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Antibacterial reactive liposomes encapsulating coupled enzyme systems

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Abstract

Liposomes have been prepared from phospholipid mixtures of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol (PI) encapsulating the enzymes chloroperoxidase (CPO) and lactoperoxidase (LPO) in combination with glucose oxidase (GO) by both extrusion (VET) and/or reverse phase evaporation (REV). The liposomes were characterised in terms of the protein content and activity of the encapsulated enzymes. The antibacterial activity of these reactive liposomes arises from hydrogen peroxide and oxyacids produced in the presence of the substrates glucose and chloride or thiocyanate ions. The liposomes were targeted to biofilms of *Steptococcus gordonii*, an oral bacterium and their antibacterial activity was measured both as a function of liposome-biofilm incubation time and incubation time with the substrates. Bacterial inhibition increases with both liposome-biofilm and substrate-biofilm incubation time and with the extent of enzyme encapsulation. The reactive liposomes also display antibacterial activity in the presence of saliva. The reactive liposomes have potential value in the context of oral hygiene.

Keywords: Liposomes; Enzymes; Chloroperoxidase; Lactoperoxidase; Glucoseoxidase; Bactericide; *Streptococcus gordonii*

1. Introduction

In the fields of cosmetics, toiletries and oral hygiene, the adaptation of natural bactericidal or bacteriostatic systems is a desirable objective. Natural antibacterial systems often depend on the production and subsequent decomposition of hydrogen peroxide by peroxidase enzymes such as lactoperoxidase and myeloperoxidase present in milk, tears and saliva (Klebanoff et al., 1966; Klebanoff, 1968). The antibacterial activity of the lactoperoxide-hydrogen peroxide-thiocyanate system towards oral streptococci has been extensively studied (Thomas et al., 1983, 1991, 1994; Thomas,

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Table 1
Characterisation of liposomes (composition DPPC-PI (17 mol%)) encapsulating coupled enzyme systems

Liposome-enzyme system	Weight ratio	Initial μg protein per μmol of lipid	Final μg per μmol of liquid	$\bar{d}_w$	% Encapsulation
REV (CPO-GO)	0:1	45.12	11.91	212	26.4
REV (CPO-GO)	1:1	45.12	13.15	204	29.2
REV (CPO-GO)	1:1	90.24	18.97	186	21.0
REV (CPO-GO)	1:3	90.24	22.38	227	24.8
REV (CPO-GO)	1:2	135.4	37.26	193	27.5
REV (CPO-GO)	1:3	180.5	42.58	237	23.6
VET (CPO-GO)	1:1	45.12	1.75	98	3.88
VET (CPO-GO)	1:2	67.68	3.03	106	4.48
VET (CPO-GO)	1:3	90.24	3.97	112	4.40
VET (CPO-GO)	1:1	90.24	3.32	111	3.67
VET (CPO-GO)	1:1.5	112.8	3.88	103	3.44
REV (LPO-GO)	1:1	45.12	10.59	243	23.5
REV (LPO-GO)	1:2	67.68	15.89	201	23.5
REV (LPO-GO)	1:3	90.24	22.12	237	24.5

1985). Lactoperoxidase in combination with glucose oxidase has been used as the basis of a commercial preservative system (Myavert C) for cosmetics and toiletries (Guthrie et al., 1992).

A number of bactericides including Triclosan® (Jones et al., 1993, 1994, 1997), vancomycin (Onyeji et al., 1994; Sanderson and Jones, 1996), ciprofloxacin (Nicholov et al., 1993), gentamicin (Nightingale et al., 1993; Sanderson and Jones, 1996) ampicillin (Bakker-Woudenberg et al., 1989) and chlorhexidine (Jones et al., 1997) have been targeted to bacteria using liposomes and the treatment of infections using liposomes has been reviewed by Bergers et al. (1995). The use of liposomes to carry the coupled enzyme system glucose oxidase-horse radish peroxidase (GO/HRP) and which produce bactericidal species in response to a glucose substrate has been described by Hill et al. (1997). Such reactive liposomes were found to be effective in inhibiting the growth of the oral bacterium *Streptococcus gordonii* from immobilised biofilms. The present work extends this approach to other enzyme systems including glucose oxidase-chloroperoxidase GO/CPO which in the presence of glucose and chloride ions results in the production of oxyacids with bactericidal properties according to the following scheme:

$$\text{glucose} \xrightarrow{\text{GO}} H_2O_2 + \text{gluconic acid lactone}$$

$$H_2O_2 \xrightarrow{\text{CPO}} H_2O + ClO^- \rightarrow \text{other oxyacids anions}$$

where the other oxyacids anions include chlorite (ClO_2^-), chlorate (ClO_3^-) and perchorate (ClO_4^-). The bactericidal properties of GO-lactoperoxidase (LPO) towards biofilms of oral bacteria have also been investigated. The liposomes have been prepared by reverse phase evaporation (REVs) (Szoka and Papahadjopoulos, 1978) and extrusion (VETs) (Mayer et al., 1986) from dipalmitoyl or dimyrstoylphosphatidylcholine (DPPC, DMPC) incorporating phosphtidylinositol (PI) which has been found to target the liposome to various oral bacteria (Jones et al., 1993).

