A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs

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Abstract We have developed a method for producing sterically stabilized immunoliposomal drugs (SIL) readily applicable to a 'mix and match' combinatorial approach for the simple manufacture of a variety of ligand-targeted liposomal drugs. Ligands coupled to the terminus of polyethylene glycol (PEG) in micelles formed from PEG-lipid derivatives ($mPEG_{2000}$-DSPE) could be transferred into preformed, drug-containing liposomes from the micelles in a temperature- and time-dependent manner. Antibody densities up to 100 μg antibody/μmol of phospholipid, and up to 3 mol% of $mPEG_{2000}$-DSPE, could be simultaneously transferred from the ligand-coupled micelles into the liposomal outer monolayer with negligible drug leakage from liposomes during transfer and good stability in human plasma. Transfer of anti-CD19 into SIL resulted in a three-fold increase in binding of these liposomes to $CD19^+$ human B cell lymphoma cells.

Key words: Long circulating liposome; Targeted drug delivery; Polyethylene glycol micelle; Liposomal doxorubicin; Immunoliposome; Antibody transfer

1. Introduction

Liposomes are receiving widespread acceptance as carriers of therapeutic drugs. Insertion of polyethylene glycol (PEG)-derivatized phospholipids (PL) into liposomal membranes, forming sterically stabilized liposomes (SL), decreases liposome clearance and increases liposome accumulation in disease sites [1–3], which can result in increased therapeutic indices for the entrapped drug (for reviews see [4–6]). Further increases in selectivity of liposomal drugs can be achieved through the use of targeting moieties attached at the liposome surface, and several investigators have devised methods for covalently attaching targeting molecules such as antibodies (Abs) to the terminus of polymer-derivatized PL (reviewed in [5,7–9]). If sterically stabilized targeted liposomes (SIL) are to be useful in the clinic, ways of tailoring the SIL to the patient's disease profile are needed that do not require a separate manufacturing line for each ligand and drug combination. We therefore borrowed from combinatorial chemistry to develop an approach which allows a variety of SIL to be made by a simple mixing procedure from a small number of starting products (Fig. 1).

Our aim was to develop a simple protocol to produce SIL by transferring ligand-coupled PEG-lipid derivatives into preformed liposomes from a micelle phase. These experiments are an extension of our previous work in which we demonstrated that $mPEG_{2000}$, covalently linked to distearoylphosphatidylethanolamine ($mPEG_{2000}$-DSPE), could be transferred from a micellar phase into preformed classical liposomes (CL) transforming them into SL [10]. Maleimide-terminated PEG_{2000}-DSPE (MAL-PEG_{2000}-DSPE) is a coupling lipid, which has previously been used to directly couple ligands to the PEG terminus of SL, converting them into SIL [11]. It can also be incorporated into PEG micelles, allowing coupling of ligands to the micellar lipids, which could then, in theory, be transferred into preformed liposomes, forming SIL.

The functionality of this approach was tested by transferring anti-CD19 PEG_{2000}-DSPE into preformed SL and examining the binding of the resulting SIL to $CD19^+$ human B lymphoma cells. In our previous work, treatment of murine models of human B lymphoma with CD19-targeted SIL containing entrapped DXR resulted in significantly increased life spans of the animals compared to non-targeted liposomal DXR or free drug [12]. A long-term aim is to reproduce these results using the micelle transfer method for formation of SIL described in this paper.

Liposome DXR, sterically stabilized with $mPEG_{2000}$-DSPE (Caelyx/Doxil), is currently approved for clinical use in the treatment of refractory Kaposi's sarcoma and ovarian cancer [13–15]. It would be useful, from a manufacturing point of view, if products such as this could be simply transformed into patient-selective immunoliposomes in order to increase their therapeutic index in the treatment of solid tumors or hematological malignancies. For the micelle transfer method of forming SIL to be clinically useful, it is important that the process of transfer of $mPEG_{2000}$-DSPE and ligand-PEG_{2000}-DSPE into preformed drug-containing liposomes not result in premature release of the drug. As a test of this, we examined the rate of release of entrapped DXR from preformed liposomes when they were exposed to IgG-PEG_{2000}-DSPE micelles. We further tested the ability of ligand-PEG_{2000}-DSPE to transfer into SL, i.e. into liposomes which already had $mPEG_{2000}$-DSPE incorporated symmetrically into liposomal membranes during their manufacture. In this regard, we measured the ability of IgG-PEG_{2000}-DSPE micelles to transfer both PEG and IgG into Caelyx/Doxil.

