Chapter 11

PREPARATION OF STREPTAVIDIN-LIPOSOMES FOR USE IN LIGAND-SPECIFIC TARGETING APPLICATIONS

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I. INTRODUCTION

Numerous methods are available for attaching proteins such as antibodies to liposomes for the purpose of targeting entrapped drugs to cells. The most versatile approach has been to prepare protein-liposome conjugates which subsequently bind the targeting antibody of interest. For example. protein A liposome conjugates have been prepared. taking advantage of the ability of protein A to bind the Fc portion of certain IgG classes.' Similarly. the conjugation of anti-mouse IgG to liposomes facilitates the rapid binding of any mouse immunoglobulin to liposomes.' These procedures are restricted to employing immunoglobulins as targeting ligands. The premise of generating a single well-characterized liposomal system which can bind a variety of targeting ligands therefore has significant merit.

The focus of our work has been to develop such a general method of attaching protein molecules to liposomes. Recently, we described a "sandwich" protocol for the preparation of targeted iiposome systems which utilizes the high affinity binding of streptavidin for biotin.[3-5] Streptavidin can be attached to liposomal systems. either covalently or noncovalently (Figure 1). Subsequently, biotinated targeting ligands are readily associated with streptavidin coated vesicles. Since biotination of antibodies (or other bioactive molecules) does not significantly influence their binding properties.',' this "sandwich" protocol for the preparation of targeted liposome systems is extremely versatile and applicable to numerous targeting ligands of interest.

This chapter describes optimized conditions for attaching streptavidin to liposomes. and associated procedures which result in physically and chemically well-characterized protein-liposome conjugates. The resulting streptavidin-coated liposomes are stable with respect to size and retain their capacity to bind biotinated targeting ligands. resulting in a flexible targeted vesicle system that can be utilized for both ***in vitro*** and ***in vivo*** targeting regimes.

II. NONCOVALENT COUPLING OF STREPTAVIDIN TO BIOTIN-PHOSPHATIDYLETHANOLAMINE (BIOTIN-PE) LIPOSOMES

Streptavidin can be attached noncovalently to liposomes containing biotinated phosphatidyiethanolamine (biotin-PE). Below we outlined conditions which lead to maximum streptavidin to lipid coupling ratios. while avoiding significant problems such as aggregation and precipitation which can arise due to intervesicle crosslinking.

A. PREPARATION OF BIOTIN-PE LIPOSOMES

Large unilamellar liposomes (LUVs; egg phosphatidylcholine [EPC]; Avanti Polar Lipids, Alabama) containing biotin-PE (0.1 mol%; Molecular Probes. Oregon, or Avanti Polar Lipids. Alabama), are prepared by extrusion techniques

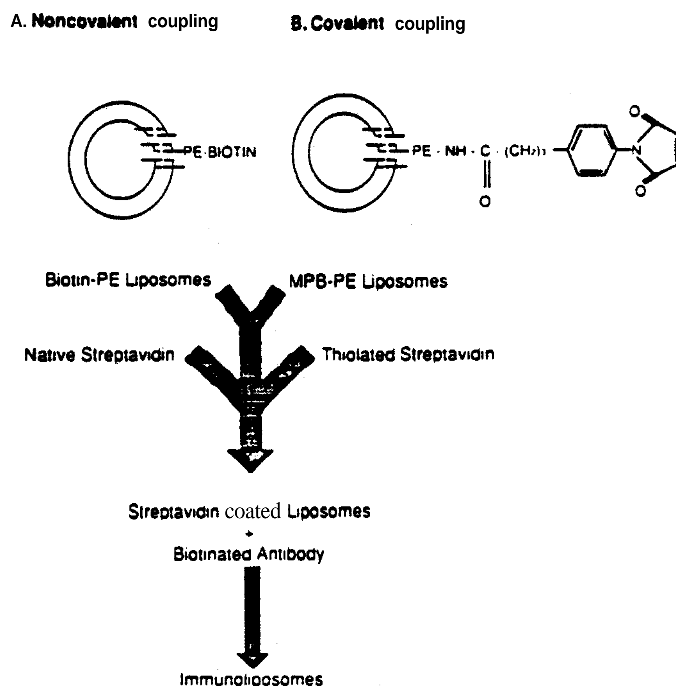


FIGURE 1. Noncovalent and covalent methods for preparing streptavidin-liposomes.

as described by Hope et al." Briefly, appropriate amounts of lipid mixtures dissolved in chloroform are deposited in a tube and dried to a lipid film under a stream of nitrogen followed by high vacuum for 2 h. Lipid samples are routinely hydrated in 150 mM NaCl. 25 mM N-(2-hydroxyethyl)piperazine-N-3-propanesulfonic acid (EPPS), pH 8. For targeting experiments. a fluorescent marker. such as carboxyfluorescein. is included in the hydration buffer (15 mM). The resulting multilamellar vesicles are frozen and thawed 5 times and extruded 10 times through 2 stacked 100-nm filters.

