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Protein-liposome conjugates with defined size distributions

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Conjugation of protein to liposomes by two coupling protocols is shown to result in vesicle aggregation. The degree of aggregation is directly related to the levels of protein conjugated to the liposomes. In an attempt to develop a method of generating stable, homogeneously sized protein-conjugated vesicles, highly aggregated liposome-protein conjugates were extruded through filters of defined pore size. The extrusion procedure is shown to allow the efficient production of protein-liposome conjugates of defined size distributions, with no loss of protein binding. The extruded samples are relatively stable with respect to size and are easily prepared for various protein to lipid ratios. Liposome size has been shown to be a major factor in determining the in vivo blood circulation times of liposomes. A corresponding, significant enhancement in the blood circulation lifetimes for extruded versus aggregated streptavidin-liposome conjugates is observed. Furthermore, the stability of streptavidin-liposome conjugates in vivo was shown by the binding of biotin to liposomes isolated from plasma 1 and 4 h post-injection. In conclusion, extrusion of the aggregated systems obtained on coupling proteins to liposomes provides a convenient and general method for generating homogeneously sized protein-liposome conjugates.

Introduction

Numerous techniques for the conjugation of proteins to liposomes have been developed over the last decade (for review see Refs. 1–3). These protein liposome conjugates have been utilized for a variety of purposes such as the targeting of drugs via immunoliposomes [4–6], diagnostic protocols [7,8] and liposomal vaccines [9]. These studies emphasize the requirement for a general technique of coupling proteins to liposomes. Particular areas of interest concern optimization of the coupling efficiency, the stability of the cross-link between the protein and liposome and the in vitro capabilities of the conjugates to bind and deliver liposomally encapsulated materials to cells (reviewed in Refs. 1, 10 and 11).

In this study we demonstrate, by two physical techniques, that the attachment of protein to liposomes by various coupling protocols results in vesicle aggregation. The amount of liposomally attached protein was shown to determine the extent of vesicle cross-linking. We report a novel method for the generation of protein-liposome conjugates with defined size distributions, obtained by the extrusion of protein-coupled vesicles through filters of defined pore size. This procedure provides a relatively gentle method of producing protein-liposome conjugates of stable size. No significant denaturation of the attached protein is observed. With regard to developing protein coupled liposomes for in vivo applications, the influence of the size of protein-liposome conjugates on blood clearance behavior was investigated in mice. The results indicate that the size of the conjugate is a major factor determining the circulation of liposome conjugates.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC), and dipalmitoylphosphatidylethanolamine (DPPE) were obtained from

Abbreviations: SMPB, *N*-succinimidyl-4-(*p*-maleimidophenyl)butyrate; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate, MPB-DPPE, *N*-(4-(*p*-maleimidophenyl)butyryl)dipalmitoylphosphatidylethanolamine; CHOL, cholesterol; HBS, 25 mM Hepes, 150 mM NaCl (pH 7.5); DTT, dithiothreitol; biotin EPE, biotin egg phosphatidylethanolamine; Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; QELS, quasi-elastic light scattering.

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Avanti Polar Lipids. Biotin egg phosphatidylethanolamine (biotin EPE), *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), *N*-succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) were obtained from Molecular Probes. Streptavidin, FITC-cellite, *N*-ethylmaleimide, dithiothreitol (DTT), cholesterol, β-mercaptoethanol, *N*-(2-hydroxyethyl)piperazine-*N*-3-propanesulphonic acid (Epps), 2-(*N*-morpholino)ethanesulphonic acid (Mes), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (Hepes) and Sephadex G-50 were obtained from Sigma. Anti-human erythrocyte IgG was obtained from Cappel and Sepharose CL-4B from Pharmacia. [^{14}C]Cholesterol and [^{3}H]cholesteryl hexadecyl ether were obtained from NEN. [^{3}H]- and [^{14}C] biotin were obtained from Amersham. Female DBA/2J mice (6 weeks of age) were obtained from Jackson Laboratories.

Synthesis of N-(4-(p-maleimidophenyl)butyryl)dipalmitoylphosphatidylethanolamine (MPB-DPPE)

MPB-DPPE was prepared by a modification of the method of Martin et al. [12] as previously described [13]. Briefly, MPB-DPPE was synthesized by reacting DPPE (69 mg) with SMPB (65 mg) in chloroform (5 ml) containing triethylamine (10 mg) at 40°C. After 2 h, TLC on silica showed conversion of DPPE to a faster running product (solvent system: chloroform/methanol/acetonitrile/water (75 : 16 : 5 : 4, v/v); R_f 0.6). The solution was diluted with chloroform (10 ml) and washed several times with NaCl (0.9%) to remove by-products of the reaction. The solution was further concentrated in vacuo and the solid residue was triturated and recrystalized from diethylether to remove unreacted SMPB. Further recrystalization from diethyl ether/acetonitrile yielded a pure product as indicated by ^{1}H-NMR analysis (Bruker W40, 400 MHz) and low resolution mass spectroscopy (KRATOS MS 50).

