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Biochimica et Biophysica Acta 1510 (2001) 56–69

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Selective protein interactions with phosphatidylserine containing liposomes alter the steric stabilization properties of poly(ethylene glycol)

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Received 7 June 2000; received in revised form 31 August 2000; accepted 14 September 2000

Abstract

Incorporation of 5 mol% poly(ethylene glycol)-conjugated lipids (PEG-lipids) has been shown to extend the circulation longevity of neutral liposomes due to steric repulsion of PEG at the membrane surface. The effects of PEG-lipids on protein interactions with biologically reactive membranes were examined using phosphatidylserine (PS) containing liposomes as the model. Incorporating 15 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-PEG 2000 into PS liposomes resulted in circulation lifetimes comparable to that obtained with neutral liposomes containing 5 mol% DSPE-PEG 2000. These results suggested that 15 mol% DSPE-PEG 2000 may be effective in protecting PS liposomes from the high affinity, PS-mediated binding of plasma proteins. This was determined by monitoring the effects of PEG-lipids on calcium-mediated blood coagulation protein interactions with PS liposomes. Prothrombin binding and procoagulant activity of PS liposomes could be inhibited $>80\%$ when 15 mol% DSPE-PEG 2000 was used. These results are consistent with PS on membrane surfaces forming transient nucleation sites for protein binding that may result in lateral exclusion of PEG-lipids incorporated at <10 mol%. These nucleation sites may be inaccessible when PEG-lipids are present at elevated levels where they adopt a highly compressed brush conformation. This suggests that liposomes with reactive groups and PEG-lipids may be appropriately designed to impart selectivity to protein interactions with membrane surfaces.

Keywords: Poly(ethylene glycol); Phosphatidylserine; Steric stabilization; Protein binding; Liposome; Drug delivery

1. Introduction

The physical, chemical and biological properties of membrane surfaces derivatized with poly(ethylene glycol) (PEG) have been extensively studied over the past decade. Much of this interest arose from

Abbreviations: PEG, poly(ethylene glycol); PEG-lipids, poly(ethylene glycol)-lipid conjugates; PE-PEG, poly(ethylene glycol)-derivatized phosphatidylethanolamine; MPS, mononuclear phagocytic system; PS, phosphatidylserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; PC, phosphatidylcholine; SM, sphingomyelin; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG 2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]; DSPE-PEG 750, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 750]; Chol, cholesterol; CHE, cholesterylhexadecyl ether; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine

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0005-2736/01/$ – see front matter
PII: S0005-2736(00)00335-7

attempts to utilize polymers as 'cloaking' agents in order to extend blood residence times of various macromolecule-based pharmaceutical formulations. For liposomal delivery systems, poly(ethylene glycol)-derivatized phosphatidylethanolamine (PE-PEG) provided a synthetic alternative to ganglioside G_{M1}, a naturally occurring glycosylated lipid that had been shown to significantly increase the circulation longevity of 100 nm diameter liposomes into which it had been incorporated [1]. This effect was correlated with the ability of surface polymers to reduce plasma protein binding to liposomes, thus minimizing their recognition and removal from the blood by the cells of the mononuclear phagocytic system (MPS) through a process referred to as opsonization [2].

It is generally accepted that the characteristics of reduced protein binding and MPS clearance associated with PEG containing liposomes are due to the 'steric stabilization' properties imparted by the extension of PEG polymers away from the liposome surface [3,4]. This characteristic arises from the high degree of water binding by the surface PEG polymers and the ability of the random coil conformation to provide a 'mushroom-shaped' physical barrier to many proteins and larger biological structures [5,6]. The degree of surface binding inhibition provided by PEG on the membrane surface depends on the molecular weight of the PEG polymer, the concentration of poly(ethylene glycol)-lipid conjugate (PEG-lipid) in the bilayer and the molecular weight (size) of the membrane binding ligand. For example, liposome-cell interactions can be reduced with as little as 0.5 mol% PE-PEG 2000 incorporation whereas inhibition of liposome binding for small macromolecules (i.e. $M_r < 20\,000$) requires between 5 and 7 mol% PE-PEG 2000 or between 10 and 20 mol% PE-PEG 750 [7,8]. It is in these ranges of incorporation into phospholipid bilayers that the PEG molecules begin to adopt a more ordered and extended 'brush' state. It may not be surprising, then, that the sterically stabilized liposome composition most typically employed for intravenous drug delivery contains 5 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000] (DSPE-PEG 2000) in order to limit liposome interactions with cells and reduce binding of small molecular weight components present in plasma.

The vast majority of studies with PEG containing membranes have been performed with liposomes composed of lipids exhibiting no net charge such as phosphatidylcholines, sphingomyelins and cholesterol. Indeed, when more reactive lipids such as the negatively charged phospholipid phosphatidylserine (PS) were incorporated into liposomes containing PE-PEG 2000, these previously sterically stabilized liposomes became readily recognized by the MPS and were removed very rapidly from the circulation [9,10]. Current trends in the development of liposomal delivery vehicles are moving increasingly towards the inclusion of reactive lipid species to assist in entrapment/retention of agents (e.g. drugs, DNA, peptides) or to facilitate the transformation of liposomes from being stable in the circulation to being reactive or unstable once they have reached their intended target. Consequently, there is a need to better characterize the steric stabilization effects of PEG-lipids in liposomes that display reactive membrane surfaces in order to control their exposure under conditions where they may be employed.

The negatively charged phospholipid PS has long been recognized for its involvement in numerous biological processes including blood coagulation, complement activation and removal of senescent cells from the blood [11–13]. As described above, liposomes containing PS bind high amounts of plasma proteins and are removed from the circulation within minutes after i.v. injection. They accumulate primarily in the liver where Kupffer cells phagocytose the opsonized liposomes [14]. In the studies described here, we have investigated the steric stabilization properties of PE-PEG incorporated into liposomes containing PS as the model reactive lipid species. We have utilized the well-characterized binding interactions of blood coagulation proteins with PS containing liposomes as a model for evaluating the surface properties of PEG on such liposomes. With this system, the influence of PEG composition and content on PS containing lipid surfaces could be evaluated at several levels: (1) quantitative evaluation of the binding of specific proteins, (2) determination of functional activity of membrane bound proteins and protein complexes, and (3) correlation of protein binding with liposome clearance properties after i.v. injection. We demonstrate here that the binding barrier properties of PEG in liposomes containing PS

are substantially modified from those observed with neutral liposomes. These results reveal opportunities to design liposomes exhibiting selective surface chemistry based on inclusion of specific reactive lipid components and appropriate amounts of PEG.

