

On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes

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Abstract

We investigated the molecular mechanisms by which pH-sensitive liposomes surpass the cytoplasmic and endosomal membranes to deliver their aqueous contents into the cytoplasm. Various liposome formulations were evaluated for their efficacy to mediate intracellular delivery of encapsulated material, including a novel sterically stabilized pH-sensitive formulation ((DOPE:CHEMS:DSPE-PEG₂₀₀₀ (6:4:0.3)) that was previously developed in our laboratories. In an attempt to fully characterize the nature of liposome–cell interactions different approaches based on a dual-labeling fluorescence assay were used. Our results indicate that the efficacy of interaction of pH-sensitive liposomes, both plain and sterically stabilized, with cells is strongly determined by the inclusion of DOPE in their composition, independently of the type of the amphiphilic stabilizer used. In fact, DOPE-containing liposomes shown to be non-pH sensitive by biophysical assays, mediated cytoplasmic delivery of their contents as efficiently as well known pH-sensitive formulations (e.g. DOPE:CHEMS). However, among the different formulations studied, DOPE:CHEMS liposomes were those exhibiting the highest extent of cell association. Moreover, our results with cells pretreated with metabolic inhibitors or lysosomotropic agents clearly indicate that DOPE-containing liposomes are internalized essentially by endocytosis and that acidification of the endosomes is not the only mechanism involved in the destabilization of the liposomes inside the cell. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Liposomes exhibiting pH sensitivity and composed

of phosphatidylethanolamine (PE) and protonatable amphiphiles have been developed in an attempt to promote the intracellular delivery of highly charged water-soluble compounds [1–4]. A series of PE stabilizers, including phospholipids, acylated amino acids, fatty acids, cholesterol derivatives and double-chain amphiphiles have been used in the preparation of PE-containing pH-sensitive liposomes [5].

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Head group protonation of these compounds caused by a pH decrease (like in the endosomes) results in liposomal destabilization, because PE molecules revert from a bilayer to an inverted hexagonal II phase (non-bilayer phase). The type of stabilizer, as well as its molar content with respect to PE, play a major role in the final properties of the pH-sensitive liposomes [6]. Such properties involve the extent of internalization, their fusogenic capacity, pH sensitivity and stability in biological fluids. In this regard, cholesterol hemisuccinate is considered to be a good candidate since its cholesterol group confers higher stability to the DOPE-containing vesicles as compared to other commonly used amphiphilic stabilizers (e.g. oleic acid) [7]. Recently, the ability of pH-sensitive liposomes to mediate the cytoplasmic delivery of a large variety of molecules, including antisense oligonucleotides, has been demonstrated in various *in vitro* studies [8–14]. Nevertheless, realization of this potential for *in vivo* purposes has been hampered because, due to interaction with serum proteins, this type of liposomes are either rapidly removed from circulation or undergo destabilization, thus losing their aqueous contents. In a previous work we have reported that incorporation of poly(ethylene glycol) (2000)-distearoylphosphatidylethanolamine into the membrane of DOPE:CHEMS (6:4 molar ratio) liposomes confers steric stability to these vesicles, thus resulting in prolonged circulation times upon *i.v.* administration into rats [15]. However, this modification was shown to significantly decrease the pH dependent release of a charged water-soluble fluorophore, calcein, from liposomes suspended in either buffer or cell culture medium whose pH was adjusted to acidic values. Surprisingly, the efficacy of such liposomes to promote intracellular delivery of their aqueous contents upon incubation with differentiated THP-1 cells remained unaltered, as demonstrated by a novel flow cytometry assay involving dual fluorescence labeling of the liposomes [15]. This lack of correlation between data obtained from biophysical assays, generally used to assess pH sensitivity, and the efficiency of the liposomes to interact with cells clearly suggests that the mechanisms by which pH-sensitive liposomes, both plain and sterically stabilized, mediate intracellular delivery of their contents are not simply dictated by the decrease of pH in the endosomal lu-

men. The knowledge of the molecular mechanisms by which these liposomes surpass the cytoplasmic and endosomal membranes to deliver their aqueous contents into the cytoplasm is therefore of crucial importance.

Aiming at clarifying the role of DOPE we investigated the effect of the liposome composition (namely the type of the amphiphilic stabilizer used) as well as the effect of pretreating differentiated THP-1 cells with different drugs that interfere with the endocytotic pathway on the efficacy of intracellular delivery.

2. Material and methods

2.1. Reagents

Egg phosphatidylcholine, dioleoylphosphatidylethanolamine (DOPE), lissamine rhodamine B-phosphatidylethanolamine (egg) (Rh-PE), phosphatidylserine (PS), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC) and poly(ethylene glycol) (2000)-distearoylphosphatidylethanolamine (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesteryl hemisuccinate (CHEMS), calcein, MES, TES, EDTA, phorbol 12-myristate 13-acetate, Triton X-100, propidium iodide, bafilomycin A₁, NaN₃, NaF, and NaCl were obtained from Sigma (St. Louis, MO, USA). Antimycin A was obtained from Calbiochem (La Jolla, CA, USA).

2.2. Liposomes

Chloroform solutions of lipids were placed into glass tubes, and the solvent was evaporated under a stream of argon. Residues of solvent were removed in a vacuum oven at room temperature. Lipid films (20 μ mol total lipid) were dissolved in 0.8 ml of prewashed diethyl ether, and 0.26 ml of an 80 mM calcein solution (in 10 mM TES buffer, pH 8.2, 1 mM EDTA, adjusted to 300 mOsm by adding NaCl) was added. Liposomes were prepared by reverse phase evaporation [16] as described [17] and extruded 21 times through two polycarbonate filters of 100 nm pore diameter (Costar, Cambridge, MA, USA), using a LiposoFast device (Avestin, Ottawa, Canada).

Non-encapsulated calcein was removed by dialysis at 4°C against three changes of 4 l of TES-buffered saline (TBS, 140 mM NaCl, 10 mM TES, pH 7.4) containing 0.1 mM EDTA. Lipid phosphate concentration was measured according to Bartlett [18]. Molar ratios of lipids are given throughout the paper.

2.3. Physico-chemical characterization of liposomes

Encapsulation efficiency (EE, %) and drug loading (DL, µg of calcein/µmol of phospholipid) of the liposomes were determined as described previously [19]. ζ potential and size measurements of the different liposomes were performed with a Coulter DELSA 440 instrument (Coulter Electronics, Hialeah, FL, USA). The DELSA 440 is a laser-based multiple angle particle electrophoresis analyzer that measures the electrophoretic mobility and ζ potential distribution simultaneously with the hydrodynamic size of the particles in suspension [20,21]. Samples were prepared in 50 mM HEPES, 145 mM NaCl, pH 8.2 (final phospholipid concentration of 1.2 mM) and placed in the measuring cell, whose position was adjusted to cover a previously determined stationary layer and an electric field of 3.0 mA was applied. Data were recorded and the ζ potential calculated for each scattering angle (8.6°, 17.1°, 25.6° and 34.2°). For size measurements no current was applied [22]. Data represent the mean ± S.D. obtained for the different angles of two measurements.