B. BINDING OF STREPTAVIDIN TO BIOTIN-PE LIPOSOMES

Liposomes (99.9 mol% EPC, 0.1 mol% biotin-PE; 1 μmol total lipid) are normally incubated with streptavidin (1 μCi ^{125}I/mg, 4 mg/ml in 20 mM EPPS. 150 mM NaCl pH 8.0; Sigma Chemical Co.) at room temperature for 30 mm. Samples are then chromatographed on Sepharose CL-4B (Pharmacia. Uppsala. Sweden) which is equilibrated with EPPS. pH 8.0 to separate liposomally bound streptavidin from free. The ratio of streptavidin bound per μmole lipid is determined by counting ^{125}I for streptavidin and a standard phosphate assay for phospholipid.[9] Under the above conditions, a maximum of 5.8 μg of streptavidin binds per μmole of lipid. The amount of streptavidin associated with vesicles is increased further by increasing the mol% of biotin-

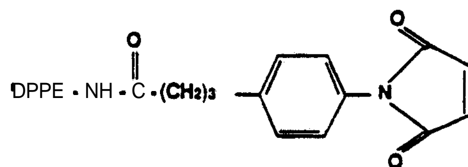
PE in the vesicles (up to *0.3* mol% of the total lipid) or by the inclusion of cholesterol (Chol) in the vesicle preparation (50 mol%). To maximize the coupling **efficiency,** a constant ratio of streptavidin to total lipid is maintained. Streptavidin-liposomes with up to 30 **μg** of protein bound per pmol of lipid can readily be achieved in this manner. Further increases in the levels of biotin-PE in liposomes lead to a significant loss of lipid due to aggregation and precipitation of vesicles. Under the optimal incubation conditions outlined above. protein to lipid coupling is rather inefficient. Only 3% of the initial protein becomes lipid associated. For this reason. the rapid interaction of streptavidin with biotinated components may be more efficiently exploited by indirect targeting procedures. whereby cells are sequentially labeled with a biotinated ligand. streptavidin. and finally, biotin-PE liposomes (see Section V).

III. COVALENT COUPLING OF STREPTAVIDIN TO N-(4-p-MALEIMIDOPHENYL)-BUTYRYL DIPALMITOYL PHOSPHATIDYLETHANOLAlMINE (MPB-DPPE) LIPOSOMES

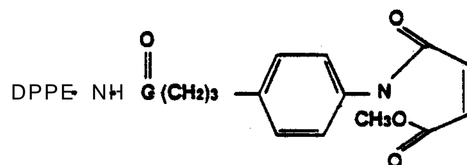
In an attempt to develop more efficient methods of attaching streptavidin to liposomes. we investigated covalent chemical coupling methods based on the two thiol reactive lipid derivatives N-(4-(p-maleimidophenyl)-butyryl] egg phosphatidylethanolamine (MPB-PE) and N-{3-(2-pyridyldithio)-propionyl] egg phosphatidylethanolamine (PDP-PE). When incorporated in liposomal systems. these lipid derivatives have been employed to couple proteins which contain endogenous thiol groups.'" or have had thiol groups introduced by modification with the amine reactive heterobifunctional reagent **SPDP.**[11-14] We have shown that the maleimide lipid derivative functions as a bettercross-linking reagent.' This may be due to the presence of a longer spacer arm associated with this lipid. Characterization of the maleimide lipid derivative, however, indicated that previous protocols for the synthesis of MPB-PE were contaminated by a ring open by-product. This contaminant is generated by methanolic cleavage of the maleimide moiety (Figure 2). In this section. we outline a recently developed method for synthesizing a pure form of this lipid.' Optimal conditions for the efficient coupling of streptavidin to liposomes containing pure MPB-PE are then described.

