Biochimica et Biophysica Acta, 1147 (1993) 185–193

BBAMEM 75942

Coupling of ligands to liposomes independently of solute entrapment: observations on the formed vesicles

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(Received 2 September 1992)

Key words: Liposome; Ligand; Targeting; Photon correlation spectroscopy; Freeze-fracture; Electron microscopy

Bovine serum albumin (BSA), employed as a model ligand, was covalently linked (about 16% of the amount used) to small unilamellar vesicles (SUV) composed of phospholipid, cholesterol and *N*-(*p*-aminophenyl)stearylamide (APSA) (molar ratios 1 : 1 : 0.05). SUV with bound BSA were then used to generate dehydration-rehydration vesicles (DRV) in the presence of tetanus toxoid and/or carboxyfluorescein (CF). Nearly all of the SUV-bound BSA (about 15% of the original amount) was recovered in the multilamellar DRV formed, with a considerable proportion (42–62%) of the ligand becoming available on the outer bilayers. This apparent spatial reorientation of BSA within DRV also caused the entrapped toxoid to shift to some extent to the liposomal surface. There was no significant difference in the *z* average mean size between DRV with and without coupled BSA (543 and 555 nm diameter, respectively). Percent number diameter distribution data revealed that 71.2 (BSA-free) and 76.4% (BSA-containing DRV) of the vesicles had diameters of about 300–440 and 330–420 nm, respectively. However, in terms of percent mass diameter distribution, 69.5% (BSA-free) and 65.2% (BSA-containing DRV) of the mass was in vesicles with corresponding ranges of diameter of 1381–2975 and 1086–2840 nm. Vesicle size heterogeneity in both preparations was confirmed by freeze-fracture electron microscopy which also indicated that structures with or without bound BSA, were mostly vesicular of the multilamellar type. Judging from CF latency values, ligand-bearing DRV were stable on incubation with blood plasma at 37°C for 24 h. Stability was, however, reduced significantly when the amount of ligand bound was excessive. The present approach allows for the coupling of ligands to and the entrapment of antigens and other labile solutes in liposomes independently, thus avoiding potential damage of such solutes by the coupling reagents.

Introduction

Increasing interest in receptor-mediated targeting of liposomes [1] has led to the development of a variety of procedures for the coupling of ligands (e.g., antibodies, glycoproteins, etc.) to preformed liposomes [2–10]. Unfortunately, reagents employed during coupling [2] may destabilize or penetrate the vesicle bilayers and inactivate or modify entrapped agents, including peptide hormones, enzymes, antigens and cytokines. This problem can be avoided by the coupling of ligands to lipids which are then incorporated into liposomes [9]. However, organic solvents or detergents participating in the coupling reaction can damage certain ligands, for instance alter their tertiary structure, and thus diminish or abolish effective binding to the relevant receptors. Moreover, some of the detergent required [9] for the incorporation of lipid-bound ligands into liposomes is usually retained [11] by the latter thus rendering their in vivo use questionable.

Preparation of liposomes by the dehydration-rehydration method (dehydration-rehydration vesicles; DRV) [12,13] has recently formed the basis for the incorporation of antibodies into such liposomes independently of solute entrapment [14]: antibody is covalently linked to appropriately formulated 'empty' (water-containing) small unilamellar vesicles (SUV) which, on removal of excess reagents, are mixed with the solute destined for entrapment and then processed [12,13] to generate DRV. DRV formation, leading to high yield solute entrapment [12,13,15–18], is carried out in the absence of organic solvents, sonication or detergents. In the present report we have investigated this approach further using albumin as a model protein ligand and carboxyfluorescein and tetanus toxoid as model solutes for entrapment. Results show that nearly all of the originally SUV-bound ligand is recovered with the generated DRV with much of the ligand

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becoming available on the DRV surface. Although the presence of the ligand in DRV does not affect the extent of toxoid entrapment, accessibility of the toxoid to externally added protease increases in direct relation to the amount of ligand bound. Increased amounts of ligand also appear to reduce bilayer stability in the presence of blood plasma, especially on prolonged incubation. Further, photon correlation spectroscopy of DRV with or without bound BSA revealed no difference in their z average mean size (543 and 555 nm diameter, respectively). Vesicle size similarity was supported by freeze-fracture electron microscopy which also indicated that formed structures, although highly heterogeneous in size, were vesicular and multilamellar. The approach should be useful for the preparation of targeted liposomes containing sensitive agents, provided that the amount of ligand bound is not excessive.

