

PROTEIN-MEMBRANE INTERACTIONS IN THE COMPLEX BIOLOGICAL MILIEU

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Alterations in the lipid composition of biological membranes can have dramatic effects on their ability to interact with soluble proteins. In a series of studies employing large unilamellar vesicles (LUVs) produced by an extrusion technique, we have characterized the influence of membrane components on protein-membrane interactions. Much of our understanding of how and why proteins interact with seemingly inert membrane surfaces stems mainly from studies involving one or two protein component systems. These studies, however, do not accurately reflect the interactions that occur in the complex biological milieu (reviewed by Horbett and Brash, 1987). There have been very few studies reported on the interactions of proteins with liposomal systems incubated with whole blood. There are two main reasons for this. First, the large majority of studies on the association of plasma proteins with liposomes *in vitro* have been performed employing multilamellar systems. Due to the variable lamellarity of liposomes of different lipid compositions, quantification of the amount of various proteins associated per liposome has not been possible. Second, convenient techniques have not been available for the isolation of liposomes, particularly LUVs, from blood components. We have recently described a rapid method for the isolation of well-defined large unilamellar liposomes from the blood of liposome-treated mice (Chonn et al., 1991). With such a procedure now available, we have started to biochemically and immunologically characterize the amount and type of proteins associated with liposomes exposed to the complex biological milieu.

Liposomes, prepared by an extrusion technique (Hope et al., 1985; Nayar et al., 1991) and having an average size of 100 nm in diameter, were intravenously administered into CD1 mice at a dose level of 200 $\mu\text{mol/kg}$ and recovered from the blood 2 min post-injection by

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employing a "spin column" procedure as previously described in detail (Chonn et al., 1991). The proteins associated with the recovered liposomes were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining to visualize the proteins or by immunoblot analysis. Further, the amount of protein associated with the liposomes was quantitated using the micro bicinchoninic acid protein assay.

Several molecular properties of blood proteins that are considered to have a major influence on their surface adsorption properties include surface charge (most charged residues reside on external surfaces of proteins), size (proteins and other macromolecules are thought to form multiple contact points when adsorbed to a surface), stability of the proteins in plasma (unfolding of proteins at the surface would increase the number of adsorption sites), and carbohydrate content (Horbett and Brash, 1987). As well, the relative concentration of the proteins in plasma should affect the distribution of proteins adsorbed on surfaces.

Our findings, however, suggest that electrostatic interactions do not play a dominant role in protein-membrane interactions in complex protein mixtures. As shown in Figures 1 and 2, liposomes composed of different anionic phospholipids, but having similar overall net surface charge, have very different abilities to interact with soluble proteins. This is dramatically demonstrated by the observation that LUVs composed of 20 mol% bovine liver phosphatidylinositol have a 6 fold greater capacity to bind proteins than those composed of 20 mol% plant phosphatidylinositol. This finding suggests that fatty acyl composition of the phospholipid can markedly influence blood protein/membrane interactions. Further, these membranes exhibit very different biological properties, as measured here by their clearance property from the circulation. It is apparent that membranes that are highly reactive with soluble blood proteins are cleared very rapidly.

As shown in Figure 2, size does not appear to be an overriding factor differentiating the surface activity of soluble proteins; a complex profile of proteins of varying molecular sizes is associated with LUVs. Further, there are blood proteins which have a specific affinity for membranes composed of certain anionic phospholipids, namely cardiolipin, phosphatidic acid or phosphatidylserine. A striking example here is a protein that migrates with a molecular weight corresponding to approximately 53 000. The levels of this protein binding to cardiolipin- or phosphatidic acid-containing LUVs corresponds to similar or even greater levels than those for albumin (66 kDa). By N-terminal region protein sequence analysis and

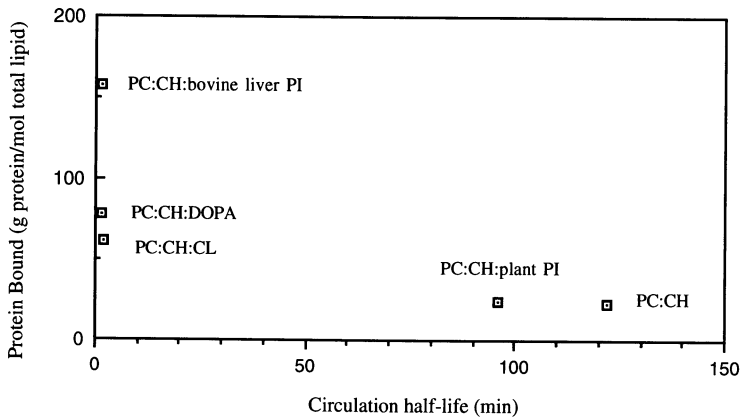


Figure 1. Amount of protein associated with liposomes composed of various anionic phospholipids and the relationship to circulation half-life.

