pH-sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules

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Negatively charged liposomes are endocytosed by the coated vesicle system and accumulate in acidic intracellular vesicles. Liposomes that become unstable at acidic pH improve cytoplasmic delivery of membrane-impermeant macromolecules such as calcein (CAL) and FITC dextran (18 or 40 kDa). Oleic acid (OA): phosphatidylethanolamine (PE) (3:7 mole ratio) liposomes become permeable to CAL at pH <7.0. Control liposomes of phosphatidylserine:PE or OA:phosphatidylcholine are stable at pH 4–8. OA:PE liposomes promote cytoplasmic delivery of encapsulated CAL to CV-1 cells, as evidenced by the emergence of diffuse, cytoplasmic CAL fluorescence. Delivery requires metabolic energy and is partially inhibited by chloroquine or monensin, which raise the pH of intracellular vesicles.

Liposome *Endocytosis* *Microinjection* *Drug carrier* *Calcein* *Membrane fusion*

1. INTRODUCTION

Liposomes have raised considerable interest as carriers for the cytoplasmic delivery of biologically active molecules. Nucleic acids, cytotoxic agents, and a variety of macromolecules have been encapsulated in liposomes and have been delivered to cells in a functional form [1–3]. Previously we demonstrated that negatively charged liposomes are endocytosed by the coated vesicle pathway and that encapsulated molecules with a propensity to become membrane-permeant at low pH can gain access to the cytoplasm. Large or highly charged molecules unable to exploit the low-pH environment of the endocytic pathway remain sequestered in intracellular vacuoles such as secondary lysosomes or dense bodies [4]. Thus lysosomal accumulation and subsequent degradation may be the fate of a large percentage of liposome-encapsulated, biologically active molecules delivered to cells.

To promote cytoplasmic delivery of molecules which would otherwise remain sequestered within vesicles of the endocytic pathway, we have produced liposomes from lipid constituents that can induce bilayer instability at mildly acidic pH ([15, 16]; and submitted), and have examined the ability of such liposomes to deliver charged or large molecules to the cytoplasm of cultured cells. Our strategy was to use the zwitterionic phospholipid phosphatidylethanolamine (PE) as the principal liposome constituent. The protonated species of PE ($pK \geq$ pH 8) [7] is unable to form bilayer vesicles near neutral pH, although vesicles can be formed above pH 9 [8,9]. Moreover, the presence of PE in liposome membranes facilitates membrane fusion induced by divalent cations [10]. To promote formation of stable PE-rich liposomes near neutral pH, we chose oleic acid (OA; *cis* 18:1), a negatively charged amphiphile with a pK close to neutral when incorporated into phospholipid membranes [11]. Protonation of OA at a pH within the range expected for the endocytic pathway ($4.5 <$ pH < 6.5) [12,13] should result in destabilization of a bilayer rich in PE, leading to liposome aggregation and/or membrane-membrane fusion. An additional benefit of OA is that it imparts a net negative charge on the liposomes at

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pH 7.4. Considerable evidence suggests that negatively charged liposomes are superior to neutral and positively charged liposomes in functional delivery of a variety of materials to cells [14–16].

2. MATERIALS AND METHODS

Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), and PE transesterified from egg PC were obtained as in [10]. OA, cholesterol (Chol), dextran T-20 (average 18 kDa), and fluoresceinated dextran (FTC-D) of 40 kDa were purchased from Sigma (St. Louis, MO); Chol was purified by recrystallization. Carboxyfluorescein (CF; Eastman Kodak) was purified as in [17,18]. CF, calcein (CAL; Alfa/Ventron Corp., Danvers, MA), fluoresceinated dextran T-20, and FTC-D of 40 kDa were prepared for encapsulation as in [4].

Liposomes were prepared by evaporating 10 μmol of a given lipid mixture from chloroform onto the wall of a glass tube and resuspending by vigorous vortex mixing in ½ ml of the aqueous solution to be encapsulated. The multilamellar liposomes thus formed were extruded through polycarbonate membranes (pore diameter 0.2 μm) [19,20]. Liposomes were separated from free CF, CAL, or FTC-D by gel chromatography or by centrifugation as described in [4]. Phospholipid concentration was determined as in [21].

