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Influence of poly(ethylene glycol) grafting density and polymer length on liposomes: Relating plasma circulation lifetimes to protein binding

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Abstract

The incorporation of poly(ethylene glycol) (PEG)-conjugated lipids in lipid-based carriers substantially prolongs the circulation lifetime of liposomes. However, the mechanism(s) by which PEG-lipids achieve this have not been fully elucidated. It is believed that PEG-lipids mediate steric stabilization, ultimately reducing surface-surface interactions including the aggregation of liposomes and/or adsorption of plasma proteins. The purpose of the studies described here was to compare the effects of PEG-lipid incorporation in liposomes on protein binding, liposome-liposome aggregation and pharmacokinetics in mice. Cholesterol-free liposomes were chosen because of their increasing importance as liposomal delivery systems and their marked sensitivity to protein binding and aggregation. Specifically, liposomes containing various molecular weight PEG-lipids at a variety of molar proportions were analyzed for in vivo clearance, aggregation state (size exclusion chromatography, quasi-elastic light scattering, cryo-transmission and freeze fracture electron microscopy) as well as in vitro and in vivo protein binding. The results indicated that as little as 0.5 mol% of 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) modified with PEG having a mean molecular weight of 2000 (DSPE-PEG_{2000}) substantially increased plasma circulation longevity of liposomes prepared of 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC). Optimal plasma circulation lifetimes could be achieved with 2 mol% DSPE-PEG_{2000}. At this proportion of DSPE-PEG_{2000}, the aggregation of DSPC-based liposomes was completely precluded. However, the total protein adsorption and the protein profile was not influenced by the level of DSPE-PEG_{2000} in the membrane. These studies suggest that PEG-lipids reduce the in vivo clearance of cholesterol-free liposomal formulations primarily by inhibition of surface interactions, particularly liposome-liposome aggregation.

Keywords: Cholesterol-free; Liposomes; PEG; Protein binding; Plasma elimination

Abbreviations: ANOVA, analysis of variance; AUC, area-under-the-curve; BCA, bicinchoninic acid; CH, cholesterol; CHE, cholesteryl hexadecyl ether; DDP, didodecylphosphate; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanol-amine; ^{3}[H], tritium radiolabel; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HBS, HEPES buffered saline, pH 7.4; LUV, large unilamellar vesicle; MPS, mononuclear phagocytic system; PAGE, polyacrylamide gel electrophoresis; P_B, protein binding (μmol protein/μmol lipid); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); PK, pharmacokinetic; QELS, quasielastic light scattering; SDS, sodium dodecyl sulphate; *T*c, phase transition temperature

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1. Introduction

Two factors are of paramount importance in the development of liposomal drug delivery carriers for intravenous use: liposome circulation lifetime and the release of encapsulated contents at a rate optimized for efficacy and/or toxicity endpoints. The presence of poly(ethylene glycol) (PEG) on the liposome exterior surface, typically achieved by formulation with PEG-modified lipids, is well known to significantly enhance liposome circulation lifetime [1–5]. The significance of the addition of PEG-modified lipids led to the realization of longer circulation half-lives that could facilitate higher levels of drug accumulation within sites of tumor growth [6–8] associated with improved antitumor activity [9–11].

The mechanisms governing these PEG-lipid mediated effects remain controversial. The most widely accepted hypothesis to explain the ability of PEG to prolong circulation time of liposomes is based on "steric stabilization" [12–16] that can lead to reductions in liposome aggregation [2,17] and plasma protein adsorption [18,19]. Evidence for this hypothesis has included measurements of the repulsive pressure between lipid membranes in the presence and absence of surface grafted polymers, which demonstrated that there was a larger interbilayer spacing (4 nm) in membranes modified with polymers as compared to unmodified bilayers [20]. Kenworthy et al. analyzed electron density profiles to show the distance between apposing DSPC/DSPE-PEG lipid bilayers varied as a function of the concentration of PEG-lipid in the bilayer and size of the grafted PEG chain. The extension of the PEG chain from the bilayer surface was 1.0, 2.8, 6.0 and 10.0 nm for PEGs with average molecular weights of 350, 750, 2000 and 5000, respectively [14].

Although some evidence suggests that PEG on liposome surfaces can reduce specific protein interactions [21,22], other studies have indicated that the clearance of liposomal formulations from the circulation is not correlated with total plasma protein binding to liposomes [23]. In view of the uncertainties regarding the mechanism by which PEG-lipid confers steric stabilization to liposomes, the research described here compares surface–surface interactions, including self-association (aggregation) and plasma protein adsorption, in cholesterol-free liposomes. We have chosen to perform these studies in cholesterol-free liposomes for two reasons. First, cholesterol-free liposomes have recently been recognized as an important new class of lipid-based drug delivery vehicles. Typically, the addition of cholesterol is viewed as essential for the generation of stable formulations of hydrophilic drugs. However, the studies of Dos Santos et al. [24] demonstrated that removal of cholesterol was associated with dramatic improvements in retention of the hydrophobic anthracycline idarubicin [24]. Interestingly, the liposomal formulations prepared with gel phase lipids such as DSPC ($Tc = 55$ °C) and surface stabilizing PEG-modified lipids exhibited long plasma circulation lifetimes, essentially identical to sterically stabilized cholesterol-containing liposomes. These results place a far greater importance on the role of PEG-lipids than cholesterol in governing the fate of intravenously administered liposomal formulations. Secondly, it is generally believed that cholesterol is a liposome "stabilizing" component which causes a reduction of protein binding following intravenous injection as well as an enhanced plasma circulation lifetime. Therefore, it was believed that the absence of cholesterol from these formulations would facilitate the evaluation of PEG-modified lipids on protein adsorption to the liposome surface and on liposome–liposome aggregation.

2. Materials and methods

2.1. Materials

1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) and (DSPE)-conjugated poly(ethylene glycol) lipids (PEG average molecular weights 350, 550, 750, 2000 and 5000) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol (CH) was obtained from the Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). ^{3}[H]-cholesteryl hexadecyl ether (CHE) (51 Ci/mmol) and ^{14}[C]-CHE (50.6 mCi/mmol) were obtained from PerkinElmer, Inc. (Boston, MA, USA). ^{3}[H]-DSPE-PEG$_{2000}$ was custom synthesized by Northern Lipids Inc. (Vancouver, BC, Canada). HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]), citric acid, Sephadex G-50 (medium), Sepharose CL-4B beads and all other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Picofluor-15 scintillation fluid was obtained from Packard Bioscience (Groningen, The Netherlands).

