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Protein-induced CF release from liposomes in vitro and its correlation with the BLOOD/RES biodistribution of liposomes

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Abstract

The kinetics of protein-induced CF release from liposomes of different lipid composition in vitro was studied, including human serum proteins HSA, IgG and HDL. CF release patterns appeared to be similar in the presence or absence of protein in the liposome dispersion medium. The presence of protein merely accelerated CF release in a degree dependent on liposome composition. The rate of CF release depended on both the liposome composition and the type of protein involved. CF release appeared to be multiphasic. By processing the release data, a 'Destabilization Index' (D.I.) was developed, which is a quantitative expression for the destabilization effect of proteins on liposomes. According to the D.I. values obtained from the different liposome compositions, the destabilization effect of the proteins decreased with an increase in liposome membrane rigidity or with the inclusion of GM_1 or PEG in liposome membrane. With all three proteins, an inverse relationship was found between the % CF released after 1 h liposome-protein incubation in vitro and the BLOOD/RES ratio of the liposomes 2 min after i.v. administration in mice (Panagi et al., 1996).

Keywords: Liposomes; Serum proteins; Protein-induced CF release; Destabilization index; Liposome biodistribution; BLOOD/RES ratio

1. Introduction

The interaction of liposomes with serum proteins or plasma in vitro has been shown to enhance the leakage of solutes from liposomes (Zborowski et al., 1977; Kirby and Gregoriadis,

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1981; Sherphof and Morselt, 1984). The extent of solute leakage from liposomes incubated in a specific medium (e.g. blood or serum) in vitro has been used as a measure of liposome stability in that medium (Senior and Gregoriadis, 1982). It appears, however, that the in vitro solute leakage kinetics in the presence of serum proteins have not been adequately analysed and the in vitro destabilization effect of proteins on liposomes have not yet been described in quantitative terms. On the other hand, the interaction of liposomes with serum proteins in vivo is considered to result in liposome clearance from systemic blood circulation (Chonn et al., 1992). It appears possible therefore that a correlation may exist between the kinetics of solute leakage from liposomes in vitro and certain biodistribution properties of liposomes in vivo.

In the present work, the kinetics of carboxyfluorescein release from liposomes of different composition in buffer and in buffer containing HSA (human serum albumin), IgG (human serum immunoglobulin) or HDL (human serum high density lipoprotein) was studied. HSA was selected because it has been found to affect liposome structure in vitro (Zborowski et al., 1977), whereas IgG and HDL were included in the study because they have been implicated in liposome clearance from blood via opsonization (IgG) or disintegration (HDL) procedures (Woodle and Lasic, 1992).

The aim of the study was twofold. First, to reveal the effects that these proteins have on CF release on liposomes in vitro and to develop a quantitative expression for the destabilization effects of proteins on liposomes, which would be based on the data of protein-induced CF release from liposomes. Secondly, to investigate if the protein-induced CF release from liposomes in vitro correlates with the BLOOD/RES biodistribution of liposomes in vivo.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), cholesterol (CH), bovine brain monosialganglioside (GM_1) and 5(6)-carboxyfluorescein (CF), were obtained from Sigma (St. Louis, MO). Poly(ethyleneglycol)-distearoylphosphatidylethanolamine (DSPE-PEG), molecular weight ca 2691, was purchased from Genzyme (Haverhill, Suffolk, UK). Human serum albumin (HSA), human serum immunoglobulin (IgG), and human serum high density lipoprotein (HDL) were obtained from Sigma (St. Louis, MO). Tris-aminomethane was obtained from Merck-Schuchardt (Darmstadt, Germany). Biogel A-15 m 200–400 mesh and Ultrogel AcA 34 chromatographic gels were obtained from Serva (Athens, Greece). Miscellaneous reagents were obtained from Sigma (St. Louis, MO) and solvents, all of analytical grade, were obtained from Farmitalia Carlo Erba (Milan, Italy).

2.2. Preparation of CF containing liposomes

A chloroform solution of lipids was slowly evaporated in a rotary evaporator (Buchi RE111, Switzerland) to prepare a thin lipid film in the inner wall of a 50-ml volumetric flask. The lipid film was hydrated with 1 ml of a 50 mM CF solution in 10 mM Tris-buffered saline (10 mM Tris, 140 mM NaCl, pH 7.4). The resultant suspensions of multilamellar vesicles were subjected to two sonication periods of 10 min each in a bath type sonicator (Branson 1200, USA), at a temperature 10°C higher than the phase transition tem-

Table 1
Size of liposomes according to photon correlation spectroscopy

Liposome composition	Mean size (nm)	Polydispersity
DMPC/DMPG (7:3)	114.3	0.486
PC/CH (2:1)	194.1	0.581
DSPC/CH (2:1)	199.4	0.441
DSPC/CH/GM_1 (2:1:0.33)	151.2	0.411
DSPC/CH/DSPE-PEG (2:1:0.33)	112.1	0.342