Efflux of calcein from liposomes was observed as the increase in fluorescence as self-quenched dye was released and diluted in the external medium, as described in the legend to fig.1.

CV-1, an established line of African Green Monkey Kidney cells, was maintained in monolayer culture and prepared for experiments as described in [4,22] and as described in the legends to figs 3 and 4.

3. RESULTS AND DISCUSSION

We evaluated the pH-sensitivity of liposomes by monitoring efflux of the anionic fluorescent dye calcein, which we demonstrated previously to be an impermeant aqueous space marker for PS:Chol (2:1 mole ratio) liposomes over the pH range 4.0–7.5 [4]. Here, we found that liposomes of PS:PE (3:7 mole ratio) were also stable over the pH range 4.0–7.5. CAL release was not observed at pH 5.3 (dashed line, fig.1), but could be detected at pH 2.9 (about 10% of the total encapsulated material over 4 min; see legend to fig.1). Proton-induced leakage was only slightly greater for OA:PC liposomes (3:7 mole ratio). Fig.1 shows the time course of CAL efflux from OA:PC liposomes from pH 6.1 to pH 4.2. At pH 4.2, approx. 10% of the encapsulated CAL was released over 3 min.

In contrast to the stability of PS:PE or OA:PC liposomes, OA:PE liposomes became destabilized and leaked to CAL at mildly acidic pH. Substantial CAL efflux from OA:PE liposomes (3:7 mole ratio) was observed upon lowering the pH to 6.1 (fig.2, solid line). Over 80% of the internal contents leaked in 3 min, roughly 8-fold more than from OA:PC liposomes at the same pH (fig.2, dashed line). In contrast to proton-destabilized liposomes described in [23], release of contents from OA:PE liposomes could be observed at temperatures outside the range for bilayer gel/

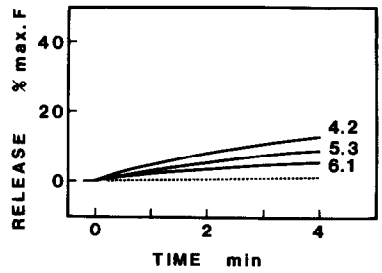

Fig.1. pH dependence of liposome stability: CAL release from OA:PC and PS:PE liposomes. 50 nmol liposomes containing self-quenched CAL (60 mM; pH 7.4) were suspended in isotonic buffer (pH 7.4) in a fluorometer cell at 20°C. At time 0, 10 μl citrate or Mes buffer adjusted to specific pH was injected to lower the pH of the suspension to the values indicated in the figure. The final pH was measured with a pH electrode, and all measurements were repeated at least 3 times. Fluorescence emission was monitored continuously at $\geq$530 nm on an SLM fluorometer (Urbana, IL) and is scaled in the figure so that 100% is the maximum fluorescence upon liposomes lysis with 0.1% Triton X-100. CAL release from OA:PC liposomes (3:7 mole ratio) at the pH indicated; (– – –) CAL release from PS:PE liposomes (3:7 mole ratio) at pH 5.3. For the sake of clarity, the trace for PS:PE liposomes at pH 2.9 has been omitted, as it was nearly identical to the curve shown for OA:PC at pH 5.3. Efflux for PS:PE at pH 2.9 was about 10% of total over 4 min.

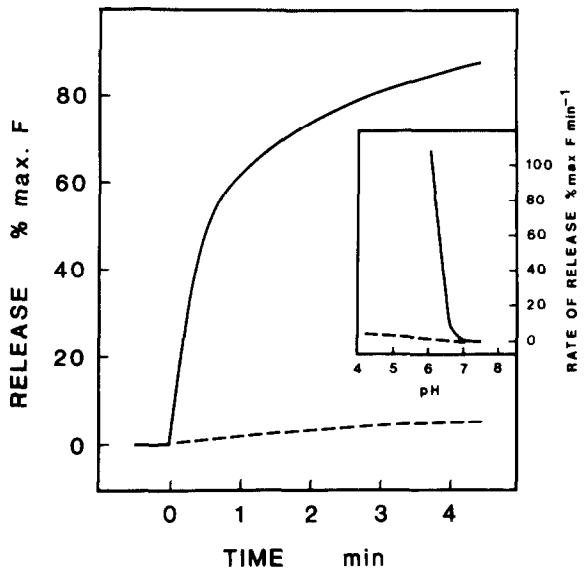

Fig.2. CAL release from OA:PE (——) and OA:PC (– – –) liposomes upon lowering the pH to 6.1. The pH was lowered as in fig.1. The inset shows the initial rate of release from OA:PE and OA:PC liposomes as a function of pH, given as the percent of maximum fluorescence per minute.

