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The effect of surface-bound protein on the permeability of proteoliposomes

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Proteoliposomes have been prepared from mixtures of dipalmitoylphosphatidylcholine and phosphatidylinositol by sonication (SUV) and reverse phase evaporation (REV) and conjugated with succinyl concanavalin A (sConA). The proteoliposomes were characterised in terms of size and composition and covered a range of size (weight-average diameter) from approx. 80 to 300 nm and surface-bound sConA (weight-average number of protein molecules per liposome) from approx. 200 to 1800. The permeabilities of the proteoliposomes to encapsulated D-glucose have been measured and found to increase linearly with protein conjugation. The D-glucose permeability also increases with temperature and passes through a maximum in the region of the gel to liquid-crystalline phase transition temperature. Conjugation has no effect on the chain-melting temperature but slightly decreases the enthalpy of the transition consistent with the withdrawal of some phospholipid participation in chain-melting. The D-glucose permeabilities and thermotropic properties of the proteoliposomes are discussed in terms of the dislocation of the bilayer by the possible off-axis motion of the lipid which anchors the protein to the liposomal surface.

Introduction

In recent studies we have described the preparation and characterisation of proteoliposomes in which the lectin, wheat germ agglutinin was convalently conjugated to the liposomal surface [1]. These liposomes target to a model biosurface of adsorbed glycophorin A [2] and have been shown to retain their structural integrity on adsorption [3]. Proteoliposomes with site-directing properties conferred by surface-bound lectins or antibodies have potential uses as drug-delivery vehicles [4,5] and in this context the permeability characteristics of the proteoliposomes are important. It was demonstrated by Blok et al. [6] that the permeability of liposomes to water-soluble solutes depends on the composition of the phospholipid bilayer and increases markedly in the region of the gel to liquid-crystalline transition temperature. This property was utilized in the development of temperature-sensitive liposomes [7,8] which can be designed to release their encapsulated contents at specific temperatures. It would be expected that the anchoring of protein to a liposomal surface by conjugation to a phospholipid or an acyl chain such as palmitoyl [9] would have some effect on the permeability characteristics of the liposome. The incorporation of glycophorin in dioleoylphosphatidylcholine liposomes increased bilayer permeability [10] and there have also been reports that addition of globular proteins such as lysozyme and insulin to liposome suspensions results in increased glucose permeability [11,12].

In this study we have examined the effect of covalently linking succinyl concanavalin A (sConA) to the surface of liposomes on their permeability characteristics using D-glucose as the entrapped permeant. For these proteoliposomes it is possible to control the surface density of the protein on the liposome and hence relate this parameter to the permeability.

Materials and Methods

Succinyl concanavalin A (sConA) product No. L3885 was obtained from Sigma Chemical Company, Poole, Dorset, U.K. D-[U-^{14}-C]Glucose (specific activity, 270

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mCi/mmol) product No. CFB.96 was from Amersham International, U.K. All the other materials were as previously described [1–3].

Preparation of proteoliposomes. Vesicles (liposomes) were prepared by sonication (SUV) and reverse phase evaporation (REV) from mixtures of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol (from wheat germ) (weight ratio 9:1) incorporation 0–14 mol% of the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoylphosphatidylethanolamine (PEMBS) and tracer levels of [^{3}H]DPPC. The liposomes were prepared in phosphate-buffered saline (PBS) (pH 7.4) and [^{14}C]glucose was encapsulated when the lipid film was initially dispersed at a level of 20 μCi per 5 ml of lipid dispersion for SUV and 10 μCi per 3 ml of lipid dispersion for REV, each lipid dispersion contained a total of 11 mg of lipid. The unencapsulated [^{14}C]glucose was removed by gel filtration using a Sephadex G-200 column (30 × 2 cm) previously equilibrated with PBS, at a flow rate of 0.2 ml min^{-1}. The liposomes containing encapsulated [^{14}C]glucose eluted in the void volume. Fractions (2 ml) were collected and aliquots (100 μl) counted by scintillation counting for [^{3}H]DPPC. The liposome fractions containing the highest ^{3}H counts were used for conjugation with derivatised sConA.

