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Plasma dependent pH sensitivity of liposomes containing sulfatide

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In this study we investigated the possibility to define relatively plasma-stable liposomal preparations in which the sensitivity to moderate drops of pH (i.e., from 7.4 to 6.8) would be induced by the presence of plasma itself. The liposome stability was monitored by determining the release of entrapped 5,6-carboxyfluorescein (CF). Using small unilamellar vesicles composed of egg phosphatidylcholine (EPC) and bovine brain sulfatide (CS) (4:1, molar ratio), the amount of CF released at pH 6.8 in the presence of 50% plasma was 3-fold that at pH 7.4, whereas no significant differences in the amount of CF released were observed when the same liposomes were incubated in buffer at pH 7.4 and 6.8, respectively. The increase in plasma induced leakage as a consequence of a drop in the pH medium, seems to specifically depend on the presence of sulfatide molecule in the bilayer since neither the acidic cholesterol 3-sulfate nor galactocerebroside, are able to induce pH sensitivity in EPC liposomes. Of all the plasma components considered (VLDL, LDL, HDL, protein fraction), VLDL seemed preferentially involved in the pH sensitivity induced by CS since they promoted an almost complete release of CF from EPC/CS small unilamellar vesicles. Thus, these liposomes are potentially a useful tool for a specific drug delivery to those pathological tissues such as tumors, inflammation sites and ischemic areas in which it is known that a lowering of the pH can occur.

Introduction

In constructing pH-sensitive liposomes the general strategy has been to include lipids containing a pH-sensitive group such as *N*-palmitoylhomocysteine (PHC) [1], cholesterylhemisuccinate [2], oleic acid (OA) with phosphatidylethanolamine (PE) as auxiliary lipid [3,4], *N*-succinyldioleoylphosphatidylethanolamine [5] and dipalmitoylsuccinylglycerol (DPSG) [6], in the bilayer. When exposed to an acidic environment, such lipids are able to destabilize the lipid bilayer with a consequent release of water soluble trapped molecules; the destabilization can result in liposome-liposome fusion [7] or liposome-liposome aggregation [2]. Although the potential use of pH sensitive liposomes remains to be evaluated, recent in vitro studies [3,8] indicate that they are effective in enhancing the cytoplasmic delivery of trapped molecules to the target cells, as a consequence of their association with the acidic environment of cellular endosomes and lysosomes. Antitumor drugs [9], toxins [10] and DNA [11] have been efficiently delivered to target cells using PE/OA liposomes.

Besides their use for improving the intracellular drug delivery of liposome encapsulated compounds to a variety of cell types, an important application of pH-sensitive liposomes lies in the possibility of selective drug release to pathological tissues such as tumors, metastases, sites of inflammation and ischemic areas that have an ambient pH considerably lower than that of normal tissue. This difference could be utilized if liposomes could be constructed so that they are relatively stable in the circulating fluids at physiological pH but release encapsulated drugs when passing through a region of lower pH. The use of many of the pH sensitive molecules mentioned above could be hampered by their potential exchange with serum components, in manner analogous to that reported for natural corresponding molecules [12], with a consequent destabilization and loss of pH sensitivity by liposomes before they reach their designated target. Different approaches have been used to increase the serum

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Abbreviations: CF, 5,6-carboxyfluorescein; EPC, egg phosphatidylcholine; CS, bovine brain sulfatide; CB, cerebroside; PHC, *N*-palmitoylhomocysteine; Chol-3S, cholesterol 3-sulfate; OA, oleic acid; PE, phosphatidylethanolamine; DPSG, dipalmitoylsuccinylglycerol; DOPE, dioleoylphosphatidylethanolamine; $P_{10}PC$, 1-palmitoyl-2-(10-pyrene)decanoylphosphatidylcholine; $P_{10}CS$, *N*-(12-(1-pyrene) decanoyl)galactosyl sphingosine I^3 sulfate; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

stability of pH-sensitive liposomes: the increase of the vesicle curvature by using small liposomes ($d \leq 200$ nm) in which the insertion of stabilizing serum apoproteins is favored [13,14], but these liposomes are no longer pH sensitive [14]; the use of pH-sensitive molecules such as PHC [1] and DPSG [6] for which a partial serum stability as well as a remaining pH sensitivity after exposure to serum have been demonstrated [6].

