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# Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo

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## Abstract

Liposomes and lipid-based drug delivery systems have been used extensively over the last decade to improve the pharmacological and therapeutic activity of a wide variety of drugs. More recently, this class of carrier systems has been used for the delivery of relatively large DNA and RNA-based drugs, including plasmids, antisense oligonucleotides and ribozymes. Despite recent successes in prolonging the circulation times of liposomes, virtually all lipid compositions studied to date are removed from the plasma compartment within 24 h after administration by the cells and tissues of the reticuloendothelial system (RES). Plasma proteins have long been thought to play a critical role in this process but only a few efforts were made to evaluate the relevant importance of plasma protein–liposome interactions in the clearance process. Strategies to increase the bioavailability of liposomal drugs have included altering lipid compositions and charge, increasing lipid doses, and incorporating surface coatings. All of these modifications can influence membrane–protein interactions. In this article, we will focus on our experiences with liposome–blood protein interactions and how alterations in the chemical and physical properties of the carrier system influence the interactions with blood proteins and circulation times. © 1998 Elsevier Science B.V.

*Keywords:* Opsonins; Plasma proteins

**Contents**

1. Introduction ... 4
2. Protein binding studies in vivo ... 4
   2.1. Protein binding ($P_B$) values ... 4
3. Factors influencing plasma protein–liposome interactions ... 6
   3.1. Surface charge ... 6
   3.2. Surface coatings ... 8
   3.3. Lipid dose ... 9
4. Plasma protein involvement in liposome clearance ... 10
   4.1. Albumin ... 11
   4.2. Complement and related proteins ... 11
   4.3. Immunoglobulins ... 11
   4.4. Fibronectin ... 12
   4.5. Apolipoproteins ... 12
   4.6. C-reactive protein ... 12
   4.7. β2-Glycoprotein I ... 12
5. Conclusions ... 13
6. Abbreviations ... 13
References ... 14

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## 1. Introduction

Very soon after the initial discovery of liposomes it became apparent that when lipid vesicles are administered in vivo they are rapidly removed from the circulation by the cells and organs comprising the reticuloendothelial system. Early studies demonstrated that several physical and chemical properties of liposomes, such as size, lipid composition, surface charge, and surface coatings, are known to influence the clearance pharmacokinetics of the vesicles (see Ref. [1] for an excellent review). Early on, attempts were made to rationalize these findings based on liposome–plasma protein interactions. Several investigators demonstrated that plasma proteins rapidly interact with lipid membranes in plasma or serum incubations in vitro. These studies generally indicated that liposome–plasma protein interactions result in destabilization and breakdown of the vesicle or in opsonization, resulting in enhanced clearance properties.

Early observations regarding the effects of membrane composition on liposome clearance suggested that liposome clearance was related to a biophysical property of the bilayer, such as membrane permeability [2,3]. Other groups demonstrated that proteins rapidly associate with liposome surfaces in incubations with isolated plasma or serum [4–6]. Therefore, it is reasonable to suggest that proteins will alter the physicochemical properties of liposomes, thereby resulting in altered stability and clearance properties in the biological milieu.

## 2. Protein binding studies in vivo

A number of methods have been used to evaluate plasma protein–liposome interactions. The earliest studies employed multilamellar vesicles (MLVs) in in vitro incubations [4–6]. MLVs had the advantage that they could be easily centrifuged and washed to remove non-associated proteins. A problem with this type of analysis in vivo is that MLVS are rapidly cleared from the circulation due to their large and heterogeneous size distribution. Furthermore, adsorbed proteins may be lost during washes. Technical difficulties in isolating unilamellar vesicles from serum or blood slowed much of this work until recently.

In order to facilitate quantitative determinations of protein–liposome interactions employing 100 nm large unilamellar vesicles (LUVs), however, a convenient method was required to rapidly isolate LUVs from plasma/serum incubation mixtures in vitro; or from whole blood isolated from liposome-treated mice. To this end, we introduced a 'spin-column' procedure that enabled the rapid isolation of LUVs from plasma components, including lipoproteins [7] (Fig. 1). In our studies, we typically administer liposomes via the dorsal tail vein of CD1 mice at a dose of 100 mg lipid/kg body weight, recover the circulating LUVs from the blood after 2 min post-injection, and analyze the proteins associated with the liposome membranes. Noteworthy is that this procedure can be performed in the absence of coagulation inhibitors, which may affect protein/liposome interactions [7]. Isolated LUVs can then be analyzed for their protein content by various quantitative protein assays to derive protein binding values, or by SDS-polyacrylamide gel electrophoretic analysis, immunoblot analysis and ELISA assays to identify and quantitate the various proteins associated with the LUVs. Determination of the lipid concentration of the recovered liposome suspensions is facilitated by the inclusion of a non-exchangeable lipid marker in the injected LUVs, such as [$^3$H]cholesterylhexadecyl ether.