A. SYNTHESIS OF MPB-DPPE

MPB-DPPE is synthesized by reacting dipalmitoylphosphatidylethanolamine (DPPE) (69 mg; Avanti Polar Lipids, Alabama) with N-succinimidyl 4-(p-maieimidophenyl) butyrate (SMPB, 65 mg; Molecular Probes, Oregon) in chloroform (5 ml) containing triethylamine (10 mg) at **40°** to 60°C. Full conversion of DPPE to a faster running product is confirmed by thin layer chromatography on silica after two h incubation (solvent system: chloroform/



Structure of pun MPB • DPPE



Structure of ring open form of MPB • DPPE

FIGURE 2. Structure of two different MPB-PE lipid derivatives.

methanol/acetonitrile/watcr, 65:26:5:4: R:0.6). The solution is diluted with chloroform (10 ml), washed several times with NaCl (0.9%) to remove by-products of the reaction and concentrated in vucuo. The resulting solid residue is triturated and recrystallized from diethylether to remove unreacted SMPB. Further recrystallization from diethylether/acetonitrile yields a pure product.

B. COUPLING OF STREPTAVIDIN TO MPB-DPPE LIPOSOMES

The chemical conjugation of streptavidin to liposomes containing pure MPB-DPPE requires prior introduction of protein thiol groups. This is readily achieved by modification of streptavidin (10 mg/ml in 25 mM N-2 hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 150 mM NaCl. pH 7.5. HBS) with the amine reactive reagent, N-succinimidyl-3-(2-pyridyldithio) propionate, (SPDP, Molecular Probes) according to published procedures.[15] We routinely modify streptavidin at a tenfold excess of SPDP to protein, which results in 5 to 6 pyridyldithio-propinate (PDP) groups per streptavidin.

Liposomes composed of 54 mol% EPC, 45 mol% Chol. and, 1 mol% MPB-DPPE, which have been sized through filters of 100-nm pore size, are generally employed in coupling reactions. Typically, freshly reduced PDP-streptavidin is incubated with liposomes at a ratio of 100 μg of protein/μmole lipid at pH 7.5. Minimal degradation of the maleimide moiety of MPB-DPPE is detected after 8 h under these conditions.' The reaction is quenched at the required time by the addition of an excess of N-ethylmaleimide (500-fold excess to protein). The extent of streptavidin-coupling to liposomes is measured after gel chromatography on Sepharose CL-4B equilibrated with HBS,

by the binding of ^{14}C biotin (Amersham. U.K.) to streptavidin. Briefly, streptavidin-liposomes (0.25 μmol lipid in 0.5 ml) are incubated with ^{14}C biotin (3.85 nmol in 25 μl. 15.4 nmol/μCi) for 10 min. and unbound biotin is removed by gel filtration on 5 ml Sepharose CL-4B columns equilibrated with HBS. The extent of binding of biotin to a streptavidin standard (100 μg) after gel chromatography on Sephadex G-50 is used as a reference for the calculation of coupling ratios. Various factors. such as the final lipid concentration and the length of incubation. determine the amount of streptavidin conjugated to vesicles. We routinely prepare streptavidin-liposome conjugates at relatively high lipid concentrations (5 mM) for short incubation times (4 h). Under these conditions, approximately 50% of the protein is attached to the liposomes. We have observed little effect on protein-to-lipid coupling levels when dioleoyiphosphatidylcholine or distearoylphosphatidylcholine is substituted for EPC. and when acidic lipids such as bovine phosphatidylserine (up to 5 mol%) are included in the liposomal preparation. Furthermore. similar levels of streptavidin are bound to liposomes extruded through filters of various pore sizes (50 to 200 nm).[28]

IV. CHARACTERIZATION OF STREPTAVIDIN-LIPOSOME CONJUGATES

In the previous section, we outlined conditions for the efficient coupling of streptavidin to MPB-DPPE liposomes. We have consistently observed by various physical techniques that these conjugates are susceptible to aggregation during the coupling procedures. which is particularly important at high protein to lipid coupling ratios (Figures 3 and 4). This increased degree of aggregation for highly coupled liposome systems can be attributed to intervesicle cross-linking via the protein molecule. After extended periods of incubation. a significant number of large vesicles (>200 nm) are observed which presumably arise due to fusion events following aggregation. Vesicle aggregation can significantly affect lipid recovery of chromatographed strep tavidin-liposome conjugates, and may also limit their use *in vitro* and *in vivo.*

