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Reactive liposomes encapsulating a glucose oxidase-peroxidase system with antibacterial activity

Kate J. Hill [a], Michael Kaszuba [a], Jonathan E. Creeth [b], Malcolm N. Jones [a,*]

[a] *School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK*
[b] *Unilever Research, Port Sunlight Laboratory, Bebington, Wirral, Merseyside L63 3JW, UK*

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Abstract

Liposomes were prepared from phospholipid mixtures of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol (PI), encapsulating the enzymes glucose oxidase (GO) and GO in combination with horse radish peroxidase (HRP) by both extrusion (VET) and reverse-phase evaporation (REV). The optimum level of PI in DPPC/PI liposomes for targeting to biofilms of the oral bacterium *Streptococcus gordonii* has been established. The liposomes were characterised in terms of the content and activity of the encapsulated enzymes. The antibacterial activity of these 'reactive' liposomes arising from hydrogen peroxide and oxyacids in the presence of the substrates glucose and iodide ions, after targeting to the biofilms, were 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: Liposome; Glucose oxidase; Horse radish peroxidase; *Streptococcus gordonii* antibacterial activity

1. Introduction

It has been demonstrated that phospholipid liposomes can be targeted to bacteria either by incorporation of particular lipids such as phosphatidylinositol (PI) [1–4] and stearylamine [5–7] or by surface-bound lectins [1,4,8] or antibodies [9]. There have been a number of examples of the delivery of bactericides to bacteria by liposomes, specifically Triclosan® [1,10], vancomycin [11], ciprofloxacin [12], gentamicin [13] and ampicillin [14] and the use of liposomes as delivery systems in the treatment of infections has been reviewed by Bergers et al. [15].

In the field of cosmetics, toiletries and oral hygiene the exploitation of 'natural' bactericidal or bacteriostatic systems is a desirable aim. Natural antibacterial systems arising from hydrogen peroxide decomposition by peroxidase enzymes such as lactoperoxidase and myeloperoxidase are present in milk, tears and saliva [16,17]. The antibacterial activity of the lactoperoxidase-hydrogen peroxidase-thiocyanate system has been studied extensively by Thomas et al. (see, e.g., Refs. [18,19]) and shown to be effective against oral streptococci [20,21] and in combination with glucose oxidase a commercial preservative system (Myavert C) for cosmetics and toiletries has been developed [22].

In this work we report an investigation to exploit

* Corresponding author. Fax: +44 161 2755082.

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targeted liposomes encapsulating the coupled enzyme system glucose oxidase (GO)–horse radish peroxidase (HRP) as an antibacterial system against the oral bacterium *Streptococcus gordonii* (formerly *S. sanguis* CR2b [23]), using glucose as the primary substrate together with iodide. The combination of hydrogen peroxide (H_2O_2) and iodide (I^-) results in the production of oxyacids (hypoiodous (HIO), iodic (HIO_3) and periodic (HIO_4)) which have antibacterial properties. The reaction scheme may be summarised as follows:

$$\text{glucose} \xrightarrow{\text{GO}} H_2O_2 + \text{gluconic acid lactone} \quad (1)$$

$$H_2O_2 \xrightarrow[I^-]{\text{HRP}} H_2O + IO^- \rightarrow \text{other oxyacids}$$

The reactive liposomes were prepared from mixtures of DPPC and PI, by both reverse phase evaporation (REV [24]) and extrusion (VETs [25]), encapsulating GO alone and in combination with HRP and were targeted to biofilms of the bacterium on microtitre plates.

2. Materials and methods

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) and o-dianisidine tablets (product no. D9154) were obtained from Sigma, Poole, Dorset, UK. Phosphatidylinositol (PI) from wheat germ, grade I was from Lipid Products, South Nutfield, UK. [^{3}H]DPPC (spec. act. 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 Ltd, Basingstoke, Hants, UK. Casein (product no. 44020) was from British Drug Houses (BDH) Poole, Dorset, UK. Filters for preparing VETs were from Poretics, Livermore CA, USA. Chloroform and methanol (Analar grade) were from BDH, they were distilled before use and stored over molecular sieves type 4A from Fisons Plc, Loughborough, UK. All other reagents were made up with double-distilled water.