2. Materials and methods

2.1. Materials

All lipids were obtained from Avanti Polar Lipids except for 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and [^{3}H]DSPE-PEG 2000 which was obtained from Northern Lipids (Vancouver, BC). The [^{3}H]- and [^{14}C]cholesterylhexadecyl ether (CHE) were from NEN/Dupont. Cholesterol, ellagic acid and Sepharose CL-4B were purchased from Sigma. All blood coagulation proteins were purchased from ICN (Aurora, OH), and the thrombin chromogenic substrate S-2238 was purchased from Chromogenix (Molndal, Sweden). Bio-Gel A-15m and A-5m size exclusion gel and gel filtration standards were obtained from Bio-Rad (Mississauga, ON). The micro bicinchoninic acid (micro BCA) protein assay kit was purchased from Pierce (Rockford, IL). The Oregon green 514 protein labeling kit was purchased from Molecular Probes (Eugene, OR).

2.2. Preparation of large unilamellar liposomes

Lipids were prepared in chloroform solution and subsequently dried under a stream of nitrogen gas. The resulting lipid film was placed under high vacuum for a minimum of 2 h. The lipid film was hydrated in HEPES 20 mM/NaCl 150 mM buffer (pH 7.5) at 65°C to form multilamellar vesicles. The resulting preparation was frozen and thawed five times prior to extrusion ten times through two stacked 0.1 μm polycarbonate filters (Poretics, Canada) with an extrusion apparatus (Lipex Biomembranes, Vancouver, BC) [15,16]. The extrusion temperature was kept at 65°C. The size of the liposomes was determined by quasi-elastic light scattering using a Nicomp 370 submicron particle sizer operating at a wavelength of 632.8 nm. Incorporation and retention of DSPE-PEG 2000 in liposomes after preparation and subsequent in vivo administration were determined by size exclusion chromatography. Briefly, liposomes with traces of [^{14}C]CHE (as a general lipid marker) and [^{3}H]DSPE-PEG 2000 (as the PEG-lipid marker) were applied to a 42 cm × 1.3 cm Bio-Gel A-15m column (50–100 mesh) at various concentrations, and eluted with HEPES 20 mM/NaCl 150 mM buffer (pH 7.5) at a flow rate of 0.5 ml/min regulated by a peristaltic pump. Aliquots from the 1 ml column fractions were counted directly in 5.0 ml scintillation fluid.

2.3. Plasma pharmacokinetics and tissue distribution of liposomes

Liposomes, labeled with [^{3}H]CHE as a non-exchangeable, non-metabolizable lipid marker [17], were injected via lateral tail vein with a lipid dose of 50 mg/kg and an injection volume of 200 μl into approx. 22 g female CD-1 mice. At various times, three mice from each group were terminated by CO_2 asphyxiation. Blood was collected by cardiac puncture, and placed into EDTA-coated or heparin-coated microtainer collection tubes (Becton-Dickinson). After centrifuging the blood samples at 4°C for 15 min at 1000 × *g*, plasma was isolated and visually showed no hemolysis. Aliquots of the plasma obtained were counted directly in 5.0 ml scintillation fluid. Liver, spleen and lungs were harvested from each group of mice to determine the biodistribution of liposomes. Briefly, 0.5 ml Solvable (Packard BioScience) was added to whole organs (spleen and lungs) or tissue homogenate (liver), and the mixture was incubated at 50°C overnight. After cooling to room temperature, 50 μl EDTA 200 mM, 200 μl hydrogen peroxide 30%, and 25 μl HCl 10 N were added, and the mixture was incubated for 1 h at room temperature. The mixture was added with 5.0 ml scintillation fluid and counted 24 h later.

2.4. In vivo recovery and analysis of liposome-associated proteins

Blood from mice injected with radiolabeled liposomes was collected into heparin-coated microtainers, and plasma was isolated and pooled from four mice. The plasma (600 μl) was applied to a 18 cm × 1 cm Sepharose CL-4B column, and eluted with HEPES 20 mM/NaCl 150 mM/$CaCl_2$ 2.5 mM buffer (pH 7.5) at a flow rate of 5.87 ml/h regulated

by a peristaltic pump. Column fractions were analyzed for radioactivity to determine the fractions containing liposomes, and the two fractions with the highest radioactivity were pooled for the quantification of total protein associated with the recovered liposomes. The proteins associated with the recovered liposomes were extracted and delipidated according to the procedure described by Wessel and Flugge since lipids interfere with most protein assays [18]. The extracted proteins were resuspended in 20 μl distilled water. Quantification of the proteins was performed using the micro BCA protein assay procedure. Ten microliters of the extracted proteins was diluted to 0.15 ml with Milli-Q water, and 0.15 ml protein assay reagent was added. The mixture was incubated for 2 h at 37°C. After the mixture was cooled to room temperature, the absorbance at 570 nm was measured with a plate reader. The extracted proteins were quantified by a standard curve consisting of known amounts of bovine serum albumin which was linear in the range of 0–17.5 μg/ml.

2.5. Prothrombin binding to liposomes

Bovine prothrombin was labeled with the fluorescent dye Oregon green 514, containing a reactive succinimidyl ester moiety that reacts with primary amines of the protein to form dye-protein conjugates. The fluorescently labeled prothrombin was incubated with various liposome compositions at lipid concentrations of 0.2 and 0.4 mg/ml in the presence of 2.0 mM Ca^{2+} at 37°C for 15 min. The mixture was then separated using Microcon 100 ultrafiltration devices by centrifugation at $3000 \times g$ for 15 min. The filtrate, containing free protein, was measured for fluorescence with excitation and emission wavelengths set at 506 and 526 nm, respectively. The amount of prothrombin bound to liposomes was determined using a calibration curve constructed with known amounts of fluorescent-labeled prothrombin and correcting for protein recovery using liposome-free prothrombin solutions.

2.6. In vitro chromogenic assay for factor Xa activity

Formation of catalytically active prothrombinase protein complexes (factor Xa and factor Va) on liposome surfaces was determined employing a chromogenic substrate that is cleaved by enzymatically active thrombin [19]. Briefly, the 'prothrombinase complex cocktail' contained the components for the prothrombinase conversion under the following conditions: 8.0 nM (0.2 unit) factor Xa, 0.2 nM factor Va, 6 mM $CaCl_2$, and liposomes at various concentrations. These mixtures were incubated in Tris 50 mM/NaCl 120 mM buffer (pH 7.8) for 5 min at 37°C. Prothrombin (1 mM) was added to the cocktail, and the final mixture (150 μl) was incubated for 3 min. The conversion of prothrombin to thrombin was stopped by the addition of EDTA (15 mM final concentration). S-2238, which is a specific chromogenic substrate of thrombin, was added at 0.4 mM, and the rate of chromogen formation was monitored at 405 nm with a plate reader equipped with kinetic analysis software (Dynex Technologies, Chantilly, VA). A calibration curve was obtained under the same conditions with known amounts of thrombin, and the amount of thrombin formed in the assay was determined from the calibration curve.

