

Effects of phosphatidylserine on membrane incorporation and surface protection properties of exchangeable poly(ethylene glycol)-conjugated lipids

Gigi N.C. Chiu^{a,c}, Marcel B. Bally^{b,c}, Lawrence D. Mayer^{a,c,*}

^a Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC, Canada

^b Department of Pathology and Laboratory Medicine, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada

^c Department of Advanced Therapeutics, British Columbia Cancer Research Center, 601 West 10th Ave., Vancouver, BC, Canada V5Z 1L3

Received 17 July 2001; received in revised form 28 November 2001; accepted 5 December 2001

Abstract

Liposomes containing the acidic phospholipid phosphatidylserine (PS) have been shown to avidly interact with proteins involved in blood coagulation and complement activation. Membranes with PS were therefore used to assess the shielding properties of poly(ethylene glycol 2000)-derivatized phosphatidylethanolamine (PE-PEG₂₀₀₀) with various acyl chain lengths on membranes containing reactive lipids. The desorption of PE-PEG₂₀₀₀ from PS containing liposomes was studied using an *in vitro* assay which involved the transfer of PE-PEG₂₀₀₀ into multilamellar vesicles, and the reactivity of PS containing liposomes was monitored by quantifying interactions with blood coagulation proteins. The percent inhibition of clotting activity of PS liposomes was dependent on the PE-PEG₂₀₀₀ content. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-PEG₂₀₀₀ which transferred out slowly from PS liposomes was able to abolish >80% of clotting activity of PS liposomes at 15 mol%. This level of DSPE-PEG₂₀₀₀ was also able to extend the mean residence time of PS liposomes from 0.2 h to 14 h. However, PE-PEG₂₀₀₀ with shorter acyl chains such as 1,2-dimyristyl-*sn*-glycero-3-phosphoethanolamine-PEG₂₀₀₀ were rapidly transferred out from PS liposomes, which resulted in a 73% decrease in clotting activity inhibition and 45% of administered intravenously liposomes were removed from the blood within 15 min after injection. Thus, PS facilitates the desorption of PE-PEG₂₀₀₀ from PS containing liposomes, thereby providing additional control of PEG release rates from membrane surfaces. These results suggest that membrane reactivity can be selectively regulated by surface grafted PEGs coupled to phosphatidylethanolamine of an appropriate acyl chain length. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Poly(ethylene glycol); Lipid transfer; Phosphatidylserine; Steric stabilization; Liposome; Drug delivery

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; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; POPE-PEG₂₀₀₀, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]; DSPE-PEG₂₀₀₀, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]; DPPE-PEG₂₀₀₀, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]; DMPE-PEG₂₀₀₀, 1,2-dimyristyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]; Chol, cholesterol; CHE, cholesterylhexadecyl ether; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine; EPC, egg phosphatidylcholine; MLV, multilamellar liposome; LUV, large unilamellar liposome

* Corresponding author. Fax: +1-604-877-6011. E-mail address: lmayer@bccancer.bc.ca (L.D. Mayer).

1. Introduction

Phosphatidylserine (PS) has long been recognized for its involvement in the blood coagulation cascade reactions [1]. Recently, solid tumor regression through selective thrombosis of tumor blood vessels has been achieved using the ‘coaguligand’ which is a truncated tissue factor linked to a monoclonal antibody targeted against the tumor vasculature [2,3]. With the ability of PS containing membranes to bind the high affinity clotting factors and subsequently activate the blood coagulation reactions, designing a liposomal carrier for PS may potentially have similar therapeutic applications in anticancer therapy. However, several obstacles have to be overcome in the development of a PS liposomal formulation capable of triggering selective tumor thrombosis.

First, the thrombogenic PS liposome surface should be protected in the bloodstream to prevent disseminated intravenous coagulation (DIC). Also, including reactive lipid species such as the negatively charged PS in liposomes enhances mononuclear phagocytic system (MPS) recognition, and causes rapid elimination of the liposomes from bloodstream, resulting in less accumulation of the liposomes in the target site [4,5]. It is widely known that the poly(ethylene glycol) (PEG) polymer conjugated to the liposome surface can be used as a steric barrier against plasma protein binding and cellular surface interactions at a surface density of 5 mol% to extend the circulation times of neutral liposomes composed of phosphatidylcholines (PC), sphingomyelins and cholesterol (Chol) [6,7]. However, this surface density of 5 mol% PEG is not effective in protecting the PS containing liposomes [6,8]. Recently, we have been able to extend the circulation longevity of 10% 1,2-dioleoyl-*sn*-glycero-3-[phosphatidylserine] (DOPS) liposomes by incorporating >10 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000] (DSPE-PEG₂₀₀₀), indicating that elevated levels of DSPE-PEG₂₀₀₀ are required to effectively protect PS liposomes from high affinity, PS-mediated plasma protein interactions [9].

Once the liposomes have reached the target site, i.e. tumor blood vessels, exposure of the thrombogenic PS liposome surface is necessary to trigger the

blood coagulation reactions. Thus, the thrombogenic PS liposomes need to be designed such that they are only temporarily protected by PEG-conjugated lipids (PEG-lipids) to prevent DIC in the general circulation. The transformation of the well-protected PS liposome surface to one that is reactive toward various clotting factors is dependent on the retention of the steric PEG barrier on the PS liposome surface. It has been shown that 5 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE)-PEG₂₀₀₀ is less effective in extending the circulation time of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/Chol liposomes than the same mole percentage of DSPE-PEG₂₀₀₀ present in the liposomes [10]. This is mainly due to the increased exchange of the entire poly(ethylene glycol 2000)-derivatized phosphatidylethanolamine (PE-PEG₂₀₀₀) conjugate out of the DSPC/Chol liposome when POPE was used as the lipid anchor [10]. Other reports have demonstrated that the rate of PEG-lipid exchange is dependent on the acyl chain composition [11–14]. The exchangeability of PEG-lipids in and out of membrane bilayers has also been demonstrated in studies where the acquisition of the PEG barrier on liposome surface was achieved by the spontaneous insertion of free micellar PEG-lipids into pre-formed liposomes [15–17]. This PEG-lipid exchange phenomenon has been utilized to provide controlled drug release for improved therapeutic activity [18], and controlled intracellular delivery for antisense oligonucleotides [19].

The aim of our present study is to use PEG₂₀₀₀-conjugated PEs with acyl chain lengths varying from 14 to 18 carbons to investigate the kinetics of PE-PEG transfer into and out of 10% DOPS liposomes, as well as how this affects PS membrane surface reactivity. These properties were evaluated by determining: (1) the clotting activity of 10% DOPS liposomes as PE-PEGs exchanged in and out of the membrane, and (2) the plasma levels of 10% DOPS liposomes stabilized by PE-PEGs following intravenous administration. We have measured the level of PE-PEGs in PS liposomes during the transfer processes, and found that the degree of protection of the PS liposome was correlated to the PE-PEG levels in the liposomes. We demonstrate here that PE-PEG₂₀₀₀ with a lipid anchor of appropriate chain length can be used to regulate membrane surface reactivity of

liposomes containing reactive lipid species such as PS. This may have important implications in (1) the delivery of biologically active lipids, and (2) the design of liposomes containing reactive surfaces for therapeutic applications, and, in particular, the application pursued here towards the development of liposomes that can induce tumor-specific thrombosis triggered by controlled exposure to PS, a lipid known to initiate the blood coagulation cascade.

2. Materials and methods

2.1. Materials

All lipids were obtained from Avanti Polar Lipids except for DSPC, [^3H]DSPC-PEG₂₀₀₀, [^3H]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE)-PEG₂₀₀₀ and [^3H]-1,2-dimyristyl-*sn*-glycero-3-phosphoethanolamine (DMPE)-PEG₂₀₀₀ which were obtained from Northern Lipids (Vancouver, BC, Canada). The [^3H]cholesterylhexadecyl ether (CHE) and [^{14}C]CHE were from NEN Life Science Products (Boston, MA, USA). Cholesterol and ellagic acid were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Bio-Gel A-15m size exclusion gel was obtained from Bio-Rad (Mississauga, ON, Canada).

