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A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells

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Abstract

The development of long-circulating formulations of liposomes (S-liposomes), sterically stabilized with lipid derivatives of poly(ethylene glycol) (PEG), has increased the likelihood that these liposomes, coupled to targeting ligands such as antibodies, could be used as drug carriers to deliver therapeutic drugs to specific target cell populations in vivo. We have developed a new methodology for attaching monoclonal antibodies to the terminus of PEG on S-liposomes. A new end-group functionalized PEG-lipid derivative pyridylthiopropionoylamino-PEG-distearoylphosphatidylethanolamine (PDP-PEG-DSPE) was synthesized for this purpose. Incorporation of PDP-PEG-DSPE into S-liposomes followed by mild thiolysis of the PDP groups resulted in formation of reactive thiol groups at the periphery of the lipid vesicles. Efficient attachment of maleimide-derivatized antibodies took place under mild conditions even when the content of the functionalized PEG-lipid in S-liposomes was below 1% of total lipid. The resulting S-immunoliposomes showed efficient drug remote loading, slow drug release rates and increased survival times in circulation compared to liposomes lacking PEG. When antibodies recognizing several different tumor-associated antigens were coupled to the PEG terminus of S-liposomes, a significant increase in the in vitro binding of liposomes to the target cells was observed. The binding of S-immunoliposomes containing entrapped doxorubicin to their target cell population resulted in increased cytotoxicity compared to liposomes lacking the targeting antibody.

Keywords: Sterically stabilized liposome; Poly(ethylene glycol); Liposome targeting; Doxorubicin; Immunoliposome; Selective toxicity; Toxicity

1. Introduction

Site-specific delivery of drugs to diseased cells can lead to significant reductions in drug toxicity, and increased therapeutic effects. Therapeutic applications of liposomal drug delivery systems have been extensively explored in recent years [1–5], and several methods have been developed for attachment of antibodies (Ab) at the liposome surface [6–9] in attempts to target the liposomes in vivo to specific sites of drug actions. Attachment of Ab at the surface of classical formulations of liposomes results in their rapid removal from circulation by the cells of the mononuclear phagocyte system (MPS, also termed reticuloendothelial system) [10,11]. New formulations of sterically stabilized liposomes (SL) have been developed recently which have extended blood circulation times as a result of reduced rates and extents of uptake by MPS cells [12–15]. SL contain, as the key component, methoxy poly(ethylene glycol) distearoylphosphatidylethanolamine

Abbreviations: mPEG, methoxy poly(ethylene glycol); MPS, mononuclear phagocyte system; SL, sterically stabilized (Stealth^R) liposomes; mPEG-DSPE, methoxy poly(ethylene glycol) distearoylphosphatidylethanolamine; PDP-PEG-DSPE, pyridyldithiopropionoylamino poly(ethylene glycol) distearoylphosphatidylethanolamine; SIL, sterically stabilized immunoliposomes; DSPE, distearoylphosphatidylethanolamine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)proprionate; DSC, disuccinimidylcarbonate; SC, succinimidylcarbonate; Boc, *tert*-butyloxycarbonyl; TLC, thin-layer chromatography; TEA, triethylamine; PDP, pyridyldithioproprionate; HSPC, hydrogenated soy phosphatidylcholine; SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate; MPB-PE, maleimidophenylbutyratephosphatidylethanolamine; ^{3}H-CHE, [^{3}H]cholesteryl hexadecyl ether; ^{125}I-TI, [^{125}I]tyraminylinulin; Ab, polyclonal antibody; mAb, monoclonal antibody; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; CHOL, cholesterol; DTT, dithiothreitol; DXR, doxorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; KLN 205, murine squamous lung carcinoma; Caov-3, human ovarian adenocarcinoma; HCT-15, human colon adenocarcinoma; mAb 174H.64, antibody recognizing KLN 205; mAb B43.13, antibody recognizing Caov-3; mAb M170, antibody recognizing HCT-15.

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(mPEG-DSPE) (4–8 mol% of the total lipid). This results in coverage of the lipid vesicle surfaces with grafted amphipathic polymer chains. It is believed that the high mobility of the mPEG chains, associated with their conformational flexibility, and also their water-binding ability, contribute to the steric stabilization which is responsible for their prolonged survival times in circulation [16,17].

We were interested in developing methods for attachment of Ab to the surface of SL which would meet a number of important criteria for 'ideal immunoliposomes'. These include: simplicity and ease of preparation, maintenance of prolonged circulation half-lives, retention of target recognition for the Ab, high coupling efficiency of the Ab to the liposomes, the ability to achieve high Ab densities at the liposome surface, the ability to achieve efficient drug remote loading, appropriate drug release characteristics, and compatibility with use in humans.

Attachment of Ab at the surface of SL, in the phospholipid headgroup region, can result in some decrease in coupling efficiency and some loss of Ab recognition, particularly in the presence of high molecular weights of mPEG which can sterically hinder access of the Ab to the liposome surface during coupling procedures and/or access of the Ab binding region to its epitope after its attachment to the liposomes [8,18,19]. Because some of the antibody coupling methods in the literature fell considerably short of the ideal, when applied to SL, we have developed a new coupling method, specifically for use with SL, which forms S-immunoliposomes (SIL) by covalently coupling antibodies to the liposomes via a thioether bond at the PEG terminus (Fig. 1), rather than to the phospholipid headgroup region on the liposome surface. This new method comes close to meeting the criteria outlined above for ideal SIL. Attachment of proteins to the distal end of PEG terminus using a PEG-COOH construct has recently been described by two different groups, with retention of long-circulating half-lives and target binding [20,21].

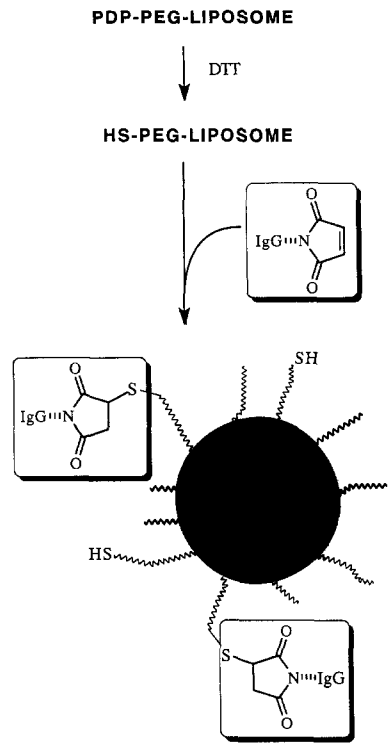

Fig. 1. Schematic diagram of the coupling of a maleimide-activated antibody (MPB-Ab) with sterically stabilized liposomes containing PDP-PEG-DSPE.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylethanolamine (DSPE) was purchased from Sygena, Cambridge, MA, disuccinimidylcarbonate (DSC) from Fluka (Ronkonkoma, NY), and *N*-succinimidyl-3-(2-pyridyldithio)proprionate (SPDP) from Bioaffinity Systems (Rosco, IL). Heterobifunctional α-amino-ω-hydroxy-PEG was prepared by partial conversion of hydroxy end groups of PEG-2000 (Fluka) into primary amines [22], followed by ion-exchange purification according to Furukawa [23]. Amino-PEG-DSPE was prepared as described elsewhere [24].