2.1. Preparation and characterisation of liposomes encapsulation enzymes

REV were prepared by a modification of the method of Szoka and Papahadjopoulos [24], DPPC (27 mg) plus the desired amount of PI (1–10 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. 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 mixture (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 (approx. 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 [26]. The size distribution of the liposomes was determined by photon correlation spectroscopy (PCS) using a Malvern Autosizer, model no. RR146.

2.2. Enzyme activity in reactive liposomes

The activities of GO, GO co-encapsulated with HRP and HRP co-encapsulated with GO were assayed both before and prior to disruption of the liposomes with Triton X-100 and the results com-

pared with 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 [27] as was the assay of HRP [28] with H_2O_2 as substrate. GO co-encapsulated with HRP was assayed using glucose and o-dianisidine without additional HRP. HRP alone and in the presence of GO was assayed with o-dianisidine plus H_2O_2 in the absence of glucose. The assay procedures for GO and HRP were as follows.

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 HRP (60 units ml^{-1}) and 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

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

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 HRP 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 HRP activity was calculated from the equation

$$\text{HRP activity (units/mg)} = \frac{\Delta A_{460\,nm}/\text{min}}{11.3 \times (\text{mg HRP/ml})} \quad (3)$$

For the assay of GO coencapsulated with HRP and HRP co-encapsulated with GO the total protein content of the liposomes was assayed by the Lowry microassay [25] and it was assumed that there was no change in the mass ratio of the enzymes from that in the initial mixture in order to determine their activities from Eq. (2) and Eq. (3).

2.3. Growth of bacteria

Brain heart infusion (BH1, 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 sterilized by autoclaving (15 lbs 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 10 screwtops 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. Targeting of liposomes to biofilms

Aliquots (200 μl) of *S. gordonii* suspension (absorbance 0.5 at 550 nm) were incubated in the wells of microtitre plates (Dynatech M1298) for 18 h at room temperature. After incubation the bacterial suspension was removed and the remaining immobilised biofilm was washed twice with sterile PBS and blotted dry by inversion. Potential vacant binding sites were then blocked with 300 μl of 0.02% w/v casein in PBS for 30 min at room temperature followed by 2× 300 μl PBS washes. Aliquots (200 μl) of liposomes were incubated with the biofilms at 37°C for 2 h, after which the liposome suspensions were removed and the wells washed 3× with 300 μl PBS and blotted dry. The biofilm was dispersed by addition of 220 μl sodium *n*-dodecylsulphate (1% w/v) followed by a brief sonication and incubated for 30 min at room temperature. Aliquots (200 μl) were

taken for scintillation counting. Control wells containing only bacteria, only PBS and only liposomes suspensions were used to monitor background levels of activity. The results of the targeting assay are expressed in terms of percentage apparent monolayer coverage (% amc) given by

$$\% \text{ amc} = \frac{N_{\text{obs}}}{L_{\text{a}}} \times 100 \quad (4)$$

where N_{obs} is the observed number of mol of lipid adsorbed to the biofilm and L_{a} is the number of mol of lipid which would be absorbed if the film was covered with a close-packed layer of liposomes. L_{a} was calculated from the equation.

$$L_{\text{a}} = \frac{A_{\text{bf}}}{\pi\left(\bar{d}_{\text{w}}/2\right)^2} \bar{N}_{\text{w}} \quad (5)$$

where $\bar{d}_{\text{w}}$ is the weight average diameter of the liposomes having a weight average number of lipid molecules per liposome of $\bar{N}_{\text{w}}$ and A_{bf} is the geometric area of the biofilm. For unilamellar liposomes $\bar{N}_{\text{w}}$ can be calculated from $\bar{d}_{\text{w}}$ assuming an area per lipid molecule in the liposomal bilayer (taken as 50 nm^2) and a bilayer thickness (taken as 7.5 nm) as previously described [29]. The area of the biofilm was taken as 2.202×10^{-4} m^2 which was measured in a previous study for the surface of microtitre plate wells exposed to 200 μl of solution [30].