liquid-crystalline transition (T_c; unpublished). Fig.2 (inset) also shows the initial rate of CAL release as a function of pH for OA:PE (solid line) and OA:PC liposomes (dashed line). With an apparent threshold for efflux around pH 6.6 (fig.2), it is clear that endocytosed OA:PE liposomes should be destabilized soon after endocytosis, as the pH of the early endocytic compartments is believed to reach pH 5.0 within 20 min of vacuole formation [24].

Previously we demonstrated the utility of CAL as an impermeant, pH-insensitive aqueous space marker for the study of liposome-cell interaction [4]. We found that CV-1 cells endocytosed PS:Chol liposomes, and that encapsulated CAL remained confined to intra- or extracellular vesicles. Here, we obtained similar results with CAL-containing liposomes of PS:PE. That is, cells showed only vesicular (punctate) fluorescence (fig.3a) which corresponds to CAL in surface-bound liposomes or intracellular pinocytic vesicles. Fig.3b is a phase contrast micrograph of the field shown in fig.3a. Some apparent diffuse fluorescence occasionally seen in photomicrographs (cf. fig.3a), particularly in the region of the nucleus, arises from fluorescent vesicles outside the focal plane of the microscope. No diffuse cytoplasmic fluorescence could be detected upon changing the focal plane, however, indicating that little CAL gained access to the cytoplasm.

By substituting the weakly acidic dye CF for CAL [4], we verified that PS:PE liposomes are indeed endocytosed and exposed to an acid pH. As anticipated, CF in PS:PE liposomes gave rise to intense diffuse intracellular fluorescence (not shown) that represents dye free in the cytoplasm [25]. The emergence of cytoplasmic CF fluorescence was inhibited both by chloroquine and by azide/2-deoxyglucose. Thus PS:PE liposomes were endocytosed by CV-1 cells and it is the physical properties of CAL, rather than a block of liposome endocytosis, that prevented CAL from gaining access to the cytoplasm.

OA:PE liposomes (3:7) gave rise to diffuse intracellular CAL fluorescence, in contrast to the exclusively vesicular CAL fluorescence resulting from PS:PE liposomes. Fig.3c shows an even, diffuse fluorescence in many cells in the field, indicating CAL delivery to the cytoplasm. Fig.3d is a phase contrast micrograph of the field shown in fig.3c. Fig.3c also shows many fluorescent vesicles, some quite large and bright, associated with the cells. These vesicles correspond either to endocytic vesicles into which dye has leaked from endocytosed liposomes, or to surface-bound liposomes that have leaked some of their CAL with a resulting reduction in CAL self-quenching.

The intensity of diffuse fluorescence, as well as the proportion of cells displaying such fluorescence, was observed to vary with the confluence of CV-1 monolayers. At low cell density, cytoplasmic fluorescence was apparent in approx. 0.1–1% of the cells. In confluent monolayers (fig.3c), a high proportion of cells (35–70%, depending on the field) showed unambiguous diffuse fluorescence. The source of variation with monolayer confluence is unknown at present, although variation in endocytic index with cell density [26] is a plausible explanation currently under investigation.

