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International Journal of Pharmaceutics 257 (2003) 125–140
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The antibacterial properties of solid supported liposomes on *Streptococcus oralis* biofilms

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Received 30 August 2002; received in revised form 27 January 2003; accepted 5 February 2003

Abstract

A novel system for the delivery of drugs to bacterial biofilms has been developed. The system is based on the use of anionic and cationic liposomes as drug carriers adsorbed on the surface of zinc citrate particles. The adsorption process results in the formation of solid supported vesicles (SSVs) which aids the stabilisation of the liposomes. Anionic liposomes have been prepared by incorporation of phosphatidylinositol (PI) into dipalmitoylphosphatidylcholine (DPPC) liposomes and cationic liposomes have been prepared by incorporation of dioctadecyldimethylammonium bromide (DDAB) into DPPC plus cholesterol liposomes. The liposomes were adsorbed onto zinc citrate particle and targeted to immobilised biofilms of the oral bacterium *Streptococcus oralis*. The liposomes were used to carry the bactericides, Triclosan®, a lipid-soluble agent, and the aqueous-soluble penicillin-G, and their ability to inhibit bacterial growth from immobilised biofilms was accessed. Zinc citrate is itself a bactericide and is used in the formulation of toothpastes. The SSVs carrying the drugs have therapeutic properties. To trace the origin of these properties, each component of the SSV was investigated alone and in combination in binary systems. Some combinations showed synergistic (or additive) antibacterial effects while others showed regressive effects compared with their components.

Keywords: Solid supported liposomes (or vesicles) (SSVs); Zinc citrate particles; Triclosan®; Penicillin-G; *Streptococcus oralis* biofilms; Antibacterial properties

1. Introduction

The adsorption of liposomes and the delivery of bactericides to bacterial biofilms have been extensively studied (Kim et al., 1999; Jones et al., 1993, 1997). Liposomes naturally target to the bacterial cell wall more or less effectively depending on the liposome composition. In addition to this property, liposomes are biocompatible and biodegradable so they have been widely used as drug delivery tools (Jones and Chapman, 1994). Liposomes have the potential to carry hydrophobic and hydrophilic drugs over long periods of time and also to decrease drug side effects by protecting the environment from direct contact with the drugs. Liposomes containing the antibacterial agent Triclosan® have been found more effective against the oral bacteria *Streptococcus sanguis* and *Streptococcus mutans* than when being used as a free drug (Jones et al., 1993). Toxic antibacterial agents, such as vancomycin, have also been successfully encapsulated in cationic liposomes and applied to *Staphylococcus aureus* (Kim et al., 1999).

It is illustrated in the literature that liposomes need to be stable when used as drug delivery tools in vivo. There are three forms of liposome stability to con-

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doi:10.1016/S0378-5173(03)00136-4

sider in relation to drug delivery: chemical, physical and biological stabilities (Lasic, 1998). Stability can be controlled by manipulating factors, such as pH, size distribution and ionic strength, or by using the alternative method of coating liposomes with inert hydrophilic polymers (Stealth® liposomes). In this paper, we describe a new approach to chemical and physical liposome stabilisation by adsorbing them on solid surfaces. When liposomes are adsorbed on solid surfaces, adsorption is irreversible. Liposomes can either disrupt on adsorption or adsorb intact or a combination of the two processes can occur. Previous studies have shown that liposomes adsorb predominantly intact on solid particulates of zinc citrate (Catuogno and Jones, 2000).

The oral bacterium *Streptococcus oralis* is a Gram-positive species; a member of the normal oral flora associated with initial bacterial colonisation of the tooth surface (Frandsen et al., 1991). Oral microorganisms adhere to the enamel and gradually form dental plaque. If it is not removed regularly, the plaque can become calcified to form calculus or tartar by enrichment in calcium and phosphate ions. Acid-producing species in the plaque are responsible for dental caries and plaque leads to other periodontal diseases. The simplest remedy for preventing oral diseases is to maintain basic oral hygiene by a combination of brushing and flossing. The incorporation of antibacterial agents in toothpaste, such as Triclosan® and chlorhexidine, also improves dental hygiene (Marsh, 1992).

The idea to develop a new dental product that incorporates stable therapeutic liposomes is presented in this article. In this study, we report the antibacterial effect of liposomes adsorbed on solid particulate zinc citrate, a material widely used in toothpaste as a bacterial growth inhibitor (Bradshaw et al., 1993). In addition to the solid supported liposomes (SSVs), two antibacterial compounds were chosen to be delivered by liposomes: Triclosan®, because it is widely used in combination with zinc citrate in the dental hygiene field and benzyl penicillin (penicillin-G or pen-G) due to its efficacy towards most Gram-positive bacteria. The antibacterial agents have been encapsulated in anionic and cationic liposomes prior to adsorption on solid particles. A study of liposomal lipid adsorption on zinc citrate has been reported (Catuogno and Jones, 2000). The results of liposomes targeted to *S. oralis* biofilms are presented here together with the antibacterial properties of liposomes, particles and antibacterial agents investigated individually on *S. oralis* and in combination in zinc citrate particles with adsorbed liposomes carrying antibacterial agents.

2. Materials and methods

1, 2- Dipalmitoyl- *sn*- glycerol-phosphatidylcholine (DPPC) was purchased from Sygena Ltd., Eichenweg, Switzerland (product code LP-04-012). Phosphatidylinositol (PI) lipid was purchased from Lipid Products, South Nutfield, Redhill, Surrey, UK. *N*-Dioctadecyldimethylammonium bromide (DDAB, code D2779), cholesterol (code C8667), Sepharose 4B (code 4B200) and benzyl penicillin (code penK) were obtained from Sigma Chemical Company, Poole, Dorset, UK. Tritiated DPPC (^{3}H-DPPC, code TRK 673), ^{14}C-DPPC (code CFA 604) and ^{14}C-pen-G (code CFA 244) were purchased from Amersham, Buckinghamshire, UK. Zinc citrate particles were obtained from Unilever Research, Port Sunlight Laboratories, Bebington, Wirral, UK (code 5569).

Brain heart infusion, yeast extract and defibrinated horse blood were purchased from Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK (product codes CM255, B60645 and SR050B, respectively). *Streptococcus oralis* and Triclosan® were a gift from Unilever Research, Port Sunlight Laboratories, Wirral, UK. ^{3}H-Triclosan® was prepared by alkali-catalysed tritium exchange using tritiated water. Sucrose was purchased from AnalaR, BDH Laboratory Supplies, Poole, Dorset, UK (code 102745C).

2.1. Liposome preparation and adsorption on solid particles

The method of adsorbing liposomes on zinc citrate particles has been previously described in detail (Catuogno and Jones, 2000). However, a brief description is presented below. Liposomes were prepared using the extrusion technique to produce largely unilamellar liposomes of approximately 100 nm diameter (Hope et al., 1985). A lipid mixture containing either anionic or cationic lipids (total mass 30 or 38 mg) and the appropriate radioactive marker (5 μCi/ml) were dissolved in a mixture of methanol and chloroform (volume ratio 1:4) at 60 °C. Liposomes were made either anionic using 14 mol% PI together with DPPC

or cationic with 9 mol% of cationic surfactant DDAB together with DPPC and cholesterol. A PBS solution at 60 °C (pH 7.3) was added to the dry lipid film obtained after rotary evaporation of the organic solvent. The liposomes were stored at room temperature in PBS.

Liposomes encapsulating Triclosan® or pen-G were made by mixing ^{3}H-Triclosan® (1 ml, 2 μCi/ml) or ^{14}C-benzyl penicillin (1 ml, 5 μCi/ml) and 10% (w/w) cold pen-G with the PBS solution added to the dry lipid film.

Zinc citrate was supplied as a fine white powder. Before use, 5 and 0.1% (w/v) of powder were mixed with PBS buffer (pH 7.3) to constitute the zinc citrate dispersion. The final pH was measured and adjusted to neutral if necessary. Aliquots (250 μl) of dispersion were added to an equal volume of liposomes and left for 2 h on a rotating wheel. The free liposomes were discarded after centrifugation of the samples and the liposomes adsorbed to the solid particles were resuspended in PBS.

