Poly(ethylene glycol)-Modified Phospholipids Prevent Aggregation during Covalent Conjugation of Proteins to Liposomes

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Liposome aggregation is a major problem associated with the covalent attachment of proteins to liposomes. This report describes a procedure for coupling proteins to liposomes that results in little or no change in liposome size. This is achieved by incorporating appropriate levels of poly(ethylene glycol)-modified lipids into the liposomes. The studies employed thiolated avidin-D coupled to liposomes containing the thio-reactive lipid *N*-(4-(*p*-maleimidophenyl)butyryl)dipalmitoyl phosphatidylethanolamine (1 mol % of total lipid) and various amounts of MePEG–S–POPE (monomethoxypoly(ethylene glycol) linked to phosphatidylethanolamine via a succinate linkage). The influence of PEG chain length and density was also assessed. The presence of PEG on the surface of liposomes is shown to provide an effective method of inhibiting aggregation and the corresponding increase in liposome size during the covalent coupling of avidin-D. A balance between the size of the PEG used and the amount of PEG–lipid incorporated into the liposome had to be achieved in order to maintain efficient coupling. Optimal coupling efficiencies in combination with minimal aggregation effects were achieved using 2 mol % $MePEG_{2000}$–S–POPE (PEG of 2000 MW) or 0.8 mol % $MePEG_{5000}$–S–POPE (PEG of 5000 MW). At these levels, the presence of PEG did not affect the biotin binding activity of the covalently attached avidin. The ability of the resulting liposomes to specifically target to biotinylated cells is demonstrated.

INTRODUCTION

Liposome-based drug carrier systems which accumulate at regions of disease are actively being developed. It is now well established that small liposomes and associated contents accumulate preferentially in sites of infection, inflammation, and cancer following iv administration *(1–10)*. The level of entrapped contents delivered to these diseased sites increases with liposome circulation longevity as well as optimized drug retention characteristics *(9–11)*. Early studies evaluating the pharmacokinetic behavior of liposomes following intravenous administration demonstrated that liposome size was a critical determinant of circulation longevity *(12, 13)*. Phosphatidylcholine–cholesterol liposomes exhibiting mean size distributions between 50 and 150 nm, for example, are retained in the circulation for extended time periods *(14, 15)*. Retention of entrapped contents is dependent on the lipid composition employed as well as the nature of the entrapped material *(11, 16, 17)*. Liposomes prepared using phospholipids with long chain saturated fatty acyl chains and cholesterol exhibit improved retention of hydrophilic compounds following iv administration.

Research has focused in three areas to develop liposomal drug carriers that have an increased propensity to accumulate in disease sites. The first concerns the use of lipids that engender extended circulation lifetime. Incorporation of the ganglioside G_{M1} or poly(ethylene glycol)-modified phospholipids in liposomes, for example, decreases uptake in the liver and increase circulating blood levels *(18–20)*. Several studies have shown that these liposomes accumulate efficiently in sites of tumor growth *(5, 8, 9)*. The second area of interest concerns the biological elements that mediate movement of liposomes from the blood compartment to an extravascular site. Recent studies have shown that such delivery to tumors occurs through blood vessels that are hyperpermeable to circulating macromolecules *(9, 10)*. Finally, it is reasonable to assume that the extent of accumulation within disease sites, such as tumors, will be dependent on an equilibrium between circulating liposomes and liposomes in the extravascular space. Targeting liposomes to specific elements or cells within the extravascular space should shift the equilibrium in favor of further liposome accumulation at the target site.

Although approaches for attaching targeting proteins to the surface of liposomes are well established *(21–24)*, the resulting proteoliposomes often do not maintain optimal characteristics. It has been shown, for example, that protein–liposome conjugation procedures based on the use of heterobifunctional reagents lead to liposome–liposome crosslinking (i.e., increases in carrier size) which results in dramatically reduced circulation lifetimes. Further, leakage of entrapped contents is also observed *(25, 26)*. Using drug entrapment procedures based on transmembrane pH gradients, where a drug is loaded into preformed liposomes *(27)*, eliminates problems associated with drug leakage during coupling. The most significant limitation to the use of these coupling procedures is liposome aggregation.

Aggregation is generally caused by the covalent crosslinking of liposomes via a multivalent protein bridge. In addition, noncovalent protein–protein interactions can lead to further aggregation. Although aggregation can be minimized by reducing the concentration of reactants and by limiting the number of reactive groups present on both the liposomes and/or the protein, these steps

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significantly reduce coupling efficiency (28, 29). The strategy developed here is to inhibit covalent crosslinking of liposomes by incorporating poly(ethylene glycol)-modified phospholipids. It is well established that incorporation of hydrophilic polymers in liposomes provides a steric barrier inhibiting surface association of serum proteins (30). Further, studies published elsewhere suggest that vesicle size can be maintained following coupling reactions when PEG-modified lipids are incorporated into the liposomes (31, 32). It is demonstrated here that efficient conjugation of thiolated avidin to MPB–PE incorporated in liposomes containing PEG-modified phosphatidylethanolamine can be achieved with no aggregation of the liposomes. It is further demonstrated that the biotin binding activity of liposome associated avidin is maintained and that the circulation lifetime of the resulting liposome is significantly improved.

