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pH sensitivity and plasma stability of liposomes containing *N*-stearoylcysteamine

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Abstract

In this study, we investigated the pH sensitivity of different liposomal formulations containing 10 mol% *N*-stearoylcysteamine, as pH sensitive molecule. Liposome stability was monitored by determining the release of different entrapped water soluble molecules, 5,6-carboxyfluorescein (CF) being the marker of leakage mainly used. Small unilamellar vesicles composed of egg phosphatidylcholine (EPC) and *N*-stearoylcysteamine (9:1 molar ratio) incubated at 20°C in citrate phosphate buffer released, at pH 6.8, 2.5 fold the amount of CF released at pH 7.4. The addition of plasma to the incubation medium and an increase of temperature to 37°C led to significantly increased the CF release from EPC/*N*-stearoylcysteamine SUV, both at pH 7.4 and 6.8. The addition of cholesterol had a stabilizing effect on liposomal vesicles with respect to both temperature and plasma, without affecting pH sensitivity. In fact, at 37°C and in 25% plasma the ternary mixture showed the highest CF release, as a consequence of the moderate acidification of the medium from 7.4 to 6.8. Thus, these liposome formulations are potentially a useful tool for specific drug delivery to pathological tissues such as tumours, inflammation sites and ischemic areas where it is known that a lowering of the pH can occur. © 1997 Elsevier Science B.V.

Keywords: *N*-Stearoylcysteamine; Liposome; Drug carrier; pH sensitivity

1. Introduction

The general strategy for constructing pH-sensitive liposomes has been to include in the bilayer lipids containing pH-sensitive groups such *N*-palmitoylhomocysteine (PHC) [1–3], cholesterylhemisuccinate [4], oleic acid (OA) with phosphatidylethanolamine (PE) as auxiliary lipid [5–7], *N*-succinyldioleoyl phosphatidylethanolamine [8], diacylsuccinylglycerols [7,9] and tocopherol hemisuccinate [10]. These molecules have the ability to destabilize the lipid bilayer when exposed to an acidic environment, with a consequent release of water soluble trapped molecules.

Although the potential use of pH-sensitive liposomes remains to be evaluated, in vitro studies [1,11–15] indicate that they are effective in enhancing the cytoplasmatic delivery of trapped molecules to target cells, as a consequence of their association with the acidic environment of cellular endosomes and lysosomes.

An important application of pH sensitive liposomes is represented by their possible use for selective drug release to those pathological tissues such as tumours, metastases, inflammation sites and ischemic

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areas that have an ambient pH considerably lower than that of normal tissues.

The in vivo application requires the definition of pH sensitive formulations relatively stable in circulating fluids at physiological pH, but able to release encapsulated drugs in response to an acidification of surrounding medium [9,16]. The use of amphipatic molecules containing SH as the pH sensitive group was widely investigated [1–3,17], but unfortunately many of the reported pH sensitive liposomal formulations are not sufficiently stable and/or do not maintain the pH sensitivity in plasma. In this study, we investigated the pH sensitivity of different liposomal formulations containing 10 mol% *N*-stearoylcysteamine as the pH sensitive molecule, using different water soluble molecules as markers of leakage.

For liposomal formulations that exhibited a marked pH sensitivity in buffer, we also evaluated the plasma stability at pH 7.4, together with the capacity to maintain a sensitivity to moderate plasma pH drop (i.e., 6.8).

2. Materials and methods

2.1. Chemicals

Analytical grade chemicals, distilled solvents and doubly distilled water were used. Egg phosphatidylcholine, cholesterol, dipalmitoylphosphatidylcholine, 5,6-carboxyfluorescein, calcein, and NADH were purchased from Sigma, Sepharose 4B from Pharmacia, 1-palmitoyl-2-(10-pyrene)decanoyl phosphatidylcholine and 12-(1-pyrene)dodecanoic acid were obtained from Molecular Probe. D-^{14}C-glucose and ^{3}H-inulin from Amersham and the liquid scintillation counting mixture (Ultimagold) from Packard.

2.1.1. Chemical syntheses

General. All melting points are uncorrected. ^{1}H-NMR spectra (500 MHz) were recorded on a Bruker AM-500 instrument in $CDCl_3$ and are reported in *d* units relative to $CHCl_3$ fixed at 7.24 ppm. TLC was performed on precoated silica gel G plates (Merck, HF_{254}); spots were visualised by spraying with 70% sulphuric acid and heating.

2.1.2. Synthesis of the N-stearoylcysteamine (*1*)

A mixture of cystamine dihydrochloride (4.5 g, 20 mmol), stearoyl chloride (12.12 g, 40 mmol), 4-(*N,N*-dimethylamino)pyridine (0.5 g) and pyridine (100 ml) was stirred at room temperature for 24 h. The reaction mixture was then poured into a stirred mixture of brine (100 g) and aqueous HCl (250 ml, 6 M). The resulting solid was filtered, washed with water and dried. Crystallisation from ethanol afforded the pure *N,N*-distearoylcystamine (**2**) (9.86 g, 72% yield): mp 132–133°C; TLC (eluent CH_2Cl_2–MeOH 100:5 v:v), $R_f = 0.38$; ^{1}H-NMR: δ 6.17 (2H, t, $J = 6.0$ Hz, $2 \times$ NH), 3.55 (4H, dt, $J = 6.0$ and 6.0 Hz, $2 \times$ NH–CH_2–), 2.81 (4H, t, $J = 6.0$ Hz, $2 \times$ –CH_2–S), 2.19 (4H, t, $J = 7.5$ Hz, $2 \times$ –CH_2CO) and 0.86 (6H, t, $J = 7.0$ Hz, $2 \times$ –CH_3). Anal. Calcd. for $C_{40}H_{80}N_2O_2S_2$: C, 70.12; H, 11.77; N, 4.09. Found: C, 69.76; H, 11.52; N, 4.21.

