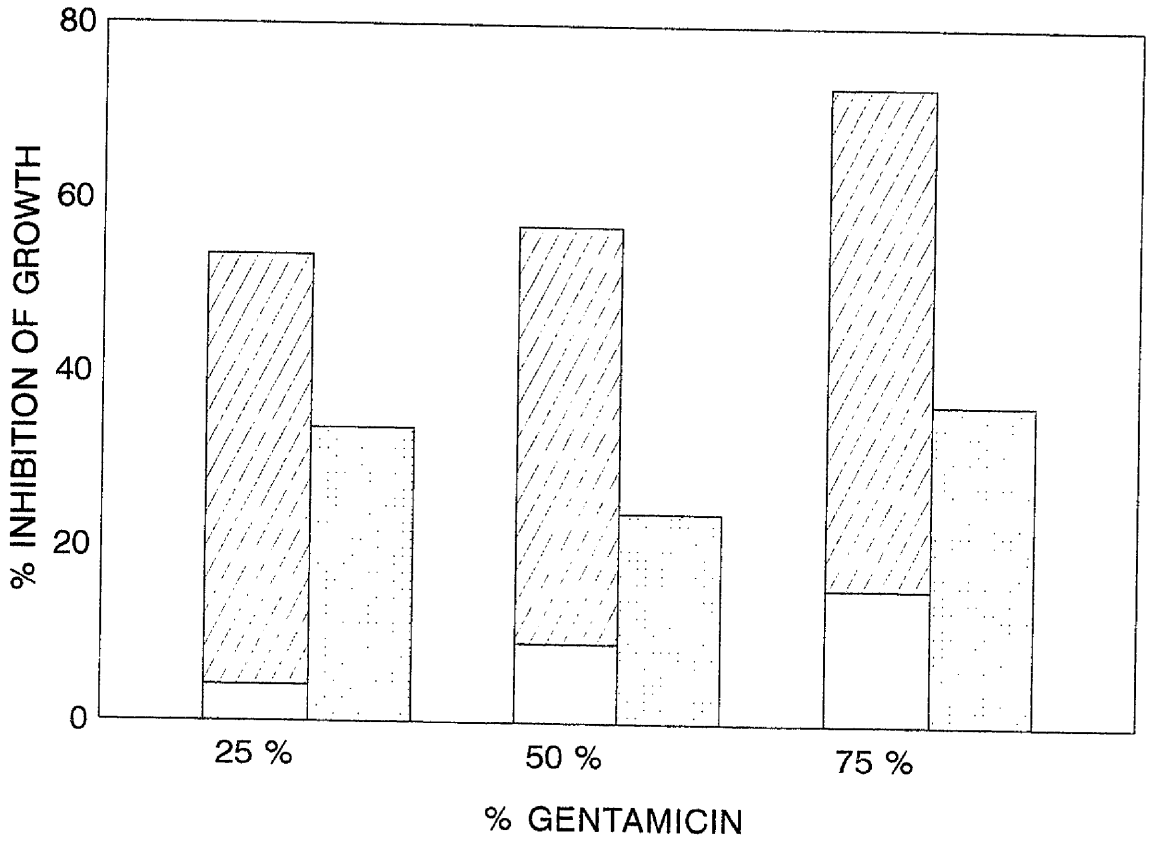


Fig. 3.2.27 Effect of gentamicin and vancomycin used in combination on inhibition of bacterial growth. Left-hand bar indicates the sum of the inhibition caused by the two drugs in isolation (shaded portion of the bars indicate inhibition caused by vancomycin; empty portion of the bars represents inhibition caused by gentamicin). Right-hand bar indicates inhibition caused by the two drugs in combination. Incubation time 15 minutes, total drug concentration 40 $\mu g\ ml^{-1}$.

caused only 33.5 % (± 0.3 %) inhibition of growth. After obtaining these results, it was decided not to try and entrap a combination of the agents within vesicles.

CHAPTER FOUR

DISCUSSION

Discussion

4.1 INTERACTION OF LIPOSOMES WITH BACTERIA

4.1.1 Characterization of Liposome Size

The size of the liposomes was relatively independent of liposome composition (see Table 3.1). The peak size in most cases was in the region of 115-130 nm, reflecting the pore size of the filters used in the extruder (100 nm). The fact that the vesicle diameters were slightly larger than the pore size could be an indication of the ability of the liposomes to "squeeze" through the pores. With the DDAB liposomes, there was a small increase in the peak size as the relative amount of DDAB in the membrane was increased from 2.81 % to 12.62 % (compositions C-G). The only composition that gave a significantly different peak size was with the DDAB liposomes where cholesterol was omitted altogether (composition L; 85.4 mole% DPPC, 14.6 mole% DDAB) which had a peak size of 96.3 nm, substantially smaller than with similar liposomes which did contain cholesterol (e.g. composition J with 28.1 mole% cholesterol; peak size 122 nm). This could be a reflection of the fact that the cholesterol-free liposomes required a larger amount of nitrogen gas pressure for extrusion than the vesicles incorporating the sterol did (500 psi c.f. 200-300 psi with cholesterol present). A slight dependency of vesicle size on extrusion pressure has previously been noted when 100 nm pore filters have been used [Kölchens et al, 1992], with larger pressure producing smaller vesicles, but not to the extent seen here. The presence of the cholesterol could have led to the formation of larger vesicles on extrusion, although Elorza *et al* [1993] reported little difference in size when analyzing extrusion of PC liposomes, with and without cholesterol, through 100 nm filters. Unfortunately, their work does not indicate the mole% of cholesterol included in the vesicles they used and so it is difficult to draw any conclusions on this point from their results.

4.1.2 Targeting Assays

For the targeting assays, the liposome samples were applied to biofilms of bacteria formed by adsorption onto the surface of microtitre plate wells from *S.epidermidis* suspensions which had an absorbance 0.5 at 550nm, leading to a close packed multilayer of cells that can be visualized by electron microscopy [Kaszuba et al, 1995]. Labelling of the bacteria with [^{3}H]-thymidine showed that each well contained approximately 5.16 x 10^6 bacterial cells (see Fig. 3.1.15). The extent of adsorption of liposomes to this surface is calculated in terms of %amc (see Section 3.1.2). The area of the bacterial surface is known and because the diameter of the liposomes has been measured (see Section 4.1.1), it is possible to calculate how many vesicles would need to adsorb to completely cover this surface, giving a %amc of 100%. It should be noted that the fact that some of the values of %amc obtained are over 100% does not necessarily imply that the liposomes are forming a multilayer on the biofilm. As the %amc is calculated with reference to this geometric surface area of the biofilm (microtitre plate well), these calculations will, depending on the surface roughness of the biofilm, 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.

4.1.3 Adsorption of Stearylamine-VETs to bacteria

Liposomes incorporating SA have previously been demonstrated to adsorb to *S.epidermidis* biofilms [Jones et al, 1994a]. The authors of that work showed that there was a certain level of specificity to the interaction. At a liposome concentration of 2.1 mM, and with an exposure time of 2 hours, the adsorption of these cationic vesicles to *S.epidermidis* was far in excess of that seen with the other bacteria they studied; the oral bacteria *Streptococcus mutans* and *Streptococcus sanguis* and the

skin-associated bacterium *Proteus vulgaris*. The smallest amount of targeting (20% monolayer coverage) was seen with *Streptococcus sanguis*.

These differences must be caused by variations in the biofilms; either in the characteristics of the bacterial cell surfaces or in the constituents of the extracellular polysaccharide slime.

Staphylococci are Gram-positive bacteria with relatively thick cell walls (around 30 nm) that make up around 10 % of the total volume of the cell (See Fig. 1.2.1). Around 50-60 % of the wall material consists of peptidoglycans, a network of polysaccharide chains (primarily composed of glucosamine and muramic acid) linked by short peptides. Covalently linked to the peptidoglycans are a variety of what are known as "accessory polymers", the most common of which are the group of polyoyl phosphate compounds known collectively as teichoic acids. These polymeric acids are very strongly anionic and give a negative charge to the surface of the bacteria. The components of the cell wall teichoic acids vary depending on the species of bacteria. For *S.epidermidis*, the repeating unit of the polymer chain consists of glycerol with its two primary alcohol groups linked to neighbouring residues by phosphodiester bonds, with glucose (either α-linked or β-linked) as a glycosyl substituent. As a comparison, the teichoic acids of *Streptococcus mutans* (a bacteria to which the stearylamine VETs show relatively little affinity) are also glycerol-based, but with galactose as the substituent [Rogers, 1983].

The extracellular slime is a loose, amorphous substance composed of low-weight and high-weight polymers. The slime is largely held together through ionic interactions. Generally, the polysaccharide substance is made up of amino sugars, uronic acids, polyols and neutral monosaccharides. The major components of the slime produced by *S.epidermidis* are glucose, galactose, glycerol, hexosamine, phosphorous, glycine, alanine and phenylalanine.

4.1.4 Effect of Liposome Concentration on Adsorption

Adsorption of SA-containing liposomes to biofilms of *S.epidermidis* was found to be dependent on liposomal lipid concentration (see Fig. 3.1.5). The %amc increased with liposome concentration, giving values of well over 100%. The plot of adsorption as a function of liposomal concentration is hyperbolic and so can be described in terms of a Langmuir adsorption isotherm [Moore, 1956]. This model is based on the theoretical assumption that free molecules in solution can adsorb to a surface and cover it until a complete monolayer is formed, after which there is no further adsorption. It was originally formulated for the adsorption of gases on solids, e.g. oxygen on tungsten or nitrogen on mica. The isotherm applies to the liposome-bacteria interaction studied here, where an equilibrium exists between adsorbed and bulk-state liposomes. With increasing concentration of liposomes, there is an increase in the %amc coverage of the biofilm. As saturation of the surface is approached, this increase becomes smaller and the %amc levels off, with most of the "sites" for liposome attachment on the bacteria already occupied and so increasing the liposomal concentration has little effect. At saturation, the maximum monolayer coverage possible - designated $(\%amc)_{max}$ - has been attained and, from the concentration of liposomes which gives %amc that is half this maximal value, we can derive a dissociation constant (K_d), as shown in Fig. 4.1.1. If θ is the fraction of the surface area that is covered by adsorbed liposomes in equilibrium with liposomes in the bulk phase (liposomal concentration C) then the equilibrium equation can be written as;

$$\underset{(1-\theta)}{\text{sites vacant}} + \underset{(C)}{\text{liposomes}} \underset{K_1}{\overset{K_2}{\leftrightarrow}} \underset{(\theta)}{\text{sites occupied}} \qquad (8)$$

So the rate of adsorption of liposomes on the surface of biofilm is proportional to the area that is not already covered (1-θ) and to the rate at which the liposomes come into

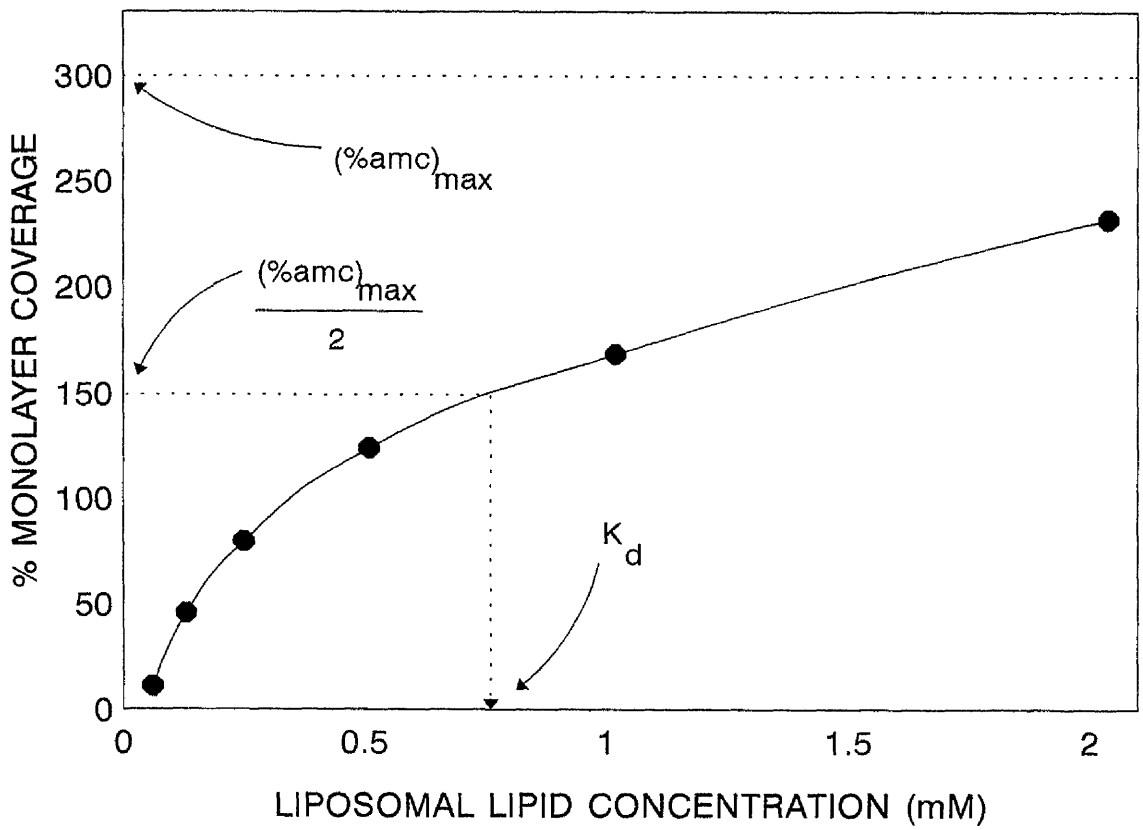


Fig. 4.1.1 The maximum theoretical monolayer coverage and the dissociation constant for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

contact with the bacteria, which for a given temperature varies directly with concentration (C);

$$\text{Rate of adsorption} = K_2\, C(1-\theta) \qquad (9)$$

The desorption of liposomes from the bacterial surface is proportional to the extent of vesicle coverage (θ), assuming that a liposome can desorb from a well covered biofilm as easily as from an almost bare one;

$$\text{Rate of desorption} = K_1\, \theta \qquad (10)$$

We can therefore define association and dissociation constants for the equilibrium;

$$K_d = \frac{K_1}{K_2} \qquad (11)$$

$$K_a = \frac{K_2}{K_1} \qquad (12)$$

At equilibrium, the rate of adsorption of liposomes to the surface equals the rate of desorption, so;

$$K_2\, C(1-\theta) = K_1\, \theta \qquad (13)$$

and so

$$C(1-\theta) = K_d\, \theta \qquad (14)$$

therefore

$$C - C\theta = K_d\, \theta \qquad (15)$$

which gives

$$\theta = \frac{C}{K_d + C} \qquad (16)$$

and so

$$\frac{1}{\theta} = \frac{K_d + C}{C} = K_d \cdot \frac{1}{C} + 1 \qquad (17)$$

The fraction of sites occupied (θ) can also be defined as;

$$\theta = \frac{\%amc}{(\%amc)_{max}} \qquad (18)$$

and by substituting this into equation (17)

$$\frac{(\%amc)_{max}}{\%amc} = K_d \cdot \frac{1}{C} + 1 \qquad (19)$$

and by dividing through with (%amc)max, the final form of the equation is;

$$\frac{1}{\%amc} = \frac{K_d}{(\%amc)_{max}} \cdot \frac{1}{C} + \frac{1}{(\%amc)_{max}} \qquad (20)$$

Values for $(\%amc)_{max}$ and K_d can be derived from this equation if a double reciprocal plot of 1/C against 1/%amc is constructed (Fig. 4.1.2). The slope of the line is equivalent to $K_d/(\%amc)_{max}$. At the point where the line crosses the y-axis, 1/C = 0 and so $(\%amc)_{max}$ = %amc, therefore this intersection gives the value for $(\%amc)_{max}$. K_d can then be derived from the slope; it can also be deduced from the x-axis intersection because at that point, 1/%amc = 0 and so $1/C = -K_d$.

The extent of liposomal adsorption can also be measured in terms of the number of liposomes attached to each bacteria in the biofilm. The number of bacteria in the biofilm on the surface of a microtitre plate well was estimated by labelling the

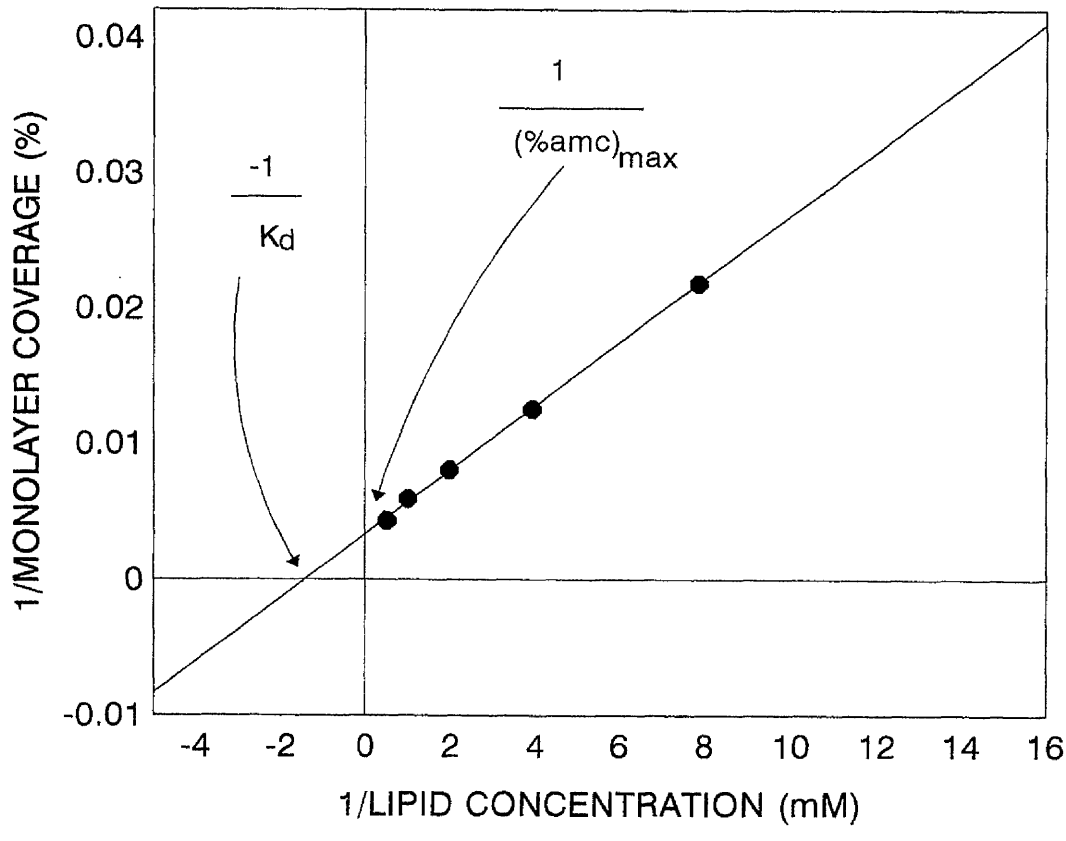


Fig. 4.1.2 Double-reciprocal plot of the data in Fig. 4.1.1 for the derivation of the maximum theoretical monolayer coverage and the dissociation constant for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

bacteria with [^{3}H]-thymidine and using 10% SDS (w/v) to disperse the subsequently formed bilayer. This method gave the number of cells attached per well as 5.16×10^6 ($\pm 0.24 \times 10^6$) as shown in Fig. 3.1.15

This amount is considerably lower than that previously reported by Kaszuba *et al* [1995]. In that paper, the number of attached *S.epidermidis* cells was calculated by measuring the diameter, and hence the projected surface area, of a bacterium using photon correlation spectroscopy. Using the Autosizer, the bacteria were found to have a weight-average diameter of 1518 nm. The number of bacteria was calculated from the exposed area of the microtitre plate well (2.202×10^{-4} m^2) divided by the projected area of one bacterium, giving 1.22×10^8 cells per well, a value more than twenty times greater than the one obtained here using radioactive labelling.

The value obtained by Kaszuba *et al* is unlikely to represent the number of attached cells, principally because the figure they obtained is actually much higher than the number of cells in the 200 μl of bacterial suspension ($OD_{550} = 0.5$) that was exposed to the wells (1.8×10^7 cells).

There are three possible explanations for this discrepancy. Firstly, on attaching to the plate material, the bacterial cells could stretch out, flattening to occupy a greater portion of the biofilm than the projected diameter would suggest. Secondly, the figures could reflect the fact that the biofilm is not wholly composed of bacterial cells. The microorganisms are surrounded by and embedded in the secreted extracellular polysaccharide slime substance which could make up a large proportion of the biofilm surface that the liposomes are exposed to. Thirdly, the method used for dispersing the biofilm for subsequent scintillation counting may only be partially effective. It is possible that the SDS solution used is not of a sufficiently high concentration to detach all the bacteria; non-covalently attached *S.epidermidis* may be removed, but where covalent interactions are involved (e.g. with the bacterial

adhesins), some cells may remain attached. Also, the biofilm may offer some protection from the effects of the detergent. The actual value is probably somewhere between the amount obtained in this work and the result calculated by Kaszuba *et al.*

For these cationic liposomes (21.3 % stearylamine), the mean value of the weight-average diameter was 126.479 nm (± 1.104 nm). Therefore, the projected surface area of one of these liposomes is calculated as;

$$\text{Surface area} = \pi (d_w/2)^2 = \pi (1.26 \times 10^{-7}\ \text{m}/2)^2$$

$$= 1.26 \times 10^{-14}\ \text{m}^2$$

$$\text{Number of liposomes giving 100 \% amc} = \frac{2.202 \times 10^{-4}\ \text{m}^2}{1.26 \times 10^{-14}\ \text{m}^2}$$

$$= 1.75 \times 10^{10}\ \text{liposomes}$$

$$\text{Therefore, liposomes per cell} = \frac{1.75 \times 10^{10}\ \text{liposomes}}{5.16 \times 10^{6}\ \text{bacteria}}$$

$$= 3387\ \text{liposomes per bacterium (at 100 \% amc)}$$

From this value, the number of liposomes attached per bacterium can be calculated for the range of liposomal concentrations used (Fig. 4.1.3).

4.1.5 Effect of Ionic Strength of Media

At each of the medium ionic strengths, the effect of liposome concentration was studied and gave hyperbolic plots of concentration against %amc (see Fig. 3.1.6).

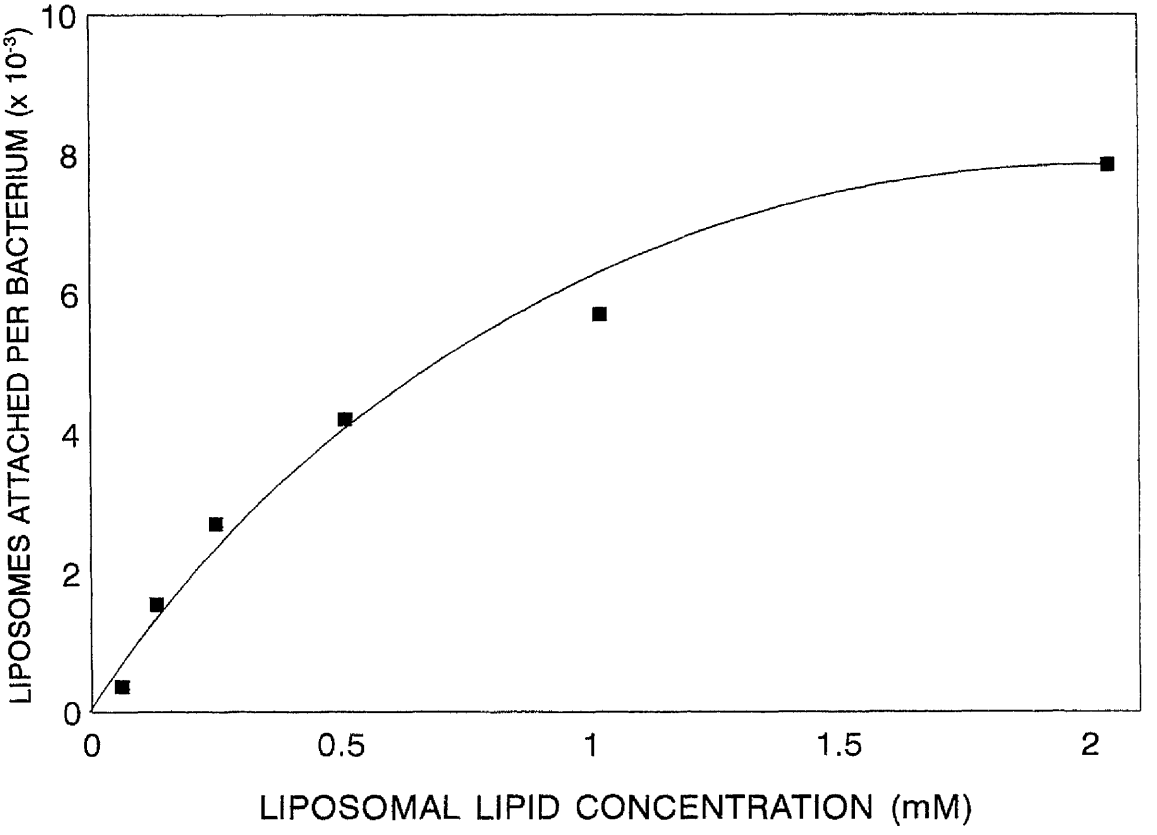


Fig 4.1.3 The dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on liposomal lipid concentration in terms of number of liposomes adsorbed per bacterial cell.

This would indicate that increasing the ionic strength did not affect the ability of individual liposomes to adsorb to and desorb from the biofilm surface, as required by the Langmuir model.

Over the range of liposomal concentrations examined, the effect of increasing the ionic strength was to decrease the level of liposomal adsorption. Thus, the highest levels of attachment were seen at 188 mM and the lowest levels at 696 mM. If these four sets or results are replotted as a double reciprocal graph (Fig. 4.1.4), values for $(\%amc)_{max}$ and K_d can be derived at each ionic strength. Also, from the equation

$$\Delta G_d = -RT \ln K_d \qquad (21)$$

where T is the absolute temperature and R the gas constant, the Gibbs free energy change of dissociation (ΔG_d) can be derived. These sets of values are shown in Table 4.1.1.

The four lines on the double-reciprocal plot cross the y-axis (1/%amc) at roughly the same point, giving similar values for $(\%amc)_{max}$. Therefore $(\%amc)_{max}$ was independent of ionic strength within experimental error. The mean value of $(\%amc)_{max}$ over the four strengths was 300.0 % (± 15.0 %). This means that when the liposomal concentration is extrapolated to infinity (i.e. at the y-intercept, where 1/C = 0) the ionic strength effect is abolished and increasing the ionic strength would not cause a decrease in the extent of liposomal adsorption at this theoretical concentration. It therefore follows that as the liposome concentration approaches infinity, the effect of increasing ionic strength is diminished.

The dissociation constant did vary with ionic strength; K_d was larger at higher values of I (Fig. 4.1.5). In other words, increasing the ionic strength increased the dissociation (the desorption of liposomes from the bacterial surface).

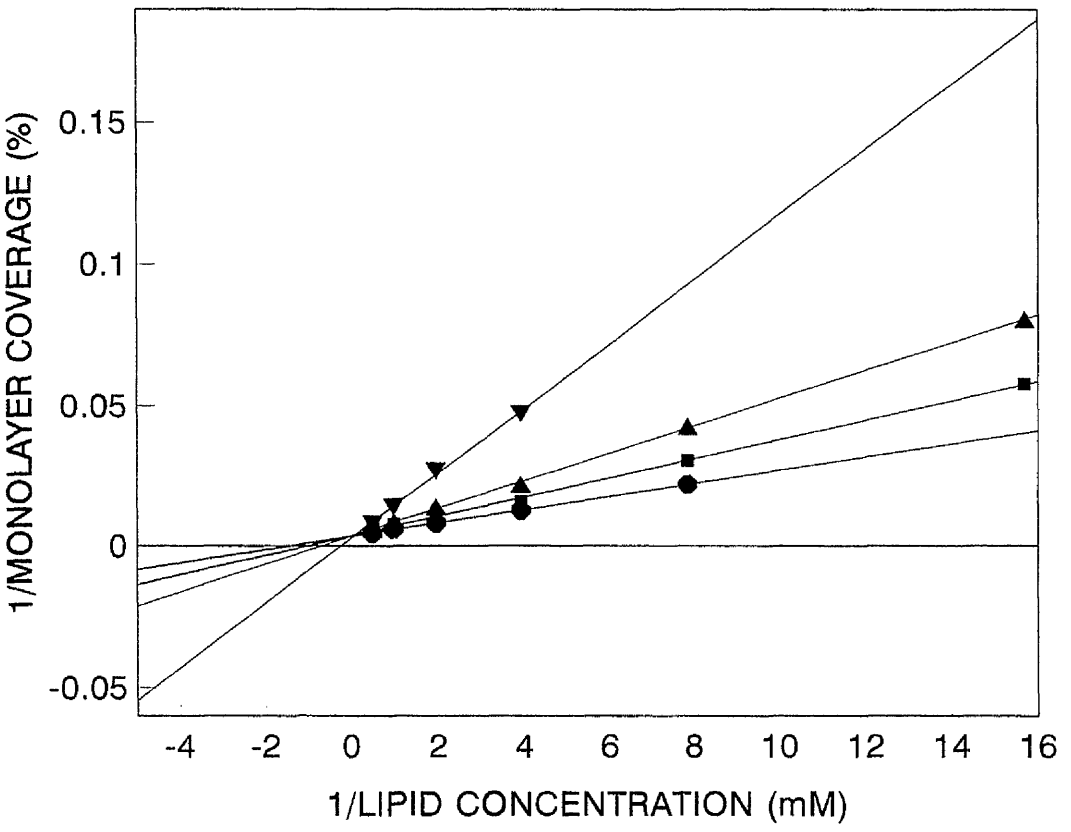


Fig. 4.1.4 Double-reciprocal plot of the data in Fig. 3.1.6, showing the dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours, temperature 37^{o}C.

IONIC STRENGTH (mM)	$(\%amc)_{max}$ (%)	K_d (mM)	ΔG_d (KJ mol^{-1})
188 mM	303.3 (± 24.2)	0.729 (± 0.061)	+18.628 (± 0.060)
357 mM	294.1 (± 36.5)	1.015 (± 0.127)	+17.774 (± 0.057)
526 mM	283.6 (± 32.5)	1.403 (± 0.162)	+16.938 (± 0.055)
696 mM	319.5 (± 117.1)	3.628 (± 1.340)	+14.489 (± 0.047)

Table 4.1.1 Calculated values of the maximum theoretical monolayer coverage ($(\%amc)_{max}$), the dissociation constant (K_d) and the Gibbs free energy change of dissociation (ΔG_d) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms in media of various ionic strengths. Incubation time 2 hours, temperature 37°C.

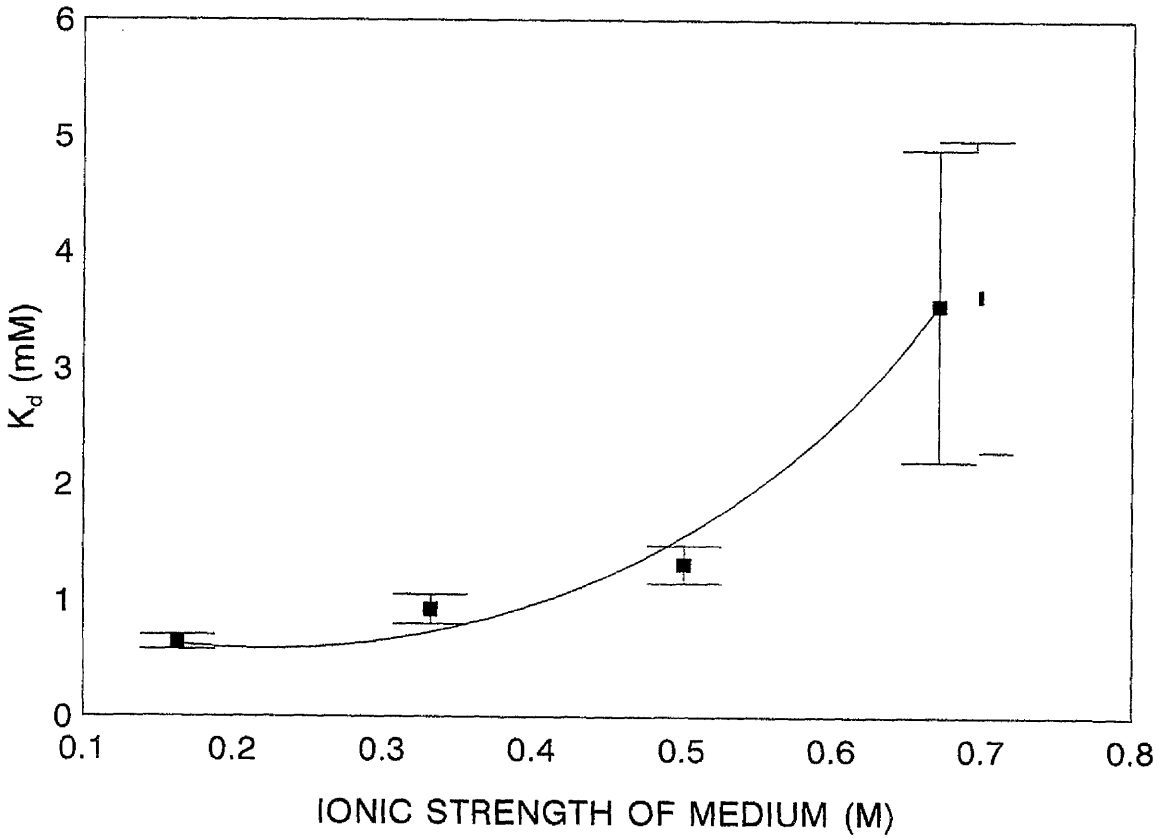


Fig. 4.1.5 Dependence of the dissociation constant (K_d) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms on the ionic strength of the incubation medium. Incubation time 2 hours, temperature 37^{o}C.