2.2. Bacteriology

2.2.1. Preparation of blood agar plates

Brain heart infusion (3.7 g) was added to 1 l of distilled water and mixed. Bacterial agar (15 g) was then added and the final solution autoclaved (121 °C, 1 atm, 40 min). The mixture was allowed to cool down before adding 5% (v/v) defibrinated horse blood. Homogenisation was done by rolling the flask and finally the medium was poured into sterile Petri dishes which were left to set at room temperature and dried in a 37 °C oven with the lid partly off for 15 min. Once dried, the plates were either sealed and stored at 4 °C or used for bacterial inoculation.

2.2.2. Inoculation of solid medium

Streptococcus oralis (formerly *S. sanguis* strain 209) bacteria were stored in semi-skimmed milk at −70 °C. The blood agar plates were streaked with defrosted bacterial solution using sterile plastic loops and incubated in a 37 °C room overnight. The colonies observed were cream coloured, round shaped and about 1 mm in diameter. The plates were then sealed and stored at 4 °C. Within a month a single colony was resuspended and used to streak a fresh blood agar plate in order to maintain the bacteria.

2.2.3. Cultivation in a liquid medium

Brain heart infusion (3.7 g), yeast extract powder (0.3 g) and sucrose (1 g) were mixed in 100 ml distilled water and autoclaved (121 °C, 40 min). Prior to use, the mixture was poured into four sterile Falcon tubes under sterile conditions. Bacteria were then transferred from the solid agar plate to the nutrient broth using a sterile disposable plastic loop. The Falcon tubes were left overnight on an agitator at 37 °C. The tubes were then centrifuged at 2000 rpm for 10 min using an MSE microcentaur bench centrifuge. The supernatant was eliminated and the pellet washed with 20 ml sterile PBS. The process was repeated three times. The bacterial suspension was then diluted in PBS in order to give an optical density (OD) of 0.5 at 550 nm.

2.2.4. Biofilm preparation

Aliquots (200 μl) of the bacterial suspension (OD_{550} = 0.5) were poured into a 96-well flat-bottomed microtitre plate using a multi-pipette loaded with sterile tips. Columns 1–12 of the plate were left free of *S. oralis* to check contamination. The surface of the plate was sealed with a plastic cover and left overnight at room temperature. The bacterial suspension was then removed and the biofilm spread at the bottom of the plate was washed three times with sterile PBS. The biofilm resulting from this procedure is constituted of metabolically active close-packed cells which multiply on addition of growth medium (Kaszuba et al., 1995, 1997).

2.2.5. Liposome targeting to Streptococcus oralis biofilms

The targeting of liposomes is expressed by the percent of apparent monolayer coverage (%amc) which compares the maximum number of liposomes (or moles of lipid) adsorbed on the biofilm to form a close-packed monolayer (calculated as described below) to the real number of liposomes or moles of lipid bound. Radiolabelled liposomes containing four compositions of anionic and cationic lipids (4, 9, 14, 19 mol% of PI and DDAB lipids) were targeted to the *S. oralis* biofilm. Two hundred microlitres of liposomes was exposed to the biofilm for 2 h and removed. The plate was washed with sterile PBS and blotted dry. Aliquots (200 μl of a 5% solution of sodium *n*-dodecyl sulphate (SDS)) were poured into the wells and left for 10 min before taking samples

for scintillation counting. Results were expressed as the %amc calculated from the following equation:

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

where N_{obs} is the observed number of moles of lipid adsorbed to the biofilm, L_{a} is the number of moles of lipid which would be adsorbed if the biofilm was completely covered with a close-packed monolayer of liposomes. L_{a} is calculated from the following equation:

$$L_{\text{a}} = \frac{A_{\text{bf}} \bar{N}_{\text{w}}}{\pi(\bar{d}_{\text{w}}/2)^2} \quad (2)$$

$\bar{d}_{\text{w}}$ is the weight average diameter of the liposomes. $\bar{N}_{\text{w}}$ is the weight average number of moles of lipid per liposome. $\bar{N}_{\text{w}}$ was calculated from $\bar{d}_{\text{w}}$ (Hutchinson et al., 1989). $A_{\text{bf}} = 2.202 \times 10^{-4}\,\text{m}^2$ is the surface area of the biofilm measured from the surface area of the microtitre plate wells exposed to a 200 μl of solution (Chapman et al., 1990).

2.2.6. SSVs targeting to Streptococcus oralis biofilms

SSVs were prepared as described earlier. After centrifugation, an aliquot of supernatant was counted and the supernatant was removed and replaced by PBS. The pellet was resuspended and the particle dispersion was applied to the biofilm for 1 h. The plate was then inverted for 10–20 min in order to eliminate the SSVs that had not adsorbed to the biofilm. The wells were emptied and finally washed with PBS three times. SDS (200 μl) was added and an aliquot (50 μl) was removed for radioactive counting. The method of calculation of the adsorbed liposomes was slightly modified from the previous method (Jones et al., 1997) to take into account the space required by the particles when calculating the maximum amount of lipid adsorbed to the biofilm. The first step (step 1) was the estimation of the projected area of a particle covered with one monolayer of intact liposomes ($P_{\text{p+2l}}$) and the deduction of the maximum number of particles plus monolayer systems (S_{pm}) which could be adsorbed on the surface of the well (A_{w}). The maximum number of moles of lipid adsorbed on a particle was then calculated (step 2) from which was deduced the maximum number of moles of lipid per well ($L_{\text{b max/well}}$). This last number was compared to the measured number of moles of lipid bound estimated by radioactive counting ($L_{\text{b exp}}$, step 3).

Step 1 : Maximum number of particle plus monolayer systems

$$P_{\text{p+2l}} = \pi(R_{\text{p}} + R_{\text{2l}})^2 \quad (3)$$

where R_{p} is the particle radius and R_{2l} the diameter of the adsorbed liposomes. The maximum number of particles plus monolayer systems per well (S_{pm}) is given by

$$S_{\text{pm}} = \frac{A_{\text{w}}}{P_{\text{p+2l}}} \quad (4)$$

Step 2 : Maximum number of moles of lipid adsorbed per well ($L_{\text{b max/well}}$)

$$\frac{\text{Number of liposome}}{\text{Particle}} = \frac{A_{\text{p}}}{A_{\text{l}}} = \frac{4\pi(R_{\text{p}})^2}{\pi(R_{\text{l}})^2} \quad (5)$$

where R_{l} is the radius of the liposomes.

$$L_{\text{b max/well}} = \frac{\text{Number of liposome/Particle} \times \bar{N}_{\text{w}}}{N_A} \times S_{\text{pm}} \quad (6)$$

Step 3 : %amc

$$\%\text{amc} = \frac{L_{\text{b exp}}}{L_{\text{b max/well}}} \times 100 \quad (7)$$

2.3. Cell growth analysis

The OD of the cell suspension is proportional to the number of bacterial cells in the well. The cell growth data were analysed by measuring the time necessary to reach a fixed OD and the time was plotted against OD. If the bacteria were highly inhibited, the time necessary to reach a certain OD (corresponding to a certain number of cells) would be longer than if the bacteria were not restricted in growth. The regrowth curves obtained were fitted by regression analysis to a three parameters sigmoidal equation. The correlation coefficient R^2 indicates how well the data fit the equation. An R^2 value near 1 indicates that the equation is a good description of the data, an R^2 value lower than 0.950 indicates a medium to poor correlation.

The sigmoidal model is presented by the following equation:

$$f(x) = \frac{a}{1 + e^{-((x-x_0)/b)}} \quad (8)$$

where a, b and x_0 are constants. When x tends to infinity, $f(x)$ tends to a; a is the value of the OD when in the stationary phase. When $x = x_0$, $f(x) = a/2$, which corresponds to the time to reach an OD half that in the stationary phase.

In the following figures, the a values represent the OD and are experimental data. A convenient way to compare the time to reach an OD of $a/2$ for each curve is to compare the x_0 values.