Up to 30 mol% cholesterol as a liposome constituent (OA:PE:Chol) did not inhibit cytoplasmic delivery by OA:PE liposomes. The diffuse intracellular fluorescence with OA:PE:Chol liposomes was slightly more intense than with OA:PE

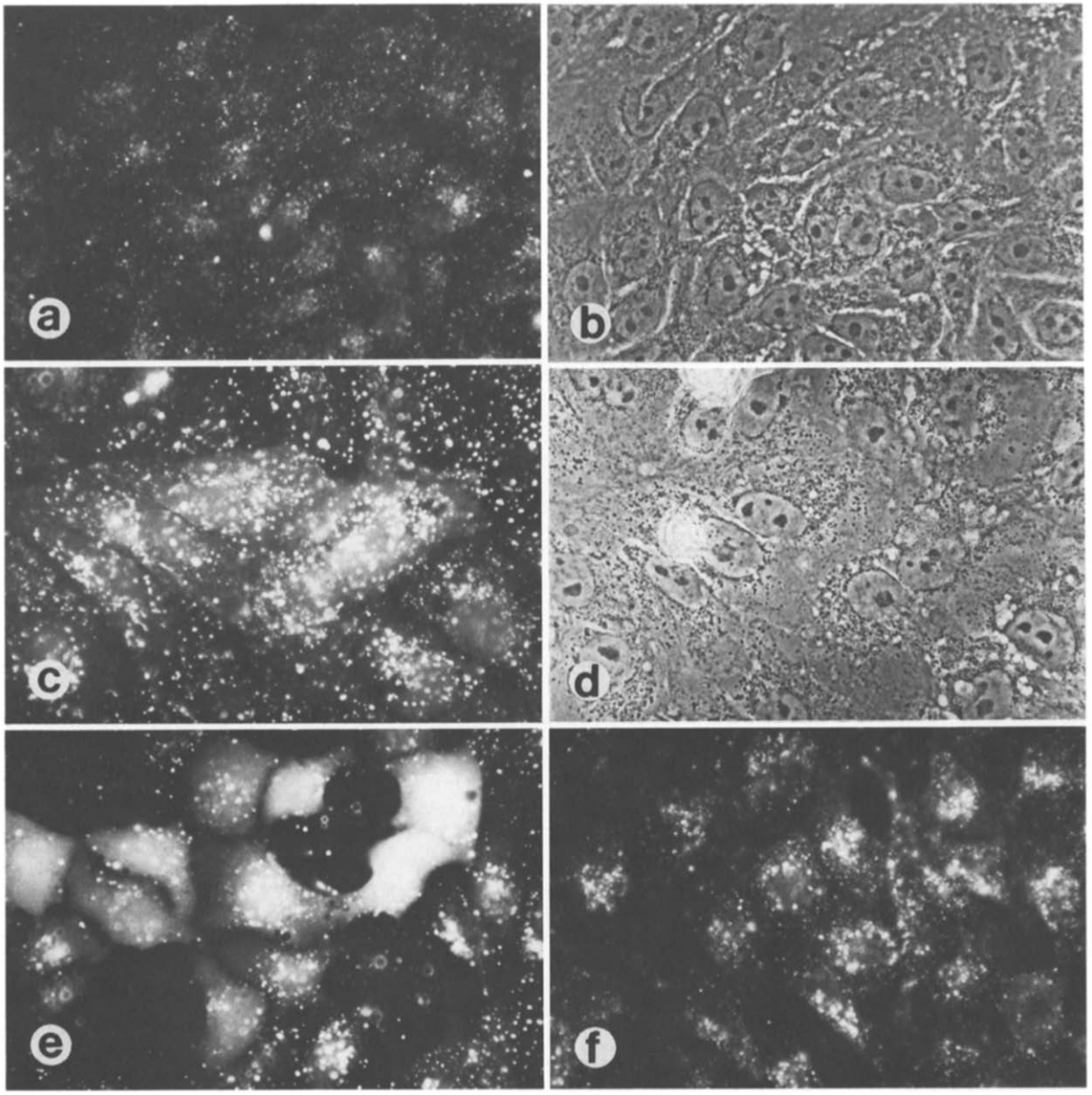

Fig.3. Cellular localization of liposome-entrapped CAL: cells ($0.5–4.0 \times 10^6$ per 66 mm dish) were washed twice with phosphate-buffered saline containing 0.4 mM Ca^{2+} and Mg^{2+} (PBS-CM), incubated for 10 min at 20°C in PBS-CM with 200 μl of CAL-containing liposomes (500 mM phospholipid), and then incubated for an additional 20 min at 37°C. Monolayers were washed twice in PBS-CM to remove free liposomes, and examined in the same buffer using a Leitz fluorescence microscope equipped with a water-immersion objective (25×). (a) Vesicular CAL fluorescence resulting from incubation with PS:PE liposomes. (b) Phase-contrast image of the field shown in (a). (c) Diffuse (cytoplasmic) and vesicular CAL fluorescence in cells incubated with OA:PE liposomes. (d) Phase-contrast image of the field shown in (c). (e) Intense diffuse CAL fluorescence in cells treated with OA:PE liposomes as in (c) and post-treated with prewarmed 21% (v/v) glycerol for 5 min at 37°C. (f) Vesicular CAL fluorescence in cells treated with PS:PE liposomes as in (a) and post-treated with glycerol. The apparent dim diffuse fluorescence in the region of the nucleus arises from vesicular fluorescence out of the focal plane of the microscope objective.

(not shown). Increased retention of encapsulated contents by cholesterol-rich liposomes [14,27] is a likely explanation for enhanced cytoplasmic delivery of CAL by OA:PE:Chol liposomes, and this possibility will be examined in future, more quantitative experiments.

Replacement of PE by PC abolished the cytoplasmic delivery of CAL. OA:PC liposomes (3:7 mole ratio) promoted only vesicular cell-associated fluorescence (similar to fig.3a; not shown), suggesting the importance of the physicochemical properties of the neutral phospholipid used. Overall, the intensity of cell-associated fluorescence was considerably less than that arising from PS:PE or OA:PE liposomes.