In the simplest terms, these effects can be explained in terms of "competition". If the attachment of the vesicles is mediated by electrostatic interaction between a negatively charged surface (the biofilm) and a positively charged surface (the cationic liposomes) then increasing the ionic strength increases the level of free cations and anions in the system. These ions have the ability to compete for the electrostatically charged sites on the two surfaces, diminishing the level of liposome-bacteria interaction. Characteristic of a competition reaction is the fact that extrapolating the liposome concentration to infinity presents a theoretical situation where the saturation level of the vesicles prevents the effect of the competitors (the free ions), so the maximal value of interaction is not affected but the dissociation constant is, as desorption is enhanced by the presence of free ions.

Another way to consider the interaction is that the attraction between the two oppositely charged surfaces is mediated by electrical double layer effects. When a charged surface, such as the outer membrane of the cationic liposomes or the surface of a biofilm of *S.epidermidis*, is in contact with a medium containing ions (e.g. PBS) it will attract a layer of oppositely charged counter-ions and will repel co-ions of the same charge. The concentration of the counter-ions will increase progressively towards the charged surface whilst the co-ion concentration will decrease. Such a region of unequal positively and negatively charged ions is known as a diffuse electrical double layer [Jones, 1975].

Compression of the diffuse double layer and increased ionic screening at the higher ionic strengths weakens the attraction. The screening of ionic interactions is related to the thickness of this 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 [Hunter, 1991] and is related to ionic strength (I) for a symmetrical electrolyte by the equation

$$\frac{1}{\kappa} = \left(\frac{\varepsilon_o \, \varepsilon_r \, k \, T}{2 \, N \, 10^3 \, e^2} \right)^{1/2} \cdot \frac{1}{\sqrt{I}} \qquad (22)$$

N	=	Avogadro's constant
e	=	electronic charge
ε_o	=	permittivity of free space (vacuum)
ε_r	=	relative permittivity of the medium
k	=	Boltzmann constant
T	=	absolute temperature

It follows that for an electrostatic interaction, adsorption would be expected to decrease as the ionic strength increases and the double layers are compressed.

Thus the adsorption constant K_a (= $1/K_d$) might be expected to be related to the thickness of the ionic atmosphere ($1/\kappa$) and hence to $1/\sqrt{I}$. Fig. 4.1.6 shows the plot of K_a vs. $1/\sqrt{I}$ in which K_a decreases linearly with increasing $\sqrt{I}$. The intercept on the x-axis when $K_a = 0$ is 0.72, corresponding to an ionic strength of approximately 1.95 M, at which no adsorption would occur.

At all the ionic strengths studied, the Gibbs energy change is positive (i.e. the process is endergonic). This indicates that dissociation is not energetically favoured, but that the opposite reaction (adsorption) is. Increasing ionic strength decreases ΔG_d. At higher ionic strengths the adsorption is not so strongly favoured due to the screening effects of the ions in solution.

Aggregation of cationic liposomes induced by the presence of increased levels of phosphate in the higher ionic strength solutions probably had little effect on the liposomal adsorption. The patterns of aggregation observed (see Fig. 3.1.7) did not

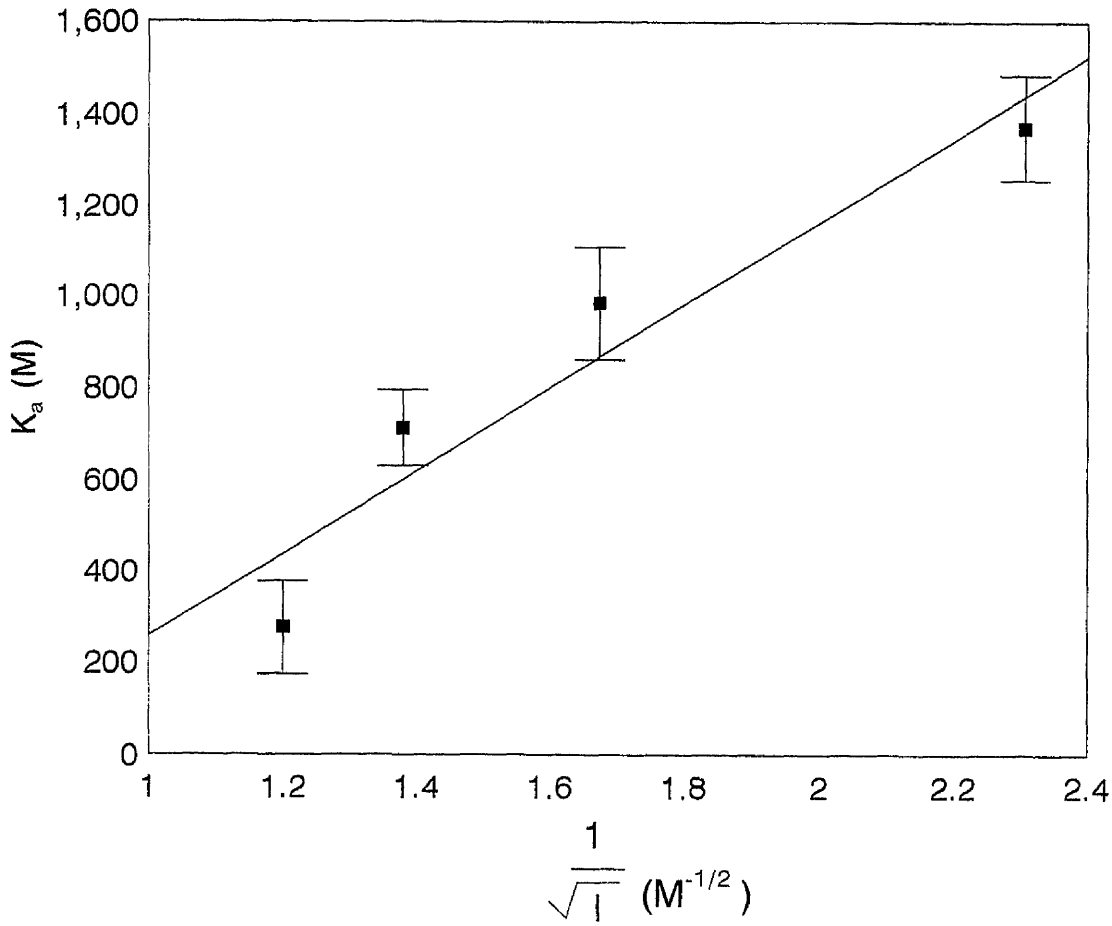


Fig 4.1.6 Dependence of the association constant (K_a) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms on the reciprocal of the square root of the ionic strength of the incubation medium. Incubation time 2 hours, temperature 37^{o}C.

reflect the changes in adsorption. For example, there was no aggregation observed at 188 mM or 357 mM, even though adsorption was significantly lower at 357 mM compared to that seen at 188 mM with all the liposomal concentrations studied.

4.1.6 Reversibility of Liposome Adsorption

The previous section dealt with the adsorption of liposomes to bacteria in the presence of increasing concentrations of free ions. Further experiments were performed to observe whether these high ionic strength solutions had any effects on biofilms that already had cationic liposomes attached. When liposomes were incubated with the biofim in PBS buffer, giving the highest possible level of coverage with the range of ionic strengths used, followed by washing and addition of the higher ionic strength solutions, the %amc fell to levels similar to those found when the liposomes were initially applied in these high strength solutions (see Fig. 3.1.8) within experimental error. In other words, the increased levels of free ions in solution still demonstrated the ability to decrease the level of liposomal attachment even when the vesicles were already adsorbed to the biofilm. The attraction of the free ions for the charged sites on the two surfaces must be strong enough to overcome established interactions between the liposomes and the bacteria; alternatively, the increased ionic atmosphere lessens the charge differential between the two surfaces.

This reversibilty of adsorption is consistent with the notion of an equilibrium between adsorbed and bulk-state liposomes mentioned above. Effectively, at each ionic strength a new position in this equilibrium is established.

4.1.7 Effect of Temperature on Liposomal Adsorption

The adsorption of cationic liposomes to *S.epidermidis* biofilms was also examined at two temperatures lower than 37^{o}C; 4^{o}C and 25^{o}C (see Fig. 3.1.9) in PBS buffer. The reciprocal graph of these values (Fig. 4.1.7) gave the values for $(\%amc)_{max}$ and K_d (Table 4.1.2). Over the range of liposome concentrations studied, the level of

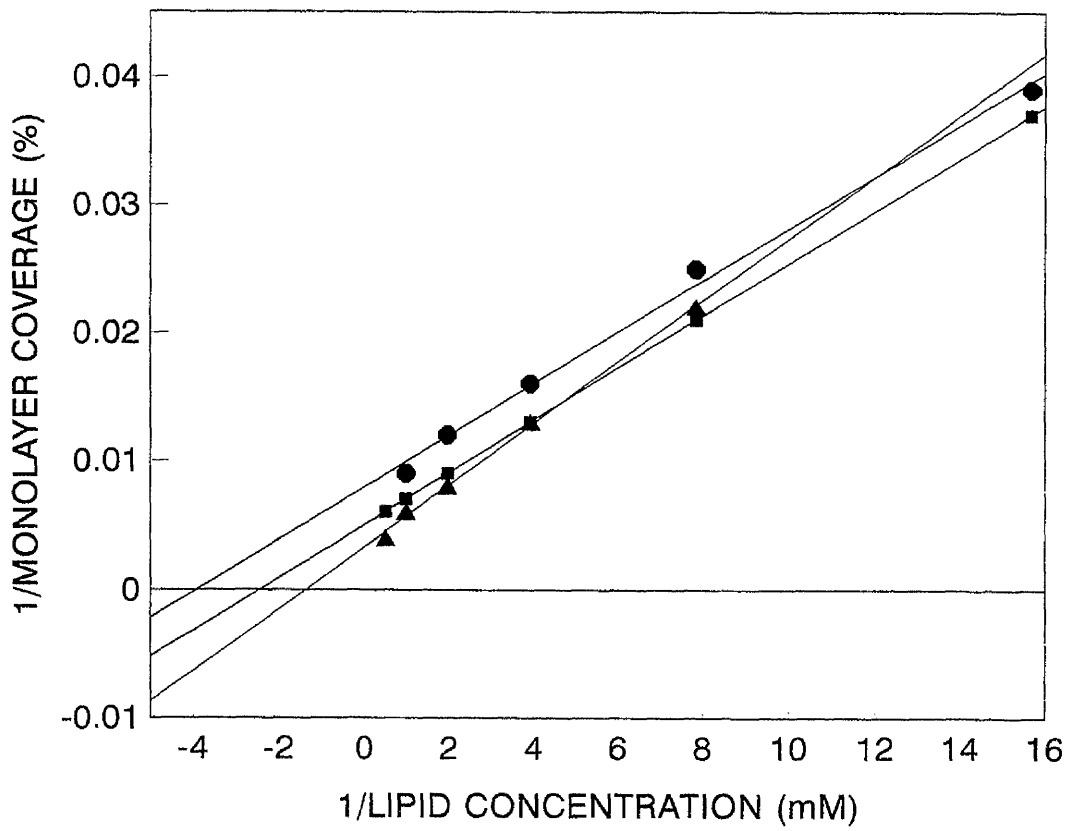


Fig. 4.1.7 Double-reciprocal plot of the data in Fig. 3.1.9, showing the dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the temperature of incubation. ●, 4°C; ■, 25°C; ▲, 37°C. Incubation time 2 hours, ionic strength 188 mM.

TEMPERATURE (°C)	$(\%amc)_{max}$ (%)	K_d (mM)	ΔG_d (KJ mol^{-1})
4	126.3 (± 10.3)	0.255 (± 0.023)	+19.065 (± 0.069)
25	199.9 (± 0.1)	0.408 (± 0.001)	+19.347 (± 0.065)
37	303.3 (± 24.2)	0.729 (± 0.061)	+18.628 (± 0.060)

Table 4.1.2 Calculated values of the maximum theoretical monolayer coverage ($(\%amc)_{max}$), the dissociation constant (K_d) and the Gibbs free energy change of dissociation (ΔG_d) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms at various incubation temperatures. Incubation time 2 hours, ionic strength 180 mM.

adsorption increased with increasing temperature. The values of $(\%amc)_{max}$ and K_d also increased between 4°C and 37°C (Figs. 4.1.8 and 4.1.9). The greater extent of adsorption at the higher temperatures could be explained by the fact that the liposomes would be moving at a larger velocity at the warmer temperatures, increasing the frequency of the collisions between the liposomes and the bacterial surface that are essential for their attachment.

The increase in the dissociation constant between 4°C and 37°C could perhaps indicate that there is a greater "turnover" between the adsorbed and the bulk-state liposomes at the higher temperatures. What this means is that even though there is a larger number of vesicles attached at the higher temperatures, the strength of the interactions is lower; the liposomes are less tightly bound than at the colder temperatures. So, even though collisions are more likely, the warmer temperatures facilitate the breaking of the interactions of the liposomes that are already attached.

Another explanation for these differences could be that the temperature affects the conformation of the surface of the bacterial biofilm. As the temperature is reduced, the biofilm could condense, perhaps making sites of attachment for the cationic liposomes less accessible.

4.1.8 Effect of Ionic Strength at Different Temperatures

The effect of increasing ionic strength on the adsorption isotherm was examined at 4°C (see Fig. 3.1.10) and 25°C (see Fig. 3.1.11). In both cases, the same pattern was seen as at 37°C; increasing the ionic strength lowered the level of adsorption. Again, double-reciprocal plots were constructed (Figs. 4.1.10 and 4.1.11), giving values for $(\%amc)_{max}$ and K_d. At each ionic strength studied, increasing the ionic strength had little effect on $(\%amc)_{max}$ but increased the dissociation constant (Fig. 4.1.12). The plot of K_a ($=1/K_d$) vs. $1/\sqrt{I}$ (section 4.1.5; Fig. 4.1.6), reflecting the effect of ionic screening at 37°C was expanded to include the values obtained at 4°C and 25°C

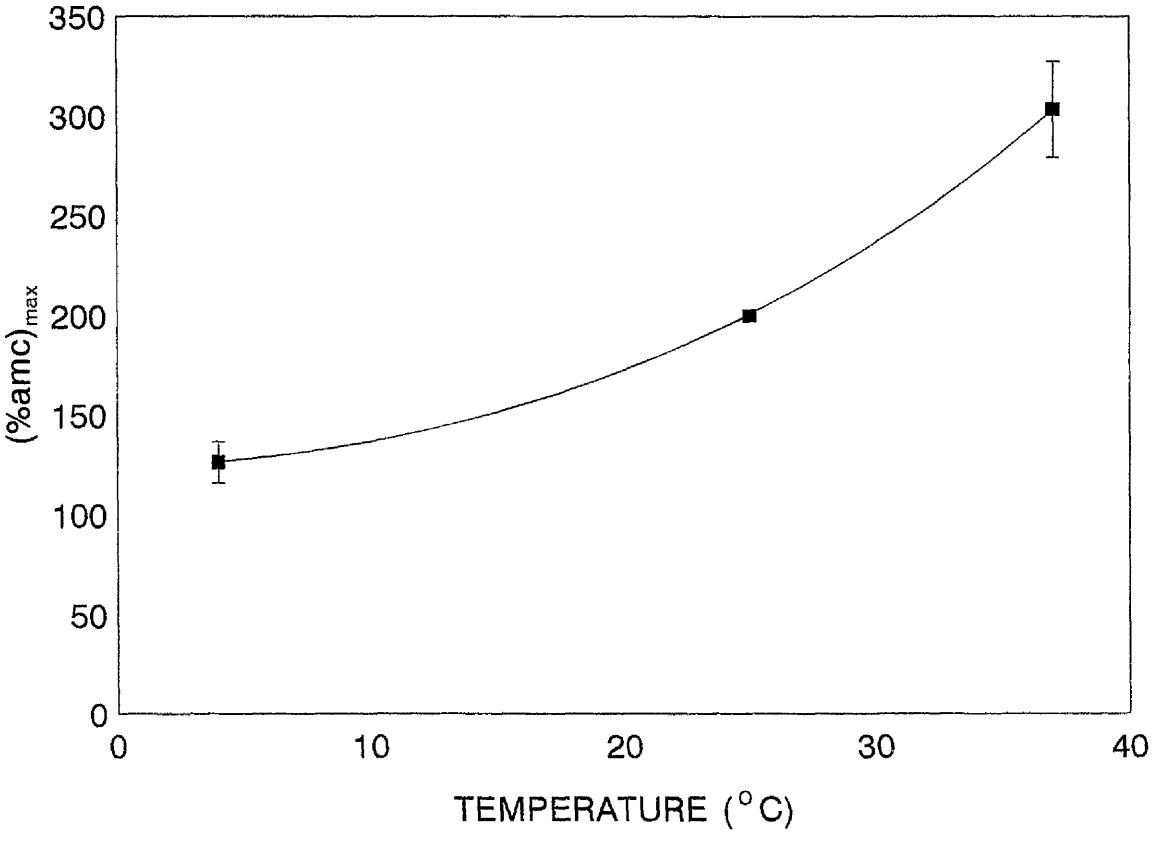


Fig. 4.1.8 Dependence of the maximum theoretical monolayer coverage ($(\%amc)_{max}$) of *S.epidermidis* biofilms by cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) on the temperature of incubation. Incubation time 2 hours, ionic strength 188 mM.

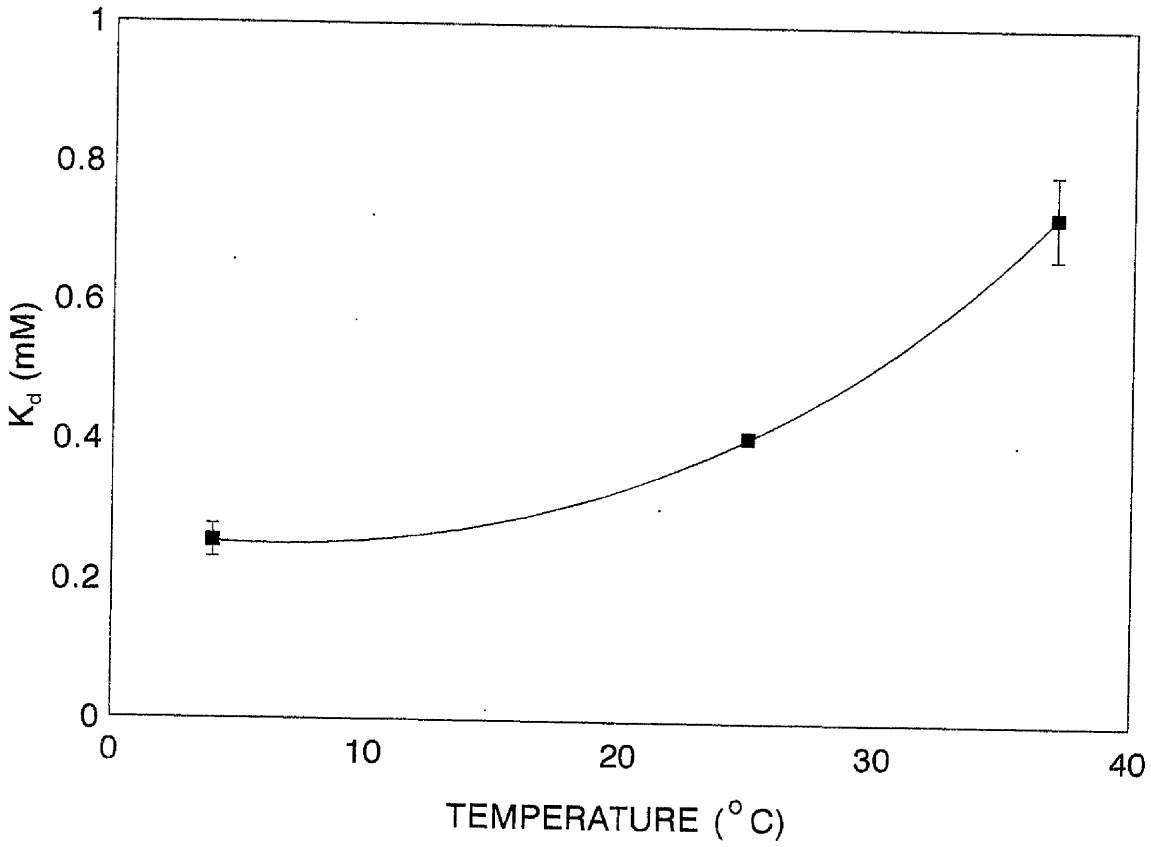


Fig. 4.1.9 Dependence of the dissociation constant (K_d) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms on the temperature of incubation. Incubation time 2 hours, ionic strength 188 mM.

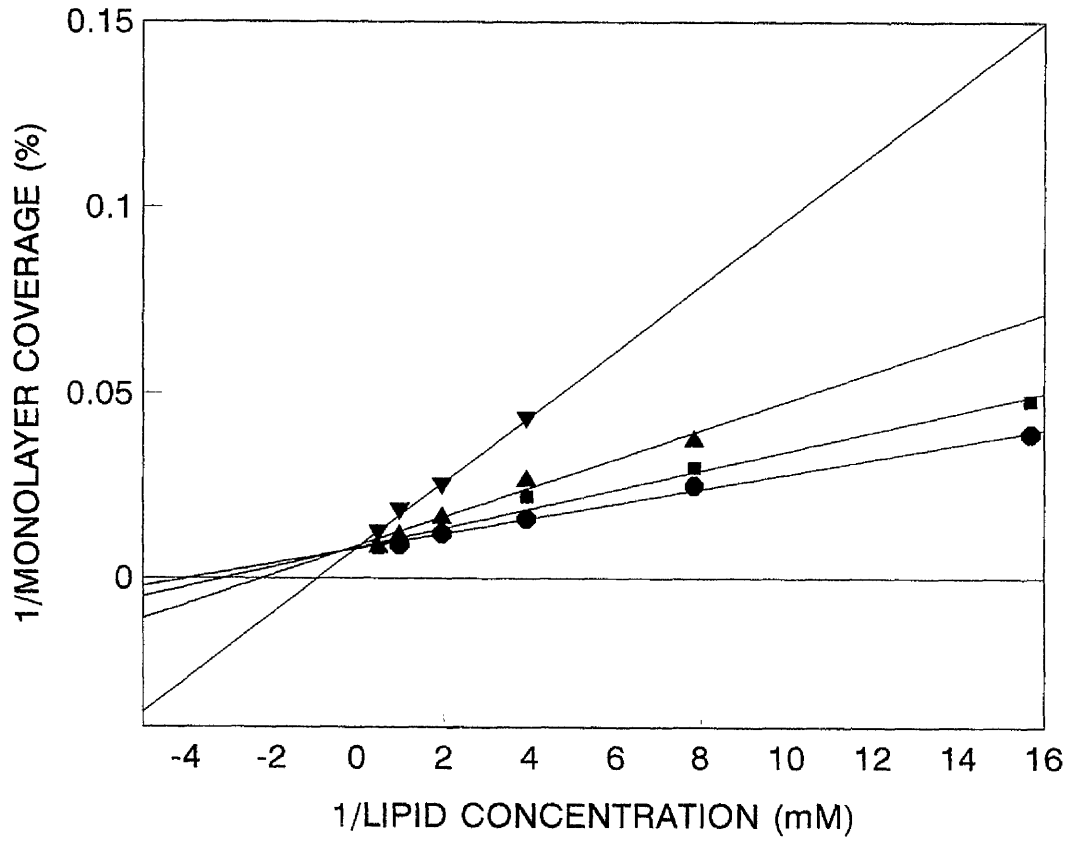


Fig. 4.1.10 Double-reciprocal plot of the data in Fig. 3.1.10, showing the dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours, temperature 4°C.

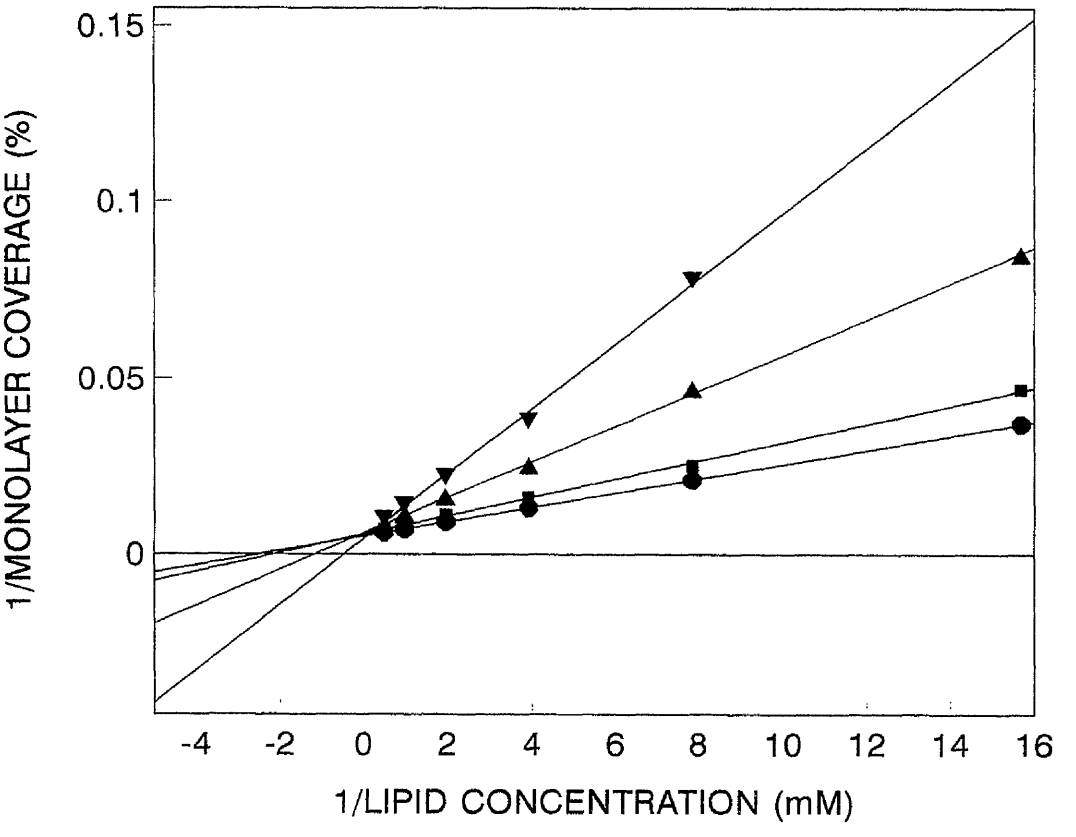


Fig. 4.1.11 Double-reciprocal plot of the data in Fig. 3.1.11, showing the dependence of adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to *S.epidermidis* biofilms on the ionic strength of the incubation medium. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours, temperature 25°C.

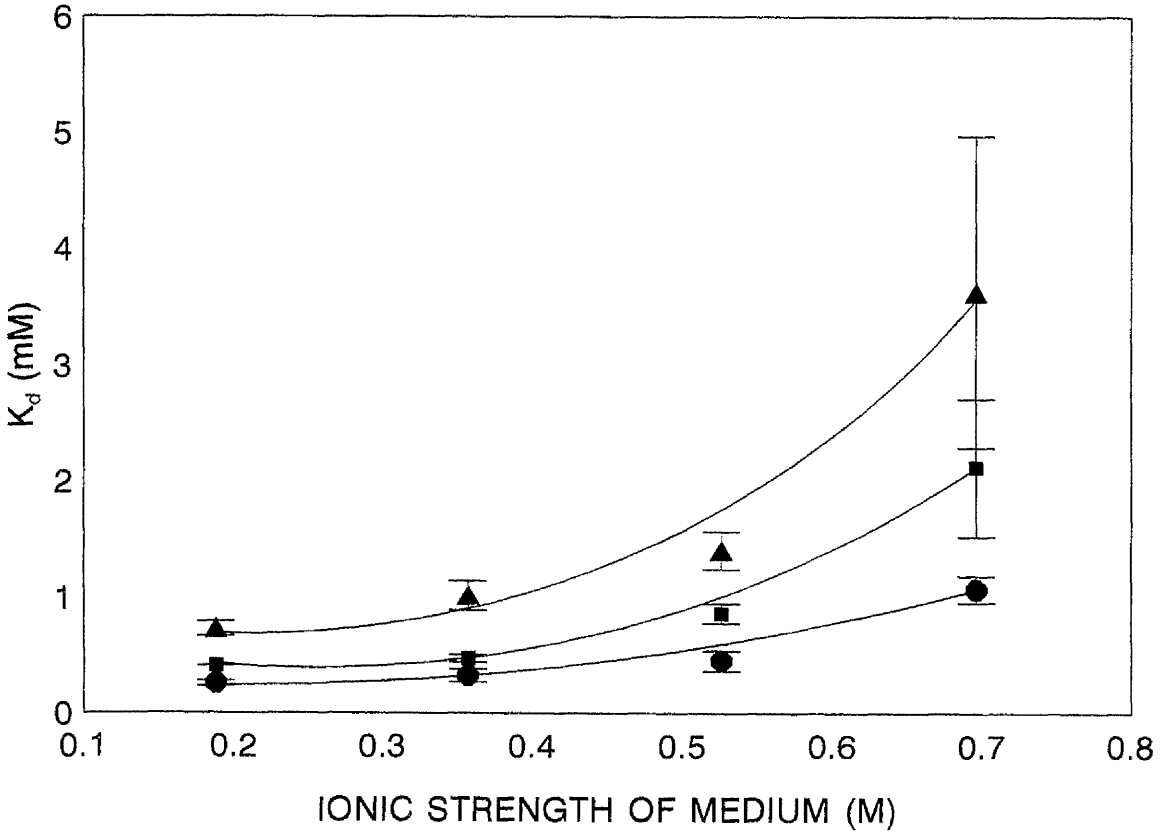


Fig. 4.1.12 Dependence of the dissociation constant (K_d) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms on the ionic strength of the medium and the temperature of incubation. ●, 4°C; ■, 25°C; ▲, 37°C. Incubation time 2 hours.

(Fig. 4.1.13). All three lines cross the x-axis ($1/\sqrt{I}$) at around the same point, corresponding to an ionic strength of around 1.95 M at which association does not occur. Therefore, this value is independent of temperature over the range studied in this work.

ΔG_a is related to ΔH_a by the equation

$$\left[\frac{\partial\,(\Delta G_a/T)}{\partial\,(1/T)}\right]_P = \Delta H_a \qquad (23)$$

Therefore, at each ionic strength studied, plotting $\Delta G_a/T$ against the reciprocal of the absolute temperature gave a straight line, the slope of which is ΔH_a (Fig. 4.1.14). With ΔG_a and ΔH_a known, values for $T\Delta S_a$ and ΔS_a were calculated from the equation;

$$\Delta G_a = \Delta H_a - T\Delta S_a \qquad (24)$$

These values are given in Table 4.1.3.

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}. All the values of ΔH_a are negative; the adsorption of liposomes is an exothermic process, liberating heat energy. It follows that the dissociation process must be endothermic (i.e. ΔH_d will be positive) under these conditions and that heat energy needs to be taken in for the breaking of the interactions between the vesicles and the biofilm. This would explain why the values of K_d increased with increasing temperature; as the heat energy input is greater at the higher temperatures, the dissociation of the liposomes from the biofilm surface is favoured.