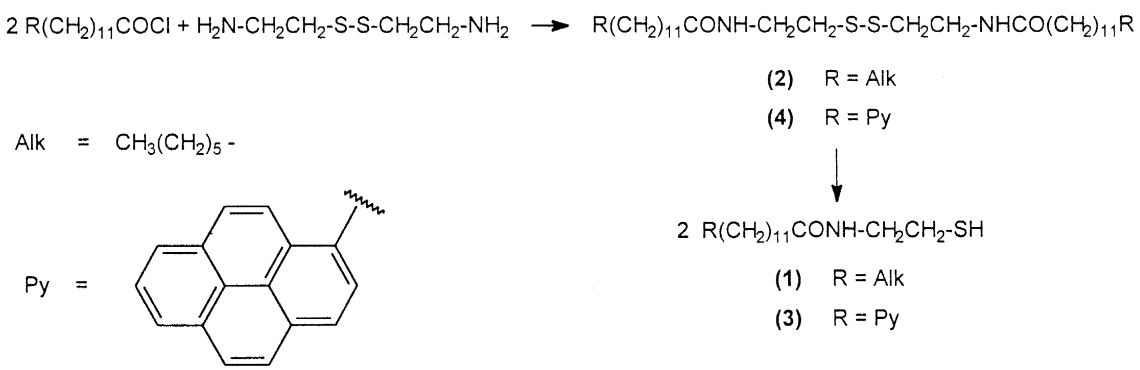
A mixture of compound **2** (5 g, 7.3 mmol), acetic acid (250 ml) and zinc dust (5 g) was refluxed under vigorous stirring for 30 min. At this time it was cooled to room temperature and 1,2-dichloroethane (250 ml) was added. The resulting mixture was filtered and the solution was evaporated under reduced pressure to afford a residue which was tritured with MeOH–H_2O (100 ml 1:1, v:v). The suspension was filtered and the crude product obtained was crystallised from ethanol to give 4.2 g (84% yield) of the *N*-stearoylcysteamine (**1**): mp 88–90°C; TLC (eluent CH_2Cl_2–MeOH 100:5 v:v), $R_f = 0.52$; ^{1}H-NMR: δ 5.80 (1H, t, $J = 6.0$ Hz, NH), 3.42 (2H, dt, $J = 6.0$ and 6.0 Hz, NH–CH_2–), 2.65 (2H, dt, $J = 8.5$ and 6.0 Hz, –CH_2–SH), 2.17 (2H, t, $J = 7.5$ Hz, CH_2CO), 1.31 (1H, t, $J = 8.5$ Hz, SH) and 0.86 (6H, t, $J = 7.0$ Hz, –CH_3); mass spectrum: m/z 343 (1.76, M^+), 284 (12.50), 267 (1.58), 171 (1.34), 149 (8.64), 137 (7.98) and 119 (15.5). Anal. Calcd. for $C_{20}H_{41}NOS$: C, 69.91; H, 12.03; N, 4.08. Found: C, 70.12; H, 11.87;, N, 3.95.

2.1.3. Synthesis of N-[12-(1-pyrenyl)dodecanoyl]cysteamine (*3*)

A solution of 12-(1-pyrenyl)dodecanoic acid (20 mg) in thionyl chloride (1 ml) containing dimethylformamide (10 μl) was refluxed for 1 h and then evaporated under reduced pressure. The residue was dissolved in toluene (2 ml) and evaporated under

reduced pressure twice for a complete removal of thionyl chloride. The residue was dissolved in pyridine (1 ml) containing 4-(*N*,*N*-dimethylamino)pyridine (2 mg) and reacted with cystamine dihydrochloride (20 mg). The solution was stirred at room temperature for 48 h and then poured into a stirred mixture of brine (1 g) and aqueous HCl (2.5 ml, 6 M). Extraction with AcOEt, drying (Na_2SO_4) and removal of the solvent under reduced pressure afforded a crude product which was purified by TLC (eluent CH_2Cl_2–MeOH 100:10 v:v, $R_f = 0.55$), to give the *N*,*N*-di-[12-(1-pyrenyl)dodecanoyl]cystamine (**4**) (15 mg): ^{1}H-NMR: δ 8.96 (2H, d, $J = 9.5$ Hz), 8.67 (2H, d, $J = 8.3$ Hz), 8.31–8.26 (6H, overlapping), 8.195 (2H, d, $J = 9.0$ Hz), 8.19 (2H, d, $J = 7.5$ Hz), 8.085 (2H, t, $J = 7.5$ Hz), 8.08 (2H, d, $J = 9.0$ Hz), 6.17 (2H, t, $J = 6.0$ Hz), 3.55 (4H, dt, $J = 6.0$ and 6.0 Hz,), 2.81 (4H, t, $J = 6.0$ Hz,) and 2.19 (4H, t, $J = 7.5$ Hz).

A stirred mixture of compound **4** (10 mg), acetic acid (1 ml) and zinc dust (20 mg) was refluxed for 30 min, cooled to room temperature, diluted with dichloroethane (2 ml), filtered and evaporated to dryness. The residue was purified by TLC (eluent CH_2Cl_2–MeOH 100:5 v:v, $R_f = 0.45$) to afford 6 mg of *N*-[12-(1-pyrenyl)dodecanoyl]cysteamine (**3**); ^{1}H-NMR: δ 8.97 (1H, d, $J = 9.5$ Hz), 8.69 (1H, d, $J = 8.2$ Hz), 8.34–8.29 (3H, overlapping), 8.22 (2H, d, $J = 8.5$ Hz), 8.12 (1H, d, $J = 8.5$ Hz), 8.11 (1H, t, $J = 7.5$ Hz), 5.80 (1H, t, $J = 6.0$ Hz), 3.42 (2H, dt, $J = 6.0$ and 6.0 Hz), 2.65 (2H, dt, $J = 8.5$ and 6.0 Hz), 2.17 (2H, t, $J = 7.5$ Hz).



