

# Sterically Stabilized pH-sensitive Liposomes

INTRACELLULAR DELIVERY OF AQUEOUS CONTENTS AND PROLONGED CIRCULATION *IN VIVO*\*

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Liposomes that destabilize at mildly acidic pH are efficient tools for delivering water-soluble drugs into the cell cytoplasm. However, their use *in vivo* is limited because of their rapid uptake from circulation by the reticuloendothelial system. Lipid-anchored polyethylene glycol (PEG-PE) prolongs the circulation time of liposomes by steric stabilization. We have found that addition of PEG-PE to the membrane of pH-sensitive liposomes composed of cholesteryl hemisuccinate (CHEMS) and dioleoylphosphatidylethanolamine (DOPE) confers steric stability to these vesicles. This modification significantly decreases the pH-dependent release of a charged water-soluble fluorophore, calcein, from liposomes suspended in buffer or cell culture medium. However, the ability of such liposomes to release calcein intracellularly, measured by a novel flow cytometry technique involving dual fluorescence labeling, remains unaltered. As expected, the release of calcein from liposomes endocytosed by cells is inhibited upon pretreatment of the cells with NH<sub>4</sub>Cl, an inhibitor of endosome acidification. The unique properties of these liposomes were also demonstrated *in vivo*. The distribution kinetics of <sup>111</sup>In-containing CHEMS/DOPE/PEG-PE liposomes injected intravenously into rats has pharmacokinetic parameters similar to control, non-pH-sensitive, sterically stabilized CHEMS/distearoylphosphatidylcholine/PEG-PE liposomes. In contrast, regular pH-sensitive liposomes lacking the PEG-PE component are cleared rapidly. Sterically stabilized pH-sensitive liposomes may therefore be useful for the intracellular delivery *in vivo* of highly negatively charged molecules such as genes, antisense oligonucleotides, and ribozymes for the treatment of various diseases.

Ever since their invention (1), liposomes have been considered possible vehicles for delivering into cells molecules that would otherwise not be readily internalized. Realization of this potential, however, has been hampered because either (i) the liposomes are rapidly removed from the circulation by the reticuloendothelial system, or (ii) they do not deliver large or highly charged molecules into cells (2–4). The first of these

handicaps has been overcome by the inclusion of poly(ethylene glycol)-derivatized phosphatidylethanolamine (PEG-PE)<sup>1</sup> in the liposome membrane, resulting in much longer half-lives *in vivo*, compared with conventional liposomes (5–7). These “sterically stabilized,” or “Stealth®,” liposomes were successfully used for the treatment of experimental cancers *in vivo* (8–10), and liposomes containing the antitumor drug doxorubicin have been approved recently for the treatment of Kaposi’s sarcoma (2). The second handicap has been addressed by developing liposomes that destabilize at mildly acidic pH (pH-sensitive liposomes) and can deliver highly charged encapsulated materials into cells more efficiently than non-pH-sensitive liposomes (11, 12). After liposomes enter cells via endocytosis, the acidic pH inside the endosomes causes pH-sensitive liposomes to release their aqueous contents into the cytoplasm, most likely by destabilizing the endosome membrane (13). Like other non-sterically stabilized liposomes, however, pH-sensitive liposomes have very short circulation times *in vivo* (14). Here we report the development of pH-sensitive, sterically stabilized, liposomes that deliver their contents into cells in a pH-dependent manner and have prolonged circulation *in vivo*. A preliminary account of our results has appeared previously (15).

## EXPERIMENTAL PROCEDURES

**Reagents**—Phospholipids: egg phosphatidylcholine, dioleoylphosphatidylethanolamine (DOPE), lissamine rhodamine B-phosphatidylethanolamine (egg) (Rh-PE), and distearoylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (CHEMS), calcein, MES, TES, EDTA, phorbol 12-myristate 13-acetate, diethylenetriaminepentaacetic acid (DTPA), Triton X-100, propidium iodide, and NaCl were obtained from Sigma. Poly(ethylene glycol) (2000)-distearoylphosphatidylethanolamine (PEG-PE) was synthesized as described before (16). <sup>111</sup>In-Oxine (<sup>111</sup>In-oxoquinoline solution, which can penetrate lipid membranes, 1.0 mCi/ml) was obtained from MPI Pharmacy Services Inc. (Sunnyvale, CA).

**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. Lipids (20 μmol total) were dissolved in 0.8 ml of 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 NaCl, was added. Liposomes were prepared by reverse phase evaporation (17) as described (18) and extruded 21 times through two polycarbonate filters of 100-nm pore diameter (Costar, Cambridge, MA), using a LiposoFast device (Avestin, Inc., Ottawa, Canada) (19).

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<sup>1</sup> The abbreviations used are: PEG-PE, poly(ethylene glycol)distearoylphosphatidylethanolamine; CHEMS, cholesteryl hemisuccinate; DOPE, dioleoylphosphatidylethanolamine; DSPC, distearoylphosphatidylcholine; DTPA, diethylenetriaminepentaacetic acid; FBS, fetal bovine serum; MES, (2-[N-morpholino]ethanesulfonic acid); Rh-PE, lissamine rhodamine B-phosphatidylethanolamine (egg); TES, (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) sodium salt; TBS, TES-buffered saline.

