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Effect of grafted polyethylene glycol (PEG) on the size, encapsulation efficiency and permeability of vesicles

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Abstract

Liposomes have been prepared by the vesicle extrusion method (VETs) from mixtures of dipalmitoylphosphatidylcholine (DPPC), phosphatidylinositol (PI) and dipalmitoylphosphatidylethanolamine with covalently linked poly(ethylene glycol) molecular mass 5000 and 2000 (DPPE-PEG 5000 and DPPE-PEG 2000) covering a range of 0–7.5 mole%. The encapsulation of D-glucose has been studied and found to be markedly dependent on the mole% DPPE-PEG. The permeability of the liposomes to D-glucose has been measured both as a function of temperature and liposome composition. The permeability coefficients for D-glucose increase with mole% DPPE-PEG 5000 and with temperature over the range 25–50°C. The activation energies for glucose permeability range from 90 to 23 kJ mol^{-1}. The decrease in activation energy with increasing temperature is attributed to an increasing number of bilayer defects as the liposome content of PEG-grafted lipid is increased. The dependence of D-glucose encapsulation as a function of PEG-grafted lipid content is discussed in terms of the conformation of the PEG molecules on the inner surface of the bilayer. For liposomes containing DPPE-PEG 5000 the relative percentage encapsulation of glucose, assuming that the PEG surface layer excludes glucose, is comparable to that predicted from the mushroom and brush conformational models. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Polyethylene glycolated liposome; Glucose encapsulation; Liposome permeability; Permeability coefficient; Activation energy

1. Introduction

The value of using vesicles (liposomes) with surface coatings of water-soluble polymers as a means of stabilizing liposomes in the blood circulation is now well established [1–5]. In particular the incorporation of phospholipid-grafted polyoxyglycol (PEG) to form Stealth liposomes has been extensively studied [6–11]. Depending on the surface density and molecular mass of the PEG grafted to the pegylated lipid molecules in the liposomal bilayer, several regimes of PEG surface conformation can be identified [12,13]. The controlling factor is the distance between the PEG chains in the lipid bilayer (D) relative to their Flory dimension, R_F. The Flory dimension is given by $R_F = aN^{3/5}$, where 'a' is the monomer size (persistence length) and N is the number of monomer units in the polymer. Three regimes can be defined: (1) when $D > 2R_F$ (interdigitated mushrooms); (2) when $D < 2R_F$ (mushrooms); and (3) when $D < R_F$ (brushes). For liposomes incorporating phosphatidylethanolamine with grafted PEG of molecular mass 2000 (DPPE-PEG 2000) it has been found that the permeabilities of the liposomes to

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glucose at 15°C pass through a maximum when the liposomes contain approximately 4 mole% DPPE-PEG 2000 [14,15]. This concentration corresponds to the range of the two-dimensional phase transition of the grafted PEG 2000 from the mushroom to the brush regime [16]. The increase in permeability was attributed to the spontaneous formation of defects (holes) in the bilayer [17] which form more easily in the transition region between the two conformation regimes. Support for the transition between mushrooms and brushes was found from the study of the thickness of foam bilayers which showed a marked increase at 4 mole% DPPE-PEG 2000 as the brushes forced the phospholipid monolayers apart [18].

Increasing the molecular mass of the grafted PEG extends the existence of the brush regime and lowers the mole% of pegylated lipid at which the mushroom to brush transition occurs. For DPPE-PEG 5000 the transition is predicted to occur at less than 2 mole% [13]. In this study we have investigated the encapsulation properties of PEG 5000 and PEG 2000 liposomes and the permeabilities of liposomes incorporating a range of DPPE-PEG 5000 mole% at several temperatures. It has been found that the extent of encapsulation of glucose in the liposomes is markedly dependent on liposome composition.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was from Sygena Ltd. (Liestal, Switzerland); phosphatidylinositol (PI) (from wheat germ, sodium salt (grade I)) was from Lipid Products (South Nutfield, Surrey, UK); 1,2-dipalmitoylethanolamine-*N*-[poly-(ethylene glycol)-5000] (DPPE-PEG 5000) product no. 880200 and DPPE-PEG 2000 product no. 880160 were from Avanti Polar Lipids Inc. (Alabaster, AL, USA); L-3-phosphatidyl-[*N*-methyl-^{3}H]-choline, 1,2-dipalmitoyl (^{3}H-DPPC), product no. TRK 673, specific activity 40–80 Ci/mmole and D-[U-^{14}C]-glucose, product no. CFB 96, specific activity 270 mCi/mmole were from Amersham International plc. (Aylesbury, Bucks., UK). These materials were used as supplied. The organic solvents, chloroform and methanol, were of analar grade (BDH, Poole, Dorset, UK) and were distilled and stored over molecular sieves. The inorganic reagents were of analytical grade and the aqueous solutions were made up with double distilled water.

2.2. Liposome preparation

Liposomes were prepared from mixtures of DPPC, PI and DPPE-PEG 5000 or DPPE-PEG 2000 of the required molar ratio. A thin lipid film (total lipid mass 30 mg plus 5 μCi [^{3}H]-DPPC) was formed in a 50 ml round bottomed flask by rotary evaporation from 5 ml of organic solvent (chloroform-methanol, volume ratio 4:1), followed by drying at 60°C in a stream of nitrogen gas. The film was rehydrated with 3 ml of Krebs-Henseleit phosphate buffer (KHP) or phosphate buffered saline, pH 7.4 (PBS) in the case of liposomes incorporating DPPE-PEG 2000, containing 20 μCi [^{14}C]-glucose at 60°C and vigorously vortexed to produce multilamellar liposomes (MLV). The KHP buffer had composition 120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgS0_4$, 16 mM Na_2HPO_4 adjusted to pH 7.4. The MLVs were transferred to a thick-walled glass tube, then frozen in liquid nitrogen (−196°C) and thawed (60°C) five times. The resulting freeze-thawed suspension (FATMLV) was extruded at 60°C through two stacked polycarbonate filters (0.1 μm pore size, Poretics) under nitrogen pressure (50–100 psi) with a Lipex Biomembranes Inc. extruder [19]. After extrusion the vesicles (hereafter called VETs; vesicles by the extrusion technique) were separated from unencapsulated glucose by passing them through 5 cm A-15 m Bio-Gel spin columns as described by Chonn et al. [20] previously equilibrated with KHP buffer or down a Sephadex G50 column equilibrated with PBS. Aliquots of the liposome fractions were assayed for ^{14}C and ^{3}H by scintillation counting to obtain their glucose and lipid concentrations.