3. Results and discussion

3.1. Targeting of liposomes and SSVs to Streptococcus oralis biofilms

A preliminary experiment showing that adsorption of liposomes did not vary significantly when the free liposomes were removed from the supernatant was undertaken (figure not shown). The free lipid concen-

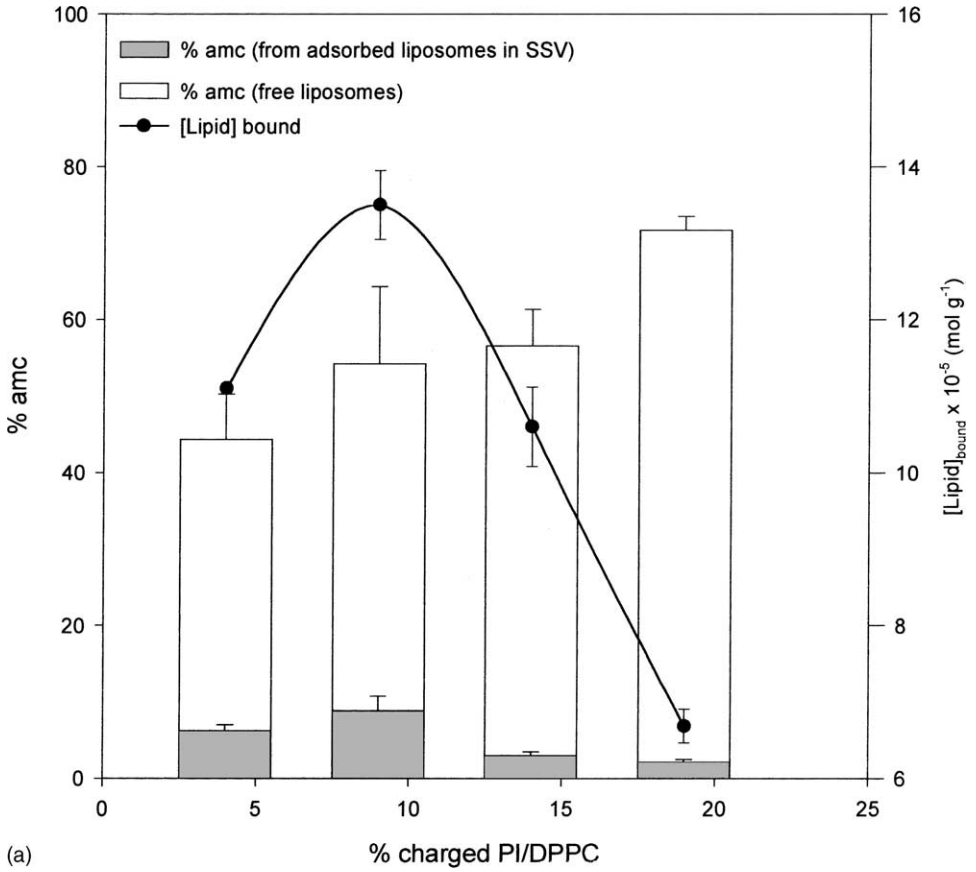

Fig. 1. (a) Adsorption of anionic liposomes on *Streptococcus oralis* biofilm (%amc) as a function of the mol% PI. Both free liposomes (□) and solid supported liposomes (■) were incubated with the bacterial biofilm for 2 h at room temperature. After incubation the bacterial biofilm was washed and solubilised with SDS in order to count the radioactivity remaining from the adsorbed lipids. The lipid bound concentration was also plotted as a function of the mol% PI (●). The error bars on the lipid bound curve and the %amc values are standard deviations calculated from experiments carried out in triplicate. (b) Adsorption of cationic liposomes on *S. oralis* biofilm (%amc) as a function of the percent of DDAB lipid. Both free liposomes (□) and solid supported liposomes (■) were incubated with the bacterial biofilm for 2 h at room temperature. After incubation the bacterial biofilm was washed and solubilised with SDS in order to count the radioactivity remaining from the adsorbed lipids. The lipid bound concentration was also plotted as a function of the mol% DDAB (●). The error bars on the lipid bound curve and the %amc values are standard deviations calculated from experiments carried out in triplicate.

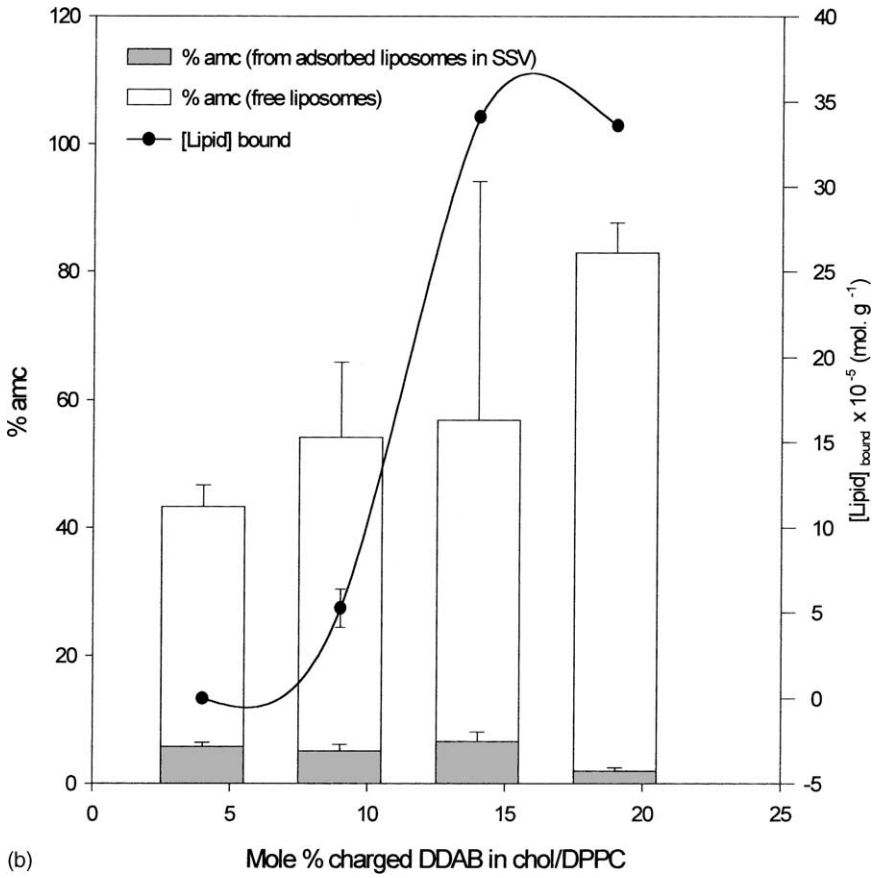

Fig. 1. (*Continued*)

tration found in the supernatant was plotted against the corresponding amount of lipid bound (adsorption isotherm). Adsorption isotherms for the anionic and cationic liposomes of the same composition as used here on zinc citrate particles have been previously reported (Catuogno and Jones, 2000). The isotherms show that the concentration of free lipid remains very small regardless of the amount of lipid bound.

The targeting of anionic and cationic liposomes (4–19 mol% of charged lipid) to *S. oralis* biofilms was investigated. Results from three independent measurements are shown in Fig. 1a for anionic liposomes and Fig. 1b for cationic liposomes. For anionic liposomes, the %amc increases with the PI content: the most adsorption was obtained with 19 mol% PI liposomes (71.72 ± 1.75 %amc) followed by 14 mol% PI (56.56 ± 4.75 %amc). When the liposomes were adsorbed on solid particles, adsorption followed a similar pattern and a peak was reached for 9 mol% PI (8.90 ± 1.88 %amc) after which targeting decreased (for 14 mol% PI/DPPC, %amc = 2.99 ± 0.46 and for 19 mol% PI/DPPC, %amc = 2.10 ± 0.40). The targeting of adsorbed liposomes was compared to the adsorption of lipid on zinc citrate and it was found that targeting increases with increase of lipid bound. For anionic liposomes, 9 mol% PI was found to be the composition giving the most adsorption ($1.35 \times 10^{-4} \pm 4.50 \times 10^{-6}$ mol/g) followed by 4 mol% PI ($1.11 \times 10^{-4} \pm 5 \times 10^{-8}$ mol/g).