2. Materials and methods

2.1. Materials

L-α-dipalmitoylphosphatidylcholine (DPPC, product no. P0763), glucose oxidase (GO, Type VII-S, product no. G7016), horse radish peroxidase (HRP, Type 1, product no. P8125), chloroperoxidase (product no. D9154) were obtained from Sigma, Dorset, UK. Phosphatidyli-

Table 2
Enzymic activities in free solution and encapsulated in VETs (DPPC-PI (17 mol%))

Enzyme assayed	Weight ratio	Initial solution activity (units mg^{-1})	Encapsulated activity (units mg^{-1})	Activity (units mg^{-1}) after Triton X-100 release	% Recovery of activity
GO	CPO:GO 0:1	69.3 ± 2.2	4.85 ± 1.01	71.1 ± 3.2	103
CPO	CPO:GO 1:0	1168 ± 242	80 ± 14.8	1135 ± 332	97
CPO	CPO:GO 1:1	1168 ± 242	76.5 ± 24.0	1147 ± 390	98

nositol (PI) from wheat germ, grade I was from Lipid Products, South Nutfield, UK. [^{3}H]DPPC (specific activity 55 Ci/mmol) was from Amersham International, Amersham, UK. Bacteriological agar No. 1 (code L11), brain heart infusion (BHI, code M 255), yeast extract powder (code L 21) and phosphate-buffered saline (PBS) tablets (code BR 14a) were from Oxoid, Hants, UK. Casein (product no. 44020) was from British Drug Houses (BDH) Dorset, UK. Filters for preparing VETs were from Poretics, Livermore, CA. Chloroform and methanol (Analar grade) were from BDH, they were distilled before use and stored over molecular sieves type 4A from Fisons, Loughborough, UK. All other reagents were made up with double distilled water.

2.2. Methods

2.2.1. Preparation and characterisation of liposomes encapsulation enzymes

REV were prepared by a modification of the method of Szoka and Papahadjopoulos (1978), DPPC (27 mg) plus of PI (6.4 mg) and [^{3}H]DPPC (5 μCi) was dissolved in 3 ml of chloroform–methanol (4:1 by volume) in a 50 ml round-bottomed flask. This liposome composition with 17.1 mol% PI gave optimum targeting to *S. gordonii* (Hill et al., 1997). The organic phase was removed by rotary evaporation at 60°C and the resulting film was dispersed in chloroform–methanol (4:1 by volume) and 3 ml of buffer solution (PBS) containing the desired enzyme (total concentration 1 mg ml^{-1}) previously held at 60°C. The mixture was gently shaken and then sonicated for 5 min using a bath sonicator (Decon FS100). The resulting homogeneous emulsion was rotary evaporated at 60°C until an aqueous suspension formed (≈ 10 min). The suspension was purged with nitrogen for 15 min at 60°C and kept at 60°C for a further 15 min to anneal the REV.

VETs were prepared by dissolving the required lipid mixtures (as for REV) in 3 ml of chloroform–methanol (4:1 by volume) in a 500 ml round bottomed flask. Following rotary evaporation to form a thin lipid film, the film was dispersed in 3 ml of nitrogen saturated PBS containing the required enzymes at 60°C and vigorously agitated to form multilamellar vesicles (MLV). The MLVs were extruded $10\times$ through two stacked polycarbonate 100 nm pore size Poretic filters at a pressure of 200 psi.

To remove unencapsulated enzymes the REV and VETs were fractionated by Sepharose 4B gel filtration. The liposome fractions were assayed for lipid by liquid scintillation counting of [^{3}H]DPPC and for protein by a Lowry microassay (Wang and Smith, 1975). The size distribution of the liposomes was determined by photon correlation spectroscopy (PCS) using a Malvern Autosizer, model no. RR146.