2.5. Antibacterial activity of reactive vesicles

S. gordonii biofilms were prepared as described in Section 2.4. Liposomes were added to the wells (200 μl) at 37°C for the desired time period (1 to 60 min). Following incubation, the plate wells were emptied of liposomes suspension and washed $2\times$ with sterile PBS and blotted dry. Glucose, 200 μl (18% w/v) was added followed by 50 μl of sodium iodide solution at the required concentration to give a final concentration in the range 1–10 mM. The exposure time to glucose and iodide was varied from 1 to 30 min. Addition of 1 M glucose shrinks the liposomes by approx. 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 $2\times$ 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-incubated with PBS followed by broth which gave maximal bacterial growth, 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 (see Fig. 6). 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 sterilized human saliva was used instead of iodide this was pooled saliva from 10 donors, stored on ice and clarified by centrifugation ($10\,000 \times g$ for 5 min). It was sterilized by heat treatment and diluted to 10% using PBS.

3. Results

3.1. Targeting of DPPC-PI liposomes to S. gordonii biofilms

To establish the optimum level of PI in DPPC-PI liposomes for targeting to the biofilms, a range of REVs and VETs were prepared in which the PI content of the bilayer was varied from approx. 3 to 20 mol%. Figs. 1 and 2 show the apparent monolayer coverage for REVs and VETs respectively. For both types of liposome, targeting was optimal at approx. 17 mol% PI although for the VETs the optimum was not as sharply defined as for the REVs. The optimum composition for targeting was used in all subsequent studies.

3.2. Characterisation of DPPC-PI liposomes encapsulating enzymes

Table 1 shows the size and extents of enzyme encapsulation for the liposomes. For the REVs the average encapsulation of GO was $24.3 \pm 2.5\%$ of the initial protein, for GO/HRP mixtures in REVs $28.6 \pm 4.1\%$ and for GO/HRP mixtures in VETs $4.06 \pm 0.62\%$. The figures for REVs suggest that the extent of encapsulation is the same within experimental

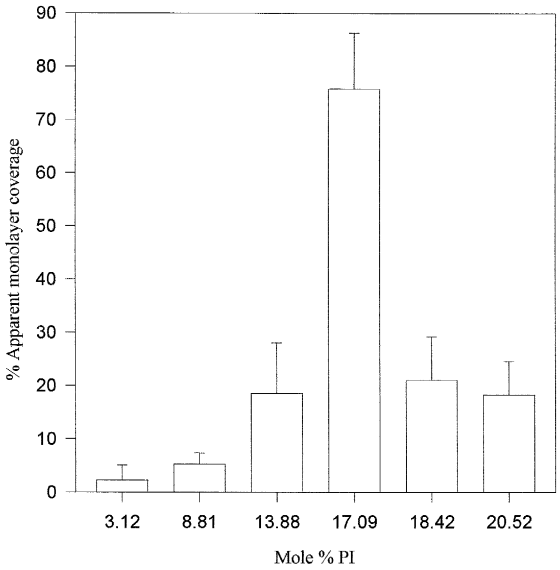

Fig. 1. Dependence of adsorption of DPPC/PI REV liposomes to *Streptococcus gordonii* biofilms on mol% PI content. The liposomes in PBS were incubated with the biofilm for 2 h. The liposomal lipid concentration was 0.3 mM and the average weight average diameter ($\bar{d}_w$) was 186 nm.