MATERIALS AND METHODS

Materials. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids, and *N*-(4-(*p*-maleimidophenyl)butyryl)dipalmitoylphosphatidylethanolamine (MPB-DPPE) was synthesized as published previously (24). The synthesis and characterization of various MePEG–lipid conjugates has been described elsewhere (33), and these lipids are now commercially available through Northern Lipids, Inc. (Vancouver, B.C.). Avidin-D was obtained from Vector Laboratories and neutravidin from Pierce. Cholesterol (Chol), *N*-ethylmaleimide (NEM), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), dithiothreitol (DTT), *N*,*N*′-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma. Biotinylated Thy 1.2 antibody was obtained from Cedar Lane Laboratories. Radiolabeled *d*-[carbonyl-^{14}C]biotin and [^{3}H]cholesteryl hexadecyl ether (^{3}H-CHE) were obtained from Amersham. Female CD1 mice were purchased from Charles River Laboratories (Ontario).

Preparation of Liposomes. Large unilamellar vesicles were prepared as described by Hope et al. (34). Lipid mixtures consisting of DSPC, cholesterol, MePEG–S–POPE, and MPB-DPPE were prepared in chloroform and subsequently concentrated to a homogeneous lipid film under a stream of nitrogen gas. The lipid film was then placed under high vacuum for at least 4 h prior to hydration at 65 °C with 300 mM citrate pH 4.0. The resulting multilamellar vesicle preparation was frozen and thawed five times (35) before the sample was extruded 10 times through stacked 100 nm polycarbonate filters (Nuclepore) employing an extrusion device (Lipex Biomembranes, Inc., Vancouver, Canada) at 65 °C. The resulting liposomes were sized by QELS using a Nicomp 270 submicron particle sizer operating at 632.8 nm.

Thiolation of Avidin-D. Avidin-D (5 mg/mL in HBS 25 mM Hepes; 150 mM NaCl, pH 7.5) was modified with the amine reactive reagent SPDP according to procedures described for streptavidin (24, 25). Briefly, SPDP (25 mM in methanol, 1–10 mol equiv) was incubated with avidin-D at room temperature for 30 min. The reaction mixture was then reduced with DTT (25 mM, 10 min), and the thiolated product was isolated by gel filtration on Sephadex G-50 equilibrated with HBS pH 7.5 and used immediately in coupling experiments. The extent of modification of avidin-D was determined by estimating the protein concentration at 280 nm (molar extinction coefficient at 280 nm of 9.52×10^4) prior to the addition of DTT and the 2-thiopyridone concentration at 343 nm (molar extinction coefficient at 343 nm of 7550) 10 min after the addition of DTT.

Coupling of Thiolated Avidin-D to Liposomes. The coupling reaction was performed by incubating thiolated avidin-D with MPB-liposomes at a ratio of 150 μg of protein per μmol of lipid (6–7 mM final lipid concentration) at pH 7.5 with stirring at room temperature. Liposomes prepared at pH 4.0 (300 mM citrate) were passed down a Sephadex G-50 column equilibrated with HBS (pH 7.5) prior to addition of the thiolated avidin-D. At selected time points coupling was stopped by the addition of β-mercaptoethanol followed by (10 min after β-mercaptoethanol addition) the addition of excess NEM. Samples were then passed down a Sepharose CL-4B column equilibrated with HBS to remove any unassociated protein. The amount of avidin coupled to the liposomes was determined by a modification of the fluorescamine assay for protein (36). Briefly, avidin–liposome conjugates were lysed by addition of 10 mM OGP before addition of 0.2 M borate buffer (pH 9.0) to raise the pH. Fluorescamine (1 mg per 5 mL of anhydrous acetone) was added with immediate vortex mixing. Standards were prepared as above using known quantities of the thiolated avidin and uncoupled liposomes. Fluorescence was then determined at an excitation wavelength of 390 nm and emission wavelength of 480 nm using a Perkin-Elmer LS50 luminescence spectrometer.

Doxorubicin Encapsulation. Doxorubicin was encapsulated in selected liposome preparations using the transmembrane pH gradient driven loading procedure as described previously (37). The liposome preparation (prepared at pH 4.0 prior to coupling avidin at pH 7.5) was heated to 60–65 °C for 10 min prior to addition to a preheated (60 °C for 10 min) solution of doxorubicin (5–6 mM in saline). A final drug-to-lipid ratio of 0.2 was typically employed. This mixture was incubated with periodic mixing for 10 min at 60 °C. Unencapsulated doxorubicin was removed by passing the sample through a Sephadex G-50 column, and the doxorubicin-to-lipid ratio was measured as described previously (16, 37).

Biotin Binding Activity. The biotin binding activity of the avidin–liposome conjugates was determined as described for streptavidin (24, 25). Briefly, avidin-liposomes (0.5 μmol of lipid in 0.5 mL) were incubated with a 10-fold excess of [^{14}C]biotin for 10 min at room temperature. Unbound biotin was removed by gel filtration on a Sepharose CL-4B column equilibrated with HBS. The extent of binding of biotin to a thiolated-avidin standard (100 μg) after gel chromatography on Sephadex G-50 was used as a reference for the calculation of coupling ratios.

Targeting to Biotin-Labeled P388 Cells. In vitro quantification of cell-associated lipid after targeting avidin-D and neutravidin-coated LUVs with 2 mol % of PEG_{2000}–DSPE to P388 cells was performed as follows. Avidin or neutravidin LUVs (51 and 63 μg/μmol of lipid, respectively) incorporating PEG_{2000}–DSPE were prepared as described above. P388 cells (10^7) were incubated with or without biotinylated anti-mouse Thy 1.2 antibody (10 μg) for 30 min at 4 °C. Cells were then washed (three 10 min centrifugations at 800*g*) with PBS prior to addition (2 mM final concentration) of either avidin or neutravidin LUVs. After 30 min incubation at 4 °C, the cells were further washed, and cell-associated lipid was determined via a ^{3}H-CHE lipid marker.