The adsorption of cationic liposomes to *S. oralis* biofilms, shown in Fig. 1b, was optimum when 19 mol% DDAB (82.97 ± 4.69 %amc) was used. Results were found comparable for 14 mol% DDAB (56.83 ± 37.28 %amc) and 9 mol% DDAB (54.12 ±

11.45 %amc). When liposomes were adsorbed on particles and incubated with the biofilm, the most adsorption was obtained with 14 mol% DDAB (5.56 ± 1.51 %amc) and 4 mol% DDAB (5.75 ± 0.68 %amc). Slightly less adsorption was achieved with 9 mol% DDAB (5.05 ± 1.01) and about three times less with 19 mol% DDAB (1.92 ± 0.51 %amc). Overall, both the curves for adsorption and the %amc for adsorbed liposomes follow the same pattern for DDAB liposomes with a slight difference for anionic liposomes. Most adsorption was obtained with 14 mol% DDAB ($4.41 \times 10^{-4} \pm 2 \times 10^{-6}$ mol/g) and 19 mol% DDAB ($3.36 \times 10^{-4} \pm 1 \times 10^{-6}$ mol/g). Adsorption was found to be negligible with 4 mol% DDAB. Results for adsorption and targeting are summarised in Table 1 in which additional data on liposomes size are presented.

3.2. Antibacterial effect of free Triclosan® and free benzyl penicillin

Experiments were carried out to test the bactericide action of the antibacterial agents Triclosan® and

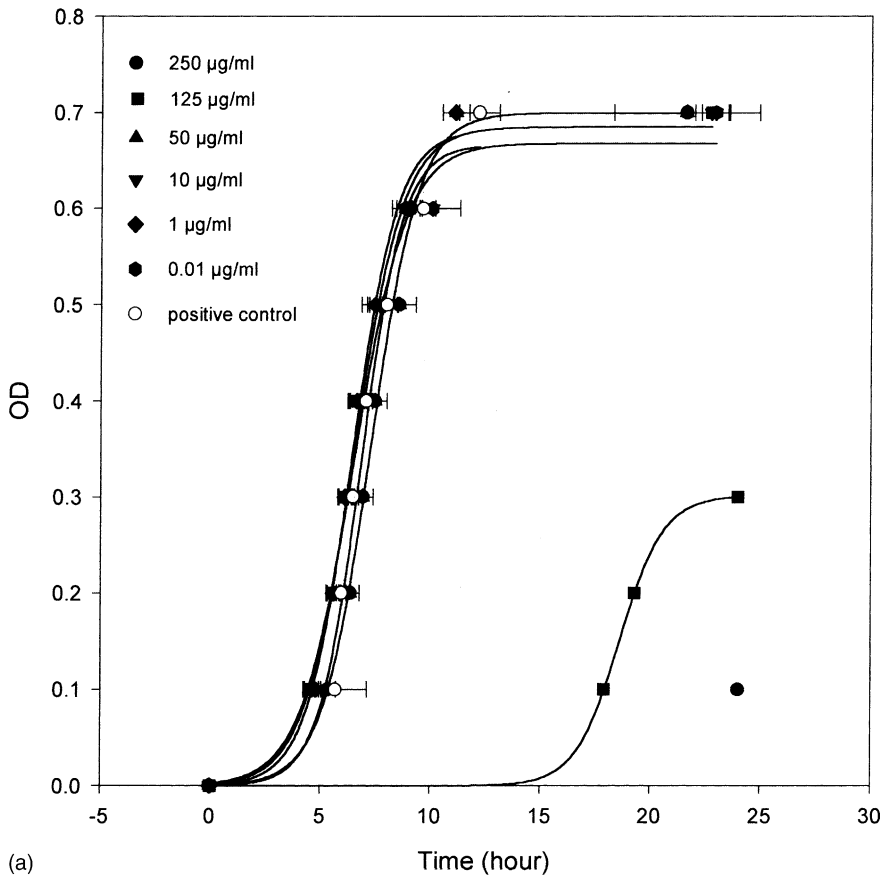

Fig. 2. (a) Effect of Triclosan® on *Streptococcus oralis* biofilm. Two hundred microlitres of each dilution of Triclosan® was added to six wells with adsorbed biofilm. The plate was left at room temperature for 1 h and agitated at low speed (300 rpm). The antibacterial solutions were removed and the plate washed two to three times with PBS. Bacterial regrowth was initiated by added nutrient broth and the OD of the cell suspension was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. The error bars are the standard deviations from six measurements on independent biofilms. (b) Effect of penicillin-G on *S. oralis* biofilm. Two hundred microlitres of each dilution of penicillin-G was added to six wells with adsorbed biofilm. The plate was left at room temperature for 1 h and agitated at low speed (300 rpm). The antibacterial solutions were removed and the plate washed two to three times with PBS. Bacterial regrowth was initiated by added nutrient broth and the OD of the cell suspension during bacterial regrowth was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. The error bars are the standard deviations from six measurements on independent biofilms.

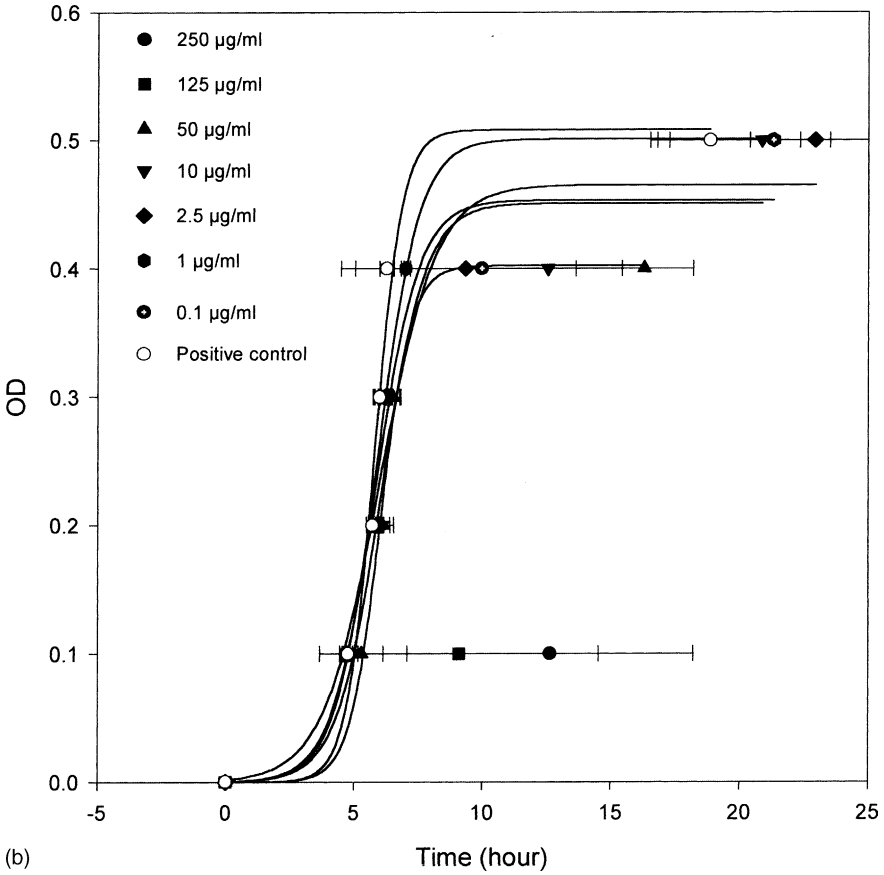

Fig. 2. (*Continued*).

pen-G against cell growth from *S. oralis* biofilms. Satisfactory results were obtained when the free drugs were applied for at least an hour to the biofilm. The effect of the free drugs on *S. oralis* biofilm was investigated as a function of drug concentration and results are shown in Fig. 2a for Triclosan® and Fig. 2b for pen-G. The positive control curve was obtained when only PBS was added to the biofilm for an hour. The data fitted the sigmoidal curves at drug concentrations up to 50 μg/ml with good correlation coefficients (on average $R^2 = 0.994 \pm 0.003$). This indicates a rapid increase in the number of cells in the stationary phase for about 2 h and slowly reached a maximum after 12 h. The positive control x_0 value was 6.83 ± 0.16 h which was very close to the values obtained when Triclosan® concentrations were below 125 μg/ml. For concentration values higher than 50 μg/ml, the curves were displaced by about 12 h or more. We defined a parameter called the minimum inhibitory concentration for regrowth (MIC_R) as compared to the minimum inhibitory concentration (MIC) used to characterise drugs in pharmacy. The MIC_R for Triclosan® was estimated to be between 50 and 125 μg/ml.