In this study we tried to define relatively plasma stable liposomal formulations in which the pH sensitivity would be induced by the presence of plasma. For this purpose we evaluated the possibility that sulfatide, an acidic glycolipid carrying a sulfate group on the galactose moiety, could induce sensitivity to moderate drops in plasma pH in EPC small unilamellar vesicles. This hypothesis is based on our previous studies in which we demonstrated that: (i) sulfatide can give rise to lateral phase separation phenomena in EPC bilayers for molar concentrations greater than 5 mol% [15,16], this segregation resulting in structural discontinuities in the bilayer; (ii) the diffusion of H^+ ions from EPC liposomes increases when more than 5 mol% of sulfatide was present in the bilayer [17]; (iii) serum albumin promoted aggregation and fusion of liposomes containing more than 5 mol% of sulfatide at acidic but not at physiological pH values without subtracting sulfatide from liposomes [18]. On the other hand calcitonin, an amphipathic peptide which is positively charged at physiological pH, promoted CF release from sulfatide containing liposomes without evidence of liposome aggregation and fusion [19].

Materials and Methods

Analytical grade chemicals, distilled solvents and doubly distilled water were used. Egg phosphatidylcholine (EPC), cholesterol 3-sulfate (Chol-3S) and 5,6 carboxyfluorescein (CF), were purchased from Sigma. 1-Palmitoyl-2-(10-pyrene)decanoylphosphatidylcholine ($P_{10}PC$) and pyrene decanoic acid came from Molecular Probe (Junction City, OR); Sepharose 4B from Pharmacia (Uppsala Sweden). ^{14}C-labeled glucose was purchased from Amersham International (UK). Bovine brain sulfatide (CS) and cerebroside (CB) were purified following the procedure of Hara and Radin [20]. *N*-(12-(1-Pyrene)decanoyl)galactosyl sphingosine I^3 sulfate ($P_{10}CS$) was prepared from galactosyl sphingosine I^3 sulfate following the procedure of Marchesini et al. [21].

Preparation of small unilamellar vesicles

Pure egg phosphatidylcholine (EPC) and binary mixtures (80:20, molar ratio), made by EPC and sulfatide, cerebroside or cholesterol 3-sulfate, respectively, were dissolved in chloroform:methanol (2:1, v/v). The lipid mixtures were evaporated under a nitrogen stream, dried under vacuum for 30 min and resuspended above the lipid transition temperature (T_m) in the appropriate buffer in order to obtain multilamellar vesicles (MLVs). Small unilamellar vesicles (SUV) were obtained by sonicating MLVs, following the procedure of Barenholz et al. [22].

Trapped volume determinations

The volumes of the internal, solute available, aqueous compartments of the four vesicles considered here were determined by 'trapping' 5,6-carboxyfluorescein (CF). Vesicles containing trapped marker were prepared by sonication of lipids in 10 mM Tris-HCl (pH 7.4), 100 mM CF and gel filtration through a 1.5 cm × 20 cm Sepharose 4B column at room temperature using as elution buffer 10 mM Tris-HCl (pH 7.4), 154 mM NaCl.

Trapped CF was determined fluorimetrically at 520 nm with excitation at 490 nm, after the rupture of chromatographed vesicles with SDS 2% final concentration. The molar concentration of lipids in the vesicle sample was determined, respectively, as inorganic phosphate for phospholipids [23], by the methods of Radin [24] for sulfatide, and Kushwaha and Kates [25] for cerebroside. The trapped aqueous volume was calculated as:

$$V_t = \frac{M_i / M_0}{P}$$

where V_t is expressed as μl of trapped volume per μmole of vesicle lipid, M_i and M_o are the concentrations of trapped solute in the chromatographed vesicle sample and in the sonication buffer, respectively, and P is the molar concentration of lipids in the vesicle sample.

Liposome stability determinations

The stability of the liposomes was monitored by determining the time-dependent leakage of 5,6-carboxyfluorescein (CF) from small unilamellar vesicles of different lipid composition prepared in 10 mM Tris-HCl (pH 7.4), 100 mM CF and separated from extravesicular by gel filtration on Sepharose 4B. In this case the CF containing vesicles were eluted with 10 mM citric acid, 20 mM Na_2HPO_4, 154 mM NaCl at pH 7.4 or 6.8 in order to obtain a liposomal suspension at the pH used in pH-dependent leakage determinations.

