Biochimica et Biophysica Acta, 978 (1989) 17–24
Elsevier

BBA 74256

The characterisation of liposomes with covalently attached proteins

Fiona J. Hutchinson [1], Sheila E. Francis [1], Ian G. Lyle [2] and Malcolm N. Jones [1]

[1] *Biomolecular Organisation & Membrane Technology Group, Department of Biochemistry & Molecular Biology, University of Manchester, Manchester and* [2] *Unilever Research, Port Sunlight Laboratory, Bebington, Wirral, Merseyside (U.K.)*

(Received 2 August 1988)

Key words: Proteoliposome; Photon correlation spectroscopy; Wheat germ agglutinin; Liposome size distribution

The problem of characterising liposomes with covalently attached proteins has been analysed theoretically in terms of a normal weight distribution of liposome diameters. The polydispersity of protein conjugation is considered in terms of the width (standard deviation) of the liposome size distribution. It is shown that the weight-average number of proteins per liposome is a convenient parameter to use to define the protein content of proteoliposomes. Two types of proteoliposome have been prepared (small unilamellar vesicles and reverse phase evaporation vesicles) in which wheat germ agglutinin is covalently coupled to the liposomal surface. The liposomes cover a range of weight average diameter from 65 to 240 nm and of polydispersity (weight to number average diameter ($\bar{d}_w/\bar{d}_n$) from 2.6 to 11.4. The liposomes have been characterised by chemical analysis and photon correlation spectroscopy and the results are discussed in terms of the theoretical consequences of an equivalent normal weight distribution of diameters.

Introduction

The use of liposomes as carriers of drugs, vaccines and other therapeutic agents [1–3] has led to numerous studies concerned with the covalent coupling of antibodies and other proteins to their surfaces [4–21]. The objective of many of these studies is to increase the affinity of the liposomes for specific target sites and hence to enhance the efficiency of liposomes as a delivery system. Numerous factors might affect the efficiency of targeting of such systems to a particular site; clearly the type of liposome, its size and lipid composition and the liposomal surface density and nature of the covalently-linked site-directing macromolecule will all be of importance. However, it is clear from the literature that there are very wide variations in the extents of protein coupling found by different workers. Table I summarises results from some of the recently reported studies. While different chemical methods and the nature of the protein might well lead to variations in the degree of coupling it is clear that in terms of the mass of protein coupled per mole of liposomal lipid the range is extremely wide (approximately four orders of magnitude taking the extreme values and at least one order of magnitude taking more conservative limits). The conversion of the protein to lipid composition into a figure for the number of protein molecules per liposome is critically dependent on a detailed knowledge of the size of the liposome. Some authors have refrained from quoting a figure but as Table I shows the quoted figures for LUV and REV of comparable size cover two orders of magnitude.

The difficulties of characterising such systems become apparent when consideration is given to the problem of size and particularly size distribution in the context of defining the extent of protein coupling to liposomes. The object of this paper is to address this problem theoretically and to present some experimental characterisation data on the coupling of a lectin (wheat germ agglutinin) to phospholipid liposomes. We have used photon correlation spectroscopy for characterising the liposomes in terms of size and size distribution and while the theoretical analysis is directly appropriate to this technique it nevertheless illustrates some of the difficulties which will be encountered using any method of size and size distribution analysis.

Abbreviations: SUV, small unilamellar vesicles; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; REV, reverse phase evaporation vesicles.

Correspondence: M.N. Jones, Biomolecular Organisation & Membrane Technology Group, Department of Biochemistry & Molecular Biology, University of Manchester, Manchester, M13 9PT, U.K.

Theoretical background

In photon correlation spectroscopy (PCS) or dynamic light scattering, use is made of the Doppler shifts in frequency of scattered light arising from Brownian motion to determine the translational diffusion coefficients of the scattering particles by autocorrelation techniques. For a monodisperse system the autocorrelation function is related to the diffusion coefficient by a single exponential term. However, for a polydisperse system there will be a distribution of diffusion coefficients but unfortunately there is no reliable way of transforming the correlation function which for a polydisperse system is a sum of exponentials into a distribution of diffusion coefficients [22]. The correlation function can be related to the z-average diffusion coefficient defined as

$$\bar{D}_z = \frac{\sum_i n_i M_i^2 D_i}{\sum_i n_i M_i^2} \tag{1}$$

where n_i, M_i and D_i are the number, mass and diffusion coefficient of a given species i. For spherical particles obeying Stokes law the diffusion coefficient can be simply related to particle diameter (d) by the relation $D = kT/3\pi\eta d$ where k, T and η are the Boltzmann constant, absolute temperature and solvent viscosity, respectively. In the system we have used (Malvern Autosizer) the observed scattering is fitted to a normal weight distribution of particle sizes which would give equivalent scattering. The equivalent normal weight distribution gives a measure of the degree of polydispersity in the system.

The normal weight distribution $W(d)$ of particle diameters (d) can be written as follows [23].

$$W(d) = \frac{1}{\sigma_w\sqrt{2\pi}} \exp\left(\frac{-(d-\bar{d}_w)^2}{2\sigma_w}\right) \tag{2}$$

where $\bar{d}_w$ is the median value of the distribution and σ_w the standard deviation. The normal distribution is a bell-shaped curve identical with the Gaussian law of errors [24]. The corresponding number distribution of diameters $N(d)$ is related to $W(d)$ by the equation

$$N(d) = W(d)/\frac{\rho\pi d^3}{6} \tag{3}$$

where ρ is the particle density. The number distribution is less symmetrical than a normal distribution and if the weight distribution only slightly overlaps the $d = 0$ axis then the distribution is very unsymmetrical with $N(d)$ rising steeply as $d \to 0$. This behaviour is illustrated in Fig. 1 for two values of σ_w.