2.2. Preparation of various liposomes

Lipids were dissolved 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. For making 'heavy' multilamellar liposomes (MLVs), the lipid film (egg phosphatidylcholine (EPC)/Chol 55:45) was hydrated in 300 mM sucrose to a concentration of 100 mg/ml at 65°C with vortex mixing. The resulting preparation was centrifuged at $1600\times g$ for 10 min in Eppendorf tubes, and the bottom sucrose layer was removed with a 1 cm³ syringe and 20 gauge needle. The MLVs were then washed three times with 0.5 ml 20 mM HEPES/150 mM NaCl buffer (pH 7.5) at $1600\times g$ for 10 min, and the pellet was resuspended in 20 mM HEPES/150 mM NaCl buffer. The recovery of MLVs in the pellet was always greater than 95%. For making large unilamellar liposomes

(LUVs), the lipid film was hydrated in 20 mM HEPES/150 mM NaCl buffer (pH 7.5) or in citrate 300 mM (pH 4.0) at 65°C. The resulting preparation was frozen and thawed five times prior to extrusion 10 times through stacked polycarbonate filters (Nucleopore, Canada) at 65°C with an extrusion apparatus (Lipex Biomembranes, Vancouver, BC, Canada). The resulting mean liposome diameter obtained following extrusion was 100–120 nm as determined by quasi-elastic light scattering using the Nicomp submicron particle sizer model 370/270 [20,21].

2.3. Incorporation and retention of PEG-lipids in liposomes

Incorporation of PE-PEGs in liposomes after hydration and extrusion (thin film method) was determined by size exclusion chromatography. Briefly, liposomes labeled with trace levels of [^{14}C]CHE and tritiated PE-PEGs were applied to a 42 cm \times 1.3 cm Bio-Gel A-15m column (50–100 mesh), and eluted with 20 mM HEPES/150 mM NaCl buffer (pH 7.5) at a flow rate of 0.5 ml/min regulated by a peristaltic pump. To elute under acidic conditions, citrate 300 mM solution (pH 4) was chosen as the elution buffer. Aliquots (0.5 ml) from the 1 ml column fractions were mixed with 5.0 ml Pico-fluor 15 scintillation fluid (Packard Biosciences, The Netherlands) and the level of radioactivity was determined using a Packard scintillation counter model 1900 TR. To determine the amount of PE-PEG transferred from pure PE-PEG micelles to liposomes, 5 μmol 10% DOPS/DSPC/Chol liposomes were incubated with micellar PE-PEGs (0.882 μmol) with a final volume of 0.4 ml for various times, and the resulting mixture was fractionated on a 27 cm \times 0.7 cm Bio-Gel A-15m column by gravity. The amount of PE-PEGs incorporated into liposomes by the micelle transfer method and by the thin film method was expressed in μmol PE-PEG/ μmol total lipid. The external PE-PEG grafting density for the pegylated liposomes prepared by micelle transfer method was estimated as follows: $\text{mol}\% \text{ PE-PEG} = 2 \times (\text{mol}_{\text{PE-PEG}} / \text{mol}_{\text{total lipid}}) \times 100\%$, based on the assumptions that PEG-lipids are inserted only into the outer monolayer of the LUVs during the transfer process and phospholipids are distributed equally between the two monolayers.

2.4. *In vitro* PEG-lipid transfer assay

Donor liposomes (LUVs) containing various types of tritiated PEG-lipids were incubated with acceptor MLVs (EPC/Chol) at 37°C for various times at a donor to acceptor mole ratio of 1:100. Donor LUVs were separated from the acceptor MLVs by centrifugation at $1600\times g$ for 10 min. The sample was washed twice with 0.5 ml 20 mM HEPES/150 mM NaCl buffer at $1600\times g$ for 10 min. Both the supernatant (donor LUVs) and the pellet (acceptor MLVs) were collected for scintillation counting. Recovery, as determined by radioactivity, was over 95% for donor and acceptor liposomes, respectively.

2.5. *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 buffer. Human citrated plasma (50 μ l) was preincubated with 10^{-5} M ellagic acid (50 μ l) and liposomes (50 μ l) for 2 min at 37°C. Calcium was then added to initiate the clotting reaction, and the mixture was gently shaken. 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.

2.6. Correlation of PEG surface density and inhibition of clotting activity

Correlation of PEG surface density and the percent inhibition of clotting activity was derived from data in Figs. 2A and 3B. The calculations were described using DMPE-PEG as the example. The DMPE-PEG levels at 0 and 24 h were 0.1048 and 0.0650 μ mol/ μ mol lipid, respectively. Therefore, the total DMPE-PEG loss was 0.0398 μ mol/ μ mol lipid. Assume there was 1 μ mol lipid and the phospholipids were distributed equally between the two monolayers [22], the amount of DMPE-PEG on the outer monolayer was equal to 0.0524 μ mol. Assume that only DMPE-PEG on the outer monolayer were available for transfer, the amount of DMPE-PEG remaining on the outer monolayer was 0.0126 μ mol. The surface density of DMPE-PEG on the outer monolayer was calculated as follows: surface

density (in mol%) = $0.0126 \mu\text{mol PEG} / 0.5 \mu\text{mol lipid} \times 100\% = 2.52$. The same calculations were conducted for DPPE-PEG. The percent inhibition of clotting activity (from Fig. 3B) was then correlated with the surface density of each of the PE-PEGs (derived from Fig. 2A). Since the levels of DSPE-PEG in the 10% DOPS LUVs were relatively constant, indicating a very small amount of PE-PEG has desorbed from the LUVs, the DSPE-PEG levels at $t = 0$ h and 24 h were correlated with the corresponding percent inhibition of clotting activity directly.

2.7. Plasma pharmacokinetics and tissue distribution of liposomes

Liposomes, labeled with traces of [14 C]CHE and various [3 H]PEG-lipids, were injected via the lateral tail vein into 20–22 g female Balb/c mice. The total lipid dose was 50 mg/kg with an injection volume of 200 μ l. At various times after liposome injection, 25 μ l blood was collected into microcapillary tubes pre-rinsed with 200 mM EDTA solution by nicking the tail vein. The blood was then added to 200 mM EDTA solution (200 μ l) and centrifuged at $1000\times g$ for 10 min. Aliquots of the supernatant were counting directly in 5 ml Pico-fluor 40 scintillation fluid (Packard Biosciences). At 4 and 24 h, three mice were terminated by CO₂ asphyxiation. Blood was collected by cardiac puncture, and was placed into EDTA-coated microtainer collection tubes (Becton-Dickinson). After centrifuging the blood samples for 15 min at $1000\times g$, 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 at 4 and 24 h to determine liposome accumulation in these organs. Briefly, 0.5 ml Solvable (Packard Bio-Science) was added to whole organs (spleen and lungs) or 50% w/v tissue homogenate (liver), and the mixture was incubated at 50°C overnight. After cooling to room temperature, 50 μ l EDTA 200 mM, 200 μ l hydrogen peroxide 30%, and 25 μ l HCl 10 N were added, and the mixture was incubated for 1 h at room temperature. The mixture was added with 5.0 ml Pico-fluor 40 scintillation fluid and counted 24 h later. It should be noted that all of the *in vivo* studies were completed using protocols approved by the University of British Columbia's Animal Care Com-

mittee. These studies met or exceeded the current guidelines of the Canadian Council of Animal Care. Pharmacokinetic parameters were calculated using the software WinNonlin version 1.5.