2.2. Preparation of liposomes

Small unilamellar vesicles (SUVs) of egg phosphatidylcholine (EPC), dipalmitoylphosphatidylcholine (DPPC) or EPC/cholesterol containing or not 10 mol% of *N*-stearoylcysteamine were prepared by sonication as previously described [18]. Large unilamellar vesicles (LUVs) of the same lipid composition were prepared by reverse-phase evaporation as described by Szoka et al. [19].

2.3. Light scattering measurements

Light scattering experiments were carried out with a Jasco FP777 spectrophotofluorimeter. The Raleigh peak from unpolarized incident light at 450 nm was measured to monitor the 90° light scattering of lipid suspension. The data are expressed as A/A_0, where A_0 is the light scattering of reference sample and A that of the analysed sample.

2.4. Trapped volume determinations

The volumes of internal, solute available, aqueous compartments of 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 (or other water soluble molecules) and gel filtration through a $1,5 \times 18$ Sepharose 4B column at room temperature using as elution buffer 10 mM citric acid, 20 mM Na_2HPO_4, 154 mM NaCl pH 7.4. Trapped CF was determined fluorimetrically at 520 nm with excitation at 490 nm, after the rupture of chromatographed vesicles with SDS 1% (w/v) final concentration.

The molar concentration of phospholipids in the vesicle sample was determined according to Bartlett [20]; the molar concentration of cholesterol was determined using a reagent kit for Boehringer Mannheim.

The trapped aqueous volume was calculated as:

$$V_t = (M_i/M_0)/P$$

where V_t is expressed as μl of trapped volume per μmol of vesicle lipid, M_i and M_0 are concentrations of trapped solute in the chromatographed vesicle sample and the sonication buffer, respectively, and P is the molar concentration of lipids in the vesicle sample.

2.5. Liposome stability determinations

The stability of liposomes was monitored by determining the time-dependent leakage of CF from liposomes with different lipid composition prepared in 10 mM Tris–HCl (pH 7.4), 100 mM CF and separated from extra vesicular CF by gel filtration on Sepharose 4B. The CF containing vesicles were eluted with 10 mM citric acid, 20 mM Na_2HPO_4, 154 mM NaCl pH 7.4. 100 μl of eluted vesicles, corresponding to 20 nmol of lipids, were then diluted to 1.0 ml with the elution buffer at pH 7.4 or 6.8 with or without 25% human plasma. 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 selfquenching [21], was monitored by measuring the time dependent leakage of this molecule from liposomes. The data are expressed as:

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

were F is the fluorescence intensity at the given time and F_t is the total CF fluorescence measured after the rupture of vesicles with SDS 1% final concentration.

The procedure described above for CF, was also used to determine the liposome stability using calcein as the water soluble trapped molecule.

The pH-dependent stability of liposomes was also determined using ^{14}C-glucose, ^{3}H-inulin and NADH as trapped molecules. In this case, vesicles containing one of these markers, previously separated from the extra vesicular one, were incubated for 30 min in the elution buffer at pH 7.4 or 6.8. 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 at pH 7.4 as elution buffer; 1 ml fractions were collected and each fraction analyzed for lipid and trapped molecule content. NADH was determined fluorimetrically (Ex $\lambda = 340$ nm; Em $\lambda = 460$ nm). Liposome stability determinations were done at least in triplicate.

2.6. Membrane fluidity

The membrane fluidity of different liposomes containing *N*-stearoylcysteamine was evaluated by measuring the fluorescence anisotropy (r_s) of 1,6-diphenyl-1,3,5-hexatriene (DPH) as a function of temperature [22].

2.7. Excimer to monomer ratio determinations

The possible fusion of vesicles, containing $NC_{18}SH$, as a consequence of a moderate drop in pH values (i.e., 6.8) was evaluated by determining the excimer (475 nm) to monomer (379 nm) fluorescence intensity ratio (E/M) of $P_{10}PC$ at different times of incubation. For this purpose small unilamellar vesicles of EPC or EPC/$NC_{18}SH$, (9:1 molar ratio) were labelled with $P_{10}PC$ (10 mol% of total lipid).

The distribution of $NC_{18}SH$ in EPC small unilamellar vesicles was evaluated by monitoring the fluorescence emission spectrum of a pyrene acyl derivative of cysteamine, namely *N*-12-(1-pyrenyl) dodecanoyl cysteamine ($NPC_{12}SH$, molecule **3** in the scheme of synthesis) that represented 10 mol% of total liposomal lipids. Concentrations of $NPC_{12}SH$ in SUVs were determined by extracting liposomes eluted from a Sepharose 4B column, with 20 vol of $CHCl_3/CH_3OH$ 2:1 (v:v) and reading the fluorescence in the lipid extract using $NPC_{12}SH$ or free pyrene standard solutions for the calibration curves.

2.8. Fluorescence measurements

All fluorescence measurements were carried out with a Jasco FP777 spectrofluorimeter equipped with a cuvette holder, the temperature being monitored by a Haake GD3 thermostatic circulating bath and monitored with a Subline PT 100 digital thermometer.

3. Results

Trapped volume determinations and gel chromatography profiles determined by light scattering measurements (data not shown) indicated that the presence of 10 mol% $NC_{18}SH$ did not alter the structural properties of EPC small unilamellar vesicles (SUVs). Higher molar concentrations of $NC_{18}SH$ had a destabilizing effect on these liposomal formulations which resulted in aggregation and a poor reproducibility of their elution profiles (data not shown). The trapped volumes determined with CF loaded vesicles were 0.63 ± 0.08 and 0.716 ± 0.09 for EPC and EPC/$NC_{18}SH$ (9:1 m/m) small unilamellar vesicles, respectively.

The kinetics of CF release from EPC and EPC/$NC_{18}SH$ vesicles at 20°C are reported in Fig. 1. The spontaneous CF release from EPC vesicles at pH 7.4 showed a biphasic kinetics with an initial fast leakage in the first 5 min, followed by a more prolonged slow release which was monitored for 45 min. The spontaneous release of CF from EPC small unilamellar vesicles was very low, only 3.8% of entrapped molecule being released after 30 min. The kinetics evaluated with the same vesicles at pH 6.8 are almost identical to those obtained at pH 7.4 (data not shown).

