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N-Palmitoylphosphatidylethanolamine stabilizes liposomes in the presence of human serum: effect of lipidic composition and system characterization

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Abstract

Liposomes containing negatively-charged phospholipid, *N*-palmitoylphosphatidylethanolamine (NPPE) were examined for stability in the presence of human serum, using the release of the entrapped 5,6-carboxyfluorescein as an aqueous marker. Either small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) were used. Incorporation of NPPE into PC SUV decreases leakage in the presence of serum or phosphate-buffered saline, no strictly related to size increase observed and to the surface negative charge present. The stabilizing effect of NPPE and Chol were synergistic. Inhibition of destabilization induced by serum of PC/Chol liposomes was observed when NPPE concentrations were above 12 mol%. Change in the membrane fluidity or incorporation of a monosialoganglioside into liposomes do not significantly change the half-life of liposomes in the presence of a high NPPE concentration. Incorporation of NPPE into PC/Chol liposomes increases membrane rigidity which does not change after serum incubation. The presence of NPPE in liposomes decreases lipid transfer/exchange between liposomes and lipoproteins although the same amount of serum proteins were incorporated as in PC/Chol liposomes. As expected, these proteins are accessible to trypsin digestion. In accordance with these results, the liposome agglutination assay shows no steric barrier activity. As a whole, the results obtained in this paper suggest a complex mechanism for stabilization of NPPE containing liposomes in human serum.

Keywords: *N*-Acylphosphatidylethanolamine; Liposome stability; Membrane fluidity; Ganglioside; Serum protein; Serum–liposome interaction

1. Introduction

The use of liposomes as delivery systems has been studied extensively in recent years due to their therapeutic efficacy and the reduction of toxicity [1,2]. However, their potential use as drug carriers by intravenous injection is limited by their low stability in the bloodstream. Firstly, phospholipid exchange and transfer to lipoproteins, mainly HDL, destabilizes and disintegrates liposomes, with subsequent loss of content [3,4]. Moreover, liposomes are eliminated from circulation by association with certain plasma proteins, called opsonins, which promotes macrophage uptake, or by direct union to the cell surface and ultimate phagocytosis by macrophages in liver and spleen [5].

The stability of liposomes has been widely studied through experiments in vitro and in vivo. This stability in the presence of serum is a first approximation to the behaviour of liposomes in biological fluids. Several laboratories have tried to obtain liposomes with long circulation time and which can be targeted to non-reticulo endothelial system tissues in a specific manner. By manipulation of some liposome properties such as lipid composition [6,7], particle size [6,7] and surface charge [8], improved results have been obtained.

Abbreviations: BSA, bovine serum albumin; CF, 5,6-carboxyfluorescein; Chol, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; GM1, monosialoganglioside; HDL, high-density lipoprotein; HPC, hydrogenated egg phosphatidylcholine; LUV, large unilamellar liposomes; MPS, mononuclear phagocytic system; NPPE, *N*-palmitoylphosphatidylethanolamine; PC, egg phosphatidylethanolamine; PS, phosphatidylserine; RES, reticulo-endothelial system; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SPH, sphingomyelin; SUV, small unilamellar liposomes; TMA-DPH, ammonium salt of DPH.

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The inclusion of lipids with long and saturated hydrocarbon chains in these liposomes, which contain cholesterol (Chol), increases their half-life and decreases the liposome leakage [4], inhibits the phospholipid exchange of liposomes with HDL [9–11] and association with serum proteins, which facilitates the uptake of liposomes by the reticulo endothelial system (RES) [12,13].

Liposomes with a shielded negative charge, like phosphatidylinositol, GM1 [12–14] or with phospholipids covalently coupled to a hydrophilic polymer poly(ethylene glycol) [15] leak less and the union of some plasma protein is impaired, therefore these liposomes can avoid uptake by the mononuclear phagocytic system (MPS) and achieve longer circulation times.

In order to find liposomes with long circulation time, we propose the introduction of a natural tricatenary, negatively-charged phospholipid, *N*-acylphosphatidylethanolamine (*N*-acylPE). This was found as minor constituent in a wide spectrum of life forms [16] including mammalian cells and tissues [17,18]. But they can accumulate under pathological conditions involving degenerative membrane changes, as in the infarcted areas of canine myocardium after coronary artery ligation [19,20]. In these tissues, *N*-acylPEs have long *N*-acyl chains, mainly saturated (16:0 and 18:0) [16].