Materials and Methods

Sources and grades of egg phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), cholesterol (CHOL) and carboxyfluorescein (CF) have been described [12,13]. Proteinase (*Streptococcus griseus*, type VI, 4 units mg^{-1}), *p*-phenylenediamine, *p*-nitrophenylstearate and bovine serum albumin (BSA) were from Sigma, London, UK. *N*-(*p*-Aminophenyl)stearylamide (APSA) was synthesized [13,19] from *p*-phenylenediamine and *p*-nitrophenylstearate. Immunopurified tetanus toxoid ($1.2 - 6.3$ mg ml^{-1}) was purchased from Wellcome Biotech., Beckenham, Kent, UK, dialysed against 2 l of distilled water and aggregates removed by centrifugation at $100000 \times g$ for 60 min. ^{131}I-labelled BSA (specific activity 2.2 MBq mg^{-1}) and ^{125}I-labelled tetanus toxoid (specific activity 2.0 MBq mg^{-1}) were prepared as previously described [12,13].

Preparation of DRV liposomes containing tetanus toxoid and / or CF

SUV of varying lipid composition without or with bound BSA (see later) were prepared by probe sonication at approriate temperatures [12,13] and used to generate DRV in the presence of solute(s) destined for entrapment. In brief, SUV were mixed with an equal volume of tetanus toxoid (0.5 mg) into which tracer ^{125}I-labelled toxoid ($4 \cdot 10^4$ cpm) had been added, 0.06 M CF or 0.06 M CF also containing 0.5 mg toxoid and tracer, and freeze-dried overnight (13 PA). The dry powder was then rehydrated in a controlled fashion [12] with 0.1 ml distilled water followed by 0.9 ml 4.5 mM Na phosphate buffer (pH 7.4) containing 0.8% NaCl and 0.02% $MgCl_2$ (PBS). The suspension consisting of liposome-entrapped and free solute(s) was diluted with 7 ml PBS and centrifuged in the cold (4°C) at $20000 \times g$ for 15 min. The liposomal pellet was washed twice in 8 ml PBS by centrifugation and suspended in 1 ml PBS. Entrapment of toxoid was estimated by the assay [12] of ^{125}I radioactivity and of CF by measuring the dye in the presence of Triton X-100 [20].

Covalent coupling of BSA to liposomes

The method of diazotization [19] as modified [13,14] was used to couple BSA to liposomes. In brief, SUV composed of PC (15 μmoles) or DSPC (15 μmoles), cholesterol (15 μmoles) and APSA (0.75 μmoles) and prepared as above, were activated by the addition of 0.16 ml cold (4°C) 1.0 M $NaNO_2$ and 0.16 ml 1.0 M HCl/NaCl. After diazotization, SUV were rapidly separated by centrifugation through Sephadex G-25 (Pharmacia) minicolumns [21] and reacted with 1.0 ml BSA (0.5–5.0 mg) mixed with ^{131}I-labelled BSA tracer (10^4 cpm). SUV with covalently linked BSA were separated from free BSA by chromatography through Sepharose 4B (Pharmacia) and used to generate DRV in the presence of solutes as above [(SUV-BSA)DRV]. In some experiments, BSA was linked by diazotization directly to preformed solute-containing DRV generated from SUV of the same lipid compositions (DRV-BSA). A variety of control preparations were also made. These included: (a) DRV generated (in the presence or absence of toxoid) from APSA-incorporating SUV which were diazotized but not interacted with BSA; (b) DRV generated (in the presence or absence of toxoid) from APSA-free SUV which were interacted or not interacted with BSA; (c) DRV (generated in the presence or absence of toxoid from APSA-free SUV) which were interacted or not interacted with BSA.

Measurement of vesicle size

Particle z-average mean size and percent number and mass diameter distributions were measured [22] by photon correlation spectroscopy of samples diluted in PBS, using a Malvern Model 4700 apparatus (Malvern Instruments, Malvern, UK) equipped with a 25 mW helium/neon laser. The performance of the instrument was checked with monodisperse polystyrene latex suspensions (Polysciences, UK) and mixtures of such latex suspensions to verify the ability of the apparatus to accurately measure polydisperse or bidisperse systems.

Freeze-fracture electron microscopy

SUV or DRV liposome samples were examined [23] by freeze-fracture electron microscopy after quenching at room temperature (23°C) using the sandwich technique and liquid propane. The specimens were fractured and shadowed in a Balzers BAF 400D freeze-fracture device at −150°C. The cleaned replicas were examined in a Jeol JEM 100B on a Tesla BS 500 electron microscope.

Treatment with proteinase

Tetanus toxoid-containing doubly labelled (SUV-BSA)DRV and DRV-BSA (up to about 100 μg of each ^{125}I-labelled toxoid and ^{131}I-labelled BSA) and control preparations devoid of either entrapped toxoid or bound BSA, were incubated in the presence of proteinase (4 units ml^{-1}) at 37°C for 60 min. In a single experiment, (SUV-BSA)DRV containing both toxoid and 0.06 M CF were also exposed to proteinase as above. At the end of the incubation period samples were, when appropriate, assayed for CF latency [20] the latter being a reliable indicator of bilayer stability [24]. Alternatively, samples were centrifuged in the cold (4°C) at 20000 $\times g$ for 15 min, washed with PBS three times and pellets assayed for BSA (^{131}I) or toxoid (^{125}I) radioactivity. In preliminary work it was found that amounts of free toxoid or BSA 2-fold greater than those contained in the incubated samples, could be fully digested by the enzyme under the conditions used.