100 μl of eluted vesicles, corresponding to 100 nmoles of lipids, were then diluted to 1.0 ml with either the corresponding elution buffer, plasma or plasma components at the same pH, and the fluorescence emission at 520 nm was determined (zero time fluorescence, F_0) setting the excitation wavelength at 490 nm.

The increase in the fluorescence emission of CF, a highly fluorescent substance which undergoes a concentration dependent self-quenching [26], was monitored by measuring the time-dependent leakage of this molecule from liposomes. The data are expressed as:

$$\% \text{ CF release} = 100 \times (F - F_0)/(F_t - F_0)$$

where F is the fluorescence intensity at a given time and F_t is the total CF fluorescence measured after the rupture of vesicles with SDS, 2% final concentration.

The pH-dependent stability of EPC/CS (80:20, molar ratio) small unilamellar vesicles in the presence of plasma was also determined using [^{14}C]glucose as the trapped molecule. In this case vesicles containing entrapped [^{14}C]glucose (1052 ± 50 dpm/μmol lipid), previously separated from extravesicular [^{14}C]glucose, were incubated for 30 min at pH 7.4 or 6.8 either alone or in presence of 50% human plasma. At the end of incubation the vesicles were separated by gel filtration on Sepharose 4B using 10 mM citric acid, 20 mM Na_2HPO_4, 154 mM NaCl (pH 7.4) as elution buffer; 1 ml fractions were collected and each fraction analyzed for EPC, sulfatide and glucose content.

Liposome stability determinations were done at least in triplicate.

Treatment of liposomes with plasma and plasma components

Liposome suspensions containing 100 nmol lipids in 500 μl of 10 mM citric acid, 20 mM Na_2HPO_4, 154 mM NaCl at pH 7.4 or 6.8 were incubated with an equal volume of human plasma conditioned at the same pH of liposome suspension by adding appropriate amounts of 100 mM citric acid.

The different lipoprotein classes (VLDL, LDL, HDL) were obtained by ultracentrifugation on discontinuous KBr gradient [27]. The plasma fraction remaining after lipoprotein separation was used as protein fraction, the albumin representing more than 90%.

The different lipoprotein classes and the plasma protein fraction were dialyzed for 24 h against 10 mM citric acid, 20 mM Na_2HPO_4, 154 mM NaCl at pH 7.4 and 6.8 in order to eliminate KBr and bring the plasma components to the same pH as the liposome suspension. The incubation of liposomes with plasma components was performed using amounts of lipoproteins and proteins corresponding to a 50% plasma dilution.

Excimer to monomer ratio determinations

The possibility of vesicle-vesicle or vesicle-VLDL fusion as a function of pH and vesicle lipid composition was evaluated by determining the Excimer (475 nm) to Monomer (379 nm) fluorescence intensity ratio (E/M) of P_{10}PC or P_{10}CS at different times during incubation. Small unilamellar vesicles of EPC or EPC/CS (80:20, molar ratio) were labelled with P_{10}PC or P_{10}CS, in both cases the probe represented 10 mol% of the total lipids. Fluorescent small unilamellar vesicles (10^{-4} M final lipid concentration) were incubated with VLDL (corresponding to 50% dilution of plasma) at pH 7.4 and 6.8 both in the absence and presence of the same non fluorescent vesicles.

TABLE I

Internal solute available aqueous compartment of different SUV formulations

Results are the mean of six experiments.

Formulation	Lipid composition (molar ratio)	Trapped volume (μl/μmol)
EPC	100	0.62 ± 0.07
EPC/CS	80:20	0.85 ± 0.08
EPC/Chol-3S	80:20	0.90 ± 0.10
EPC/CB	80:20	0.63 ± 0.08

Fluorescence measurements

All fluorescence measurements were carried out with a Jasco FP 700 spectrophotofluorimeter equipped with a cuvette holder the temperature being maintained by a Haake GD3 thermostatic circulating bath and monitored with a Subline PT 100 digital thermometer.

Results

Small unilamellar vesicles (SUV) were prepared by sonication in 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM CF. They had the following lipid composition: pure EPC, EPC/CS (80:20, molar ratio), EPC/CB (80:20, molar ratio) and EPC/Chol-3S (80:20, molar ratio). The different SUV preparations were then separated from extravesicular CF by gel filtration and used for trapped volume determinations; the results are reported in Table I and indicate that the presence of an acidic lipid in the liposomal membrane determines an increase in the internal, solute available, aqueous compartment of the vesicles. The internal compartments determined for SUV containing CS or Chol-3S were 0.9 and 0.85 μl/μmol lipid, respectively, representing a 45% increase in the trapped volume if compared with values obtained with 'uncharged' vesicles: 0.62 and 0.63 μl/μmol lipid for EPC and EPC/CB vesicles, respectively.

