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Optimized procedures for the coupling of proteins to liposomes

H.C. Loughrey [1], L.S. Choi [1], P.R. Cullis [1,2] and M.B. Bally [1,2]

[1] *The University of British Columbia, Faculty of Medicine, Department of Biochemistry, 2146 Health Sciences Mall, Vancouver, B.C. V6T 1W5, Canada, and* [2] *The Canadian Liposome Company, Suite 308, 267 West Esplanade, North Vancouver, B.C. V7M 1A5, Canada*

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A general, optimized method for coupling proteins to liposomes is presented. This procedure utilizes streptavidin covalently coupled to liposomes to allow the subsequent attachment of a variety of biotinated proteins of interest. In the first part of this study, covalent methods for coupling proteins to liposomes which contain the lipid derivatives MPB-PE and PDP-PE were examined. The maleimide lipid derivative MPB-PE was found to allow more efficient coupling. Thin layer chromatography however revealed that during the standard synthesis of MPB-PE, an impurity was generated which can constitute 40% or more of the derivatized PE. An improved method for the synthesis and isolation of pure MPB-PE is presented here. Subsequently, optimized conditions for the covalent coupling of streptavidin to liposomes containing pure MPB-PE were determined. The flexibility of the streptavidin-liposome system for the preparation of various types of ligand bearing liposomes is demonstrated by the rapid association of a variety of biotinated proteins to streptavidin-liposome systems. The ability of these conjugates to target to specific cell populations in vitro as directed by defined biotinated monoclonal antibodies is demonstrated.

Key words: Liposome; Streptavidin; Biotin; Targeted-liposome

Correspondence to: M.B. Bally, The Canadian Liposome Company, Suite 308, 267 West Esplanade, North Vancouver, B.C. V7M 1A5, Canada.

Abbreviations: EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; DPPE, dipalmitoyl phosphatidylethanolamine; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; SMPB, *N*-succinimidyl 4-(*p*-maleimidophenyl) butyrate; PDP-EPE, *N*-[3-(2-pyridyldithio)-propionyl] egg phosphatidylethanolamine; MPB-EPE, *N*-[4-(*p*-maleimidophenyl)-butyryl] egg phosphatidylethanolamine; MPB-DPPE, *N*-[4-(*p*-maleimidophenyl)-butyryl] dipalmitoyl phosphatidylethanolamine; NHS-biotin, *N*-hydroxysuccinimidyl biotin; DTT, dithiothreitol; EPPS, *N*-(2-hydroxyethyl) piperazine-*N'*-3-propanesulphonic acid; MES, 2-(*N*-morpholino) ethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DTNB, dithiobis-2-nitro-benzoic acid; SATA, *N*-succinimidyl *S*-acetylthioacetate; NEM, *N*-ethylmaleimide; EDTA, ethylenediamine tetraacetate; PBS, phosphate-buffered saline; TLC, thin layer chromatography on silica; HBS, 25 mM Hepes, 150 mM NaCl, pH 7.5.

Introduction

The possible use of liposomes as targeted vesicles for the delivery of entrapped drugs to specific sites has stimulated considerable interest in recent years. Significant progress has been made. For example, liposomes of defined size and lamellarity can now be reproducibly prepared in a manner which allows for the efficient entrapment of a variety of biologically active molecules (Szoka and Papahadjopoulos, 1980; Hope et al., 1985; Mayer et al., 1986). Targeting of such liposomes can be achieved by coupling antibodies to the vesicle surface. Leserman and co-workers (Barbet et al., 1981; Leserman et al., 1984) have described a procedure whereby a thiolated IgG is covalently attached to liposomes containing *N*-[3-(2-pyridyldithio)-propionyl] phosphatidylethanolamine

(PDP-PE) via a disulphide bond. A generalized version of this technique was developed by coupling protein A to vesicles (Leserman et al., 1980), which takes advantage of the ability of protein A to bind the Fc portion of IgGs of certain classes. A limitation is that many monoclonal antibodies are not of the appropriate classes. An alternative coupling procedure is that of Martin and Papahajopoulos (1982) who covalently attached thiolated antibodies and Fab' fragments to liposomes containing *N*-[4-(*p*-maleimidophenyl)-butyryl] phosphatidylethanolamine (MPB-PE) by formation of a thio-ether linkage, a linkage which is less susceptible to reducing conditions found in serum. These protocols and various modifications (Martin et al., 1981; Goundalkar et al., 1984; Wolff and Gregoriadis, 1984) represent the most versatile approaches to coupling currently available. Difficulties with these latter protocols include the fact that certain thiolated proteins do not react with MPB-PE containing liposomes (Leserman et al., 1984) and that the thiolation procedure can result in loss of binding activity (Heath et al., 1983).

