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Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures

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Abstract

Several coupling methods for binding antibodies (Ab) to liposomes have previously been developed. We were interested in examining if some of these methods would be suitable for attaching Ab to long-circulating formulations of liposomes (SL), sterically stabilized with poly(ethylene glycol) (PEG). We studied three 'classical' coupling methods in which Ab was attached at the bilayer surface of SL, and two new coupling methods in which Ab was attached at the PEG terminus. Parameters examined included binding efficiency, antibody surface density, the ability of the immunoliposomes to remote-load the anticancer drug doxorubicin, and the specific binding of the resulting immunoliposomes to target cells. The non-covalent biotin-avidin coupling method resulted in low Ab densities at the cell surface, as did a coupling method in which maleimide-derivatized Ab was attached to the liposome surface through a thiolated phospholipid incorporated into the liposomes. The low levels of Ab achieved in these method was likely due to interference by PEG with the access of the Ab to the liposome surface. However, when a maleimide-derivatized Ab was coupled to thiolated PEG, moving the coupling reaction away from the liposome surface, very high coupling efficiencies were achieved, and these immunoliposomes achieved good specific binding to their target cells. Oxidizing the Fc region of the Ab and coupling it to the PEG terminus through a hydrazone bond was a less efficient coupling method, but had the advantage of retaining Ab orientation. Efficient remote-loading of doxorubicin was found for immunoliposomes in which Ab was attached at the PEG terminus.

Keywords: Sterically stabilized liposome; Antibody conjugation; Poly(ethylene glycol); Drug delivery system; Targeted drug delivery

1. Introduction

The utility of liposomes as drug sustained release systems or as drug delivery systems for passive targeting is now well established, with several liposome-drug formulations in the clinic or in late clinical trials (reviewed in [1–3]). Recently, the description of long-circulating, sterically stabilized liposomes (SL) containing lipid derivatives of polyethylene glycol (PEG) [4–7] has increased the opportunities for specific, ligand-mediated, targeting of liposome-entrapped drugs to diseased tissues. The properties of sterically stabilized liposomes, as compared to 'classical' liposomes (CL), with their shorter circulation half-lives, have been widely reviewed [8–10].

One of the most effective ways to specifically target cells, e.g., neoplastic cells, is through the high affinity binding of monoclonal antibodies (mAb) to their specific

Abbreviations: MPS, mononuclear phagocyte system; SL, sterically stabilized (Stealth^R) liposomes; SIL, sterically stabilized (Stealth^R) immunoliposomes formed by attachment of Ab to SL; CL, 'classical' liposomes with short circulation half-lives and dose-dependent pharmacokinetics; CIL, immunoliposomes formed from attachment of Ab to CL; PL, phospholipid; mPEG-DSPE, methoxypolyethylene glycol of M_r 2000 covalently attached by a carbamate bond to distearoylphosphatidylethanolamine; PDP-PEG-DSPE, *N*-(3′-(pyridyldithio)propionoyl)amino-poly(ethylene glycol)-distearoylphosphatidylethanolamine; Hz-PEG-DSPE, hydrazide-poly(ethylene glycol)-distearoylphosphatidylethanolamine; MPB-DOPE, *N*-(4′-(4″-maleimidophenyl)butyroyl)-dioleoylphosphatidylethanolamine; PDP-DOPE, *N*-(3′-(pyridyldithio)-propionoyl)dioleoylphosphatidylethanolamine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)proprionate; HSPC, fully hydrogenated soy phosphatidylcholine; SMPB, H-succinimidyl-4-(*p*-maleimidophenyl)butyrate; Ab, polyclonal antibody; mAb, monoclonal antibody; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CHOL, cholesterol; DXR, doxorubicin; Hepes buffer, 25 mM Hepes, 140 mM NaCl, pH 7.4.

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antigens. Several methods for binding antibodies (Ab) to liposomes have been developed [11–18]. Some of the most efficient coupling chemistry, originally developed for CL, involved conjugation of thiolated Ab to liposomes grafted with either thiol or maleimide groups. In these methods, thiol groups were generated on the Ab either by interaction of the Ab with heterobifunctional crosslinkers such as SPDP or by reducing native disulfide bonds in the whole Ab to expose sulfhydryl groups [16–18]. In several studies, immunotargeted CL achieved an increased localization in cells in vitro and tumours in vivo [19–23]. However, it was also observed that 'classical' immunoliposomes (CIL) were very rapidly removed from circulation by the cells of the mononuclear phagocyte system (MPS) [21,22] which greatly reduced the opportunities for their specific binding to most target tissues in vivo. In order to attach Ab to SL, we have explored some established methods, concentrating on coupling methodologies involving the thioether bond, as well as developed some new coupling methodologies, in an effort to overcome these short-comings of CIL. Our aim was to develop long-circulating, sterically stabilized, immunoliposomes (SIL) which could lead to significantly increased levels of in vivo targeting and to improved therapeutic effects.

To develop a targeted drug delivery system using sterically stabilized liposomes we began by outlining what we felt to be the major criteria for 'ideal' immunoliposomes. These criteria fell into two main groups. The first was related to optimizing the conjugate chemistry. Ideally, coupling methods should be both simple and rapid, producing a stable, non-toxic bond. A wide range of Ab densities should be achievable at the liposome surface in order to optimize immunoliposome binding to their target cells, as each different Ab will have a different affinity for its antigen. During conjugation the antibody should retain antigen recognition and the liposomes should not lose their structural integrity. The second set of criteria dealt with developing sterically stabilized immunoliposomes (SIL) as drug delivery systems. Drugs, such as the antineoplastic drug doxorubicin, need to be loaded efficiently into the SIL and, once at the target site, released at rates which will result in improved therapeutic effects over that achievable for the free drug. SIL should survive in circulation for sufficient time to find and bind to their targets. Recognition of the target cells by the immunoliposomes should not be reduced significantly by the associated drugs or by the PEG coating at the liposome surface. The carrier should not be toxic to the host and the drug-loaded SIL should have in vivo therapeutic efficacy.

We have used two basic strategies for the development of SIL (Fig. 1). The first strategy was to adapt the covalent Ab conjugation techniques described for CL for use with SL. In these procedures, the previously described linker-lipids *N*-(4′-(4″-maleimidophenyl)butyroyl)dioleoylphosphatidylethanolamine (MPB-DOPE) or *N*-(3′-(pyridyldithio)propionoyldioleoylphosphatidylethanolamine (PDP-DOPE) were incorporated into liposomes, sterically stabilized with mPEG-distearoylphosphatidylethanolamine (mPEG-DSPE), and Ab was coupled to the resulting SL to form SIL. The second strategy was to develop new covalent conjugation procedures for coupling Ab to the terminus of PEG using the novel functionalized PEG-lipids, *N*-(3′-(pyridyldithio)propionoylamino-PEG-DSPE (PDP-PEG-DSPE) [23] and hydrazide-PEG-DSPE (Hz-PEG-DSPE) [24]. In addition, we have included some data comparing a non-covalent (biotin-avidin) method of attachment of Ab to SL [25] with the covalent methods. In this report we have compared the different coupling procedures, relative to a number of the criteria outlined in the previous paragraph, and have shown that the coupling procedures which most closely approaches the ideal are those in which Ab is coupled at the PEG terminus. In addition, we have demonstrated specific binding of SIL, coupled to mAb against cancer-associated antigens, to their target epitopes on neoplastic cells in vitro.