The curves obtained when the penicillin-G concentration was less than 125 μg/ml were found to be similar to the positive control curve (x_0 varying from 6.00 ± 0.11 h for 50 μg/ml to 5.80 ± 0.26 h for 0.1 μg/ml) showing no growth inhibition. Above 50 μg/ml, the curves showed a slower growth rate indicating bacterial inhibition. The MIC_R was found to be between 50 and 125 μg/ml for penicillin-G. Both bactericides were thus effective against *S. oralis* growth when used at high concentration above the MIC_R.

Table 1
Adsorption parameters for free liposomes and adsorbed liposomes

Systems	$[Lipid]_{bound}$ (mol/g) ($\times 10^{-5}$)	Liposomes characterisation		Solid supported liposomes adsorption on biofilm		Free liposomes adsorption on biofilm	
		Diameter (nm)	NTW	$[Lipid]_{bound}$ (mol/well)	%amc	$[Lipid]_{bound}$ (mol/well)	%amc
4 mol% PI	11.10 ± 0.00	71.1 ± 0.6	52349.99	$1.17 \times 10^{-9} \pm 1.51 \times 10^{-10}$	6.21 ± 0.80	$2.14 \times 10^{-9} \pm 2.86 \times 10^{-10}$	44.34 ± 5.94
9 mol% PI	13.50 ± 0.45	68.0 ± 0.5	46122.99	$1.62 \times 10^{-9} \pm 3.43 \times 10^{-10}$	8.90 ± 1.88	$2.52 \times 10^{-9} \pm 4.70 \times 10^{-10}$	54.22 ± 10.14
14 mol% PI	10.60 ± 0.52	137.0 ± 0.9	210175.4	$5.94 \times 10^{-10} \pm 9.17 \times 10^{-11}$	2.99 ± 0.46	$1.18 \times 10^{-8} \pm 1.01 \times 10^{-9}$	56.56 ± 4.75
19 mol% PI	6.69 ± 0.22	142.2 ± 1.0	231799.29	$4.27 \times 10^{-10} + 8.00 \times 10^{-10}$	2.10 ± 0.40	$5.76 \times 10^{-9} \pm 3.96 \times 10^{-9}$	71.72 ± 1.75
4 mol% DDAB	0	166.2 ± 0.6	291468.56	$1.07 \times 10^{-9} \pm 1.27 \times 10^{-10}$	5.75 ± 0.68	$5.75 \times 10^{-9} \pm 3.60 \times 10^{-9}$	43.25 ± 3.45
9 mol% DDAB	5.28 ± 1.12	157.5 ± 1.7	278980.57	$1.01 \times 10^{-9} \pm 1.95 \times 10^{-10}$	5.05 ± 1.01	$9.19 \times 10^{-10} \pm 4.38 \times 10^{-10}$	54.12 ± 11.75
14 mol% DDAB	34.10 ± 0.020	167.7 ± 0.2	255915.9	$1.05 \times 10^{-9} \pm 2.41 \times 10^{-10}$	5.56 ± 1.51	$3.67 \times 10^{-9} \pm 1.75 \times 10^{-9}$	56.83 ± 37.28
19 mol% DDAB	36.60 ± 0.10	174.4 ± 1.4	343700.74	$3.36 \times 10^{-10} \pm 1.80 \times 10^{-10}$	1.92 ± 0.51	$6.20 \times 10^{-9} \pm 2.26 \times 10^{-9}$	82.97 ± 4.69

The errors are the standard deviations from experiments done in triplicate. NTW is the weight average number of lipid molecules per liposome.

Adsorption of Triclosan® by bacterial cells is rapid and is closely associated with the lipid fraction of the cell wall. However, the major determinant of antimicrobial activity is possibly the increase of permeability of the bacterial cell wall induced by Triclosan® (Scheie, 1989). At low bacteriostatic concentrations, the action of Triclosan® on the membrane interferes with uptake of amino and nucleic acids and is directed against RNA and protein synthesis. At higher bactericidal concentrations, the action of Triclosan® on the membrane induces release of cytoplasmic material. The resulting membrane lesions permit leakage of cellular contents and lead to cell death. Additive inhibitory effects of zinc and Triclosan® on acid production during glucose metabolism by *S. mutans* have been reported (Cummins and Watson, 1989; Cummins, 1991). Triclosan® also has inhibitory effects on glycolysis and protease activity, and additive effects were found when low concentrations of zinc salts and Triclosan® were combined. Triclosan® efficiency against *S. oralis* in vivo has been widely shown by the toothpaste industry when used in combination with zinc citrate. The most commonly used concentrations in toothpaste is 0.2% Triclosan® combined with 0.5% zinc citrate. In our studies, the MIC_R was found to be between 50 and 125 μg/ml (50–125 mg/l). Consequently, the bacterial inhibition in vivo must be due to the combine effect of Triclosan® and zinc citrate as demonstrated by Bradshaw et al. (1993).

Teng et al. (1998) studied the frequency of penicillin-resistant isolates in the species of viridans streptococci. *Streptococcus oralis* showed the highest frequency (35%) of high level penicillin resistance (MIC $\geq$ 4 mg/l) compared to other oral streptococci (20% for *Streptococcus mitis*, 0% for *S. sanguis*). Five percent of *S. oralis* isolates showed intermediary resistance to penicillin (MIC = 0.25–2 mg/l) and 60% were susceptible. Although MIC and MIC_R are two different entities, the MIC_R found in our studies was at least 30 times higher than the MIC. A possible explanation resides in the method of cultivation of the bacteria. Teng used an agar plate-based method to determine the MIC on which bacterial colonies were inoculated and we grew biofilms on immunological plates before adding the antibacterial agents. If the layers of bacterial biofilms are composed of close-packed cells, as demonstrated by Kaszuba et al. (1997), the bottom layers of cells are more protected

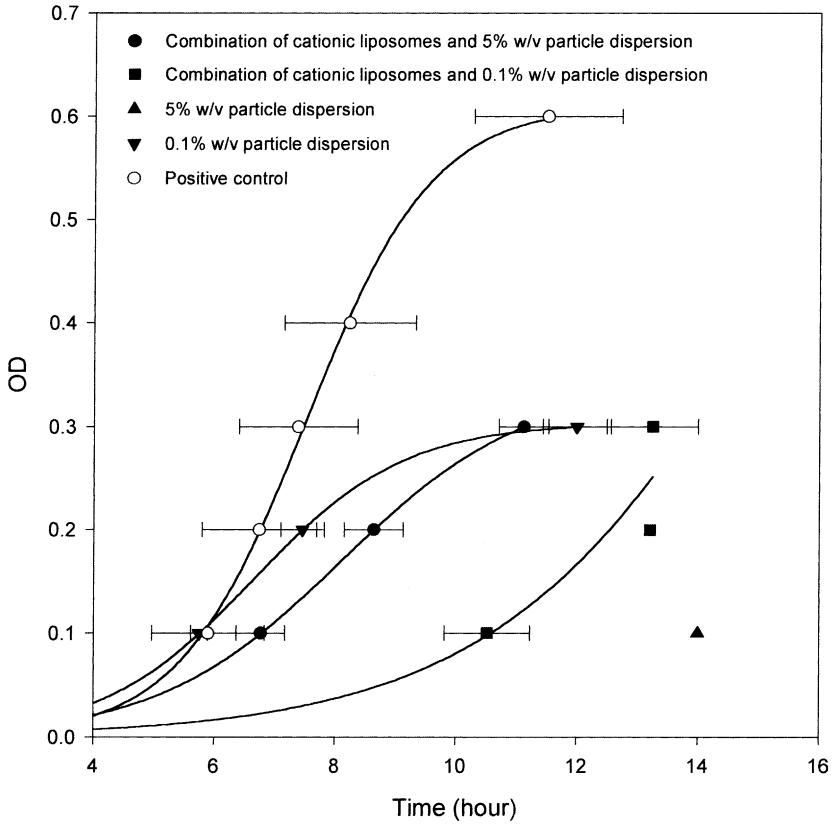

Fig. 3. Comparative effect of particle dispersions and their combination with cationic liposomes on *Streptococcus oralis* biofilm cell growth. The test system (200 μl) was incubated with the biofilm for 1 h at room temperature. The plate was slowly agitated at 300 rpm. The solutions were then removed and the biofilm washed two to three times with PBS. Nutrient broth was added and the OD of the cell suspension during bacterial regrowth was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. The error bars are the standard deviations from six measurements on independent biofilms.