Hydrogenated soy phosphatidylcholine (HSPC) and methoxy (polyethylene glycol) (2000) distearoylphosphatidylethanolamine (mPEG-DSPE) were obtained from Liposome Technology (Menlo Park, CA). mPEG-DSPE synthesis was described in [14]. Cholesterol (CHOL) and pyridyldithioproprionate dioleoylPE (PDP-DOPE) were purchased from Avanti Polar Lipids (Birmingham, AL). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-succinimidyl-4-(*p*-maleimidophenyl)-butyrate (SMPB) and sheep IgG, used as a source of antibody (Ab) were purchased from Sigma (St. Louis, MO). Doxorubicin was obtained from Adria Laboratories (Mississauga, Ontario). $Na^{125}I$ and cholesteryl [1,2(n)-^{3}H]hexadecyl ether, 1.48–2.22 TBq/mmol (^{3}H-CHE) was purchased from New England Nuclear (Mississauga, Ontario). Tyraminylinulin was synthesized and [^{125}I]tyraminylinulin (^{125}I-TI) was prepared as previously described [25]. Iodobeads were purchased from Pierce (Rockford, IL). Monoclonal antibodies (mAb) 174H.64, B43.13 and M170 and the cell lines KLN 205 and Caov-3 were generous gifts of Biomira, Edmonton, AB. The cell line HCT-15 was purchased from American Type Culture Collection (Rockville, MD). All other chemicals were analytical grade.

2.2. Synthesis

General methods

TLC on silica gel G were visualized with iodine vapour, ninhydrin and Dragendorff [26] spray reagents, when ap-

propriate. NMR spectra was recorded on a 360 MHz Nicolet instrument. When it was important to detect the terminal OH group of PEG, DMSO-d_6 was used as a solvent [27], otherwise $CDCl_3$ was used.

Preparation of PDP-PEG-OH

SPDP (100 mg, 0.32 mmol) and α-amino-ω-hydroxy-PEG (0.55 g, 0.275 mmol) dissolved in acetonitrile (2 ml) reacted at 25° C for 4 h. TLC of the reaction mixture ($CHCl_3/CH_3OH$, 8:2) showed disappearance of H_2N-PEG-OH with appearance of a less polar, UV-absorbing new material ($R_f = 0.80$). The solvent was rotary evaporated and ethyl ether (50 ml) added. After overnight storage at 4° C white solid was collected and dried in vacuo over P_2O_5. Yield: 0.5 g (90%).

H-NMR (DMSO-d_6): $\delta \approx 2.5$ (CH_2S, overlap w/ DMSO), 3.01 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.20 (m, CH_2NH, 2H), 3.51 (s, PEG, ≈ 18OH), 4.52 (t, HO-PEG, 1H), 7.24 (m, pyr, 1H), 7.76 (m, pyr, 1H), 7.82 (m, pyr, 1H), 7.99 (br m, NH, 1H), 8.45 (m, pyr, 1H) ppm. H-NMR ($CDCl_3$): δ 2.62 (t, $J = 7$ Hz, CH_2SS, 2H), 3.08 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.47 (m, CH_2N, 2H), 3.64 (s, PEG, ≈ 18OH), 6.78 (br s, NH, 1H), 7.10, (m, pyr, 1H), 7.66 (m, pyr, 2H), 8.49 (m, pyr, 1H) ppm.

Preparation of PDP-PEG-SC

Meticulously dried PDP-PEG-OH (0.4 g, 0.18 mmol) was dissolved in acetonitrile (0.5 ml) and treated with DSC (81 mg, 0.31 mmol) and pyridine (62 ml, 0.79 mmol) overnight at 25° C. The product was precipitated with ethyl ether (40 ml) at 4° C, redissolved in ethyl acetate (4 ml) and precipitated with equal volume of ethyl ether at 4° C. The product was collected by filtration and dried in vacuo over P_2O_5. Yield: 330 mg, (85%).

H-NMR (DMSO-d_6): $\delta \approx 2.5$ (CH_2SS, overlap w/ DMSO), 2.81 (s, SC, 4H), 3.01 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.20 (m, CH_2NH, 2H), 3.51 (s, PEG, ≈ 18OH), 4.45 (t, CH_2-SC, 2H), 7.24 (m, pyr, 1H), 7.8 (m, pyr, 2H), 8.0 (br m, NH, 1H), 8.45 (m, pyr, 1H) ppm. H-NMR ($CDCl_3$): δ 2.62 (t, $J = 7$ Hz, CH_2SS, 2H), 2.83 (s, SC, 4H), 3.09 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.44 (m, CH_2N, 2H), 3.64 (s, PEG, ≈ 18OH), 4.46 (m, CH_2-SC, 2H), 6.73 (br s, NH, 1H), 7.10, (m, pyr, 1H), 7.66 (m, pyr, 2H), 8.48 (m, pyr, 1H) ppm.

Preparation of PDP-PEG-DSPE

Method A. DSPE (36 mg, 0.043 mmol) was added to a solution of PDP-PEG-SC (100 mg, 0.042 mmol) in chloroform (1 ml), followed by TEA (33 μl, 0.237 mmol). The reaction mixture became clear during incubation at 40° C for 10 min. The solvent was evaporated and replaced with acetonitrile (5 ml). The cloudy solution was kept at 4° C overnight. To remove traces of insoluble DSPE the solution was centrifuged and the clear solution separated. It was then rotary evaporated under reduced pressure, and the residue was dried in vacuo over P_2O_5. Yield: 130 mg (quantitative).

Method B. To a solution of amino-PEG-DSPE hydrochloride [24] (198 mg, 0.07 mmol) in acetonitrile (2 ml), SPDP (26 mg, 0.085 mmol) was added followed by TEA (30 ml, 0.42 mmol). The solution was stirred overnight. The product (200 mg) in chloroform was loaded onto a silica (11 g) column. It was eluted with a step gradient of CH_3OH in chloroform (5, 10, 15, and 20%, 100 ml each step). Yield: 106 mg of pure product and 77 mg of a product contaminated with a more polar material ($R_f = 0.125$).