2. Materials and methods

2.1. Materials

HSPC, PDP-PEG-DSPE, Hz-PEG-DSPE and mPEG-DSPE were generous gifts of SEQUUS Pharmaceuticals (formerly Liposome Technology Inc.) (Menlo Park, CA). The synthesis of PDP-PEG-DSPE [23], Hz-PEG-DSPE [24] and methoxyPEG-DSPE (mPEG-DSPE) [6] has been previously described. PEG of molecular weight 2000 was used in all PEG-containing compounds. Cholesterol, MPB-DOPE, PDP-DOPE and biotinylated PE were purchased from Avanti Polar Lipids (Alabaster, AL). Sephadex G-50, Sephadex G-25, Sepharose CL-4B, SMPB, SPDP, avidin (egg), Mes (2-(*N*-morpholino)ethanesulfonic acid), sodium periodate, rabbit anti-bovine IgG (whole molecule) antibody, mouse anti-rabbit (whole molecule) horseradish peroxidase conjugate, *N*-hydroxysuccinimide-biotin, bovine IgG, sheep IgG and Tes (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) were purchased from Sigma (St. Louis, MO). Doxorubicin (DXR, Adriamycin RDF) was obtained from Adria Laboratories (Mississauga, Ontario). $Na^{125}I$ and ACS scintillation fluid was purchased from Amersham (Oakville, Ontario). [1α,2α(n)-^{3}H]Cholesteryl hexadecyl ether, 1.48–2.22 TBq/mmol (^{3}H-CHE) was purchased from New England Nuclear (Mississauga, Ontario). Fetal bovine serum was purchased from Gibco BRL (Burlington, Ontario). Centricon 100 (M_r cut-off 100000) concentrators were purchased from Amicon (Beverly, MA). Falcon 6-well and Corning 96-well plates were purchased from Becton Dickinson (Lincoln Park, NJ), and Corning (Corning, NY), respectively. Bio-Rad protein microassay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The cell line KLN 205 (murine squamous lung carcinoma) was

a generous gift of Biomira, Edmonton, Alberta. The mammalian squamous carcinoma mAb 174H.64 [26] (IgG_1, recognizing KLN 205), and the pan adenocarcinoma mAb 170H.82 [27] (IgG_1, recognizing HCT-15) were generous gifts of Biomira. The human colon adenocarcinoma cell line HCT-15 (also binding with mAb 170H.82) was purchased from American Type Tissue Culture (Rockville, MD). All other chemicals were analytical grade.

2.2. Preparation of antibodies

Monoclonal antibody solutions were concentrated and the storage buffer exchanged with 25 mM Hepes, 140 mM NaCl, pH 7.4 (Hepes buffer), using a Centricon 100 concentrator (Amicon, USA) by centrifugation at 3000 rpm in a Sorvall S34 rotor. Protein content was determined for the concentrated solution using the Bio-Rad protein microassay kit.

For preparation of iodinated Ab, solutions (10 mg/ml) of sheep IgG, bovine IgG, 174H.64 mAb or 170H.82 mAb were prepared in Hepes buffer. The Ab solution (200–300 μl) was then mixed with 185 MBq of $Na^{125}I$ in a 2 ml reaction vial with 7 Iodo-Beads (Pierce, Rockford, IL) at 22°C for 1 h. The resulting ^{125}I-Ab was desalted by gel filtration over Sephadex G-25 in the above buffer.

Thiolated Ab was prepared from an Ab solution (10 mg/ml) in Hepes buffer containing a trace amount of ^{125}I-Ab. PDP-Ab was formed by adding SPDP (25 mM in 95% ethanol) slowly to the antibody solution at a SPDP/Ab molar ratio of 10:1, and the mixture was then incubated for 30 min at 22°C. Unbound SPDP was removed, and the pH was lowered, by passing the Ab mixture over a Sephadex G-50 column eluted with 100 mM sodium acetate, 100 mM NaCl, pH 4.7. The PDP group on the antibody was then reduced (forming a thiol group) by adding DTT at a final concentration of 20 mM DTT. DTT was removed by passing the thiolated Ab over a Sephadex G-50 column with 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7.

The extent of PDP substitution on the PDP-Ab was determined by measuring the release of 2-thiopyridone ($\varepsilon_{343} = 8300\ M^{-1}\ cm^{-1}$) at OD 343 nm after reduction of the PDP-Ab with 20 mM DTT.

Maleimide-Ab (MPB-Ab) was prepared by adding SMPB (25 mM in dimethylformamide) slowly to an antibody solution (10 mg/ml in Hepes buffer) at a molar ratio of 20:1, SMPB/Ab, then incubated for 1–2 h at 22°C. Unbound SMPB was removed by passing the MPB-Ab over a Sephadex G-50 column and eluted with 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7.

The number of maleimide residues was determined indirectly by assaying for the binding to MPB-Ab of a thio-containing fluorescent probe, SAMSA fluorescein (5-((2-(and 3-)-*S*-(acetylmercapto)succinoyl)amino)fluorescein, λ_{ex}495 nm and λ_{em}520 nm, Molecular Probes, Eugene, OR). SAMSA fluorescein binds to maleimide residues in a 1:1 molar ratio. SAMSA fluorescein (2 mg) was dissolved in 200 μl of 0.1 M NaOH. After 15 min at 22°C the solution was neutralized with 2 μl of 6 M HCl and 40 μl of 0.5 M $NaPO_4$, pH 7.0, buffer. The activated probe was then incubated at a 10-times molar excess with MPB-Ab for 2 h at 22°C. Unbound SAMSA fluorescein was removed by using a Sephadex G-25 column equilibrated with Hepes buffer. The degree of SAMSA fluorescein substitution of the labelled Ab was determined by measuring the absorbance at 495 nm on a Titertek Multiskan PLUS MK II plate reader (Flow Laboratories, Mississauga, Ontario, Canada) and interpolating from a SAMSA fluorescein standard curve or calculating from the molar extinction coefficient of 80 000 $cm^{-1}\ M^{-1}$ at 495 nm (the molar extinction coefficient of SAMSA fluorescein did not appear to change significantly in the presence of bound Ab).

Oxidized Ab was prepared by a modification of the procedure of Chua et al. [15] by oxidizing the carbohydrates on the Fc portion of the antibodies through addition of sodium periodate (0.2 M in distilled H_2O) to the Ab solution (10 mg/ml in 123 mM sodium citrate, pH 5.5) to give a final periodate concentration of 10 mM. After 1 h at 22°C, periodate was quenched with 50 mM *N*-acetylmethionine (0.5 M in distilled H_2O).

Biotinylated Ab was prepared by incubating *N*-hydroxysuccinimide-biotin (20 mg/ml in dimethylsulfoxide) with the Ab (5 mg/ml) at a ratio of 5:1 (w/w) at room temperature for 4 h. Unbound material was separated by gel filtration over a Sephadex G-25 column using 10 mM Tes, 150 mM NaCl, pH 7.4 buffer.

2.3. Liposome preparation

Liposomes were composed of HSPC/CHOL (2:1 molar ratio) with or without various amounts of mPEG-DSPE and with or without the following linker lipids: PDP-PEG-DSPE, Hz-PEG-DSPE, PDP-DOPE, MPB-DOPE or biotinylated PE. In some experiments trace amounts of ^{3}H-CHE were added as a lipid label. Liposomes were prepared by hydrating dried lipid films in an appropriate buffer at a phospholipid concentration of 10 mM. Using a Lipex extruder (Lipex Biomembranes, North Vancouver, BC), the liposomes were passed through 0.1 μm polycarbonate membranes (Nuclepore, Pleasanton, CA or Poretics, Livermore, CA) to give primarily unilamellar vesicles of approx. 100 nm in diameter [28,29]. The average size of the liposomes were measured by dynamic light scattering using a Brookhaven BI90 submicron particle sizer (Brookhaven Instruments, Holtsville, NY).

2.4. Doxorubicin loading and leakage

DXR was loaded into liposomes via an ammonium sulfate gradient [30]. Briefly, liposomes were hydrated at

10 mM phospholipid in 155 mM ammonium sulfate, pH 5.5. The external buffer was exchanged by passing the liposomes over a Sephadex G-50 column and eluting with 123 mM sodium citrate, pH 5.5. DXR was then incubated with the liposomes (0.2 mg DXR per mg phospholipid) for 1 h at 65°C. Any unloaded DXR was separated over Sephadex G-50 in the appropriate buffer and the amount of DXR was determined from its absorbance at 492 nm. Leakage of DXR from the liposomes was measured by dialysing the DXR liposomes in 25% human plasma against a large volume of 25% human plasma at 37°C, sampling the contents of the dialysis bag at increasing time intervals and determining the absorbance as above.

2.5. Antibody conjugation

Liposomes (HSPC/CHOL, 100 nm diameter), containing 0.1 mol% biotin-DOPE, were hydrated in 10 mM Tes, 150 mM NaCl, pH 7.4 buffer and incubated with avidin at a biotin-DOPE to avidin molar ratio of 1:1 for 30 min at 22°C (Fig. 1A). Unbound avidin was removed by gel filtration using Sepharose CL-4B and the above buffer. The avidin-liposomes were then mixed for 30 min at 22°C with biotin-Ab at a 1:1000 (biotin-Ab/PL) molar ratio.

Liposomes (HSPC/CHOL, 100 nm diameter) containing either PDP-DOPE (Fig. 1C) or PDP-PEG-DSPE (Fig. 1E) were prepared in either Hepes buffer or 155 mM ammonium sulfate (pH 5.5) when DXR was to be loaded into the liposomes. The PDP groups were reduced to form free thiols by adding DTT to the liposomes to give a final concentration of 20 mM DTT and the preparations were incubated for 30 min at room temperature. DTT was separated by passing the liposomes over a Sephadex G-50 column and eluting with 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7. The thiolated liposomes were then incubated overnight at 22°C with MPB-Ab.