Table 2
Half-maximum growth time (time (x_0)) for particulate, anionic liposome and zinc ion systems

Systems	x_0 (positive control) (h)	x_0 (system) (h)
Liposomes and particles		
Anionic liposomes 0.1% (w/v) particles	6.64 ± 0.03	9.21 ± 0.11
Anionic liposomes 5% (w/v) particles	6.64 ± 0.03	~7.40
Zinc citrate particle solutions		
0.1% (w/v) particle dispersion	4.57 ± 0.04	6.65 ± 0.03
0.1% (w/v) particle dispersion aqueous part	4.57 ± 0.04	7.48 ± 0.32
5% (w/v) particle dispersion	4.57 ± 0.04	14
5% (w/v) particle dispersion aqueous part	4.57 ± 0.04	12
Zinc ion solutions		
50 mM zinc concentration	8.60	12.80
5 mM zinc concentration	8.60	11.84 ± 0.47
1 mM zinc concentration and 0.5 mM zinc concentration	8.60	8.72 ± 0.11

than the top layer. Consequently, the difference of accessibility creates a drug concentration gradient across the layers. When concentrations were increased, the concentration gradients between the layers would also be increased. Under such conditions, it was observed with both Triclosan® and pen-G that the growth inhibition was also increased. According to Fick's law of diffusion, the number of molecules passing through a cross-section of the film (flux) is proportional to the concentration gradient. Therefore, when the concentration gradient is increased more molecules access the biofilm and more bacterial inhibition is expected. This hypothesis is in agreement with the results.

3.3. Antibacterial properties of liposomes and zinc citrate particles in combination

The liposome antibacterial effect, as well as the cumulative effect of liposomes and zinc citrate particles, was investigated. Fig. 3 shows results for cationic

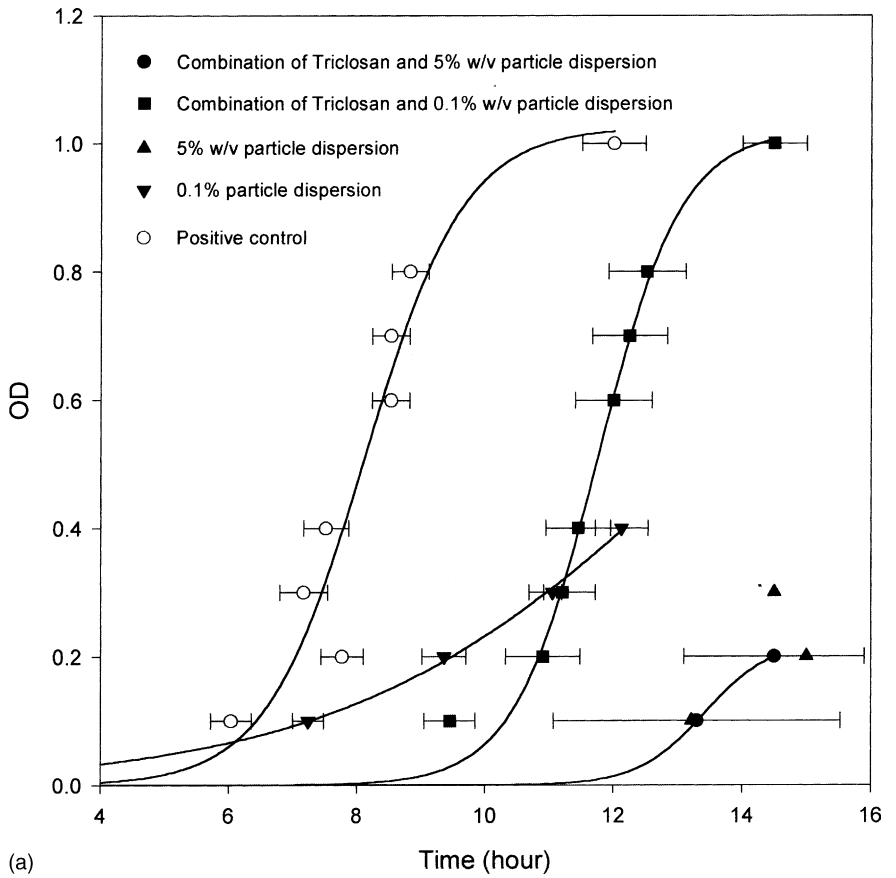

Fig. 4. (a) Comparative effect of 125 μg/ml Triclosan® used in combination with zinc citrate particles on *Streptococcus oralis* biofilms. The drug (100 μl) was incubated with 100 μl zinc citrate particle dispersion for 1 h at room temperature under low speed agitation (300 rpm). The solutions were then removed and the biofilm washed two to three times with PBS. Nutrient broth was finally added and the OD of the cell suspension during bacterial regrowth was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. The error bars are the standard deviations from six measurements on independent biofilms. (b) Comparative effect of 125 μl/ml penicillin-G and particles on *S. oralis* biofilms. Penicillin (100 μl) and particle dispersion (100 μl) were added to six wells of an immunological plate. Alternatively, 200 μl of particle dispersion was incubated with the biofilms. The plate was left at room temperature for 1 h and agitated at low speed (300 rpm). The solutions were removed and the plate washed two to three times with PBS. Bacterial regrowth was initiated by adding nutrient broth and the OD of the cell suspension was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. The error bars are the standard deviations from six measurements on independent biofilms.

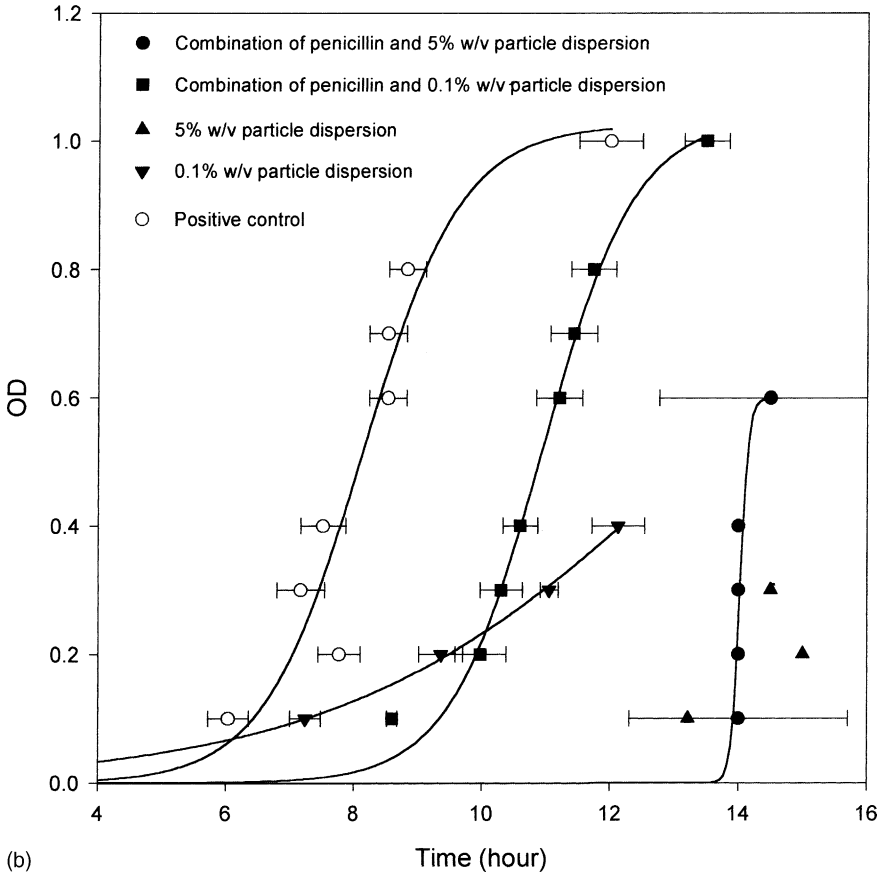

Fig. 4. (*Continued*).