Non-encapsulated calcein was removed by dialysis at 4 °C against three changes of 4 liters 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 Ref. 20. Molar ratios of lipids are given throughout the paper.

**pH Sensitivity of Liposomes**—Calcein was encapsulated in liposomes at a concentration of 80 mM, at which concentration its fluorescence is self-quenched (21). 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  $\mu\text{M}$ ) 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. After 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  $\mu\text{l}$  of 10% (v/v) Triton X-100, using an LS-5B fluorometer (Perkin-Elmer) operated with a Softways (Morena Valley, CA) computer program. Fluorescence intensities obtained at acidic pH values were corrected for the slight effect of pH on calcein fluorescence. To calibrate the assay, 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:  $((I_{\text{pH}} - I_0)/(I_{100} - I_0)) \times 100\%$ , where  $I_0$  is the fluorescence at neutral pH, and  $I_{\text{pH}}$  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 by a modification of the method of Liu and Huang (22). Liposomes were added to the medium at a final concentration of 100  $\mu\text{M}$  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 ( $I_{\text{pH}}$ ), 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 the formula 1.

**Interaction of Liposomes with Cells**—Monocytic human THP-1 cells (23) were cultured in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS and maintained as described (24). They were differentiated to macrophage-like cells by incubation with 160 nM phorbol 12-myristate 13-acetate in 24-well culture plates ( $10^6$  cells per well). Five or six 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  $\mu\text{M}$ . In some experiments 20 mM  $\text{NH}_4\text{Cl}$  was added to the cells 30 min before the liposomes, to inhibit the acidification of endosomes (25). 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).

For flow cytometric analysis (26) the cells were detached from plastic by adding 0.5 ml of dissociation buffer (Life Technologies, Inc.) and mixed with 0.5 ml of PBS with divalent cations, containing 2% FBS and 1  $\mu\text{g}/\text{ml}$  propidium iodide used to assess cell viability in flow cytometry. Rhodamine and calcein fluorescence were detected with a Becton Dickinson FACStar Plus flow cytometer, controlled by a Hewlett-Packard computer with Lysis II software (Becton Dickinson, San Jose, CA). 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, and 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, without any endocytosis, was obtained by pretreating the cells for 30 min with 1  $\mu\text{g}/\text{ml}$  antimycin A, 10 mM NaF, and 0.1%  $\text{NaN}_3$  to inhibit endocytosis (27).

**Stability of Liposomes in Plasma**—Calcein fluorescence in plasma is significantly quenched, thus precluding the use of calcein as a tracer. Therefore, the stability of liposomes in plasma was measured by the leakage of the encapsulated radioactive marker,  $^{111}\text{In}$ . Gamma-emitting isotopes are convenient aqueous phase markers for *in vivo* studies as well (28). Liposomes were made as described above, except that TBS containing 5 mM DTPA was used as the aqueous phase for the subsequent chelation of  $^{111}\text{In}$ . Liposomes were extruded through polycarbonate filters of 100-nm pore size. The mean diameter of liposomes was

measured by light scattering (Coulter model N450). Unencapsulated DTPA was removed by dialysis against three changes of 4 liters of TBS overnight. Fifty microliters of  $^{111}\text{In}$ -oxine were added to 0.3 ml of liposomes, and the mixture was incubated for 1 h at room temperature for irreversible chelation of  $^{111}\text{In}$  to DTPA inside liposomes. After chelation of excess  $^{111}\text{In}$  with 10 mM EDTA, liposomes were purified by gel filtration on a Sephadex G-75 column. Fifty to 95% of the added isotope was encapsulated, depending on the lipid composition. To label the lipid phase of the liposomes, 0.2 mol % of DTPA conjugated to distearoyl phosphatidylethanolamine (DTPA-DSPE) (29) was added to the lipid mixture in chloroform. Reverse phase evaporated vesicles were prepared using 100 mM NaCl, 50 mM TES, pH 7.8, as the aqueous phase. Liposomes were extruded through polycarbonate filters of 100-nm pore size, and 50  $\mu\text{l}$  of  $^{111}\text{In}$  was added to 300  $\mu\text{l}$  of the liposome suspension. After incubation for 1 h at ambient temperature unchelated  $^{111}\text{In}$  was removed by purification through Econo-Pac 10DG Desalting Columns (Bio-Rad). About 95% of  $^{111}\text{In}$  was recovered in the liposome-containing fractions.

To test the stability of liposomes in rat plasma, 5  $\mu\text{l}$  of liposomes encapsulating  $^{111}\text{In}$  were added to 95  $\mu\text{l}$  of rat plasma and incubated for various times at 37 °C. The mixture was then applied to a Sephadex G-75 column, and the radioactivity of liposomal and free fractions was measured in a gamma counter. Leakage of  $^{111}\text{In}$  was calculated as the percentage of the isotope found in the free  $^{111}\text{In}$  peak compared with the total radioactivity applied to the column.