For liposome dispersions, given an equivalent normal weight distribution and the related number distribution, the calculation of the number of coupled proteins per liposome is critically dependent on the type of averaging used unless the standard deviation σ_w of the weight distribution is very small. To a first approximation, if we assume that the molar ratio of protein to lipid (P/L) in the liposomes is not a function of liposome size, then the distribution of the number of proteins per liposome of diameter d can be calculated for both the equivalent normal weight and corresponding number size distributions. From the distributions both the weight-average

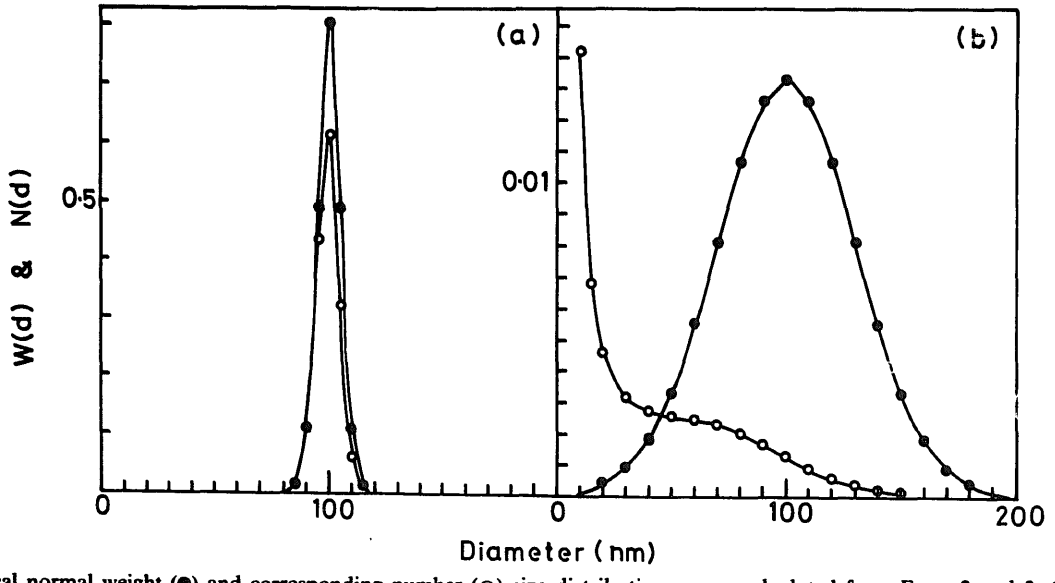

Fig. 1. Theoretical normal weight (●) and corresponding number (○) size distribution curves calculated from Eqns. 2 and 3. (a) Mean particle diameter 100 nm, S.D. 5 nm. (b) Mean particle diameter 100 nm, S.D. 30 nm.

TABLE I

Characterisation of proteoliposomes

SUV, small unilamellar vesicles; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; REV, reverse phase evaporation vesicles.

Liposome type	Protein	Liposome size	Protein/lipid ratio (μg/μmol)	No. of proteins/ liposome	Ref.
SUV	Monoclonal IgM	-	340-675	-	4
SUV	IgG	44-80	20	-	5
SUV	Horse-radish peroxidase	25-50	200-250	-	6
MLV	Egg albumin	-	39.7±7.5	-	7
MLV	Tetanus toxoid	-	20-50	-	8
LUV	Bovine-γ-globulin	400	300	-	9
LUV	Bovine serum albumin	-	4	-	10
LUV	Monoclonal H2K^{k}IgG	40-200	-	12-15	11
LUV	Pullulan-IgMs fragment	109	0.032	35	12
LUV	Monoclonal H-2^k IgG	90	-	67	13
LUV	G(IgG)	100	~ 74	48	14
REV	IgG	200	100-200	200	15
REV	IgG	-	290	-	16
REV	Fab' fragment	200	70-584	> 3000	17
REV	RbIg	200	180-200	-	18
REV	Horse-radish peroxidase	-	65	-	6
REV	Fab' fragment	50-500	100-600	up to 6000	19
Dialysis	Asialofetuin	450-500	34-68	-	20

number ($\bar{P}_w$) and the number-average number ($\bar{P}_n$) of proteins per liposome can be defined by the equations

$$\bar{P}_w = \frac{\sum_i P_i w_i}{\sum_i w_i} = \frac{\sum_i P_i W(d_i)}{\sum_i W(d_i)} \quad (4)$$

$$\bar{P}_n = \frac{\sum_i P_i n_i}{\sum_i n_i} = \frac{\sum_i P_i N(d_i)}{\sum_i N(d_i)} \quad (5)$$

where P_i, w_i and n_i are the number of proteins per liposome, weight and number of liposomes of species, i, respectively. P_i can be calculated from the molar ratio of protein to lipid (P/L) and the number of lipid molecules per liposome (N^L). For single-walled spherical liposomes of radius R_i, bilayer thickness h, in which the area per lipid molecule in the bilayer is a^L, N_i^L is given by

$$N_i^L = \frac{4\pi}{a^L} R_i^2 + \frac{4\pi}{a^L}(R_i - h)^2 = \frac{4\pi}{a^L} h(2R_i - h) \quad (6)$$