CF-loaded vesicles were also used to study the pH sensitivity of liposomes by measuring the increase in CF fluorescence intensity when vesicles were incubated at different pH in buffer or in 50% human plasma. The use of 50% plasma dilution was based on previous evidence [28] that indicated that almost all the effects observable in full serum, are found even in 25% serum.

The kinetics of CF release from EPC vesicles are reported in Fig. 1 (panel A). The spontaneous CF release from EPC vesicles at pH 7.4 showed a biphasic kinetics with an initial fast leakage in the first 5 minutes, followed by a more prolonged slow release which was monitored for up to 45 min. The spontaneous release of CF from EPC small unilamellar vesicles was very low, only 1.4 and 3.35% of the entrapped molecule were released after 5 and 45 min of incubation, respectively. Kinetics evaluated with the same vesicles at pH 6.8 are almost identical to those obtained at pH 7.4. When EPC vesicles were incubated in a medium containing 50% plasma at pH 7.4, the amount of CF released from liposomes was increased in both the fast and the slow phases of the kinetics. At 5 and 45 min the amount of CF released was, respectively, 3.8 and 9% of the entrapped molecule. The acidification of the medium at pH 6.8 determined only a small increase in the extent of CF leakage: at the same times at pH 7.4 the CF release was, respectively, 5.8 and 10% of the entrapped molecule.

The presence of 20 mol% of sulfatide in EPC vesicles (panel B) determines a stabilization of the vesicle bilayer which results in a reduction of the spontaneous leakage of entrapped CF in buffer, both at pH 7.4 and 6.8. At all the times considered the CF diffusion from sulfatide containing vesicles is less than that determined with EPC vesicles. At 5 and 45 min the CF release was 0.57 and 1.58%, respectively, thus indicating that the presence of sulfatide determines a 60% increase in the stability of CF-containing liposomes in buffer. When EPC/CS vesicles were incubated in the presence of 50% human plasma at physiological pH, the kinetics of CF release were very similar to those obtained with EPC liposomes, apart from a small increase in the amount of leaked CF at all the times considered. In fact the values determined at 5 and 45 min were 4.9 and 10.5%, respectively. In contrast to

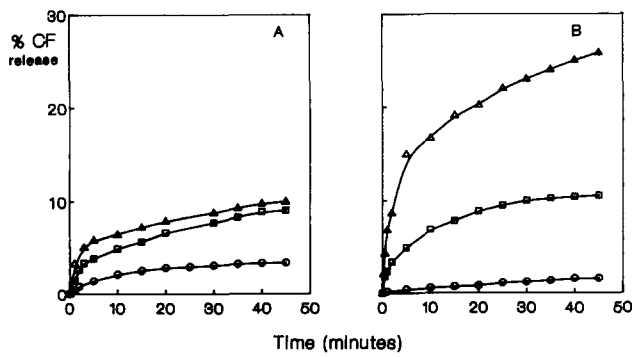

Fig. 1. Extent of spontaneous (○) and serum induced carboxyfluorescein (CF) release at pH 7.4 (□) and 6.8 (△), from EPC (panel A) and EPC/CS (80:20, molar ratio (panel B) small unilamellar vesicles. Data are expressed as % CF release calculated as described in Materials and Methods. Data reported are the means of at least three experiments.

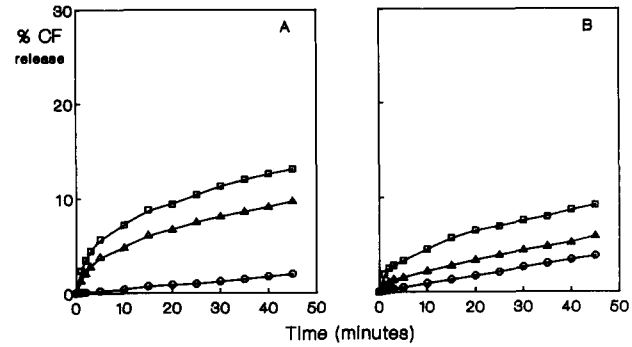

Fig. 2. Extent of spontaneous (○) and serum induced carboxyfluorescein (CF) release at pH 7.4 (□) and 6.8 (△), from EPC/Chol3S (80:20, molar ratio (panel A) and EPC/CB 80:20, molar ratio (panel B) small unilamellar vesicles. Data are expressed as % CF release calculated as described in Materials and Methods. Data reported are the means of at least three experiments.