2.3. Liposome size determination

Liposome size distributions were determined by photon correlation spectroscopy (PCS) using a Malvern autosizer model RR146 or a Zetamaster 3000 (Malvern, Enigma Park, South, Malvern, Worcs WR14 1AT, UK) and by transmission electron mi-

croscopy with negative staining (2% w/v phosphotungstic acid) using a Philips 400 electron microscope. PCS measurements of the weight-average diameters ($\bar{d}_w$) of the liposomes were made on MLVs and FATMLVs during their preparation and on the final VET preparations.

2.4. Liposome permeability measurements

The permeabilities of the liposomes to encapsulated [^{14}C]-glucose were measured using a similar dialysis technique to that of Johnson and Bangham [21]. Immediately after separation of the unencapsulated [^{14}C]-glucose on the spin columns the VET suspensions (lipid concentration 2–3 mg ml^{-1} encapsulating 0.07–6.0 μg of glucose) in KHP buffer (1.1 ml) of known [^{3}H]-DPPC and [^{14}C]-glucose activity were placed in dialysis bags cut from Spectra Pore tubing (No. 3, molecular mass cut-off 3500, Spectrum Medical Industries, LA, USA). The dialysis bags were sealed with plastic clips, rinsed with KHP buffer, blotted externally with tissue and placed in 25 ml screw-cap sample tubes for incubation in a water bath at the required temperature. The time interval between collecting the VET suspensions from the spin columns and the start of dialysis was typically 15–20 min. Glucose diffusion from the dialysis bags was assayed from 2 min to 24 h. The permeability of the dialysis bag material was determined by measurement of the diffusion of free glucose (0.2 μCi [^{14}C]-glucose in 1.1 ml KHP).

The experiments were interpreted using the equation [21]

$$\frac{\mathrm{dpm_o}\,v_0}{\mathrm{dpm_i}\,v_2} = 1-\frac{1}{K-L}\left\{(1-\alpha)K\mathrm{e}^{-Lt} - (1-\alpha K)\mathrm{e}^{-Lt}\right\} \quad (1)$$

where $\mathrm{dpm_o}$ and $\mathrm{dpm_i}$ are the count rates outside and inside the bag respectively. v_0 is the total volume of the system and v_2 the volume outside the dialysis bag. The parameters K and L relate to the permeabilities of the dialysis bag (p_1) and liposomes respectively. According to the following relationships

$$K = \frac{p_1 v_0}{v_1 v_2} \quad (2)$$

$$L = \frac{p}{v_c} \quad (3)$$

where v_1 and v_c are the volumes inside the bag and the liposomes respectively. The factor α is the fraction of the initial counts inside the bag (α $\mathrm{dpm_i}$) that diffused out of the liposomes before the start of the experiment.

The value of K for the bag material can be obtained using only free glucose initially inside the bag. Under these conditions α = 1 and $L = 0$ hence Eq. 1 becomes

$$\ln\left(1-\frac{\mathrm{dpm_o}\,v_0}{\mathrm{dpm_i}\,v_2}\right) = -Kt \quad (4)$$

Table 1
Effect of increasing DPPE-PEG 5000 content (mol%) in DPPC:PI VET preparations on liposome diameters (d_w) of MLVs, FATMLVs and VETs as measured by photon correlation spectroscopy (PCS) and electron microscopy (EM)

Liposome composition: DPPC:PI:DPPE-PEG 5000 (mol%)	$\bar{d}_w$ (nm) (PCS)			Equivalent normal weight distribution (nm)[b]	$\bar{d}_n$ (nm) (EM)	($\bar{d}_w$ (50°C) after 24 h/d_w (37°C)) × 100
	MLV	FATMLV	VET			
(91:9:0)	3210.1 ± 1591.8	2605.0 ± 1023.4	112.8 ± 9.3	34.4 ± 2.6	122.4 ± 14.1	99.7 ± 2.8
(90:9:1)	2934.5 ± 2050.0	1020.4 ± 777.7	121.5 ± 3.1	30.8 ± 0.8	123.4 ± 18.2	96.5 ± 7.8
(88.5:9:2.5)	643.0 ± 206.4	475.2 ± 331.3	113.9 ± 11.3	35.3 ± 1.8		94.2 ± 5.6
87.25:9:3.75)	572.4 ± 256.0	260.8 ± 78.7	116.5 ± 4.1	36.7 ± 0.8	131.7 ± 18.0	90.3 ± 8.0
(86:9:5)	441.6 ± 153.7	379.3 ± 263.2	96.8 ± 15.8	41.6 ± 5.3	115.3 ± 29.6	87.0 ± 5.1
(85:9:6)[a]	225.7 ± 19.0		95.9 ± 1.6	39.0 ± 3.0		73.5 ± 20.0
(83.5:9:7.5)[a]	249.9 ± 22.8		108.9 ± 6.2	46.0 ± 2.0		79.8 ± 31.6

[a]Not freeze-thawed preparation.
[b]Standard deviation.

Under condition when $\alpha = 0$ (i.e. no leakage) before the start of the experiment and $p_1 \gg p$ Eq. 1 becomes

$$\ln\left(1-\frac{\mathrm{dpm_o}\,v_0}{\mathrm{dpm_i}\,v_2}\right) = -Lt \tag{5}$$

The permeability of the liposomes calculated from L from Eq. 1 or 5 can be used to calculate the permeability coefficients, P (cm h^{-1}) from

$$P = \frac{Lv_c}{S_c} \tag{6}$$

where S_c is the liposome surface area.