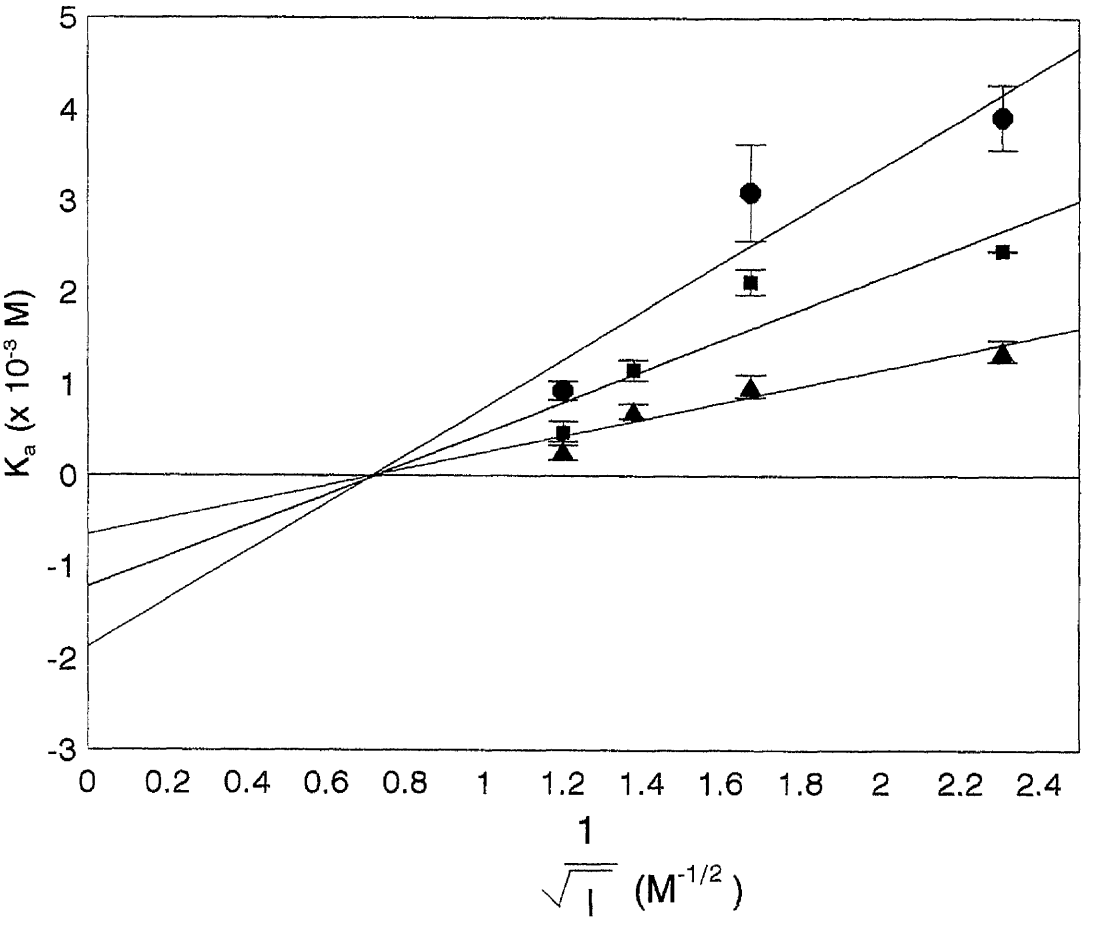


Fig. 4.1.13 Dependence of the association constant (K_a) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms on the reciprocal of the square root of the ionic strength of the medium and the temperature of incubation. ●, 4°C; ■, 25°C; ▲, 37°C. Incubation time 2 hours.

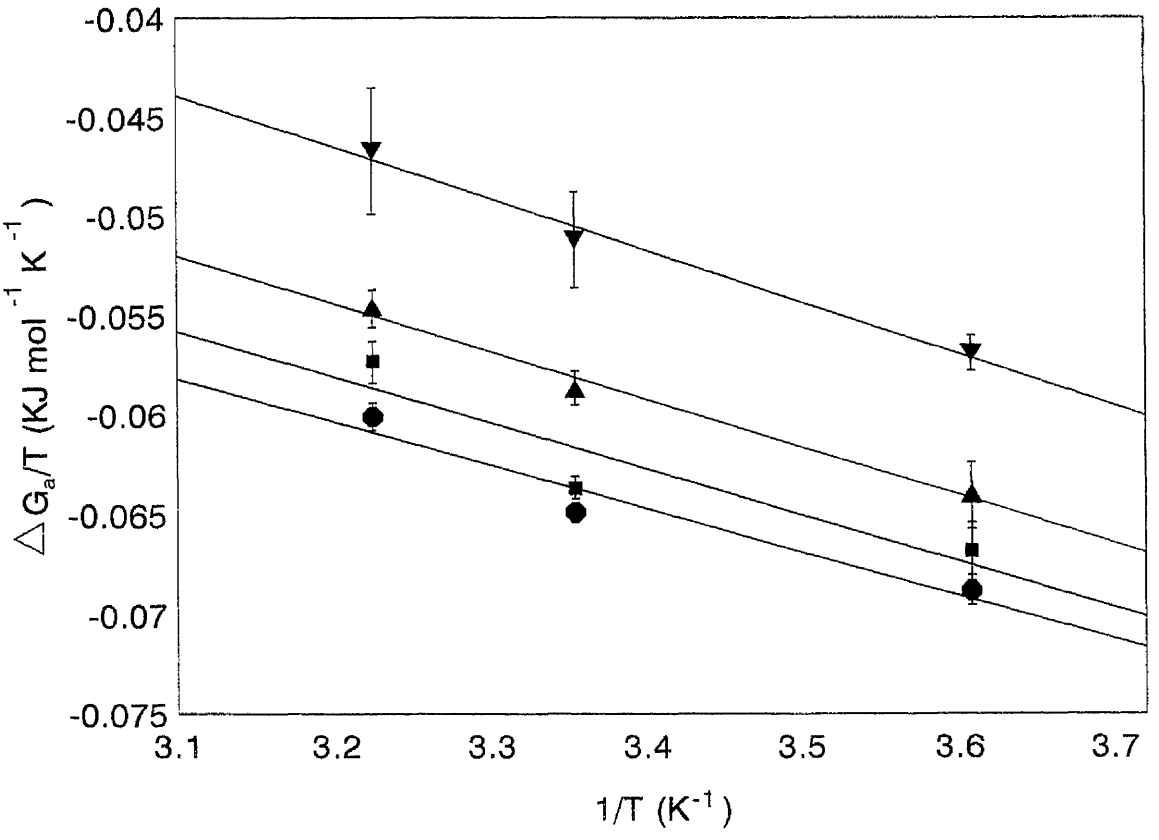


Fig. 4.1.14 Plot of $\Delta G_a/T$ against reciprocal temperature for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and *S.epidermidis* biofilms. The slopes of these lines gave values for the enthalpy change of association (ΔH_a) of liposomes with biofilms at each ionic strength. ●, ionic strength 188 mM; ■, 357 mM; ▲, 526 mM; ▼, 696 mM. Incubation time 2 hours.

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})
188 mM	277.15	126.3 (± 10.3)	3919.66 (± 354.64)	-19.065 (± 0.209)	-21.651 (± 5.390)	-2.587	-0.00933
	298.15	199.9 (± 0.1)	2452.63 (± 1.64)	-19.347 (± 0.002)	-21.651 (± 5.390)	-2.304	-0.00773
	310.15	303.3 (±24.2)	1372.29 (± 115.50)	-18.628 (± 0.218)	-21.651 (± 5.390)	-3.023	-0.00975
357 mM	277.15	123.3 (± 19.5)	3097.99 (± 530.05)	-18.523 (± 0.398)	-23.015 (± 9.252)	-4.492	-0.01620
	298.15	180.5 (± 11.4)	2118.60 (± 139.62)	-18.984 (± 0.164)	-23.015 (± 9.252)	-4.031	-0.01350
	310.15	294.1 (± 36.5)	985.37 (± 123.51)	-17.774 (± 0.325)	-23.015 (± 9.252)	-5.240	-0.01690
526 mM	277.15	115.3 (± 19.5)	2211.99 (± 425.79)	-17.746 (± 0.449)	-23.982 (± 2.669)	-6.236	-0.02250
	298.15	170.0 (± 13.5)	1159.91 (± 114.57)	-17.491 (± 0.246)	-23.982 (± 2.669)	-6.491	-0.02180
	310.15	283.6 (± 32.5)	712.54 (± 82.07)	-16.938 (± 0.298)	-23.982 (± 2.669)	-7.044	-0.02270
696 mM	277.15	121.1 (± 12.2)	934.74 (± 101.55)	-15.762 (± 0.251)	-25.995 (± 3.042)	-10.234	-0.03690
	298.15	229.8 (± 64.2)	471.63 (± 132.54)	-15.260 (± 0.716)	-25.995 (± 3.042)	-10.735	-0.03600
	310.15	319.5 (± 117.1)	275.61 (± 101.81)	-14.489 (± 1.000)	-25.995 (± 3.042)	-11.506	-0.03710

Table 4.1.3 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) and *S.epidermidis* biofilms in media of various ionic strengths and at three different temperatures. Incubation time 2 hours.

Over the range of ionic strengths studied in this work, the enthalpies and entropies of association are negative; they are working against each other. As $|\Delta H_a|$ is greater than $|T\Delta S_a|$ the association is primarily enthalpy-driven. However, as the ionic strength was increased, the $T\Delta S_a$ term became more dominant as ΔS_a became larger.

Entropy is a measure of the disorder in a system. At all the temperatures and ionic strengths studied, the entropy change of association (ΔS_a) was negative. This is because the adsorbed liposomes were more ordered than when in solution; attached to the surface of the biofilm, they had less freedom of movement and hence the entropy was lower.

With increasing ionic strength, ΔS_a increased. This indicates that there was a larger difference between the disorder of the adsorbed liposomes and the free liposomes when the concentration of ions in the solution was higher. This reflects either an increase in the order of the adsorbed vesicles, perhaps due to anions binding to the positive charges on the portions of the adsorbed liposomes not involved in the attachment to the biofilm which could lessen repulsion between adjacent adsorbed vesicles; or it could indicate a greater degree of disorder amongst the liposomes in solution. This could also explain why the magnitude of ΔH_a increased with increasing ionic strength; more heat energy would be needed for desorption of the liposomes.

The Gibbs free energy change of association decreased with increasing ionic strength at each temperature studied. ΔG_a was negative under all conditions, which indicates that it is the association reaction that occurs spontaneously. The drop in ΔG_a with increasing ionic strength could reflect the decrease in K_a as the saturating concentration of ions where $K_a = 0$ is approached (I = 1.95 M; see Section 4.1.5). As K_a tends towards zero, ΔG_a becomes positive (Fig. 4.1.15), indicating that it is the reverse process (desorption) that would occur spontaneously at these high ionic strengths. The decrease in ΔG_a was accompanied by an increase in the size of the

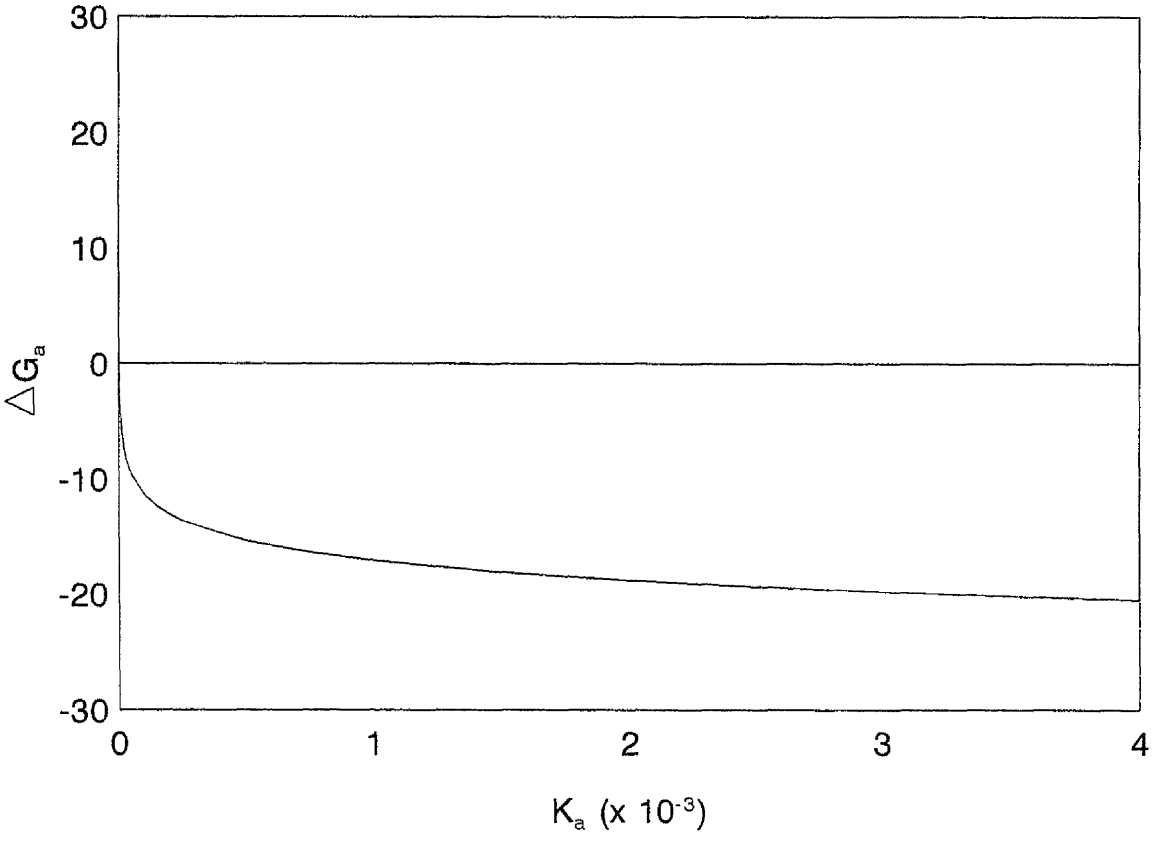


Fig. 4.1.15 Dependence of the Gibbs free energy change of association (ΔG_a) on the association constant (K_a) at 37°C (310 K).

$T\Delta S_a$ term as the ionic strength was increased. ΔG_a becomes positive at the point where the $T\Delta S_a$ term is greater in magnitude than ΔH_a, i.e. when the reaction becomes entropy-driven. ΔG_a was slightly lower at 37°C compared to 4°C and 25°C, reflecting a slight increase in the entropy change at the higher temperature.

4.1.9 Hydrophilic and Hydrophobic Subpopulations

The adsorption isotherms obtained from attachment of vesicles to *S.epidermidis* (NCTC 11047) was compared with the data attained when the cationic liposomes were incubated with biofilms of subpopulations of this bacteria that had been separated on the basis of their hydropathy (see Fig. 3.1.12).

Over the range of liposomal concentrations that were studied, the greatest level of adsorption was seen with the hydrophobic subpopulation and the lowest level with the hydrophilic subpopulation, with the pooled population somewhere between these two extremes. From the double-reciprocal plots of these three sets of data (Fig. 4.1.16), values for $(\%amc)_{max}$ and K_d were derived (Table 4.1.4). Both the maximum theoretical level of adsorption 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;

$$(0.7 \times 244.7\ \%) + (0.3 \times 418.2) \quad = \quad 296.8\ \%$$

then the figure obtained is similar to the $(\%amc)_{max}$ for the total population (303 %). This implies that the relative abundance of the two subpopulations attached to the plate reflected their proportion in the bacterial pool.

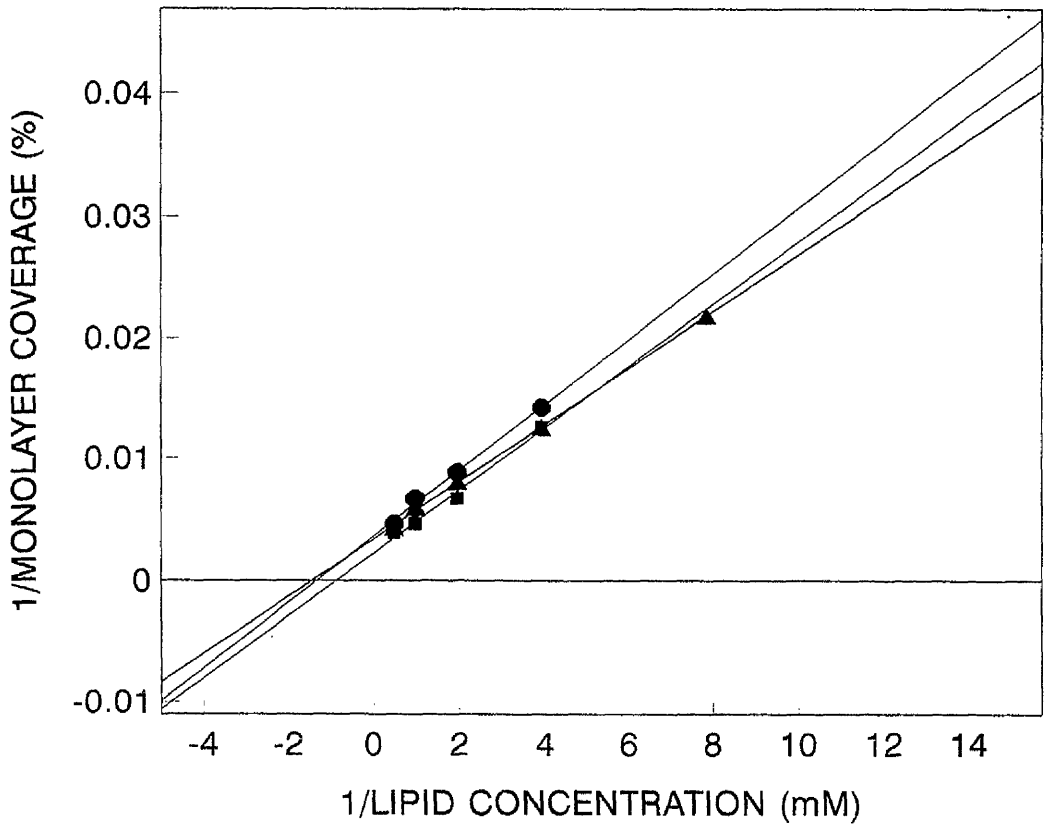


Fig. 4.1.16 Double-reciprocal plot of the data in Fig. 3.1.12, showing the adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to biofilms of hydrophobic and hydrophilic subpopulations of *S.epidermidis*. ▲, total population; ■, hydrophobic subpopulation; ●, hydrophilic subpopulation. Incubation time 2 hours, ionic strength 188 mM, temperature 37^{O}C.

S.epidermidis SUBPOPULATION	$(\%amc)_{max}$ (%)	K_d (mM)	ΔG_d (KJ mol^{-1})
HYDROPHOBIC	418.2 (± 74.2)	0.1.106 (± 0.211)	+17.553 (± 0.057)
TOTAL POPULATION	303.3 (± 24.2)	0.729 (± 0.061)	+18.628 (± 0.060)
HYDROPHILIC	244.7 (± 19.8)	0.621 (± 0.062)	+19.041 (± 0.061)

Table 4.1.4 Calculated values of the maximum theoretical monolayer coverage ($(\%amc)_{max}$), the dissociation constant (K_d) and the Gibbs free energy change of dissociation (ΔG_d) for the interaction between cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) and biofilms of the total population of *S.epidermidis* NCTC 11047 and the hydrophobic and hydrophilic subpopulations. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

For these results to be valid, it was essential to check whether there was any difference between the two subpopulations in the level of the attachment to the plates. With [^{3}H]-thymidine labelling, these levels were calculated (see Fig. 3.1.15). The hydrophilic bacteria attached to the surface of the microtitre plate in lower numbers than the hydrophobic bacteria (3.88×10^6 c.f. 5.16×10^6 cells per well), indicating that, as might be expected, their surface characteristics have a bearing on their ability to attach to the polystyrene surface of the microtitre plates. The hydrophobic bacteria did not bind to the plate in greater numbers than the total population as would have been predicted, considering the fact that 70 % of the bacteria were the less extensively attaching hydrophilic subpopulation. This could indicate that the number of hydrophobic bacteria attached (5.16×10^6 cells) represented the maximum number of *S.epidermidis* cells that could attach to the well and that the total population contained a large enough number of hydrophobic bacteria (30 % of the total) to reach this maximum level. The fact that the hydrophilic bacteria bound to the plate at only 75 % of the level seen by the total pool supports the idea mentioned above that the bacteria are attaching to the plate in the same relative proportion that exists in the total population.

For a better comparison of the relative levels of liposomal attachment, the values of $(\%amc)_{max}$ were converted into the numbers of liposomes that gave that amount of coverage (based on the weight-average diameters of the vesicles) and hence the number of vesicles binding per cell could be calculated. As shown above (Section 4.1.4), it has been estimated that at 100 % monolayer coverage there are 1.75×10^{10} liposomes attached to each bacterial population and so;

Hydrophobic subpopulation = 7.30×10^{10} liposomes attached

= 14,152 liposomes per cell

Total population	=	5.31×10^{10} liposomes attached
	=	10,296 liposomes per cell
Hydrophilic subpopulation	=	4.28×10^{10} liposomes attached
	=	11,015 liposomes per cell

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 is significantly higher.

4.1.10 Adsorption to Adherence-Defective Mutants

Adherence-defective mutants of *S.epidermidis* were generated using transposon mutagenesis. There was a much lower degree of attachment of liposomes to biofilms of the mutant bacteria compared to the wild-type (see Fig. 3.1.13) and this was reflected in the values of $(\%amc)_{max}$ and K_d obtained from the double-reciprocal plot (Fig. 4.1.17). The maximum theoretical coverage was 119.2 % (± 23.4 %), compared to 303.3 % for the wild-type, and the dissociation constant was 0.426 mM (± 0.087 mM), lower than that seen for the wild-type (0.729 mM).

However, reflecting the decreased adherence of these mutants, the number of cells attached to the microtitre plate wells was much lower than with the wild-type (4.73×10^5 cells c.f. 5.16×10^6 cells per well). Therefore, when the maximum theoretical coverage is reached, the number of liposomes attached to each bacteria is much higher with the mutant bacteria;

Total population	=	5.31×10^{10} liposomes attached
	=	10,296 liposomes per cell

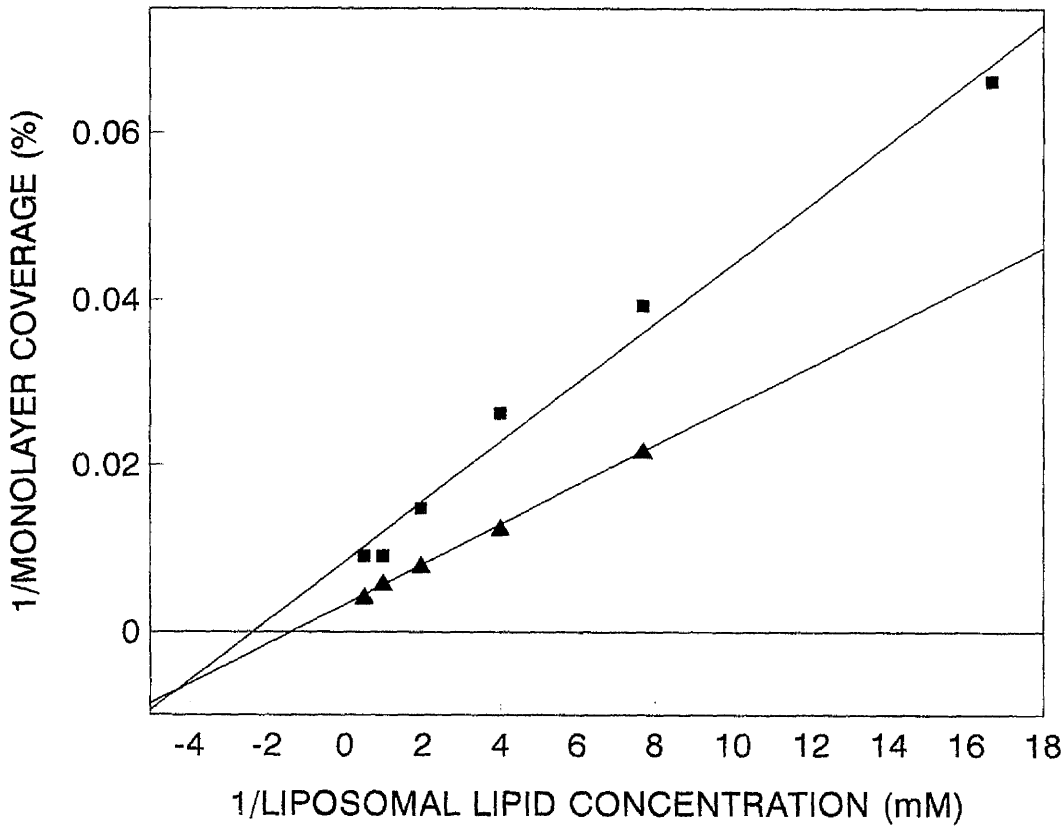


Fig. 4.1.17 Double-reciprocal plot of the data in Fig. 3.1.13, showing the adsorption of cationic liposomes (DPPC/Chol/SA VETs, molar ratio 1:0.49:0.4) to biofilms of adherence-defective mutants *S.epidermidis*. ▲, *S.epidermidis* NCTC 11047 wild-type; ■, *S.epidermidis* M3 mutant. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

Mutants	=	2.09×10^{10} liposomes attached
	=	44,065 liposomes per cell

This is perhaps an indication that, either the adhesin molecules involved in attachment to the plate restricted attachment of the vesicles, or that the liposomes primarily adsorb to moieties on the extracellular slime rather than on the bacterial cells themselves.

4.2 EFFICIENCY OF ENCAPSULATED AGENTS

4.2.1 Adsorption of cationic DDAB-VETs to *S.epidermidis*

From the results of the liposomal adsorption as a function of lipid concentration for the liposomes incorporating various amounts of cationic DDAB (see Fig. 3.2.1), a double-reciprocal plot was constructed (Fig. 4.2.1). From the points at which the lines crossed the y-axis, values of $(\%amc)_{max}$ were derived (Fig. 4.2.2). With the compositions studied, 14.77 mole% DDAB seemed to be the optimum level of the cationic molecule, with $(\%amc)_{max}$ of 370.8 % (± 133.7%).

4.2.2 Encapsulation of Antibacterial Agents

Both vancomycin and gentamicin were successfully encapsulated within liposomes. The encapsulation efficiency was, at best, around 4 %. Freezing and thawing of the multilamellar vesicles prior to extrusion has been reported to increase the level of encapsulation [Mayer et al, 1986]. No such effect was seen using that protocol in this work, possibly due to the relatively low concentrations of lipid used (10-17 mg ml^{-1}).

The encapsulation efficiency was lower when cholesterol was not included within the liposomal membrane of the cationic liposomes. One possible reason for this could be the smaller size of these vesicles (peak diameter 96.3 nm compared with 132.3 nm for the liposomes with cholesterol), as it follows that the internal aqueous volume of the VETs incorporating cholesterol will be about two and a half times larger (i.e. a diameter of 96.3 nm corresponds to a volume of 0.47 x 10^6 nm^3 and a diameter of 132.3 nm corresponds to a volume of 1.21 x 10^6 nm^3). Another possible explanation for this discrepancy could be because at temperatures above the chain-melting point (41°C for DPPC), high levels of cholesterol decrease the permeability of liposomal membranes [New, 1990]. The VETs were produced at 60°C, well above the T_c, and so at this temperature the liposomes incorporating cholesterol may have retained more of their entrapped contents.

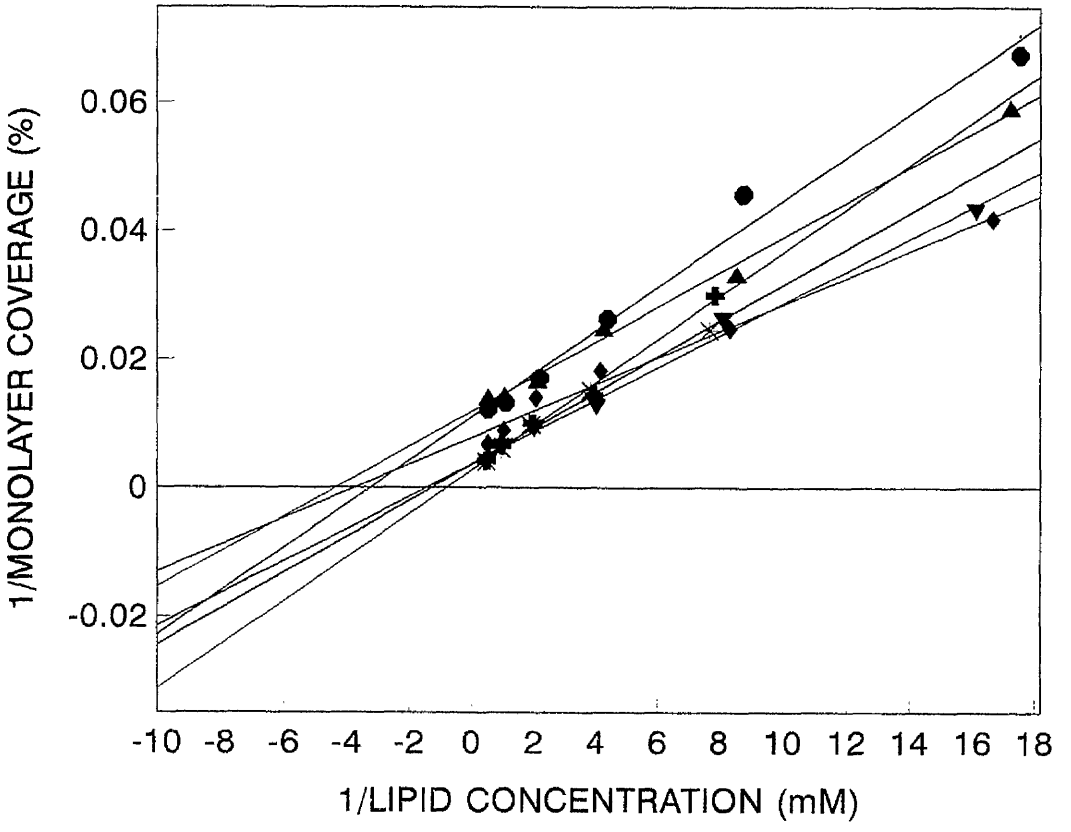


Fig. 4.2.1 Double-reciprocal plot of the data in Fig. 3.2.1, showing the liposome concentration-dependent adsorption of DPPC/Chol/DDAB VETs to *S.epidermidis* biofilms as a function of mole% DDAB in the liposome membrane. ■, 2.81 mole% DDAB; ●, 5.46 mole% DDAB; ▲, 7.97 mole% DDAB; ◆, 10.35 mole% DDAB; ▼, 12.62 mole% DDAB; ✚, 14.77 mole% DDAB; ✳, 16.81 mole% DDAB. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

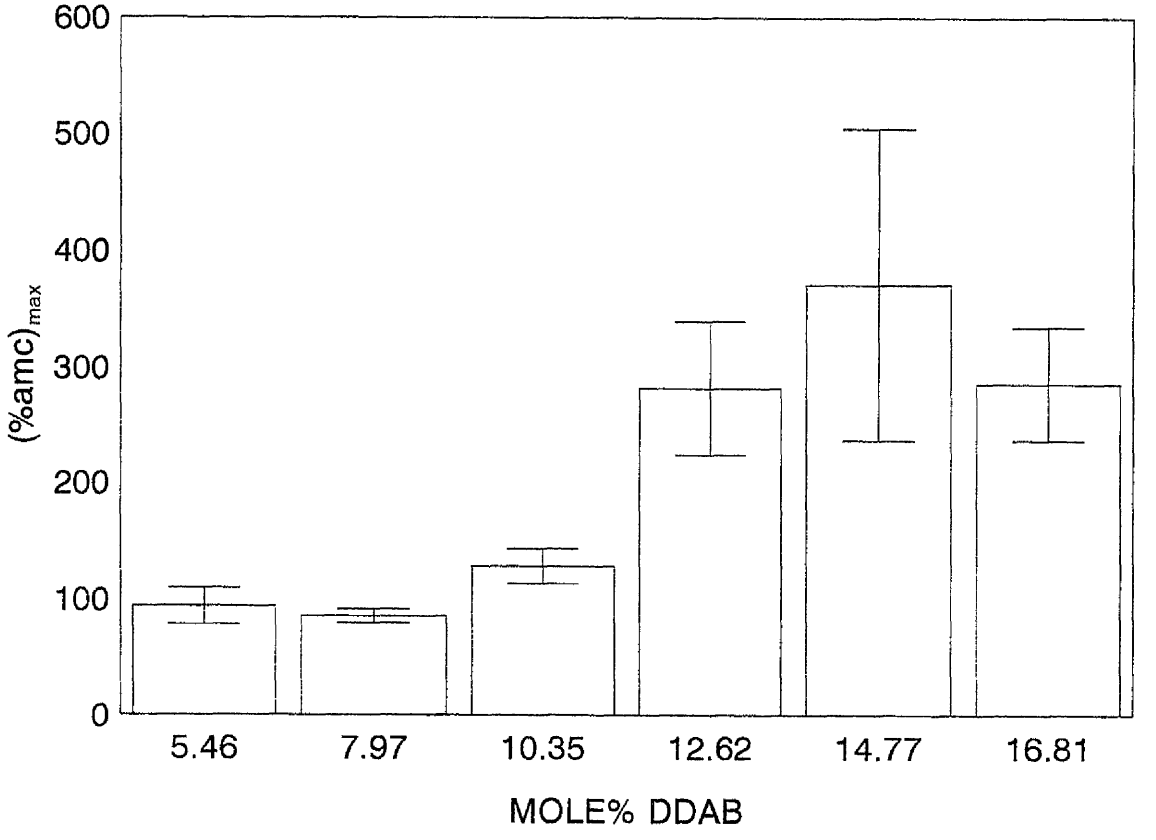


Fig. 4.2.2 Dependence on the maximum theoretical adsorption of cationic liposomes incorporating DDAB on the mole% of DDAB in the liposomal membrane. Incubation time 2 hours, ionic strength 188 mM, temperature 37°C.