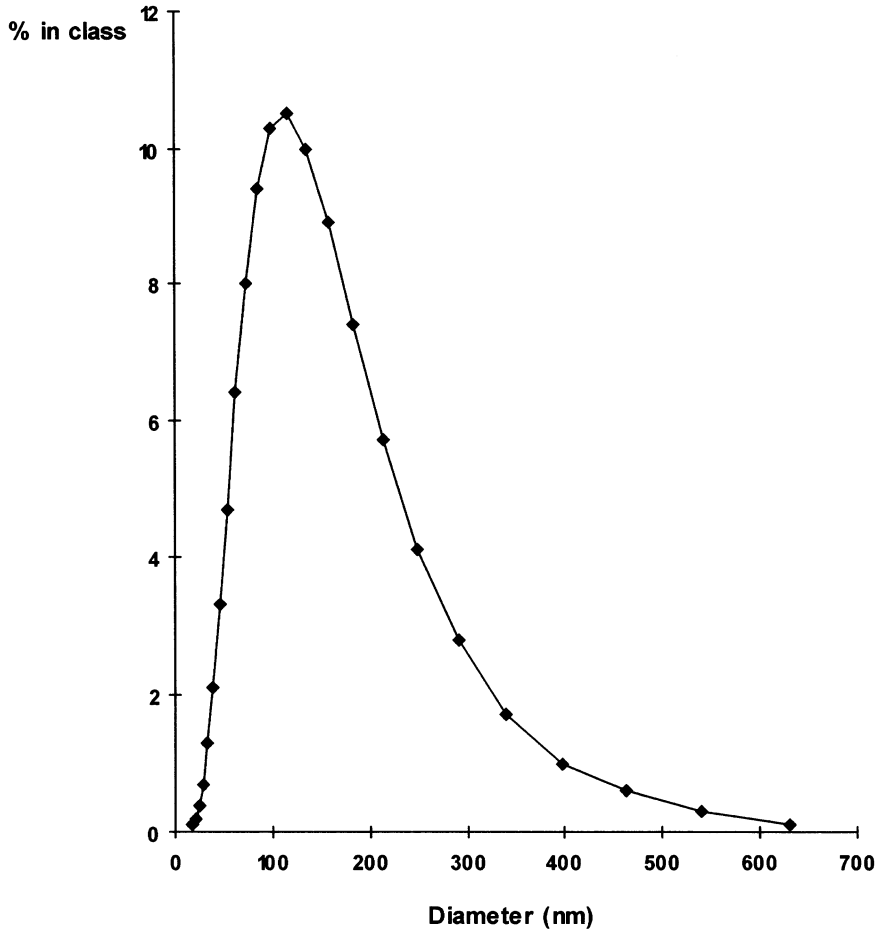


Fig. 1. Size distribution of DSPC/CH/DSPE-PEG (2:1:0.33) liposomes.

perature (T_c) of the lipid component with the highest T_c, interrupted by a 10-min resting period. A similar procedure was reported to provide unilamellar vesicles in the range of 100–200 nm (Tari et al., 1994). The vesicles were aged for 1 h at room temperature before the non-entrapped CF in liposomes was removed by gel filtration through an Ultrogel AcA34 column (1 × 25 cm) equilibrated with Tris-buffered saline. The separation of the non-entrapped CF was performed at room temperature except in the case of DMPC/DMPG (7:3) liposomes where the separation was performed at 4°C. The size and size distribution of liposomes obtained with the above method was determined by photon correlation spectroscopy using a Malvern 5000 zetasizer (Malvern Instruments, UK). The mean size of the different liposome compositions ranged from 112.2 to 199.4 nm (Table 1) and the size distributions obtained were unimodal and positively skewed (e.g. Fig. 1).

CF containing liposomes of the following composition were prepared: PC/CH (2:1), DMPC/DMPG (7:3), DSPC/CH (2:1), DSPC/CH/GM_1 (2:1:0.33), and DSPC/CH/DSPE-PEG (2:1:0.33). The values in parentheses represent the lipid molar ratio in each composition.