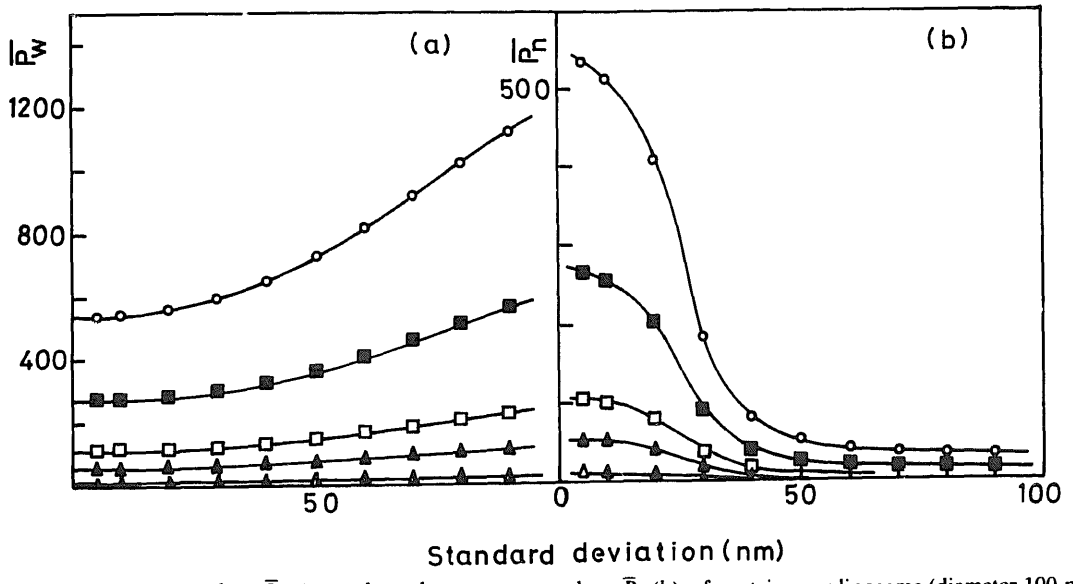

Fig. 2. Theoretical weight average number, $\bar{P}_w$ (a), and number average number, $\bar{P}_n$ (b), of proteins per liposome (diameter 100 nm) as a function of the standard deviation of the normal weight distribution of liposome diameters (σ_w) and the molar ratio of protein to lipid. The protein to lipid molar ratios are as follows: △, $1 \cdot 10^{-4}$; ▲, $5 \cdot 10^{-4}$; □, $1 \cdot 10^{-3}$; ■, $2.5 \cdot 10^{-3}$; ○, $5 \cdot 10^{-3}$.

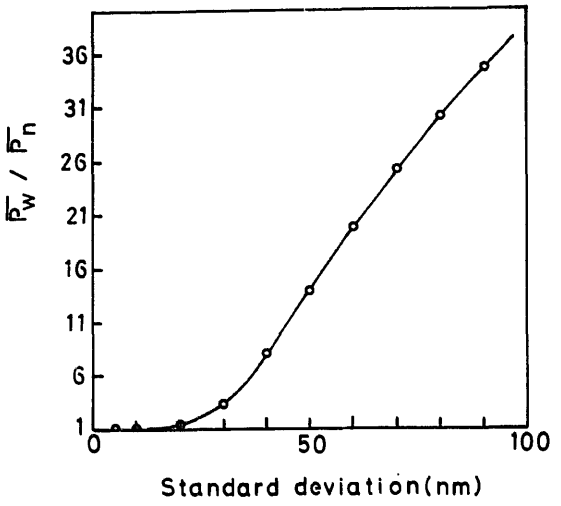

Fig. 3. Theoretical weight to number average number of proteins per liposome (diameter 100 nm), $\bar{P}_w/\bar{P}_n$, as a function of the standard deviation of the normal weight distribution of liposome diameters (σ_w). This curve is independent of the protein-to-lipid molar ratio.

It has been assumed here that the area per lipid molecule in the liposomal bilayer (a^L) is 0.50 nm^2 [25] and the bilayer thickness is 7.5 nm [26]. Thus $P_i = (P/L)\, N_i^L$.

The dependence of $\bar{P}_w$ and $\bar{P}_n$ on the standard deviation of the equivalent normal weight distribution of liposome diameters are shown in Fig. 2 for a range of values of (P/L). In Fig. 2a it is seen that $\bar{P}_w$ increases with σ_w particularly for high (P/L) values although not dramatically. In contrast $\bar{P}_n$ not only decreases with σ_w but is extremely sensitive to distribution width. The dependence of the ratio of $\bar{P}/\bar{P}_n$ on σ_w is independent of (P/L) as shown in Fig. 3. The great sensitivity of $\bar{P}_n$ to the distribution width is of course a consequence of the rise in $N(d)$ at low d as σ_w increases.

These theoretical calculations illustrate the difficulty of characterising proteoliposomes in terms of the number of proteins per liposome unless the size distribution is very narrow. The wide range of values of protein per liposome found in the literature might in part be related to the problems of obtaining protein conjugated liposomes with narrow size distributions. On the basis of Fig. 2 it would seem reasonable to suggest that the weight-average number of proteins per liposome ($\bar{P}_w$) is a sensible parameter to use, firstly because photon correlation data can be used to derive the equivalent normal weight distribution of size and secondly because it is much less dependent on σ_w than is $\bar{P}_n$.