2.2.2. Enzyme activity in reactive liposomes

The activities of GO and CPO were assayed both before and prior to disruption of the liposomes with Triton X-100 and the results compared to the initial activities of the enzymes. The assays were based on the HRP catalysed oxidation of *o*-dianisidine by H_2O_2. The method for the assay of GO using *o*-dianisidine in combination with HRP with glucose as the substrate was a modification of a previously described method (Worthington Enzyme Manual Freehold, 1972) as was the assay of CPO with H_2O_2 as substrate (Klebanoff, 1965). The assay procedures for GO and CPO were as follows.

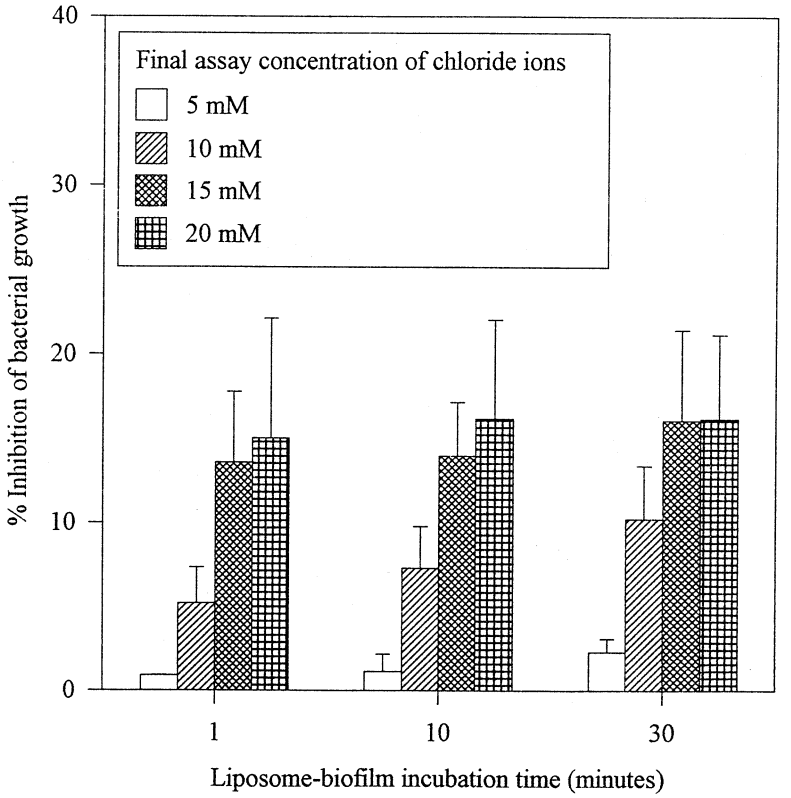

Fig. 1. Antibacterial activity to *S. gordonii* biofilms of VETs encapsulation CPO + GO as a function of the liposome-biofilm incubation time and the exposure of the liposome-biofilm to 1 M glucose plus chloride ion concentration (5–20 mM). The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 3.03 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w = 106$ nm. Error bars (standard deviations) for $n = 3$.

For GO, 12 ml of phosphate buffer (0.1 M pH 6) was mixed with 100 μl *o*-dianisidine (1% w/v) to give a stock dye solution. Aliquots (2.5 ml) of dye solution, 300 μl glucose solution (18% w/v), 100 μl of liposome sample were mixed and the absorbance was measured at 460 nm for 2–4 min against a blank containing no liposomes. The activity of the GO was calculated from the relation

$$\text{GO activity (units/mg)} = \frac{\Delta A_{460\,nm}/\text{min}}{11.3(\text{mg GO/ml})} \quad (1)$$

where the extinction coefficient of oxidised *o*-dianisidine is 11.3×10^3 M^{-1} cm^{-1}. For assays in the presence of Triton X-100 the liposomes were disrupted by the addition of 100 μl of 1 mM Triton X-100, the dye solution volume being reduced to 2.4 ml.

For CPO the assay mixture contained 300 μl phosphate buffer (0.1 M, pH 6.0), 300 μl of H_2O_2 (0.01 M), 50 μl *o*-dianisidine (0.02 M in methanol, freshly prepared) 2.25 ml of water and 50 μl of liposome sample. The absorbance was followed as for GO using a blank containing no liposomes. For assays in the presence of Triton X-100 the liposomes were disrupted by addition of 50 μl of 1 mM Triton X-100. The CPO activity was calculated from the equation

$$\text{CPO activity (units/mg)} = \frac{\Delta A_{460\,nm}/\text{min}}{11.3(\text{mg CPO/ml})} \quad (2)$$

For the assay of CPO co-encapsulated with GO, the total protein content of the liposomes was assayed by the Lowry microassay (Wang and Smith, 1975) and it was assumed that there was no change in the mass ratio of the enzymes from