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Abbreviations: Ab, antibody; Caelyx/Doxil, doxorubicin-containing sterically stabilized liposomes; CHOL, cholesterol; ^{14}C-$mPEG_{2000}$-DSPE, [carbamoyl-^{14}C]$mPEG_{2000}$-DSPE; ^{3}H-CHE, cholesteryl-(1,2-[^{3}H](*N*)]-hexadecyl ether; CL, classical liposomes (liposomes without $mPEG_{2000}$-DSPE); CMC, critical micellar concentration; DSPE, distearoylphosphatidylethanolamine; DXR, doxorubicin; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HSPC, hydrogenated soy phosphatidylcholine; mAb, monoclonal antibody; MAL-PEG_{2000}-DSPE, maleimide-terminated polyethylene glycol (M_r 2000) covalently coupled to DSPE; $mPEG_{2000}$-DSPE, methoxypolyethylene glycol (M_r 2000) covalently coupled to DSPE; PL, phospholipid; SL, sterically stabilized (Stealth) liposomes; SIL, sterically stabilized (Stealth) immunoliposomes

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2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC), $mPEG_{2000}$-DSPE and sterically stabilized liposomes containing entrapped doxorubicin (DXR-SL, Caelyx/Doxil) were generous gifts from SEQUUS Pharmaceuticals, Inc. (Menlo Park, CA, USA). MAL-PEG_{2000}-DSPE was custom synthesized by Shearwater Polymers, Inc. (AL, USA). Cholesterol (CHOL) was purchased from Avanti Polar Lipids (AL, USA). Sephadex G-50 and Sepharose CL-4B were purchased from Pharmacia Biotech (Uppsala, Sweden). Cholesteryl-[1,2-[^{3}H](*N*)]-hexadecyl ether (^{3}H-CHE) and $Na^{125}I$ were purchased from Mandel Scientific (Ontario, Canada). [Carbamoyl-^{14}C]$mPEG_{2000}$-DSPE (^{14}C-$mPEG_{2000}$-DSPE) was custom synthesized by Chemsyn Science Laboratories (KS, USA). 2-Iminothiolane, metrizamide and sheep IgG (reagent grade) were purchased from Sigma (MO, USA). Murine mAb anti-CD19 was prepared by us from the FMC-63 murine CD19 hybridoma cell line obtained from Dr. H. Zola (Children's Health Research Institute, Australia). Iodination of IgG was performed according to the method described elsewhere [12]. The human B cell lymphoma line Namalwa (ATCC CRL 1432) was obtained from American Type Culture Collection (MD, USA). All other chemicals were of analytical grade and purity.

2.2. Preparation of liposomes

Liposomes were prepared as previously described [11]. CL were composed of HSPC:CHOL (2:1, molar ratio). SL additionally contained 2 or 4 mol% $mPEG_{2000}$-DSPE as a ratio to total PL (SL:2 mol% and SL:4 mol%). Caelyx/Doxil is HSPC:CHOL:$mPEG_{2000}$-DSPE, 2:1:0.184. Liposomes, including Caelyx/Doxil, had diameters in the range of 90–100 nm. ^{3}H-CHE was added as a non-metabolized and non-exchangeable lipid tracer in some experiments [16]. When applicable, DXR-loaded liposomes were prepared by remote loading using ammonium sulfate gradients at a DXR:PL ratio of 0.2:1 (w/w), as previously described [17]. The concentration of the liposome-entrapped DXR was determined by spectrophotometry ($\lambda = 490$ nm), and PL concentrations were determined using the Fiske-Sabbarow colorimetric assay [18].

