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Influence of dose on liposome clearance: critical role of blood proteins

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Abstract

It is well established that the circulation half-life of liposomes increases with increasing dose. This effect is commonly attributed to 'saturation' of the fixed and free macrophages of the reticuloendothelial system resulting in reduced clearance rates. However, it is also known that the clearance rate of liposomes is dependent on the amount of associated blood protein, leading to the possibility that dose-dependent increases in circulation lifetimes could be due to decreases in the amount of blood protein associated per liposome. In order to test this hypothesis, the protein binding and clearance properties of large unilamellar liposomes composed of distearoylphosphatidylcholine/cholesterol and egg phosphatidylcholine/dioleoylphosphatidic acid/cholesterol were examined in mice. Liposomes were injected over a dose range of 10 to 1000 mg lipid/kg body weight, and the circulation lifetime and liver and spleen accumulation monitored. As expected, longer circulation half-lives were observed at higher doses for both liposome compositions. However, it was also found that at higher liposome doses, significantly less protein was bound per liposome. The results indicate that there is a limited pool of blood proteins that is able to interact with liposomes of a given composition. At higher lipid doses these blood proteins are distributed over more liposomes resulting in lower protein binding values and longer circulation lifetimes.

Keywords: Liposome; Blood protein; Protein; Reticuloendothelial system; Opsonin; Phagocytosis; Biodistribution

1. Introduction

Liposomes are important vehicles for drug delivery, particularly for anticancer drugs [1], antifungal agents [2] and antibacterial drugs [3]. Liposomes also exhibit considerable potential as gene and oligonucleotide delivery systems [4]. However, a basic understanding of mechanisms whereby liposomes are recognized and cleared from the circulation has not been achieved. It has been shown that liposome clearance rates are dependent on factors such as lipid composition, vesicle size, and lipid dose [5–7]. It is well known that increased doses of liposomes lead to longer circulation lifetimes [8–12]. This effect is generally believed to result from 'saturation' of the fixed and free macrophages of the reticuloendothelial system (RES), which play a dominant role in the clearance process, resulting in decreased clearance rates.

It is also well established that liposomes exhibit strong interactions with blood proteins [13–16]. This includes opsonins such as IgG [17], complement component C3 [13,15,18], and fibronectin [19]. Furthermore, liposomes have been shown to bind blood proteins in amounts inversely related to liposome clearance rates [15,20], where liposomes with low levels of bound blood proteins exhibit extended circulation lifetimes. This relation indicates that liposomes coated with a large amount of total blood protein have a higher probability of being recognized and cleared by RES cells. This leads to the possibility that increased doses of liposomes result in a decrease in the binding of proteins critical to immune recognition, which leads to extended circulation lifetimes.

In the present study we have investigated liposome blood–protein interactions over a range of doses for two liposome formulations. These are egg phosphatidylcholine/cholesterol/dioleoylphosphatidic acid (PC/CHOL/DOPA) which binds high levels of blood proteins, and distearylphosphatidylcholine/cholesterol (DSPC/CHOL), which binds much lower blood protein levels. Our

Abbreviations: RES, reticuloendothelial system; PC, egg phosphatidylcholine; CHOL, cholesterol; DOPA, dioleoylphosphatidic acid; DSPC, distearylphosphatidylcholine; LUV, large unilamellar vesicle; CHE, cholesteryl hexadecyl ether; HBS, Hepes-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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results indicate that dose-dependent increases in the circulation lifetimes of both liposome formulations were accompanied by decreases in the amount of protein associated per vesicle, suggesting that reduced clearance rates at high doses are directly attributable to decreased levels of blood proteins associated with the individual liposomes.