Preparation of liposomes

Large unilamellar liposomes were prepared as described by Hope et al. [14]. Briefly, appropriate amounts of lipid mixtures in chloroform were deposited in a tube and dried to a lipid film under a stream of nitrogen followed by high vacuum for 2 h. Lipid was then hydrated in 25 mM Mes, 25 mM Hepes, 150 mM NaCl (pH 6.5) and extruded through two stacked 100 nm or 50 nm filters ten times. Prior to coupling experiments, samples were titrated to pH 7.5 with NaOH. Lipid was estimated either by the colorimetric method of Fiske and SubbaRow [15] or by incorporating trace amounts of [^{14}C]cholesterol or [^{3}H]cholesteryl hexadecyl ether in the lipid mixture. Such samples were assayed by scintillation counting in a Packard Tri-Carb liquid or a Beckmann model LS 3801 scintillation analyzer.

Preparation of proteins for coupling

Streptavidin (10 mg/ml in HBS (25 mM Hepes, 150 mM NaCl, pH 7.5)) was modified with the amine reactive reagent, SPDP according to published procedures [16]. Briefly, SPDP (25 mM in methanol) was incubated at a 10 molar ratio to streptavidin at room temperature for 30 min. To estimate the extent of modification, a portion of the reaction mixture was passed down Sephadex G-50 equilibrated with HBS to remove unreacted SPDP. The extent of modification of streptavidin was determined by estimating the protein concentration at 280 nm (molar extinction coefficient at 280 nm (ϵ_{280}): $1.847 \cdot 10^5$) prior to the addition of dithiothreitol (DTT) and the 2-thiopyridone concentration at 343 nm (ϵ_{343}: 7550) 10 min after the addition of DTT (25 mM). The remainder of the reaction mixture was reduced with DTT (25 mM, 10 min) and the thiolated product was isolated by gel exclusion on Sephadex G-50 equilibrated with 25 mM Mes, 25 mM Hepes, 150 mM NaCl (pH 7.5). The product was immediately used in coupling experiments. In the case of IgG (20 mg/ml in HBS; ϵ_{280}: $2.025 \cdot 10^5$), following the modification of the protein with SPDP, the protein was fluorescently labelled with FITC-cellite (50% weight of IgG in 150 mM NaCl, 0.2 M $NaHCO_3$ (pH 8.8), 20 min). Prior to the treatment of the protein with DTT, the sample was separated from unreacted reagents on Sephadex G-50 equilibrated with an acetate buffer (100 mM NaCl, 100 mM Na acetate (pH 5.0)), to protect against the reduction of the intrinsic disulphides of the molecule. The sample was concentrated to 5 mg/ml by dehydration with aquacide prior to coupling experiments. The extent of modification of streptavidin was 5–6 SPDP molecules per protein while the modification of the antibody preparation resulted in 2–3 molecules of SPDP per protein.

Coupling of proteins to liposomes

The coupling of proteins to liposomes was performed by incubating the reduced PDP-modified protein with liposomes (54 mol% EPC, 45 mol% cholesterol, 1 mol% MPB-DPPE, sized through filters of 50 or 100 nm pore size), at a ratio of 100 μg protein/μmol lipid (5–30 mM final lipid concentration) at pH 7.5 as described elsewhere [13]. The reaction was quenched at various times by the addition of *N*-ethylmaleimide (500 molar ratio to protein, methanol stock). For in vivo experiments, samples were further quenched with β-mercaptoethanol (10 molar ratio with respect to *N*-ethylmaleimide) after a 2 h incubation of the reaction mixture with *N*-ethylmaleimide. Uncoupled protein was removed by gel filtration on Sepharose CL-4B equilibrated with HBS. The extent of coupling of streptavidin to liposomes was measured by the binding of [^{3}H]- or [^{14}C]biotin to streptavidin. Briefly, streptavidin-liposomes (0.25 μmol lipid in 0.5 ml) were in-

cubated with [^{3}H]- or [^{14}C]biotin (3.85 nmol in 25 μl, 15.4 nmol/μCi) for 10 min and unbound biotin was removed by gel filtration on Sepharose CL-4B equilibrated with HBS. The extent of binding of biotin to a streptavidin standard (100 μg) after gel chromatography on Sephadex G-50 was used as a reference for the calculation of coupling ratios. For the determination of the extent of antibody coupled to liposomes, samples (200 μl) were dissolved in ethanol (1.8 ml) and the liposome associated fluorescence was correlated to a known quantity of fluorescein labelled antibody. Fluorescence was monitored at 520 nm using a SLM-500C spectrofluorometer with an excitation wavelength of 495 nm.

Prior to the non-covalent attachment of streptavidin to liposomes, streptavidin was fluorescently labelled with FITC-cellite as described above for IgG. Subsequently, streptavidin (4.1 mg) was incubated for 10 min. with liposomes (54.75 mol% EPC, 45 mol% cholesterol, 0.25 mol% biotin EPE) at a 10 molar excess to biotin EPE as described previously [17]. The extent of coupled streptavidin was determined after gel filtration on Sepharose CL-4B as described for IgG.