3. Results

3.1. Kinetics of PE-PEG insertion into PS liposomes from micelle addition

The incorporation of various PE-PEGs into neutral and PS containing liposomes can be achieved using two different methods. In one procedure of incorporation, PE-PEGs were added as a separate micelle solution to pre-formed liposomes (micelle transfer method). The transfer of various PE-PEG from micelles into PS containing liposomes as a function of time is shown in Fig. 1. Pure PE-PEG micelles were added to pre-formed 10% DOPS containing liposomes to achieve a final concentration of 2.205 mM, which was above the CMC reported for

DSPE-PEG₂₀₀₀ (CMC of 2.2–10.8 μM [15]). At a PE-PEG to liposome mole ratio of 0.176:1, not all of the PE-PEGs were transferred into the PS containing liposomes, and any unincorporated PE-PEG was separated from the liposomes with Bio-Gel A-15m column according to established procedures [9]. Mixing PE-PEG micelles with liposomes did not have any significant effect on liposome size, which remained in the range of 100–120 nm during the 24 h incubation period with the PE-PEG micelles. After 4 h, only 0.012 μmol DSPE-PEG₂₀₀₀ has been inserted into 1 μmol 10% DOPS/DSPC/Chol liposomes, as compared to 0.038 μmol DMPE-PEG₂₀₀₀ and 0.045 μmol DPPE-PEG₂₀₀₀. After 24 h, the transfer of DMPE-PEG₂₀₀₀ and DPPE-PEG₂₀₀₀ into the 10% DOPS liposomes was almost complete. The amounts of DMPE-PEG₂₀₀₀ and DPPE-PEG₂₀₀₀ transferred into 10% DOPS liposomes after 24 h were 0.045 and 0.056 μmol PE-PEG/ μmol total lipid, respectively. The transfer of DSPE-PEG₂₀₀₀ into 10% DOPS liposomes did not reach a similar level of 0.058 μmol PE-PEG/ μmol total lipid until after 72 h.

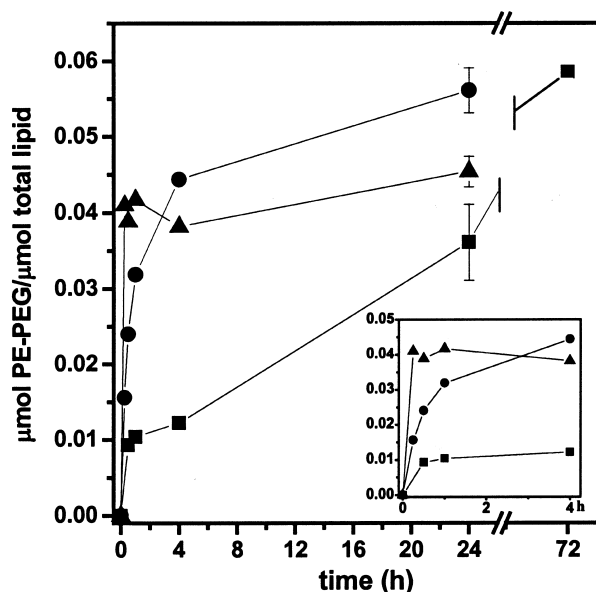


Fig. 1. Time course for DMPE-PEG₂₀₀₀ (▲), DPPE-PEG₂₀₀₀ (●) and DSPE-PEG₂₀₀₀ (■) transfer from micelles into 10% DOPS/DSPC/Chol liposomes at 37°C. The inset highlights differences in the rates of PE-PEG transfer over the first 4 h. The amount of PEG-lipids transferred into liposomes is expressed as μmol PE-PEG per μmol total lipid with each data point representing a single determination except at 24 h where the amount of PEG-lipid transferred was determined in triplicates as described in Section 2.

3.2. Incorporation of PE-PEGs into PS and neutral liposomes

The second procedure of PE-PEG incorporation involved the addition of PE-PEGs to the lipid mixture before making the dried lipid film for hydration (thin film method). The pegylated liposomes produced by the thin film method would have an approximately equal distribution of the PE-PEGs in the inner and outer monolayer [23], whereas PE-PEG incorporation from addition of micelles results in asymmetric PE-PEG distribution, where the PE-PEGs are found exclusively in the outer monolayer [11,24]. Table 1 summarizes the results of PEG-lipid incorporation into liposomes by the two different methods. The incorporation of DPPE- and DSPE-PEG₂₀₀₀ into 10% DOPS/DSPC/Chol and DSPC/Chol liposomes was approx. 14 mol% using the thin film method. This is reflected by the near quantitative (> 90%) incorporation of the PEG-lipids into the liposomes. These results are consistent with previous studies by Kenworthy et al. and Hristova et al. [25–27]. The incorporation of DMPE-PEG₂₀₀₀ into 10% DOPS/DSPC/Chol liposomes was less efficient compared to that of DPPE- and DSPE-PEG₂₀₀₀,

Table 1
Incorporation of various PEG-lipids into PS and neutral liposomes^a

	10% DOPS/DSPC/Chol		DSPC/Chol	
	$\mu\text{mol PE-PEG}/\mu\text{mol total lipid}$	mol%	$\mu\text{mol PE-PEG}/\mu\text{mol total lipid}$	mol%
<i>Thin film method</i>				
DMPE-PEG ₂₀₀₀ ^b	0.105 ± 0.006	10.5 ^{d,e}	0.120 ± 0.009	12.0 ^d
DMPE-PEG ₂₀₀₀ (pH 4)	0.123 ± 0.003	12.3 ^d		
DPPE-PEG ₂₀₀₀	0.132 ± 0.001	13.2 ^e	0.139 ± 0.009	13.9
DSPE-PEG ₂₀₀₀	0.140 ± 0.003	14.0	0.144 ± 0.004	14.4
<i>Micelle transfer method^c</i>				
DMPE-PEG ₂₀₀₀ ^b	0.045 ± 0.002	9.08 ^e		
DPPE-PEG ₂₀₀₀	0.056 ± 0.003	11.2 ^e		
DSPE-PEG ₂₀₀₀	0.058 (72 h)	11.7		

^aReported values represent means of three independent determinations and the standard deviations. Statistical analysis was performed using ANOVA and Newman–Keuls test with $P < 0.05$.

^bDMPE-PEG₂₀₀₀ was incorporated into 10% DOPS/DSPC/Chol liposomes hydrated at pH 7.5 and 4.0. Other PEG-lipids were incorporated into liposomes hydrated at pH 7.5.

^cThe 10% DOPS/DSPC/Chol liposomes were incubated with PE-PEG micelles for 24 h (DMPE-PEG₂₀₀₀ and DPPE-PEG₂₀₀₀) or 72 h (DSPE-PEG₂₀₀₀). The mixture was separated on Bio-Gel A-15m column as described in Section 2 to determine the amount of PEG-lipids incorporated into the liposomes by the micelle transfer method, which was calculated as follows: $\text{mol\% PE-PEG} = 2 \times (\text{mol}_{\text{PE-PEG}}/\text{mol}_{\text{total lipid}}) \times 100\%$ to reflect the PE-PEG density on the outer monolayer, based on the assumptions that PE-PEG are inserted only into the outer monolayer of LUVs during the transfer process and phospholipids are distributed equally between the two monolayers.

^dIncorporation of DMPE-PEG₂₀₀₀ in 10% DOPS liposomes was statistically different from that in DSPC/Chol liposomes and from that incorporated at pH 4.

^eIncorporation of DMPE-PEG₂₀₀₀ in 10% DOPS liposomes by the thin film method was not statistically different from that incorporated by the micellar transfer method, while incorporation of DPPE-PEG₂₀₀₀ in 10% DOPS liposomes by the thin film method was statistically different from that incorporated by the micellar transfer method.

where the liposomes after hydration and extrusion contained 10.5 mol% DMPE-PEG₂₀₀₀ (Table 1). This lower level of PEG-lipid incorporation was reflected by a peak of free micellar DMPE-PEG₂₀₀₀ observed in the column elution profile (data not shown). Similarly, incorporation of DMPE-PEG₂₀₀₀ into DSPC/Chol liposomes was lower than the other two types of PEG-lipids.