2.2. Liposome preparation

All liposome formulations were prepared by the extrusion technique [25,26]. Briefly, the specified lipids were dissolved in chloroform and mixed together in a test tube at the indicated molar ratios. ^{3}[H]-CHE was added as a non-exchangeable, non-metabolizable lipid marker [27,28]. The chloroform was evaporated under a stream of nitrogen gas and the sample was placed under high vacuum overnight to remove residual solvent. The lipid films were hydrated in HBS by gentle mixing and heating. When preparing cholesterol-containing formulations, these were also subjected to five cycles of freeze (liquid nitrogen) and thaw (65 °C) prior to extrusion. Multilamellar vesicles (cholesterol-free and cholesterol-containing) were passed 10 times through an extruding apparatus (Northern Lipids Inc., Vancouver, BC, Canada) containing two stacked 100 nm Nucleopore® polycarbonate filters (Northern Lipids Inc., Vancouver, BC, Canada). The mean diameter and size distribution of the liposome preparations were analyzed by a NICOMP model 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA, USA) operating at 632.8 nm, and were typically 100±30 nm in the volume-weighting mode.

2.3. Pharmacokinetic analysis

Balb/c mice (breeders) weighing 20–22 g, were purchased from Charles River Laboratories (St. Constant, QC, Canada) and then bred in-house. Mice were housed in microisolator cages and given free access to food and water. All animal studies were conducted according to procedures approved by the University of British Columbia's Animal Care Committee and in accordance with the current guidelines established by the Canadian Council of Animal Care.

The plasma elimination of liposomes containing trace quantities of ^{3}[H]-CHE radiolabel was assessed. In brief, Balb/c mice were administered intravenously, into the lateral tail vein, with liposomes at a lipid dose of 165 μmol/kg. At various time points up to 24 h after drug administration, blood was collected by tail nick (collected in microfuge tubes) or cardiac puncture (collected in liquid EDTA coated tubes) and centrifuged at 1000×*g* for 10 min to isolate the plasma fraction. Lipid concentration was subsequently measured by liquid scintillation counting. Plasma area-under-the-curve values were calculated using WinNonlin pharmacokinetic software (Version 1.5, Pharsight Corp., Mountain View, CA). Biodistribution studies of tissue samples and plasma correction factors were applied as previously published [29].

2.4. Freeze-fracture electron microscopy

For freeze-fracture analysis, aliquots of each liposome preparation were mixed with glycerol (to final proportion of 25% v/v) as a cryoprotectant and incubated for 30 min. A small droplet of sample was loaded into the well of a Balzers gold freeze fracture specimen holder (BAL-TEC, BU 012 130-T) such that the sample protruded 2 mm above the top of the holder. The holder was inverted and plunged into liquid propane (cooled to ~−160 °C with liquid nitrogen). After immersion, specimen were transferred to dry liquid nitrogen cooled cryovials and stored in liquid nitrogen. For fracturing and replica preparation, specimen were loaded onto the freeze fracture specimen table under liquid nitrogen, then inserted into a Balzers BAF 060 freeze fracture apparatus equipped with a quartz thin-film monitor (BAL-TEC, Balzers, Liechtenstein, Germany), which had been pre-cooled to −170 °C. Specimen were then warmed to −110 °C to −115 °C and fractured without etching, followed immediately by replication using unidirectional platinum/carbon shadowing at 45° (2.3 nm) and carbon backing at 90° (2.2 nm). The holders containing the replicated samples were removed from the freeze fracture unit, thawed and slid gently into commercial bleach in the wells of a porcelain spotting plate. Cleaning required 48 h in bleach at room temperature, with 3 changes of bleach. Replicas were then washed extensively with NANOpure water (Barnstead/Thermolyne, Dubuque, IA, USA) mounted on Formvar-coated 100/hex copper grids and viewed at an accelerating voltage of 80 kV in a Hitachi H7600 transmission electron microscope (Tokyo, Japan) equipped with an AMT Advantage HR digital CCD camera (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

2.5. Size exclusion chromatography

For evaluating the mean diameter and structure of liposomes containing increasing concentrations of DSPE-PEG lipids, DSPC/DSPE-PEG_{2000} (20 mM lipid) liposome formulations were dually radiolabeled with ^{14}[C]-CHE and ^{3}[H]-DSPE-PEG_{2000} and passed down a Sepharose CL-4B column (40 ml, 22 cm × 1.5 cm) at a flow rate of 0.5 ml/min. ^{3}[H]-DSPE-PEG_{2000} micelles were passed down the Sepharose CL-4B column as a reference for the elution volume of pure micelles.

2.6. Protein binding assays

For *in vivo* protein binding studies, Balb/c mice were administered intravenously with various liposomal formulations, via the lateral tail vein, at a lipid dose of 165 μmol/kg. Five minutes after injection, blood samples were obtained by cardiac puncture. Serum was isolated from whole blood by allowing the blood to clot for 30 min then centrifuging for 10 min at 1000×*g*. Liposomes with adsorbed proteins were separated from free proteins by analyzing a 500 μl aliquot of serum by size exclusion chromatography as described below. For *in vitro* protein binding studies, various liposomal formulations (0.7 μmol lipid) were incubated with mouse serum (400 μl) for 10 min 37 °C. This lipid concentration was chosen to approximate the *in vivo* lipid concentration after liposome administration at 165 μmol lipid/kg, assuming a blood volume of 2 ml per 22 g mouse and a hematocrit of 0.48. Liposomes with adsorbed proteins were separated from free proteins by analyzing a 500 μl aliquot of serum by size exclusion chromatography as described below.

The size exclusion method for separation of liposome-adsorbed proteins from free proteins was modified from previous reports [4,30–32]. The *in vivo* and *in vitro* samples described above were separated by size exclusion chromatography on a Sepharose CL-4B column (40 ml, 22 cm × 1.5 cm) eluted with HBS (pH 7.4) at a flow rate of 0.5 ml/min and the collection of 0.5 ml fractions. In the initial analysis, each fraction was assayed for lipid concentration by liquid scintillation counting and protein concentration using 20 μl for micro BCA assay. Based on these results, and providing that there was good separation between lipid and bulk plasma protein peaks, 3–5 fractions were pooled from the lipid peak. These pooled fractions were then re-assayed for lipid and protein as follows. Due to the fact that lipid tends to interfere with many protein assays, a lipid extraction was performed to separate the protein for quantification by micro-BCA acid assay, as described previously [33]. Briefly, 400 μl of cold methanol was added to 100 μl of pooled sample (in triplicate) in 1.5 ml microfuge tubes. The samples were vortexed and centrifuged at 9000×*g* for 3 min. 200 μl of chloroform was added to the samples, followed by vortexing and centrifugation at 9000×*g* for 3 min. 300 μl of H_20 was added to each sample, followed by vortexing and centrifuging at 9000×*g* for 4 min. The upper phase was discarded (approximately 700 μl) from this two-phase system then 300 μl of methanol was added to the lower phase and the sample was subsequently vortexed and centrifuged at 9000×*g* for 4 min. Most of the supernatant was removed and the residual supernatant was dried down with nitrogen for 2 h. Protein was present as a dry pellet at the bottom of the microfuge tube and was resuspended in 110 μl water at 50 °C–60 °C. For determination of the protein concentration, 110 μl of micro-BCA working reagent (Pierce, Rockford, IL, USA) was added to each sample and measured at 570 nm on MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA, USA) and was compared to a bovine serum albumin standard curve. For the quantification of lipid, 20 μl aliquots (in triplicate) of pooled sample were measured by liquid scintillation counting then the amount of lipid in the recovered sample was calculated from the specific activity. Protein binding values (P_B) were measured as μg protein/μmol liposomal lipid; values represented the mean and standard deviations from three experiments.