2.7. In vitro clotting time assay

This assay was based on the activated partial thromboplastin time. An ellagic acid solution was used freshly prepared and diluted in 20 mM HEPES/150 mM NaCl. Human citrated plasma (50 μl) was preincubated with 10^{-5} M ellagic acid (50 μl) and liposomes (50 μl) for 2 min at 37°C. Calcium was then added to initiate the clotting reaction. The time at which the mixture changed from a liquid to a viscous gel was recorded, and was noted as the time for the clotting reaction to be completed.

3. Results

3.1. Effect of PEG-lipids on the plasma pharmacokinetics and the tissue distributions of PS containing liposomes

Liposomes containing PS are rapidly eliminated from the circulation, a phenomenon believed to be due to extensive binding of plasma proteins and uptake by the MPS [14]. This can be seen in Fig. 1, which presents the plasma pharmacokinetics of 10 mol% PS liposomes containing various amounts of

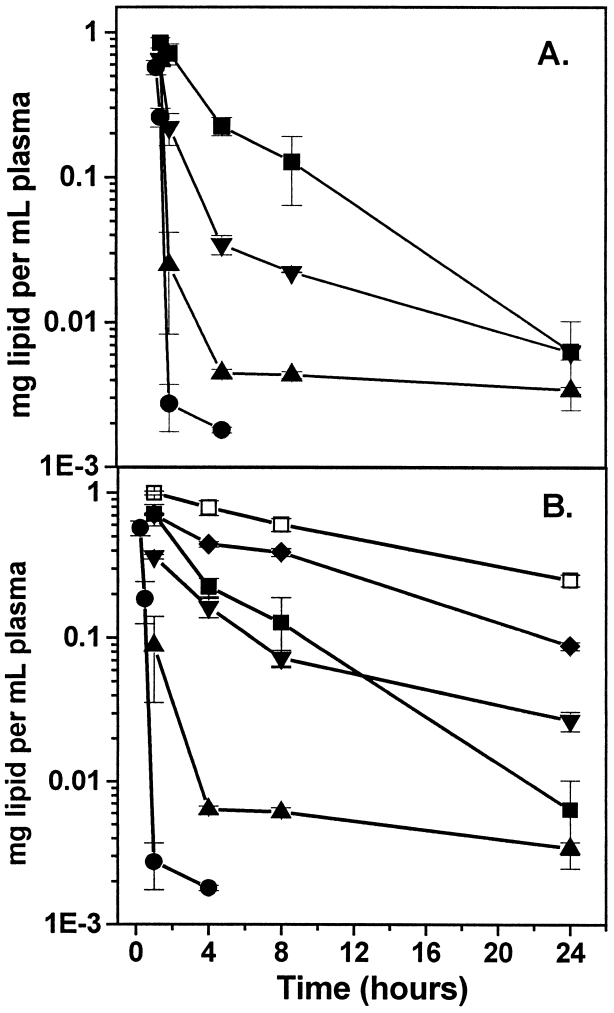


Fig. 1. The effect of incorporating (A) DSPE-PEG 750 and (B) DSPE-PEG 2000 on the plasma elimination of PS liposomes. Three mice were used for each data point, and the error bars represent the standard errors. The plasma elimination curves for the following liposomes were determined and plotted in (A): DSPC/Chol 55:45 (■), DOPS/DSPC/Chol 10:45:45 (●), DSPE-PEG 750 incorporated at 10 mol% (▲) and 20 mol% (▼) in DOPS 10%/DSPC/Chol 45%. The plasma elimination curves for the following liposomes were determined and plotted in (B): DSPC/Chol 55:45 (■), DOPS/DSPC/Chol 10:45:45 (●), DSPE-PEG 2000 incorporated at 5 mol% (▲), 10 mol% (▼) and 15 mol% (♦) in DOPS 10%/DSPC/Chol 45%, and DSPE-PEG 2000/DSPC/Chol 5:50:45 (□).

DSPE-PEG 750 (Fig. 1A) or DSPE-PEG 2000 (Fig. 1B). Whereas conventional neutral DSPC/cholesterol (Chol) liposomes exhibited a monophasic plasma elimination curve with $>10\%$ of the injected dose remaining at 8 h post injection, inclusion of 10 mol% PS in DSPC/Chol liposomes changed the plasma elimination curve to a biphasic shape with $<1\%$ of the liposomes in plasma within 1 h. The circulation longevity of DSPC/Chol liposomes was dramatically decreased with the inclusion of 10 mol% PS, as reflected by the concentration of liposomes remaining in the circulation which was reduced from 0.23 mg/ml to 0.0019 mg/ml 4 h post injection. The mean AUC_{0-24h} for DSPC/Chol liposomes was also reduced from 4.02 to 0.65 mg ml^{-1} h with the inclusion of 10 mol% PS.

The incorporation of DSPE-PEG 750 or DSPE-PEG 2000 generally provided protection of the PS liposomes from plasma clearance, reflected by the trend that increasing PEG-lipid content in the PS liposomes increased the circulation longevity of the PS liposomes (Fig. 1A,B). However, the protecting effect of DSPE-PEG against liposome clearance was significantly increased for PEG 2000 compared to PEG 750. Incorporation of DSPE-PEG 750 into the liposomes at 20 mol%, which is the maximum concentration without inducing micelle formation [20], increased the circulation lifetime of PS liposomes as reflected by an increase in the 4 h plasma lipid concentration to 0.035 mg/ml. This was significantly lower than the 4 h plasma lipid concentration of 0.23 mg/ml observed for DSPC/Chol liposomes. Similar results were observed when 5 mol% DSPE-PEG 2000 was incorporated into PS containing liposomes (Fig. 1B). In contrast, incorporation of 10 mol% of DSPE-PEG 2000 into PS containing liposomes resulted in plasma elimination curves that were comparable to those obtained for DSPC/Chol liposomes devoid of PS (Fig. 1B). Furthermore, increasing the DSPE-PEG 2000 content to 15 mol%, which is the maximum incorporation level of DSPE-PEG 2000 for bilayer stability, resulted in plasma liposome elimination curves that approached those observed for PS-free sterically stabilized liposomes (5 mol% DSPE-PEG 2000 in DSPC/Chol liposomes). Inclusion of 15 mol% DSPE-PEG 2000 into 10 mol% PS liposomes increased the mean AUC_{0-24h} by 12-fold from 0.65 mg ml^{-1} h to 7.97 mg ml^{-1} h.

The trends observed in liposome plasma elimination time correlated closely with liposomal lipid levels observed in the major MPS organs, the liver and spleen. Specifically, the plasma liposome levels were inversely proportional to the liver and spleen liposome recovery 4 h after i.v. injection. The inclusion

of 10 mol% PS in DSPC/Chol liposomes increased the amount of lipid accumulated in liver from 0.29 to 0.63 mg/g tissue and in spleen from 1.27 to 2.47 mg/g tissue. Incorporating DSPE-PEG 750 or DSPE-PEG 2000 into 10 mol% PS liposomes decreased the amount of lipid accumulated in liver and spleen. For example, when 15 mol% DSPE-PEG 2000 was incorporated into the PS liposomes, a five-fold reduction in the amount of lipid accumulated in liver and spleen was observed (data not shown).

