Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 1373–1379

BIOORGANIC & MEDICINAL CHEMISTRY

# Spectroscopic Detection of Endovesiculation by Large Unilamellar Phosphatidylcholine Vesicles: Effects of Chlorpromazine, Dibucaine, and Safingol

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Received 28 September 1998; accepted 15 December 1998

**Abstract**—Endovesiculation by large unilamellar vesicles (LUVs) induced by cationic amphiphiles is described in this work. A recent procedure to monitor phagocytosis of vesicles by macrophages by determining the amount of the simultaneously internalized water-soluble fluorescent dye HPTS with external quencher was adapted to LUVs (Daleke, D. L.; Hong, K.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1990**, *1024*, 352). Compared to dibucaine and safingol, the local anesthetic chlorpromazine (CPZ) was found to be the most efficient inducer of HPTS-internalization by LUVs. Control experiments using LUVs with entrapped HPTS indicated that the observed dye-internalization does not originate from transient lysis. A strong increase in activity above the critical micelle concentration of CPZ implies the importance of CPZ-micelles for endovesiculation. The significantly less efficient CPZ-induced HPTS-internalization by LUVs with 68 nm compared to 176 nm diameter further diminishes the likelihood of a micelle/bilayer fusion mechanism and supports the presence of 'zipper-type' endovesiculation by LUVs with diameters as small as 68 nm. 

## Introduction

Large unilamellar vesicles (LUVs) are well-defined model systems that have substantially contributed to our comprehension of the molecular mechanisms of complex biological processes that occur at cell membranes.[1,2] In contrast to other cell membrane model systems such as black lipid membranes or Langmuir films, LUVs are also compatible with conventional spectroscopic methods, a property that facilitates the examination of structure–activity relationships (SAR) of natural products and synthetic therapeutics that interact with lipid bilayers.[1] Beyond SAR, LUVs serve as excellent models to quantify the effect of specific parameters of the lipid bilayer on activity (e.g. membrane potential,[3–6] membrane curvature,[7] charge of the membrane surface,[6,8–10] phase behavior,[7–12] osmotic pressure,[13–16] and membrane thickness [17,18]), because the (glyco)proteins, glycolipids or complex lipid mixtures that complicate the interpretation of whole-cell assays are absent. However, despite the clear advantages of these biomembrane models, some essential transport mechanisms have yet to be investigated with LUVs. For instance, we are not aware of model studies on the molecular mechanism of endocytosis using LUVs.

Endocytosis is an intriguing signal transduction process that in most cases involves the formation of protein-coated intracellular vesicles upon binding of an external ligand to a cell surface receptor.[19] Although appealing, the reconstitution of such complex supramolecular systems with, in part, poorly identified components in LUVs is currently unrealistic. The few examples of clathrin-independent receptor-mediated endocytosis follow perhaps a simpler mechanism, but the involved proteins are not well characterized.[20,21] Thus, only a few endocytosis inducers exist today that can be used as positive controls for the development of an LUV-model system to study endocytosis.[22–24]

Local anesthetics such as chlorpromazine (CPZ) are best characterized among the rare examples of small organic molecules that presumably cause endocytosis without further assistance of membrane proteins.[22] CPZ is an amphiphilic phenothiazine tranquilizer, and the effects of CPZ on lipid bilayers have attracted a great deal of attention because biomembranes are a potential site of action.[11,22,25–35] Concerning endocytosis, detailed

Abbreviations: CPZ: chlorpromazine; DMPC: dimyristoylphosphatidylcholine; DMSO: dimethyl sulfoxide; DPX: *p*-xylene-bis-pyridinium bromide; EYPC: egg yolk phosphatidylcholine; HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPTS: 8-hydroxypyrene-1,3,6-trisulfonic acid; LUV: large unilamellar vesicle; RBC: red blood cell; SAR: structure–activity relationship; SUV: small unilamellar vesicle

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0968-0896/99/$ - see front matter 
PII: S0968-0896(99)00076-0

microscopic studies by Hägerstrand and Isomaa on red blood cells (RBCs) treated with various amphiphiles revealed an apparently unique property of CPZ: endovesicles within the lumen of endovesicles with diameters below 1 μm were seen in the electron micrograph of RBCs treated with 200 μM CPZ at pH 7.4 (Fig. 3(f) in ref. 22). This recent observation is in disagreement with the unverified hypothesis that the increased curvature of the membrane in LUVs would inhibit endovesiculation. Here, we report for the first time the spectroscopic detection of endovesiculating vesicles with diameters down to 68 nm, using cationic amphiphiles as mediators.