2.7. Electrophoretic analysis of liposome-bound proteins

After the separation of liposomes from bulk plasma proteins and delipidation (lipid extraction/protein precipitation) as described above, samples were solubilized in SDS-reducing buffer (0.0625 M Tris–HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.125% (w/v) bromophenol blue) heated to 95 °C, cooled and centrifuged. Approximately 0.1 μmol of total lipid was loaded on a SDS-polyacrylamide gel (PAGE) prepared on a Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Proteins were visualized by silver staining. Silver stain SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Richmond, CA, USA) were used to estimate the molecular weights of proteins.

2.8. Statistical analysis

A standard one-way analysis of variance (ANOVA) was used to determine statistically significant differences of the means. For multiple comparisons, post-hoc analysis using the Tukey–Kramer test was performed. A value of $p<0.05$ was considered significant.

3. Results

3.1. Effect of DSPE-PEG_{2000} composition on the circulation lifetime of cholesterol-free liposomes

Pharmacokinetic studies were performed as a functional test for assessing the effects of different amounts and molecular weights of surface-grafted PEG on the circulation lifetimes of DSPC (cholesterol-free) liposomes. The effects of 0.5 to 5 mol % of DSPE-PEG_{2000} in DSPC liposomes are shown in Fig. 1. These data indicate that the addition of DSPE-PEG_{2000}, at proportions as low as 0.5 mol%, to DSPC liposomes significantly enhanced liposome circulation longevity. Specifically, the plasma area-under-the-curve ($AUC_{0-24\ h}$) value for DSPC liposomes containing only 0.5 mol% of DSPE-PEG_{2000} was 5.7-fold higher than that for 100 mol% DSPC liposomes. Further increases in plasma $AUC_{0-24\ h}$ were observed as the DSPE-PEG_{2000} concentration was increased and reached a plateau for DSPC liposomes prepared with 2 and 5 mol% DSPE-PEG_{2000}, with plasma $AUC_{0-24\ h}$ values of 41.3 and 45.8 $\mu mol \cdot h \cdot ml^{-1}$, respectively (Table 1). The percent of the injected lipid dose that was remaining in the circulation 24 h after injection was 30%, 26%, 18% and 13% for liposomes

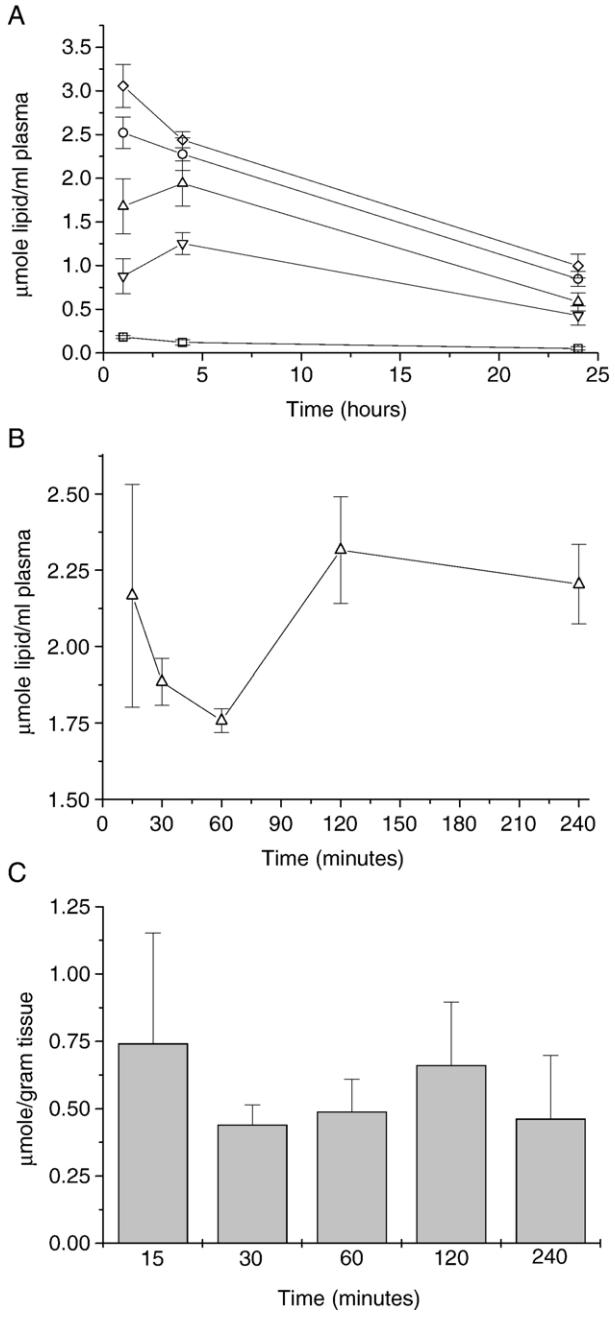

Fig. 1. The effect of mol% DSPE-PEG$_{2000}$ proportion on the plasma clearance of DSPC liposomes. (A) Liposome formulations composed of DSPC and varying amounts of DSPE-PEG$_{2000}$; 0 mol% (□), 0.5 mol% (▽), 1 mol% (△), 2 mol% (○), and 5 mol% (◇) were administered as a single i.v. bolus injection of 165 µmol/kg total lipid in female Balb/c mice (injected dose=3.3 µmol lipid/ml plasma). (B, C) DSPC/DSPE-PEG$_{2000}$ (99:1 mol ratio) liposomes were administered as a single i.v. bolus injection of 165 µmol/kg total lipid in male Balb/c mice and (B) plasma lipid concentrations and (C) lung tissue distribution was measured. Each data point represents the average total lipid plasma concentration±standard deviation of 3 mice.

containing 5, 2, 1 and 0.5 mol% DSPE-PEG$_{2000}$, respectively. All of these values were significantly ($p<0.05$) higher than those observed for liposomes prepared in the absence of added DSPE-PEG$_{2000}$. Further pharmacokinetic analysis was performed with DSPC liposomes containing 1 mol% PEG lipids to further dissect the elimination curve. A similar decrease in plasma lipid concentration was observed between 15 min and 1 h, followed by an increase at the 2 h time point (Fig. 1B). Biodistribution studies indicated that a peak of 0.74 µmol lipid/gram lung tissues occurred 15 min following injection (Fig. 1C). Lipid accumulation in the MPS organs, including the liver and spleen peaked at 2 h post injection with 2.23 µmol lipid/gram and 2.59 µmol lipid/gram, respectively (data not shown).