2.3. Preparation of IgG-coupled PEG_{2000}-DSPE micelles

Sheep IgG or anti-CD19 mAb was coupled to micelles containing $mPEG_{2000}$-DSPE and the coupling lipid MAL-PEG_{2000}-DSPE at a 4:1 molar ratio with trace amounts of ^{14}C-$mPEG_{2000}$-DSPE. $mPEG_{2000}$-DSPE was necessary to prevent intermicellar crosslinking during coupling with IgG. Dried lipid film containing $mPEG_{2000}$-DSPE and MAL-PEG_{2000}-DSPE were hydrated in HEPES buffer (pH 7.4) at a concentration above the critical micellar concentration (CMC) of the lipids with gentle agitation and heating at 60°C. Coupling of IgG to MAL-PEG_{2000}-DSPE in the preformed micelles was carried out according to a previously described method [11].

2.4. Transfer of IgG-PEG_{2000}-DSPE into preformed liposomes

Transfer of IgG-PEG_{2000}-DSPE into preformed liposomes was initiated by mixing aliquots of the IgG-micelles with preformed liposomes for varying times and temperatures at various ratios of liposomal PL to PEG_{2000}-DSPE followed by chromatography on a Sepharose CL-4B columns eluted with HEPES buffer (pH 7.4). The amounts of transferred IgG, transferred PEG_{2000}-DSPE and liposomal PL in the liposome fractions were assayed. The size of resulting liposomes was also determined.

2.5. Cell binding experiments

SIL[anti-CD19] for cell binding experiments were prepared by transferring, at 60°C for 1 h, anti-CD19 mAb-PEG_{2000}-DSPE micellar lipid into preformed SL:4 mol% at a liposomal PL:micellar PEG_{2000}-DSPE molar ratio of 100:3. SL were radiolabelled with ^{3}H-CHE. Namalwa cells (human B lymphoma cells) were plated at 1×10^6 cells/300 μl RPMI 1640 supplemented with 10% (v/v) FBS in 24-well tissue culture plates. SL:4 mol%, with or without transferred anti-CD19 PEG_{2000}-DSPE, were added to each well (200–800 nmol/ml in a total volume of 600 μl) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 1 h incubation, the cells were washed three times with cold PBS (pH 7.4), and the amount of ^{3}H-CHE-liposomes associated with the cells was determined by scintillation counting (Aqueous Scintillant scintillation fluid) in a Beckman LS-6800 counter. The amount of liposomes bound (in pmol PL) was calculated from the initial specific activity of ^{3}H-CHE-liposomes.

2.6. In vitro doxorubicin leakage experiments

Liposomes containing entrapped DXR were chromatographed on a Sephadex G-50 column immediately prior to use to remove any residual free DXR. IgG-coupled micelles were added to DXR-containing liposomes (1.5 mM) at a PL molar ratio of 3:100 and incubated at either 37°C or 60°C. Liposomes contained DXR at concentrations sufficient to cause fluorescence quenching and DXR leakage was determined by fluorescence dequenching on a CytoFluor 2350 fluorimeter (Millipore, Bedford, MA, USA) at excitation and emission wavelengths of 485 nm and 590 nm, respectively. Total DXR (100% dequenched) was measured by lysing the liposomes with 5% (v/v) Triton X-100.

2.7. Dissociation of IgG-PEG_{2000}-DSPE and $mPEG_{2000}$-DSPE from SIL in vitro

SIL were prepared by transferring IgG from micelles into SL:4 mol% (1.2 mM) followed by incubation in human plasma (50% v/v) at 37°C for 48 h. IgG-PEG_{2000}-DSPE or $mPEG_{2000}$-DSPE dissociated from the SIL was determined by chromatography on a Sepharose CL-4B columns in HEPES buffer, pH 7.4.

2.8. Statistics

Statistic analyses were performed using StatView software (Abacus Concepts, Inc., CA, USA).