In Vivo Clearance Studies. Coated LUVs composed of DSPC/Chol/$MePEG_{2000}$–S–DSPE/MPB-DPPE (52:45:2:1) containing either avidin or neutravidin were prepared as outlined previously. CD1 mice were injected iv at 30 mg of lipid/kg with one of the above protein-coated LUVs. Whole blood was collected at 1, 4, and 24 h

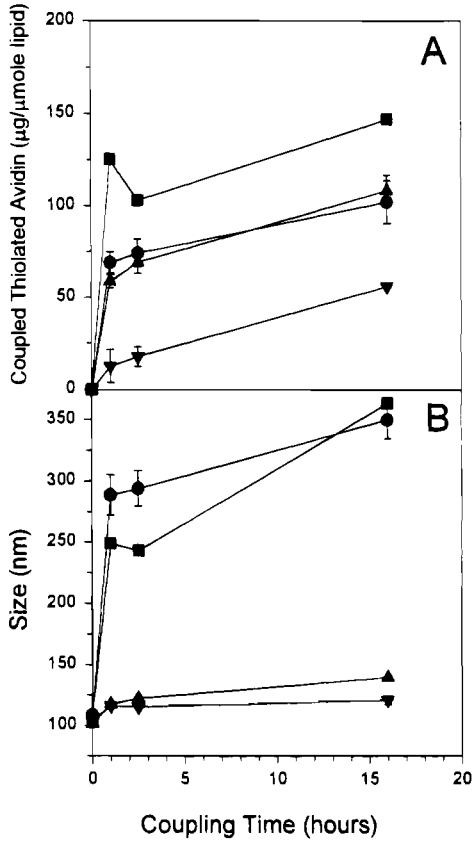

Figure 1. Effect of different MePEG–S–POPE on the coupling reaction of thiolated avidin (3.6 SH equiv, 150 μg per μmol of lipid) with MPB-liposomes (DSPC:Chol:MPB-DPPE:MePEG–S–POPE, 52:45:1:2; 6.57 mM). Panel A shows the amount of protein (expressed as μg of thiolated avidin per μmol of lipid) coupled to MPB–liposomes as a function of coupling reaction time. Panel B shows the size (nm) of the corresponding proteoliposomes as measured by QELS: control (●); $MePEG_{550}$–S–POPE (■); $MePEG_{2000}$–S–POPE (▲); and $MePEG_{5000}$–S–POPE (▼). At 16 h, LUV size for both $MePEG_{2000}$ and $MePEG_{5000}$ were significantly different from control liposomes ($p < 0.001$ and $p < 0.005$, respectively). Points: mean of three assays. Error bars: SD of at least three experiments.

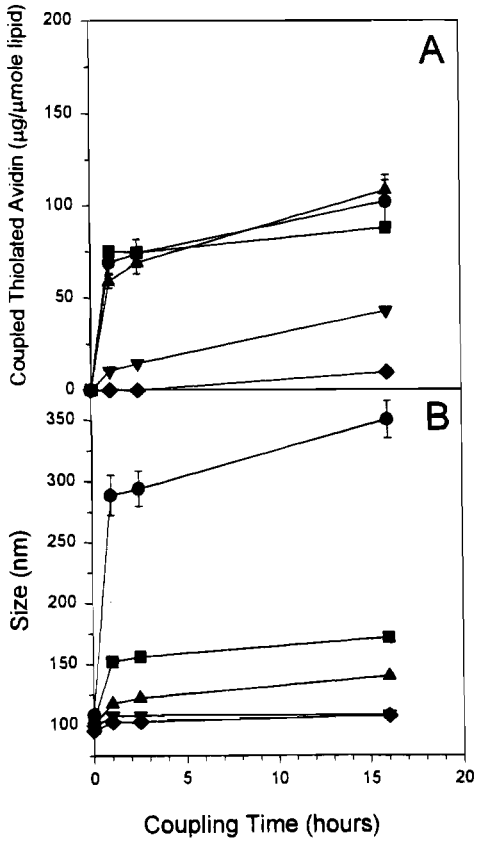

Figure 2. Effect of different levels of the polymer $MePEG_{2000}$ on the coupling reaction of the thiolated avidin (3.9 SH equiv, 150 μg per μmol of lipid) with MPB-liposomes (6.54 mM) containing various concentrations of $MePEG_{2000}$–S–POPE: 0% (●); 1% (■); 2% (▲); 5% (▼); and 8% (◆). Coordinates for panels A and B are the same as in Figure 1. At 16 h, all treatment groups were statistically significant from control ($p < 0.05$). Points: mean of three assays. Error bars: SD of at least three experiments.

intervals via cardiac puncture and collected in EDTA-coated tubes. Plasma was subsequently prepared by centrifuging at 1500*g* for 10 min. Lipid was then assayed via a ^{3}H-CHE lipid marker.