Materials and Methods

L-α-Dipalmitoylphosphatidylethanolamine (DPPE) product No. P-0890, L-α-dipalmitoylphosphatidylcholine (DPPC) product No. P-0763 and wheat germ agglutinin (from *Triticum vulgaris*) product No. L-9640, were obtained from Sigma Chemical Company, Poole, Dorset, U.K. *N*-Succinimidyl-*S*-acetylthioacetate (SATA) was from Calbiochem, Cambridge, U.K. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was from Pierce Warriner Ltd., Chester, U.K. Phosphatidylinositol (PI) Grade I was from Lipid Products, South Nutfield, Surrey, U.K. ^{3}H-DPPC was from Amersham International, U.K. The organic solvents, chloroform and methanol were of Analar grade (BDH, Poole, Dorset, U.K.) and were distilled from sodium metabisulphite and stored over molecular sieves (type 4A, Fisons plc, Loughborough, U.K.). All inorganic reagents were of analytical grade and aqueous solutions were made up with double distilled water.

Derivatisation of WGA with SATA [27]. The SATA derivative of WGA was prepared by addition of SATA (0.69 to 6.9 μmol in 50 μl dimethylformamide) to WGA (0.28 μmol in 2.5 ml of phosphate (50 mM)-EDTA (1 mM) buffer (pH 7.5)) at room temperature. The solution always contained less than 10 μl of dimethylformamide per ml. The reaction was complete in 15 min after which time derivatized WGA was separated from unreacted SATA by gel filtration on a Sephadex G-50 column (15 × 2 cm). Fractions (2 ml) were collected and the WGA fractions (detected at A_{280nm}) were pooled and stored at 4°C. The extent of derivatisation of the WGA was determined after deacetylation of the sulphydryl group of the SATA by reaction with a solution of hydroxylamine (0.5 M plus 25 mM EDTA and solid Na_2HPO_4 to pH 7.5). The sulphydryl content of the deprotected WGA derivative was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB) as described by Ellman [28]. Protein was assayed by a Lowry assay [29] using a WGA (0–200 μg/100 μl) standard. The extent of derivatisation of WGA reached a limiting value of approx. 3 SATA per WGA dimer (mol. wt. 36000 [30,31]), comparable to that found for other globular proteins [27]. Routinely a reaction mole ratio of 10:1 SATA to WGA was used, giving approximately two reactive sulphydryl groups per dimeric WGA.

Synthesis of the MBS derivative of DPPE. DPPE (40 mg) was dissolved in a mixture of dry chloroform (16 ml), dry methanol (2 ml) and dry triethylamine (20 mg). MBS (20 mg) was added and the reaction mixture was stirred under nitrogen at room temperature for 24 h, after which time the organic phase was washed three times with phosphate-buffered saline (PBS) pH 7.3 to remove unreacted MBS. The DPPE-MBS derivative was recovered from the organic phase by rotary evaporation and analysed by TLC using a silica plate and a solvent mixture containing chloroform, methanol and glacial acetic acid, volume ratio 65:25:13. TLC confirmed that there was negligible contamination of DPPE-MBS (R_f 0.78) with DPPE (R_f 0.56). The DPPE-MBS was stored in a chloroform/methanol (9:1, v/v) mixture at 4°C.

Vesicle preparation and WGA conjugation. For SUV, DPPC (9 mg), PI (1 mg) and variable amounts of DPPE-MBS (0.4–4.5 mg) plus 100 μl of [^{3}H]DPPC (4 μCi/ml) were dissolved in chloroform (20 ml) and methanol (5 ml) in a 1 litre round-bottomed flask. The mixture was rotary evaporated at 60 °C to yield a thin lipid film. The lipid film was dispersed in 5 ml of nitrogen-saturated PBS at 60 °C, vigorously shaken and transferred to a glass test-tube, purged with nitrogen, sealed with a suba-seal and sonicated in a bath sonicator (Decon FS100) at 60 °C until visibly clear (1 h). The liposome suspension was then applied to a Sephadex G-200 column (30 × 2 cm) previously equilibrated with PBS (pH 7.3) at a flow rate of 0.2 ml · min^{-1}. Fractions (2 ml) were collected and aliquots (100 μl) of the fractions taken for scintillation counting.

The liposome fractions containing the highest [^{3}H]DPPC counts were mixed with derivatised WGA in appropriate proportions and reacted overnight at 4 °C. The reaction mixture was applied to a Sephadex G-200 column to separate the conjugated liposomes from unreacted WGA and fractions (2 ml) collected. The fractions were assayed for protein content [29] and lipid from the [^{3}H]DPPC count.

REV were prepared by a modification of the method of Szoka and Papahadjopoulos [32]. A lipid film was prepared using 3 ml chloroform/methanol (4:1, v/v) as above in a 50 ml round-bottomed flask. The film was re-dispersed in 6 ml of 4:1 chloroform/methanol (4:1, v/v), 3 ml of nitrogen-saturated 1:10 PBS was added at 60 °C and the mixture gently shaken, followed by 3 min sonication under nitrogen at 50 °C. The resulting emulsion was rotary evaporated at 60 °C. After a viscous gel phase and 'frothing' an aqueous phase formed spontaneously (2–4 min). The aqueous suspension was then purged with nitrogen for a further 15 min at 50 °C to remove traces of organic solvent. The resulting liposome suspension was applied to a Sephadex G200 column and peak fractions coupled with derivatized WGA at 4 °C for 2 h.