The sConA was derivatised with *N*-succinimidyl-*S*-acetylthioacetate (SATA) [13,14] routinely using a molar ratio of SATA to sConA of 10:1. Specifically 2.5 μl of a stock solution of 9.08 mg SATA in 50 μl of dimethylformamide was added to sConA (10 mg in 2.5 ml phosphate (50 mM)-EDTA (1 mM) buffer (pH 7.5)) at room temperature. After reaction (approx. 15 min) the derivatised sConA was separated from unreacted SATA by gel filtration on a Sephadex G-50 column (15 × 2 cm). Fractions were collected (2 ml) and both derivatised sConA and unreacted SATA detected spectrophotometrically at 280 nm and the protein was assayed by a Lowry microassay [15] using sConA as the standard. The derivatised sConA fractions were pooled and stored at 4°C. The extent of derivatisation was determined after deacylation of the sulphydryl groups of the SATA by reaction with hydroxylamine hydrochloride (200 μl of a stock solution containing 0.1 M hydroxylamine plus 2.5 mM EDTA and solid Na_2HPO_4 to pH 7.5). The sulphydryl content of the deacylated derivative was determined by the method of Ellman [16]. This procedure resulted in derivatised sConA with 1.21 ± 0.05 -SH groups per protein molecule (molecular weight 51 000 [17]). For conjugation with the liposomes the derivatised sConA was mixed with liposome fractions in appropriate proportions and reacted at room temperature for 2 h or overnight at 4°C after which the reaction mixture was applied to a Sepharose 4B column to separate the proteoliposomes from unreacted protein. The lipid and protein content of the proteoliposomes were determined from the [^{3}H]DPPC counts and Lowry assay, respectively, the size distribution of the liposomes was determined by photon correlation spectroscopy using a Malvern autosizer and these data were combined to calculate the weight-average numbers of protein molecules per liposome ($\bar{P}_w$) as previously described [1].

Permeability measurements. The permeability of the proteoliposomes was determined from the release of encapsulated [^{14}C]glucose by a method similar to that of Johnson and Bangham [18]. The proteoliposome suspensions (1 ml) were placed in a dialysis bag made from Spectrapor membrane tubing (No 1, molecular weight cut-off 6000–8000, Spectrum Medical Industries, Inc., Los Angeles) and incubated at the desired temperature in 10 ml of PBS with stirring. Aliquots (100 μl) were removed from the external medium for scintillation counting ^{14}C at time intervals.

If the internal volume of the proteoliposomes is v_c and they contain N counts, the bag volume is $v_1 (\gg v_c)$ and the external volume is v_2, then the rate of appearance of counts (n_2) into the medium total volume, $v_o = (v_1 + v_2)$ from the proteoliposomes is given (Ref. 18) by

$$\frac{dn_2}{dt} = p\left(\frac{N - n_2}{v_c} - \frac{n_2}{v_o}\right) \quad (1)$$

where p is the proteoliposome permeability (volume t^{-1}). Since $n_2 v_c \ll n_2 v_o$

$$\frac{dn_2}{dt} = \frac{p}{v_c}(N - n_2) \quad (2)$$

On integration

$$\ln\left(1 - \frac{n_2}{N}\right) = -\frac{p}{v_c} \cdot t \quad (3)$$

If $(dpm)_o$ is the count rate outside the dialysis bag (volume 10 ml) then the count rate outside the proteoliposomes is $(dpm)_o \times 11/10$ since the bag volume is 1 ml. If the initial count rate for the proteoliposome suspension is $(dpm)_i$ then Eqn. 3 can be written as

$$\ln\left(1 - \frac{(dpm)_o}{(dpm)_i} \cdot \frac{11}{10}\right) = -\frac{p}{v_c} \cdot t \quad (4)$$

This assumes that there is no leakage of radiolabel in the short time interval (30 min) between taking an aliquot of proteoliposomes off the final gel filtration column and setting-up the suspension in the dialysis bag. It is also assumed that the dialysis bag is not a permeability barrier to [^{14}C]glucose. The left hand side of Eqn. 4 is a linear function of time and has slope p/v_c. The permeability coefficients (P) in cm h^{-1} were

calculated from the slopes of the semi-log plots based on Eqn. 4 and the proteoliposome sizes as follows

$$P = \frac{p}{\text{liposome area}} = \frac{\text{slope } v_c}{a} \quad (5)$$

$$P = \frac{\text{slope}}{4\pi R^2} \tfrac{4}{3}\pi(R-h)^3 = \text{slope}\frac{(R-h)^3}{3R^2} \quad (6)$$

where R is the radius of the proteoliposome (in cm) and h the bilayer thickness (in cm). R was taken as half the measured weight-average diameter ($\bar{d}_w/2$) and h as $75 \cdot 10^{-8}$ cm [19].