Although there are few studies on the physical properties of *N*-acylPEs, it has been observed that a *N*-acyl chain containing 10 or more carbon atoms penetrates the bilayer and increases of the hydration degree of interfacial and phosphate regions [21–23]. This results in a bilayer stabilizing effect on unsaturated phosphatidylethanolamine which is a non-bilayer lipid [21,23,24]. In our laboratory we have also observed that incorporation of *N*-acylPE with a long *N*-acyl chain into eggPC liposomes produces a decrease in permeability due to an increase in rigidity caused by the third hydrocarbon chain, a surface modification and a size increase [25].

In this paper, we have examined the effect of incorporation of *N*-acylPE of natural origin to liposomal bilayers on serum-induced leakage of liposome contents in vitro. With these experiments, we demonstrate that the incorporation of *N*-acylPE to liposomes can provide them with a long half-life in the presence of serum due to the changes in fluidity and surface modification observed previously. Moreover, the fact that *N*-acylPE is present only as a minor component in biological membranes suggests that its exchange may be limited which would increase stability. *N*-PalmitoylPE (NPPE) was used for several reasons. Palmitoyl is the *N*-acyl fatty acid found in greatest proportion and this lipid can form stable closed vesicles with a size near 100 nm [25]. We have tested the effect of incorporation of NPPE in small unilamellar liposomes (SUV) and in large unilamellar liposomes (LUV), based on eggPC. We have also tested the effect of incorporation of GM1, which helps to avoid uptake by the RES, and the importance of system fluidity.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (PC), transphosphatidylated egg yolk phosphatidylethanolamine, hydrogenated egg yolk phosphatidylcholine (HPC), *N*-biotinylPE and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids. Bovine brain sphingomyelin (SPH), bovine brain monosialoganglioside (GM1) and bovine serum albumin (BSA) and streptavidin were from Sigma and cholesterol (Chol), palmitoyl chloride and TLC plates were from Merck. 6(5)-Carboxyfluorescein (CF), from Eastman-Kodak, was purified as described elsewhere [26]. Ficoll, Sephadex-G50, SDS-PAGE gel electrophoresis and the molecular weight standards for electrophoresis were from Pharmacia. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and its ammonium salt (TMA-DPH) were obtained from Molecular Probes. All inorganic reagents were analytical grade and aqueous solutions were made up with double distilled water and deionized water. Human serum was obtained from clotted transfusion blood obtained from the Sección de Hematología of Clinica Asepeyo, Barcelona. Before use the serum was clarified by centrifugation for 2 min in a Heraeus microfuge.

2.2. Synthesis and characterization of NPPE

NPPE was synthesized from transphosphatidylated egg yolk phosphatidylethanolamine and purified as described elsewhere [25]. The final product was characterized by proton-NMR, IR spectroscopy and fatty acid composition (data not shown). Lipid concentrations were determined by phosphorus analysis [27].

2.3. Preparation of unilamellar vesicles

The lipid films were hydrated in 2 ml of phosphate-buffered saline pH 7.4 (PBS) or of CF 50 mM in PBS (the osmolarity of the CF solution was adjusted to 320 mosM/kg with PBS, pH 7.4). Small unilamellar vesicles (SUV) were prepared by sonication of a lipid suspension following a standard procedure [25]. In order to obtain vesicles of a definite size, the lipid suspensions were extruded through polycarbonate filters of definite size (manufactured by Nuclepore) using an extrusion device (Lipex Biomembranes) [28]. Non-encapsulated CF was separated from liposomes on a spin column of Sephadex G-50 medium with isotonic PBS as eluent. Liposomes of different composition were characterized by particle size analysis (determined by dynamic laser light scattering using a PCS41 optics unit, Malvern Autosizer IIC) and entrapped volume determination in l/mol lipid (obtained by the ratio of fluorescence of encapsulated CF in non-

quenching conditions and lipid concentration quantified by phosphorus analysis [29]).