2.4. pH sensitivity of liposomes

Calcein was encapsulated in liposomes at a concentration of 80 mM, at which concentration its fluorescence is self-quenched [23]. Leakage of calcein from the liposomes and its dilution in the buffer results in an increase of fluorescence. One microliter of calcein-loaded liposomes (final phospholipid concentration between 5 and 6 µM) was added to 2 ml of MES-buffered saline (140 mM NaCl, 10 mM MES) at various pH values, in a fluorometer cuvette at 37°C under constant stirring. Following a 10 min incubation calcein fluorescence was measured at $\lambda_{\text{ex}} = 490$ and $\lambda_{\text{em}} = 520$ nm, before and after the addition of 20 µl of 10% (v/v) Triton X-100, using an LS-5B fluorometer (Perkin-Elmer, Mountain View, CA, USA) operated with a Softways (Morena Val-

ley, CA, USA) computer program. Fluorescence intensities obtained at acidic pH values were corrected for the slight effect of pH on calcein fluorescence. To calibrate the fluorescence scale, 100% leakage was achieved by the addition of Triton X-100 (final concentration 0.1%). The percentage of calcein leakage was calculated according to formula 1: % leakage = $((I_{\text{pH}} - I_0)/(I_{100} - I_0)) \times 100$, where I_0 is the fluorescence at neutral pH, I_{pH} is the corrected intensity at acidic pH before the addition of Triton X-100, and I_{100} is the totally dequenched calcein fluorescence at neutral pH. Leakage induced by the acidic pH of the cell culture medium was measured as previously described [15]. Liposomes were added to the medium at a final concentration of 100 µM phospholipid and incubated for 1 h at 37°C at pH 7.4 or 5.5. The intensity of calcein fluorescence was measured right after the addition of liposomes to the medium (time 0, I_0) and again after a 1 h incubation (I_{pH}), by taking a 0.10 ml aliquot and diluting it in 1.9 ml of MES-buffered saline, pH 7.4. Leakage of calcein was expressed by formula 1.

2.5. Cells

Monocytic human THP-1 cells were cultured in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% FBS and maintained as described [24]. They were differentiated to macrophage-like cells upon incubation with 160 nM phorbol 12-myristate 13-acetate in 24-well culture plates (10^6 cells per well). Five or 6 days after differentiation, the culture medium was replaced with fresh medium. Liposomes encapsulating 80 mM calcein and containing 1 mol% Rh-PE in their membrane were added to the cells at a final phospholipid concentration of 100 µM. Cells were incubated with liposomes for various times at 37°C and then washed twice with phosphate-buffered saline (PBS) without calcium or magnesium ions (Irvine Scientific).

2.6. Assessment of liposome–cell interactions

For flow cytometric analysis the cells were detached from plastic by adding 0.5 ml of dissociation buffer (Gibco, BRL, Gaithersburg, MD, USA) and mixed with 0.5 ml of PBS containing divalent cations, 2% FBS and 1 mg/ml propidium iodide, the

latter being used to assess cell viability. Rhodamine and calcein fluorescence was detected with a Becton Dickinson FACStar Plus flow cytometer, controlled by a Hewlett-Packard computer with Lysis II software (Becton Dickinson, San Jose, CA, USA) [15]. Samples were analyzed for lissamine rhodamine using excitation at 528 nm and emission at 575 nm, and for calcein using excitation at 488 nm and emission at 520 nm with a 0.1 neutral density filter. Ten thousand events were recorded for each sample. Forward scatter and propidium iodide fluorescence signals were used to gate the cell subset of interest and eliminate debris, dead cells, and cell aggregates. Mean rhodamine fluorescence values reflect the binding and uptake of liposomes, whereas the mean calcein fluorescence reflects the intracellular dequenching of the dye. The calculated ratio of calcein to rhodamine fluorescence was taken to measure the amount of aqueous marker released intracellularly per cell-associated liposome. The initial calcein to rhodamine fluorescence ratio of liposomes bound to the cells, in the absence of endocytosis, was obtained by incubating the liposomes with the cells at 4°C [25].

For image analysis, THP-1 cells were differentiated in Lab-Tek Chambered Coverglasses for Tissue Culture, obtained from Nunc (Naperville, IL, USA). Cells were washed with cold RPMI medium without phenol red, containing 20 mM HEPES buffer, pH 7.4. Liposomes were added to cells at a final phospholipid concentration of 200 μM and incubated for 1 h at 4°C. After prebinding, cells were washed with cold medium and the initial calcein and rhodamine fluorescence images were recorded using a Photon Technology International (PTI) ratio imaging system. To evaluate the kinetics of calcein dequenching, medium at 37°C was added to the chambers and cells were incubated at this temperature for various times. After incubation, cells were washed with cold medium, and the calcein and rhodamine fluorescence images were recorded. Cells were observed in a Nikon Diaphot epifluorescence microscope (Melville, NY, USA) using a 100 \times objective and filters for FITC/TXRD obtained from Molecular Probes (Eugene, OR, USA). Averages of 16 snapshots were taken to reduce background. Ratio images were produced using PTI software. To evaluate the degree of calcein dequenching per cell, histograms of calcein to rhodamine ratio for each cell were determined

using a square of 100 \times 100 pixels, representing the approximate area of one cell at the magnification used. Average histograms for each liposome composition and time points were then calculated using Excel software. Medians for each average histogram were calculated from cumulative curves and used to follow calcein dequenching under different experimental conditions. To calculate the growth of the area occupied by dequenched calcein, the highest ratio in the control experiment (incubation at 4°C) was taken as a cutoff. The sum of the pixels with ratios higher than the cutoff ratio was taken to estimate the dequenched area at various time points.

For fluorimetric measurements, 5×10^6 cells were differentiated in 5 ml of medium in six-well culture plates. Cells were incubated with liposomes (final phospholipid concentration of 150 μM) for different times at 37°C, and then washed as described above. Following their incubation with 2 ml of dissociation buffer for 10 min at 37°C, cells were detached from plastic with disposable scrapers (Costar) and transferred into disposable fluorimetric cuvettes (Hughes & Hughes, Tonedale, New Zealand). Fluorescence measurements were performed in a SPEX Fluorolog 2 fluorometer (SPEX Industries, Edison, NJ, USA). Calcein fluorescence was read at excitation and emission wavelengths of 490 and 520 nm respectively, using 0.5 mm excitation and 1.0 mm emission slits, whereas rhodamine fluorescence was read at excitation and emission wavelengths of 568 and 600 nm respectively, using 0.5 mm excitation and 1.5 mm emission slits. The sample chamber was adjusted to the front face configuration (to prevent light scattering contributions to the fluorescence) and equipped with a magnetic stirrer. The temperature was maintained at 20°C with a thermostatic circulator.