### Terms and definitions

'Endocytosis' is used here without mechanistic implications:[19] 'Uptake of material into a cell by its internalization in an endovesicle.' 'Endovesiculation' is used for the identical process occurring with vesicles instead of cells (i.e. 'uptake of material into a *vesicle* by its internalization in an endovesicle'). Also, we do not differentiate between the terms 'vesicle' and 'liposome' and use 'vesicle' only.[2] Unilamellar vesicles have been classified as large (LUVs) for 100–200 nm diameter and small (SUVs) for 30–50 nm diameter;[2] unilamellar vesicles with intermediate diameters down to 68 nm are named here LUVs for convenience only.

## Results

### CPZ-induced HPTS-internalization by LUVs with 176 nm diameter

Papahadjopoulos and co-workers have recently pointed out that the water-soluble, pH-sensitive fluorophore HPTS has superb spectroscopic properties to measure endocytosis.[36] Namely, the intensity of the excitation maxima at 403 nm increases with increasing pH, while the one at 450 nm decreases, and excitation intensity at the isosbestic point at 413 nm is pH-independent. Internalized HPTS can be quantified by quenching the external HPTS with water-soluble DPX and measuring the remaining HPTS-emission intensity using the pH-independent excitation at 413 nm. The pH-dependent emissions of HPTS can further be used to detect pH-changes in the environment of HPTS during endocytosis or endovesiculation in a ratiometric manner.

To optimize the conditions to study endovesiculation by LUVs using the fluorophore HPTS and the quencher DPX, the following parameters were considered: High salt concentration, namely 100 mM NaCl, is advantageous to enhance the emission intensity of HPTS, i.e. the sensitivity of the measurement.[37] The buffer capacity of 100 mM HEPES at pH 7.1 is sufficient to prevent significant pH-changes by addition of CPZ as a HCl salt.[28] In 100 mM NaCl, 100 mM HEPES, pH 7.1, the emission intensity of HPTS was maximal around 90 μM.

In all cases, suspensions of uniformly sized unilamellar EYPC vesicles were used at a lipid concentration of 5 mM. Under these conditions, the ratio of external/internal volume is ~19 for 176 nm LUV-diameter and ~52 for 68 nm diameter.[38] Thus, the quenching efficiency of external DPX is a critical parameter for the detection of relatively small amounts of internalized HPTS, i.e. the sensitivity of the experiment.[36] For a single fluorimetric analysis of internalized HPTS after a given period of time, 20 μL of the vesicular suspension were added to 1980 μL of DPX-containing buffer. During this operation, external HPTS is being diluted to 900 nm while the concentration of internalized HPTS remains 90 μM. To optimize the detectability of small amounts of internal HPTS under these conditions, the emission intensity of 90 μM HPTS entrapped in LUVs (176 nm diameter) was measured at low LUV-concentrations in the presence of 900 nm external HPTS and increasing concentrations of external DPX. 99.7%-Quenching of 900 nm external HPTS by 40 mM DPX proved sufficient to unambiguously detect internal HPTS of 500 nm EYPC-LUVs with entrapped 90 μM HPTS. This indicates that internalized HPTS (90 μM) occupying ≤1% of the internal volume of 5 mM LUVs is measurable.

Figure 1 shows the HPTS emission spectra of a representative experiment. Suspensions containing 5 mM EYPC-LUVs with a diameter of 176 nm, external HPTS (90 μM) and external CPZ at concentrations ranging from 0 to 400 mM were incubated at 22 °C. After defined periods of incubation time (i.e. 1, 24, 48 h), external HPTS was quenched by adding 20 μL of this suspension to 40 μM water-soluble DPX (1980 μL), and the internalized amount of HPTS was detected by measuring the emission intensity ($I$) at 510 nm (Fig. 1A). To normalize the obtained values for internalized fluorophore ($F_{in}$), the emission intensity ($I_0$) of fully quenched HPTS was measured after lysis with 40 μL 10% triton X-100 (Fig. 1B). A plot of the ratio of externally versus completely quenched HPTS emission ($F_{in} = I/I_0 - 1$) as a function of CPZ-concentration is shown in Figure 2.

