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Intermembrane transfer of polyethylene glycol-modified phosphatidylethanolamine as a means to reveal surface-associated binding ligands on liposomes

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Abstract

In order to explore the use of exchangeable poly(ethylene glycol) (PEG)-modified diacylphosphatidylethanolamines (PE) to temporarily shield binding ligands attached to the surface of liposomes, a model reaction based on inhibition and subsequent recovery of biotinylated liposome binding to streptavidin immobilized on superparamagnetic iron oxide particles (SA magnetic particles) was developed. PEG-lipid incorporation into biotinylated liposomes decreased liposome binding to SA magnetic particles in a non-linear fashion, where as little as 0.1 mol% PEG-PE resulted in a 20% decrease in binding. Using an assay based on inhibition of binding, PEG_{2000}-PE transfer from donor liposomes to biotinylated acceptor liposomes could be measured. The influence of temperature and acyl chain composition on the transfer of PEG-diacyl PEs from donor liposomes to acceptor liposomes, consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, cholesterol and *N*-((6-biotinoyl)amino)hexanoyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (54.9:45:0.1 mole ratio), was measured. Donor liposomes were prepared using 1,2-distearoyl-*sn*-glycero-3-phosphocholine (50 mol%), cholesterol (45 mol%) and 5 mol% of either PEG-derivatized 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE-PEG_{2000}), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE-PEG_{2000}), or 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG_{2000}). Transfer of DSPE-PEG_{2000} to the donor liposomes was not detected under the conditions employed. In contrast, DMPE-PEG_{2000} was transferred efficiently even at 4°C. Using an acceptor to donor liposome ratio of 1:4, the time required for DMPE-PEG_{2000} to become evenly distributed between the two liposome populations (T_{EQ}) at 4°C and 37°C was approx. 2 and < 0.5 h, respectively. An increase in acyl chain length from C14:0 to C16:0 of the PEG-lipid resulted in a significant reduction in the rate of transfer as measured by this assay. The transfer of PEG-lipid out of biotinylated liposomes was also studied in mice following intravenous administration. The relative rates of transfer for the various PEG-lipids were found to be comparable under in vivo and in vitro conditions. These results suggest that it is possible to design targeted liposomes with the targeting ligand protected while in the circulation through the use of PEG-lipids that are selected on the basis of exchange

Abbreviations: PEG, poly(ethylene glycol); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; biotin-X-DSPE (Bx-DSPE), *N*-((6-biotinoyl)amino)hexanoyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DMPE-PEG_{2000}, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; DPPE-PEG_{2000}, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*n*-methoxy(polyethylene glycol)-2000]; DSPE-PEG_{2000}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; Chol, cholesterol; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; HBS, HEPES-buffered saline

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characteristics which result in exposure of the shielded ligand following localization within a target tissue.

Keywords: Poly(ethylene glycol); Intermembrane transfer; Biotin; Liposome; Ligand binding

1. Introduction

Intermembrane transfer of conjugated lipids is of interest due to the active roles such lipids play in controlling surface reactions. Biologically relevant exchange processes include membrane fusion [1] and intracellular signaling mediated by the transfer of glycosylphosphatidylinositol (GPI)-linked peptides [2]. Lipid transfer is also becoming an important attribute in the development of liposomes as biopharmaceutical delivery systems. Two areas of particular interest concern: (i) membrane dissociation of antibody/phospholipid conjugates following interaction with a target ligand [3] and (ii) applications involving controlled loss of poly(ethylene glycol) (PEG) lipids to achieve a transformation of the liposome's surface characteristics [4,5]. It has been established that PEG-modified lipids can transfer out of liposomal membranes, a property that is dependent on the acyl chain composition of the PEG-modified lipids as well as the size of the PEG moiety [6]. Formulations can be designed where PEG-lipid transfer is associated with a change in the liposomal properties and this is perhaps best illustrated by the transformation of a stable liposome to one that is highly fusogenic [4,5,7].

The concept that PEG-lipid transfer could transform the properties of a liposome is novel and many practical applications of this technology can be considered. For example, despite the beneficial effect of PEG-lipid incorporation on liposome circulation lifetime [8–11], the development of liposomes with surface-grafted PEG moieties and attached targeting ligands has been restricted because of PEG-mediated inhibition of binding reactions between the targeting ligand and its binding site [12,13]. In order to address this problem, investigators have been coupling the targeting ligand to the distal end of PEG moieties which are chemically attached to lipids incorporated in the liposomes [14–19]. The rationale for the latter approach can be questioned given the propensity of the resulting liposome to be immunogenic [20]. In addition, the stability of the ligand-conjugated PEG-lipid in the liposomal membrane is potentially problematic given the tendency of PEG-lipids to exchange out of liposomes [6]. This problem may be exacerbated with the additional forces that play a role in dissociation/extraction of a lipid linked to an antibody following antibody antigen interaction [3]. An alternative approach to address this problem is to employ PEG-modified lipids that can be transferred from the lipid bilayer thereby slowly exposing small, surface-associated, targeting ligands.

In this regard, studies on lipid transfer are of particular interest in the development of liposomes with surface-active groups, such as a targeting ligand or charged moiety. Intervesicle transfer of phospholipids has been well documented in the past [6,21–23]. Conjugation of hydrophilic macromolecules, including PEG, to lipid anchors is known to affect the rate of transfer of the entire phospholipid out of the bilayer [6]. In this study, we describe a method to measure PEG-lipid transfer based on a functional assay for quantifying liposomal PEG-lipid content in the acceptor liposome. More specifically, we utilized well characterized biotin–streptavidin interactions as a tool to study (i) PEG-lipid transfer as measured by the generation of a surface shield that prevents biotinylated liposome binding to streptavidin immobilized on 1 μm superparamagnetic iron oxide particles and (ii) the effect of PEG-grafted polymers on biotinylated liposome binding to a defined surface. The assay described provides a very sensitive means to assess PEG-lipid transfer. From the perspective of using exchangeable PEG-modified lipids to shield small targeting ligands on the surface of liposomes, the results indicate that even small amounts (<1 mol%) of residual PEG-grafted lipids can lead to significant reductions in specific target binding.

2. Materials and methods

2.1. Materials

Cholesterol (Chol) and streptavidin immobilized on superparamagnetic iron oxide particles were purchased from Sigma (St. Louis, MO). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(-polyethylene glycol)-2000] (DMPE-PEG_{2000}), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] (DPPE-PEG_{2000}), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine-*n*-[methoxy(polyethylene glycol)-2000 (DOPE-PEG_{2000}) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Birmingham, AL). *N*-((6-Biotinoyl)amino)hexanoyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (Bx-DSPE), ^{3}H-DSPE-PEG_{2000}, ^{3}H-DPPE-PEG_{2000}, and ^{3}H-DMPE-PEG_{2000} were purchased from Northern Lipids (Vancouver, Canada). C_{14}-Cer-PEG_{2000} was a gift from Inex Pharmaceuticals (Vancouver, Canada). Pico-Fluor 40 scintillation cocktail was obtained from Canberra-Packard Canada (Mississauga, ON). Nuclepore polycarbonate filters were obtained from Watman (Clifton, NJ). Biogel A15M size exclusion gel was obtained from Bio-Rad (Mississauga, ON). ^{3}H-Cholesteryl hexadecyl ether (CHE) was obtained from NEN Life Science (Boston, MA). RAG-2 mice and Balb/c mice were bred in-house at the BC Cancer Agency animal facility (Vancouver, Canada).

2.2. Preparation of large unilamellar vesicles

Liposomes were prepared using the extrusion method of Mayer et al. [24]. Lipid mixtures (100–150 μmoles total lipid) were first dissolved in chloroform. ^{3}H-Cholesteryl hexadecyl ether, used as a liposome label, was added to the lipid mixture to obtain a specific activity of 0.1 μCi/μmole. A lipid film was formed from the lipid mixture by first drying under nitrogen gas and then under high vacuum for at least 3 h. The lipid film was hydrated at 65°C in 1.5 ml of HEPES-buffered saline (HBS; pH 7.4). The resulting multilamellar vesicles were subjected to five freeze–thaw cycles and then extrusion (10 times) using two 0.08 μm and one 0.1 μm polycarbonate membranes with an extrusion device (Lipex Biomembranes, Vancouver, Canada) which was thermoregulated at 65°C. Liposome size was determined by quasielastic light scattering (QELS) using a Nicomp 370 submicron particle sizer operating at a wavelength of 632.8 nm. The liposomes used in this study exhibited diameters of 100–120 nm.