Incubation with plasma

CF-containing (SUV-BSA)DRV (0.1 ml) were incubated with 0.5 ml fresh mouse (Balb/c) plasma (or PBS in control experiments) at 37°C. At time intervals, samples were assayed for liposomal stability in terms of CF latency [20].

Results and Discussion

Binding of BSA to liposomes

Table I and legend show that following diazotization of APSA-incorporating SUV and interaction with BSA (5 mg), nearly all [15.1% (PC) and 15.5% (DSPC) of the amount used for coupling] of the protein originally bound to SUV (16.1 and 16.4%, respectively, of the amount used for coupling) was recovered with the subsequently formed (SUV-BSA)DRV. Although these values were obtained with 15 μmoles total liposomal phospholipid, coupling experiments in which lesser amounts (7.5 and 4.4 μmoles) of phospholipid and correspondingly reduced amounts of APSA were used for the same amount of BSA, showed that percent protein binding remained essentially unchanged (15.0 and 13.2% for PC and 15.4 and 14.1% for DSPC DRV) (results not shown). These findings are in agreement with those reported [14] for much smaller amounts (0.04–0.4 mg) of immunoglobulin G linked to SUV which were then used to generate DRV. Similar BSA binding values to those seen for (SUV-BSA)DRV were observed when BSA was coupled to preformed DRV (Table I). Corresponding values for BSA binding to various control DRV preparations as described in Materials and Methods, were low (1.4–4.2% of the amount used; legend to Table I).

Vesicle size of DRV formulations

Photon correlation spectroscopy of DRV preparations showed (see legend to Fig. 1) a z-average mean size for BSA-free DRV of 555 nm. This remained unaltered in DRV with bound BSA and entrapped toxoid (543 nm diameter). However, polydispersity values were high (0.586 and 0.651; legend to Fig. 1) signifying highly heterogeneous populations of vesicles. Furthermore, percent number diameter distribution data (Fig. 1) revealed that many of the vesicles in both formulations (71.2 and 76.4% for BSA-free and BSA-containing DRV) had diameters in the range of 300–440 and 330–420 nm, respectively. The remaining vesicles had a range of diameters with a measurable upper limit of about 3500 nm. On the other hand, in terms of mass, its percent diameter distribution favoured vesicles with size ranges of 1381–2975 (69.5%; BSA-free)

TABLE I

Covalent coupling of albumin to DRV liposomes and surface availability

Values for BSA binding to the various control DRV preparations were 1.4–4.2% of the amount used. For other details see Materials and Methods.

Composition of liposomes [a]	(SUV-BSA)DRV			DRV-BSA		
	% coupled BSA	% BSA released		% coupled BSA	% BSA released	
		− toxoid	+ toxoid		− toxoid	+ toxoid
PC/CHOL/APSA	15.1 [b] (16.1) [c]	42.1	45.8	16.4 [d] (18.2) [e]	69.0	63.5
DSPC/CHOL/APSA	15.5 [b] (16.4) [c]	61.8	60.0	15.0 [d] (16.8) [e]	81.9	72.6

[a] (SUV-BSA)DRV or DRV-BSA liposomes with or without entrapped tetanus toxoid and composed of PC or DSPC, cholesterol and APSA (molar ratios of 1:1:0.05) were coupled to ^{131}I-labelled BSA (5 mg used). BSA coupling or release after protease treatment, were estimated by the assay of ^{131}I radioactivity.

[b] Values (% of BSA used for coupling to SUV and subsequently recovered with (SUV-BSA)DRV) correspond to 50.3 (PC) and 51.8 (DSPC DRV) μg BSA per μmole phospholipid.

[c] Values denote % of the protein used originally coupled to SUV.

[d] % Values denote % of BSA used coupled to toxoid-free DRV and correspond to 54.6 (PC) and 50.0 (DSPC DRV) μg BSA per μmole phospholipid.

[e] Values denote BSA coupling to toxoid-containing DRV.

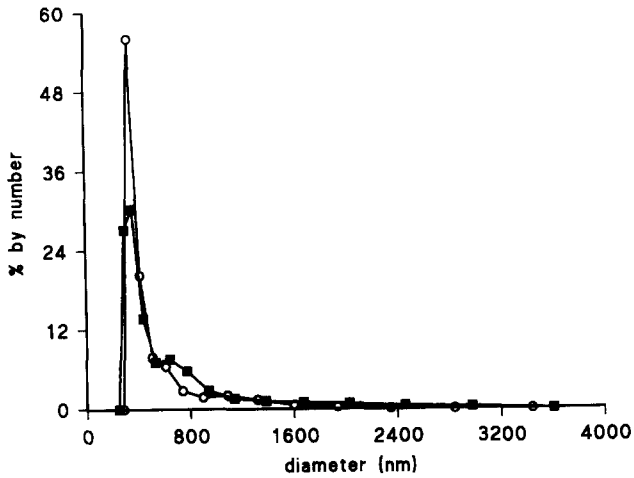

Fig. 1. Size distribution (% number) of BSA-free DRV (■) and DRV with bound BSA and entrapped toxoid (○). *z*-Average mean diameter values for the two formulations as measured by photon correlation spectroscopy were 555.3 and 543.3 nm and polydispersity values were 0.586 and 0.651, respectively. For other details see Materials and Methods.