Differential scanning calorimetry (DSC). The thermal characteristics of liposomes (REV) and proteoliposomes (REV) with different amounts of covalently bound sConA were examined by DSC using a Perkin-Elmer DSC-4 coupled to a system 4 thermal analysis microprocessor controller, data system and interface. The calorimeter was calibrated with indium. Liposomes and proteoliposomes were prepared and pelleted by centrifugation for 1 h at 40000 rpm ($100000 \times g$) at 20°C. The supernatant was removed and 5–10 mg of pellet accurately weighed and sealed in aluminium DSC pans. The samples were scanned using a heating rate of 5 C° min^{-1}. After data acquisition the pans were carefully cut-open and left overnight in 1% (w/v) sodium *n*-dodecylsulphate solution to disperse the contents. Aliquots (100 µl) were then taken for counting ^3H and the counts used to calculate the amount of lipid present with reference to a lipid standard.

Results

The extent of conjugation of sConA on the proteoliposome surface can be controlled by varying the amount of the reactive lipid in the initial liposome formulation. Fig. 1 shows the dependence of mass of protein per mole of lipid in proteoliposomes covering a range of weight-average diameters ($\bar{d}_w$) from approx. 70 to 250 nm. The curve is linear which shows that the surface density of protein is independent of proteoliposome size as was found for proteoliposomes with surface bound wheat germ agglutinin [1]. For proteoliposomes of the same size (selected from appropriate fractions after gel filtration) the weight-average number of sConA molecules per proteoliposome ($\bar{P}_w$) also increases linearly with mol% PEMBS (Fig. 2).

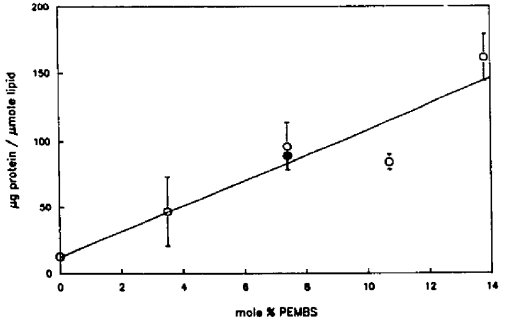

Fig. 1. Extent of conjugation of succinyl concanavalin A (sConA) to dipalmitoylphosphatidylcholine (DPPC)-phosphatidylinositol (PI) (mole ratio 10.4:1) liposomes as a function of mol% PEMBS. ●, SUV; ○, REV. The weight-average diameter ($\bar{d}_w$) of the proteoliposomes was 150–250 nm (REV) and 67 nm (SUV).

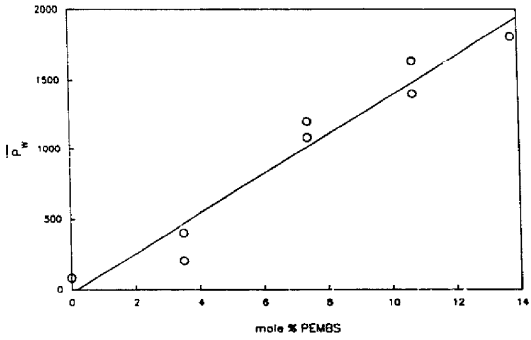

Fig. 2. The dependence of the weight-average number of succinyl concanavalin A (sConA) molecules per liposome ($\bar{P}_w$) on the mol% PEMBS in dipalmitoylphosphatidylcholine (DPPC)-phosphatidylinositol (PI) (mole ratio 10.4:1) liposomes of weight-average diameter ($\bar{d}_w$) of 217 ± 20 nm.