### 2.1. Protein binding ($P_B$) values

In order to quantitate the surface adsorption properties of liposome membranes to proteins in solution, we have introduced a parameter that we have termed '$P_B$ value' indicating 'protein binding value' [8]. $P_B$ values represent the total protein binding ability of liposomes, expressed in 'g of protein/mol of lipid'. In estimating $P_B$ values, it is desirable to employ well defined large unilamellar vesicles (LUVs) having a narrow size distribution because vesicle size affects both liposome stability and clearance rates [9,10]. Typically, we employ LUVs that have been prepared by an extrusion procedure through 100 nm pore-sized filters [11]. The vesicle population obtained by this procedure is essentially unilamellar, thereby maximizing the membrane surface area that has access to the protein solution and, depending on the lipid composition, remains stable. In contrast, SUVs are inherently

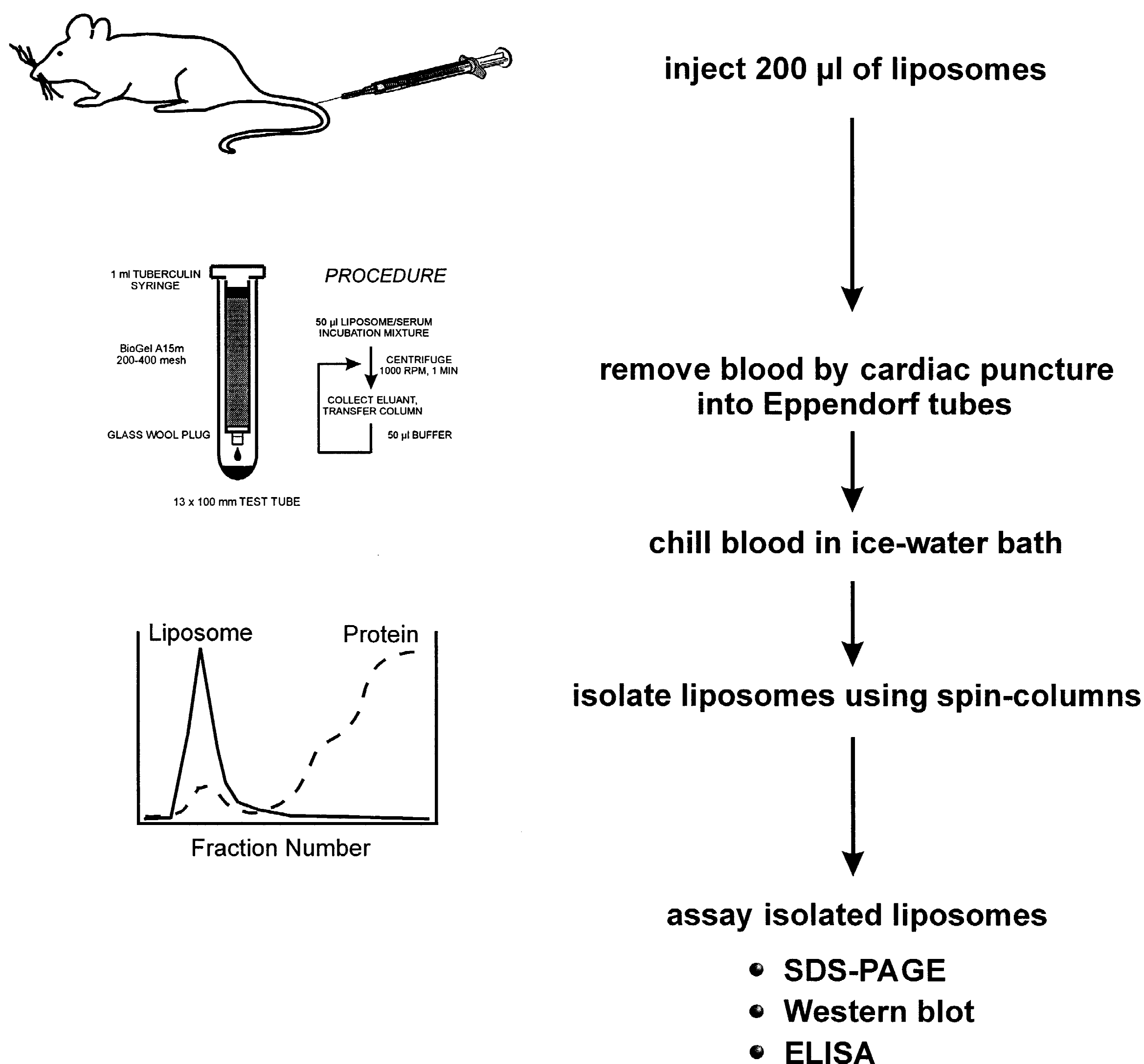


Fig. 1. Isolation and characterization of plasma proteins associated with liposomes.

unstable due to their highly strained, curved bilayers [12–15].

The $P_B$ values determined from in vitro incubation mixtures of LUVs with isolated human serum exhibit similar trends to those determined from LUVs isolated from the blood of liposome-treated mice at 2 min post-injection [7,8]. Comparable with the in vivo findings, anionic LUVs that are cleared extremely rapidly from the circulation of mice have the highest in vitro $P_B$ values; whereas LUVs that are cleared less rapidly have moderate in vitro $P_B$ values. This finding suggests that in vitro $P_B$ value determinations should be somewhat predictive of the in vivo clearance behavior of LUVs, at least in mice.

The SDS-PAGE profiles of the proteins associated with the various anionic LUVs are more complex for LUVs isolated from in vivo incubations compared with LUVs isolated from in vitro incubations. This most likely reflects the more complex nature of the in vivo system. An interesting point to consider here is that some of these proteins may represent cell-derived proteins [16] and/or proteolytic fragments generated by the activation of blood pathways, such as the complement and coagulation systems.

Noteworthy are two proteins with apparent molecular weights of 22 000 and 14 000 that are associated with LUVs in vivo but are absent from recovered LUVs in vitro [8].