The presence of 10 mol% EPC/$NC_{18}SH$ in EPC vesicles determined a partial destabilization of the vesicle bilayer, which resulted in an increase of spontaneous leakage of entrapped CF at pH 7.4. The acidification of the medium at pH 6.8 promoted a strong increase in the CF release from EPC vesicles containing $NC_{18}SH$. At all the considered times, the CF diffusion, from EPC/$NC_{18}SH$ containing vesicles, was 2.5-fold higher at pH 6.8 than at 7.4, thus indicating that $NC_{18}SH$ promotes a pH sensitivity in these liposomal formulations.

The pH-dependent increase in CF leakage could depend on liposomal fusion promoted by the acidification of the incubation medium. To ascertain this possibility we incubated EPC/$NC_{18}SH$ SUVs containing 10 mol% $P_{10}PC$ with a large excess of non-fluorescent SUVs of the same lipid composition. The ability of pyrene to form excimer with a characteristic shift in the fluorescence spectrum has, in fact, been used to demonstrate membrane fusion: Probe ($P_{10}PC$) was incorporated into the bilayer of the donor vesicles at excimerising concentrations (i.e., 10 mol% of total lipids). Labelled vesicles were then mixed with unlabelled vesicles. Fusion of the vesicles gives the pyrene derivative a greater area in which to diffuse, reducing the number of excimers formed and thus reducing the E/M ratio. We monitored the E/M ratio, at pH 7.4 and 6.8, for the same time period used for CF release determinations. E/M did not vary in these experimental conditions (data not shown), thus excluding possible vesicle fusion at acidic pH.

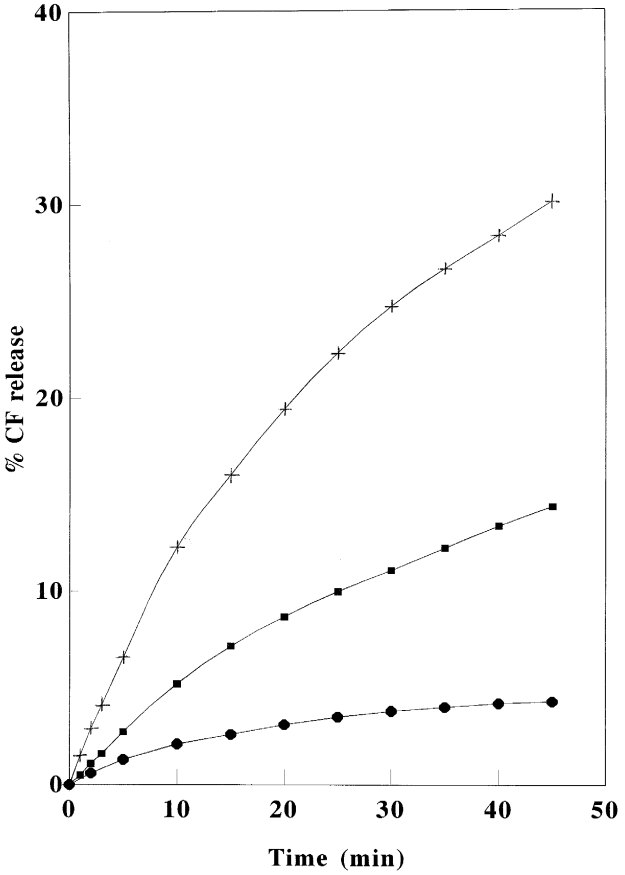


Fig. 1. Extent of spontaneous carboxifluorescein (CF) release from EPC SUVs (●) at pH 7.4 and from EPC/$NC_{18}SH$ SUVs (9:1 molar ratio) at pH 7.4 (■) and 6.8 (+) respectively. Data are expressed as % CF release calculated as described in Section 2. Data reported are the means of at least three experiments.

In order to elucidate the contribution of the head group interactions to the pH sensitivity observed with EPC vesicles containing $NC_{18}SH$, the CF release of EPC/$NC_{18}SH$ SUVs (9:1 molar ratio) was evaluated in the presence of 500 mM NaCl, 500 mM urea, 25

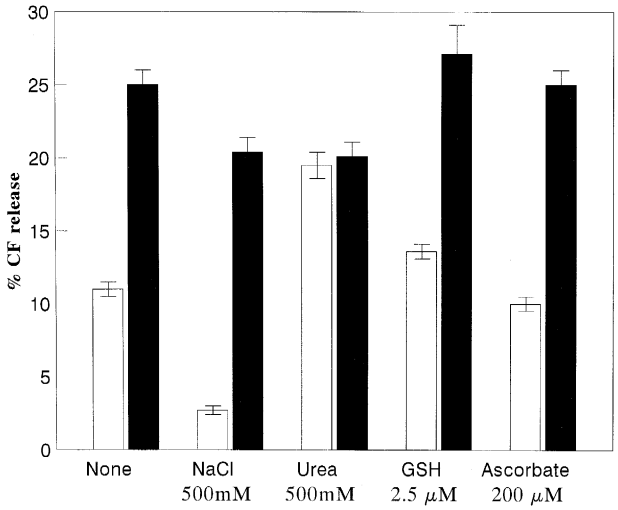


Fig. 2. Extent of CF release from EPC/$NC_{18}SH$ SUVs (9:1 molar ratio) after 30 min of incubation at pH 7.4 (□) and 6.8 (■) in presence of NaCl 500 mM, urea 500 mM, reducing glutathione 25 μM or ascorbate 200 μM. Data are expressed as % CF release calculated as described in Section 2. Data reported are the means ± SD of at least three experiments.