Fig. 3 shows the % leakage of [^{14}C]glucose from several types of liposome at 20°C and 37°C. The corresponding semilogarithmic plots based on Eqn. 4 are shown in Fig. 4. The % leakage is less than 5% from DPPC-PI (mole ratio 10.4:1) REV with permeability coefficient $(0.08 \pm 0.02) \cdot 10^{-7}$ cm h^{-1}, DPPC-PI-PEMBS (mole ratio 10.4:1:0.91, 7.4 mol% PEMBS) REV with permeability coefficient $(0.12 \pm 0.02) \cdot 10^{-7}$

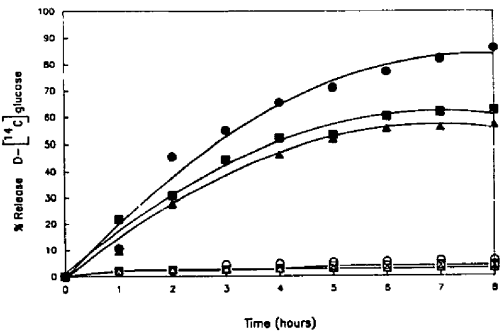

Fig. 3. Percentage release of D-[^{14}C]glucose as a function of time from dipalmitoylphosphatidylcholine (DPPC)-phosphatidylinositol (PI) (mole ratio 10.4:1) liposomes. Open symbols 20°C, filled symbols 37°C. △, ▲, DPPC-PI; □, ■, DPPC-PI plus 13.8 mol% PEMBS; ○, ●, DPPC-PI-PEMBS (13.8 mol%) conjugated with succinyl concanavalin A ($\bar{P}_w = 1807$).

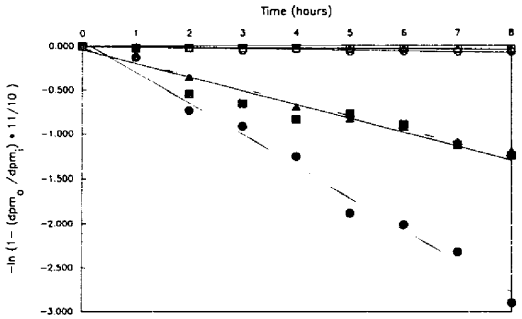

Fig. 4. Semilogarithmic permeability plot based on Equation 4 of the data in Fig. 3. Symbols etc. as Fig. 3.

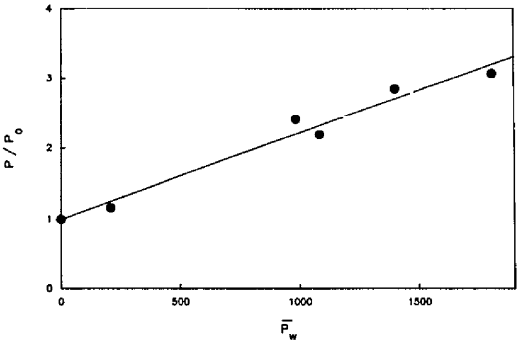

Fig. 5. Dependence of the relative permeability of proteoliposomes with surface-bound concanavalin A (P/P_o) on $\bar{P}_w$.

cm h^{-1} and REV with surface bound sConA ($\bar{P}_w = 1807$, permeability coefficient $(0.24 \pm 0.03) \cdot 10^{-7}$ cm h^{-1}) at 20 °C, but increases markedly at 37 °C. Thus at both temperatures addition of PEMBS to DPPC-PI liposomes increases the % leakage of [^{14}C]glucose and it is further increased on conjugation with sConA although at 20 °C the differences are very small.

Table I shows characterisation data and permeability coefficients of [^{14}C]glucose at 20 °C for liposomes and proteoliposomes with surface-bound sConA. For liposomes with weight-average diameters up to approx. 200 nm the permeability coefficients are independent of liposome size. The permeability coefficient is an intrinsic property of the liposomal bilayer and would be expected to be independent of liposome size in the absence of effects of curvature. On conjugating sConA the permeability coefficients increase with $\bar{P}_w$. Fig. 5 shows the dependence of the relative permeabilities coefficient $P/P(\bar{P}_w = 0)$ on $\bar{P}_w$ and demonstrates that there is a linear increase in relative permeability on increasing the number of surface-bound sConA molecules.