We recently demonstrated that highly aggregated liposome conjugates with various amounts of bound protein can be readily extruded through filters of various pore sizes (50 to 100 nm. Figures 5A and 5B).[5] We routinely extrude streptavidin-conjugated liposomes at a final lipid concentration of 5 mM prior to chromatography on Sepharose CL-4B. The resulting population of conjugates has a narrow size distribution. Importantly, loss of lipid on extrusion of protein-liposome conjugates is minimal: up to 90% of total lipid is recovered for protein-liposome conjugates which have a range of initial size distributions (150 to 500 nm in diameter as estimated by quasi-elastic light scattering techniques: corresponding protein to lipid ratios of 25 to 60 μg/μmol lipid). Extruded samples fall within a narrow size range (120 to 140 nm) whose size is only slightly larger than the initial liposomal preparation

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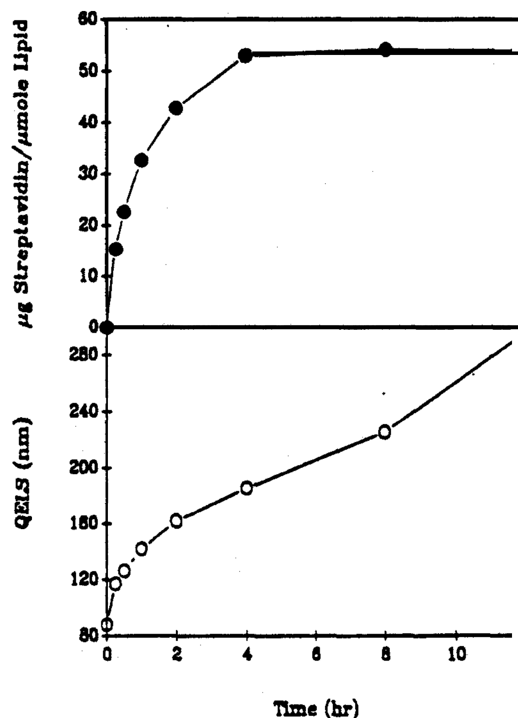

FIGURE 3. Effect of coupling streptavidin to liposomes on vesicle size. Liposomes (54 mol% EPC. 45 mol% Chol. 1mol% MPB-DPPE. 5 mM final lipid concentration. 100 nm) were incubated with streptavidin (100 µg protein/µmol lipid) overtime at pH 7.5. At various time points. the reaction was quenched by addition of N-ethylmaleimide (500 molar ratio to protein). and free streptavidin was removed by gel filtration on Sepharose CLJB. Extent of coupled streptavidin was determined by ^{3}H biotin binding (A. ●), and vesicle size was estimated by quasi-elastic light scattering techniques (QELS: B. ○). (From Loughrey H. et al.. *Biochim. Biophys. Acta.* 1028. 73. 1990. With permission.)

(110 nm). This small increase in size may be attributed to the presence of dimers in the extruded sample (Figure 5B).

After extrusion of highly aggregated liposome conjugates, reaggregation does occur. This is, however, minimal when compared to aggregation during coupling reactions, and can be minimized by storing samples at 4°C or freezing samples immediately after the extrusion process. The extrusion procedure is a gentle method for preparing small homogeneously-sized streptavidin-coupied vesicles. This is shown by the retained ability of extruded streptavidin-liposome conjugates to bind biotin. This approach of preparing sized protein Iiposome conjugates is applicable to other coupling procedures. For example, we have shown that highly aggregated streptavidin-biotin-PE liposomes (Section II) can be easily extruded through filters of small pore size (Figures 5C and SD). Significant loss of lipid (50%). however, is common after extrusion of these vesicles.