2.7. Effect of drugs on liposome–cell interactions

In some experiments, before the addition of liposomes, differentiated THP-1 cells were incubated for 30 min at 37°C, in the absence of serum, with either (i) a mixture of antimycin A (1 $\mu\text{g/ml}$), NaF (10 mM) and NaN_3 (0.1%) to inhibit endocytosis [15,25], (ii) chloroquine (100 μM) to reduce acidification of endocytotic vacuoles [26,27], or (iii) bafilomycin A_1 (200 nM) to specifically prevent acidification of the endosomes and lysosomes [27–30]. Cells were further

incubated for different times at 37°C with the different liposome formulations in the presence of the various drugs. The viability of the cells treated in the presence of these agents was evaluated and compared to that of untreated control cells.

3. Results

3.1. Physico-chemical characterization of the different liposome formulations

The physico-chemical properties of the lipids are crucial in the formation and final features of liposomes [17]. Table 1 illustrates the results obtained for the encapsulation parameters, size and charge of some of the formulations tested.

As can be observed, no significant differences in the encapsulation efficiency, drug loading or size of the liposomes were observed independently of their composition, suggesting that these properties are essentially determined by the mode of liposome preparation. In fact, the reverse phase evaporation (REV) method followed by extrusion has been described as leading to the formation of unilamellar vesicles (LUVs) exhibiting an homogeneous size distribution, whose mean size depends mainly on the pore diameter of polycarbonate membranes used for extrusion.

The low values of encapsulation efficiency can be justified by the low available aqueous space of liposomes within this size range (± 100 nm) when prepared by the REV method [31,32]. As determined by ζ potential measurements, DOPE:CHEMS liposomes exhibited a net negative charge which is con-

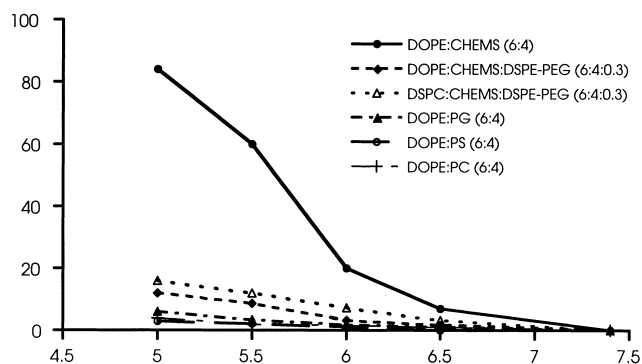


Fig. 1. pH sensitivity of DOPE-containing liposomes. Calcein-encapsulated liposomes (final lipid concentration of 5–6 μ M) were incubated for 10 min at 37°C in MES-buffered saline at different pH values. The fluorescence intensity of calcein was measured before and after the addition of Triton X-100 (final concentration of 0.1%). Leakage of calcein was calculated as described in Section 2. The results shown are from one representative experiment.

ferred by the amphiphilic stabilizer (CHEMS). However, incorporation of DSPE-PEG conjugates into the membrane of these vesicles masked their negative surface charge, the liposomes thus becoming neutrally charged and therefore sterically stabilized.

3.2. Influence of the lipid composition on the pH sensitivity of liposomes

In a previous work we reported that incorporation of DSPE-PEG into DOPE:CHEMS liposomes strongly reduces the ability of these vesicles to destabilize under acidic conditions [15]. This effect was attributed to the stabilizer effect of PEG. It should be noted that the pH sensitivity of the referred lipo-

Table 1
Effect of lipid composition on the physico-chemical properties of liposomes^a

Lipid composition (molar ratio)	EE (%)	DL (μ g/ μ mol)	Diameter (nm)	ζ potential (mV)
DOPE:CHEMS (6:4)	2.2 \pm 0.1	27.36 \pm 1.24	121 \pm 8	-31.66 \pm 1.62
DOPE:CHEMS:DSPE-PEG (6:4:0.3)	2.7 \pm 0.2	30.5 \pm 2.26	135 \pm 12	0.575 \pm 2.7
DSPC:CHEMS:DSPE-PEG (6:4:0.3)	3.0 \pm 0.14	33.9 \pm 1.58	129 \pm 6	n.d.
PC:CHEMS (6:4)	4.45 \pm 0.6	55.35 \pm 7.46	130 \pm 3	n.d.
DOPE:PS (6:4)	2.8 \pm 0.2	34.83 \pm 2.48	134 \pm 6	-29.66 \pm 2.02
DOPE:PG (6:4)	2.9 \pm 0.2	32.5 \pm 3.43	125 \pm 4	n.d.

^aLiposomes were prepared by the REV method and extruded 21 times through two polycarbonate filters of 100 nm pore diameter. Non-encapsulated calcein was removed by dialysis at 4°C against three changes of 4 l of TES-buffered saline. Encapsulation efficiency (EE, %) and drug loading (DL, μ g of calcein/ μ mol of phospholipid) of the obtained liposomes were determined as described previously. ζ potential and size measurements of the different liposomes were performed with a Coulter DELSA 440 instrument as described in Section 2. The data indicate the mean \pm S.D. and are representative of two independent experiments. n.d., not determined.

somes was evaluated using a simple biophysical assay. As described in Section 2, this assay consists of incubating the liposomes in a cuvette containing buffer solutions whose pH was adjusted at different values, the extent of leakage of the liposomes being evaluated in terms of percentage of released calcein. Surprisingly, no correlation could be established between these data and those obtained upon incubation of the liposomes with cells. In fact, DOPE:CHEMS:DSPE-PEG liposomes were as effective as plain DOPE:CHEMS liposomes in promoting the intracellular release of their aqueous contents. These intriguing results prompted us to extend these studies to other DOPE-containing liposome formulations, but using different amphiphilic stabilizers. Fig. 1 illustrates the extent of calcein leakage from the different liposomes tested. As can be observed, DOPE:CHEMS liposomes exhibited a significant pH sensitivity since almost 60% of their aqueous contents is released at pH 5.5. As previously reported, incorporation of 5 mol% of DSPE-PEG strongly decreased the extent of leakage of calcein as a function of pH. As expected, replacement of CHEMS with PC, PG or PS completely abolished the pH sensitivity of the DOPE-containing liposomes. Similarly, DSPC:CHEMS:DSPE-PEG liposomes were completely insensitive to the pH of the milieu.