4.2.3 Regrowth Assays

The aim of treating problem-causing organisms such as *S.epidermidis* with antibacterial agents is, of course, to kill them. However, with microorganisms it is difficult to define the point at which they are actually dead. The results obtained from this work were assessed on the principle that a living (viable) organism is defined as one that is capable of continuing cell division, and hence the definition of a dead (non-viable) organism is of one that cannot multiply, even though it may still be metabolically active [Wilkinson, 1986]. The relative effectiveness of the various preparations used in this work was gauged in this fashion, with antibacterial 'killing' determined as the inhibition of growth of treated bacteria that have been re-incubated with nutrient broth for 18 hours.

4.2.4 Effect of Vancomycin

Vancomycin is a complex tricyclic glycopeptide antibiotic (Mwt ~ 1500) that was discovered in the 1950s. It is effective mainly against Gram-positive bacteria, including methicillin-resistant staphylococci [Rang and Dale, 1991]. The outer membranes of Gram-negative microorganisms are not permeable to this large antibiotic. It is not absorbed from the gut and so is administered intravenously [Gillies et al, 1986]. Vancomycin works by inhibiting the synthesis of cell walls in dividing bacteria by binding to precursors of the walls (Fig. 4.2.3). The antibiotic has a hydrophobic cleft that binds with high affinity to the sequence D-alanine-D-alanine at the free carboxyl end of UDP-pentapeptide precursors of the wall [Cuperus et al, 1995; Gilman et al, 1990], causing the precursors to accumulate. The binding is initiated by an interaction between the peptide carboxylate group and protonated amine on the vancomycin [Coulson, 1994]. The drug may also cause a change in the permeability of the cell membrane and could cause interference with RNA synthesis. Vancomycin has been shown to have a synergistic effect when used in conjunction with aminoglycosides such as gentamicin or tobramycin against *Staphylococcus aureus* [Gilman et al, 1990; Meredith et al, 1995].

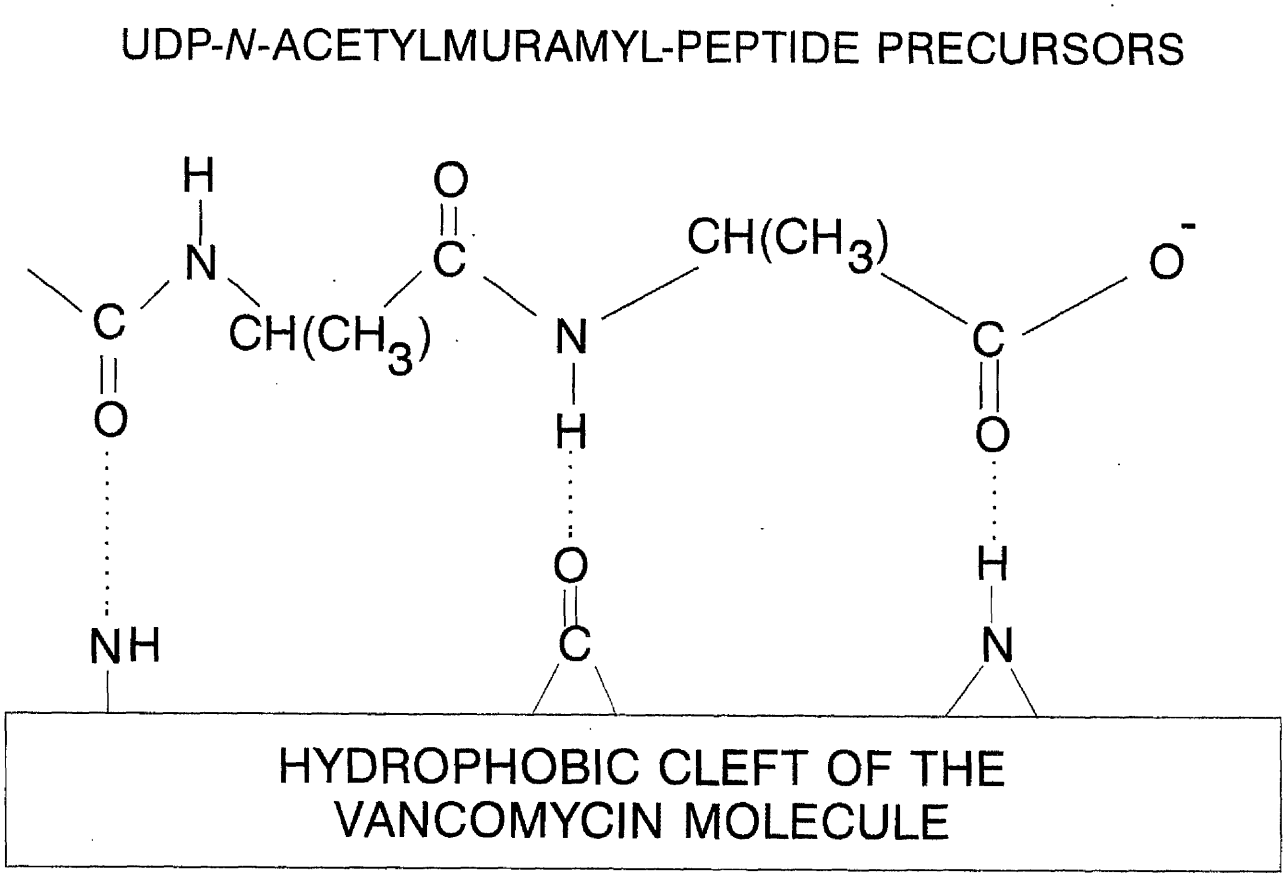


Fig. 4.2.3 Binding of precursors of the bacterial cell wall to the hydrophobic cleft of vancomycin. The drug binds to alanine residues at the C-terminal of the peptides.

Harmful side effects of the drug include fever, rashes, proteinuria and local phlebitis (inflammation of the walls of veins) or venous thrombosis (clotting) at the site of injection [Gillies et al, 1986; Rang and Dale, 1991]. High plasma levels of the drug have been associated with nephrotoxicity and ototoxicity - auditory impairment is frequently, but not always, permanent [Leader et al, 1995]. This toxicity makes vancomycin a 'second line' antibiotic - only used where other, less toxic, agents are unsuitable, for example with methicillin resistance.

Because the drug works by acting on a structural component of the cell wall, rather than on an enzyme (e.g. as β-lactam antibiotics such as penicillin do), it was thought that resistance to vancomycin would be unlikely to develop. However, in the late 1980s, the first cases of vancomycin-resistant enterococci (VRE) appeared. Enterococci, a major component of the normal gastrointestinal microflora, are important pathogens, not least because of their rapid acquisition of a range of antimicrobial resistance traits which makes them nearly impossible to eradicate [Low et al, 1995; Muhammad et al, 1993]. Despite its low virulence, enterococcus is the third most common pathogen found in bloodstream infections and it is even more frequently found in cases of superinfection where it arises due to ineffective antibacterial treatment of an entirely different microorganism that has caused the initial infection [Low et al, 1995]. Some strains of enterococcus were found to be resistant to all available agents except vancomycin, which was therefore used as the universal treatment. However, various outbreaks of VRE have been reported. One method of resistance involves the expression of a transposon-based gene (*vanA*) that produces cell wall precursors terminating in D-alanine-D-lactic acid instead of D-alanine-D-alanine [Rice and Shales, 1995]. This terminus is too large for vancomycin to bind properly, enabling proper synthesis of the cell wall. Gastrointestinal colonization with vancomycin-resistant *Enterococcus faecium* and the use of antimicrobial agents with significant activity against anaerobes have been implicated as risk factors for the development of VRE bacteraemia [Edmond et al, 1995].

There is evidence that transfer of antimicrobial resistance takes place between enterococci and staphylococci [Rice and Shales, 1995] and the spread of vancomycin resistance by plasmid to staphylococci - particularly to methicillin-resistant *Staphylococcus aureus* - would be very dangerous [Murray, 1995; Shay et al, 1995]. Steps are being taken to minimize the damage done if this resistance does cross over to the staphylococci - as has been demonstrated under laboratory conditions [Rice and Shales, 1995] although, as yet, no resistant clinical isolates have been reported. However, resistance to vancomycin in staphylococci has been shown to occur *in vitro* by serial passaging of the bacteria in media containing increasing concentrations of the agents [Moreira and Daum, 1995]. Since experiments such as these show that the use of vancomycin is itself a risk factor in the development of VRE infection, use of the drug is being regulated and its use is discouraged to limit the selective pressure on bacteria already unaffected by most antimicrobials. This reduction in the use of vancomycin includes treatment for patients with a single blood culture positive for CONS such as *S.epidermidis* if other blood cultures are negative [Murray, 1995]. Attention may now focus on other drugs such as chloramphenicol which has been successfully used against vancomycin-resistant enterococci [Norris et al, 1995].

In the results detailed in Chapter Three, vancomycin was found to be effective against *S.epidermidis*, inhibiting growth after only a two minute incubation at concentrations as low as 20 $\mu g\ ml^{-1}$. The *S.epidermidis* bacteria produce slime and, *in vivo*, the polysaccharide of the slime interacts with host factors to form a matrix that enhances the stability of the biofilm on the surface of the implanted device [Dunne Jr. et al, 1993]. The thickness of this barrier can limit the diffusion of antibiotics, lowering their effective concentration. Obviously, with the *in vitro* system used in this work - where the biofilm is established on the surface of the wells of microtitre plates - there were no host factors present and so the biofilms may not have been as stable as they would have been had they formed *in vivo*.

From the results obtained (see Fig. 3.2.6), the diffusion through the microtitre plate-based biofilms after two minutes incubation must have been sufficient to allow a significant effective concentration of vancomycin at the site of the bacteria. Increasing the amount of time that the free drug was exposed to the biofilm for caused greater inhibition of bacterial growth, probably due to an increase in the effective concentration of vancomycin because of a higher degree of diffusion through the biofilm.

4.2.5 Effect of Liposomal Vancomycin

Vancomycin was passively encapsulated within both neutral and cationic liposomes. Both types of vesicle were capable of inhibiting bacterial growth. The fact that the neutral liposomes had an effect indicates that the attachment of vesicles to the bacterial biofilm was not essential for antibacterial activity of encapsulated vancomycin. Even though there was negligible adsorption of the neutral vesicles, nearly 60 % inhibition of growth was observed after 30 mins incubation of these liposomes with the bacteria (see Fig. 3.2.16). This suggests that these uncharged vesicles affected the bacteria by releasing their contents by leakage through the liposomal membrane into the surrounding medium, where the vancomycin could come into contact with the biofilm.

Encapsulation of vancomycin within these neutral liposomes effectively lowered the antibacterial activity of the agent against *S.epidermidis* because the free drug was more effective at all concentrations studied after both 15 mins (see Fig. 3.2.16) and 30 mins incubations (see Fig. 3.2.20). This was because the effective level of vancomycin was lower when encapsulated in neutral liposomes as a proportion of the drug would have remained inside the aqueous core of the vesicles, whereas with the free drug all of the agent was exposed to the biofilm throughout the incubation period.

As noted in section 3.2.5, the level of entrapment of vancomycin within the neutral liposomes had an effect on the antibacterial activity. This phenomenon could be seen more clearly if the growth inhibition was studied as a function of the concentration of the liposomal lipid (Figs. 4.2.4 and 4.2.5). At a fixed concentration of vancomycin, increasing the number of liposomes needed to attain that level of the drug led to a decrease in the effectiveness of the antibacterial liposomes. In other words, the higher the level of entrapment, the more effective the antibacterial activity; a lower number of highly-encapsulated liposomes inhibited growth more than a higher number of vesicles with relatively low encapsulation. This implied that the rate of release of vancomycin from the neutral liposomes was dependent on the level of encapsulation - the higher the concentration of vancomycin in the aqueous core, the greater the release into the surrounding medium.

The positively-charged liposomes that had vancomycin encapsulated within were more effective than the neutral ones and, in some cases, more effective than the free drug (see Section 3.2.6). This enhancement of inhibition was most probably due to the ability of the cationic VETs to adsorb to the biofilm during the incubation period (see Figs. 3.2.2-3.2.4) because of the electrostatic attraction between the two surfaces. This attachment gives the cationic vesicles two advantages over the uncharged liposomes. Firstly, because the cationic liposomes were in direct contact with the biofilm surface, the released contents would have had a greater chance of diffusing into the biofilm than the free drug in solution would have had. Secondly, unlike the free drug, the adsorbed liposomes would not have been washed off the biofilm after the incubation period and so would have been able to carry on releasing their contents during the 18 hour regrowth period, providing enhanced antibacterial action. Such enhancement has been demonstrated elsewhere [Swenson et al, 1988].

It is unlikely that the adsorbed liposomes would have fused with the bacterial cells. As well as the fact that the biofilm has a barrier effect that would probably have kept

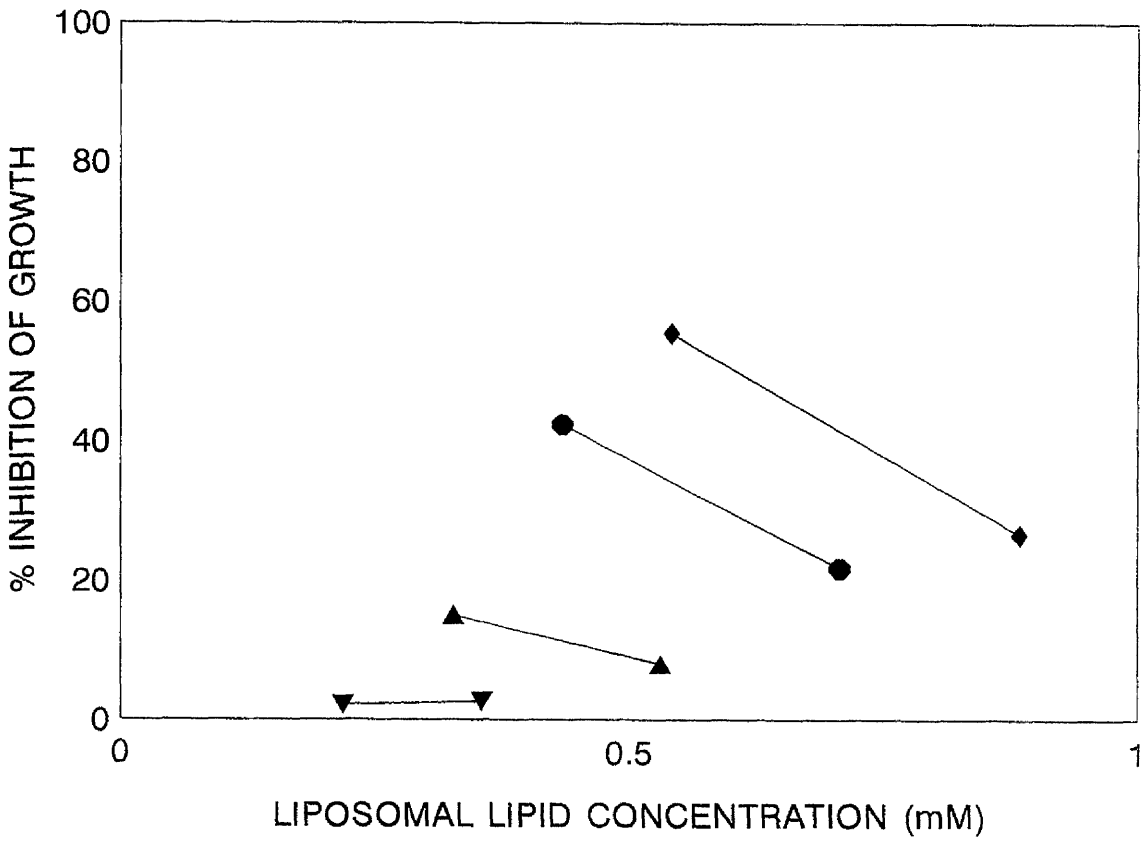


Fig. 4.2.4 Effect of liposomal lipid concentration on inhibition of bacterial growth by neutral liposomes (DPPC/Chol, molar ratio 1:0.21) encapsulating vancomycin. ▼, 20 μg vancomycin ml^{-1}; ▲, 30 μg ml^{-1}; ●, 40 μg ml^{-1}; ◆, 50 μg ml^{-1}. Incubation time 15 mins, room temperature.

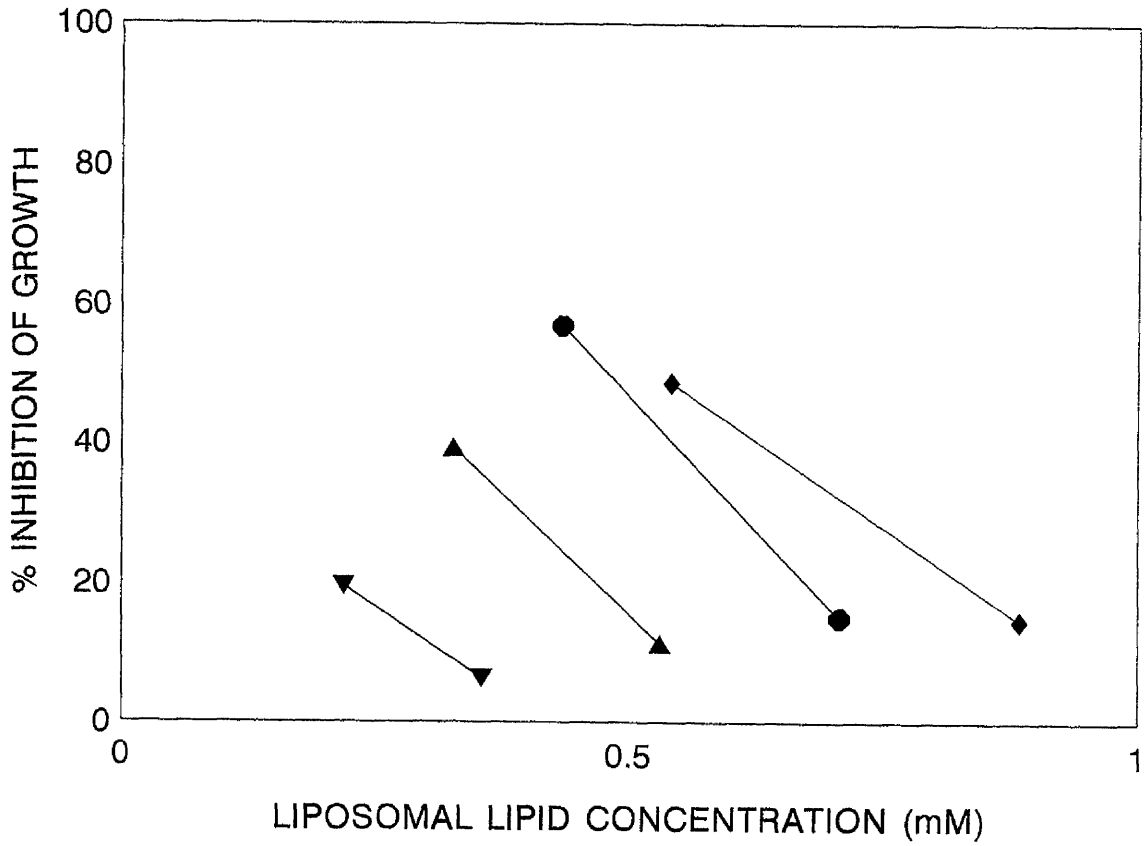


Fig. 4.2.5 Effect of liposomal lipid concentration on inhibition of bacterial growth by neutral liposomes (DPPC/Chol, molar ratio 1:0.21) encapsulating vancomycin. ▼, 20 μg vancomycin ml^{-1}; ▲, 30 μg ml^{-1}; ●, 40 μg ml^{-1}; ◆, 50 μg ml^{-1}. Incubation time 30 mins, room temperature.

the liposomes from coming into direct contact with the bacterial cells, only allowing released drug to diffuse through its matrix, the *Staphylococcus* bacteria are surrounded by relatively thick Gram-positive cell walls which have well-defined molecular size exclusion limits [Hancock and Poxton, 1988] which would prevent interaction between the liposomes and the bacterial cell membrane.

The presence of cholesterol in the liposomal membrane had an effect on antibacterial activity. This could have been due to the effect of the sterol on membrane permeability, as the absence of cholesterol did not cause any decrease in adsorption of cationic liposomes to the biofilm (see Fig. 3.2.2). Cholesterol has a rigidifying effect on liposomal membranes, so leakage of the drug would be much faster out of the liposomes without this steroid in the membrane, especially in the initial period between making the liposomes and performing the regrowth experiment (around one hour). Such rapid release reduces the beneficial effects of the adsoprtion of the cationic vesicles to the bacteria (see above). The most effective liposomes were those with the low cholesterol content; especially with vancomycin concentrations lower than 10 $\mu g\ ml^{-1}$, where the free vancomycin had no effect after 15 mins but the encapsulated drug led to a significant inhibition in bacterial growth (Fig. 4.2.6).

As with the neutral liposomes, the effectiveness of the cationic liposomes was dependent on the level of entrapment of the drug within, at least at some concentrations of vancomycin. Using the liposomes incorporating a low level of cholesterol, for example, after 15 mins incubation (Fig. 4.2.7) the antibacterial activity of the vesicles was dependent on the degree of encapsulation at vancomycin concentrations of 5-30 $\mu g\ ml^{-1}$ but was relatively independent of the entrapment level with higher drug concentrations (above 50 $\mu g\ ml^{-1}$). Using the same liposomes for a longer incubation time (30 mins), the inhibition was dependent of encapsulation with drug concentrations between 4 and 10 $\mu g\ ml^{-1}$ but was now independent of entrapment at vancomycin levels as low as 20 $\mu g\ ml^{-1}$ (Fig. 4.2.8).

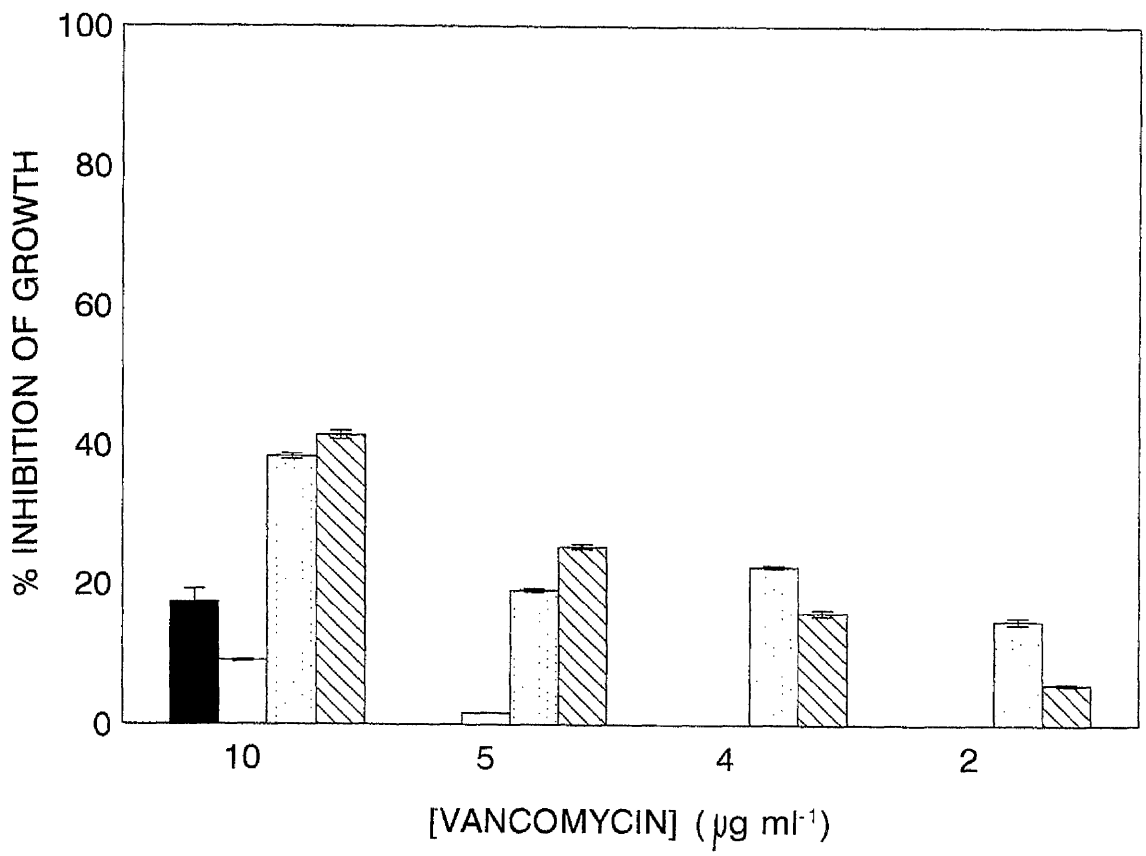


Fig. 4.2.6 Enhancement of antibacterial activity using vancomycin encapsulated in cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) compared to free vancomycin. Closed bar, free vancomycin; empty bar, liposomes (0.080 mg vancomycin/mg liposomal lipid); dotted bar, liposomes (0.129 mg/mg lipid); shaded bar, liposomes (0.187 mg/mg lipid). Incubation time 15 mins.

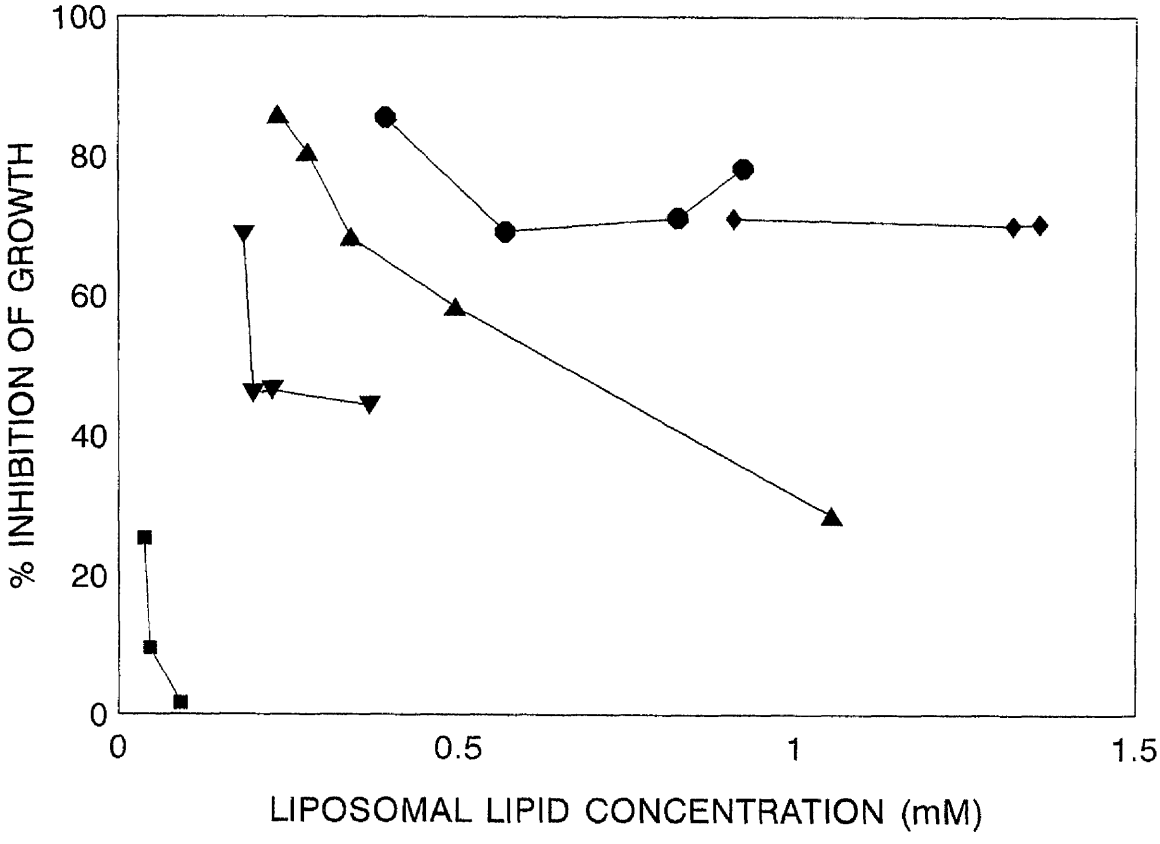


Fig. 4.2.7 Effect of liposomal lipid concentration on inhibition of bacterial growth by cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) encapsulating vancomycin. ■, 5 μg vancomycin ml^{-1}; ▼, 20 μg ml^{-1}; ▲, 30 μg ml^{-1}; ●, 50 μg ml^{-1}; ◆, 80 μg ml^{-1}. Incubation time 15 mins, room temperature.

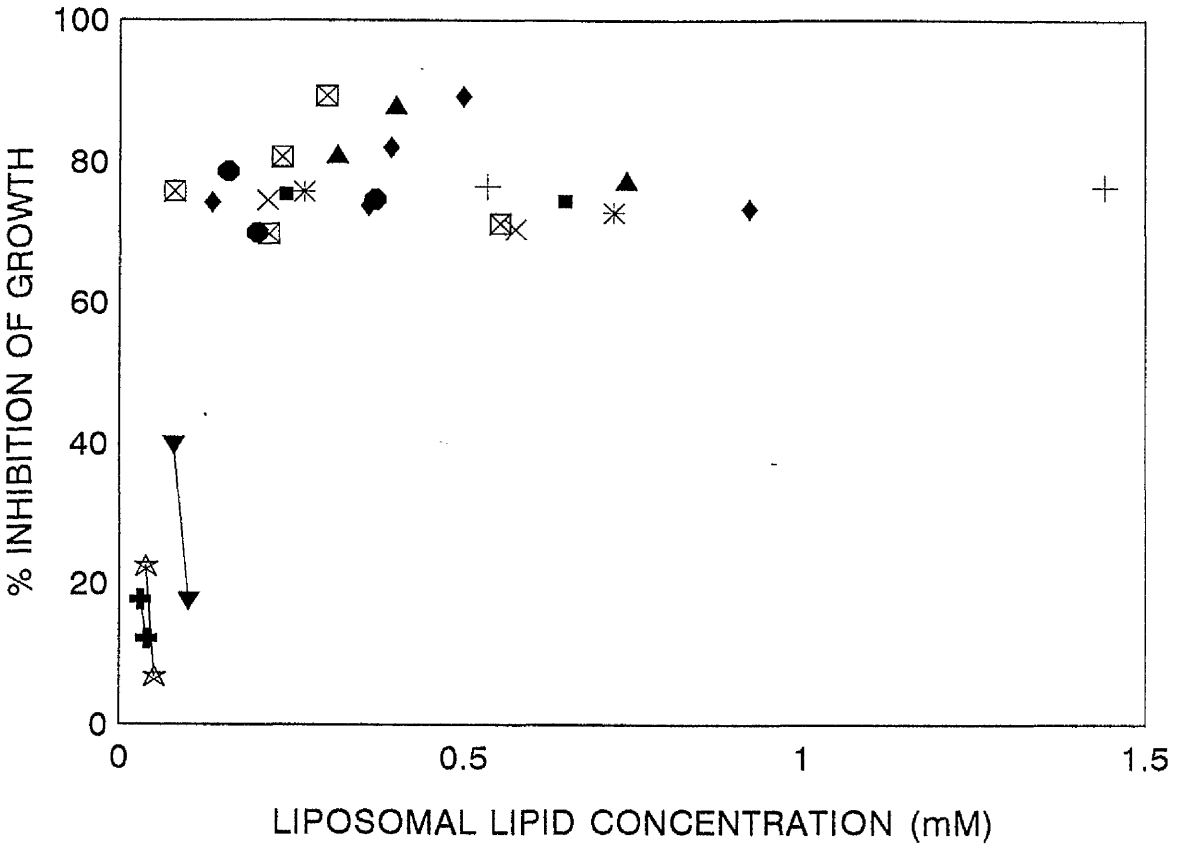


Fig. 4.2.8 Effect of liposomal lipid concentration on inhibition of bacterial growth by cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) encapsulating vancomycin. ✚, 4 μg vancomycin ml^{-1}; ☆, 5 μg ml^{-1}; ▼, 10 μg ml^{-1}; ●, 20 μg ml^{-1}; ☒, 30 μg ml^{-1}; ▲, 40 μg ml^{-1}; ◆, 50 μg ml^{-1}; ✶, 80 μg ml^{-1}; ■, 90 μg ml^{-1}; ✳, 100 μg ml^{-1}; +, 200 μg ml^{-1}. Incubation time 30 mins, room temperature.