In this investigation we develop a general procedure for coupling biotinated proteins to vesicles via conjugation with streptavidin covalently coupled to the vesicles. This procedure utilizes the tight association of biotin to streptavidin and has a number of potential advantages. For example, it is known that biotination of IgGs does not significantly influence binding to antigens (Heitzman and Richards, 1974; Bayer et al., 1979). In addition, coupling of biotinated proteins to streptavidin requires only gentle incubation procedures as opposed to exposure to relatively harsh reducing conditions during covalent coupling protocols.

The studies presented here are developed in two stages. First, optimized procedures for the covalent coupling of streptavidin to liposomes are developed. It is shown that the procedure employing MPB-PE allows more efficient coupling than does the PDP-PE derivative, and is also less sensitive to the presence of cholesterol. Further it is shown that previous protocols for synthesizing MPB-PE are subject to contamination by a lipid by-product and a new procedure for the synthesis of pure MPB-PE is presented. Optimized coupling conditions for the conjugation of thiolated streptavidin to liposomes are then developed. Subsequently it is shown that these streptavidin coated liposomes rapidly and efficiently bind biotinated proteins and lead to liposome conjugates which exhibit specific targeting properties in vitro.

Materials and methods

Materials

Egg phosphatidylcholine (EPC), egg phosphatidylethanolamine (EPE), and dipalmitoyl phosphatidylethanolamine (DPPE) were obtained from Avanti Polar Lipids. *N*-succinimidyl 3-(2-pyridyldithio)propionic acid (SPDP), *N*-succinimidyl 4-(*p*-maleimidophenyl)butyric acid (SMPB) were from Molecular Probes and *N*-hydroxysuccinimide biotin (NHS-biotin) was from Pierce. Dithiothreitol (DTT), Triton-X-100, 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), FITC-cellite, ethylenediamine-tetraacetic acid (EDTA), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), *N*-ethylmaleimide (NEM), bovine serum albumin (BSA), carboxyfluorescein, streptavidin, biotinated-protein A, biotinated-alkaline phosphatase, biotinated-succinylated concanavalin A and Sephadex G-50 were obtained from Sigma. Anti-human erythrocyte IgG was purchased from Cappel, and biotinated anti-B 1 (PAN-B, IgG2a) and biotinated anti-T 11 (E rosette, IgG1) were obtained from Coulter Electronics. 2-amino-2-hydroxy-methylpropane-1,3-diol (Tris) was obtained from Bio-Rad, Sepharose CL-4B and Ficoll-Paque were obtained from Pharmacia, ^{3}H-biotin was from Amersham and ^{14}C-cholesterol was from NEN.

Synthesis of N-[3-(2-pyridyldithio)-propionyl] phosphatidylethanolamine (PDP-PE) and N-[4-(p-maleimidophenyl)-butyryl] phosphatidylethanolamine (MPB-PE)

PDP-EPE was synthesized as described by Leserman et al. (1980). Briefly, 50 μmol EPE was dissolved in 3.5 ml chloroform/methanol (9 : 1) and added to 1.5 ml methanol containing 60 μmol SPDP and 100 μmol triethylamine. After a 4 h incubation at room temperature, analysis by thin

layer chromatography on silica (TLC, running solvent: chloroform/methanol/water, 65 : 25 : 4), indicated 99% conversion of EPE to a faster running product. The reaction mixture was washed with 10 ml of phosphate-buffered saline (PBS). This was repeated three times prior to removal of the organic phase under reduced pressure. Analysis by two dimensional TLC and proton NMR indicated a single product which was greater than 98% pure. PDP-PE was stored under nitrogen in chloroform at −20°C for several months.

MPB-EPE was initially synthesized according to the method of Martin et al. (1982) with minor modifications. EPE (100 μmol) was dissolved in 5 ml of anhydrous methanol containing 100 μmol of freshly distilled triethylamine and 50 mg of SMPB. The reaction was carried out at room temperature under nitrogen and its progress monitored by TLC (running solvent: chloroform/methanol/water, 65 : 25 : 4). Following an 18 h incubation, 95% of the EPE was converted to a faster running product. Methanol was removed under reduced pressure, the sample was dissolved in chloroform and washed extensively with 1% NaCl to remove by-products of the reaction. TLC analysis using the solvent system employed by Martin et al. (1980) indicated that the lipid product ran as a single component which was ninhydrin-insensitive and phosphate positive. However, further characterization by TLC with the solvent system: chloroform/methanol/acetonitrile/water: 75 : 16 : 5 : 4, revealed that in addition to the expected MPB-PE derivative (R_f: 0.6), an additional lipid product was generated (R_f: 0.5) which comprised up to 40% of the total lipid fraction.

Pure 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 with the modified solvent system described above. The lipid products generated following the reaction of DPPE with SMPB were identical to those observed when employing EPE as the starting material. 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 diethylether/acetonitrile yielded a pure product as indicated by ^{1}H-NMR analysis (Bruker W40, 400 MHz; p.p.m. downfield from the internal standard tetramethylsilane) and fast atom bombardment (FAB) mass spectroscopy (AEI MS9, analyzed at the B.C. Regional Mass Spectroscopy Centre).

Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared as described by Hope et al. (1985). Briefly, appropriate amounts of lipid mixtures dissolved 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. Normally lipid samples (50–54 mol% EPC, 45 mol% cholesterol, 1–5 mol% thiol reactive lipid) were hydrated in 150 mM NaCl, 25 mM Hepes, 25 mM MES, pH 6.5 and extruded ten times through two stacked 100 nm filters. Just prior to coupling experiments, samples were titrated to the appropriate pH with NaOH. For studies on the thiol dependence of the coupling procedure, liposomes containing 1 mol% (for coupling) or 5 mol% (for maleimide reactivity) pure MPB-DPPE were prepared at pH 6.5 as described above, titrated to pH 7.5 with NaOH and an aliquot was incubated with β-mercaptoethanol for 5 min at a molar ratio of 10 mol β-mercaptoethanol/mol of maleimide containing lipid. Liposomes were separated from free β-mercaptoethanol on Sephadex G-50 equilibrated with 25 mM Hepes, 25 mM MES, 150 mM NaCl, pH 7.5. The coupling efficiency and the reactivity of the maleimide group of quenched liposomes was compared to that of unquenched samples. Lipid was estimated either by the colorimetric method of Fiske and Subbarrow (1925) or by trace amounts of ^{14}C-cholesterol present in the lipid mixture. This was performed by scintillation counting in a Packard Tri Carb liquid scintillation analyzer.

Assay for maleimide reactivity

Reactivity of the maleimide group of MPB-PE lipids was estimated by the thiol binding of β-mercaptoethanol to lipid derivatives and back titration of unbound β-mercaptoethanol with Ellman's reagent, DTNB as described by Sedlack et

al. (1968). Liposomes (5 mol% MBP-DPPE, 50 mol% EPC, 45 mol% CHOL, 1 μmol in 200 μl) were incubated with β-mercaptoethanol (100 μl of 1 mM) at pH 8.2 (0.2 M Tris-Cl, 20 mM EDTA, 1% Triton X-100 (w/v), pH 8.2, 1.6 ml) for 30 min at room temperature. DTNB (100 μl, 20 mM in methanol) was added and the absorbance was measured at 412 nm after 30 min.

Preparation of proteins for coupling

Streptavidin (5 mg/ml in 25 mM Hepes, 150 mM NaCl, pH 7.5, HBS), was modified with the amine reactive reagent, SPDP according to published procedures (Carlsson et al., 1978). Briefly, SPDP (25 mM in methanol) was incubated at a 10 molar ratio to streptavidin at room temperature for 30 min. Unreacted SPDP was removed by gel filtration on Sephadex G-50 equilibrated with HBS. PDP-modified streptavidin was reduced with DTT (25 mM, 10 min). The thiolated product was isolated by gel chromatography on Sephadex G-50 equilibrated with the relevant buffer and was immediately used in coupling experiments. The extent of modification of streptavidin was determined by estimating the concentration of the protein at 280 nm (molar extinction coefficient, E_{280}: 1.8×10^5) prior to the addition of dithiothreitol (DTT) and the 2-thiopyridone concentration at 343 nm (E_{343}: 7550) 10 min after addition of DTT. In the case of IgG (5 mg/ml in HBS, E_{280}: 2.025×10^5), after modification with SPDP as described for streptavidin, the protein was fluorescently labelled by incubating IgG with FITC-cellite for 20 min at a ratio of 2 : 1 (w/w) respectively. 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 sodium acetate, pH 5.0) to protect against the reduction of the intrinsic disulphides of the molecule. Both protein preparations were modified to the same extent with SPDP (5-6 SPDP molecules per protein).

Coupling of proteins to liposomes

The coupling of proteins to liposomes was performed by incubating the reduced PDP-modified protein with liposomes containing PDP-PE, MPB-EPE, or pure MPB-DPPE at a ratio of 100 μg protein/μmol lipid (1 mM final lipid concentration). As noted by other investigators (Bredehorst et al., 1986) the coupling reactions employed here can result in significant vesicle-vesicle crosslinking. The reaction conditions employed (low protein and low lipid) minimize this problem. Further, a procedure to generate protein-liposome conjugates of defined size is described elsewhere (Loughrey et al., 1990). Following the coupling reaction unassociated protein was removed by gel filtration on Sepharose CL-4B equilibrated with HBS. The extent of coupling of streptavidin to liposomes was assayed by monitoring the binding of ^{3}H-biotin to streptavidin. Briefly, streptavidin-liposomes (0.25 μmol lipid in 0.5 ml) were incubated with ^{3}H 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 ^{3}H biotin to a streptavidin sample (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 liposome associated fluorescence was correlated to a known quantity of fluorescein-labelled antibody. Fluorescence was monitored at 520 nm using a SLM-aminco SPF-500C spectrofluorometer with an excitation wavelength of 495 nm.