and 1088–2840 nm (65.2% of the mass; BSA-containing DRV) (not shown). Similar *z* average mean sizes (about 520–580 nm) and mean number and mass diameter distribution data (not shown) were obtained for control DRV preparations as described above.

Morphological observations on DRV formulations

Fig. 2 shows a freeze-fracture electron micrograph of (PC) SUV with bound BSA prior to their use for DRV generation. The range of vesicle sizes in this representative micrograph confirms previous findings [25,26] of a size range of 30–100 nm for conventional SUV prepared by the same method. DRV produced from BSA-free (Fig. 3a) or BSA-incorporating SUV in the presence of toxoid (Fig. 3b) are again representative of the two types of vesicles formed by the dehydration-rehydration procedure, show no dissimilarity in appearance and their multilamellar structure is clearly illustrated. Lower magnification micrographs of BSA-free (Fig. 4a) and BSA-incorporating DRV (Fig. 4b) show representative populations of vesicles of varying sizes. Many of the vesicles fall within the size limits shown in Fig. 1 although some are very large (up to 7000 nm in diameter; without the accuracy limits of the apparatus used for photon correlation spectroscopy). Again, there is no difference in vesicle appearance between the two DRV formulations.

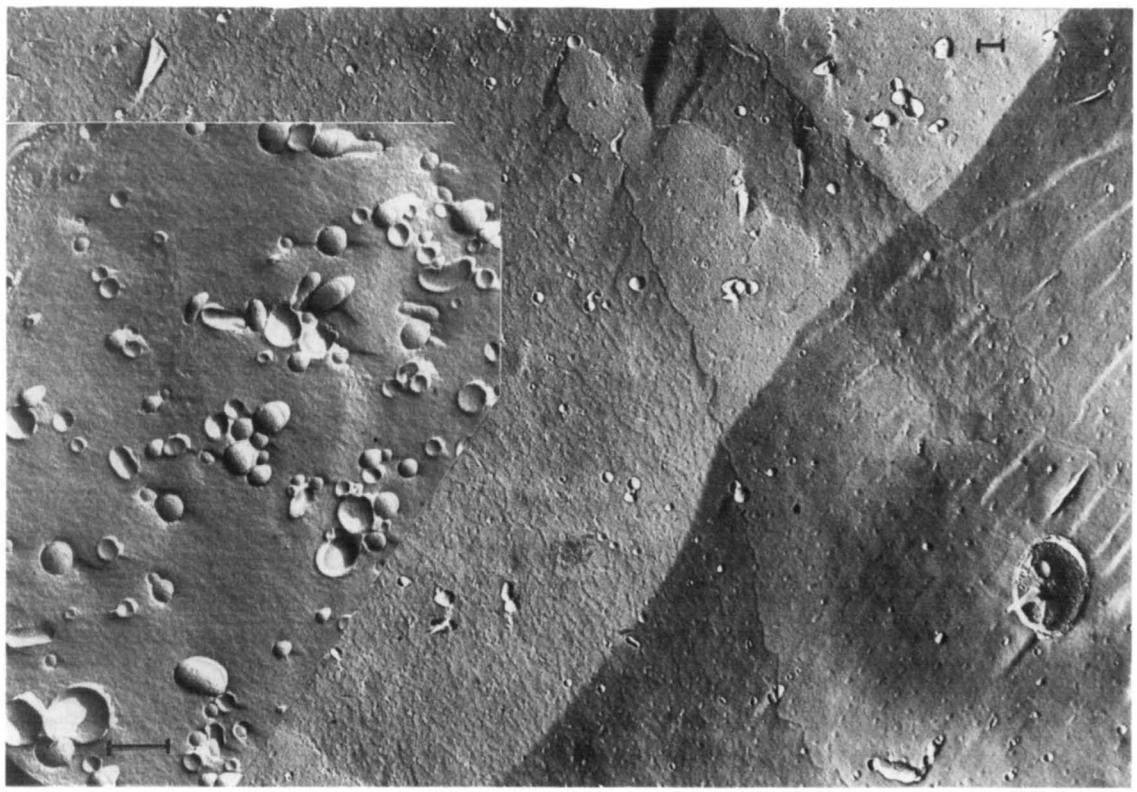

Fig. 2. Freeze-fracture electron micrographs of SUV wit bound BSA. Inset shows the SUV-BSA in a higher magnification. Bars 200 nm; shadowing direction is from bottom to top.