The effect of increasing CPZ-concentrations on the internalization of 90 μM external HPTS is not linear (Fig. 2). CPZ-concentrations of up to 250 μM induced slow HPTS-internalization that never exceeded $F_{in} = 2$ over time. Identical LUVs that were filled to 100% with 90 μM HPTS gave $F_{in} \sim 40$ after 1 h of incubation, followed by decreasing values in the presence and absence of CPZ to $F_{in} \sim 30$ within 2 days due to spontaneous collapse of ~25% of the LUVs (vide infra, Fig. 3). Thus, CPZ-induced HPTS-internalization of $F_{in} \leq 2$ indicates that external solute occupies up to 7% volume of intact LUVs. Higher values are presumably not observed due to competing lysis increasingly dominating after more than two days incubation. However, at CPZ-concentrations from 250 up to 400 μM, significantly increased internalized HPTS was observed. LUVs filled up to 35% with external buffer were found with 400 μM CPZ after 2 days incubation.

### CPZ-induced leakage of HPTS from LUVs with 176 nm diameter

To investigate whether the trends seen in Figure 2 originate indeed from endovesiculation, we prepared LUVs

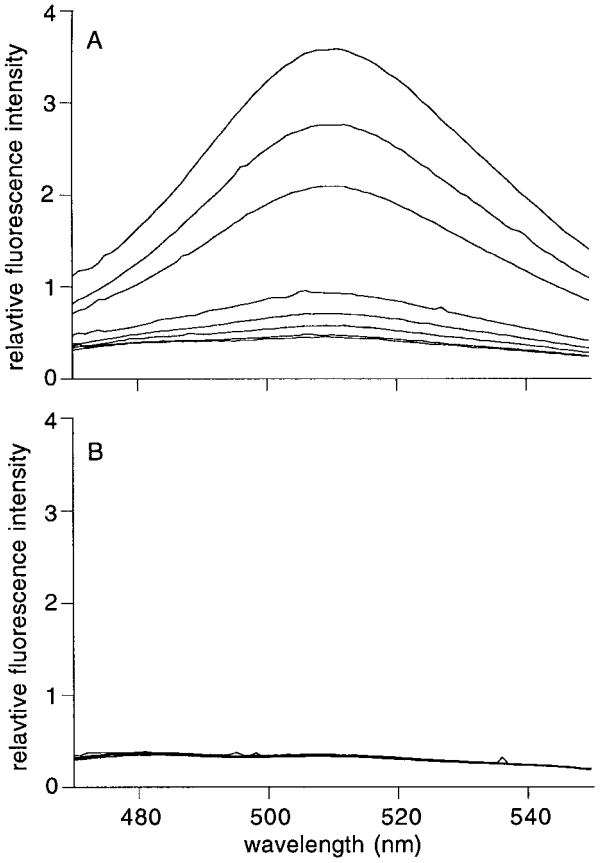


**Figure 1**. (A) Influence of increasing chlorpromazine concentrations on the intensity of HPTS emission (excitation: 413 nm) after incubation in the presence of 5 mM LUVs (176 nm diameter) for 48 h followed by quenching of extravesicular HPTS with 40 mM DPX. The CPZ concentrations following decreasing emission intensity were 400, 350, 300, 250, 200, 150, 100, and 50 μM. (B) The intensity of HPTS emission (excitation: 413 nm) for all samples shown in Figure 1A after the addition of 50 μL 10% triton X-100. The scale for the relative emission intensity in both figures is identical.

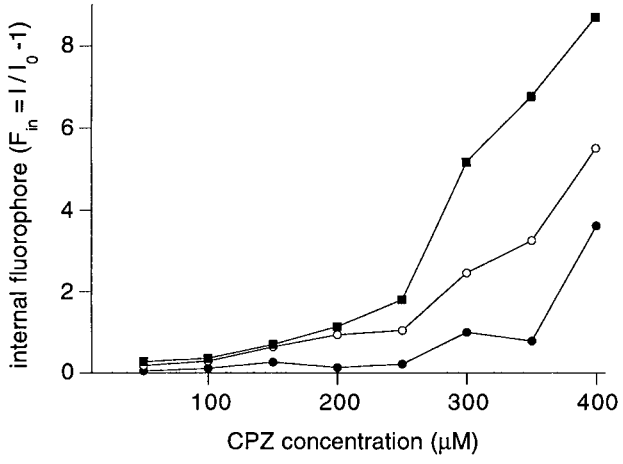


**Figure 2**. CPZ-induced HPTS-internalization by LUVs (176 nm diameter) as a function of chlorpromazine concentration and time (incubation: (●) 1 h, (○) 24 h, (□) 48 h). Normalized values ($F_{in} = I/I_0 - 1$) were obtained from the ratio of the emisson maxima of HPTS before ($I$, Fig. 1A) and after the addition of 10% triton X-100 ($I_0$, Fig. 1B).