$P_B$ values have turned out to be very useful in assessing the relation between the surface adsorption properties and the clearance behavior of liposomes. Our studies to date indicate that there is an inverse relationship between $P_B$ values and the circulation half-lives of liposomes. In particular, liposomes that have greater than 50 g protein/mol lipid associated with their membranes are cleared very rapidly from the circulation (half-lives, $t_{1/2}$, of less than 2 min); whereas liposomes that have less than 20 g of associated protein/mol lipid exhibit more extended circulation times ($t_{1/2}$ greater than 2 h). Thus, the blood proteins that associate with liposomes in the circulation dramatically influence the clearance behavior of liposomes in vivo. We have systematically looked at the effects of various aspects of liposome design on $P_B$ values in order to determine how extensive this relationship applies. Following is a brief summary of our recent findings.

$P_B$ values determined from circulating LUVs recovered shortly after in vivo injection are likely an underestimate of the actual protein binding ability of the liposomes, since LUVs expressing the most surface-bound protein are also most likely to be cleared first. Since $P_B$ values are indicative of the probability of liposomes being recognized and cleared from the circulation, it would be of interest to determine how $P_B$ values change over time for liposome compositions having relatively long circulation times. To date, all in vivo $P_B$ estimates have been made at 2 min post-injection. These values should give reasonable estimates of protein binding for most liposome compositions.

Differences in the protein profiles of liposomes can be observed based on the method of isolating the vesicles from blood or serum. Liposomes isolated using column procedures typically exhibit more extensive protein profiles than those isolated by ultracentrifugation procedures. This may reflect the increased potential for protein loss during the multiple washing steps required in the latter procedure. In our investigations we have routinely used either spin column or conventional column procedures for isolating liposomes from blood.

The proteins found to associate with the LUVs recovered from blood using the 'spin column' procedure most likely represent proteins that are tightly bound to the liposome surface. In our experience, the protein profiles of the liposomes recovered after 2 min in the circulation of mice using the spin column procedure have always been very reproducible, both qualitatively (as determined by SDS-polyacrylamide gel electrophoretic analysis) and quantitatively (as determined by $P_B$ values). This indicates that the proteins are not readily desorbed or displaced. It has been suggested that this irreversibility in protein binding to the liposome surface is a result of multiple adsorption sites between proteins and the liposome membrane such that the probability that all the binding sites will be broken at the same time is minimal [17]. However, proteins that are readily displaceable are probably not recovered with the liposomes using the spin column procedure or by ultracentrifugation procedures as a result of dilution of the proteins in buffer. The displacement of adsorbed proteins has been observed by several investigators and has been termed the 'Vroman Effect' [17,18]. These proteins may also play a role in liposome clearance, either by enhancing liposome/cell interactions, or by blocking liposome-associated opsonin/cellular receptor interactions, thus providing a 'dysopsonin' effect. It would therefore be of interest to develop methods to characterize these readily displaceable proteins that may associate with liposomes in blood.

## 3. Factors influencing plasma protein–liposome interactions

A number of factors have been reported to influence plasma protein–liposome interactions and clearance rates, including surface charge, surface coatings and lipid dose. Some of these findings are summarized below.

### *3.1. Surface charge*

The recent interest in cationic lipid vectors and cationic lipid-based systems for the delivery of plasmids, antisense, and ribozymes makes it of interest to examine how some of these systems

interact with blood proteins. Several reports have indicated that these systems can have significant toxicity in vivo. This is consistent with our findings that cationic liposomes show considerably more complement activation in an in vitro human complement assay compared to neutral liposomes. Furthermore, our recent studies on the clearance properties and $P_B$ value determinations of cationic liposomes (100 nm LUVs) composed of 1,2-dioleyl-3-*N*,*N*,*N*-trimethylaminopropane chloride (DOTMA):DOPC (1:1 mol mol$^{-1}$) and DOTMA:DOPE (1:1 mol mol$^{-1}$) are consistent with a significant role of proteins in mediating liposome clearance. These cationic liposomes acquire $P_B$ values in excess of 500 g protein/mol lipid in vivo and are cleared extremely rapidly from the circulation of mice (our unpublished results). These findings are in agreement with earlier in vitro findings that liposomes containing 50 mol% DOTMA exhibit extensive interactions with plasma, resulting in immediate clot formation at charge concentrations $>0.5$ μmol ml$^{-1}$ [19]. A similar system employing the cationic lipid DODAC and DOPE (1:1) acquired in excess of 800 g protein/mol lipid in vivo (Fig. 2). The circulation time for this liposome formulation was in the order of minutes. Recently, Oku and co-workers examined vesicles containing several different cationic lipids, including DMRIE (~5 mol%) [20]. In these systems, significant serum turbidity and protein binding (400–1100 g protein/mol total lipid) was observed. These results are not unexpected since the majority of plasma proteins carry a net negative charge at physiological pH.

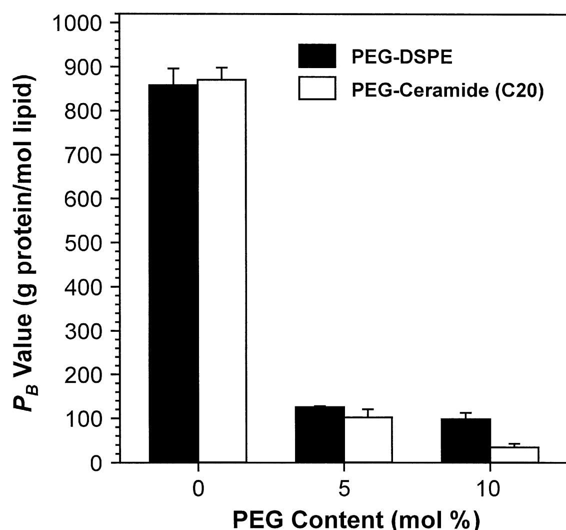


Fig. 2. Influence of cationic lipid and PEG on protein–liposome interactions.

