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Antibody Conjugation Methods for Active Targeting of Liposomes

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1. Introduction

Liposomes are useful drug delivery vehicles since they may protect encapsulated drugs from enzymatic degradation and rapid clearance in vivo, or alter biodistribution, potentially leading to reduced toxicities *(1,2)*. A major limitation to the development of many specialized applications is the problem of directing liposomes to tissues where they would not normally accumulate. Consequently, a great deal of effort has been made over the years to develop liposomes that have targeting vectors attached to the bilayer surface. These vectors have included ligands such as oligosaccharides *(3,4)*, peptides *(5,6)*, proteins *(7,8)* and vitamins (9). Most studies have focused on antibody conjugates since procedures for producing highly specific monoclonal antibodies (MAbs) are well established. In principle it should be possible to deliver liposomes to any cell type as long as the cells are accessible to the carrier. In practice it is usually not this simple since access to tissue, competition, and rapid clearance are formidable obstacles. It has also been shown that antibodies become immunogenic when coupled to liposomes *(10,11)*, although in similar experiments with ovalbumin we have demonstrated that immunogenicity can be suppressed by formulating the liposomes with the cytotoxic drug doxorubicin (12). Such issues as these suggest that the development of antibody-targeted liposomes for in vivo applications will present difficult challenges.

In addition to addressing the challenges related to use of antibody-conjugated carriers, there must be a fundamental understanding regarding why a targeted ligand is being attached. The concept, in its simplest form, is to

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enhance binding of the carrier to a defined cell population or a selected tissue element. It is essential, therefore, that the binding attributes of the targeting ligand be retained during the procedures that are used to prepare the targeted conjugate. Binding attributes can be determined in vitro using target cells in culture or in vivo by careful evaluation of the carrier's distribution characteristics. With respect to the latter, targeting should increase association of the carrier with a target cell, which may be reflected by enhanced delivery to the site where the cell is located, enhanced retention of the carrier within the site where the cell is located; a redistribution of the carrier within the site where the target cells are located, and/or target binding-mediated changes in the characteristics of the carrier.

Numerous procedures for the conjugation of antibodies to lipcsomes have been developed (13-16). These fall into four general categories defined by the particular functionality of the antibody being modified, namely amine modification, carbohydrate modification, disulfide modification, and noncovalent conjugation, each of which will be discussed below. These procedures are very similar to those used to prepare affinity columns *(17)*, except for modifications made to accommodate the fact that the substrate is in the solution phase rather than the solid phase.

Antibodies consist of two pairs of light and heavy chains that are held together by intrachain disulfide bonds. There are two isoforms of the light chain and five of the heavy chain. The type of heavy chain defines the class of antibody, namely IgG, IgM, IgA, IgE, or IgD. Most liposome conjugates are produced using IgG and occasionally IgM. Various classes of antibodies exist as monomers or multimeric structures; for example, IgG exists as a monomer whereas the soluble form of IgM generally exists as a pentamer. These size differences may be significant when conjugating the antibody to liposomes, particularly when molecules, such as polyethylene glycol (PEG)-lipids, have been incorporated into the liposome membrane, affecting such parameters as conjugation efficiency and liposome aggregation. In order to illustrate potential sites of modification a schematic diagram of an IgG molecule is detailed in **Fig. 1.** Proteolytic degradation of antibodies can be used to generate smaller functional antigen-binding proteins. For example, treatment of IgG with papain or pepsin is used to generate Fab *(18)* and $F(ab')_2$ *(19)* fragments, respectively. Antibody fragments such as these are attractive for targeting purposes since they should not have the problems associated with the effector functions of the Fc chain, such as Fc receptor binding *(20)* and complement activation. It is also possible to generate chimeric antibodies to avoid problems associated with species differences in the conserved regions of the antibody (21).

Many chemistries have been used to conjugate antibodies to liposomes. The most useful of these involve modification of the antibody and a lipid in the