2. Materials and methods

2.1. Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared at various concentrations by extrusion of freeze-thawed multilamellar vesicles through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA, USA) using an extrusion device (Lipex Biomembranes, Vancouver, BC, Canada) [21]. DSPC/CHOL (55:45, mol%) and PC/CHOL/DOPA (35:45:20, mol%) liposomes were prepared in Hepes-buffered saline solution (HBS; 20 mM Hepes, pH 7.4, 145 mM NaCl) at 2–200 mM total lipid concentrations. The average size of these liposomes was 100 ± 30 nm as determined by quasielastic light scattering analysis using a Nicomp Model 270 Submicron Particle Sizer (Nicomp Instruments, Santa Barbara, CA, USA). Liposomes were radiolabelled using a lipid marker, [^{3}H]cholesteryl hexadecyl ether (CHE) (10 μCi/30 μmol of total lipid) (NEN Research Products, Mississauga, Ont, Canada). This label is non-exchangeable and non-metabolizable in mice due to low levels of cholesteryl ester transfer protein activity [22–24]. Specific activities of liposome preparations were determined by standard liquid scintillation counting methods using a Beckman LS 3801 Liquid Scintillation System (Beckman Instruments, Fullerton, CA, USA), and a colorimetric phosphorous assay [25]. Lipids (DSPC, DOPA and PC) were purchased from Avanti Polar Lipids (Pelham, AL, USA). Cholesterol (CHOL) was purchased from Sigma, St. Louis, MO, USA. All lipids were used without further purification.

2.2. In vivo mouse biodistribution studies

Liposome preparations (200 μl) were administered via the dorsal tail vein of female CD-1 mice (20–25 g, Charles River, St. Constant, Que, Canada). At appropriate time points, mice were killed by exposure to carbon dioxide. Blood was removed by cardiac puncture and collected in ice-cold 1.5 ml microcentrifuge tubes, immediately cooled to 0°C to prevent coagulation and centrifuged to separate plasma from blood cell components (12 000 rpm, 2 min, 4°C). Plasma liposome concentrations were determined from the specific activity of the injected liposomes, using standard liquid scintillation counting techniques. Plasma volume was assumed to be 5% of total body weight. The principle organs of the RES (liver and spleen) were individually collected, weighed, and homogenized (10% homogenates in saline) for 2 min using a Polytron homogenizer (Brinkman Instruments, Rexdale, Ont, Canada). Aliquots of the tissue homogenates (400 μl) were added to 500 μl of Solvable (NEN Research Products, Mississauga, Ont, Canada) in 7 ml glass scintillation vials and digested for 3 h at 50°C. Samples were subsequently decolorized for 3 h at room temperature by the addition of 100 μl of 30% hydrogen peroxide. Finally, 5 ml of Ultima Gold scintillation fluid (Packard Instrument, Meriden, CT, USA) was added to the vials and the levels of [^{3}H]cholesteryl hexadecyl ether (CHE) in the digested tissue samples were determined by standard liquid scintillation analysis. All in vivo analyses used four mice per time point.

2.3. Isolation of liposomes from blood components

Liposomes were retrieved from mouse plasma using an established 'spin column' procedure, described in detail elsewhere [26]. Briefly, Bio-Gel A-15m, 200–400 mesh (Bio-Rad, Richmond, CA, USA) was equilibrated with HBS and packed in 1.0 ml Tuberculin syringes with glass wool plugs. Aliquots of plasma (50 μl), isolated at 2 min post injection, were immediately applied to these columns. Column fractions were collected in glass 13×100 mm culture tubes by the repetitive addition of 50 μl of HBS to the spin columns followed by centrifugation (1000 rpm for 1 min). The eluate from each centrifugation step was considered to be one fraction. Liposomes eluted in fractions 6 and 7, and were pooled for subsequent use in protein analysis.

2.4. Quantification of the amount of total protein associated with recovered liposomes

Liposome-associated proteins were extracted and delipidated according to the procedure described by Wessel and Flugge [27]. Delipidation was required to prevent interference with protein assay results. Protein was quantified using the Micro BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL, USA) in 0.5% SDS in Milli-Q (0.22 μm) filtered water. Briefly, 1.0 ml of protein assay working reagent was added to 1.0 ml 0.5% SDS protein solution, and following a 60 min incubation at 60°C, the sample absorbance at 562 nm was compared to a bovine serum albumin standard curve (0–16 μg/ml). Protein binding values (P_B; g protein/mol lipid) were calculated from the lipid concentrations of the recovered liposomes. A minimum of three P_B determinations were made for each lipid composition and dose.

