Influence of Cholesterol on the Association of Plasma Proteins with Liposomes†

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ABSTRACT: The *in vivo* association of blood proteins with large unilamellar liposomes composed of saturated phosphatidylcholines was analyzed to determine the effect of membrane fluidity and hydrocarbon chain length on liposome−plasma protein interactions and liposome clearance. Liposomes composed of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and diarachidoylphosphatidylcholine (DAPC) were administered via the lateral tail vein of CD-1 mice and were subsequently isolated from the blood at 2 min postinjection. The protein binding ability (P_B, grams of protein bound per mole total lipid) of the liposomes was quantified and related to their circulation half-lives. Liposomes composed of long-chain saturated phospholipids that exist in the gel (frozen) state at 39 °C (DPPC, DSPC, and DAPC) bound large quantities of blood proteins, in excess of 48 g of protein per mole total lipid, and were found to be rapidly cleared from the circulation. The incorporation of cholesterol into DSPC liposomes resulted in significantly decreased P_B values and enhanced circulation lifetimes for this lipid system. This cholesterol effect plateaued at 30 mol % cholesterol, corresponding to the loss of the gel−liquid crystalline phase transition, and resulted in P_B values of 23−28 grams of protein per mole of total lipid. The types of blood proteins binding to DSPC liposomes were not significantly altered by the inclusion of cholesterol. This is the first demonstration of rapid clearance of neutral large unilamellar liposomes having high levels of bound protein.

The mechanisms responsible for the recognition and clearance of liposomes, such as large unilamellar vesicles (LUVs),[1] from the circulation *in vivo* are presently not well-understood. A variety of blood proteins are known to bind to liposomes *in vitro* and *in vivo*, some of which can cause membrane destabilization and leakage of entrapped contents (Krupp *et al*., 1976; Scherphof *et al*., 1978; Kirby *et al*., 1980; Chonn *et al.*, 1992). Moreover, high levels of protein binding are associated with rapid clearance of liposomes from the circulation. In this regard, vesicles composed of saturated phosphatidylcholines and cholesterol have been shown to possess relatively long circulation lifetimes and are highly stable to the release of entrapped aqueous markers in the presence of blood components (de Gier *et al.*, 1969; Inoue, 1974; Gregoriadis & Davis, 1979; Kirby *et al.*, 1980; Allen & Cleland, 1980).

Although the precise role of cholesterol in biological membranes remains to be defined, the inclusion of cholesterol in liposome membranes has been shown to result in increased packing densities of phospholipid molecules (Demel & de Kruijff, 1976), reduced bilayer permeability to ions and solutes (Demel *et al*., 1972; Paphadjopoulos *et al*., 1973; Corvera *et al.*, 1992), decreased binding of proteins and serum opsonins (Papahadjopoulos *et al*., 1973; Moghimi & Patel, 1989), and increased circulatory lifetimes of liposomes (Senior & Gregoriadis, 1982; Kirby *et al*., 1980; Senior, 1987). Furthermore, it has been suggested that the cholesterol-dependent increase in the circulation half-lives of liposomes is a result of a dual functionality of membrane cholesterol (Patel *et al*., 1983). First, as mentioned previously, cholesterol decreases membrane permeability and affords the bilayer a greater resistance to destabilization by blood components. Second, it has been demonstrated that cholesterol-rich liposomes possess a lower affinity for uptake by hepatic Kupffer cells, suggesting that cholesterol inhibits the binding of serum opsonins to liposomes (Patel *et al*., 1983; Dave & Patel, 1986; Classen & Van Rooijen, 1984).