3.3. Interaction of liposomes with cells

Although the replacement of CHEMS by the other amphiphilic stabilizers decreased the *in vitro* pH sensitivity of the DOPE-containing liposomes, we examined whether this would affect their ability to deliver encapsulated molecules to differentiated THP-1 cells. The cellular uptake of liposomes was followed by including Rh-PE in the lipid phase and measuring cell-associated rhodamine fluorescence. As previously reported, the presence of Rh-PE did not affect the pH sensitivity of DOPE:CHEMS liposomes in buffer [15]. The release of the aqueous contents of liposomes inside the cells was monitored by encapsulating calcein at self-quenching concentrations and measuring the fluorescence dequenching as the fluorophore was diluted [1,2]. This fluorescence value reports both the quenched encapsulated calcein into liposomes bound to, or internalized by cells and the dequenched dye released inside the cells. To control

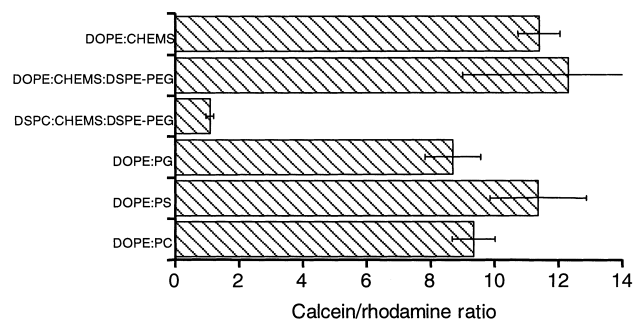


Fig. 2. Efficacy of intracellular delivery mediated by different liposome formulations. Calcein-encapsulated liposomes (final lipid concentration of 100 μM) were incubated with differentiated THP-1 cells in RPMI medium supplemented with 10% FBS for 30 min at 37°C. Cells were washed twice with PBS and detached from plastic with enzyme-free cell dissociation buffer. They were then transferred into polypropylene tubes for flow cytometry and mixed with an equal volume of PBS, containing 2% FBS and 1 $\mu\text{g/ml}$ propidium iodide. Flow cytometric analysis was performed as described in Section 2. The data represent the mean \pm S.D. obtained from duplicates of three independent experiments.

for the contribution of bound or internalized liposomes to the total calcein fluorescence, the calcein to rhodamine fluorescence ratio was calculated. This ratio provides a measure of the intracellular fluorescence dequenching of calcein per liposome, particularly when it is compared with that of cell-bound but not internalized liposomes obtained in control experiments performed at 4°C. Therefore, this calcein/rhodamine ratio represents a useful tool to estimate the global efficacy of liposome–cell interactions.

As illustrated in Fig. 2, no significant differences in the efficacy of intracellular delivery mediated by the different DOPE-containing liposome formulations were observed upon their incubation with THP-1 cells for 30 min. This incubation time was selected since it is considered to be physiologically more appropriate to evaluate the pH sensitivity of the liposomes inside the cell, thus preventing their degradation in the lysosomes [33]. The calcein/rhodamine fluorescence ratio determined for DOPE:CHEMS liposomes was essentially identical to that observed for DOPE:CHEMS:DSPE-PEG and DOPE:PS and only slightly larger than that for DOPE:PG and DOPE:PC formulations. In contrast, the intracellular delivery mediated by DSPC:CHEMS:DSPE-

PEG was shown to be a very inefficient process. It seems therefore that the absence of pH sensitivity exhibited by some of the DOPE-containing liposomes (as assessed by the calcein release assay) does not affect their ability to deliver their aqueous contents into the cell cytoplasm.

In an attempt to better understand these unexpected findings, we analyzed separately the fluorescence intensities of both rhodamine and calcein as a function of time (up to 60 min). As illustrated in Fig. 3A, the mean fluorescence intensity of rhodamine (reflecting the extent of binding and cellular uptake) upon incubation of THP-1 cells with DOPE:CHEMS liposomes was significantly larger than that observed for DOPE:CHEMS:DSPE-PEG or DOPE:PS liposomes, independently of the incubation time. In addition, the extent of binding and internalization of DOPE:CHEMS liposomes increased continuously and linearly with time, while no significant increase was observed for the other formulations.

As expected, sterically stabilized pH-sensitive liposomes exhibited the lowest levels of rhodamine fluorescence, clearly indicating that this type of liposomes can avoid uptake by macrophage-like cells such as differentiated THP-1 cells. Although DOPE:PS liposomes are not sterically stabilized, they were internalized by these cells to a much lower extent compared to DOPE:CHEMS liposomes, this being particularly evident at the longer incubation times.

Fig. 3B shows that the extent of calcein release from the liposomes (reflecting the extent of intracellular delivery of their aqueous contents) upon their incubation with differentiated THP-1 cells followed essentially the same pattern as that described for rhodamine fluorescence. In fact, the highest levels of calcein fluorescence were observed when the cells were incubated with DOPE:CHEMS liposomes. However, in contrast to what was described for the rhodamine experiment, no significant difference among the formulations tested was observed for the kinetics of intracellular delivery of calcein.

The similarity of calcein to rhodamine fluorescence ratio observed for the different formulations at any incubation time (data not shown; Fig. 2 illustrates the results for a 30 min incubation) clearly suggests that the efficacy of intracellular delivery of calcein

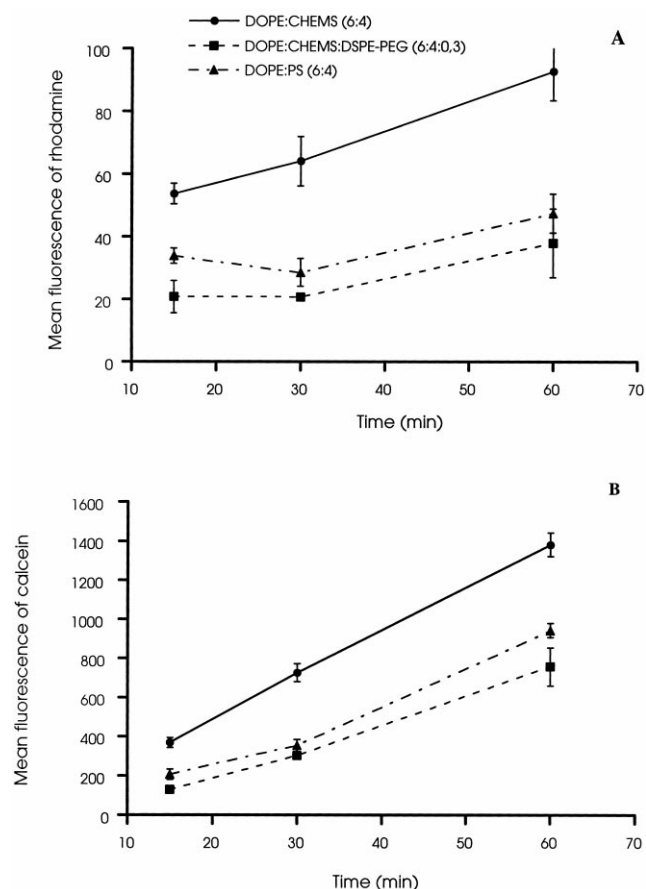


Fig. 3. Kinetics of uptake (A) and intracellular delivery (B) of calcein-containing liposomes labeled with Rh-PE. Liposomes (final lipid concentration of 100 μ M) were incubated with differentiated THP-1 cells in RPMI medium supplemented with 10% FBS at 37°C for different periods. Cells were analyzed by flow cytometry as described in the legend to Fig. 2. The data represent the mean \pm S.D. obtained from duplicates of three independent experiments.

mediated by liposomes is essentially determined by the extent of their uptake by THP-1 cells.