2.4. Liposome stability assay

An aliquot (40–50 μl) of CF-liposomal suspension (50–120 nmol as total lipids) was added to 450 μl of prewarmed (37° C) human serum (lipid/serum ratio of 0.1 μmol/ml) or PBS (pH 7.4). At different times, 10 μl of the suspensions were diluted 200 times with PBS and the fluorescence intensity (λ_{ex} = 492 nm and λ_{em} = 520 nm) was measured using a Kontron SFM25 spectrofluorimeter before and after liposome lysis. To lyse liposomes, 100 μl of 5% deoxycholate was added. Low liposome concentrations were chosen in order to approximate the ratio of liposomes to the serum components which would be expected if liposomes were injected in vivo [14].

Release of the entrapped dye from liposomes was calculated from the equation described by Liu and Huang [30]. Half-lives ($T_{1/2}$) for CF leakage in the presence or absence of human serum were determined by the technique of Allen and Cleland [31]. The kinetics of leakage was linear on a semi-logarithmic plot for all compositions assayed.

2.5. Association of serum components with liposomes

To determine serum protein binding to liposomes, we used a method described by Liu and Huang [30]. Unilamellar vesicles without CF and extruded through 0.1 μm pore size were used. Briefly, aliquots of liposomes (5 μmol of lipids) were incubated with the same volume of human serum at 37° C for 1 h. The unbound proteins was separated on discontinuous gradient of Ficoll (in PBS, pH 7.4) by centrifugation in a Beckman L-70 ultracentrifuge with a swinging-bucket 40-TI rotor for 1 h at 10 000 rpm at room temperature. The liposomes which floated at the PBS/15% Ficoll interface were collected and subjected once more to an identical procedure. In the collected suspension the protein concentration was determined using the BCA protein assay (Pierce) with BSA as standard. As control, in all our experiments human serum without liposomes was treated in the same way. The absorbance of the blanks was always lower than 15% of that of the samples. The same samples were used for analysis of proteins bound to liposomes. The proteins were solubilized [32] and subjected to SDS-PAGE electrophoresis, using 7.5% polyacrylamide under denaturing conditions [33] in a Phast-System electrophoretic apparatus (Pharmacia LKB Biotechnology), followed by silver staining. Prestained SDS-PAGE molecular weight standards were used to estimate the molecular weights of the proteins. Lipid compositions of incubated liposomes were examined by extraction of the lipids [34] followed by TLC analysis on silica gel plate using chloroform/methanol/water (65:25:4) as developing solvent. The individual phospholipid content was determined by a phosphorus assay [27] and the cholesterol was quantified using the Boehringer-Mannheim Kit based on an enzymatic method (Chol oxidase) [35].

2.6. Interaction of serum components with liposomes

Liposomes treated in the same way as those in the preceding section were separated from excess of serum proteins by chromatography on Bio-Gel A-15M, 200–400 mesh size (Bio-Rad), in spin columns as described by Chonn et al. [36]. 100 μl of liposome/serum incubation mixtures was then applied and immediately centrifuged (2000 rpm, 1 min at 4° C). Column fractions were collected in glass tubes by applying 100 μl of PBS. The liposomes, usually, eluted within the first 2–5 fractions and did not present serum protein contamination. The column fractions were analyzed for protein content as described above.

Aliquots of serum-treated liposomes (0.26 μmol in 0.2 ml of PBS) were further incubated with equal volume of PBS containing trypsin (0.26 mg/ml) for 1 h at 37° C. Trypsin inhibitor (0.35 mg/ml final) was added to stop the reaction. Liposomes were chromatographed as described, to remove free proteins.

Aliquots of serum-treated and trypsin-treated liposomes were diluted to a final concentration of 0.25 mg/ml and labelled with DPH and TMA-DPH for 1 h at 40° C. The molar ratio of probes to lipid was 1:375. Fluorescence depolarization of probes in liposomes was measured by a similar method to that described by Shinitzky and Barenholz [37]. The fluorescence was measured with excitation at 360 nm and emission at 430 nm. In all experiments, corrections of light-scattering blank were made by measuring the excitation/emission ratio in the absence of the fluorophore.