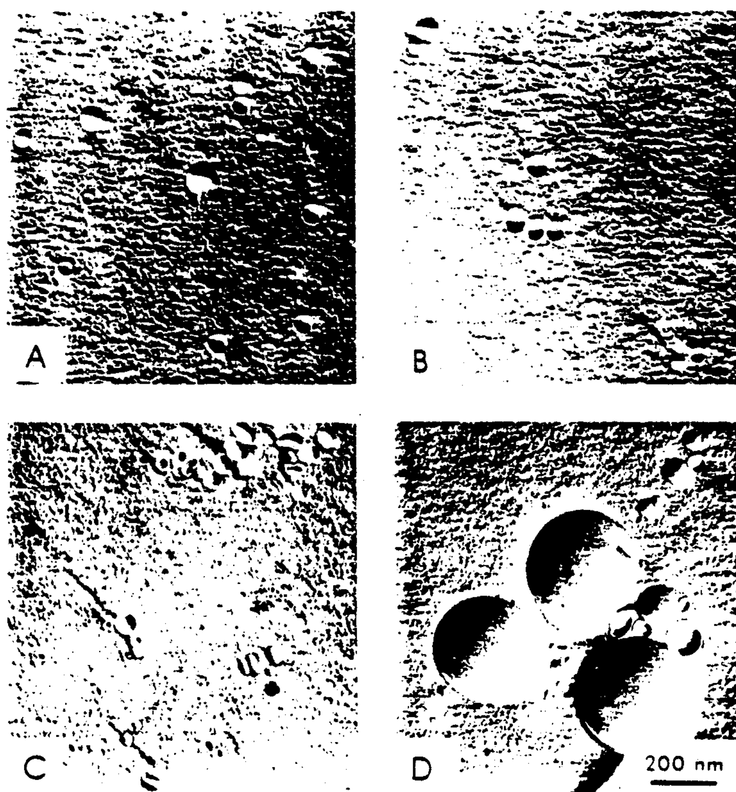

FIGURE 4. Freeze fracture of streptavidin-liposome preparations Strcptavidin-liposome samples quenched with N-ethylmaleimide at 0.5 (A). 2 (B), 4 (C) and IX (D) h were prepared as described in Figure 3 and examined by freeze fracture (From Loughrey, H. et al.. *Biochim. Biophys. Acta. 1028. 73. 1990.* With permission.)

V. TARGETING OF STREPTAVIDIN-LIPOSOMES

The final step in the preparation of target-specific immunoliposomes involves the attachment of biotinated antibody to streptavidin-coated liposomes. Many antibodies of interest are commercially available in a biotinated form. Alternatively, antibodies can easily be derivatized with biotin according to the procedure of Bayer et al.[16] This reaction involves modifying primary amine groups in the protein with a N-hydroxysuccinimidyl derivative of biotin (NHS-biotin: Pierce. Rockford. IL). Briefly. NHS-biotin in 50 μl of dimethylformamide (DMF) is incubated with antibody (10 mg in 0.95 ml), in 1 M NaCI. 0. 1M $NaHCO_3$ pH 8.0 for 4 h at room temperature. The molar ratio of NHS-biotin to antibody in the reaction depends on the number of biotins required to be bound per antibody. The reaction is quenched with an excess