If the liposomes have an internal radius R

$$P = \frac{4\pi R^3 L}{3}/4\pi R^2 = \frac{LR}{3} \tag{7}$$

For a liposome of bilayer thickness h_b the internal radius is given by

$$R = \frac{\bar{d}_w}{2} - h_b \tag{8}$$

For a liposome incorporating grafted PEG with a polymer layer thickness h_p the internal radius (R_c) is given by

$$R_c = \frac{\bar{d}_w}{2} - (h_b - 2h_p) \tag{9}$$

3. Results

3.1. Liposome sizes

Table 1 shows the weight-average diameter of the liposome preparations ($\bar{d}_w$) incorporating DPPE-PEG 5000 as measured by photon correlation spectroscopy (PCS) at three stages of preparation and the number-average diameters ($\bar{d}_n$) of some of the preparations as measured by electron microscopy on negatively stained samples. Both the MLVs and the MLVs after freeze-thawing (FAT-MLVs) decrease in size as the DPPE-PEG 5000 content is increased. At 6 and 7.5 mole% the MLVs were sufficiently small to be directly extruded. In contrast to the MLVs and FAT-MLVs the sizes of the VETs were independent of the DPPE-PEG 5000 content. As the VETs were to be used for permeability studies up to a temperature of 50°C their sizes were measured after incubation for 24 h to check on their integrity. Incubation for 24 h at 37°C gave diameters relative to those of the initial preparation of 99.5 ± 4.6%, incubation for 24 h at 25°C gave relative diameters of 98.7 ± 3.7%.

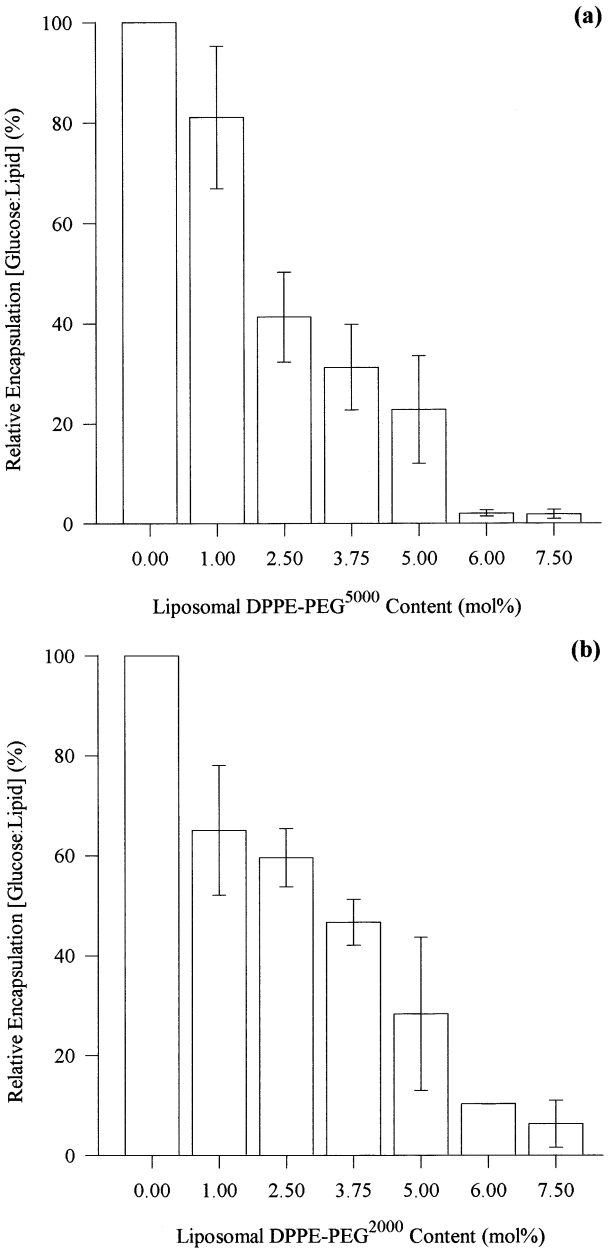

Fig. 1. Encapsulation of glucose in DPPC:PI:DPPE-PEG 5000 (a) and DPPE-PEG 2000 (b) liposomes as a function of the mole% DPPE-PEG. The compositions of the liposomes (DPPC:PI content) are given in Table 1. Encapsulation is expressed as glucose to lipid mass ratio as a percentage of that in liposomes containing no DPPE-PEG.