2.7. Agglutination assay

Large unilamellar vesicles were prepared from PC/Chol/NAPE (with different molar ratio) and 2.5% *N*-biotin-cap-PE. 50 μl liposomes (1 mg/ml) were mixed with 0.5 ml PBS in a microcuvette and 5 μg streptavidin was added. Increase in turbidity was monitored as the optical density at 440 nm [8].

3. Results

The stability of the liposome preparations used was tested by photon correlation spectroscopy. The liposome preparations, stored at 4° C, were stable for at least a week without any change in their structure (mean diameter and size distribution), which is the longest time for which they were used (data not shown).

Table 1
Effect of incorporation of NPPE, Chol or their combination on the stability of PC-based liposomes

Lipid composition (molar ratio)	$T_{1/2}$ (h) [a]		D_{mean} [a] (nm)	Trapped volume [a] (l/mol)
	buffer	serum		
PC	15.4 ± 1.7	0.4 ± 0.1	40.2 ± 3.5	0.43 ± 0.08
PC/Chol 2:1	46.8 ± 4.7	10.4 ± 2.3	56.1 ± 2.4	0.62 ± 0.14
PC/NPPE 10:1	30.7 ± 2.2	0.7 ± 0.1	43.5 ± 3.0	0.48 ± 0.12
PC/NPPE 3:1	45.4 ± 4.9	13.7 ± 2.9	58.2 ± 2.5	0.65 ± 0.13
PC/Chol/NPPE 12:7:2	79.2 ± 3.3	48.7 ± 5.0	73.4 ± 5.8	0.87 ± 0.16
PC/Chol/NPPE 6:4:2	89.0 ± 4.7	111.8 ± 13.5	91.6 ± 6.5	1.10 ± 0.22
PC/Chol/NPPE 4:3:2	94.7 ± 6.0	116.0 ± 14.3	98.4 ± 6.9	1.36 ± 0.19

[a] Results are expressed as means ± S.D. ($n \geq 5$), n = number of assays.

3.1. Effect of NPPE on the stability of liposomes in human serum

Stability studies were performed in vitro in buffer (PBS) or in the presence of human serum at 37° C at lipid/serum ratio of 0.1 μmol lipid/ml serum. As reference system we used SUV of PC, well characterized in the bibliography [4,14]. As expected, the leakage of aqueous solute from liposomes containing only PC was higher in the presence of serum than in buffer (Table 1). $T_{1/2}$ for leakage of CF from SUV composed by PC/NPPE with different molar ratios was determined (Table 1). Incorporation of 9 mol% NPPE to PC liposomes decreased the leakage 2-fold in the presence of serum or buffer. Increase in NPPE concentration up to 22 mol% raised $T_{1/2}$ 28-fold in serum, but the increase was less marked in buffer. This stabilization is higher than observed for 33 mol% Chol (Table 1), in accord with previous studies [4,31].

Incorporation of 33 mol% Chol and 10 mol% negative charge improved stability of liposomes in biological fluids [31,38]. Thus, we tested liposomes containing Chol and we added NPPE as a negatively-charged phospholipid. For a molar ratio PC/Chol/NPPE 10:5:1, we obtained a longer $T_{1/2}$ in buffer than in binary formulations (Table 1). A more marked stabilization effect was observed in the presence of serum, and the leakage from PC liposomes decreased 45-fold. As expected [14,25], the incorporation of NPPE or Chol to PC liposomes increased the trapped volumes (Table 1). No differences were observed in trapped volumes of PC/Chol/NPPE 10:5:1, PC/Chol 2:1 and PC/NPPE 10:1 vesicles. These results were confirmed by quasi elastic light scattering (Table 1).

The effect of increasing NPPE concentration on the leakage of CF from SUV containing Chol was assessed next. In PBS, the incorporation of increasing amounts of NPPE resulted in a progressive increase in $T_{1/2}$ but from 10 mol% NPPE no significant improvement was observed (Table 1). In the presence of serum, optimal values were obtained when the molar fraction of NPPE was 15–22% (Table 1). Incorporation of increasing amounts of NPPE considerably enhanced liposome entrapped volumes and these values were in agreement with the sizes obtained by dynamic light scattering (Table 1).