2.3. Biotinylated liposome binding assay

Biotinylated liposomes (DOPC/Chol/Bx-DSPE; 55:45:0.1) binding to target was determined by incubating the liposomes (100 nmoles in 100 μl HBS) for 20 min at room temperature with 100 μl of streptavidin immobilized on iron oxide particles prepared in a suspension provided by the manufacturer (suspension in 0.85% NaCl, 0.01 M phosphate, pH 8.0, containing 0.1% bovine serum albumin, 15 mM sodium azide). Unbound liposomes in the supernatant were removed from the mixture after separation using a magnetic separator (Advanced Magnetics, Cambridge, MA). SA magnetic particles with bound liposomes were then collected after several washes and the radioactivity was counted using 5 ml of Pico-Fluor 40 and a Beckman LS 3801 scintillation counter. The radioactivity counted was a direct measure of the amount of liposomal lipid bound to streptavidin. The radioactivity of ^{3}H-CHE associated with SA magnetic particles was measured in the liposomal PEG-lipid transfer assay and the radioactivity of ^{14}C-CHE was measured in the micellar PEG-lipid transfer assay. The data obtained were averaged from duplicates within the same experiment unless otherwise stated.

2.4. PEG-lipid transfer experiments

Intervesicle transfer of PEG-lipids was determined indirectly by measuring the inhibition of biotinylated liposomes (acceptor liposomes) binding to streptavidin due to the incorporation of PEG-lipids in these liposomes. DSPC/Chol liposomes containing 5 mol% of either DSPE-PEG_{2000}, DPPE-PEG_{2000}, or DMPE-PEG_{2000} were used as donor liposomes. In the transfer assay, acceptor liposomes were incubated with donor liposomes using conditions specified in each

experiment. Subsequently, 100 μl of SA magnetic particles were added to the liposome mixture to measure biotinylated liposome binding to SA magnetic particles as described above. PEG-lipid transferred to biotinylated acceptor liposomes was estimated based on the residual amount of biotinylated liposomes bound to streptavidin which was converted to liposomal PEG-lipid content using a standard curve (see Results) with the following assumptions: (1) PEG-lipid is transferred only into the outer membrane of acceptor liposomes; (2) reduction in biotinylated liposome binding to streptavidin is attributed to PEG-lipids in the outer leaflet; (3) PEG-lipids are symmetrically distributed on the inner and outer leaflet of liposomes if PEG-lipids were incorporated in the initial step of liposome making. With these assumptions, reductions in biotinylated liposome binding obtained in Fig. 1B were attributed to 50% of the total PEG-lipid content. This was considered when extrapolating PEG-lipid content in acceptor liposomes after transfer from donor liposomes. Thus, the amount of PEG-lipid transferred to biotinylated acceptor liposome was determined by extrapolating, based on the level of target binding after transfer and using Fig. 1B, the liposomal PEG-lipid content which was then divided by two. Data points presented are averages between two individual measurements in each experiment with less than 5% standard deviation.

2.5. PEG-lipid transfer from micelles

PEG-lipid transfer from micelles was done using DSPE-PEG_{2000}. PEG-lipid micelles were prepared by dissolving 4 μmoles of DSPE-PEG_{2000} in chloroform with trace amount of ^{3}H-DSPE-PEG_{2000}. After the lipid mixture was dried under N_2 gas and vacuum for at least 3 h, micelles were formed by adding HBS to the dried lipid to make a 5 mM solution. In the transfer experiment, 500 nmoles of biotinylated acceptor liposomes (with ^{14}C-CHE as liposome label) were incubated with the indicated amount of PEG-lipid micelles under conditions specified for each experiment. After PEG-lipid transfer, liposome binding to target was assessed using the binding assay described above. Transfer of micellar PEG-lipids into liposomes was also monitored by following the elution of the PEG-lipid label in a size exclusion column. ^{3}H-DSPE-PEG micelles were incubated with non-radiolabeled biotinylated acceptor liposomes under the conditions specified in the experiment. Micelles and liposomes were then separated by size exclusion chromatography using a Biogel A15m (100–200 mesh) column (1.5×42 cm) with a flow rate of 0.5 ml/min. The amount of ^{3}H radioactivity in the liposomal peak would indicate the amount of DSPE-PEG transferred to acceptor liposomes. Total amount of phospholipid in the liposomal fractions was determined by organic phosphate measurement [25].

2.6. PEG-lipid exchange in vivo

RAG-2 mice were injected i.v. with DSPC/Chol/Bx-DSPE liposomes (3 μmoles/200 μl/mouse) containing 5 mol% of either C_{14}-Cer-PEG_{2000}, DMPE-PEG_{2000}, DOPE-PEG_{2000} or DSPE-PEG_{2000}. At 16 h after injection, the mice were euthanized and the plasma was collected. Since the presence of plasma did not affect the binding of Bx-liposomes to SA magnetic particles (data not shown), liposome binding to target can be assessed directly using the plasma samples collected. The plasma was pooled from three mice in each treatment group and then diluted accordingly using untreated control mice plasma to obtain the same lipid concentration for all groups. One hundred microliters of plasma (containing 42.2 nmoles of lipid) was incubated with 100 μl of SA magnetic particles for 2 h. Liposomes bound to SA magnetic particles were then recovered using a magnetic separator and the amount of liposome bound was determined as described above. Recovery of liposome binding to streptavidin was used as an indirect measure of PEG-lipid released from liposomes. Alternatively, ^{3}H-labeled PEG-lipids were used to monitor the retention of PEG-lipid in liposomes. Balb/c mice were injected i.v. with DSPC/Chol liposomes (3.3 μmoles/mouse) containing 5 mol% of either DSPE-PEG_{2000}, DPPE-PEG_{2000} or DMPE-PEG_{2000} with trace amounts of the respective ^{3}H-PEG-lipid. ^{14}C-CHE was incorporated in these liposomes as a liposome label. Circulating levels of both ^{3}H-PEG-lipid and ^{14}C-CHE were determined in blood samples collected at 1, 4, and 24 h after liposome injection.

3. Results

3.1. PEG-lipid-mediated reductions in biotinylated liposome binding to streptavidin iron oxide particles

It is well established that incorporation of PEG-modified lipids into liposomes which contain biotinylated lipids can interfere with streptavidin binding [12,13,26]. In these studies, biotinylated liposomes binding to streptavidin immobilized on microtiter plates was much reduced when PEG-lipids were incorporated into liposomes. For this reason, we proposed that PEG-modified lipid exchange assays could be developed on the basis of PEG-lipid-mediated reductions in the interactions between biotinylated liposomes and streptavidin immobilized on magnetic particles. The effect of PEG-lipid content on the inhibition of biotin-mediated binding of liposomes to streptavidin immobilized on magnetic particles was measured using DOPC/Chol liposomes containing 0.1 mol% Bx-DSPE and DMPE-PEG_{2000} at a concentration ranging from 0 to 5 mol%. The results (shown in Fig. 1) indicate that the amount of biotinylated liposomes bound to SA magnetic particles decreased as the PEG grafting density increased. Instead of a proportional decrease in binding as a function of PEG-lipid content, a biphasic decrease in liposome binding was observed. Fig. 1A was determined under conditions where 500 nmoles of liposomal lipid were incubated with 100 μl of streptavidin SA magnetic particles. When PEG-lipids were not present, 40 nmoles of liposomes were bound to the magnetic particles after incubation for 20 min at which time binding had reached equilibrium. Since the binding efficiency was only 8% under these conditions, we repeated the assay using lower liposome concentrations in an effort to demonstrate improved binding efficiency. When 100 nmoles of biotinylated liposomes were incubated with the SA magnetic particles, 25 nmoles bound, as shown in Fig. 1B, and this was equivalent to approx. 25% of the added liposomal lipid. Whether using 100 nmoles or 500 nmoles of liposomes, the PEG-lipid-dependent inhibition of binding was comparable (Fig. 1A,B). Inclusion of 0.5 mol% DMPE-PEG_{2000} caused a 30 and 33% reduction in binding to streptavidin, respectively. However, doubling the PEG-lipid content to 1 mol% only caused a further 8–10% decrease in binding. When a PEG-lipid content of 5 mol% was used, there was a 90% reduction in biotinylated liposomes bound to SA magnetic particles, suggesting efficient steric stabilization of the liposome surface can be achieved at this PEG grafting density, a result that is similar to previous findings [27].