liposomes. When the diluted particle dispersion 0.1% (w/v) was used, the combination of particles and liposomes was more efficient at inhibiting bacterial growth than the particles alone, i.e. the growth curve for particles plus liposomes was shifted to the right relative to the growth curve for particles alone. When liposomes were incubated on the biofilm with 0.1% (w/v) particle dispersions, the regrowth curve was displaced by about 1.5 h (x_0 (control) $= 6.64 \pm 0.03$ h and x_0 (combination) $= 8.10 \pm 0.03$ h). Results for anionic liposomes, presented in Table 2, show similar results. In Table 2, the inhibitory effect of the aqueous part of the zinc citrate particle dispersions (0.1 and 5% (w/v)) is also shown. In order to check the origin of this inhibitory effect, different zinc concentrations were applied during bacterial regrowth (see Table 2). The zinc ion concentration of the aqueous part of a 5% (w/v) zinc citrate dispersion was measured by microanalysis and found to be 24 mM. This concentration explains the inhibitory effect of the 5% (w/v) particle dispersion but not of the dilute 0.1% (w/v) dispersion (no regrowth inhibition occurred with 0.5 mM zinc ions). Consequently, the inhibitory effect at 0.1% (w/v) particle concentration comes from the combination of particles and liposomes. These results confirmed the antibacterial action of DDAB found for other bacterial systems (Campanha et al., 1999). The action of the liposome and particle combination can arise from synergetic effects or may be due to separate actions. In contrast to DDAB-containing liposomes, no antibacterial effects have been reported for PI-containing liposomes. The idea that PI is contributing to the antibacterial action rather than being responsible for it is more realistic. One hypothesis of synergetic action involves

the OH residues of the PI headgroup that can interact with the polysaccharide part of the teichoic acids. This would result in strong H-bonding between the liposomes and the biofilm. The accumulation of negative charges on the biofilm from the liposomes may attract more zinc ions at the bacterial surface and this may result in an increase in the antibacterial effect. Furthermore, binding of liposomes may induce some conformation changes in the biofilm and facilitate the access of the particles and zinc ions on the active site of the biofilm. It is probable that the effects of liposomes and particles are additive rather than synergetic effects.

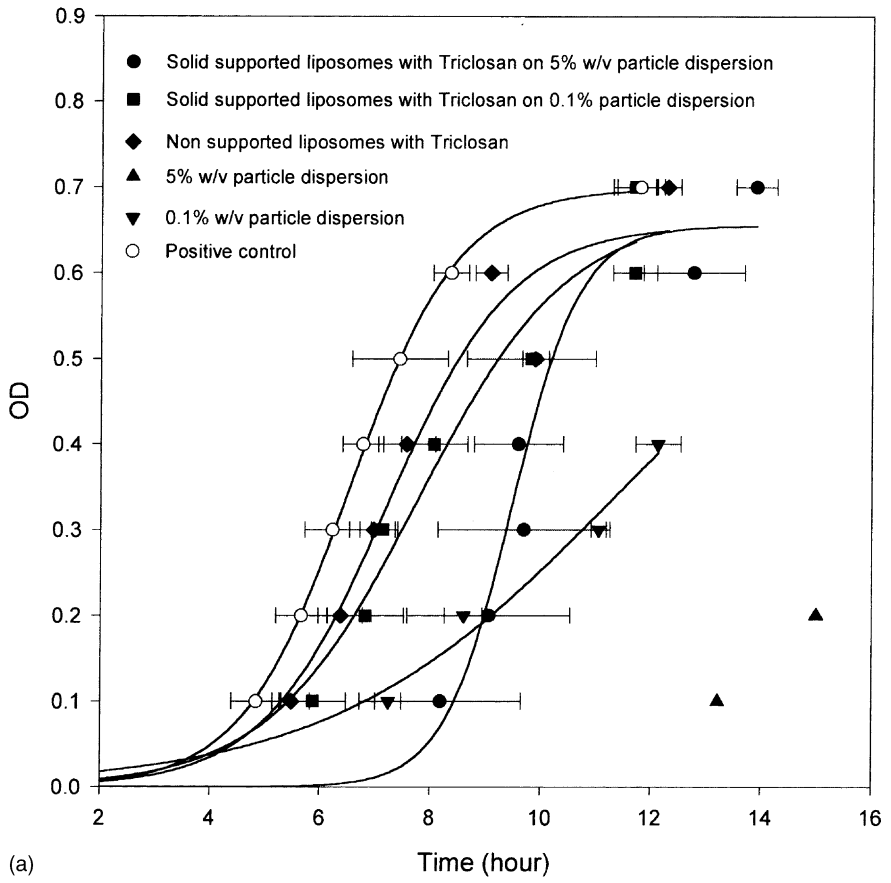

Fig. 5. (a) Effect of solid supported cationic liposomes (SSVs) (9 mol% DDAB/DPPC/cholesterol) encapsulating Triclosan® on *Streptococcus oralis* biofilms. Triclosan® (108.45 μg/ml) was encapsulated in liposomes ([lipid] = 7.31×10^{-9} M) and the liposomes left to adsorb on zinc citrate particles. Two hundred microlitres of SSVs ($[\text{lipid}]_{\text{bound}} = 6.00 \times 10^{-10}$ M on 5% (w/v) particles and 2.7×10^{-10} M on 0.1% (w/v) particles) was incubated with the biofilms for 1 h at room temperature under low speed agitation (300 rpm). The solutions were then removed and the biofilm washed two to three times with PBS. Nutrient broth was finally added and the OD of the suspension was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. For comparison, curves were obtained with particles alone. The error bars are the standard deviations from six measurements on independent biofilms. (b) Effect of solid supported cationic liposomes (SSVs) (9 mol% DDAB/DPPC/cholesterol) encapsulating penicillin-G on *S. oralis* biofilms. Penicillin-G (6 μg/ml) was encapsulated in the aqueous core of the liposomes ([lipid] = 4.44×10^{-9} M) and the liposomes left to adsorb on zinc citrate particles. Two hundred microlitres of SSVs ($[\text{lipid}]_{\text{bound}} = 1.63 \times 10^{-9}$ M on 5% (w/v) particles and 1.98×10^{-9} M on 0.1% (w/v) particles) was incubated with the biofilm for 1 h at room temperature under low speed agitation (300 rpm). The solutions were then removed and the biofilm washed two to three times with PBS. Nutrient broth was finally added and the OD of the suspension was measured using a Dynex plate reader 3000 at a constant temperature of 37 °C. For comparison, curves were obtained with particles alone. The error bars are the standard deviations from six measurements on independent biofilms.