Anionic lipids have been incorporated into liposomes to increase the trapped volume and improve the loading of various molecules and drugs, such as plasmids. In our studies the incorporation of 20 mol% negative surface charge in the form of different anionic phospholipids triggers rapid clearance behavior in CD1 mice [8]. However, the clearance is not solely dependent on the negative surface charge since LUVs expressing phosphatidylserine (PS), phosphatidic acid (PA) or cardiolipin (CL) on their outer surface are all cleared very rapidly from the circulation; whereas liposomes expressing phosphatidylglycerol (PG) or phosphatidylinositol (PI) circulate for longer periods. Upon analyzing the $P_B$ values of the various recovered LUVs, we observed that there was a dramatic difference in the ability of these anionic liposome membranes to interact with blood proteins. The biophysical basis for this difference is not entirely clear; what can be derived from these experiments, however, is that the presence of surface charge alone does not necessarily facilitate protein–membrane interactions since all of the liposomes studied expressed similar membrane surface charge. Specific lipid head group and acyl chain composition must also influence liposome–protein interactions. The importance of acyl chain composition is illustrated by the observation that liposomes containing different sources of PI are cleared at significantly different rates. LUVs containing bovine liver PI have $P_B$ values of 158 g protein/mol lipid and are cleared from the circulation in minutes, whereas LUVs containing plant PI have a circulation half-life of approximately 90 min and $P_B$ value of 27 g protein/mol lipid. The major difference between these two types of PI is in the saturation and length of the acyl chains, with the major acyl chain species being 18:0 and 18:2 for bovine liver PI and plant PI, respectively.

A study by Hernandez and co-workers [21] determined that the stability of various anionic MLV formulations to carboxyfluorescein release in the presence of human serum is strongly dependent on the types of anionic phospholipids used. This study went on to further describe a similar inverse relation

between the quantity of protein binding to the vesicles and liposome stability. These observations further support the hypothesis that plasma proteins play a key role in mediating liposome membrane destabilization and opsonization.

Recently, we have investigated various neutral liposomal systems. The influence of acyl chain length and saturation on the clearance and protein binding ability of phosphatidylcholine LUVs was evaluated using a series of saturated phosphatidylcholine derivatives [22]. Somewhat unexpectedly, liposomes composed of neutral saturated lipids with acyl chain lengths greater than 16 carbons bound large quantities of blood protein, in excess of 90 g mol$^{-1}$, and were very rapidly cleared from the circulation. DMPC (14:0/14:0) vesicles had $P_B$ values of ~30 g mol$^{-1}$ and experienced enhanced circulation times, $t_{1/2}$ of 80 min, compared to longer chain versions. Liposomes composed of DPPC (16:0/16:0) were intermediate with respect to both protein binding and circulation times. These effects were attributed to the presence of membrane packing defects that occur in saturated phosphatidylcholine vesicle systems at temperatures below the phase transition temperature of the constituent phospholipid. These defects result in the occurrence of hydrophobic domains at the surface of the vesicles. Many plasma proteins, particularly IgG and albumin, have strong affinities for hydrophobic domains and, consequently, become rapidly adsorbed to these regions [23,24].

Cholesterol has long been known to have a stabilizing, and in some systems, a fluidizing effect, on lipid bilayers. When 30–50 mol% cholesterol was incorporated into DSPC LUVs, the result is a relatively circulation stable vesicle formulation ($t_{1/2}$ 5–6 h) having a low $P_B$ value, 22–27 g mol$^{-1}$ [25]. In the absence of cholesterol, pure DSPC vesicles are cleared almost immediately ($t_{1/2} < 2$ min) from the circulation and bind significant quantities of protein, 90 g mol$^{-1}$. This finding suggests that an important function of cholesterol may be to prevent the formation of membrane surface defects, thus eliminating potential sites for protein adsorption. These studies are consistent with previous in vitro findings that indicate more extensive protein interactions with cholesterol-poor membranes [26,27]. Furthermore, studies utilizing cholesterol-rich and cholesterol-poor membranes have suggested the existence of liver and spleen-specific opsonins that possess different affinities for these membranes [28,29].

### 3.2. Surface coatings

Possibly one of the most widely used methods of enhancing the circulation properties of liposomes is the inclusion of amphipathic poly(ethyleneglycols) (PEG) in the membrane [30–33]. These polymers are typically 2000–5000 molecular weight (M.Wt.) and are usually coupled to phosphatidylethanolamine. Decreased adsorption of plasma proteins to liposome surfaces containing PEG has also been demonstrated using aqueous two-phase partitioning techniques [34,35]. Our studies have shown that the inclusion of 5% DSPE-PEG (average M.Wt. 2000) in liposomes decreases $P_B$ values considerably compared to identical vesicles lacking PEG (Fig. 3). $P_B$ values for DSPC:CH (55:45, mol/mol) and PC:CH:DOPA (35:45:20, mol/mol/mol) were 19 and 46, respectively, in the absence of PEG; in the presence of 5% DSPE-PEG, these values were 7 and 25, respectively. In cationic lipid systems, the inhibition of protein binding was even more dramatic (Fig. 2). Plasma protein binding was reduced by approximately eight-fold in PEG-containing vesicles; how-