3.4. Effect of inhibitors of the endocytotic pathway on the efficacy of intracellular delivery mediated by liposomes

In order to better define the mechanisms involved in the internalization and intracellular trafficking of the different DOPE-containing liposomes, we evaluated the efficacy of intracellular delivery mediated by the liposomes upon treatment of THP-1 cells with drugs that interfere with the endocytotic pathway. These include (i) bafilomycin A₁, a specific inhibitor

of both the endosomal and lysosomal ATPase proton pump, that prevents acidification of the endocytotic pathway [27–30]; (ii) chloroquine, a weak base that by sequestering protons prevents non-specific acidification of the endocytotic vacuoles [26,27]; and (iii) a mixture of antimycin A, sodium azide and sodium fluoride, which, being potent metabolic inhibitors, lead to a strong inhibition of both receptor and non-receptor mediated endocytosis [15,25]. Fig. 4 shows the intracellular delivery efficacy of the different liposomes tested following their incubation with the cells for 30 min. These results are expressed in terms of percentage of calcein/rhodamine fluorescence ratio with respect to the controls, i.e. cells that have not been pretreated with the drugs.

As can be observed, the specific inhibition of the endosomal and lysosomal acidification by bafilomycin A₁ resulted in approx. 50% inhibition of the intracellular delivery efficacy for all the formulations tested. However, the increase in the endosomal/lysosomal pH due to the presence of chloroquine in the medium resulted in a much more significant reduction of the calcein to rhodamine fluorescence ratio.

Finally, it is interesting to note that the inhibition of the endocytotic pathway by the mixture of drugs composed of antimycin A, sodium azide and sodium fluoride resulted in approx. 90% reduction of the cytoplasmic release of calcein, independently of the liposome formulation tested. This clearly shows that the endocytotic pathway plays a major role in the process of internalization.

It should be noted that the presence of the different drugs did not affect the extent of liposome–cell association expressed in terms of rhodamine fluorescence (data not shown). Therefore, the effect of the drugs reflects only the reduction in the levels of calcein fluorescence (Fig. 4). For the particular case of the cells treated with inhibitors of endocytosis, this indicates that although cellular internalization is hampered the liposomes are able to bind to the cells to the same extent. On the other hand, the similarity of the calcein/rhodamine fluorescence ratios determined either in the presence of those agents or under conditions where endocytosis is blocked (incubation at 4°C) suggests that liposome–cell binding does not lead to leakage of calcein due to liposome destabilization (data not shown).

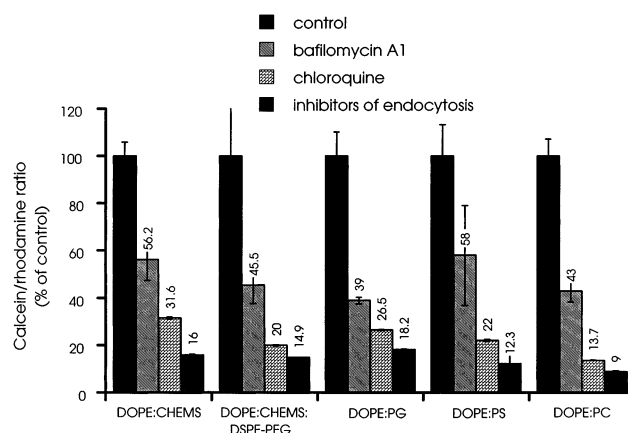


Fig. 4. Effect of inhibitors of the endocytotic pathway on the efficacy of intracellular delivery of different liposome formulations. Differentiated THP-1 cells were incubated for 30 min at 37°C in RPMI medium containing HEPES buffer (20 mM) without serum, with either (i) a mixture of antimycin, NaF and NaN₃, (ii) chloroquine or (iii) bafilomycin A₁. Cells were further incubated for 30 min at 37°C with the different liposome formulations in the presence of the various drugs. Cells were analyzed by flow cytometry as described in the legend to Fig. 2. The data represent the mean \pm S.D. obtained from duplicates of three independent experiments.

3.5. Quantification of liposome–cell interactions by image analysis

The capacity of the different types of liposomes to mediate the intracellular delivery of calcein was also confirmed by the direct observation of the cells using fluorescence microscopy. Following incubation of THP-1 cells with the liposomes for different periods an intense and diffuse fluorescence in the cytoplasm was observed for any of the formulations tested. This was attributed to an increase in calcein fluorescence due to its release from the endosomes and consequent dilution into the cell cytoplasm. It should be noted, however, that for the formulations DOPE:CHEMS:DSPE-PEG, DOPE:PG, DOPE:PS and DOPE:PC, the levels of fluorescence intensity were lower than those observed for DOPE:CHEMS liposomes. This was attributed to the lower extent of internalization observed for those formulations, as assessed by following rhodamine fluorescence (data not shown).

Aiming at confirming the results obtained by flow cytometry, we have performed parallel experiments to those described above, regarding the effect of the