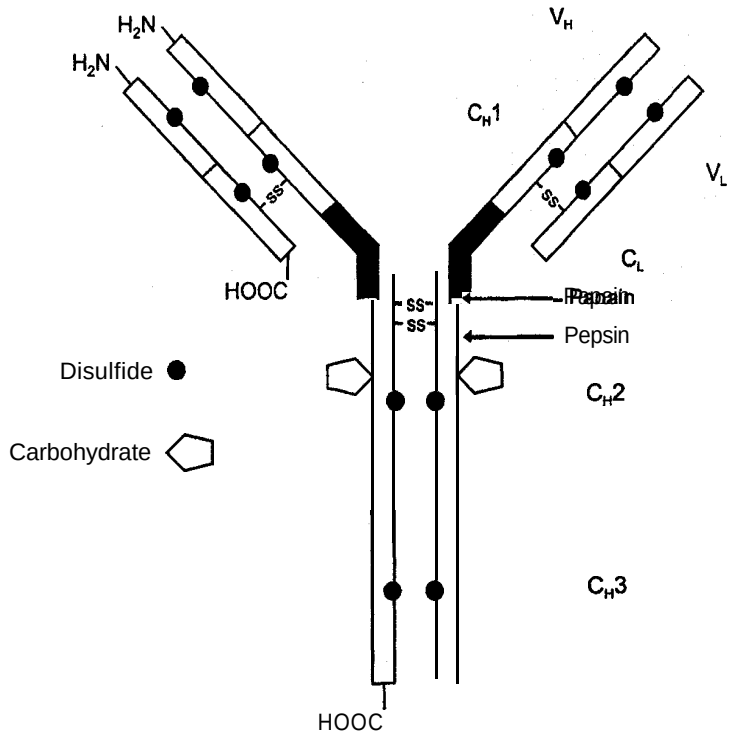

Fig. 1. A schematic representation of IgG depicting sites available for modification. Amino groups in the form of lysine residues may be scattered throughout the antibody. Arrows depict the sites of proteolytic lysis by papain and pepsin.

liposome with crosslinkers, which, when activated, react with each other to form a permanent covalent link. The most widely used approach has been the reaction of sulflhydryl groups with maleimide groups, as detailed in **Fig. 2A.** This reaction has the advantage of being relatively clean, fast, and efficient, and has been adapted to the modification of all of the antibody functional groups in the preparation of liposome conjugates. Selection of a particular chemistry and site of modification should be made depending on what procedures are compatible with the antibody in question. Different antibodies may be more sensitive to some procedures than others and it may be necessary to attempt a number of protocols. The recommended general procedure (because it is very well characterized) involves the thiolation of antibodies with 3-(2-pyridyldithio)propionic acid-N-hydroxysuccinimide ester (SPDP), followed by deprotection with dithiothreitol (DTT) and conjugation to liposomes containing maleimide-derivatized 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE).

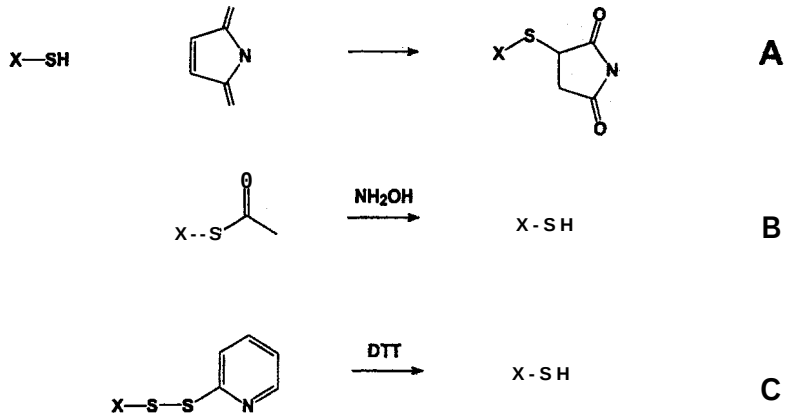

Fig. 2. Important reactions involved in the generation of thiol-maleimide links. All of these reactions proceed under neutral conditions. **(A)** Free sulfhydryl groups undergo additional reactions with maleimide groups. **(B)** Thioacetate protection groups may be hydrolyzed with hydroxylamine. (C) 2-Pyridyldisulfide protecting groups are reduced by DTT.

2. Materials

1. Crosslinkers are available from Molecular Probes, Inc. (P.O. Box 22010, Eugene, OR 97402-0469, Tel. 541-465-8300, Fax 541-344-6504, or Pierce (P.O. Box 117, Rockford, IL 61105, Tel. 815-968-0747, Fax 815-968-8148).
2. Lipids are available from Avanti Polar Lipids, Inc. (700 Industrial Park Drive, Alabaster, AL 35007, Tel. 800-227-0651, Fax 800-229-1004, or Northern Lipids, Inc. (2660 Oak Street, Vancouver, BC, V6H 3Z6, Canada, Tel. 604-875-4836, Fax 604-875-4979).
3. Extruders are available from Lipex Biomembranes Inc. (3550 W 11th Ave., Vancouver, BC, V6R 2K2, Canada, Tel. 604-734-8263, Fax 604-734-2390).
4. *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) buffered saline (HBS): 20 m*M* HEPES, 150 m*M* NaCl, pH 7.4; sodium acetate buffered saline (SAS): 100 m*M* NaOAc, 50 m*M* NaCl, pH 4.4; phosphate-buffered saline (PBS): 20 m*M* Na_2HPO_4, 145 m*M* NaCl, pH 7.4.