Photon correlation spectroscopy. Liposome size and size distributions were determined using a Malvern autosizer at four stages during their preparation: as initially prepared, after gel filtration (G-200), after reaction with derivatised WGA and after final gel filtration (G-200). Checks of the accuracy of the autosizer were made using monodisperse latex beads with nominal diameters of 50 nm, 150 nm and 244 nm supplied by Polysciences Inc., Warrington, PA, U.S.A. (Cat. Nos. 8691, 8216 and 15717, respectively). Measurements were also made on filtered REV using Millipore (type AAWP, pore size 0.8 μm, Cat. No. AAWP 01300, 67120 Molsheim, France) and Nuclepore (pore size 0.4 μm, Cat. No. 110607, 7035 Commerce Circle Pleasanton, Ca 94566, U.S.A.) membranes and on SUV fractions from a Sepharose 4B column (30 cm × 1.5 cm diameter). All these measurements were made on liposomes dispersed in PBS (pH 7.3).

Results

Liposome size distribution

Fig. 4 shows some typical equivalent normal weight distributions of diameters of protein free SUV (panels (c) and (d)) and REV (panels (e) to (h)) in comparison with diameter distributions of latex dispersions of comparable weight-average diameter. For the three commercial monodisperse dispersions analysed of nominal diameters 50 nm, 150 nm and 244 nm we obtained weight-average diameters of 50.9 ± 1.4 nm, 163.5 ± 4.4 nm and 237.9 ± 9.4 nm with weight to number diameter ratios ($\bar{d}_w/\bar{d}_n$) of 1.23 ± 0.09, 1.09 ± 0.07 and 1.24 ± 0.18, respectively. Both SUV and REV have relatively wide size distributions as initially prepared. Gel filtration of SUV on a Sephadex G-200 column slightly sharpens the distribution as panels (c) and (d) show. The size distributions of the REV were found to be

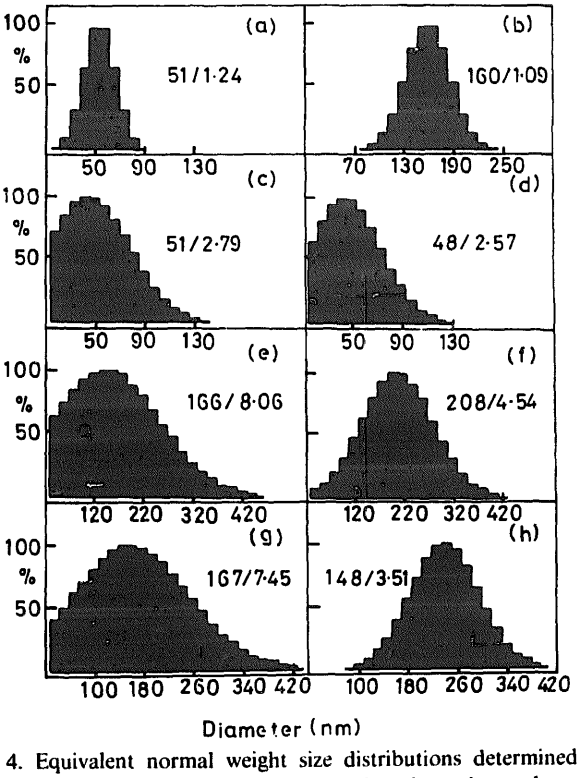

Fig. 4. Equivalent normal weight size distributions determined by photon correlation spectroscopy. The numbers in each panel are the weight-average diameter and the ratio of weight- to number-average diameter ($\bar{d}_w/(\bar{d}_w/\bar{d}_n)$). (a) and (b) standard latex particles of nominal diameter 50 and 150 nm. (c) SUV (composition DPPC/PI, 9:1, by wt.) plus 7.4 mol% DPPE-MBS as initially prepared and (d) after elution from a Sephadex G-200 column. (e) REV (composition DPPC/PI, 9:1 by wt. plus 7.4% DPPE-MBS) as initially prepared and (f) after filtration through a 0.8 μm pore size Millipore filter. (g) REV (composition DPPC/PI, 9:1 by wt. plus 24 mol% DPPE-MBS) as initially prepared and (h) after filtration through a 0.4 μm pore size Nuclepore filter.