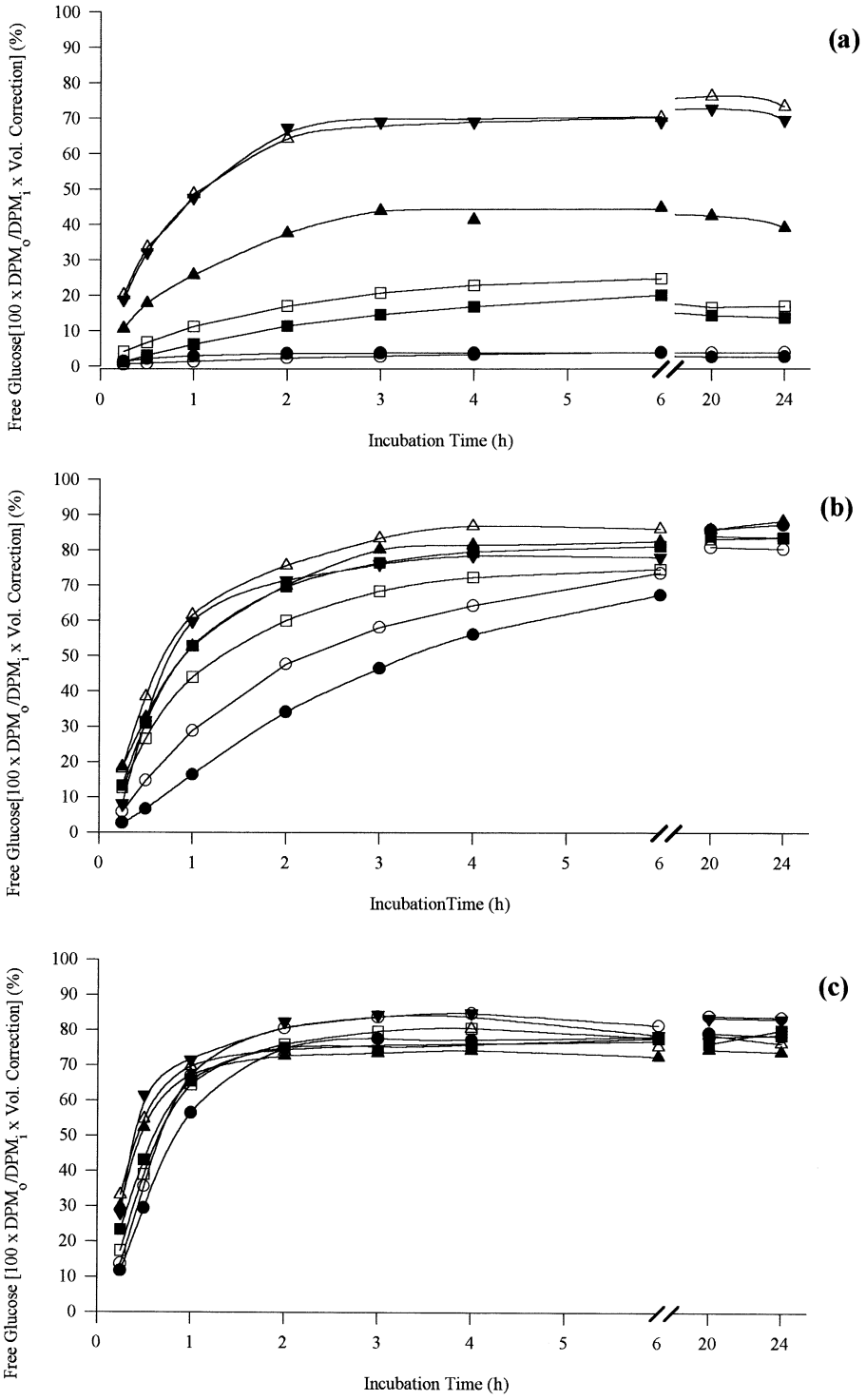

Fig. 2. The release of glucose after encapsulation within DPPC:PI:DPPE-PEG 5000 liposomes as a function of time at (a) 25°C; (b) 37°C; and (c) 50°C, and liposome composition. The liposome compositions (mole%) were as follows: ●, 91:9 (DPPC:PI); ○, 90:9:1 (DPPC:PI:DPPE-PEG 5000); ■, 88.5:9:2.5; □, 87.25:9:3.75; ▲, 86:9:5; △, 85:9:6; ▼, 83.5:9:7.5.