3. Methods

3.1. *Liposome Preparation*

The most commonly used method of liposome preparation (see **Note 1)** involves hydration of a lipid mixture in buffer, followed by extrusion through a press of some description. A number of devices are commercially available for this purpose, the most widely used being the "Extrude? from Lipex Biomembranes. Homogenous lipid mixtures can be prepared by drying the

sample down from a chloroform solution, or by lyophilization from an organic solvent, such as benzene. We usually use the chloroform procedure when making liposomes, as follows:

1. Dissolve lipid mixture of the appropriate composition in chloroform (~1 mL per 50-100 μmole of lipid) in a glass tube. In some cases it may be necessary to add a minimum amount of methanol to dissolve all of the lipid.
2. Dry the lipid down to a thin film on the tube surface using a stream of nitrogen gas. It is advisable to use a reservoir of warm water to heat the solution during this process. This facilitates evaporation of the solvent and minimizes the possibility of lipid crystallizing or precipitating from the solution. The process should be repeated if there is any evidence that this has happened. It is important to produce as thin a film as possible since large lumps can be extremely difficult to hydrate.
3. Lightly cap the tube with tissue paper and dry the lipid overnight on a lyophilizer. Some foaming may occur because of residual solvent in the lipid film.
4. Add buffer to the tube and vortex until the lipid has fully dispersed. It may be necessary to warm the sample in a waterbath set at above the phase transition temperature of the primary lipid to facilitate this.
5. Transfer the suspension to a cryovial.
6. Freeze the suspension in liquid nitrogen for 5 min and then thaw it for 5 min in a water bath set above the phase transition temperature of the dominant lipid.
7. Repeat **step** 6 four more times.
8. Pass the suspension though a stacked pair of polycarbonate 100-nm filters (Nuclepore) using an extruder. The suspension should be allowed to equilibrate for 5 min inside the extruder before pressure is applied in cases where the extrusion is performed above room temperature.
9. Repeat **step 8** nine more times. Equilibration is not usually required after the first pass.
10. Examine the filters when dismantling the extruder. Any sign of solids or gels indicates that some of the lipid has phase separated and consequently the lipid composition may have changed.
11. Analyze the lipid content using an incorporated label or by using a phosphate assay.
12. Dilute the lipid to the desired concentration with buffer.

3.2. Amine Modification

Modification of the protein amine groups is the procedure most frequently used to produce antibody-liposome conjugates. Early procedures used crosslinking agents, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) *(27,28)* in the presence of preformed liposomes containing a lipophilic carboxylic acid. Condensing agents like these tend to form protein-protein polymers. Control of these reactions is typically difficult and complex, and as a result separation of the liposomes from protein polymers is a

major problem. Other early approaches involved direct modification of antibodies with activated fatty acids, such as the *N*-hydroxysuccinimide (NHS) ester of palmitic acid, prior to incorporation into a liposome membrane, typically by detergent dialysis procedures (29). Reagents, such as EDC, have been used in conjunction with NHS to activate acidic functions on liposomes, which were then conjugated to the amino groups on antibodies (30). Better control of the conjugation reaction can been achieved using hetereobifunctional crosslinkers, which efficiently introduce a unique and selective reactive function, such as a protected thiol or maleimide group. Examples of these crosslinkers are SPDP *(31)*, S-acetylthioglycolic acid *N*-hydroxysuccinimide ester (SATA) *(32,33)* and 4-(p-maleimidophenyl)butyric acid *N*-hydroxy–succinimide ester (SMPB) (34). Antibodies which have been activated by these crosslinkers can, after deprotection where appropriate, react with activated lipids in liposome bilayers. Maleimide and protected thiol-derivatized lipids are available from commercial sources for this purpose.