Increase in liposomes size produces a fall in permeability and makes liposomes less susceptible to the action of serum components [39]. The $T_{1/2}$ of liposomes prepared by the extrusion method increases as size increases, at least up to 200 nm, and were longer than those obtained for SUV (Tables 1 and 2).

In order to determine whether the leakage decrease from liposomes containing NPPE was attributed only to its negative charge, we replaced NPPE by other lipids with negative charge like GM1 or phosphatidylserine (PS), which stabilize liposomes in vitro [14]. No difference was observed in buffer; GM1 and NPPE showed a similar effect and PS had a smaller stabilizing effect on liposomes

Table 2
Importance of negative charge and liposome size on the stability of NPPE liposomes

Lipid composition (molar ratio)	$T_{1/2}$ (h) [a]		D_{mean} [a] (nm)	Trapped volume [a] (l/mol)
	buffer	serum		
PC/Chol/NPPE 10:5:1	69.9 ± 8.1	27.5 ± 3.4	59.7 ± 3.8	0.66 ± 0.14
PC/Chol/PS 10:5:1	46.7 ± 7.6	10.1 ± 1.1	n.d.	0.53 ± 0.12
PC/Chol/GM1 10:5:1	55.7 ± 6.6	12.5 ± 0.8	n.d.	0.66 ± 0.11
PC/Chol 2:1				
LUV 0.1 μm	90.8 ± 7.3	36.9 ± 5.0	104 ± 7.5	1.39 ± 0.16
PC/Chol/NPPE 10:5:1				
LUV 0.1 μm	113.6 ± 9.5	68.6 ± 5.0	96.6 ± 4.5	1.52 ± 0.13
LUV 0.2 μm	113.1 ± 6.6	88.3 ± 5.0	133.6 ± 5.1	2.57 ± 0.22
LUV 0.4 μm	96.5 ± 8.9	81.6 ± 6.1	314.4 ± 9.9	3.45 ± 0.19

[a] Results are expressed as means ± S.D. ($n \geq 5$), n = number of assays.

Table 3
Modulation of NPPE containing liposomes stability related to membrane fluidity and presence of GM1

Lipid composition (molar ratio)	$T_{1/2}$ (h)		D_{mean} (nm)	Trapped volume (l/mol)
	buffer	serum		
HPC/Chol/NPPE 4:3:2	47.0 ± 3.6	67.1 ± 10.0	n.d.	n.d.
SPH/Chol/NPPE 4:3:2	95.2 ± 15.0	116.6 ± 13.5	94.6 ± 3.0	1.38 ± 0.15
PC/SPH/Chol/NPPE 2:2:3:2	97.5 ± 6.6	125.1 ± 11.7	84.0 ± 2.2	0.93 ± 0.11
PC/SPH/Chol/NPPE 5:5:5:1	52.9 ± 5.6	60.3 ± 2.3	58.5 ± 3.4	0.60 ± 0.08
PC/Chol/NPPE/GM1 10:5:1:1	54.1 ± 6.0	40.7 ± 3.6	57.9 ± 3.0	0.65 ± 0.10
PC/Chol/NPPE/GM1 4:3:2:1	96.0 ± 14.1	122.2 ± 17.9	86.1 ± 3.4	0.94 ± 0.12

[a] Results are expressed as means ± S.D. ($n \geq 5$), n = number of assays.

than the other two negatively-charged lipids. However, in the presence of serum liposomes containing NPPE had a higher $T_{1/2}$ than liposomes with GM1 or PS (Table 2).

3.2. Effect of membrane fluidity and presence of GM1 on the stability of NPPE containing liposomes

Liposomes composed by hydrogenated eggPC (HPC), Chol and NPPE, in a 4:3:2 molar ratio, showed a higher leakage than PC/Chol/NPPE 4:3:2 vesicles in buffer and in serum (Tables 1 and 3). This could be related to these liposomes are in a gel phase at 37° C and some membrane imperfections which decrease their stability can appear. When we used sphingomyelin (SPH) as rigidifying component, the total or partial substitution of PC by SPH in PC/Chol/NPPE 4:3:2 liposomes had no significant effect on leakage (Table 3). However, when the NPPE concentration was lower (6.25 mol%), incorporation of SPH in a molar ratio SPH:PC 1:1 had a greater stabilizing effect in the presence of serum. The SPH effect was not related to size modifications since no changes in trapped volumes or in liposome size were observed (Table 3).