Preparation and characterization of extruded protein-liposome samples

Protein-liposome conjugates (5 mM or 20 mM final lipid concentration) were extruded ten times through two stacked Nuclepore filters (0.1 or 0.05 μm in pore size). Lipid recovery was estimated by scintillation counting of an aliquot of the extruded sample. Freeze fracture was performed on samples containing 25% (v/v) glycerol as a cryoprotectant. Samples were frozen in liquid nitrogen, prepared using a Balzers BAF 301 apparatus and replicas were viewed employing a Philips 400 electron microscope. The size of the protein-coupled vesicles before and after extrusion was estimated by quasi-elastic light scattering (QELS) using a Nicomp Model 270 submicron particle sizer operating at 632.8 nm and 5 mW.

In vivo studies of liposome preparations

For in vivo studies streptavidin-liposome conjugates were prepared at a final lipid concentration of 30 mM and a coupling period of 15 min, essentially as described above. A portion of the sample was extruded ten times through two stacked 50 or 100 nm filters immediately prior to injection. As controls, liposomes containing MPB-DPPE (54 mol% EPC, 45 mol% cholesterol, 1 mol% MPB-DPPE) were prepared at pH 6.5 as described above. An aliquot of the lipid sample was titrated to pH 7.5 with NaOH, quenched with β-mercaptoethanol (10 molar excess to MPB-DPPE) and free β-mercaptoethanol was removed by gel filtration on Sephadex G-50 equilibrated with HBS. Unquenched MPB-DPPE liposomes were exchanged on Sephadex G-50 equilibrated with HBS prior to in vivo experiments. Control liposomes (55 mol% EPC, 45 mol% cholesterol) were prepared in HBS.

For in vivo plasma lipid level determinations, mice (4–8/time point) were injected via the tail vein at a dose of 100 mg total lipid/kg (injected volume of 200 μl). Blood was collected by cardiac puncture into EDTA treated microcontainers (Becton-Dickenson). Plasma was prepared by centrifuging (200 × *g*) whole blood for 10 min in a clinical centrifuge. Total plasma volume per animal was taken to be 4.55% of mean body weight. Liposomal lipid was quantified employing the non-metabolizable, non-exchangeable lipid marker [^{3}H]cholesteryl hexadecyl ether (specific activity: 0.23 μCi/mg total lipid) [18,19]. For scintillation counting, 50–100 μl plasma was added to 5 ml Pico-Fluor 40 (Packard, Canada) scintillation cocktail and samples were counted in a Beckman model LS 3801 scintillation counter. Control blood samples containing known amounts of liposomes showed that only a minor fraction of the liposomal lipid was associated with the pelleted blood cells. The recovery of liposomes was similar if determined from whole blood or from plasma. The levels of streptavidin associated with liposomes in vivo was determined by the binding of [^{14}C]biotin to a plasma sample isolated 1 and 4 h post injection.

Results

Initial efforts were centered on characterizing the influence of protein conjugation on vesicle size. As shown in Fig. 1, an increase in the amount of protein bound to liposomes results in a significant increase in vesicle size as recorded by QELS. The initial rapid coupling of streptavidin to vesicles correlates with a rapid increase in the size distribution of the preparation. In order to confirm this observation employing a different technique and to examine the morphology of the larger systems, aliquots of the same coupling system were examined by freeze-fracture. The results presented in Fig. 2, clearly show that the increase in size as measured by QELS is related to vesicle aggregation. However, after extended periods of incubation, a significant number of large vesicles (> 200 nm) are observed, presumably due to fusion events following aggregation.

An optimized coupling protocol should not affect the overall size distribution of the conjugated system. In an attempt to achieve small, homogeneously sized protein-liposome conjugates, the effects of extruding aggregated, conjugated vesicles through filters with 100 nm pore size were examined for liposomes with attached streptavidin (Fig. 3) or antibody (Fig. 4). The coupling reaction mixtures were quenched with *N*-ethylmaleimide at various times and the size of the coupled samples prior to and after extrusion was estimated by

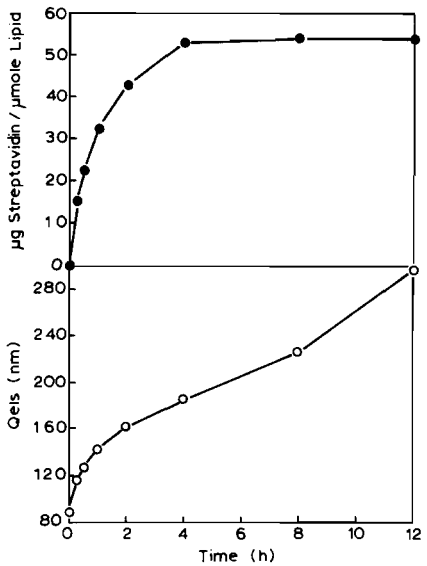

Fig. 1. Effect of coupling streptavidin to liposomes on vesicle size. Liposomes (54 mol% EPC, 45 mol% CHOL, 1 mol% MPB-DPPE, 5 mM final lipid concentration, 100 nm) were incubated with streptavidin (100 µg protein/µmol lipid) over time at pH 7.5. At various time points, the reaction was quenched by addition of *N*-ethylmaleimide (500 molar ratio to protein) and free streptavidin was removed by gel filtration on Sepharose CL-4B. Extent of coupled streptavidin was determined by [^{3}H]biotin binding (top figure) and vesicle size was estimated by QELS (bottom figure).