The PE-PEGs used in the present studies were conjugated through a carbamate linkage that imparts a net negative charge on the phosphate moiety at physiological pH [28,29]; thus, the negative surface charge on PS could potentially impede the incorporation of DMPE-PEG₂₀₀₀ into the PS containing liposomes due to charge repulsion. We examined the effect of protonating the carboxyl moiety on PS on DMPE-PEG₂₀₀₀ incorporation. At pH 4.0, approx. 97% of the carboxylate PS head groups are protonated (based on $pK = 5.5$ [30]), and this led to a modest yet statistically significant increase in the incorporation of DMPE-PEG₂₀₀₀ into 10% DOPS/

DSPC/Chol liposomes from 10.5 mol% to 12.3 mol%, which was similar to that obtained with neutral DSPC/Chol liposomes (12.0 mol%, see Table 1).

The incorporation of PE-PEGs into 10% DOPS/DSPC/Chol liposomes by the micelle transfer method described in Section 3.1 is also summarized in Table 1. The values reported for PE-PEG incorporation using the micellar transfer method were PE-PEG levels observed in the 10% DOPS liposomes after 24 h for DMPE-PEG₂₀₀₀ and DPPE-PEG₂₀₀₀ and after 72 h for DSPE-PEG₂₀₀₀, time points where the transfer process achieved >90% of the estimated maximum. Assuming that PEG-lipids were inserted only into the outer monolayer of the LUVs during the transfer process and phospholipids were distributed equally between the two monolayers [11,24], the amount of PEG-lipids transferred to the liposomes was first calculated as mole percentage of total lipid, and this value was then multiplied by two to approximate the PEG-lipid density in the outer monolayer which was equivalent to that achieved when preparing the

liposomes by the thin film method (see Table 1). The external grafting density of DMPE-PEG₂₀₀₀ in 10% DOPS liposomes was comparable using the two different methods. However, DPPE-PEG₂₀₀₀ and DSPE-PEG₂₀₀₀ had slightly lower yet statistically significant external grafting densities in the outer monolayer of 10% DOPS liposomes using the micelle transfer method as compared to the thin film method. The grafting densities achieved in the outer monolayer for DPPE-PEG₂₀₀₀ and DSPE-PEG₂₀₀₀ were 11 and 12 mol% respectively using the micelle transfer method, as compared to 13 and 14 mol% respectively using the thin film method.

3.3. Kinetics of PE-PEG transfer from neutral and anionic LUVs into MLVs

When liposomes are injected intravenously, membranes from the vascular endothelium and other cellular components in the bloodstream constitute a large lipid pool that may interact with liposome membranes, leading to possible interbilayer exchange of lipids. In order to model this, the loss of various PE-PEGs from neutral DSPC/Chol and PS containing LUVs was examined by incubating the LUVs with a 100-fold molar excess of EPC/Chol MLVs. The MLVs provided a lipid pool to accept the PEG-lipids when transferring out of the LUVs (see Fig. 2). A rapid decrease in DMPE-PEG₂₀₀₀ membrane content was observed in both 10% DOPS liposomes (Fig. 2A) and neutral DSPC/Chol liposomes (Fig. 2B), with approx. 40% of DMPE-PEG₂₀₀₀ transferred out of the two types of liposomes within 15 min following the addition to the MLVs.

As the acyl chain length of the lipid anchor increased from 14 carbons (DMPE) to 18 carbons (DSPE), the rate of transfer of the PEG-lipids from LUVs to MLVs was reduced such that the level of DSPE-PEG₂₀₀₀ in 10% DOPS liposomes and in DSPC/Chol liposomes remained relatively constant over 24 h. This observation is consistent with results from previous studies where the rate of PEG-lipid transfer was decreased with increasing acyl chain length [11–14]. Desorption of various PE-PEGs out of the LUVs in the absence of MLVs under similar conditions was negligible, with no micellar PE-PEGs detected in fractions isolated following column chromatography (data not shown). It is interesting to

note that the amount of DPPE-PEG₂₀₀₀ transferred out from 10% DOPS LUVs was higher than that of DSPC/Chol LUVs, with 55% of DPPE-PEG₂₀₀₀ remaining in 10% DOPS liposomes after 24 h compared to 85% in DSPC/Chol liposomes.

3.4. Regulation of membrane reactivity by exchangeable PE-PEGs

Since protection of the lipid membrane surface from protein and cellular binding is dependent on

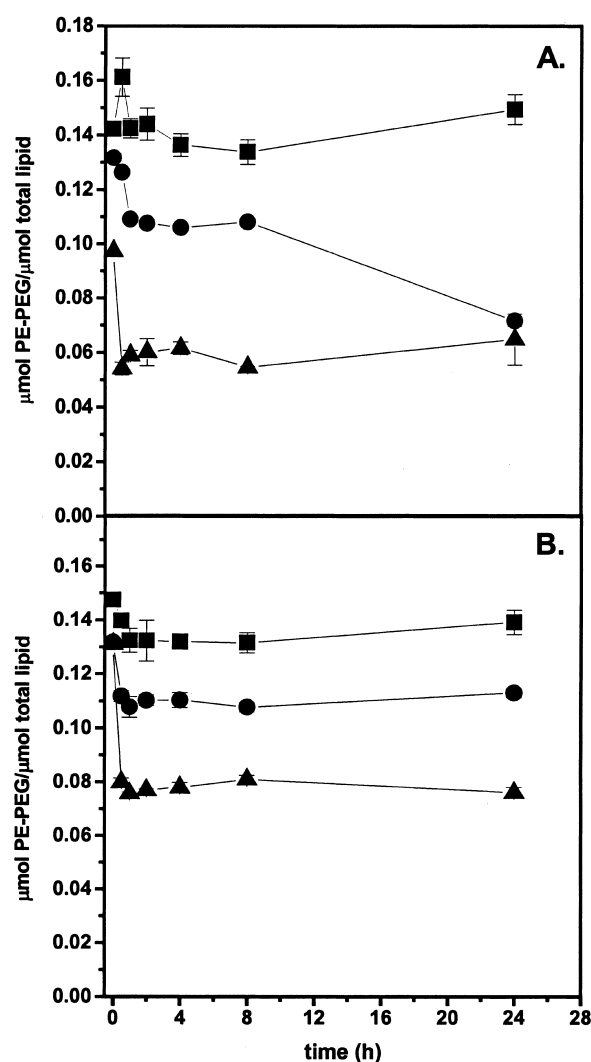


Fig. 2. Time course for DMPE-PEG₂₀₀₀ (▲), DPPE-PEG₂₀₀₀ (●) and DSPE-PEG₂₀₀₀ (■) transferred from 10% DOPS/DSPC/Chol LUVs (A) or DSPC/Chol LUVs (B) to EPC/Chol MLVs at 37°C. Data points were determined in triplicate using the *in vitro* PE-PEG transfer assay as described in Section 2, and the error bars represent standard deviations.