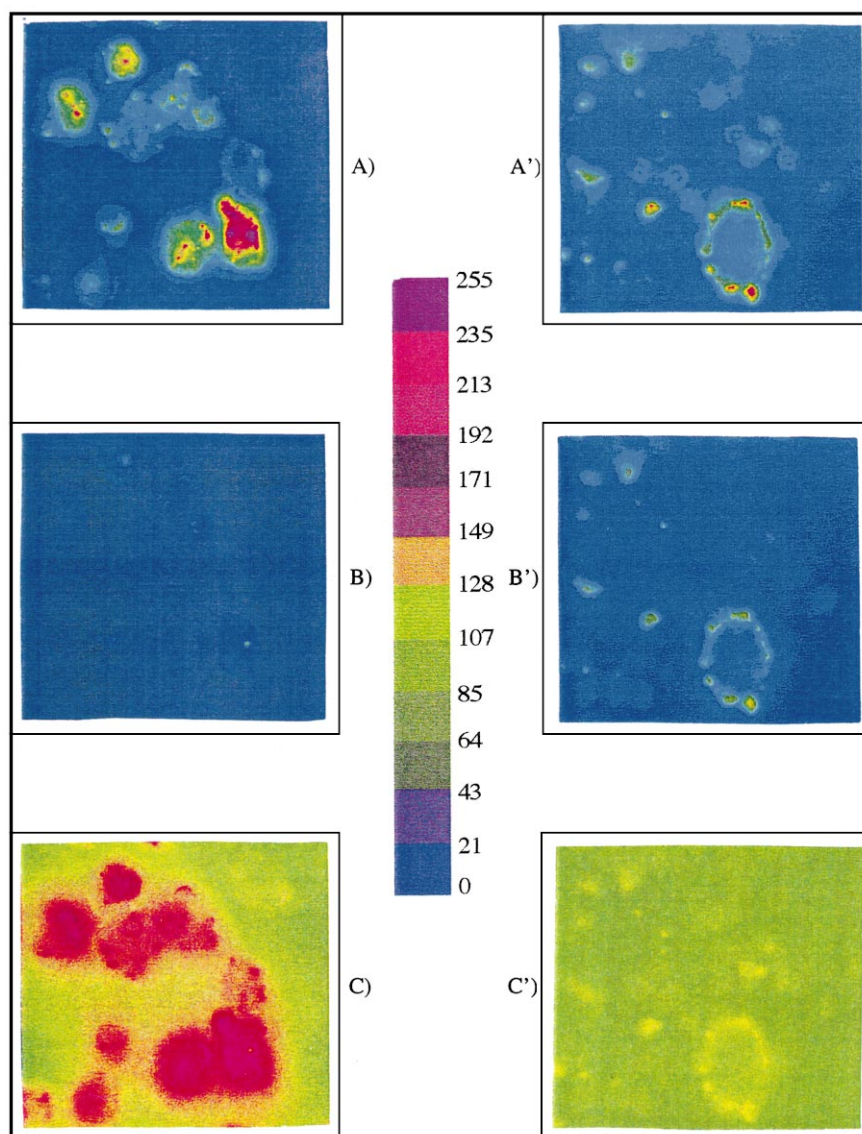


Fig. 5. Fluorescence micrographs of differentiated THP-1 cells incubated with calcein-loaded DOPE:CHEMS liposomes labeled with Rh-PE for 30 min at 37°C. The liposomes were added to cells (final phospholipid concentration of 200 μ M) in the absence or presence of inhibitors of endocytosis composed of a mixture of antimycin A (1 μ g/ml), NaF (10 mM) and NaN₃ (0.1%) to inhibit endocytosis, as described in Section 2. After incubation, cells were washed with cold medium, and the calcein and rhodamine fluorescence images were recorded. (A,B,C) Digitalized images obtained for calcein, rhodamine and calcein/rhodamine ratio, respectively. (A',B',C') Images obtained from parallel experiments performed in the presence of inhibitors of endocytosis.

different drugs on liposome–cell interactions, utilizing fluorescence microscopy and image analysis.

Fig. 5 illustrates the results obtained from a representative experiment on the interaction of DOPE:CHEMS liposomes with THP-1 cells in the presence or absence of inhibitors of endocytosis. These are digital images of calcein and rhodamine fluorescence as well as its ratio, acquired following liposome–cell

incubation for 30 min at 37°C. Incubation of the liposomes with the cells resulted in an intense and diffuse calcein fluorescence in the cytoplasm (green to red in the color scale), reaching saturation in some cases (purple color) (Fig. 5A). In contrast, the levels of rhodamine fluorescence, corresponding to the presence of the liposome membrane in the endocytotic vacuoles, were only barely detectable (Fig. 5B).

Fig. 5C illustrates the levels of color intensity resulting from the ratio of calcein to rhodamine fluorescence. The larger fluorescence intensity of calcein as compared to rhodamine leads to an orange-red color pattern in the cell cytoplasm, whereas regions corresponding to identical calcein and rhodamine fluorescence (background) are shown in green. Upon treatment of the cells with inhibitors of endocytosis the levels of calcein fluorescence intensity in the cytoplasm were drastically reduced, and only traces of punctate fluorescence were observed at the cell periphery (which are attributed to liposome aggregates containing calcein at self-quenching concentrations) (Fig. 5A'). The presence of such aggregates is also detectable by following rhodamine fluorescence (Fig. 5B'). The low fluorescence intensity of calcein observed under these conditions resulted in a low color intensity for the ratio of calcein and rhodamine images (Fig. 5C'), which differs slightly from the background.

Since it was possible to obtain a ratio of images with image analysis software, reflecting the global efficacy of liposome–cell interaction (similar to what was obtained with flow cytometry), we developed a novel methodology to quantitate this efficacy. Briefly, this approach consisted of determining cumulative distribution curves based on average histograms of 16 cells as described in Section 2. The median (I_{50}) determined from cumulative curves constitutes a numerical parameter that facilitates the precise comparison of the efficacy of intracellular calcein delivery mediated by different liposome formulations under different experimental conditions. Fig. 6 illustrates the cumulative distribution curves of calcein/rhodamine ratio fluorescence intensities obtained when DOPE:CHEMS liposomes were incubated with THP-1 cells for 30 min in the absence or presence of bafilomycin A₁ or inhibitors of endocytosis. From those curves the value of the medians (I_{50}), corresponding to a cumulative frequency of 50% (i.e. 50% of the pixels exhibit an intensity equal to or lower than that value), were determined and are presented in Table 2.

The cumulative curve obtained for DOPE:CHEMS liposome formulations in the absence of any treatment (control) was significantly shifted to high values of the ratio calcein/rhodamine, reflecting the higher fluorescence intensity of calcein as com-

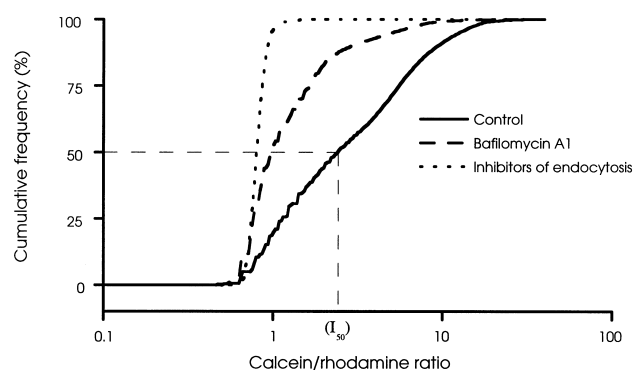


Fig. 6. Cumulative distribution curves of calcein/rhodamine ratio fluorescence intensities. DOPE:CHEMS liposomes (final phospholipid concentration of 200 μ M) were incubated with THP-1 cells for 30 min at 37°C in the absence or presence of bafilomycin A₁ or inhibitors of endocytosis. The curves are based on average histograms of 16 cells obtained from different experiments, as described in Section 2. The mode of calculation of the median (I_{50}) is illustrated for the case of untreated cells (Control).

pared to rhodamine. The inhibitory effect of pretreating the cells with bafilomycin A₁ on the intracellular delivery of calcein was evident, since the respective cumulative curve was markedly shifted to lower values of ratio intensities. This shift was even more pronounced when liposomes were incubated with THP-1 cells that were pretreated with inhibitors of endocytosis. In addition, it should be noted that for the cases of treated cells, narrow distributions of ratio intensities were observed, suggesting that calcein and rhodamine fluorescence was restricted to punctates either inside the cell (corresponding to intracellular vacuoles) or to liposome aggregates at the cell surface.