Binding of biotinated proteins to streptavidin-lipsomes

Anti-erythrocyte IgG was biotinated according to the method of Bayer et al. (1976). All biotinated proteins were fluorescently labelled with FITC-cellite as described above for IgG. Proteins were incubated at a two-fold molar ratio to streptavidin coupled to liposomes for 10 min. Unassociated protein was removed by gel chromatography on Sepharose CL-4B pre-equilibrated with HBS. The extent of liposome associated protein was determined as described above for fluorescently labelled IgG. Background binding of all biotinated proteins was shown to be negligible.

In vitro targeting of streptavidin-liposome conjugates

Liposomes with entrapped carboxyfluorescein (15 mM) were coupled to thiolated streptavidin as

described above at pH 7.5 and a final lipid concentration of 2.5 mM. The coupling reaction was quenched with *N*-ethylmaleimide (500 molar ratio to streptavidin) after 4 h, streptavidin-liposome conjugates were isolated by gel chromatography on Sepharose CL-4B and levels of liposomally associated streptavidin were determined as described previously.

For targeting experiments, human blood was collected in EDTA (25 mM in PBS). Human peripheral blood leukocytes were isolated by standard protocols using Ficoll-Paque (Böyum, 1968) and suspended in PBS containing 2% BSA (w/v) and 0.01% Na-azide (w/v) at 4°C prior to binding studies. Cells (10^6) were aliquoted into round bottom microtitre wells, washed and incubated with antibody (T11 and B1, 5 and 10 μg respectively in 100 μl PBS) or alone in PBS for 1 h at 4°C. After washing twice with PBS, cells were incubated with streptavidin-liposome conjugates (0.2 μmol in 200 μl PBS) for a further hour at 4°C. The cells were then washed three times with PBS and analyzed by flow cytometry.

Flow cytometry

Cell associated fluorescence was measured with an Epics Profile Analyzer (Coulter Electronics). Cells were illuminated with the 488 nm line of an argon ion laser. Fluorescence was measured behind a 515–530 nm band-pass filter. Fluorescence signals were gated on the basis of a right angle versus forward light scatter cytogram to restrict analysis to signals from single cells. Amplifiers were set in the log area mode. For statistical analysis of histograms, region 1 was arbitrarily set (min. 2.705, max. 1023) with the lower channel at the base of the right shoulder of the histogram of the control sample.

Results

Two thiol reactive lipids PDP-EPE and MPB-EPE, were synthesized according to published procedures (Leserman et al., 1980; Martin et al., 1982). We first determined which EPE derivative was the more efficient protein-liposome coupling reagent. As shown in Fig. 1, significant coupling of thiolated IgG to liposomes containing PDP-EPE did not occur until greater than 20 mol% cholesterol was incorporated into liposomes. In contrast, levels of 12 μg IgG/μmol lipid were obtained for liposomes containing MPB-EPE, even in the absence of cholesterol. The level of liposomally conjugated protein increased linearly with respect to amounts of cholesterol incorporated into vesicles. Significantly higher coupling ratios were obtained for the maleimide derivative of EPE under all conditions examined. For this reason further work concentrated on the use of the MPB-EPE derivative.

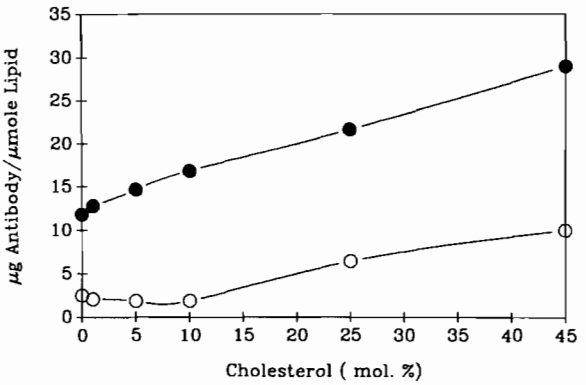

Fig. 1. Coupling of anti-rat erythrocyte IgG to EPC liposomes containing thiol reactive lipids (5 mol%) as a function of cholesterol content (0–45 mol% of total lipid). Thiolated IgG (four SPDP groups/IgG, 100 μg) was fluorescently labelled and incubated with PDP-EPE (○) or MPB-EPE (●) liposomes (1 μmol lipid, 1 mM final lipid concentration, labelled with ^{3}H-DPPC) overnight in EPPS pH 8.0 buffer. After removal of uncoupled IgG by gel chromatography on Sepharose CL-4B, the ratio of IgG bound/μmol lipid was determined by measuring the levels of liposomally associated fluorescence for protein and scintillation counting for lipid.