The temperature dependence of liposome and proteoliposome permeabilities is shown in Fig. 6 for both SUV and REV. These liposomes and proteoliposomes have the same lipid composition and differ only in size and

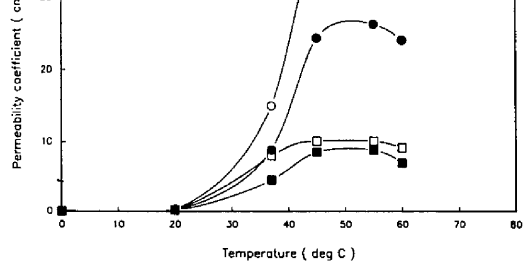

Fig. 6. Effect of temperature on the permeability coefficients of D[^{14}C]glucose for SUV and REV liposomes and proteoliposomes. ■, SUV (DPPC-PI-PEMBS, mole ratio 10.4:1:0.91); □, SUV proteoliposomes (DPPC-PI-PEMBS-sConA, mole ratio 10.4:1:0.91, $\bar{P}_w$ = 800); ●, REV (DPPC-PI-PEMBS, mole ratio 10.4:1:0.91); ○, REV proteoliposomes (DPPC-PI-PEMBS-sConA, mole ratio 10.4:1:0.91, $\bar{P}_w$ = 1084). The weight average diameter ($\bar{d}_w$) was 78 ± 8 nm (SUV) and 198 ± 38 nm (REV).

TABLE I

Characterisation and permeability coefficients of D-glucose at 20 °C for liposomes and proteoliposomes with surface-bound succinyl concanavalin A

Liposome composition (mole ratio)	$\bar{d}_w$ (nm)	σ_w (nm)	$\bar{P}_w$	$10^7 P$ (cm h^{-1})
DPPC-PI (10.4:1)	80	40	0	0.15 ± 0.009
DPPC-PI (10.4:1)	169	91	0	0.12 ± 0.011
DPPC-PI (10.4:1)	187	118	0	0.14 ± 0.008
DPPC-PI (10.4:1)	309	200	0	0.26 ± 0.010
DPPC-PI-PEMBS (10.4:1:0.41)	210	132	209	0.16 ± 0.012
DPPC-PI-PEMBS (10.4:1:0.91)	211	137	984	0.33 ± 0.012
DPPC-PI-PEMBS (10.4:1:0.91)	203	112	1084	0.30 ± 0.050
DPPC-PI-PEMBS (10.4:1:1.36)	245	142	1400	0.39 ± 0.040
DPPC-PI-PEMBS (10.4:1:1.83)	211	106	1807	0.42 ± 0.040

TABLE II

Characterisation and thermal properties of liposomes and proteoliposomes with surface-bound succinyl concanavalin A

Liposome composition (mole ratio)	$\bar{d}_w$ (nm)	σ_w (nm)	$\bar{P}_w$	T_c (K)	ΔH (kJ mol^{-1})
DPPC	436	298	0	39.4	33.6
DPPC-PI (10.4:1)	204	130	0	39.4	33.9
DPPC-PI-PEMBS (10.4:1:0.91)	261	175	0	40.7	30.2
DPPC-PI-PEMBS (10.4:1:0.91)	459	294	741	41.0	30.4
DPPC-PI-PEMBS (10.4:1:0.91)	306	188	815	41.0	27.4
DPPC-PI-PEMBS (10.4:1:0.91)	320	189	992	41.0	27.7

hence in the case of the proteoliposomes in the amount of surface bound sConA. The permeability coefficients increase with temperature and pass through a maximum in the region of the phase transition of DPPC. The dependence of the enthalpy and temperature of the gel to liquid-crystalline phase transition (transition peak maximum) on the extent of conjugation of proteoliposomes are shown in Table II. The enthalpy of the gel to liquid-crystalline phase transition of DPPC and DPPC-PI liposomes is in good agreement with literature values [20]. Introduction of the reactive lipid (PEMBS) reduces the transition enthalpy as do high levels of protein conjugation although the effects are not large.

Discussion

It is well established and further confirmed from the permeability data in Fig. 4 that diffusion of a hydrophilic permeant such as D-glucose through the lipid bilayer is facilitated in the region of the phospholipid chain-melting temperature. This suggests that the permeability is greatest when both gel ($L_{\beta'}$, $P_{\beta'}$) phase and the liquid-crystalline phase (L_{α}) coexist implying that bilayer dislocations enhance diffusion. In the gel phase ($L_{\beta'}$) at 20 °C the permeabilities of D-glucose increase with increase in the number of sConA molecules covalently bound to the liposomal surface (Fig. 3). In relative terms the increase up to 3-times that of 'naked' liposomes is large although the absolute values of the permeabilities are small relative to the values in the liquid-crystalline phase.