3.2. Evaluation of liposome-protein interactions after in vivo injection

The extended circulation time of sterically stabilized liposomes has been attributed to the ability of PEG-lipids to reduce protein binding and cell interactions [3,4]. In this study, the amount of protein associated with liposomes recovered from mice 30 min post injection was determined with the micro BCA protein assay (Table 1). Liposomes with 10 mol% PS in the absence of PEG-lipids had the highest amount of associated protein per mole of recovered liposomal lipid (20.0 g protein/mol lipid). In contrast, the amount of protein associated with the sterically stabilized 5 mol% DSPE-PEG 2000/DSPC/Chol liposomes was 5.86 g protein/mol lipid. The incorporation of 10 mol% DSPE-PEG 2000 into the 10 mol% PS liposomes provided moderate reduction in the amount of liposome-associated protein (7.49 g protein/mol lipid). When 15 mol% DSPE-PEG 2000 was incorporated into the 10 mol% PS liposomes, the amount of associated protein was reduced to 4.03 g protein/mol lipid, which reflected a five-fold reduction when compared to values of non-pegylated PS containing liposomes (Table 1). Thus, as the mole percentage of DSPE-PEG 2000 was increased in the PS liposomes, the circulation time of PS liposomes was extended with reduced plasma protein binding and decreased accumulation in the MPS organs.

3.3. Incorporation of DSPE-PEG 2000 into PS liposomes

From the results of the in vivo studies, DSPE-PEG 2000 needed to be incorporated at 15 mol% in PS liposomes to reduce plasma protein interactions. At such high levels, there are potential concerns regarding the incorporation efficiency of the PEG-lipids. In particular, formation of micelles has been demonstrated when the level of DSPE-PEG 2000 was more than 15 mol% in liposomes [20–22], which is close to the level used here for extending the circulation longevity of PS liposomes. Therefore, the incorporation of 15 mol% DSPE-PEG 2000 into 10 mol% PS liposomes was examined with size exclusion chromatography to separate liposomes from DSPE-PEG 2000 micelles. The ability of the Bio-Gel A-15m gel filtration column to resolve liposomes from DSPE-PEG 2000 micelles was first confirmed by applying liposomes and DSPE-PEG 2000 micelles to the column immediately after mixing. As shown in the inset of Fig. 2, liposomes eluted at the exclusion volume of the column while DSPE-PEG 2000 micelles eluted near the inclusion volume. Further separation on a Bio-Gel A-5m gel filtration column and comparison with molecular weight standards indicated that the micelles exhibited an average size of approx. 670 000 Da (data not shown). This demonstrated that DSPE-PEG 2000 micelles could be separated from liposomes in mixed solutions, and this

Table 1
P_B values[a] for liposomes recovered from murine plasma 30 min post injection of liposomes

Liposomes	In vivo P_B value (g protein/mol lipid)
DSPE-PEG 2000/DSPC/Chol 5:50:45	5.86
1,2-Dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS)/DSPC/Chol 10:45:45	20.0
DSPE-PEG 2000/DOPS/DSPC/Chol 10:10:40:45	7.49
DSPE-PEG 2000/DOPS/DSPC/Chol 15:10:30:45	4.03

[a] P_B value was defined as the amount of protein associated with each mole of lipid recovered from murine plasma 30 min post injection in a single determination. Due to low recovery of lipids from animals, plasma from four mice was pooled, and chromatographed on a Sepharose CL-4B column under similar conditions. Column fractions containing the liposomes were pooled, and the amount of protein was determined by the micro BCA assay as described in Section 2.