The effect of the molecular weight of the PEG moiety conjugated to DSPE on the circulation longevity of cholesterol-free liposomes was used as a functional assay of surface protection. Balb/c mice were injected with DSPC liposomes containing either 2 mol% or 5 mol% of DSPE-PEG where the mean PEG molecular weight was 350 (DSPE-PEG$_{350}$), 550 (DSPE-PEG$_{550}$), 750 (DSPE-PEG$_{750}$), or 2000 (DSPE-PEG$_{2000}$). Although DSPC liposomes containing 5 mol% (Fig. 2A) or 2 mol% (Fig. 2B) of DSPE-PEG$_{2000}$ had higher concentrations of liposomal lipid in the plasma 24 h after i.v. administration, the differences between these liposomes and those prepared with lower molecular weight PEGs were not significant. That is, liposomes containing DSPE-PEG$_{750}$, DSPE-PEG$_{550}$, or DSPE-PEG$_{350}$ had similar circulation lifetimes regardless of whether the level of incorporation was 5 mol % or 2 mol%.

3.2. In vitro and in vivo adsorption of proteins to liposomes

Further studies were conducted with the intent of correlating pharmacokinetic behaviour of liposomal formulations having a range of different clearance characteristics with protein binding and/or aggregation results. In the absence of distinct pharmacokinetic effects attributable to the low molecular weight PEGs (Fig. 2), we have used DSPC-based liposomes containing 0–5 mol% of DSPE-PEG$_{2000}$ for the correlation to protein binding and aggregation results.

Protein binding studies were completed using liposomes exposed to plasma proteins *in vivo* and *in vitro*. For these studies, the recovered plasma was fractionated on a size exclusion column to separate liposome-associated proteins from free, bulk, protein. Fig. 3 shows a typical elution profile of liposomes and bulk plasma proteins from the size exclusion column. The peak fractions of the eluted liposomes, at elution volumes between 14 and 16 ml, were pooled and the lipid was extracted to avoid interference in the micro-BCA assay used to measure protein. For the *in vivo* studies, the recovery of liposomes from the serum at 5 min after intravenous injection was between 75% and 95%.

The protein binding (P_B) values, given as µg of protein bound per µmol lipid, for DSPC liposomes prepared with increasing amounts of DSPE-PEG$_{2000}$ are summarized in Table 1. P_B values obtained with cholesterol-free liposomes were compared to conventional liposomes (DSPC/CH; 55/45 mole ratio) and to sterically stabilized (DSPC/CH/PEG; 50/45/5 mole ratio) liposomes. The data presented in Table 1 emphasize three points. First, the P_B values determined from liposomes incubated *in vitro* were essentially identical ($p>0.05$) to those determined for liposomes recovered after *in vivo* incubation.

Table 1
Summary of protein binding values to liposomes determined using *in vitro* and *in vivo* methods, calculated liposome surface coverage provided by PEG and clearance properties of liposomes from the circulation of mice

Formulation [a]	Protein Binding (P_B) (μg protein/μmol lipid)		PEG coverage (%) [b]	$AUC_{0-24\ h}$ (μg·h/ml)
	In vitro [c]	In vivo [d]		
DSPC	N/D [e]	N/D [e]	0	3.9
DSPC/DSPE-PEG_{2000} (99.5/0.5)	N/D [e]	N/D [e]	N/D [e]	22.1
DSPC/DSPE-PEG_{2000} (99/1)	35.4 (10.9)	42.6 (6.1)	N/D [e]	33.2
DSPC/DSPE-PEG_{2000} (98/2)	39.4 (5.4)	N/D [e]	100	41.3
DSPC/DSPE-PEG_{2000} (95/5)	39.9 (6.6)	29.5 (6.6)	100	45.8
DSPC/DSPE-PEG_{750} (98/2)	N/D [e]	N/D [e]	43	32.6
DSPC/DSPE-PEG_{750} (95/5)	N/D [e]	N/D [e]	100	36.1
DSPC/DSPE-PEG_{550} (98/2)	N/D [e]	N/D [e]	31	33.9
DSPC/DSPE-PEG_{550} (95/5)	N/D [e]	N/D [e]	79	45.6
DSPC/DSPE-PEG_{350} (98/2)	N/D [e]	N/D [e]	17	30.8
DSPC/DSPE-PEG_{350} (95/5)	N/D [e]	N/D [e]	45	41.1
DSPC/CH (55/45)	29.9 (5.5)	29.9 (5.7)	0	23.3
DSPC/CH/DSPE-PEG_{2000} (50/45/5)	34.2 (8.7)	23.8 (3.6)	100	44.7

[a] Liposome formulations with molar ratios of lipid components indicated in parentheses.
[b] From Ref. [12].
[c] For *in vitro* groups, data represent the means of 3–5 experiments (±standard deviation).
[d] For *in vivo* groups, data represent the means of 3 mice (±standard deviation).
[e] N/D, indicates not determined.

This indicates that P_B values obtained from either the *in vitro* or *in vivo* approaches can be used to evaluate protein adsorption to liposomes. Second, all liposome formulations evaluated, regardless of the proportion of PEG-modified lipids in the preparations, had comparable *in vitro* P_B values. That is, the P_B values ranged from 29.9 (±5.5) for DSPC/CH (55/45 mole ratio) liposomes to 39.9 (±6.6) for DSPC/DSPE-PEG_{2000} (95/5 mole ratio). Third, there was no discernible correlation of *in vitro* P_B values with the known clearance characteristics (Fig. 1) of the liposomes. Specifically, the DSPC/DSPE-PEG_{2000} (99/1 mole ratio) liposomes (P_B values of 35.4±10.9) were eliminated more rapidly from the circulation than were liposomes containing either with 2 mol% (P_B values of 39.4±