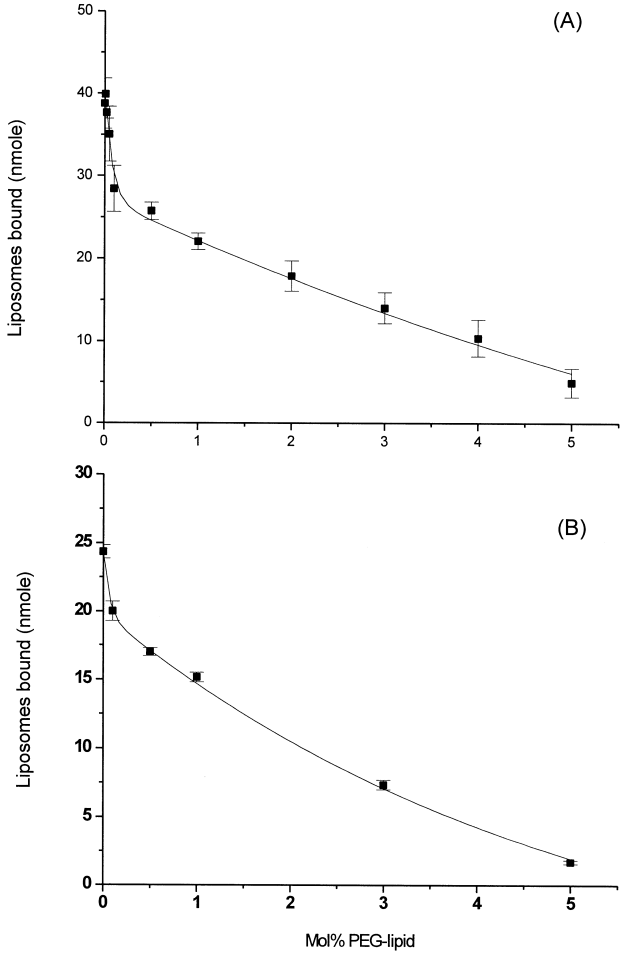

Fig. 1. Biotinylated liposome binding to streptavidin immobilized on superparamagnetic particles (SA magnetic particles) as a function of liposomal PEG-lipid content. DOPC/Chol liposomes containing 0.1% biotin-X-DSPE and 0–5% DMPE-PEG_{2000} were prepared as described in Section 2. Liposomes bound to SA magnetic particles were measured using either 500 nmoles (A) or 100 nmoles of liposomes (B) which were incubated at room temperature with SA magnetic particles as described in Section 2. The data represents averaged results ± S.D. obtained from 2–4 liposome preparations.

3.2. Assessing PEG-lipid transfer from donor liposomes to biotin-labeled acceptor liposomes

A previous study using a fluorescence resonance energy transfer-based assay demonstrated that PEG-modified lipids can transfer from one liposome population (donor) to another liposome population (acceptor) [4]. The rate of transfer is dependent on the ratio of donor to acceptor liposomes as well as other parameters including the size of the PEG moiety, the acyl chain composition of the PEG-modified lipid as well as incubation conditions. The transfer assay developed in this study relies on the inhibition of biotinylated liposomes binding to SA magnetic particles. Therefore, it was important to determine whether the presence of donor (DSPC/Chol) liposomes changed the binding attributes of these liposomes. As shown in Fig. 2, at all acceptor to donor liposome ratios tested there was little effect on the amount of biotinylated (acceptor) liposomes bound to SA magnetic particles. Since the data suggests that an acceptor to donor liposome ratio of 1:10 (mol/mol) can cause a 10% reduction in binding, subsequent experiments studying the transfer of PEG-modified lipids were completed using an acceptor to donor ratio of 1:4.

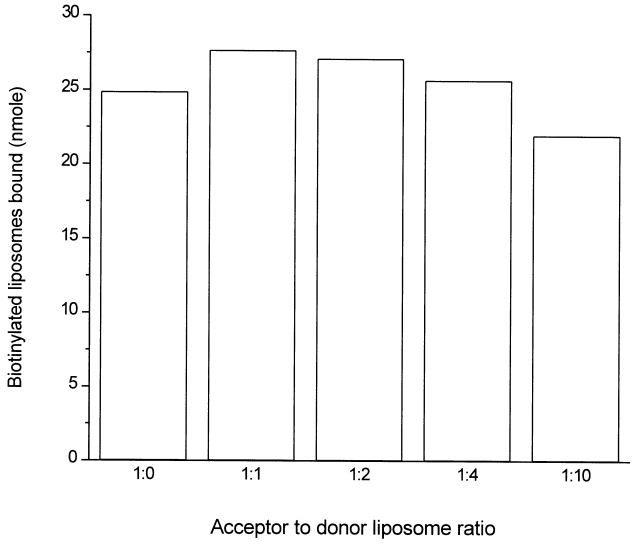

Fig. 2. Effect of DSPC/Chol liposomes on the binding of biotinylated liposomes to SA magnetic particles. The binding of biotinylated liposomes to streptavidin can be interfered by the presence of donor liposomes. DOPC/Chol/Bx-DSPE (49.9:45:0.1) liposomes (100 nmoles) were incubated with DSPC/Chol (55:45) liposomes at the indicated acceptor to donor liposome ratios for 24 h at 37°C. After the incubation, SA magnetic particles (100 μl) were added to the liposome mixture to measure the binding of biotinylated liposomes as described in Section 2. Data presented are averages between two individual measurements in each experiment with less than 5% standard deviation.

To measure PEG-lipid transfer from DSPC/Chol liposomes to biotinylated DOPC/Chol liposomes, reductions in biotinylated liposome binding to SA magnetic particles were measured following incubation of these acceptor liposomes with donor liposomes containing 5 mol% of either DMPE-PEG_{2000}, DPPE-PEG_{2000}, or DSPE-PEG_{2000}. PEG-lipid transfer was measured at 4°C and 37°C as shown in Fig. 3. The data presented in Fig. 3A shows the amount of biotinylated liposomes bound to SA magnetic particles after incubation with the donor liposomes for the indicated time periods. Fig. 3B represents the calculated amount (nmoles) of PEG-lipid transferred to acceptor liposomes, a value determined from the binding data in Fig. 3A and the inhibition curves shown in Fig. 1B for data conversion. The results demonstrate that when the acceptor liposomes were incubated with donor liposomes containing DMPE-PEG_{2000} for 30 min, there was a 53% and 86% reduction in acceptor liposome binding to SA magnetic particles when the incubation temperature was 4°C and 37°C, respectively. The amount of PEG-lipid in the biotinylated acceptor liposomes was calculated using the assumption that reductions in binding were due solely to PEG-lipid transfer to the liposome's outer leaflet. It was estimated that 2.2 nmoles of PEG-lipid (22% of total available PEG-lipid) was transferred to the acceptor liposomes within 30 min or less at 37°C. At 4°C, maximum transfer was observed at the 2 h time point, but the amount of transfer (1.6 nmoles of DMPE-PEG_{2000}) was less than that observed at 37°C. The half-times of transfer for DMPE-PEG_{2000} at 4°C and 37°C, as determined by this assay, were less than 30 and 15 min, respectively.