Liposomes (HSPC/CHOL, 100 nm diameter) containing MPB-DOPE (Fig. 1B) or Hz-PEG-DSPE (Fig. 1D) were prepared in 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7 buffer or 123 mM sodium citrate, pH 5.5 buffer, respectively. For DXR loading experiments, these liposomes were prepared with 155 mM ammonium sulfate (pH 5.5). MPB-DOPE or Hz-PEG-PE liposomes were mixed with thiolated-Ab or oxidized-Ab respectively, and incubated overnight at 22°C.

In each case, unbound antibody was removed by passing the Ab-liposome mixture over a Sepharose CL-4B column in Hepes buffer. Conjugation results were expressed as μg bound Ab per μmol phospholipid as routinely determined by assaying for ^{3}H-CHE (lipid) and ^{125}I-Ab. In some instances phospholipid assay was also performed according to method of Bartlett [31]. Protein concentration in solutions of free Ab, prior to conjugation, was in some instances determined by the Bio-Rad protein assay.

2.6. ELISA (antibody capture assay)

Bovine IgG (antigen) was plated on 96 well plates at 500 ng per well (100 μl, 5 μg IgG/ml in Hepes buffer) and was allowed to stand overnight. After washing three times with Hepes buffer, excess binding sites were saturated with 200 μl of 0.8% gelatin in distilled H_2O for 2 h at 22°C. Rabbit anti-bovine IgG (primary antibody) was modified with SPDP, SMPB or periodate as described above. In a separate experiment, oxidized antibody was incubated at various concentrations with hydrazide liposomes (HSPC/CHOL/Hz-PEG-DSPE, 2:1:0.1 molar ratio) to achieve a range of Ab densities at the liposome surface. The plates were washed five times with Hepes buffer and the PDP-Ab, MPB-Ab, oxidized Ab or Ab-liposomes were plated at 1.25–40 ng per well or 0.025–0.8 mM phospholipid per well for 2 h at 22°C. After washing the plates, mouse anti-rabbit horse radish peroxidase conjugate was added to each well for 2 h at 22°C. The plates were then washed as above, and colour was developed by adding either the substrate TMB (3,3′,5,5′-tetramethylbenzidine, Gibco BRL, Gaithersburg, MD) or ABTS (2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid, KPL, Gaithersburg, MD). The plates were read after 10–30 min at OD 650 nm (TMB) or OD 405 nm (ABTS) on a Titertek Multiskan PLUS MK II plate reader (Flow Laboratories, Mississauga, Ontario).

2.7. In vitro cell binding studies

Binding of immunoliposomes to cultured cells was compared for three different coupling procedures (MPB-DOPE, Hz-PEG-DSPE and PDP-PEG-DSPE). Murine squamous lung carcinoma (KLN 205) and human colon adenocarcinoma (HCT-15) cells were grown as monolayers in RPMI 1640 media. Media was supplemented with 10% fetal bovine serum, and cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. On day 1, cells were plated in triplicate into 6-well plates at $(1.5–2) \cdot 10^5$ cells/well. SIL were prepared as above by conjugating mAb either to the PEG terminus (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE, 2:1:0.08:0.02 or HSPC/CHOL/Hz-PEG-DSPE, 2:1:0.1 molar ratio), or to the maleimide group on the surface of SL (HSPC/CHOL/mPEG-DSPE/MPB-DOPE, 2:1:0.1:0.02). The mAb were specific to each cell line, i.e., mAb 174H.64 coupled to SL (SIL[174H.64]) for experiments with the KLN 205 cell line, or 170H.82 coupled to SL (SIL[170H.82]) for experiments involving the HCT-15 cell line. On the fourth day SIL[174H.64] or SIL[170H.82] (0.1–0.4 μmol PL/ml Hepes buffer), labelled with ^{3}H-CHE, were added to each well of the appropriate cell lines. After 1 h incubation at 37°C, cells were washed three times with phosphate-buffered saline, pH 7.4, trypsinized with 0.5 ml of 0.05% trypsin, and the levels of bound ^{3}H-CHE-labelled liposomes were determined by placing

the cells in ACS scintillation fluid, and counting in a Beckman LS-6800 counter. Competition for binding to KLN 205 cells between free mAb and liposome-coupled specific mAb or isotype-matched non-specific antibodies (NSAb) (B27.29, an murine IgG_1 from Biomira), coupled via the PDP-PEG-DSPE method, was determined by addition of 20-fold excess of free Ab 10 min before adding ^{3}H-CHE-labelled SIL.

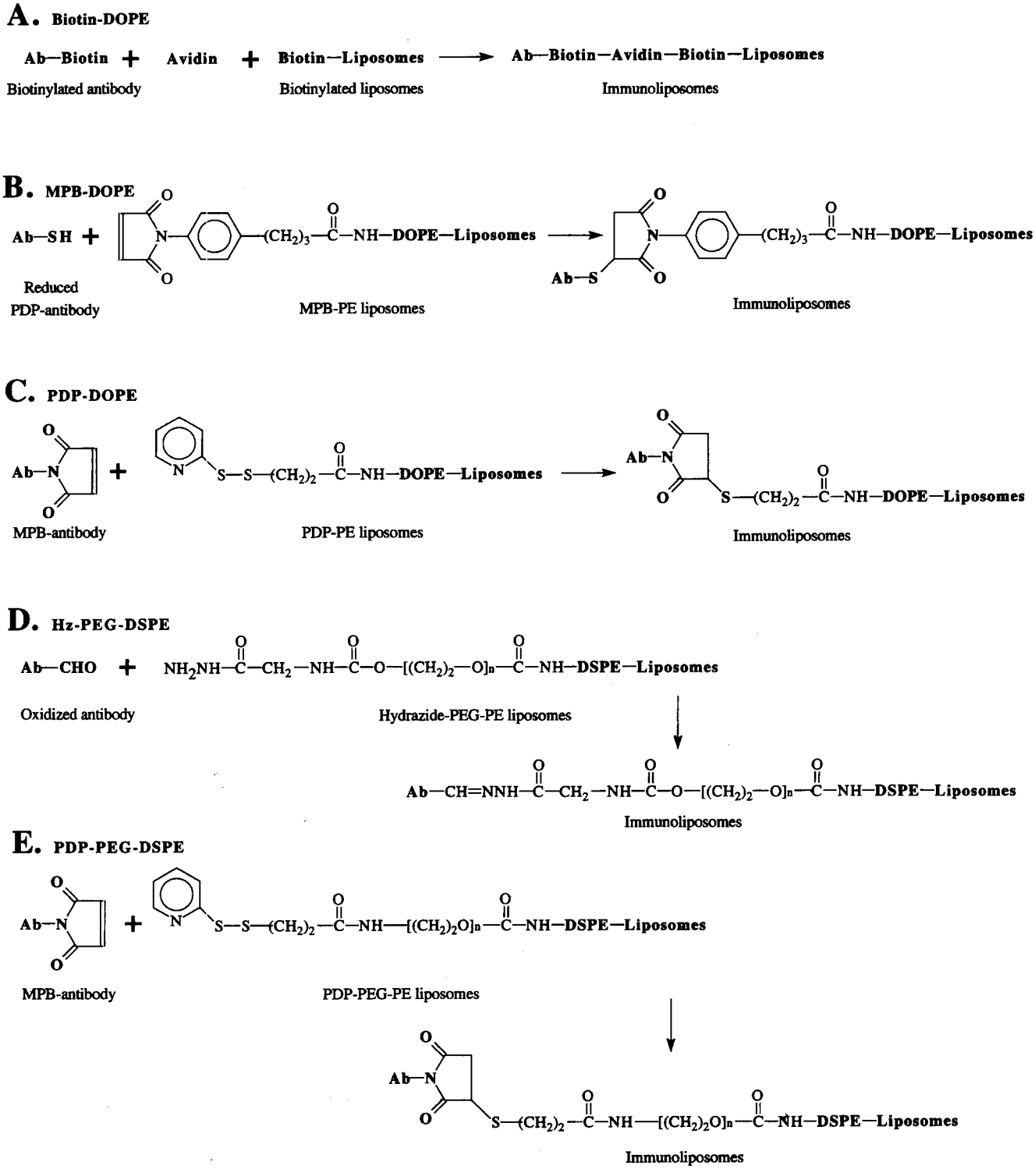

Fig. 1. Schematic diagram of the different coupling methods between SL and Ab. (A) biotin-avidin, (B) MBP-DOPE, (C) PDP-DOPE, (D) Hz-PEG-DSPE, (E) PDP-PEG-DSPE.