The I_{50} values presented in Table 2 are numerical parameters that reflect the differences in the profiles of cumulative curves referred to above, and therefore represent efficacy indexes of the intracellular delivery observed under different experimental conditions. As can be observed, that efficacy was significantly de-

Table 2
Medians (I_{50}) of cumulative distribution curves of calcein/rhodamine ratio fluorescence intensities

Experimental condition	Median (I_{50})
Control	2.47
Bafilomycin A ₁	0.97
Inhibitors of endocytosis	0.8

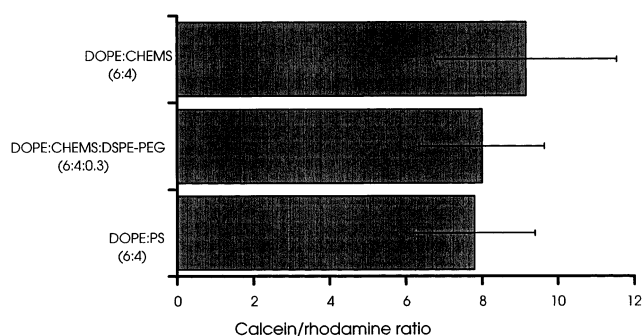


Fig. 7. Efficacy of liposomes in mediating intracellular delivery of encapsulated material as evaluated by spectrofluorimetry. DOPE-containing liposomes (final phospholipid concentration of 150 μ M) were incubated with 5×10^6 differentiated THP-1 cells for 30 min at 37°C in RPMI 1640 supplemented with 10% FBS. Cells were washed twice with PBS and detached with 2 ml of dissociation buffer and transferred to disposable fluorimetric cuvettes. Fluorescence measurements were performed as described in Section 2. The data represent the mean \pm S.D. obtained from duplicates of three independent experiments.

creased upon inhibition of endosome acidification or blockage of cellular internalization. Overall, these data are in close agreement with those obtained from flow cytometry studies presented in the previous section, thus reinforcing the validity and importance of this methodology to assess liposome–cell interactions.

3.6. Spectrofluorimetric studies

We also examined the mechanisms of liposome–cell interactions by spectrofluorimetry measurements. This technique is particularly simple and less cumbersome than flow cytometry and image analysis. Fig. 7 shows typical results obtained for the ratios of calcein/rhodamine fluorescence intensity when different liposome formulations were incubated with differentiated THP-1 cells for 30 min at 37°C. As illustrated, no differences were observed between the efficacies of intracellular delivery mediated by the different formulations tested. In these experiments we used approaches and methodologies that differ significantly from those described above, since spectrofluorimetric measurements reflect the overall cell suspension. Nevertheless, these results were strikingly similar to those observed when flow cytometry was used, the calcein/rhodamine values being identical, thus leading to the same conclusions. These ex-

periments thus demonstrate that spectrofluorimetry constitutes not only a useful tool for this type of studies, but that it may also be used to validate results obtained from other approaches.

4. Discussion

In the present work, we carried out studies aiming at clarifying the mechanisms by which pH-sensitive liposomes interact with cells and mediate the intracellular delivery of their aqueous contents. We have demonstrated that the pH sensitivity of DOPE-containing liposomes in buffer is mainly determined by the type of the amphiphilic stabilizer used. In contrast to what we have observed for DOPE:CHEMS liposomes, liposomes composed of DOPE:PG, DOPE:PS, or DOPE:PC did not exhibit any pH sensitivity, even when incubated under very acidic conditions. As previously reported [15], an increase in liposomal stability under these conditions can also be obtained upon incorporation of 5 mol% DSPE-PEG into DOPE:CHEMS liposomes. However, this lack of pH sensitivity does not appear to affect the capacity of the different liposomal formulations to mediate cytoplasmic delivery of their aqueous contents following their internalization.

These findings suggest that the processes underlying the intracellular efficacy of the different DOPE-containing liposomes involve more complex mechanisms than the mere decrease of the endosomal pH. On the other hand, the combination of the present results with those previously reported [15] indicates that the presence of DOPE is the crucial factor determining the ability of such liposomes to undergo destabilization upon acidification of the endosomes. In fact, in those studies non-pH-sensitive liposomes composed of DSPC:CHEMS:DSPE-PEG were the only ones where a correlation between a lack of pH sensitivity in buffer and inability to mediate intracellular delivery was observed.

In this context, it is interesting to note that studies performed by Vidal and Hoekstra [34] on fusion between endocytotic vesicles isolated from reticulocytes and liposomes with different compositions showed that the presence of PE when compared with other phospholipids, namely PC, is crucial to the fusion process.

This specific effect of PE or DOPE to promote liposome–endosome interactions can be explained by the low hydration of its polar head group as compared to the significant repulsive forces associated with the hydration layer of PC or DSPC polar head groups [35]. Therefore, the presence of DOPE enhances the hydrophobicity of the liposomal membrane, thus facilitating energetically favorable interactions between lipid bilayers. Furthermore, DOPE tends to assume a hexagonal inverted phase (H_{II}) leading to the formation of non-lamellar structures [8]. This may constitute a key element to trigger endosomal destabilization, thus leading to cytoplasmic delivery of their contents. This proposal partially explains our observations on the striking similarity of the efficacy of intracellular delivery mediated by all DOPE-containing formulations.

Vidal and Hoekstra [34] have also demonstrated that treatment of endocytotic vesicles with trypsin strongly reduced their interaction with PE-containing liposomes, thus providing evidence that endosome-associated proteins play a major role in this process. Based on these findings, these authors suggest that such proteins may not only be involved in the process of membrane fusion, but also promote liposome aggregation which favors their destabilization. Assuming that this hypothesis can be extended to liposome–endosome interactions at the luminal surface of endocytotic vesicles, it could justify the disparity of results between the biophysical studies (in buffer) and those involving cultured cells, in terms of acidification-induced destabilization. Similar conclusions were obtained in lipid mixing studies involving pH-sensitive liposomes and human erythrocyte ghosts, where a decrease in the extent of lipid mixing was observed by cleaving the sialic acid residues of the glycocalyx [36].

