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Hydrogen peroxide production from reactive liposomes encapsulating enzymes

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Abstract

Reactive cationic and anionic liposomes have been prepared from mixtures of dimyristoylphosphatidylcholine (DMPC) and cholesterol incorporating dimethyldioctadecylammonium bromide and DMPC incorporating phosphatidylinositol, respectively. The liposomes were prepared by the vesicle extrusion technique and had the enzymes glucose oxidase (GO) encapsulated in combination with horseradish peroxidase (HRP) or lactoperoxidase (LPO). The generation of hydrogen peroxide from the liposomes in response to externally added D-glucose substrate was monitored using a Rank electrode system polarised to +650 mV, relative to a standard silver–silver chloride electrode. The effects of encapsulated enzyme concentration, enzyme combinations (GO+HRP, GO+LPO), substrate concentration, electron donor and temperature on the production of hydrogen peroxide have been investigated. The electrode signal (peroxide production) was found to increase linearly with GO incorporation, was reduced on addition of HRP and an electron donor (*o*-dianisidine) and showed a maximum at the lipid chain-melting temperature from the anionic liposomes containing no cholesterol. To aid interpretation of the results, the permeability of the non-reactive substrate (methyl glucoside) across the bilayer membranes was measured. It was found that the encapsulation of the enzymes effected the permeability coefficients of methyl glucoside, increasing them in the case of anionic liposomes and decreasing them in the case of cationic liposomes. These observations are discussed in terms of enzyme bilayer interactions.

Keywords: Encapsulated enzyme; Anionic liposome; Cationic liposome; Hydrogen peroxide production; Glucose oxidase; Lactoperoxidase

1. Introduction

It has been demonstrated that reactive liposomes encapsulating coupled enzyme systems such as glucose-oxidase (GO) with horseradish peroxidase (HRP) [1,2], chloroperoxidase (CPO) or lactoperoxidase (LPO) [3] in the presence of glucose with iodide, chloride or thiocyanate respectively, will produce bactericidal species effective against biofilms of oral bacteria. The primary step in the reaction sequence leading to bactericidal species is the production of hydrogen peroxide (H_2O_2) from glucose as follows:

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

The decomposition of hydrogen peroxide by peroxidases in the presence of halides and pseudo-halides generates oxyacids, which, like hydrogen peroxide itself, are toxic to bacteria. These systems

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resemble natural antibacterial systems, which use hydrogen peroxide decomposition by peroxidase enzymes, such as lactoperoxidase and myeloperoxidase, in milk, tears and saliva [4,5]. The glucose oxidase–lactoperoxidase system forms the basis of a commercial preservative (Myavert C) for cosmetics and toiletries [6].

In liposome-encapsulated enzyme systems, numerous factors would be expected to effect the production of bactericidal species from a specific substrate, such as the level of enzyme encapsulation, the permeability of the liposomal bilayer to substrate and bactericides produced, temperature and liposomal bilayer composition. In this paper, we have taken a first step to investigate these factors with respect to the production of hydrogen peroxide from reactive liposomes with incorporated GO and peroxidases in the presence of a glucose substrate. Specifically, we have studied the production of hydrogen peroxide from cationic and anionic phospholipid liposomes by use of a Rank electrode system. To investigate the effects of substrate permeability across the liposomal bilayers, we have also made permeability measurements using a non-reactive substrate analogue methylglucopyranoside.

2. Materials and methods

L-α-Dipalmitoylphosphatidylcholine (DPPC, product no. P 0763), dimethyldiotadecyl ammonium bromide (DDAB, product no. D 2779), cholesterol (product no. C 8667), glucose oxidase (GO type VII-S, product no. G 7016), horse radish peroxidase (HRP, type I, product no. P 8125), lactoperoxidase (LPO, product no. L 2005), methyl (*d*-D-[U-^{14}C]gluco)pyranoside (specific activity 293 Ci/mol), 30% hydrogen peroxide and *o*-dianisidine tablets (product no. D 9154) were obtained from Sigma, Poole, Dorset, UK. Phosphatidylinositol (PI) from wheat germ, grade 1 was from Lipid Products, South Nutfield, UK. [^{3}H]DPPC (specific activity 55 Ci/mmol) was from Amersham International, Amersham, UK. D(+)-Glucose (analar grade) was from BDH, Poole, Dorset, UK. Spectra Por (cellulose ester) dialysis tubing, molecular mass cut-off 3500; was obtained from Pierce and Warriner, Chester, UK. Phosphate-buffered saline (PBS) tablets (code BR 14a) were from Oxoid, Basingstoke, Hants, UK. All other reagents were of analytical grade and solutions were made up with double-distilled water.