The derivatization of phosphatidylethanolamine (EPE or DPPE) with SMPB is a straightforward reaction that readily generates a lipid product which runs as a single component under certain solvent conditions as analyzed by thin layer chromatography on silica (Martin et al., 1982). However, further characterization of the reaction products by TLC using an alternative solvent system indicated that this product contained a significant impurity (up to 40%) which has subsequently been identified as a ring open form of MPB-DPPE (Choi et al., 1989). A new method for the preparation of a pure MPB-DPPE lipid derivative was thus developed for the synthesis and

isolation of MPB-DPPE (see materials and methods section). The purity of this lipid product was shown by ^{1}H-NMR analysis as illustrated in Fig. 2*B*. For example, the ^{1}H-NMR spectra of the pure product exhibited low field resonances attributed to the aromatic protons of the phenyl groups (chemical shift *d*: 7.3) and vinyl protons (*d*: 6.86) of SMPB with loss of the signal attributed to *N*-hydrosuccinimide group (*d*: 2.86). Mass spectroscopy studies confirmed the purity of the lipid derivative by the presence of a molecular ion at 955 which corresponds to a molecular formula of $C_{51}H_{84}O_{11}PNa$ for the Na salt of MPB-DPPE.

Optimal conditions for coupling thiolated streptavidin to liposomes containing pure MPB-DPPE were investigated. It is well established that the stability of the maleimide group of SMPB is sensitive to pH (Kitagawa et al., 1981). For this reason, the pH dependence of the binding of thiolated-streptavidin to MPB-DPPE liposomes and the stability of the maleimide function were initially established. As shown in Fig. 3, the amount of liposomally conjugated protein increased rapidly at pH values greater than 7.0. However, incubation of liposomes containing MPB-DPPE at pH values of 7.0 and above resulted in a corresponding rapid degradation of the maleimide group of the derivatized lipid. At pH 7.5 after 18 h incubation, significant levels of streptavidin were coupled to liposomes (45%) with acceptable loss of maleimide reactivity (65% remaining). For this reason, a pH of 7.5 was chosen for further optimization of the coupling reaction.

In Fig. 4, a time course relating streptavidin binding to liposomes and reactivity of the maleimide lipid is presented. The results indicate that optimal levels of streptavidin conjugated to liposomes (approx. 37 μg/μmol lipid) were obtained

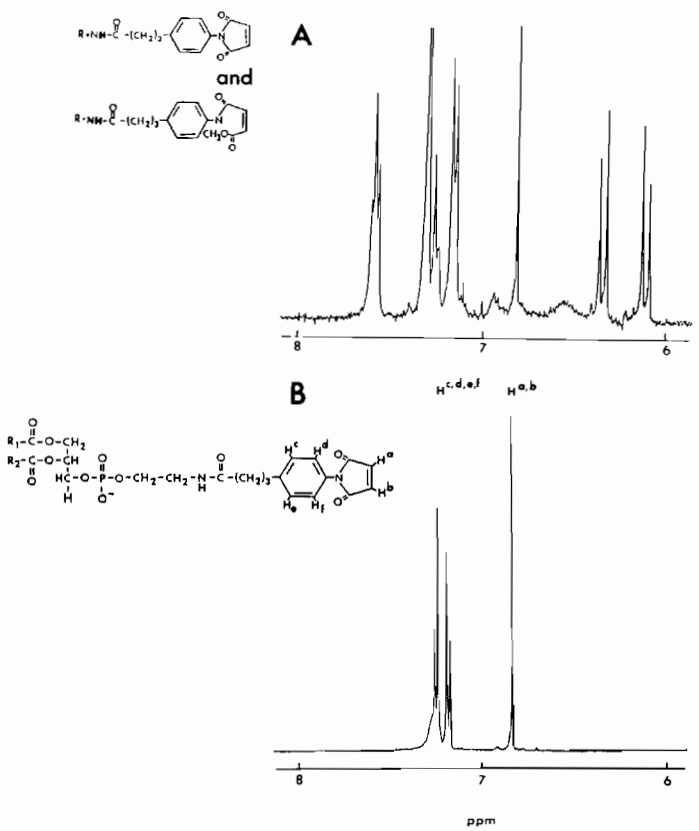

Fig. 2. Low field region of ^{1}H-NMR spectra of reaction products generated following the derivatization of phosphatidylethanolamine with SMPB (*A*). The ^{1}H-NMR spectra of pure MPB-PE is indicated in *B*. The synthesis and purification of MPB-PE are described in the materials and methods section.

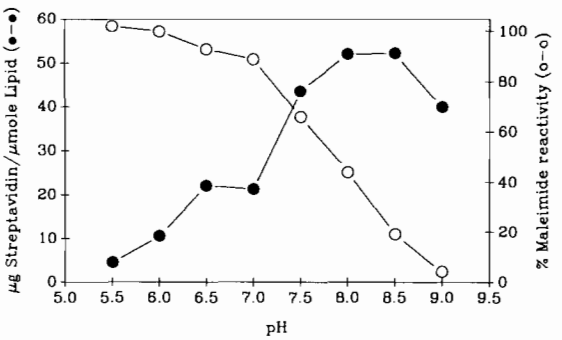

Fig. 3. Effect of pH on the levels of streptavidin conjugated to liposomes containing pure MPB-DPPE and on the stability of the maleimide moiety. Liposomes (1 or 5 mol% MPB-DPPE, 54 or 50 mol% EPC, 45 mol% cholesterol, trace amounts of ^{14}C cholesterol) were incubated with thiolated streptavidin (1 mol% MPB-DPPE liposomes) or alone (5 mol% MBP-DPPE liposomes for maleimide assay) at various pHs (pH: 5.5–9.0) overnight. The level of streptavidin conjugated to liposomes (●) and the stability of the maleimide group (○) were determined as detailed in the materials and methods section.