3. Results

3.1. Coupling antibodies to sterically stabilized liposomes: comparison of the different methods

The basic reaction schemes for the four covalent coupling methods and the non-covalent biotin-avidin method are diagrammed in Fig. 1. In the biotin-avidin method, avidin, with its four biotin binding sites, functions to crosslink biotinylated Ab to biotinylated lipid at the liposome surface. The MPB-DOPE and PDP-DOPE methods involve the formation of a covalent thioether bond between thiol and maleimide groups, near the liposome surface, while the PDP-PEG-DSPE method involved the formation of a thioether bond at the PEG terminus. In the fourth method a covalent hydrazone bond is formed between a oxidized carbohydrate in the Fc region of an Ab and a hydrazide group at the PEG terminus on the liposome.

For the preparation of SL, the total PEG-lipid content was always maintained at 5 mol% of the phospholipids (PL), i.e., HSPC. Liposomes containing 5 mol% Hz-PEG-DSPE, therefore, contained no mPEG-DSPE. All linker lipids incorporated readily into liposomes and resulted in no alterations in liposome size or stability (results not shown). The complete incorporation of both PDP-DOPE and PDP-PEG-DSPE at a concentration of 1 mol% PL was shown by the 100% release of 2-thiopyridone from liposomes following reduction of PDP with DTT as determined spectrophotometrically.

Table 1 compares the μg sheep IgG bound to the liposomes per μmol phospholipid for the non-covalent biotin-avidin method, and the four covalent coupling methods in the presence and absence of mPEG-DSPE. Linker lipids were incorporated into liposomes at 1 mol% of PL content except for Hz-PEG-DSPE which was incorporated at 5 mol% of PL and biotin-DOPE which was incorporated at 0.1 mol% of PL. Using the biotin-avidin method, low levels of Ab attached to the liposome surface (14 μg Ab/μmol PL) with low efficiency. For the MPB-DOPE and PDP-DOPE methods where the antibody is bound to the liposome surface, in the absence of mPEG-DSPE, comparably high amounts of protein were bound to the liposomes (112 and 108 μg Ab/μmol PL, respectively). An insignificant reduction occurred in the amount of Ab bound to MPB-DOPE liposomes in the presence of mPEG-DSPE (97 μg Ab/μmol PL). However, when 5 mol% mPEG-DSPE was incorporated into PDP-DOPE-liposomes a 72% reduction in Ab binding occurred (15 μg Ab/μmol PL), likely a result of steric hinderance by PEG to the access of the MPB-Ab to the small thiol group at the liposome surface. Furthermore, when the conjugation reaction occurred at the PEG terminus using PDP-PEG-DSPE, which has the same reaction chemistry as the PDP-DOPE method, conjugation was not hindered by PEG (96 and 93 μgAb/μmol PL in the presence or absence of 4 mol% mPEG-DSPE, respectively). All coupling reactions involving the formation of a thioether bond, with the exception of the PDP-DOPE method in the presence of mPEG-DSPE, had a coupling efficiency of approx. 60–70% at comparable reaction conditions (Table 1). Although the Hz-PEG-DSPE method was the simplest of the coupling methods, Ab densities (25.7 μg Ab/μmol PL) and coupling efficiencies (17%) were lower than for the MPB-DOPE and PDP-PEG-DSPE methods. Indeed, when the linker lipid Hz-PEG-DSPE was included in the liposomes at 1 mol%, Ab levels associated with the liposomes were not significantly elevated above background levels, therefore all experiments were done with 5 mol% Hz-PEG-DSPE in the liposomes.

Table 1 also shows the amount of DXR loaded in 1 h by an ammonium sulfate gradient method [30] into liposomes containing linker lipids, with and without mPEG-DSPE. DXR could be loaded efficiently (> 95%) at 65°C into all liposomes except those containing MPB-DOPE

Table 1
Effect of coupling method and mPEG on binding of Sheep IgG to liposomes

Liposome composition [a] (molar ratio)	mol% mPEG	Ab binding μg/μmol PL	Binding efficiency (%)	approx. No. Ab per liposome [b]	DXR loading (%)
HSPC/CHOL/biotin-DOPE (2:1:0.002)	5	14 ± 5	9 ± 3	7 ± 3	> 95
HSPC/CHOL/MPB-DOPE (2:1:0.02)	5	97 ± 15	63 ± 10	49 ± 8	variable [c]
	0	113 ± 11	72 ± 7	56 ± 6	variable [c]
HSPC/CHOL/PDP-DOPE (2:1:0.02)	5	15 ± 1	10 ± 1	7 ± 1	> 95
	0	108 ± 11	69 ± 7	54 ± 5	> 95
HSPC/CHOL/PDP-PEG-DSPE (2:1:0.02)	4	96 ± 22	61 ± 14	48 ± 11	> 95
	0	93	60	46	> 95
HSPC/CHOL/Hz-PEG-DSPE (2:1:0.02)	0	26 ± 4	17 ± 3	13 ± 2	> 95

[a] Liposomes preparation, conjugation procedures and DXR loading were according to Section 2. Sheep IgG was coupled to 100 nm liposomes at an Ab/PL molar ratio of 1:1000, and a phospholipid concentration of 2 mM. The total PEG content of the liposomes was kept constant at 5 mol% of PL. DXR was loaded at 65°C for 1 h.

[b] The conversion from μg IgG/μmol phospholipid to the approximate number of Ab per liposomes was based on the following assumptions: the area per polar head group for HSPC is 72 Å^2 and for CHOL is 19 Å^2 [2] with a combined area per phospholipid for a 100 nm, HSPC/CHOL (2:1, mol/mol) liposome of 81.5 Å^2 and that there are approx. $4 \cdot 10^{12}$ antibodies per μg protein and $7.8 \cdot 10^{12}$ liposomes per μmol PL.

[c] A precipitate of DXR was often observed, complicating the calculations (see text).

Table 2
% DXR loading into liposomes as a function of mol% MPB-PE

Mol% MPB-DOPE in liposomes	% DXR loading, 1 h at 65°C
1.0	40
0.5	73
0.1	80
0.05	> 95
0	> 95

(i.e., the comparatively large, hydrophobic MPB group at the liposome surface) where DXR loading was highly variable and unpredictable, ranging from a maximum of 40% loading at 1 mol% MPB-DOPE to > 95% of the initial DXR entrapped at 0.05 mol% MPB-DOPE or less (Table 2). With the ammonium sulfate loading method at 1 mol% MPB-DOPE in the liposomes, a DXR precipitate was sometimes observed which complicated the calculation of the % entrapped (or associated) DXR. This is thought to be caused by DXR reacting with ammonium sulfate leaking from the liposomes in the presence of 1 mol% MPB-DOPE (but not lower concentrations of MPB-DOPE) to form a $(DXR\text{-}NH_3)_2SO_4$ complex which has a low solubility [32,33]. The difficulties experienced with DXR remote loading in the presence of 1 mol% MPB-DOPE in the liposomes also occurred when we employed the pH gradient method [34]. Increasing the incubation time to 6 h in the presence of 1 mol% MPB-DOPE could sometimes increase the amount of DXR loading, but this was highly variable, and we have not yet been able to determine if the DXR was in the liposome interior or associated with the bilayer. Furthermore, longer incubation times at high temperatures led to the degradation of some of the maleimide groups, compromising Ab coupling efficiency (results not shown) [1]. In contrast, for the PDP-PEG-DSPE method, DXR loading was > 95% and the $T_{1/2}$ for DXR leakage in 25% human plasma at 37°C was approx. 88 days in the unconjugated liposomes and over 15 days in the conjugated liposomes (88 μg sheep IgG/μmol PL).

3.2. Factors affecting Ab coupling efficiency

We have investigated, for the MPB-DOPE method, three main factors affecting coupling of Ab to SL: (1) the concentration of linker lipid incorporated into the liposomes, (2) the Ab concentration and (3) the lipid concentration during the coupling reaction (Fig. 2A–C). A 10-fold increase in the amount incorporated MPB-DOPE, from 0.1 to 1.0 mol% of PL resulted in a 5-fold increase in the amount of bound Ab (Fig. 2A). Similarly, as the Ab/PL ratio increased the total amount of bound Ab also increased substantially (Fig. 2B). Fig. 2C demonstrates that coupling is much less dependent on PL concentration than on the concentration of linker lipid or Ab. A 5-fold increase in PL concentration during Ab coupling resulted in only a 1.5-fold increase in Ab binding.