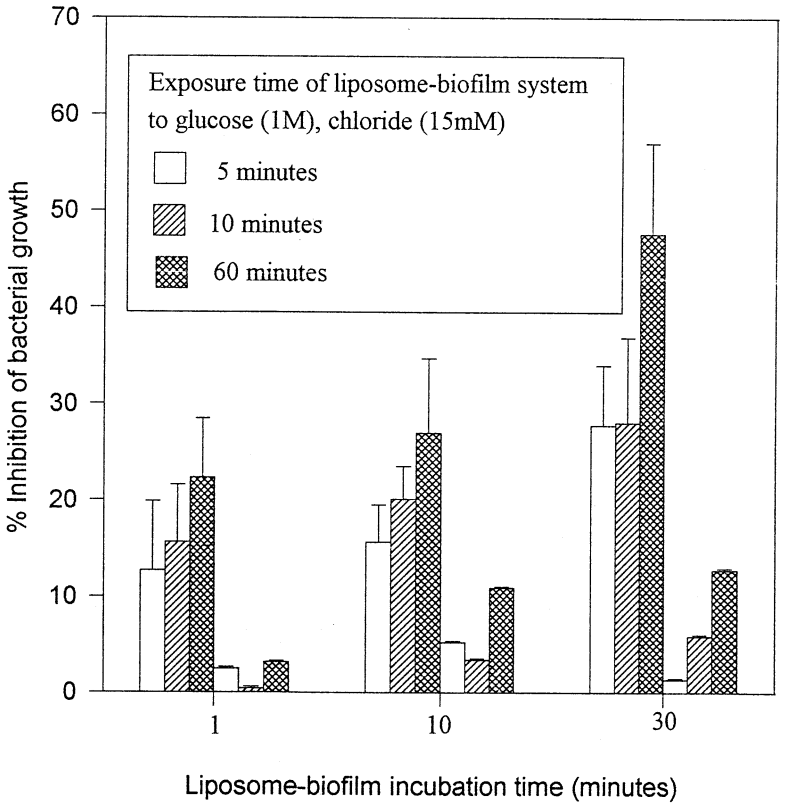

Fig. 2. Antibacterial activity to *S. gordonii* biofilms of VETs encapsulating CPO + GO (mass ratio 1:1) as a function of the liposome-biofilm incubation time and incubation time in the presence of glucose (1 M) plus 15 mM chloride ion. The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 3.97 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w = 112$ nm. The three bars to the right of each group are data for enzyme-free VETs, liposomal lipid concentration 0.3 mM, VET weight average diameter $\bar{d}_w = 102$ nm. Error bars (standard deviations) for $n = 3$.

that in the initial mixture in order to determine their activities from Eqs. (1) and (2).

2.3. Growth of bacteria

Brain heart infusion (BHI, 3.7 g) was mixed in 100 ml of water and 1.5 g of bacteriological agar added. The mixture was boiled to dissolve the agar and sterilised by autoclaving (15 lb pressure, 15 min). Defibrinated horse blood (5% v/v) was added and the resulting mixture used to charge sterile petri dishes which were then incubated at 37°C for 15 min. The plates were inoculated with *S. gordonii* and incubated for 18 h at 37°C in a candle jar. The bacterial colonies were used to inoculate sterile growth medium containing 3.7 g BHI, 0.3 g yeast extract powder, 2 g sucrose in 100 ml of water divided between ten screw-top jars. The jars were placed in a candle jar and incubated at 37°C for 18 h. The cell suspensions were centrifuged (2000 rpm for 5 min, MSE super minor with swing-out rotor) to pellet the cells and the supernatant discarded. The cells were resuspended in PBS at 4°C and re-pelleted and the process repeated 3 × and the cells finally diluted to give an absorbance of 0.5 at 550 nm.

2.4. Antibacterial activity of reactive vesicles

S. gordonii biofilms were prepared as described in Section 2.3. Liposomes were added to the wells (200 μl) at 37°C for the desired time period (1–60 min). Following incubation, the plate wells were emptied of liposome suspensions and washed 2 ×