μM GSH or 200 μM ascorbate. The % of CF release after 30 min of incubation at pH 7.4 or 6.8 is reported in Fig. 2. The pH sensitivity was maintained in the presence of reducing agents like GSH and ascorbate, or in presence of high ionic strength obtained with NaCl. In contrast, urea determined an increase in CF leakage at pH 7.4 from vesicles containing $NC_{18}SH$. The amount of CF released after 30 min at pH 7.4, was similar to that released at pH 6.8. None of the molecules considered affected the permeability of pure EPC vesicles at both pHs (data not shown).

The distribution of $NC_{18}SH$ in the EPC bilayer was evaluated using EPC SUVs containing 10 mol% of *N*-[12-(1-pyrenyl)dodecanoyl]cysteamine ($NPC_{12}SH$). The fluorescence emission spectra of liposomal dispersions at pH 7.4 or 6.8 are shown in Fig. 3. The spectra at both the pH considered are characterized by the presence of two emission peaks at 379 and 399 nm, that correspond to the typical fluorescence emission pattern of non-interacting pyrene molecules. The absence of an additional peak in the region of 470 nm, that corresponds to fluorescence emission of excimer-forming aggregates, indicated that all $NPC_{12}SH$ molecules are present in the bilayer in monomeric form, thus indicating the absence of local enriched domains of $NC_{18}SH$ in the EPC bilayer at both considered pHs.

In order to investigate whether or not the structural modifications at the basis of the pH sensitivity of these liposomal formulations are a reversible phenomenon, we incubated EPC/$NC_{18}SH$ SUVs for 5 min at pH 6.8, then the pH of incubation medium was brought up to 7.4 (Fig. 4B). In a parallel experiment vesicles of the same lipid composition were incubated for 5 min at pH 7.4, then the pH was lowered to 6.8 (Fig. 4A). In both cases the rate of CF release responded to changes in the pH of the incubation medium.

The influence of the physicochemical state of the bilayer was evaluated using SUVs composed of DPPC and $NC_{18}SH$ (9:1 m/m) that have a gel to liquid

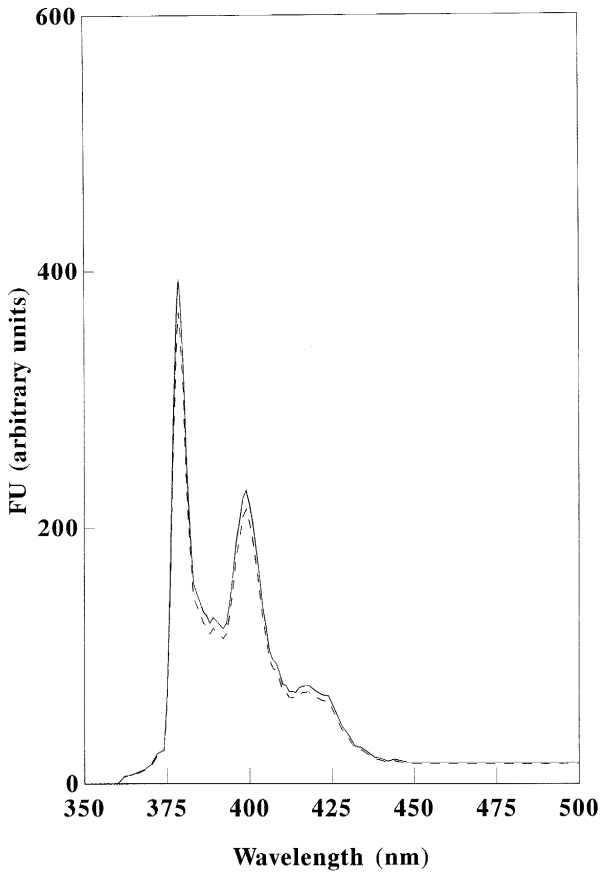


Fig. 3. Fluorescence emission spectra of EPC SUVs containing 10 mol% of $NPC_{12}SH$ at pH 7.4 (————) and 6.8 (---). Excitation wavelength = 343 nm.

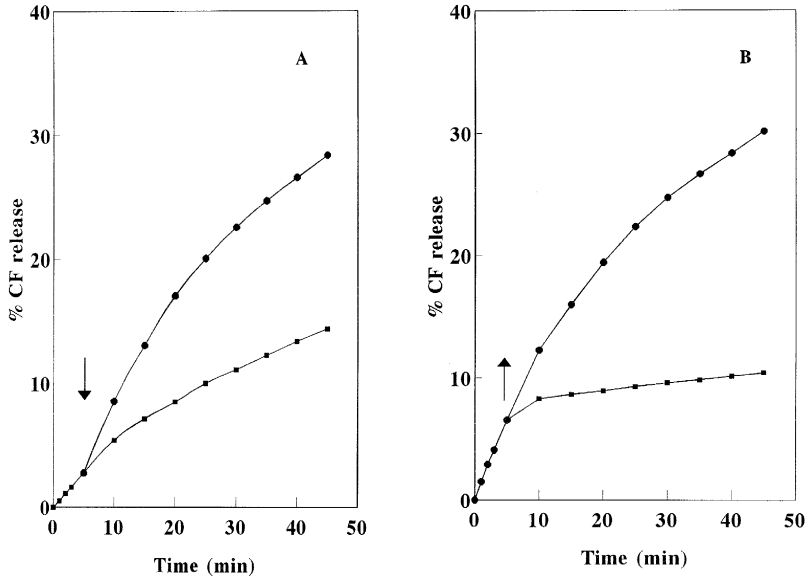


Fig. 4. Extent of CF release from EPC/NC_{18}SH SUVs (9:1 molar ratio) preincubated at pH 7.4 (■), after lowering (↓) the pH of incubation medium to 6.8 (●) (panel A) or preincubated at pH 6.8 (●) after increasing (↑) the pH of incubation medium up to 7.4 (■) (panel B). In panels A and B, the kinetics of CF release from vesicles not submitted to pH changes are also reported. Data are expressed as %CF release calculated as described in Section 2. Data reported are the means of at least three experiments.

