Biochimica et Biophysica Acta, 987 (1989) 212–216
Elsevier

BBAMEM 74683

The integrity of proteoliposomes adsorbed on a biosurface

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(Received 20 March 1989)
(Revised manuscript received 19 September 1989)

Key words: Proteoliposome; Wheat germ agglutinin; Adsorption; Liposome integrity; Glycophorin

Proteoliposomes encapsulating [^{14}C]glucose have been prepared from a mixture of dipalmitoylphosphatidylcholine and phosphatidylinositol by sonication (SUV) and reverse phase evaporation (REV) and conjugated with wheat germ agglutinin (WGA). The proteoliposomes were characterised in terms of size and composition and covered a range of size (weight-average diameter) from approximately 60–330 nm and surface-bound WGA (weight-average number of protein molecules per liposome) from approximately 70 to 3000. Methods have been developed for assessing the extent of adsorption and integrity of the proteoliposomes when targeted to glycophorin A-coated microtitre wells. From the amount of [^{14}C]glucose released by detergent disruption from the adsorbed proteoliposomes it is found that the extent of adsorption increases with proteoliposome size and WGA conjugation and that the integrity of the proteoliposomes remains intact on adsorption. The results can be explained in terms of monolayer coverage of the surface with preferential adsorption of larger proteoliposomes from the size distribution.

Introduction

In recent studies we have considered the implications of size distribution on the characterisation of liposomes with covalently attached protein and developed an ELISA system for assessing the targeting efficiency to a model biosurface consisting of adsorbed glycoprotein [1,2]. In the application of proteoliposomes as carriers of therapeutic agents [3] the integrity of the liposomes after adsorption to a biosurface will play an important part in determining the fate of the encapsulated therapeutic agent. It has been demonstrated that when dipalmitoylphosphatidylcholine vesicles are adsorbed on a 'high energy' surface such as the surface of clean glass beads extensive disruption occurs with loss of encapsulated material [4] and the surface is covered with a lipid multilayer or monolayer depending on the temperature of adsorption relative to the phase transition temperature of the lipid [5]. In contrast liposomes adsorb intact onto octadecylsilyl monolayers on glass surfaces [6] and, on heating DPPC bilayers adsorbed on silicon above the phase transition temperature, expansion generates sufficient lateral pressure to cause liposomes of various shapes to form at the interface [7].

We report here a study of the integrity of proteoliposomes prepared from dipalmitoylphosphatidylcholine (DPPC) and a natural phosphatidylinositol (PI) with wheat germ agglutinin (WGA) covalently conjugated to their surface, when they are adsorbed to a glycophorin A-coated substrate. The liposomes cover a range of size, from small unilamellar vesicles (SUV) to large vesicles prepared by reverse phase evaporation (REV), and a range of protein content. As well as demonstrating the integrity of proteoliposomes on the biosurface, the data yield some information on the relationship between proteoliposome composition/size and their adsorption characteristics. A preliminary abstract of this work has been given [8].

Methods

Preparation of proteoliposomes

SUV and REV were prepared from a mixture of DPPC and PI (from wheatgerm) (weight ratio 9:1) incorporating 7–11% by weight of the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoylphosphatidylethanolamine (PE-MBS) [9]. The *N*-succinimidyl-*S*-acetylthioacetate (SATA) derivative of wheat germ agglutinin was prepared, activated using hydroxylamine and coupled to the liposomes by reaction of the thiol group with the maleimido group of the PE-MBS. The vesicles also contained tracer levels of [^{3}H]DPPC. The materials used and preparative details

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TABLE I

Characterisation of phospholipid vesicles (DPPC/PI, weight ratio 9:1) at stages during their preparation and conjugation with wheat germ agglutinin

	SUV			REV		
	$\bar{d}_w$ (nm)	σ (nm) [a]	$\bar{d}_w/\bar{d}_n$	d_w (nm)	σ (nm) [a]	$\bar{d}_w/d_n$
Initial preparation	44	22	2.2	172	95	7.62
Peak fraction (G-200) after [^{14}C]glucose encapsulation	53	28	2.6	173	101	7.93
After WGA conjugation	84	51	4.1	186	102	8.22
Peak fraction (G-200) after conjugation	111	70	5.4	194	112	8.83
Mol WGA/mol lipid	$1.37 \cdot 10^{-4}$			$1.47 \cdot 10^{-4}$		
Encapsulated volume litre/mol lipid	2.09 (2.06) [b]			5.61 (4.14) [b]		
Weight-average number of proteins/liposome ($\bar{P}_w$)	241			652		