It has been shown that the incorporation of GM1 stabilizes liposomes in vitro [14] and in vivo [12,38]. In buffer, the $T_{1/2}$ values obtained in the presence of GM1 were similar to those obtained in its absence (Table 3). In serum, the incorporation of 6 mol% GM1 to liposomes containing 6 mol% NPPE enhanced $T_{1/2}$ 1.5-fold. The increase was lower when GM1 was incorporated to liposomes with a higher NPPE concentration (Table 3).

3.3. Interaction of liposomes with serum components

NPPE may reduce the serum induced destabilization of liposomes by providing a steric barrier due to a higher hydration of headgroup [22,23]. To test this hypothesis, we have studied the agglutination, induced by streptavidin, of liposomes containing biotin-cap-PE measuring the increase of sample turbidity. The agglutination requires a close apposition of the neighbouring liposomes and thus the decrease in turbidity, compared to control liposomes, directly reflects the degree of steric barrier produced on the liposome surface. These experiments (Fig. 1) show that introduction of NPPE (6% or 22%) in PC/Chol 2:1 liposomes does not inhibit liposome agglutination. However GM1, as was described previously [8], produced an important decrease in turbidity.

The binding of serum proteins to the liposome surface depends on the liposome composition and affects liposome stability [30,32,40]. Liposomes of different lipid compositions (PC, PC/Chol 2:1, PC/Chol/NPPE 4:3:2) were incubated for 1 h with serum at 37° C and isolated from other serum components on a discontinuous Ficoll gradient and analyzed.

Different amounts of protein were bound to liposomes (Table 4). As expected [40]), the presence of Chol de-

Table 4
Influence of lipid composition on protein binding and membrane fluidity of serum-treated liposomes

Lipid composition (molar ratio)	Liposome treatment	Protein/lipid ratio [a] (mg protein/mg lipid)	Fluorescence anisotropy [a]	
			DPH	TMA-DPH
PC	PBS	n.d.	0.067 ± 0.003	0.212 ± 0.007
	serum	0.142 ± 0.025	0.069 ± 0.005	0.215 ± 0.006
	serum then trypsin	0.023 ± 0.006	0.071 ± 0.003	0.214 ± 0.005
PC/Chol 2:1	PBS	n.d.	0.148 ± 0.001	0.227 ± 0.008
	serum	0.097 ± 0.013	0.148 ± 0.001	0.243 ± 0.007
	serum then trypsin	0.019 ± 0.009	0.153 ± 0.005	0.237 ± 0.006
PC/Chol/NPPE 4:3:2	PBS	n.d.	0.167 ± 0.002	0.225 ± 0.004
	serum	0.094 ± 0.011	0.170 ± 0.003	0.232 ± 0.006
	serum then trypsin	0.022 ± 0.006	0.182 ± 0.004	0.242 ± 0.008

[a] Results are expressed as means ± S.D. ($n \geq 3$), n = number of assays.

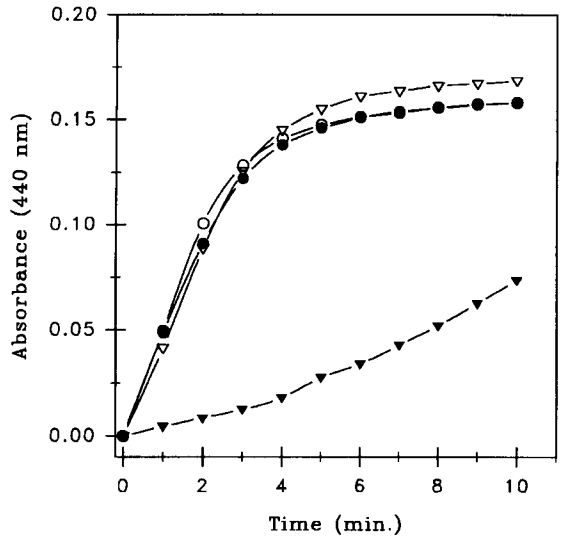

Fig. 1. Effect of NPPE on the streptavidin-induced agglutination of liposome containing *N*-biotinyl-PE. The turbidity increase (absorbance at 440 nm) was measured with time for PC/Chol 2:1 (○), PC/Chol/NPPE 10:5:1 (▽), PC/Chol/NPPE 4:3:2 (●) and PC/Chol/GM1 10:5:1 (▼) liposomes.