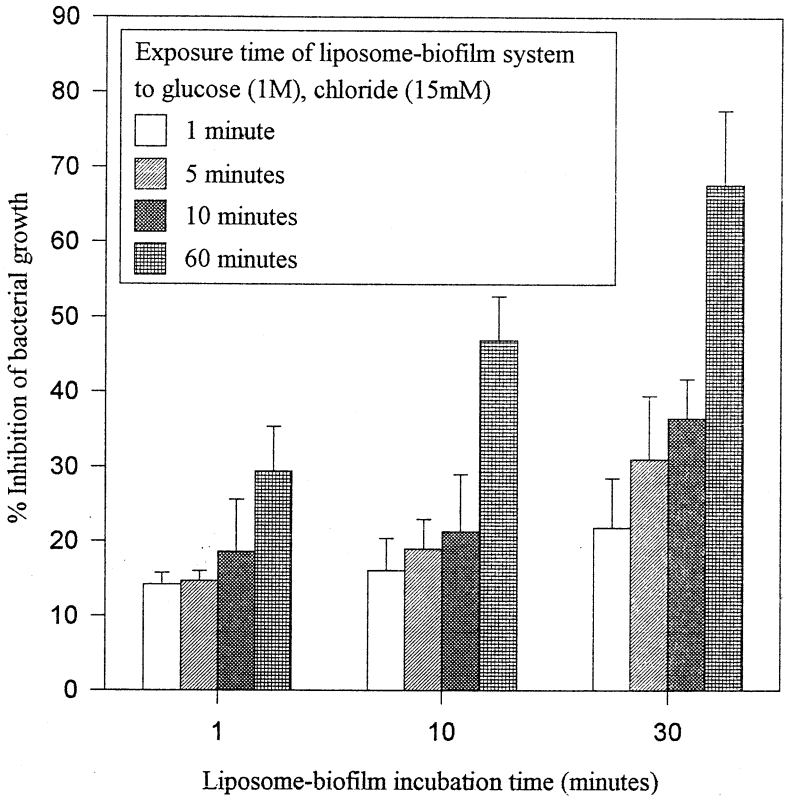

Fig. 3. Antibacterial activity to *S. gordonii* biofilms of REVs encapsulating CPO + GO (mass ratio 1:1) as a function of liposome-biofilm incubation time and the time of incubation with glucose (1 M) and chloride ions (15 mM). The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 18.97 μg (μmol lipid)$^{-1}$, REV weight average diameter $\bar{d}_w = 186$ nm. Error bars (standard deviations) for $n = 3$.

with sterile PBS and blotted dry. Glucose, 200 μl (18% w/v) was added followed by 50 μl of sodium chloride or thiocyanate solution at the required concentration to give a final concentration in the range 1–20 mM. The exposure time to glucose and chloride was varied from 1 to 60 min. Addition of 1 M glucose shrinks the liposomes by approximately 20% but we have no evidence that they do not remain intact in the presence of 1 M glucose. After the required incubation time the plate was washed 2 × with sterile PBS and blotted dry. Sterile nutrient broth (200 μl) was added to each well and the plate incubated for 18 h at 37°C in a candle jar. The absorbance of each well was measured at 630 nm using a Dynatech MR 610 plate reader coupled to an Apple IIe microcomputer. The increase in absorbance over the 18 h period was taken as a measure of bacterial growth. Controls used were bacteria-free wells with PBS or liposomes exposed to glucose and iodide which were taken as the background level. Controls with enzyme-free liposomes were also carried out (Fig. 2). These showed that some inhibition of growth could arise with empty liposomes which was significant particularly for short liposome-biofilm incubation times (1 min). When sterilised human saliva was used instead of iodide this was pooled saliva from ten donors, stored on ice and clarified by centrifugation (10 000 × g for 5 min). It was sterilised by heat treatment and diluted to 10% using PBS.