[a] Standard deviation of the equivalent normal weight distribution. [b] Calculated from the geometric volume using $\bar{d}_w$.

have been fully described previously [1]. The only variation in procedure pertaining to the proteoliposome preparations described here was the encapsulation of [^{14}C]glucose. This was added to the phosphate-buffered saline (PBS) pH 7.4 used to disperse the lipid film in the initial stages of the preparation at a level of 20 μCi per 5 ml of dispersion (concentration 14.8 μM). 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) of the fractions taken for scintillation counting of both ^{3}H and ^{14}C. The liposome fractions containing the highest ^{3}H counts were mixed with SATA-derivatized WGA and reacted overnight at 4°C. The resulting conjugated liposomes were applied to the Sephadex G-200 column and fractions (2 ml) collected and analysed for protein by the method of Lowry et al. [10] and lipid from the ^{3}H count. The size distributions of the liposomes were monitored at each stage in the preparation by photon correlation spectroscopy. The size distributions of the final proteoliposome preparations together with the molar ratios of protein to lipid were used to obtain the weight-average numbers of protein molecules per liposome ($\bar{P}_w$) as previously described [1] and the encapsulated volumes were determined from the [^{14}C]glucose and ^{3}H-lipid counts and the specific activities of glucose and lipid.

For SUV the encapsulated volume calculated from the radioactive counts was in good agreement with that calculated from the geometric volume of the vesicles as determined from the measured diameters (Table I). For the REV the encapsulated volume was larger than calculated from the weight-average diameter. This is partly due to the width of the distribution, since volume increases as the cube of the diameter larger vesicles make a greater contribution to the encapsulated volume.

Proteoliposome adsorption

The adsorption of the proteoliposomes on plastic microtitre plates coated with glycophorin A from blood group B (Sigma Chemical Co., product no. G5911) was carried out by a similar procedure to that previously described for assessing targeting efficiency to glycophorin A-coated microtitre wells [2]. Glycophorin A was allowed to adsorb on the wells of microtitre plates (Dynatech M129B) from 200 μl of a solution of concentration 0.02 mg · ml^{-1} in PBS by incubation at 4°C for a period of 12 h. Glycophorin A is soluble in PBS but readily adsorbs on plastic microtitre plates. The antigenic response of adsorbed glycophorin A in an ELISA system [2] increases with adsorbed glycophorin A reaching a limiting value on incubation with a glycophorin A solution of concentration 0.05 mg · ml^{-1}. The wells were then washed three times with 300 μl of PBS and vacant sites were blocked by incubation with 300 μl of 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37°C followed by a wash with 300 μl of 0.1% (w/v) BSA in PBS. Aliquots of the proteoliposome solution (200 μl) were added and incubated at 37°C for periods up to 2 h after which 100 μl aliquots were removed for scintillation counting (^{3}H and ^{14}C). The remaining 100 μl were removed and the wells again washed with 300 μl of 0.1% (w/v) BSA in PBS. The adsorbed proteoliposomes were then disrupted by addition of 100 μl of 1% (w/v) sodium *n*-dodecylsulphate (SDS) for a period of 30 min at room temperature with gentle shaking to ensure that the sides of the wells were washed with the detergent solution. Aliquots (80 μl) were removed from the wells for scintillation counting (^{3}H and ^{14}C). An SDS concentration of 1% (w/v) is well above the critical micelle concentration of SDS and will release all the encapsulated [^{14}C]glucose [11].

Results and Discussion

Fig. 1 shows the elution profiles of a typical preparation of SUV and REV encapsulating [^{14}C]glucose from Sephadex G-200 columns after conjugation of WGA to the liposome surfaces. The coincidence of the peaks of ^{3}H-lipid, [^{14}C]glucose and protein at the void volume of

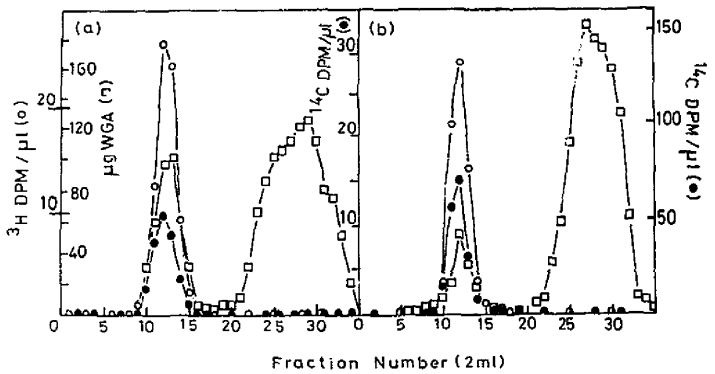

Fig. 1. Elution profile of DPPC/PI (weight ratio 9:1) SUV(a) and REV(b) encapsulating [^{14}C]glucose from a Sephadex G-200 column after conjugation with wheat germ agglutinin. The ^{3}H-labelled vesicles elute in the void volume of the column.