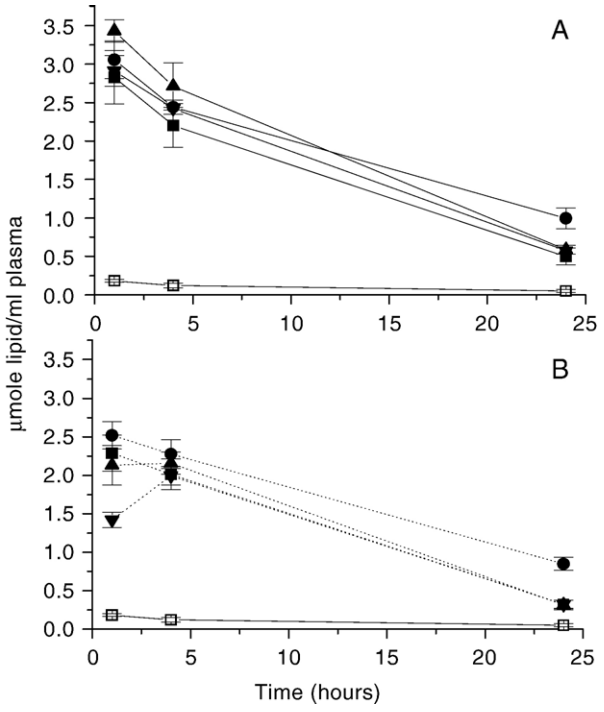

Fig. 2. The effect of PEG-lipid molecular weight on the plasma clearance of DSPC liposomes. Liposomal formulations were composed of DSPC containing 5 mol% (A) and 2 mol% (B) PEG-lipids of varying molecular weights. Symbols represent DSPE-PEG_{2000} (●), DSPE-PEG_{750} (■), DSPE-PEG_{550} (▲), or DSPE-PEG_{350} (▼). Unfilled squares indicate DSPC (100 mol%) liposomes. Female Balb/c mice were administered a single i.v. bolus injection of 165 μmol/kg total lipid (injected dose=3.3 μmol lipid/ml plasma). Each data point represents the average total lipid plasma concentration±standard deviation for 3 mice.

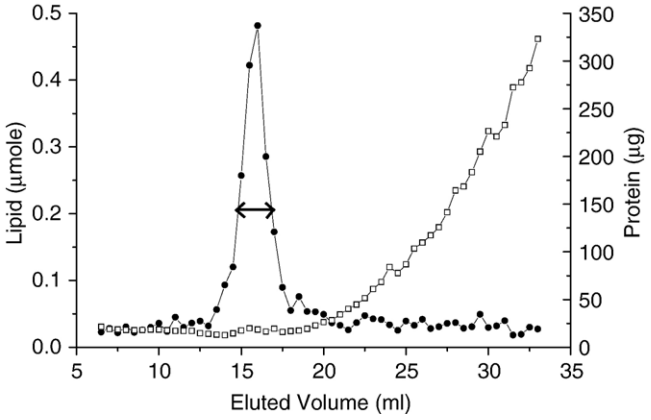

Fig. 3. Separation of DSPC/DSPE-PEG_{2000} (95:5 mol/mol) liposomes from bulk mouse plasma proteins by size exclusion chromatography. Mouse serum and liposomes were incubated at 37 °C for 10 min. The mixture was passed down a Sepharose CL-4B column (40 ml, 22 cm×1.5 cm) with HBS at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and assayed for liposomal lipid concentration (●) by liquid scintillation counting and for protein concentration (□) by micro-BCA assay. Under these conditions, optimal separation of liposomes and bulk serum proteins was achieved. The peak fractions, indicated between the arrows, were pooled to determine the amount of plasma protein bound to liposomes.

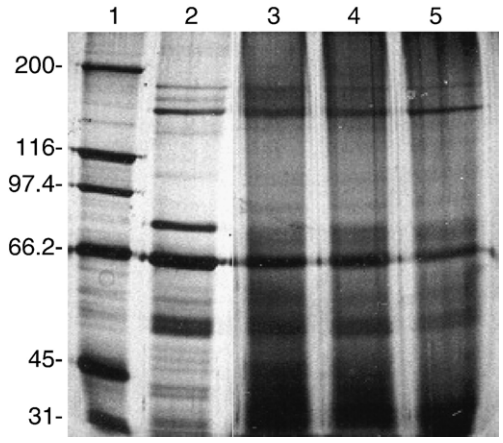

Fig. 4. PAGE analysis of liposome-associated plasma proteins. Protein was extracted by precipitation from column-purified liposomes (0.1 μmol total lipid) and separated on 7.5% polyacrylamide gels. Proteins were visualized by silver stain. Lane 1, high molecular weight markers; Lane 2, total mouse serum (200× diluted); Lane 3, DSPC/CH/DSPE-PEG_{2000} (50:45:5 mol ratio); Lane 4, DSPC/DSPE-PEG_{2000} (99:1 mol ratio); Lane 5, DSPC/DSPE-PEG_{2000} (95:5 mol ratio).

5.4) or 5 mol% (P_B values of 39.9±6.6) of DSPE-PEG_{2000}. Therefore, it is concluded from these results that protein binding in these formulations does not correlate with liposome clearance from the circulation after intravenous administration.

Although the P_B values suggest that PEG incorporation does not have a significant impact on non-specific plasma protein adsorption, it is possible that the binding of specific protein types were different, a parameter that could be qualitatively assessed by PAGE analysis of the liposome-associated proteins. The protein binding profiles were investigated by recovering plasma proteins that were bound to 0.1 μmol DSPC/CH/DSPE-PEG_{2000} (50/45/5 mole ratio), DSPC/DSPE-PEG_{2000} (99/1 mole ratio) and DSPC/DSPE-PEG_{2000} (95/5 mole ratio) liposomes. Subsequently the isolated proteins were separated on 7.5% acrylamide gel and visualized by silver stain. A representative study is shown in Fig. 4. The profiles suggest that proteins exhibiting molecular weights similar to serum albumin (mol. wt. ~66,000) and IgG (mol. wt. = 150,000) were bound at comparable levels for DSPC/CH/DSPE-PEG_{2000} (50/45/5 mole ratio) and DSPC/DSPE-PEG_{2000} (99/1 and 95/5 mole ratios) liposomes and there were no readily identifiable differences in