**Pharmacokinetic Studies in Vivo**— $^{111}\text{In}$ -loaded liposomes were injected intravenously into rats. At various times post-injection, animals were anesthetized, and blood samples were obtained. The concentration  $c(t)$  of  $^{111}\text{In}$  was calculated as a function of time,  $t$ , and given as the percent of injected dose,  $(c(t)/c(0)) \times 100\%$ . A nonlinear, weighted, least-squares curve-fitting program (RSTRIP, Micromath, Salt Lake City, UT) was used to fit mean values of the percentage of injected dose in blood versus time data, to calculate values for the area under the curve, and to estimate the terminal half-lives ( $t_{1/2}$ ). Animals were sacrificed 24 h post-injection, and selected tissues were removed, weighed, and counted with a gamma counter to measure the amount of  $^{111}\text{In}$ . Total radioactivity of urine collected in 24 h was estimated as well. The total label remaining *in vivo* gives an indication of the label retained in liposomes during circulation or delivered to tissues, since any  $^{111}\text{In}$ -DTPA released from liposomes is removed rapidly from blood by the kidneys.

## RESULTS

**pH Sensitivity of Liposomes in Buffer and in Serum-containing Medium**—To develop sterically stabilized pH-sensitive liposomes, a large number of liposome compositions were examined for the leakage of encapsulated calcein at mildly acidic pH and for stability of liposomes in serum (15). These studies showed that of all pH-sensitive compositions tested liposomes composed of CHEMS/DOPE (30) are the most stable in serum. Since inclusion of PEG-PE in various liposome compositions confers steric stability (28), we examined how the addition of PEG-PE to the membrane of CHEMS/DOPE liposomes affected the leakage of calcein as a function of pH. The pH sensitivity of the liposomes decreased as the mol percentage of PEG-PE was increased (Fig. 1). For example, inclusion of only 1 mol % PEG-PE (with respect to the total phospholipid) decreased the release of calcein from liposomes at pH 5 by a factor of 2. Although the pH-dependent leakage from liposomes containing 5 mol % of PEG-PE was decreased significantly compared with CHEMS/DOPE liposomes, it was still more than in controls lacking the DOPE component. Control liposomes containing 1 mol % Rh-PE instead of PEG-PE had the same leakage characteristics as CHEMS/DOPE liposomes (Fig. 1).

As a primer for their use in cell culture experiments (described below), we examined whether CHEMS/DOPE liposomes with or without 5 mol % PEG-PE were stable in cell culture medium supplemented with 10% FBS and whether they were pH-sensitive. The leakage of calcein from these liposomes, incubated in cell culture medium at pH 7.4 for 1 h at 37 °C, was negligible. At pH 5.5, however, calcein leakage from CHEMS/DOPE liposomes was significantly decreased in the presence of serum ( $29.7 \pm 2.9\%$  in 1 h), compared with that in

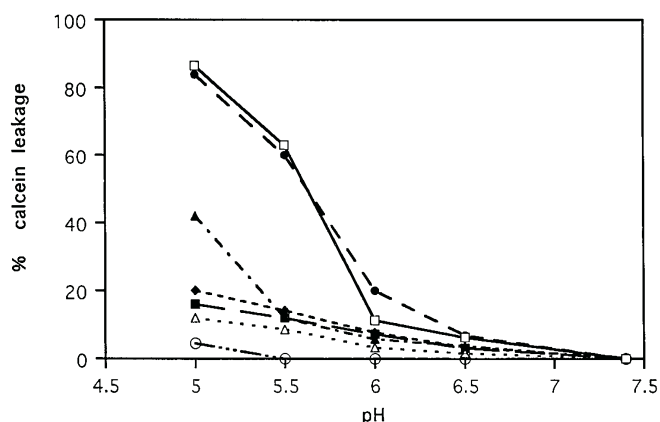


FIG. 1. Influence of PEG-PE on the pH sensitivity of CHEMS/DOPE liposomes. Calcein-containing liposomes (final lipid concentration, 10  $\mu$ M) were incubated for 10 min at 37  $^{\circ}$ C in MES-buffered saline at different pH values. The fluorescence intensity of calcein was measured before and after addition of Triton X-100 (final concentration 0.1%). Leakage of calcein was calculated as described under "Experimental Procedures." ●, CHEMS/DOPE (4:6); □, CHEMS/DOPE/Rh-PE (4:6:0.06); ▲, CHEMS/DOPE/PEG-PE (4:6:0.06); ◆, CHEMS/DOPE/PEG-PE (4:6:0.15); ■, CHEMS/DOPE/PEG-PE (4:6:0.3); △, CHEMS/DSPC/PEG-PE (4:6:0.3); ○, CHEMS/PC/Rh-PE (4:6:0.06). The results shown are from one representative experiment.

buffer (84%). Calcein leakage from CHEMS/DOPE/PEG-PE liposomes at pH 5.5 in the culture medium and in buffer were similar ( $5.8 \pm 4.8\%$  and  $6.8\%$ , respectively).