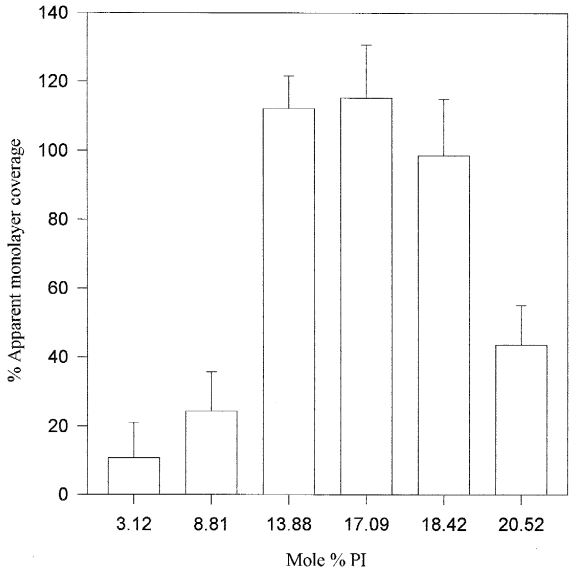

Fig. 2. Dependence of adsorption of DPPC/PI VETs to *Streptococcus gordonii* biofilms on mol% PI content. The liposomes in PBS were incubated with the biofilm for 2 h. The liposomal lipid concentration was 0.3 mM and the average weight average diameter ($\bar{d}_w$) was 112 nm.

error for GO and GO + HRP, 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 % encapsulation is independent of the initial protein to lipid concentration ratio in the approximate range 45 to 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 % encapsulation attainable under the conditions of preparation. The ratio of

Table 1
Characterisation of liposomes (composition DPPC-PI (17 mol %)) encapsulating enzymes

Liposome type	Enzyme wt ratio GO:HRP	Initial μg protein per μmol of lipid	Final μg protein per μmol of lipid	$\bar{d}_w$ (nm)	% Encapsulation
REV	1:0	45.12	11.91	212	26.4
REV	1:0	67.68	13.98	218	20.7
REV	1:0	90.24	22.97	210	25.5
REV	1:0	135.4	33.49	178	24.7
REV	1:1	45.12	11.21	215	24.9
REV	1:1	90.2	23.82	208	26.4
REV	1:0.7	112.8	40.00	224	35.5
REV	1:1	135.4	39.19	218	28.9
REV	1:1	180.5	48.81	199	27.1
VET	1:1	45.12	2.19	97	4.84
VET	1:0.5	67.68	3.01	103	4.45
VET	1:1	90.24	3.61	98	4.00
VET	1:1.5	112.8	3.63	108	3.22
VET	1:0.7	112.8	4.30	106	3.81

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

Enzyme wt ratio GO:HRP	Enzyme assayed	Initial solution activity (units mg^{-1})	Encapsulated activity (units mg^{-1}) prior to Triton X-100 release	Activity (units mg^{-1}) after Triton X-100 release	% recovery of activity
1:0	GO	69.3 ± 2.2	4.85 ± 1.01	71.1 ± 3.2	103
0:1	HRP	246.1 ± 11.4	43.4 ± 5.21	191.7 ± 12.4	77.8
1:1	GO	69.3 ± 2.2	9.12 ± 0.23	52.8 ± 9.4	76.2
1:1	HRP	246.1 ± 11.4	61.8 ± 29.7	271.6 ± 76.7	110

the internal volumes of the REVs to the VETs is on average $(209/102)^3 = 8.6$, so from the average % encapsulation of the VETs (4.06%) the REVs would be expected to encapsulate 35% of the protein which compares with the observed values of, on average 27 ± 4%. This disprepancy may arise from some 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 HRP encapsulated independently after treating the liposomes with Triton X-100, the recovery of activity was 103 and 78% respectively. Recovery was also high after coencapsulation of the enzymes. The low figures for the activity of the enzymes before Triton treatments possibly arose from the disruption of a small proportion of the liposomes during the assay. The results demonstrate that there was no major loss in enzymic activity during encapsulation.