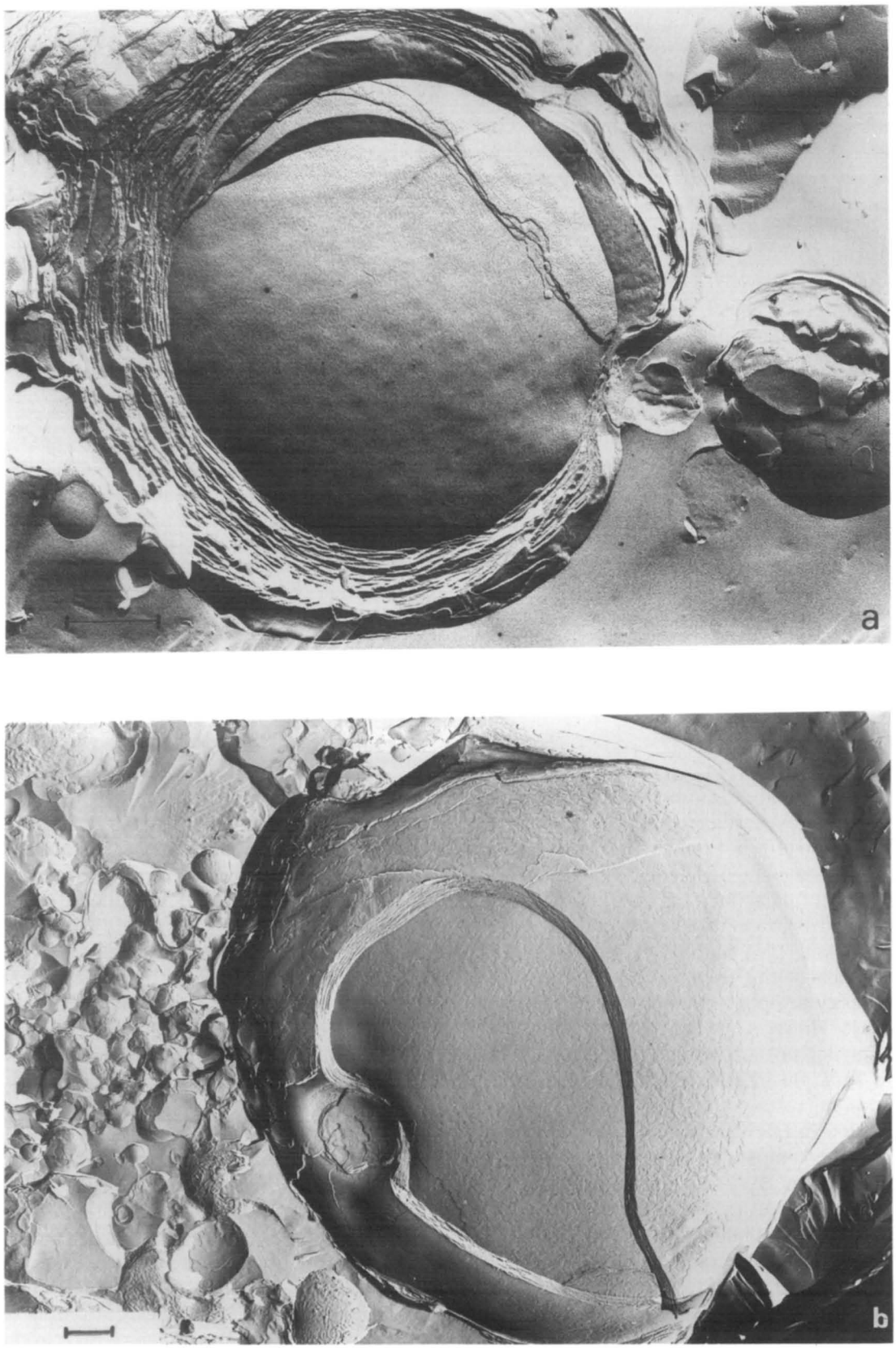

Fig. 3. Freeze-fracture electron micrographs of BSA-free DRV (a), and BSA-incorporating toxoid-containing DRV (b). Note multilamellar structure of DRV. Bars 500 nm; shadowing direction is from bottom to top.

BSA presence on the surface of DRV

In studies designed to establish the extent to which BSA bound to SUV is exposed on the surface of the subsequently formed DRV [(SUV-BSA)DRV], the latter were incubated with proteinase in PBS. According to results from a typical experiment (Table I), 42.1% of the total DRV-incorporated BSA could be digested (in terms of released radioactivity) by the enzyme for PC, and considerably more (61.8%) for DSPC liposomes. Further, the presence of toxoid within the DRV did not appear to influence BSA accessibility to the enzyme (45.8% and 60.0% of BSA digested for PC and DSPC liposomes, respectively) (Table I). These values of availability (to proteinase) of BSA on the DRV surface are much higher than anticipated from the multilamellar nature (Figs. 3a, b) of the vesicles. Indeed, judging from the incomplete digestion of BSA coupled directly to the surface of preformed DRV (63.5%–81.9% released radioactivity; Table I) and presumably entirely external to the vesicles, values are probably an underestimate: In the case of (SUV-BSA)DRV, BSA originally bound to the outer surface of the APSA-incorporating SUV, would have been expected to be evenly distributed in the bilayers of DRV formed on rehydation of the freeze-dried SUV, with only a small fraction of the protein exposed on the outer bilayer. However, a similar extensive recovery of the ligand on the DRV surface has also been observed for immunoglobulin G [14] and *p*-aminophenyl α-D-mannopyranoside [27], both of which were initially linked to SUV.