Many of the studies that have investigated the role of phospholipid composition and cholesterol content on the interaction of liposomes with serum components have involved single proteins, such as albumin (Hernández *et al.*, 1989), clotting proteins (Zwaal, 1978), apo A-I (Pownall, 1978; Swaney, 1980), and IgG (Weissmann, 1974). While providing insight on the interactions of purified protein systems with membranes, these isolated systems do not accurately reflect membrane−protein interactions in complex protein mixtures, such as blood (Andrade & Hlady, 1987). Consequently, we developed a spin column procedure that allows the rapid isolation of LUVs from blood components (Chonn *et al*., 1991). Using this procedure, we have demonstrated that the clearance rates of large unilamellar vesicles are inversely related to the amount of protein associated with the liposomes (Chonn *et al.*, 1992). To further test this relation, in this study, we investigate the *in vivo* interactions of LUVs composed of saturated phosphatidylcholines and cholesterol with plasma proteins. Our results demonstrate that liposomes composed of long-chain saturated phosphatidylcholines bind high levels of blood protein and

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[1] Abbreviations: CHE, cholesterylhexadecyl ether; CHOL, cholesterol; DAPC, diarachidoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; HBS, Hepes-buffered saline; P_B, protein binding ability (grams of protein per mole of total lipid); LUVs, large unilamellar vesicles; RES, reticuloendothelial system.

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are very rapidly cleared from the circulation. Cholesterol is shown to modulate this effect by reducing the total amount of protein that is bound to the vesicles.

MATERIALS AND METHODS

Lipids and Chemicals. Saturated phosphatidylcholines were purchased from Avanti Polar Lipids (Pelham, AL), cholesterol was purchased from Sigma, and [^{3}H]cholesteryl-hexadecyl ether ([^{3}H]CHE) was purchased from DuPont (Boston, MA). Lipids were used without further purification. All other chemicals were purchased from BDH (Vancouver, BC).

Preparation of Liposomes. LUVs were prepared as described previously (Hope *et al*., 1985; Chonn *et al*., 1991). Briefly, multilamellar vesicles (MLVs) were prepared by hydration of a dried lipid film in 20 mM Hepes (pH 7.4) and 145 mM NaCl (HBS) at approximately 10 °C above the gel−liquid crystalline phase transition temperature (T_c) of the particular phospholipid (Nayar *et al.*, 1989). Frozen-and-thawed MLVs (five cycles, freeze in liquid nitrogen and thaw above T_c) were extruded 10 times through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA) at temperatures above the phase transition temperature of the phospholipid using a thermostatted extruder (Lipex Biomembranes, Vancouver, BC), specifically, DMPC at 30 °C, DPPC at 50 °C, DSPC at 65 °C, and DAPC at 85 °C. These vesicles were extruded immediately prior to administration and were incubated for 10 min at 39 °C before injection. The size distribution of the vesicle population was found to be stable for at least 2 h after preparation as monitored by quasi-elastic light scattering using a NICOMP Model 270 submicron particle sizer. Liposome recoveries and biodistribution were determined by the inclusion of [^{3}H]-CHE, a nonexchangeable, nonmetabolizeable lipid marker (Stein, 1980). Lipid compositions are expressed as molar ratios.

In Vivo Plasma Distribution Studies. Briefly, 200 μL aliquots of liposome preparations were administered intravenously via the lateral tail vein of CD-1 mice (female, 6−8 weeks old, Jackson Laboratory Animals). At given times, mice were sacrificed by exposure to carbon dioxide and blood was withdrawn by cardiac puncture and collected into 1.5 mL microcentrifuge tubes. To prevent coagulation, the blood was immediately cooled to 0 °C in an ice−water bath and centrifuged (12 000 rpm, 2 min, and 4 °C) to remove blood cells. Plasma was separated into cold glass tubes and was mixed gently. Aliquots of plasma were measured for radioactivity, using standard liquid scintillation methods, to determine liposome recovery. Plasma volume was assumed to be 5.0% of the total body weight. For the 2 min time point, 50 μL aliquots of plasma were applied to spin columns (five columns per mouse) and the liposomes were isolated as described previously (Chonn *et al.*, 1991).