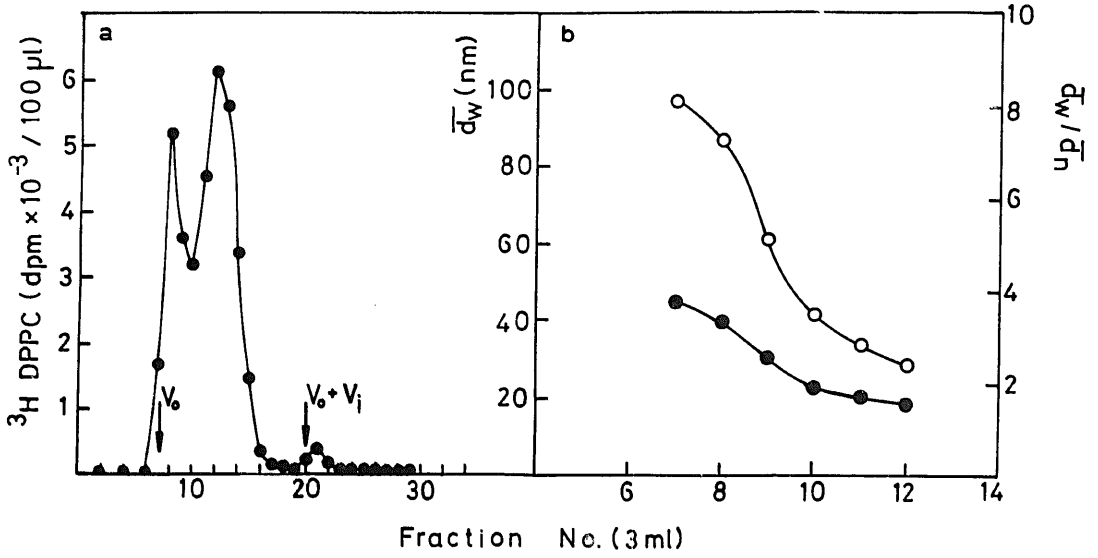

Fig. 5. (a) Elution profile of SUV (composition DPPC/PI, 9:1 by wt.) plus 7.5 mol% DPPE-MBS) from a Sepharose 4B column, 3 cm^3 fractions were collected. The void (V_o) and inner volume (V_i) of the column are indicated. (b) Weight-average diameters ($\bar{d}_w$, ○) and weight to number average diameters ($\bar{d}_w/\bar{d}_n$, ●) of liposomes in the fractions eluted from the Sepharose 4B column.

wider than SUV and could be sharpened to some degree by filtration through Millipore (0.8 µm) or Nuclepore (0.4 µm) membranes. We were unable to reduce the polydispersity of REV further by filtration through 0.6 µm Millipore or 0.2 µm Nuclepore membranes. The latter observation is consistent with that of Huang et al. [33].

Fig. 5 shows the results of a fractionation of a sample of SUV (initial weight-average diameter 59.9 nm, $\bar{d}_w/\bar{d}_n$ 2.95) using a Sepharose 4B column. The elution profile (Fig. 5a) shows a sharp peak at the void volume (V_o), a broad peak within the inner volume (V_i) which constitutes the bulk of the sample, and a very small peak eluting at ($V_i + V_o$) which probably represents small lipid aggregates from liposome breakdown. Fig. 5b shows the size distribution analysis of the fractions from the major peak. The weight-average diameters show a sigmoidal decrease from approx. 98 nm to 26 nm and $\bar{d}_w/\bar{d}_n$ decreases from 2.95 to 1.57. The size distributions of the fraction were not significantly sharpened by reducing the fraction size from 3 cm^3 to 2 cm^3. Similar experiments with REV showed that these largely eluted in the void volume of the column and could not be satisfactorily fractionated using Sepharose 4B.

These data demonstrate the difficulty of generating liposome dispersions with very narrow size distributions. It follows that due consideration must be given to liposome size distribution when characterising proteoliposomes.

Proteoliposome composition

Fig. 6 shows the dependence of the extent of protein conjugation on the mol% of DPPE-MBS for SUV and REV. The vesicle composition was DPPC/PI (weight ratio 9:1) plus variable amounts of the reactive derivative DPPE-MBS. The data cover a range of vesicle size and polydispersity; for the SUV the weight-average diameters were in the range 65–150 nm and for the REV 160–240 nm, the polydispersities ($\bar{d}_w/\bar{d}_n$) were in the ranges 2.6–4.3 (SUV) and 6.6–11.4 (REV). For both types of liposome there is a linear relationship between the extent of protein conjugation and the mol% of DPPE-MBS. Regression analysis of the data gives a gradient of 4.87 ± 0.39 (µg protein/µmol lipid)/mol% DPPE-MBS for the SUV and 4.45 ± 0.27 (µg protein/µmol lipid)/mol% DPPE-MBS for the REV. The data for the REV differed from that of the SUV in showing evidence of some physically adsorbed WGA when experiments were done with liposomes with no DPPE-MBS. Within experimental error the gradients of the plots are the same. This observation confirms the assumption made in the above theoretical treatment that the extent of protein conjugation is not a function of liposome size and hence that for a given mol% of reactive lipid the average surface density of conjugated protein is constant. The efficiency of targeting of SUV of the type described here to a model biosurface has been described previously [34].

Fig. 7 shows the weight-average number of protein molecules per liposome ($\bar{P}_w$) as a function of mol% DPPE-MBS for SUV and REV with approximately constant weight-average diameters and polydispersities of 83 ± 4 nm ($\bar{d}_w/\bar{d}_n = 3.9 \pm 0.3$) and 163 ± 6 nm ($\bar{d}_w/\bar{d}_n = 7.5 \pm 0.4$), respectively. $\bar{P}_w$ is seen to be linearly related to the mol% of reactive lipid incorporation. In this analysis the values of $\bar{P}_w$ for REV were corrected for physically adsorbed protein using the regression line intercept from Fig. 6b.

In Fig. 8 the ratio of weight-average number of proteins per liposome to number-average number ($\bar{P}_w/\bar{P}_n$) is shown as a function of the standard devia-