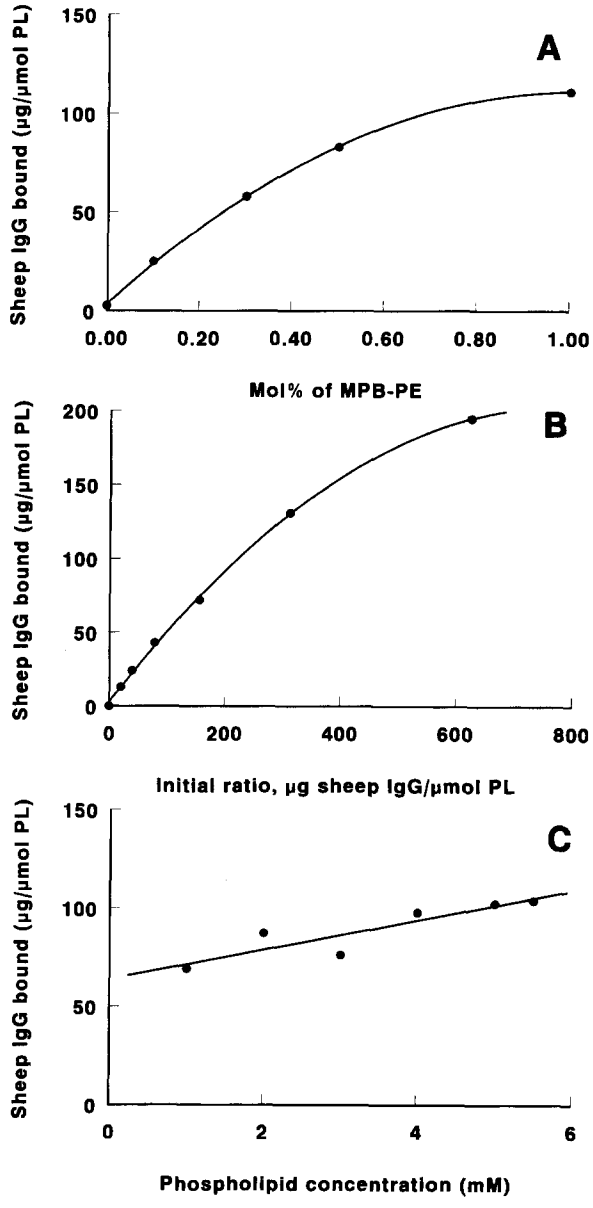

Fig. 2. Factors affecting the coupling of Ab (sheep IgG) to SL (MPB-DOPE method). Liposomes were 100 nm in diameter, composed of HSPC/CHOL/mPEG-DSPE, 2:1:0.1 molar ratio. PDP-Ab (sheep IgG) was prepared using SPDP as outlined in Section 2. (A) Effect of MPB-DOPE content. Liposomes containing 0 to 1 mol% MPB-DOPE were incubated with PDP-Ab at an Ab/PL molar ratio of 1:1000. (B) Effect of Ab concentration. Liposomes (1 mol% MPB-DOPE) were incubated with various amounts of Ab at a constant phospholipid concentration of 2 mM. (C) Effect of lipid concentration. Various concentrations of liposomes (1 mol% MPB-DOPE) were incubated with PDP-Ab at a molar ratio of 1:1000 (Ab/PL).

[1] Not only did we have problems with the loading and leakage of DXR in the presence of 1 mol% MBP-DOPE, but we could not perform biodistribution experiments with these liposomes using ^{125}I-tyraminy-linulin as an aqueous space label because it bound to the outside of the liposomes.

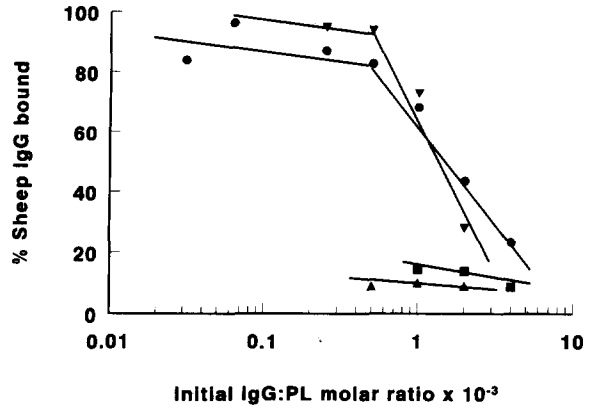

Fig. 3. Efficiency of sheep IgG binding to SL for different coupling procedures. Liposomes (100 nm in diameter) were incubated at 2 mM PL with the appropriate Ab at a varying Ab/PL molar ratios. (●) HSPC/CHOL/mPEG-DSPE/MPB-DOPE (2:1:0.1:0.02), (▼) HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02), (▲) HSPC/CHOL/mPEG-DSPE/PDP-DOPE (2:1:0.1:0.02), (■) HSPC/CHOL/Hz-PEG-DSPE (2:1:0.1).

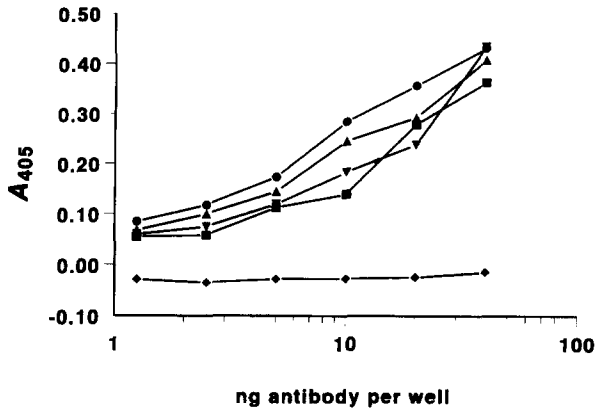

Fig. 4. Effect of various antibody coupling treatments on binding of anti-bovine IgG to bovine IgG-coated ELISA plate wells. The mAb was prepared as described in Section 2. (●) untreated mAb, (▲) sodium periodate oxidized mAb, (■) SMPB-treated mAb, (▼) SPDP-treated mAb, (♦) non-specific Ab (sheep IgG).

Fig. 3 compares the efficiency of coupling of Ab to SL by the various coupling methods. For both the PDP-PEG-DSPE and MPB-DOPE methods, maximum coupling efficiency, approaching 100%, occurred at a Ab/PL ratio of approx. 1:1000 or lower, corresponding to an Ab to linker lipid molar ratio of about 1:10 or lower. Above this ratio a steep dependence of % bound Ab on Ab/PL molar ratio was observed. As seen previously (Table 1) the coupling efficiency for the PDP-DOPE method was low, and increasing the Ab/PL molar ratio did not lead to a significant increase in coupling efficiency (Fig. 3). The Hz-PEG-DSPE method also had lower coupling efficiencies than the PDP-PEG-DSPE or MPB-DOPE methods and demonstrated a shallow dependence on Ab/PL molar ratios.

3.3. Effect of chemical manipulation of an Ab on its antigen binding activity

An ELISA was used to determine the affinity of anti-bovine IgG (Ab) for bovine IgG (antigen) after treatment of the Ab with either SMPB, SPDP or oxidization with periodate. The results, over a 40-fold concentration range, demonstrate an increase in ELISA signal with increasing Ab concentration (Fig. 4). As expected a nonspecific Ab (sheep IgG) was not recognized in this assay (Fig. 4). Chemical manipulation of the Ab caused a slight loss in antigen binding affinity for all methods used, as shown by the reduction in ELISA signal at 405 nm (Fig. 4).

3.4. Effect of Ab surface density on SIL on target binding

We have used liposomes with increasing amounts of bound Ab (Hz-PEG-DSPE method) to investigate the relationship between the liposome surface density of Ab and the level of target binding. ELISA (Fig. 5) was used to compare the degree of binding of SIL to the target antigen as a function of both increasing lipid concentration and Ab surface density. A direct dependence, both of Ab surface density as well as liposome concentration, was found for binding of SIL to their target antigens. In order to examine this relationship for cellular epitopes, the effects of liposomal surface densities of mAb 174H.64 was determined on the KLN 205 cell line for both the MPB-DOPE and PDP-PEG-DSPE methods as shown in Fig. 6A and B, respectively. For both methods the effect of Ab density on SIL binding in vitro was modest (Fig. 6A) or appeared to be absent (Fig. 6B), as compared to the ELISA experiments.