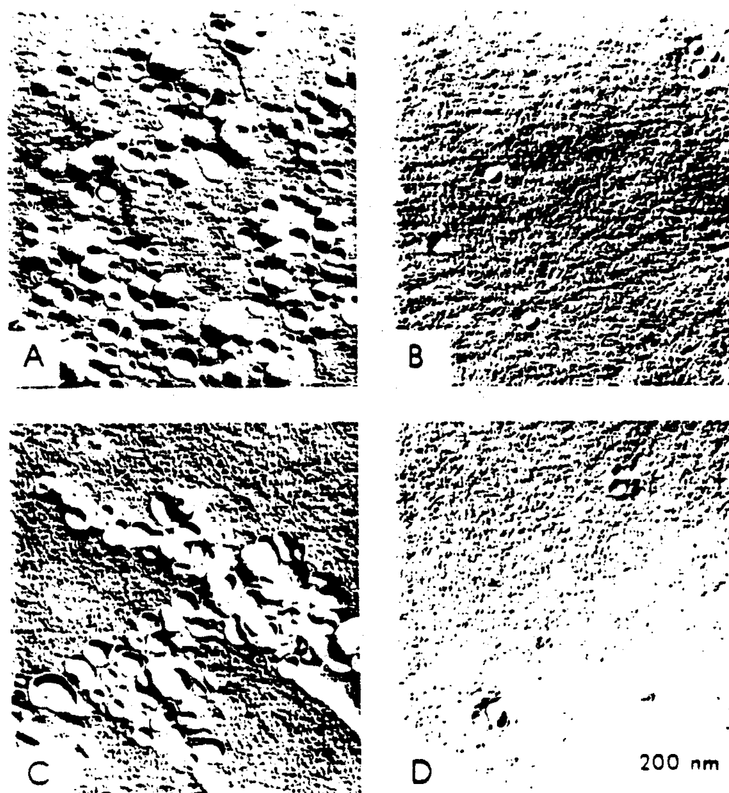

FIGURE 5. **Freeze fracture of streptavidin-liposomes before and after extrusion. Streptavidin was coupled to liposomes at a final lipid concentration of 20 mM for 8 h. The sample was diluted to 5μmol/ml prior to extrusion Noncovalent attachment of strptavidin to liposomes containing biotin EPE (0.25 mol%) was periotmed as described in Section III 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 PE before (C) and after (D) extntsion. (From Loughrey, H. et al.. Biochim.** ***Biophys Acta,*** **1028. 73. 1990. With permission.)**

of Tris (25 mM final concentration), and the product is purified by chromatography on PD 10 columns (Pharmacia. Uppsala. Sweden). We usually estimate the extent of biotination of antibodies by employing trace amounts of tritiated NHS-biotin (Amersham). and assume an extinction coefficient, E_{280} of 1.02 x 10^5 for antibodies. Under the above conditions using anti-erythrocyte IgG (Organon Teknika. Ontario) we have obtained values of 1 to 5 mol of biotin bound per mole protein.

To prepare immunoliposomes. biotinated antibodies in HBS are usually incubated at room temperature with streptavidin-coated liposomes (1 mM final lipid concentration) at a twofold excess of antibody to streptavidin. A

fluorescent label is introduced into the biotinated antibody by modification of residual amine groups with FITC cellite. in order to estimate the extent of antibody coupling to vesicles.[17] The association of biotinated antibodies with streptavidin-liposomes is rapid (<5 min) and is pH independent (pH 6 to 9).[3] Antibody-coupled vesicles are purified by gel filtration on Sepharose CL-4B. and the antibody to lipid coupling ratio is estimated by measuring the amount of liposome-associated fluorescence. Under the above conditions. approximately 1 mol of biotinated antibody binds per 1.5 mol of streptavidin. The modification of antibody with biotin does not need to be extensive; as little as a single biotin molecule coupled to IgG is sufficient for the efficient coupling to streptavidin-coated liposomes.' Our experience is limited to the use of antibodies modified with NHS-biotin. Binding of biotinated antibodies to streptavidin-coated vesicles may be further enhanced by using a biotin derivative such as NHS-LC-biotin. that has an extended spacer arm. This may alleviate any steric hindrance imposed by the antibody on the binding of the biotin moiety to streptavidin-coupled vesicles.

An alternative method of achieving targeting is by indirectly associating liposomes with cells which have been prelabeled with a biotinated targeting ligand. The potential of this approach is illustrated by the specific binding of streptavidin-liposome conjugates to lymphocyte populations via defined biotinated monoclonai antibodies (Figure 6). In this experiment. incubation of liposome-streptavidin conjugates (containing encapsulated carboxyfluorescein (15 mM)) with cells prelabeled with a biotinated monoclonai antibody specific for peripheral B cells (B 1: Coulter Electronics). resulted in the fluorescent labeling of approximately 20% of the total lymphocyte population (Figure 6B). In comparison. similar studies with a biotinated anti-T cell antibody (T1 1: Coulter Electronics, resulted in the labeling of approximately 90% of lymphocytes (Figure 6C). These results are consistent with the expected cell distribution of the antigens defined by T11[18] and B I.[19] The specificity of these conjugates is indicated by the negligible back-mound binding of streptavidin-liposome conjugates to lymphocytes in the absence of biotinated antibodies (Figure 6A).

VI. *IN VIVO* PROPERTIES OF SIZED STREPTAVIDIN-LIPOSOME CONJUGATES

A major application envisioned for antibody-liposome conjugates is to target liposomally encapsulated material to defined sites ***in vivo.*** Limited success has been achieved in this regard due in part to the rapid sequestration of antibody conjugated liposomes from the circulation by, the reticulo-endothelial system.[20] Recent work from this lab indicates that this rapid clearance behavior could be partly due to aggregation of liposome conjugates. For example. we have shown that aggregated streptavidin liposomes (>530 nm in diameter) when injected intravenously. are rapidly removed from the cir-