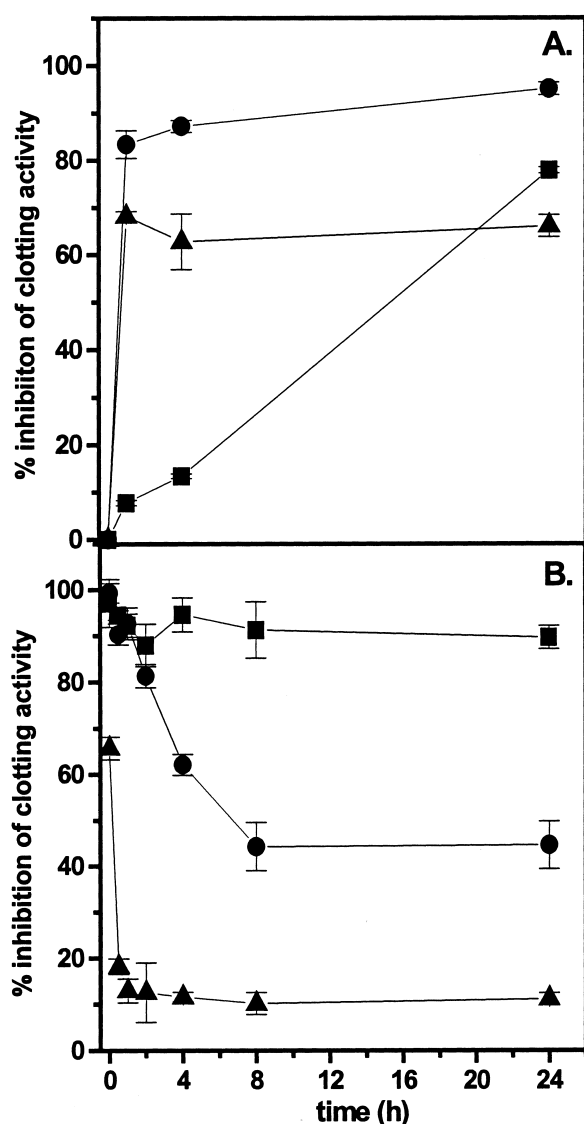


Fig. 3. Regulation of the clotting activity of 10% DOPS/DSPC/Chol liposomes incorporated with various PE-PEGs by the micelle transfer method (A) or by the thin film method (B). Panel A shows the % inhibition of clotting activity as DMPE-PEG₂₀₀₀ (▲), DPPE-PEG₂₀₀₀ (●) or DSPE-PEG₂₀₀₀ (■) transferred into the 10% DOPS liposomes from micelles over 24 h. Panel B shows the % inhibition of clotting activity as DMPE-PEG₂₀₀₀ (▲), DPPE-PEG₂₀₀₀ (●) and DSPE-PEG₂₀₀₀ (■) transferred out from the 10% DOPS liposomes into EPC/Chol MLV over 24 h. The % inhibition of clotting activity was calculated as follows: $\% \text{ inhibition} = (t_{\text{PEG}} - t_{\text{PS}}) / (t_{\text{Blank}} - t_{\text{PS}}) \times 100$, where t represents the clotting time of each type of liposome as determined by the in vitro clotting time assay. Data points were determined in triplicate, and the error bars represent standard deviations.

the retention of the PEG polymer barrier on the membrane surface, the transfer of PEG-lipids into or out of liposomes should also modulate the reactivity of a biologically active membrane surface which is capable of eliciting protein binding reactions. In this study, the control of surface reactivity of PS containing liposomes by PE-PEGs was evaluated by monitoring the clotting activity of 10% DOPS liposomes at various times following PE-PEG insertion and desorption. The transfer of micellar DMPE-PEG₂₀₀₀ and DPPE-PEG₂₀₀₀ into 10% DOPS liposomes resulted in rapid inhibition of clotting activity (Fig. 3A). The percent inhibition of clotting achieved following the incubation of PS liposomes with micellar DMPE-PEG₂₀₀₀ and DPPE-PEG₂₀₀₀ reached levels of 65% and 92% within 1 h, respectively. In contrast, the transfer of micellar DSPE-PEG₂₀₀₀ into 10% DOPS liposomes was slow, and this resulted in much slower generation of clotting activity inhibition, with only 8% inhibition achieved after 1 h. It is of interest to note that the inhibition of clotting activity achieved 1 h following the insertion of DPPE-PEG₂₀₀₀ was higher than that achieved using DMPE-PEG₂₀₀₀ even though the level of the DPPE-PEG₂₀₀₀ present in the 10% DOPS liposomes was lower than that of DMPE-PEG₂₀₀₀ (compare Figs. 1 and 3A).

Next, the clotting activity of 10% DOPS liposomes containing various types of PEG-lipids was examined as the PEG-lipids were transferring out of the liposomes (Fig. 3B). The transfer of approx. 40% DMPE-PEG₂₀₀₀ out of 10% DOPS liposomes within 15 min resulted in a dramatic decrease in the inhibition of clotting activity from 67% to 18%. In contrast, the percent inhibition of clotting activity for DSPE-PEG₂₀₀₀ remained constant for 24 h. This is consistent with the data presented in Fig. 2B which indicates that DSPE-PEG₂₀₀₀ is not readily lost from the PS liposomes. For DPPE-PEG₂₀₀₀ which has a rate of transfer that is intermediate between those of DMPE-PEG₂₀₀₀ and DSPE-PEG₂₀₀₀, the inhibition of clotting activity gradually decreased from 99% to 45% within 8 h (Fig. 3B).

Fig. 4 presents a composite of blood clotting activity obtained as a function of the PEG-lipid content in 10% DOPS liposomes derived from the various systems studied. Results from this study have demonstrated a trend which reflects a dependence

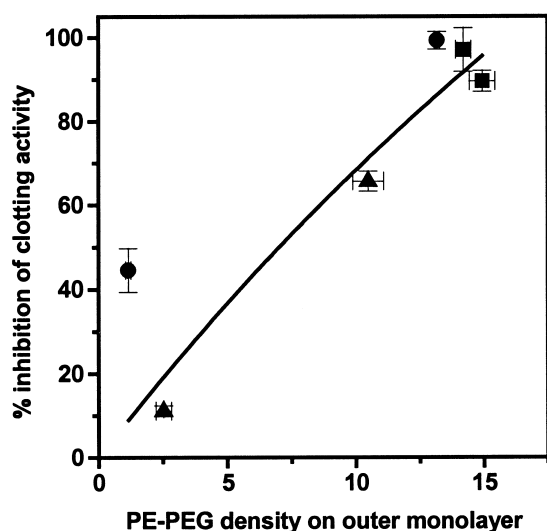


Fig. 4. The % inhibition of clotting activity as a function of PEG-lipid level in 10% DOPS/DSPC/Chol liposomes. Data points represent the % inhibition of clotting activity and the PE-PEG level in the 10% DOPS/DSPC/Chol liposomes before or after 24 h incubation with the EPC/Chol MLV (derived from Figs. 2A and 3B). The 10% DOPS liposomes were made with DMPE-PEG₂₀₀₀ (\blacktriangle), DPPE-PEG₂₀₀₀ (\bullet) or DSPE-PEG₂₀₀₀ (\blacksquare) incorporated by the thin film method. Error bars represent standard deviations.

of the inhibition of clotting on the PE-PEG surface density in the DOPS membrane. As the PEG surface density increased in the DOPS membrane, it resulted in higher percent inhibition of clotting activity. Results from a previous study also demonstrated a similar trend where inhibition of clotting activity depends on the PEG surface density [9].

3.5. Effect of exchangeable PEG-lipids on the plasma levels of PS and neutral liposomes after intravenous injection

To examine the effect of PE-PEG transfer on the plasma elimination profiles of PS containing LUVs and DSPC/Chol LUVs, these two types of liposomes with various PE-PEGs incorporated at >10 mol% were injected into mice, and plasma liposome concentrations were determined by monitoring a non-exchangeable lipid label, [¹⁴C]CHE [31] (see Fig. 5). The rapid transfer of DMPE-PEG₂₀₀₀ out of both types of liposomes resulted in rapid removal of liposomes from circulation, with approx. 10% and 35% of the injected liposomal lipid remaining in plasma

after 1 h for 10% DOPS liposomes and DSPC/Chol liposomes, respectively. The plasma levels of 10% DOPS liposomes and DSPC/Chol liposomes containing the slowly exchanged DSPE-PEG₂₀₀₀ were much higher, with 66% and 82% of the liposomal lipid remaining in the plasma after 1 h, respectively. After 24 h, the plasma liposome levels for these systems were 10% and 30%, respectively (Fig. 5). The plasma elimination profile of 10% DOPS liposomes contain-