3.5. Binding of SIL, formed by different coupling methods, to neoplastic cells

The comparative binding by KLN 205 cells of SIL[174H.64], conjugated by two different coupling proce-

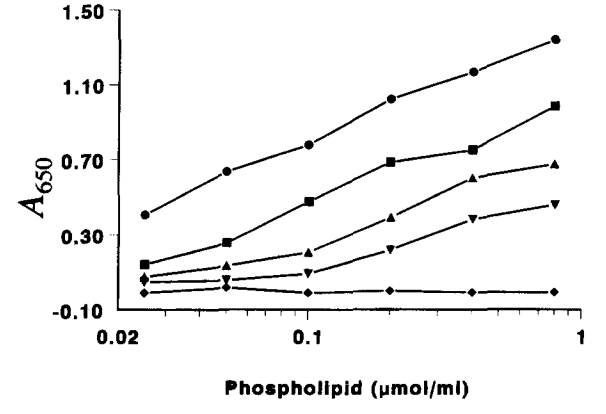

Fig. 5. Effect of antibody density on binding of SIL[anti-bovine IgG] to bovine IgG-coated ELISA plates. Various amounts of oxidized anti-bovine IgG was coupled to HSPC/CHOL/Hz-PEG-DSPE (2:1:0.1) liposomes, as described in Section 2. Antibody densities : (●) 52, (■) 31, (▲) 19, (▼) 11, (♦) 0 μg mAb/μmol PL.

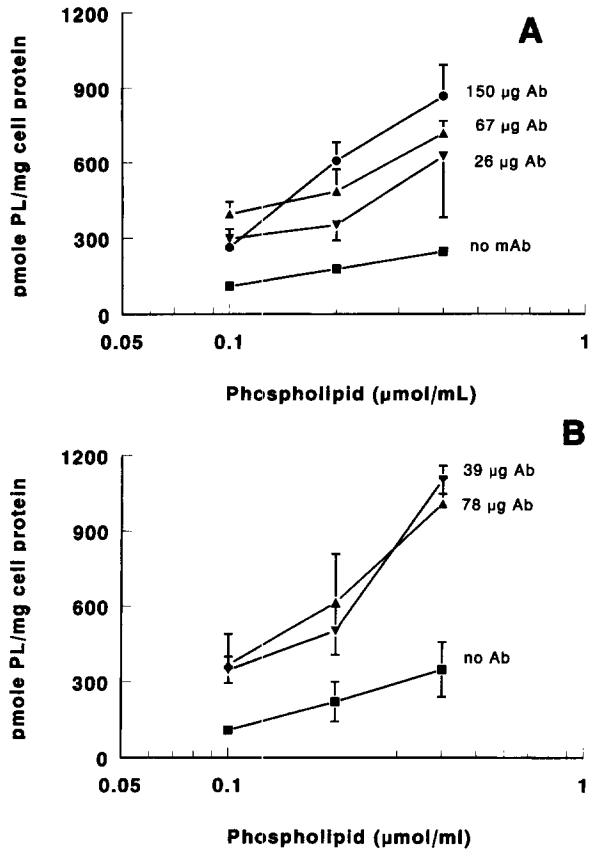

Fig. 6. Binding of SIL[174H.64] with different antibody surface densities, by KLN 205 cells, using MPB-DOPE or PDP-PEG-DSPE coupling procedures. PDP-mAb and MPB-mAb were prepared for conjugation as described in methods. SIL were labelled with ^{3}H-CHE and incubated for 1 h at 37°C with KLN 205 cells at a liposome concentration of 0.1–0.4 µmol PL/ml. (A) HSPC/CHOL/mPEG-DSPE/MPB-DOPE (2:1:0.1:0.02); (●) 150, (▲) 67 (▼) 26 and (■) 0 µg mAb/µmol PL. (B) HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02), (▲) 78, (▼) 39 and (■) 0 µg mAb/µmol PL. Binding is expressed as pmol PL/mg cell protein. Means ± S.D., $n = 3$.

dures (Hz-PEG-DSPE and PDP-PEG-DSPE), is shown in Fig. 7. The PDP-PEG-DSPE method resulted in significantly increased binding of SIL to KLN 205 cells, as compared to controls, while the HZ-PEG-PE method gave poor binding results with this model system, even with almost twice the Ab surface density (Fig. 7). In experiments where we incubated free mAb174H.64 for different times with increasing concentrations of periodate, we have observed that this particular mAb, unlike some other mAb or sheep IgG (Fig. 4) loses its binding activity under oxidizing conditions, which explains the low binding to its target cells for the Hz-PEG-DSPE method.

The binding of SIL[170H.82], formed by three coupling procedures at similar Ab densities, by HCT-15 cells is shown in Fig. 8. The PDP-PEG-DSPE and MPB-DOPE methods resulted in significantly increased binding (approximately double) of SIL for both cell lines, while the HZ-PEG-PE method, while resulting in increased binding, was not as high as the other two methods (approx. 1.5-fold).

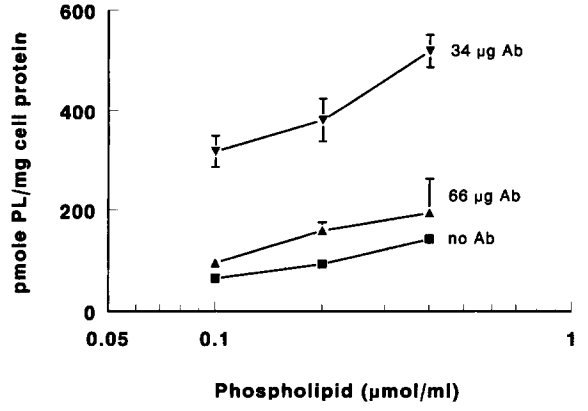

Fig. 7. Comparative binding of SIL[174H.64], prepared by two different coupling procedures, by KLN 205 cell line. Preparation of liposomes and the mAb 174H.64 for conjugation and binding procedures are described in Section 2. (▼), HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02), 34 µg mAb/µmol PL; (▲) HSPC/CHOL/Hz-PEG-DSPE (2:1:0.1), 66 mg mAb/µmol PL; (■) HSPC/CHOL/mPEG-DSPE (2:1:0.1), no mAb. Binding is expressed as pmol PL/mg cell protein. Means ± S.D., $n = 3$.

To investigate whether enhanced binding of liposomes was through specific recognition of antibodies, competition studies were performed, using the PDP-PEG-DSPE method, and results are shown in Table 3. SIL[174H.64] had significantly higher binding to KLN 205 cells than control liposomes with no antibody, or liposomes attached to an isotype-matched non-specific Ab SIL[B27.29]. Incubation of KLN 205 cells with SIL[174H.64] in the presence of a 20-fold excess of free mAb174H.64 caused a significant reduction in binding, while incubation with excess of free mAb B27.29 caused no signification reduction in binding.

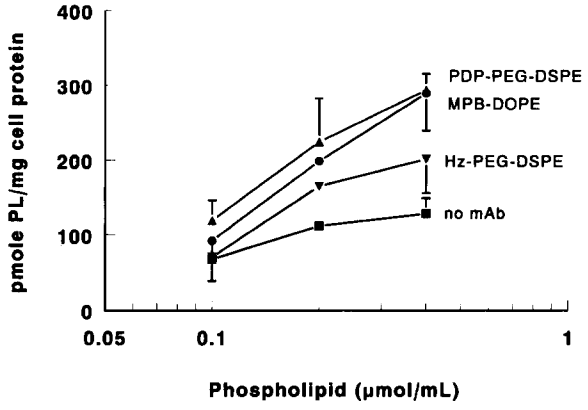

Fig. 8. Comparative binding of SIL[170H.82], prepared by three different coupling procedures, by the HCT-15 colon adenocarcinoma cell line. SIL were labelled with ^{3}H-CHE and incubated with cells for 1 h at 37°C with cells at a liposome concentration of 0.1–0.4 µmol PL/ml media. (●) HSPC/CHOL/mPEG-DSPE/MPB-DOPE (2:1:0.1:0.02), 59 µg mAb/µmol PL; (▲) HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02), 63 µg mAb/µmol PL; (▼) HSPC/CHOL/Hz-PEG-DSPE (2:1:0.1), 66 µg mAb/µmol PL; (■) HSPC/CHOL/mPEG-DSPE (2:1:0.1), no mAb. Binding is expressed as pmol PL/mg cell protein. Means ± S.D., $n = 3$.