RESULTS

The first set of experiments was aimed at determining the influence of different sizes of PEG polymer on the coupling reaction between MPB-liposomes and thiolated avidin. Monomethoxypoly(ethylene glycol) (MePEG) of three different molecular weights (550, 2000, and 5000) was linked via a succinate bond to POPE to form the respective MePEG–lipid conjugates ($MePEG_{550}$–S–POPE, $MePEG_{2000}$–S–POPE, and $MePEG_{5000}$–S–POPE) *(33)*. These MePEG–lipid conjugates were then incorporated into MPB-containing liposomes (1 mol % MPB–DPPE/54 mol % DSPC/45 mol % Chol) at a level of 2 mol % of the total lipid. Incubation of these MePEG-coated MPB-containing liposomes with thiolated avidin resulted in the covalent attachment of protein to the liposomes (Figure 1). The rate and extent of coupling was dependent on the molecular size of the poly(ethylene glycol) incorporated. In the absence of MePEG–S–POPE substantial levels of avidin (70 μg of avidin/μmol of lipid) were conjugated to the liposomes within 1 h (Figure 1A). Subsequently, the coupling reaction occurred at a reduced rate ultimately leading to levels of approximately 100 μg of avidin/μmol of lipid observed at 16 h. As observed in previous studies *(25, 26)*, these liposomes exhibited a dramatic increase in size, indicative of liposome–liposome crosslinking (Figure 1B). The time course for size increase was similar to that observed for coupling. As shown in Figure 1B, incorporation of $MePEG_{2000}$– and $MePEG_{5000}$–S–POPE substantially reduced time dependent increases in liposome size. While the rate of avidin coupling to liposomes was reduced significantly when 2 mol % $MePEG_{5000}$–S–POPE was present, both the rate and extent of coupling obtained for liposomes with 2 mol % $MePEG_{550}$–S–POPE was identical to controls.

The results illustrated in Figure 1 indicated that a hydrophilic polymer coating imparted by incorporation of 2% $MePEG_{2000}$ was the most suitable for preparation of proteoliposomes in terms of protein-coupling efficiency and effectiveness in inhibiting vesicle aggregation. The next series of experiments were designed to determine whether 2 mol % $MePEG_{2000}$–S–POPE was optimal. Four different levels of $MePEG_{2000}$–S–POPE (1%, 2%, 5%, and 8%) were studied and compared with control liposomes. The different quantities of thiolated avidin that could be coupled to each type of liposome are illustrated in Figure 2A. The presence of 5 and 8 mol % $MePEG_{2000}$ significantly reduced the amount of protein that could be conjugated to the surface of the liposomes. In contrast, addition of 1 or 2 mol % $MePEG_{2000}$ did not influence the coupling reaction. For these liposomes, 70–

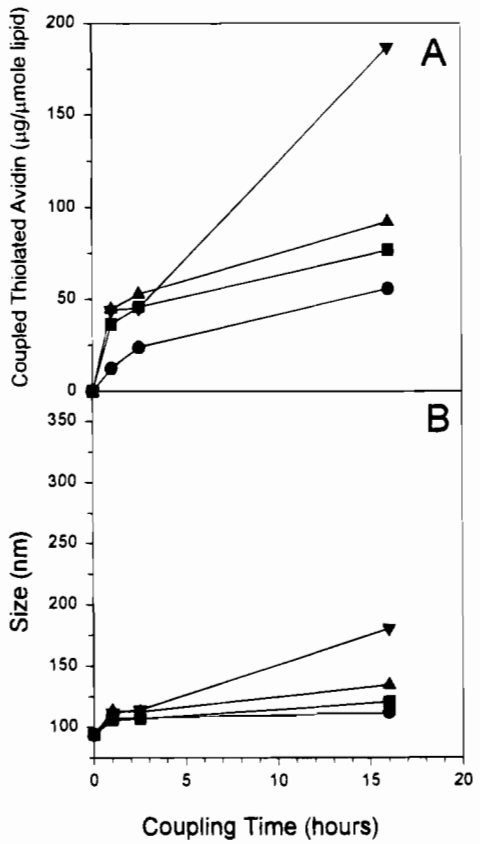

Figure 3. Effect of the degree of protein thiolation on the coupling reaction of thiolated avidin (150 μg per μmol of lipid) with pegylated liposomes (DSPC:Chol:MPB-DPPE:MePEG$_{2000}$–S–POPE, 52:45:1:2; 6.49 mM). 1.2 SH equiv (●); 1.9 SH equiv (■); 4.5 SH equiv (▲); and 5.3 SH equiv (▼). Coordinates for panels A and B are the same as in Figure 1. Points: mean of three assays. Error bars: SD of at least three experiments.

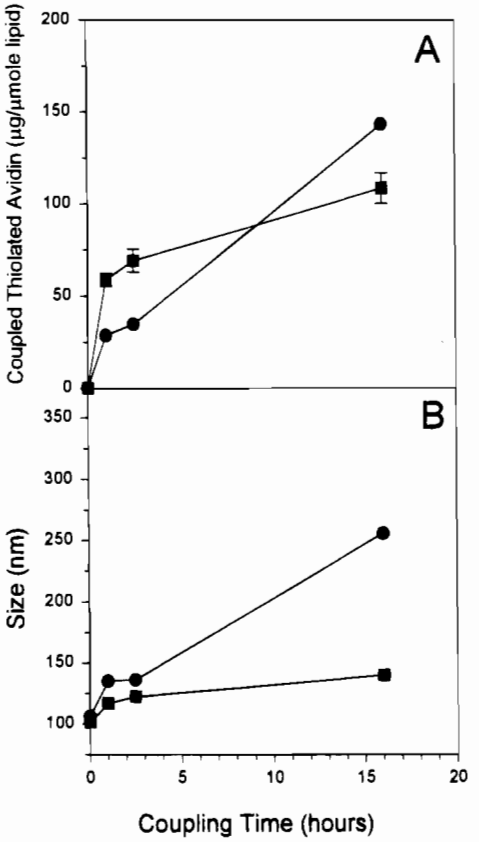

Figure 4. Effect of different polymer coatings on the coupling reaction of the thiolated avidin (4.2 SH equiv, 150 μg per μmol of lipid) with MPB-liposomes (6.15 mM) containing 8% PEG$_{550}$–S–POPE (●) or 2% MePEG$_{2000}$–S–POPE (■). Coordinates for panels A and B are as in Figure 1. Points: mean of three assays. Error bars: SD of at least three experiments.