Deprotection of 3-pyridyl disulfides is usually effected by DTT **(Fig. 2C)**, and occasionally by some other mercaptan. Particular care should be exercised when removing these reagents after the reaction since they could potentially interfere in subsequent coupling reactions. Mercaptan-based deprotection reagents also damage the disulfide bonds in antibodies. This damage can be minimized by performing the deprotection at low pH (35) with short reaction times (36). Thioacetate-protected crosslinkers have been used less frequently. These reagents can be deprotected with hydroxylamine buffers **(Fig. 2B)** under conditions that do not damage the disulfide bonds. In theory this should allow better control of the thiolation process and simplify some of the workup steps.

Once deprotected, sulfhydryl groups can react with maleimide (for example, SMPB-modified conjugates) or iodo (for example, iodoacetic acid *N*-hydroxysuccinimide ester (SIAA)-modified conjugates) groups. Maleimide groups are recommended since iodo functions can react with amino groups in either of the substrates, leading to undesirable side products. Deprotection is not required for these reagents.

Liposomes containing excess maleimide or thiol groups after the conjugation reaction may exhibit undesirable qualities, such as aggregation, reactions in vitro and in vivo, and immunogenicity. In general, these aspects are not well studied and the effects of these groups on the liposome surface cannot therefore be predicted. It is possible to quench these reactive functions with reagents containing iodo, maleimide, or sulfhydryl groups where appropriate. This is likely to be a particularly serious problem for thiolated liposomes, consequently we recommend that the antibody be thiolated and that a maleimide modified lipid be incorporated into the liposomes in order to generate the appropriate reactive entities for the final conjugation reaction.

3.2.1. SPDP-Coupling Protocol

The most widely used procedure for generating protein-liposome conjugates involves modification of the protein with SPDP, followed by deprotection with DTT and conjugation to SMPB-derivatized liposomes. We use the following protocol to generate such conjugates using IgG (see **Note** *2).*

1. Prepare liposomes incorporating 1% *N*-(4-(p-maleidophenyl)butyryl)-1,2-*sn*-distearoylphosphatidylethanolamine (MPB-DSPE) as described above. This should be done not more than 1 d prior to use.
2. Prepare a solution of IgG (10–20 mg/mL) in PBS or HBS at pH 7.4.
3. Prepare a 1 m*M* solution of SPDP in HBS by diluting 80 µL of an ethanol stock solution (3.9 mg SPDP/mL ethanol) with 920 µL HBS.
4. Add an aliquot of the SPDP (5 mol equivalents) solution to the IgG solution. Stir at room temperature for 20 min.
5. Pass the solution down a Sephadex G-50 column (10 mL gel/mL of solution) equilibrated in SAS (pH 4.4). Collect and combine fractions with an absorbance >1.O at 280 nm.
6. Add DTT (3.8 mg/mL of solution) and stir at room temperature for 20 min.
7. Pass the solution down a Sephadex G-50 column (10 mL gel/mL of solution) equilibrated in HBS (pH 7.4). Collect and combine fractions with an absorbance >1.0 at 280 nm. In cases where the yield is low it may be necessary to collect fractions with lower absorbances as well.
8. Determine an approximate IgG concentration from the absorbance at 280 nm (mg/mL = A_{280}/1.35).
9. Add an aliquot of the IgG solution to an aliquot of the liposome solution (75 µg protein per µmol of lipid). Stir at room temperature for 16 h.
10. Pass the mixture down a Sepharose CL-4B column (10 mL gel/mL solution). Fractions containing liposomes are easily detected against a dark background because of the turbidity of the solution.
11. Determine the coupling efficiency using protein and lipid assays.
12. Determine the size of the conjugates using a particle sizer, if one is available.

3.3. Carbohydrate Modification

Oxidation of the carbohydrate functions on antibodies with sodium periodate generates aldehyde groups that can be used to conjugate the proteins to liposomes. This approach is attractive since it is known that procedures that derivatize antibody amino groups or disulfide bonds may damage some of the antigen-binding sites, whereas modification of the carbohydrate functions do not (37). Glycosylation on antibodies occurs at various points on the structure depending on the antibody class. Most applications are likely to use IgG-type antibodies, which are glycosylated in the CH_2 region of the heavy chain. Modification of these functions therefore does not directly affect antigen binding, although it is known to affect the structure of the Fc chains and various effector functions of IgG, such as complement activation and Fc receptor binding.