2.1. Preparation and characterisation of liposomes encapsulating enzymes

Liposomes were prepared by the vesicle extrusion technique (VETs) [7]. The required lipid mixtures including [^{3}H]DPPC (5 μCi) (total mass 30 mg) was dissolved in 3 ml of chloroform–methanol (4:1, v/v) or 3 ml of *tert*-butyl alcohol in the case of cationic liposomes in a 500-ml round-bottomed flask. The organic solvent was removed by 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 multilamallar vesicles (MLV). The MLVs were extruded 10× through two stacked polycarbonate 100-nm pore size Poretic filters (Livermore, CA) at a pressure of 200–500 psi. The unencapsulated enzymes were removed by gel filtration using a Sepharose 4B gel. The liposome fraction was assayed for lipid by liquid scintillation counting of [^{3}H]DPPC and for protein by a Lowry microassay [8]. The size distribution (weight average diameter, d_w) of the liposomes was determined by photon correlation spectroscopy (PCS) using a Malvern Autosizer, model no RR 146.

2.2. Measurement of hydrogen peroxide production from liposomes

The production of hydrogen peroxide from liposomes encapsulating enzyme systems was monitored from the electrical current produced by a Rank electrode polarised at +650 mV (versus Ag/Ag Cl_s reference). The Rank electrode was connected to a potentiostat via a 5-pin DIN plug and coaxial adaptor. The electrode signal was continuously recorded on a chart recorder. The electrode compartment contained liposome suspensions in PBS of variable concentration, but of constant volume of 1 ml. The compartment was thermostatted with a water jacket through which water was circulated at the required temperature. Aliquots of glucose substrate were added to the liposomes to give a final concentration of 10 or 100 mM as required. The electrode was

calibrated using 1 ml of hydrogen peroxide in the concentration range 0–60 mmol/ml in PBS at 20°C. The electrode response in nA after 5 min was a linear function of the hydrogen peroxide concentration and obeyed the relationship:

$$\text{nA (at 5 min)} = 0.7647 + 2.6353 \times 10^6\ [H_2O_2] \qquad (2)$$

where $[H_2O_2]$ is the molar concentration of hydrogen peroxide. The regression coefficient $r^2 = 0.999$.

It has previously been demonstrated that enzymic activity was fully recoverable after encapsulation, on releasing encapsulated enzyme with Triton X-100 [2].

2.3. Measurement of permeability coefficients

The permeability of the VET bilayers to the non-reactive methylglucoside was measured using the method of Johnson and Bangham [9]. The release of [^{14}C]methylglucoside encapsulated in VETs containing GO, HRP and GO plus HRP, in dialysis bags, was monitored as a function of time as described by Francis et al. [10]. VETs were prepared as described, except that the [^{3}H]DPPC was excluded. The required enzymes in PBS at a concentration of 1 mg/ml both when required singly or in combination (1 mg/ml GO plus 1 mg/ml HRP) plus [^{14}C]methylglucoside (5 μCi in 25 μl) were added to the dry lipid film. After extrusion, the liposome dispersion was applied to a Sepharose 4B column to separate unencapsulated enzymes and methylglucoside. The peak VET fraction (2 ml) was immediately placed in a Spectra Por dialysis tube clipped at both ends and immersed in 10 ml of PBS at 20°C. Aliquots (300 μl) were removed from the 10 ml external buffer for scintillation counting and replaced with 300 μl of PBS to maintain the volume at 10 ml. Dialysis was followed for 24 h.

The permeabilities (p) in volume per unit time (cm^3/h) were calculated using the equation:

$$\ln\{1-(\text{dpm})_o/(\text{dpm})_i)\ (12/10)\} = -(p/v_c)t \qquad (3)$$

where $(\text{dpm})_o$ was the count rate at time t outside the dialysis bag (volume 10 ml), $(\text{dpm})_i$ the initial count rate for the VET suspension (volume 2 ml) and v_c the internal volume of the liposomes. The initial slope of the plot of the left-hand side of Eq. 3 vs t was used to calculate p/v_c from which the permeability coefficient P (cm/h) was calculated from the expression [10]:

$$P = (p/v_c)\ (R-h)^3/3R^2 \qquad (4)$$

where R is the radius of the liposomes (taken as $d_w/2$) and h the bilayer thickness taken as 7.5 nm [11].