The transfer of DPPE-PEG_{2000} and DSPE-PEG_{2000} was less efficient than that of DMPE-PEG_{2000}, consistent with reports suggesting that the transfer of PEG-lipids is dependent, in part, on the acyl chain length of the chemically modified phosphatidylethanolamine used. Transfer of DPPE-PEG_{2000} was not detectable at 4°C (Fig. 3B); how-

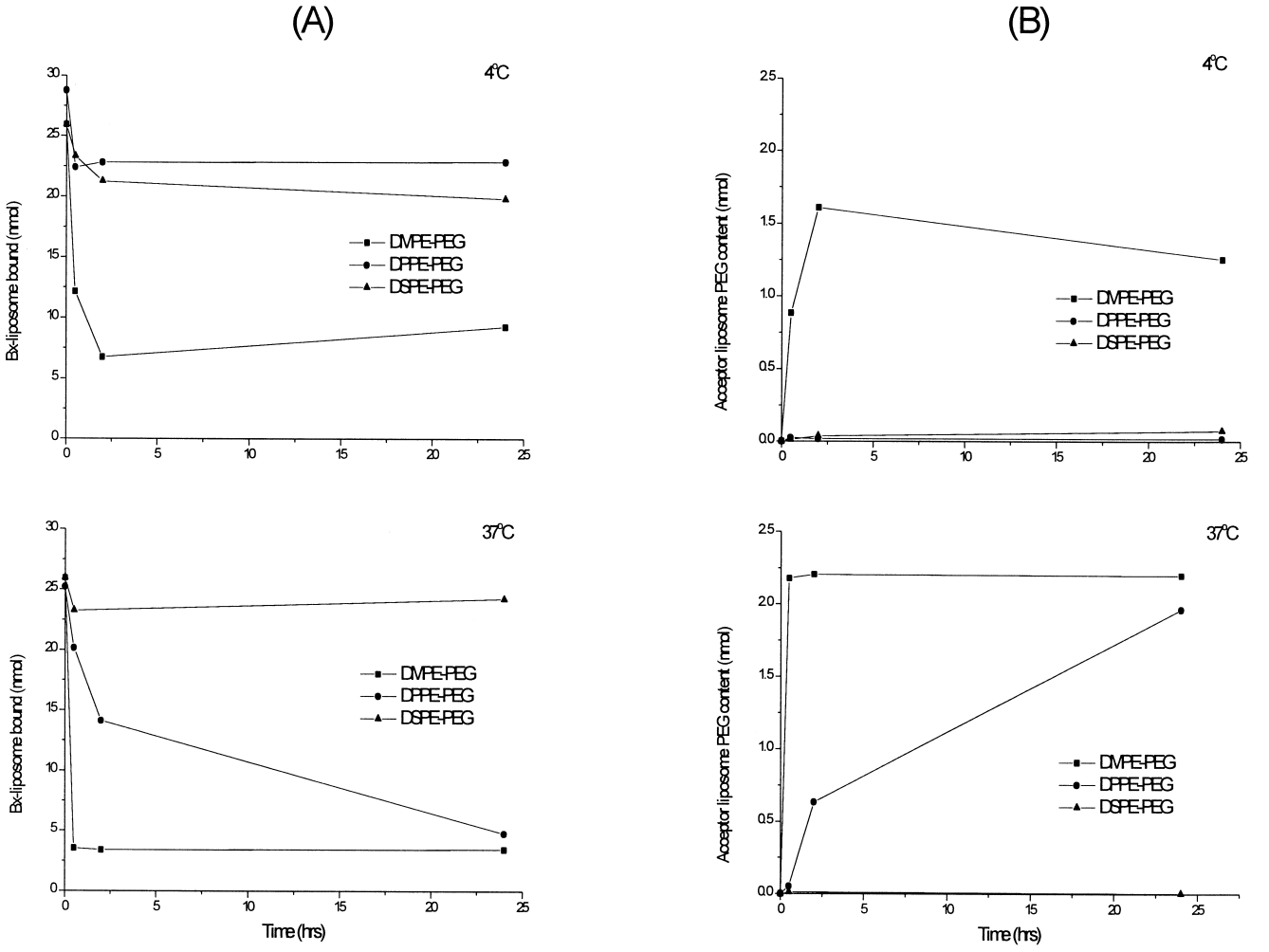

Fig. 3. Inhibition of biotinylated liposome binding to SA magnetic particles mediated by the transfer of PEG-lipids into liposomes. (A) Inhibition of biotinylated liposome binding to streptavidin as a function of incubation time with donor liposomes containing DMPE-PEG_{2000} (■), DPPE-PEG_{2000} (●), or DSPE-PEG_{2000} (▲). To measure intervesicle PEG-lipid transfer, 100 nmoles of acceptor liposomes containing DOPC/Chol/Bx-DSPE (54.9:45:0.1) were incubated at either 4°C (top) or 37°C (bottom) with donor DSPC/Chol (50:45) liposomes (400 nmoles) containing 5 mol% of the indicated PEG-lipid. After incubation for the indicated time period, SA magnetic particles were added to the liposome mixture to measure PEG-lipid-mediated inhibition of biotinylated acceptor liposome binding to streptavidin as described in Section 2. (B) PEG-lipid content in acceptor liposomes estimated based on the amount of liposomes bound to SA magnetic particles at the end of incubation with donor liposomes. Reduction in biotinylated liposome binding to streptavidin was converted to liposomal PEG-lipid content using Fig. 1B as described in Section 2. The transfer of PEG-lipid at 4°C and 37°C is presented in the top and bottom panel respectively.

ever, at 37°C the transfer of DPPE-PEG_{2000} was significant as judged by an 80% reduction in acceptor liposome binding to the SA magnetic particles (Fig. 3A). The transfer of DSPE-PEG_{2000}, on the other hand, could not be enhanced by increasing the temperature to 37°C. As shown in Fig. 3B, DSPE-PEG-lipid transfer was negligible at both 4°C and 37°C (<0.08 nmoles of PEG-lipid). It should be noted, however, that there was a consistent 10–20% reduction in the amount of acceptor liposome binding to SA magnetic particles observed at the first time point following the addition of donor liposomes containing DSPE-PEG_{2000}. Because this assay is sensitive to even very small amounts of PEG-lipids, this reduction in liposome binding, when converted to amount of PEG-lipid transferred, represents only 0.8% of total available DSPE-PEG_{2000}.

The amount of DMPE-PEG_{2000} transferred to ac-

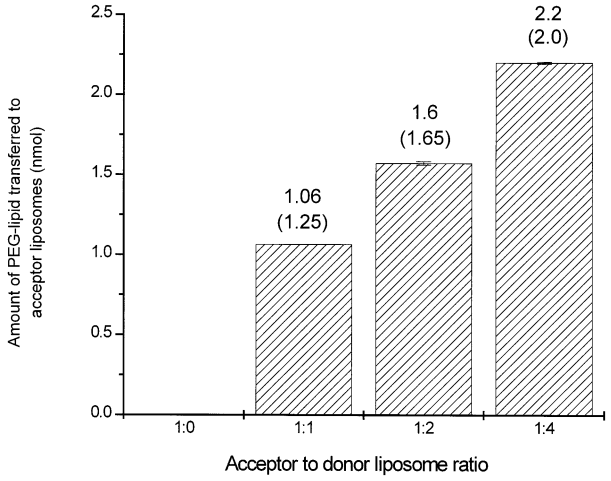

Fig. 4. PEG-lipid transfer as a function of donor liposome concentration. Biotinylated acceptor liposomes (100 nmoles) were incubated at 37°C with donor liposomes containing 5 mol% of DMPE-PEG_{2000} at the indicated ratios to allow PEG-lipid transfer. When the transfer had reached equilibrium after 2 h, the amount of PEG-lipid present on acceptor liposomes at equilibrium was determined by measuring liposome bound to SA magnetic particles as described for Fig. 3.

ceptor liposomes was also measured at equilibrium as a function of acceptor to donor ratio and the results, plotted as the calculated amount of PEG-lipid transfer, are shown in Fig. 4. With increasing quantities of donor liposomes (up to 1:4 ratio), more PEG-lipid was transferred to acceptor liposomes. At an acceptor to donor liposome ratio of 1:1, 1:2, and 1:4, the PEG-lipid content in the acceptor liposomes at equilibrium was determined to be 1.06, 1.6, and 2.2 nmoles, respectively. These levels of DMPE-PEG_{2000} were comparable to the amount predicted on the basis of three assumptions: (i) the donor liposomes have 50% of the PEG-modified lipid in the outer leaflet and 50% retained in the inner leaflet of the liposome bilayer, (ii) only the DMPE-PEG_{2000} within the outer leaflet are available for transfer, and (iii) there is equal distribution of DMPE-PEG_{2000} among donor and acceptor liposomes when the transfer has reached equilibrium. On the basis of these assumptions, there should be 1.25, 1.65, and 2.0 nmoles of DMPE-PEG_{2000} transferred to the acceptors when the donor to acceptor liposome ratios are 1:1, 1:2, and 1:4, respectively. As shown in Fig. 4, the measured and theoretical values are comparable.