QELS (see insets for Figs. 3 and 4). The extent of coupled protein was determined after extrusion of conjugated samples. Irrespective of the amount of protein coupled to the liposomes, both types of coupled vesicles were readily extruded and the resulting preparations fell within a narrow size range. For example, extrusion of liposomes with attached streptavidin (25–60 µg/mol lipid) resulted in a vesicle sizes of 120–140 nm in diameter as compared to initial size distributions of 150 to more than 590 nm. Similarly, extrusion of antibody-liposome conjugates (15–35 µg protein/µmol lipid) resulted in smaller vesicles of narrow size distribution (90–110 nm) compared to a size range of 130–230 nm prior to extrusion. Importantly, the loss of lipid for both protein coupled vesicles during the extrusion process was minimal (85–90% lipid recovery). The extrusion procedure did not significantly reduce the level of protein association. For IgG coupled liposomes, approx. 10% of the associated protein (as measured by FITC labelled IgG) was lost following extrusion. For streptavidin coupled liposomes a 5% to 10% increase in protein association was typically observed after extrusion (see Table II). Streptavidin was measured by a functional assay, [^{14}C]biotin binding, and this increase in association may reflect an enhanced accessibility of biotin for streptavidin associated with non-aggregated (extruded) liposomes. These results demonstrate that highly aggregated preparations of vesicles with high levels of conjugated protein can be extruded efficiently and the resulting preparations are of a similar size to the precursor vesicles. Furthermore, the extrusion of protein-liposome aggregates represents a gentle method of preparing size protein conjugated vesicles. This was illustrated by the retention of streptavidin-liposome conjugates to bind biotin after extrusion. In addition, studies employing liposomes with encapsulated markers such as 5, 6-carboxylfluorescein and calcein suggest that these trapped agents are retained to a large extent following-extrusion (unpublished observations).

The observation that liposomes aggregate during protein coupling to liposomes is not unique to the covalent procedure described above. Vesicle aggregation also occurs during the non-covalent attachment of streptavidin to liposomes containing biotin EPE [17]. To demonstrate the general application of the extrusion process as a means of generating sized populations of protein-liposome conjugates, the effect of extrusion of streptavidin coupled covalently to liposomes containing MPB-DPPE or non-covalently bound to liposomes containing biotin EPE, was examined by freeze-fracture (Fig. 5). Both types of streptavidin-liposome conjugates were observed to be highly aggregated prior to extrusion. After extrusion the coupled vesicles existed as monomers or dimers with the maximum aggregate observed to be a conglomerate of four vesicles. Approx. 50% of the resulting liposomes existed as monomers as judged by analysis of the freeze-fracture micrograph. In the case of the non-

TABLE I

Factors affecting the aggregation of extruded streptavidin-liposomes

Sample characteristics		QELS size estimates of streptavidin-liposome conjugates (nm)		
µg streptavidin /µmol lipid	Lipid concn. (mM)	before extrusion	after extrusion	
			0 h	8 h
0 [c]	2.5	110	104	104
17.1 [a]	2.5	177	109	119
31.6 [a]	2.5	232	119	140
45.3 [a]	2.5	286	123	154
45.1 [b]	5.0	403	174	197
45.1 [b]	15.0	403	174	197
45.1 [b,d]	5.0	403	174	182
45.1 [b,e]	5.0	403	174	188

[a] Liposome samples (54 mol% EPC, 45 mol% CHOL, 1 mol% MPB-DPPE) were prepared with different levels of coupled streptavidin by quenching the coupling mixture (20 mM final lipid concentration) with *N*-ethylmaleimide at various time points.

[b] Streptavidin-liposomes were prepared at a final lipid concentration of 30 mM and an incubation period of 15 min.

[c] Streptavidin (50 µg) quenched with *N*-ethylmaleimide was extruded with liposomes (1 µmol, 2.5 mM final lipid concentration) containing 1 mol% MPB-DPPE.

[d] Extruded samples were kept on ice for 3 h prior to QELS measurements.

[e] Extruded samples were frozen immediately after extrusion and thawed just prior to QELS measurements.