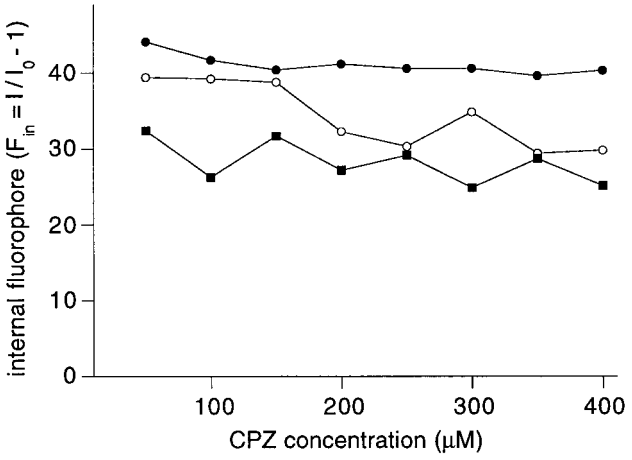


**Figure 3**. CPZ-induced dye leakage from LUVs (176 nm diameter) loaded with 90 μM HPTS as a function of chlorpromazine concentration and time (incubation: (●) 1 h, (○) 24 h, (□) 48 h). Normalized values for the remaining internal HPTS ($F_{in} = I/I_0 - 1$) were obtained from the ratio of the emisson of HPTS at 510 nm before ($I$, compare Fig. 1A) and after the addition of 10% triton X-100 ($I_0$, compare Fig. 1B); excitation: 413 nm.

with entrapped 90 μM HPTS, and measured dye leakage as a function of time and CPZ-concentration as described above for dye internalization. Figure 3 shows that the concentration of internal HPTS, $F_{in}$, decreases from ~40 to ~28 within two days. Increasing CPZ concentrations slightly accelerated the collapse of HPTS-filled LUVs in a roughly linear manner, while significant effects comparable to the increased internalization above CPZ-concentrations of ~200 μM were, however, not observed. The absence of simultaneous leakage indicates that CPZ-induced internalization is not the result of transient lysis with rapid bilayer resealing[15] and thus supports the view of endovesiculating LUVs.

## CPZ-induced HPTS-internalization by LUVs with 68 nm diameter

Concerns that the increased curvature of the membrane in LUVs would hinder or even inhibit endovesiculation are perhaps the major reason endovesiculation by LUVs has not been investigated before. To assess the effect of increasing membrane curvature on CPZ-induced endovesiculation, LUVs with a diameter of 68 nm were prepared and studied. As depicted in the representative experiment in Figure 4, LUVs with 68 nm generally gave more than 10-times lower values for internalized HPTS compared to 176 nm diameter (Fig. 2).

This reduced HPTS-internalization with increased membrane curvature needs to be interpreted with caution, because the internal volume of vesicles per mole of lipid decreases with size. EYPC-LUVs of 68 nm have an internal volume of 1940 mL/mole lipid, EYPC-LUVs of 176 nm have one of 5350 mL/mole lipid.[38] For internalized HPTS $F_{in} = 5.3$ in LUVs with 176 nm after 24 h with 400 μM CPZ (Fig. 2), for example, $F_{in} = 1.9$ would be expected for LUVs with 68 nm with identical activity. However, $F_{in} = 0.7$ was observed instead of 1.9, indicating that the efficiency of CPZ-induced endovesiculation