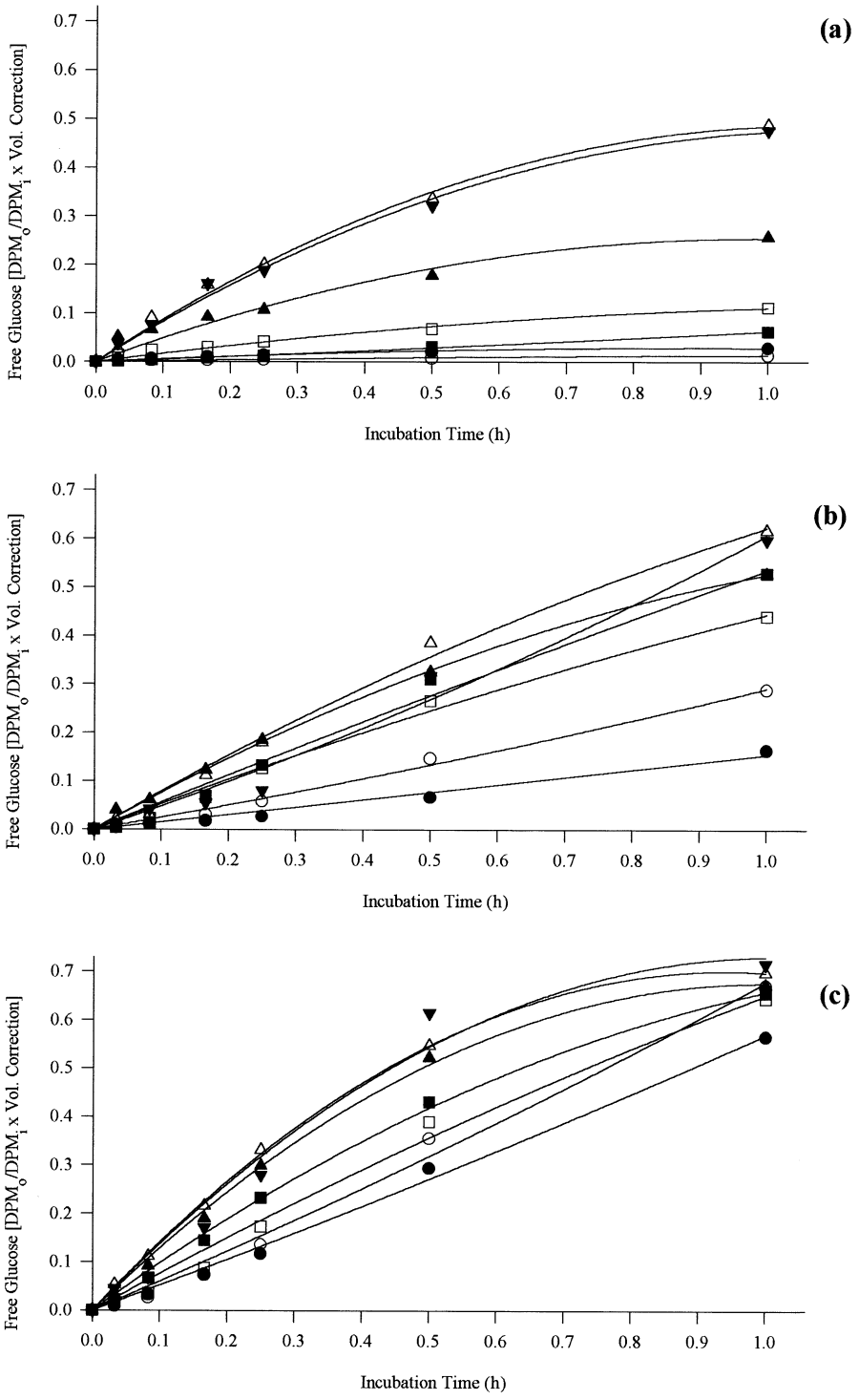

Fig. 3. The release of glucose after encapsulation within DPPC:PI:DPPE-PEG 5000 liposomes as a function of time at (a) 25°C; (b) 37°C; and (c) 50°C, and liposome composition fitted to Eq. 1. The solid lines are the non-linear fits and the symbols correspond to those in Fig. 2.

Table 2
Permeability coefficients at 25, 37 and 50°C of DPPC:PI:DPPE-PEG 5000 VET

Incubation temp. (°C)	Liposome composition: DPPC:PI:DPPE-PEG 5000 (mol%) (R, R_c (nm))	α (%)	L (h^{-1})	$10^7 P_R$ (cm h^{-1})	$10^7 P_{R_c}$ (cm h^{-1})
25	($K = 1.73 \pm 0.02$)				
	(91:9:0) (50, 50)	10.64 ± 6.84	0.065 ± 0.063	1.21 ± 1.17	1.21 ± 1.17
	(90:9:1) (51, 45)	1.36 ± 0.51	0.007 ± 0.004	0.089 ± 0.066	0.077 ± 0.057
	(88.5:9:2.5) (49, 34)	2.59 ± 0.06	0.128 ± 0.019	2.08 ± 0.41	1.46 ± 0.32
	(87.25:9:3.75) (53, 32)	33.47 ± 26.89	0.202 ± 0.126	2.57 ± 1.07	1.19 ± 0.21
	(86:9:5) (41, 16)	29.35 ± 17.99	0.654 ± 0.172	9.87 ± 1.68	4.42 ± 0.25
	(85:9:6) (42, 14)	77.57 ± 2.98	0.894 ± 0.050	12.67 ± 0.63	4.18 ± 0.15
	(83.5:9:7.5) (49, 17)	13.51	1.229	19.91	6.80
37	($K = 1.77 \pm 0.02$)				
	(91:9:0) (50, 50)	9.29 ± 7.16	0.24 ± 0.05	4.06 ± 0.97	4.06 ± 0.97
	(90:9:1) (51, 45)	13.38 ± 7.52	0.45 ± 0.07	7.77 ± 1.09	6.79 ± 0.94
	(88.5:9:2.5) (50, 35)	21.04 ± 12.74	1.71 ± 0.25	28.01 ± 1.61	19.77 ± 0.76
	(87.25:9:3.75) (51, 30)	17.28 ± 10.20	1.59 ± 0.22	26.93 ± 4.71	16.07 ± 3.20
	(86:9:5) (42, 17)	39.41 ± 9.37	1.74 ± 0.91	32.75 ± 15.14	14.38 ± 10.51
	(85:9:6) (42, 14)	28.23 ± 10.57	2.92 ± 2.16	40.48 ± 29.29	12.79 ± 8.80
	(83.5:9:7.5) (49, 17)	35.00 ± 8.95	1.91 ± 1.61	31.02 ± 26.05	10.68 ± 8.88
50	($K = 2.23 \pm 0.03$)				
	(91:9:0) (51, 51)	14.22 ± 9.54	1.23 ± 0.45	20.27 ± 6.89	20.27 ± 6.89
	(90:9:1) (51, 45)	9.12 ± 5.19	1.61 ± 0.22	27.43 ± 4.24	23.95 ± 3.76
	(88.5:9:2.5) (47, 32)	38.02 ± 7.85	2.05 ± 0.67	31.81 ± 9.63	21.89 ± 6.42
	(87.25:9:3.75) (47, 27)	17.77 ± 10.10	1.96 ± 0.004	31.75 ± 0.15	18.34 ± 0.18
	(86:9:5) (34, 24)	44.77 ± 5.34	5.05 ± 3.65	59.01 ± 48.51	25.29 ± 19.91
	(85:9:6) (35, 6.8)	0.02	4.14	55.43	16.08
	(83.5:9:7.5) (38, 6.1)	7.70	4.19	58.79	14.05

However at 50°C there was a decrease in diameter for VETs containing higher mole% DPPE-PEG 5000 as shown in Table 1. This size decrease was taken into account in the calculations of the values of R and R_c and in the permeabilities. Similar data were obtained for VETs incorporating DPPE-PEG 2000, these VETs had similar diameters ($\bar{d}_w$) to the DPPE-PEG 5000 VETs with a mean value of 117 ± 5 nm.

3.2. Encapsulation efficiency

Fig. 1a and b show the relative encapsulation of glucose at 4°C into the VETs as a function of DPPE-PEG 5000 mole% and DPPE-PEG 2000 mole% respectively. The values were calculated as a percentage of the glucose to lipid ratio for VETs with 0 mole% DPPE-PEG 5000 and DPPE-PEG 2000 for which the glucose to lipid weight ratio was $0.1991(\pm 0.0281)\times 10^{-2}$ and $0.275(\pm 0.002)\times 10^{-4}$ respectively. Addition of 7.5 mole% DPPE-PEG 5000 brought the weight ratio down to 0.0037 ± 0.0020 (i.e. down to 1.87 ± 0.94% of the value in the absence of pegylated lipid). Fig. 1a shows that the addition of pegylated lipid very markedly reduces the amount of glucose encapsulation in the VETs; the decrease in encapsulation is particularly large between 0 and 2.5 mole% pegylated lipid. For DPPE-PEG 2000 VETs (Fig. 1b) a similar marked reduction in encapsulated glucose occurs but not until the VETs contain 4–6 mole% pegylated lipid.