what was obtained with EPC vesicles, the acidification of the incubation medium to pH 6.8 determined a dramatic increase in the amount of CF released from liposomes containing sulfatides. The increase in the rate of the initial fast leakage was greater than that of the slow release phase: after 5 min of incubation at pH 6.8 in the presence of 50% plasma the CF released was 16.7% of the entrapped molecule, a 3-fold greater value than that obtained at pH 7.4. After 45 min of incubation at pH 6.8 a 2.5-fold greater release than at physiological pH was determined. The capacity of liposomes containing sulfatide to exhibit a sensitivity to small modifications of plasma pH could depend on the acidic character of this molecule which carries a sulfate group on the 3 position of the galactose moiety. In order to elucidate if the presence of a sulfate group is the only requirement for determining a pH sensitivity of EPC small unilamellar vesicles, the CF release in buffer and 50% plasma at pH 7.4 and 6.8, was evaluated using EPC/Chol-3S (80:20, molar ratio) small unilamellar vesicles. The results reported in Fig. 2A demonstrate that the presence of Chol-3S increases the liposome stability in buffer, just as the presence of sulfatide did. In fact the CF diffused from liposomes after 5 and 45 min of incubation in buffer at both pH values was 0.4 and 2%, respectively, of the entrapped dye. The kinetics obtained in 50% plasma at physiological pH were very similar to those obtained with pure EPC vesicles, the percent release at 5 and 45 min being 5.7 and 13%, respectively. Nevertheless in this case, unlike to that determined with sulfatide, the acidification of the medium to pH 6.8 did not determine any increase but rather a small decrease in the CF leakage; in this case the amount of CF released at 5 and 45 min of incubation was 4.8 and 9.7%, respectively of the entrapped molecule.

It has also been considered the contribution that the galactose moiety made to the plasma and the pH

dependent destabilization observed with EPC/CS liposomes. Data (Fig. 2B), demonstrate that the presence of 20 mol% of cerebroside, a neutral glycolipid which differs from sulfatide in the absence of the sulfate group, does not increase the EPC liposome stability in buffer. The amount of CF released in the medium from these liposomes after 5 and 45 min of incubation was 0.47 and 3.8%, respectively. The addition of 50% plasma to the incubation medium at pH 7.4 determines kinetics analogous to those obtained with EPC vesicles; also in this case the percent of CF released at pH 6.8 at all the times considered is less than those determined at physiological pH.

Of all the molecules considered sulfatide is the only one that seems to induce a plasma-dependent sensitivity to small pH modifications in liposomes. To confirm that this characteristic does not depend on the nature of the water soluble molecule trapped within the aqueous compartment, CF was substituted with [^{14}C]glucose. This molecule does not permit a continuous monitoring of its release from EPC/CS vesicles, thus the [^{14}C]glucose concentration associated to liposomes was evaluated radiometrically after 30 min incubation of [^{14}C]glucose-loaded liposomes in the presence of 50% plasma at pH 7.4 and 6.8. The liposome associated [^{14}C]glucose was 950 ± 50 and 740 ± 40 dpm/μmol lipid at pH 7.4 and 6.8, respectively, the initial liposomal [^{14}C]glucose concentration being 1052 ± 50 dpm/μmol lipid. The 3-fold increase in [^{14}C]glucose leakage at pH 6.8 with respect to that determined at pH 7.4, is in good agreement with the data obtained with CF-loaded liposomes.

The pH sensitivity demonstrated by EPC/CS vesicles in the presence of plasma could be related to a specific component of the human serum. In order to evaluate which of the plasma components is mainly responsible for the plasma-dependent pH sensitivity demonstrated by liposomes containing sulfatide, CF loaded EPC/CS vesicles and, for comparison, EPC vesicles were incubated at pH 7.4 and 6.8 in the presence of the different lipoprotein classes (VLDL, LDL, HDL) and plasma proteins. The amount of lipoproteins and proteins used in these experiments corresponded to their concentration in the plasma volume used in previous experiments. It should be noted that the amounts of CF released during incubation of liposomes with all the plasma fractions are always greater than those obtained during incubation with plasma.