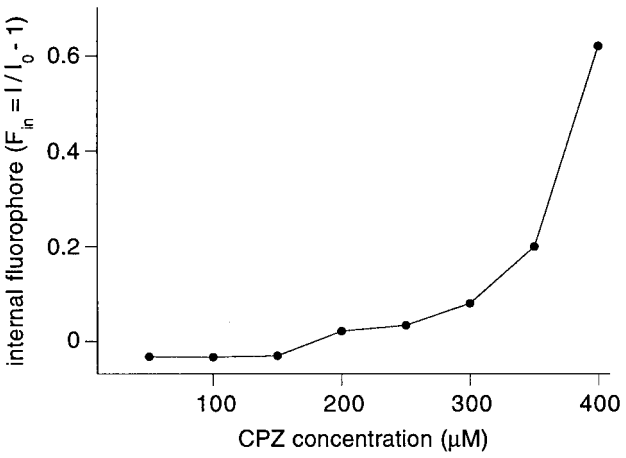


**Figure 4**. CPZ-induced HPTS-internalization by LUVs (68 nm diameter) as a function of chlorpromazine concentration (incubation time: 24 h). Normalized values ($F_{in} = I/I_0 - 1$) were obtained from the ratio of the emisson maxima of HPTS before ($I$, compare Fig. 1A) and after the addition of 10% triton X-100 ($I_0$, compare Fig. 1B).

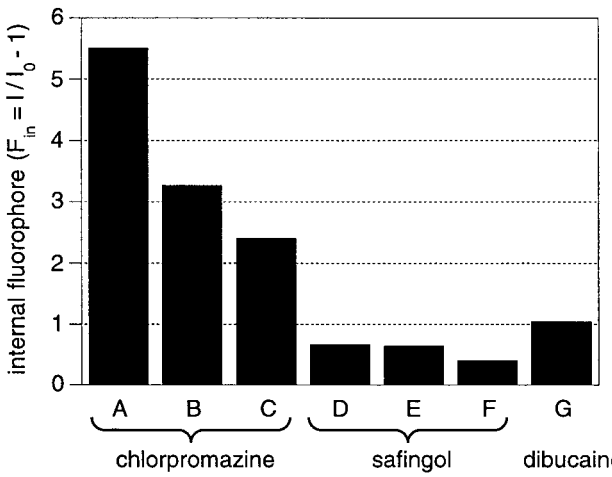


**Figure 5**. HPTS-internalization by LUVs (176 nm diameter) after 24 h incubation at 22 ± 1 °C in the presence of the cationic amphiphiles chlorpromazine, safingol, and dibucaine at biochemically relevant concentrations. (A) 400 μM, (B) 350 μM, (C) 300 μM chlorpromazine; (D) 100 μM, (E) 50 μM, (F) 25 μM safingol; (G) 600 μM dibucaine.

of unilamellar vesicles indeed depends significantly on the size of the vesicle.

### Dibucaine- and safingol-induced HPTS-internalization by LUVs with 176 nm diameter

The capacity of cationic amphiphiles other than CPZ to induce HPTS-internalization was explored using dibucaine and safingol as examples. Dibucaine was selected because of its similarity to CPZ with respect to structure and function.[4,22] Dibucaine at a concentration of 600 μM was shown to induce endocytosis by RBCs.[22] In contrast to CPZ, vesicles within the lumen of endovesicles within RBCs were not reported, although endovesicles with invaginations were seen.[22] In EYPC-LUVs, dibucaine induced HPTS-internalization significantly less efficiently than CPZ (Fig. 5). For instance, treatment of LUVs with 600 μM dibucaine for 24 h resulted in $F_{in} = 1.0$ compared to $F_{in} = 5.5$ for 400 μM CPZ (Fig. 5A and G). Due to competing lysis, the values for internalized HPTS never exceeded $F_{in} = 2$. Even weaker HPTS-internalization was observed in the presence of 25–100 μM safingol (i.e. L-*threo*-dihydrosphingosine, Fig. 5D–F). We investigated safingol because of the recent finding that apoptosis of mitomycin C-treated gastric cancer cells is potentiated in the presence of 50 μM of this cationic lipid.[39] Although not considered by the authors, our preliminary results do not exclude the possibility of safingol-induced internalization of mitomycin C as the molecular mechanism of this synergism.