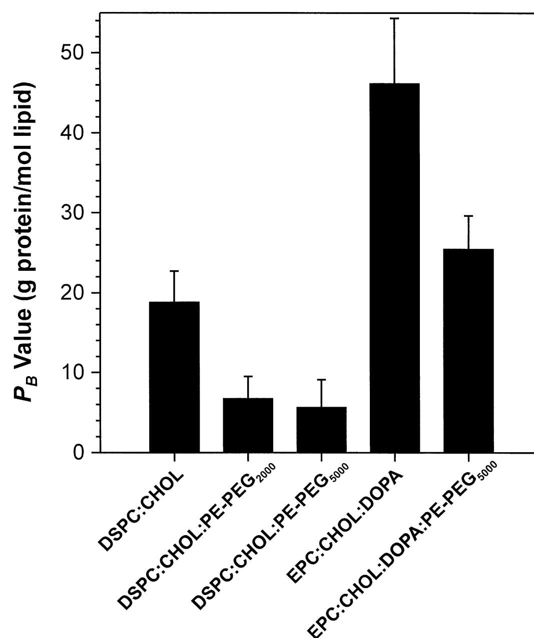


Fig. 3. Influence of PEG on protein binding to liposomes.

ever, major differences in clearance properties were not observed since the PEG-containing vesicles still bound significant ( > 100 g $mol^{-1}$ lipid) quantities of protein. Despite the net decrease in protein binding to PEG-containing LUVs, however, the protein profiles of these liposomes were not significantly different than controls, other than a general decrease in the intensity of all observable bands. This suggests that the mechanism by which PEG decreases protein interactions is non-specific.

A second membrane surface component that has received considerable attention for its ability to enhance the circulation times of liposomes is ganglioside $G_{M1}$. From the studies examining the mechanism by which lipids such as ganglioside $G_{M1}$ extend the circulation lifetime of liposomes, it was shown that the inclusion of ganglioside $G_{M1}$ reduces the total protein binding to LUVs in a relatively non-specific manner [8]. The inclusion of ganglioside $G_{M1}$ creates a surface that is not interactive with blood proteins; therefore, membrane-associated reactions such as complement activation do not occur [36–39].

### 3.3. Lipid dose

It has been a matter of debate for some time whether the increased circulation times that are typically observed with higher lipid doses are a result of saturation of RES uptake or depletion of blood opsonins. Recently, we have investigated the influence of lipid dose on protein binding and interactions in vivo [40]. In these studies both neutral DSPC:CHOL and anionic EPC:CHOL:DOPA vesicles were evaluated for the ability to interact with blood proteins in vivo at doses ranging from 10 to 1000 mg $kg^{-1}$. It was found, in both instances, that longer circulation times were observed with higher doses and that this corresponded to a decrease in protein binding. Furthermore, while total liver uptake increased for each lipid dose, total protein binding appeared saturable when total protein was adjusted to lipid dose. In this regard there appeared to be specific pools of protein available to interact with neutral DSPC:CHOL vesicles (~70 μg total) and EPC:CHOL:DOPA vesicles (~650 μg total). The relative importance of specific proteins within these pools remains unclear. These results suggest that increased lipid doses result in decreased protein levels on the surface of the liposome which can then lower the probability of uptake by receptor-mediated mechanisms (Fig. 4A).

In an attempt to further verify the concept of opsonin depletion a variety of doses and pre-doses were administered. When the fate of a 100 mg $kg^{-1}$ dose of EPC:CHOL:DOPA liposomes was followed after the administration of 1000 mg $kg^{-1}$ doses of either DSPC:CHOL or EPC:CHOL:DOPA, only the EPC:CHOL:DOPA pre-dose produced a significant increase in circulation half-life (10 min in the absence of a predose compared to ~2 h with the predose; our unpublished results). One interpretation of these results is that the pre-dose binds the plasma proteins that are relevant to clearance and conse-

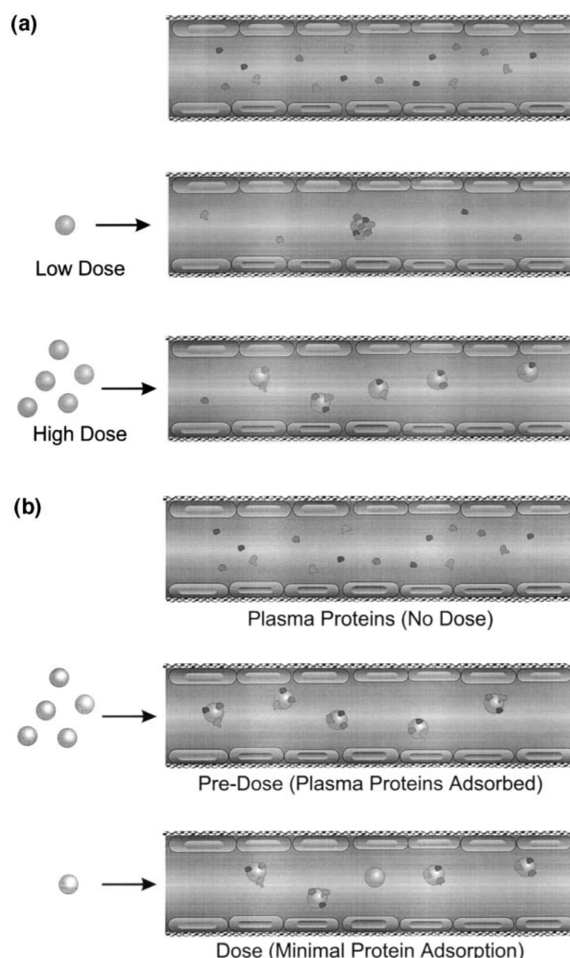


Fig. 4. (A) Depletion of plasma opsonins. (B) Influence of pre-dosing on plasma protein binding to a subsequent dose.

quently reduces the $P_B$ value of the subsequent dose (Fig. 4B).