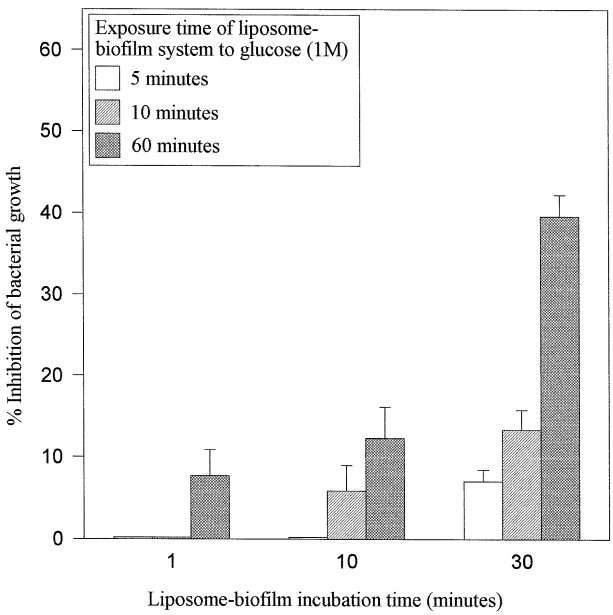

Fig. 3. Antibacterial activity to *Streptococcus gordonii* biofilms of REVs encapsulation GO as a function of the liposome-biofilm incubation time and the exposure time of the liposome-biofilm to 1 M glucose. The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 26.47 μg (μmole lipid)$^{-1}$, REV weight average diameter $\bar{d}_w = 215$ nm.

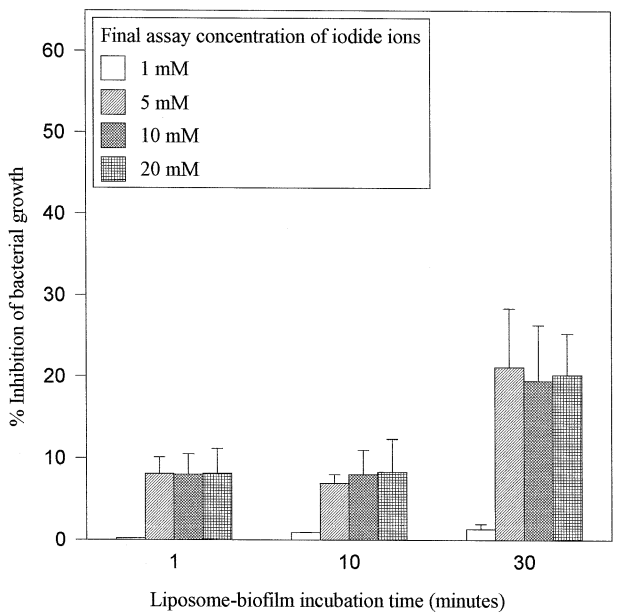

Fig. 4. Antibacterial activity to *Streptococcus gordonii* biofilms of VETs encapsulating GO/HRP (mass ratio 1:1) as a function of the liposome-biofilm incubation time and the concentration of iodide ions in the presence of glucose (1 M). The liposome-biofilm system was exposed to glucose and iodide for 10 min. The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 3.61 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w = 98$ nm.

3.3. Antibacterial activity of liposomes encapsulating enzymes

The antibacterial activity of the reactive liposomes encapsulating GO alone (Fig. 3) and in combination with HRP was assessed as a function of the time of exposure of biofilm to the liposomes and the time in the presence of the enzyme substrates glucose and iodide ions (Figs. 4–6) and glucose in the presence of saliva (Fig. 7). Fig. 3 shows the antibacterial activity of liposomes encapsulating GO increases with the time of incubation of the biofilm with the liposomes and the time of exposure to the substrate (glucose). For this system the bactericidal action will come only from hydrogen peroxide. Fig. 4 shows the antibacterial activity of reactive liposomes containing both GO and HRP in the presence of iodide ions. The activity of the reactive VETs increases between 1 and 5 mM iodide concentration, remaining constant above 5 mM iodide. Using an iodide concentration of 5 mM, the activity of reactive REVs after various times of exposure to substrates are shown in Fig. 5 and the