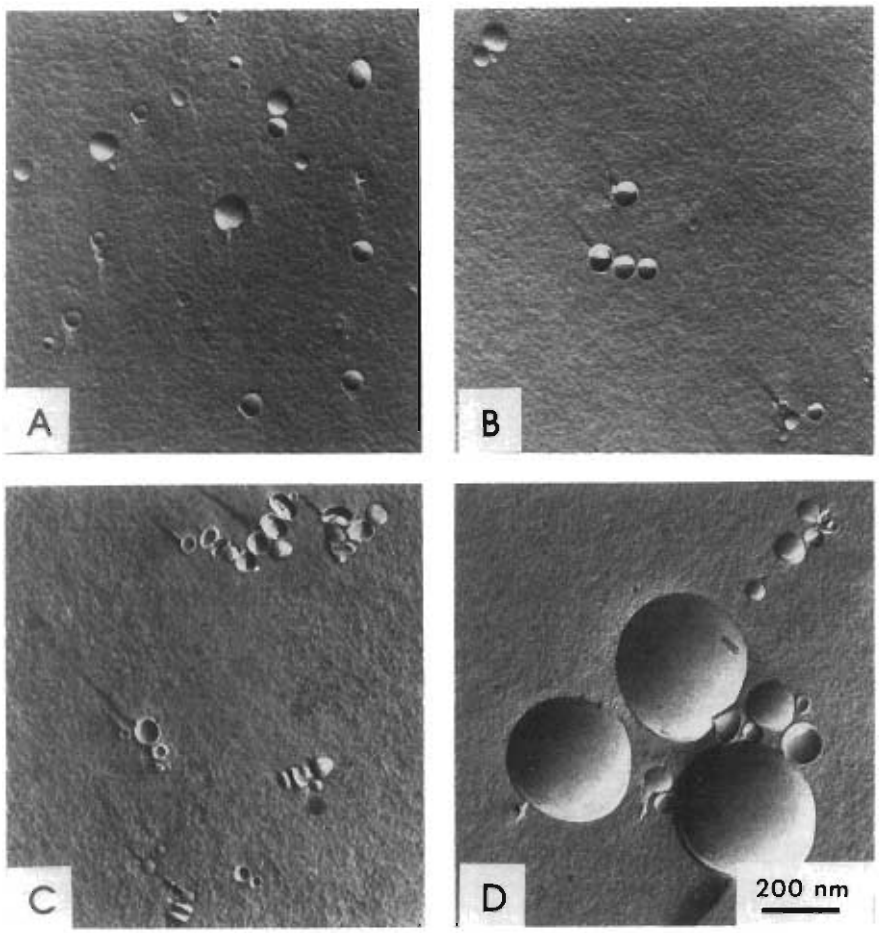

Fig. 2. Freeze-fracture of streptavidin-liposome preparations. Streptavidin-liposome samples quenched with *N*-ethylmaleimide at 0.5 (A), 2 (B), 4 (C) and 18 (D) h were prepared as described in Fig. 1 and examined by freeze-fracture.

covalent coupling procedure (Figs. 5C and 5D), significant loss of lipid occurred (50%) during the extrusion of coupled vesicles. The initial protein to lipid ratio, however, was maintained for the extruded sample.

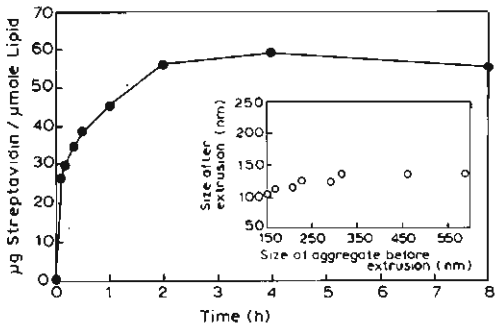

Fig. 3. Extrusion of streptavidin-liposome conjugates. Streptavidin was coupled to liposomes (100 nm) containing 1 mol% MPB-DPPE at a final lipid concentration of 20 mM. At various time points aliquots of the reaction mixtures were quenched with *N*-ethylmaleimide and diluted to 5 mM lipid concentration before extrusion through 100 nm filters. The extent of coupled streptavidin (●) was estimated by [^{3}H]biotin binding to streptavidin-liposomes after extrusion and gel exclusion of lipid samples on Sepharose CL-4B. The size of the liposome streptavidin conjugates was estimated by QELS before and after (inset, ○) extrusion as described in Materials and Methods.

The stability of extruded samples containing covalently bound streptavidin with respect to size is addressed in Fig. 6. QELS measurements indicate an initial

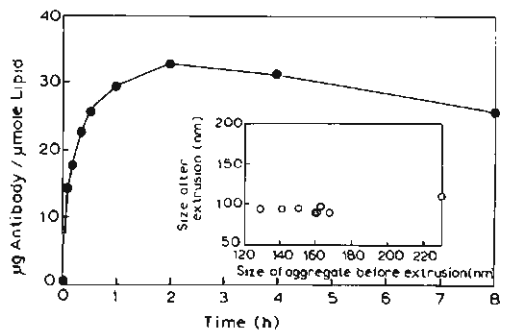

Fig. 4. Extrusion of antibody-liposome conjugates. Fluorescein-labelled antibody was coupled to liposomes (100 nm) containing 1% MPB-DPPE at a final lipid concentration of 20 mM. At various time points aliquots of the reaction mixtures were quenched with *N*-ethylmaleimide and diluted to 5 mM lipid concentration before extrusion through 100 nm filters. The extent of coupled antibody (●) was determined by estimating the levels of liposomally associated fluorescence after extrusion and exchange of lipid samples on Sepharose CL-4B. The size of the antibody-liposome conjugates was estimated by QELS before and after (inset, ○) extrusion as described in Materials and Methods.