3. Results and discussion

The CMC of $mPEG_{2000}$-DSPE and MAL-PEG_{2000}-DSPE was determined by monitoring micelle turbidity at 240 nm. Regression analysis indicated that the CMCs for $mPEG_{2000}$-DSPE and MAL-PEG_{2000}-DSPE were approximately 6.3 and 2.3 μM, respectively (not shown). Kanda et al. have previously demonstrated that a nitroxide spin-labeled diacyl ganglioside, which has a similar CMC, could be inserted from the micelle phase into preformed multilamellar vesicles [19]. Micelles composed of $mPEG_{2000}$-DSPE and MAL-PEG_{2000}-DSPE (4:1, mol/mol) had a diameter of 30–50 nm. Following coupling with IgG, the micelle diameter increased to approximately 65–80 nm.

Coupling efficiencies of IgG to preformed micelles were determined by separating IgG-micelles from free IgG by ultracentrifugation on a metrizamide gradient. Free IgG was located at the bottom of the gradient (Fig. 2). The main peak of IgG-micelles (86%) was located at the middle of the gradient,

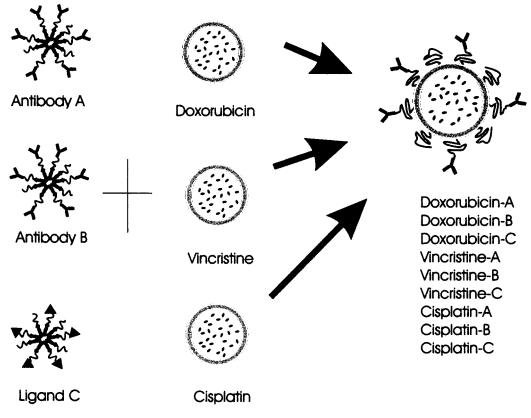

Fig. 1. Cartoon depicting the combinatorial approach to the formation of ligand-targeted liposomal anticancer drugs.

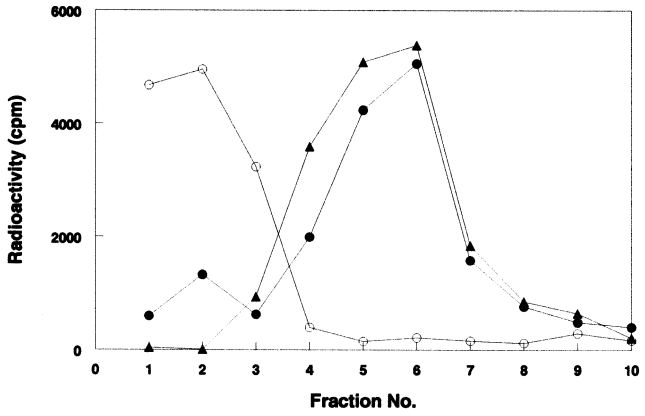

Fig. 2. Fractionation of IgG-micelles on a metrizamide gradient. IgG-micelles containing ^{125}I-labeled IgG and ^{14}C-labeled mPEG$_{2000}$-DSPE were fractionated on a 40/50/60% (3/4/3 ml) discontinuous metrizamide gradient at 37 000 rpm for 14 h in a SW41 rotor using an L8-70 Ultracentrifuge (Beckman, CA, USA). Free IgG spiked with tracer ^{125}I-IgG and was also fractionated, for a control. Ten 1 ml fractions were collected starting from the bottom of the gradient and counted for ^{125}I and ^{14}C. ^{125}I counts from IgG-PEG$_{2000}$-DSPE micelles (●); ^{14}C counts from IgG-PEG$_{2000}$-DSPE micelles (▲); ^{125}I counts from free IgG (○).

similar to the peak for the micelles. This shows that most of the IgG coupled to the preformed micelles.