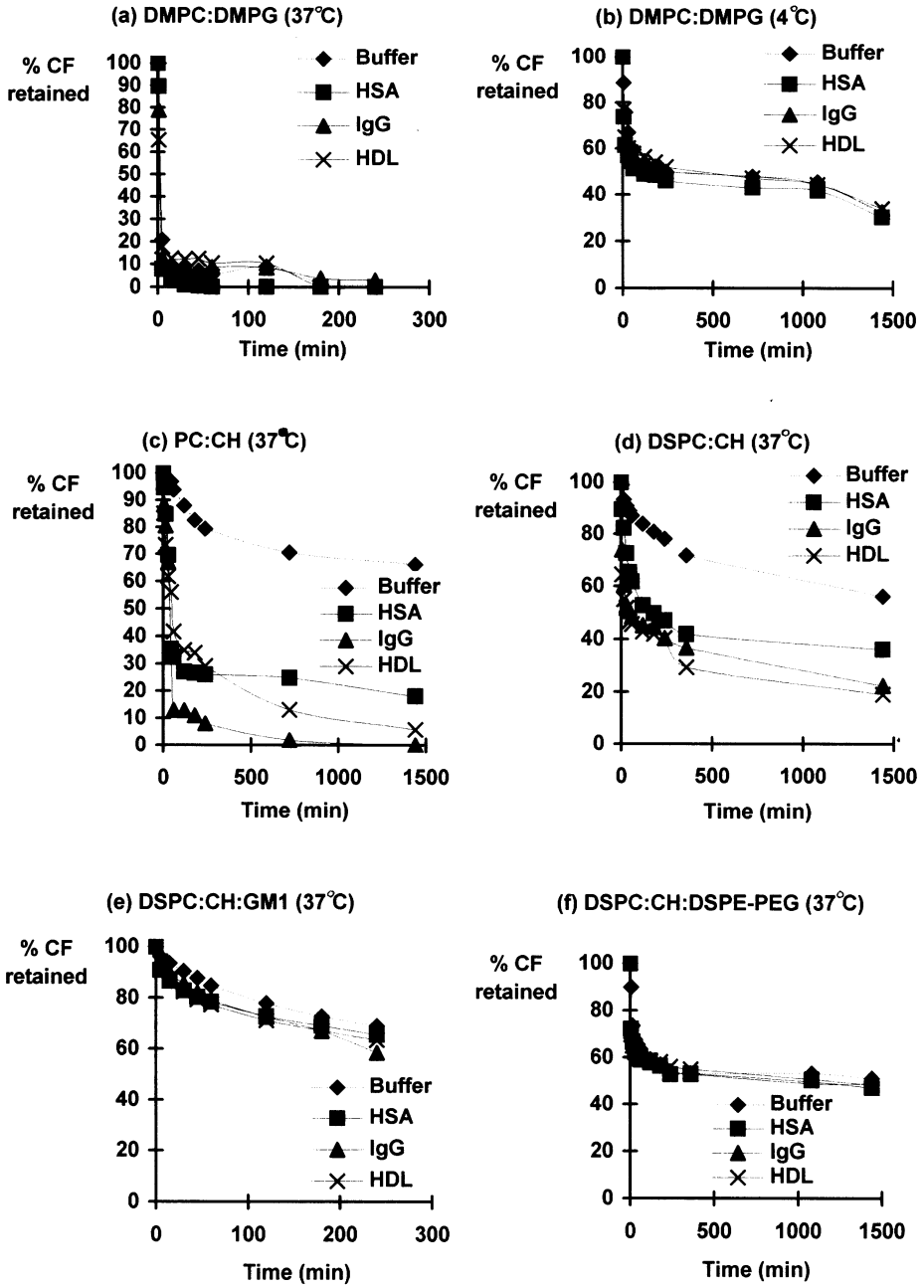


Fig. 2. CF release kinetics from liposomes of different composition in buffer (◆) and in buffer containing HSA (■), IgG (▲) or HDL (×) protein.

2.3. Determination of CF release kinetics

A liposome dispersion (60 μl) containing 80 μg lipids was mixed with 3 ml prewarmed at 37°C Tris-buffered saline, or with 3 ml of Tris-buffered saline containing 400 μg HSA, IgG or HDL, and incubated at 37°C in a mildly shaking water bath. The release of CF from liposomes increases the fluorescence intensity of the liposome dispersion due to fluorescence dequenching. The fluorescence intensity (excitation at 490 nm, emission at 520 nm) of liposomes was measured at regular time intervals up to 24 h using a Shimadzu RF-1501 (Japan) spectrofluorophotometer. After the final measurement, the total CF content of liposomes was determined after liposome lysis with Triton X-100 (2% final concentration). The % CF release was calculated using the following equation:

$$\% \text{ CF release} = 100(F - F_o)/(F_t - F_o) \quad (1)$$

where F, F_o, and F_t are the fluorescence intensity at time t, at time $t = 0$, and after Triton X-100 addition, respectively.

The effect of HDL concentration on CF release from PC/CH (2:1) liposomes was studied by varying the amount of HDL added in 3 ml PC/CH (2:1) liposome dispersions in the range 80–800 μg. The lipid content of the PC/CH (2:1) dispersions used was 80 μg.

Table 2
Rate constants of CF release k_{out} (min^{-1}) and CF uptake k_{in} (min^{-1}) from liposomes obtained with the different liposome compositions after fitting the release data in Eq. (1)

	Buffer	HSA	IgG	HDL
DMPC/DMPG (7:3), 37°C				
k_{out}	0.237	0.320	0.360	0.449
k_{in}	0.010	0.000	0.029	0.059
r^2	0.9525	0.9630	0.9839	0.9995
DMPC/DMPG (7:3), 4°C				
k_{out}	0.019	0.054	0.051	0.048
k_{in}	0.019	0.051	0.058	0.055
r^2	0.9785	0.9312	0.9128	0.9116
PC/CH (2:1), 37°C				
k_{out}	0.001	0.020	0.022	0.020
k_{in}	0.003	0.006	0.001	0.010
r^2	0.9907	0.9463	0.9402	0.9810
DSPC/CH (2:1), 37°C				
k_{out}	0.002	0.013	0.055	0.097
k_{in}	0.002	0.013	0.050	0.080
r^2	0.9333	0.9887	0.9766	0.9547
DSPC/CH/GM_1 (2:1:0.33), 37°C				
k_{out}	0.003	0.008	0.005	0.007
k_{in}	0.007	0.017	0.008	0.014
r^2	0.9872	0.9254	0.9572	0.9702
DSPC/CH/DSPE-PEG (2:1:0.33), 37°C				
k_{out}	0.051	0.063	0.070	0.089
k_{in}	0.064	0.080	0.089	0.118
r^2	0.9574	0.8649	0.8719	0.8952

r^2 = squared correlation coefficient.