3. Results and discussion

3.1. Effect of enzyme concentration on hydrogen peroxide production

The electrode signal arising from production of hydrogen peroxide from cationic liposomes encapsulating GO and a combination of GO and HRP (molar ratio 0.26) in the presence of 10 mM glucose substrate added externally is shown in Fig. 1. The signal increases almost linearly as a function of liposome encapsulation GO concentration. In the concentration range of encapsulated enzyme studied, we found no evidence for the electrode signal saturating,

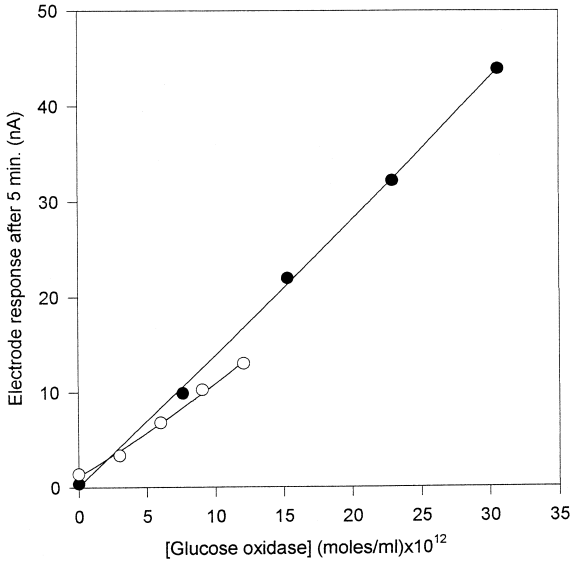

Fig. 1. Dependence of electrode response to 10 mM D-glucose (nA after 5 min) as a function of concentration of glucose oxidase (GO) encapsulated in cationic liposomes. The liposomes had a lipid composition DMPC/DDAB/cholesterol (mole ratio 1:0.16:0.45) and contained GO (●) and GO plus horseradish peroxidase (HRP) (○). The liposome weight average diameters were 138 nm (●) and 143 nm (○). The molar ratio of GO:HRP was 0.261 and the liposomal lipid concentration was in the range 0–5.484 μmol/ml. Electrode signals were measured in triplicate and had standard deviations of less than 1 nA.

although this might be expected at very high encapsulated enzyme concentrations. From the electrode calibration data (Eq. 2) the production of hydrogen peroxide from liposomes encapsulating GO is 509 ± 38 mol H_2O_2/mol GO; addition of HRP reduces the production to 372 ± 31 mol H_2O_2/mol GO. This 27% decrease arises from the decomposition of peroxide by HRP, which occurs before it can diffuse out of the liposomes. The production of hydrogen peroxide from anionic liposomes encapsulating GO and GO+HRP (molar ration 0.26) in the presence of 100 mM glucose substrate added externally is shown in Fig. 2. The production of peroxide is 9220 ± 620 mol H_2O_2/mol GO and 18030 ± 570 mol H_2O_2/mol GO in the presence of HRP. The amounts of peroxide are considerably larger due to the increased substrate concentration, but, surprisingly, HRP results in an increase in peroxide from the liposomes; however, the increase probably relates to the effect of GO plus HRP on the bilayer permeability to substrate and peroxide (see below).

Comparison of the enzyme combinations GO/HRP and GO/LPO on peroxide production from anionic liposomes in the presence of 10 mM glucose substrate is shown in Fig. 3. The peroxide production from the GO/HRP-containing liposomes is 6456 ± 689 mol H_2O_2/mol GO and that from the GO/LPO-containing liposomes 3027 ± 234 mol H_2O_2/mol GO which suggests that the LPO more effectively decomposes peroxide than the HRP; however, the molar ratio of HRP:GO is 3.83 and LPO:GO is 1.97. Dividing the peroxide productions by the enzyme ratio gives for the GO/HRP system 1686 mol H_2O_2/mol HRP and for the GO/LPO system 1537 mol H_2O_2/mol LPO, which shows that both systems are behaving similarly.