It has been shown that symmetrical inclusion of 3–10 mol% mPEG$_{2000}$-DSPE (approximately 1.8–6 mol% PEG in the outer monolayer) in liposomes resulted in prolonged liposome circulation times [20]. Above Ab densities of approximately 75 μg/μmol PL, an increased rate of clearance of SIL from circulation has been reported [21]. Therefore, we aimed to achieve approximately 3 mol% of mPEG$_{2000}$-DSPE in the outer monolayer and an Ab density of less than 75 μg/μmol PL following transfer of IgG-PEG$_{2000}$-DSPE and mPEG$_{2000}$-DSPE into preformed liposomes. mPEG$_{2000}$ already included in the preformed liposomes might be expected to reduce the transfer of additional IgG-PEG$_{2000}$-DSPE and mPEG$_{2000}$-DSPE to the liposomes. To address this, transfer from IgG-micelle conjugates into liposomes was examined for liposomes containing 0, 2, 4 or 9.2 mol% of mPEG (Fig. 3). Transfer experiments were carried out at 60°C for 1 h which is above the phase transition for HSPC [10]. The amount of IgG (Fig. 3A) and mPEG$_{2000}$-DSPE (Fig. 3B) transferred to the liposomes increased in proportion to the concentration of IgG-coupled micelles added. In the presence of mPEG$_{2000}$-DSPE, the transfer of IgG-PEG$_{2000}$-DSPE and mPEG$_{2000}$-DSPE to liposomes significantly decreased (Fig. 3A,B). Therefore, if the liposomes already contain mPEG$_{2000}$-DSPE, the additional insertion of micellar IgG-PEG$_{2000}$-DSPE into the liposomes was inhibited. However, except at the highest concentration of mPEG$_{2000}$-DSPE, found in Caelyx/Doxil, sufficient transfer of IgG into the preformed liposomes occurred that the resulting SIL should show good target binding as well as long circulation times in vivo (Fig. 3A). For Caelyx/Doxil, the presence of mPEG$_{2000}$-DSPE in the outer monolayer (approximately 5 mol%) allowed only low levels of insertion of IgG-PEG$_{2000}$-DSPE from micelles, which would be borderline for targeting applications. The upper limit for introducing mPEG$_{2000}$-DSPE into bilayers appears to be approximately 6 mol% [10], consistent with our present findings. Zalipsky et al. have also shown that 2 mol% of sialyl Lewis^x oligosaccharide-PEG$_{2000}$-DSPE conjugate transferred into preformed SL already containing 2.8 mol% outer monolayer PEG$_{2000}$-DSPE, resulting in 5.3 mol% PEG$_{2000}$-DSPE incorporated into outer monolayer following transfer [22].

It cannot be determined if there is net transfer of ligand and/or mPEG$_{2000}$-DSPE into the liposomes at high concentrations of pre-existing PEG$_{2000}$-DSPE in the liposomal membrane, of if there is a component of exchange/transfer with some of the existing PEG$_{2000}$-DSPE leaving the liposomes to allow for the insertion of new PEG$_{2000}$-DSPE or ligand-PEG$_{2000}$-DSPE-conjugates.

We determined changes in mean liposome diameter following transfer of IgG-PEG$_{2000}$-DSPE into liposomes, to ensure that the product was within an acceptable diameter for extravasation into diseased tissues, generally accepted as 150 nm in diameter or less. Larger liposomes are rapidly removed from the circulation into tissues of macrophage origin and do not achieve significant levels in other tissues of the body (reviewed in [23]). The mean diameters of the 100 nm CL and SL increased by 32–36 nm and 22–26 nm, respectively. The extension length of the grafted PEG on the surface of phosphatidylcholine liposomes containing 6 mol% mPEG$_{2000}$-DSPE is 6 nm [24]. The maximal linear dimension of IgG is about 10.5 nm in the electron microscope by shadowing and