## Discussion

Our results demonstrate CPZ-induced HPTS-internalization by large unilamellar vesicles (Figs 2 and 4). The absence of HPTS-efflux comparable to HPTS-internalization explicitly excludes the possibility of transient lysis[15] and strongly implies that the HPTS-internalization, particularly in the presence of > 200 μM CPZ, originates from CPZ-induced endovesiculation by LUVs. The smaller effects of ≤200 μM CPZ may have the same origin, but other mechanisms can not be excluded at this stage. The nonlinear change in activity of CPZ with increasing concentrations can be best explained with the formation of CPZ-micelles above 200 μM. However, the critical micelle concentration (cmc) of CPZ has been a controversial topic during the last decade, and a broad variety of reported values ranging between 10 μM and 2 mM illustrates well the high dependence of the cmc of CPZ on experimental conditions.[11,28] In support of an increased HPTS-internalization above 200 μM due to induction by CPZ-micelles, a cmc of 200 μM at pH 7.3 and 22 °C was found in a comprehensive study by Louro and co-workers.[28] Reduced CPZ-activity with increased temperature and decreased pH further supports the importance of CPZ-micelles for HPTS-internalization (not shown).

Although less likely, CPZ-induced HPTS-internalization by a mechanism other than endovesiculation (Fig. 6A) can not be excluded (Fig. 6B). Namely, mixed HPTS/CPZ-micelles may fuse with the lipid bilayers and subsequent mixing of CPZ and lipids may trigger spontaneous release of HPTS along its concentration gradient. However, micelle/membrane-fusion should be unaffected or rather facilitated by increased membrane curvature, which is not the case (Figs 2 and 4). The observation of Luxant and Galla that the partition coefficient of CPZ in DMPC vesicles dramatically decreases above the cmc of CPZ further disfavors micelle/membrane-fusion.[11] CPZ-induced endovesiculation is further in agreement with recent reports on endovesicles (and exovesicles) seen after the treatment of RBCs with CPZ,[22] and frequent observations of endovesicles within various cell types induced by polycationic particles.[36,40,41]

The mechanism of CPZ-induced endovesiculation by LUVs may be comparable to 'zipper-type' endocytosis (Fig. 6A). Inspired by viral infection, zipper endocytosis

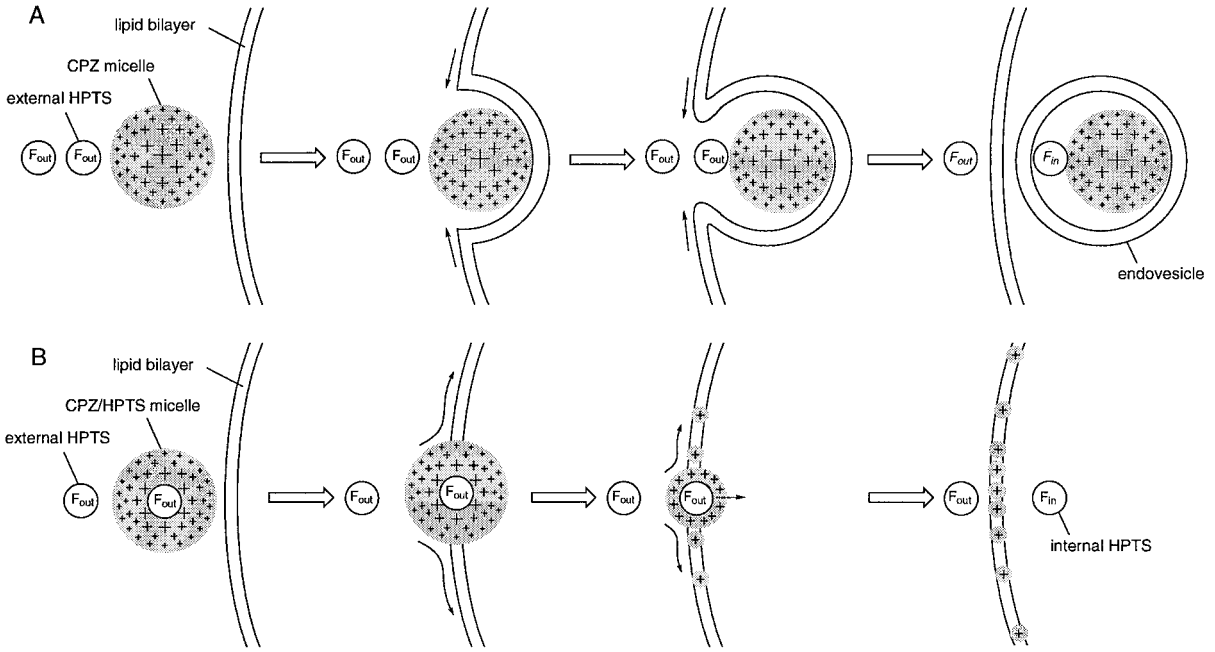


**Figure 6**. Two possible mechanisms from CPZ-induced HPTS-internalization by LUVs. (A) 'Zipper' endovesiculation (adapted from ref 24). (B) Micelle/membrane fusion.