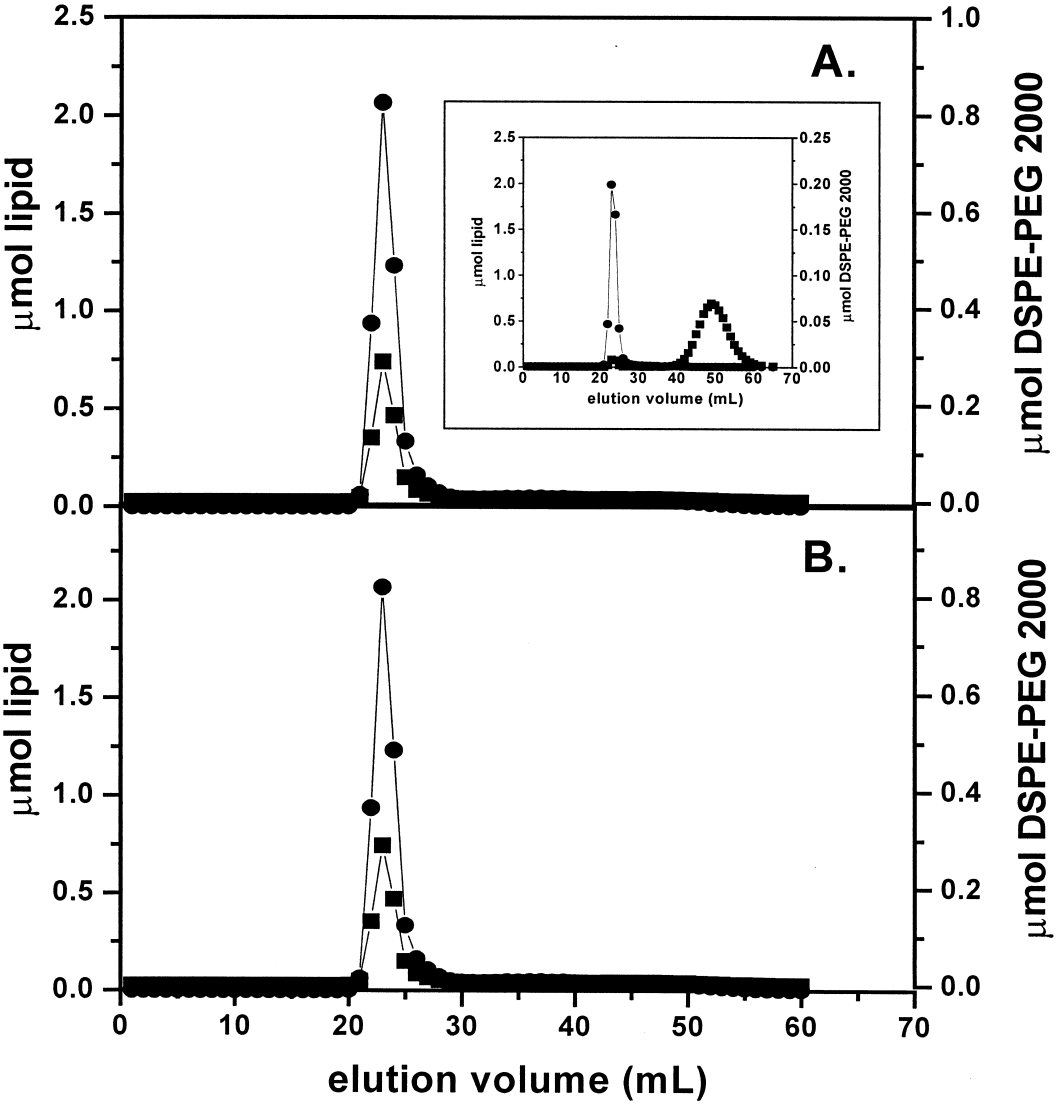


Fig. 2. Size exclusion chromatography with Bio-Gel A-15m column of (A) 10 mol% PS liposomes and (B) DSPC/Chol liposomes, each containing 15 mol% DSPE-PEG 2000, at stock concentrations (54 mM). Both types of liposomes were labeled with traces of [^{14}C]CHE (●) as a lipid marker and [^{3}H]DSPE-PEG 2000 (■). The calibration of the Bio-Gel A-15m column with DOPS/DSPC/Chol 10:45:45 liposomes (●) and DSPE-PEG 2000 micelles (■) is shown in the inset.

could be used to determine the retention of DSPE-PEG 2000 in liposomes when prepared at high incorporation levels.

Fig. 2A shows that no micelle peak was observed in the elution profile of the stock preparation (54 mM) of 10 mol% PS liposomes containing 15 mol% DSPE-PEG 2000. Identical elution profiles were obtained when the stock liposomes were diluted to the concentrations used in in vitro (0.2 mM) and in vivo (6.2 mM) experiments (data not shown). Based on the ratio of the radiolabeled DSPE-PEG 2000 and liposome marker ([^{14}C]CHE), the amount of DSPE-PEG 2000 present in PS liposome containing fractions reflected a DSPE-PEG 2000 composition of 14 mol% for all of the liposome concentrations. For comparison, DSPC/Chol liposomes containing 15 mol% DSPE-PEG 2000 were assayed with the same technique, and a similar elution profile was observed, with over 14 mol% DSPE-PEG 2000 being incorporated into the liposomes (Fig. 2B). These data indicate that PEG-lipid content up to 15 mol% can be incorporated into liposomes, and

Table 2
Mole percentage of DSPE-PEG 2000 remaining in liposomes recovered from murine plasma at various hours post injection of liposomes

	Time (h)	Mole% of DSPE-PEG 2000 remaining in liposomes (±S.D.)[a]
DSPE-PEG 2000/DOPS/DSPC/Chol 15:10:30:45[b]	1	14.3±0.1
	4	13.9±0.2
	24	13.4±0.1
DSPE-PEG 2000/DSPC/Chol 15:40:45[b]	1	14.5±0.4
	4	14.1±0.1
	24	12.6±0.2

[a]Data represent the average ± S.D. of three mice. The injected lipid dose was 50 mg/kg.
[b]The liposomes were dual-labeled with [^{14}C]CHE (as a general lipid marker) and [^{3}H]DSPE-PEG 2000 and prepared as described in Section 2.

that PEG-lipid micelles could not be detected during the preparation or the dilution of liposomes containing up to 15 mol% DSPE-PEG 2000.

The retention of DSPE-PEG 2000 in liposomes after intravenous administration was also investigated, since the loss of PEG-lipids from liposomes could compromise the shielding of PS liposomes from plasma components. Ten mol% PS liposomes and PS-free DSPC/Chol liposomes, both containing 15 mol% DSPE-PEG 2000, were injected into mice to determine the amount of DSPE-PEG 2000 retained by the liposomes in plasma at various times post injection (Table 2). Approx. 13 mol% DSPE-PEG 2000 remained associated with the 10 mol% PS liposomes and DSPC/Chol liposomes in the circulation over 24 h. The incorporation and the retention of 15 mol% DSPE-PEG 2000 were not specific to PS liposomes, since similar gel filtration column profiles (see Fig. 2B) and in vivo DSPE-PEG 2000 retention were observed with 15% DSPE-PEG 2000/DSPC/Chol liposomes (see Table 2).

3.4. *Effects of DSPE-PEG 2000 on prothrombin to PS liposomes*

The results from our in vivo studies have shown that an elevated PEG-lipid content (15 mol%) was effective in reducing plasma protein binding of PS liposomes, and that the protection of PS liposomes was dependent on the molecular weight of the PEG polymer, with higher molecular weight (PEG 2000) being more effective. In light of these results, we postulated that M_r 2000 PEG-lipids, incorporated at an elevated level (15 mol%), may be effective in protecting the PS liposome surface from the high affinity, PS-mediated binding of plasma proteins. We investigated this concept by utilizing the well-characterized interactions of blood coagulation proteins with PS membranes as a model to evaluate the 'steric stabilizing' effect of PEG-lipids on such reactive membranes.

Calcium-dependent prothrombin binding to 10 mol% PS liposomes was determined using fluorescently labeled protein and separating free and liposome bound pools under equilibrium conditions with ultrafiltration devices [23]. Fluorescent derivatization did not significantly alter the binding properties of prothrombin to PS containing liposomes as free vs. bound protein fractions were similar to those for prothrombin binding to PS containing liposomes reported previously using light scattering techniques [23]. Negligible prothrombin association with liposomes was observed in the absence of PS under conditions where between 25% and 40% of the protein in solution was bound to 10 mol% PS liposomes with initial prothrombin to lipid weight ratios of 0.25:1 and 0.1:1 respectively. Incorporation of DSPE-PEG 2000 at 5 mol% in DSPC/Chol (50:45 molar ratio) liposomes resulted in 28% and 37% inhibition of prothrombin binding at protein/lipid weight ratios of 0.25:1 and 0.1:1, respectively (Fig. 3). Increasing the amount of DSPE-PEG 2000 to 10 mol% and 15 mol% significantly enhanced the inhibition of prothrombin binding to the 10% PS liposomes where an 85% decrease in protein binding was observed using 15% DSPE-PEG 2000 at a protein to lipid weight ratio of 0.1:1 and a 75% protein binding decrease was obtained at the 0.25:1 protein to lipid ratio.