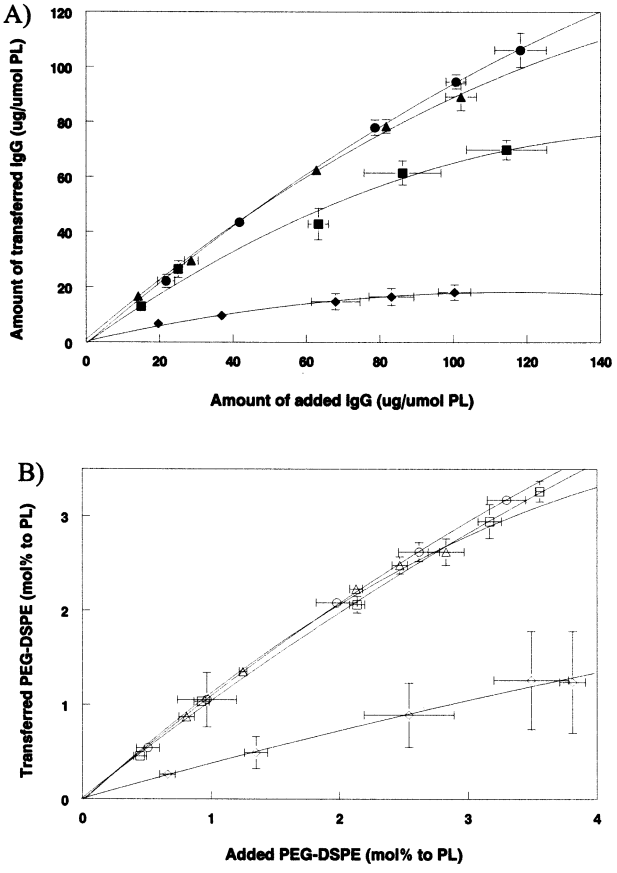

Fig. 3. Transfer of IgG-PEG$_{2000}$-DSPE into preformed liposomes. Transfer of IgG-PEG$_{2000}$-DSPE into liposomes (HSPC:CHOL, 2:1 molar ratio, 100 nm in diameter, ± mPEG$_{2000}$-DSPE) was initiated by mixing aliquots of IgG-micelles with the liposomes at specified ratios of liposomal PL to micellar PEG$_{2000}$-DSPE at 60°C for 1 h. A: Transferred IgG. B: Transferred PEG$_{2000}$-DSPE. CL (●,○); SL:2 mol% (▲,△); SL:4 mol% (■,□); Caelyx/Doxil (◆,◇). Data are given as mean ± S.D. (n = 3).

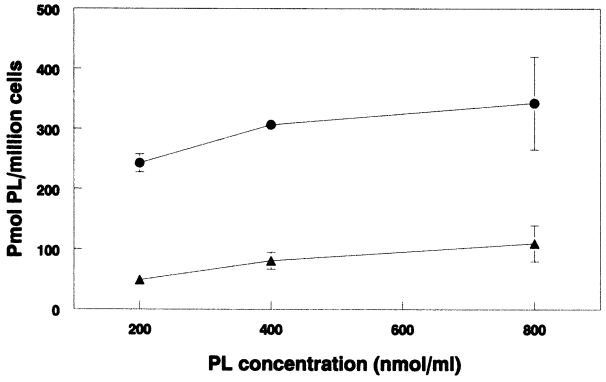

Fig. 4. Binding of SIL[anti-CD19] by CD19$^+$ human B lymphoma cells (Namalwa cells) as a function of liposome concentration. Liposomes were composed of HSPC:CHOL:mPEG$_{2000}$-DSPE (2:1:0.08 molar ratio; 120 nm in diameter) ± transferred anti-CD19-PEG$_{2000}$-DSPE. SIL[anti-CD19] (●) or SL (▲) were incubated with 1×10^6 Namalwa cells at 37°C. Data are expressed as pmol PL ± S.D./10^6 cells ($n=3$).

by negative contrast [25]. Therefore, our direct measurement was consistent with the 33 and 21 nm estimate inferred from these reports.

Time and temperature dependence of the transfer of lipid from 55 μg IgG-micelles into 1 μmol HSPC:CHOL liposomes was determined. At 37°C, after 1 h incubation small amounts of IgG-PEG$_{2000}$-DSPE (10.4%) transferred to the liposomes. After 24 h incubation the amount increased by approximately four-fold to 45.1%, approximately one-half of the amount transferred at 60°C for 1 h. The inefficient transfer might be because the transfer was done below the T_c of HSPC (58°C) [10]. As seen for incubations at 60°C (Fig. 3) the amount of transfer at 37°C was reduced in the present of pre-existing mPEG$_{2000}$-DSPE in the bilayers (not shown). In order to determine if IgG-PEG$_{2000}$-DSPE, not free IgG, was transferred into the liposomes, the non-specific adsorption of free IgG on liposomes was also examined under similar incubation times and temperatures. Less than 4% free IgG associated with liposomes ($P<0.05$ compared to IgG-PEG$_{2000}$-DSPE for 1 h at 37°C). This finding suggests that the lipid anchor of polymer-derivatized lipid (DSPE) coupled to IgG is responsible for the transfer of IgG into the preformed liposomal membrane.