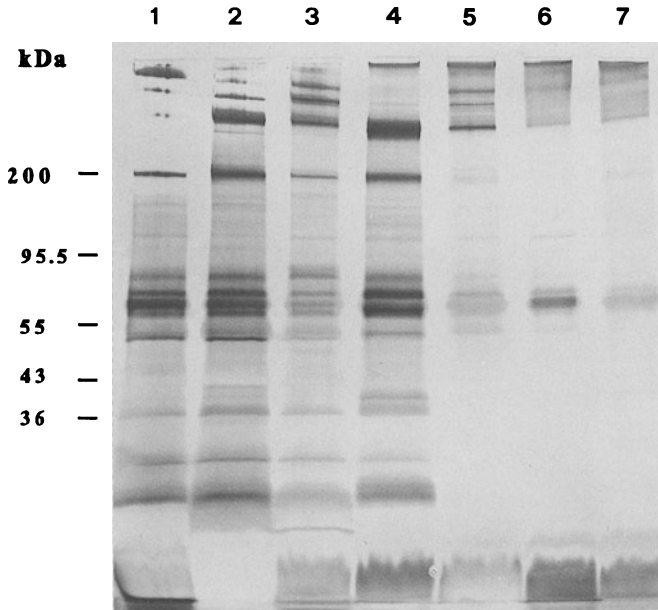


Figure 2. Silver-stained nonreducing SDS-polyacrylamide gels of proteins associated with liposomes recovered from the circulation of mice after 2 min post-injection. The lanes contain 25 nmol of total lipid of liposomes composed of the following: PC:CH:CL (35:45:10, lane 1), PC:CH:DOPA (35:45:20, lane 2), PC:CH:DOPS (35:45:20, lane 3), PC:CH:PI (bovine liver, 35:45:20, lane 4), PC:CH:PI (plant, 35:45:20, lane 5), PC:CH:PG (35:45:20, lane 6) and PC:CH (55:45, lane 7).

immunological analysis, this protein has been identified as the mouse equivalent to human β 2-glycoprotein I. The reported values for the concentration of β 2-glycoprotein I in rats and humans is approximately 0.2 mg/ml, 200 fold less than the plasma concentration of serum albumin. If one assumes that the association of albumin to these vesicles is non-specific, then this finding would indicate that β 2-glycoprotein I is greatly concentrated on these anionic membranes. It is interesting to note that β 2-glycoprotein I has recently been shown to be a cofactor for the binding of some antiphospholipid antibodies. Inasmuch as cardiolipin, phosphatidic acid and phosphatidylserine normally reside intracellularly and become expressed when cells undergo programmed cell death or apoptosis, such protein-membrane interactions may be involved in distinguishing "nonself" from "self" membranes.

In subsequent studies, we further investigated the effect of fatty acyl composition on the protein binding ability of LUVs composed solely of phosphatidylcholines. The total amount of protein bound to saturated phosphatidylcholine LUVs increased as the length of the fatty acyl chain increased (Table 1). This was a somewhat unexpected result because conceptually, it was widely believed that it would be more difficult for proteins to insert into tightly packed, highly ordered membranes. In support of this hypothesis is the observation that vesicles composed of gel state lipids are very stable to the release of entrapped solutes in the presence of serum (Senior and Gregoriadis, 1982). However, our findings indicate that LUVs composed solely of saturated phospholipids exhibit very rapid clearance kinetics from the circulation of mice.

Table 1. Amount of protein associated with LUVs composed of saturated phosphatidylcholines isolated from the blood of CD1 mice.

Liposome composition	Fatty Acid Species	Phase Transition Temperature (°C)	Protein Binding Index (g/mol lipid) ^a
DMPC	14:0/14:0	23	23 ± 4
DPPC	16:0/16:0	41.5	48 ± 6
DSPC	18:0/18:0	54.5	96 ± 8
DAPC	20:0/20:0	66	101 ± 9

^a Values represent average and standard deviation from 2 independent determinations, each using 8 mice.

Why blood proteins should strongly interact with solid phase saturated PC vesicles is not inherently obvious. The lack of net surface charge and the uniformity of the lipid head group in the liposomes studied suggests that membrane fluidity and fatty acyl chain composition are significant determinants of liposome-protein interactions. It has been demonstrated that homogeneous vesicles composed of gel state phosphatidylcholines in the absence of cholesterol develop packing defects upon cooling below their phase transition temperatures. These defects are thought to expose hydrophobic domains on the surface of the bilayer that increase the contact between water and the hydrophobic fatty acyl chains.

Table 2. Influence of cholesterol on the protein binding ability of distearoylphosphatidylcholine (DSPC) LUVs.

Composition of LUVs	Protein Binding Index (g/mol lipid)
DSPC	96 ± 8
DSPC:CH (9:1)	86 ± 6
DSPC:CH (8:2)	54 ± 5
DSPC:CH (7:3)	23 ± 3
DSPC:CH (6:4)	27 ± 4
DSPC:CH (5:5)	28 ± 4

^a Values represent average and standard deviation from 2 independent determinations, each using at least 4 mice.

The inclusion of increasing amounts of cholesterol in DSPC LUVs dramatically reduces the protein binding ability (Table 2). Cholesterol is known to eliminate the sharp gel-liquid crystalline phase transition in homogeneous saturated phosphatidylcholine vesicles resulting in a permanent liquid crystalline state at cholesterol concentrations greater than 30 mol%. Thus, the inclusion of cholesterol likely eliminates potential membrane defects and consequently reduces the likelihood of the interaction of blood proteins with these liposomes.

These findings suggest that protein-membrane interactions can be regulated by alterations in the lipid composition of membranes. Exposure of hydrophobic domains in the membrane as a result of fatty acyl packing defects or by inclusion of certain anionic

phospholipids leads to membrane surfaces that are highly interactive with soluble proteins. This interaction leads to protein unfolding on the surface of the membranes resulting in stable protein-membrane interactions. The general properties of membrane-reactive proteins, determined from studies involving simple systems, do not for the most part apply to complex protein mixtures.

Acknowledgments

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