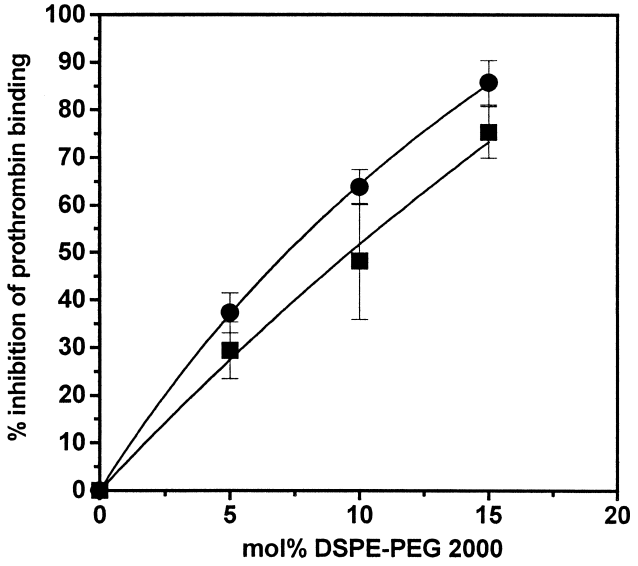


Fig. 3. The effect of DSPE-PEG 2000 on the binding of prothrombin to 10 mol% PS liposomes at two different prothrombin to liposome ratios: 0.1 w/w (●) and 0.25 w/w (■). The % inhibition was calculated as follows: % inhibition = $(F_{PEG}-F_{PS})/(F_{control}-F_{PS})\times 100$, where F represents the fluorescence of the unbound protein in the filtrate after centrifugation with the Microcon 100 ultrafiltration device and the subscripts represent the different types of liposomes tested as described in Section 2.

3.5. *Effects of PEG-lipids on the functional activity of membrane bound blood coagulation proteins*

The results above demonstrate that the degree of inhibition of prothrombin binding to PS liposomes is related to increasing PEG-lipid content. We then extended these observations to examine effects of PEG-lipids on the functional activity of membrane bound blood coagulation proteins. We first investigated the effect of PEG-lipids on the catalytic activity of the prothrombinase complex, which consists of factors Xa and Va assembled on negatively charged membrane surfaces and is responsible for the proteolytic activation of prothrombin to thrombin. The rate of thrombin formation by the prothrombinase complex in the presence of liposomes was monitored using a chromogenic substrate that is activated by thrombin. In DSPC/Chol liposomes (0 mol% PS), the rate of thrombin formation was negligible, and no substrate activation was observed in the absence of liposomes. Incorporating 10 mol% PS into DSPC/Chol liposomes resulted in a rate of thrombin formation of 1.94 mol thrombin/min/mol factor Xa (Table 3). The effectiveness of DSPE-PEG 750 and DSPE-PEG 2000 in inhibiting the assembly and the catalytic activity of the prothrombinase complex on the PS membrane surface were evaluated. With 10 mol% DSPE-PEG 750 or 5 mol% DSPE-PEG 2000 in 10 mol% PS liposomes, the rates of thrombin formation were 2.48 and 1.96 mol thrombin/min/mol factor Xa respectively, which were similar to those for the non-pegylated PS liposomes. Only by elevating the PEG-lipid content to 20 mol% for DSPE-PEG 750 or 10–15 mol% for DSPE-PEG 2000 could the rate of thrombin formation be substantially reduced to <0.465 mol thrombin/min/mol factor Xa (minimum detectable rate).

In addition to the prothrombinase complex, the PS membrane surface is required for the proteolytic activation of several additional blood coagulation proteins and propagation of the blood coagulation cascade [11,24]. Also, full clot formation requires the release of thrombin from the prothrombinase complex which can then enzymatically convert fibrinogen to fibrin. We therefore determined the impact of PEG-lipids on the comprehensive procoagulant activity of PS liposomes using an assay which measured the activated partial thromboplastin time where exogenously added liposomes provided the catalytic membrane surface. First, the clotting times

Table 3
Rate of thrombin formation in the presence of various liposomes assayed by the in vitro chromogenic assay

Liposomes	Mol thrombin/min/mol factor Xa ±S.D.[a]
DSPC/Chol 55:45	N.D.[b]
DOPS/DSPC/Chol 10:45:45	1.94 ± 0.24
DSPE-PEG 2000/DOPS/DSPC/Chol 5:10:40:45	1.96 ± 0.34
DSPE-PEG 2000/DOPS/DSPC/Chol 10:10:35:45	N.D.
DSPE-PEG 2000/DOPS/DSPC/Chol 15:10:30:45	N.D.
DSPE-PEG 750/DOPS/DSPC/Chol 10:10:35:45	2.48 ± 0.18
DSPE-PEG 750/DOPS/DSPC/Chol 20:10:25:45	N.D.

[a]Data represent the averages ± S.D. of three experiments. Liposome concentrations used were 75 μM.
[b]N.D. represents non-detectable levels which were characterized by the assay system to be below 0.46 mol thrombin/min/mol factor Xa.

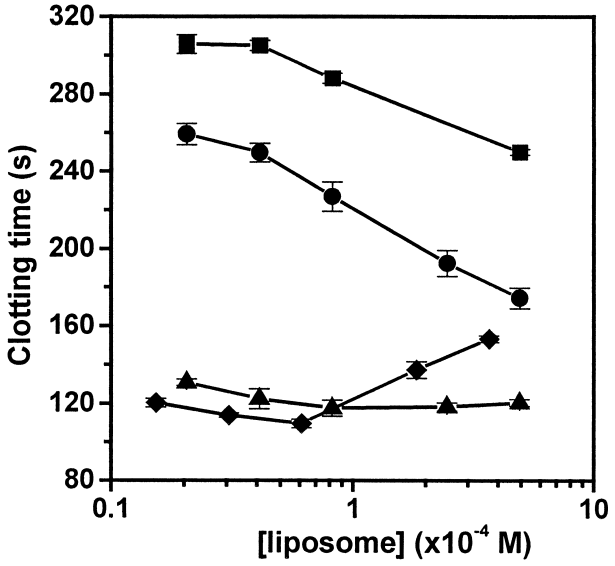
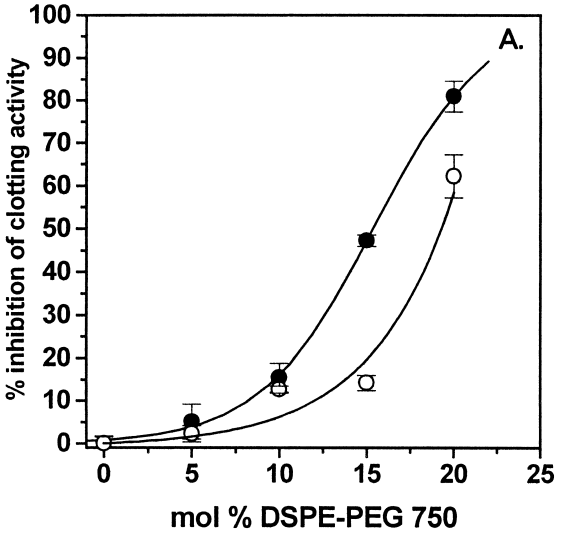