Dissociation and/or exchange of the ligand-PEG$_{2000}$-DSPE and/or mPEG$_{2000}$-DSPE from the SIL would result in loss of their ability to bind to their target cells and would increase their clearance in vivo. To address this, we determined the off-rate of IgG-PEG$_{2000}$-DSPE and mPEG$_{2000}$-DSPE from SIL (60 μg IgG/μmol PL) after incubation in human plasma. Even after a 48 h incubation, the transferred IgG and mPEG$_{2000}$-DSPE remained associated with the SIL (102.3 ± 2.4% and 99.4 ± 8.2%, respectively). Parr et al. have reported that a primary factor for retention of the PEG coating is the lipid anchor and DSPE is one of the better lipid anchors [26]. The in vitro half-life of the exchange rate of mPEG$_{1900}$-DSPE out of the bilayer is reported to be 70 h at 37°C [27]. However, the rate at which IgG-lipid derivatives or mPEG$_{2000}$-DSPE dissociate from the liposomes under in vivo conditions has not yet been adequately addressed. Recent experience in our laboratory with anti-CD19 mAb attached to the PEG terminus of liposomes by hydrazide or maleimide linkages suggests that the in vivo stability of these immunoliposomes is good [12].

Addition of IgG-PEG$_{2000}$-DSPE to the outer monolayer of liposomes may cause transient membrane defects resulting in some release of liposome-encapsulated drugs. Therefore, the release of DXR was studied in the presence or absence of IgG-PEG$_{2000}$-DSPE micelles. At 60°C, approximately 4% of DXR was released during a 6 h incubation in the absence of IgG-micelles and this increased only 2–3% in the presence of IgG-micelles, when 87% of IgG was transferred to liposomes. Although 45% of IgG-PEG$_{2000}$-DSPE transferred from micelles to liposomes at 37°C over 24 h, there was less than a 4% release of DXR under these conditions.

Following the transfer of IgG-PEG$_{2000}$-DSPE into preformed liposomes at 60°C, reduction of the binding activity of IgG is possible if some denaturation of IgG occurs at this temperature. In vitro binding studies with SIL containing transferred anti-CD19 (SIL[anti-CD19]) were conducted with CD19$^+$ Namalwa (human B lymphoma) cells (Fig. 4). There was a three-fold increase in the association of SIL[anti-CD19] to Namalwa cells compared to that of SL [no mAb], which is similar to the fold increase previously reported for anti-CD19 coupled directly to liposomes [12]. These results show that the transferred IgG can retain sufficient binding activity to achieve selective binding of the SIL to targeted cells.

We showed in this study that IgG-PEG$_{2000}$-DSPE and mPEG$_{2000}$-DSPE are transferred into the membrane of preformed liposomes from IgG-micelle conjugates, with very little DXR release during the transfer and only small increases in liposome diameters. The resulting liposomes would be expected to have long circulation times and selective binding to target cells in vivo. The IgG-PEG$_{2000}$-DSPE transfer method described here is a simple and commercially acceptable method to produce targeted sterically stabilized liposomal drugs. Several liposomal preparations of anticancer drugs are approved for clinical use or are in clinical trials, including Caelyx/Doxil. As we envision it, the combinatorial approach for making many different formulations of targeted liposomes would involve a simple procedure, requiring only the selection of a vial of ligand-PEG-micelles for a one-step incubation with the appropriate liposomal drug formulation (Fig. 1). Our transfer method holds out the possibility for the transfer of a variety of antibody, peptide, and other ligands into a variety of preformed drug-containing liposomes to create a custom library of targeted liposomal drugs tailored to individual patient needs.

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