3.3. Liposome permeabilities

The percentage release of glucose from VETs as a function of time at 25°C, 37°C and 50°C is shown in Fig. 2. At 25°C and 37°C at the highest pegylated lipid concentration (7.5 mole%) equilibrium is reached after 3–4 h. In the absence of PEG the lipo-

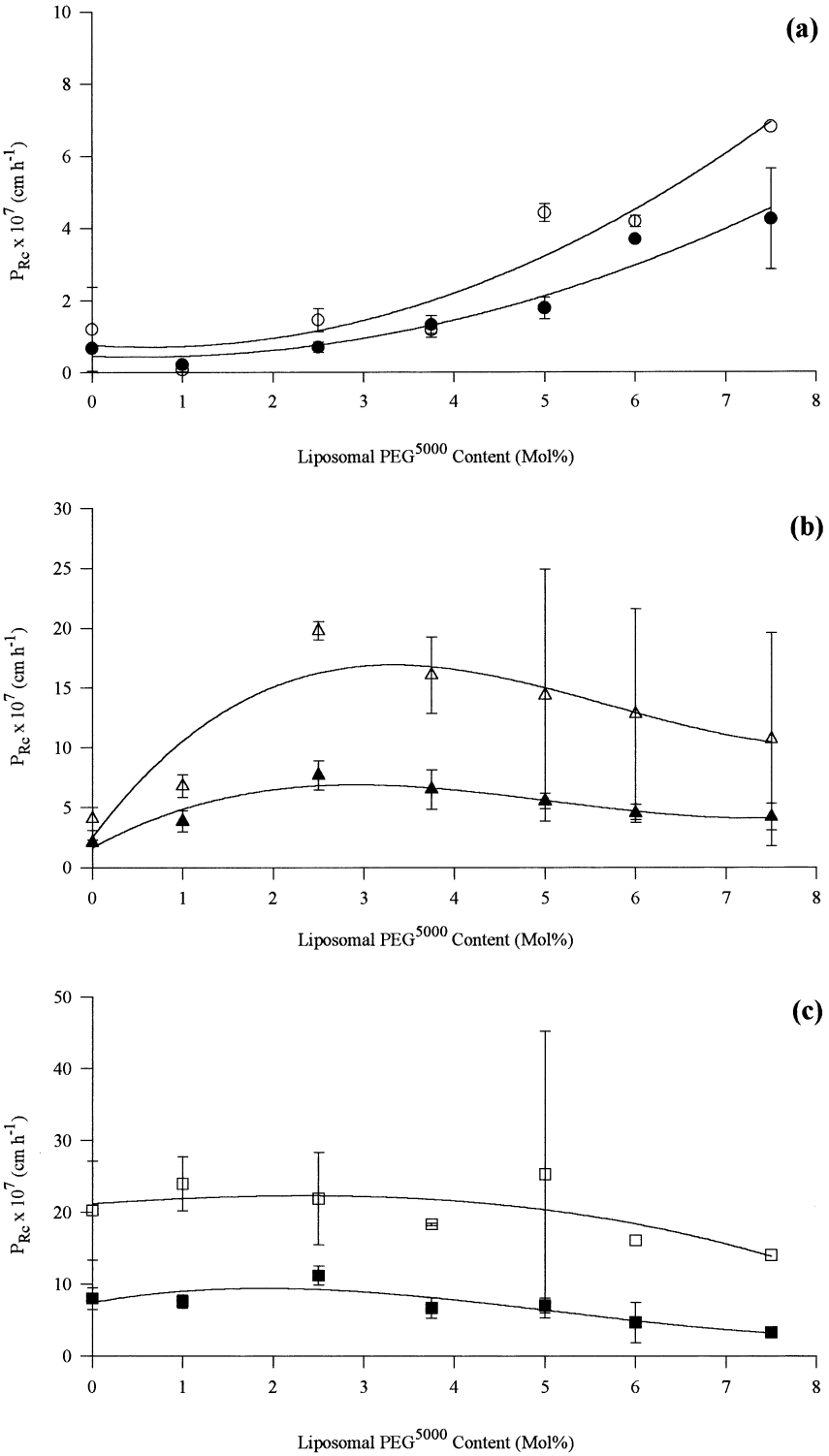

Fig. 4. Permeability coefficients (P_{R_c} (h^{-1})) for the release of glucose from DPPC:PI:DPPE-PEG 5000 liposomes as a function of mole% DPPE-PEG 5000 at (a) 25°C; (b) 37°C; and (c) 50°C. The open points were calculated by application of Eq. 1 and the solid points by application of Eq. 5.