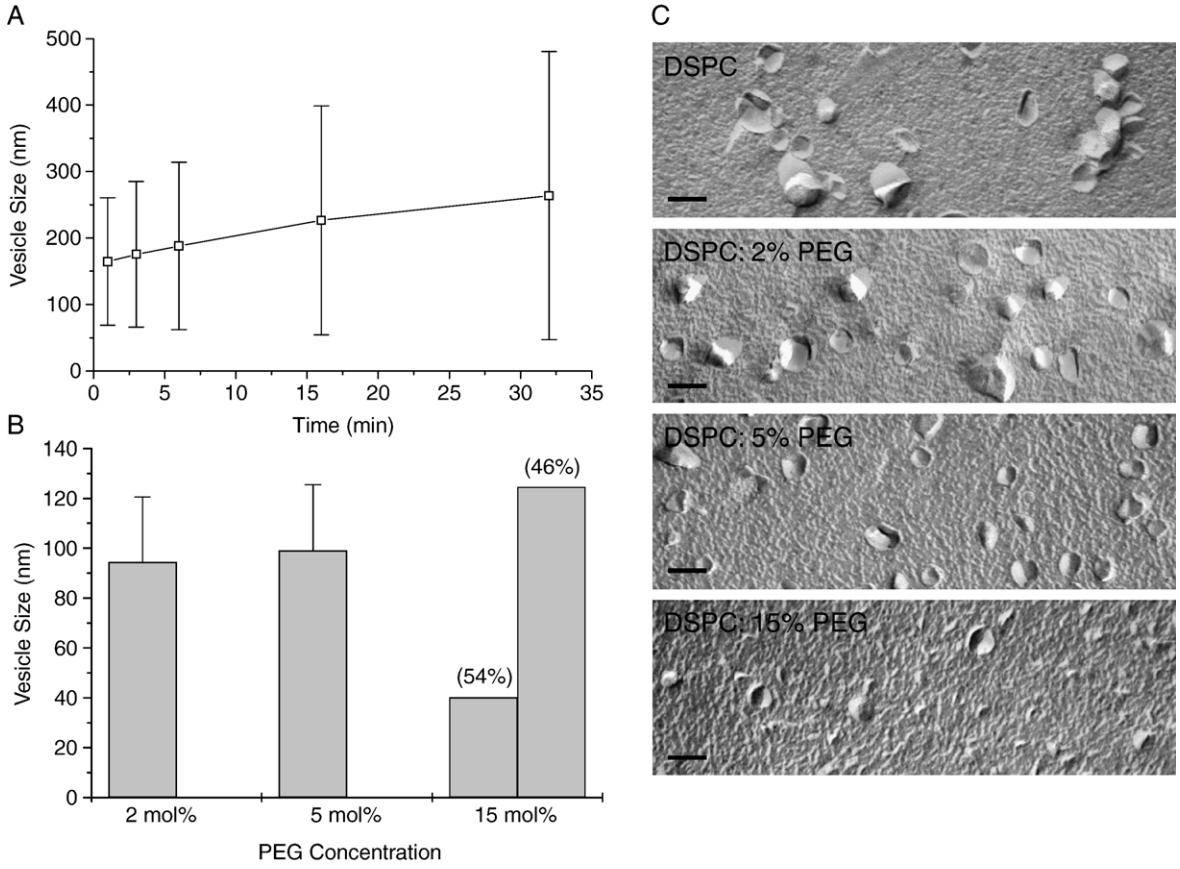

Fig. 5. The effect of DSPE-PEG_{2000} on liposome aggregation as determined by QELS and freeze-fracture analysis. (A) The apparent mean liposome diameter (bars represent the standard deviation of the liposome population) of DSPC liposomes (without DSPE-PEG) following extrusion through two stacked 100-nm polycarbonate filters was measured by QELS analysis over a 30-min time course. (B) The effect of DSPE-PEG_{2000} on the diameter of DSPC liposomes. The size of DSPC liposomes containing 2, 5 and 15 mol% DSPE-PEG_{2000} was determined by QELS (bars represent the standard deviation of the liposome population). Liposomes containing 15 mol% DSPE-PEG_{2000} exhibited a bimodal distribution with % of total liposome population indicated in parentheses. (C) Freeze-fracture electron micrographs of DSPC liposomes containing 0 (upper panel), 2 (second panel), 5 (third panel) or 15 (lower panel) mol% DSPE-PEG_{2000}. Bars indicate 100 nm.

appearance of proteins from the different liposomes used. Better separation and protein visualization techniques will be required to determine minor (but perhaps significant) changes in protein binding that were not detected by the methods used here. Collectively, the most significant conclusion from these protein binding studies suggest that proteins adsorb onto the surface of liposomes at comparable levels and in comparable patterns regardless of liposome composition.

3.3. Effect of DSPE-PEG on aggregation of cholesterol-free liposomes

The effects of PEG-lipid incorporation on liposome diameter and aggregation behaviour were determined by QELS and freeze-fracture electron microscopy. For DSPC liposomes lacking DSPE-PEG_{2000}, a time-dependent increase of the measured size occurred during the 30 min after liposome preparation by extrusion (Fig. 5A). The increase of the apparent liposome size was immediate, as indicated by an average particle diameter at 1 min after extrusion of 164.7±96 nm (chi-squared 0.435) (Fig. 5A). The measured size further increased to 263.8±216.9 nm (chi-squared 2.990, suggestive of a multimodal size distribution) after 32 min of incubation at room temperature. When these liposomes were heated to 65 °C, the size returned to mean diameters between 100 and 200 nm, suggesting that aggregation/self-association rather than fusion caused the vesicle size increase. This interpretation was directly confirmed by examination of DSPC liposomes using freeze-fracture electron microscopy (Fig. 5C, upper panel) showing the presence of large aggregates comprised of multiple liposomes having individual diameters of approximately 100 nm. These independent methods both support the conclusion that liposome–liposome association occurs in the DSPC preparations lacking DSPE-PEG_{2000}.

In contrast to the results described above, DSPC liposomes containing either 2 mol% or 5 mol% DSPE-PEG_{2000} had mean diameters of between 95 and 100 nm (with a standard deviation of <20% and a chi-squared value of 0.250 indicative of a unimodal vesicle population) (Fig. 5B). The size of these liposomes were directly confirmed by freeze-fracture electron microscopy showing discrete, non-aggregrated, liposomes with mean diameters of approximately 100 nm (Fig. 5C, two middle panels). When DSPC liposomes were prepared with 15 mol% DSPE-PEG_{2000}, QELS analysis indicated a bimodal distribution with structures exhibiting a mean diameter of 40 nm (54% of the liposome population) and 125 nm (46% of the liposome population). The existence of the smaller vesicle population represents bilayer disks and/or mixed micelles and is consistent with the bilayer destabilizing effects of PEG-modified lipids when incorporated at high levels. Freeze fracture electron micrographs of these formulations (Fig. 5C, last panel) suggest that there were fewer liposomes present within a given fracture plane when compared to samples prepared from liposomes composed of 2 and 5 mol% DSPE-PEG_{2000} (note that all samples were prepared with 10 mM total lipid concentration). Previous studies have indicated that it is difficult to distinguish micelles by freeze fracture techniques due to the lack of a fraction plane between an inner and outer leaflet which comprises the lipid bilayer [34].

To further assess whether the presence of DSPE-PEG_{2000} at levels in excess of 5 mol% led to the formation of mixed micelles, DSPC liposomal formulations composed of 5–20 mol% DSPE-PEG_{2000} were analyzed by size exclusion chromatography (Fig. 6). The elution profiles were compared to those observed using 100 nm DSPC/cholesterol liposomes and to pure DSPE-PEG_{2000} micelles. For size exclusion chromatography studies, dual-labeled DSPC liposomes with increasing levels of DSPE-PEG_{2000} were passed down a Sepharose CL-4B column at a flow rate of 0.5 ml/min. One peak eluted between 24 and 30 ml for liposomes composed of 5 mol% PEG, the same elution profile observed for control DSPC/cholesterol. liposomes. For formulations containing 10 mol% DSPE-PEG_{2000} the bulk of the lipid eluted between 24 and 30 ml (consistent with liposomes), however there was significant tailing in the elution profile. This tailing was increased as the amount of DSPE-PEG_{2000} increased and appeared as a distinct peak in fraction 32–35 ml for those formulations prepared using 20 mol% DSPE-PEG_{2000}. It should be noted that the second peak was distinct from that observed when using pure PEG micelles, which eluted in fractions 48–62 ml. For all elution peaks, both ^{3}[H]-PEG-lipids and ^{14}[C]-CHE were present, suggesting that all liposome populations represented by the different elution peaks contained both the bulk phospholipids and PEG components.