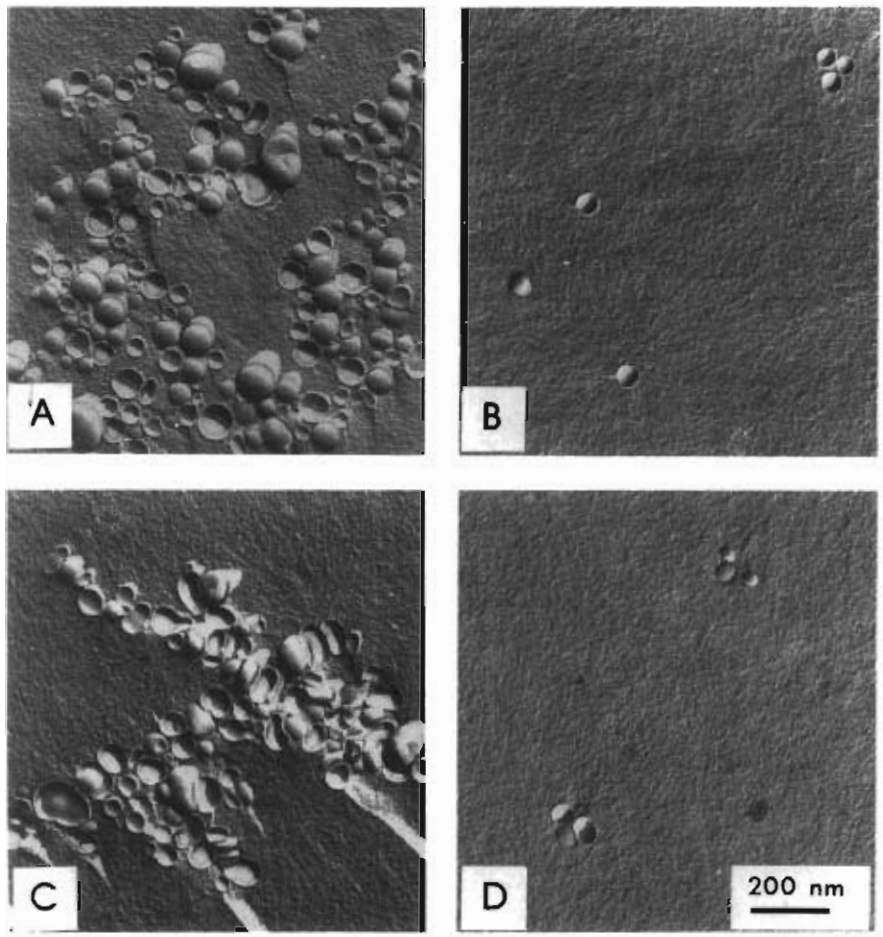

Fig. 5. Freeze-fracture of streptavidin-liposomes before and after extrusion. Streptavidin was coupled to liposomes at a final lipid concentration of 20 mM for 8 h as described in Fig. 1. The sample was diluted to 5 μmol/ml prior to extrusion. Non-covalent attachment of streptavidin to liposomes containing biotin EPE (0.25 mol%) was performed as described in Materials and Methods at a final lipid concentration of 5 mM. Samples were examined by freeze-fracture before and after extrusion through 100 nm filters. Streptavidin-liposomes containing MPB-DPPE before (A) and after (B) extrusion; streptavidin-liposomes containing biotin EPE before (C) and after (D) extrusion.

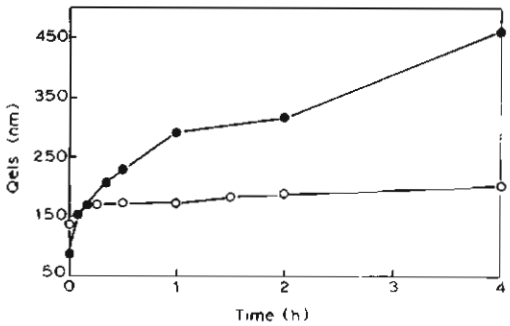

Fig. 6. Examination of the stability of extruded streptavidin-liposome conjugate by QELS. Streptavidin-liposomes with approx. 51 μg protein/μmole lipid were prepared by incubating thiolated streptavidin with liposomes containing 1 mol% MPB-DPPE for 8 h at a final lipid concentration of 20 mM. After removal of unbound streptavidin by gel filtration on Sepharose CL-4B, the sample was diluted to 5 mM lipid and extruded 10 times through two stacked 100 nm filters. At various time points the size of the extruded preparation was determined by QELS (○). Size of streptavidin-liposome conjugates prepared as in Fig. 1 are shown for comparison (●).

small (30 nm) rapid increase in the size of the preparation after extrusion. This was reflected by increased aggregation of the extruded vesicles as indicated by freeze-fracture (results not shown). As shown in Table I, the level of reaggregation observed 3 h after extrusion of various streptavidin-liposome conjugates was minimal when compared to the aggregated state of the samples prior to extrusion. Such reaggregation of liposomes was not observed when MPB-DPPE liposomes were extruded with thiolated-streptavidin which had been quenched by prior incubation with *N*-ethylmaleimide (see Table I). This indicates that reaggregation was not due to non-specific association of protein with liposomes. It was found that the incorporation of negatively charged lipids such as phosphatidylserine, or the presence of low or high ionic strength buffers did not prevent reaggregation (data not shown). Reduction of the amount of streptavidin coupled to vesicles (Table I)

resulted in a corresponding decrease in the extent of reaggregation observed 3 h after extrusion. Varying the lipid concentration of the extruded sample did not significantly affect reaggregation. Streptavidin coupled to liposomes which were frozen immediately after extrusion, maintained their original size distribution on thawing. Finally, storage of the extruded samples at 4°C resulted in increased stability of liposome size.