Quantification of Total Protein Associated with Recovered Liposomes. The protein associated with recovered LUVs was extracted and delipidated according to the procedure described by Wessel and Flugge (1984). Delipidation was required due to lipid interference in the protein assay (Kessler & Fanestil, 1986). Extracted proteins were resuspended in 0.2 mL of 1% sodium dodecyl sulfate (SDS) in Milli-Q water, followed by the addition of 0.8 mL of micro bicinchoninic acid protein reagent (Pierce, Rockford, IL).

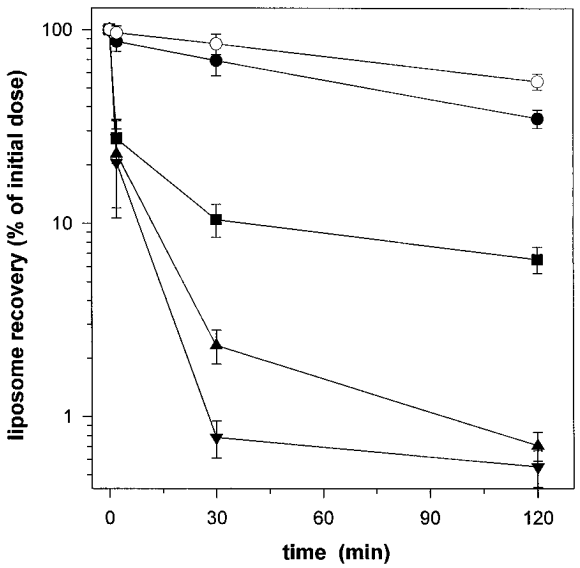

FIGURE 1: Plasma clearance of LUVs composed of saturated phosphatidylcholines. Large unilamellar vesicles (100 nm) containing [^{3}H]CHE were administered intravenously via the lateral tail vein of CD-1 mice. Liposome administration was at an approximate dose of 20 μmol of total lipid per 100 g of mouse weight. At various times, mice were sacrificed and the liposome recovery in the plasma was determined by standard liquid scintillation methods as described in Materials and Methods. The liposomes were composed of pure, saturated phosphatidylcholines as follows: (●) DMPC, (■) DPPC, (▲) DSPC, and (▼) DAPC. The final liposome formulation was composed of an unsaturated phosphatidylcholine, DOPC (○). The data points represent the average plasma recovery and standard deviations from at least four mice.

The mixture was incubated at 60 °C for 60 min, and after the mixture was cooled to room temperature, the absorbance was measured spectrophotometrically at a wavelength of 562 nm. A standard curve consisting of known quantities of bovine serum albumin (Pierce) was used to quantify the total protein in the extracted samples and was found to be linear in the range of 0−16 μg/mL. The amount of lipid in the recovered sample was calculated from the specific activity of the initial liposome suspension and the volume of the recovered liposomes used in the extraction procedure. For *in vivo* experiments, at least two determinations of protein binding for each lipid composition were performed.

Electrophoretic Analysis of Liposome-Associated Proteins. Identification of proteins associated with liposomes isolated from circulation was facilitated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) employing a Mini Protean-II electrophoretic apparatus (Bio-Rad), under nonreducing conditions (Laemmli, 1970). Silver stain SDS−PAGE molecular weight standards (Bio-Rad) were used to estimate the molecular weights of the liposome-bound proteins. Proteins were visualized by silver staining (Rabilloud *et al.*, 1988). The silver-stained SDS−PAGE results are representative of two or three analyses.