4. Discussion

The rapid removal of CIL from circulation has greatly limited their use for in vivo target binding to cells such as cancer cells. Several recent studies have demonstrated that immunoliposomes could achieve significantly increased in vivo target binding when the immunoliposomes contained either GM_1 or PEG, which increased their circulation half-lives [35–37]. In these studies mAb was associated with liposomes by methods such as the *N*-glutaryl PE detergent dialysis method [14,35] or the non-covalent biotin-avidin method [25,37]. We have previously demonstrated that increased target binding in vitro can lead to increased cytotoxicity [38] and in vivo can lead to improved therapeutic effects [37]. Although good target binding could be obtained by attaching Ab in the phospholipid headgroup region of SL, target recognition was sensitive to the chain length of PEG incorporated into the liposomes [39,40]. Mori et al. [39] have shown that this steric barrier effect is directly related to the chain length of PEG. They reported that target binding of immunoliposomes to the surface of vascular endothelial cells in the lung was reduced for liposomes containing PEG5000-DSPE but not for the smaller molecule GM_1 or lower molecular weights of PEG. When Ab was attached to functional groups at the surface of liposomes containing PEG5000, the PEG substantially prevented antibody recognition of antigens as determined by an ELISA assay [41]. However when the length of the PEG is reduced to PEG2000, the size of the Ab molecule (molecular weight 150000) appears to be sufficient to allow it to bind antigens to a similar degree to that found for liposomes lacking PEG-DSPE [41].

The mechanism by which PEG-lipids prolong the circulation times of liposomes is proposed to be that of imparting a hydrophillic steric barrier to the surface of the bilayer [9,42] which is thought to prevent binding of serum protein opsonins to the liposome surface [43,44]. PEG may, by a similar mechanism, interfere with the binding of Ab or avidin to the surface of SL, resulting in the low binding efficiency which we have observed for some of the coupling methods. However, covalent binding of antibodies to the PEG terminus does not appear to cause any interference with antibodies binding to their respective antigens, and furthermore, the PEG coating of the liposome surface apparently continues to retard opsonization of the liposomes, leading to the extended circulation half-lives which we observed for SIL as compared to CIL [23]. Thus the Hz-PEG-DSPE and the PDP-PEG-DSPE coupling methods have significant advantages over other methods for binding Ab to SL. The observation that ligands can be coupled to the PEG terminus with retention of the circulation half-lives and binding affinity has also been made for other proteins [45].

The Hz-PEG-DSPE method has the advantage of being the simplest of the coupling methods and, because binding occurs through oxidized carbohydrate in the Fc region of the antibody, is the only method in which Ab orientation is maintained [15] (i.e., no modification of the Fab binding region occurs). This, therefore, would be the method of choice where lack of interference with the Fab binding region and/or site-specific conjugation were important experimental parameters. Rodwell et al. [46] has shown increased localization of ^{111}In-labelled Ab conjugates in target tumours when modification and conjugation occurred through a oligosaccharide label when compared to methods using direct amino acid labels. However, the formation of the hydrazone bond is much less efficient that the formation of a thioether bond, which is reflected in the lower coupling efficiencies seen for the Hz-PEG-DSPE method compared to the PDP-PEG-DSPE coupling method.

By ELISA, modification of the antibodies by the various coupling methods resulted in only a modest reduction of Ab activity (Fig. 4) using rabbit anti-bovine IgG. However, SIL prepared by the Hz-PEG-DSPE method had little binding to KLN 205 cells or HCT-15 cells as compared either to the MPB-DOPE or the PDP-PEG-DSPE methods (Figs. 7 and 8). In other words, some antibodies appear to be more sensitive than others to the oxidizing conditions of this method. In other targeting experiments, e.g., those involving binding of SIL[anti-CD19] to B cells, however, we have achieved similar target binding for both the Hz-PEG-DSPE and the PDP-PEG-DSPE methods [47]. Although the binding results were consistent within each model system studied, the variation between systems suggests that each Ab should be examined for its sensitivity to oxidation before use of the Hz-PEG-DSPE method.

Ideally, we would want to achieve close to 100% coupling efficiencies for an Ab coupling method in order not to waste mAb, which can be very costly to produce. The coupling method which best meets this criteria uses one of the most stable and efficient reactions in conjugate chemistry, the formation of a thioether bond by interaction of sulfhydryl and maleimide groups [16] (Figs. 2 and 3). High coupling efficiencies approaching 100% can be achieved for both the MPB-DOPE and PDP-PEG-DSPE coupling methods when the Ab to linker lipid ratio is approx. 1:10 (Fig. 3).

We were interested to observe that little reduction in coupling efficiency was observed for the MPB-DOPE coupling method, in contrast to the great reduction in efficiency for the PDP-DOPE method, in the presence of mPEG-DSPE in the liposomes. Since both approaches utilize the same thiol plus maleimide coupling reaction, this result is quite surprising. The explanation could lie in the difference between the two linker lipids at the liposome surface. In the PDP-DOPE method, coupling proceeds via a thiol group at the liposome surface, which is much smaller and more hydrophillic than the MPB group in the MPB-PE method. Is it possible that the bulkier, more hydrophobic MPB group may cause perturbation of the hydrophillic mPEG-DSPE chains, allowing access of the thiolated Ab to the liposome surface? The explanation

will require further experimentation. As noted above, the MPB group also appears to interact with doxorubicin, slowing the remote-loading of this drug into liposomes, and also results in binding of the aqueous space marker ^{125}I-tyraminylinulin to the liposomes surface, thus preventing the use of this marker in pharmacokinetic and biodistribution studies. In addition, non-specific binding to red blood cells of immunoliposomes containing MPB-DOPE has been observed and could be overcome by blocking the unreacted maleimide with DTT [48]. All four lines of evidence point to significant effects of the MPB group at the liposome surface at concentrations of 1 mol% of phospholipid. These problems can be handled either by reducing the mol% of MPB at the liposome surface (which, however, decreases coupling efficiencies), by switching to a more hydrophilic maleimide-based linker lipid such as (*N*-maleimidomethyl)cyclohexane-1-carboxylate-DOPE (MCC-PE) (Avanti Polar Lipids), or by 'reversing' the reaction and moving it to the PEG terminus away from the liposome surface, i.e., the PDP-PEG-DSPE method. We prefer the last solution, as we have been able to achieve high coupling efficiencies, long circulation times, efficient remote-loading of doxorubicin, and good target binding for all mAb systems examined with this method, which of all the methods we have examined to date comes closest to the ideal.

This study and others suggest that large amounts of bound Ab may not be necessary for efficient target binding of SIL to cells in vitro [35,38] and in vivo [37]. The relationship between Ab density and Ab binding seen in ELISA studies (Fig. 5) was more pronounced than that seen with cells growing in culture (Fig. 6A,B) where many of the binding sites may not be accessible to the SIL, depending on the degree of cell confluence and the degree of attachment of the cells to the substrata. Factors such as these may account for the some of the variability between level of SIL binding seen in replicate experiments done on different days (e.g., Fig. 6B and 7, and Table 3 for SIL[174H,64]). Our observations that we have been able to achieve higher levels of binding of SIL to cells grown as suspension cultures [47] compared to cells grown on solid substrates, e.g., this study, may be relevant to the choice of in vivo targets (see below). If the Ab is sufficiently exposed and binding is unhindered by the PEG steric barrier, Ab density may not be as critical a factor in the efficient binding of immunoliposomes to their target cells, as access of SIL to their binding sites. The ability to achieve good target binding in vivo at low Ab densities is important because the rate of elimination of SIL from circulation increases with increased Ab density on liposomes, particularly above 70–80 μg Ab/μmol PL [23].

Several questions involving in vivo applications of SIL remain to be answered. To mention only a few, our ability to achieve in vivo targeting will depend on whether the target is one accessible from the vasculature [35,36,47], where very long circulation half-lives for SIL may be less critical, or an extravascular target such as a solid tumor, either present as an easily accessible micrometastatic lesion, where SIL appear to have good therapeutic effects [37], or as a more developed mass, where SIL appear to be significantly less effective (experiments in progress). Localization of SL (no Ab) in small solid tumors appears to increase as the circulation times of the liposomes increases [8]. However, in the case of well-developed solid tumors, access of SIL, as well as SL, to the bulk of the tumor cells may be considerably compromised, and the use of SIL to treat advanced solid tumors may not be feasible. Further questions about in vivo applications of SIL which need to be answered center around: the in vivo stability and toxicity of the conjugates; the tendency of the SIL to precipitate immune reactions, e.g., anti-idiotypic reactions; the relative advantages of internalizing versus non-internalizing Ab; and the optimum rate of release of drug for the SIL. Relevant to the last point are recent experiments by Gaber et al. demonstrating that mild hyperthermia can lead to the rapid release of entrapped doxorubicin from SL which have localized in tumor microvascular [49]. In other words, the rate of release of liposome contents is amenable to manipulation, depending on the desired results.