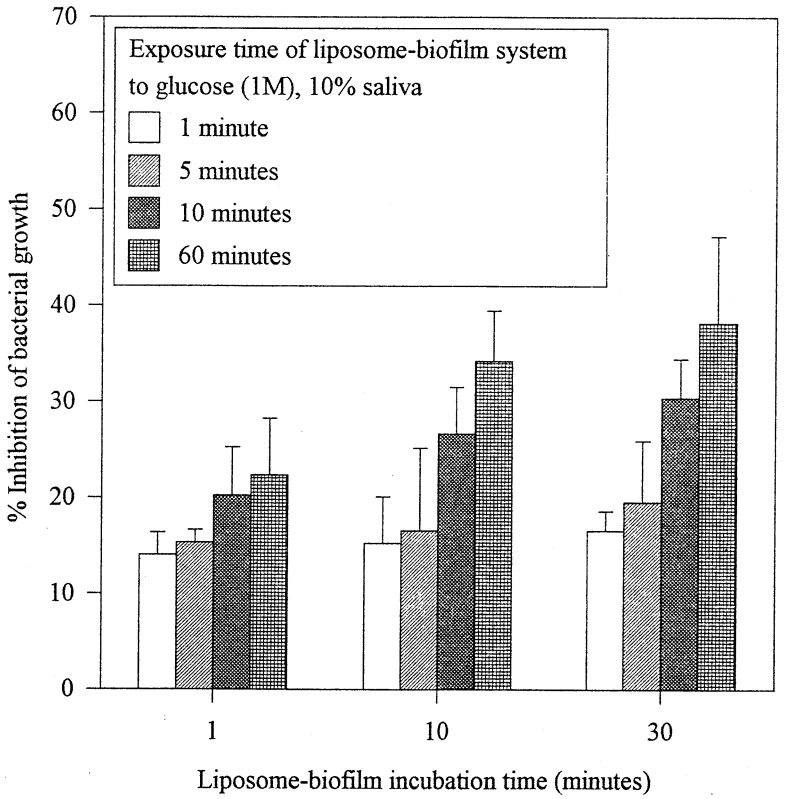

Fig. 4. Antibacterial activity to *S. gordonii* biofilms of VETs encapsulating CPO + GO (mass ratio 1:1) as a function of liposome-biofilm incubation time and time of incubation with glucose (1 M) and saliva (10%). The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 3.87 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w = 103$ nm. Error bars (standard deviations) for $n = 3$.

3. Results

3.1. Characterisation of DPPC-PI liposomes encapsulating enzymes

Table 1 shows the size and extent of enzyme encapsulation for the liposomes encapsulating GO, CPO-GO and LPO-GO. For the REVs, the average encapsulation of GO was 26.4% of the initial protein, for CPO-GO mixtures in REVs $25.2 \pm 3.2\%$, for LPO-GO mixtures in VETs $23.8 \pm 3.2\%$ and for CPO-GO mixtures in VETs $3.97 \pm 0.45\%$. The figures for REVs suggest that the extent of encapsulation is the same within experimental error for GO and GO plus CPO or LPO suggesting that the enzymes are being encapsulated independently, the amount of encapsulation being determined by the liposome internal volume. The amount of protein encapsulated increases with protein to lipid ratio, however, the percentage encapsulation is independent of the initial protein to lipid concentration ratio in the approximate range 45–180 μg protein per μmol of lipid. These observations suggest that while the internal volumes of the liposomes are not saturated with protein there are limits to the percentage encapsulation attainable under the conditions of preparation. The ratio of the internal volumes of the REVs to the VETs is on average $(209/102)^3 = 8.5$ so from the average percentage encapsulation of the VETs (3.97%) the REVs would be expected to encapsulate 34% of the protein which compares with the observed values of, on average $25 \pm 3\%$. This discrepancy may arise from some

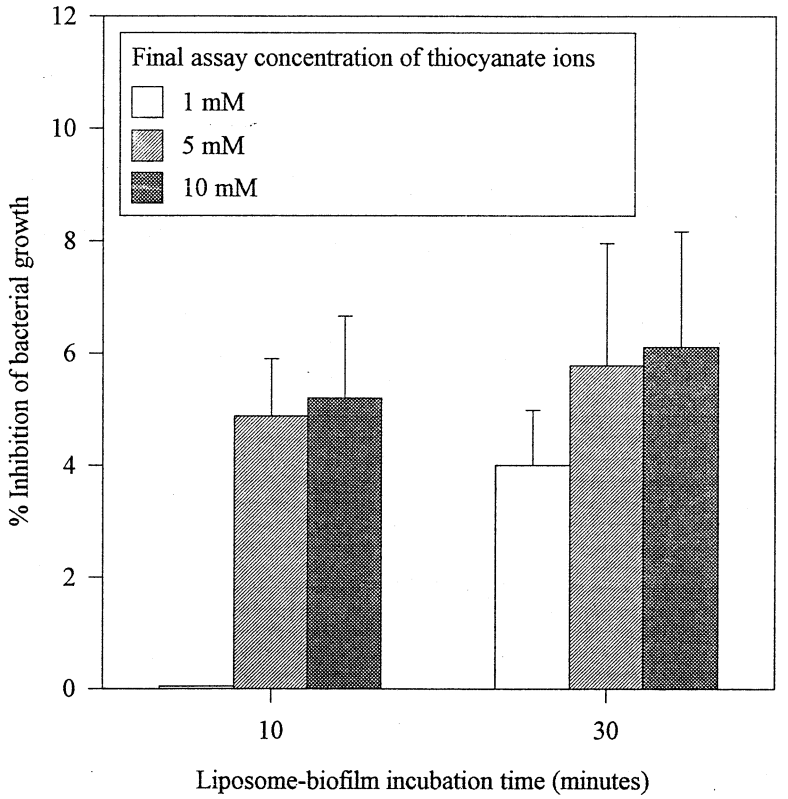

Fig. 5. Antibacterial activity to *S. gordonii* biofilms of VETs encapsulating CPO + GO (mass ratio 1:1) as a function of liposome-biofilm incubation time and time of incubation with glucose (1 M) plus thiocyanate ions (1–10 mM). The liposomal lipid concentration was 0.3 mM encapsulated protein concentration 3.31 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w = 111$ nm. Error bars (standard deviations) for $n = 3$.

multilamellarity of the REVs which would lead to a smaller internal diameter and volume and hence a smaller extent of encapsulation.