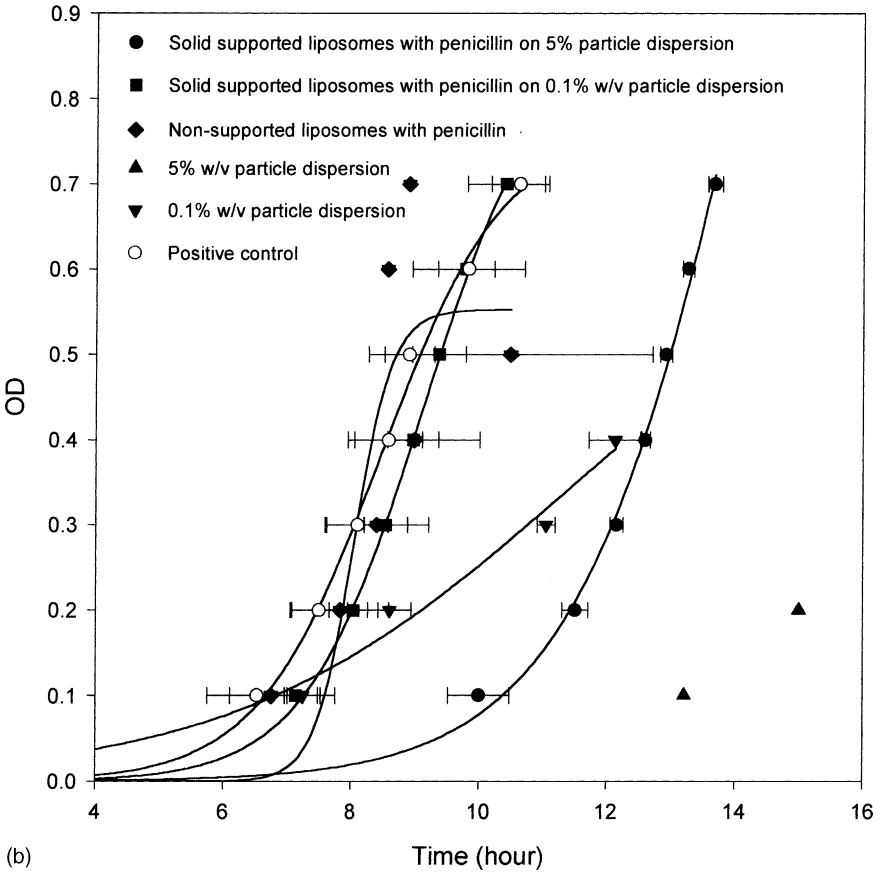

Fig. 5. (*Continued*).

3.4. Antibacterial properties of drug and particle combination

Zinc citrate particles (5 and 0.1% (w/v) particle dispersions) and free Triclosan® were mixed together and applied to *S. oralis* biofilms to investigate the role of drug and particles in combination on bacterial regrowth. Fig. 4a obtained with Triclosan® and particles, shows that the curves are grouped in two distinctive sets. In the first set is the 0.1% (w/v) particle solution used alone or in combination with Triclosan®. Both show some inhibition of the bacterial growth with a 4-h delay between the first set of curves ($x_0 = 11.75 \pm 0.07$ h for Triclosan® and 0.1% (w/v) particle) and the positive control ($x_0 = 8.12 \pm 0.23$). When the particle concentration was increased, the bacterial growth was delayed by about 11 h compared to the positive control.

Free penicillin-G (125 μg/ml) was also mixed with the particles and applied on *S. oralis* biofilm. Curves obtained with penicillin-G show a similar pattern to Fig. 4a with two groups of curves ($x_0 = 10.93 \pm 0.07$ h for pen-G and 0.1% (w/v) particle). The initial pen-G concentration was taken at 125 μg/ml, so a strong inhibitory effect was expected. As seen in Fig. 2b a 10-h delay was observed when the same concentration of free penicillin was used on the biofilm. The free drug was slightly more effective than when it was mixed with 0.1% (w/v) particles indicating interferences between drug and particles. The second group of curves corresponds to results obtained with 5% (w/v) particle dispersion and its combination with benzyl penicillin. Both curves indicate a long bacterial growth delay of about 7 h compared to the positive control. Results obtained with the 5% (w/v) dispersion combination

were probably largely due to the presence of the particles.

Zinc citrate particles have a natural bactericide effect due to the presence of zinc ions. A strong inhibition of growth was expected when the particles were used at high concentration. When the particles are used in combination with a high concentration of antibacterial agent, inhibition is not significantly enhanced over that of the particles alone. The effects of particles and bactericide are not additive under theses conditions. Possibly, the particles have an inhibitory effect on antibacterial agents.

3.5. Effect of adsorbed liposome and drug on *Streptococcus oralis* biofilms and SSV drug delivery

Triclosan® is a lipid-soluble drug and, therefore, it was entrapped in the liposome lipid bilayer while benzyl penicillin is encapsulated in the aqueous core of cationic liposomes. Cationic liposomes entrapping drugs were adsorbed on zinc citrate particles and the systems were applied to *S. oralis* biofilm to investigate the antibacterial effect. Fig. 5a shows results for SSV encapsulating Triclosan® and Fig. 5b for SSV encapsulating pen-G.

A high concentration of Triclosan® (108.45 μg/ml) was entrapped in the lipid bilayer of the liposomes. These liposomes when adsorbed on 0.1% (w/v) particles have a better inhibitory effect ($x_0 = 7.76 \pm 0.42$ h, $R^2 = 0.972$) than the positive control ($x_0 = 6.52 \pm 0.03$ h, $R^2 = 1$) and the free liposomes ($x_0 = 7.20 \pm 0.33$ h). There is a 5-h delay between the regrowth curve obtained for liposomes adsorbed on 5% (w/v) particles and for the particles used alone.

Liposomes encapsulating 6 μg/ml of penicillin (freeze–thraw method) and adsorbed on 0.1% (w/v) particles gave results similar to the positive control and the free liposomes-containing pen-G, all systems showing little inhibition of the bacterial regrowth. Under similar conditions, better results were obtained with Triclosan®. The positive control gives an x_0 value of 8.46 ± 0.12 h ($R^2 = 0.996$) and the SSV on 0.1% (w/v) particle dispersion gave an x_0 value of 9.11 ± 0.07 h ($R^2 = 0.999$). The diluted particles used alone had a lower inhibitory effect compared to the 5% (w/v) particle dispersion and the SSV on 5% (w/v) particle dispersion.

It is interesting to note that the results obtained with particles alone show a stronger antibacterial effect than the systems particles/liposomes/penicillin. This suggests that the presence of liposomes (or lipid) is inhibiting the particle action. The reasons for this inhibition are numerous and could indicate either the liposomes or the drugs or the combination of the two are implicated.

4. Conclusions

4.1. Targeting of liposomes and SSV to *Streptococcus oralis* biofilm

Anionic and cationic liposomes successfully targeted *S. oralis* biofilms. Although some ionic interactions are involved, they do not lead the adsorption process, as targeting of PI and DDAB liposomes was found to be similar. Adsorption of anionic liposomes arises from a balance of hydroxy attractive interactions and repulsive electrostatic effects with a predominance of H-bonding interactions(Jones and Kaszuba, 1994). Cationic liposome adsorption increased with the DDAB content, indicating that attractive electrostatic effects are involved. The targeting of SSVs was found to be dependent on the amount of lipid adsorbed. This confirms that H-bonding between the PI headgroups and the biofilm predominates and that electrostatic interactions are of only secondary importance.

4.2. The antibacterial properties of zinc citrate particulate systems

Triclosan® and benzyl penicillin were successfully encapsulated in the liposome bilayer and aqueous compartment, respectively. Both drugs may diffuse out of the liposomes to reach the bacterial biofilm. Free Triclosan® and penicillin were found to be effective against *S. oralis* when used at a concentration of 125 μg/ml. Zinc citrate particles were found to have some antibacterial properties when used at high concentration (5% (w/v)). This effect probably arises from the high concentration of zinc ions. Free empty liposomes had a small inhibition activity on the bacterial growth. Cationic liposomes containing DDAB have been shown in the past to have an-

tibacterial properties (Campanha et al., 1999), while the enhanced antibacterial properties of PI lipids in combination with zinc citrate particles probably only arise from their attraction for zinc ions. Therapeutic liposomes have shown effects due to the combination of liposomes and antibacterial agents. Each one of the components of SSV (particles, liposomes and drugs,) was investigated alone and in combination with the other components in binary systems. Some combinations have shown synergistic (or additive) antibacterial effects and other regressive effects compared with the systems used alone. Particles and drugs have an inhibitory effect on each other while the particles and free liposomes have an additional or synergetic effect. SSVs did not improve the antibacterial activity of the systems used alone and it was found that the presence of drugs or liposomes could inhibit the bactericidal effects of the particles, although the particles increase the stability of the liposomes.

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