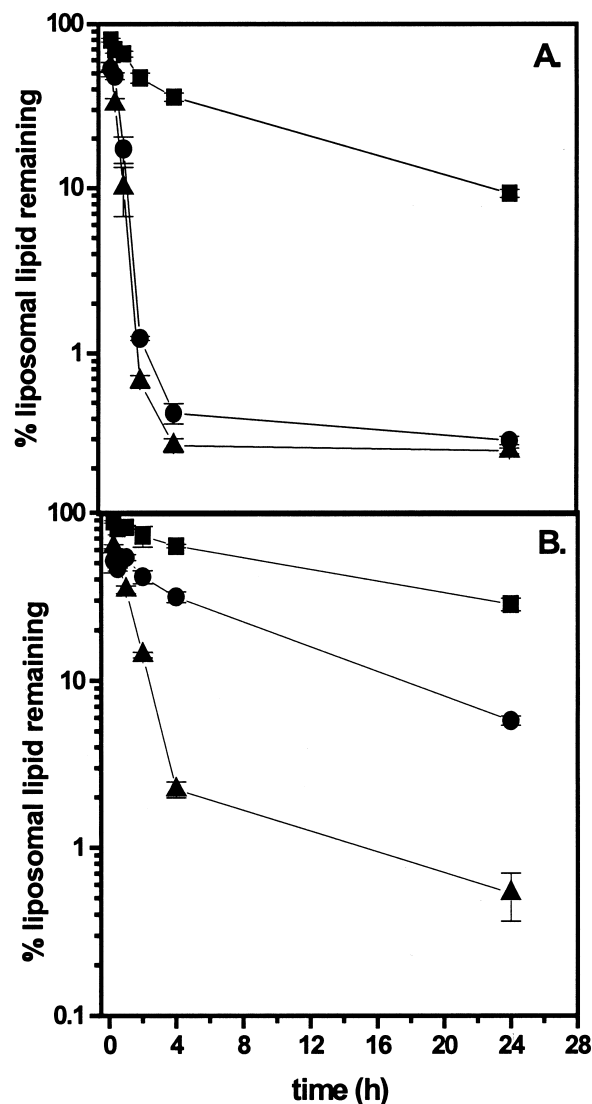


Fig. 5. The effect of DMPE-PEG₂₀₀₀ (\blacktriangle), DPPE-PEG₂₀₀₀ (\bullet) and DSPE-PEG₂₀₀₀ (\blacksquare) on the plasma elimination of 10% DOPS/DSPC/Chol liposomes (A) and DSPC/Chol liposomes (B). Plasma levels of the liposomal lipids were expressed as percentages of the amount of liposomal lipids injected. Three Balb/c mice were used for each data point, and the error bars represent the standard errors.

ing PE-PEGs was similar for systems incorporating DPPE-PEG₂₀₀₀ and DMPE-PEG₂₀₀₀. This is in contrast to the results obtained with neutral DSPC/Chol liposomes where DPPE-PEG₂₀₀₀ containing systems were removed from the bloodstream much slower than DMPE-PEG₂₀₀₀ liposomes (32% vs. 2% remaining in plasma, respectively, after 4 h). This PS effect was reflected by the fact that for liposomes prepared with the same amount of DPPE-PEG₂₀₀₀, 6% of the

injected liposomes remained in plasma for DSPC/Chol liposomes compared to <0.5% for 10% DOPS liposomes after 24 h.

The amount of PE-PEGs remaining in plasma as a percentage of the PEG-lipid injected was also monitored in order to correlate liposome elimination properties with retention of the PEG-lipids in the liposomes. Fig. 6 demonstrates that the plasma elimination of the three PEG-lipids studied followed the same trend as that of the liposomal lipids where DMPE-PEG₂₀₀₀ itself and the liposomes containing this PEG-lipid had the fastest removal from the bloodstream. DSPE-PEG₂₀₀₀ and the liposomes containing this PEG-lipid were removed most slowly from circulation. After 24 h, the plasma levels of DSPE-PEG₂₀₀₀ were 4-fold and 10-fold higher than those of DMPE-PEG₂₀₀₀ in 10% DOPS liposomes and DSPC/Chol liposomes respectively. It is interesting to note that the relative amount of PE-PEG in the plasma was higher than the relative plasma levels for liposomes when these liposomes were eliminated rapidly from bloodstream. For instance, the percentage of DMPE-PEG₂₀₀₀ remaining in plasma at 24 h for both 10% DOPS liposomes and DSPC/Chol liposomes was approx. 2% compared to <0.5% of liposomal lipid remaining for the two types of liposomes, as determined from [¹⁴C]CHE. Since DMPE-PEG₂₀₀₀ is able to transfer out of liposomes rapidly, it may possibly interact with plasma components that act as an acceptor for DMPE-PEG₂₀₀₀, leading to longer circulation times of the PEG-lipid. This is supported by a previous finding that DMPE-PEG₂₀₀₀ can exchange into the plasma lipoprotein pool [32].

4. Discussion

In the development of thrombogenic PS liposomes for selective thrombosis in tumor blood vessels, controlled exposure of the thrombogenic surface is crucial and this requires transient shielding of the liposomal PS surface. Retention of a surface grafted PEG polymer coating is necessary for the protection of the thrombogenic PS liposome surface in the general circulation. However, the PEG polymer coating on the PS liposome surface may prevent interactions with the clotting factors and the initiation of blood coagulation once they have localized in the tumor

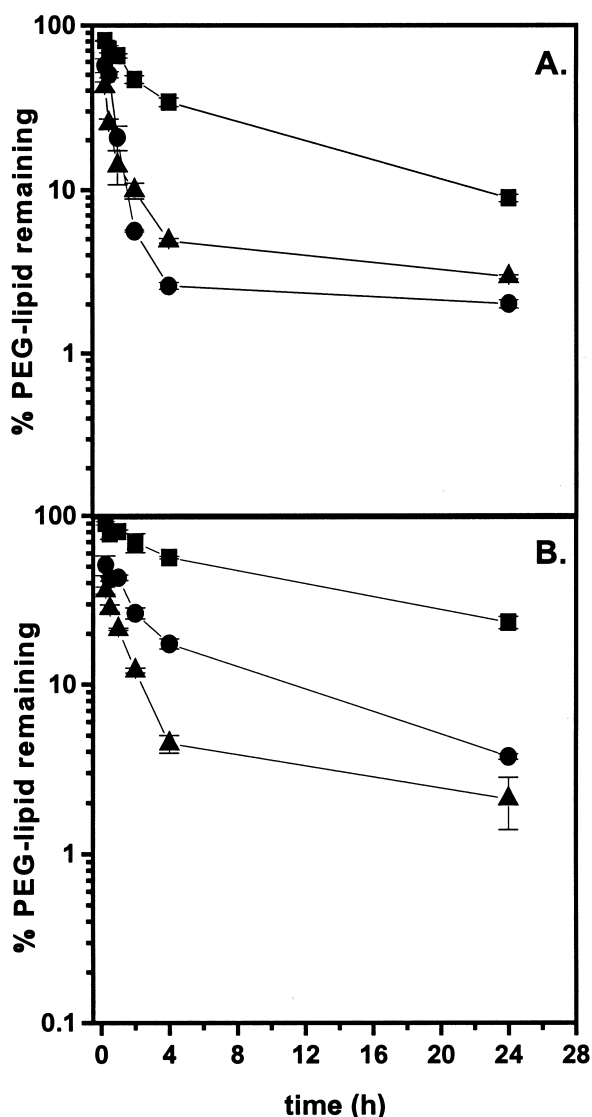


Fig. 6. Plasma levels of DMPE-PEG₂₀₀₀ (▲), DPPE-PEG₂₀₀₀ (●) and DSPE-PEG₂₀₀₀ (■) in Balb/c mice expressed as percentages of the amount of PEG-lipids injected, with 10% DOPS/DSPC/Chol (A) and DSPC/Chol (B) as carriers for the PEG-lipids. Three mice were used for each data point, and the error bars represent the standard errors.

vasculature. Controlled loss of the PEG coating is therefore necessary to trigger the blood coagulation reactions mediated by the PS membrane surface within the tumor blood vessels. Indeed, current trends in designing liposomes with surface grafted PEG are to achieve: (1) temporary stabilization of liposomes in circulation to allow adequate accumulation in target site, and (2) subsequent transformation of liposomes to a reactive or unstable form to facilitate target cell interaction/fusion and content release through the loss of PEG polymer [11,14,18,19,33–35]. Furthermore, in designing pegylated liposomes, it is possible to control membrane-dependent enzymatic activity by controlling the access of proteins/enzymes to the liposome surface via inclusion of appropriate levels of the surface grafted PEG polymer coating [36,37]. In this study, we have demonstrated the ability to control the surface reactivity of PS containing membranes toward high affinity clotting factors in a time-dependent fashion through the use of exchangeable PE-PEGs. This enhances our understanding of how to design liposomes for inducing tumor selective thrombosis.