phase transition at about 40°C (T_c), as determined from r_s vs. temperature curves (Fig. 5B). The time dependent CF release observed at 25°C, i.e., below T_c, was similar and negligible at both the pH considered. When the same liposomal formulations were incubated above T_c, i.e., at 45°C, we obtained a

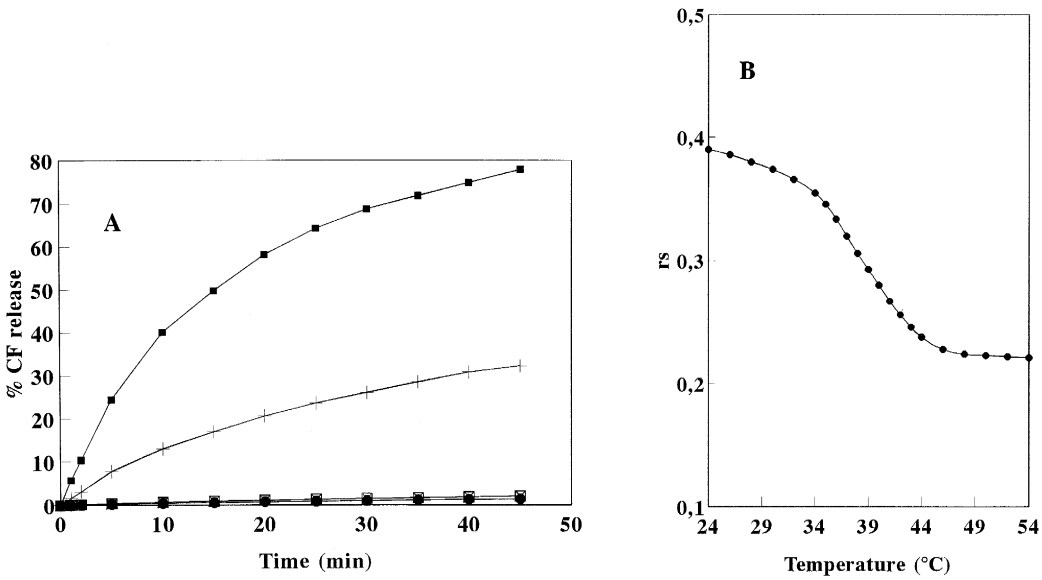


Fig. 5. (A) Extent of CF release from DPPC/ NC_{18}SH SUVs (9:1 molar ratio) incubated at 25°C at pH 7.4 (●) and 6.8 (□) or at 45°C at pH 7.4 (+) and 6.8 (■). Data are expressed as % CF release calculated as described in Section 2. Data reported are the means of at least three experiments. (B) Fluorescence polarisation measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH). The steady state anisotropy, r_s, calculated as described in Section 2, was continuously monitored vs. temperature.

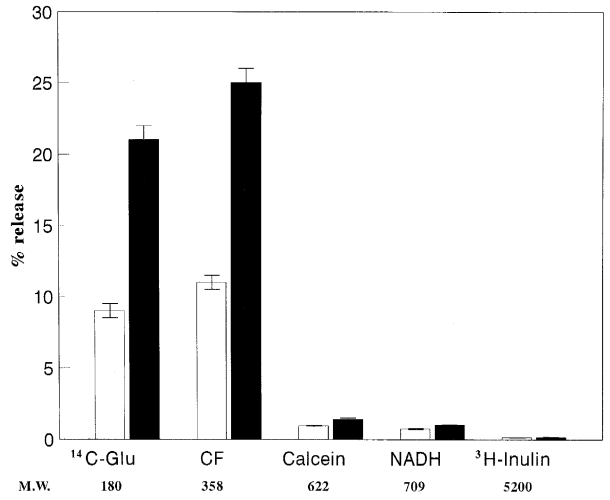


Fig. 6. Extent of release of trapped molecules of different molecular weight from EPC/NC_{18}SH SUVs (9:1 molar ratio) after 30 min of incubation at pH 7.4 (□) and 6.8 (■). Data are expressed as % of release of trapped molecules calculated as described Section 2. Data reported are the means of at least three experiments.

time-dependent CF release at pH 6.8 higher than at pH 7.4 (Fig. 5A). After 30 min of incubation at 45°C, DPPC/NC_{18}SH SUV released 26% and 67% of entrapped CF at pH 7.4 and 6.8, respectively. This seems to indicate that NC_{18}SH promotes a significant response to slight pH variations only in membranes in the fluid state.

We also investigated if the pH sensitivity of liposomes containing NC_{18}SH could depend on the nature of water soluble molecule trapped within the aqueous compartment. For this purpose, CF was substituted with ^{14}C-glucose. This molecule does not permit a continuous monitoring of its release from EPC/NC_{18}SH vesicles, thus the ^{14}C-glucose concentration associated to liposomes was evaluated radiometrically after 30 min incubation of ^{14}C-glucose-loaded liposomes at pH 7.4 and 6.8. The liposome associated ^{14}C-glucose was 5.2 and 4.45 dpm nmol^{-1} lipid at pH 7.4 and 6.8, respectively, the initial liposomal ^{14}C-glucose concentration being 5.7 dpm nmol^{-1} lipid. The about 2.5-fold ^{14}C-glucose leakage at pH 6.8 compared to that determined at pH 7.4, is in good agreement with the data obtained with CF-loaded liposomes (Fig. 6). Release of calcein, NADH and ^{3}H-inulin, from EPC/NC_{18}SH SUV after 30 min of incubation at pH 7.4 and 6.8 was also determined (Fig. 6). The release of molecules with molecular weight greater than CF was very low at both pH considered, thus indicating a size selectivity leakage of molecules from EPC/NC_{18}SH SUV.