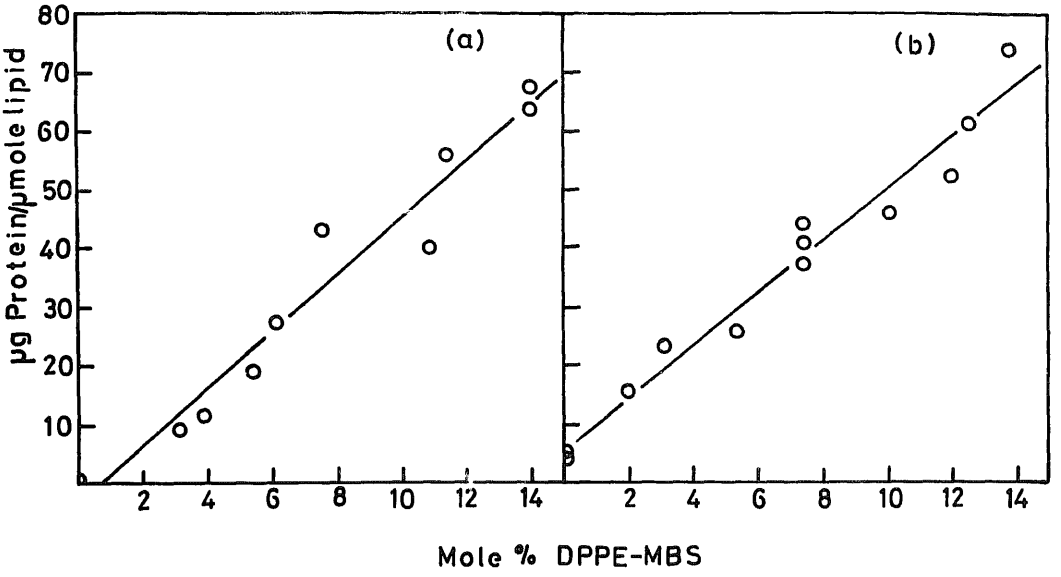

Fig. 6. Relationship between the extent of conjugation of wheat germ agglutinin (μg protein per μmol lipid) to DPPC/PI (9:1, by wt.) liposomes as a function of DPPE-MBS incorporation. (a) SUV, (b) REV.

tion of liposome diameter (σ_w). The data points correspond to those in Fig. 6 for SUV and REV. All the points lie on the same curve as expected from the theoretical calculations shown in Fig. 3.

Discussion

Although there are considerable theoretical difficulties in the application of photon correlation spectroscopy to polydisperse systems [22] the fitting of the scattering data to an equivalent normal weight distribution of size has been found to be a convenient method of analysing liposome dispersions. The data obtained with monodisperse latex particles confirm the accuracy of the measurement of weight-average diameters and the assessment of polydispersity. It should be noted however, that the low end of the distribution histograms obtained corresponds to particles of very small diameters (approx. 10 nm) which may not have physical significance in terms of a stable liposomal structure. This is a consequence of the use of the 'equivalent' normal weight distribution.

The analysis of size distributions of filtered liposomes and liposomes fractionated by gel filtration on a Sepharose 4B column suggests that it is very difficult to obtain liposome dispersions with very narrow size distributions; the narrowest distributions obtained ($\bar{d}_w/\bar{d}_n \approx 1.6$) were found for the smaller liposomes eluted from a Sepharose 4B column (Fig. 5b). Given this problem the determination of the number of proteins conjugated to liposomal surfaces will be critically dependent on the

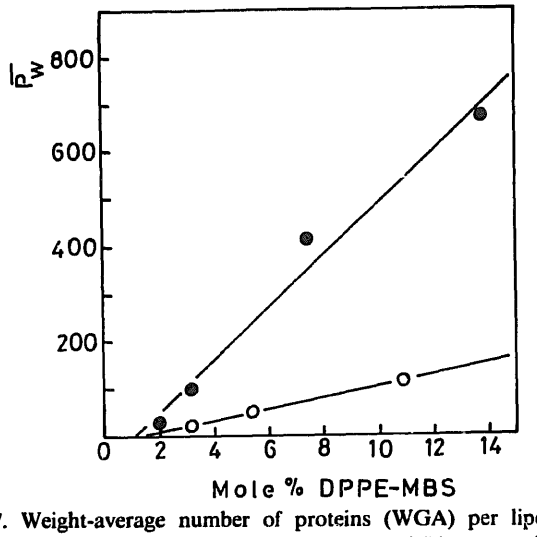

Fig. 7. Weight-average number of proteins (WGA) per liposome (DPPC/PI 9:1, by wt.) as a function of DPPE-MBS incorporation. ●, REV ($\bar{d}_w = 163 \pm 6$ nm); ○, SUV ($\bar{d}_w = 83 \pm 4$ nm).

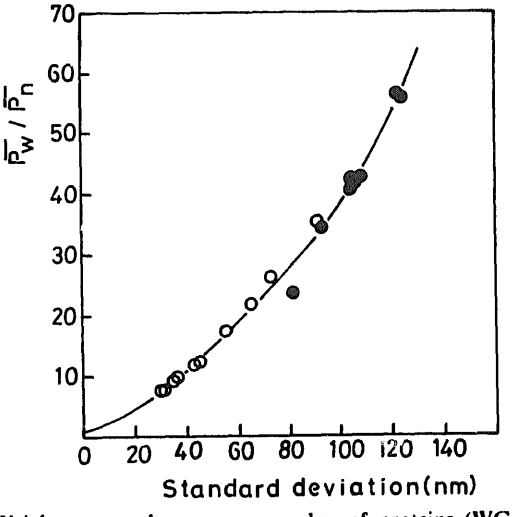

Fig. 8. Weight- to number-average number of proteins (WGA) per liposome ($\bar{P}_w/\bar{P}_n$) as a function of the standard deviation of the equivalent normal weight distribution of liposome diameters (σ_w). The data points correspond to those in Fig. 6. ○, SUV; ●, REV.

liposome size distribution and the nature of the type of averaging process used to characterise it. The data in Fig. 6 shows that the extent of protein conjugation increases in proportion to the mol% of reactive lipid incorporation in the liposomes at least up to 15 mol%. Above this level characterisation becomes difficult due to aggregation arising because of the high level of surface bound protein. The extent of conjugation when measured in mass or moles of protein per mol of lipid is independent of the size of the liposomes since this is a measure of the surface density of conjugated protein. A linear relationship between the weight-average number of protein molecules per liposome ($\bar{P}_w$) and the mol% of reactive lipid can only be obtained for liposomes of approximately constant size and polydispersity (Fig. 7).