2.5. SDS-polyacrylamide gel electrophoretic analysis of proteins associated with liposomes

Protein analysis was facilitated by SDS-polyacrylamide gel electrophoresis using a Mini-PROTEAN II Dual Slab

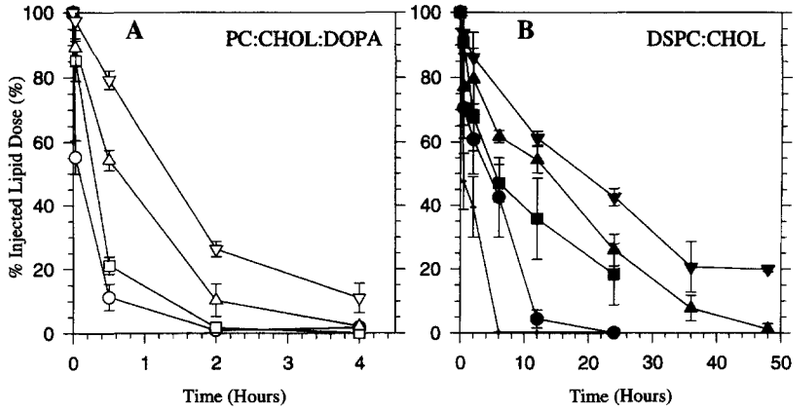

Fig. 1. Plasma clearance (%) of liposomes of varying dose. LUVs (100 nm) containing trace amounts of [^{3}H]cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, plasma levels of LUVs were measured by analyzing aliquots of plasma using standard scintillation counting methods. Panel A depicts PC/CHOL/DOPA (35:45:20, mol%): 100 mg/kg, (○); 200 mg/kg, (□); 500 mg/kg, (△); 1000 mg/kg, (▽). Panel B depicts DSPC/CHOL (55:45, mol%) liposomes of varying dose: 10 mg/kg, (+); 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). Each data point represents the average plasma recovery and standard deviation from four mice.

Gel electrophoretic apparatus (Bio-Rad, Richmond, CA, USA). Delipidated protein samples were solubilized in SDS-reducing buffer (0.0625 M Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.125% (w/v) Bromophenol blue), heated at 95°C, cooled and centrifuged prior to application on the gel. Plasma proteins were separated using Bio-Rad Mini-PROTEAN II Ready Gels, 4–20% polyacrylamide gradient, 0.375 M Tris-HCl, pH 8.8 with a 4% stacking gel (75 × 75 × 1.0 mm). The gels were run at 150 V for 60 min. Protein molecular weights were estimated by comparison to High and Low Range Silver Stain SDS-PAGE Standards (Bio-Rad). The proteins were detected using an optimized silver-staining procedure [28]. All gel mixtures and buffer solutions were prepared in Milli-Q (0.22 μm filtered) water and degassed. Gel and buffer reagents were purchased from Bio-Rad.

3. Results

3.1. Biodistribution of increasing doses of DSPC / CHOL and PC / CHOL / DOPA liposomes

The first set of experiments was aimed at characterizing the circulation longevity and tissue distribution of 100 nm diameter LUVs composed of PC/CHOL/DOPA (35:45:20, mol%) and DSPC/CHOL (55:45, mol%). These two lipid compositions were selected because of their dramatically different circulation clearance characteristics