has been first proposed by Behr to explain the delivery of hydrophilic drugs to intracellular targets by polycationic particles.[24] Zipper endocytosis is thought to be initiated by electrostatic contact of a polycationic particle with a negatively charged plasma membrane, which induces the inversion of the membrane curvature to maximize multivalent, nonspecific electrostatic interactions with the charged surface of the particle, invagination and finally the release of an endovesicle. Because CPZ-micelles are polycationic particles comparable to synthetic gene transfer vectors, zipper endovesiculation may apply for CPZ-induced HPTS-internalization. In contrast to Behr's model though, CPZ-induced endovesiculation apparently does not require a negatively charged membrane for function. The absence of charges at the surface of the zwitterionic EYPC-LUVs, together with other parameters such as membrane curvature and/or membrane potential,[4] may affect the rate of endovesiculation.[22,36] However, it is the distinct advantage of the LUV-method introduced here that the influence of parameters such as charges, membrane curvature and potential on endocytosis can be studied, identified, and quantified separately. Structural and functional similarity with CPZ implies that dibucaine-induced HPTS-internalization may follow the same 'zipper' mechanism (Fig. 6A). However, the apparent lack of reports on endocytosis induced by safingol-micelles together with the lipidic nature of dihydrosphingosine rather support the possibility of a mixed micelle/membrane fusion mechanism for the observed weak HPTS-internalization instead (Fig. 6B).

Exovesicles have been seen together with endovesicles in electron micrographs of RBCs treated with 200 μM CPZ,[22] and Luxant and Galla have found that DMPC-vesicles and CPZ-micelles form low molecular weight mixed micelles which may be interpreted as exovesicles.[11] Although not detectable, it is therefore likely that CPZ-induced exovesiculation occurs simultaneously with endovesiculation. The absence of HPTS-leakage implies that exovesiculation of LUVs is either disfavored compared to endovesiculation or that the resulting exovesicles do not release the entrapped HPTS.

In summary, we have demonstrated that CPZ induces internalization of HPTS by unilamellar vesicles with diameters as small as 68 nm, dependent on concentration, cmc, and membrane curvature, and concluded that these effects originate with all likelihood from CPZ-induced 'zipper-type' endovesiculation. Other cationic amphiphiles had reduced activity compared to CPZ. Future studies of endocytosis inducers and mediators (e.g. vesicles,[36] dendrimers,[42] natural and synthetic amphiphiles,[24,43] cell surface receptors[19–21] and potential models[44]) with the here introduced, well-defined model system may provide insights into the molecular mechanism(s) of endocytosis and facilitate rational design and evaluation of drug delivery systems.

## Experimental

### Materials

Egg yolk phosphatidylcholine (EYPC) was purchased from Avanti Polar Lipids, 8-hydroxypyrene-1,3,6-trisulfonic acid (trisodium salt, HPTS) and *p*-xylene-bispyridinium bromide (DPX) were obtained from Molecular Probes. Safingol, detergents, all salts, and buffers were of the best grade available from Sigma and used without further purification. Dibucaine (hydrochloride) and chlorpromazine (hydrochloride, CPZ) were purchased from Aldrich Co.