RESULTS

Effect of Fatty Acyl Chain Length on the Circulation Clearance Lifetimes and Protein Binding Abilities of Saturated Phosphatidylcholine LUVs. The first set of experiments was aimed at monitoring the clearance properties of gel state LUVs and measuring the associated proteins. Figure 1 shows the clearance properties of LUVs composed of pure, saturated phosphatidylcholines injected intravenously at a

Table 1: P_B Values for LUVs Composed of Various Phosphatidylcholines Isolated from the Plasma of CD-1 Mice[a]

liposome composition	phase transition temperature (°C)[b]	circulation half-life ($t_{1/2}$) (min)	P_B (grams of protein per mole of total lipid)
DMPC	23	74	32.3 ± 3.7
DPPC	41.5	1.2	48.5 ± 5.6
DSPC	54.5	0.9	96.2 ± 8.1
DAPC	66	0.9	101.2 ± 9.8
DOPC	−22	>120	23.4 ± 2.5

[a] Values represent the average and standard deviation from two independent determinations, each using eight mice. [b] Values taken from *CRC Handbook of Lipid Bilayers* (1990).

lipid dose of 200 μmol/kg. Liposomes composed of lipids, such as DAPC, DSPC, or DPPC, which exist in the gel state prior to injection were cleared very rapidly from the circulation (Figure 1, and Table 1). Liposomes containing lipid (DMPC) in the liquid crystalline state, however, possessed relatively long lifetimes. Comparison was also made with liposomes composed of DOPC (18:1/18:1). DOPC vesicles are liquid crystalline prior to injection and, like DMPC vesicles, exhibit relatively long circulation times.

To determine whether the clearance behavior is related to the level of serum protein binding, injected liposomes were isolated from the blood of CD-1 mice at 2 min postinjection. Table 1 lists the P_B values for LUVs composed of various phosphatidylcholines. It is interesting to note that liposomes composed of phosphatidylcholines that normally exist in the gel state at 39 °C (DPPC, DSPC, or DAPC) were cleared very rapidly from the circulation and had large quantities of associated blood protein, in excess of 48 g of protein per mole of total lipid. Conversely, LUVs composed of phosphatidylcholines that exist in the liquid crystalline state at 39 °C (DMPC or DOPC) had less associated protein. These recovered liposomes were then analyzed by SDS−PAGE to examine the types of proteins binding to the vesicles. The silver-stained protein profiles of the liposomes recovered at 2 min postinjection are depicted in Figure 2. To facilitate simple qualitative analysis of the amount and types of protein bound to the various liposome compositions, equivalent amounts of lipid were added to each lane. It is evident that the liposomes composed of gel state phosphatidylcholines (prior to entering the circulation) bound the most total protein, while the liposomes composed of liquid crystalline phosphatidylcholines clearly bound less. Although the P_B values significantly varied for the LUVs composed of different phosphatidylcholine species, the electrophoretic profiles of the proteins associated with these LUVs were similar.

It should be emphasized that the LUVs were recovered from blood at 2 min postinjection to analyze the protein−liposome interactions that occurred *in vivo*. As such, changes in the lipid composition of the LUVs due to lipid exchange were predicted to be minimal, particularly in mice, which lack cholesteryl ester transfer protein. Lipid exchange between lipid donors and lipid acceptors and/or cells has been demonstrated to occur on a time scale of hours for phosphatidylcholines (Tall, 1986) and cholesterol (Rodrigueza *et al*., 1995). To ascertain whether this was indeed the case, the Bligh and Dyer extracted lipids from DSPC LUVs recovered from the blood of eight mice at 2 min postinjection were analyzed by thin layer chromatography followed by staining for both phospholipids and cholesterol, using well-established procedures. Cholesterol or additional phospholipid was not detectable in the lipid extract (results not shown).