The purified products obtained by both methods were identical.

TLC ($CHCl_3/CH_3OH/H_2O$ 90:18:2) $R_f = 0.52$. H-NMR ($CDCl_3$): δ 0.89 (t, $J = 6.8$ Hz, CH_3, 6H), 1.26 (s, CH_2, 56H), 1.58 (br m, $CH_2CH_2C=O$, 4H), 2.28 (2 overlapping t, $CH_2C=O$), 2.62 (t, $J = 7$ Hz, CH_2SS, 2H), 3.09 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.36 (br m, OCH_2CH_2N, 2H), 3.44 (m, CH_2N, 2H), 3.64 (s, PEG, ≈ 18OH), 3.94 (br m, CH_2CH_2OP, 2H), 4.17 (dd, $J = 7.0$, 12 Hz, glycerol CH_2OP, 2H), 4.21 (m, CH_2-O_2CN, 2H), 4.39 (dd, $J = 3.2$, 12 Hz, glycerol $CH_2OC=O$, 2H), 5.20 (m, CH, 1H), 6.73 (br, NH, 1H), 7.10, (m, pyr, 1H), 7.66 (m, pyr, 2H), 8.48 (m, pyr, 1H) ppm. ^{13}C-NMR ($CDCl_3$): δ 14.0 (CH_3), 22.7 (CH_2CH_3), 24.9 ($CH_2CH_2C=O$), 29.7 ($CH_2CH_2CH_2$), 31.9 ($CH_2CH_2CH_3$), 34.1 and 34.3 ($CH_2C=O$), 34.7 ($SCH_2CH_2C=O$), 35.6 ($SCH_2CH_2C=O$), 39.4 ($CH_2NHC=O$), 42.4 (CH_2NHCO_2), 62.8 ($CH_2OC=O$), 63.4 (CH_2OPO_3), 64.2 ($CH_2OC=ON$), 69.9 (CHOC = O), 70.6 (PEG), 120.0 (C2 pyr), 137.0 (C3 pyr), 120.8 (C4 pyr), 149.7 (C5 pyr), 156.6 (C = O of urethane), 159.8 (C1 pyr), 170.7 (C = O of amide), 173.0 and 173.3 (C = O of esters) ppm.

2.3. Preparation of immunoliposomes

Liposome preparation

Liposomes were prepared by hydrating dry lipid films in an appropriate buffer, at a lipid concentration of 10 mM. The resulting multivesicular preparations were then passed through 0.08–0.1 μm polycarbonate membranes (Nuclepore, Pleasanton, CA or Poretics, Livermore, CA) using a Lipex extruder (Lipex Biomembranes, Vancouver, BC), to give primarily unilamellar vesicles of approx. 100 nm in diameter [28,29]. The resulting liposomes were sized by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instruments, Holtsville, NY).

Iodination of antibody

Ab (2 mg) was dissolved in 200 μl of 25 mM Hepes, 140 mM NaCl, pH 7.4 buffer. The Ab solution was mixed with 185 MBq of $Na^{125}I$ in a 2 ml reaction vial with 5 Iodobeads for 1 h at room temperature (22° C). ^{125}I-Ab was purified by passage over a Sephadex G25 gel filtration column, eluting with the above buffer.

Preparation of maleimidophenylbutyrate-Ab (MPB-AB)

Ab was dissolved in 25 mM Hepes, 140 mM NaCl, pH 7.4 at concentration of 10 mg/ml and trace amounts of ^{125}I-Ab were added. SMPB (25 mM in dimethylformamide) was slowly added to the Ab solution at a molar ratio of 20:1 (SMPB/Ab) for 30 min at room temperature. Unbound SMPB was removed and the pH lowered by passing the solution over a Sephadex G50 column in 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7 buffer.

Antibody conjugation

Liposomes were composed of HSPC/CHOL/PDP-PEG-DSPE at a molar ratio of 2:1:0.02 with or without 4 mol% mPEG-DSPE (total PEG lipid, 5 mol% of PL) or of HSPC/CHOL/PDP-DOPE, at a molar ratio of 2:1:0.02 with or without 5 mol% mPEG-DSPE. In experiments to determine the coupling efficiency of the PDP-PEG-PE method, the ratio of PDP-PEG-DSPE and mPEG-DSPE were varied, keeping the total amount of PEG-lipids in the liposomes constant at 5 mole% of phospholipids (PL). Trace amounts of ^{3}H-CHE was added to each liposome preparation and the PL concentration was calculated from the specific activity using a Beckman LS6800 Scintillation counter.

Liposomes were hydrated with 100 mM sodium acetate, 70 mM NaCl, pH 5.5 buffer. The pyridyldithio groups were reduced by the addition of dithiothreitol (DTT) to a final concentration of 20 mM for 30 min at room temperature. DTT was separated and the pH raised by passing the liposomes over a Sephadex G50 column eluted with 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7 buffer.

Thiolated liposomes (2 μmol PL) were incubated overnight at room temperature with MPB-Ab (0.25–8 nmol) at Ab/PL molar ratios ranging from 1:250 to 1:8000, at a final PL concentration of 2–4 mM. Unbound Ab was removed by passing the liposomes over a Sepharose CL-4B column with pH 7.4 buffer (25 mM Hepes, 140 mM NaCl). The Ab to PL ratio was determined by counting for ^{125}I-IgG and ^{3}H-CHE. The amount of ^{125}I in the ^{3}H channel was subtracted from the total ^{3}H counted and this overlap was kept below 10% of ^{3}H.

2.4. Properties of immunoliposomes

Liposomes were composed of HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02 molar ratio) or HSPC/CHOL/PDP-DOPE (2:1:0.02 molar ratio). Liposomes were hydrated with the aqueous space label ^{125}I-TI [30] and prepared as above, with diameters of 98–130 nm. Free ^{125}I-TI was separated from the liposomes by chromatography over a Ultragel AcA 34 column (IBF Biotechnics, France) with 25 mM Hepes, 140 mM NaCl, pH 7.4 buffer. MPB-Ab was then conjugated to PDP-containing liposomes at various ratios to give Ab surface densities of 0–140 μg Ab/μmol PL.