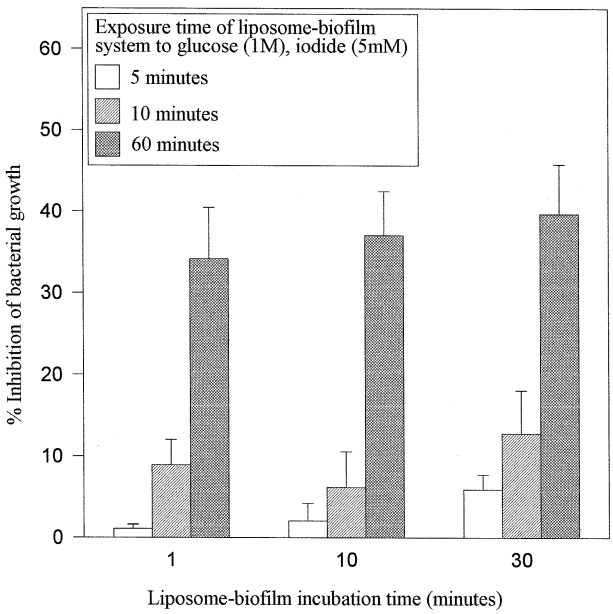

Fig. 5. Antibacterial activity to *Streptococcus gordonii* biofilms of REVs encapsulating GO/HRP (mass ratio 1:1) as a function of liposome-biofilm incubation time and the time of incubation with glucose (1 M) and iodide ions (5 mM). The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 23.82 μg (μmol lipid)$^{-1}$, REV weight average diameter $\bar{d}_w$ = 208 nm.

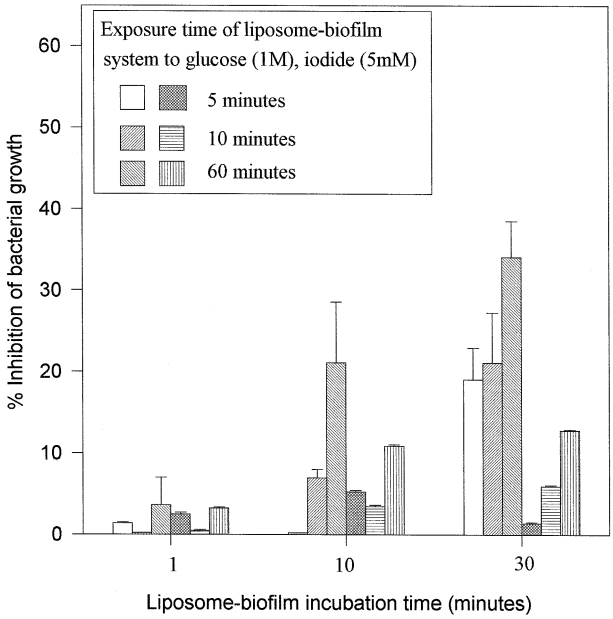

Fig. 6. Antibacterial activity to *Streptococcus gordonii* biofilms of VETs encapsulating GO/HRP (mass ratio 1:1) as a function of liposome-biofilm incubation time and time of incubation with glucose (1 M) and iodide ions (5 mM). The liposomal lipid concentration was 0.3 mM, encapsulated protein concentration 3.63 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w$ = 108 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.

corresponding data for reactive VETs in Fig. 6. Also shown in Fig. 6 are the effects of enzyme-free liposomes on growth inhibition. Some inhibition was observed by 'empty' liposomes, but this was significantly smaller than the effects of liposomes-encapsulating enzyme.

Comparison of the data in Fig. 3 for REVs encapsulating only GO with the data in Fig. 5 for REVs encapsulating both GO and HRP shows that the coupled enzyme system is more effective at inhibiting growth for shorter times of exposure of the biofilms to the liposomes; e.g., for 1- and 10-min exposure times followed by 60 min in contact with substrate, the REV-GO liposomes inhibit 7.7% and 12.30% respectively, whereas the REV-GO/HRP liposome inhibit 34.2% and 37.1% respectively. For long exposure times (30 min), both systems show similar inhibition effects.