the column separated from the peaks of unreacted protein demonstrate the integrity of proteoliposomes with encapsulated [^{14}C]glucose. Table I shows the characterisation data for typical batches of vesicles at stages throughout their preparation as obtained by photon correlation spectroscopy and protein/lipid analysis. Characterisation data of this type were obtained for all the preparations of SUV and REV. In general the weight-average diameters and polydispersity of SUV increase after conjugation; in contrast the size and polydispersity of the REV remain approximately constant throughout the preparation. The increase in the size of SUV is probably due to a degree of aggregation. Surface bound WGA would not increase the size (diameter) by more than approximately 10 nm even if the WGA molecules were close-packed since WGA has a diameter of 4–6 nm depending on orientation [12]. Electron microscopy of the REV suggest that the vesicle walls were largely unilamellar or consisted of only a small number of bilayers.

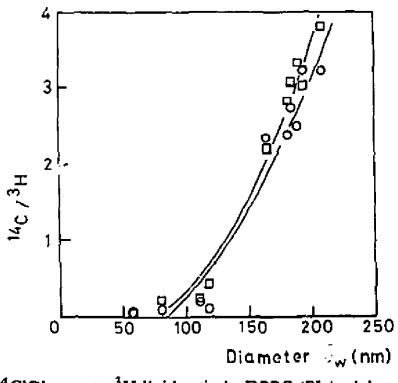

Fig. 2. [^{14}C]Glucose to ^{3}H-lipid ratio in DPPC/PI (weight ratio 9:1) vesicles conjugated with wheat germ agglutinin as a function of weight average diameter ($\bar{d}_w$) as initially prepared (□) and after exposure to glycophorin A-coated microtitre wells (○) for 2 h.

Fig. 2 shows the count ratios of [^{14}C]glucose to ^{3}H-lipid in the proteoliposomes as initially prepared prior to adsorption on the glycophorin A surface and in the supernatant in the microtitre wells after 2 h incubation at 37 °C, as a function of liposome size. No adsorption occurs on microtitre plates blocked with BSA in the absence of glycophorin A. Since the bulk volume to surface area is large the percentage of proteoliposomes adsorbed in these experiments is small (approx. 3–4% of the total). In principle the ^{14}C: ^{3}H ratio in the dispersion should not change after adsorption provided that the adsorbed proteoliposomes remain intact, their permeability to glucose is negligible and that adsorption is not size-dependent. If however a proportion of the

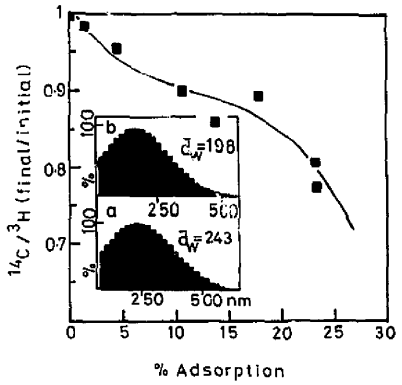

Fig. 3. The ratio of ^{14}C: ^{3}H in DPPC/PI (weight ratio 9:1) wheat germ agglutinin conjugated vesicles encapsulating [^{14}C]glucose in the bulk phase $(^{14}C/^{3}H)_f$ after targeting to a glycophorin A-coated surface relative to the initial ratio $(^{14}C/^{3}H)_i$, as a function of the % adsorption to the glycophorin A surface. The inset shows the equivalent normal weight size distribution for a control sample of proteoliposomes (a) and after targeting to a glycophorin A-coated surface (b). For these proteoliposomes the weight-average number of wheat germ agglutin molecules per liposome was 745 and adsorption was 23%.