2.4. Determination of protein effect on structural integrity of liposomes

In order to obtain information on the mechanism with which the proteins facilitate CF leakage from liposomes, the effect of proteins on the structural integrity of PC/CH (2:1) LUVs was investigated. The effect of HSA, IgG or HDL on the structural integrity of liposomes was assessed from the phospholipid loss from the vesicles occurring during liposome incubation with the protein. PC/CH (2:1) blank (containing no CF) LUVs in VBS (10 mM sodium barbital, 154 mM NaCl, pH 7.4) were prepared as described previously (Panagi et al., 1996). Various amounts (1.56–156 mg) of protein were added in 1 ml liposome dispersions in VBS (15.6 mg lipid/ml), and the mixtures were incubated for 1 h at 37°C in a mildly shaking water bath. Then, 50-μl samples from the mixtures were applied to mini (1 ml) Biogel (A-15m, 200–400 mesh) columns in order to separate the liposomes from the protein nonbound on liposomes. Details on the preparation of the mini columns have been reported elsewhere (Chonn et al., 1991). Immediately after the sample application, the columns were centrifuged at 3500 rev./min for 2 min using a Labofuge A (Heraeus Christ) bench centrifuge. The column fractions were collected in glass tubes by applying 50 μl of VBS to the columns and centrifuging at 3500 rev./min for 2 min. Both the pooled liposome and the pooled protein fractions were analysed for protein using the Bradford protein assay (Bradford, 1976) and for phospholipid using a colorimetric phosphorus assay (Bartlett, 1959). This procedure allowed for the calculation of the %

Table 3
Areas under the % CF retained in liposomes vs time curves up to 60 min (AUC_{60}, min) obtained with the different liposomes using Eq. (2)

Liposome composition	Buffer	HSA	IgG	HDL
DMPC/DMPG (7:3), 37°C	637.525	305.387	472.587	879.097
DMPC/DMPG (7:3), 4°C	4196.355	3413.625	3613.812	3665.829
PC/CH (2:1), 37°C	5791.088	3744.473	3386.704	3855.779
DSPC/CH (2:1), 37°C	5717.622	4497.883	3359.187	3028.812
DSPC/CH/GM_1 (2:1:0.33), 37°C	5488.351	5104.696	5270.351	5098.938
DSPC/CH/DSPE-PEG (2:1:0.33), 37°C	3734.105	3664.051	3634.959	3623.189

phospholipids from those applied in the column which were recovered in the liposome fractions (column fractions 3–5).

3. Results and discussion

In the present work, the protein-induced CF release from liposomes in vitro and its possible correlation with the BLOOD/RES biodistribution of liposomes in vivo was investigated. In addition, this work aimed at developing a quantitative expression for the in vitro destabilization effect of proteins on liposomes, based on the data of protein-induced CF release from liposomes in vitro.

The rate of CF release was found to depend on both the liposome composition and the type of protein involved. CF release patterns appeared to be similar in the presence or absence of protein in the liposome dispersion medium. The presence of protein merely accelerated CF release in a degree which depended on liposome composition (Fig. 2).

An increase in liposome membrane fluidity increased the rate of CF release from liposomes in the presence of protein. Thus, the DMPC/DMPG (7:3) vesicles exhibited much higher CF release rates at the fluid state (at 37°C) than at the gel ('solid') state (at 4°C), and the PC/CH (2:1) vesicles released CF faster than the more rigid DSPC/CH (2:1) vesicles in the presence of protein (Fig. 2). A more fluid and less tightly packed liposome membrane is probably more easily destabilized by the protein bound on liposome, and this may account for the observed effect of liposome membrane fluidity on the protein-induced CF release from liposomes.

The addition of GM_1 in liposome composition, caused a significant decrease in the rate of protein-induced CF release from liposomes. Thus, in the presence of protein, the DSPC/CH/GM_1 (2:1:0.33) vesicles exhibited lower rates of CF release than the DSPC/CH (2:1) vesicles (Fig. 2). This effect may mainly be attributed to the increased hydrophilicity of lipid membranes containing GM_1 (Woodle and Lasic, 1992), which would reduce the hydrophobic interactions between the liposomes and the protein. Also, the addition of DSPE-PEG in the same type of liposomes brought about a decrease in the rate of protein-induced CF release from liposomes (Fig. 2). The steric hindrance to protein approach on liposome surface posed by the chains of PEG extending from the liposome surface (Blume and Cevc, 1993) probably accounts for this result.

The improvement in membrane packing (from PC/CH to DSPC/CH), and especially the presence of GM_1 or DSPE-PEG in liposome membrane not only reduced the rate of protein-induced CF release, but also decreased the effect of protein on CF release rate (Fig. 2).

CF release from liposomes appeared to be multiphasic, with apparent equilibrium (plateau) regions separating the two successive phases. It may be considered that the plateau regions of the release curves was the result of an equilibrium reached, i.e. of equivalence of the rate of CF uptake from liposomes with the rate of CF release from liposomes. Multiphasic CF release from liposomes has also been reported by Frezard et al. (1994). The reasons for such a release pattern are

not clear at present and need further investigation. One should, however, note that CF can penetrate liposome membrane in both directions (Kirby and Gregoriadis, 1981) and that the released CF was not removed from the liposome dispersion during the release experiment. Therefore, the plateau regions in the release curves following the fast first release phase may reasonably be attributed to an equilibrium reached between the release and the uptake of CF from liposomes. This equilibrium was followed, sooner or later depending on liposome inherent stability, by further CF leakage due to instability of liposomes.