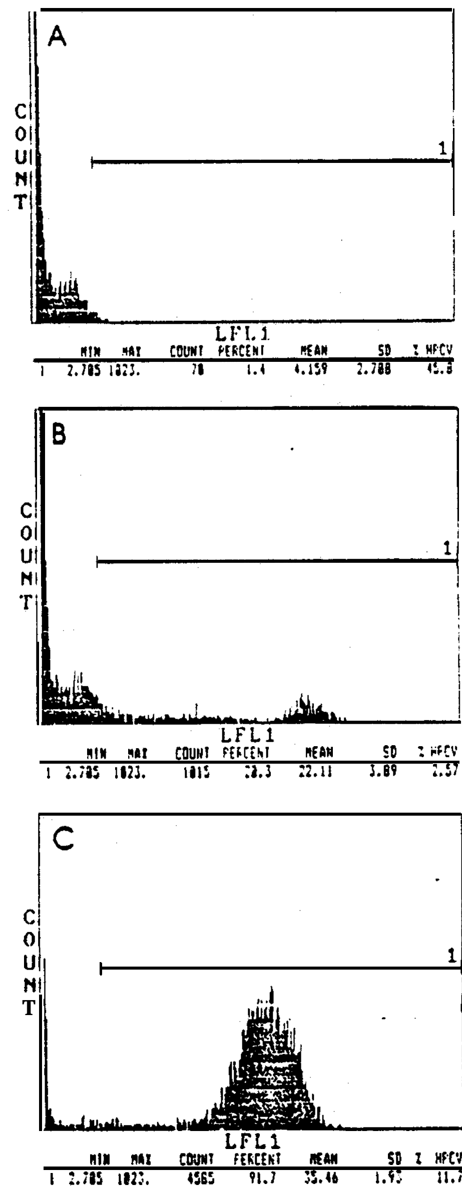

FIGURE 6. Targeting of streptavidin-liposome conjugates via biotinated monoclonal antibodies to human peripheral lymphocytes Streptavidin-liposome conjugates (38.8 μg streptavidin per μmol lipid) with entrapped carboxyfluorescein (15 mM) were prepared as described in Section III. Lymphocytes (10^6) were 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 an hour at 4°C, and washed three times with PBS. Samples were subsequently examined for cell-associated fluorescence by flow cytometry (LFL1, log of fluorescence). (From Loughry H. et al.. J. Immuno1. Methods 132. 25. 1990. With permission.)

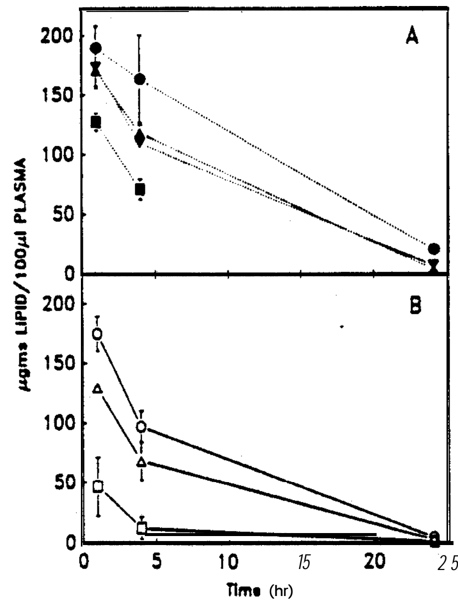

FIGURE 7. In vivo clearance of protein-liposome conjugates. Streptavidin was coupled to liposomes (50 and 100 nm). at a final lipid concentration of 30 mM and incubation period of l5 min. quenched with N-ethylmaleimide for 2 h, followed by an overnight incubation with β-mercapoethanol Control liposomes containing MPB-DPPE were titrated to pH 7.5 and exchanged on Sephadex G-50 equilibrated with HBS. EPC/Chol liposomes were made up in HBS. Mice (4 to 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. Size of extruded samples were determined by QELS. (A): EPC:Chol. 125 nm (●); EPC/Chol. 197 nm (■); MPB-DPPE liposomes. 170 nm (quenched A. 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 (○). (From Loughrey, H. et al., Biochim. *Biophys. Acta*, 1028. 73. 1990. With permission.)

culation *(<3%)* remaining after 4 h.[5] In comparison. extended 'circulation times are obtained for extruded conjugates; 32 and 48% of the initial lipid dose remained in the circulation 4 h after injection for samples of 187 nm and 139 nm in diameter respectively (figure 7). These small homogeneously sized streptavidin-liposome conjugates which have favorable circulation half-lives within the blood importantly retain their capacity to bind biotin up to 4 h after injection. These observations Suggest that extruded preparations of streptavidin coupled liposomes will be capable of binding to biotinated molecules in blood.