Fig. 4. Clotting activity of DSPC/Chol liposomes with different mol% DOPS as determined by the in vitro clotting time assay. The clotting times were measured for 0 mol% (■), 5 mol% (●), 10 mol% (▲) and 20 mol% (♦) of DOPS incorporated with DSPC/Chol as the bulk lipids. Data points were determined in triplicate, and the error bars represent standard deviations.

of liposomes with various mol% PS were compared in order to establish baseline procoagulant activity of the liposomes as well as conditions for comparative experiments (Fig. 4). In the absence of phospholipids, the time to form the fibrin clot was approx. 350 s (data not shown), while the addition of neutral DSPC/Chol liposomes at 0.02 mM and 0.5 mM decreased the clotting time to 310 s and 255 s, respectively. The inclusion of 10–20 mol% PS in DSPC/Chol liposomes further decreased the clotting times to approx. 120 s when the liposome concentration was below 0.1 mM. This reduction in clotting times reflected that the presence of a negatively charged phospholipid membrane surface enhanced the blood coagulation reactions. The clotting time of 20 mol% PS liposomes increased to 152 s as the liposome concentration was increased to 0.36 mM; however, the clotting times of 10 mol% PS liposomes were maintained at approx. 120 s throughout the range of liposome concentrations used in the assay. The increase in clotting time observed with 20 mol% PS liposomes as the liposome concentration increased is consistent with an excess of the catalytic surface. This excess catalytic surface results in surface-dilution effects of the blood coagulation proteins leading to decreased thrombogenic activity [11]. These results demonstrated that both 10 and 20 mol% PS liposomes had significant procoagulant activity, and were utilized to evaluate the effectiveness of DSPE-PEG 750



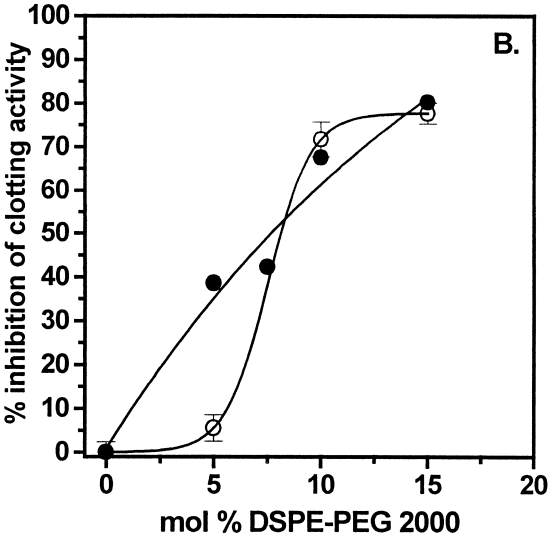


Fig. 5. The use of (A) DSPE-PEG 750 and (B) DSPE-PEG 2000 to inhibit the clotting activity of PS liposomes. The % inhibition was calculated as follows: % inhibition = $(t_{PEG}-t_{PS})/(t_{blank}-t_{PS})\times 100$, where t represents the clotting time of each type of liposome as determined by the in vitro clotting time assay. Liposomes with 10 mol% (●) and 20 mol% (○) of DOPS in DSPC/Chol were compared. The liposome concentrations used in A and B were 0.4 mM and 0.2 mM respectively. Data points were determined in triplicate, and the error bars represent standard deviations.

and DSPE-PEG 2000 in impeding the propagation of the blood coagulation cascade.

The percent inhibition of clotting activity of 10 and 20 mol% PS liposomes by the PEG-lipids is presented in Fig. 5A,B. When incorporated at ≤ 10 mol%, DSPE-PEG 750 inhibited approx. 15% of the clotting activity for 10 and 20 mol% PS liposomes. The inhibitory effect of DSPE-PEG 750 was sigmoidal, as reflected by the increase in the percent inhibition of the clotting activity for 10 and 20 mol% PS liposomes. When the level of DSPE-PEG 750 in PS liposomes was increased to 20 mol%, the inhibition of procoagulant activity was increased to 85% and 65% for 10 and 20 mol% PS liposomes, respectively (Fig. 5). A similar trend was observed with DSPE-PEG 2000 where low levels of the PEG-lipid in the PS liposomes provided only modest inhibition to the clotting activity. Specifically, when DSPE-PEG 2000 was incorporated at 5 mol%, 40% and 8% of the clotting activity was inhibited for 10 and 20 mol% PS liposomes, respectively. This inhibitory effect was increased to approx. 80% when the level of DSPE-PEG 2000 was increased to 15 mol% in the two PS liposomes. These results agreed well with those obtained for prothrombin binding and prothrombinase complex activity determined with the chromogenic assay.

4. Discussion

Surface modification of liposomes with the hydrophilic polymer PEG has provided a major advance in drug delivery applications due to the ability of this polymer to reduce protein binding and plasma elimination of liposomes. The mechanism by which the PEG polymer protects the liposome surface has been attributed to (1) the hydrophilicity of the polymer that effectively provides a 'water shell' to the liposome surface, and (2) the flexibility of the polymer that gives a large number of conformations, resulting in a 'statistical cloud' which sterically protects the liposome surface [5,25]. Most of the studies on PEG-lipids have focused on the protection of neutral liposome surfaces from plasma protein binding and subsequent MPS uptake. In this context, several studies have demonstrated that 5–7 mol% DSPE-PEG 2000 is optimal for protection of such liposomes [5,10]. However, the use of PEG-lipids to protect a biologically active membrane surface has not been well documented. In this study, liposomes containing PS were used as the model for a biologically active membrane, and the ability of the PEG-lipids to protect this liposome surface was tested under both in vitro and in vivo conditions.

One of the first questions that arose in this study was the feasibility of incorporating PEG-lipids at elevated levels into liposomes. This is due to reports that DSPE-PEG 2000 incorporation beyond 7–8 mol% into phospholipid liposomes can lead to alternate lipid phases primarily arising from DSPE-PEG micelle formation [5,10]. However, our results demonstrated DSPE-PEG 2000 can be readily incorporated into DSPC/Chol-based liposomes up to 15 mol% without the generation of micelles. Further, the in vivo retention and stability of the PEG-lipids in liposomes exposed to the biological milieu argue against micelle formation. These results are in good agreement with another study which demonstrated that DSPE is a more stable anchor for PEG 2000 than 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine (POPE) with respect to PE-PEG retention in liposomes [26].