Very few reports have been made using this approach to synthesize antibody-liposome conjugates. Early work (38) showed that simple acyl hydrazides in liposomes could be used to conjugate periodate-oxidized IgM in good yield. More recently, a lipid-PEG-hydrazide derivative has been reported that was used to couple oxidized IgG to liposomes *(34)*, albeit in low yield. Subsequent work has reported optimized conditions that resolve these problems and minimize liposome-liposome crosslinking *(10)*. We have recently reported a variation of these procedures in which a protected thiol-hydrazide crosslinker was used to thiolate oxidized IgG and subsequently to effect coupling to maleimide derivatized liposomes (36).

3.3.1. PDPH Coupling Protocol

The following is the procedure that we have developed to thiolate IgG through modification of the carbohydrate functions. The procedure is very similar to the SPDP protocol after the initial thiolation process (see **Note** 3).

1. Prepare liposomes incorporating 1% MPB-DSPE as described above. This should be done not more than 1 d prior to use.
2. Prepare a solution of IgG (10–20 mg/mL) in PBS or HBS at pH 7.4.
3. Prepare a 0.1 *M* suspension of 3-(2-pyridyldithio)propionic acid hydrazide (PDPH) in ethanol.
4. Dissolve sodium metaperiodate (1 mg/mL of final volume) in 0.3 mL distilled water.
5. Immediately add the antibody solution to the sodium periodate solution and stir at room temperature for 1 h.
6. Pass the solution down a Sephadex G-50 column (10 mL gel/mL of solution) equilibrated in SAS (pH 4.4). Collect and combine fractions with an absorbance >1.O at 280 nm.
7. Add an aliquot of the PDPH suspension (vortex immediately prior to taking the aliquot)(40 µL PDPH stock per milliliter of IgG solution). Stir at room temperature for 5 h.
8. Pass the solution down a Sephadex G-50 column (10 mL gel/mL of solution) equilibrated in SAS (pH 4.4). Collect and combine fractions with an absorbance >1.O at 280 nm.
9. Add DTT (3.8 mg/mL of solution) and centrifuge at 3000 rpm at room temperature for 20 min.
10. Pass the supernatant down a Sephadex G-50 column (10 mL gel/mL of solution) equilibrated in HBS (pH 7.4). Collect and combine fractions with an absorbance >1.0 at 280 nm. In cases where the yield is low it may be necessary to collect fractions with lower absorbances as well.
11. Determine an approximate IgG concentration from the absorbance at 280 nm (mg/mL = A_{280}/1.35).
12. Add an aliquot of the IgG solution to an aliquot of the liposome solution (75 µg protein per µmol of lipid). Stir the reaction mixture at room temperature for 16 h.

13. Pass the mixture down a Sepharose CL-4B column (10 mL gel/mL solution). Fractions containing liposomes are easily detected against a dark background because of the turbidity of the solution.
14. Determine the coupling efficiency using protein and lipid assays.
15. Determine the size of the conjugates using a particle sizer, if one is available.

3.4. Disulfide Modification

Treatment of antibodies, or abbreviated antibodies, such as Fab or $F(ab')_2$ fragments, with reducing agents, such as DTT or 2-mercaptoethylamine, cleaves disulfide bonds in the antibody. These sulfhydryl groups have been used to conjugate the antibodies to maleimide-derivatized liposomes ***(39-41).*** However, it is important to remember that some of the disulfide bonds are responsible for maintaining the structure of the variable region and damage to these by reducing agents may result in some loss of binding activity.

3.5. Indirect Methods

Liposomes may be targeted indirectly using antibodies if a secondary receptor-ligand system is used. For example, liposome–avidin conjugates have been shown to effectively target biotinylated antibodies ***(42,43)*** that have previously been bound to antigens on cells. A similar approach has frequently been used with protein A/G-liposome conjugates targeted to the Fc chain of antibodies ***(44,45)***. The latter approach is not suitable for in vivo applications because of competition from the general IgG population. These systems can be used to generate liposome-antibody conjugates before targeting ***(46)***, although these are likely to be more complex than direct conjugation procedures.

3.6. Analytical Protocols

Reliable lipid and protein analysis of the prepared antibody-liposome conjugate is essential for proper characterization and subsequent interpretation of results obtained in the application of the conjugates. In addition, we strongly advise that the liposome conjugate size be determined with a particle sizer, if one is available, since liposome size plays such an important role in the pharmacokinetics of the system in vivo.