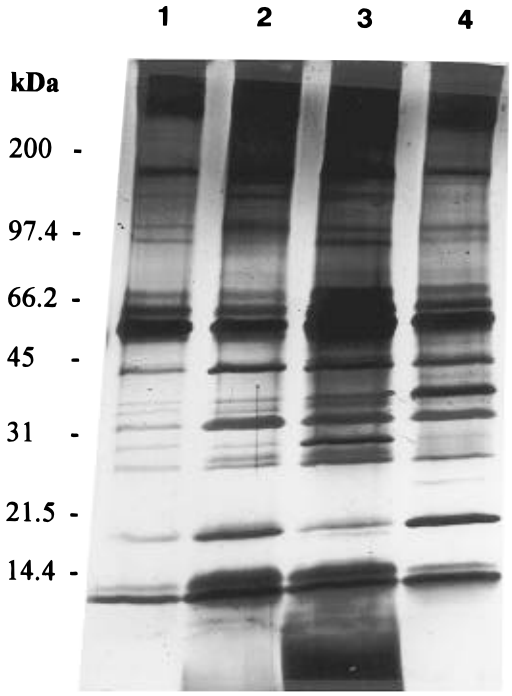

FIGURE 2: Nonreducing SDS−PAGE analysis of the proteins associated with saturated phosphatidylcholine LUVs after recovery from the circulation of mice at 2 min postinjection. The proteins associated with plasma-recovered liposomes were separated on a 4 to 20% SDS−PAGE gradient gel under nonreducing conditions and were visualized by silver staining as described in Materials and Methods. The lanes contain the proteins associated with 50 nmol of total lipid of the following liposome compositions (lane 4 contains 25 nmol of total lipid): DMPC (lane 1), DPPC (lane 2), DSPC (lane 3), and DAPC (lane 4).

Effect of Cholesterol on the Circulation Lifetimes and Protein Binding Ability of DSPC LUVs. The inclusion of cholesterol in DSPC liposomes at various molar ratios has a profound effect on the clearance rates of these vesicles (Figure 3). At 20 mol % cholesterol, the circulation half-life is roughly 30 min, as compared to seconds for pure DSPC vesicles. The inclusion of 30 mol % cholesterol further extended the half-life of the vesicles to 5 h. The cholesterol effect plateaus at 30 mol %, at which point the further addition of cholesterol did not result in any significant changes in the clearance behavior of the liposomes.

An increase in the membrane cholesterol content was accompanied by a marked decrease in the total amount of protein which bound to the vesicles (Table 2). A 4-fold reduction in the binding of protein occurred as the cholesterol content was increased from 0 to 50 mol %. This reduction in protein binding, as a result of the addition of cholesterol, correlates well with the significant increase in the circulatory lifetimes of DSPC:CHOL liposomes. In order to examine the specificity of the reduction in protein binding, the recovered liposomes were solubilized and loaded at equivalent protein concentrations on SDS gels under nonreducing conditions. The resulting protein profiles were very similar for each different cholesterol concentration (Figure 4). This suggests that the cholesterol-mediated reduction in protein binding was nonspecific. These protein binding results, in combination with the observed clearance profiles, strongly

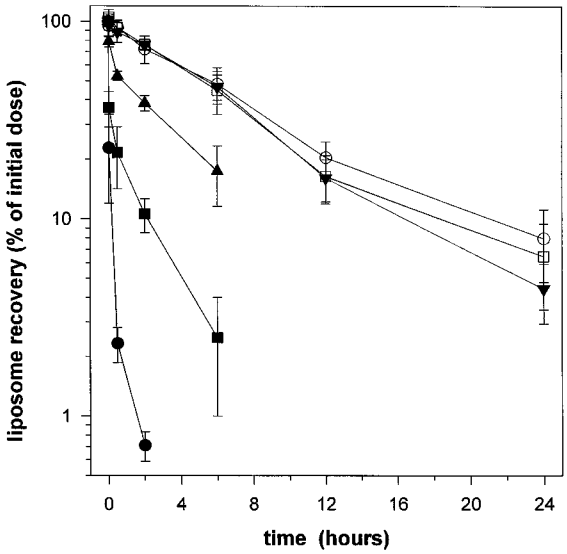

FIGURE 3: Influence of cholesterol on the plasma clearance of DSPC liposomes. LUVs (100 nm) containing a radioactive lipid marker, [^{3}H]CHE, were administered via the lateral tail vein of CD-1 mice, at an approximate dose of 100 mg of total lipid per kilogram of mouse body weight. Liposome recovery in the plasma was determined by standard liquid scintillation methods as described in Materials and Methods. Liposomes were composed of DSPC and cholesterol in the following molar ratios (DSPC:CHOL): (●) DSPC, (■) 9:1, (▲) 8:2, (□) 7:3, (○) 6:4, and (▼) 5:5. Data points represent the average plasma recovery and standard deviations from at least four mice.