The CF released up to equilibrium and the duration of equilibrium appeared to depend on the liposome composition and on the protein involved. The release process up to equilibrium could be represented by the scheme:

$$CF_{\text{in}} \underset{k_{\text{in}}}{\overset{k_{\text{out}}}{\rightleftharpoons}} CF_{\text{out}},$$

where CF_{in} and CF_{out} are the amounts of CF inside and outside the liposomes respectively, and k_{out} and k_{in} are the rate constants for the release and the uptake process, respectively. The rate of change of CF concentration inside the liposomes is:

$$\frac{\mathrm{d}[CF_{\text{in}}]}{\mathrm{d}t} = -k_{\text{out}}[CF_{\text{in}}] + k_{\text{in}}[CF_{\text{out}}]$$

$$= -k_{\text{out}}[CF_{\text{in}}] + k_{\text{in}}([CF_{0,\text{in}}] - [CF_{\text{in}}])$$

$$= -(k_{\text{out}} + k_{\text{in}})[CF_{\text{in}}] + k_{\text{in}}[CF_{0,\text{in}}]$$

where $[CF_{0,\text{in}}]$ is the initial concentration of CF inside the liposomes. The solution of this differential equation is:

$$[CF_{\text{in}}] = \frac{(k_{\text{in}} + k_{\text{out}}\mathrm{e}^{-(k_{\text{in}} + k_{\text{out}})t})}{k_{\text{in}} + k_{\text{out}}}[CF_{0,\text{in}}]$$

or in percentage concentration terms:

$$\%CF_{\text{ret}} = \frac{(k_{\text{in}} + k_{\text{out}}\mathrm{e}^{-(k_{\text{in}} + k_{\text{out}})t})}{k_{\text{in}} + k_{\text{out}}}100 \qquad (2)$$

where % CF_{ret} is the % CF remaining in liposomes at time t.

The kinetic data for the first release phase (up to equilibrium) were fitted in Eq. (2) using non-linear regression (STATGRAPHICS Vs. 2.6 software) in order to obtain estimates for the k_{out} and k_{in} values for the different liposome compositions (Table 2). These estimates were derived by considering that the first release phase lasted 1 h and 18 h for DMPC/DMPG (7:3) at 37°C and 4°C, respectively, 24 h and 3 h for PC/CH (2:1) in the absence or presence of protein respectively, 24 h and 4 h for DSPC/CH (2:1) in the absence or presence of protein respectively, 4 h for DSPC/CH/GM_1 (2:1:0.33) and 24 h for DSPC/CH/DSPE-PEG (2:1:0.33).

The stability of liposomes, as judged by the rate of CF leakage from liposomes, could quantitatively be described by the area under the % CF_{ret} vs time curves up to a certain time T (AUC_{T}). This area can be calculated by integrating Eq. (2):

$$AUC_{\text{T}} = \int_0^T \frac{(k_{\text{in}} + k_{\text{out}}\mathrm{e}^{-(k_{\text{in}} + k_{\text{out}})t})}{k_{\text{in}} + k_{\text{out}}}100$$

$$= \frac{k_{\text{in}}100}{k_{\text{in}} + k_{\text{out}}}T + \frac{k_{\text{out}}100}{(k_{\text{in}} + k_{\text{out}})^2} - \frac{k_{\text{out}}100}{(k_{\text{in}} + k_{\text{out}})^2}\mathrm{e}^{-(k_{\text{in}} + k_{\text{out}})T} \qquad (3)$$

The higher this area the higher the stability of liposomes. The areas under the $\%CF_{\text{ret}}$ vs time curves up to 60 min (AUC_{60}) for the different liposome compositions were calculated using Eq. (3), and the values obtained showed that the different liposome compositions exhibited different stability and that the liposome stability was generally lower in the presence of protein (Table 3).

Table 4
Destabilization Indices (D.I.) for HSA, IgG and HDL obtained with liposomes of different composition

Liposome composition	HSA	IgG	HDL
DMPC/DMPG (7:3), 4°C	0.187	0.139	0.126
DMPC/DMPG (7:3), 37°C	0.521	0.259	−0.379
PC/CH (2:1), 37°C	0.353	0.415	0.334
DSPC/CH (2:1), 37°C	0.213	0.412	0.470
DSPC/CH/GM_1 (2:1:0.33), 37°C	0.070	0.040	0.071
DSPC/CH/DSPE-PEG (2:1:0.33), 37°C	0.019	0.027	0.030