Large liposomes are rapidly removed from the blood circulation when compared to small preparations [20,21]. It is therefore possible that the rapid clearance observed for targeted systems in vivo [22,23] could be partly due to aggregation of liposomes. The time required for clearance from the blood of certain control liposome preparations (Fig. 7A) as well as aggregated and extruded streptavidin-liposome conjugates (Fig. 7B) in mice were therefore examined. Aggregated streptavidin-liposomes (530 nm in diameter as indicated by QELS) were cleared rapidly from the circulation; only 3% of the initial lipid dose remained in the circulation 4 h after injection. Extrusion of these protein-vesicle conjugates through 50 or 100 nm polycarbonate filters

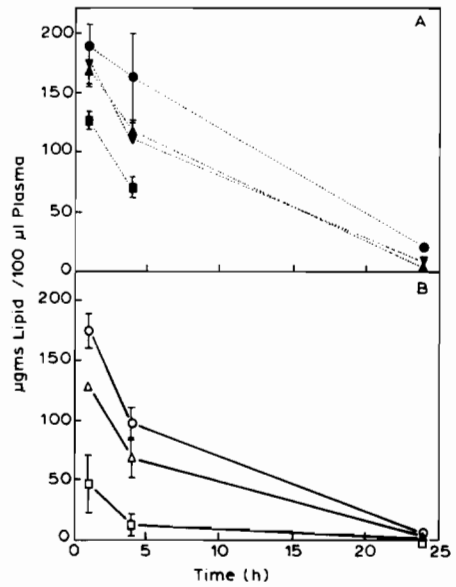

Fig. 7. In vivo clearance rates of protein-liposome conjugates. Streptavidin was coupled to liposomes (50 and 100 nm), at a final lipid concentration of 30 mM and incubation period of 15 min, quenched with *N*-ethylmaleimide for 2 h followed by an overnight incubation with β-mercaptoethanol. Control liposomes containing MPB-DPPE were titrated to pH 7.5 and exchanged on Sephadex G-50 equilibrated with HBS. EPC/cholesterol liposomes were made up in HBS. Mice (4–8 mice per time point) were injected with lipid at a dose of 100 mg/kg. Plasma was prepared from EDTA treated whole blood at specific time points and aliquots were analyzed by scintillation counting (see Materials and Methods for details). Size of extruded samples were determined by QELS. (A) EPC/cholesterol, 125 nm (●); EPC/cholesterol, 197 nm (■); MPB-DPPE liposomes, 170 nm (quenched ▲, unquenched ▼). (B) Aggregated 100 nm streptavidin-liposomes, 530 nm (□); streptavidin-liposomes extruded through 100 nm, 187 nm (△); streptavidin-liposomes extruded through 50 nm, 139 nm (○).

TABLE II

Stability of streptavidin-liposome conjugates in vivo

The amount of streptavidin attached to liposomes was determined by the binding of [^{14}C]biotin to lipid samples or pooled plasma samples from three mice, 1 and 4 h post-injection (see Materials and Methods).

Streptavidin-liposome conjugate (QELS size estimates)	µg streptavidin/µmol lipid		
	prior to administration	after administration	
		1 h	4 h
Aggregated (> 530 nm)	42.9 ± 0.1	43.1 ± 0.8	29.8 ± 0.8
Extruded (187 nm)	41.1 ± 2.8	35.4 ± 0.2	32.9 ± 0.3
Extruded (139 nm)	47.1 ± 0.5	44.5 ± 1.4	39.1 ± 0.6

resulted in preparations with size distributions of 139 and 187 nm respectively. Both of these preparations showed extended blood circulation times in vivo, with 48 and 32% of the initial dose remaining in circulation after 4 h. When compared to EPC/CHOL vesicles (125 nm), the presence of covalently bound protein on liposomes of similar size (139 nm), increased the clearance of liposomes from the circulation (80 and 48%, respectively, remaining in circulation at 4 h). The presence of MPB-DPPE (normal or quenched with β-mercaptoethanol, 170 nm in diameter) did not appear to influence the clearance of liposomes. The stability of the function of covalently conjugated streptavidin-liposomes in vivo was demonstrated by the binding of biotin to liposome samples isolated from plasma at 1 and 4 h post-injection (Table II). A slight loss of biotin binding capacity of streptavidin-coupled liposomes was observed for samples isolated from plasma, which could arise due to the absorption of serum components to the vesicles, the inactivation of streptavidin by proteolysis or the binding of endogenous biotin to the preparation.