The kinetics of CF release obtained by incubating liposomes with VLDL are reported in Fig. 3. Also in this case biphasic kinetics were obtained with an initial fast leakage in the first 5 min, followed by a slow and more prolonged release which was monitored for 45 min. With EPC liposomes the CF release curves at pH 7.4 and 6.8 are very similar, with a small reduction for values observed at pH 6.8. After 5 min of incubation

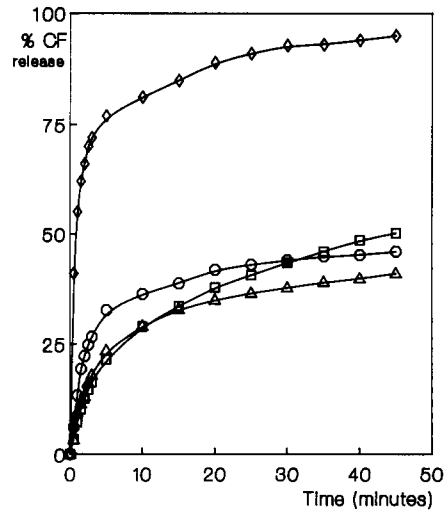

Fig. 3. Extent of VLDL induced CF release from EPC and EPC/CS (80:20, molar ratio) small unilamellar vesicles at pH 7.4 and 6.8. EPC: pH 7.4 (○), pH 6.8 (□); EPC/CS: pH 7.4 (△), pH 6.8 (◇). Data are expressed as % CF release calculated as described in Materials and Methods. Data reported are the means of at least three experiments.

the % CF release at pH 7.4 and 6.8 were, respectively, 30 and 25%; this difference decreased after 45 min when the observed values were, respectively, 50 and 46%. When EPC/CS vesicles were incubated with VLDL at physiological pH, the kinetics of CF release were very similar to those obtained with EPC vesicles; on the contrary, the pH lowering determined a dramatic increase in the amount of CF released from EPC/CS liposomes in the presence of VLDL. After 5 min of incubation at pH 6.8 77% of liposome-en-

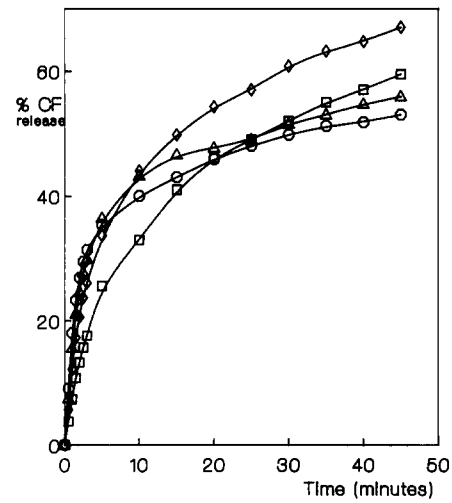

Fig. 4. Extent of LDL induced CF release from EPC and EPC/CS (80:20, molar ratio) small unilamellar vesicles at pH 7.4 and 6.8. EPC: pH 7.4 (○), pH 6.8 (□); EPC/CS: pH 7.4 (△), pH 6.8 (◇). Data are expressed as % CF release calculated as described in Materials and Methods. Data reported are the means of at least three experiments.

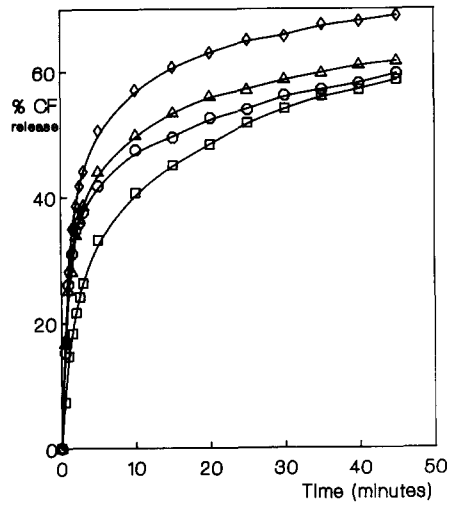

Fig. 5. Extent of HDL induced CF release from EPC and EPC/CS (80:20, molar ratio) small unilamellar vesicles at pH 7.4 and 6.8. EPC: pH 7.4 (○), pH 6.8 (□); EPC/CS: pH 7.4 (△), pH 6.8 (◇). Data are expressed as % CF release calculated as described in Materials and Methods. Data reported are the means of at least three experiments.

trapped CF was released in the medium and after 45 min this value reached 95%.