The effect of the electron donor *o*-dianisidine on the GO/HRP system was investigated and the results are shown in Fig. 4. The electron donor should facilitate the decomposition of peroxide by HRP. Thus the peroxide production from VETs encapsulating GO and HRP in the presence of 10 mM glucose substrate was reduced from 6460 ± 690 mol H_2O_2/

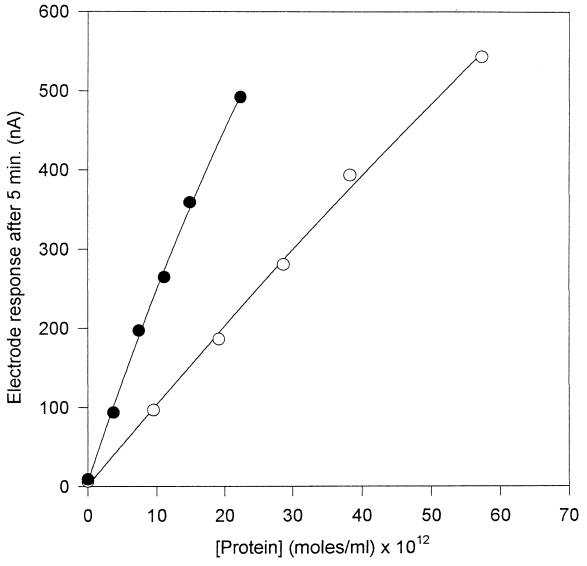

Fig. 2. Dependence of electrode response to 100 mM D-glucose (nA after 5 min) as a function of concentration of glucose oxidase (GO) encapsulated in anionic liposomes. The liposomes had a lipid composition DMPC/PI (mole ratio 1:0.97) and contained GO (●) and GO plus horseradish peroxidase (HRP) (○). The liposome weight average diameters were 124 nm (●) and 120 nm (○). The molar ratio of GO:HRP was 0.261 and the liposomal lipid concentration was in the range 0–0.825 μmol/ml. Electrode signals were measured in triplicate and had standard deviations of less than 20 nA.

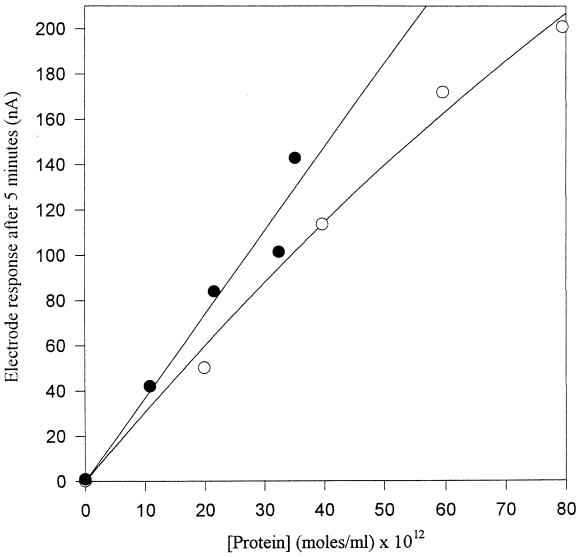

Fig. 3. Comparison of electrode response to 10 mM D-glucose (nA after 5 min) as a function of total enzyme concentration in anionic liposomes. The liposomes had a lipid composition DMPC/PI (mole ratio 1:0.97) and contained glucose oxidase (GO) plus horseradish peroxidase (HRP) (●) or GO plus lactoperoxidase (LPO) (○). The liposome weight average diameters were 113 nm (●) and 109 nm (○). The molar ratios were GO:HRP 0.261 and GO:LPO 0.507. The liposomal lipid concentrations were in the range 0–0.762 μmol/ml (●) and 0–3.225 μmol/ml (○). Electrode signals were measured in triplicate and had standard deviations of less than 4 nA.