Comparison of the data for REV and VETs encapsulating GO + HRP in Figs. 5 and 6 show that the

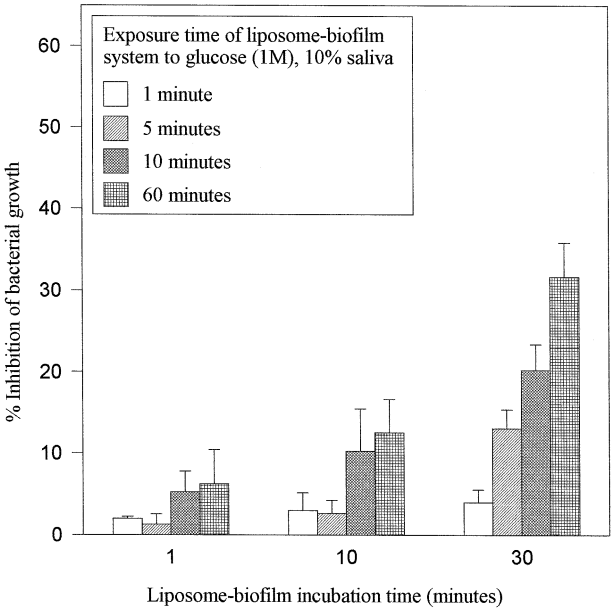

Fig. 7. Antibacterial activity to *Streptococcus gordonii* biofilms of VETs encapsulating GO/HRP (mass ratio 1:1) as a function of liposome-biofilm incubation time and time of incubation with glucose (1 M) and saliva (10%). The liposomal lipid concentration was 0.3 mM encapsulated protein concentration 4.29 μg (μmol lipid)$^{-1}$, VET weight average diameter $\bar{d}_w = 106$ nm.

REV are more antibacterial than the VETs for the shorter exposure times (1 and 10 min) of liposomes to biofilm. It should be noted that the enzyme content of the REV is considerably larger (23.82 μg/μmol lipid) than the VETs (3.63 μg/μmol lipid).

Fig. 7 shows that the reactive liposomes have significant antibacterial activity in the presence of saliva with only glucose as the substrate. The iodide level in saliva is very low (13.8 ± 8.5 μM [31]) and would not be expected to play a significant role; however, bacteriostatic activity from hypothiocyanite ions ($OSCN^-$) arising from the oxidation of thiocyanate, a substrate for lactoperoxidase in saliva, would be expected to arise [21] since the thiocyanate concentration in saliva is relatively high at 1.2 ± 0.7 mM [31].

4. Discussion

The objectives of this study were to prepare and characterise reactive liposomes which would both target to bacterial biofilms and, in the presence of appropriate substrates, inhibit subsequent bacterial growth. The coupled enzyme system selected, glucose oxidase plus horse radish peroxidase in the presence of glucose and halide ion produces hydrogen peroxide which oxidises iodide ions to oxyacids. Both hydrogen peroxide and the oxyacids have antibacterial activity to oral streptococci. The data in Figs. 1 and 2 show that the incorporation of PI into the bilayer of DPPC liposomes effectively targets the liposomes to *Streptococcus gordonii* and the optimum level of PI is the same for both liposomes prepared by extrusion (VETs) and reverse-phase evaporation (REV), although the extent of adsorption (% apparent monolayer coverage) is greater for REV than for VETs. It is possible that the higher apparent monolayer coverage found for REV may arise as a consequence of a higher degree or percentage of multilamillarity in these preparations. The reason for the existence of an optimum PI level for liposome targeting to biofilm has been discussed previously [2,4]. The optima arise as a consequence of possibly hydrogen bonding interactions both within and between the polyhydroxy head groups of the liposomal lipids and the teichoic acids in the bacterium surface. Consideration of these interactions on the basis of a lattice model [2] shows that there is a balance between 'self-interactions' within the liposome and bacterium surfaces and 'cross-interactions' between the surfaces. Such a model predicts that there is an optimum level of polyhydroxy lipid (PI) at which the cross-interactions predominate over the self-interactions which can give rise to a minimum in the potential energy-separation curve and hence a maximum in the extent of adsorption.