3.6.1. Lipid Analysis

Lipid analysis is typically performed either by incorporating a nonexchangeable radiolabel marker, such as (^{14}C) or (^{3}H) cholesteryl hexadecyl ether (CHE), into the vesicle membrane, or by analyzing the phosphate content and extrapolating the result according to the original composition of the vesicles. Both approaches assume that the label concentration and vesicle composition do not change on vesicle preparation or subsequent manipulation. The phosphate assay is carried out as follows:

1. All glassware used in the assay should be washed with phosphate-free detergents.
2. Prepare a molybdate solution by dissolving 4.4 g of ammonium molybdate in 2000 mL of distilled water and 40 mL sulfuric acid.
3. Prepare Fiske reagent as follows: Dissolve 150 g sodium bisulfite and 5 g sodium sulfite in 1 L of distilled water. Add 2.5 g 1-amino-2-naphthol-4-sulfonic acid and warm to 40°C until the material has dissolved (keep the solution covered during this process). Allow to stand at room temperature overnight in the dark. Filter to remove the crystalline material formed and store in a dark bottle.
4. Prepare a 2 m*M* sodium phosphate standard solution.
5. Add aliquots (0, 25, 50, 100 µL) of the standard to 16 x 150-mm Pyrex test tubes.
6. Add aliquots in triplicate of the sample to be tested to 16 x 150-mm Pyrex test tubes. The aliquots should contain 100-200 nmol of phosphate. It may be necessary to dilute some of the sample prior to the assay.
7. Add 0.7 mL of perchloric acid to each tube.
8. Close the tube by placing a marble on the top. Digest the sample on heating blocks set at 180°C for 1 h **(Caution!** Hot perchloric acid is highly corrosive and potentially explosive. This procedure should be carried out in a suitable fumehood behind a blast shield).
9. Allow the tubes to cool. Add 7 mL of the molybdate solution followed by 0.7 mL of the Fiske reagent. Vortex to thoroughly mix the solutions.
10. Heat the tubes in a boiling water bath for 30 min. Cool to room temperature.
11. Assay the samples at 815 nm in a spectrometer using a standard curve (0, 50, 100, 200 nmol).

3.6.2. Sulfhydryl Analysis

Sulfhydryl content can be determined directly by using any thiol assay, typically by using Ellman's reagent, although other more sensitive procedures exist *(47)*, or indirectly by release of some reporter molecule on deprotection of crosslinkers. For example, many reports use the absorbance of 2-thiopyridone at 343 nm after treatment of pyridyldithio-modified proteins with DTT. Extreme caution should be used when interpreting results from these indirect methods since in our experience treatment of antibodies with DTT, even under mild conditions, results in subtle changes in the baseline absorbance at 343 nm and consequent erroneous estimates of thiol content.

1. Add 60 µL Ellman's reagent (4 mg/mL solution in HBS) to 600 µL aliquots of a control and the samples.
2. Allow to stand at room temperature for 20 min.
3. Measure the absorbance at 280 nm in a spectrophotometer using the control to zero the instrument.
4. Determine the thiol content using the formula SH = 1.1 $A_{412}/13{,}600\,C_p$, where A_{412} is the absorbance at 412 nm, C_p is the protein concentration, and SH is the number of thiol equivalents.

3.6.3. Protein Analysis

Protein is assayed using a modification of the protocol for the micro BCA protein assay kit from Pierce. Pierce claims that some lipids may interfere with the assay; therefore, it is advisable to periodically assay control liposomes to ensure that the protein assay is returning reliable results. This is particularly important when using new lipid formulations or buffers. Interference from lipids should not exceed 1 μg protein/μmol lipid.

1. Prepare a set of bovine serum albumin (BSA) standards containing 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 μg of protein in 5 mL tubes.
2. Add aliquots of the sample containing 0.333 μmol of lipid to 5-mL tubes in triplicate (if there is enough material).
3. Add 100 μL of 5% Triton X-100 solution to the standards and samples.
4. Dilute the standards and samples with distilled water to a final volume of 1 .O mL.
5. Prepare the assay reagent according to the instructions in the Pierce micro BCA assay kit.
6. Add 1 .O mL of the assay reagent to the standards and samples. Cap the tubes and vortex to ensure complete mixing.
7. Incubate the tubes in a water bath at 60°C for 1 h.
8. Cool the tubes to room temperature.
9. Determine the protein content of the samples using a standard curve obtained at 562 nm.