As alluded to elsewhere [14,27], one possible explanation for this apparently disporportional distribution of ligand on the DRV surface would be that (SUV-BSA)DRV are a mixture not only of DRV vesicles of various sizes (and number of lamellae) but also of a large proportion of lamellar sheets and membrane fragments, formed because of a possible interference of the APSA-bound BSA with DRV generation. In such a case, much of the BSA associated with these putative non-vesicular open structures would be accessible to protease. However, no such structures (at least to a significant degree) are suggested in the data of Fig. 1 or are apparent in a number of samples examined by electron microscopy (e.g., Figs. 4a, b). Furthermore, a reduction in DRV formation would be inconsistent with (a) the comparable CF entrapment values of 35.2 and 32.8% for BSA-free and BSA-incorporating PC DRV (see legend to Table II) which are also similar to those (30%) obtained with conventional PC DRV [12]; (b) the toxoid entrapment values of 47.4% and 40.0% (PC and DSPC (SUV-BSA)DRV, respectively; Table II) which are similar not only to those (42.2% and 38.1% observed with PC and DSPC BSA-free liposomes (Table II)) but also to values (47.5 ± 7.4; 12 preparations) obtained by entrapping the toxoid in conventional APSA-free PC/CHOL DRV using practically the same amount (16 μmoles) of phospholipid [13]. On the other hand, it is possible that during rehydration of the lyophilised SUV to generate DRV, much of the APSA-BSA complex originally embedded into the SUV bilayers is forced, by an inability to accommodate itself in the narrow water spaces between the bilayers of the forming DRV, to incorporate itself in the outer bilayers and thus assume surface exposure.

TABLE II

Entrapment of tetanus toxoid in DRV liposomes and surface availability

^{125}I-labelled tetanus toxoid (0.5 mg used) (and 0.06 M CF) was entrapped in (SUV-BSA)DRV liposomes without or with bound BSA (5 mg used in the coupling reaction). Liposomes were composed of phospholipid, cholesterol and APSA (molar ratio 1:1:0.05). Toxoid entrapment or release after protease treatment were estimated by the assay of ^{125}I radioactivity. CF entrapment values for BSA-free and BSA incorporating PC DRV were 35.2 and 32.8% of the amount used, respectively. For other details see Materials and Methods.

Composition of liposomes	(SUV-BSA)DRV			
	% toxoid entrapped		% toxoid released	
	−BSA	+BSA[a]	−BSA	+BSA[a]
PC/CHOL/APSA	42.2	47.4	31.2	47.5
DSPC/CHOL/APSA	38.1	40.0	28.7	59.7

[a] For amount of BSA bound see Table I.

The effect of DRV-bound BSA on entrapped solute localization and vesicle stability

It was thought conceivable that a significant movement (as suggested above) of BSA, originally bound to SUV, to the surface of DRV formed from the remains of such vesicles may at the same time alter the spatial arrangement of solutes entrapped in DRV during their formation and, also, perturb the DRV bilayers thus affecting their stability. These possibilities were examined respectively by (a) measuring the extent to which entrapped tetanus toxoid becomes available on the DRV surface; (b) monitoring the latency of entrapped CF in the presence of PBS and blood plasma.

With regard to toxoid availability on the surface of DRV, as ascertained by exposing liposomes to proteinase, Table II shows that for DRV made of either (PC or DSPC) phospholipid but with no BSA bound on the precursor SUV, a significant amount (31.2 and 28.7%, respectively) of toxoid is already exposed on the liposomal surface. This is probably the result of regions of entrapped toxoid molecules protruding through the bilayers rather than adsorption of protein during DRV formation: very little (< 4%) of the protein was recovered in (SUV-BSA)DRV or plain DRV when these