A similar pattern was seen with the cationic liposomes without cholesterol (Figs. 4.2.9 and 4.2.10) and with the liposomes with a high cholesterol content (Figs. 4.2.11 and 4.2.12) after 15 and 30 mins incubation, as summarised in Table 4.2.1.

With cationic vesicles, increasing the incubation time lowered the threshold vancomycin concentration at which the inhibition became independent of the concentration of encapsulated drug. This dependence on entrapment level seemed to be eliminated at those drug concentrations where even vesicles with relatively low encapsulation levels (i.e. where high liposomal lipid concentrations were needed) were causing high levels of inhibition of bacterial growth (70 - 80 % inhibition) and so increasing the entrapment did not have much scope for increasing efficiency.

With the longer incubation time (30 mins), the liposomes could adsorb to the biofilm to a greater extent and the encapsulated contents had a longer time to leak out, so the drug concentration at which the less highly encapsulated vesicles can cause high levels of inhibition was reduced, hence the lowering of the threshold drug concentration.

The effect of entrapment can be examined in terms of how inhibition is affected by the monolayer coverage. For example, examining the results with the cationic liposomes with low cholesterol after 15 mins exposure (see Fig. 4.2.7), the inhibition observed at vancomycin levels where inhibition was dependent on drug entrapment (5-30 $\mu g\ ml^{-1}$) was seen to decrease with increased monolayer coverage at a fixed total drug concentration (Fig. 4.2.13), because as the coverage increases, the number of liposomes is greater and so the entrapment level is lower and the liposomal preparation less effective. Similarly, if liposomes with a similar entrapment level are compared (Fig. 4.2.14) then increasing the monolayer coverage, and hence the total drug concentration, increases the level of inhibition. Because the monolayer coverage and the level of entrapment are known, we can examine the inhibition in terms of the

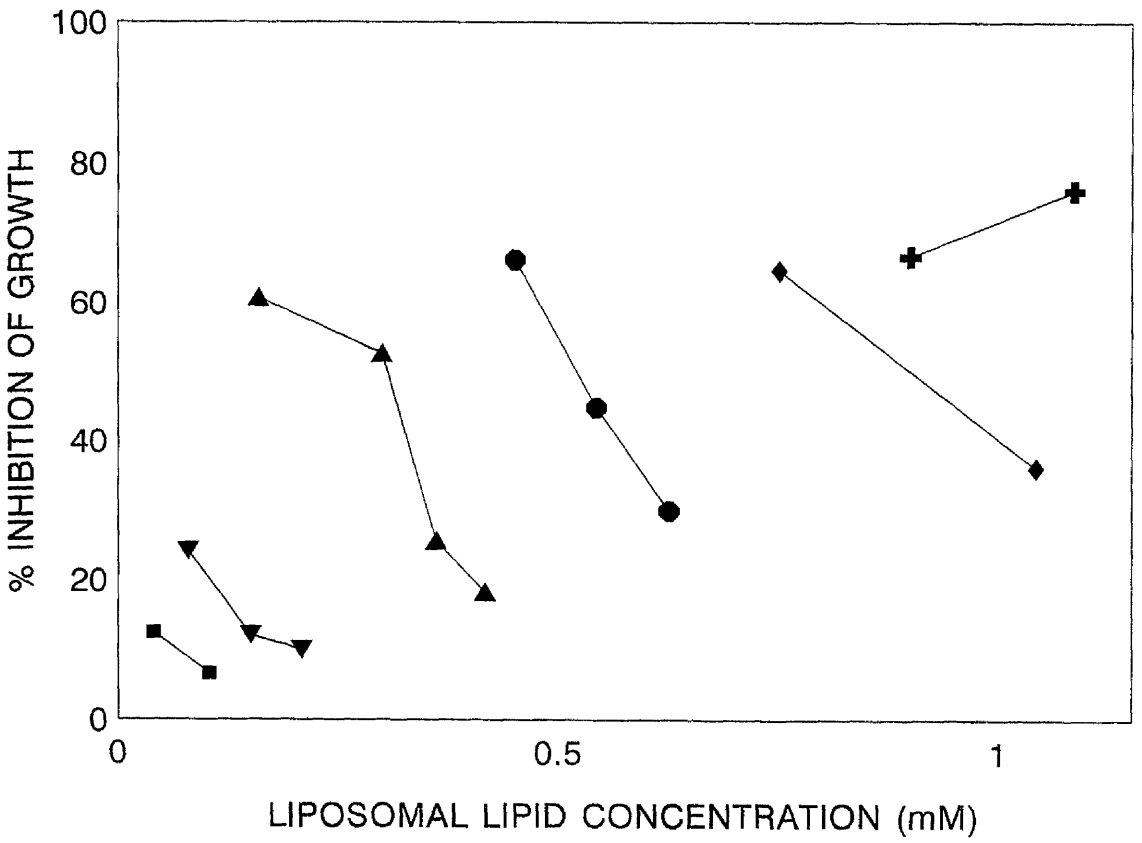


Fig. 4.2.9 Effect of liposomal lipid concentration on inhibition of bacterial growth by cationic liposomes without cholesterol (DPPC/DDAB, molar ratio 1:0.17) encapsulating vancomycin. ■, 5 μg vancomycin ml^{-1}; ▼, 10 μg ml^{-1}; ▲, 20 μg ml^{-1}; ●, 30 μg ml^{-1}; ◆, 50 μg ml^{-1}; ✚, 60 μg ml^{-1}. Incubation time 15 mins, room temperature.

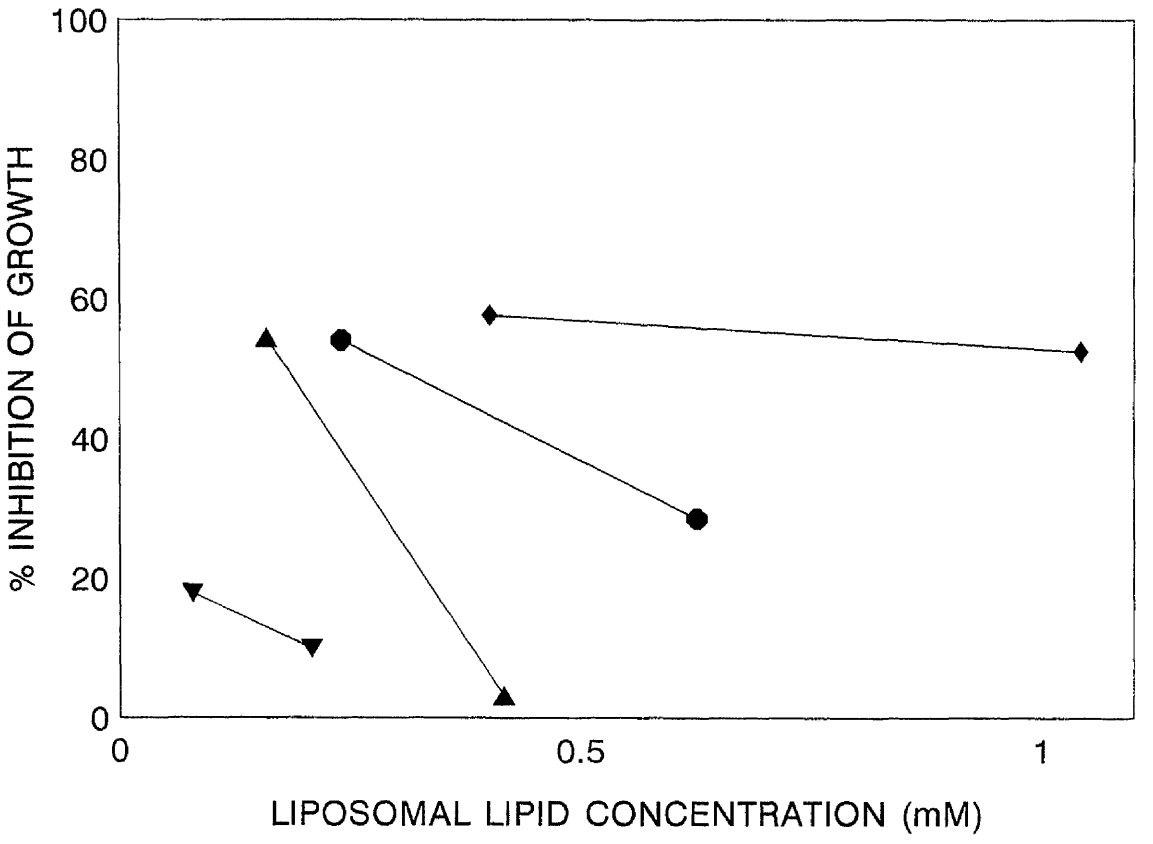


Fig. 4.2.10 Effect of liposomal lipid concentration on inhibition of bacterial growth by cationic liposomes without cholesterol (DPPC/DDAB, molar ratio 1:0.17) encapsulating vancomycin. ▼, 10 μg vancomycin ml^{-1}; ▲, 20 μg ml^{-1}; ●, 30 μg ml^{-1}; ◆, 50 μg ml^{-1}. Incubation time 30 mins, room temperature.

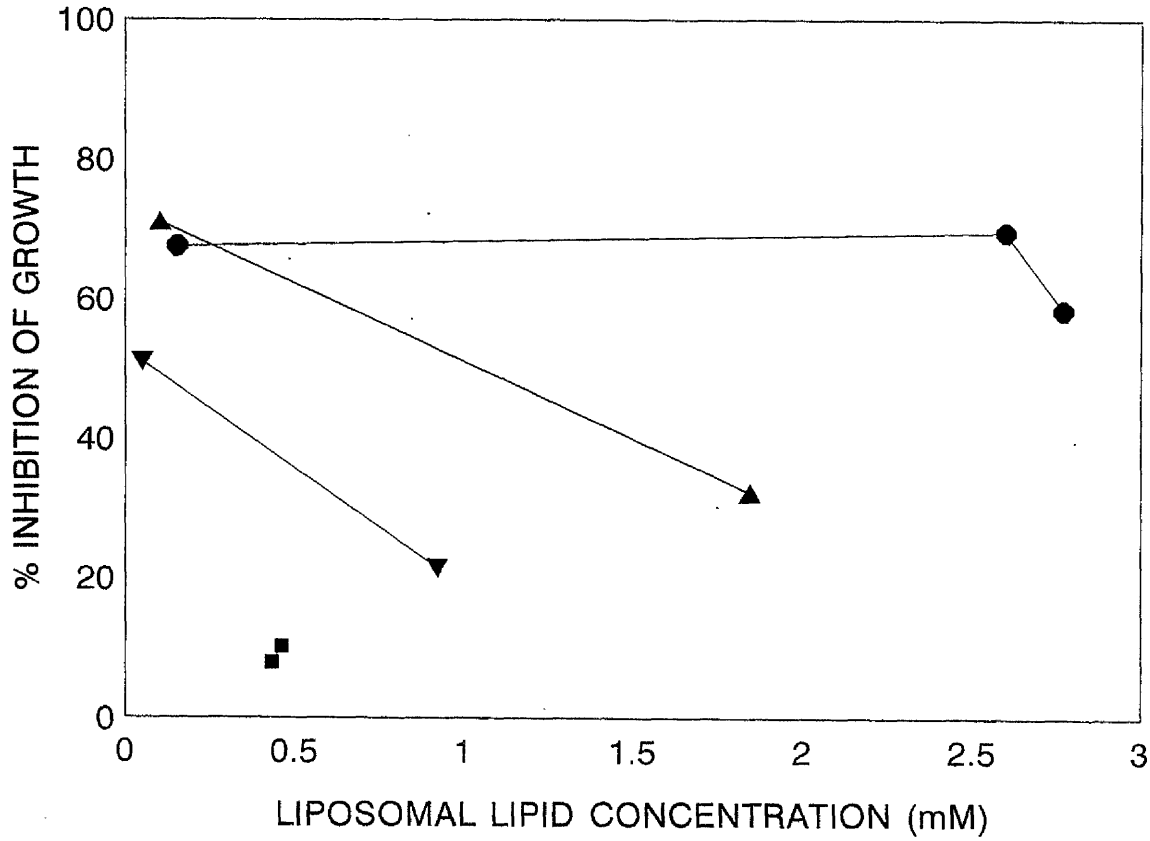


Fig. 4.2.11 Effect of liposomal lipid concentration on inhibition of bacterial growth by cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34) encapsulating vancomycin. ■, 5 μg vancomycin ml^{-1}; ▼, 10 μg ml^{-1}; ▲, 20 μg ml^{-1}; ●, 30 μg ml^{-1}. Incubation time 15 mins, room temperature.

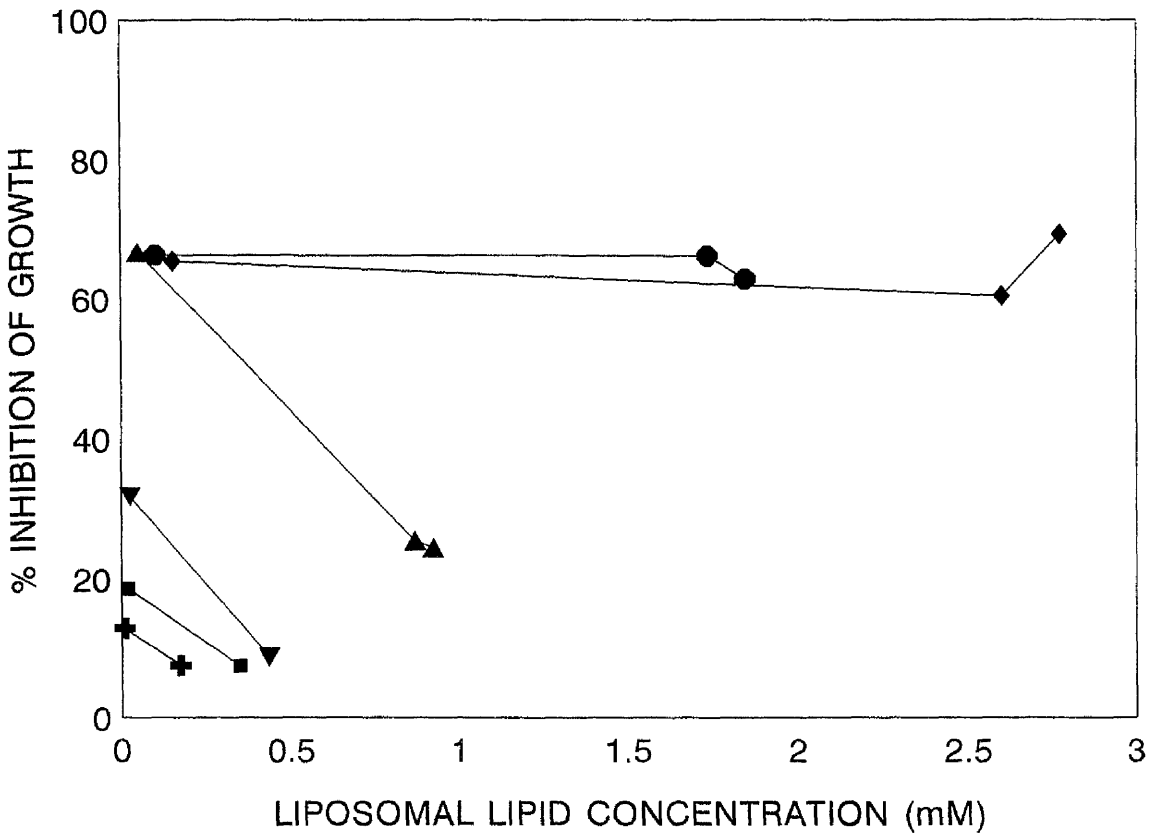


Fig. 4.2.12 Effect of liposomal lipid concentration on inhibition of bacterial growth by cationic liposomes with high cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.98:0.34) encapsulating vancomycin. ✚, 2 μg vancomycin ml^{-1}; ■, 4 μg ml^{-1}; ▼, 5 μg ml^{-1}; ▲, 10 μg ml^{-1}; ●, 20 μg ml^{-1}; ◆, 30 μg ml^{-1};. Incubation time 30 mins, room temperature.

LIPOSOME TYPE	EXPOSURE TIME	[VANCOMYCIN] WHERE INHIBITION WAS DEPENDENT ON ENTRAPMENT	[VANCOMYCIN] WHERE INHIBITION WAS INDEPENDENT OF ENTRAPMENT
NEUTRAL	15 MINS	20 - 50 μg ml-1	-
	30 MINS	20 - 50 μg ml-1	-
CATIONIC (NO CHOL)	15 MINS	5 - 50 μg ml-1	60 μg ml-1
	30 MINS	10 -30 μg ml-1	50 μg ml-1
CATIONIC (LOW CHOL)	15 MINS	5- 30 μg ml-1	50 - 80 μg ml-1
	30 MINS	4 - 10 μg ml-1	20 - 200 μg ml-1
CATIONIC (HIGH CHOL)	15 MINS	5 -20 μg ml-1	30 μg ml-1
	30 MINS	4-10 μg ml-1	20 - 30 μg ml-1

Table 4.2.1 Dependence of the antibacterial activity of liposomal vancomycin on the level of drug entrapment.

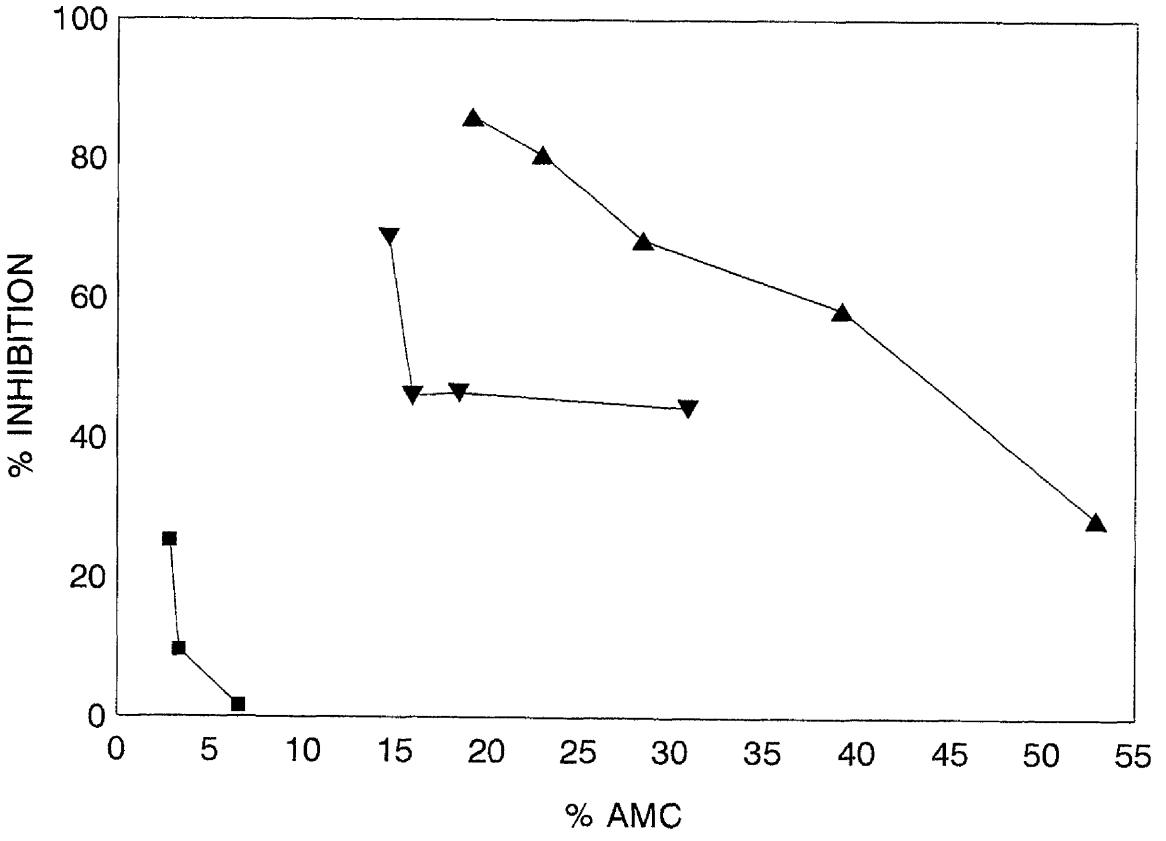


Fig. 4.2.13 Effect of monolayer coverage on inhibition of bacterial growth by cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) encapsulating vancomycin at various drug concentrations. ■, 5 μg vancomycin ml^{-1}; ▼, 20 μg ml^{-1}; ▲, 30 μg ml^{-1}. Incubation time 15 mins, room temperature.

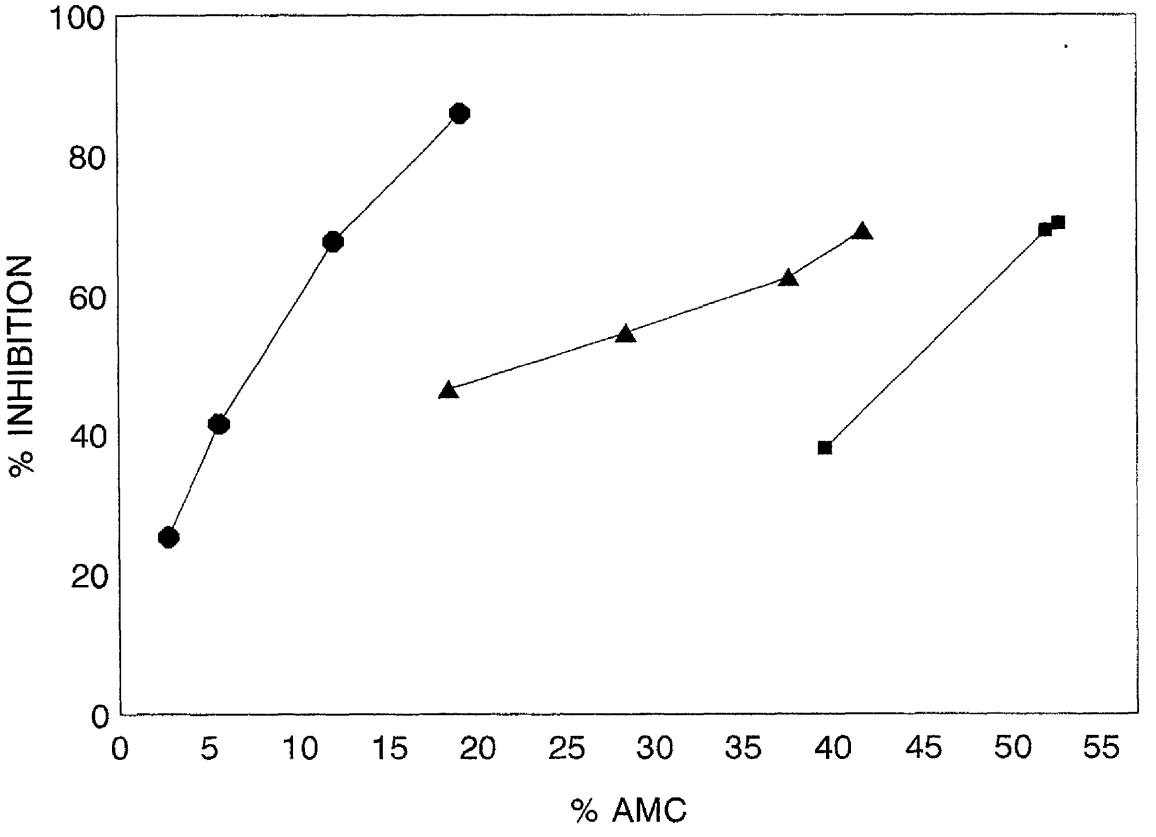


Fig. 4.2.14 Effect of monolayer coverage on inhibition of bacterial growth by cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) encapsulating vancomycin at various levels of drug entrapment. ■, 0.086 mg vancomycin/mg liposomal lipid; ▲, 0.129 mg/mg lipid; ●, 0.187 mg/mg lipid. Incubation time 15 mins, room temperature.

effective amount of vancomycin, i.e. the total amount of drug in the liposomes that have adsorbed to the bacterial biofilm (mg liposomal lipid adsorbed multiplied by the mg vancomycin per mg liposomal lipid). When the inhibition is plotted against effective amount of vancomycin (Fig. 4.2.15), hyperbolic plots are seen. At a given effective amount of vancomycin, the greatest inhibition of growth is seen with the liposomes with the highest level of entrapment (0.187 mg/mg lipid in this case). Thus, it is not the number of adsorbed liposomes nor the amount of liposomal vancomycin that is adsorbed at the biofilm surface that primarily affects the level of inhibition; it is the concentration of vancomyin within those vesicles. This implies that the factor that determines the efficiency of the liposomal preparations is the drug concentration gradient across the liposomal membranes; the greater the concentration of drug within the liposomes, the greater the extent of release from the liposomes.

The encapsulation of vancomycin within liposomes presents two advantages over the use of the free drug,. Firstly, the vesicles could be made part of a cleaning gel or cream applied to the skin at the site where a device is to be inserted. The liposomes will enhance the penetration of vancomycin into layers of the skin which can be a problem when the free drug is used in such a way [Bandyk and Esses, 1994]. Secondly, where infection of an implanted device is already established, a suspension of the vesicles could be injected into the patient in the vicinity of the implant. Hopefully, the cationic liposomes should target to the bacteria on the surface of the device and so reduce the general distribution of the drug in the body, as toxicity is a problem when the free drug is used [Entenza et al, 1994].

Liposomal vancomycin may become useful in the near future if strains of *S.epidermidis* develop that are vancomycin-resistant (see Section 4.2.4). Chowdhury *et al* [1981] demonstrated that the encapsulation of penicillins in cationic liposomes (egg PC/Chol/SA, molar ratio 2:1.5:0.22) had an antibacterial effect on penicillin-resistant bacteria, including a strain of *Staphylococcus aureus*. Cationic liposomes

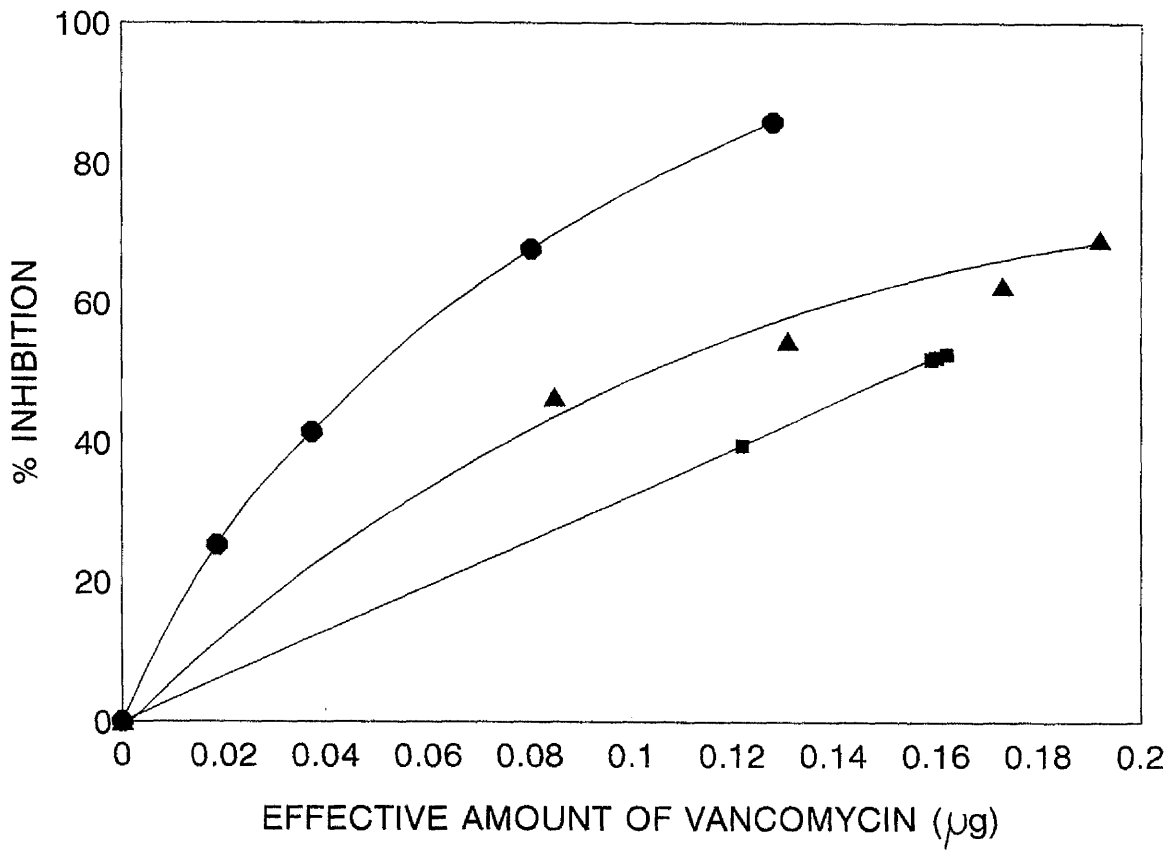


Fig. 4.2.15 Effect of total amount of vancomycin in liposomes adsorbed to the bacterial biofilm on inhibition of bacterial growth by cationic liposomes with low cholesterol content (DPPC/Chol/DDAB, molar ratio 1:0.28:0.22) encapsulating vancomycin at various levels of drug entrapment. ■, 0.086 mg vancomycin/mg liposomal lipid; ▲, 0.129 mg/mg lipid; ●, 0.187 mg/mg lipid. Incubation time 15 mins, room temperature.

containing 30 μg ml^{-1} of penicillin were shown to inhibit growth of *S.aureus* by up to 77.8 % compared to 30 % with neutral liposomes. However, 'empty' cationic liposomes with no encapsulated penicillin were also found to inhibit growth, by up to 25.8 %. This is perhaps an indication of the toxicity of stearylamine; 'empty' DDAB liposomes did not cause any inhibition of *S.epidermidis* growth (see Section 3.2.3). On the basis of this work by Chowdhury *et al*, liposomal vancomycin has potential use in the clinical treatment of resistant bacteria.

Vancomycin has previously been encapsulated in liposomes for the treatment of *S.aureus* resident in human macrophages [Onyeji et al, 1994a]. The drug was encapsulated because the vesicles would be taken up by the macrophages, increasing the intracellular vancomycin concentration to enhance the antibacterial action. The antibiotic was passively encapsulated within neutral SUVs (egg PC/Chol) produced by sonication, with encapsulation efficiencies of 4-8 %. Although the vesicles enhanced the activity of vancomycin against the intracellular pathogen, when they were used against free *S.aureus* the antibacterial effect was lower than that seen with the unencapsulated drug. That finding is similar to the result obtained in this work (see Section 3.2.5), where neutral liposomes were used to treat *S.epidermidis*.

4.2.6 Effect of Liposomal Gentamicin

Gentamicin is one of a family of antibiotics, the aminoglycosides [Coulson, 1994]. Under aerobic conditions they are bactericidal, i.e. they kill bacteria rather than just inhibiting their growth. These drugs are taken up by ribosomes where they interfere with protein synthesis. They are effective against a wide range of pathogens and are used in severe staphylococcal infections when other treatments have failed [Gillies et al, 1986]. Toxicity mainly affects the ears and kidneys.