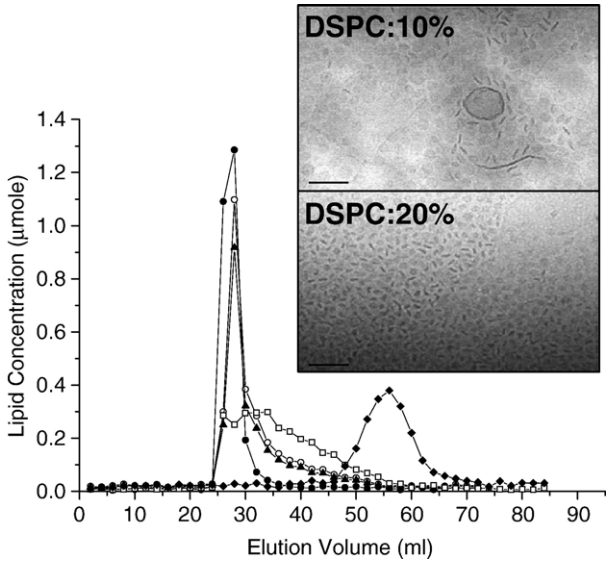

Fig. 6. Size exclusion chromatography analysis of DSPC liposomes prepared with 5–20 mol% DSPE-PEG_{2000}. DSPC liposomes (20 mM lipid) containing 5–20 mol% DSPE-PEG_{2000} were dual radiolabeled with tracer quantities of ^{14}[C]-CHE and ^{3}[H]-DSPE-PEG_{2000} were prepared and subsequently passed down a Sepharose CL-4B column (40 ml, 22 cm×1.5 cm) at a flow rate of 0.5 ml/min. The formulations containing varying amounts of PEG conjugated lipids are indicated by the following symbols, 5 mol% (●), 10 mol% (○), 15 mol% (▲), 20 mol% (□). ^{3}[H]-DSPE-PEG_{2000} micelles were passed down the column as a control (◆). Inset: Representative cryo-transmission electron micrographs of mixed micelles composed of DSPC and 10 or 20 mol% DSPE-PEG_{2000}. Bar represents 100 nm.

To further characterize the mixed micelle peak present in DSPC liposomes prepared using 10 and 20 mol% DSPE-PEG_{2000}, cryo-transmission electron micrographs of these samples were prepared. Representative micrographs of DSPC liposomes prepared with 10 mol% and 20 mol% DSPE-PEG_{2000} (Fig. 6 inset) indicated that small structures were present. In particular, bilayer disks (indicated by many "edge on" orientations) were evident as described previously by Edwards et al. for cholesterol-containing systems [35] and Ickenstein et al. for cholesterol-free systems [36]. Taken together, these studies indicate that DSPC liposomes, incorporating between 2 and 5 mol% DSPE-PEG_{2000}, can form stable preparations that exhibit extended circulation lifetimes. Bilayer disks or mixed micelles were present when the amount of DSPE-PEG_{2000} was equal to or greater than 10 mol%.

4. Discussion

The incorporation of surface grafted PEGs on liposomes has had a significant impact on the development of liposomal technology for the delivery of chemotherapeutic agents. In spite of the widespread use of PEG-conjugated lipids in liposomal formulations, there remains a great deal of speculation about the mechanism(s) by which surface-exposed PEGs increase the circulation lifetime of i.v.-administered liposomes. It is generally believed that this effect can be attributed to PEG's ability to reduce plasma protein binding; an observation originally documented for solid surfaces with surface-grafted PEG moieties [37,38]. A reduction of plasma protein adsorption to the liposome surface is believed to minimize the recognition of injected liposomes by cells of the mononuclear phagocytic system (MPS), which are known to play a significant role in the elimination of particulate delivery systems from the blood compartment.

In recent years, there has been a growing body of evidence suggesting that PEG does not reduce plasma protein adsorption on liposomes [32,39]. An alternative mechanism of action of the PEGs could be via the inhibition, or reduction, of liposome–liposome aggregation and maintenance of the injected liposome population as discrete particles with a small (about 100 nm) diameter optimized for prolonged circulation lifetime [40 Mayer, 1993 #344]. In this study, a functional pharmacokinetic assay, based on PEG-mediated alterations in the circulation lifetime of DSPC liposomes following i.v. administration in mice, was used to measure how the proportion and molecular weight of PEG conjugated to DSPE influenced the behaviour of DSPC liposomes. The results from this functional assay were correlated with studies on the effects of PEG on surface–surface interactions including plasma protein adsorption and liposome aggregation.

Previous studies of the plasma elimination of intravenously-injected liposomes have suggested that the PEG lipid anchor [41], PEG density [42], PEG molecular weight [43,44] affect the circulation longevity of cholesterol-containing liposomes [9]. However, in the pharmacokinetic studies reported here, there were no significant differences in the circulation longevity of cholesterol-free liposomes containing 2 mol% and 5 mol% (Fig. 1) to 10 mol% (data not shown) of DSPE-PEG_{2000} or in liposomes containing 5 mol% PEG having molecular weights between 350 and 2000. There were no additional improvements in the circulation longevity of liposomes prepared with PEG (molecular weight 2000) at concentrations greater than 2 mol%; a polymer concentration that is argued to provide sufficient surface coverage for a 100 nm liposome [23]. Assuming that the PEG-lipids homogenously distribute within the DSPC lipid matrix, surface coverage calculations for DSPC/DSPE-PEG_{2000} liposomes [23] indicate that 100% surface coverage of a 100 nm liposome is obtained with both 2 mol% DSPE-PEG_{2000} and 5 mol% DSPE-PEG_{750}. However, as indicated in Fig. 2A, liposomes containing 5 mol% DSPE-PEG_{550} or DSPE-PEG_{350} had similar elimination profiles ($AUC_{0-24\ h}$ values of 45.6 and 41.1 μg·h/ml, respectively, Table 1), yet calculations estimating surface coverage with these shorter length polymers would be quite different (79% and 45%, respectively, Table 1). For example, liposomes composed of 2 mol% DSPE-PEG_{350} have only 17% surface coverage, yet had a 7.9-fold higher plasma $AUC_{0-24\ h}$ compared to that for 100 mol% DSPC liposomes (Table 1) [45,46].

The correlation between surface protection provided by PEG and prolonged circulation lifetime is shown in Fig. 7. This scatter plot of calculated surface coverage values vs. measured $AUC_{0-24\ h}$ values (from Table 1) for cholesterol-free liposomes containing various PEG-PE molecular species shows a weak ($r^2 = 0.56$) relationship. Taken together, these data clearly indicate that several formulations of DSPC liposomes have surface area coverage less than 100% but which also have prolonged circulation lifetimes (Figs. 1 and 2). We interpret these results to suggest that complete surface coverage is not required for PEG to cause increased circulation time of liposomes and that other PEG-mediated effects are involved.