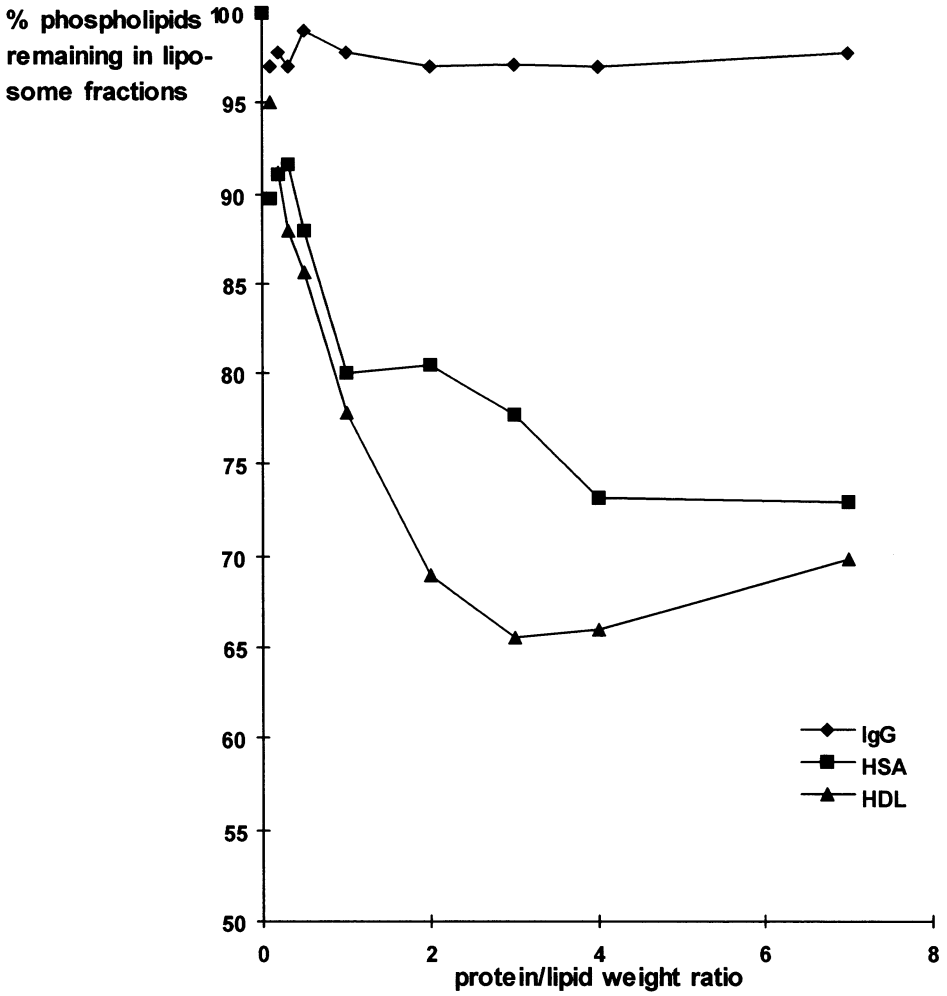


Fig. 3. Effect of IgG (◆), HSA (■), and HDL (▲) human serum proteins on PC/CH (2:1) liposome integrity after incubating the liposomes with each protein for 1 h at 37°C. The protein effect on integrity was quantitated by the % phospholipid loss from liposome column fractions.

A Destabilization Index (D.I.) may then be defined (Eq. (4)) to quantitatively express the destabilization effect of a protein on liposomes.

$$\text{D.I.} = 1 - \frac{(AUC_T)_{\text{protein}}}{(AUC_T)_{\text{buffer}}} \quad (4)$$

where $(AUC_T)_{\text{protein}}$ and $(AUC_T)_{\text{buffer}}$ are the areas under the $\%CF_{\text{ret}}$ vs time curves up to time T obtained in buffer and in buffer containing the protein, respectively.

Theoretically, the D.I. defined in Eq. (4) takes values in the range 0–1. The 0 value corresponds to the minimum (zero) destabilization effect of the protein, whereas the 1 value corresponds to the maximum destabilization effect. A similar procedure with that described above can be applied in order to obtain D.I. values for agents other than the proteins.

According to the D.I. values obtained using Eq. (4) (Table 4), a decrease in liposome membrane fluidity (comparison of PC/CH with the more tightly packed DSPC/CH vesicles) or the incorporation of GM_1 or PEG in liposome membrane (comparison of DSPC/CH with DSPC/CH/GM_1

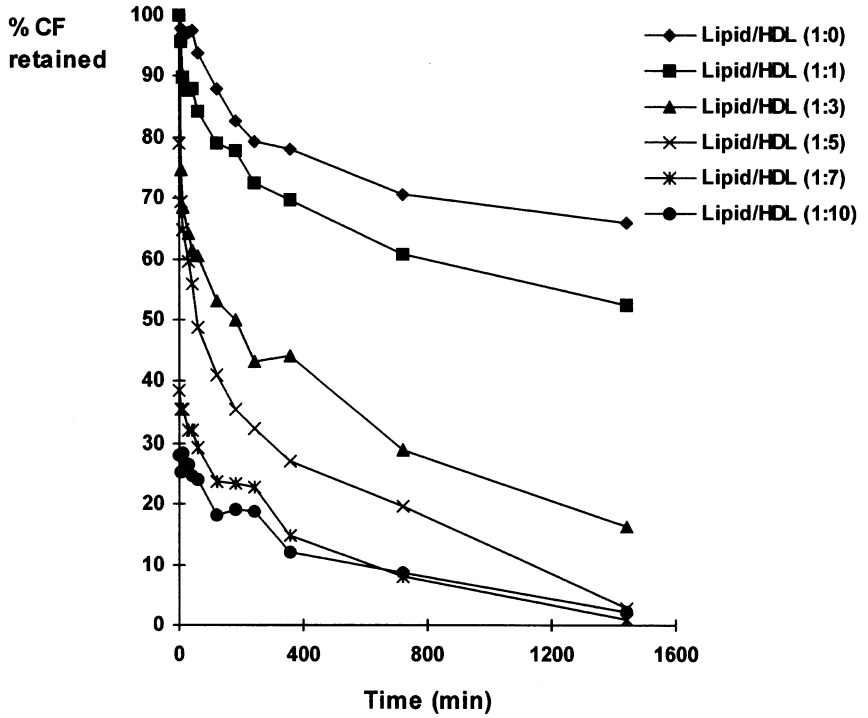


Fig. 4. Effect of HDL concentration on CF release from PC/CH (2:1) liposomes.

and with DSPC/CH/DSPE-PEG liposomes), generally caused a decrease in the destabilization effect of the proteins on liposomes. The DMPC/DMPG (7:3) vesicles leaked their CF content too rapidly at 37°C (Fig. 2) to give meaningful D.I. values.