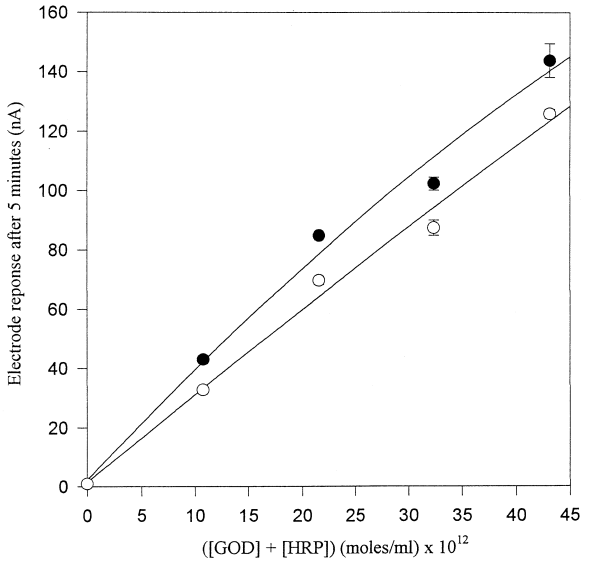

Fig. 4. Comparison of electrode response to 10 mM D-glucose (nA after 5 min) as a function of total enzyme concentration in anionic liposomes in the presence (○) and absence (●) of *o*-dianisidine (5 μM) electron donor. The liposomes had a lipid composition DMPC/PI (mole ratio 1:0.97). The liposome weight average diameters were 114 nm. The molar ratio of GO:HRP was 0.261 and the liposomal lipid concentration was 0–0.762 μmol/ml.

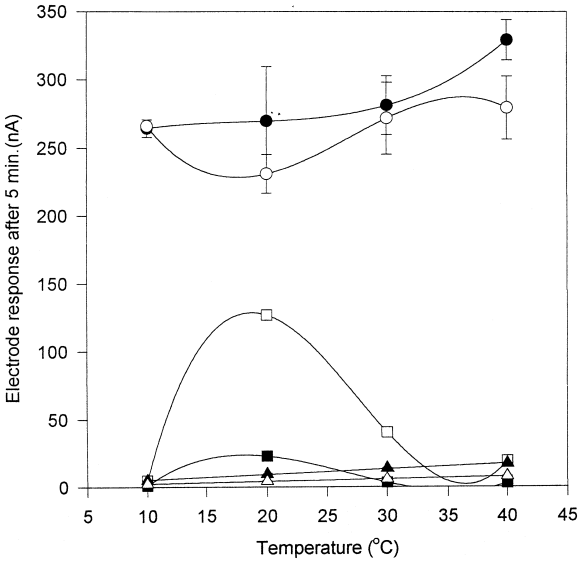

Fig. 5. Effect of temperature on the electrode response to 10 mM D-glucose (nA after 5 min) of free enzymes (glucose oxidase (GO) concentration 1.31×10^{-11} mol/ml (●) and GO concentration 1.31×10^{-11} mol/ml plus horseradish peroxidase (HRP) concentration 5×10^{-11} mol/ml (○)) and enzymes encapsulated in anionic and cationic liposomes. The anionic liposomes had composition DMPC/PI (molar ratio 1:0.088), weight average diameter 112 nm, GO concentration 1.185×10^{-11} mol/ml, liposomal lipid concentration 0.855 μmol/ml (■); weight average diameter 115 nm, GO concentration 1.002×10^{-11} mol/ml, molar ratio GO:HRP 0.261, liposomal lipid concentration 0.884 μmol/ml (□). The cationic liposomes had composition DMPC/DDAB/cholesterol (mole ratio 1:0.16:45), weight average diameter 134 nm, GO concentration 6.830×10^{-12} mol/ml, liposomal lipid concentration 13.72 μmol/ml (▲), weight average diameter 146 nm, GO concentration 2.376×10^{-11} mol/ml, GO:HRP ratio 0.261, liposomal lipid concentration 8.059 μmol/ml (△).

mol GO to 5290 ± 380 mol H_2O_2/mol GO (i.e. ~18%) when 5 μM *o*-dianisidine was added to the VETs.

3.2. *Effect of temperature and liposome composition on hydrogen peroxide production*

The electrode signal from the free enzymes, GO and GO/HRP also anionic and cationic liposomes encapsulating the enzymes was measured in the temperature range 10–40°C; the data are shown in Fig. 5. At 20°C, the current from free GO gives a peroxide production of 7790 mol H_2O_2/mol GO and in the presence of HRP 6670 mol H_2O_2/mol GO from 10 mM glucose. There is only a small variation in the signal with temperature. For the anionic liposomes, the signals pass through maxima at approximately 20°C, consistent with the onset of the chain-melting temperature of DMPC at ~23°C [12,13]. For cationic liposomes in which the bilayers contain approximately 28% cholesterol, no maxima are found consistent with the absence of a chain-melting temperature in the presence of cholesterol [14].