## 4. Plasma protein involvement in liposome clearance

We have thus far described a method for quickly screening liposome formulations for total protein interactions. For all formulations studied to date there appears to be a good correlation between total protein binding and liposome clearance. While, in general terms, the amount of blood protein that associates with a lipid vesicle is important in determining its clearance rate, the individual proteins profiles may be very significant. The protein binding ability of liposomes appears to markedly affect their clearance behavior, indicating that in order to achieve effective opsonization or liposome/cell interactions, proteins must be adsorbed on liposome surfaces. The rapid clearance of liposomes having large amounts of surface-associated protein suggests that at least some of these proteins promote liposome/cell interactions. Indeed, a study of the proteins associated with rapidly cleared LUVs revealed that these membranes are enriched for blood proteins known to function as opsonins [8,41]. The importance of blood proteins in mediating liposome uptake is further substantiated by the studies of Kiwada and co-workers employing a perfused rat liver model system [42,43] (see also this issue). Their findings suggest that liposomes can pass freely through the liver in the absence of blood components, indicating that liposome uptake by the liver is plasma-dependent. This model may be very important in further defining the role and relative importance of specific proteins in the clearance process.

Liposome uptake by phagocytic cells is for the most part believed to involve opsonic receptors; however, the role of scavenger receptors in mediating the uptake of liposomes by mouse or guinea pig peritoneal macrophages has recently been described [44] (see also this issue). Macrophages in culture were found to actively incorporate and metabolize PC:CH liposomes containing small amounts of acidic phospholipids such as PS, PI, or PA and to store the fatty acyl chains and cholesterol in triacylglycerol and cholesteryl ester form in their cytosol. Competition studies using various ligands for the scavenger receptor showed that acetylated low density lipoprotein, dextran sulfate, or fucoidan was able to compete for up to 60% of the binding of PS-containing vesicles, and that copper-oxidized low density lipoproteins competed for more than 90% of the vesicle binding. On the other hand, PS-containing vesicles were able to compete for more than 90% of the binding of acetyl low density lipoproteins. These results indicate that acidic phospholipids are recognized by scavenger receptors on the surface of macrophages and that more than one scavenger receptor exists on mouse peritoneal macrophages.

From our in vivo studies, blood proteins become immediately associated with PS-containing membranes. These adsorbed proteins, some of which are specific for the PS-headgroup, could potentially mask the headgroup and block interactions with scavenger receptors. Consequently, the importance of these receptors in mediating liposome clearance in vivo is unclear. Moreover, Papahadjopoulos and co-workers were unable to observe increases in the cellular uptake of anionic liposomes using phorbol-ester induced Chinese hamster ovary cells and smooth muscle cells [45]. Increases in the uptake of chemically modified low density lipoproteins was observed for both cell types, indicating a lack of specificity for anionic liposomes.

Adherence and/or internalization of liposomes by various cells, particularly the resident macrophages of the liver and spleen, however, is not the only mechanism involved in liposome clearance. Numerous studies (for reviews see Refs. [46,47]) have established that destabilization of liposome membranes resulting in loss of membrane integrity may significantly contribute to liposome clearance. Indeed, this may be the case for systems that are capable of evading the reticuloendothelial system and reside in the circulation for extended times. Liposome interactions with high density lipoproteins or low density lipoproteins [48–54], apolipoproteins A-1 or E [55–59], various lipid transfer proteins [60–62], and complement components [63–70] result in increased leakage of entrapped solutes and/or net transfer or release of phospholipids from liposomes.

A recent report by Moghimi and co-workers [71] indicated that two heat-stable serum components, one having a molecular weight less than 30 000 and the other greater than 100 000, can inhibit the uptake of poloxamine-coated microspheres by isolated liver sinusoidal cells. It has been suggested that these

proteins function as 'dysopsonins'. Dysopsonins would presumably function by inhibiting the association of opsonic proteins with the lipid membrane or by blocking interactions with receptors.

Following is a brief review of the key blood proteins that associate with liposomes in the circulation and are believed to enhance the probability of liposome destabilization and/or uptake by cells of the reticuloendothelial system (for more extensive reviews see Refs. [72–74]).

### *4.1. Albumin*

Albumin is typically the most abundant protein associated with liposomes recovered from the circulation. This includes both neutral and charged liposomes [8]. Albumin has been implicated in liposome destabilization.