The absolute values of the permeability coefficients of liposomes (Table I) are small compared with the permeability coefficients of D-glucose through planar bilayer membranes for which values of the order of $(2–10) \cdot 10^{-8}$ cm s^{-1} have been reported [21–23] compared with the values here for protein-free liposomes of the order of $5 \cdot 10^{-12}$ cm s^{-1}. Several factors might contribute to the smaller values of the permeability coefficients of liposomes relative to planar bilayers including bilayer curvature which will restrict the transport of D-glucose across the inner monolayer of the bilayer and the phospholipid composition (the measurements on planar bilayers relate to total erythrocyte lipid extracts and egg phosphatidylcholine-cholesterol mixtures). The use of weight-average dimensions in the calculations (Eqn. (6) will bias the data to larger values although this would increase the permeability coefficients by less than an order of magnitude.

Perhaps the most significant question raised by these observations is the extent to which a surface bound protein covalently linked to a bilayer lipid dislocates the packing of the bilayer lipids in its immediate environment and how such dislocation is brought about. The size of the protein (molecular weight 55 000) relative to the lipid (PEMBS, molecular weight 922) suggests that the thermal motion of the protein molecule will cause the lipid anchor to move within the bilayer. It has been demonstrated that restricted rotational movement of membrane proteins which are partially embedded in the bilayer results in off-axis motion or 'wobbling in a cone' [24]; motion of this type would, in the case of a lipid anchor, create free volume in the bilayer and hence a possible pathway for permeants.

The extent of coverage of the liposomal surface by covalently-linked protein can be estimated from the dimensions of protein and liposome. The diffusion coefficient of ConA in water at 20 °C is $5.43 \cdot 10^{-11}$ m^2 s^{-1} [25] which gives an equivalent hydrodynamic radius (R) of 3.95 nm (using the Stokes equation $D = kT/6\pi\eta R$, with $\eta = 10.02 \cdot 10^{-4}$ N m^{-2} s) and a projected area (πR^2)of $4.89 \cdot 10^{-17}$ m^2. Using this projected area, for the proteoliposome dimensions given in Table I and the values of $\bar{P}_w$ in the range 984–1635 it follows that the protein covers approx. 34–40% of the liposomal surface. However, the influence of the protein on the underlying bilayer is largely restricted to the immediate environment of the phospholipid anchoring the protein. The enthalpy of the gel to liquid-crystalline phase transition is slightly decreased by surface bound protein due to the withdrawal of phospholipid from participation in the phase transition. For example, for proteoliposomes with $\bar{P}_w = 992$, $\bar{d}_w = 320$ nm (Table II) the number (N) of phospholipid molecules per protein molecule withdrawn from participation in the gel to liquid-crystalline phase transition can be estimated from the relation [26]

$$\Delta H = \Delta H_0 \left(1 - \frac{[\text{sConA}]}{[\text{Lipid}]} N\right) \quad (7)$$

where ΔH_0 is the enthalpy of the transition in 'naked' liposomes. For these proteoliposomes ($\bar{P}_w = 992$) the molar ratio [sConA]/[Lipid] is $1.37 \cdot 10^{-3}$ and hence $N \cong 60$. Taking the area per lipid molecule in the liposomal bilayer as 0.50 nm^2 [27] for a proteoliposome with $\bar{d}_w = 320$, the surface area is $3.22 \cdot 10^{-13}$ m^2 and the fraction of the surface occupied by lipids withdrawn from the gel to liquid crystalline phase transition by the dislocation caused by the lipid anchors will be $(60 \times 0.50 \cdot 10^{-18}/3.22 \cdot 10^{-13}) \times 992 = 0.092$ or approx. 9%. Thus, this value is significantly less than the approx. 40% of the surface covered by protein and is consistent with the location of the protein projecting from the liposomal surface into the aqueous phase and causing only local dislocations in the bilayer around the phospholipid anchor.

In summary the results demonstrate that the permeability coefficients of proteoliposomes depend on the amount of surface-bound protein due to the dislocations introduced into the bilayer by the lipid molecules which anchor the protein, so creating free volume avail-

able for the passage of encapsulated solutes. Proteoliposomes become more permeable in the region of the phospholipid chain melting temperature, in this context it is noteworthy in relation to drug delivery by liposomes, that interactions between serum components, particularly high density lipoproteins result in enhanced release of encapsulated solutes in the region of the lipid phase transition temperature [28,29].

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