Discussion

Protein-liposome conjugates have many potential applications, ranging from systems of diagnostic ability [8] to systems specifically targeted to disease sites in vivo [10]. Efforts in this laboratory have been directed towards developing general procedures for the generation of protein-liposome conjugates which allow efficient and straightforward coupling of a variety of proteins to liposomes and which result in conjugated systems of defined size distributions and protein-lipid ratios. As indicated elsewhere [13,17] the coupling of streptavidin to liposomes results in a flexible basic system which subsequently allows the straightforward conjugation of a wide variety of biotinated proteins. In the present investigation it is shown that such conjugates are susceptible to aggregation during the coupling procedure, which is particularly notable at high protein to lipid ratios. A major finding is that aggregated systems can be re-extruded through filters of defined pore size,

resulting in smaller, homogeneously sized systems with enhanced size stability and corresponding decreased blood clearance rates in vivo.

The observation that liposome-protein conjugates tend to aggregate during the conjugation process (particularly at high protein to lipid ratios) is consistent with previous observations. For example, Bredhorst et al. [24] have found that increased amounts of protein (Fab′ fragments) conjugated to liposomes resulted in an increase in the polydispersity of vesicle populations. It has also been observed that conditions which increase the coupling efficiency of protein to liposomes, such as lipid concentration and the ratio of protein to lipid in the coupling incubation step, affects the extent of vesicle-aggregation observed by negative staining [25]. The increased degree of aggregation obtained for highly coupled liposome systems can be attributed to inter-vesicle cross-linking via the protein molecule. The presence of large liposome systems (> 200 nm in diameter) as observed by freeze-fracture after extended coupling periods, also indicates that liposome fusion can occur subsequent to the aggregation step associated with the coupling of protein to liposomes.

The demonstration that highly aggregated liposome conjugates with various amounts of bound protein, can be readily extruded through filters of 50 or 100 nm in pore size yielding a population of conjugates with narrow size distribution is a primary finding of this investigation. The size of the re-extruded preparations as determined by QELS, were slightly larger than the initial liposome preparation, which is consistent with the presence of vesicle dimers as observed by freeze-fracture. For the chemically coupled liposome-protein conjugates, greater than 85% of lipid was recovered after extrusion of conjugates. The retention of the binding capacity of streptavidin-liposomes for biotin indicates that extrusion does not result in protein denaturation and loss of binding activity.

After extrusion of highly aggregated liposome preparations, reaggregation of vesicles occurs (Fig. 6). As indicated by the QELS and freeze-fracture studies, this reaggregation is relatively minor (size increases of 20–30 nm over 3 h) as compared to the aggregation observed during the preparation of protein-liposome conjugates. This reassociation is insensitive to a variety of protocols. For example, stable liposome-coupled samples with different levels of attached protein (17–50 μg/μmol lipid) were prepared at lipid concentrations ranging from 5 mM to 30 mM (Table I) with little influence on reaggregation. Further, minimal reaggregation was observed when extruded samples were stored at 4°C or frozen immediately after the extrusion process.

It is possible that during the coupling process some vesicle aggregation corresponds to cross-linking of protein to the lipid MPB-DPPE on two or more vesicles. Extrusion of such an aggregate may result in smaller systems, with exposed protein associated MPB-DPPE removed from previously adjacent vesicles. Exposure of such hydrophobic groups would be expected to lead to aggregation.

The rate of clearance of liposomes from the circulation is dependent on the size of the preparation; the larger the liposome the faster it is removed [20,21]. In this regard, limited success has been achieved for the targeting of protein-liposome conjugates to specific sites in vivo, due in part to their rapid sequestration from the circulation by the reticuloendothelial system [20]. As shown here, the extent of aggregation of the coupled-liposomes significantly alters the blood clearance behavior of the conjugated preparations. For example, aggregated streptavidin-liposomes (> 530 nm in diameter) were rapidly removed from the circulation (< 3% remaining after 4 h). In comparison, extended circulation times were obtained for extruded conjugates, i.e., 32 and 48% of the initial lipid dose remained in circulation 4 h post-injection for samples of 187 nm and 139 nm in diameter respectively. The enhanced circulation times observed for smaller protein-liposome conjugates indicates that aggregation of the preparation can be a major factor that determines the lifetimes of conjugates in vivo. It should be noted, however, that the clearance of protein-liposome conjugates from the blood was always greater than for control samples of similar size, indicating that the presence of protein on liposomes contributes to some extent to an enhanced clearance of liposomes from the circulation. The presence of the thiol reactive coupling lipid MPB-DPPE in liposomes does not significantly affect their in vivo clearance behavior when compared to EPC/cholesterol liposomes, suggesting that the binding of thiol containing serum proteins does not affect the in vivo properties of liposomes.

In summary, the major focus of our work has been to develop a general method of attaching protein molecules to liposomes to achieve well characterized liposome-protein systems for targeting applications. Here we present a technique for the generation of sized protein-liposome conjugates by extrusion through filters of defined pore size. The procedure allows easy manipulation of the physical size of protein coupled liposomes without affecting the binding activity of the protein. Stable protein-vesicle conjugates of defined size distribution can readily be prepared with various amounts of protein attached to liposomes by this technique. The enhanced blood circulation times of extruded conjugates and the retention of the biotin binding capacity of extruded streptavidin-liposomes 4 h post-injection in mice, indicate that extruded preparations of streptavidin coupled liposomes will be capable of binding to biotinated molecules in vivo. Further studies addressing the feasibility of this procedure in targeting of liposomes to specific cell populations in vivo are in progress.

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