Previously we demonstrated that brief treatment of CV-1 cells with glycerol solutions enhances the effective cytoplasmic delivery of a number of molecules encapsulated in PS:Chol liposomes [4,14]. The enhancement is mediated by an energy-dependent, chloroquine-sensitive mechanism [4] involving extensive ruffling of the plasma membrane [28] and perhaps macropinocytosis of surface-bound liposomes [14]. Since glycerol does not promote appreciable cytoplasmic CAL delivery from PS:Chol liposomes [4], the principal effect of glycerol may be to promote liposome internalization, rather than membrane permeabilization by osmotic shock.

In this study, glycerol treatment did not result in cytoplasmic CAL delivery when cells were incubated with PS:PE (fig.3f) or OA:PC liposomes (not shown). As in fig.3a, the apparent weak diffuse fluorescence in fig.3f, particularly over the nucleus, arises from defocused vesicular fluorescence outside the focal plane of the microscope objective. In contrast, glycerol treatment did enhance considerably the intensity of diffuse cytoplasmic CAL fluorescence in cells treated with OA:PE (fig.3e) or OA:PE:Chol (not shown) liposomes.

Weakly basic amines such as chloroquine and ammonium chloride, as well as the proton-carrying ionophore monensin reduced considerably the production of cytoplasmic CAL fluorescence from OA:PE or OA:PE:Chol liposomes, in the presence or absence of glycerol. Thus a low-pH intracellular compartment is implicated in the pathway by which OA:PE liposomes deliver membrane-impermeant molecules to the cytoplasm. The failure of lysosomotropic amines to inhibit completely the emergence of diffuse fluorescence suggests that intracellular delivery may not require an endosomal pH very much below approx. 6.2, the maximum pH which lysosomes are believed to reach in the presence of chloroquine [13]. As can be seen from fig.2 (inset), pH 6.2 is well within the range of pH which promotes significant CAL leakage from OA:PE liposomes.