### Vesicle preparation

Large unilamellar vesicles (LUVs) were prepared using the dialytic detergent removal method[38,45] using a Mini Lipoprep® (Sialomed) which gives reproducibly uniformly sized vesicles. For EYPC-LUVs with 176 ± 3 nm diameter, a solution of EYPC (50 mg, 66 μmol) in ethanol (50 μL) was mixed with *n*-octyl β-D-glucopyranoside (96.4 mg, 330 μmol) and buffer (950 μL, 100 mM HEPES (pH 7.1), 100 mM NaCl). The resulting clear solution was dialyzed against buffer (1 L, 100 mM HEPES (pH 7.1), 100 mM NaCl) for 12 h. All dialyses were carried out at room temperature in the dark. The resulting suspension was purified by gel filtration (Sephadex G-50, 1×27 cm) and diluted with 100 mM HEPES (pH 7.1), 100 mM NaCl to 6 mL to give 10 mM vesicle stock solution. For EYPC-LUVs with 176 ± 3 nm diameter and entrapped HPTS, a solution of EYPC (50 mg, 66 μmol) in ethanol (50 μL) was mixed with *n*-octyl β-D-glucopyranoside (96.4 mg, 330 μmol) and 950 μL of buffer (100 mM HEPES (pH 7.1), 100 mM NaCl) containing 0.1 mM HPTS. The resulting clear solution was dialyzed against 150 mL of buffer (100 mM HEPES (pH 7.1), 100 mM NaCl) containing 0.1 mM HPTS for 5 h and then against 1 L of the same buffer without HPTS for 12 h. Extravesicular HPTS was removed by gel filtration (Sephadex G-50, 1×27 cm) and the resulting suspension was diluted with 100 mM HEPES (pH 7.1), 100 mM NaCl to 6 mL to give 10 mM vesicle stock solution. For EYPC-LUVs with 68 ± 3 nm diameter, a solution of EYPC (50 mg, 66 μmol) in ethanol (50 μL) was mixed with sodium cholate (22.4 mg, 52 μmol) and buffer (950 μL, 100 mM HEPES (pH 7.1), 100 mM NaCl). The resulting clear solution was dialyzed against buffer (1 L, 100 mM HEPES (pH 7.1), 100 mM NaCl) for 12 h. The resulting suspension was purified by gel filtration (Sephadex G-50, 1×27 cm) and diluted with 100 mM HEPES (pH 7.1), 100 mM NaCl to 6 mL to give 10 mM vesicle stock solution.

### CPZ/HPTS-LUV incubation

For CPZ-induced HPTS-internalization by EYPC-LUVs, the above described vesicle suspensions without internal HPTS (500 μL, 10 mM in 100 mM HEPES (pH 7.1), 100 mM NaCl) were diluted with 500 μL buffer (100 mM HEPES (pH 7.1), 100 mM NaCl) and treated with 20 μL HPTS (4.6 mM, $H_2O$) and 20 μL amphiphile (CPZ, dibucaine, or safingol at various concentrations in DMSO). The resulting suspensions were incubated in the dark at controlled temperature (22 ± 1°C unless otherwise indicated) using an Eppendorf Thermomixer 5437 (Fisher Scientific). In all experiments, fluorimetric analysis was carried out by taking 20 μL of the suspension after defined periods of incubation time. For CPZ-induced HPTS-leakage from EYPC-LUVs, the above described vesicle suspensions with internal HPTS (500 μL, 10 mM in 100 mM HEPES (pH 7.1), 100 mM NaCl) were diluted with 500 μL buffer (100 mM HEPES (pH 7.1), 100 mM NaCl) and treated with 20 μL CPZ (various concentrations in DMSO). The resulting suspensions were incubated and analyzed as described above.

### Fluorimetric analysis

Fluorescence was monitored with a SPEX Fluoromax 2 equipped with a thermostated cell holder and a house-made injector port. To detect the amount of internal(ized) HPTS after defined periods of incubation, 20 μL of the suspensions described above (5 mM lipids (LUVs), 90 μM HPTS (external or internal), amphiphile at various concentrations (CPZ, safingol, dibucaine), 100 mM HEPES (pH 7.1), 100 mM NaCl) were added to a gently stirred solution of 40 mM DPX (1980 μL, 100 mM HEPES (pH 7.1), 100 mM NaCl). The intensity of the HPTS emission at 510 nm was measured using the pH-independent excitation wavelength of 413 nm ($I_0$). The intensity of the HPTS emission at 510 nm was measured using excitation at 413 nm was remeasured after the addition of 50 μL of 10% aqueous triton X-100 ($I$), and the relative amount of internal(ized) HPTS was calculated ($F_{in} = I_0/I - 1$). All reported experiments were performed at least three times. However, in all cases discussed below, results from one representative experiment and not average values are reported to illustrate (a) trends that were reproducible for at least three times, and (b) hardly avoidable irregular values for single, presumably contaminated samples within a sequence.

### Acknowledgements

This work is dedicated to the memory of Demetrios Papahadjopoulos. We thank NIH (GM56147), the donors of the Petroleum Research Fund, administered by the American Chemical Society, Research Corporation (Research Innovation Award), Suntory Institute for Bioorganic Research (SUNBOR Grant), and Georgetown University for support of this work.

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