Several features have been identified in governing the transfer rate of PEG-lipids, including the head group and the acyl chain of PEG-lipids, the liposomal lipid composition and the concentration of the acceptor membrane [12,13,38–40]. The hydrophilic PEG polymer (MW 2000) on the various lipid anchors used in this study was conjugated to the amino group on PE, resulting in the generation of a negatively charged PE-PEG at physiological pH [28,29]. The negative charge on PE-PEGs has a modest effect on reducing DMPE-PEG₂₀₀₀ membrane incorporation into the negatively charged 10% DOPS membrane. This is likely due to the charge repulsion between the DMPE-PEG₂₀₀₀ and DOPS head groups. Although the effect of the molecular weight of the PEG polymer was not investigated in this study, it has been documented that an increase in the molecular weight of the PEG polymer would lead to an increase in the desorption rate of PEG-lipids from vesicles [13].

Among the three types of PE-PEGs used, DMPE-PEG₂₀₀₀ was observed to have the lowest incorporation into both neutral and PS containing liposomes and the fastest rate of transfer into and out of the liposomes. This result is consistent with previous

studies [11–14]. Considering the acyl chain lengths of DMPE-PEG₂₀₀₀ and of the remaining lipid components (DSPC/Chol \pm 10 mol% DOPS), formation of packing defects in the lipid bilayer is probable because of the four-carbon mismatch in the acyl chain lengths [41,42]. These packing defects may make the incorporation of DMPE-PEG₂₀₀₀ into the liposomes less favorable, thus limiting the amount of DMPE-PEG₂₀₀₀ that can be incorporated into liposomes. Alternatively, the smaller length/width axial ratio of this PEG-lipid may result in a more preferred partitioning into highly curved micellar structures [24,43]. Desorption of lipids or lipid conjugates involves the formation of a transition state which entails a significant displacement of the acyl chain into the aqueous phase [44]. This requires energy to disrupt the lipid–lipid van der Waals interactions and the hydrogen-bonded water to accommodate the lipid acyl chain in the aqueous phase. Thus, increasing the hydrophobicity by extending the acyl chain of the lipid anchor would make the desorption of PEG-lipids energetically more unfavorable.

Under *in vitro* conditions in the absence of an acceptor membrane, PE-PEGs did not desorb from 10% DOPS LUVs or from DSPC/Chol LUVs. The presence of an acceptor membrane is thus necessary for the desorption of PEG-lipids from LUVs. In this study, 20 mM MLVs were used in the *in vitro* transfer assay, and under high acceptor vesicle concentrations, it is possible to enhance the rate of PE-PEG desorption. This may be due to an increase in the collisional frequency of the pegylated LUVs and the acceptor MLVs that may facilitate transfer of the lipid [39,40]. Although fusion-mediated lipid transfer has been suggested previously as an alternative means of PE-PEG transfer [45], our data do not support this mechanism because recovery of the LUVs in the supernatant, as determined by the [¹⁴C]CHE lipid marker, was always >95%. Given that membrane–membrane contact can be inhibited by surface grafted PEG polymer at 1–2 mol% [11], fusion-mediated lipid transfer may be sterically hindered in our case where PEG surface density was elevated (>10 mol%). Other possible mechanisms whereby PE-PEG can be transferred into MLVs include direct transfer of the PE-PEG monomer or transfer through the formation of intermediate PE-PEG micelles or oligomers after desorption of the

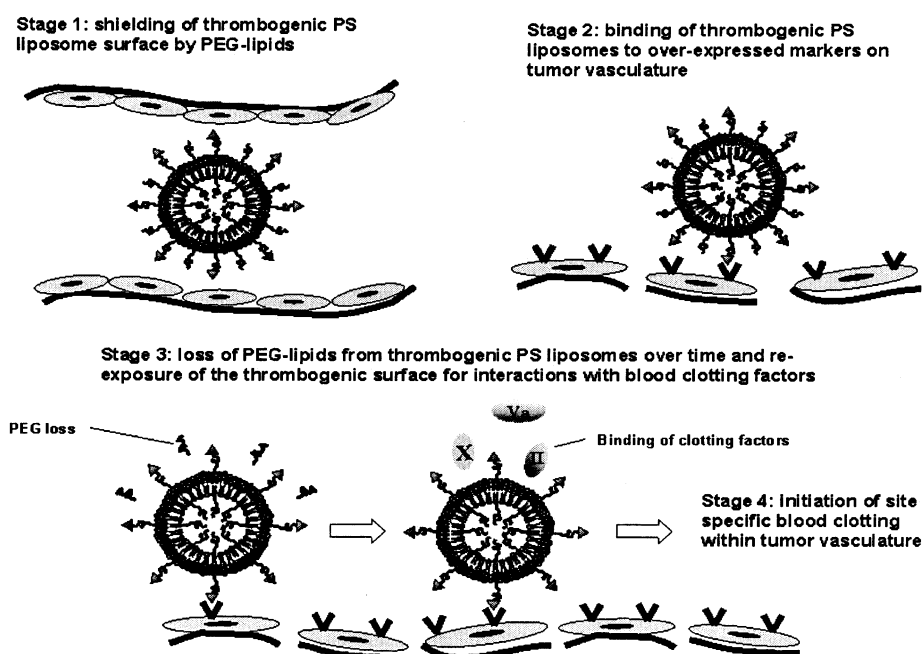


Fig. 7. A schematic illustration of the thrombogenic PS liposomes targeted to over-expressed markers on the tumor vasculature.

PE-PEG monomers from the LUV bilayer. The MLVs in the *in vitro* PEG-lipid transfer assay act as a lipid pool, which provides a large surface area to accept the PEG-lipids as they are desorbing from liposomes, similar to the large membrane surface from the vascular endothelium and other cellular components in the bloodstream. Another potential PEG-lipid acceptor *in vivo* is the lipoprotein family which is known to be involved in lipid transfer [32,46]. Our data showed that the relative amount of DMPE-PEG₂₀₀₀ in plasma was higher than that for the liposomal lipids which were eliminated rapidly from the bloodstream. It is possible that DMPE-PEG₂₀₀₀ was transferred to the lipoproteins, resulting in longer circulation lifetimes.

Our *in vivo* data clearly demonstrated that the negatively charged PS liposomes containing DMPE-PEG or DPPE-PEG were eliminated from the bloodstream more rapidly than the neutral DSPC/Chol liposomes containing either of these PE-PEGs. It is well documented that the plasma elimination of PS containing liposomes is very rapid (>90% of injected dose within minutes) due in part to PS-mediated plasma protein interactions with the liposome surface and subsequent MPS recognition. As DMPE-PEG and DPPE-PEG exchanged out of the PS liposomes, PS would be exposed to the high bind-

ing affinity plasma proteins. It is likely that the interaction between the high affinity plasma proteins and PS liposomes (with a free energy of association of about -9 kcal/mol for factor X [47]) may provide additional energy to overcome the energy barrier for PE-PEG desorption from PS membranes. This may result in further PE-PEG loss from PS liposomes and more PS being exposed to plasma proteins for interactions.