proteoliposomes disrupted on adsorption then the $^{14}C:{}^{3}H$ ratio in the bulk phase could increase due to release of encapsulated [^{14}C]glucose. The data in Fig. 2 raised the possibility that the $^{14}C:{}^{3}H$ ratio may decrease on adsorption, but because the proportion of adsorbed proteoliposomes was small the decrease was within the experimental error. To investigate this further the $^{14}C:{}^{3}H$ ratios before $(^{14}C/^{3}H)_i$ and after $(^{14}C/^{3}H)_f$ adsorption were measured as a function of proteoliposome dilution. At high dilutions the number of proteoliposomes adsorbed relative to the number in the bulk volume increases. Fig. 3 shows $(^{14}C/^{3}H)_f/(^{14}C/^{3}H)_i$ as a function of the % adsorption; if adsorption was independent of size this ratio would be unity. It is clear that the ratio decreases with % adsorption so that the supernatant in the microtitre wells becomes depleted in larger proteoliposomes encapsulating more [^{14}C]glucose and hence it follows that these are preferentially adsorbed. The inset in Fig. 3 shows the equivalent normal weight size distribution obtained by photon correlation spectroscopy of a sample of proteoliposomes after incubation in glycophorin-coated microtitre wells for 2 h at 37°C (inset b) compared with the control sample (inset a) incubated in the absence of the adsorbing surface. The weight-average diameter of the proteoliposomes after targeting decreased by approx. 20% and the weight to number-average diameters was changed from 11.6 to 9.4 by adsorption. The extent of adsorption was 23% in these experiments.

The decrease in the $^{14}C:{}^{3}H$ ratio in the bulk phase after adsorption implies that the proteoliposomes with the larger internal volumes are lost from the bulk phase and also suggests that the proteoliposomes are not disrupted on adsorption since disruption would lead to an increase in the $^{14}C:{}^{3}H$ ratio in the bulk phase. It should be noted however that there is a possibility that both the processes of preferential adsorption and disruption could be occurring simultaneously and that the fall in the $^{14}C:{}^{3}H$ ratio in the bulk phase might be a consequence of the fact that the extent of preferential adsorption of intact large proteoliposomes merely exceeds the extent of disruption of proteoliposomes from the whole population. However the fact that [^{14}C]glucose can only be released from the adsorbed layers by the action of detergent suggests that the integrity of the proteoliposomes is largely retained on adsorption.

The release of [^{14}C]glucose by detergent from adsorbed proteoliposomes is shown as a function of initial incubation time in Fig. 4 for vesicles of two different sizes and initial $^{14}C:{}^{3}H$ ratios. The amount of [^{14}C]glucose released by SDS treatment remains constant after approx. 30 min incubation suggesting that the surface becomes saturated with proteoliposomes after this time period. In routine experiments incubation times of 2 h were used to ensure proteoliposome saturation coverage. Figs. 5 and 6 show the [^{14}C]glucose released from

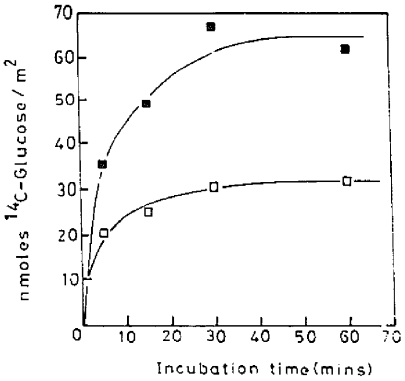

Fig. 4. Detergent release of encapsulated [^{14}C]glucose (nmol/m^2) from DPPC/PI (weight ratio 9:1) wheat germ agglutinin conjugated vesicles after targeting to a glycophorin A-coated surface as a function of incubation time. ■, weight-average diameter ($\bar{d}_w$) 332 nm, initial $^{14}C:{}^{3}H$ ratio 3.3; □, weight-average diameter ($\bar{d}_w$) 198 nm, initial $^{14}C:{}^{3}H$ ratio 1.5.

adsorbed layers of SUV and REV as a function of proteoliposome size (Fig. 5) and as a function of the weight-average number of WGA molecules per liposome (Fig. 6). In these experiments the initial $^{14}C:{}^{3}H$ ratios varied from 0.04 to 3.8.

These data demonstrate that the extent of proteoliposome adsorption to a glycophorin A surface increases with the size of the liposomes (Fig. 5) and with the extent of protein coupling as expressed in terms of the weight-average number of wheat germ agglutinin molecules per liposome ($\bar{P}_w$) (Fig. 6).