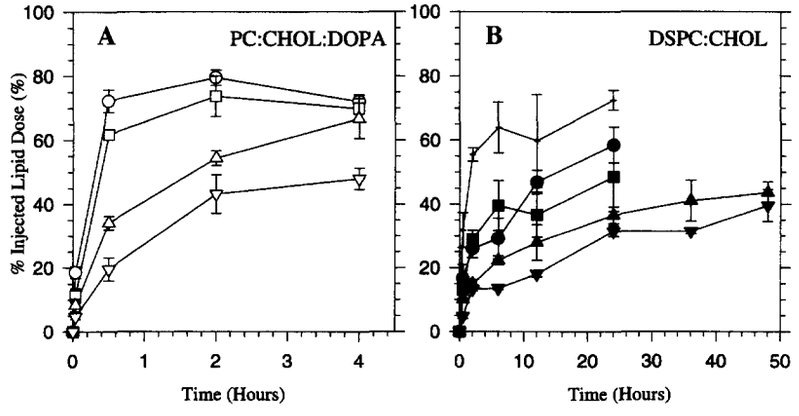

Fig. 2. RES uptake (%) of liposomes of varying dose. LUVs (100 nm) containing trace amounts of [^{3}H]cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, liposome recovery in the liver and spleen was measured by counting aliquots of 10% HBS organ homogenates solubolized using a Solvable digestion procedure, using standard scintillation counting methods. Panel A depicts PC/CHOL/DOPA (35:45:20, mol%) liposomes of varying dose: 100 mg/kg, (○); 200 mg/kg, (□); 500 mg/kg, (△); 1000 mg/kg, (▽). Panel B depicts DSPC/CHOL (55:45, mol%) liposomes of varying dose: 10 mg/kg, (+); 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). Each data point represents the average combined organ and spleen recovery and standard deviation from four mice.

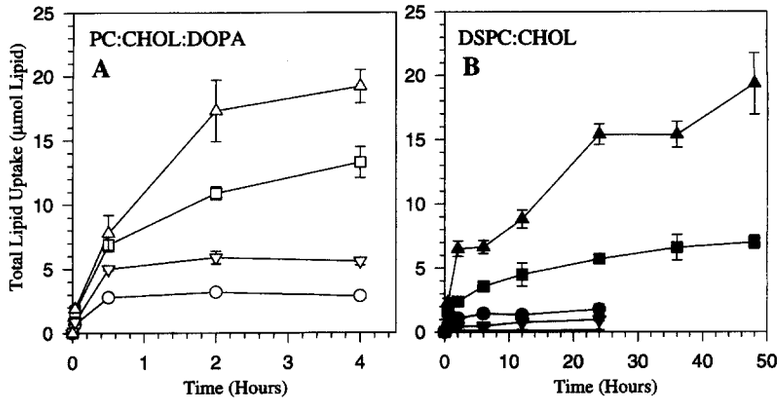

Fig. 3. Total lipid uptake by the RES varying liposome dose. LUVs (100 nm) containing trace amounts of [^{3}H]cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, liposome recovery in the liver and spleen was measured by counting aliquots of 10% HBS organ homogenates solubolized using a Solvable digestion procedure, using standard scintillation counting methods. Panel A depicts PC/CHOL/DOPA (35:45:20, mol%) liposomes of varying dose: 100 mg/kg, (○); 200 mg/kg, (▽); 500 mg/kg, (□); 1000 mg/kg, (△). Panel B depicts DSPC/CHOL (55:45, mol%) liposomes of varying dose: 10 mg/kg, (+); 50 mg/kg, (▼); 100 mg/kg, (●); 500 mg/kg, (■); and 1000 mg/kg, (▲). Each data point represents the average combined organ and spleen recovery and standard deviation from four mice.

and protein binding behavior. The effects of increasing lipid dose up to 1000 mg lipid/kg body weight on the clearance from the circulation and organ biodistribution of the liposomes were examined. As shown in Fig. 1, the clearance of DSPC/CHOL and PC/CHOL/DOPA LUVs from the circulation of CD-1 mice is consistent with earlier findings [15], in that DSPC/CHOL liposomes experience relatively long circulation lifetimes (Fig. 1B), while vesicles containing DOPA are very rapidly cleared (Fig. 1A). In both cases longer circulation lifetimes are observed as the dose is increased. Circulation half-lives for the 1000 mg/kg doses of DSPC/CHOL and PC/CHOL/DOPA LUVs were 1200 and 80 min, respectively, as compared to 360 min and 4 min for the 100 mg/kg doses.