80 μg of avidin was bound to the liposomes within 1 h after addition of the thiolated protein, representing a coupling efficiency of approximately 50%. As expected, liposomes that did not efficiently couple protein showed no size increases (Figure 2B). Efficient coupling with only minimal increases in vesicle size was observed for liposomes prepared with 2% MePEG$_{2000}$.

The degree of protein thiolation is also known to have an effect on protein-coupling reactions mediated by MPB-modified lipids. In order to assess whether incorporation of 2 mol % MePEG$_{2000}$–S–POPE inhibited aggregation regardless of the extent of protein thiolation, coupling of modified avidin having approximately 1, 2, 4, and 5 thio equiv was determined (Figure 3A, B). As expected, protein association was dependent on the degree of thiolation. The presence of 2–4 thio equiv appeared optimal for efficient coupling with no associated changes in vesicle size. The amount of protein-coupling was significantly enhanced when using avidin with 5 thiol equiv; however, increases in vesicle size were observed for this system even in the presence of 2 mol % MePEG$_{2000}$–S–POPE.

Further investigations on the importance of the effect of molecular size of the polymer chain were conducted by comparing the coupling reaction of thiolated avidin with MPB-liposomes containing 8% MePEG$_{550}$–S–POPE or 2% MePEG$_{2000}$–S–POPE. It can be estimated that these liposomal preparations should exhibit similar numbers of PEG units on the surface of the liposomes. However, as illustrated in Figure 4A, the initial rates of protein conjugation are quite different. With 8% MePEG$_{550}$, the higher density of the shorter PEG$_{550}$ molecules on the liposomal surface initially inhibited the coupling reaction with thiolated avidin. Alternatively, MPB-liposomes with 2% MePEG$_{2000}$–S–POPE did not exhibit any noticeable barrier to chemical coupling of thiolated avidin (Figure 4A). As indicated before, the presence of this lipid did provide a substantial barrier in terms of inhibition of intervesicular crosslinking and liposome aggregation (Figure 4B). As the coupling reaction proceeded further (16 h), the steric stabilization effect of the longer chain MePEG$_{2000}$ was apparent.

The effect of incorporating decreased quantities of the longer MePEG$_{5000}$–S–POPE on the coupling reaction between MPB-liposomes and thiolated avidin is illustrated in Figure 5. Results indicate that incorporation of 1.2, 0.8, and 0.4 mol % MePEG$_{5000}$–S–POPE effectively inhibits aggregation of the avidin–liposome conjugates (Figure 5B) without hindering the protein-coupling efficiency (Figure 5A). It should be noted that studies were initiated to determine whether G_{M1}, a ganglioside that is similar to PEG-modified lipids in that it can prolong the *in vivo* circulation lifetime of liposomes, prevents coupling-induced aggregation. The results (not shown) indicate that at levels of 10 mol % G_{M1} the coupling reaction was not effected. Specifically, the rate and extent of coupling were identical to control liposomes, and there is a coupling dependent increase in vesicle size.

The results presented thus far demonstrate that incorporation of either MePEG$_{2000}$ or MePEG$_{5000}$ at appropriate levels inhibits vesicle–vesicle crosslinking that occurs when coupling thiolated protein to MPB-PE-containing liposomes. Optimal coupling, in terms of reaction rates and coupling efficiency, are achieved when using 2 mol % MePEG$_{2000}$–S–POPE or 0.8 mol %

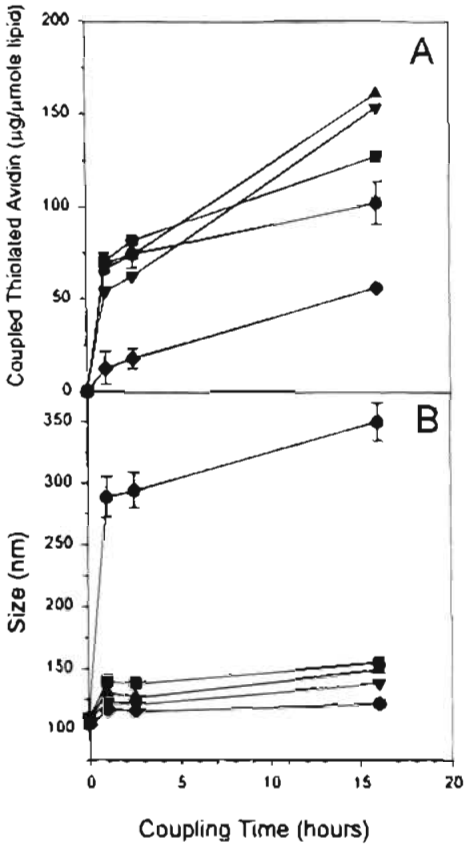

Figure 5. Effect of different levels of the polymer $MePEG_{5000}$ on the coupling reaction of thiolated avidin (3.6 SH equiv, 150 μg per μmol of lipid) with MPB-liposomes (6.2 mM) containing various concentrations of $MePEG_{5000}$–S–POPE: 0% (●); 0.4% (■); 0.8% (▲); 1.2% (▼); and 2% (◆). Coordinates for panels A and B are as in Figure 1. At 16 h, all treatment groups were statistically significant from control ($p < 0.05$). Points: mean of three assays. Error bars: SD of at least three experiments.