From the size of the proteoliposomes, the number of lipids per liposome (N^L) and the surface area of the microtitre wells exposed to the dispersion, it is possible to calculate the amount of radiolabelled lipid which would be released from the surface if a monolayer of

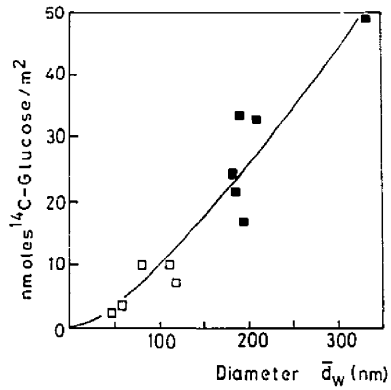

Fig. 5. Detergent release of encapsulated [^{14}C]glucose (nmol/m^2) from DPPC/PI (weight ratio 9:1) wheat germ agglutinin conjugated vesicles after targeting to a glycophorin A-coated surface as a function of weight-average diameter ($\bar{d}_w$). □, SUV; ■, REV.

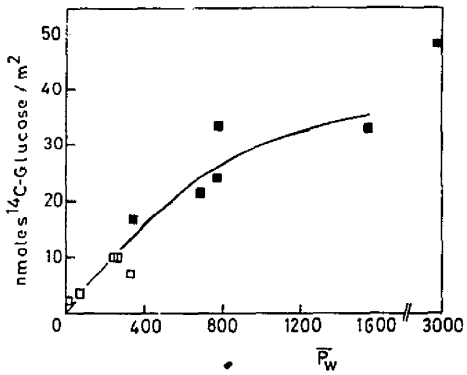

Fig. 6. Detergent release of encapsulated [^{14}C]glucose (nmol/m^2) from DPPC/PI (weight ratio 9:1) wheat germ agglutinin conjugated vesicles after targeting to a glycophorin A-coated surface as a function of the weight-average number of site-directing wheat germ agglutinin molecules per liposome ($\bar{P}_w$). □, SUV; ■, REV.

close-packed spherical proteoliposomes formed. Table II shows the results of such calculations. For the protein-conjugated SUV the surface coverage relative to that of the close-packed monolayer is high (78%) although due to the relatively low counts involved and the variation in the size and extent of conjugation (surface protein density on the liposome surface (nm^2/WGA)) there is considerable variation. For the protein-conjugated REV the corresponding figures are significantly lower (8.4%). This effect can only partially be explained in terms of the preferential adsorption of larger proteoliposomes since the apparent area per lipid in the adsorbed layers (a^L) decreases by only 16% on increasing the weight-average diameter of the proteoliposomes from 58 to 209 nm. The differences in coverage relative to monolayer coverage between SUV and REV proteoliposomes might arise if the REV occupied a larger area than that of a sphere projected on the surface, i.e. if there was some 'flattening' on adsorption. We cannot, however, rule out the possibility that neither SUV nor REV form 'close-packed' monolayers on adsorption and the greater apparent coverage for SUV arises as a consequence of the presence of a proportion of small multilamellar proteoliposomes.

It is clear however that there is no evidence for multilayer adsorption and that a monolayer model adequately describes the adsorption of the proteoliposomes targeted to the glycophorin A surface and that the integrity of the proteoliposomes is largely maintained on adsorption. This ability of proteoliposomes to retain their integrity on adsorption to a biosurface could be an advantageous property in the context of controlled drug delivery. In the in vivo situation intact proteoliposomes adsorbed to a cellular surface could then undergo fusion and/or endocytosis with concomitant delivery of the encapsulated contents [15].

TABLE II

Adsorption of DPPC/PI (weight ratio 9:1) wheat germ agglutinin conjugated vesicles targeted to a glycophorin A surface

$\bar{d}_w$ (nm)	$N^L(\times 10^{-4})$ [a]	nm^2/ WGA	Coverage (%) monolayer coverage	a^L (nm^2) [b]
SUV				
58	3.28	148	86	0.0807
111	13.53	161	66	0.0715
118	15.38	133	82	0.0711
			Mean 78 ±11	
REV				
182	38.35	135	12	0.0678
185	39.80	157	5.5	0.0675
190	41.83	145	5.5	0.0677
194	43.63	347	8.1	0.0677
209	50.83	88.7	11	0.0675
			Mean 8.4±3.0	

[a] N^L is the number of lipid molecules per proteoliposome calculated from the weight-average diameter ($\bar{d}_w$) assuming the liposomes are spherical.

[b] a^L is the area per lipid molecule in the adsorbed layer calculated from the projected area (π ($\bar{d}_w/2$)^2) and N^L. In the calculations the area per lipid molecule in the liposome bilayer was taken as 0.50 nm^2 [13] and the bilayer thickness as 7.5 nm [14].

Acknowledgements

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

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