The potential use in vivo of these pH sensitive liposomal formulations depends on their plasma stability and on the maintenance of the pH sensitivity in plasma. The plasma stability of EPC/NC_{18} SH SUV was evaluated incubating at 37°C CF-loaded vesicles at different pHs in 25% human plasma. The use of 25% plasma dilution was based on previous evidence

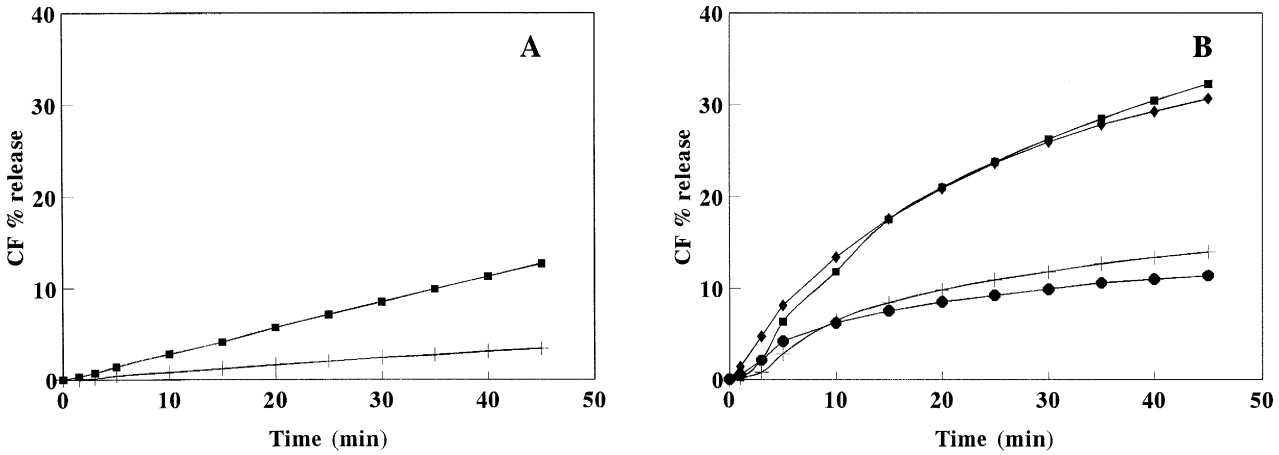


Fig. 7. Extent of CF release from EPC/Chol/NC_{18}SH SUVs (6:3:1 molar ratio) in buffer at pH 7.4 (+) and 6.8 (■) incubated at 25°C (panel A) or at 37°C (panel B) and in 25% human plasma at pH 7.4 (●) and 6.8 (♦) incubated at 37°C (panel B). Data are expressed as %CF release calculated as described in Section 2. Data reported are the means of at least three experiments.

[23] that indicates that almost all the effects observable in full plasma are found even in 25% plasma. The addition of 25% plasma to the incubation medium and the increase of temperature determined a significant increase of CF release from vesicles both at pH 7.4 and 6.8. After 30 min of incubation, the CF release at pH 7.4 and 6.8 was 46 and 58%, respectively. The considerable increase in CF leakage at pH 7.4 makes these liposomal formulations unsuitable for an in vivo utilization. It is known that cholesterol strongly influences lipid membrane properties and when incorporated into pH-sensitive liposomes it increases their stability at neutral pH values [5]. Thus, we investigated the effect of the addition of cholesterol to EPC/NC_{18}SH SUV on their plasma stability and pH sensitivity.

Kinetics of CF release from EPC/Chol/NC_{18}SH SUV (6:3:1 molar ratio) were determined at 25°C (Fig. 7A). These vesicles are still pH sensitive in buffer but the presence of cholesterol strongly stabilizes the vesicles at both the pHs considered. In fact, after 30 min of incubation at pH 7.4 and 6.8 the CF released represented 2.4 and 8.5%, respectively.

Kinetics of CF release from EPC/Chol/NC_{18}SH SUVs were also determined at 37°C in the presence or absence of 25% human plasma (Fig. 7B). The CF release observed at pH 7.4 and 6.8 at this temperature was not strongly affected by the presence of 25% plasma. In fact after 30 min of incubation at pH 6.8 it was 2–2.6 fold that released at pH 7.4 irrespective of the presence of 25% plasma. The trapped volume of this liposomal formulation was 0.63 ± 0.06 μl μmol^{-1} of lipids ($n = 5$). At 37°C, 50 mol% of cholesterol further stabilized SUVs containing NC_{18}SH both at pH 7.4 and 6.8 (data not shown). Thus, reducing the amount of trapped molecule released in consequence to an acidification of the medium.

EPC/Chol/NC_{18}SH (6:3:1 molar ratio) SUVs were incubated at 37° 5 min at pH 6.8, then the pH of the medium was brought up to 7.4. The rate of CF release responded to changes in the pH of the incubation medium also in the presence of plasma (data not shown).

In order to increase the volume of entrapped aqueous space per mol of lipid, we prepared large unilamellar vesicles of EPC/Chol/NC_{18}SH (6:3:1 molar ratio) by reverse-phase evaporation. The trapped volume of these LUVs was 7.4 ± 0.66 μl μmol^{-1} of lipids ($n = 5$), but the release after 30 min of incubation at 37°C in 25% plasma at pH 7.4 and 6.8 of trapped molecule (^{14}C-glucose) was 60% and 70%, respectively (Fig. 8). The decrease in stability at both the considered pH make LUVs unsuitable for selective drug release.