In designing 'transiently stabilized' liposomes, it is important to balance the necessity of achieving circulation longevity for target accumulation, and the importance of transforming the liposomes to a form that is capable of initiating site-specific reactions such as drug release, cell fusion or selective access of proteins. For pegylated liposomes, this balance is dependent on the rate at which the grafted PEGs desorb from the liposomes. In this study, we have demonstrated that the membrane surface reactivity of PS containing liposomes can be controlled in a time-dependent fashion by using exchangeable PEG-lipids of various acyl chain lengths. This has greatly contributed to our development of liposomes capable of triggering tumor site-specific thrombosis. For our intended application, PS containing liposomes must also achieve tumor site-specific accumulation and localization of PS to minimize the poten-

tial for disseminated intravascular coagulation (see Fig. 7). We are currently conducting studies to target these liposomes to tumor vasculature by conjugating monoclonal antibodies specific for antigen expressed on endothelial cells of the tumor neovasculature. Results from this study have important implications in the design of more sophisticated, multi-functional liposomes for targeted drug delivery. Specifically, the amount and chemical attributes of steric stabilizing lipids for such liposomes may be significantly different from those used for purposes of simply reducing liposome elimination rates.

Acknowledgements

We are grateful to Dana Masin, Hong Yan and Sophia Tan for their technical assistance in the animal studies. This research project is supported by a research grant from the Canadian Institute of Health Research. G.N.C.C. is supported by a graduate fellowship from the University of British Columbia.

References

- [1] R.F.A. Zwaal, P. Comfurius, E.M. Bevers, *Biochim. Biophys. Acta* 1376 (1998) 433–453.
- [2] S. Ran, B. Gao, S. Duffy, L. Watkins, N. Rote, P.E. Thorpe, *Cancer Res.* 58 (1998) 4646–4653.
- [3] F. Nilsson, H. Kosmehl, L. Zardi, D. Neri, *Cancer Res.* 61 (2001) 711–716.
- [4] T.M. Allen, P. Williamson, R.A. Schlegel, *Proc. Natl. Acad. Sci. USA* 85 (1988) 8067–8071.
- [5] A. Gabizon, D. Papahadjopoulos, *Biochim. Biophys. Acta* 1103 (1992) 94–100.
- [6] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, *Biochim. Biophys. Acta* 1066 (1991) 29–36.
- [7] K. Maruyama, T. Yuda, A. Okamoto, C. Ishikura, S. Kojima, M. Iwatsuru, *Chem. Pharm. Bull.* 39 (1991) 1620–1622.
- [8] A. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, *Biochim. Biophys. Acta* 1062 (1991) 142–148.
- [9] G.N. Chiu, M.B. Bally, L.D. Mayer, *Biochim. Biophys. Acta* 1510 (2001) 56–69.
- [10] M.J. Parr, S.M. Ansell, L.S. Choi, P.R. Cullis, *Biochim. Biophys. Acta* 1195 (1994) 21–30.
- [11] J.W. Holland, C. Hui, P.R. Cullis, T.D. Madden, *Biochemistry* 35 (1996) 2618–2624.
- [12] J.R. Silvius, R. Leventis, *Biochemistry* 32 (1993) 13318–13326.
- [13] J.R. Silvius, M.J. Zuckermann, *Biochemistry* 32 (1993) 3153–3161.
- [14] W.M. Li, L. Xue, L.D. Mayer, M.B. Bally, *Biochim. Biophys. Acta* 1513 (2001) 193–206.
- [15] P.S. Uster, T.M. Allen, B.E. Daniel, C.J. Mendez, M.S. Newman, G.Z. Zhu, *FEBS Lett.* 386 (1996) 243–246.
- [16] K. Sou, T. Endo, S. Takeoka, E. Tsuchida, *Bioconjug. Chem.* 11 (2000) 372–379.
- [17] H. Yoshioka, *Biomaterials* 12 (1991) 861–864.
- [18] G. Adlakha-Hutcheon, M.B. Bally, C.R. Shew, T.D. Madden, *Nat. Biotechnol.* 17 (1999) 775–779.
- [19] Q. Hu, C.R. Shew, M.B. Bally, T.D. Madden, *Biochim. Biophys. Acta* 1514 (2001) 1–13.
- [20] L.D. Mayer, M.J. Hope, P.R. Cullis, A.S. Janoff, *Biochim. Biophys. Acta* 817 (1985) 193–196.
- [21] L.D. Mayer, M.J. Hope, P.R. Cullis, *Biochim. Biophys. Acta* 858 (1986) 161–168.
- [22] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, *Biochim. Biophys. Acta* 812 (1985) 55–65.
- [23] I. Szleifer, O.V. Gerasimov, D.H. Thompson, *Proc. Natl. Acad. Sci. USA* 95 (1998) 1032–1037.
- [24] M.C. Woodle, D.D. Lasic, *Biochim. Biophys. Acta* 1113 (1992) 171–199.
- [25] A.K. Kenworthy, S.A. Simon, T.J. McIntosh, *Biophys. J.* 68 (1995) 1903–1920.
- [26] K. Hristova, D. Needham, *Macromolecules* 28 (1995) 991–1002.
- [27] K. Hristova, A. Kenworthy, T.J. McIntosh, *Macromolecules* 28 (1995) 7693–7699.
- [28] M.C. Woodle, L.R. Collins, E. Sponsler, N. Kossovsky, D. Papahadjopoulos, F.J. Martin, *Biophys. J.* 61 (1992) 902–910.
- [29] M.S. Webb, D. Saxon, F.M. Wong, H.J. Lim, Z. Wang, M.B. Bally, L.S. Choi, P.R. Cullis, L.D. Mayer, *Biochim. Biophys. Acta* 1372 (1998) 272–282.
- [30] G. Cevc, A. Watts, D. Marsh, *Biochemistry* 20 (1981) 4955–4965.
- [31] Y. Stein, G. Halperin, O. Stein, *FEBS Lett.* 111 (1980) 104–106.
- [32] G. Adlakha-Hutcheon, in: *Doctoral thesis, Department of Pharmacology and Therapeutics, University of British Columbia, Vancouver, 1999*, p. 183.
- [33] D. Kirpotin, K. Hong, N. Mullah, D. Papahadjopoulos, S. Zalipsky, *FEBS Lett.* 388 (1996) 115–118.
- [34] S. Zalipsky, M. Qazen, J.A.n. Walker, N. Mullah, Y.P. Quinn, S.K. Huang, *Bioconjug. Chem.* 10 (1999) 703–707.
- [35] P. Harvie, F.M. Wong, M.B. Bally, *J. Pharm. Sci.* 89 (2000) 652–663.
- [36] C. Vermehren, T. Kiebler, I. Hylander, T.H. Callisen, K. Jorgensen, *Biochim. Biophys. Acta* 1373 (1998) 27–36.
- [37] C. Vermehren, K. Jorgensen, S. Frokjaer, *Int. J. Pharm.* 183 (1999) 13–16.
- [38] J.W. Nichols, R.E. Pagano, *Biochemistry* 20 (1981) 2783–2789.
- [39] J.D. Jones, T.E. Thompson, *Biochemistry* 28 (1989) 129–134.
- [40] J.D. Jones, T.E. Thompson, *Biochemistry* 29 (1990) 1593–1600.

- [41] W.C. Wimley, T.E. Thompson, *Biochemistry* 30 (1991) 1702–1709.
- [42] S.J. Slater, C. Ho, F.J. Taddeo, M.B. Kelly, C.D. Stubbs, *Biochemistry* 32 (1993) 3714–3721.
- [43] J.W. Holland, P.R. Cullis, T.D. Madden, *Biochemistry* 35 (1996) 2610–2617.
- [44] J.W. Nichols, *Biochemistry* 24 (1985) 6390–6398.
- [45] R.E. Brown, *Biochim. Biophys. Acta* 1113 (1992) 375–389.
- [46] G. Scherphof, B. Van Leeuwen, J. Wilschut, J. Damen, *Biochim. Biophys. Acta* 732 (1983) 595–599.
- [47] D.A. Plager, G.L. Nelsestuen, *Biochemistry* 33 (1994) 7005–7013.