Both types of liposome were produced in which the enzymes were encapsulated. There is no evidence to suggest for the coupled enzyme system that both enzymes do not encapsulate in the proportion present in the initial mixture used for encapsulation. The data in Table 2 demonstrate that the enzymic activity is substantially similar in the aqueous core of the liposomes to that in free solution and is largely recovered after disruption of the liposomes with Triton X-100.

The reactive liposomes have antibacterial activity towards biofilms of *Streptococcus gordonii*. The regrowth assay of activity involved initial adsorption of the liposomes on the biofilm, a period of incubation with substrates, followed by addition of growth

medium. Antibacterial activity will depend on the extent of adsorption of the liposomes to the biofilm and the generation and action of bactericidal or bacteriostatic species during the period of incubation with substrates. The function of the reactive liposomal system is two-fold: retention of the enzymes in close contact with the biofilm and the production of bacterial growth inhibitors. The data clearly show that the extent of growth inhibition depends on the time of incubation of the liposomes with the biofilm. It is also dependent on the enzyme content of the liposomes (REV inhibit more than VETs) and would be expected to be proportional to the extent of targeting to the biofilm. Thus to a first approximation, assuming a constant time of incubation with substrates, we may expect the % inhibition to be given by a relation of the form

$$\%\ \text{inhibition} = C\,[E]\,[t]\,[\text{targeting }\%] \qquad (6)$$

where $[E]$ is the enzyme concentration in the liposomes, $[t]$ the time of exposure of the liposomes to the biofilm and C is the constant of proportionality. Eq. (6) assumes that targeting increases in proportion to the time of liposome-biofilm incubation so that the apparent monolayer coverage at short times is proportional to that at saturation. It is also assumed that the liposomes remain intact on adsorption to the biofilms, as suggested by previous studies on proteoliposomes [32], and hence the substrates react within the liposomes to produce the bactericidal species which diffuse out into the biofilms. Fig. 8 shows a test of Eq. (6) for the different systems studied, all the data relating to a 60-min incubation with substrates. It is seen that for the VETs the % inhibition increases in proportion to the parameter $[E][t]$ [targeting %]. The REV data suggest that for these liposomes, inhibition has almost reached limiting values. Only for the liposomes incorporating the single enzyme GO does inhibition increase markedly with time of exposure of liposomes to biofilm (Fig. 3), indicating that the reactivity of these liposomes is below the limiting value.

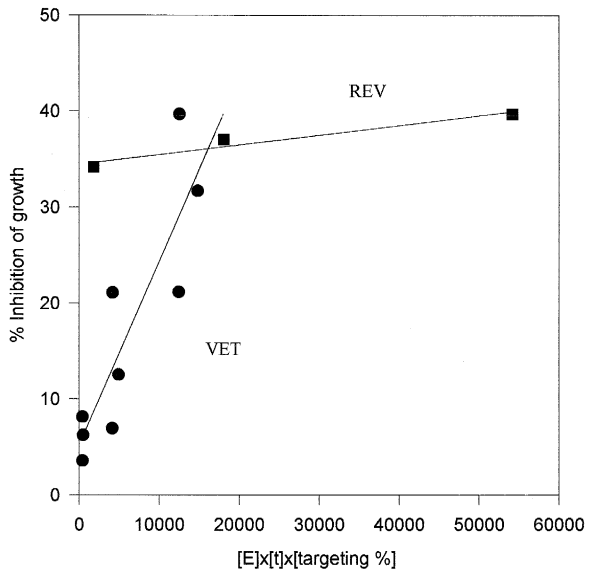

Fig. 8. Inhibition of bacterial growth from *Streptococcus gordonii* biofilms as a function of the parameter $[E][t]$ [targeting %] (see text).

In practical terms the experiments cover the realistic situation of a brief exposure of the biofilm to the reactive liposomes followed by a longer period of exposure to natural substrates, as might be conceived during use of a preparation for oral hygiene. Such reactive liposomes may also exploit substrates present in saliva for their antibacterial activity (Fig. 7).

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