3.7. Application of Steric Barrier Molecules

A major problem in the preparation of protein-liposome conjugates is the tendency of the conjugates to crosslink, resulting in the formation of large aggregates that are cleared rapidly in vivo. The crosslinking effect is intrinsic to all reactions involving multivalent ligands and can be controlled by reducing the number of reactive functions on the liposome or antibody (for example, reducing the degree of thiolation when using SPDP, or by reducing the initial protein/lipid ratio). These approaches, however, generally require extensive optimization and result in lower coupling efficiencies. We have recently developed an alternative approach to this problem *(48)*, where we use PEG-lipids to control the crosslinking reactions. PEG-lipids were originally developed to reduce liposome clearance in circulation *(49,50)*, an effect that is achieved by reducing the absorption of serum proteins onto liposome surfaces. These proteins are believed in part to mediate clearance of the liposomes by the reticuloendothelial system *(51,52)*. Access to the liposome surface in the presence of PEG-lipids is strongly affected by size; consequently, single antibodies may penetrate the polymer cloud and react at the surface but the much larger antibody-PEGylated liposome conjugates will not, because of steric interactions between the PEG-lipids incorporated into the membranes. Small size increases are typically observed when coupling

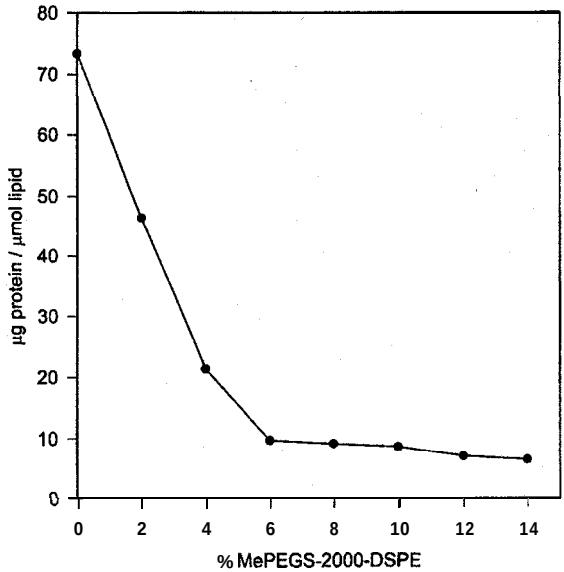

Fig. 3. The coupling efficiency of SPDP-modified human IgG to DSPC/Chol/MPB-DSPE/*N*-(2'-(ω-monomethoxypolyethyleneglycol$_{2000}$)succinoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (MePEGS-2000-DSPE) (54-n:45: 1 :n) liposomes. The initial protein-to-lipid ratio was 150 µg protein/µmol lipid.

antibodies to PEGylated liposomes. In a typical case we see 100 nm vesicles increasing to ~120 nm in the presence of PEG. Similar 100 run vesicles without PEG-lipids form aggregates with measured average diameters of 160 nm or larger.

Although this technique is a powerful method for preventing aggregation, it does have its drawbacks. First, since PEG-lipids reduce total protein binding at equilibration in vivo because of steric inhibition, they will also limit the amount of antibody that can be conjugated to the liposomes. The coupling efficiency is reduced in a manner dependent on PEG-lipid concentration and polymer size, typically reaching a base level at about 6% PEG-lipid (based on a PEG molecular weight of 2000) with 100 nm vesicles. The effect of PEG-lipid concentration is illustrated in **Fig. 3.** A second problem with the use of PEG-lipids in antibody targeting systems is the tendency of the polymer to act as a steric barrier, which inhibits interaction with the cellular target sites (53). Again, this is dependent on PEG-lipid concentration, with binding levels reaching control levels at 6% PEG-lipid.

We typically use 2% PEG-lipids (PEG molecular weight 2000) in our antibody-conjugated formulations in order to control aggregation but still retain reasonable binding and coupling efficiency.

More recently, PEG-lipids have been used as tethers between liposomes and antibodies ***(10,30,34)*** (see **Fig. 4).** The basic idea behind this approach has