3.3. Transfer of PEG-modified lipids from micelles

As shown in Fig. 3A there was a significant, 20%, reduction in the amount of acceptor liposome binding to SA magnetic particles observed even following addition of donor liposomes prepared with DSPE-PEG_{2000}. This inhibition could be due to the presence of low levels of PEG-lipid that did not incorporate into the liposomes during their preparation. Since it has been shown by others that PEG-modified lipids which do not incorporate into the liposomal membrane will form micelles [28], we investigated the possibility that the reduction in biotinylated liposome binding to SA magnetic particles was due, in part, to the presence of PEG-lipid micelles. DSPE-PEG_{2000} micelles were prepared as described in Section 2 and then incubated with 500 nmoles of biotinylated acceptor liposomes. Subsequently, liposome binding to SA magnetic particles was measured. Fig. 5A shows the amount of biotinylated liposome bound to SA magnetic particles as a function of the amount of DSPE-PEG_{2000} added, as micelles, just before the binding assay. Following a 20 min incubation with SA magnetic particles at room temperature, there was less than a 5% reduction in biotinylated liposome binding observed even in the presence of 30 nmoles of added DSPE-PEG_{2000} (Fig. 5A). When the acceptor liposomes were incubated for 3 h with DSPE-PEG_{2000} micelles prior to binding with SA magnetic particles, there was a concentration-dependent reduction in binding. Similar to the results obtained with pre-formed PEGylated liposomes (Fig. 1), the inhibition of binding appeared biphasic. When the amount of added DSPE-PEG_{2000} was increased from 0 to 2 nmoles, there was a 35% reduction in liposome binding to streptavidin. However, when DSPE-PEG_{2000} was increased from 2 to 25 nmoles (more than 10-fold increase), liposome binding to streptavidin was reduced further by only 36%. The results suggest that the added DSPE-PEG_{2000} was transferred into the lipid bilayer of biotinylated liposomes following incubation, consistent with previous studies demonstrating the transfer of PEG-lipids from micelles onto liposomes [29–31].

To confirm that the inhibition in binding mediated by PEG-lipid micelles is due to the insertion of DSPE-PEG_{2000} into pre-formed liposomes, DSPE-

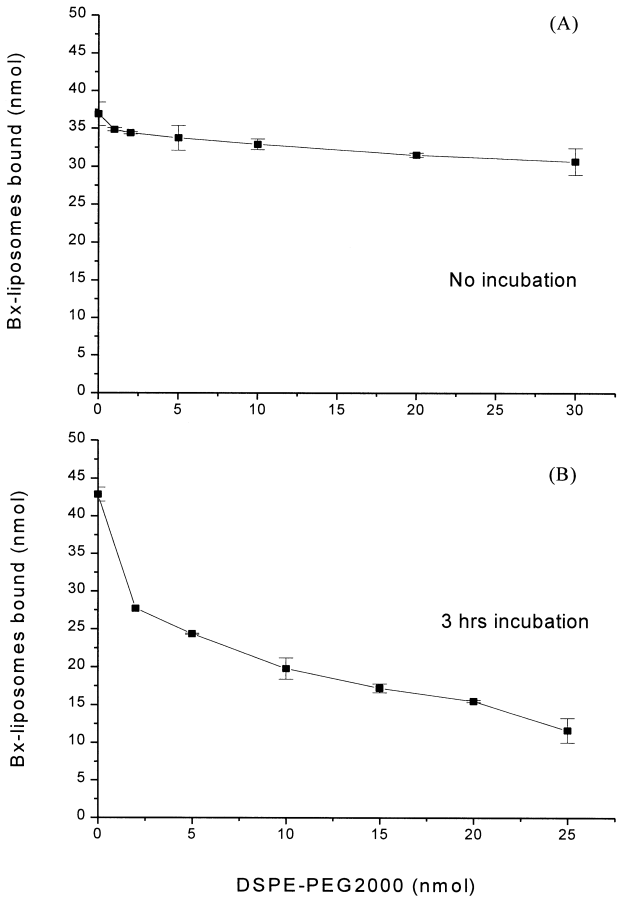

Fig. 5. Effect of micellar PEG-lipid on biotinylated liposome binding to SA magnetic particles as a function of the amount of added DSPE-PEG_{2000}. Binding to SA magnetic particles was measured using 500 nmoles of biotinylated liposomes immediately after adding DSPE-PEG_{2000} micelles (A) or after incubation for 3 h at 37°C (B) with the indicated amounts of PEG-lipids.

PEG_{2000} micelles with trace amounts of ^{3}H-DSPE-PEG_{2000} were first incubated with acceptor liposomes and subsequently fractionated on a size exclusion column to separate micellar DSPE-PEG- and liposomal PEG-lipid. Using previously published conditions [7], ^{3}H-labeled DSPE-PEG_{2000} micelles can be separated from ^{14}C-labeled biotinylated liposomes by size chromatography on a Biogel A15m column. As shown in Fig. 6, when ^{3}H-labeled DSPE-PEG micelles (250 nmoles) were added to acceptor liposomes (5 μmoles) and then immediately chromatographed, the majority ($>98\%$) of the PEG-lipid eluted in the micellar peak (Fig. 6B). However, if the DSPE-PEG micelles were incubated for 24 h with acceptor liposomes before column separation, 100% of the ^{3}H-labeled DSPE-PEG-lipid was recovered in the liposomal fractions indicating complete transfer of the PEG-lipid into the membrane bilayer of acceptor liposomes (Fig. 6C). When the amount of DSPE-PEG_{2000} micelles used was increased to 750 nmoles (15% of the total liposomal lipid), it was found that DSPE-PEG_{2000} was recovered in both the liposomal peak and the micellar peak, suggesting that the amount of DSPE-PEG_{2000} transfer into liposomes was saturable. By measuring the total amount of phosphate in the liposomal peak, it was estimated that 7 mol% of PEG-lipid was incorporated into liposomes, presumably in the outer leaflet of the liposomal membrane.

3.4. PEG-lipid exchange in vivo

Our in vitro experiments demonstrate that in the presence of acceptor liposomes PEG-lipid transfer can be measured using a functional assay that relies on the binding of 0.1 μm liposomes to 1 μm magnetic particles. These experiments were conducted under conditions where an acceptor membrane was present in excess; however, these conditions can be viewed as inadequate when considering the membrane and lipoprotein pools that exist in vivo. To assess the transfer of PEG-lipids in vivo, an assay based on similar principles of biotin-mediated binding of liposomes to SA magnetic particles was used. DSPC/Chol/Bx-DSPE liposomes with and without PEG-lipids were injected into mice and then recovered from plasma 16 h later. Recovered liposomes were incubated with SA magnetic particles to determine the binding attributes of these liposomes which in turn are dependent on exposure of biotin due to the loss of PEG-lipids. Recovery of target binding, which was used as an indirect measure of PEG-lipid loss, is expressed as a ratio of the binding level before and after injection for each of the liposome formulations studied. A binding ratio of 1 would indicate no change in target binding after circulation in vivo. As expected, liposomes without PEG-lipids had a reduction in binding to SA particles, likely a result of serum protein binding to the liposome surface, making biotin less accessible for binding to streptavidin. Similarly for liposomes containing DSPE-PEG_{2000}, there was a reduction rather than an in-