$MePEG_{5000}$–S–POPE. It is important to demonstrate, however, that the resulting liposomal preparations exhibit appropriate characteristics required for *in vivo* drug delivery applications. For this reason the following experiments characterized four important parameters, namely the drug loading characteristics, the biotin binding capacity, the *in vitro* cell-targeting efficiencies, and the *in vivo* plasma clearance behavior of the avidin-coated liposomes. The drug loading characteristics of avidin-D coated liposomes was assessed using the transmembrane pH gradient mediated loading procedure to encapsulate the anticancer drug doxorubicin (*10*, *27*). The pH gradient was established by preparing liposomes at pH 4.0 (300 mM citrate buffer) prior to adjusting the external pH to 7.5 (as required for the protein-coupling reaction). Efficient doxorubicin loading was achieved for the avidin-D-coated liposomes prepared with different amounts of either $MePEG_{2000}$ or $MePEG_{5000}$–S–POPE (results not shown), where greater than 95% of the added doxorubicin (a drug to lipid weight ratio of 0.2) was encapsulated within 5 min at a incubation temperature of 65 °C. The resulting liposomes retain drug over storage periods (at 4 °C) in excess of 48 h.

As shown in Figure 6, the biotin binding capacity of liposomes that have bound avidin-D in the presence of incorporated $MePEG_{2000}$–S–POPE was well retained at levels below 5 mol %. At levels of 8 mol % $MePEG_{2000}$–S–POPE, a level shown to inhibit avidin-D coupling, there was a greater than 50% loss of biotin binding activity of the surface-associated avidin-D. A further indication of the biotin binding capacity of avidin-D

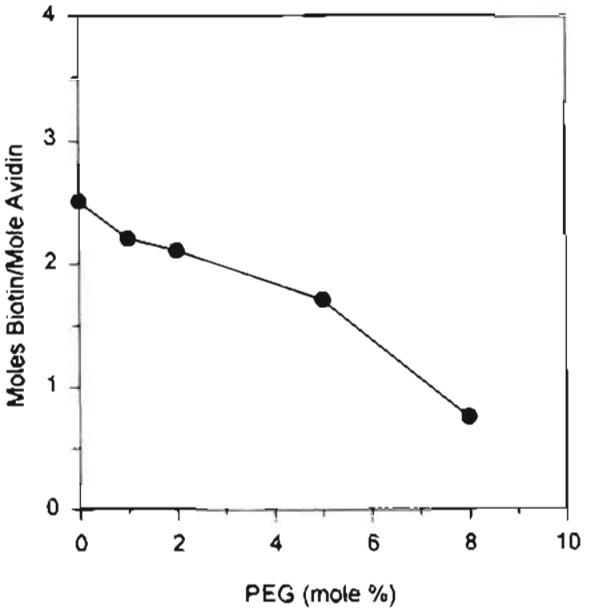

Figure 6. Biotin binding activity of proteoliposomes formed by the coupling of thiolated avidin (3.9 SH equiv, 150 μg per μmol of lipid) with various levels of $MePEG_{2000}$–S–POPE. Avidin–liposomes (1 μmol/mL) were incubated with [^{14}C]biotin (10-fold excess) for 10 min at room temperature. Unbound biotin was removed on a sepharose CL-4B column, and the extent of biotin binding was evaluated against a thiolated-avidin standard as a reference for the calculation of coupling ratios.

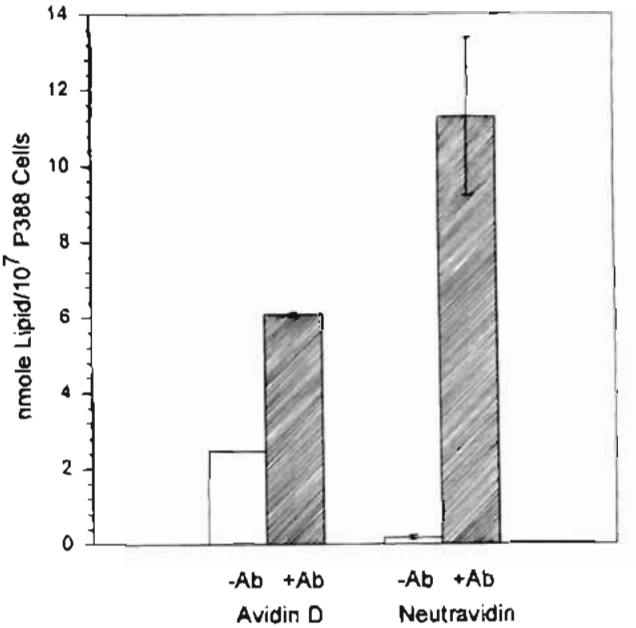

Figure 7. Quantification of cell associated lipid after targeting avidin-D and neutravidin-coated LUVs with 2 mol % PEG_{2000}–DSPE to P388 cells in vitro. Avidin-D or neutravidin LUVs (51 and 63 μg/μmol of lipid, respectively) incorporating PEG_{2000}–DSPE were prepared as described in the Materials and Methods. P388 cells (10^7) incubated with (hatched) or without (empty bars) biotinylated anti-mouse Thy 1.2 antibody (10 μg) for 30 min at 4 °C followed by a further 30 min incubation with avidin or neutravidin LUVs (2 mM final concentration). Cell-associated lipid was determined by a ^{3}H-CHE lipid marker. Neutravidin + Ab was significantly different from avidin + Ab, $p < 0.05$. Points: mean of three assays. Error bars: SD of at least three experiments.

coupled liposomes with 2 mol % $MePEG_{2000}$ is illustrated in Figure 7. Briefly, avidin-D liposomes were targeted *in vitro* to P388 cells (a murine lymphocytic leukemia cell line) prelabeled with biotinylated anti-Thy 1.2 antibody.