**Interaction of Liposomes with Cells**—Although the inclusion of PEG-PE in the membrane of CHEMS/DOPE liposomes decreased their pH sensitivity *in vitro*, 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 shown above, the presence of Rh-PE did not affect the pH sensitivity of CHEMS/DOPE liposomes in buffer (Fig. 1). The release of the aqueous contents of liposomes inside the cells was monitored by encapsulating calcein at a self-quenching concentration and measuring the fluorescence dequenching as the fluorophore was diluted (31, 32). This fluorescence value reports both the quenched calcein in liposomes bound to, or internalized by, cells and the dequenched dye released inside the cells. To control for the contribution of bound or internalized liposomes to the total calcein fluorescence, we measured the calcein to rhodamine fluorescence ratio. This ratio provides a measure of the intracellular dequenching of calcein per liposome, particularly when it is compared with that of cell-bound but not internalized liposomes obtained in control experiments with inhibitors of endocytosis. The cytoplasmic delivery of calcein was also ascertained by observing the kinetics of calcein release into the cytoplasm, using fluorescence microscopy, and measuring the calcein to rhodamine fluorescence ratio inside the cells by ratio fluorescence imaging (Photon Technology International, Monmouth Jct., NJ).<sup>2</sup> Representative micrographs of intracellular calcein fluorescence taken 4 h after the addition of liposomes to differentiated THP-1 cells are shown in Fig. 2. Diffuse cytoplasmic fluorescence was observed when pH-sensitive CHEMS/DOPE liposomes were used (Fig. 2, panel 1A). In contrast, only some punctate fluorescence was visualized in the cells treated with control liposomes (CHEMS/DSPC/PEG-PE) (Fig. 2, panel 3A). Incubation of the cells with pH-sensitive sterically stabilized liposomes composed of CHEMS/DOPE/

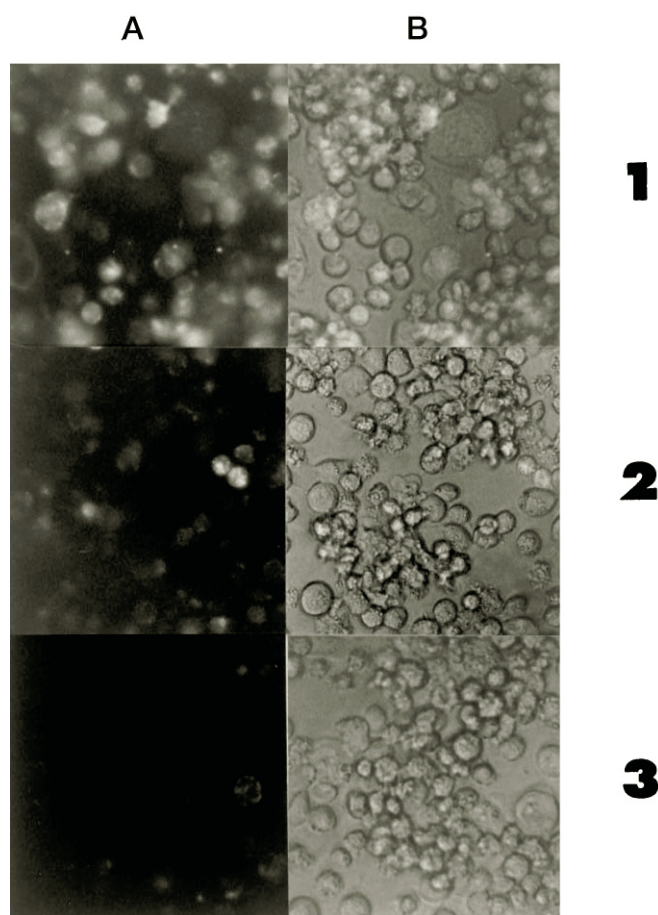
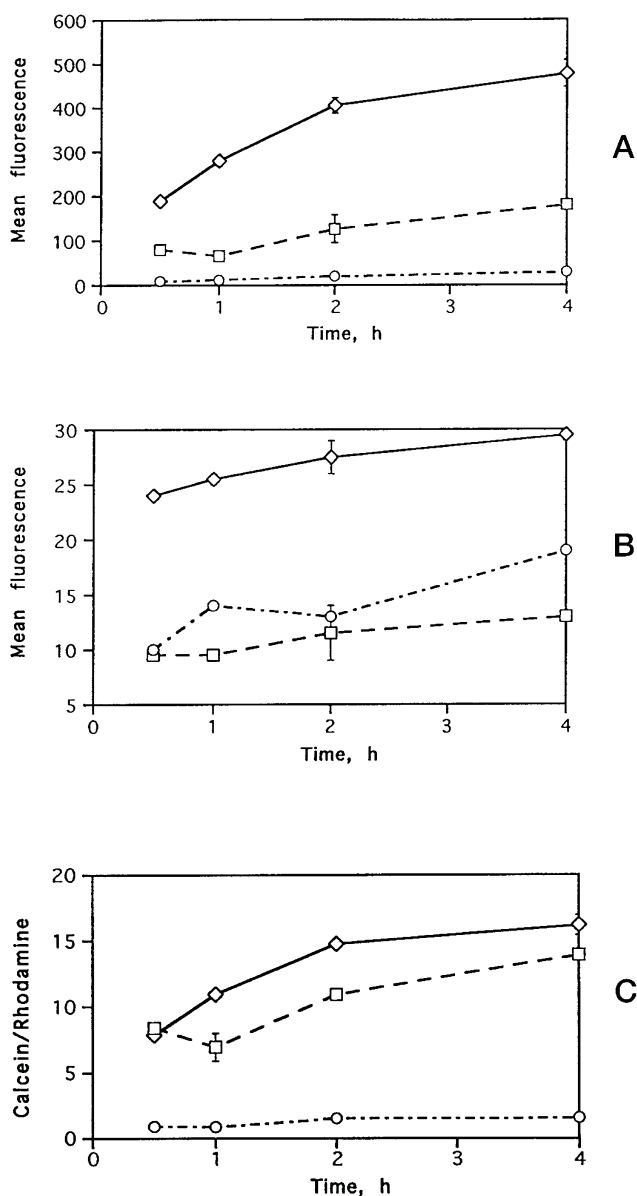