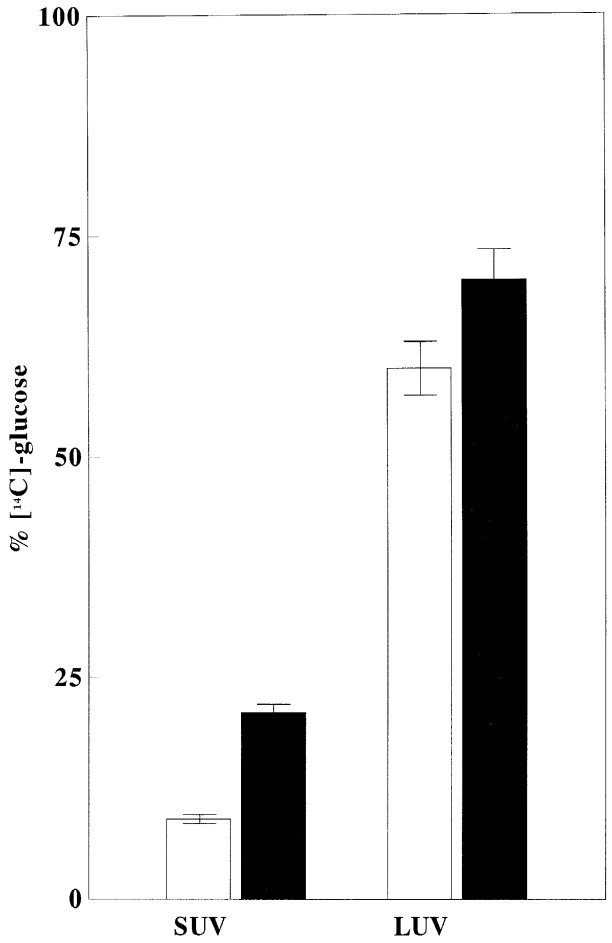


Fig. 8. Extent of ^{14}C-glucose release from SUVs or LUVs of EPC/Chol/NC_{18}SH (6:3:1 molar ratio) after 30 min of incubation in 25% human plasma at 37°C at pH 7.4 (□) and 6.8 (■).

4. Discussion

In this study, we investigated the pH sensitivity of different liposomal formulations containing 10 mol% *N*-stearoylcysteamine as the pH sensitive molecule,

using different water soluble molecules as markers of leakage. For liposomal formulation that exhibited pH sensitivity in buffer, we also evaluated the plasma stability at pH 7.4 together with the capacity to maintain a sensitivity to a moderate drop in plasma pH (i.e., 6.8).

The results demonstrated that $NC_{18}SH$ promotes pH sensitivity in citric acid/Na_2HPO_4 buffer in different liposomal formulations using CF as marker of leakage. The increased release of CF at pH 6.8 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 units below the pH used. Furthermore the results obtained with ^{14}C-glucose loaded liposomes clearly indicate that the pH sensitivity of EPC/$NC_{18}SH$ liposomes is independent of the ionic nature of the trapped molecule.

The pH sensitivity promoted by $NC_{18}SH$ does not depend on liposomal fusion, as demonstrated by the pyrene E/M based membrane fusion assay performed at pH 7.4 and 6.8. Furthermore, the sensitivity to changes in the pH of the medium demonstrated for liposomes containing $NC_{18}SH$ excluded any possible contribution of liposomal fusion to that phenomenon.

The results obtained in the presence of urea (Fig. 2) indirectly suggest that a rearrangement of hydrogen bonds at the surface of the bilayer could contribute to the pH sensitivity associated with these liposomal formulations.

A contribution of modification in the redox status of $NC_{18}SH$ and in the electrostatic interactions at the level of the head groups to the pH sensitivity of these liposomes can also be excluded. In fact, significant differences observed in CF leakage at pH 6.8 vs. 7.4 were maintained when the release was determined in the presence of reducing agents such as GSH and ascorbate or NaCl 500 mM (concentration able to decrease the strength of electrostatic interactions) (Fig. 2).

The decrease in pH of the incubation medium determined a size selective permeability of the liposome bilayer. In fact, only the water soluble molecules trapped within the aqueous compartment with molecular weight lower than 400 (i.e., CF and ^{14}C-glucose) were released from EPC/$NC_{18}SH$ SUVs (Fig. 6). This behaviour is not related to the formation of $NC_{18}SH$ enriched domains in EPC membranes because the fluorescence emission spectra obtained with *N*-acylsteamine carrying a pyrene group ($NPC_{12}SH$) did not show a peak in the region of 470 nm, that corresponds to the fluorescence emission of excimer-forming aggregates (Fig. 3). The defect points generated in the bilayer as a consequence of lowering the pH could depend on a rearrangement of the $NC_{18}SH$ head group. In fact at pH 6.8, SH is prevalently in a undissociated form, thus increasing the possibility of intramolecular hydrogen bonds.

The potential use in vivo of pH sensitive liposome formulations depends on their plasma stability and on the maintenance of the pH sensitivity in plasma [15,16]. The addition of plasma to the incubation medium and the increase of temperature from 25 to 37°C determined a significant increase of CF release from EPC/$NC_{18}SH$ SUVs at both pH 7.4 and 6.8.

The addition of cholesterol to the binary mixture stabilizes the vesicles in the presence of plasma at pH 7.4 without modifying their pH sensitivity (Fig. 7). In fact, at 37°C and in 25% plasma the ternary mixture showed the highest increase in CF release as a consequence of the moderate acidification of the medium from 7.4 to 6.8; moreover the pH sensitivity of EPC/Chol/$NC_{18}SH$ SUVs in plasma responded to variations of the pH of the medium, as observed for EPC/$NC_{18}SH$ liposomes in buffer.

In order to increase the volume of entrapped aqueous space per mole of lipid, we used large unilamellar vesicles, but observed a decrease in stability of LUVs at both pHs considered; this is in agreement with Liu et al. [24,25] who demonstrated that the increase of vesicle curvature, by using small unilamellar liposomes ($d \leq 200$ nm) in which the insertion of stabilizing serum apoprotein is favoured, increase the plasma stability of pH sensitive liposomes.

The behaviour of EPC/Chol/$NC_{18}SH$ SUVs in the presence of plasma makes this liposomal formulation the most promising for possible clinical applications. From our results with plasma in vitro, we could in fact predict that drug release from these liposomes will be significantly increased if the liposomes pass through a region with a pH lower than physiological. Thus, these liposomes could become a clinically relevant means of drug delivery to pathological tissues such as tumoral tissues, inflammation or infection sites and ischemic areas in which it is well known that a lowering of the pH can occur.

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