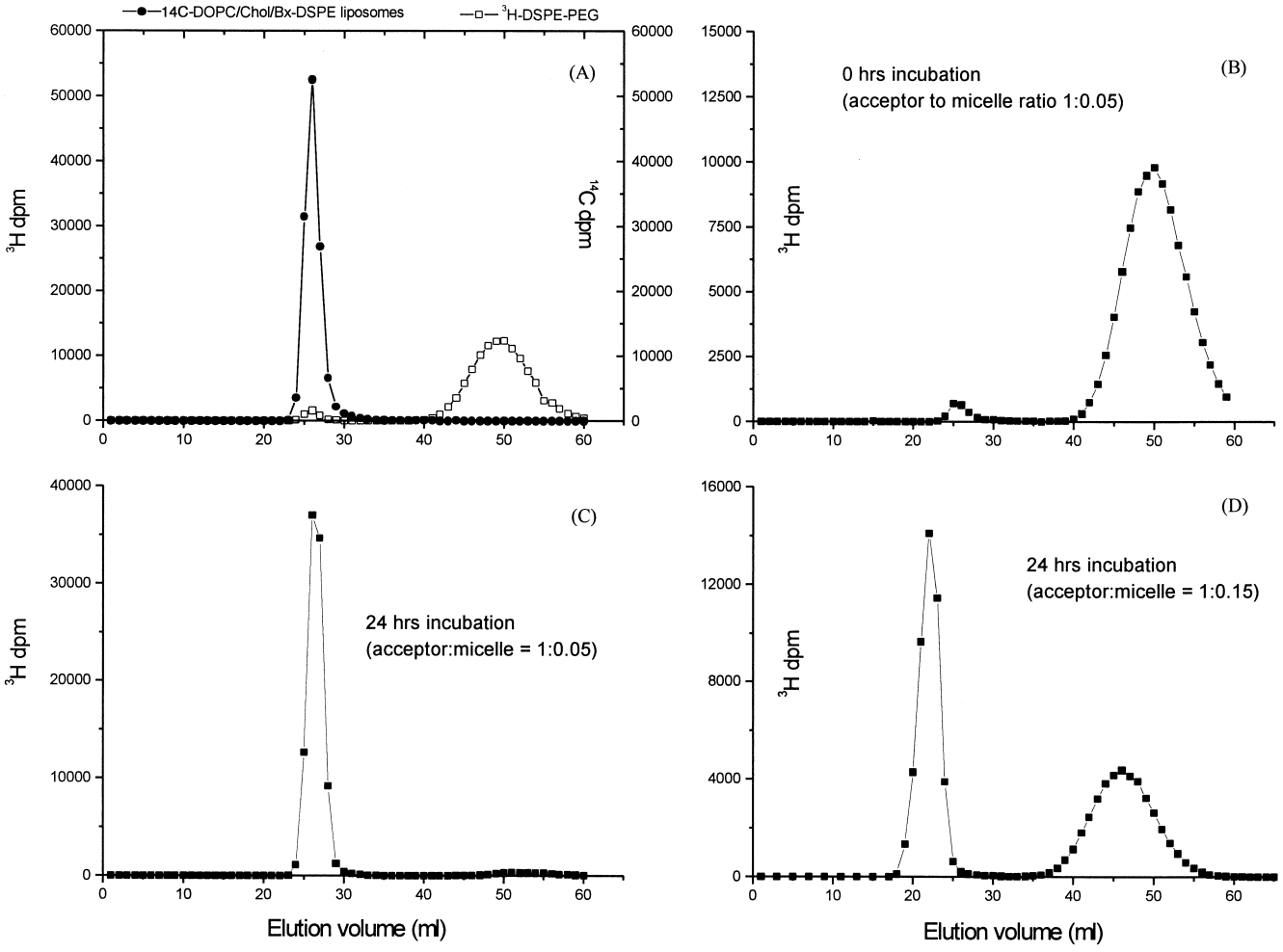

Fig. 6. Time-dependent transfer of PEG-lipids from micelles to liposomes. Transfer of DSPE-PEG_{2000} from micelles was followed using trace amounts of ^{3}H-labeled PEG-lipid which was incorporated into PEG-lipid micelles. (A) Separation of liposomes from micelles by size exclusion chromatography. ^{3}H-Labeled DSPE-PEG_{2000} micelles and biotinylated liposomes containing ^{14}C-CHE (used as a liposome label) were loaded separately onto a Biogel A15m (100–200 mesh) column (1.5×42 cm) and then chromatographed at a flow rate of 0.5 ml/min. (B) Elution profile of ^{3}H-labeled DSPE-PEG_{2000} which was chromatographed immediately after mixing the micelles (250 nmoles) with non-radiolabeled biotinylated acceptor liposomes (5 μmoles). (C) Elution profile of ^{3}H-labeled DSPE-PEG_{2000} which was chromatographed after mixing the micelles (250 nmoles) with biotinylated liposomes (5 μmoles) and incubated at 37°C for 24 h. (D) Elution profile of ^{3}H-labeled DSPE-PEG_{2000} which was chromatographed after mixing excess amounts of micelles (750 nmoles) with biotinylated liposomes (5 μmoles) and incubated at 37°C for 24 h.

crease in target binding after injection into mice. This finding is consistent with our in vitro data indicating that DSPE-PEG_{2000} is retained well in the liposomal membrane. For all other formulations, a binding ratio of greater than 1 was obtained, indicating recovery of binding after circulation in vivo. Changing the acyl chain composition, either by adding double bonds (e.g. DOPE-PEG_{2000}), or changing the acyl chain length, could influence the exchangeability of the PEG-lipid as reflected by the different binding ratios obtained (Fig. 7A). Liposomes made with DOPE-PEG_{2000} had a binding ratio of 3.7, corresponding to a 41% recovery in binding when compared to that observed for non-PEGylated formulations after injection into mice. Decreasing the acyl chain length to C_{14} caused an increase in the binding ratio to 8 and 11 for C_{14}-Cer-PEG_{2000} and DMPE-PEG_{2000}, respectively. When compared with non-PEG-

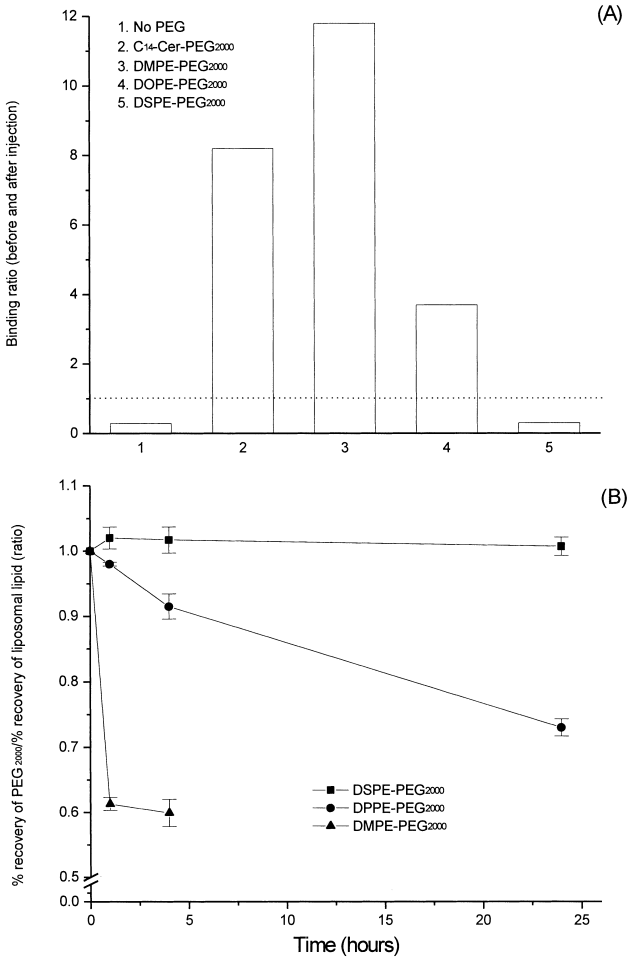

Fig. 7. PEG-lipid exchange in vivo. (A) PEG-lipid exchange as represented by recovery of target binding after circulation in vivo. Mice were injected with DSPC/Chol/Bx-DSPE liposomes with or without the indicated PEG-lipids (5 mol%). Liposomes were recovered from plasma after 16 h to measure binding to SA magnetic particles as described in Section 2. Recovery in binding is expressed as a ratio of the level of target binding before and after injection into mice. Data shown represent the mean of two individual measurements in the binding assay using plasma pooled from three mice for each liposome formulation. (B) PEG-lipid exchange as measured using radiolabeled PEG-lipids. DSPC/Chol liposomes containing 5 mol% of either DSPE-PEG_{2000}, DPPE-PEG_{2000}, or DMPE-PEG_{2000} with trace amounts of the respective ^{3}H-PEG-lipid were injected into mice to monitor the elimination of both ^{3}H-PEG-lipid and ^{14}C-CHE which was used as a liposome label. A ratio of the % recovery of ^{3}H-PEG-lipid/^{14}C-CHE was obtained at 1, 4, and 24 h after liposome injection as described in Section 2. Data shown represent averaged results obtained from four mice ± S.D.

ylated liposomes recovered from mice, these liposomes have recovered 72 and 63% of their biotin binding capacity, respectively.