somes release glucose more slowly. At 50°C, equilibrium is reached after approximately 2 h for all the liposomes. The data were fitted to Eq. 1 up to a period of 1 h using the kinetic fitting program 'Multi Fit' [22]. Fig. 3 shows the data fitted to Eq. 1 and the resulting values of L, K and α are tabulated in Table 2. The values of α, the loss of glucose before the start of the experiments, have an average value of $22.1 \pm 17.9\%$. Two values of the permeability coefficients were calculated, one based on a radius R, calculated from the measured weight-average diameter (d_w) and a bilayer thickness of 7.5 nm [23], and based on R_c in which the polymer layer thickness is taken into account (Eqs. 8 and 9 respectively). The values of P_R and P_{R_c} are given in Table 2. The polymer layer thickness (h_p) as a function of pegylated lipid was estimated from the data of Kuhl et al. [24] for the layer thickness of bilayer incorporating DSPE-PEG 2000. A factor 5/2 was used to take into account the longer PEG chain. The data of Kuhl et al. fit the relationship

$$h_p = 1.32c - 0.06c^2 \quad (10)$$

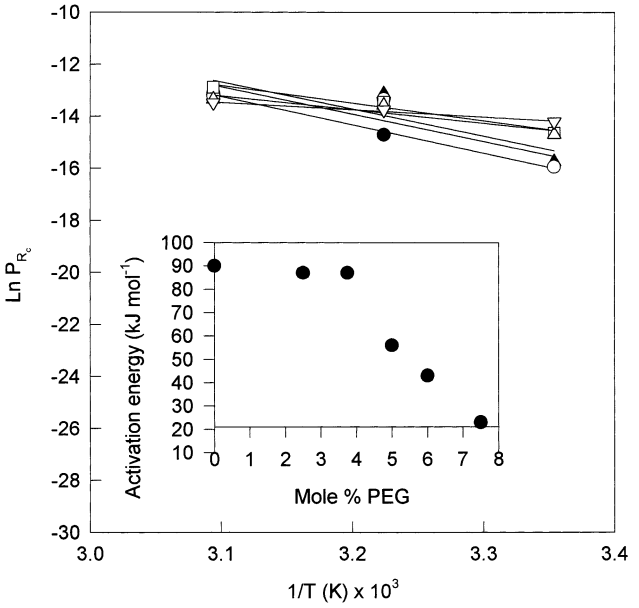

Fig. 5. Dependence of ln(permeability coefficient) on reciprocal temperature for DPPC:PI:DPPE-PEG 5000 liposomes. The liposome compositions (mole%) were as follows (DPPC:PI:DPPE-PEG 5000): ●, 91:9; ▲, 88.5:9:2.5; ○, 87.25:9:3.75; □, 86:9:5; △, 85:9:6; ▽, 83.5:9:7.5. The inset graph shows the activation energy for permeability as a function of mole% DPPE-PEG 5000.

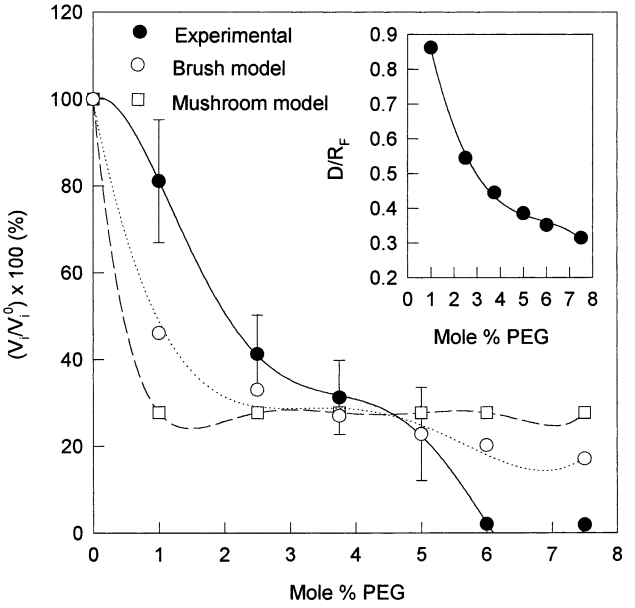

Fig. 6. Encapsulation of glucose in DPPC:PI:PEG 5000 liposomes as a function of mole% DPPE-PEG 5000 $(V_i/V_i^0) \times 100$ is the encapsulation expressed as glucose:lipid mass ratio as a percentage of that in liposomes containing no DPPE-PEG 5000. ●, experimental values (from Fig. 1); ○, calculated on the brush model; □, calculated on the mushroom model as described in the text. The inset curves show the ratio of the separation between graft points relative to the Flory radius (D/R_F) as a function of mole% DPPE-PEG 5000.

where c is the mole% of pegylated lipid. Also shown in Table 2 are the values of the bag permeabilities to free glucose, K, determined from independent experiments based on Eq. 4. The permeability coefficients are smaller when the polymer layer thickness is taken into account, especially at higher mole% pegylated lipid.

The permeability coefficients are plotted as a function mole% pegylated lipid in Fig. 4. Also shown in Fig. 4 are the permeability coefficients calculated from R_c and Eq. 5 which neglects leakage before the start of the experiment. Both methods of calculation give curves showing the same trends of permeability with pegylated lipid content. Permeability increases with both pegylated lipid content at 25°C and temperature.

4. Discussion

The dependences of the permeabilities of the lipo-

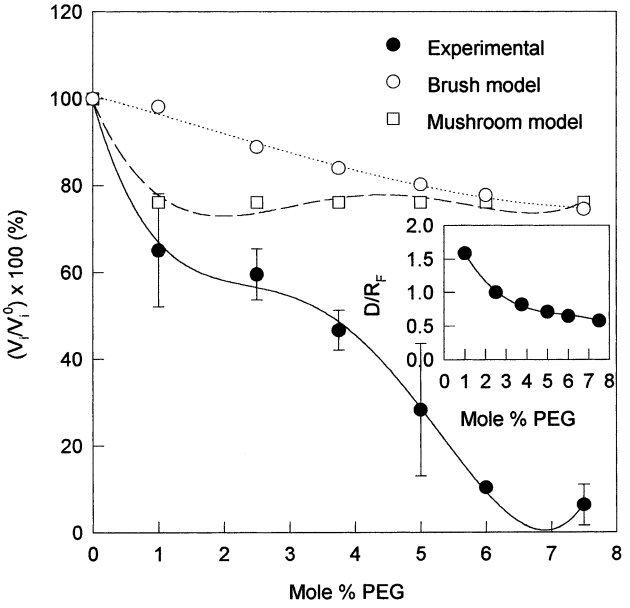

Fig. 7. Encapsulation of glucose in DPPC:PI:PEG 2000 liposomes as a function of mole% DPPE-PEG 5000 $(V_i/V_I^0)\times 100$ is the encapsulation expressed as glucose:lipid mass ratio as a percentage of that in liposomes containing no DPPE-PEG 2000. ●, experimental values (from Fig. 1); ○, calculated on the brush model; □, calculated on the mushroom model as described in the text. The inset curves show the ratio of the separation between graft points relative to the Flory radius (D/R_F) as a function of mole% DPPE-PEG 2000.