The enzymic activities of the enzymes after encapsulation relative to their initial values were assessed and shown in Table 2. For GO and CPO encapsulated independently and co-encapsulated, after treating the liposomes with Triton X-100 the recovery of activity was high. The results demonstrate that there was no major loss in enzymic activity during encapsulation.

3.2. Antibacterial activity of liposomes encapsulating enzymes

The antibacterial activity of reactive VETs encapsulating CPO plus GO was investigated as a function of liposome-biofilm incubation time and as a function of chloride ion concentration plus glucose substrate for a constant incubation time of 10 min. The data are shown in Fig. 1 and demonstrate that the antibacterial activity increases with chloride ion concentration up to 15–20 mM. An incubation time with glucose and chloride of 10 min results in approximately 15% growth inhibition even when the liposome-biofilm incubation time is short (1 min). Increasing the substrate incubation time at optimum chloride concentration to 60 min results in a significant increase in growth inhibition approaching 50% as shown in Fig. 2. Also shown in Fig. 2 are results for empty vesicles, some growth inhibition is observed for empty liposomes but this is significantly smaller than the effects of liposomes encapsula-

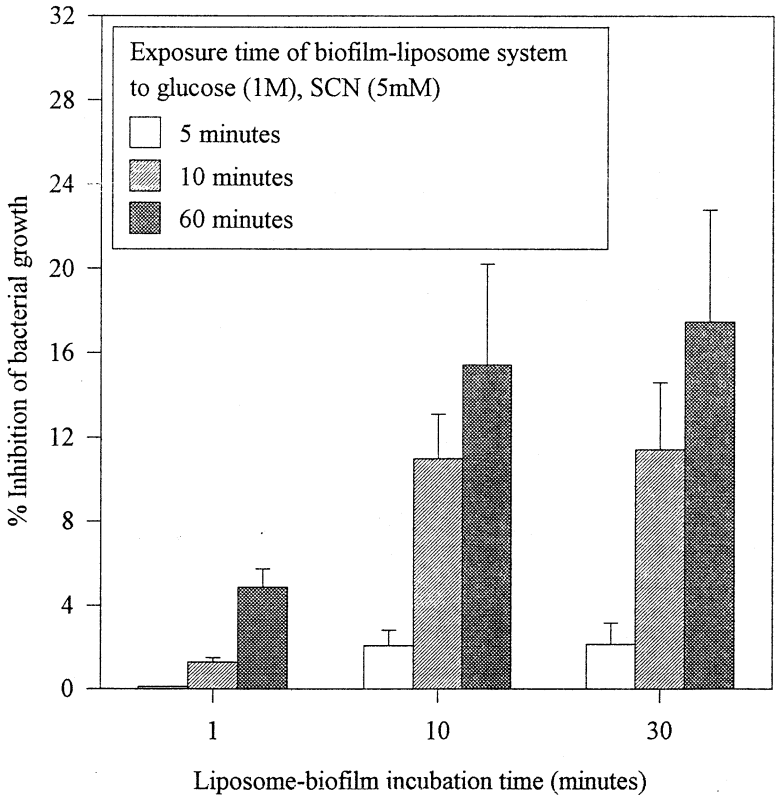

Fig. 6. Antibacterial activity to *S. gordonii* biofilms of REV encapsulating LPO + GO (mass ratio 1:1) as a function of liposome-biofilm incubation time and time of incubation with glucose (1 M) and thiocyanate ion (5 mM). The liposomal lipid concentration was 0.3 mM encapsulated protein concentration 22.12 μg (μmol lipid)$^{-1}$, REV weight average diameter 237 nm. Error bars (standard deviations) for $n = 3$.

tion, the enzyme system. Fig. 3 shows data for growth inhibition by REV. In general, growth inhibition is greater using REV due to the larger amounts of enzymes immobilised by the liposomes on the biofilm.

The effects of saliva on the functioning of the reactive liposomes are shown in Fig. 4. A comparison of the data with those in Fig. 2 shows that 10% saliva give on average a 7% increase in growth inhibition compared to 15 mM chloride ions which is not statistically significant, so that there is no significant inhibition of antibacterial activity brought about by the presence of saliva.