LUVs which are rapidly cleared from the circulation are accumulated by the RES organs (liver and spleen), as illustrated in Fig. 2. The lower percentage of liposome uptake observed with high liposome doses suggests saturation of the RES uptake pathways (Fig. 2). However, Fig. 3 shows that despite the decreasing percentage of total liposome uptake observed, the amount of liposomes taken up by the principal organs of the RES (liver and spleen) still increases at higher doses. This pattern is consistent for both short- and long-lived lipid compositions (Fig. 3A and Fig. 3B), and is inconsistent with RES saturation. Estimates of initial liposome uptake rates for the liver also reflect the unsaturated nature of the RES, as illustrated in Fig. 4, which shows that the rates of liver uptake increased with the injected lipid dose over the full dose range for both compositions studied.

3.2. Effect of liposome dose on P_B values

As previously noted, an inverse relationship exists between the amount of protein bound to liposomes and their

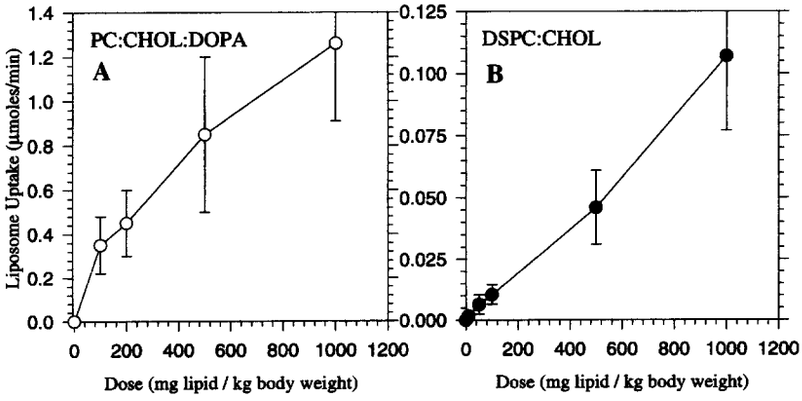

Fig. 4. Initial rate of liver uptake of liposomes of varying dose. LUVs (100 nm) containing trace amounts of [^{3}H]cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. Liposome recovery in the liver was subsequently measured and plotted over time. Estimates of the initial rates of liposome uptake by the liver were determined based on initial slope determinations from the uptake curves. Error bars represent the approximate degree of uncertainty associated with the slope determination. Panel A (○) depicts PC/CHOL/DOPA (35:45:20, mol%) liposomes of varying dose. Panel B (●) depicts DSPC/CHOL (55:45, mol%) liposomes of varying dose.

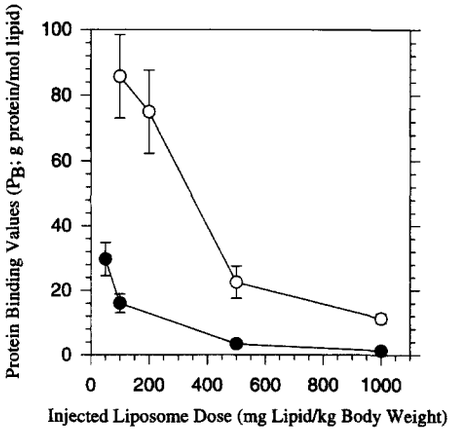

Fig. 5. Relation of liposome-associated protein and the injected lipid dose. Aliquots of recovered liposomes were delipidated, and the extracted proteins quantified using the micro BCA protein assay (see Section 2). The liposomes were composed of PC/CHOL/DOPA (35:45:20, mol%) (○) and DSPC/CHOL (55:45, mol%) (●). The data points represent the average and standard deviation obtained from three separate trials.