VII. DISCUSSION

The conjugation of liposomes to antibody confers on liposomes the ability to bind in a specific manner with soluble or membrane-associated antigens. Such targeted liposomal systems have diverse applications. In order to exploit the full potential of antibody targeted carrier systems. a versatile and reliable methodology for coupling is required. Our efforts have focused on methods which exploit the high-affinity interaction of biotin with streptavidin-coupled vesicles. This approach facilitates the attachment of any biotinated antibody of interest to a single liposomal system in a rapid and gentle manner. The resulting targeting system is highly versatile with respect to *in rdvo* and *in vitro* applications.

In this chapter. we outline covalent and noncovalent methods of attaching streptavidin to liposomes. The noncovalent approach is rapid. but care needs to be employed in maintaining a high ratio of streptavidin to biotin-PE in liposomes to avoid precipitation of lipid. As a consequence. coupling efficiencies are relatively low. The covalent coupling approach is a more efficient coupling procedure. Coupling efficiencies of up to 50% are readily achieved under the optimized coupling conditions described here. Importantly. coupling of protein to liposomes containing low levels of purified MPB-PE does not influence liposome stability. This may be of critical imponance for applications which are dependent on the retention of small molecules such as fluorescent compounds and hydrophilic drugs. within the aqueous compartment of the targeted liposomes.

The chemical method that we have outlined here for the efficient preparation of streptavidin-liposome conjugates is designed in such a way that the protein can only react with the liposome surface. Water-soiuble carbodimides which react with amine groups on both liposomes and protein have also been -used to conjugate streptavidin to vesicles." These procedures are inefficient due to the formation of protein-protein conjugates. With regard to other chemical covalent coupling methods for the preparation of protein-liposome conjugates. equivalent coupling efficiencies have been reported in a limited number of cases.[11]

In contrast to other laboratories,[23,24] we have chosen to use streptavidin over other biotin-binding proteins. such as avidin, in coupling protocols. In our hands. highly purified avidin can induce nonspecific aggregation of vesicles during conjugation reactions.[28] This can be attributed to the basic nature of avidin,[25] and is avoided by employing streptavidin. which is neutral under physiological conditions.[26] A further advantage is that streptavidin is a nonglycosyiated protein. This may be important in *in' vivo* targeting applications, since the presence of sugar moieties may alter the in *vivo* behavior of the protein-liposome conjugates.

It has been observed by our group and others that conditions which increase the coupling efficiency of protein to liposomes: such as lipid con-

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centration. and the ratio of protein to lipid in the coupling incubation mixture. affects the extent of vesicle aggregation.'.“.” Many applications of targeted liposome conjugates require small-sized vesicle systems. Extrusion **procedures** which allow easy manipulation of the physical size of protein-coupled liposomes without affecting the binding activity of the protein were subsequently developed with this aim in mind. Small-sized conjugates exhibit long half-lives within the circulation when administrated intravenously and retain their capacity to bind biotin in plasma. Therefore. extruded streptavidin-liposome conjugates should be able to bind free or cellular bound biotinated target ligands in the circulation.

The “sandwich” protocol described here for the preparation of targeted immunotiposomes is particularly attractive since it allows investigators to pursue two approaches for targeting. First. biotinated targeting ligands may be attached directly to streptavidin-liposome conjugates creating target specific liposomes. Alternatively. cells may be prelabeled with biotinated targeting antibodies followed by addition of stteptavidincoated liposomes. With respect to *in vivo* targeting regimes. the latter indirect approach may have significant advantages. Foremost. it is expected that the clearance behavior of preformed targeted liposomes will be dependent on the type of targeting ligand attached to the vesicle surface. In the case of the noncovalent components would result in efficient usage of reagents and avoid precipitation problems arising due to aggregation of vesicles. Our current efforts are focused on extensively characterizing the pharmacological behavior of covalently coupled streptavidin-liposomes and biotin-PE containing liposomes in the circulation. The results of these studies will define the potential utility of these liposomal systems for *in vivo* targeting applications.

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