FIG. 2. Fluorescence (A) and phase-contrast (B) micrographs of differentiated THP-1 cells incubated with calcein-loaded liposomes for 4 h at 37  $^{\circ}$ C. Liposomes were added to THP-1 cells cultured in 48-well plates at a final phospholipid concentration of 100  $\mu$ M in 1 ml of RPMI medium supplemented with 10% FBS. After incubation, cells were washed twice with PBS and observed under a Nikon Diaphot epi-fluorescence microscope (40 $\times$  objective). Final magnification is  $\times 156$ . 1, CHEMS/DOPE liposomes; 2, CHEMS/DOPE/PEG-PE liposomes; 3, CHEMS/DSPC/PEG-PE liposomes.

PEG-PE resulted in lower fluorescence intensity than regular pH-sensitive liposomes (Fig. 2, panel 2A), most likely because their uptake was inhibited by the presence of PEG-PE. Nevertheless, diffuse cytoplasmic fluorescence could be detected in a large fraction of the cells.

Cell-associated calcein fluorescence was quantified by flow cytometry as a function of time for different liposome preparations (Fig. 3A). It was reduced in the case of CHEMS/DOPE/PEG-PE liposomes, compared with CHEMS/DOPE liposomes. This reduction could be accounted for by the decreased cellular uptake of PEG-PE-containing liposomes, measured by rhodamine fluorescence (Fig. 3B). Nevertheless, the release of aqueous contents of liposomes into the cells relative to the amount of cell-associated liposomes, as expressed by the calcein/rhodamine ratio (Fig. 3C), was comparable for both of these pH-sensitive liposomes. This release was considerably higher than that of control CHEMS/DSPC/PEG-PE liposomes. The calcein/rhodamine ratios obtained in control experiments with cells pretreated with endocytosis inhibitors and incubated with CHEMS/DOPE or CHEMS/DOPE/PEG-PE liposomes was about 0.5; the value obtained with control CHEMS/DSPC/PEG-PE liposomes was 0.4. Therefore, the fluorescence ratios of around 8–10 obtained with CHEMS/DOPE/PEG-PE liposomes reflect significant intracellular dequenching of calcein fluorescence, part of which is due to diffuse cytoplasmic fluo-

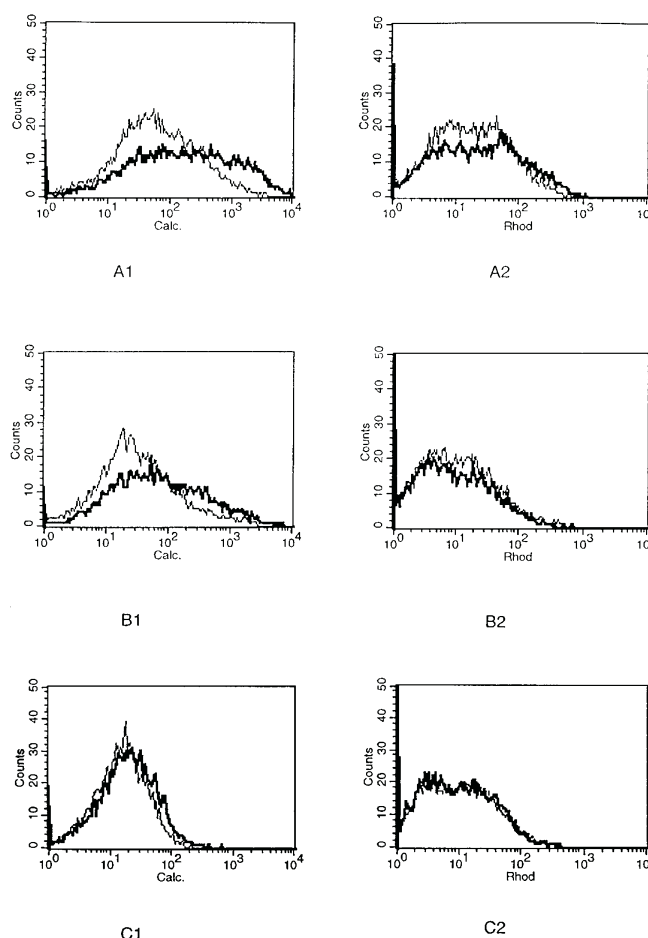
<sup>2</sup> Slepushkin, V. A., Simões, S., Dazin, P., Pedroso de Lima, M. C., and Düzgüneş, N. (1996) *Mol. Biol. Cell* 7, 268a.