In these experiments, the amount of Ab bound to the liposomes was determined by an adaptation of a fluorescamine assay, where an increase in fluorescence is observed when fluorescamine binds to a free amino group on a protein [31,32]. Briefly, 10 mg fluorescamine was dissolved in 50 ml of dry acetonitrile. One ml samples of SIL (0.2–1 mM PL) were mixed with 1 ml of fresh fluorescamine reagent for 15 min at room temp then 1 ml of ethanol was added. The relative fluorescence was determined on a Perkin-Elmer MPF-4 spectrofluorimeter (EM 475 nm, EX 395 nm). Protein concentrations were determined from a standard curve using known concentrations of MPB-Ab (5–160 μg/ml).

Outbred female CD$_1$(ICR)BR mice were purchased from Charles River Canada (St. Constant, Quebec) and maintained in standard housing. Mice (three per group) were given a single bolus i.v. injection via the tail vein of 0.2 ml of SIL (0.5 μmol PL/mouse) containing $(1–3)\cdot 10^5$ cpm of ^{125}I-TI. At different times post-injection, animals were anaesthetized with halothane and sacrificed by cervical dislocation. Major organs (liver, spleen, lung, heart and kidney), blood (100 μl), thyroid and carcass (remainder of the animal) were collected and counted for ^{125}I label in a Beckman 8000 gamma counter. Blood correction factors [33], were applied to all samples. The data is expressed as the percentage of counts in each organ relative to the total counts remaining in vivo at each time point [30].

The blood elimination half life of SIL was calculated using the polyexponential curve stripping and least squares parameter estimation program RSTRIP (Micromath, Salt Lake City, USA).

Doxorubicin loading and leakage studies

For DXR loading experiments, liposomes were hydrated in 155 mM ammonium sulfate at pH 5.5 [34]. The liposomes were then passed over a Sephadex G50 column equilibrated in 123 mM sodium citrate, pH 5.5. DXR (0.2 mg/mg PL) was then added to the liposomes and the mixture was incubated at 65° C for 1 h. DXR-containing liposomes were separated from any remaining free DXR by passage over a Sephadex G50 column and eluted with 123 mM sodium citrate pH 5.5 buffer.

For leakage experiments, DXR-loaded liposomes, composed of HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02 molar ratio), were labelled with trace amounts of ^{3}H-CHE. Liposomes were coupled with ^{125}I-Sheep IgG (SIL[sheep IgG]) to give 88 μg Ab/μmol PL, then mixed with 25% human plasma in 25 mM Hepes, 140 mM NaCl, pH 7.4 at 0.6–1.2 mM PL and dialysed (Spectra/Por 2, 12 000–14 000 M_r cut-off; Spectrum, Los Angeles, Ca) against 50 volumes of 25% human plasma. At various times, samples were removed and the external plasma volume exchanged. The amount of DXR remaining in the liposomes was determined by extracting the DXR in methanol and determining the absorbance at A_{492} nm.

Uptake and cytotoxicity studies

Murine squamous lung carcinoma (KLN 205), human ovarian adenocarcinoma (Caov-3), and human colon

adenocarcinoma (HCT-15) cell lines were grown as monolayers in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL, Burlington, Ontario, Canada) at 37° C in a humidified atmosphere of 5% CO_2 in air. Cells were plated in triplicate onto 6-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at $(0.15–1) \cdot 10^6$ cells/well on day 1. SIL were prepared by conjugating (see above), to the surface of SL (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE, 2:1:0.08:0.02 molar ratio), mAb specific to each cell line, i.e., mAb 174H.64, B43.13 or M170 for experiments involving KLN 205, Caov-3 or HCT-15, respectively. On the fourth day (or at the point where the cells just reach confluence), SIL[174H.64], SIL[B43.13] or SIL[M170], labelled with ^{3}H-CHE (0.1–0.4 μmol/ml), in PBS were added to each well of their targeted cell lines, respectively. In some instances, binding of SIL to cells was also measured in the presence of 10% FBS. 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, placed in ACS scintillation fluid (Amersham, Oakville, Ontario), and counted in a Beckman LS-6800 counter.

A colorimetric assay using a tetrazolium salt was used for the measurement of surviving and/or proliferating cells according to Mosmann [35]. Cells (KLN 205) were plated in 96-well plates (Corning, Corning, NY) on day 1. On the third day, cells were incubated with either free DXR, DXR entrapped in non-targeted liposomes (DXR-SL) or DXR entrapped in SIL[174H.64] (DXR-SIL[174H.64]) and the cells were incubated for 1 h or 24 h at 37° C in a humidified 5% CO_2 atmosphere. At the end of the incubation, free or liposomal DXR was removed by gentle washing with phosphate-buffered saline, pH 7.4, and the cells were further incubated for 47 h or 24 h respectively, for a total of 48 h. At the end of the incubation, a solution of MTT (0.5 mg MTT/ml media) was added to each well, and the mixture was incubated at 37° C for 4 h. Acid-isopropanol (100 μl of 0.04 M HCl in isopropanol) was added to each well and mixed thoroughly until all crystals were dissolved. The plates were read immediately on a Titertek Multiskan PLUS MK II plate reader (Flow Laboratories, Mississauga, Ontario, Canada), using a test wavelength of 570 nm, and a reference wavelength of 650 nm.

3. Results and discussion

3.1. Synthesis of PDP-PEG-DSPE

For preparation of SILs we chose the conjugation strategy shown in Fig. 1 for the following reasons. Reaction between thiol and maleimide groups is one of the most useful and efficient reactions in bioconjugate chemistry [6,36]. It takes place at close to neutral pH, at ambient temperature, within short periods of time, and often results in satisfactory yields of conjugates, even when relatively