In contrast to neutral liposomes such as DSPC/Chol where 5–7 mol% DSPE-PEG 2000 provides protection for protein binding, biologically active liposomes containing PS with these levels of PEG-lipids are still capable of interacting with relatively large proteins. Only when the PEG-lipids were present at higher mole percentages was prothrombin binding significantly inhibited. This observation is somewhat surprising since studies by Needham et al. [7] have demonstrated that the k_{on} for similarly sized proteins or lysolipid micelles (compared to 1800 $Å^2$ per molecule surface coverage for prothrombin, see [27]) interacting with a membrane surface is decreased approx. 100-fold when DSPE-PEG 750 or DSPE-PEG 2000 are incorporated into phosphatidylcholine (PC) bilayers at 12% and 6%, respectively. The fact that 20% DSPE-PEG 750 or 15% DSPE-PEG 2000 are required to significantly inhibit prothrombinase protein interactions with PS containing liposomes indicates that these protein molecules can penetrate through the PEG layer at lower polymer levels (e.g. 5 mol% DSPE-PEG 2000). Similar PEG content dependences observed for inhibition of pro-

thrombin binding, chromogenic prothrombinase activity and complete clotting activity indicate that even the large ternary protein prothrombinase complex (in excess of 7000 Å^2 surface coverage) can associate on PS membranes containing $<10\%$ PEG 2000.

Three key features may account for the altered steric stabilization effects of PEG-DSPE observed for the protein membrane interactions studied here. First, the electrostatic attraction that exists between negatively charged PS molecules and binding ligands on the coagulation proteins could provide some of the energy necessary for the additional work required to move proteins through the PEG layer. This could decrease the effect of PEG on k_{on} for PS coagulation protein binding compared to non-specific protein-neutral membrane interactions. Second, the high binding affinities of coagulation proteins for PS containing liposomes (K_d values ranging from 7×10^{-8} M to 2.5×10^{-10} M, see [28,29]) arise primarily from significant decreases in k_{off} where collisional binding efficiencies $>30\%$ have been documented for proteins of the prothrombinase complex [28]. This is in contrast to the very inefficient contact phase of protein adsorption to neutral membrane surfaces [28,29]. Consequently, the ability of prothrombin and other blood coagulation proteins to bind to PS containing liposomes in the presence of 5% PEG 2000 may be kinetically driven due to the very slow release of protein once initial contact with the membrane has been made through transient openings in the steric PEG barrier that have been shown to occur at PEG 2000 concentrations as high as 10 mol% [30].

The third membrane binding feature for prothrombin, factor X and factor V, that may impact on the steric stabilization properties of PEG-lipids is their multivalent interaction with PS at the membrane surface. Eight PS molecules are required to bind one prothrombin molecule and the total PS requirement for binding all three proteins of the prothrombinase complex is between 40 and 50 molecules [31]. Assuming an average area per PS molecule of approx. 60 Å^2, 27% of the area covered by one prothrombin molecule is occupied by PS, reflecting a 2.7-fold enrichment of PS in this region. Similarly, the approx. 7000 Å^2 covered by the complete prothrombinase complex (prothrombin, factor X and factor V) would be predicted to have a lipid composition consisting of between 40 and 50 mol% PS. Previous studies have demonstrated that these proteins are capable of inducing lateral separation of PS in PS/PC liposomes [23]. Such membrane microdomains enriched in PS would be largely devoid of DSPE-PEG, thereby providing potential nucleation sites for protein binding where the steric barrier associated with PEG-lipids would be absent.

We propose that compression of the packing area per PEG molecule at the liposome surface is necessary as DSPE-PEG 2000 is being excluded from the protein binding sites under this PS-enriched protein binding microdomain model. This prediction can be made due to the fact that prothrombinase protein binding is mediated through the PS head group at the lipid/water interface and does not require protein insertion into the hydrophobic portion of the bilayer [32]. Previous studies have documented that the packing area per PEG molecule was 960 Å^2 and 480 Å^2 for levels of 5 and 10 mol% incorporation respectively, based on a PEG volume of 31 500 Å^3 [33]. We propose that DSPE-PEG 2000 can be further compressed to a PEG packing area of 330 Å^2 per molecule and an extension of 100 Å from surface at 15 mol% incorporation (see Fig. 6). For PS liposomes containing 5 and 10 mol% DSPE-PEG 2000, this means that 67% and 33% of liposome surface can be made available for protein binding if the PEG molecule is compressed to 330 Å^2. The level of prothrombin binding to PS liposomes with 5 and 10 mol% DSPE-PEG 2000 was approx. 70% and 30%, respectively, of that obtained in the absence of PEG which is consistent with the PEG compression model described above. The combined effects of densely packed PEG and extension of the polymer barrier from the membrane surface under these conditions appear to provide steric inhibition of even high affinity protein-membrane interactions. It remains to be determined whether much smaller molecules are able to penetrate to the liposome surface under these densely packed conditions.

It is of particular interest that the relationship of DSPE-PEG 2000 incorporation with in vivo circulation lifetime and protein binding closely matched the results of specific in vitro assays for inhibition of coagulation protein-PS binding. This suggests that the altered steric stabilization effects of PEG in membranes containing significant concentrations of reac-

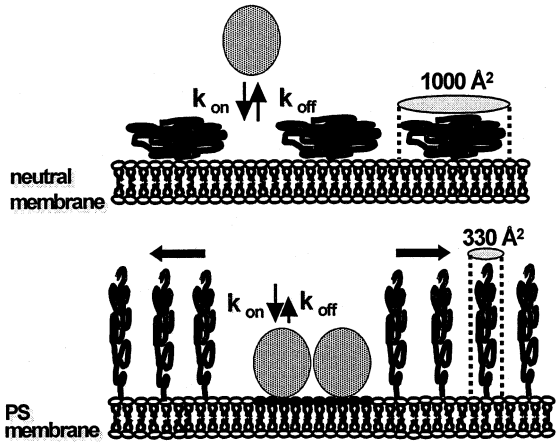


Fig. 6. An illustration of the PS-enriched protein binding microdomain model. DSPE-PEG 2000 is laterally excluded from the protein binding site (indicated by black arrows) when proteins bind to the PS membrane surface mediated by the PS head group (black circles). Compression of the packing area per PEG molecule at the liposome surface from 1000 Å^2 (at 5 mol% DSPE-PEG 2000) to 330 Å^2 is proposed to be necessary for accommodating the bound proteins.

tive lipid species may have significant implications beyond the interactions of blood coagulation proteins. The pharmacokinetic studies here are the first to document effective protection of PS liposomes from plasma protein binding and removal from circulation by MPS in the absence of phagocytic poisons. Specifically, 10% PS liposomes containing 15% DSPE-PEG 2000 displayed plasma elimination curves as well as liver and spleen uptake that were comparable to those for traditional 'stealth' DSPE-PEG 2000 5%/DSPC/Chol liposomes. This suggests that liposomes containing surface grafted PEG and reactive lipids may be appropriately designed to select for specific interactions with plasma components based on molecular size and membrane binding affinities. Such applications may provide additional flexibility in developing therapeutic liposomes with improved targeting and disease site specific activation properties.

Acknowledgements

We are grateful to Dana Masin, Natashia McIntosh and Rebecca Ng for their technical assistance in the animal studies. This research project is supported by a research grant from the Medical Research Council of Canada. G.N.C.C. is supported by a graduate fellowship from the University of British Columbia.

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