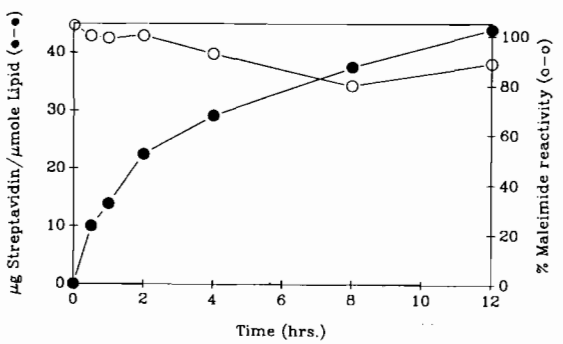

Fig. 4. Optimal coupling conditions for the conjugation of streptavidin to liposomes containing pure MPB-DPPE. Liposomes (1 or 5 mol% MPB-DPPE, 54 or 50 mol% EPC, 45 mol% cholesterol) were incubated with thiolated streptavidin (1 mol% MPB-DPPE liposome) or alone (5 mol% MBP-DPPE liposomes for maleimide assay) at pH 7.5. At various times streptavidin-liposome conjugates were separated from free streptavidin by gel exclusion on Sepharose CL-4B and the levels of protein conjugated to liposomes (●) and the stability of the maleimide group (○) were determined as detailed in the materials and methods section.

with minimal degradation of the maleimide group after an incubation period of 8 h at pH 7.5 and at room temperature.

The requirement for protein associated thiol groups in the coupling procedure is illustrated in

TABLE I

THIOL DEPENDENCE OF THE COUPLING OF STREPTAVIDIN TO LIPOSOMES CONTAINING PURE MPB-DPPE

Liposomes (1 or 5 mol% MPB-DPPE, 54–50 mol% EPC, 45 mol% cholesterol) were quenched with β-mercaptoethanol ten-fold molar excess to MPB-DPPE) for 5 min at pH 7.5, exchanged on Sephadex G-50 equilibrated with HBS and incubated with streptavidin or alone as described in Fig. 4. After 8 h incubation, the extent of streptavidin conjugated to liposomes and the reactivity of the maleimide group was determined for control (unquenched MPB-DPPE liposomes or unthiolated streptavidin) and quenched samples (see materials and methods section for details).

Sample	% maleimide reactivity		μg streptavidin/μmol lipid
	0 h	8 h	8 h
MPB-DPPE liposomes	100	73	36.0
β-mercaptoethanol-treated MPB-DPPE liposomes	11	0	2.5
MPB-DPPE liposomes + unthiolated streptavidin	100	77	0

TABLE II

BINDING OF BIOTINATED PROTEINS TO STREPTAVIDIN-LIPOSOMES

Streptavidin-liposomes with 45.2 μg protein bound/μmol lipid were prepared as described in Fig. 4. Fluorescein-labelled biotinated proteins were incubated with conjugated liposomes at a two-fold molar excess to streptavidin for 10 min at pH 7.5. The extent of coupling of biotinated proteins to streptavidin-liposomes was determined after gel chromatography of samples on Sepharose CL-4B by measuring the levels of fluorescence associated with liposomes for protein and scintillation counting for lipid.

Protein	μg/μmol lipid	nmol/μmol lipid	Molar ratio protein : streptavidin
Anti-human erythrocyte IgG (MW: 150,000)	62.6	0.417	1 : 1.68
Alkaline phosphatase (MW: 140,000	77.7	0.555	1 : 1.25
Protein A (MW: 43,000)	20.3	0.482	1 : 1.46
Succinylated concanavalin A (MW: 55,000)	26.4	0.480	1 : 1.46

Table I. Prior exposure of MPB-DPPE liposomes to β-mercaptoethanol resulted in a decrease in the extent of liposomally conjugated streptavidin when quenched samples were compared to control MPB-DPPE liposomes. This was paralleled by a decrease in the detectable reactivity of the maleimide group of the lipid derivative. Furthermore, native streptavidin did not associate with liposomes containing the maleimide lipid.