**FIG. 3. Kinetics of uptake of calcein-containing liposomes by differentiated THP-1 cells.** Liposomes (final phospholipid concentration  $100 \mu\text{M}$ ) were incubated with cells in RPMI medium supplemented with 10% FBS at  $37^\circ\text{C}$  for different periods. 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  $2 \mu\text{g}/\text{ml}$  propidium iodide. Flow cytometric analysis was performed as described under "Experimental Procedures." *A*, mean fluorescence of calcein. (Mean autofluorescence of unstained cells = 4.1). *B*, mean fluorescence of Rh-PE. (Mean autofluorescence of unstained cells = 4.2). *C*, ratio of calcein to rhodamine fluorescence. ◇, CHEMS/DOPE/Rh-PE (4:6:0.06) liposomes; □, CHEMS/DOPE/PEG-PE/Rh-PE (4:6:0.3:0.06) liposomes; and ○, CHEMS/DSPC/PEG-PE/Rh-PE (4:6:0.3:0.06) liposomes. The initial calcein to rhodamine fluorescence ratio for all preparations incubated with cells pretreated with inhibitors of endocytosis for 0.5 h was  $0.6 \pm 0.2$ . The averages of duplicates and standard deviations are shown. The results shown are from one representative experiment.

rescence. The significant result shown in Fig. 3C is that the release of calcein per liposome is not significantly decreased by the inclusion of PEG-PE in CHEMS/DOPE liposomes. Thus, effective release of contents inside cells can be achieved by pH-sensitive liposomes altered so as to prevent rapid clearance by the reticuloendothelial system.

Although the pH sensitivity of CHEMS/DOPE liposomes was



**FIG. 4. Flow cytometry histograms of calcein and rhodamine fluorescence of differentiated THP-1 cells incubated in RPMI medium supplemented with 10% FBS with various liposomes for 0.5 h in the presence (*thin line*) and absence (*thick line*) of  $\text{NH}_4\text{Cl}$ .** In experiments with  $\text{NH}_4\text{Cl}$ , liposomes (phospholipid concentration  $100 \mu\text{M}$ ) were added to cells preincubated for 0.5 h with 20 mM  $\text{NH}_4\text{Cl}$ . *A*, CHEMS/DOPE/Rh-PE (4:6:0.06) liposomes. *B*, CHEMS/DOPE/PEG-PE/Rh-PE (4:6:0.3:0.06) liposomes. *C*, CHEMS/DSPC/PEG-PE/Rh-PE (4:6:0.3:0.06) liposomes. *1*, calcein fluorescence; *2*, rhodamine fluorescence.

reduced in serum-containing medium, the calcein/rhodamine fluorescence ratio in THP-1 cells incubated with these liposomes for 0.5 h was about the same in RPMI medium with and without 10% FBS ( $2.56 \pm 0.05$  and  $2.21 \pm 0.1$ , respectively). In contrast, the ratio for CHEMS/DOPE/PEG-PE liposomes, which had similar sensitivities to low pH in buffer and in serum-containing medium, was significantly increased in the absence of serum ( $1.9 \pm 0.17$  and  $4.1 \pm 0.16$ , respectively). These data show that the intracellular delivery of the contents of pH-sensitive liposomes is more complex than just the simple release of their contents in response to the low pH inside of endosomes (33) and that this process cannot be simulated adequately by *in vitro* experiments. It is likely that cell membrane proteins modulate the pH sensitivity of the liposomes, as discussed below.

To assess whether calcein was released from pH-sensitive liposomes in response to the low pH inside endosomes, we examined the dependence of this process on pretreatment of cells with  $\text{NH}_4\text{Cl}$ , an inhibitor of endosome acidification.  $\text{NH}_4\text{Cl}$  did not influence significantly the association of liposomes with the cells (Fig. 4, panels A2, B2, and C2). In contrast, calcein fluorescence in the case of pH-sensitive liposomes (Fig. 4, panels A1 and B1) was inhibited significantly by the pretreatment

TABLE I  
Stability of CHEMS-containing liposomes in rat plasma

Lipid composition	% of $^{111}\text{In}$ associated with liposomes after incubation in 95% rat plasma for		% of $^{111}\text{In}$ associated with liposomes after incubation in buffer for 19 h
	2 h	19 h	
CHEMS/DOPE (4:6)	53	31	64
CHEMS/DOPE/PEG-PE (4:6:0.3)	80	68	95
CHEMS/DSPC/PEG-PE (4:6:0.3)	85	88	97

with  $\text{NH}_4\text{Cl}$ . Calcein dequenching in cells incubated with non-pH-sensitive liposomes was not affected by  $\text{NH}_4\text{Cl}$  (Fig. 3, panel C1). The intracellular release of calcein, measured as the ratio of calcein to rhodamine mean fluorescence intensities, was also inhibited by  $\text{NH}_4\text{Cl}$  in the case of pH-sensitive, but not control, liposomes. After a 0.5-h incubation with cells, these ratios were  $8.7 \pm 0.1$  for CHEMS/DOPE liposomes and  $9.1 \pm 0.5$  for CHEMS/DOPE/PEG-PE liposomes in the absence of  $\text{NH}_4\text{Cl}$  and  $3.8 \pm 0.2$  and  $3.4 \pm 0.2$ , respectively, in the presence of  $\text{NH}_4\text{Cl}$ .