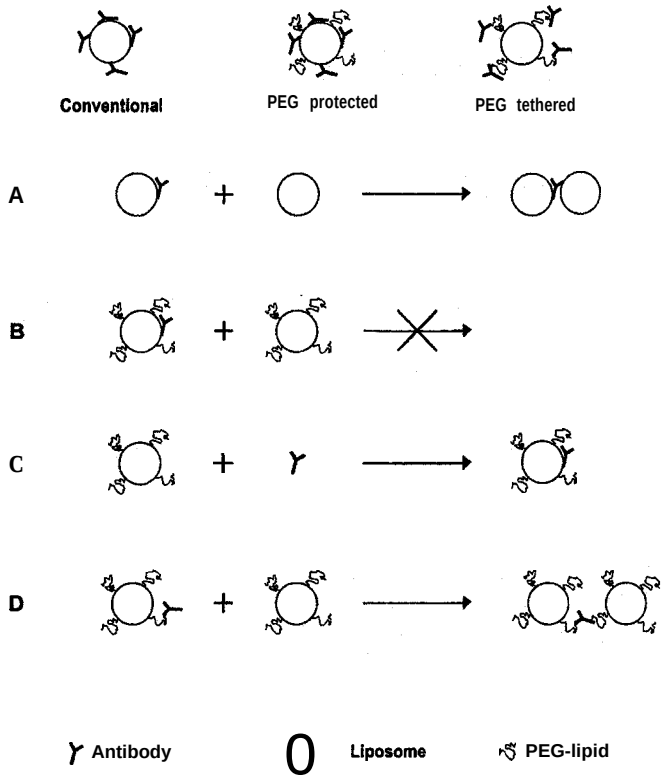

Fig. 4. Aggregation reactions associated with different classes of antibody-liposome conjugates. (A) Antibody-liposome conjugates may react further with other liposomes to form aggregates. **(B)** The presence of PEG-lipids prevents these crosslinking reactions through steric hindrance. (C) Individual proteins may penetrate the PEG cloud to react with the liposome surface. (D) Antibodies tethered on the distal end of PEG may react with the distal ends of PEG molecules on a second liposome, resulting in crosslinking.

been to remove the targeting vector from the surface to allow free access to binding sites but at the same time to retain the protection from serum proteins afforded by PEG lipids. Systems of this type are likely to suffer from the same crosslinking reactions as non-PEGylated systems, although not as severely since some steric inhibition will be effected by the PEG tether. Careful optimization has been shown in at least one case to minimize these problems ***(10)***.

4. Notes

1. Numerous techniques for the preparation of liposomes have been described. Typical procedures involve the hydration of lipid mixtures in buffer, resulting in the formation of large multilamellar vesicles (MLV). These are of limited use in

active targeting applications, particularly in vivo, because a strong correlation between size and clearance by the reticuloendothelial system exists. Larger vesicles tend to be cleared more rapidly than small vesicles. Unilamellar vesicles are prepared from MLVs in a number of ways. Sonication of MLVs results in the smallest thermodynamically stable vesicles, typically about 25 nm in size (22). These vesicles tend to be unstable and may not retain their contents, which is a significant problem when developing a drug-delivery system. Large unilamellar vesicles (LUV) can be prepared by extrusion of MLVs through sizing filters (23). Extrusion techniques result in narrow size distributions, which are determined primarily by the particular pore size of the filter used. Optimal formulation and clearance characteristics in vivo are typically observed with 100 nm vesicles, which are most conveniently prepared by extrusion methods. Other methods for liposome preparation include reverse-phase *(24)* and detergent dialysis (25) techniques.

Several important considerations should be addressed when coupling antibodies to liposomes. Activated lipids that are incorporated into liposomes often have limited stability and consequently the liposomes should be used as soon as possible after preparation. Suitable care should also be taken when selecting buffers, pH, and temperature to ensure compatibility with the lipids being used. Most applications of targeted systems are likely to involve liposomes that encapsulate some drug. In these situations it is important to select conditions that do not facilitate leakage or degradation of the drug. A commonly used method of drug encapsulation involves active loading of liposomes using pH gradients *(26)*, typically with a low pH inside and neutral conditions outside the vesicle. Once drugs have been loaded in this manner it is important to maintain the pH gradient; consequently, the coupling reaction should be carried out before or after loading depending on the pH required for coupling.

2. It should be noted that in our experience the liposome conjugation efficiency varies for different MAbs; therefore, it is necessary to perform preliminary experiments to determine optimal initial antibody/lipid ratios. Other factors affecting conjugation efficiency include maleimide concentration, degree of thiolation, presence of PEG-lipids, and initial reagent concentration.
3. It is important to note that oxidation of IgG makes the protein more prone to aggregation and adhesion to surfaces. It is essential that the modified IgG be centrifuged prior to liposome coupling to ensure removal of aggregated IgG since this material is extremely difficult to remove after coupling and will interfere with assays used to characterize the final conjugate. Further, it appears important that the protein not be subjected to concentration procedures after the oxidation step since these often lead to the loss of large quantities of the antibody. The procedure is best suited for solutions with concentrations in the range 10-20 mg/mL, preferably 15 mg/mL, since this allows complete processing without the intervention of concentration steps.

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