### *4.2. Complement and related proteins*

Liposomes coated with complement are taken up more readily than non-coated vesicles by cultured macrophages [37,39,75,76]. For example, ingestion of MLVs containing galactosyl ceramide and coated with IgM antibodies directed against galactosyl ceramide by cultured mouse peritoneal macrophages is enhanced five- to 10-fold by addition of guinea pig complement [75]. The major opsonic fragments generated by complement activation are C3b and iC3b, both generated by proteolytic degradation of C3. In addition to the classical and alternative pathways of complement activation, C3 products can be generated by interactions between complement components and certain membrane-bound plasma proteins, including pentraxins and mannose-binding protein.

Characterization of the blood proteins associated with liposomes recovered from the blood of liposome-treated mice indicate that LUVs are capable of activating complement in vivo, resulting in the association of opsonic C3 fragments with their membranes [36]. Moreover, the amount of C3 associated per liposome relates to their observed circulation clearance properties in vivo; liposomes bearing elevated C3 levels per liposome are cleared more rapidly. PC:CH:CL (35:45:10 mol/mol/mol) LUVs, which exhibit very rapid clearance properties, bind 46.2 nmol C3/mmol lipid, as measured using a competitive C3 ELISA; DSPC:CH (55:45 mol/mol) LUVs, which exhibit more extended circulation times, bind 3.2 nmol C3/mmol lipid [41].

Deposition of C3 fragments, derived by classical pathway activation of complement, on anionic liposome surfaces has been described by a number of investigators [41,77,78]. Activation of the classical pathway by negatively charged liposomes has been demonstrated to occur even in the absence of immunoglobulin, suggesting that liposomes may become opsonized by complement components regardless of the presence of IgG or IgM [78]. Another report has studied in detail the assembly and regulation of human classical complement C3 convertases using purified complement components and PG-containing SUVs [79]. This study demonstrates in vitro that stable C3 convertases can be assembled on PG-containing SUVs, and lead to C3 activation and C3b deposition.

The generation of C3 by the alternative pathway has also been described [80–82]. Funato and coworkers detected the presence of C3b and iC3b on the surface of MLVs composed of PC:CH:dicetylphosphate, in fresh rat plasma [80]. In addition, Mold has demonstrated that liposomes containing greater than 20 mol% PE will covalently bind C3 [81].

Mannose-binding protein, a serum lectin, has been shown to activate the classical pathway of complement through the interaction and activation of the $C1r_2C1s_2$ complex, independent of the presence of antibody and C1q (reviewed in Ref. [83]). This interaction can result in the deposition of opsonic C3 fragments on the surface of liposomes. Moreover, mannose-binding protein itself has been shown to possess opsonic activity, having an affinity for the C1q receptor [84]. These observations may be of importance for liposome clearance since mannose-binding protein has been shown to associate with LUVs containing CL, PA and PS [85].

The importance of complement and related proteins in mediating liposome stability and clearance is discussed in greater detail by Devine and Bradley in this issue.

### *4.3. Immunoglobulins*

The opsonic role of IgG antibodies in mediating the phagocytic uptake of liposomes by macrophages has been established in vitro and in vivo. For instance, the covalent attachment of rabbit IgG to

LUVs resulted in a five-fold increase in liposome uptake by rat liver macrophages (Kupffer cells) compared to noncoated liposomes [86]. Specific anti-dinitrophenol antibody/dinitrophenylcaproyl PE antigen complexes assembled on liposomal surfaces markedly increases the rate and extent of phagocytosis by cultured macrophages [87,88]. Similarly, endocytosis of IgG anti-dinitrophenyl liposomes by Fc receptor positive phagocytic murine tumor cells was enhanced [89]. Studies using antibody-coupled immunoliposomes have demonstrated that these vesicles are cleared much more rapidly from the circulation than normal liposomes [90,91], most likely by Fc receptor-mediated or complement-mediated mechanisms.

The nature of the immunoglobulin/liposome interaction may be specific for certain lipids or nonspecific. Anti-phospholipid antibodies have been described to exist in plasma of patients with systemic lupus erythematosus, other immunological, neoplastic, or infective disorders, and apparently normal people with no evidence of underlying disease [92–95]. Antibodies which are capable of recognizing phospholipids on the basis of the membrane structure they form have also been described [96]. In particular, antibodies against hexagonal phase phospholipids, including natural and synthetic forms of PE, have been characterized as having anticoagulant activity as measured by a prolonged partial thromboplastin time assay. Alternatively, a report by Senior and co-workers indicates that nonspecific adsorption of mouse IgG antibodies to the surface of liposomes composed of equimolar PC and CH can be considerable (34–89%) [97].

### 4.4. Fibronectin

Fibronectin, and other extracellular matrix proteins such as laminin and serum amyloid P component, can enhance the phagocytic activity of peripheral blood leukocytes [98]. This phagocyte-enhancing effect requires direct interaction of these proteins with phagocytic cells and occurs through cell surface receptors for these molecules (reviewed in Ref. [99]). Fibronectin also affects the function of monocyte complement receptors [98,100]. Purified fibronectin has been shown to bind to liposomes of various compositions and can augment liposome uptake 10-fold [87].

### 4.5. Apolipoproteins

Apolipoproteins A-I, A-II, A-IV, B, C, and E have been shown to interact with liposome membranes [55,101] and may function as opsonins for the uptake of liposomes by macrophages [102]. Liposomes bearing apolipoprotein E have been shown to efficiently compete for β-very low density lipoproteins receptors in cultured macrophages [103]. Apolipoproteins have also been suggested to play a role in the uptake of liposomes by hepatocytes via apolipoprotein B or apolipoprotein E receptors [104,105]. The importance of lipoproteins and apolipoproteins in liposome destabilization and clearance is discussed in greater detail by Rodrigueza et al. in this issue.