3.3. *Decomposition of hydrogen peroxide by horse radish peroxidase*

The decomposition of hydrogen peroxide by free and encapsulated HRP was investigated in the presence and absence of *o*-dianisidine donor. Fig. 6 shows the electrode response to 20 μM peroxide as a function of HRP concentration for free HRP and HRP encapsulated in anionic liposomes. The decomposition of free HRP reaches 1.98 mol H_2O_2/mol HRP at an HRP concentration of 1 μM, addition of 1 μM *o*-dianisidine increases the decomposition and gives a more linear response with a decomposition of 4.3 ± 0.5 mol H_2O_2/mol HRP. Encapsulated HRP at the same overall bulk concentration plus

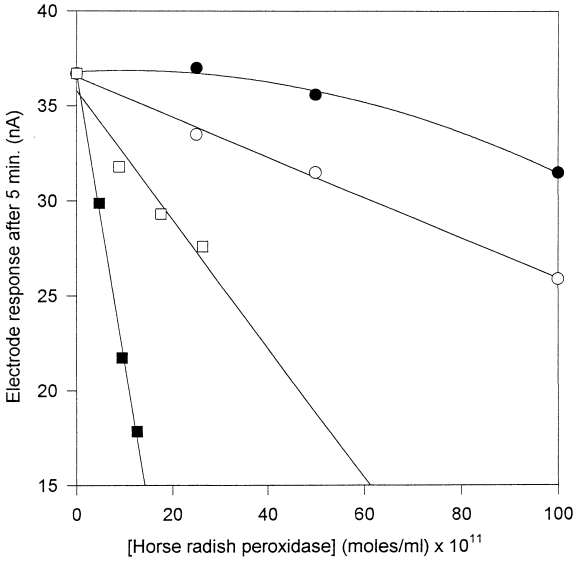

Fig. 6. Comparison of the decrease in electrode signal (nA after 5 min) from hydrogen peroxide (20 mmol/ml) as a function of free horseradish peroxide (HRP) and liposome encapsulated HRP concentration in the absence and presence of the electron donor (*o*-dianisidine). Free HRP (●); free HRP plus 1 μM *o*-dianisidine (○); HRP encapsulated with 1 μM *o*-dianisidine in DMPC/PI (mole ratio 1:0.088) liposomes (weight average diameter 123 nm), liposomal lipid concentration 0–1.202 μmol/ml (□); HRP encapsulated in DMPC/PI (mole ratio 1:0.088) liposomes (weight average diameter 123 nm), liposomal lipid concentration 0–1.202 μmol/ml with externally added 1 μM *o*-dianisidine (■).

encapsulated 1 μM *o*-dianisidine increases the decomposition to 16 ± 4 mol H_2O_2/mol HRP and 57 ± 3 mol H_2O_2/mol HRP in the presence 1 μM free *o*-dianisidine. The greater rate of decomposition found when the donor is added externally to the liposomes suggests that in contrast to the donor being encapsulated, the small amount inside the liposomes limits peroxide decomposition. The encapsulated volume of the liposomes is approximately 3% of the total bulk volume, so that inside the liposomes, the HRP will be greatly concentrated, compared with an equivalent bulk concentration, which is consistent with greater peroxide decomposition rates found for the liposomes in comparison with the free enzyme.

3.4. Permeability of liposomes to a substrate analogue

The permeability of anionic and cationic liposome bilayers to the glucose substrate would be expected to have some influence on the hydrogen peroxide production. The data in Fig. 5 at 20°C show that peroxide production increases with liposome composition and enzyme encapsulation in the series DMPC/DDAB/cholesterol/GO/HRP < DMPC/DDAB/cholesterol/GO < DMPC/PI/GO/HRP. Due to the decomposition of the glucose substrate inside the liposomes, it is not possible to measure glucose permeability of liposomes encapsulating the enzymes, so the permeability of the non-metabolised substrate