The protein effect on PC/CH (2:1) integrity is shown in Fig. 3, where the % phospholipids recovered in liposome fractions at different protein/lipid weight ratios in the liposome-protein incubation mixtures is presented. In the case of HSA and HDL, an increase in protein/lipid ratio up to 4:1 and 3:1, respectively decreased phospholipid recovery, but a further increase in protein/lipid ratio did not significantly alter phospholipid recovery. In the case of IgG, there was similar, high, phospholipid recovery at all IgG/lipid ratios. Considering that the lower the phospholipid recovery the higher the destructive effect of the protein on the structural integrity of liposomes, HDL was more destructive on liposomes than HSA, whereas IgG did not have any significant destructive effect on liposome structural integrity (Fig. 3).

An increase in HDL/lipid weight ratio in the incubation mixture of PC/CH (2:1) with HDL caused an increase in the rate of CF release from liposomes (Fig. 4). However, the % CF released and % phospholipid loss from PC/CH (2:1) after 1-h incubation with HDL at different HDL/lipid ratios (Figs. 3 and 4) were not linearly correlated (Fig. 5). This result would indicate that HDL induced CF release mainly through liposome membrane destabilization than through liposome disintegration. The same mechanism of CF leakage facilitation should also apply in the case of HSA and IgG which are less destructive to liposome structure.

According to literature data (Gabizon and Papahadjopoulos, 1988; Allen et al., 1991), the liposome compositions found in the present study to exhibit reduced protein-induced CF leakage in vitro, such as those having tighter membrane packing or those containing GM_1 or DSPE-PEG,

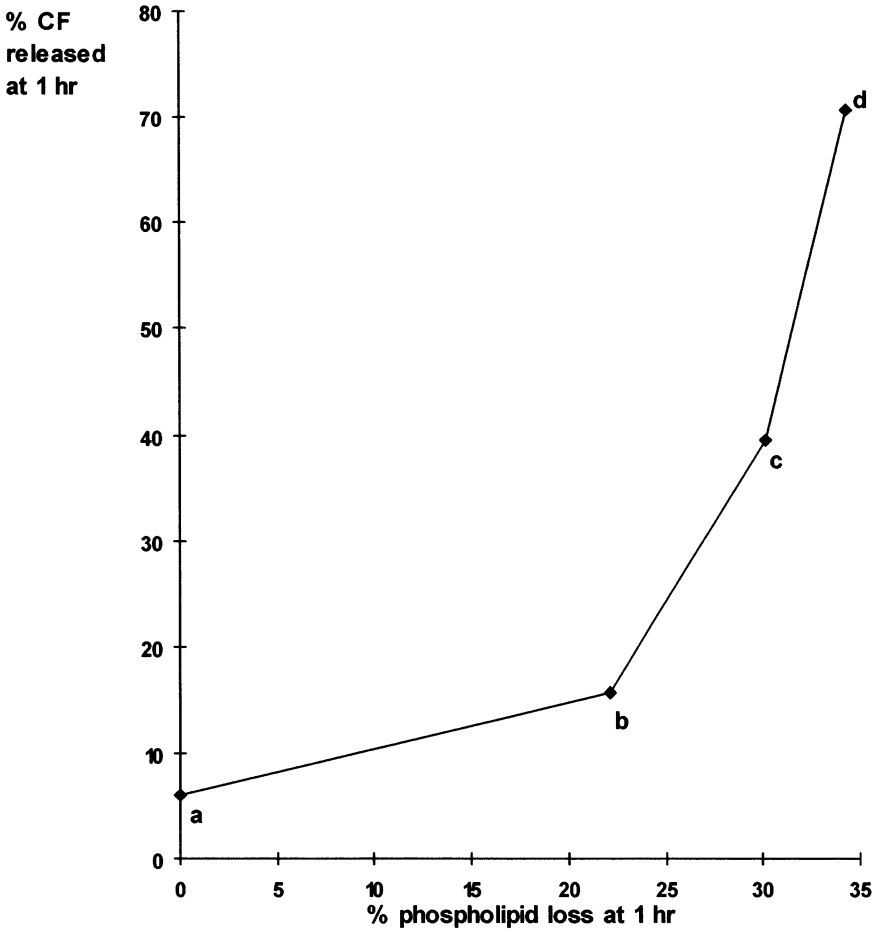
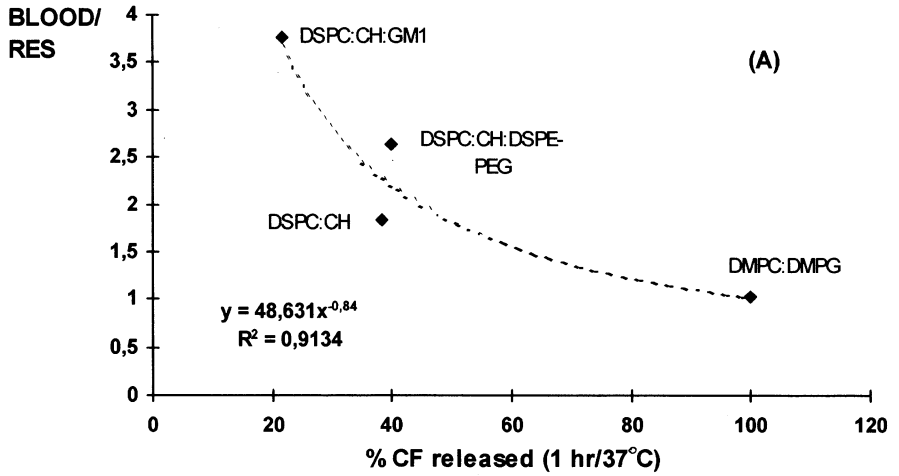
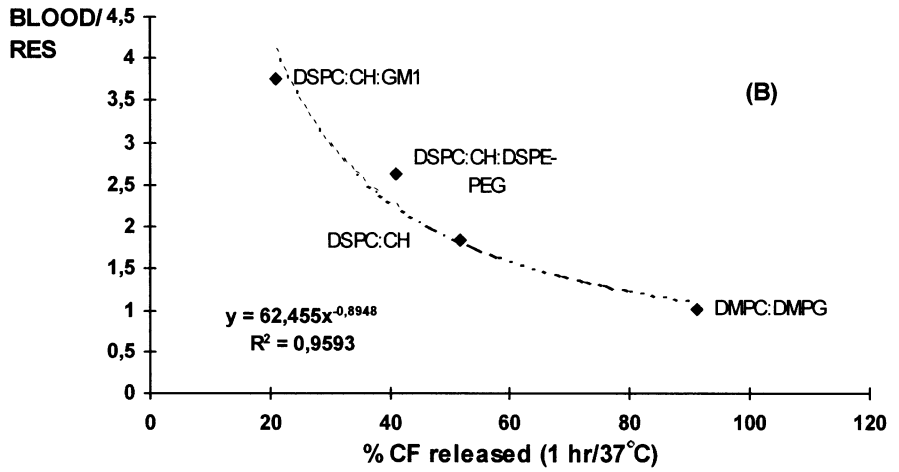