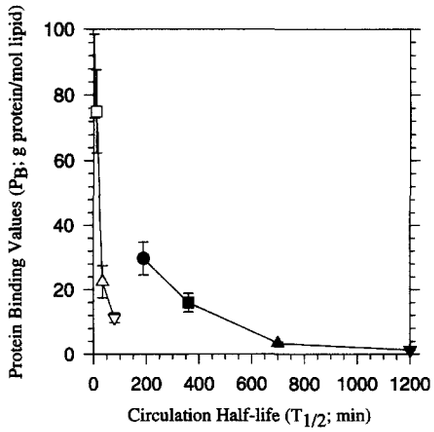

Fig. 6. Relation of protein bound to liposomes and the circulation half-lives. Aliquots of recovered liposomes were delipidated, and the extracted proteins quantitated using the micro BCA protein assay (see Section 2). The liposomes were composed of PC/CHOL/DOPA (35:45:20, mol%): 100 mg/kg, (○); 200 mg/kg, (□); 500 mg/kg, (△); 1000 mg/kg, (▽) and DSPC/CHOL (55:45, mol%) liposomes: 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). The data points represent the average and standard deviation obtained from three separate trials. Half-lives were determined by analysis of the plasma clearance data of Fig. 1.

clearance rates from the circulation [15]. It was therefore of interest to determine whether the more slowly cleared high dose liposomes bound less blood protein. Liposomes were recovered from the blood at 2 min post-injection, and were subsequently delipidated and analyzed for associated protein using a BCA protein assay. Fig. 5 shows the P_B values as a function of lipid dose, for both compositions studied. A significant decrease in protein binding values is immediately apparent as the dose of each composition increases. When the protein binding values are expressed as a function of the liposome circulation half-lives (Fig. 6), the results are consistent with earlier observations [15]. High liposome doses exhibit lower P_B values and significantly increased circulation lifetimes. These results therefore support an inverse relationship between circulation half-lives and the amount of liposome-bound protein even for the same liposomes administered at different doses.

Fig. 6 also displays a unique pattern of P_B versus half-life for each liposome composition. Liposome preparations with the same P_B value do not necessarily experience the same clearance kinetics. At a P_B value of 12 g/mol, DOPA-liposomes (1000 mg/kg) possessed a 75 min half-life, while DSPC/CHOL (200 mg/kg) exhibits a half-life of 450 min. Clearly, liposome clearance is dependent on the type, as well as the quantity, of protein bound.

An important corollary of the observation that the amount of protein bound per LUV decreases as the dose increases, is that the total amount of blood protein available for binding is limited. The total blood protein (μg total protein) bound to each dose of the two compositions

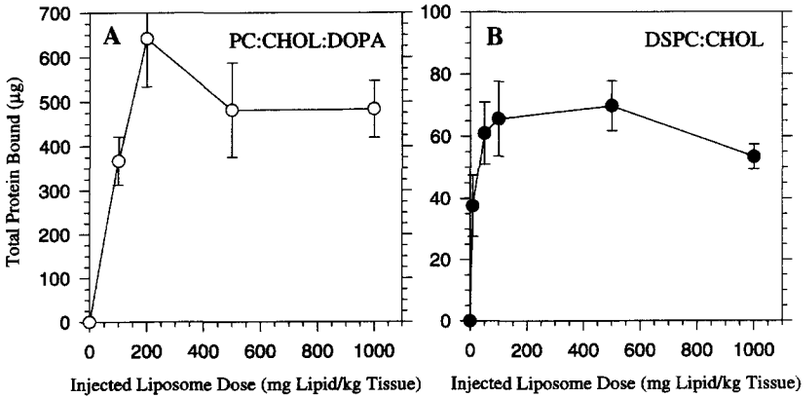

Fig. 7. Relation of total protein bound to circulating liposomes and the injected dose. Aliquots of recovered liposomes were delipidated, and the extracted proteins quantitated using the micro BCA protein assay (see Section 2). Based on the P_B values obtained, the total protein bound to each of the injected lipid samples was determined and plotted versus the injected lipid dose. Panel A (○) depicts liposomes composed of PC/CHOL/DOPA (35:45:20, mol%). Panel B (●) depicts liposomes composed of DSPC/CHOL (55:45, mol%). The data points represent the average and standard deviation obtained from three separate trials.