In summary, we have examined a number of coupling methods for covalent or avidin-biotin mediated attachment of Ab to the surface of SL, endeavouring to fulfill a number of criteria for 'ideal' immunoliposomes. We have identified methods which have excellent coupling efficien-

Table 3
Competitive binding of SIL[174H.64] in the presence of free mAb

Liposome composition	Binding (pmol PL/mg cell protein ± S.D.)	Significance relative to binding of SIL[174H.64]
SIL[174H.64]	792 ± 68	
SIL[B27.29]	355 ± 63	$P < 0.001$
SL (no mAb)	272 ± 40	$P < 0.001$
SIL[174H.64] + free 174H.64	507 ± 40	$P < 0.01$
SIL[174H.64] + free B27.29	684 ± 179	NS

KLN 205 cells were grown to confluence in 6-well tissue culture plates, as described in Section 2. SIL, prepared by the PDP-PEG-DSPE method, were added to KLN 205 cells 10 min after a 20-fold excess of the same mAb, or a type-matched non-specific mAb (B27.29), in a total volume of 0.3 ml in each well. Concentration of SIL was 0.2 μmol PL/ml in all wells. Binding (pmol PL/mg cell protein, $n = 3$) was compared to that of SIL[174H.64] incubated with cells in the absence of competing or non-specific mAb. Statistics were done by analysis of variance using the ANOVA program.

cies, good drug remote-loading properties, and which results in high Ab densities at the liposomes surface. Several of these methods result in significantly increased levels of binding of SIL to their respective target cells lines. Of the methods examined, those in which Ab is attached at the PEG terminus appear to have the most useful combinations of properties, and in particular we favour the PDP-PEG-DSPE method in which a maleimide-modified Ab is covalently attached to a thiol group at the PEG terminus via a thiolether bond. We are currently using both the Hz-PEG-DSPE liposomes [47] and the PDP-PEG-DSPE liposomes to answer some of the important questions outlined above which will help to determine the best therapeutic niches for SIL in in vivo applications.

Acknowledgements

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References

[1] Gregoriadis, G. and Florence, A.T. (1993) Drugs 45, 15–28.
[2] Lasic, D.D. (1993) Liposomes: from physics to applications, Elsevier Science, Amsterdam.
[3] Kim, S. (1994) Drugs 46, 618–638.
[4] Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
[5] Blume, G. and Cevc, G. (1990) Biochim. Biophys. Acta 1029, 91–97.
[6] Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
[7] Senior, J., Delgado, C., Fischer, D., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1066, 77–82.
[8] Papahadjopoulos, D., Allen T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.L., Lee, D.-D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
[9] Woodle, M.C. and Lasic, D.D. (1992) Biochim. Biophys. Acta 1113, 171–199.
[10] Allen, T.M. and Papahadjopoulos, D. (1993) in Liposome Technology, 2nd Edn., Vol. III (Gregoriadis, G., ed.), pp. 59–72, CRC Press, Boca Raton.
[11] Torchilin, V.P. and Klibanov, A.L. (1993) Drug Target. Del. 2, 227–238.
[12] Weiner, A.L. (1990) in Targeted Therapeutic Systems (Tyle, P. and Ram, B.P., eds.), pp. 305–336, Marcel Dekker, New York.
[13] Bogdanov Jr., A.A., Klibanov, A.L. and Torchilin, V.P. (1988) FEBS Lett. 231, 381–384.
[14] Holmberg, E., Maruyama, K., Litzinger, D.C., Wright, S., Davis, M., Kabalka, G.W., Kennel, S.J. and Huang, L. (1989) Biochem. Biophys. Res. Commun. 165, 1272–1278.
[15] Chua, M-M., Fan, S-T. and Karush, F. (1984) Biochim. Biophys. Acta 800, 291–300.
[16] Martin, F.J., Wayne, L.H. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238.
[17] Martin, F.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.
[18] Leserman, L.D., Barbet, J., Kourilsky, F. and Weinstein, J.N. (1980) Nature 288, 602–604.
[19] Wright, S. and Huang, L. (1989) Adv. Drug Del. Rev. 3, 343–389.
[20] Sullivan, S.M., Connor, J. and Huang, L. (1986) Med. Res. Rev. 6, 171–195.
[21] Aragnol, D. and Leserman, L.D. (1986) Proc. Natl. Acad. Sci. USA 83, 2699–2703.
[22] Debs, R.J., Heath, T.D. and Papahadjopoulos, D. (1987) Biochim. Biophys. Acta 901, 183–190.
[23] Allen, T.M., Brandeis, E., Hansen, C.B., Kao, C.Y. and Zalipsky, S. (1995) Biochim. Biophys. Acta 127, 99–108.
[24] Zalipsky, A. (1993) Bioconj. Chem. 4, 296–299.
[25] Loughrey, H., Bally, M.B. and Cullis, P.R. (1987) Biochim. Biophys. Acta 901, 157–160.
[26] Samuel, J., Noujaim, A.A., Willans, D.J., Brzezinska, G.S., Haines, D.M. and Longenecker, B.M. (1989) Cancer Res. 49, 2465–2470.
[27] Longenecker, B.M., Willans, D.J., MacLean, G.D., Selvaraj, S., Mavanur, R.S. and Noujaim, A.A. (1987) J. Nat. Cancer Inst. 78, 489–496.
[28] Olson, T., Hunt, C.A., Szoka, F.C., Vail, W.I. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
[29] Mayer, L.D., Hope, M.I. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
[30] Haran, G., Cohen, R., Bar, L.K. and Barenholz, Y. (1993) Biochim. Biophys. Acta 1151, 201–215.
[31] Bartlett, G.R. (1959) J. Biol. Chem. 234, 446–468.
[32] Lasic, D.D., Frederik, P.M., Stuart, M.C.A., Barenholz, Y. and McIntosh, T.J. (1992) FEBS Lett. 312, 255–258.
[33] Bolotin, E.M., Cohen, R., Bar, L.K., Emanuel, N., Ninio, S., Lasic, D.D. and Barenholz, Y. (1994) J. Liposome Res. 4, 455–479.
[34] Mayer, L.D., Bally, M.B. and Cullis, P.R. (1986) Biochim. Biophys. Acta 857, 123–126.
[35] Maruyama, K., Kennel, S.L. and Huang, L. (1990) Proc. Natl. Acad. Sci. USA 87, 5744–5748.
[36] Torchilin, V.P., Klibanov, A.L., Huang, L., O'Donnell, S., Nossiff, N.D. and Khaw, B.A. (1992) FASEB J. 6, 2716–2719.
[37] Ahmad, I., Longenecker, M., Samuel, J. and Allen, T.M. (1993) Cancer Res. 53, 1484–1488.
[38] Ahmad, I. and Allen, T.M. (1992) Cancer Res. 52, 4817–4820.
[39] Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) FEBS Lett. 284, 263–266.
[40] Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P. and Huang, L. (1992) Biochim. Biophys. Acta 1062, 142–148.
[41] Allen, T.M., Agrawal, A.K., Ahmad, I., Hansen, C.B. and Zalipsky, S. (1994) J. Liposome Res., 4, 1–25.
[42] Blume, G. and Cevc, G. (1993) Biochim. Biophys. Acta 1146, 157–168.
[43] Chonn, A., Semple, S.C. and Cullis, P.R. (1992) J. Biol. Chem. 267, 18759–18765.
[44] Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov Jr., A.A., Trubstskoy, V.S., Herron, J.N. and Gentry, C.A., (1994) Biochim. Biophys. Acta 1195, 11–20.
[45] Blume, G., Cevc, G., Crommelin, D.J.A., Bakkerwoudenberg, I.A.J.M., Klutt, C. and Storm, G., (1993) Biochim. Biophys. Acta 1149, 180–184.
[46] Rodwell, J.D., Alvarez, V.L., Lee, C., Lopes, A.D., Goers, J.W.F., King, H.D., Powsner, H.J. and McKearn, T.J. (1986) Proc. Natl. Acad. Sci. USA 83, 2632–2636.
[47] Lopes de Menezes, D., Pilarski, D.E. and Allen, T.M. (1995) Proc. Am. Assoc. Cancer Res., 36, 307.
[48] Peeters P.A.M., Oussoren, C., Eling, W.M.C. and Crommelin, D.J.A. (1990) J. Liposome Res. 1, 261–268.
[49] Gaber, M.H., Wu, N.Z., Hong, K., Huang, S-K., Dewhirst, M.W. and Papahadjopoulos, D. (1995) Proc. Am. Assoc. Cancer Res. 36, 309.