creases the bound protein and the protein/lipid ratio was about 66% of that obtained for PC-liposomes. The incorporation of NPPE does not significantly change the protein bound to liposomes. The profile of protein associated with liposomes depends only slightly on the liposome composition, in the electrophoretic conditions used (data not show).

On the other hand, lipid exchange and/or transfer between liposomes and other serum components, especially HDL, can result in loss of encapsulated material, liposome disintegration, or both [4]. Lipids of serum-treated liposomes were separated by thin-layer chromatography and compared with those of non-treated liposomes. In every sample assessed, the lipid composition of serum-treated liposomes was more complex than the original composition. In comparison with the lipids extracted from serum, the chromatogram reveals that SPH, lysoPC and neutral lipids are incorporated to liposomes (data not shown). With PC/Chol 2:1 liposomes, a decrease in the lipid molar ratio after serum incubation was observed (PC/Chol = 1.6). This fact was also observed in the case of PC/Chol/NPPE 4:3:2 liposomes. The PC/NPPE molar ratio was 1.7 and the PC/Chol ratio was 1.2, and the NPPE/Chol ratio remained constant at 0.65. This result was in agreement with PC being the phospholipid which mainly exchanges/transfers. The ratio of the total phospholipids to (SPH + lysoPC) incorporated to serum-incubated liposomes increases in order of PC < PC/Chol 2:1 < PC/Chol/NPPE 4:3:2. It seems that the presence of NPPE decreases the net transfer of serum phospholipids.

The association of proteins with liposomes could modify the membrane fluidity and its the stability in the presence of serum, as has been observed for dioleolylphosphatidylethanolamine/oleic acid liposomes [30,41]. We examined this possibility by measuring the fluorescence depolarization of DPH, in order to determine whether any serum protein was inserted in the hydrophobic area and altered the bilayer fluidity. As expected, Chol and NPPE increase the values of the anisotropy of non-treated PC liposomes (Table 4). We obtained similar anisotropy values for liposomes before and after incubation with serum, in every lipid composition assessed. Fluorescence depolarization of TMA-DPH was also measured to determine whether proteins were inserted in a more hydrophilic area (Table 4). No change between liposomes incubated or before incubation were observed. We have also incubated these liposomes with trypsin and determined the protein bound. This treatment removed the majority of proteins originally associated with liposomes and the anisotropy of membrane was unaltered, as expected (Table 4).

4. Discussion

Liposomes have been proposed as useful drug delivery systems. They are biodegradable and produce minimal toxicity, but they can be destabilized in biological fluids and eliminated from the bloodstream by MPS [12]. Lipid composition influences the stability of liposomes in vitro [14] and in vivo [8,42].

In the present work, we studied the inclusion of NPPE in the bilayer composition with the aim of obtaining liposomes with a high half-life. In vitro, addition of NPPE to PC liposomes gives a stabilizing effect, which is greater in serum, where PC liposomes are rapidly destabilized (Table 1). Increasing the NPPE molar fraction in PC liposomes decreases the leakage from them (Table 1). Besides, addition of 25 mol% NPPE to PC liposomes can produce a similar decrease in serum-induced leakage to that produced by addition of 33 mol% Chol which has been widely used to stabilize liposomes [30,31]. Chol and NPPE have a synergistic effect at the molar ratio of PC/Chol/NPPE 10:5:1. In PC/Chol/NPPE liposomes, $T_{1/2}$ in buffer and in the presence of serum increases when NPPE concentration rises up to 22 mol%. This increase is hyperbolic in buffer and sigmoidal in serum. $T_{1/2}$ at the saturation point was higher in serum than in buffer (data not shown). We did not test NPPE higher concentrations since a excess of negatively charged surface is not advisable [14].