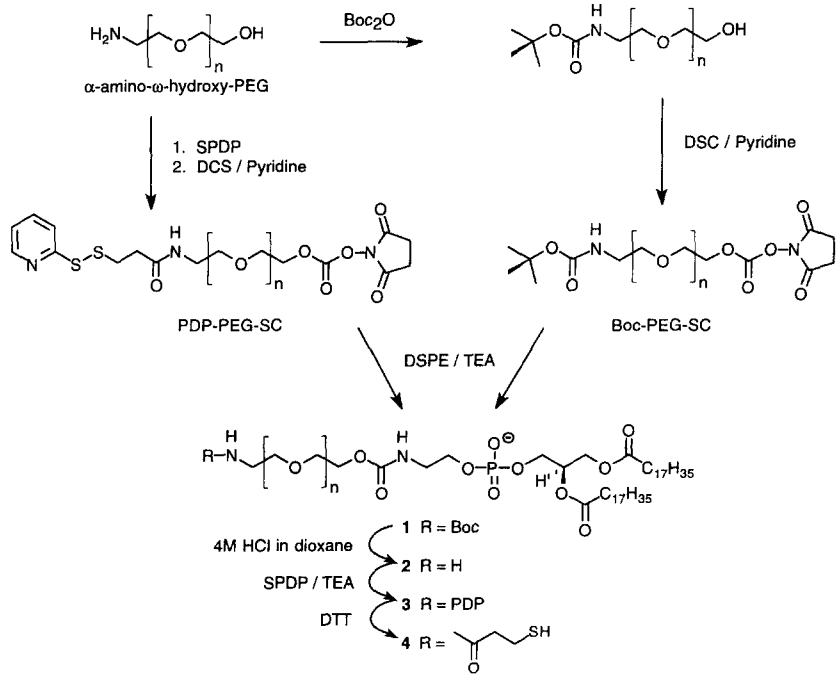

Fig. 2. Synthetic pathways for PDP-PEG-DSPE. Both PDP-PEG-DSPE synthetic pathways started with the heterobifunctional polymer, α-amino-ω-hydroxy poly(ethylene glycol) [24]. On the right, α-amino-ω-hydroxy-PEG is coupled with SPDP then with DSPE. On the left, α-amino-ω-hydroxy-PEG is first protected with Boc then bound to DSPE and subsequently Boc was removed and replaced with SPDP. Once incorporated into liposomes containing derivative **4**, a free thiol was generated by reduction of PDP with DTT (see Materials and methods).

low concentrations of the reactants are present. Thus it seems to be particularly suitable for interlinking of two macromolecular entities.

Previous experience with maleimide-PE incorporated into liposomes showed that it interfered with the drug loading procedures [8]. Maleimide is also known to undergo gradual degradation under conditions used during liposome preparation, sizing, and drug loading steps [6,37]. On the other hand, a maleimide group can be introduced onto IgG just prior to the actual conjugation step giving it no opportunity to undergo any side reactions.

Thiol groups, while very reactive towards various electrophiles, can be preserved under non-oxidizing conditions. To absolutely assure the integrity of the thiol groups, we decided to prepare a protected form of thiol group (PDP) positioned at the terminus of PEG-DSPE. Thus, our strategy involved preparation of liposomes containing the new conjugate, PDP-PEG-DSPE, first. They were conveniently formulated and drug-loaded, and after reductive deprotection of the reactive thiol groups with DTT, conjugated with maleimide-containing IgG (see Fig. 1).

The two synthetic pathways leading to PDP-PEG-DSPE are schematically depicted in Fig. 2. The synthesis started with heterobifunctional polymer, α-amino-ω-hydroxy poly(ethylene glycol), which was prepared by partial conversion of the hydroxyl end groups into primary amines [22] followed by ion-exchange purification of H_2N-PEG-OH [23]. The amino group was selectively acylated with di-*tert*-butyldicarbonate resulting in the introduction of Boc protecting group. Proton-NMR obtained in DMSO-d_6 confirmed the presence of the protecting group as well as the unaltered hydroxyl. The remaining three steps of the synthesis were performed similarly to the previously published method for preparation of hydrazido-PEG-DSPE [39]. Briefly, a succinimidylcarbonate (SC) group, introduced at the hydroxy-end of the polymer, was used to form urethane attachment of the polymer to the amino group of DSPE yielding Boc-protected amino-PEG-DSPE (**1**) at the end of the PEG chain. The primary amine functionality was regenerated by acidolytic removal of the Boc-group forming **2**. Finally, PDP-PEG-DSPE (**3**) was obtained by acylation of the terminal amino group with SPDP.

Alternatively α-amino-ω-hydroxy-PEG could be directly reacted with SPDP substituting only the amino group. Then the remaining hydroxy end group was cleanly converted into succinimidyl carbonate and the resulting macromolecular analog of SPDP (PDP-PEG-SC) was coupled to DSPE. It appears that the second approach is preferable for a direct preparation of PDP-PEG-DSPE (**3**). It is two steps shorter and it resulted in higher yields of the product. The first approach is more general and allows conversion of amino-PEG-DSPE [24] into various functionalized PEG-DSPE-derivatized conjugates via use of different heterobifunctional reagents [38].

It is important to note that the utility of the new macromolecular crosslinker, PDP-PEG-SC clearly extends beyond the current application. It is in a sense a PEG analog of SPDP and as such offers several advantages. Unlike SPDP, PDP-PEG-SC is water soluble. Since, PEG is a material of choice for rendering surfaces biocompatible, PDP-PEG-SC is perfectly suitable for linking ligands to biomaterials intended for blood plasma contact. It is ideally suited for preparation of two or more component macromolecular conjugates, e.g., protein-protein crosslinking. In this case, the PEG spacer, being very flexible and well solvated in water, would allow maximum freedom of mobility for each of the conjugated components.

Table 1
Coupling efficiency and Ab density in liposomes containing PDP-PEG-DSPE

mol% mPEG-DSPE	mol% PDP-PEG-DSPE	Initial Ab/PL molar ratio	Ab density (μg Ab/μmol PL)	Coupling efficiency (%)
3	2	1: 250	316	51
		1: 500	265	85
		1:1000	136	87
4	1	1:1000	114	73
		1:2000	63	81
4.5	0.5	1: 500	82	26
		1:1000	76	48
		1:2000	66	85
4.75	0.25	1: 500	41	13
		1:1000	36	23
		1:2000	35	45
5	0	1: 500	11	3.4
		1:1000	10	6.1
		1:2000	7	9.5
0	1	1:1000	93	60
		1:2000	69	88

Liposomes were composed of HSPC/CHOL, 2:1 molar ratio and contained 5 mol% total PEG, consisting of a combination of mPEG-DSPE and PDP-PEG-DSPE as indicated. Liposomes averaged 100 nm in diameter. The source of Ab was sheep IgG and the Ab/PL molar ratio in the coupling mixture (2 mM PL) was varied from 1:250 to 1:2000. The coupling procedure, schematically shown in Fig. 1, is described in detail in Materials and methods. The coupling efficiency is expressed as the % of initial Ab attached to the liposomes.

3.2. Coupling of antibodies to liposomes containing PDP-PEG-DSPE

The PDP-PEG-DSPE could be incorporated easily into liposomes during their formation and Ab could be efficiently coupled to the surface of liposomes containing PDP-PEG-DSPE following the reduction of the PDP group with DTT. A comparison of the effect of the PDP-PEG-DSPE content in the liposomes, and the effect of Ab/PL ratio on coupling efficiency and the Ab density conjugated at the liposome surface is given in Table 1. Higher mol% of PDP-PEG-DSPE incorporated into the liposomes resulted in higher Ab densities at the liposome surface, with the highest densities being over 300 μg Ab/μmol PL (Table 1) corresponding to approx. 150 Ab/100 nm diameter liposome, which is almost complete coverage of the

surface of the liposome. One can estimate that, at complete coverage, 200 Ab can be bound to a liposome 100 nm in diameter. These calculations were based on the following assumptions: the effective diameter of a IgG_1 molecule is 142 Å [40] (150 000 Da), the area per polar head for HSPC is 72 $Å^2$ and for CHOL is 19 $Å^2$ [41] with an combined area per phospholipid of 81.5 $Å^2$ for a HSPC/CHOL (2:1 mol/mol) liposome.