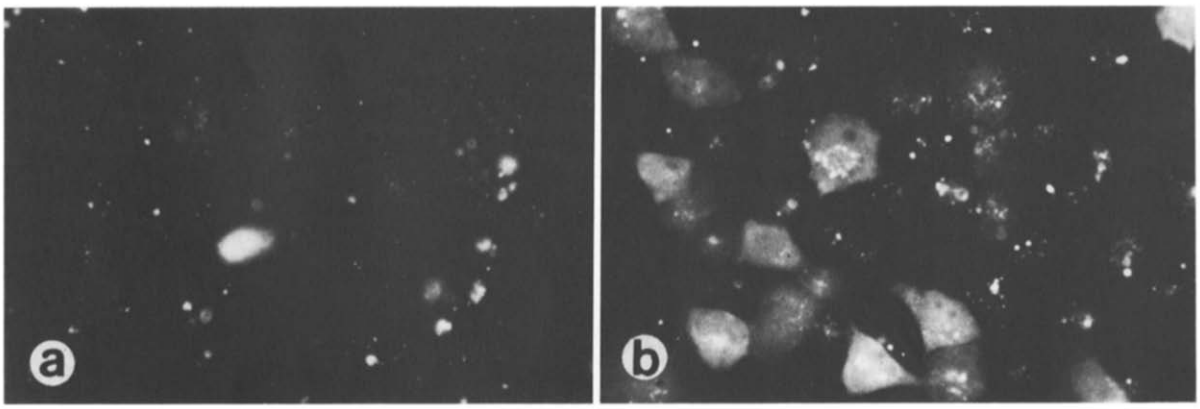

Fig.4. Cellular localization of liposome-entrapped fluoresceinated dextran. Cells were treated with liposomes as in fig.1 except that FTC-D (average 20 kDa) was substituted for CAL in liposomes. Cells were post-treated for 5 min with 21% glycerol, washed, and incubated for 24 h in growth medium before viewing on the fluorescence microscope. (a) Vesicular fluorescence resulting from incubation with PS:PE liposomes. The large fluorescent spot is debris clinging to the monolayer. (b) Diffuse fluorescence from FTC-D (average 20 kDa) delivered to the cytoplasm via OA:PE liposomes. The cell monolayer confluency and the magnification in both photographs is the same.

The emergence of cytoplasmic CAL fluorescence from OA:PE liposomes involves an energy-requiring process. Treatment of cells with 5 mM azide and 50 mM 2-deoxyglucose inhibited cytoplasmic CAL fluorescence with or without glycerol treatment, and reduced the overall level of cell-associated (vesicular) fluorescence (not shown).

Fluoresceinated dextran (average 18 or 40 kDa) was encapsulated to determine whether OA:PE liposomes also deliver large water-soluble molecules to cells. In general, cells showed vesicular fluorescence with all lipid compositions examined (not shown). It was difficult to identify unambiguous cytoplasmic fluorescence because of the high fluorescent background from surface-bound and intracellular liposomes, as well as the lower concentration of encapsulated fluor in the case of FTC-D (not shown). Following brief treatment with glycerol, bright, diffuse FTC-D fluorescence was observed in cells treated with OA:PE and OA:PE:Chol but was not observed in cells treated with PS:PE or OA:PC liposomes (not shown). As observed with cytoplasmic CAL delivery by OA:PE liposomes, the proportion of cells with diffuse FTC-D fluorescence varied with cell confluence. The frequency was 1% or less in sparsely seeded monolayers, but reached 30–50% in highly confluent monolayers. Cytoplasmic dextran fluorescence was stable, and could be observed in viable cells 24 h or more after OA:PE liposome presentation to cells (fig.4b). Over the same time period, diffuse fluorescence did not appear in cells treated with PS:PE liposomes (fig.4a). The production of FTC-D diffuse fluorescence was energy dependent, as it was inhibited by treatment of cells with azide and 2-deoxyglucose.

4. CONCLUSIONS

To promote the cytoplasmic delivery of liposome-entrapped molecules that would otherwise remain sequestered within vesicles of the endocytic pathway, we have designed liposomes which respond to mildly acidic pH by undergoing bilayer destabilization and loss of contents. When incubated with cells, such liposomes deliver their contents to the cytoplasm by a mechanism which requires metabolic energy as well as mildly acidic pH in some intracellular compartment. Here, oleic acid and phosphatidylethanolamine were found to be obligatory constituents of pH-sensitive liposomes, as substitution of PS for OA, or PC for PE drastically decreases pH-sensitivity and cytoplasmic delivery. Other negatively charged amphiphiles, such as cholesterol hemisuccinate [29] or palmitoylhomocysteine [30] can be substituted for oleic acid in making PE-rich liposomes that lose their contents in response to mildly acidic pH. However, it is as yet unknown whether liposomes of those components will be useful for cytoplasmic delivery of macromolecules. Current efforts are directed toward establishing a quantitative assay for intracellular delivery, delineating further the intracellular pathway for delivery, and evaluating pH-sensitive liposomes as carriers for intracellular delivery of biologically active macromolecules such as DNA.

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