The object of this investigation was to establish a general, efficient method of attaching various ligands to liposomes. The results to this point demonstrate that streptavidin can be efficiently coupled to liposomes containing a pure form of the maleimide lipid derivative MPB-DPPE. To show the applicability of this system in attaching various types of targeting molecules of interest to liposomes, the binding of a variety of biotinated proteins to streptavidin-liposomes was examined. As shown in Table II, on incubation of various biotinated proteins with streptavidin-conjugated liposomes, approximately two protein molecules binds for every three molecules of streptavidin. The extent of binding of biotinated proteins to streptavidin coupled vesicles is independent of the size of the biotinated protein (MW: 42,000–150,000).

Liposomes coated with streptavidin can obviously be used directly as targeted systems by attachment of a biotinated antibody (Loughrey et al., 1987). Alternatively, an indirect procedure is possible whereby biotinated antibodies are first associated with target cells and the streptavidin coated liposomes are introduced subsequently. Here we demonstrate liposome targeting employing this latter approach utilizing flow cytometry. As shown in Fig. 5, incubation of liposome-streptavidin conjugates (containing encapsulated carboxyfluorescein) with cells pre-labelled with a biotinated monoclonal antibody specific for peripheral B cells (B1), resulted in the fluorescent labelling of approximately 20% of the total lymphocyte population (Fig. 5*B*). In comparison, similar studies with a biotinated anti T cell antibody (T11) resulted in the labelling of approximately 90% of lymphocytes (Fig. 5*C*). These results are consistent with the expected cell distribution of the antigens defined by T11 (Howard et al., 1981) and B1 (Stashenko et al., 1980). The

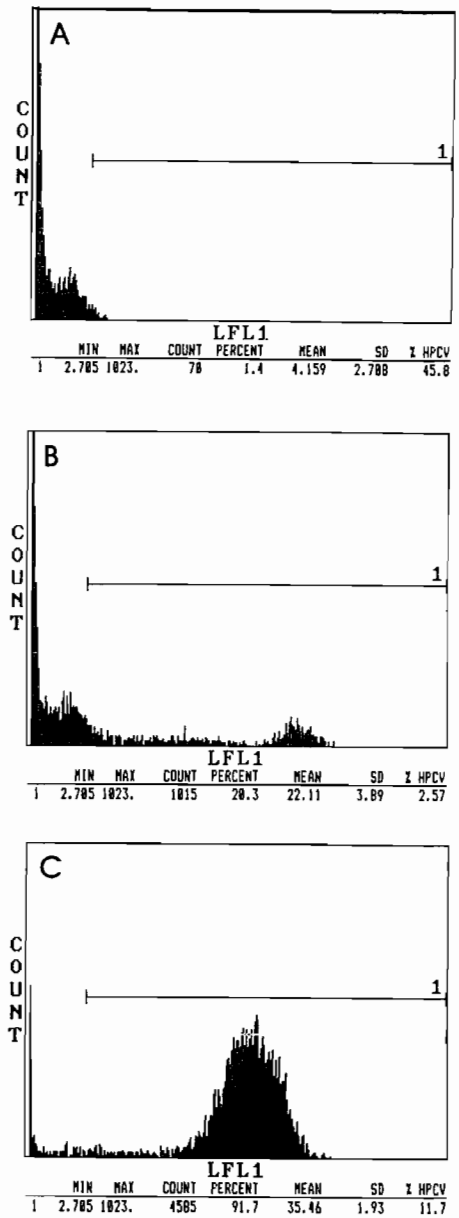

Fig. 5. Targeting of streptavidin-liposome conjugates via biotinated monoclonal antibodies to human peripheral lymphocytes. Streptavidin-liposome conjugates (38.8 μg streptavidin/μmol lipid) with entrapped carboxyfluorescein (15 mM) were prepared as described in the materials and methods section. Lymphocytes (10^6) were incubated with biotinated antibodies (10 μg, B1 (*B*); 5 μg, T11 (*C*)) or in PBS (*A*) for 1 h at 4°C. After two washes, streptavidin-liposome conjugates (0.2 μmol lipid) were added, incubated for 1 h at 4°C and washed thrice with PBS. Samples were subsequently examined for cell associated fluorescence by flow cytometry (LFL1, log of fluorescence).

specificity of these conjugates is indicated by the negligible background binding of streptavidin-liposome conjugates to lymphocytes in the absence of biotinated antibodies (Fig. 5*A*).

Discussion

Covalent attachment of liposomes to antibodies, which are directed against cell surface antigens such as those associated with transformed cells, may have therapeutic potential. However, at the present time, such targeted liposomal systems have mainly been used for in vitro applications such as diagnostic assays (Kung et al., 1985). In order to exploit the full potential of antibody targeted carrier systems a versatile and reliable methodology for coupling is required. This report assesses two major procedures currently employed for chemically coupling proteins to liposomes. Further, we describe an approach to rapidly couple IgGs and biotinated proteins to liposomal systems which relies on the tight binding of biotin to streptavidin. This technology should be applicable to a variety of other bioactive peptides.