Table 1
Permeability Coefficients for methyl glucopyranoside at 20°C

Sample	Slope of ln[1−*D*] versus time	d_w (nm)	[GO] (mol/ml)	[HRP] (mol/ml)	Permeability coefficient (cm/h) ($\times 10^7$)
'Free' methyl glucopyranoside (control)	−1.168				
DMPC, PI (Control) (mole ratio 1:0.088)	−0.5996	125.75			8.58
DMPC, PI (GO) (mole ratio 1:088)	−0.6702	122.99	6.55×10^{-11}		9.30
DMPC, PI (HRP) (mole ratio 1:0.088)	−0.8415	132.93		6.80×10^{-10}	13.02
DMPC, PI (GO, HRP) (mole ratio 1:0.088)	−0.9740	116.00	8.699×10^{-11}	3.328×10^{-10}	12.43
DMPC, DDAB, cholesterol (mole ratio 1:0.16:0.45) (Control)	−0.07153	136.97			1.15
DMPC, DDAB, cholesterol (mole ratio 1:0.16:0.45) (GO)	−0.03283	129.03	1.397×10^{-10}		0.49
DMPC, DDAB, cholesterol (mole ratio 1:0.16:0.45) (HRP)	−0.03171	132.95		2.50×10^{-10}	0.49
DMPC, DDAB, cholesterol (mole ratio 1:0.16:0.45) (GO, HRP)	−0.02822	125.11	4.06×10^{-11}	1.554×10^{-10}	0.40

$D = [(dpm)_o/(dpm)_i]$ (12/10), parameters as defined in the text.

analogue, methyl glucoside, was measured as a function of liposome composition. Fig. 7 and 8 show plots based on Eq. 3. The slopes of these plots were used to calculate the permeability coefficients of the liposome bilayers to methyl glucoside using Eq. 4. The permeability coefficient of methyl glucoside for DMPC/PI liposomes in the absence of encapsulated enzymes is higher than for glucose in DPPC/PI liposomes (molar ratio 1:0.10) at 25°C which was found to be 0.37×10^{-7} cm/h, but very similar to that for DPPC/PI liposomes at 37°C, close to the chain-melting temperature of DPPC, where a value of 8.50×10^{-7} cm/h was found [15]. These values for glucose permeability coefficients of liposomes are considerably smaller than for the permeability coefficients of planar bilayers to glucose, where values of the order of 10^{-4} cm/h have been observed [16]. The results are shown in Table 1. The anionic liposomes have a larger permeability coefficient for methyl glucoside than the cationic liposomes by a factor of approximately 7.5×. Encapsulation of GO increases the permeability coefficient of anionic liposomes by approximately 8% and encapsulation of GO and HRP by approximately 45%. This increase is largely attributable to HRP that by itself increases the permeability by 52%. These observations are consistent with the electrode signals observed in Fig. 5 for anionic liposomes encapsulating GO and GO+HRP. Encapsulation of the enzymes separately into cationic liposomes, in contrast to anionic liposomes, reduces the permeability coefficient for methyl glucoside by 57% and in combination by 65%. These observations are also consistent with the lower electrode signal from cationic liposomes (Fig. 5).

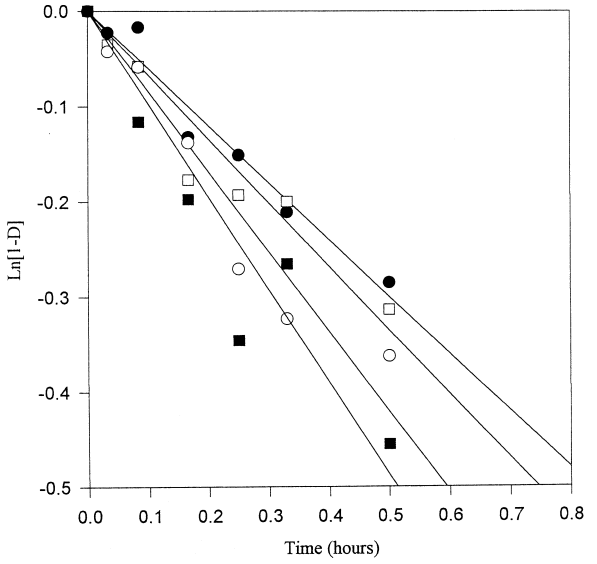

Fig. 7. Permeability plots of methyl glucoside based on Eq. 3 ln $(1-D)$ vs t where $D=(\mathrm{dpm})_o/(\mathrm{dpm})_i$ (12/10) for DMPC/PI (mole ratio 1:0.088) anionic liposomes empty (●); encapsulating glucose oxidase (GO) (□); encapsulating horseradish peroxidase (HRP) (○); encapsulating GO+HRP (■). The liposome characteristics are given in Table 1.