As the amount of PDP-PEG-DSPE in the liposomes was decreased, the Ab density at the liposome surface also decreased, but even at the lowest concentration of PDP-PEG-DSPE (0.25 mol%), substantial amounts of Ab still could be conjugated to the liposome surface (35–41 μg Ab/μmol PL) (Table 1). In the absence of PDP-PEG-DSPE, very low amounts of Ab were associated with the liposomes (approx. 10 μg Ab/μmol PL), possibly through passive absorption (Table 1).

When the initial Ab/PL molar ratios were kept constant (e.g., 1:1000), the coupling efficiency decreased as the concentration of PDP-PEG-DSPE in the liposomes decreased, from a high of 87% of the Ab attached to liposomes in the presence of 2 mol% PDP-PEG-DSPE to a low of 23% in the presence of 0.25 mol% PDP-PEG-DSPE (Table 1). Although Ab density decreased as the initial Ab/PL molar ratio decreased, the coupling efficiency increased as this ratio decreased. A plot of coupling efficiency versus PDP-PEG-DSPE/Ab ratio demonstrates that the % of initial Ab bound to the liposomes reaches a maximum of greater than 80% above PDP-PEG-DSPE/Ab ratios of 10:1 (Fig. 3). In other words, for maximum coupling efficiency to occur, the PDP-PEG-DSPE must be present in approximately a 10-fold excess.

In the absence of mPEG-DSPE in the liposomes, equivalent amounts of Ab became associated with the liposomes as in the presence of mPEG-DSPE, with similar coupling efficiencies (Table 1). This demonstrates that the Ab can gain easy access to its coupling site at the PEG terminus.

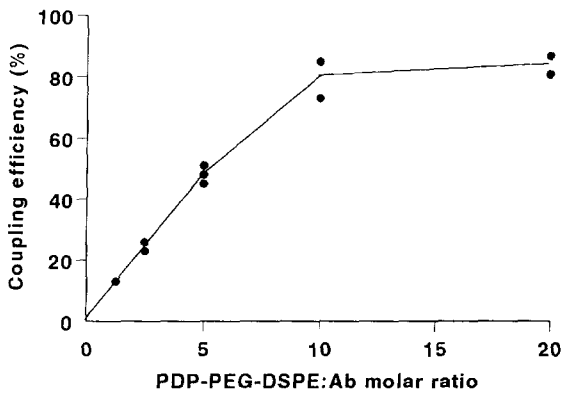

Fig. 3. Coupling efficiencies as a function of PDP-PEG-DSPE/Ab molar ratio. Liposomes (100 nm in diameter), composed of HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02 molar ratio), were coupled to Ab (sheep IgG) at various molar ratios of PDP-PEG-DSPE/Ab (2 mM phospholipid). The coupling efficiency is expressed as the % of initial Ab bound to the liposomes.

Table 2
Coupling efficiency and Ab densities for liposomes containing PDP-DOPE

Initial Ab/PL molar ratio	Ab density (μg Ab/μmol PL)	Coupling efficiency (%)
1: 500	28	9
1:1000	15	10
1:2000	7.4	9

Liposomes were composed of HSPC/CHOL, 2:1 molar ratio and contained 5 mol% mPEG-DSPE and 1 mol% PDP-DOPE. Liposomes averaged 100 nm in diameter. The source of Ab was sheep IgG and the Ab/PL molar ratio in the coupling mixture (2 mM PL) was varied from 1:500 to 1:2000. The coupling procedure was as outlined in Materials and methods. The coupling efficiency is expressed as the % of initial Ab attached to the liposomes.

This is in contrast to methods where Ab is coupled at the liposome surface when mPEG can sterically interfere with access of Ab to its coupling site [8]. Further data in support of this conclusion is presented in Table 2. For liposomes containing PDP-DOPE and mPEG-DSPE (i.e., the PDP group is at the liposome surface), the coupling efficiency and Ab densities are significantly lower than for liposomes containing PDP-PEG-DSPE. We suggest that this is due to steric hinderance by mPEG-DSPE to access of maleimide-Ab to the reduced PDP groups (thiol-PE) at the liposome surface.

3.3. Remote loading of doxorubicin and drug leakage rates

In the PDP-PEG-PE coupling method, the MPB group is attached to the Ab rather than to the liposome surface, as in coupling methods using MPB-PE [6,7]. Hence the MPB group is present in much lower concentrations, and is located much further from the immunoliposome surface after conjugation, than in the MPB-PE method. DXR could be remote loaded into SL (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE, 2:1:0.08:0.02) prior to Ab conjugation with greater than 95% efficiency after 1 h at 65° C. The half-life for release of doxorubicin from these liposomes, following conjugation of Ab was in excess of 150 h. This is in contrast to the difficulties with the remote loading of doxorubicin into liposomes containing the MPB group at the liposome surface (MPB-PE method), where the presence of the hydrophobic MPB group at the liposome surface appears to significantly decrease the rate of loading of doxorubicin and increase the rate of drug leakage [8].

3.4. Pharmacokinetics of S-immunoliposomes formed by the PDP-PEG-DSPE method

Immunoliposome clearance was determined with liposomes containing the aqueous space label, ^{125}I-tyraminyl-inulin (^{125}I-TI). Free ^{125}I-TI was removed from the body with a $t_{1/2}$ of a few minutes, while the liposome-entrapped label had a long latency time in the body with a $t_{1/2}$ of 78

h, indicating that the label had a very slow leakage rate from the liposomes in vivo. Leakage of the label was totally independent of the presence of Ab on the liposomes. The blood levels, as a function of time, for SIL containing increasing Ab densities (sheep IgG), formed by the PDP-PEG-PE method, are shown in Fig. 4A and B. Polyclonal sheep IgG was used for these experiments in order to not to deplete our limited supplies of monoclonal antibodies, however in our experience, there is no difference in the pharmacokinetics between polyclonal and monoclonal antibodies.