### 4.6. C-reactive protein

C-reactive protein is an acute-phase reactant in human plasma, belonging to a family of proteins called pentraxins, which are so named for their characteristic pentameric organization of identical subunits (for reviews see Refs. [106,107]). During trauma, infection, or inflammation, C-reactive protein levels can be upregulated by over 100-fold. In serum, C-reactive protein is able to activate complement via the classical pathway by covalent binding to phosphorylcholine residues [108–112]). Interactions between C-reactive protein and positively charged DMPC:CH:SA liposomes result in both consumption of classical complement components and release of entrapped liposomal glucose [113]. C-reactive protein-mediated complement consumption also occurs with liposomes containing egg PC and either lysoPC or lysoPE [114]. It has been demonstrated that C-reactive protein is able to enhance macrophage activity. Furthermore, Fc receptors found on monocytes and lymphocytes seem to be important in their interactions with C-reactive protein-coated vesicles [115].

### 4.7. β2-Glycoprotein I

Recently we have identified β2-glycoprotein I as a major protein component isolated from rapidly cleared liposomes [116]. While the biological function of this protein is not known there is a significant body of literature that suggests that this protein acts

as a cofactor for the binding of antiphospholipid antibodies. The amount of β2-glycoprotein I that associates with anionic liposomes correlates with their clearance rates, suggesting that β2-glycoprotein I plays a role in mediating the immune clearance of anionic liposomes and particles. This is made even more significant inasmuch as β2-glycoprotein I levels in plasma are approximately 200-fold lower than the other major protein associated with these vesicles, albumin. This suggests a preferential interaction of β2-glycoprotein I with anionic vesicles.

## 5. Conclusions

The ability to isolate small quantities of liposomes from the blood of liposome-treated mice has facilitated a significant progression in our understanding of the role of plasma proteins in liposome clearance. The importance of plasma proteins is clearly suggested by the apparent relation between liposomal $P_B$ values and circulation half-life ($t_{1/2}$), with the general trend of decreased $P_B$ value and extended $t_{1/2}$ holding true for all liposome compositions studied to date.

While it is apparent that adsorbed plasma proteins are very important in determining liposome clearance, liposome compositions that exhibit extended circulation times and low $P_B$ values are nevertheless cleared from the circulation over a period of days. This suggests that not all clearance mechanisms are protein-mediated. Indeed, serum independent uptake of liposomes has been reported to occur in mouse liver [117]. Surface adsorbed blood proteins likely enhance the probability that liposomes will interact with phagocytic cells in vivo. Consequently, the slow rate of uptake of liposomes with low $P_B$ values may simply reflect the decreased probability of liposome/cell interactions. Alternatively, this clearance may result from alterations in membrane lipid composition over time or from the net transfer of lipid components between lipoproteins and cell membranes, resulting in a net loss of membrane components that prolong circulation times. These changes in lipid composition may result in changes in the surface adsorption properties of the liposome membrane, potentially leading to increased $P_B$ values over time.

As mentioned previously, while $P_B$ values are a good predictor of liposome behaviour in vivo, the importance of specific proteins in mediating liposome clearance must not be minimized. Rapidly cleared liposomes are enriched with the blood opsonins C3 and IgG. From our analyses, β2-glycoprotein I is another major protein associated with rapidly cleared liposomes. Since there is a correlation between the amount of β2-glycoprotein I associated with liposomes and their clearance rate, β2-glycoprotein I may also function as a blood opsonin. Thus, it would be of interest to determine the significance of β2-glycoprotein I in mediating liposome clearance. This could be addressed by studying the clearance of liposomes in β2-glycoprotein I deficient animal models, or by studying the uptake of liposomes coated with purified β2-glycoprotein I by macrophages, either in culture or in liver perfusion model systems. Indeed, we have demonstrated that pre-doses of anti-β2-glycoprotein I antibody could increase the circulation time of anionic liposomes. Noting the complexity of the protein profiles associated with rapidly cleared liposomes, other proteins may also be involved in mediating clearance. The identities of these proteins may lead to new strategies for prolonging the circulation times and therapeutic benefit of liposomes.

Finally, the 'dysopsonin' hypothesis presents an attractive mechanism for enhancing the circulation times of liposomes. If 'dysopsonins' play a significant role in vivo, then liposomes that are capable of residing in the circulation for extended times should be enriched with these proteins. By identifying and characterizing these type of proteins it may be possible to pre-incubate liposomes with inert proteins to block available protein adsorption sites on the liposome membrane, thus preventing the subsequent binding of opsonins.

## 6. Abbreviations

| | |
|---|---|
| $P_B$ | Protein binding value (g blood protein bound/mol total lipid) |
| LUVs | Large unilamellar vesicles |
| SUVs | Small unilamellar vesicles |
| MLVs | Multilamellar vesicles |
| ELISA | Enzyme-linked immunosorbent assay |
| $t_{1/2}$ | Circulation half-life |
| PS | Phosphatidylserine |

| | |
|---|---|
| PA | Phosphatidic acid |
| CL | Cardiolipin |
| PG | Phosphatidylglycerol |
| PI | Phosphatidylinositol |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |
| CH | Cholesterol |
| DOTMA | 1,2-Dioleyl-3-*N*,*N*,*N*-trimethyl-aminopropane chloride |
| $G_{M1}$ | Ganglioside $G_{M1}$ |

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