With regard to the covalent coupling of proteins to vesicles, two thio-reactive lipid derivatives were employed, MPB-EPE and PDP-EPE. When incorporated in liposomal systems these lipids crosslink proteins which have thiol groups introduced by modification with the amine reactive heterobifunctional reagent SPDP (Barbet et al., 1981; Bragman et al., 1983, 1984; Leserman et al., 1984). In addition, they are reactive with IgGs following exposure of the intrinsic sulphydryl residues (Martin and Papahadjopoulos, 1982; Bredehorst et al., 1986). We demonstrate that the SMPB derivatized PE functions as a more efficient coupling reagent, which may be due to the presence of a longer spacer arm associated with this lipid. As shown here, coupling of IgG to vesicles containing either lipid species is more efficient in the presence of cholesterol. This cholesterol effect has also been observed for reactions employing Fab' fragments (Kung et al., 1985). Since covalent association of SPDP-modified streptavidin was not influenced by addition of cholesterol, this effect may be related to steric constraints associated with interactions of larger immunoglobulins at the liposomal surface.

Previous literature methods for the synthesis of the maleimide lipid derivative MPB-PE lead to the generation of a contaminant, ring open form of the expected lipid product (Choi et al., 1989). In this study, a new protocol for the synthesis of a pure SMPB derivative of phosphatidylethanolamine is presented. Coupling conditions for the conjugation of protein to liposomes were optimized such that the integrity of the maleimide function of pure MPB-DPPE was retained in order to generate well characterized protein-liposome conjugates. Significant amounts of protein were associated with MPB-DPPE liposomes (equivalent to 30 μg streptavidin/μmol lipid) at a pH of 7.5 after 8 h with minimal loss of maleimide function (approximately 85–90% reactivity remaining). This amount of liposomally associated streptavidin corresponds to about 75 copies of streptavidin bound per 100 nm vesicle.

Coupling efficiencies of up to 50% are readily achieved under the optimized conditions outlined in this study. Similar efficiencies have been attained only on incorporation of higher levels of MPB-EPE in liposomes (5 mol%) (Bragman et al., 1984). The increased efficiency observed here likely reflects the pH of the conjugation reaction rather than the impurity of the derivatized lipid. For example, conjugation of thiolated protein to MPB-DPPE liposomes at pH 6.7 (conditions used by Bragman et al., 1984) results in an approximate four-fold reduction in the levels of liposomally conjugated protein when compared to levels obtained at the optimum pH of 7.5 (Fig. 2). This efficient coupling of protein to liposomes containing low levels of MPB-PE is of particular importance as higher concentrations of this anchor molecule (< 2.5 mol%) dramatically affect liposome stability (Bredehorst et al., 1986). Finally, with regard to other covalent methods for the preparation of protein-liposome conjugates, equivalent coupling efficiencies have been reported in a limited number of cases (Barbet et al., 1981; Rosenberg et al., 1987).

The covalent method of conjugating proteins to liposomes under optimized coupling conditions requires the presence of protein thiol groups. As illustrated here, in the absence of endogenous free

sulphydryl groups, exogenous thiol groups are introduced by the modification of protein amino functions with the hetero-bifunctional reagent SPDP. The deprotection of the SPDP thiol moiety which requires exposure of the derivatized protein to a strong reducing agent such as DTT may result in the loss of the biological function of the molecule (Heath et al., 1983). In order to circumvent this problem, a sulphydryl-introducing reagent that does not require the use of such harsh reagents has been synthesized (Duncan et al., 1983). The alternative approach exploited here utilizes the high affinity of streptavidin-liposome conjugates for biotinated proteins. This sandwich method of preparing protein-liposome conjugates requires the prior modification of the protein of interest with the amine reactive derivative *N*-hydroxysuccinimide biotin, a procedure that does not appear to significantly influence the function of proteins (Heitzman et al., 1974; Bayer et al., 1979). This modification does not need to be extensive, as little as a single biotin coupled to IgG is sufficient for the efficient coupling to streptavidin coated vesicles (Loughrey et al., 1987). As indicated here, the coupling of biotinated proteins to streptavidin conjugated liposomes is rapid (< 10 min) and can be 20–40% more efficient than covalent coupling procedures. As described elsewhere (Loughrey et al., 1987) streptavidin can also be linked non-covalently to liposomes which contain biotinated phosphatidylethanolamine. The covalent procedure reported here is a much more efficient way to generate the streptavidin liposomes.

In summary, we describe the preparation of liposomes coated with streptavidin covalently bound to MPB-DPPE via an improved coupling procedure. It is demonstrated that such protein-liposome conjugates rapidly and efficiently conjugate with biotinated proteins resulting in targeted vesicle systems. The potential application of these conjugates in targeting and diagnostic regimes is illustrated by the specific binding of such conjugates to lymphocyte subpopulations via defined biotinated monoclonal antibodies. This technology should be readily extended to a large variety of antibodies and other ligands and thus satisfies many of the requirements of a general protocol for generating targeted liposomal systems.

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