Table 2: Influence of Cholesterol on the P_B Values of DSPC LUVs[a]

composition of LUVs	P_B (grams of protein per mole of total lipid)
DSPC	96.2 ± 8.1
DSPC:CHOL (9:1)	86.2 ± 5.6
DSPC:CHOL (8:2)	54.2 ± 4.8
DSPC:CHOL (7:3)	23.3 ± 3.2
DSPC:CHOL (6:4)	25.7 ± 4.4
DSPC:CHOL (5:5)	27.8 ± 4.2

[a] Values represent the average and standard deviation from two independent determinations, each using at least four mice.

suggest that the extent of the interaction of LUVs with blood proteins dictates the circulation lifetime of a given liposome composition.

DISCUSSION

It has been well documented that the lipid composition of liposomes markedly affects their clearance kinetics in the circulation (Chonn *et al*., 1992; Senior & Gregoriadis, 1982; Gregoriadis & Senior, 1980). Furthermore, liposomes exposed to plasma or serum *in vitro* have been shown to bind a complex profile of proteins (Bonte & Juliano, 1986; Juliano & Lin, 1980). In view of our earlier findings indicating that the amount of protein binding to liposomes composed of various net negatively charged phospholipids *in vivo* is inversely related to their circulation clearance rate (Chonn *et al*., 1992), it is of considerable interest to relate this protein binding to the mechanisms of liposome destabilization and clearance for other liposome systems where surface charge is not a factor.

Several *in vitro* studies have previously shown that vesicles composed of gel state lipids are very resistant to the release of entrapped solutes in the presence of serum (Senior & Gregoriadis, 1982; Gregoriadis, 1988; Allen & Cleland, 1980). Since serum components such as HDL have been shown to cause liposome leakage (Scherphof *et al*., 1978; Krupp *et al.*, 1976), the decreased leakage in vesicles composed of gel state lipids may be taken to suggest a decreased interaction with serum components. It would therefore be expected that highly ordered, gel state membranes would decrease protein interactions and thus promote prolonged circulation lifetimes.

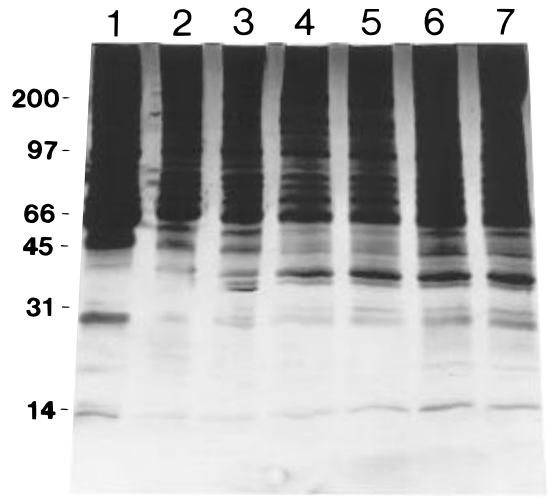

FIGURE 4: SDS−PAGE analysis of proteins associated with cholesterol-containing DSPC liposomes. The proteins associated with DSPC:CHOL liposomes were separated on a 4 to 20% SDS−PAGE gradient gel under nonreducing conditions and were visualized by silver staining as described in Materials and Methods. The lanes contain 5 μg of the proteins associated with DSPC:CHOL liposomes, containing the following molar percentages of cholesterol: normal mouse serum (25 μL of a 1:50 dilution) (lane 1), 0 mol % CHOL (lane 2), 10 mol % CHOL (lane 3), 20 mol % CHOL (lane 4), 30 mol % CHOL (lane 5), 40 mol % CHOL (lane 6), and 50 mol % CHOL (lane 7).