somes incorporating DPPE-PEG 5000 follow monotonous curves. These results are different from those found for liposomes incorporating DPPE-PEG 2000 where similar experiments showed maximum permeabilities at around 4 mole% DPPE-PEG 2000 in the region of the mushroom to brush chain conformation [15]. For a 5000 molecular mass PEG chain the transition from the mushroom to brush conformation is predicted to occur at approximately 1.7 mole% [13]. It is difficult to detect a transition at this low level of PEG where the permeability is not greatly affected by grafted PEG chains, although at 37°C (Fig. 4b) a maximum is observed in the region of 2–3 mole% and hence the results are consistent with the depression of the mushroom to brush transition to lower PEG levels.

The temperature dependence of the permeability coefficients can be related to an activation energy E_a by the equation [25]

$$\ln P(T) = -\frac{E_a}{RT} + C - \ln \sigma + \kappa\sigma \qquad (11)$$

where C and κ are constants independent of temperature and σ is the normalized surface density (A_0/A), where A is the area per lipid molecule and A_0 the area in the crystalline state. Plots of ln P vs. $1/T$ are shown in Fig. 5. All the data with the exception of 1 mole% PEG (not shown) give linear plots with regression coefficients from 0.781 (2.5 mole% PEG) to 0.993 (0 mole% PEG). The permeability data for 1 mole% PEG gave a curved plot of ln P_{R_c} vs. $1/T$, possibly because the PEG may be partially in the mushroom conformation. The activation energies range from 90 kJ mol^{-1} to 23 kJ mol^{-1} as the mole% PEG increases (inset plot Fig. 5). The decreasing activation energy for transport of glucose across the bilayer with increasing mole% PEG is consistent with an increase in bilayer disorder with the introduction of an increasing number of bilayer defects around the grafted lipids. The magnitude of the activation energies is comparable to values found from the temperature coefficients of the permeability coefficients of acetic acid for DPPC bilayers (37–25 kJ mol^{-1} [25]) and fluoride ions (64 ± 12 kJ mol^{-1} [26]).

The data in Fig. 1 show a very dramatic change in the encapsulation of glucose with pegylated lipid mole% which suggests that the PEG markedly restricts the free volume available inside the liposomes for carrying glucose. For DPPE-PEG 5000 VETs, over most of the composition range studied the PEG chains should be in the brush conformation and it is appropriate to consider how free volume varies with PEG mole% for both the mushroom and brush models. For the mushroom model the PEG Flory radius R_F is given by $R_F = aN^{3/5}$, where a is the monomer size (molecular mass 44) with persistence length (0.35 nm) and N the number of monomers per chain [13]. Then $R_F = 0.35\times 10^{-9}(5000/44)^{3/5} = 5.989$ nm. Assuming the mushrooms are close packed on the inside surface of the liposomes the free volume inside (V_i) the liposomes would then be given by

$$V_i = \frac{4}{3}\pi(R - h_b - 2R_F)^3 \qquad (12)$$

where R is the measured liposome radius (as given in Table 2). It should be noted that for a liposome of diameter 110 nm the number of close packed PEG 5000 mushrooms of radius R_F on the inside and out-

side of the liposome are 134 and 179 respectively. As the total number of lipid molecules (assuming a cross-sectional area of 0.5 nm^2 [23]) on the inside and outside of the liposome are 56 700 and 76 030 respectively, the maximum mole% PEG that could be in the mushroom conformation is 0.24 mole%, so that apart from small variations arising from changes in radius, the available free volume would be approximately constant above this mole% DPPE-PEG 5000.

For the brush model the brush length (L) is given by

$$L = aN\left(\frac{a}{D}\right)^{2/3} \quad (13)$$

where D is the separation between graft points given by (A/mole fraction PEG)$^{1/2}$, where A is the area per lipid molecule in the liposome (0.5 nm^2). For this model the free volume inside the liposome will be given by

$$V_i = \frac{4}{3}\pi(R - h_b - 2L)^3 \quad (14)$$

Fig. 6 shows the relative percentage encapsulation relative to 0 mole% PEG 5000 $(V_i/V_i^0)\times 100$ calculated by application of Eqs. 11 and 14 together with the experimental values for glucose encapsulation (Fig. 1a). The experimental data show a steep fall in the relative percentage encapsulation at low mole% PEG. Both the mushroom and brush models predict this, however as the inset shows the separation between graft points relative to the Flory radius (D/R_F) is less than unity. This requires all these data to relate to the brush regime, although the differences between the relative percentages encapsulation for the two conformational models are only small.

Fig. 7 shows similar data for liposomes incorporating DPPE-PEG 2000. For these systems D/R_F (inset) falls below unity only above approximately 2.5–3.5 mole%, so that at low mole% PEG the data relate to the mushroom regime. There is clearly a steep decrease in the relative percentage encapsulation above 3.5 mole% PEG, although both conformational models predict a greater encapsulation than experimentally observed. The origin of this disagreement is unclear; mixed micelles of pegylated phospholipid might occupy some space in the core of the liposomes, although we have no evidence of the presence of such micelles, that would elute at a greater elution volume than the liposomes, also mixed micelles would have been more likely to form PEG 5000 lipid than form PEG 2000 lipid.

The results demonstrate that PEG chains on the inside of vesicle bilayers can restrict encapsulation of water-soluble molecules. One way to avoid this problem might be to introduce the PEG lipid to preformed vesicles, such vesicles have been shown to have an extended life time in the circulation [27].

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