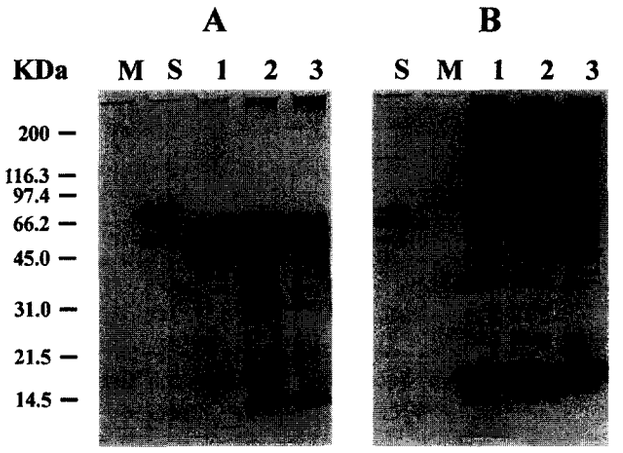

Fig. 8. Silver-stained reducing SDS-PAGE gels of proteins associated with liposomes recovered from the circulation of CD-1 mice after 2 min post-injection. The proteins associated with the liposomes were separated electrophoretically on 4–20% SDS-polyacrylamide gels and visualized by silver stain. Each lane of Panel A consists of 2 μg of total protein isolated from PC/CHOL/DOPA (35:45:20, mol%) liposomes injected at the following doses: 100 mg/kg (lane 1), 500 mg/kg (lane 2), and 1000 mg/kg (lane 3). Panel B consists of 2 μg protein samples isolated from DSPC/CHOL (55:45, mol%) liposomes injected at the following doses: 100 mg/kg (lane 1), 500 mg/kg (lane 2), and 1000 mg/kg (lane 3). Lane M contains silver-stained SDS-PAGE molecular weight standards from Bio-Rad (myosin, 200000; β-galactosidase, 116250; phosphorylase *b*, 97400; serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; trypsin inhibitor, 21500; and lysozyme, 14400). Lane S contains normal mouse serum.

is illustrated in Fig. 7. It is apparent the maximum amount of blood protein bound to the PC/CHOL/DOPA vesicles is approx. 550 ± 70 μg (Fig. 7A), whereas DSPC/CHOL vesicles bind a total of 60 ± 10 μg (Fig. 7B). This indicates that a limited pool of blood proteins is available to bind to liposomes of a given lipid composition, regardless of dose. At higher doses, these proteins are redistributed over a greater number of liposomes, resulting in lower P_B values and reduced RES uptake.

3.3. Influence of bound protein composition on clearance

Fig. 8 shows the silver stained SDS-PAGE profiles of in vivo protein samples obtained from liposomes composed of PC/CHOL/DOPA and DSPC/CHOL isolated at 2 min post-injection. Comparison of protein composition was simplified by examining equal protein quantities (2 μg total protein/lane). As may be observed, the liposome composition has a significant influence on the profile of liposome-associated blood proteins. It is interesting to note that different doses of the same lipid composition produced relatively minor changes in the plasma protein profile.

4. Discussion

In agreement with earlier work [10–12], this study reports increases in the circulation half-lives of liposomes as the lipid dose is increased. However, it was widely believed that this dose effect is a result of RES saturation [10,29]. As clearly demonstrated in this work, increased lipid dose results in decreased protein binding. Previously, we have demonstrated that P_B values are inversely correlated with circulation lifetimes [15]. Together this suggests that the prolonged circulation lifetimes observed with increasing liposome dose are the direct result of the decreased protein binding.