The kinetics of CF leakage from EPC and EPC/CS liposomes in the presence of LDL and HDL are reported in Figs. 4 and 5, respectively. In both cases the acidification of the incubation medium causes only a small increase in the amount of CF released from EPC/CS liposomes: after 45 min of incubation with LDL the % of CF leakage was 56 and 67 at pH 7.4 and 6.8, respectively, with HDL in the same conditions 62 and 69%, respectively. Also in the case of LDL and HDL the incubation at pH 6.8 determined a decrease

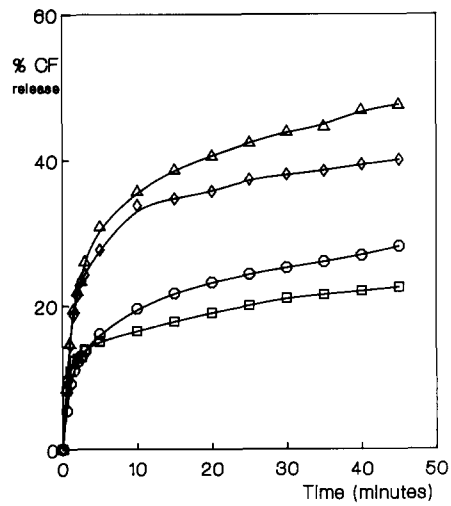

Fig. 6. Extent of plasma protein fraction induced CF release from EPC and EPC/CS (80:20, molar ratio) small unilamellar vesicles at pH 7.4 and 6.8. EPC: pH 7.4 (○), pH 6.8 (□); EPC/CS: pH 7.4 (△), pH 6.8 (◇). Data are expressed as % CF release calculated as described in Materials and Methods. Data reported are the means of at least three experiments.

in the amount of CF released from EPC liposomes in the initial fast leakage, while not modifying the subsequent slow release phase. After 45 min the release obtained with LDL and HDL at both pH values (≅ 60%) was greater than that determined incubating EPC liposomes with VLDL.

The kinetics of CF release in the presence of the plasma protein fraction (fig. 6) indicate that liposomes containing sulfatide are more destabilized than EPC vesicles, and that the CF leakage from both the liposomes is not influenced by small pH variations of the medium.

Discussion

The aim of this work was to define a liposomal formulation with a fairly large trapping efficiency, relative stability at physiological pH and in which pH sensitivity could be induced by the presence of plasma. Data (Table I) indicate that in general the presence of acidic lipids increases the aqueous volume of liposomes; in particular sulfatide determines a 50% increase in the trapped aqueous volume of EPC small unilamellar vesicles, thus demonstrating an improved trapping capacity of liposomes promoted by sulfatide.

The results obtained demonstrate that liposomes containing sulfatide can really be considered 'plasma-dependent, pH-sensitive' liposomes. In fact in the presence of 50% human plasma the amount of CF released at pH 6.8 is 3-fold that at pH 7.4 for all the times considered (Fig. 1B). The pH sensitivity of small unilamellar vesicles containing sulfatide depends on the presence of plasma in the incubation medium since the kinetics determined for incubating the same liposomes in citric acid/Na_2HPO_4 buffer at pH 6.8 were similar to those obtained in the same buffer at pH 7.4. This excludes the possibility that a pH-dependent molecular rearrangement of sulfatide with a consequent bilayer destabilization could occur. The increased release of CF at low pH is not due to the leakage resulting from the conversion of CF to the non-ionized form by H^+ ions because pK of CF is more than two pH units below the range studied and few, if any, pH-dependent differences were observed with EPC and the other liposome preparations used. Furthermore the results obtained with [^{14}C]glucose-loaded liposomes clearly indicate that the plasma-dependent pH sensitivity of EPC/CS liposomes is independent of the nature of the trapped molecule.

The pH sensitivity of liposomes containing sulfatide could depend on different interactions of the hydrophilic portion of this glycolipid with the plasma components as a consequence of a lowering of pH. The kinetics of CF release determined using EPC liposomes in which CS was replaced by cholesterol 3-sulfate (Fig. 2A) clearly demonstrate that the presence of a

sulfate group on the membrane surface is not the only requirement for inducing plasma dependent pH sensitivity in liposomes, therefore indicating that the possible electrostatic interactions of acidic lipids with plasma components did not play a primary role in determining the pH sensitivity observed with EPC/CS small unilamellar vesicles. Similarly the results obtained with vesicles containing cerebroside (Fig. 2B), seem to indicate that also the galactose residue by itself, and therefore the possibility of hydrogen bonds, is not sufficient to promote the plasma-dependent pH sensitivity exhibited by the EPC/CS liposomes.