Aminoglycoside antibiotics such as gentamicin are large, hydrophilic compounds with 3-5 aminated groups which makes the compounds polycationic [Van Bambeke

et al, 1995]. For these reasons, they cross membranes poorly and would not be expected to leak out of liposomes very much [Swenson et al, 1988]. This could explain why the liposomal form of gentamicin was far less effective than the free drug at gentamicin levels of 100 $\mu g\ ml^{-1}$ and below (see Fig. 3.2.26). Encapsulated gentamicin is probably most effective against intracellular pathogens, not extracellular microorgansims like *S.epidermdis* [Karlowsky and Zhanel, 1992]. If phagocytosed by the cells that are acting as host to the infection, the liposomal membranes would be broken down and so the low permeability would not be a problem.

The encapsulation of two or more antibiotics in a single liposome has been suggested [Swenson et al, 1988]. This would seem an ideal system for combining vancomycin and gentamicin, as the two drugs together have been demonstrated to exhibit a synergistic effect against *S.aureus* [Gilman et al, 1990]. However, no such effect was seen here against *S.epidermidis* with the free drugs and so the encapsulation was not attempted. It seemed that the two drugs actually had less effect when used together than when applied separately, implying that one of the drugs may inhibit the action of the other against *S.epidermidis*.

4.3 CONCLUSIONS

Cationic liposomes have been produced and have been shown to adsorb to biofilms of the bacteria *Staphylococcus epidermidis*. The interaction is reversible and is dependent on the length of time that the liposomes are incubated with the biofilm and on the concentration of liposomes applied. The adsorption is affected by the ionic strength of the incubation medium, the temperature of incubation and the hydrophobicity of the bacterial cells. Calculation of the Gibbs free energy change of adsorption indicates that the process is thermodynamically favourable, with the liposomes being more ordered when adsorbed than when free in solution.

The antibacterial agents vancomycin and gentamicin have been encapsulated (separately) within vesicles. Liposomal gentamicin was less effective than the free drug in inhibiting growth of *S.epidermidis*, suggesting that the encapsulation of this agent was not suited for treatment of such extracellular pathogens. Vancomycin was as effective as, and in some case more effective than, the free drug when encapsulated in cationic liposomes. The effectiveness of the preparation was determined by the composition and the concentration of drug entrapped within the vesicles. The liposomal form of the drug was effective at some low vancomycin concentrations where the free drug was ineffective, meaning that lower concentrations could be used to treat infections, lowering systemic toxicity. The encapsulated drug could potentially be used to topically treat areas of skin destined to be sites for implantation of biomedical devices, as penetration of the free drug into the skin is a problem.

CHAPTER FIVE

REFERENCES

References

Ahmad, I., Longenecker, M., Samuel, J. and Allen, T.M. (1993). Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. Cancer Research **53**, 1484-1488

Akamo, Y., Mizuno, I., Yotsuyanagi, T., Ichino, T., Tanimoto, N., Yamamoto, T., Nagata, M., Takeyama, H., Shinagawa, N., Yura, J. and Manabe, T. (1994). Chemotherapy targeting regional lymph nodes by gastric submucosal injection of liposomal adriamycin in patients with gastric carcinoma. Japanese J. Cancer Research **85**, 652-658

Aliño, S.F., Crespo, J., Bobadilla, M., Lejarreta, M., Blaya, C. and Crespo, A. (1994). Expression of human α1-antitrypsin in mouse after *in vivo* gene transfer to hepatocytes by small liposomes. Biochemical and Biophysical Research Communications **204**, 1023-1030

Allen, T.M. (1994). Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends in Pharmacological Sciences **15**, 215-220

Allen, T.M. and Chonn, A. (1987). Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Letters **223**, 42-46

Allen, T.M. and Hansen, C. (1991). Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochimica et Biophysica Acta **1068**, 133-141

Alpar, H.O., Bowen, J.C. and Brown, M.R.W. (1992). Effectiveness of liposomes as adjuvants of orally and nasally administered tetanus toxoid. Int. J. Pharmaceutics **88**, 335-344

Alving, C.R. (1987). Liposome techniques in cell biology. Nature **330**, 189-190

Alving, C.R. (1992). Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens. Biochimica et Biophysica Acta **1113**, 307-322

Alving, C.R. (1993). Lipopolysaccharide, lipid A and liposomes containing lipid A as immunologic adjuvants. Immunobiology **187**, 430-446

Anaissie, E., Paetznick, V., Proffitt, R., Adler-Moore, J. and Bodey, G.P. (1991). Comparison of the *in vitro* antifungal activity of free and liposome-encapsulated amphotericin B. Eur. J. Clinical Microbiology and Infectious Diseases **10**, 665-668

Antimisiaris, S.G., Jayasekera, P. and Gregoriadis, G. (1993). Liposomes as vaccine carriers. Incorporation of soluble and particulate antigens in giant vesicles. J. Immunological Methods **166**, 271-280

Arvaniti, A., Karamanos, N.K., Dimitracopoulos, G. and Anastassiou, E.D. (1994). Isolation and characterisation of a novel 20-kDa sulfated polysaccharide from the extracellular slime layers of *Staphylococcus epidermidis*. Archives of Biochemistry and Biophysics **308**, 432-438

Baker, L.R.I., Brown, A.L., Stephenson, J.R., Tabaqchali, S., Zatouroff, M., Parkin, J.M. and Pinching, A.J. (1993). Bacteraemia due to recurrent reinfection with *Staphylococcus epidermidis* associated with defective opsonisation and procidin function in serum. J. Clinical Pathology **46**, 398-402

Baker, R.R., Czopf, L., Jilling, T., Freeman, B.A., Kirk, K.L. and Matalon, S. (1992). Quantitation of alveolar distribution of liposome-entrapped antioxodant enzymes. Am. J. Physiology **263**, L585-L594

Bakker-Woudenberg, I.A.J.M., Lokerse, A.F., ten Kate, M.T., Melissen, P.M.B., van Vianen, W. and van Etten, E.W.M. (1993). Liposomes as carriers of antimicrobial agents or immunomodulatory agents in the treatment of infections. Eur. J. Clinical Microbiology and Infectious Diseases **12**, S61-S67

Bandyk, D.F. and Esses, G.E. (1994). Prosthetic graft infection. Surgical Infections **74**, 571-590

Banerjee, G., Bhaduri, A.N. and Basu, M.K. (1994). Mannose-coated liposomal hamycin in the treatment of experimental leishmaniasis in hamsters. Biochemical Medicine and Metabolic Biology **53**, 1-7

Bangham, A.D. (1993). Liposomes: the Babraham connection. Chemistry and Physics of Lipids **64**, 275-285

Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Molecular Biology **13**, 238-252

Baztri, S. and Korn, E.D. (1973). Single bilayer liposomes prepared without sonication. Biochimica et Biophysica Acta **298**, 1015-1019

Bennett, D.B., Tyson, E., Mah, S., de Groot, J.S., Hegde, S.G., Terao, S. and Teitelbaum, Z. (1994). Sustained delivery of detirelix after pulmonary administration of liposomal formulations. J. Controlled Release **32**, 27-35

Berg, J.T., Lee, S.T., Thepen, T., Lee, C.Y. and Tsan M.-F. (1993). Depletion of alveolar macrophages by liposome-encapsulated dichloromethylene-diphosphonate. J. Applied Physiology **74**, 2812-2819

Betageri, G.V., Jenkins, S.A. and Ravis, W.R. (1993). Drug delivery using antibody-liposome conjugates. Drug Development and Industrial Pharmacy **19**, 2109-2116

Betjes, M.G.H., Tuk, C.W., Struijk, D.G., Krediet, R.T., Arisz, L., Hart, M. and Beelen, R.H.J. (1993). Interleukin-8 production by human peritoneal mesothelial cells in response to tumor necrosis factor-α, interleukin-1 and medium conditioned by macrophages cultured with *Staphylococcus epidermidis*. J. Infectious Diseases **168**, 1202-1210

Bétrémieux, P., Donnio, P.Y. and Pladys, P. (1995). Use of ribotyping to investigate tracheal colonisation by *Staphylococcus epidermidis* as a source of bacteremia in ventilated newborns. Eur. J. Clinical Microbiology and Infectious Diseases **14**, 342-345

Block, M.C., van der Neut-Kok, E.C.M., van Deenen, L.L.M. and de Gier, J. (1975). The effect of chain length and lipid phase transitions on the selective permeability properties of liposomes. Biochimica et Biophysica Acta **406**, 187-196

Blume, A., Jansen, M., Ghyczy, M. and Gareiss, J. (1993). Interaction of phospholipid liposomes with lipid model mixtures for sratum corneum lipids. Int. J. Pharmaceutics **99**, 219-228

Blume, G., Cevc, G., Crommelin, M.D.J.A., Bakker-Woudenberg, I.A.J.M., Kluft, C. and Storm, G. (1993). Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. Biochimica et Biophysica Acta **1149**, 180-184

Bonetti, A. and Kim, S. (1993). Pharmacokinetics of an extended-release human interferon alpha-2b formulation. Cancer Chemotherapy and Pharmacology **33**, 258-261

Bonventre, P.F. and Gregoriadis, G. (1978). Killing of intraphagocytic *Staphylococcus aureus* by dihydrostreptomycin entrapped within liposomes. Antimicrobial Agents and Chemotherapy **13**, 1049-1051

Brandl, M., Bachmann, D., Augustin, A.J., Jäger, L.J.E. and Lutz, J. (1994). Depression of the phagocytic activity of the RES by liposomes. Influence of lipids. Pharmaceutical and Pharmacological Letters **4**, 1-4

Brandl, M. and Gregoriadis, G. (1994). Entrapment of haemoglobin into liposomes by the dehydration-rehydration method: vesicle characterization and in vivo behaviour. Biochimica et Biophysica Acta **1196**, 65-75

Brook, S.J. and Brook, I. (1994). Are public library books contaminated by bacteria ? J. Clinical Epidemiology **47**, 1173-1174

Calame, W., Hendrickx, R.J.B.M., Namavar, F., Oe, L.P. and Beelen, R.H.J. (1995). Effect of glucose in dialysis fluid on antibacterial defence in the peritoneal cavity. J. Infection **30**, 227-233

Campbell, M.J. (1995). Lipofection reagents prepared by a simple ethanol injection technique. BioTechniques **18**, 1029-1032

Chandrasekhar, U., Sinha, S., Bhagat, H.R., Sinha, V.B. and Srivastava, B.S. (1994). Comparative efficacy of biodegradable liposomes and microspheres as carriers for delivery of *Vibrio cholerae* antigens in the intestine. Vaccine **12**, 1384-1388

Chapman, V., Fletcher, S.M. and Jones, M.N. (1990). A simple theoretical treatment of a competitive enzyme-linked immunosorbent assay (ELISA) and its application to the detection of human blood group antigens. J. Immunological Methods **131**, 91-98

Chen, D., Li, Q.-T. and Lee, K.H. (1992). Studies on the entrapment and stability of liposomes containing sulfatide from human brain. Biochemistry International **28**, 297-304

Chonn, A., Semple, S.C. and Cullis P.R. (1992). Association of blood proteins with large unilamellar liposomes *in vivo*: relation to circulation lifetimes. J. Biological Chemistry **267**, 18759-18765

Chopra, R., Fielding, A. and Goldstone, A.H. (1992). Successful treatment of fungal infections in neutropenic patients with liposomal amphotericin (AmBisome) - a report on 40 cases from a single centre. Leukemia and Lymphoma **7**, 73-77

Chowdhury, M.K.R., Goswami, R. and Chakrabarti, P. (1981). Liposome-entrapped penicillins in growth inhibition of some penicillin-resistant bacteria. J. Applied Bacteriology **51**, 223-227

Clerc, S.G. and Thompson, T.E. (1994). A possible mechanism for vesicle formation by extrusion. Biophysical J. **67**, 475-476

Coker, R., Tomlinson, D. and Harris, J. (1991). Successful treatment of cryptococcal meningitis with liposomal amphotericin B after failure of treatment with fluconazole and conventional amphotericin B. AIDS **5**, 232-232

Connor, J., Yatvin, M.B. and Huang, L. (1984). pH-sensitive liposomes: acid-induced liposome fusion. Proceedings of the National Academy of Sciences U.S.A. **81**, 1715-1718

Coulson, C.J. (1994). "Molecular Mechanisms of Drug Action.", pp. 86-88. Taylor & Francis, U.K.

Coutelle, C. (1995). Gene therapy approaches for cystic fibrosis. Biologicals **23**, 21-25

Cowens, J.W., Creaven, P.J., Greco, W.R., Brenner, D.E., Tung, Y., Ostro, M., Pilkiewicz, F., Ginsberg, R. and Petrelli, N. (1993). Initial clinical (phase I) trials of TLC D-99 (doxorubicin encapsulated in liposomes). Cancer Research **53**, 2796-2802

Creaven, P.J., Cowens, J.W., Brenner, D.E., Dadey, B.M., Han, T., Huben, R., Karakousis, C., Frost, H., LeSher, D., Hanagan, J., Andrejico, K. and Cushman, M.K. (1990). Initial clinical trial of the macrophage activator muramyl tripeptide-phosphatidylethanolamine encapsulated in liposomes in patients with advanced cancer. J. Biological Response Modifiers **9**, 492-498

Cullis, P.R. and de Kruijff, B. (1979). Lipid polymorphism and the functional roles of lipids in biological membranes. Biochimica et Biophysica Acta **559**, 399-420

Cuperus, P.L., Van der Mei, H.C., Reid, G., Bruce, A.W., Khoury, A.E., van der Kuijl-Booij, M., Noordmans, J. and Busscher, H.J. (1995). Effects of ciprofloxacin and vancomycin on physiochemical surface properties of *Staphylococcus epidermidis*, *Escherichia coli*, *Lactobacillus casei* and *Lactobacillus acidophilus*. Microbios **82**, 49-67

Daemen, T., Dontje, B.H.J., Regts, J. and Scherpof, G.L. (1993). Chemoimmunotherapy of murine liver metastases with 5-fluorouracil in combination with liposome-encapsulated muramyl dipeptide. J. Immunotherapy **13**, 31-35

Daemen, T., Hofstede, G., Ten Kate, M.T., Bakker-Woudenberg, I.A.J.M. and Scherpof, G.L. (1995). Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages. Int. J. Cancer **61**, 716-721

Daoud, S.S. (1992). Cell membranes as targets for anti-cancer drug action. Anti-Cancer Drugs **3**, 443-453

Deamer, D.W. and Bangham, A.D. (1976). Large volume liposomes by an ether vaporization method. Biochimica et Biophysica Acta **443**, 629-634

Dean, T. (1993). Liposomes in allergy and immunology. Clinical and Experimental Allergy **23**, 557-563

Deighton, M. and Borland, R. (1993). Regulation of slime production in *Staphylococcus epidermidis* by iron limitation. Infection and Immunity **61**, 4473-4479

de Marie, S., Janknegt, R. and Bakker-Woudenberg, I.A.J.M. (1994). Clinical use of liposomal and lipid-complexed amphotericin B. J. Antimicrobial Chemotherapy **33**, 907-916

Désormeaux, A. and Bergeron, M.G. (1995). Targeting HIV with liposome-encapsulated antivirals. Zbl. Bakt. **282**, 225-231

Doi, K., Oku, N., Toyota, T., Shuto, S., Sakai, A., Itoh, H. and Okada, S. (1994). Therapeutic effect of reticuloendothelial system (RES)-avoiding liposomes containing a phospholipid analogue of 5-fluorouracil, dipalmitoylphosphatidyl-fluorouridine, in Meth A sarcoma-bearing mice. Biological and Pharmaceutical Bulletin **17**, 1414-1416

Dunne Jr, W.M. and Burd, E.M. (1992). The effects of magnesium, calcium, EDTA, and pH on the *in vitro* adhesion of *Staphylococcus epidermidis* to plastic. Microbiology and Immunology **36**, 1019-1027

Dunne Jr, W.M., Mason Jr, E.O. and Kaplan, S.L. (1993). Diffusion of Rifampin and Vancomycin through a *Staphylococcus epidermidis* biofilm. Antimicrobial Agents and Chemotherapy **37**, 2522-2526

Du Plessis, J., Weiner, N. and Müller, D.G. (1994). The influence of in vivo treatment of skin with liposomes on the topical absorption of a hydrophilic and a hydrophobic drug in vitro. Int. J. Pharmaceutics **103**, R1-R5

Edmond, M.B., Ober, J.F., Weinbaum, D.L., Pfaller, M.A., Hwang, T., Sanford, M.D. and Wenzel, R.P. (1995). Vancomycin-resistant *Enterococcus faecium* bacteremia: risk factors for infection. Clinical Infectious Diseases **20**, 1126-1133

Ellens, H., Bentz, J. and Szoka, F.C. (1984). pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. Biochemistry **23**, 1532-1538

Elorza, B., Elorza, M.A., Sainz, M.C. and Chantres, J.R. (1993). Analysis of the particle size distribution and internal volume of liposomal preparations. J. Pharmaceutical Sciences **82**, 1160-1163

Embree, L., Gelmon, K.A., Lohr, A., Mayer, L.D., Coldman, A.J., Cullis, P.R., Palaitis, W., Pilkiewicz, F., Hudon, N.J., Heggie, J.R. and Goldie, J.H. (1993). Chromatographic analysis and pharmacokinetics of liposome-encapsulated doxorubicin in non-small-cell lung cancer patients. J. Pharmaceutical Sciences **82**, 627-634

Emminger, W., Graninger, W., Emminger-Schmidmeier, W., Zoubek, A., Pillwein, K., Susani, M., Wasserer, A. and Gadner, H. (1994). Tolerance of high doses of amphotericin B by infusion of a liposomal formulation in children with cancer. Annals of Hematology **68**, 27-31

Eng, R.H.K., Hsieh, A. and Smith, S.M. (1995). Antibiotic killing of bacteria: comparison of bacteria on surfaces and in liquid, growing and nongrowing. Chemotherapy **41**, 113-120

Engel, A., Bendas, G., Wilhelm, F., Mannova, M., Ausborn, M. and Nuhn, P. (1994). Freeze drying of liposomes with free and membrane-bound cryoprotectants - the background of protection and damaging processes. Int. J. Pharmaceutics **107**, 99-110

Entenza, J.M., Fluckiger, U., Glauser, M.P. and Moreillon, P. (1994). Antibiotic treatment of experimental endocarditis due to methicillin-resistant *Staphylococcus epidermidis*. J. Infectious Diseases **170**, 100-109

Etienne, J., Brun, Y., El Solh, N., Delorme, V., Mouren, C., Bes, M. and Fleurette, J. (1988). Characterization of clinically significant isolates of *Staphylococcus epidermidis* from patients with endocarditis. J. Clinical Microbiology **26**, 613-617

Eytan, G.D. (1982). Use of liposomes for reconstitution of biological functions. Biochimica et Biophysica Acta **694**, 185-202

Farber, B.F., Hsieh, H.-C., Donnenfeld, E.D., Perry, H.D., Epstein, A. and Wolff, A. (1995). A novel antibiofilm technology for contact lens solutions. Opthalmology **102**, 831-836

Fatunmbi, O.O., Newman, J.A., Sivanandan, V. and Halvorson, D.A. (1992a). Efficacy of avridine and liposomes as adjuvants for avian influenza virus antigens in turkeys. Avian Pathology **21**, 225-237

Fatunmbi, O.O., Newman, J.J., Sivanandan, V. and Halvorson, D.A. (1992b). Enhancement of antibody response of turkeys to trivalent avian influenza vaccine by positively charged liposomal avridine adjuvant. Vaccine **10**, 623-626

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proceedings of the National Academy of Sciences U.S.A. **84**, 7413-7417

Felgner, P.L. and Ringold, G.M. (1989). Cationic liposome-mediated transfection. Nature **337**, 387-388

Fischer, G.W., Cieslak, T.J., Wilson, S.R., Weisman, L.E. and Hemming, V.G. (1994). Opsonic antibodies to *Staphylococcus epidermidis*: in vitro and in vivo studies using human intravenous immune globulin. J. Infectious Diseases **169**, 324-329

Fountain, M.W., Weiss, S.J., Fountain, A.G., Shen, A. and Lenk, R.P. (1985). Treatment of *Brucella canis* and *Brucella abortus* in vitro and in vivo by stable plurilamellar vesicle-entrapped aminoglycosides. J. Infectious Diseases **152**, 529-535

Friede, M., van Regenmortel, M.H.V. and Schuber, F. (1993). Lyophilized liposomes as shelf items for the preparation of immunogenic liposome-peptide conjugates. Analytical Biochemistry **211**, 117-122

Fujii, Y., Aramaki, Y., Hara, T., Yachi, K., Kikuchi, H. and Tsuchiya, S. (1993). Enhancement of systemic and mucosal immune responses following oral administration of liposomes. Immunology Letters **36**, 65-70

Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A. and Barenholz, Y. (1994). Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Research **54**, 987-992

Gangadharam, P.R.J., Ashtekar, D.R., Flasher, D.L. and Düzgünes, N. (1995). Therapy of *Mycobacterium avium* complex infections in beige mice with streptomycin encapsulated in sterically stabilized liposomes. Antimicrobial Agents and Chemotherapy **39**, 725-730

Gao, X. and Huang, L. (1991). A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochemical and Biophysical Research Communications **179**, 280-285

Geertsema-Doornbusch, G.I., van der Mei, H.C. and Busscher, H.J. (1993). Microbial cell surface hydrophobicity. The involvement of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). J. Microbiological Methods **18**, 61-68

Genin, I., Barratt, G., Tran, X.T., De Lattre, J. and Puisieux, F. (1994). Optimization and characterization of freeze-dried multilamellar liposomes incorporating different standardized allergen extracts. Allergy **49**, 645-652

Genne, P., Olsson, N.-O., Gutierrez, G., Duchamp, O. and Chauffert, B. (1994). Liposomal mitoxantrone for local treatment of peritoneal carcinomatosis induced by colon cancer cells in mice. Anti-Cancer Drug Design **9**, 73-84

Gilchrest, B.A., Zhai, S., Eller, M.S., Yarosh, D.B. and Yaar, M. (1993). Treatment of human melanocytes and S91 melanoma cells with the DNA repair enzyme T4 endonuclease V enhances melanogenesis after ultraviolet irradiation. J. Investigative Dermatology **101**, 666-672

Gillies, H.C., Rogers, H.J., Spector, R.G. and Trounce, J.R. (1986). "A Textbook of Clinical Pharmacology" (2nd Ed.), pp. 736-737. Hodder & Stoughton, U.K.

Gilman, A.G., Rall, T.W., Nies, A.S. and Taylor, P. (1990). "The Pharmacological Basis of Therapeutics." (8th Ed.), pp. 1138-1140. Pergamon Press, New York.

Giridhar, G., Kreger, A.S., Myrvik, Q.N. and Gristina, A.G. (1994). Inhibition of *Staphylococcus* adherence to biomaterials by extracellular slime of *S.epidermidis* RP12. J. Biomedical Materials Research **28**, 1289-1294

Goëau-Brissonnière, O (1995). Infection of arterial prostheses. Presse Medicale **24**, 454-458

Goëau-Brissonnière, O., Mercier, F., Nicolas, M.H., Bacourt, F., Coggia, M., Lebrault, C. and Pechère, J.C. (1994). Treatment of vascular graft infection by in situ replacement with a rifampin-bonded gelatin-sealed Dacron graft. J. Vascular Surgery **19**, 739-744

Gogineni, P.V., Crooks, P.A., and Murty, R.B. (1993). Gas chromatographic determination of residual levels of *tert.*-butanol from lyophilized liposomal formulations. J. Chromatography **620**, 83-88

Gokhale, P.C., Barapatre, R.J., Advani, S.H., Kshirsagar, N.A. and Pandya, S.K. (1993a). Pharmacokinetics of liposomal amphotericin B in patients. J. Antimicrobial Chemotherapy **32**, 133-139

Gokhale, P.C., Barapatre, R.J., Advani, S.H., Kshirsagar, N.A. and Pandya, S.K. (1993b). Successful treatment of disseminated candidiasis resistant to amphotericin B by liposomal amphotericin B: a case report. J. Cancer Research and Clinical Oncology **119**, 569-571

Goldbach, P., Brochart, H., Wehrlé, P. and Stamm, A. (1995). Sterile filtration of liposomes: retention of encapsulated carboxyfluorescein. Int. J. Pharmaceutics **117**, 225-230

Goldstein, B.P., Candiani, G., Arain, T.M., Romanò, G., Ciciliato, I., Berti, M., Abbondi, M., Scotti, R., Mainini, M., Ripamonti, F., Resconi, A., Denaro, M. (1995). Antimicrobial activity of MDL 63,246, a new semisynthetic glycopeptide antibiotic. Antimicrobial Agents and Chemotherapy **39**, 1580-1588

Gómez-Gutiérrez, J., Rodríguez-Crespo, I., Peterson, D.L. and Gavilanes, F. (1995). Antigenicity of hepatitis B surface antigen proteins reconstitiuted with phospholipids. Biochimica et Biophysica Acta **1233**, 205-212

Gordon, D.M. (1993). Use of novel adjuvants and delivery systems to improve the humoral and cellular immune response to malaria vaccine candidate antigens. Vaccine **11**, 591-593

Goto, R. and Ibuki, Y. (1994). Tissue distribution of liposomes prepared from synthetic amphiphiles after intraperitoneal injection into mice. Applied Radiation and Isotopes **45**, 57-62

Gray, A. and Morgan, J. (1991). Liposomes in haematology. Blood Reviews **5**, 258-271

Gregoriadis, G. (1977). Targeting of drugs. Nature **265**, 407-411

Gregoriadis, G. (1985). Liposomes for drugs and vaccines. Trends in Biotechnology **3**, 235-241

Gregoriadis, G. (1994a). Liposomes as adjuvants and vaccine carriers: antigen entrapment. Immunomethods **4**, 210-216

Gregoriadis, G. (1994b). The immunological adjuvant and vaccine carrier properties of liposomes. J. Drug Targeting **2**, 351-356

Gregoriadis, G. and Florence, A.T. (1993a). Liposomes in drug delivery. Clinical, diagnostic and ophthalmic potential. Drugs **45**, 15-28

Gregoriadis, G. and Florence, A.T. (1993b). Recent advances in drug targeting. Trends in Biotechnology **11**, 440-442

Gregoriadis, G., McCormack, B., Wang, Z. and Lifely, R. (1993a). Polysialic acids: potential in drug delivery. FEBS Letters **315**, 271-276

Gregoriadis, G., Tan, L., Ben-Ahmeida, E.T.S. and Jennings, R. (1992). Liposomes enhance the immunogenicity of reconstituted influenza virus A/PR/8 envelopes and the formation of protective antibody by influenza virus A/Sichuan/87 (H3N2) surface antigen. Vaccine **10**, 747-753

Gregoriadis, G., Wang, Z., Barenholz, Y. and Francis, M.J. (1993b). Liposome-entrapped T-cell peptide provides help for a co-entrapped B-cell peptide to overcome genetic restriction in mice and induce immunological memory. Immunology **80**, 535-540

Grit, M. and Crommelin, D.J.A. (1992). The effect of aging on the physical stability of liposome dispersions. Chemistry and Physics of Lipids **62**, 113-122

Guaglianone, P., Chan, K., DelaFlor-Weiss, E., Hanisch, R., Jeffers, S., Sharma, D. and Muggia, F. (1994). Phase I and pharmacologic study of liposomal daunorubicin (DaunoXome). Investigational New Drugs **12**, 103-110

Guiot, H.F.L., Visser, L.G., Barge, R.M.Y., Bosboom, R. and van de Klundert, J.A.M. (1994). Fatal meningitis due to catheter-related *Staphylococcus epidermidis* bacteraemia in a granulocytopenic patient without predisposing trauma. Eur. J. Clinical Microbiology and Infectious Diseases **13**, 772-773

Gustafsson, J., Arvidson, G., Karlsson, G. and Almgren, M. (1995). Complexes between cationic liposomes and DNA visualized by cryo-TEM. Biochimica et Biophysica Acta **1235**, 305-312

Hamori, C.J., Lasic, D.D., Vreman, H.J. and Stevenson, D.K. (1993). Targeting zinc protoporphyrin liposomes to the spleen using reticuloendothelial blockade with blank liposomes. Pediatric Research **34**, 1-5

Hancock, I. and Poxton, I. (1988). Structure of bacteria and their envelopes, *in* "Bacterial Cell Surface Techniques." pp. 1-20. John Wiley & Sons (U.K.).

Harashima, H., Kume, Y., Yamane, C. and Kiwada, H. (1993a). Kinetic modeling of liposome degradation in blood circulation. Biopharmaceutics & Drug Deposition **14**, 265-270

Harashima, H., Sakata, K. and Kiwada, H. (1993b). Distinction between the depletion of opsonins and the saturation of uptake in the dose-dependent hepatic uptake of liposomes. Pharmaceutical Research **10**, 606-610

Harwood, J.L. (1992). Understanding liposomal properties to aid their clinical usage. Trends in Biochemical Sciences **17**, 203-204

Harrison, M., Tomlinson, D. and Stewart, S. (1995). Liposomal-entrapped doxorubicin: an active agent in AIDS-related Kaposi's Sarcoma. J. Clinical Oncology **13**, 914-920

Hashimoto, Y. and Suzuki, S. (1992). Basic approach to application of liposomes for cancer chemotherapy. Tohuku J. Experimental Medicine **168**, 361-369

Hazemoto, N., Harada, M., Suzuki, S., Kaiho, F., Haga, M. and Kato, Y. (1993). Effect of phosphatidylcholine and cholesterol on pH-sensitive liposomes. Chemical and Pharmaceutical Bulletin **41**, 1003-1006

Hedin, G. and Löfdahl, S. (1993). Detecting methicillin-resistant *Staphylococcus epidermidis* - disc diffusion, broth breakpoint or polymerase chain reaction ? APMIS **101**, 311-318

Heeremans, J.L.M., Kraaijenga, J.J., Los, P., Kluft, C. and Crommelin, D.J.A. (1992). Development of a procedure for coupling the homing device glu-plasminogen to liposomes. Biochimica et Biophysica Acta **1117**, 258-264

Hernández-Caselles, T., Villaláin, J. and Gómez-Fernández, J.C. (1993). Influence of liposome charge and composition on their interaction with human blood serum proteins. Molecular and Cellular Biochemistry **120**, 119-126

Hoedemakers, R.M.J., Vossebeld, P.J.M., Daemen, T. and Scherphof, G.L. (1993). Functional characteristics of the rat liver macrophage population after a single intravenous injection of liposome-encapsulated muramyl peptides. J. Immunotherapy **13**, 252-260

Hope, M.J., Bally, M.B., Webb, G. and Cullis P.R. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochimica et Biophysica Acta **812**, 55-65

Hu, L., Umeda, A. and Amako, K. (1995). Typing of *Staphylococcus epidermidis* colonizing in human nares by pulsed-field gel electrophoresis. Microbiology and Immunology **39**, 315-319

Huang, S.K., Mayhew, E., Gilani, S., Lasic, D.D., Martin, F.J. and Papahadjopoulos, D. (1992). Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. Cancer Research **52**, 6774-6781

Huebner, J., Pier, G.B., Maslow, J.N., Muller, E., Shiro, H., Parent, M., Kropec, A., Arbeit, R.D. and Goldmann, D.A. (1994). Endemic nosocomial transmission of *Staphylococcus epidermidis* bacteremia isolates in a neonatal intensive care unit over 10 years. J. Infectious Diseases **169**, 526-531

Hunter, R.J. (1981). *in* "Zeta Potential in Colloid Science." pp. 24-25. Academic Press (U.K.)