To confirm that recovery in target binding was due to the loss of PEG-lipids, we conducted an in vivo experiment using ^{3}H-PEG-lipids and ^{14}C-CHE to monitor circulating levels of PEG-lipid and liposomes in vivo, respectively. Mice were injected (i.v.) with liposomes containing 5% of either DSPE-PEG_{2000}, DPPE-PEG_{2000}, or DMPE-PEG_{2000}. The relative ratios of PEG-lipid to liposomal lipid were measured over a 24 h time course following i.v. administration of the liposome (Fig. 7B). Confirming our in vitro and in vivo data (Fig. 7A), there was no measurable level of DSPE-PEG_{2000} loss from liposomes even when the liposomes were recovered from the plasma compartment 24 h after i.v. injection. In contrast, over 40% of the liposome-associated DMPE-PEG_{2000} was lost by the first time point measured (1 h). The loss of DPPE-PEG_{2000} in vivo was more gradual, with 10 and 25% loss of this lipid observed from liposomes in the plasma compartment isolated at 4 and 24 h after injection, respectively.

4. Discussion

The method described here to evaluate PEG-modified lipid transfer was based on PEG-lipid-mediated reductions in biotinylated liposome binding to streptavidin magnetic particles. This model was also developed in consideration of our goal to develop targeted liposome preparations in which the targeting ligand used is small enough to be shielded in a controlled manner by surface-associated PEG polymers. It was demonstrated that the inclusion of PEG-lipid caused a concentration-dependent decrease in biotinylated liposome binding to the SA magnetic particles. However, the decrease was not proportional to the increase in liposomal PEG content. At low PEG grafting densities, there was an abrupt reduction in streptavidin binding while the decrease was more gradual at higher PEG-lipid concentrations. PEG-mediated inhibition of non-specific protein binding to surfaces [32] and avidin binding to biotin molecules conjugated to liposomal lipids [27] has been documented previously. However, the results presented here suggest that even at low PEG concentra-

tions, much lower than those required to influence protein binding, there is significant inhibition of surface–surface binding reactions. In the model developed here, PEG-modified lipids incorporated at concentrations less than 0.1 mol% can significantly reduce the binding of 0.1 μm liposomes to 1 μm particles. This discussion is focused on how PEG acts to limit the interactions between two particles as well as the role that PEG-lipid transfer can have in the design of targeted liposomal formulations.

It was originally proposed by de Gennes [33] that surface-grafted polymers exist in two conformations depending on the grafting density. When the grafting density is low, where the Flory radius (R_f) of the polymer is smaller than the distance between grafting points on the surface (D), the polymer is envisioned to be in the 'mushroom' conformation. When the grafting density is high, where $R_f > D$, the polymer is in the 'brush' configuration [33]. Thus, the surface area covered by the polymer is predicted to be different for the two regimes [33]. Du et al. [32] reported that protein adsorption decreases steeply at PEG_{5000} concentrations below 1 mol%, very close to 0.7 mol% at which the 5000 average molecular weight polymer is predicted to undergo transition to the brush conformation, supporting that the different effectiveness of PEG on surface shielding is due to changes in polymer conformation. However, for shorter chain polymers such as PEG_{2000}, the transition between mushroom and brush conformation is not as distinct. Based on the prediction of Needham et al. [34], the transition occurs at 4 mol% for PEG_{2000}, whereas Rex et al. reported that PEG_{2000} would be in the mushroom configuration at concentrations as high as 9 mol% [35]. Regardless, it is likely that the mushroom conformation was obtained with the PEG-lipid concentrations used in this study and we still observed a pattern of non-linear surface shielding similar to the finding of others using PEG_{750} [27]. Thus, the distinctive shielding properties obtained with different grafting densities of shorter PEG cannot be simply explained by the two different conformations of the polymer.

In contrast to most studies which measure the binding of free molecules to liposome surface [27,32,36], our PEG shielding assay is based on liposome binding to a target molecule on a larger surface, mimicking the situation of a target cell. It was surprising to note that there was a significant reduction in target binding when the content of PEG-modified lipid was less than 0.1%. This indicates that even low levels of grafted PEGs are sufficient to interfere with particle–particle binding when measuring the interactions between liposomes with target molecules on a larger surface. This observation is consistent with previous studies that indicate PEG-lipids inhibited binding between antibody-targeted liposomes and their target cell population [37,38]. In contrast, it was noted that the binding of free biotin to avidin conjugated to liposomal surface was unaffected at PEG-lipid concentrations as high as 2% [36]. Thus, it appears that PEG-lipids can prevent specific interaction with molecules fixed on a larger surface better than with molecules free in solution. The differential effect of PEG in preventing adsorption of free vs. bound target molecules may be explained by the size of the target molecule, which in turn determines its mobility. As noted by Needham et al., the hydrated PEG layer provides a barrier that acts like a molecular sieve when measuring penetration and binding of molecules to liposomes [34]. Larger polymers such as PEG_{2000} and PEG_{5000} can restrict larger molecules (as in the case of molecules bound to a larger surface) while they are less effective in preventing penetration of smaller molecules. This result has implications with respect to our goal of designing ligand-targeted liposomes that rely on the use of PEG-lipid shielding of the surface-associated targeting ligand. Our laboratory believes that it is important to develop targeted liposomes with time-controlled release of PEG-lipids to protect liposomes while in the circulation, and to subsequently expose the shielded ligand for targeting when exiting the circulation.

Using the relationship obtained between the PEG content of biotinylated liposome and liposome binding to streptavidin immobilized on magnetic particles, we measured PEG-mediated inhibition of biotin liposome binding as a functional assay to determine PEG-lipid transfer. Consistent with other reports, we found that the transfer of PEG-lipids from DSPC/Chol donor liposomes was dependent on temperature and the acyl chain length of the lipid anchor [4,6,23]. Using this method, we were able to quantitate PEG-lipid transfer with reasonable accuracy. Our experimental results compared well with

theoretical predictions determined on the basis of several assumptions. These assumptions include the equal distribution of PEG-lipids in the outer and inner leaflets of the 'donor' liposomes which is supported by the prediction that the curvature of the inner leaflet of a bilayer of a 100 nm liposome would not be constrained sufficiently to affect the bilayer distribution of lipids with covalently linked PEG_{2000}. It was also assumed that only PEG-lipids in the outer leaflet would be transferred and that at equilibrium the concentration of PEG-lipids in the outer leaflet of the acceptor and donor liposomes would be equal. An equally important assumption made in the development of this model concerned the mechanism of PEG-lipid transfer. Based on the conclusions of Silvius and Zuckermann [6] as well as studies demonstrating PEG-mediated inhibition of liposome–liposome interactions [36], it is believed that PEG-lipid transfer occurred by monomer diffusion or via a micellar intermediate. It is demonstrated that DSPE-PEG_{2000} presented in micelles can facilitate DSPE-PEG_{2000} transfer to the 'acceptor' liposomes. We believe, however, that inter-liposome PEG-lipid transfer is not due to excess PEG-lipid in micellar form co-existing with donor liposomes. This interpretation is based, in part, on data suggesting that a maximum of 7 mol% PEG-lipids can be incorporated asymmetrically into pre-formed liposomes. Assuming the outer and inner leaflets of liposomes have an equivalent capacity for PEG-lipids, a total of approx. 14 mol% of DSPE-PEG_{2000} can be incorporated into liposomes before excess PEG-lipids form micelles.