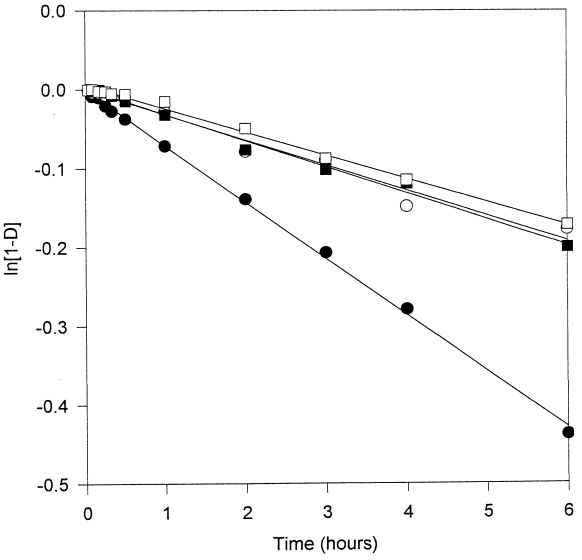

Fig. 8. Permeability plots of methyl glucoside based on Eq. 3 ln $(1-D)$ vs t where $D=(\mathrm{dpm})_o/(\mathrm{dpm})_i$ (12/10) for DMPC/DDAB/cholesterol (mole ratio 1:0.16:0.45) cationic liposomes; empty (●); encapsulating glucose oxidase (GO) (○); encapsulating horseradish peroxidase (HRP) (■); encapsulating GO+HRP (□). The liposome characteristics are given in Table 1.

The isoelectric points of GO and HRP are 4.35 [17] and 6.6 [18], respectively. At pH 7.4 (PBS) both GO and HRP will be negatively charged and might interact preferentially with a positively charged bilayer. The decrease in permeability to methyl glucoside of the cationic liposomes may result from the effect of adsorption of the enzymes on the inner surface of the bilayer leading to an effective increase in liposome wall thickness and a reduced permeability. The thickness effect must predominate over hydrophobic interactions which might lead to an increase in permeability (see below). In the case of the anionic liposomes, the enzymes, particularly HRP, cause an increase in permeability to methyl glucoside. The reason for this is not clear, the negatively charged enzymes will be repelled by the bilayer, this may lead

possibly to a decrease in lipid packing within the bilayer and an increase in permeability. As the anionic liposomes in the absence of encapsulated protein have a greater permeability to methyl glucose by a factor of 7–8×, the anionic bilayer already has looser packing due to the absence of cholesterol. Hence inhomogeneities arising from the proximity to the chain-melting temperature might make the bilayer more susceptible to changes resulting from repulsive protein interactions. Alternatively, as the isoelectric point of HRP (pH 6.6) is not too far removed from that of the bulk solution (pH 7.4), the enzyme might interact hydrophobically with the bilayer causing dislocations and an increased permeability.

4. Conclusions

The Rank electrode polarised at +650 mV relative to a standard Ag/ACl_s electrode has been shown to be a convenient method for the detection of hydrogen peroxide produced by liposomes encapsulating GO+HRP in the presence of a glucose substrate. The production of hydrogen peroxide increases almost linearly with the concentration of encapsulated GO and increases with substrate concentration. Co-encapsulation of peroxidase enzymes, HRP or LPO, that decompose hydrogen peroxide, lead to a decreased production of peroxide. This decrease is enhanced by the addition of an electron donor such as *o*-dianisidine. For anionic liposomes peroxide production goes through a maximum in the region of the lipid (DMPC) chain-melting temperature, but for cationic liposomes containing cholesterol, with no chain-melting temperature, the production of peroxide increases little, but linearly, with temperature. Liposomes encapsulating HRP alone in the presence of an electron donor (*o*-dianisidine) can decompose hydrogen peroxide and the decomposition is a linear function of the concentration of encapsulated HRP. Measurements of the permeability of liposomes encapsulating enzymes, to the non-metabolisable substrate analogue methyl glucoside, showed that the enzymes increased the permeability of anionic liposomes and decreased the permeability of cationic liposomes. The decrease in permeability would seem to relate to the interaction of the negatively charged enzymes with cationically charged liposome bilayers. The increase in permeability of anionic liposomes may arise from either repulsive ionic or hydrophobic interactions.

Acknowledgements

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