This convenient two-step targeting approach has been used previously by our group to assess the binding of streptavidin-coated liposomes to this cell line *(38)*. The results demonstrate that a 3-fold increase in liposome-cell association is achieved when incubating avidin-D coated liposomes with biotin-labeled P388 cells when compared with incubations with unlabeled P388. The increase in liposome targeting achieved is far less than that observed previously for liposomes with bound streptavidin *(38)*. The avidin-D coated liposomes exhibit significantly higher background (nonspecific) binding to P388 cells than streptavidin liposomes (results not shown), and this is believed to be a consequence of the carbohydrate groups present on avidin-D. The influence of the carbohydrate moiety on nonspecific cell association is illustrated in Figure 7. A deglycosylated version of avidin, referred to as neutravidin, coupled to liposomes using procedures identical to those used for avidin-D resulted in a liposome preparation with vastly improved specificity. It should be noted that the level of protein bound to these liposomes was 51 and 63 μg/μmol for avidin-D and neutravidin, respectively.

It was anticipated that the carbohydrate groups on avidin-D would promote liposome clearance following iv administration of the protein-coated liposomes with 2% $MePEG_{2000}$. For this reason the deglycosylated version of avidin (neutravidin) was also selected for preliminary *in vivo* clearance studies. A further modification in the liposomes used for *in vivo* studies involved the use of $MePEG_{2000}$ linked to DSPE rather than POPE. Results from this laboratory demonstrate that, *in vivo*, the POPE-based PEG_{2000} lipid conjugates rapidly exchange out of the liposomal membrane *(33)*. This study also demonstrated that DSPE-modified-PEG lipids were most appropriate for *in vivo* applications on the basis of exchangeability and stability. The DSPE–PEG lipid-containing liposomes exhibited similar protein-coupling characteristics as observed for POPE–PEG systems (results not shown). *In vivo* plasma clearance studies (in female CD1 mice), therefore, determined the circulation lifetime of protein-free, avidin-D, and neutravidin-coated liposomes with 2% $MePEG_{2000}$–S–DSPE.

The results shown in Figure 8 were obtained after iv administration of liposomes at a lipid dose of 30 mg/kg. All liposomal preparations were similar in size prior to administration, where protein-free, avidin-D, and neutravidin liposomes exhibited mean diameters (as measured by QELS) of 109, 106, 119 nm, respectively. Liposomal lipid levels were determined using [^{3}H]cholesteryl hexadecyl ether as a nonexchangeable lipid marker. Protein-free liposomes were maintained in the plasma compartment at levels greater than either of the protein-coated liposomes. The results suggest that avidin-D liposomes are removed from the circulation faster than neutravidin-coated liposomes (half-lives for avidin-coated, neutravidin-coated, and protein-free LUVs of ~2.5, 3, and 11 h, respectively). It should be noted that previous studies have already shown that in the absence of size reduction, protein-coated liposomes are rapidly cleared following iv administration *(25)*. More specifically, streptavidin liposomes prepared by the covalent coupling procedure described here, but in the absence of PEG lipids, exhibited circulation half-lives of less than 30 min *(25)*. These results suggest that the methodology developed here will be appropriate for *in vivo* targeting of liposomal carriers. Detailed characterization of the utility of $MePEG_{2000}$–S–DSPE-containing liposomes for preparation and targeting (*in vitro* and *in vivo*) of avidin-coated and IgG-coated liposomes will be provided elsewhere.

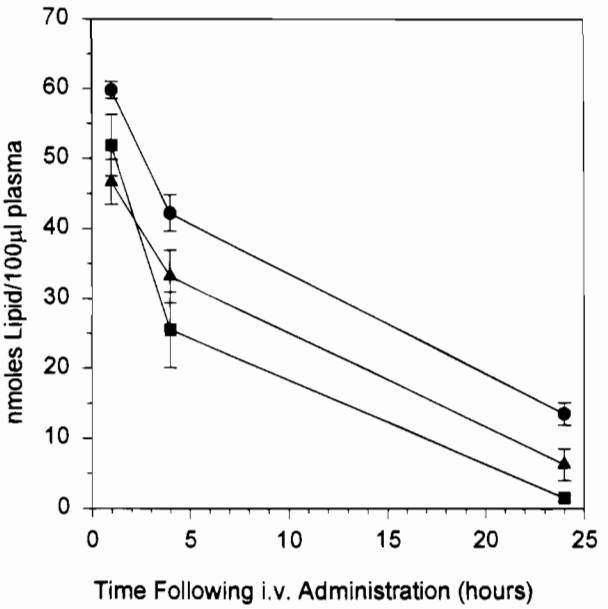

Figure 8. In vivo clearance characteristics of protein-coated LUVs composed of DSPC/Chol/$MePEG_{2000}$–S–DSPE/MPB–DPPE (52:45:2:1) containing either no protein (●), avidin (■), or neutravidin (▲) (0, 51, and 63 μg/μmol of lipid, respectively). Female CD1 mice were injected via a lateral tail vein at a dose of 30 mg of lipid/kg. Whole blood was collected at the indicated time points via cardiac puncture, and plasma was prepared as outlined in the Materials and Methods. Theoretical levels of lipid at $t = 0$ are approximately 100 nmol of lipid/100 μL of plasma based on 20 g mice. At 24 h avidin and neutravidin were statistically significant from control ($p < 0.001$ and $p < 0.05$, respectively) and from each other, $p < 0.01$. Points: mean of three assays. Error bars: SD of at least three experiments.