Our results indicate that the addition of PEG-PE to CHEMS/DOPE liposomes did not interfere significantly with their ability to release their aqueous contents inside cells in a pH-sensitive manner, although it decreased the pH sensitivity of calcein release in buffer (Fig. 1).

**Stability of the Liposomes in Plasma**—In an experiment preliminary to investigating the pharmacokinetics of pH-sensitive liposomes *in vivo*, we studied their stability in rat plasma. Liposomes were loaded with  $^{111}\text{In}$  and incubated with plasma for different times. The release of  $^{111}\text{In}$  was estimated by comparing the radioactivity of the free isotope peak with the liposome peak after gel filtration. The stability of pH-sensitive liposomes, as measured by retention of  $^{111}\text{In}$ , was greatly improved after addition of PEG-PE (Table I). Sixty-eight percent of the radioactive marker was still associated with CHEMS/DOPE/PEG-PE liposomes after a 19-h incubation in 95% rat plasma, in contrast to only 31% for CHEMS/DOPE liposomes.

**Pharmacokinetics of the pH-sensitive Liposomes in Vivo**—The blood clearance curve of  $^{111}\text{In}$  encapsulated in sterically stabilized pH-sensitive liposomes (Fig. 5A) was similar to that of previously developed liposomes with prolonged circulation time, and a substantial percentage of liposomes (8.5%) remained in the blood after 24 h. In contrast, the radioactive marker encapsulated in regular pH-sensitive liposomes was almost completely eliminated from the bloodstream within 0.5 h. The  $t_{1/2}$  of sterically stabilized pH-sensitive CHEMS/DOPE/PEG-PE liposomes and control CHEMS/DSPC/PEG-PE liposomes was similar ( $11.1 \pm 0.6$  and  $11.8 \pm 0.7$  h, respectively). The area under the curve for control liposomes was greater than that for sterically stabilized pH-sensitive liposomes ( $1071 \pm 151$  and  $629 \pm 52\%$  dose  $\times$  h/ml, respectively), presumably because a greater number of the latter were taken up rapidly by the liver and spleen (Table II and Fig. 5A, time = 0 point), and therefore did not appear in blood samples. It is important to note that the area under the curve for regular pH-sensitive liposomes ( $6.47 \pm 1.24\%$  dose  $\times$  h/ml) was about 100-fold lower than that for sterically stabilized pH-sensitive liposomes. The radioactive marker encapsulated in the former liposomes may be eliminated from the circulation either because it leaks out as a result of the interaction of plasma proteins with liposomes or due to phagocytosis of the liposomes by the cells of the reticuloendothelial system. To discriminate between these possibilities we associated  $^{111}\text{In}$  with the lipid membrane of liposomes. Blood clearance curves of lipid-labeled liposomes (Fig. 5B) showed rapid elimination from the circulation of the lipid component of the regular pH-sensitive liposomes but not that of sterically stabilized pH-sensitive liposomes.