In sharp contrast, however, the results reported here show that liposomes composed solely of gel state lipids such as DPPC, DSPC, or DAPC are very rapidly cleared from the circulation. Moreover, our results demonstrate that LUVs composed of gel state phosphatidylcholines, notably DSPC or DAPC, strongly interact with plasma proteins. Consistent with our earlier observations, an inverse relationship between protein binding and circulation half-lives exists for these net neutral lipids (Chonn *et al*., 1992).

Why blood proteins should strongly interact with long-chain saturated phosphatidylcholine vesicles is not clear. It has been demonstrated that homogeneous unilamellar vesicles composed of long-chain phosphatidylcholines, in the absence of cholesterol, develop packing defects upon cooling below their phase transition temperatures (Lee, 1977). This is particularly apparent in LUV systems (Nayar *et al*., 1989). Freeze-fracture studies of saturated phosphatidylcholine LUVs showed angular fracture planes, indicative of packing defects, whereas DSPC:CHOL LUVs possessed smooth fracture surfaces (Nayar *et al*., 1989). These bilayer-packing defects are believed to expose hydrophobic domains on the surface of the bilayer that increase the contact between water and the hydrophobic fatty acyl chains (Larrabee, 1979; Schullery *et al*., 1980). The most obvious explanation, therefore, for the enhanced protein binding to LUVs with T_c values above body temperature is that serum proteins adsorb in the defect regions, leading to rapid clearance. An

alternative possibility concerns the observation that these LUVs are somewhat unstable, especially for sonicated vesicle systems, resulting in aggregation and vesicle fusion over a period of 24 h (Larrabee, 1979), which could lead to trapping of serum proteins and more rapid clearance due to their larger size. However, the LUVs we employed were stable for at least 2 h (119 ± 45 nm) as monitored by quasi-elastic light scattering, showing size increases by 24 h (320 ± 95).

The ability of plasma proteins to bind to DSPC vesicles was reduced dramatically by the inclusion of cholesterol in the bilayer as demonstrated by decreasing P_B values as the cholesterol content increased (Table 2). At membrane concentrations greater than 30 mol %, cholesterol eliminates the gel−liquid crystalline phase transition temperature in saturated phosphatidylcholine vesicles, resulting in a highly ordered but crystalline state (Silvius, 1982; Bloom *et al*., 1991). The association of blood proteins with DSPC:CHOL mixtures occurs in an apparently nonspecific manner (Figure 4). With the exception of apparent increases in the relative quantities of two proteins (approximate molecular masses, 30 and 40 kDa), no obvious differences in the types of individual proteins were observed between cholesterol-rich and cholesterol-poor liposomes, suggesting little difference in the types of opsonins binding to these vesicles. This does not support the hypothesis of Moghimi and Patel (1989), which suggests that opsonins exist which are specific for cholesterol-rich membranes. Furthermore, our results do not indicate an enhanced association of proteins (dysopsonins) that prevent RES uptake in liposome compositions capable of long circulation times.

In an earlier study employing various negatively charged LUVs (Chonn *et al*., 1992), we demonstrated that the protein binding ability of LUVs was inversely related to their clearance rates. The present study clearly indicates that it is not the liposome charge per se that triggers rapid clearance. When LUVs composed of homogeneous saturated phosphatidylcholines are administered *in vivo*, blood proteins rapidly associate with their membranes. In addition, the correlation between plasma protein binding and liposomal clearance rates extends for systems lacking a net surface charge, further supporting a dominant role for blood proteins in the recognition and clearance of liposomes *in vivo*.

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