Thiocyanate is a pseudo halide and can take the place of chloride in some peroxidase systems (Thomas et al., 1983). Fig. 5 shows the effects of thiocyanate ions on the antibacterial effects of VETs encapsulating CPO plus GO. Little inhibition was observed when chloride was replaced by thiocyanate. Thiocyanate is more effective as a substrate with glucose for reactive VETs encapsulating LPO plus GO (Fig. 6).

4. Discussion

The results demonstrate that liposomes encapsulating CPO and GO have antibacterial activity towards *S. gordonii* in the presence of glucose and chloride substrates. The liposomes have been targeted to the biofilm by incorporation of PI which at 17.1 mol% in DPPC was found to be optimal for adsorption to *S. gordonii* biofilms (Hill et al., 1997). The antibacterial activity increases with both the time of incubation of the liposomes with the biofilm and with the time of incubation with substrate.

Table 3
Comparison of antibacterial activity of reactive liposomes to *S. gordonii*

Enzyme	Liposome type	Substrate	Protein concentration $\mu g/\mu$ mole lipid	% Inhibition[a]	% Inhibition[b]
GO	REV	1 M glucose	26.5	7 ± 3	39.6 ± 2.7
CPO+GO	REV	1 M glucose+15 mM Cl^-	19.0	29.4 ± 6.0	67.6 ± 10.0
CPO+GO	VET	1 M glucose+15 mM Cl^-	4.0	22.4 ± 6.1	47.6 ± 9.4
CPO+GO[d]	VET	1 M glucose+5 mM SCN^-	3.3	5 ± 1	6 ± 2
CPO+GO	VET	1 M glucose+10% saliva	3.9	22.4 ± 6.0	38.2 ± 9.0
HRP+GO[c]	REV	1 M glucose+5 mM I^-	23.8	34.2 ± 6.3	39.6 ± 6.1
HRP+GO[c]	VET	1 M glucose+5 mM I^-	3.6	3.6 ± 3.5	34.0 ± 4.5
HRP+GO[c]	VET	1 M glucose+10 mM I^-	3.6	8 ± 2.5	19.5 ± 6.8
HRP+GO[c]	VET	1 M glucose+10% saliva	4.3	6 ± 4	31.7 ± 4.1
LPO+GO	REV	1 M glucose+5 mM SCN^-	22.1	5 ± 1	17.5 ± 5.3

[a] Liposome-biofilm incubation time is 1 min except where indicated and 30 min incubation with substrate.
[b] Liposome-biofilm incubation time is 60 min except where indicated and 60 min incubation with substrate.
[c] Data from Hill et al. (1997).
[d]Liposome-biofilm is 10 min incubation time and 10 min incubation with substrate.

To be of practical value for inhibition of bacterial growth in the oral cavity it is desirable that the system should function after a minimal exposure time of liposomes to biofilm of the order of 1 min and continue to produce bacterial species (hydrogen peroxide and choline oxyacids) over a period of at least 30–60 min. The requirement for such a brief exposure to the oral cavity is a severe test of the system. In Table 3, data has been compared for a range of coupled enzyme systems as described in this work and also for the HRP plus GO system previously described (Hill et al., 1997). Comparison of the data for a 1 min liposome-biofilm incubation time shows that the most effective antibacterial systems are REV encapsulation CPO + GO in the presence of 15 mM chloride ion or 10% saliva and REV encapsulating HRP + GO in the presence of 5 mM iodide ion. The chloride ion concentration in saliva is approximately 17 mM (Kostlin and Rauch, 1957) and hence sufficient to give maximum CPO activity (Fig. 1). The iodide concentration in saliva is approximately 1 μM (Diem and Lentner, 1970) which is lower than required to give optimum activity for the HRP + GO system which requires approximately 5 mM iodide ion. The data in Table 3 show that antibacterial activity is increased for all the systems when the liposome-biofilm incubation time is extended to 30 min and that under these conditions the CPO + GO enzyme couple in REV gives the greatest growth inhibition approaching 70%. It should, however, be stressed that in the case of a commercial product which would be used repetitively, a system that displays a relatively low antibacterial activity after a single application could be very effective in repetitive use. For example, six applications of a system displaying only 10% inhibition would lead to an overall inhibition of 48% assuming bacteriostatic conditions between applications. In conclusion, the preparation of natural antibacterial enzyme systems encapsultated in targeted liposomes has been described and they have been demonstrated to be effective in inhibiting bacterial

growth from biofilms under conditions which simulate their application in the oral cavity.

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