Hussain, M., Hastings, J.G.M. and White, P.J. (1991). Isolation and composition of the extracellular slime made by coagulase-negative staphylococci in a chemically defined medium. J. Infectious Diseases **163**, 534-541

Hussain, M., Wilcox, M.H. and White, P.J. (1993). The slime of coagulase-negative staphylococci - biochemistry and relation to adherence. FEMS Microbiology Reviews **104**, 191-208

Hutchinson, F.J., Francis, S.E., Lyle, I.J. and Jones, M.N. (1989). The characterisation of liposomes with covalently attached proteins. Biochimica et Biophysica Acta **978**, 17-24

Imaoka, T., Date, I., Miyoshi, Y., Ono, T., Furuta, T., Asari, S., Ohmoto, T., Yasuda, T. and Tsuda, M. (1995). Preliminary results of gene transfer to central nervous system by continuous injection of DNA-liposome complex. Cell Transplantation **4**, S23-S26

Jääskeläinen, I., Mönkkönen, J. and Urtti, A. (1994). Oligonucleotide-cationic liposome interactions. A physiochemical study. Biochimica et Biophysica Acta **1195**, 115-123

Janiak, M.J., Small, D.M. and Shipley, G.G. (1976). Nature of the thermal pretransition of synthetic phospholipids: dimyristoyl- and dipalmitoyl-lecithin. Biochemistry **15**, 4575-4580

Janoff, A.S. (1992). Lipids, liposomes and rational drug design. Laboratory Investigation **66**, 655-658

Jansen, B. and Peters, G. (1993). Foreign body associated infection. J. Antimicrobial Chemotherapy **32**, 69-75

Johnson, L.G. (1995). Gene therapy for cystic fibrosis. Chest **107**, 77S-83S

Jones, M.N. (1975). The electrical double layer, *in* "Biological Interfaces." pp. 135-161. Elsevier Scientific Publishing Company (Netherlands).

Jones, M.N. and Chapman, D. (1995). The liposomal state, *in* "Micelles, Monolayers and Biomembranes." pp. 117-142. Wiley-Liss, New York.

Jones, M.N., Kaszuba, M., Hill, K.J., Song, Y.-H. and Creeth, J.E. (1994a). The use of phospholipid liposomes for targeting to oral and skin-associated bacteria. J. Drug Targeting **2**, 381-389

Jones, M.N., Kaszuba, M., Reboiras, M.D., Lyle, I.G., Hill, K.J., Song, Y.-H., Wilmot, S.W. and Creeth, J.E. (1994b). The targeting of phospholipid liposomes to bacteria. Biochimica et Biophysica Acta **1196**, 57-64

Jones, J.W. (1993). Interaction of coagulase negative staphylococci with lectins. J. Clinical Pathology **48**, 761-763

Jung, S., Huitinga, I., Schmidt, B., Zielasek, J., Dijkstra, C.D., Toyka, K.V. and Hartung, H.-P. (1993). Selective elimination of macrophages by dichlormethylene diphosphonate-containing liposomes suppresses experimental autoimmune neuritis. J. Neurological Sciences **119**, 195-202

Kadir, F. (1993). Intramuscular and subcutaneous drug delivery: encapsulation in liposomes and other methods to manipulate drug availability. Pharmacy World & Science **15**, 173-175

Kamata, H., Yagisawa, H., Takahashi, S. and Hirata, H. (1994). Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection. Nucleic Acids Research **22**, 536-537

Karamanos, N.K., Panagiotopoulou, H.S., Syrokou, A., Frangides, C., Hjerpe, A., Dimitracopoulos, G. and Anastassiou, E.D. (1995). Identity of macromolecules present in the extracellular slime layer of *Staphylococcus epidermidis*. Biochimie **77**, 217-224

Karlowsky, J.A. and Zhanel, G.G. (1992). Concepts on the use of liposomal antimicrobial agents: applications for aminoglycosides. Clinical Infectious Diseases **15**, 654-667

Karyotakis, N.C. and Anaissie, E.J. (1994). Efficacy of escalating doses of liposomal amphotericin B (AmBisome) against hematogenous *Candida lusitaniae* and *Candida krusei* infection in neutropenic mice. Antimicrobial Agents and Chemotherapy **38**, 2660-2662

Kaszuba, M., Lyle, I.G. and Jones, M.N. (1995). The targeting of lectin-bearing liposomes to skin-associated bacteria. Colloids and Surfaces B: Biointerfaces **4**, 151-158

Kim, C.-K., Lee, M.-K., Han, J.-H. and Lee, B.-J. (1994). Pharmacokinetics and tissue distribution of methotrexate after intravenous injection of differently charged liposome-entrapped methotrexate to rats. Int. J. Pharmaceutics **108**, 21-29

Kim, M.M., Lee, S.H., Lee, M.G., Hwang, S.J. and Kim, C.-K. (1995). Pharmacokinetics of methotrexate after intravenous and intramuscular injection of methotrexate-bearing positively charged liposomes to rats. Biopharmaceutics and Drug Disposition **16**, 279-293

Kim, S. (1993). Liposomes as carriers of cancer chemotherapy. Current status and future prospects. Drugs **46**, 618-638

Kirilenko, V.N. and Gregoriadis, G. (1993). Fat soluble vitamins in liposomes: studies on incorporation efficiency and bile salt induced vesicle disintegration. J. Drug Targeting **1**, 361-368

Klang, S.H., Frucht-Pery, J., Hoffman, A. and Benita, S. (1994). Physiochemical characterization and acute toxicity evaluation of a positively-charged submicron emulsion vehicle. J. Pharmacy and Pharmacology **46**, 986-993

Kleeman, K.T., Bannerman, T.L. and Kloos, W.E. (1993). Species distribution of coagulase-negative staphylococcal isolates at a community hospital and implications for selection of staphylococcal identification procedures. J. Clinical Microbiology **31**, 1318-1321

Kleinerman, E.S., Meyers, P.A., Raymond, A.K., Gano, J.B., Jia, S.-F. and Jaffe, N. (1995). Combination therapy with isofamide and liposome encapsulated muramyl tripeptide: tolerability, toxicity, and immune stimulation. J. Immunotherapy **17**, 181-193

Klemens, S.P., Cynamon, M.H., Swenson, C.E. and Ginsberg, R.S. (1990). Liposome-encapsulated gentamicin therapy of mycobacterium avium complex infection in beige mice. Antimicrobial Agents and Chemotherapy **34**, 967-974

Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990). Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Letters **268**, 235-237

Kodama, M., Miyata, T. and Takaichi, Y. (1993). Calorimetric investigations of phase transitions of sonicated vesicles of dimyristoylphosphatidylcholine. Biochimica et Biophysica Acta **1169**, 90-97

Kölchens, S., Ramaswami, V., Birgenheier, J., Nett, L. and O'Brien, D.F. (1993). Quasi-elastic light scattering determination of the size distribution of extruded vesicles. Chemistry and Physics of Lipids **65**, 1-10

Komatsu, H., Higaki, K., Okamoto, H., Miyagawa, K., Hashida, M. and Sezaki, H. (1986). Preservative activity and *in vivo* percutaneous penetration of butylparaben entrapped in liposomes. Chemical and Pharmaceutical Bulletin **34**, 3415-3422

Kono, K., Hayashi, H. and Takagishi, T. (1994). Temperature-sensitive liposomes: liposomes bearing poly(*N*-isopropylacrylamide). J. Controlled Release **30**, 69-75

Konopka, K., Davis, B.R., Larsen, C.E. and Düzgünes, N. (1993). Anionic liposomes inhibit human immunodeficiency virus type 1 (HIV-1) infectivity in $CD4^+$ A3.01 and H9 cells. Antiviral Chemistry and Chemotherapy **4**, 179-187

Krause, W., Leike, J., Sachse, A. and Schuhmann-Giampieri, G. (1993). Characterization of iopromide liposomes. Investigative Radiology **28**, 1028-1032

Kreuter, J. (1994). Drug targeting with nanoparticles. Eur. J. Drug Metabolism and Pharmacokinetics **3**, 253-256

Kropec, A., Huebner, J., Wursthorn, M. and Daschner, F.D. (1993). In vitro activity of vancomycin and teicoplanin against *Staphylococcus aureus* and *Staphylococcus epidermidis* colonizing catheters. Eur. J. Clinical Microbiology and Infectious Diseases **12**, 545-548

Kulkarni, S.B., Betageri, G.V. and Singh, M. (1995). Factors affecting microencapsulation of drugs in liposomes. J. Microencapsulation **12**, 229-246

Kunimasa, J., Inui, K., Hori, R., Kawamura, Y. and Endo, K. (1992). Mannan-coated liposome delivery of gadolinium-diethylenetriaminepentaacetic acid, a contrast agent for use in magnetic resonance imaging. Chemical and Pharmaceutical Bulletin **40**, 2565-2567

Lambe Jr., D.W., Jeffery, C., Ferguson, K.P. and Cooper, M.D. (1994). Examination of the glycocalyx of four species of *Staphylococcus* by transmission electron microscopy and image analysis. Microbios **78**, 133-143

Lambing, H.E., Wolf, B.D. and Hartsel, S.C. (1993). Temperature effects on the aggregation state and activity of Amphotericin B. Biochimica et Biophysica Acta **1152**, 185-188

Langdale, L.A., Maier, R.V., Wilson, L., Pohlman, T.H., Williams, J.G. and Rice, C.L. (1992). Liposome-encapsulated hemoglobin inhibits tumor necrosis factor release from rabbit alveolar macrophages by a posttranscriptional mechanism. J. Leukocyte Biology **52**, 679-686

Langer, R. (1993). Polymer-controlled drug delivery systems. Chemical Research **26**, 537-542

Lasic, D. (1992). Liposomes. American Scientist **80**, 20-31

Lasic, D.D., Frederik, P.M., Stuart, M.C.A., Barenholz, Y. and McIntosh, T.J. (1992). Gelation of liposome interior. A novel method for drug encapsulation. FEBS Letters **312**, 255-258

Law, T.M., Mencel, P. and Motzer, R.J. (1994). Phase II trial of liposomal encapsulated doxorubicin in patients with advance renal cell carcinoma. Investigational New Drugs **12**, 323-325

Leader, W.G., Chandler, M.H.H., Castiglia, M. (1995). Pharmacokinetic optimisation of vancomycin therapy. Clinical Pharmacokinetics **28**, 327-342

Lebeau, C., Renaud, F., Fleurette, J. and Étienne, J. (1993). Absence of distinctive characteristics between *Staphylococcus epidermidis* isolates from community acquired infective endocarditis and commensal isolates. Pathologie Biologie **41**, 583-588

Lee, H.J., Ahn, B.-N., Yoon, E.J., Paik, W.H., Shim, C.-K. and Lee, M.G. (1995). Pharmacokinetics and tissue distribution of adriamycin and adriamycinol after intravenous administration of adriamycin-loaded neutral proliposomes to rats. Int. J. Pharmaceutics **121**, 1-10

Lee, K.-D., Pitas, R.E. and Papahadjopoulos, D. (1992). Evidence that the scavenger receptor is not involved in the uptake of negatively charged liposomes by cells. Biochimica et Biophysica Acta **1111**, 1-6

Lee, R.J. and Low, P.S. (1995). Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. Biochimica et Biophysica Acta **1233**, 134-144

Lerebours, B., Wehrli, E. and Hauser, H. (1993). Thermodynamic stability and osmotic sensitivity of small unilamellar phosphatidylcholine vesicles. Biochimica et Biophysica Acta **1152**, 49-60

Li, C., Yang, D.J., Kuang, L.-R. and Wallace, S. (1993). Polyamino acid microspheres preparation: preparation, characterization and distribution after intravenous injections in rats. Int. J. Pharmaceutics **94**, 143-152

Lilley, J.C., Patterson, L.H. and Taylor, M.J. (1994). Preparation and stability of liposome encapsualted doxorubicin and mitoxantrone and their internalisation by murine peritoneal macrophages. Int. J. Pharmaceutics **107**, 149-157

Lina, B., Bes, M., Vandenesch, F., Greenland, T., Etienne, J. and Fleurette, J. (1993). Role of bacteriophages in genomic variability of related coagulase-negative staphylococci. FEMS Microbiology Letters **109**, 273-278

Lina, B., Forey, F., Tigaud, J.D. and Fleurette, J. (1995). Chronic bacteraemia due to *Staphylococcus epidermidis* after bone marrow transplantation. J. Medical Microbiology **42**, 156-160

Lina, B., Forey, F., Troncy, J., Greenland, T., Fleurette, J. and Etienne, J. (1994). Oral source of *Staphylococcus epidermidis* in a neutropenic patient. Eur. J. Clinical Microbiology and Infectious Diseases **13**, 773-774

Liu, D., Liu, F. and Song, Y.K. (1995). Monosialoganglioside GM_1 shortens the blood circulation time of liposomes in rats. Pharmaceutical Research **12**, 508-512

Lopez-Berestein, G. (1987). Liposomes as carriers of antimicrobial agents. Antimicrobial Agents and Chemotherapy **31**, 675-678

Loukas, Y.L., Jayasekera, P. and Gregoriadis, G. (1995). Novel liposome-based multicomponent systems for the protection of photolabile agents. Int. J. Pharmaceutics **117**, 85-94

Low, D.E., Willey, B.M. and McGeer, A.J. (1995). Multidrug-resistant enterococci: a threat to the surgical patient. Am. J. Surgery **169**, 8S-12S

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biological Chemistry **193**, 265-275

Lutz, J., Augustin, A.J., Jäger, L.J., Bachmann, D. and Brandl, M. (1995). Acute toxicity and depression of phagocytosis in vivo by liposomes: influence of lysophospatidylcholine. Life Sciences **56**, 99-106

Mainardi, J.-L., Shlaes, D.M., Goering, R.V., Shlaes, J.H., Acar, J.F. and Goldstein, F.W. (1995). Decreased teicoplanin susceptibility of methicillin-resistant strains of *Staphylococcus aureus*. J. Infectious Diseases **171**, 1646-1650

Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A. and Iwastsuru, M. (1992a). Characterization and application of polyethyleneglycol-liposomes. J. Pharmacobio-Dyn. **15**, s-70

Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A. and Iwastsuru, M. (1992b). Prolonged circulation time *in vivo* of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochimica et Biophysica Acta **1128**, 44-49

Mattsson, E., Rollof, J., Verhoef, J., van Dijk, H. and Fleer, A. (1994). Serum-induced potentiation of tumor necrosis factor alpha production by human monocytes in response to staphylococcal peptidoglycan: involvement of different serum factors. Infection and Immunity **62**, 3837-3843

Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986). Vesicles of variable sizes produced by a rapid extrusion procedure. Biochimica et Biophysica Acta **858**, 161-168

Mayer, L.D., Hope, M.J., Cullis, P.R. and Janoff, A.S. (1985). Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochimica et Biophysica Acta **817**, 193-196

McCormack, B. and Gregoriadis, G. (1994a). Drugs-in-cyclodextrins-in liposomes: a novel concept in drug delivery. Int. J. Pharmaceutics **112**, 249-258

McCormack, B. and Gregoriadis, G. (1994b). Entrapment of cyclodextrin-drug complexes: potential advantages in drug delivery. J. Drug Targeting **2**, 449-454

McCormack, B. and Gregoriadis, G. (1994c). Polysialic acids: in vivo properties and possible uses in drug delivery. Targeting of Drugs **4**, 139-145

McDermid, K.P., Morck, D.W., Olson, M.E., Boyd, N.D., Khoury, A.E., Dasgupta, M.K. and Costerton, J.W. (1993a). A porcine model of *Staphylococcus epidermidis* catheter-associated infection. J. Infectious Diseases **168**, 897-903

McDermid, K.P., Morck, D.W., Olson, M.E., Dasgupta, M.K. and Costerton, J.W. (1993b). Effect of growth conditions on expression and antigenicity of *Staphylococcus epidermidis* RP62A cell envelope proteins. Infection and Immunity **61**, 1743-1749

Mehta, R.T., McQueen, T.J., Keyhani, A. and López-Berestein, G. (1994). Phagocyte transport as mechanism for enhanced therapeutic activity of liposomal amphotericin B. Chemotherapy **40**, 256-264

Melissen, P.M.B., van Vianen, W. and Bakker-Woudenberg, I.A.J.M. (1992). Roles of peripheral leukocytes and tissue macrophages in antibacterial resistance induced by free or liposome-encapsulated muramyl tripeptide phosphatidyl-ethanolamine. Infection and Immunity **60**, 4891-4897

Memoli, A., Palermiti, L.G., Travagli, V. and Alhaique, F. (1993). Liposomes in cosmetics: Which kind of phospholipid ? Which kind of loading method ? J. Society of Cosmetic Chemists **44**, 123-128

Mercardal, M., Domingo, J.C., Bermudez, M., Mora, M. and De Madariaga, M.A. (1995). *N*-palmitoylphosphatidylethanolamine stabilizes liposomes in the presence of human serum: effect of lipidic composition and system characterization. Biochimica et Biophysica Acta **1235**, 281-288

Meredith, T.A., Aguilar, H.E., Shaarawy, A., Kincaid, M., Dick, J. and Niesman, M.R. (1995). Vancomycin levels in the vitreous cavity after intravenous administration. Am. J. Opthalmology **119**, 774-778

Mezei, M. and Gulasekharam, V. (1980). Liposomes - a selective drug delivery system for the topical route of administration. I. Lotion dosage form. Life Sciences **26**, 1473-1477

Middleton, P.G., Caplen, N.J., Goa, X., Huang, L., Gaya, H., Geddes, D.M. and Alton, E.W.F.W. (1994). Nasal application of the cationic liposome DC-Chol:DOPE does not alter ion transport, lung function or bacterial growth. Eur. Respiratory J. **7**, 442-445

Mills, W., Chopra, R., Linch, D.C. and Goldstone, A.H. (1994). Liposomal amphotericin B in the treatment of fungal infections in neutropenic patients: a single-centre experience of 133 episodes in 116 patients. British J. Haematology **86**, 754-760

Miranda, M., Amicarelli, F., Volpe, A.R., Poma, A., Masciocco, L. and Carmignani, M. (1993). Specific increase of L-DOPA levels in plasma upon infusion of tyrosinase containing liposomes. General Pharmacology **24**, 1319-1322

Mirchandani, H. and Chien, Y.W. (1993). Drug-delivery approaches for anti-HIV drugs. Int J. Pharmaceutics **95**, 1-21

Moghimi, S.M., Hedeman, H., Christy, N.M., Illum, L. and Davis, S.S. (1993a). Enhanced hepatic clearance of intravenously administered sterically stabilized microspheres in zymosan-stimulated rats. J. Leukocyte Biology **54**, 513-517

Moghimi, S.M., Muir, I.S., Illum, L., Davis, S.S. and Kolb-Bachofen, V. (1993b). Coating particles with a block co-polymer (poloxamine-908) suppresses opsonization but permits the activity of dysopsonins in the serum. Biochimica et Biophysica Acta **1179**, 157-165

Mönkkönen J. and Heath, T.D. (1993). The effects of liposome-encapsulated and free clodronate on the growth of macrophage-like cells *in vitro*: the role of calcium and iron. Calcified Tissue Int. **53**, 139-146

Mönkkönen J., Valjakka, R., Hakasalo, M. and Urtti, A. (1994). The effects of liposome surface charge and size on the intracellular delivery of clodronate and gallium in vitro. Int. J. Pharmaceutics **107**, 189-197

Moore, W.J. (1956). Surface chemistry, *in* "Physical Chemistry" (2nd Ed.) pp. 515-517. Longmans (U.K.)

Moreira, B.M. and Daum, R.S. (1995). Antimicrobial resistance in staphylococci. Antimicrobial Resistance in Pediatrics **42**, 619-648

Mori, A., Kennel, S.J. and Huang, L. (1993). Immunotargeting of liposomes containing lipophilic antitumour prodrugs. Pharmaceutical Research **10**, 507-514

Morris, W., Steinhoff, M.C. and Russell, P.K. (1994). Potential of polymer microencapsulation technology for vaccine innovation. Vaccine **12**, 5-11

Mosharraf, M., Taylor, K.M.G., Craig, D.Q.M. (1995). Effect of calcium ions on the surface charge and aggregation of phosphatidylcholine liposomes. J. Drug Targeting **2**, 541-545

Muhammad, G., Hoblet, K.H., Jackwood, D.J., Bech-Nielsen, S. and Smith, K.L. (1993). Interspecific conjugal transfer of antibiotic resistance among staphylococci isolated from the bovine mammary gland. Am. J. Veterinary Research **54**, 1432-1440

Mui, B.L.-S., Cullis, P.R., Pritchard, P.H. and Madden, T.D. (1994). Influence of plasma on the osmotic sensitivity of large unilamellar vesicles produced by extrusion. J. Biological Chemistry **269**, 7364-7370

Muller, E., Takeda, S., Shiro, H., Goldmann, D. and Pier, G.B. (1993). Occurence of capsular polysaccharide adhesin among clinical isolates of coagulase-negative *staphylococci*. J. Infectious Diseases **168**, 1211-1218

Murray, B.E. (1995). What can we do about vancomycin-resistant enterococci? Clinical Infectious Diseases **20**, 1134-1136

Nakamori, K., Nakajima, T., Odawara, M., Koyama, I., Nemoto, M., Yoshida, T., Oshima, H. and Inoue, K. (1993). Stable positively charged liposome during long-term storage. Chemical and Pharmaceutical Bulletin **41**, 1279-1283

Nakamura, M., Tsujii, K., Katsuragi, Y., Kurihara, K. and Sunamoto, J. (1994). Taste receptor proteins directly extracted by liposome from intact epithelium of bullfrog tongue. Biochemical and Biophysical Research Communications **201**, 415-422

Nässander, U.K., Steerenberg, P.A., De Jong, W.H., Van Overveld, W.O.W.M., Te Boekhorst, C.M.E., Poels, L.G., Jap, P.H.K. and Storm, G. (1995). Design of immunoliposomes directed against human ovarian carcinoma. Biochimica et Biophysica Acta **1235**, 126-139

Nataro, J.P., Corcoran, L., Zirin, S., Swink, S., Taichman, N., Goin, J. and Harris, M.C. (1994). Prospective analysis of coagulase-negative staphylococcal infection in hospitalized infants. J. Pediatrics **125**, 798-804

New, R.C.C. (1990). *in* "Liposomes: a practical approach." pp. 1-31. Oxford University Press (U.K.).

Ng, T.T.C. and Denning, D.W. (1995). Liposomal amphotericin B (AmBisome) therapy in invasive fungal infections. Archives of Internal Medicine **155**, 1093-1098

Nicholov, R., Khoury, A.E., Bruce, A.W. and DiCosmo, F. (1993). Interaction of ciprofloxacin loaded liposomes with *Pseudomonas aeruginosa* cells. Cells and Materials **3**, 321-326

Norris, A.H., Reilly, J.P., Edelstein, P.H., Brennan, P.J. and Schuster, M.G. (1995). Chloramphenicol for the treatment of vancomycin-resistant enterococal infections. Clinical Infectious Diseases **20**, 1137-1144

Ohta, S., Igarashi, S., Honda, A., Sato, S. and Hanai, N. (1993). Cytotoxicity of adriamycin-containing immunoliposomes targeted with anti-ganglioside monoclonal antibodies. Anticancer Research **13**, 331-336

Oku, N. and Namba, Y. (1995). Long-circulating liposomes. Critical Reviews in Therapeutic Drug Carrier Systems **11**, 231-270

Oku, N., Namba, Y., Takeda, A. and Okada, S. (1993). Tumor imaging with technetium-99m-DTPA encapsulated in RES-avoiding liposomes. Nuclear Medicine and Biology **20**, 407-412

Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979). Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochimica et Biophysica Acta **557**, 9-23

Oñaderra, M., Monsalve, R.I., Mancheño, J.M., Villalba, M., Martinez Del Pozo, A., Gavilanes, J.G. and Rodriguez, R. (1994). Food mustard allergen interaction with phospholipid vesicles. Eur. J. Biochemistry **225**, 609-615

Ono, A., Ueno, M., Zou, I. and Horikoshi, I. (1992). Basic study on hepatic artery chemoembolization using temperature-sensitive liposome. J. Pharmacobio-Dyn. **15**, s-71

Onur, M.A., Kirby, C.J., Isimer, A., Beksac, S., Basci, N., Pamir, R., Coskun, T. and Tumer, A. (1992). Effect of liposomal encapsulation of chloramphenicol on its transfer across the human placenta in a dual in vitro perfusion system. Int. J. Pharmaceutics **88**, 313-317

Onyeji, C.O., Nightingale, C.H. and Marangos, M.N. (1994a). Enhanced killing of methicillin-resistant *Staphylococcus aureus* in human macrophages by liposome-entrapped vancomycin and teicoplanin. Infection **22**, 1-5

Onyeji, C.O., Nightingale, C.H., Nicolau, D.P. and Quintiliani, R. (1994b). Activities of liposome-encapsulated azithromycin and rifabutin compared with that of clarithromycin against *Mycobacterium avium-intracellulare* complex in human macrophages. Int. J. Antimicrobial Agents **4**, 281-289

Ostro, M.J. (1987). Liposomes. Scientific American **256**, 91-99

Ostro, M.J. and Cullis, P.R. (1989). Use of liposomes as injectable-drug delivery systems. Am. J. Hospital Pharmacy **46**, 1576-1587

Owais, I.M., Ahmed, I., Krishnakumar, B., Jain, R.K., Bachhwat, B.K. and Gupta, C.M. (1993). Tuftsin-bearing liposomes as drug vehicles in the treatment of experimental aspergillosis. FEBS **326**, 56-58

Pajean, M. and Herbage, D. (1993). Effect of collagen on liposome permeability. Int. J. Pharmaceutics **91**, 209-216

Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973). Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochimica et Biophysica Acta **311**, 330-348

Parr, M.J., Ansell, S.M., Choi, L.S. and Cullis, P.R. (1994). Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. Biochimica et Biophysica Acta **1195**, 21-30

Parr, M.J., Bally, M.B. and Cullis, P.R. (1993). The presence of G_{M1} in liposomes with entrapped doxorubicin does not prevent RES blockade. Biochimica et Biophysica Acta **1168**, 249-252

Parsons, C.L., Stein, P.C., Dobke, M.K., Virden, C.P. and Frank, D.H. (1993). Diagnosis and therapy of subclinically infected prostheses. Surgery **177**, 504-506

Patel, H.M. and Ryman, B.E. (1976). Oral administration of insulin by encapsulation within liposomes. FEBS Letters **62**, 60-63

Patel, K.R., Li, M.P. and Baldeschwieler, J.D. (1983). Suppression of liver uptake of liposomes by dextran sulfate 500. Proceedings of the National Academy of Sciences U.S.A. **80**, 6518-6522

Patrick, C.C., Plaunt, M.R., Sweet, S.M., Patrick, G.S. (1990). Defining *Staphylococcus epidermidis* cell wall proteins. J. Clinical Microbiology **28**, 2757-2760

Perl, T.M., Rhomberg, P.R., Bale, M.J., Fuchs, P.C., Jones, R.N., Koontz, F.P. and Pfaller, M.A. (1994). Comparison of identification systems for *Staphylococcus epidermidis* and other coagulase-negative *Staphylococcus* species. Diagnostic Microbiology and Infectious Disease **18**, 151-155

Perotti, E. and Chanson, A. (1993). Reconstitution into liposomes of the tonoplast ATP- and pyrophosphate-dependent proton pumps from *Rubus hispidus* cell cultures. Botanica Acta **106**, 193-196

Perry, D.G. and Martin, W.J. (1995). Fluorescent liposomes as quantitative markers of phagocytosis by alveolar macrophages. J. Immunological Methods **181**, 269-285

Pfaller, M.A. and Herwaldt, L.A. (1988). Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. Clinical Microbiology Reviews **1**, 281-299

Ponchel, G. (1994). Formulation of oral mucosal delivery systems for the delivery of bioactive materials. Advanced Drug Delivery Reviews **13**, 75-87

Pons, M., Foradada, M. and Estelrich, J. (1993). Liposomes obtained by the ethanol injection method. Int. J. Pharmaceutics **95**, 51-56

Praet, M., Defrise-Quertain, F. and Ruysschaert, J.M. (1993). Comparison of adriamycin and derivatives uptake into large unilamellar lipid vesicles in response to a membrane potential. Biochimica et Biophysica Acta **1148**, 342-350

Price, C.I., Horton, J.W. and Baxter, C.R. (1994). Liposome encapsulation: a method for enhancing the effectiveness of local antibiotics. Surgery **115**, 480-487

Raad, I., Costerton, W., Sabharwal, U., Sacilowski, M., Anaissie, E. and Bodey, G.P. (1993). Ultrastructural analysis of indwelling vascular catheters: a quantitative relationship between luminal colonization and duration of placement. J. Infectious Diseases **168**, 400-407

Rahman, A., Husain, S.R., Siddiqui, J., Verma, M., Agresti, M., Center, M., Safa, A.R. and Glazer, R.I. (1992). Liposome-mediated modulation of multidrug resistance in human HL-60 leukemia cells. J. National Cancer Institute **84**, 1909-1915

Ralph, E.D., Barber, K.R. and Grant, C.W.M. (1993). Clinical experience with multilamellar liposomal amphotericin B in patients with proven and suspected fungal infections. Scandanavian J. Infectious Diseases **25**, 487-496

Rogers, H.J. (1983). The cell walls of bacteria, *in* "Bacterial Cell Structure" pp. 6-27. Van Nostrand Reinhold (U.K.).

Rang, H.P. and Dale, M.M. (1991). "Pharmacology" (2nd Ed.), pp. 825. Churchill Livingstone, U.K.

Ravindranath, M.H., Morton, D.L. and Irie, R.F. (1994). Attachment of monophosphoryl Lipid A (MPL) to cells and liposomes augments antibody response to membrane-bound gangliosides. J. Autoimmunity **7**, 803-816

Rice, L.B. and Shlaes, D.M. (1995). Vancomycin resistance in the enterococcus. Relevance in pediatrics. Antimicrobial Resistance in Pediatrics **42**, 602-618

Ringdén, O., Andström, E., Remberger, M., Svahn, B.-M. and Tollemar, J. (1994). Allergic reactions and other rare side-effects of liposomal amphotericin. The Lancet **344**, 1157

Roerdink, F.H., Daemen, T., Bakker-Woudenberg, I.A.J.M., Storm, G., Crommelin, D.J.A. and Scherphof, G.L. (1987). Therapeutic utility of liposomes, *in* "Drug delivery systems. Fundamentals and techniques." (P. Johnson and J.G. Lloyd-Jones) pp.66-80. VCH Publishers (U.K.).