Thus the increase in plasma induced leakage as a consequence of a drop in the medium pH, seems to specifically depend on the presence of sulfatide molecule in the bilayer. This behavior could be related to the glycolipid distribution in the bilayer; in fact in previous papers we demonstrated [15,16] the formation of sulfatide-enriched domains in EPC membranes for glycolipid concentrations greater than 5 mol%. The presence of sulfatide-enriched domains, which determines structural point defects at the domain boundaries as a consequence of the acyl chain heterogeneity between sulfatide and egg phosphatidylcholine [16,29], was proposed to explain the albumin promoted vesicle aggregation and fusion at acidic pH [18] as well as the increased transmembrane proton diffusion in EPC vesicles containing sulfatide [17]. Sulfatide domain formation could be the basis of the plasma-dependent pH sensitivity of EPC/CS liposomes, favoring the bilayer destabilizing interactions of some plasma apoprotein with the liposomal membrane. In order to elucidate if any one plasma components is preferentially involved in this phenomenon, the pH-dependent CF release from EPC/CS liposomes was evaluated in the presence of the various lipoprotein classes and the protein fraction of human plasma. First of all it should be noted that at both pH the incubation of EPC and EPC/CS liposomes with all the plasma fractions determines a CF release greater than that observed in the presence of whole plasma; this could be explained on the basis of a competitive effect of lipoprotein-lipoprotein collisions or lipoprotein–liposome interactions in whole plasma which could therefore reduce the amount of CF released from liposomes.

In addition a stabilization factor(s) present in whole plasma could be lost during lipoprotein preparation, in particular in the course of fraction dialysis, thus determining a greater sensitivity of EPC/CS SUV to the destabilizing effect of plasma lipoproteins and proteins.

The kinetics of CF release from EPC/CS liposomes determined with all lipoproteins at pH 6.8 were greater than the corresponding kinetics determined at pH 7.4, but only in the case of VLDL is the CF release at acidic pH 3-fold that determined at physiological pH, reaching nearly 100% release of the entrapped molecule after 45 min. The almost complete release determined at pH 6.8 with VLDL does not depend on the aggregation or fusion of liposome by themselves or with lipoprotein since the Excimer to Monomer fluorescence intensity ratio (E/M) of $P_{10}CS$ and $P_{10}PC$ inserted in EPC/CS liposomes does not decrease during the incubation of labelled liposomes with VLDL in the presence, or not, of non fluorescent vesicles (data not shown). In fact the E/M ratio of pyrene depends on the membrane concentration of the probe and should decrease if fluorescent liposomes fuse with a non fluorescent structure, with a consequent dilution of the probe in the new lipid environment.

Since the phospholipid composition of the surface envelope of different lipoprotein is very similar, it offers no explanation for the differences observed in the kinetics of CF release in the presence of lipoproteins. Therefore it could be hypothesized that the pH sensitivity exhibited by EPC/CS liposomes at pH 6.8 depends on the interaction of one or more serum apoproteins with liposomal sulfatide which can promote the destabilization of the bilayer and the increase in the CF release.

A similar mechanism has been demonstrated by Liu et al. [30]. These authors reported that the stabilizing effect of serum on dioleoylphosphatidylethanolamine (DOPE)/OA liposomes, already observed with small but not large unilamellar vesicles [13], is based on the specific interaction of $ApoA_1$ with DOPE/OA small unilamellar vesicles. Also in our case the curvature radius of vesicles could be important for the observed destabilizing effect of VLDL apoproteins. Further investigations are necessary to individuate the apoprotein involved in this mechanism and the contribution of curvature radius to the possible interactions between vesicle and apoprotein.

From our results with plasma in vitro we can predict that drug release from sulfatide containing liposomes will be significantly increased if the liposomes pass through a region with a pH lower than physiological. This property could be of great interest for some useful applications in vivo. For example, it is well known that a lowering of the pH can occur at the level of tumoral tissues, inflammation or infection sites and ischemic areas. Thus these liposomes could become a clinically relevant means of drug delivery if it will be possible to demonstrate an increased specific drug release from sulfatide containing liposome to pathological tissues.

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