Comparisons between DSPC/CHOL and PC/CHOL/DOPA liposomes at equivalent doses make it quite evident that both the total lipid accumulation and the rate of lipid uptake by the RES, are up to 10-times greater for the DOPA-containing systems (Figs. 3 and 4). If the mechanisms of clearance for the two different liposome compositions both rely on uptake by the free and fixed macrophages of the RES, then the DOPA systems reflect at most the minimum capacity of the RES to take up liposomes. This capacity is not reached by the DSPC/CHOL liposomes, suggesting that RES saturation cannot account for the increased lifetimes of higher doses of DSPC/CHOL liposomes. Consequently, either the DOPA-containing liposomes are removed from the circulation by a different mechanism or the quantity and type of blood protein that associates with each liposome is of critical importance in establishing RES phagocytic ability.

A number of recent investigations have focused on the importance of blood protein–liposome interactions [15,30–32]. Blood opsonins have long been known to be involved in the phagocytosis of foreign particulate matter [8,33], and Chonn et al. [15] demonstrated an inverse relationship between the blood protein associated with liposomes and the circulation half-lives. When dose-dependent circulation half-lives are examined, the results presented here also reflect an inverse relationship with the protein bound. Analysis of plasma protein binding over a range of dose reveals 8–12-fold increased protein binding to low doses of liposomes compared to high doses, for both DSPC/CHOL and PC/CHOL/DOPA liposomes (Fig. 5). Just as low protein binding lipid compositions have previously been associated with enhanced circulation half-lives [15], high lipid doses with low P_B values experienced similar extended lifetimes. This is likely because low P_B values decrease the probability that the liposomes can be recognized and ingested by the RES. The blood opsonins on liposomal membranes are 'diluted' over a much greater surface area at higher lipid doses, as represented by the reduced P_B values.

An important result of this work is the apparent existence of specific and finite pools of plasma proteins that are available for binding to liposomes with different lipid compositions. Previous work has clearly illustrated that lipid composition strongly influences protein binding, both in total amount of protein bound and in terms of protein profiles. By varying only liposome dose, the results presented here show that the total quantity of liposome-bound

blood protein reaches a plateau at higher doses (Fig. 7). Furthermore, the protein composition of this liposome-associated protein does not significantly vary with dose (Fig. 8). This suggests that the blood protein pools are specific in quantity and identity for each lipid composition. The DOPA-containing liposomes interact strongly with blood proteins, and the associated protein composition has been shown to be enriched in known blood opsonins [15]. DSPC/CHOL liposomes are low protein binding systems, and do not bind known opsonins with the same affinity of the high protein binding systems [15]. It should be noted that the maximum amount of blood protein bound, at any liposome composition or dose, was 0.6 mg/ml plasma. This is approx. 1% of the total plasma protein, 60–80 mg/ml, calculated based on a plasma protein concentration of 6–8 g/dl [34] and assuming a plasma volume of 5% of body weight. Although this represents only 1% of total blood protein, LUVs interact with major (albumin) [13] and minor (β_2-glycoprotein-1, IgG, and C3) [35,13,15] protein components in the blood. Some of the minor components of the blood can be depleted at these levels of protein bound, increasing the possible in vivo significance of binding otherwise small amounts of total blood protein.

In summary, the studies described here illustrate the importance of liposome-bound blood proteins in mediating liposome clearance from the blood circulation and the role of membrane lipid composition as a factor regulating blood protein interactions. Different doses of the same liposomes lead to different blood protein binding properties, consistent with the hypothesis that the amount of bound protein dictates the liposome clearance properties. Low P_B values associated with high doses of liposomes which exhibit extended circulation times, suggest that the 'saturation' phenomena may in fact be a consequence of the depletion of blood opsonins, and not a result of RES saturation. This is further substantiated by the existence of a limited pool of blood proteins that is available for binding to liposomes.

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