The stabilization of NPPE-containing liposomes in serum can be attributed to different factors such as changes in fluidity, vesicle size and vesicle surface [25]. It could also be related to the limited exchange of this liposomes with HDL due to this fact that *N*-acylPE is found in small amounts in vivo [16].

NPPE produces an increase in liposome rigidity as demonstrated by fluorescence polarization of DPH (Table 4). This rise in rigidity is due to the long saturated *N*-acyl chain of NPPE which penetrates deeply into the hydropho-

bic moiety, decreases the mobility of chains and provides a decrease in permeability [25]. The no increase in the half-life of liposomes containing 22 mol% NPPE by inclusion of more rigid lipids (HPC and SPH) suggest an optimal membrane fluidity since stabilization effect by inclusion of SPH was observed at lower molar fraction of NPPE (Table 3).

In accordance with previous results, the susceptibility of liposomes to the action of serum components decreases when liposome size increases [39] so the $T_{1/2}$ of PC/Chol/NPPE 10:5:1 liposomes prepared by the extrusion method increases as size increases (Table 2). However, the stability of NPPE containing liposomes cannot be only assigned to the size increase, since PC/Chol/NPPE 10:5:1 LUV 100 nm have a higher half-life than PC/Chol LUV 100 nm even though they have similar size (Table 2) and both have half-life lower than sonicated liposomes containing 22 mol% NPPE and sized near 100 nm, which is considered a suitable size for in vivo studies [13] (Table 1).

The surface charge is an important parameter which contributes to liposome stability [43,44]. The importance of the negative charge of NPPE has been pointed out since we observed that the introduction of NPPE into PC/Chol liposomes allows to achieve a longer half-life in vitro than that of a negatively-charged PS or a shielded negative-charged GM1 (Table 2). NPPE does not have a bulky group to protect the negative charge but this phospholipid presents the phosphate group highly hydrated [22]. The possibility of a steric barrier presented by NPPE is eliminated since no inhibition of the agglutination of liposomes containing biotin-cap PE, mediated by streptavidin, was observed in liposomes containing NPPE (Fig. 1). The absence of a steric barrier does not rule out the possibility that NPPE containing liposomes could be useful for in vivo applications, as has been demonstrated for GM1 [45]. Moreover, the surface modification produced by NPPE could maybe modify the interaction with serum components [7].

The exchange/transfer of phospholipids between liposomes and lipoproteins produce the incorporation to liposomes of SPH and lyso-PC, which decreases by the presence of Chol and NPPE (data not shown). NPPE/Chol ratio does not change after serum incubation, which indicates that probably neither NPPE nor Chol transfer has been produced. This could be another factor which contributes to the stabilization of liposomes containing NPPE.

The interactions with serum proteins can affect the stability of liposomes [46,47]. In our case, the introduction of Chol into PC liposomes decreases the association of protein by 33% (Table 4), in accord with previous studies [3,40]. The same amount and electrophoretic profile of proteins bound to PC/Chol 2:1 liposomes has been observed in PC/Chol/NPPE liposomes (Table 4 and data not shown), however these liposomes are more stable. These proteins associated with liposomes are accessible to trypsin and no increase in membrane rigidity, either in the hydrophobic moiety, measured by DPH, or in the more hydrophilic area, measured by TMA-DPH, was observed after serum incubation (Table 4). Therefore, these proteins do not seem responsible for stabilization of liposomes containing NPPE. However, we do not rule out the possibility that some low molecular proteins, which are not detected in our electrophoretic conditions, could be the responsible for the stabilization of liposomes containing NPPE.

In summary, we have obtained a ternary liposome formulation PC/Chol/NPPE including 10–22 mol% of NPPE which is greatly stable in the presence of serum. The reason for this stabilization is not attributed to a single factor because probably different parameters described in this paper are involved. In forecoming studies, we will test the behaviour of these liposomes in vivo to assess that this liposome formulation is useful as a drug carrier system. In this context, we have observed that the introduction of GM1 has no important effect on $T_{1/2}$ in vitro at a higher molar fraction of NPPE (Table 3). Therefore, if NPPE liposomes do not evade RES, the incorporation of GM1 which produces a modification in the liposome surface would permit the evasion of RES [48].

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