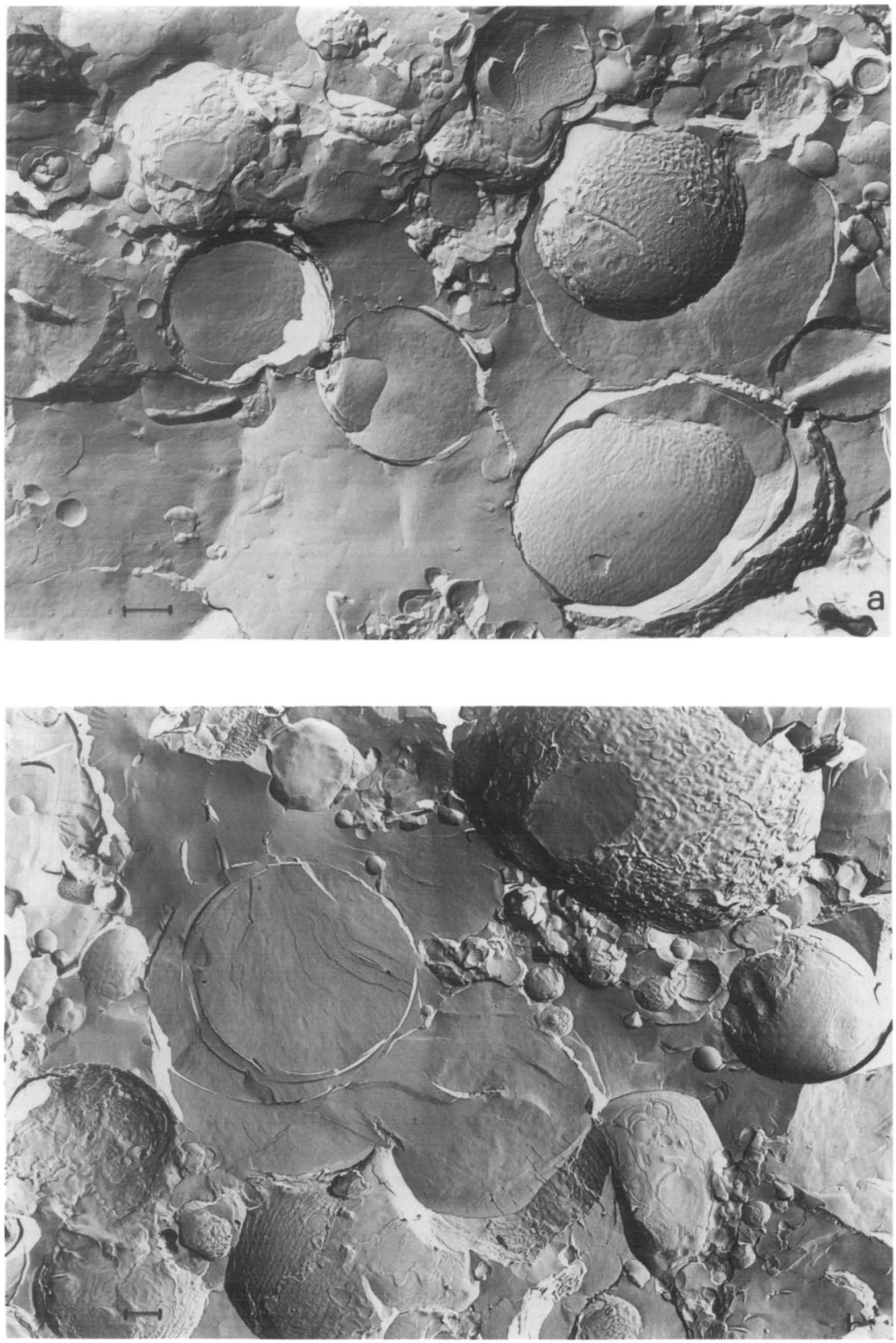

Fig. 4. Freeze-fracture electron micrographs of BSA-free (a) and BSA-incorporating, toxoid-containing DRV (b). Note heterogeneity in vesicle size. Bars 500 nm; shadowing direction is from bottom to top.

were mixed with toxoid (not shown). However, availability of entrapped toxoid to protease becomes much greater (47.5 and 59.7% for PC and DSPC liposomes, respectively) when DRV are generated (in the presence of toxoid) from BSA-coated SUV (Table II). Such findings were confirmed in a separate experiment (Fig. 5) where a linear relationship between increasing amounts of BSA bound to DSPC liposomes and increasing toxoid release on protease treatment was observed. It would thus appear that apparent re-orientation of APSA-bound BSA on the surface of DRV liposomes during their formation in the presence of toxoid, is associated with a parallel movement of the latter to the outer bilayers. Fig. 5 also shows that on the basis of constancy of CF latency values observed, removal of external toxoid (and BSA) regions by protease does not reduce bilayer integrity even for the highest amount of bound BSA where surface localization of the toxoid is greatest.