It should be noted that despite the similarity of the efficacy of intracellular delivery, evaluated in terms of calcein release, observed among the different liposomes tested, such findings cannot be extrapolated directly to other types of encapsulated molecules, particularly to those with high molecular weights [3]. In addition, we recently reported that the ability to mediate intracellular delivery of molecules with relatively high molecular weight (e.g. antisense oligonucleotides) is significantly higher for liposomes composed of DOPE:CHEMS as compared with oth-

er DOPE-containing liposomes [14]. Formulations that were shown not to be pH-sensitive in buffer (DOPE:PG, DOPE:PS and DOPE:PC) were those that have proven to be less efficient in releasing large molecules upon their destabilization triggered by acidification of the endosomal lumen and/or by interactions with the endosomal membrane.

Our results indicate that DOPE:CHEMS liposomes were internalized more extensively than either sterically (DOPE:CHEMS:DSPE-PEG) and non-sterically stabilized liposomes (e.g. DOPE:PS). As referred to above, our findings can be explained by the fact that sterically stabilized pH-sensitive liposomes can escape the capture by macrophage-like cells. However, such an explanation does not hold for the observations regarding DOPE:PS liposomes. In this context, it should be noted that liposomes with a net negative charge are internalized more extensively than uncharged liposomes [37]. On the other hand, it is known that pH-sensitive liposomes are internalized by a non-specific process and more efficiently than conventional liposomes (lacking DOPE). This has been attributed to the low degree of hydration of the DOPE polar head group and to the resulting increased affinity to the cell membrane [3,5]. Based on these considerations and since all tested formulations were negatively charged (exhibiting similar values for the ζ potential) and contained the same amount of DOPE, it remains to be clarified why DOPE:CHEMS liposomes exhibit higher affinity to THP-1 cells as compared to DOPE:PS liposomes. A possible explanation is that the polar head groups of DOPE molecules become more exposed in CHEMS-containing liposomes (due to the formation of less densely packed bilayers) favoring the interaction with the lipids of the cell membrane. In fact, CHEMS does not present a bulky head group and thus may facilitate the approach of DOPE to the cell membrane.

Despite these differences, our results also suggest that liposome composition does not affect significantly the kinetics of intracellular delivery. In fact, following an initial stage of liposome–cell incubation, the extent of cytoplasmic delivery of calcein per cell-associated liposome is already significant for all the formulations and tends to increase at a similar rate with incubation time. It should be noted, however, that for incubation times longer than 30 min, calcein

release may also result from liposome degradation inside lysosomes.

Besides the crucial role attributed to DOPE in promoting cytoplasmic delivery as illustrated by our results, it is possible that the high efficacy observed for sterically stabilized pH-sensitive liposomes (DOPE:CHEMS:DSPE-PEG) is due to the presence of poly(ethylene glycol). In fact, several studies have shown that PEG behaves as a dehydrating agent that could facilitate the close approach between membranes, thus promoting fusion [38]. Nevertheless, such a putative synergistic effect of PEG on fusion strongly depends on the presence of a fusogenic compound in the liposomal membrane (e.g. DOPE), since DSPC:CHEMS:DSPE-PEG liposomes were particularly inefficient in promoting intracellular delivery.

The results obtained from studies involving the use of agents that interfere with the endocytotic pathway also contributed to a better understanding of the mechanisms underlying liposome–cell interactions. The drastic reduction of the efficacy of intracellular delivery observed for all the formulations tested when their internalization was inhibited upon cell treatment with a mixture of drugs (antimycin A, sodium fluoride and sodium azide) indicates that such liposomes utilize the endocytotic pathway (either mediated by clathrin or non-clathrin coated vesicles) to promote the intracellular release of their contents. On the other hand, the strong inhibition observed for the calcein/rhodamine fluorescence ratio when the cells were treated with lysosomotropic agents clearly demonstrates that acidification of the endocytic vesicles is crucial to the intracellular delivery mediated by the liposomes. According to the studies of Yoshimori et al. [29], treatment of cells with bafilomycin A₁ or chloroquine at the concentrations used in the present work resulted in an identical increase of the pH to values close to 6.3 in both endosomes and lysosomes. Such increase in the pH seems therefore to be sufficient to prevent liposome destabilization and/or to reduce the potential effect of the endosomal membrane-associated proteins.

For any of the formulations tested the inhibitory effect of chloroquine was more pronounced than that observed for bafilomycin A₁. Since both drugs lead to an identical increase in pH, the differences ob-

served should be attributed to mechanisms other than preventing acidification. Chloroquine is a weak base that sequesters protons being impermeable to organelle membranes when in its protonated form. Nevertheless, a lack of specificity has been recognized to this drug since its effect is not only restricted to endosomes and lysosomes, as opposed to bafilomycin A₁. Chloroquine was also shown to exhibit the capacity to affect other cellular functions, namely those related to the internalization processes [27]. Therefore, the results obtained for the effect of bafilomycin A₁ are considered as being more reliable to describe the importance of the acidification of the endocytotic pathway in the process of intracellular delivery mediated by liposomes.

Aiming at further clarifying the molecular mechanisms by which DOPE-containing liposomes overcome the endosomal membrane, we have performed experiments to monitor lipid mixing between liposome and endosome membranes in intact cells. For this purpose, liposomes labeled with Rh-PE at a self-quenching concentration (5 mol%) were incubated with the cells and the extent of membrane fusion was measured as a function of time. Independently of the technique used (spectrofluorimetry, flow cytometry or fluorescence microscopy) no sufficient sensitivity was achieved to allow such evaluations. In this context, recent studies have shown that the permeabilization and fusion of this particular formulation of pH-sensitive liposomes occur as a result of progressive lipid reorganization upon acidification [39]. These findings are in close agreement with those from other previous studies that reported aggregation of DOPE:CHEMS liposomes as well as occurrence of lipid mixing induced by acidification of the milieu [40]. Nevertheless, it should be noted that these results were obtained from membrane fusion studies that were performed using only unlabeled liposomes as target membranes, which are too simple and therefore far from mimicking cellular membranes. It is possible that by performing lipid mixing studies using other sensitive assays, including resonance energy transfer assays (e.g. NBD-PE/Rh-PE), the ability of DOPE-containing liposomes to fuse with the endosome membranes in intact cells can be demonstrated. On the other hand, it is also possible that destabilization of endosomal membranes

mediated by pH-sensitive liposomes does not involve lipid mixing but rather pore formation, whose evaluation would require another type of assays.

Using a novel fluorescence assay involving the labeling of both the membrane and aqueous compartment of liposomes, we were able to fully characterize the extent of their interaction with cells from binding, internalization to intracellular delivery. In addition, the use of different but complementary techniques (which in some cases were optimized for this purpose) allowed us to gain insights into the role of DOPE in the process of endosomal destabilization as well as into the mechanisms by which DOPE-containing liposomes interact with cells and deliver their contents.

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