Fig. 5. Comparison of % CF released and % phospholipid loss from PC/CH (2:1) liposomes at different HDL/lipid weight ratios: (a) 0:1, (b) 1:1, (c) 3:1, and (d) 7:1.

are expected to exhibit prolonged retention in systemic blood circulation in vivo. The BLOOD/RES ratio 2 min after i.v. administration in Swiss/De mice of certain liposome compositions included in the present study have recently been reported by our group (Panagi et al., 1996). When these BLOOD/RES ratios were plotted against the % CF released from the same liposomes after 1 h of liposome-protein incubation in vitro, an inverse relationship was noted with all three proteins. This relationship could satisfactorily be described by power functions of the type $y = a \cdot x^{-b}$ (Fig. 6). The biodistribution data of liposomes 2 min after their i.v. administration were used due to their increased reliability as compared to the biodistribution data obtained at later times (Panagi et al., 1996). However, similar trends of the biodistribution data were observed at all times studied post-administration (Panagi, 1996; Panagi et al., 1996). Thus, the time of BLOOD/RES ratio determination should not affect the validity of the inverse relationship between the liposome BLOOD/RES ratio and the protein-induced CF release from liposomes, provided that the time selected lies inside the time span of liposome circulation in blood. The observed in vitro–in vivo correlation may prove useful in the prediction of certain biodistribution properties of liposomes using in vitro data of CF release from liposomes in the presence of specific serum proteins.





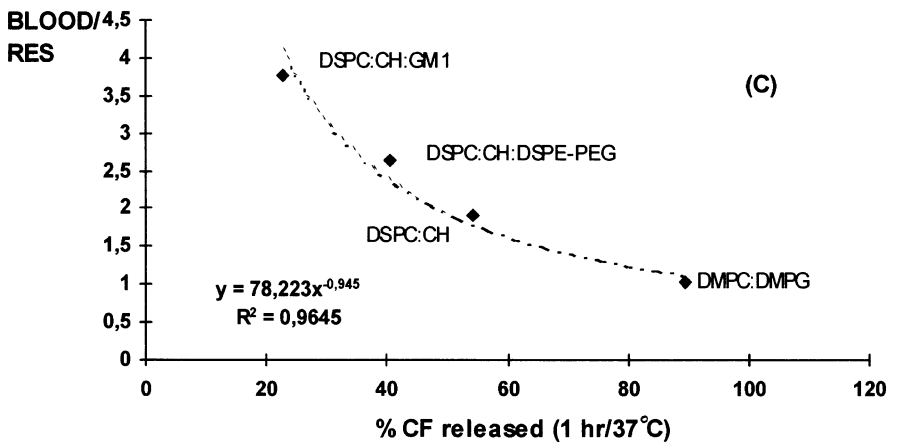


Fig. 6. Correlation of % CF released from different liposomes after 1-h incubation at 37°C in vitro with: (A) HSA, (B) IgG and (C) HDL with the BLOOD/RES ratio of these liposomes in mice.

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