The addition of PDP-PEG-DSPE into S-liposomes (no Ab) did not significantly alter their circulation times ($t_{1/2}$ of 16 h, with greater than 80% of the label remaining liposome entrapped and therefore remaining in vivo). The SIL were removed from circulation in a log-linear manner

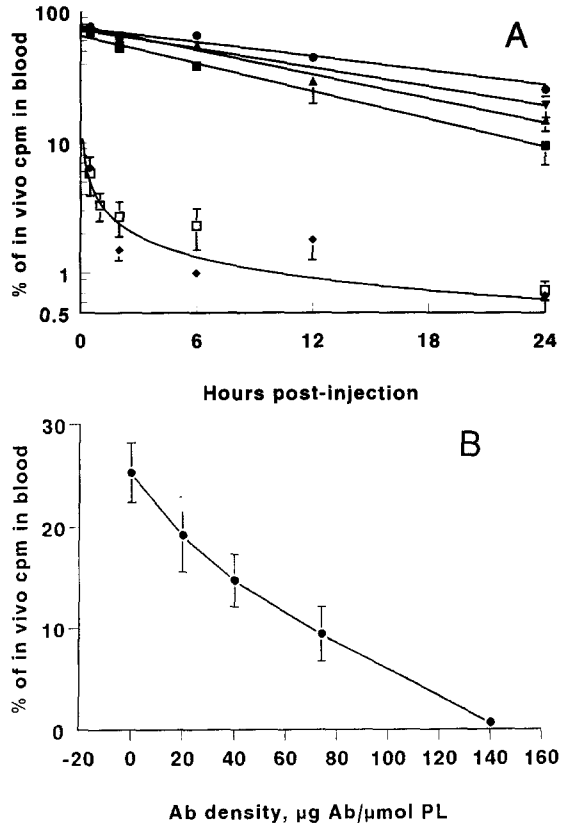

Fig. 4. Blood clearance kinetics of S-immunoliposomes in mice. Antibody (sheep IgG) was coupled at various densities to liposomes (HSPC/CHOL/PDP-PEG-DSPE, 2:1:0.02 molar ratio ±4 mol% mPEG-DSPE, 100 nm diameter) containing the aqueous space label ^{125}I-TI. The resulting S-immunoliposomes were injected i.v., via the tail vein, into CD_1 (ICR) mice. Results are expressed as label remaining in blood as a percentage of the label remaining in the body at various times post injection (means ± S.D., $n = 3$). (A) Blood clearance of S-immunoliposomes as a function of time and Ab density. Liposomes contained various Ab densities in the presence of mPEG-DSPE (●) no Ab; (▼) 20 µg Ab/µmol PL; (▲) 40 µg Ab/µmol PL; (■) 74 µg Ab/µmol PL; (◆) 140 µg Ab/µmol PL. Control liposomes (◆) contained no mPEG-DSPE, and had an antibody density of 73 µg Ab/µmol PL. (B) Blood clearance of SIL at 24 h post-injection as a function of Ab density.

at Ab densities of 74 µg/µmol PL and lower (Fig. 4A) and the level of SIL in blood at 24 h decreased with an increase in the Ab density (Fig. 4B). High Ab densities (140 µg/µmol PL) resulted in rapid removal of the SIL from circulation (Fig. 4A and B), but no change in the rate of label leakage from the liposomes. Ab densities in the range of approx. 20–80 µAb/µmol PL resulted in $t_{1/2}$ in the circulation of from 7 to 9 h compared to 16 h in the absence of Ab (with greater than 90% of the entrapped liposome label remaining in vivo), which is likely to be sufficiently long to allow in vivo targeting. By contrast, liposomes lacking mPEG (73 µg Ab/µmol PL) were rapidly removed from circulation (Fig. 4A) even at low Ab densities, with half-lives of less than 0.5 h.

3.5. Binding to, and cytotoxicity to, cancer cells in culture by S-immunoliposomes

The binding by three different cancer cell lines, of SIL conjugated by the PDP-PEG-PE method to three different mAb specific for each cell line, is shown in Fig. 5. In each case the attachment of a specific mAb to SIL (SIL[174H.64], SIL[B43.13] or SIL[M170]) resulted in 2-to 3-fold increased binding of the SIL to their respective cell lines (KLN-205, Fig. 5A; Caov.3, Fig. 5B; or HCT-15, Fig. 5C). Increasing the Ab density at the liposome surface resulted in a modest increase in the amount of SIL bound to the KLN-205 cells (Fig. 5A). We have previously shown that an isotype matched non-specific Ab (B27.29, Biomira) conjugated to immunoliposomes by the PDP-PEG-PE method showed no increase in binding to KLN-205 cells compared with antibody-free liposomes [42,43]. Excess free mAb 174H.64 would compete for binding of SIL[174H.64] to KLN-205 cells, but free mAb B27.29 was not effective in displacing binding of SIL[174H.64] [42]. Incubation of HCT-15 cells in the presence of 10% FBS reduced the binding of SL (no mAb) and SIL[M170] to the cells by approx. 2-fold, but the increase in binding of SIL compared with SL was, if anything, greater in the presence of serum than in its absence (Fig. 5D versus 5C).

The ability of SIL[174H.64] to increase the cytotoxicity of entrapped DXR to KLN-205 cells was also examined (Table 3). The IC_{50} for DXR-SIL[174H.64] at 1 h incubation was lower than that for either free DXR or DXR-SL. For this short incubation time, the free DXR and DXR-SL, both of which would be expected to have only low levels of non-specific association with the cells, were likely washed away before the drug could be taken up into cells in significant amounts. In addition, the very high IC_{50} for DXR-SL is likely also due to the sequestration of the DXR inside liposomes, with relatively little DXR released from the liposomes during the 1 h incubation period. As anticipated, free DXR and DXR-SL had significantly lower IC_{50} after a 24 h incubation, while the SIL[174H.64] appeared to have reached close to its maximum cytotoxicity after 1 h incubation and only experienced a slight decrease in IC_{50}

Table 3
Cytotoxicity of SIL[174H.64] against murine squamous lung carcinoma cells in culture

Sample	IC_{50}, μg DXR/ml of media for KLN 205 cells	
	1 h incubation	24 h incubation
Free DXR	93	15
DXR-SL	> 200	68
DXR-SIL[174H.64]	42	31

Murine squamous lung carcinoma (KLN 205) cells were plated in 96-well plates on day 1. 24 h later, free DXR, DXR-SL or DXR-SIL[174H.64] was incubated with the cells for 1 h or 24 h prior to gently washing the cells three times with phosphate-buffered saline, pH 7.4. The cells were then incubated for a further 47 or 24 h, respectively (total incubation time, 48 h). The IC_{50} (μg DXR/ml) was determined from cell viability using a tetrazolium dye assay [35].