DISCUSSION

One of the most significant problems associated with the preparation and use of protein-coated liposomes for targeting purposes concerns coupling induced liposome–liposome crosslinking. The resulting liposome aggregates release entrapped contents *(26)* and are very rapidly cleared from the circulation. The most versatile approach for attaching proteins to liposomes is based on the use of heterobifunctional reagents. The use of these coupling reagents for attaching protein to liposomes was first documented in early 1980 by the work of Leserman et al. *(21)* and Martin et al. *(22)*. However, this coupling technology has not yet resulted in a liposomal formulation that can specifically target defined cell populations *in vivo*. Research in this laboratory has focused on developing methodology that results in a protein-coated liposome preparation more appropriate for *in vivo* targeting applications. The studies described here investigate the use of PEG-modified lipids to inhibit liposome crosslinking and clearly demonstrate that efficient protein coupling to liposomes can be achieved with little or no change in liposome size when PEG-modified lipids are incorporated in the liposomes prior to coupling. The importance of providing an appropriate balance between steric inhibition of liposome–liposome crosslinking while maintaining efficient protein-coupling reactions is discussed below.

Two closely related factors are important for designing a hydrophilic-polymer coating on the surface of liposomes used for protein coupling. A balance must be reached between the polymer length of the PEG used and the density of the polymeric coating. For coupling of thiolated avidin to MPB-liposomes, either 2 mol % $MePEG_{2000}$ or 0.4–0.8 mol % $MePEG_{5000}$ on the liposomal surface is optimal for the formation of nonaggregated

avidin-D–liposome conjugates (see Figures 3 and 5). As the amount of $MePEG_{2000}$ or $MePEG_{5000}$ is increased to 5 or 2 mol %, respectively, there is a significant reduction in protein-coupling efficiencies. Lower levels are insufficient to prevent liposome crosslinking. In contrast, results with the lower molecular weight PEG ($MePEG_{550}$) suggest that this chain length is not capable of preventing liposome crosslinking at any concentration employed. Yet at levels of 8 mol % $MePEG_{550}$ there is an initial inhibition of protein coupling (Figure 4). Clearly, the higher density of the polymer coating presents a large steric barrier impeding close contact of the thiolated avidin molecule with the liposome surface. This is reflected in the initial lower amounts of protein coupled. However, once covalently conjugated, the thiolated avidin is not adequately shielded by the smaller $MePEG_{550}$ chain on the liposome surface and interliposomal crosslinking becomes more prevalent resulting in a significant increase in size of the proteoliposome.

The presence of appropriate levels of either $MePEG_{2000}$ (2%) or $MePEG_{5000}$ (0.4–0.8%) on the surface of MPB-liposomes did not impede covalent coupling of thiolated avidin; however, liposome–liposome crosslinking was inhibited. These results are consistent with studies reported by Klibanov et al. *(39)* who demonstrated that incorporation of PEG-modified lipids into liposomes prevented streptavidin-induced aggregation of biotin-labeled liposomes. It is of interest to note that the ganglioside G_{M1}, a lipid that behaves comparably to PEG-modified lipids in terms of inhibiting protein binding, reducing RES uptake and engendering long circulation lifetimes *(18)*, does not inhibit either protein-coupling reactions or protein-coupling induced liposome aggregation.

This report shows that the presence of $MePEG_{2000}$ in proteoliposomes should facilitate development of liposomes for *in vivo* targeting applications. Such liposomes must maintain an ability to efficiently encapsulate and retain drugs such as doxorubicin following covalent attachment of the selected targeting protein and still be able to bind the target antigen. As demonstrated here, the use of $MePEG_{2000}$ as a polymer coating at levels of 2 mol % did not affect binding to a target-cell population labeled with a biotinylated antibody (Figure 7). As a final consideration it is important that the proteoliposome exhibit a pharmacological behavior comparable to a liposome with no surface-associated protein. This will, in part, be dependent on the nature of the associated protein. IgG, for example, may promote liposome clearance due to Fc-mediated clearance *(38, 40)*. Alternatively, as shown here, glycoproteins attached to liposomes reduce circulation lifetime even in the presence of 2 mol % $MePEG_{2000}$. Long circulation lifetimes are particularly important when liposome targeting is attempted *in vivo* following iv administration of proteoliposomes. It has been suggested that in order to maximize liposome movement from the blood compartment to an extravascular site, the liposomes must exhibit an enhanced circulation lifetime *(9, 10)*.

Optimal levels of PEG density (required for enhanced circulation longevity) and attached targeting ligand (required for specificity) must be established for effective *in vivo* targeting to be achieved. Although it has been demonstrated that the incorporation of PEG_{2000} at 5 mol % is very effective in terms of increasing circulation lifetimes of liposome formulations *(20)*, studies presented here indicate that this level of PEG–lipid significantly reduced the quantity of protein coupled to the liposome surface (Figure 2). Further, the biotin binding capacity to covalently attached avidin–liposomes with 5 mol % PEG_{2000} was effectively reduced. This would suggest that the ability of these liposomes to bind a biotinylated-antibody would be reduced, or alternatively, the ability of the liposomes to bind an antigen expressed on a cell may be inhibited. By incorporating PEG_{2000} at lower mole percentages greater levels of protein can be coupled to the surface of the liposome (Figure 2) and adequate target specific binding can occur (Figure 6); however, this is achieved at the risk of reduced circulation lifetimes. Thus, the appropriate balance of protein content and PEG density in *in vivo* applications is a feature of targeted liposomal systems that will have to be empirically derived through experimentation.

In summary, the presence of hydrophilic polymers such as PEG on the surface of liposomes provides a general and practical method for controlling liposome size during the covalent conjugation of proteins to liposomes. A balance between the molecular size of the MePEG chain and the concentration of the polymer on the liposomal surface has been determined to allow efficient protein coupling with little or no liposome crosslinking. The resulting liposomes exhibit characteristics suitable for development of *in vivo* targeting approaches.

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