Rose, J.K., Buonocore, L. and Whitt, M.A. (1991). A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10**, 520-525

Rowe, L. and Miller, T.E. (1993). Effect of dialysis on the clearance of Staphylococcus epidermidis from the peritoneal cavity: an experimental evaluation. Clinical Nephrology **40**, 106-113

Rowland, R.N. and Woodley, J.F. (1981). The uptake of distearoylphosphatidylcholine/cholesterol liposomes by rat intestinal sacs in vitro. Biochimica et Biophysica Acta **673**, 217-223

Rupp, M.E. and Archer, G.L. (1992). Hemagglutination and adherence to plastic by *Staphylococcus epidermidis*. Infection and Immunity **60**, 4322-4327

Sachse, A., Leike, J.U., Rößling, G.L., Wagner, S.E. and Krause, W. (1993). Preparation and evaluation of lyophilized iopromide-carrying liposomes for liver tumor detection. Investigative Radiology **28**, 838-844

Sadzuka, Y., Nakai, S., Miyagashima, A., Nozwa, Y. and Hirota, S. (1995). The effect of dose on the distribution of adriamycin encapsulated in polyethyleneglycol-coated liposomes. J. Drug Targeting **3**, 31-37

Sato, T. and Sunamoto, J. (1992). Recent aspects in the use of liposomes in biotechnology and medicine. Progress in Lipid Research **31**, 345-372

Schierholz, J., Jansen, B., Jaenicke, L. and Pulverer, G. (1994). *In-vitro* efficacy of an antibiotic releasing silicone ventricle catheter to prevent shunt infection. Biomaterials **15**, 996-1000

Schmitt, H.-J. (1993). New methods of delivery of amphotericin B. Clinical Infectious Diseases **17**, S501-S506

Schneider, T., Sachse, A., Rößling, G. and Brandl, M. (1995). Generation of contrast-carrying liposomes of defined size with a new continuous high pressure extrusion method. Int. J. Pharmaceutics **117**, 1-12

Schreier, H. and Bouwstra, J. (1994). Liposomes and niosomes as topical drug carriers: dermal and transdermal drug delivery. J. Controlled Release **30**, 1-15

Schreier, H., Gonzalez-Rothi, R.J. and Stecenko, A.A. (1993). Pulmonary delivery of liposomes. J. Controlled Release **24**, 209-223

Seaman, J., Boer, C., Wilkinson, R., de Jong, J., de Wilde, E., Sondorp, E. and Davidson, R. (1995). Liposomal amphotericin B (AmBisome) in the treatment of complicated kala-azar under field conditions. Clinical Infectious Diseases **21**, 188-193

Senga, S., Onituka, A., Hirose, H., Yamamoto, K. and Niwa, K. (1990). Protective effect of liposomal encapsulated superoxide dismutase on ischemically injured liver in the rat. Transplantation Proceedings **22**, 2025-2026

Shay, D.K., Goldmann, D.A. and Jarvis, W.R. (1995). Reducing the spread of antimicrobial-resistant microorganisms. Control of vancomycin-resistant enterococci. Antimicrobial Resistance in Pediatrics **42**, 703-716

Shi, F., MacEwen, E.G. and Kurzman, I.D. (1993). *In vitro* and *in vivo* effect of doxorubicin combined with liposome-encapsulated muramyl tripeptide on canine monocyte activation. Cancer Research **53**, 3986-3991

Shibata, A., Ikawa, K., Shimooka, T. and Terada, H. (1994). Significant stabilization of the phosphatidylcholine bilayer structure by incorporation of small amounts of cardiolipin. Biochimica et Biophysica Acta **1192**, 71-78

Simon, W.C., Raptis, L., Pang, S.C. and Bennett, B.M. (1993). Comparison of liposomes fusion and electroporation for the intracellular delivery of nonpermeant molecules to adherent cultured cells. J. Pharmacological and Toxicological Methods **29**, 29-35

Song, Y.-H. and Jones, M.N. (1994). The interaction of positively charged phospholipid vesicles with bacteria. Biochemical Society Transactions **22**, 330S

Stevens, V.C. (1993). Vaccine delivery systems: potential methods for use in antifertility vaccines. Am. J. Reproductive Immunology **29**, 176-188

Stevenson, M., Baillie, A.J. and Richards, R.M.E. (1983). Enhanced activity of streptomycin and chloramphenicol against intracellular *Escherichia coli* in the J774 macrophage cell line mediated by liposome delivery. Antimicrobial Agents and Chemotherapy **24**, 742-749

Sveinsson, S.J. and Holbrook, W.P. (1993). Oral mucosal adhesive ointment containing liposomal corticosteroid. Int. J. Pharmaceutics **95**, 105-109

Swenson, C.E., Popescu, M.C. and Ginsberg, R.S. (1988). Preparation and use of liposomes in the treatment of microbial infections. Critical Reviews in Microbiology **15**, S1-S31

Szebeni, J., Wassef, N.M., Spielberg, H., Rudolph, A.S. and Alving, C.R. (1994). Complement activation in rats by liposomes and liposome-encapsulated hemoglobin: evidence for anti-lipid antibodies and alternative pathway activation. Biochemical and Biophysical Research Communications **205**, 255-263

Szoka, F. and Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proceedings of the National Academy of Sciences U.S.A. **75**, 4194-4198

Tabak, A. and Hoffer, E. (1994). Depletion of serum iron levels in rats by intravenous administration of liposome-encapsulated desferrioxamine. Acta Haematologica **91**, 111-113

Tabak, A., Hoffer, E. and Taitelman, U. (1994). Evaluation of a liposome system for the delivery of desferrioxamine to lungs in rats. J. Pharmacy and Pharmacology **46**, 789-796

Tadakuma, T., Ikewaki, N., Yasuda, T., Tsutsumi, M., Saito, S. and Saito, K. (1985). Treatment of experimental salmonellosis in mice with streptomycin entrapped in liposomes. Antimicrobial Agents and Chemotherapy **28**, 28-32

Tahara, Y. and Fujiyoshi, Y. (1994). A new method to measure bilayer thickness: cryo-electron microscopy of frozen hydrated liposomes and image simulation. Micron **25**, 141-149

Takehara, T., Hayashi, N., Miyamoto, Y., Yamamoto, M., Mita, E., Fusamoto, H. and Kamada, T. (1995). Expression of the hepatitis C virus genome in rat liver after cationic liposome-mediated *in vivo* gene transfer. Hepatology **21**, 746-751

Tanguay, S., Bucana, C.D., Wilson, M.R., Fidler, I.J., von Eschenbach, A.C. and Killion, J.J. (1994). *In vivo* modulation of macrophage tumoricidal activity by oral administration of the liposome-encapsulated macrophage activator CGP 19835A. Cancer Research **54**, 5882-5888

Taylor, K.M.G. and Newton, J.M. (1992). Liposomes for controlled delivery of drugs to the lung. Thorax **47**, 257-259

Teichman, J.M.H., Stein, P.C. and Parsons, C.L. (1993). Quaternary amine (protamine sulfate) is bactericidal to *Staphylococcus epidermidis*. J. Infectious Diseases **167**, 1500-1501

Teichman, J.M.H., Abraham, V.E., Stein, P.C. and Parsons, C.L. (1994). Protamine sulfate and vancomycin are synergistic against Staphylococcus epidermidis prostheses infection in vivo. J. Urology **152**, 213-216

Thierry, A.R., Jorgensen, T.J., Forst, D., Belli, J.A., Dritschilo, A. and Rahman, A. (1989). Modulation of multidrug resistance in Chinese hamster cells by liposome-encapsulated doxorubicin. Cancer Communications **1**, 311-316

Thierry, A.R., Vigé, D., Coughlin, S.S., Belli, J.A., Dritschilo, A. and Rahman, A. (1993). Modulation of doxorubicin resistance in multidrug-resistant cells by liposomes. FASEB J. **7**, 572-579

Tonetti, M. and De Flora, A. (1993). Carrier erythrocytes. Clinical pharmacokinetic considerations. Clinical Pharmacokinetics **25**, 351-357

Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov Jr., A.A., Trubetskoy, V.S., Herron, J.N. and Gentry, C.A. (1994a). Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochimica et Biophysica Acta **1195**, 11-20

Torchilin, V.P., Shtilman, M.I., Trubetskoy, V.S., Whiteman, K. and Milstein, A.M. (1994b). Amphiphilic vinyl polymers effectively prolong liposome circulation time in vivo. Biochimica et Biophysica Acta **1195**, 181-184

Tschaikowsky, K. (1994). Protein kinase C inhibitors suppress LPS-induced TNF production in alveolar macrophages and in whole blood: the role of encapsulation into liposomes. Biochimica et Biophysica Acta **1222**, 113-121

Uchegbu, I.F., Turton, J.A., Double, J.A. and Florence, A.T. (1994). Drug distribution and a pulmonary adverse effect of intraperitoneally administered doxorubicin niosomes in the mouse. Biopharmaceutics and Drug Disposition **15**, 691-707

Unger, E.C., Lund, P.J.L., Shen, D.-K., Fritz, T.A., Yellowhair, D. and New, T.E. (1992). Nitrogen-filled liposomes as a vascular US agent: preliminary evaluation. Radiology **185**, 453-456

Unger, E., Shen, D.-K., Fritz, T., Lund, P., Wu, G.-L., Kulik, B., De Young, D., Standen, J., Ovitt, T. and Matsunaga, T. (1993). Gas-filled liposomes as echocardiographic contrast agents in rabbits with myocardial infarcts. Investigative Radiology **28**, 1155-1159

Vaage, J., Barberá-Guillem, E., Abra, R., Huang, A. and Working, P. (1994). Tissue distribution and therapeutic effect of intravenous free or encapsulated doxorubicin on human prostate carcinoma xenografts. Cancer **73**, 1478-1484

Vaage, J., Donovan, D., Mayhew, E., Abra, R. and Huang, A. (1993). Therapy of human ovarian carcinoma xenografts using doxorubicin encapsulated in sterically stabilized liposomes. Cancer **72**, 3671-3675

Van Bambeke, F., Tulkens, P.M., Brasseur, R. and Mingeot-Leclercq, M.-P. (1995). Aminoglycoside antibiotics induce aggregation but not fusion of negatively-charged liposomes. Eur. J. Pharmacology **289**, 321-333

Vemuri, S. and Rhodes, C.T. (1994). Development and characterization of a liposome preparation by a pH-gradient method. J. Pharmacy and Pharmacology **46**, 778-783

Vemuri, S. and Rhodes, C.T. (1995). Encapsulation of a water soluble drug in a liposome preparation: removal of free drug by washing. Drug Development and Industrial Pharmacy **21**, 1329-1338

Verheyen, C.C.P.M., Dhert, W.J.A., Petit, P.L.C., Rozing, P.M. and De Groot, K. (1993). *In vitro* study on the integrity of a hydroxylapatite coating when challenged with staphylococci. J. Biomedical Materials Research **27**, 775-781

Wang, C.-S. and Smith, R.L. (1975). Lowry determination of protein in the presence of Triton X-100. Analytical Biochemistry **63**, 414-417

Wang, I., Anderson, J.M. and Marchant, R.E. (1993a). Platelet-mediated adhesion of *Staphylococcus epidermidis* to hydrophobic NHLBI reference polyethylene. J. Biomedical Materials Research **27**, 1119-1128

Wang, I., Anderson, J.M. and Marchant, R.E. (1993b). *Staphylococcus epidermidis* adhesion to hydrophobic biomedical polymer is mediated by platelets. J. Infectious Diseases **167**, 329-336

Watanabe, Y., Nomoto, H., Takezawa, R., Miyoshi, N. and Akaike, T. (1994). Highly efficient transfection into primary cultured mouse hepatocytes by use of cation-liposomes: an application for immunization. J. Biochemistry **116**, 1220-1226

Weinstein, J.N., Magin, R.L., Yatvin, M.B. and Zaharko, D.S. (1979). Liposomes and local hyperthermia: selective delivery of methotrexate to heated tumors. Science **204**, 188-191

Weiss, R.C., Cox, N.R. and Martinez, M.L. (1993). Evaluation of free or liposome-encapsulated ribavirin for antiviral therapy of experimentally induced feline infectious peritonitis. Research in Veterinary Science **55**, 162-172

Wilkinson, J.F. (1986). *in* "Introduction to Microbiology." (3rd Ed.) pp. 77-78. Blackwell Scientific Publications (U.K.).

Wolf, P., Cox, P., Yarosh, D.B. and Kripke, M.L. (1995). Sunscreens and T4N5 liposomes differ in their ability to protect against ultraviolet-induced sunburn cell formation, alterations of dendritic epidermal cells, and local suppression of contact hypersensitivity. J. Investigative Dermatology **104**, 287-292

Wolf, P., Yarosh, D.B. and Kripke, M.L. (1993). Effects of sunscreens and a DNA excision repair enzyme on ultraviolet radiation - induced inflammation, immune suppression, and cyclobutane pyrimidine dimer formation in mice. J. Investigative Dermatology **101**, 523-527

Woodle, M.C. and Lasic, D.D. (1992). Sterically stabilized liposomes. Biochimica et Biophysica Acta **1113**, 171-199

Wrobel, I. and Collins, D. (1995). Fusion of cationic liposomes with mammalian cells occurs after endocytosis. Biochimica et Biophysica Acta **1235**, 296-304

Yagi, K., Noda, H., Kurono, M. and Ohishi, N. (1993). Efficient gene transfer with less cytotoxicity by means of cationic multilamellar liposomes. Biochemical and Biophysical Research Communications **196**, 1042-1048

Yamaguchi, T. and Mizushima, Y. (1994). Lipid microspheres for drug delivery from the pharmaceutical viewpoint. Critical Reviews in Therapeutic Drug Carrier Systems **11**, 215-229

Yamauchi, H., Yano, T., Kato, T., Tanaka, I., Nakabayashi, S., Higashi, K., Miyoshi, S. and Yamada, H. (1995). Effects of sialic acid derivative on long circulation time and tumor concentration of liposomes. Int. J. Pharmaceutics **113**, 141-148

Yarosh, D.B. (1990). Topical application of liposomes. J. Photobiochemistry and Photobiology, B:Biology **6**, 445-449

Yarosh, D., Bucana, C., Cox, P., Alas, L., Kibitel, J. and Kripke, M. (1994). Localization of liposomes containing a DNA repair enzyme in murine skin. J. Investigative Dermatology **103**, 461-468

Yatvin, M.B., Krevtz, W., Horwitz, B.A. and Shinitzky, M. (1980). pH-sensitive liposomes: possible clinical implications. Science **210**, 1253-1255

Yuan, F., Leunig, M., Huang, S.K., Berk, D.A., Papahadjopoulos, D. and Jain, R.K. (1994). Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. Cancer Research **54**, 3352-3356

Zakharova, T.S., Ivanov, A.S., Echkalov, A.P., Beriozov, A.T., Khalilov, E.M. and Archakov, A.I. (1993). Interaction of cholesterol containing liposomes with blood serum lipoproteins. Biochemistry and Molecular Biology International **31**, 315-324

Zalipsky, S., Brandeis, E., Newman, M.S. and Woodle, M.C. (1994). Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine. FEBS Letters **353**, 71-74

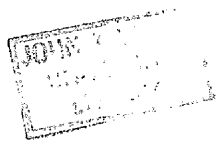
Zhang, W.-W., Fang, X., Branch, C.D., Mazur, W., French, B.A. and Roth, J.A. (1993). Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. BioTechniques **15**, 868-872

Zou, Y., Ling, Y.H., Van, N.T., Priebe, W. and Perez-Soler, R. (1994). Antitumor activity of free and liposome-entrapped annamycin, a lipophilic anthracycline with non-cross-resistance properties. Cancer Research **54**, 1479-1484

Zoubek, A., Emminger, W., Emminger-Schmidmeier, W., Peters, C., Pracher, E., Grois, N. and Gadner, H. (1992). Conventional vs. liposomal amphotericin B in immunosuppressed children. Pediatric Hematology and Oncology **9**, 187-190

Zuidam, N.J., Lee, S.S.L. and Crommelin, D.J.A. (1993). Sterilization of liposomes by heat treatment. Pharmaceutical Research **10**, 1591-1596

Zuidema, J., Kadir, F., Titulaer, H.A.C. and Oussoren, C. (1994). Release and absorption rates of intramuscularly and subcutaneously injected pharmaceuticals (II). Int. J. Pharmaceutics **105**, 189-207



CHAPTER SIX

APPENDICES

APPENDIX I : CALCULATING WEIGHT-AVERAGE DIAMETERS

The computer program "D-LIPPRO", written for BBC Basic, calculates the weight-average diameter ($\overline{d}_w$) and the number of molecules of lipid per liposome ($\overline{N}_w$, as NTW) for a population of vesicles based on the output from the Autosizer (see Section 3.1.1).

```
10 *FX6,0
15 VDU2
20 INPUT DZ,DM,S,RHO,LL,UL
25 FOR I=LL TO UL
30  Y=(1/(S*2.5066))*(EXP(-((I-DM)^2)/(2*S^2)))
35  N=Y/(I^3)
40  SUMYD=SUMYD+(Y*I)
45  SUMY=SUMY+Y
50  SUMND=SUMND+(N*I)
55  SUMN=SUMN+N
60  R=(I/2)*1.0E-9
65  NO=(4*3.142*(R)^2)/50E-20
70  NI=(4*3.142*(R-7.5E-9)^2)/50E-20
75  NT=NO+NI
80  P=NT*RHO
85  SUMYP=SUMYP+(P*Y)
90  SUMNP=SUMNP+(P*N)
95 NEXT I
100 DW=SUMYD/SUMY
105 DN=SUMND/SUMN
110 NOM=(4*3.142*((DM*1.0E-9)/2)^2)/50E-20
115 NIM=(4*3.142*(((DM*1.0E-9)/2)-7.5E-9)^2)/50E-20
120 NTM=NOM+NIM
125 PM=NTM*RHO
126 NOZ=(4*3.142*((DZ*1.0E-9)/2)^2)/50E-20
127 NIZ=(4*3.142*(((DZ*1.0E-9)/2)-7.5E-9)^2)/50E-20
128 NTZ=NOZ+NIZ
129 PZ=NTZ*RHO
130 NOW=(4*3.142*((DW*1.0E-9)/2)^2)/50E-20
135 NIW=(4*3.142*(((DW*1.0E-9)/2)-7.5E-9)^2)/50E-20
140 NTW=NOW+NIW
145 PW=NTW*RHO
150 NON=(4*3.142*((DN*1.0E-9)/2)^2)/50E-20
155 NIN=(4*3.142*(((DN*1.0E-9)/2)-7.5E-9)^2)/50E-20
160 NTN=NON+NIN
165 PN=NTN*RHO
170 PRINT"RESULTS FOR NORMAL DISTRIBUTION"
175 PRINT"WEIGHT AVE. DIAMETER="DW
180 PRINT"NUMBER AVE. DIAMETER="DN
185 PRINT"WEIGHT:NUMBER AVE. DIAMETER="DW/DN
190 PRINT"WEIGHT AVE. NUMBER OF PROTEINS PER LIPOSOME="SUMYP/SUMY
195 PRINT"NUMBER AVE. NUMBER OF PROTEINS PER LIPOSOME="SUMNP/SUMN
200 PRINT"MEAN(DM) NO. OF PROTEINS PER LIPOSOME="PM
205 PRINT"MEAN(DZ) NO. OF PROTEINS PER LIPOSOME="PZ
210 PRINT"MEAN(DW) NO. OF PROTEINS PER LIPOSOME="PW
```

```
215 PRINT"MEAN(DN) NO. OF PROTEINS PER LIPOSOME="PN
216 PRINT"NTM="NTM
217 PRINT"NTZ="NTZ
218 PRINT"NTW="NTW
219 PRINT"NTN="NTN
```

APPENDIX II : CALCULATING MONOLAYER COVERAGE

An example is given below of the calculation of monolayer coverage of bacteria by cationic liposomes (27 mg DPPC, 7 mg cholesterol, 4 mg stearylamine in 3 ml of buffer).

Mwt. of DPPC = 734 Mwt. of Chol = 386.7 Mwt. of SA = 269.5

Therefore, the average Mwt of this mixture is;

$$= \frac{27}{38} \times 734 + \frac{7}{38} \times 386.7 + \frac{4}{38} \times 269.5 = 621.1$$

A sample of the VETs (10 μl) was taken for scintillation counting.

$$\text{Moles of lipid in 10 μl} = \frac{0.038}{621.1} \times \frac{0.01}{3} = 2.04 \times 10\text{-}7 \text{ moles}$$

Scintillation count (10 μl) of the VETs = 36596.94 dpm

$$\text{Specific activity} = \frac{36596.94}{2.04 \times 10^{-7}} = 1.79 \times 10^{11} \text{ dpm mole}^{-1}$$

From the wells to which the liposome sample had been added (200 μl), after washing any liposomes that had adsorbed were disrupted by the addition of SDS (200 μl). After 30 mins at room temperature and 5 mins sonication, 180 μl of the SDS was removed for scintillation counting.

Average dpm for bacteria + VETs (180 µl) = 1867.74

Average dpm for plate + VETs (180 µl) = 64.29

Difference = 1803.45

$$\text{Moles removed in 180 µl} = \frac{\text{Difference}}{\text{Specific activity}} = \frac{1803.45}{1.79 \times 10^{11}}$$

$$= 1.005 \times 10^{-8} \text{ moles}$$

This figure has to be adjusted because of the fact that, of the 200 µl of SDS that is added to the well, only 180 µl is removed for scintillation counting. This gives the number of adsorbed moles of lipid (N_{obs});

$$\text{Moles in 200 µl} = 1.005 \times 10^{-8} \times \frac{200}{180} = 1.117 \times 10^{-8} \text{ moles}$$

From the amount of lipid added and the amount removed by the addition of SDS, it is possible to calculate the percentage of lipid that was adsorbed.

$$\text{Concentration of lipid added} = \frac{0.038}{621.1} \times \frac{1}{3} \times \frac{1}{10} = 2.039 \times 10^{-6} \text{ moles ml}^{-1}$$

$$\text{Amount of lipid added in 200 µl} = 2.039 \times 10^{-6} \times \frac{200}{1000} = 4.079 \times 10^{-7} \text{ moles}$$

$$\% \text{ adsorbed} = \frac{\text{moles in SDS}}{\text{moles added to well}} = \frac{1.117 \times 10\text{-}8}{4.079 \times 10\text{-}7} \times 100\ \%$$

$$= 2.74\ \%$$

The percentage of monolayer coverage is calculated by working out the number of liposomes adsorbed onto the bacteria, and the projected area each liposome occupies on the biofilm surface in the well. The area of the well covered by the 200 µl suspension of bacteria (A_{bf}) has previously been measured as 2.202 x 10^{-4} m^2.

$$\overline{dw} = 127.938\ \text{nm} = 127.938 \times 10^{-9}\ \text{m}$$

$$\text{Projected area covered per liposome} = \pi \left(\frac{dw}{2}\right) = \pi \left(\frac{127.938}{2}\right)^2$$

$$= 1.28 \times 10^{-14}\ m^2 \text{ per liposome.}$$

$$\text{Number of VETs in a monolayer} = \frac{\text{area of the well}}{\text{area of one VET}} = \frac{2.202 \times 10^{-4}}{1.28 \times 10^{-14}} = 1.71 \times 10^{10}$$

The number of adsorbed vesicles can be calculated by dividing the number of molecules of lipid adsorbed to bacteria in the well by the number of lipid molecules per liposomes ($\overline{N}_w$), as determined by the weight-average diameter.

$$\text{VETs adsorbed} = \frac{\text{molecules of lipid adsorbed}}{\text{molecules of lipid per VET}}$$

$$= \frac{(1.117 \times 10^{-8}) \quad (6.023 \times 10^{23})}{1.83 \times 10^{5}} = 3.676 \times 10^{10}$$

From this value, the percentage monolayer coverage is obtained.

$$\%amc = \frac{\text{VETs adsorbed}}{\text{VETs in a monolayer}} \times 100\ \%$$

$$= \frac{3.676 \times 10^{10}}{1.71 \times 10^{10}} \times 100\ \% = \underline{\mathbf{214.5\ \%}}$$

To avoid having to perform this set of calculations with every set of data, a BBC Basic computer program was written ("NEIL3") to work out the results in response to inputted values;

```
10 *FX6,0
15 LET Q$="OFF"
17 LET O$="1"
20 DPM=0:MWT=0
25 COLOUR 140
30 CLS
39 COLOUR 144:COLOUR 11
40 PRINT''"#########################################################"
50 PRINT"#                                                         #"
60 PRINT"#  MONOLAYER COVERAGE PROGRAM BY NEIL SANDERSON  V3.3  #"
70 PRINT"#                                                         #"
80 PRINT"#########################################################"
90 COLOUR 9:COLOUR 140
100 PRINT''"              OPTIONS"
110 COLOUR 7
120 PRINT''"   1. ENTER CHARACTERISTICS OF VESICLES"
130 PRINT''"   2. ENTER VALUES FROM TARGETING ASSAY"
135 PRINT''"   3. PRINTER ON/OFF (";Q$;")"
140 PRINT''"   4. QUIT PROGRAM"
150 PRINT:INPUT OPT$
155 IF OPT$="" THEN LET OPT$=O$
157 O$=OPT$
160 IF OPT$="4" THEN GOTO 3000
170 IF OPT$="1" THEN GOTO 1000
180 IF OPT$="2" THEN GOTO 2000
```

```
185 IF OPT$="3" AND Q$="OFF" THEN LET Q$="ON":GOTO 30
190 IF OPT$="3" AND Q$="ON" THEN LET Q$="OFF":GOTO 30
200 GOTO 30
1000 COLOUR 137:COLOUR 11:CLS
1005 IF Q$="ON" THEN VDU2
1010 INPUT ' ' " ENTER AVERAGE DPM FOR A 10 MICROLITRE SAMPLE OF THE VETS
";DPM
1020 INPUT ' " ENTER AVERAGE MOLECULAR WEIGHT OF THE LIPIDS ";MWT
1030 INPUT ' " ENTER MASS OF LIPID (MILLIGRAMS) ";MS
1040 INPUT ' " WHAT FACTOR WERE THE VETS DILUTED (e.g. 10 FOR A TENFOLD
DILUTION) ";DIL
1050 INPUT ' " ENTER DW (FROM D-LIPPRO) " ;DX
1060 INPUT ' " ENTER NTW (FROM D-LIPPRO) ";NTW
1150 MOLES=MS/1000/MWT*.01/3
1160ACT=DPM/MOLES
1170 PRINT ' " MOLES OF LIPID = ";MOLES;" MOLES"
1180 PRINT ' " SPECIFIC ACTIVITY = ";ACT;" DPM/MOLE"
1190 VDU3
1200 PRINT ' ' ' "                PRESS ENTER TO RETURN TO MAIN MENU"
1210 INPUT K$
1220 GOTO 25
2000 COLOUR 144:COLOUR 6:CLS
2010 IF DPM=0 AND MWT=0 THEN PRINT ' ' ' "  NO DATA HAS YET BEEN ENTERED
CHARACTERISING THE VESICLES." ' ' "       PRESS ENTER TO RETURN TO THE MAIN
MENU !!":INPUT ' ' " K$:GOTO 25
 2020 INPUT ' ' '" DPM OF SOLUBILZED VETS REMOVED FROM WELLS CONTAINING
BACTERIA ":VET
 2030 INPUT ' " DPM OF SOLUBILIZED VETS REMOVED FROM WELLS CONTAINING PBS
ONLY ";PBS
2040 DIF=VET-PBS
2050 WEL=DIF/ACT
2060 COR=WEL*10/9
2070 PLT=MS/1000/MWT/3
2080 ADD=PLT*.2/DIL
2090 ATT=COR/ADD*100
2100 DW=DX/1E9
2110 COV=(DW/2)^2*PI
2120 ADS=COR*6.023E23/NTW
2130 MON=2.202E-4/COV
2140 PC=ADS/MON*100
2150 CLS
2170 IF Q$="ON" THEN VDU2
2180 PRINT ' ' " DPM FOR BACTERIA = ";VET
2190 PRINT"  DPM FOR PBS ONLY = ";PBS
2200 PRINT "      DIFFERENCE = ";DIF
2210 PRINT ' "  ";COR;" MOLES IN 200 MICROLITRES"
2220 PRINT"  ";ADD;" VETS ADDED IN 200 MICROLITRES"
2230 PRINT"  ";ATT;"% ATTACHMENT"
2240 PRINT" PROJECTED AREA COVERED = ";COV;" M2 PER LIPOSOME"
2250 PRINT" VETS ADSORBED = ";ADS
2260 PRINT" VETS IN A CALCULATED MONOLAYER = ";MON
2265 PRINT ' '
2270 COLOUR 18:PRINT"  ";PC;" % MONOLAYER COVERAGE"
2275 COLOUR 7
2300 VDU3
2310 PRINT ' ' ' "     PRESS ENTER TO RETURN TO MAIN MENU"
2320 INPUT ' ' K$
2330 GOTO 25
2999 GOTO 9999
```

```
3000 COLOUR 144:COLOUR 7:CLS:PRINT ' ' ' ' "   EXIT TO DOS (Y/N) ?"
3010 INPUT ' ' ' ' Y$
3020 IF Y$="Y" THEN *QUIT
3030 END
9999 COLOUR 144:COLOUR 7:END
```

CHAPTER SEVEN

PUBLICATION

Interaction of cationic liposomes with skin bacteria

NEIL M. SANDERSON and MALCOLM N. JONES

School of Biological Sciences, University of Manchester, Oxford Road, M13 9PT, U.K.

The increased use of implanted biomedical devices such as catheters, prosthetic heart valves and artificial hip joints has led to a greater susceptibility to bacterial infection, most frequently with *Staphylococcus epidermidis* [1]. This is normally a non-pathogenic organism associated with human skin, but it can cause disease when it persists in sites inaccessible to host defences or if these mechanisms are defective [2]. The foreign body biomaterials of the implanted devices interfere with the methods the body normally uses to deal with bacterial infection (e.g. leukocyte phagocytosis). Most of these infections are of a nosocomial origin; that is, they originate and spread within the hospitals. The skin around the insertion site for implanted devices is considered the most common source of entry for microorganisms that cause these infections [3]. Once the skin barrier has been breached, the bacteria infect the devices by adhering to and colonizing their biomaterial surfaces where they secrete an extracellular polysaccharide (slime) which forms a protective biofilm. Such device-associated infections can lead to serious consequences, most frequently septicaemia. Replacement of the infected device is often the only option. Where infection has taken place, antibiotics such as vancomycin can be used to treat the patient. However, vancomycin is potentially toxic and cannot be given orally. Cationic liposomes, incorporating positively-charged stearylamine (SA), have previously been shown to target to biofilms of *S.epidermidis*, exploiting the negative charge on the surface of the bacteria [4]. Such vesicles could be used to deliver agents such as vancomycin to the bacteria, hopefully minimising the unwanted side-effects of the free drug.

Cationic liposomes were prepared using the extrusion method [5] in phosphate buffered saline (pH 7.4) from dipalmitoylphosphatidylcholine (DPPC), cholesterol and stearylamine (mole ratio 1.0 : 0.4 : 0.49). The liposomes, which had weight-average diameters of 127.6±1.2nm, were radiolabelled with [^{3}H]-DPPC. The interaction of the liposomes with the *S.epidermidis* bacteria was studied by examining liposome adsorption to adsorbed biofilms on microtitre plates. The biofilms were prepared by incubation of bacterial suspensions of specific optical absorbance (0.5 at 550nm) in microtitre plate wells for 18 hours. Liposome suspensions were incubated with the biofilms for 2 hours, after which the liquid phase was removed and the biofilm was dispersed in 1% w/v sodium *n*-dodecylsulphate (SDS) and analysed by scintillation counting to determine the extent of liposome adsorption. Adsorption was expressed as the percentage apparent monolayer coverage (%amc) of the biofilm, calculated from the projected diameter of the liposomes and the area of the biofilm. A figure of %amc in excess of 100% reflects the surface roughness of the biofilm since the geometric area was used in calculations.

The adsorption can be described in terms of a Langmuir adsorption isotherm, applicable to situations in which species are adsorbed as monolayers on solid surfaces. Plots of reciprocal %amc vs. reciprocal liposome concentration based on the Langmuir equation were linear and used to derive values for the maximum theoretical monolayer coverage - $(\%amc)_{max}$ - and an association constant (K_a) for the interaction. Table 1 summarises these results over a range of temperatures and ionic strengths

TABLE 1. Values for maximum theoretical %amc and associations constants

IONIC STRENGTH	180 mM		
TEMP. (K)	277.15	298.15	310.15
$(\%amc)_{max}$	126.3 ± 10.3	199.9 ± 0.1	303.3 ± 24.2
K_a (M^{-1})	3919.66 ± 354.64	2452.63 ± 1.64	1372.29 ± 115.50
ΔG_a (KJ mol^{-1})	-19.065 ± 0.209	-19.347 ± 0.002	-18.628 ± 0.218
ΔH_a (KJ mol^{-1})	-25.651 ± 5.390	-25.651 ± 5.390	-25.651 ± 5.390
$T\Delta S_a$ (KJ mol^{-1})	-2.587	-2.304	-3.023
ΔS_a (KJ K^{-1} mol^{-1})	-0.00933	-0.00773	-0.00975
IONIC STRENGTH	666 mM		
TEMP. (K)	277.15	298.15	310.15
$(\%amc)_{max}$	121.1 ± 12.2	229.8 ± 64.2	319.5 ± 117.1
K_a (M^{-1})	934.74 ± 101.55	471.63 ± 132.54	275.61 ± 101.81
ΔG_a (KJ mol^{-1})	-15.762 ± 0.251	-15.26 ± 0.716	-14.489 ± 1.000
ΔH_a (KJ mol^{-1})	-25.995 ± 3.042	-25.995 ± 3.042	-25.995 ± 3.042
$T\Delta S_a$ (KJ mol^{-1})	-10.234	-10.735	-11.056
ΔS_a (KJ K^{-1} mol^{-1})	-0.0369	-0.036	-0.0371

From the association constants, it was possible to calculate the Gibbs energy change of association ($\Delta G_a = -RT \ln K_a$) and, by application of the Gibbs-Helmholtz equation, the enthalpy and entropy changes. $(\%amc)_{max}$ is independent of I within experimental error but K_a is lower at the higher ionic strength.

The results indicate that the liposome-biofilm interaction is mediated by electrical double layer effects, in that compression of the diffuse double layer and increased ionic screening weakens the attraction. Because of this decreased interaction, ΔG_a is smaller at higher I as the increased entropy change (ΔS_a) makes a greater contribution and the enthalpy change (ΔH_a) is less dominant. ΔS_a is negative because the adsorbed liposomes are more ordered than when in solution and this change is greater at higher I. Increasing the temperature of incubation has the effect of increasing $(\%amc)_{max}$ but lowering K_a, so a larger number of liposomes are bound but with decreasing strength of interaction. ΔH_a and ΔS_a remain constant over the range of temperatures studied.

1. Finch, R.G., Hill, P. & Williams, P. (1995) Chemistry & Industry 6, 225-228
2. Jansen, B. & Peters, G. (1993) J. Antimicrobial Chemotherapy **32**, 69-75
3. Bandyk, D.F. & Esses, G.E. (1993). Surgical Infections. 74, 571-590
4. Song, Y.-H. & Jones, M.N. (1994) Biochem. Soc. Trans. **22**, 330S
5. Mayer, L.D., Hope, M.J. & Cullis, P.R. (1986) Biochim. Biophys. Acta **858**, 161-168