The finding that DSPE-PEG_{2000} from micelles, but not liposomes, is readily incorporated into preformed liposomes indicates that desorption of phospholipid is the rate-limiting step in the transfer process, in agreement with the mechanism proposed by Jones and Thompson [22]. Many factors which affect the rate of phospholipid desorption have been identified. These include the degree of unsaturation of the acyl chains of the lipid anchor [23], donor liposome lipid environment and size [22,39], temperature and ionic strength of buffer [22], and acyl chain length of the lipid anchor which seems to have the most prominent effect on the rate of transfer [23].

The time course of PEG-lipid transfer has very important implications in designing liposomes for therapeutic and diagnostic purposes. Our in vitro data indicated that PEG-lipids with short acyl chains (C14:0) exhibit the fastest rate of transfer, which occurs on a time scale of minutes at 37°C. This finding is well correlated with our in vivo results which demonstrate that liposomes made with DMPE-PEG_{2000} lost most of the PEG-lipid within 1 h. We believe that a slower rate of PEG-lipid removal is required to protect liposomes in the circulation for a sufficient period of time before leaving this compartment. The rapid removal of DMPE-PEG_{2000} from fusogenic vesicles has been shown to result in their rapid elimination from the circulation and a reduction in stability of the drug-loaded formulations prepared using this lipid [5]. DPPE-PEG_{2000} or DOPE-PEG_{2000}, on the other hand, exhibit slower rates of transfer, where lipid loss occurs over a time scale of hours according to our in vitro and in vivo data. Knowing that liposome accumulation in tumors reaches its maximum between 16 and 24 h [40,41], it is predicted that these PEG-modified lipids will provide the ideal choice for time-controlled exposure of liposomal ligand for targeting. In conclusion, our data indicate that PEG-lipid transfer can lead to recovery of liposome binding attributes and that this recovery of binding is dependent on the attributes of the PEG-modified lipid used. Further characterization of this approach, where a targeting ligand is shielded for a controlled time period following intravenous administration, is currently underway.

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References

[1] R. Jahn, T.C. Sudhof, Annu. Rev. Biochem. 68 (1999) 863–911.
[2] D.R. Jones, I. Varela-Nieto, Int. J. Biochem. Cell Biol. 30 (1998) 313–326.

[3] M.B. Bally, S.M. Ansell, P.G. Tardi, T.O. Harasym, J. Liposome Res. 7 (1997) 331–361.
[4] J.W. Holland, C. Hui, P.R. Cullis, T.D. Madden, Biochemistry 35 (1996) 2618–2624.
[5] G. Adlakha-Hutcheon, M.B. Bally, C.R. Shew, T.D. Madden, Nat. Biotechnol. 17 (1999) 775–779.
[6] J.R. Silvius, M.J. Zuckermann, Biochemistry 32 (1993) 3153–3161.
[7] J.W. Holland, P.R. Cullis, T.D. Madden, Biochemistry 35 (1996) 2610–2617.
[8] M.C. Woodle, D.D. Lasic, Biochim. Biophys. Acta 1113 (1992) 171–199.
[9] M.C. Woodle, Chem. Phys. Lipids 64 (1993) 249–262.
[10] T.M. Allen, Trends Pharmacol. Sci. 15 (1994) 215–220.
[11] V.P. Torchilin, V.G. Omelyanenko, M.I. Papisov, A.A. Bogdanov Jr., V.S. Trubetskoy, J.N. Herron, C.A. Gentry, Biochim. Biophys. Acta 1195 (1994) 11–20.
[12] H.C. Loughrey, A. Ferraretto, A.M. Cannon, G. Acerbis, F. Sudati, G. Bottiroli, M. Masserini, M.R. Soria, FEBS Lett. 332 (1993) 183–188.
[13] P. Corley, H.C. Loughrey, Biochim. Biophys. Acta 1195 (1994) 149–156.
[14] D. Kirpotin, J.W. Park, K. Hong, S. Zalipsky, W.L. Li, P. Carter, C.C. Benz, D. Papahadjopoulos, Biochemistry 36 (1997) 66–75.
[15] D.E. Lopes de Menezes, L.M. Pilarski, T.M. Allen, Cancer Res. 58 (1998) 3320–3330.
[16] J. Bestman-Smith, P. Gourde, A. Desormeaux, M.J. Tremblay, M.G. Bergeron, Biochim. Biophys. Acta 1468 (2000) 161–174.
[17] B.B. Lundberg, G. Griffiths, H.J. Hansen, Int. J. Pharm. 205 (2000) 101–108.
[18] K. Maruyama, O. Ishida, T. Takizawa, K. Moribe, Adv. Drug Deliv. Rev. 40 (1999) 89–102.
[19] K. Maruyama, Biol. Pharm. Bull. 23 (2000) 791–799.
[20] J.A. Harding, C.M. Engbers, M.S. Newman, N.I. Goldstein, S. Zalipsky, Biochim. Biophys. Acta 1327 (1997) 181–192.
[21] J.D. Jones, T.E. Thompson, Biochemistry 28 (1989) 129–134.
[22] J.D. Jones, T.E. Thompson, Biochemistry 29 (1990) 1593–1600.
[23] J.R. Silvius, R. Leventis, Biochemistry 32 (1993) 13318–13326.
[24] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[25] P.S. Chen, T.Y. Toribara, H. Warner, Anal. Chem. 28 (1956) 1756.
[26] T.E. Redelmeier, J.-G. Guillet, M.B. Bally, Drug Deliv. 2 (1995) 98–109.
[27] D.A. Noppl-Simson, D. Needham, Biophys. J. 70 (1996) 1391–1401.
[28] K. Edwards, M. Johnsson, G. Karlsson, M. Silvander, Biophys. J. 73 (1997) 258–266.
[29] H. Yoshioka, Biomaterials 12 (1991) 861–864.
[30] P.S. Uster, T.M. Allen, B.E. Daniel, C.J. Mendez, M.S. Newman, G.Z. Zhu, FEBS Lett. 386 (1996) 243–246.
[31] S. Zalipsky, N. Mullah, J.A. Harding, J. Gittelman, L. Guo, S.A. DeFrees, Bioconjugate Chem. 8 (1997) 111–118.
[32] H. Du, P. Chandaroy, S.W. Hui, Biochim. Biophys. Acta 1326 (1997) 236–248.
[33] P.G. De Gennes, in: P. Bongrand (Ed.), Physical Basis of Cell-Cell Adhesion, CRC Press, FL, 1988, pp. 39–60.
[34] D. Needham, D.V. Zhelev, T.J. McIntosh, in: Liposomes: Rational Design, Marcel Dekker, New York, 1999, pp. 13–62.
[35] S. Rex, M.J. Zuckermann, M. Lafleur, J.R. Silvius, Biophys. J. 75 (1998) 2900–2914.
[36] T.O. Harasym, P. Tardi, S.A. Longman, S.M. Ansell, M.B. Bally, P.R. Cullis, L.S. Choi, Bioconjugate Chem. 6 (1995) 187–194.
[37] A. Mori, A.L. Klibanov, V.P. Torchilin, L. Huang, FEBS Lett. 284 (1991) 263–266.
[38] G. Blume, G. Cevc, M.D. Crommelin, I.A. Bakker-Woudenberg, C. Kluft, G. Storm, Biochim. Biophys. Acta 1149 (1993) 180–184.
[39] W.C. Wimley, T.E. Thompson, Biochemistry 30 (1991) 1702–1709.
[40] A.A. Gabizon, Cancer Res. 52 (1992) 891–896.
[41] S.K. Huang, K.D. Lee, K. Hong, D.S. Friend, D. Papahadjopoulos, Cancer Res. 52 (1992) 5135–5143.