The fundamental problem of the characterisation of proteoliposomes can be seen from a consideration of Figs. 3 and 8: the polydispersity of liposome size leads directly to a polydispersity of protein content ($\bar{P}_w/\bar{P}_n$) and unless the liposome size distribution is very narrow the extreme sensitivity of $\bar{P}_n$ arising from the unsymmetrical number distribution of liposome sizes leads to large values of $\bar{P}_w/\bar{P}_n$. While extrusion [35] or detergent dialysis methods [36] of liposome production can lead to relatively narrow size distributions the possibility always exists of a distribution widening on storage over relatively short time periods. Although it might be argued that the photon correlation spectroscopic method of size analysis can give only an 'equivalent' normal weight distribution of diameters the literature data (Table I) seem to suggest that there are major difficulties in characterising proteoliposomes which it is hoped the present analysis may clarify.

Acknowledgements

We thank the SERC for financial support for F.J.H. and for a CASE studentship for S.E.F.

References

1 Knight, C.G. (ed.) (1981) Liposomes: from Physical Structure to Therapeutic Applications, Elsevier, Amsterdam.
2 Ostro, M.J. (ed.) (1987) Liposomes from Biophysics to Therapeutics, Marcel Dekker, Inc., New York.
3 Weinstein, J.N. and Leserman, L.D. (1984) Pharmac. Ther. 24, 207–233.
4 Hashimoto, Y., Sugawara, M. and Endoh, H. (1983) J. Immunol. Meth. 62, 155–162.
5 Senior, J., Waters, J.A. and Gregoriadis, G. (1986) FEBS Lett. 196, 54–58.
6 Heath, T.D., Robertson, D., Birbeck, M.S.C. and Davies, A.J.S. (1980) Biochim. Biophys. Acta 599, 42–62.
7 Snyder, S.L. and Vannier, W.E. (1984) Biochim. Biophys. Acta 772, 288–294.
8 Gregoriadis, G., Davis, D. and Davies, A. (1987) Vaccine 5, 145–151.
9 Derksen, J.T.P. and Scherphof, G.L. (1985) Biochim. Biophys. Acta 814, 151–155.
10 Yu, B.S., Choi, Y.K. and Chung, H. (1987) Biotech. Appl. Biochem. 9, 209–216.
11 Houck, K.S. and Huang, L. (1987) Biochem. Biophys. Res. Commun. 145, 1205–1210.
12 Sunamoto, J., Sato, T., Hirota, M., Fukushima, K., Hiratani, K. and Hara, K. (1987) Biochim. Biophys. Acta 898, 323–330.
13 Huang, A., Huang, L. and Kennel, S.J. (1980) J. Biol. Chem. 255, 8015–8018.
14 Huang, A., Tsao, Y.S., Kennel, S.J. and Huang, L. (1982) Biochim. Biophys. Acta 716, 140–150.
15 Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 640, 66–81.
16 Kung, V.T. and Redemann, C.T. (1986) Biochim. Biophys. Acta 862, 435–439.
17 Martin, F.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.
18 Derksen, J.T.P., Morselt, H.W.M. and Scherphof, G.L. (1987) Biochim. Biophys. Acta 931, 33–40.
19 Martin, F.J., Hubbell, W.L. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238.
20 Tsuchiya, S., Aramaki, Y., Hara T., Hosoi, K. and Okada, A. (1986) Biopharm. Drug. Dispos. 7, 549–558.
21 Hospenthal, D.R., Rogers, A.L. and Mills, G.L. (1988) Mycopathologia 101, 37–45.
22 Pusey, P.N. (1973) in Photon Correlation and Light Beating Spectroscopy (Cummins, H.Z. and Pike, E.R., eds.), pp. 387–428, Plenum Press, New York and London.
23 Herdan, G. (1960) Small Particle Statistics, 2nd Edn., Butterworth, London.
24 Topping, J. (1966) Errors of Observation and their Treatment, 3rd Edn., Chapman and Hall, London.
25 Janiak, M.J., Small, D.M. and Shipley, G.G. (1979) J. Biol. Chem. 254, 6068–6078.
26 Palmier, J.M. and Hall, D.O. (1972) Prog. Biophys. Mol. Biol. 24, 127–176.
27 Duncan, R.J.S., Weston, P.D. and Wrigglesworth, R. (1983) Anal. Biochem. 132, 68–73.
28 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
30 Nagata, Y. and Burger, M.M. (1974) J. Biol. Chem. 249, 3116–3122.
31 Allen, A.K., Neuberger, A. and Sharon, N. (1973) Biochem. J. 131, 155–162.
32 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
33 Huang, L., Huang, A. and Kennel, S.J. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 3, pp. 52–62, CRC Press Inc., Boca Raton, FL.
34 Hutchinson, F.J. and Jones, M.N. (1988) FEBS Lett. 234, 493–496.
35 Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
36 Weder, H.G. and Zumbühl, O. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 1, pp. 79–107, CRC Press Inc., Boca Raton, FL.