As anticipated from previous work [20], incubation of (BSA-free) DSPC DRV liposomes with PBS or mouse plasma for up to 24 h produces no change in their stability (Fig. 6). On the other hand, the presence of bound BSA in the same liposomes seems to reduce bilayer integrity in PBS, albeit only modestly. On addition of plasma, stability of preparations with the lower amounts (e.g., 9.5 and 23.7 μg BSA per μmole phospholipid) of bound BSA remains unchanged and it is only when larger amounts of the protein (e.g., 47.4 and 94.8 μg) are bound to liposomes that these become progressively unstable to reach a CF latency value of about 50% at 24 h for the preparation with the largest concentration of BSA. Thus, the process of incorporating BSA onto the DRV surface by previously coupling the protein to the SUV, renders DRV susceptible to destabilization by plasma when the amount of bound

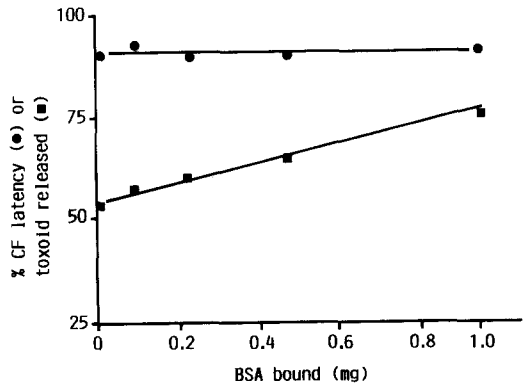

Fig. 5. Effect of BSA bound to DSPC DRV on the availability of tetanus toxoid on the liposomal surface. (SUV-BSA)DRV (0.1 ml) with increasing amounts of bound ^{131}I-labelled BSA, containing CF and ^{125}I-labelled tetanus toxoid, were exposed to protease for 60 min. Values shown are % CF latency and % ^{125}I radioactivity released for amount (mg) of BSA bound per 1.0 ml liposomes. For other details see Materials and Methods.

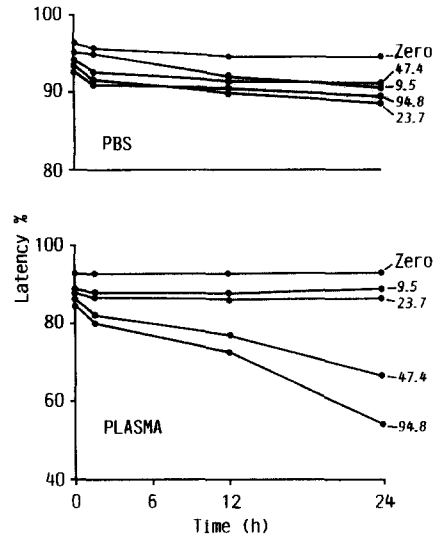

Fig. 6. Effect of BSA bound to DRV on vesicle stability in the presence of plasma. (SUV-BSA)DRV (0.1 ml) with increasing amounts of bound BSA were incubated with 0.5 ml of PBS (upper panel) or mouse blood plasma (lower panel) at 37°C. Samples of the incubation mixtures were assayed at time intervals for CF latency. Amounts (μg per μmol phospholipid) of bound BSA are shown for each preparation. For other details see Materials and Methods.

protein is sufficiently high and exposure to plasma prolonged. However, for shorter periods of exposure (e.g., 2 h) destabilization is only modest (CF latency down to 80%) even for the preparation with the highest amount of bound BSA. Taking into consideration that intravenously injected multilamellar liposomes are cleared from the circulation rapidly (more than 90% of the dose is removed from the circulation within 1 h after injection [28], contact of (SUV-BSA)DRV with plasma is expected to be relatively brief in vivo, with vesicle stability remaining essentially unaltered.

In conclusion, results indicate that after coupling of a model ligand (BSA) to SUV, a considerable proportion of it is expressed on the surface of the subsequently formed DRV. As show previously with mannosylated BSA (as a targeting ligand for liposomal vaccines) [29] and a specific antibody [14], ligands bound to the liposomal surface by the present method retain their ability to interact with the respective receptors. Since entrapment of solutes in DRV is carried out after the coupling of the ligand to the precursor 'empty' vesicles, there is no contact of coupling reagents with solutes destined for entrapment, thus avoiding their possible denaturation or inactivation. For instance, in the case of antigens [13,30], this could potentially lead to the unmasking of irrelevant epitopes or changes in their immunogenicity and, with enzymes [14] and cytokines [15], to their inactivation. Further, such ligand-bearing DRV retain much of their stability on incubation with blood plasma for at least 2 h. Stability is,

however, reduced with longer periods of incubation, but only when the amount of bound ligand is excessive.

It is suggested that the present approach of coupling ligands to liposomes is appropriate for the preparation of targeted formulations containing labile pharmacologically active agents, especially those that are relevant to liposomal vaccines [30]. Recent work [22] has shown that drug-containing DRV liposomes can be reduced in size by a microfluidization technique which ensures quantitative drug retention even by the smallest (about 100 nm diameter) vesicles produced. As small liposomes have a longer half-life in the blood circulation [20] and are therefore more suitable for intravascular targeting, work is in progress to apply the technique [22] to ligand-bearing, drug-containing DRV as such or in association with a hydrophilic surface [31].

Acknowledgements

This work was supported by a Medical Research Council project grant to G.G. N.G. was a British Council scholar during her visit in the senior author's laboratory.

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