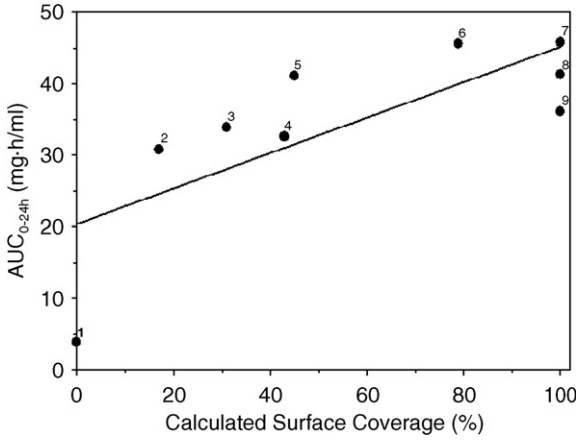

Fig. 7. Relationship between calculated surface coverage of liposomes by PEG and the observed clearance of liposomes from the circulation. Figure shows a scatter plot of the PEG coverage (%) vs. $AUC_{0-24\ h}$ values from Table 1 for the following cholesterol-free liposome formulations: 1, DSPC; 2, DSPC/DSPE-PEG_{350} (98:2); 3, DSPC/DSPE-PEG_{550} (98:2); 4, DSPC/DSPE-PEG_{750} (98:2); 5, DSPC/DSPE-PEG_{350} (95:5); 6, DSPC/DSPE-PEG_{550} (95:5); 7, DSPC/DSPE-PEG_{2000} (95:5); 8, DSPC/DSPE-PEG_{2000} (98:2); 9, DSPC/DSPE-PEG_{750} (95:5). The line represents the linear regression for these points and has a correlation coefficient, r^2, of 0.56.

Since protein binding values (Table 1) were not significantly influenced by the presence of 0–5 mol% DSPE-PEG_{2000}, in spite of significant differences in the liposome clearance from the circulation (Fig. 1), the role of protein binding in mediating liposome clearance must be reconsidered. Specifically, the adsorption of total (Table 1) and specific (Fig. 4) serum proteins were not significantly affected by the presence of either DSPE-PEG or cholesterol, despite significant pharmacokinetic differences between these liposomes (Fig. 1). These studies also demonstrated that liposomal DSPC formulations with adsorbed proteins ($\geq$35.4 μg protein/μmol lipid) had long circulation lifetimes. The absolute protein binding values obtained in these studies correlated well with those determined in previous studies [47–51]. It was not possible to assess P_B values for a pure DSPC formulation due to problems associated with liposome aggregation prior to and after mixing with serum. Overall, these results strongly suggest the absence of a causal relationship between protein adsorption on the surface of DSPC-based liposomes and the circulation lifetime of the liposome. That is, although PEG-containing formulations had longer circulation lifetimes than liposomes lacking PEG (Figs. 1 and 2) the increase of the circulation lifetime could not be directly associated with either PEG content or protein binding. Furthermore, it is apparent that long circulating liposomes or lipid/DNA complexes [52] have high amounts of protein bound to their surfaces. It could be interpreted that the liposomes prepared with DSPE-PEG did not have reduced protein binding values due to the depletion of the pool of proteins available for adsorption to the liposome surface, as observed previously [53]. However, we consider this explanation to be unlikely since the lipid doses necessary to deplete the available pool of blood proteins associated with MPS-mediated liposome clearance [42,53] are much higher than the 165 μmol/kg (approximately 130 mg/kg) used in this study. Although to further eliminate the effect of MPS-mediated liposome clearance, full dose titrations of low PEG containing liposomes are required. However, we interpret these results to suggest that PEG-mediated effects distinct from reductions of protein binding are also involved in the circulation longevity of PEG-containing liposomes.

An alternative mechanism by which PEG-modified lipids act to prolong the circulation lifetime of DSPC liposomes is to sterically preclude interactions that lead to rapid clearance from blood compartment. This could include a role for PEG in preventing interaction with Fc receptors or complement proteins [54,55], directly inhibiting cell uptake and/or preventing liposome aggregation and subsequent rapid elimination due to large particle size. The most compelling evidence for the ability of PEG-lipids to prevent surface–surface interactions, particularly aggregation, was revealed by freeze-fracture micrographs and QELS analyses (see Fig. 5). These studies demonstrate that 100 mol% DSPC liposomes can be prepared by extrusion methods, but the resulting liposomes rapidly coalesce and aggregate (Fig. 5A) into larger structures (Fig. 5C, upper panel). Previous studies have established that the clearance of liposomal drugs from the circulation is strongly dependent on the liposome size, with more rapid clearance occurring in liposomes having mean diameters of >100 nm [40,56]. Structures having greater sizes were observed for aggregated preparations of 100 mol% DSPC (Fig. 5A and C, upper panel). It is likely, therefore, that aggregation of liposomes contributed to the rapid initial phase of clearance of the DSPC liposomes from the circulation and, further, that it is possible that liposome aggregation was increased in the plasma compartment. This is consistent with the report by Ahl et al. [57] which investigated the role of non-PEG derivatives of PE on circulation longevity in DSPC liposomes and showed that aggregation was prevalent in both rat serum and plasma and, in turn, the liposome aggregation level was inversely correlated with *in vivo* circulation lifetimes. Further, previous studies investigating the effects of PEG on preventing fusion of dioleoylphosphatidylethanolamine (DOPE) or didodecylphosphate (DDP) bilayers, also concluded that the inhibition of fusion was due to PEG-mediated inhibition of liposome association [58,59]. PEG-lipids engrafted on liposomes prevent close interactions between liposomes, effectively ablating attractive Van der Waals short range forces that promote aggregation [58,59]. Since cholesterol-free liposomes have a greater tendency to aggregate than cholesterol-containing liposomes, the effects of short chain PEGs and low levels of PEG incorporation are much more apparent in these formulations. It should be noted that Edwards et al. [35] have shown with cryo-TEM that cholesterol containing liposomes have a strong tendency to aggregate and that this aggregation is effectively prevented by incorporation of PEG-lipids.

In summary, these studies indicated that levels $\leq$5 mol% DSPE-PEG_{2000} can be incorporated into cholesterol-free DSPC liposomes and have profound effects on their longevity in the circulation. Although DSPC liposomes containing between 1 and 5 mol% DSPE-PEG_{2000} had significant differences in their circulation lifetimes, the protein binding profiles for these formulations were very similar. In contrast, the addition of DSPE-PEG_{2000} prevented the aggregation of DSPC liposomes and was correlated with prolonged circulation longevity. It is concluded that the primary effect of PEG-conjugated lipids in these liposomes is mediated via a PEG-dependent steric barrier that does not influence protein binding and does not require 100% coverage of the liposome surface.

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