after 24 h. At 24 h, SIL[174H.64] were slightly less cytotoxic than free DXR, although more cytotoxic than DXR-SL. It would appear that the 1 h incubation period was sufficient for binding and/or internalization of the SIL[174H.64] to liposomes, as the longer incubation time did not result in very large increases in cytotoxicity for these liposomes. The 1 h incubation would be more similar to the in vivo situation, where free DXR would be rapidly distributed throughout the body in a large volume of distribution [44,45], with tumor cells exposed to relatively low concentrations of the drug. The SIL, on the other hand, when bound to tumor cells in vivo, might be expected to deliver higher amounts of drug to the tumor in a sustained manner, either through release of locally high concentrations of drug from SIL bound at the tumor surface and uptake of DXR through the normal uptake mechanisms, or through Ab-mediated internalization of the drug-liposome package. At present we are undertaking experiments to distinguish between the relative contribution of these two mechanisms of SIL-mediated cytotoxicity, however, independent of the mechanism of cytotoxicity, we have recently demonstrated a dramatic increase in mean survival time of mice inoculated with the KLN-205 cell line and treated with DXR-SIL[174H.64] relative to DXR-SL or free DXR [46].

In conclusion, we developed a simple 3 step synthesis of new end-group functionalized PEG-lipid, PDP-PEG-DSPE, starting from α-amino-ω-hydroxy-PEG. Incorpora-

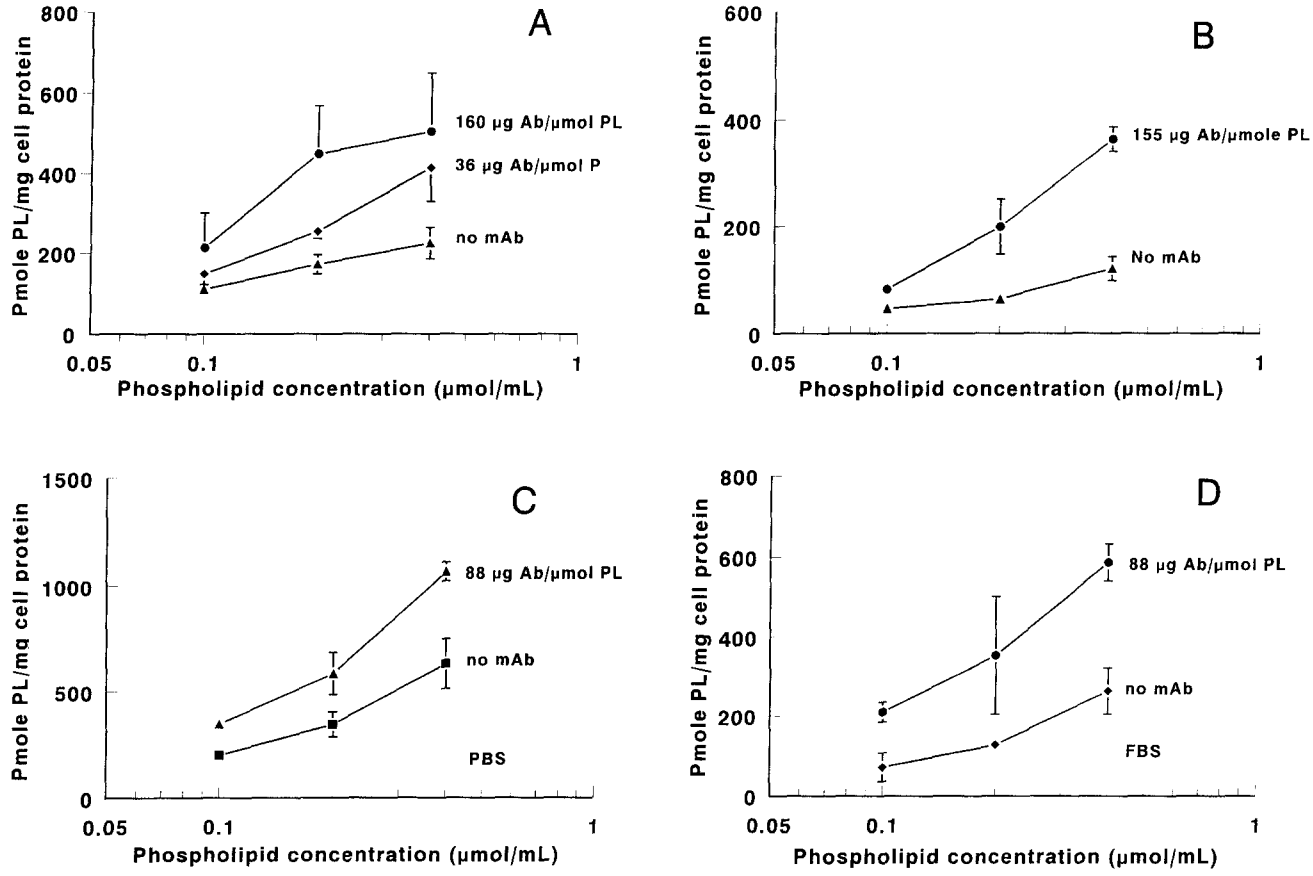

Fig. 5. Binding of S-immunoliposomes by various tumor cell lines. S-immunoliposomes in PBS (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE. 2:1:0.08:0.02 molar ratio, 100 nm in diameter) were labelled with ^{3}H-CHE and incubated for 1 h at 37° C with cells at a liposome concentration of 0.1–0.4 μmol PL/ml. (A) Murine squamous lung carcinoma (KLN205) and mAb 174H.64 coupled to SIL; (B) Human ovarian adenocarcinoma (Caov-3) and mAb B43.13 coupled to SIL; (C) human colon adenocarcinoma (HCT-15) and mAb M170 coupled to SIL. (D) HCT-15 cells and mAb M170 coupled to SIL incubated in the presence of 10% FBS. means ± S.D., $n = 3$.

tion of this PEG-lipid conjugate into liposomes allows for convenient generation of thiol groups at the distal ends of the grafted PEG chains. Incubation of the vesicles containing thiol-PEG-DSPE (**4**) with maleimide-antibodies resulted in efficient conjugation even when content of the functionalized PEG-DSPE component in the liposomes was below 1 mol%. This method realizes many of the criteria which would be desirable for 'ideal' S-immunoliposomes including simplicity, high coupling efficiency, the ability to achieve a large range of Ab densities at the liposome surface, significantly increased survival times in circulation compared to classical immunoliposomes, efficient drug loading, slow drug release rates, and retention of target binding.

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