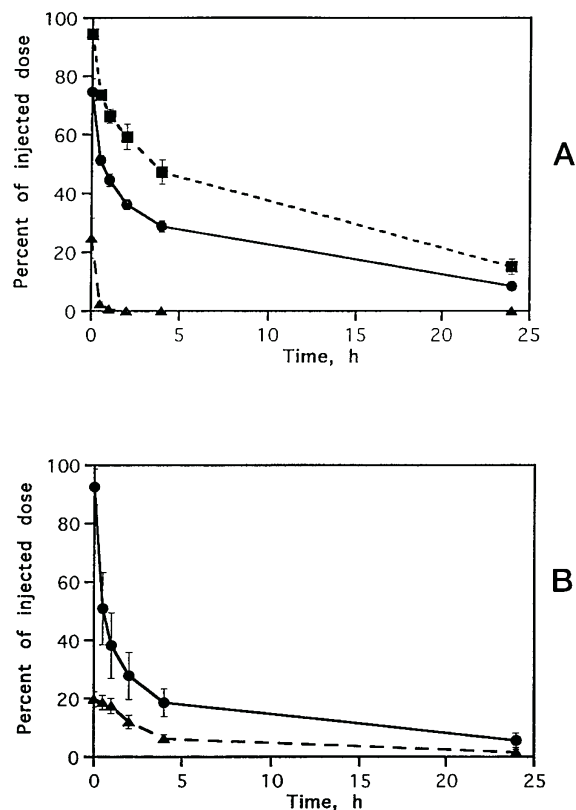


FIG. 5. Blood clearance curves of regular pH-sensitive, sterically stabilized pH-sensitive, and control sterically stabilized liposomes.  $^{111}\text{In}$ -loaded liposomes (A) or liposomes containing lipid-associated  $^{111}\text{In}$  (B) were injected intravenously into rats (0.4 ml of a liposome suspension containing approximately 1 mM phospholipid and  $1.4 \times 10^7$  cpm/ml radioactivity). Blood samples were taken after various times, and radioactivity was determined in a gamma counter (the first blood sample was drawn immediately after injection at time 0). Percent of injected dose was calculated and plotted against time. The mean diameter of liposomes in experiment A was CHEMS/DOPE,  $114 \pm 6$  nm, CHEMS/DOPE/PEG-PE,  $172 \pm 10$  nm, and CHEMS/DSPC/PEG-PE,  $103 \pm 5$  nm; in experiment B was CHEMS/DOPE,  $132 \pm 7$  nm, and CHEMS/DOPE/PEG-PE,  $98 \pm 5$  nm. ▲, CHEMS/DOPE (4:6) liposomes; ●, CHEMS/DOPE/PEG-PE (4:6:0.3) liposomes; ■, CHEMS/DSPC/PEG-PE (4:6:0.3) liposomes. Mean values and standard deviations for each experiment of four animals (two animals for control, non-pH-sensitive liposomes) are shown.

somes. This observation indicates that the elimination of the liposome-encapsulated water-soluble marker, described above, was mostly due to removal of the liposomes from the blood by the reticuloendothelial system.

$^{111}\text{In}$  encapsulated in either pH-sensitive or control liposomes had similar patterns of distribution *in vivo* in that it primarily accumulated in the spleen and liver (Table II). Injection of regular pH-sensitive liposomes resulted in a high level of  $^{111}\text{In}$  in the liver. This observation and the rapid clearance of these liposomes from the circulation (Fig. 5) indicate that most of the liposomes are taken up within an hour by liver cells and that only a fraction of the encapsulated marker leaks out and is

TABLE II  
Tissue distribution of liposome-encapsulated [ $^{111}\text{In}$ ] 24 h after intravenous injection in rats

Tissue	Percent of injected $^{111}\text{In}$ dose in tissues <sup>a</sup>			Percent of injected $^{111}\text{In}$ dose/g in tissues		
	CHEMS/DOPE	CHEMS/DOPE/PEG-PE	CHEMS/DSPC/PEG-PE	CHEMS/DOPE	CHEMS/DOPE/PEG-PE	CHEMS/DSPC/PEG-PE
Liver	45.67 ± 3.82	23.4 ± 1.25	9.18 ± 3.77	5.46 ± 1.83	2.01 ± 0.23	0.76 ± 0.28
Spleen	2.51 ± 0.21	16.7 ± 0.36	4.76 ± 1.09	4.15 ± 0.6	22.06 ± 2.68	6.27 ± 0.33
Heart	0.03 ± 0.01	0.15 ± 0.02	0.29 ± 0.06	0.03 ± 0.02	0.16 ± 0.01	0.27 ± 0.08
Kidneys	0.26 ± 0.02	0.53 ± 0.08	0.75 ± 0.04	0.12 ± 0.01	0.24 ± 0.03	0.33 ± 0.04
Lung	0.03 ± 0.01	0.23 ± 0.03	0.37 ± 0.07	0.03 ± 0.01	0.17 ± 0.02	0.25 ± 0.01
Skin	0.02 ± 0	0.08 ± 0.04	0.12 ± 0.01	0.01 ± 0	0.03 ± 0.01	0.05 ± 0.01
Bone	0.03 ± 0.01	0.39 ± 0.14	0.19 ± 0.01	0.05 ± 0.02	0.53 ± 0.17	0.25 ± 0.03
Muscle	0.02 ± 0.02	0.05 ± 0.01	0.03 ± 0	0.02 ± 0.01	0.02 ± 0	0.02 ± 0
Urine	33.23 ± 14.48	19.24 ± 7.78	34.08 ± 7.87	2.22 ± 0.94	1.36 ± 0.28	1.89 ± 0.83

<sup>a</sup> Recovery of radioactivity do not added up to 100% because  $^{111}\text{In}$  associated with the remaining carcass and feces was not determined. Mean values and standard deviations are shown.

then excreted in urine (Table II). Although 24 h after injection a higher fraction of the CHEMS/DOPE/PEG-PE liposomes was localized in the liver and, especially, spleen, compared with the non-pH-sensitive control CHEMS/DSPC/PEG-PE liposomes, the total urinary excretion of  $^{111}\text{In}$  was also about 2-fold lower compared with the control liposomes (Table II). This result, together with the observation that the  $t_{1/2}$  (and hence the clearance rate) of both sterically stabilized liposomes were similar, suggests that a higher fraction of the contents of pH-sensitive liposomes was accumulated and retained in the liver and spleen, most likely because they were delivered into the cytoplasm and thus partially avoided the usual metabolic processing of liposome contents.

#### DISCUSSION

Liposomes exhibiting pH sensitivity, and composed of phosphatidylethanolamine and protonatable amphiphiles, have been developed and used for the intracellular delivery of charged water-soluble compounds (31, 32, 34). The inclusion of PEG-PE in liposomes of various (non-pH-sensitive) compositions has been shown to overcome the problem of their rapid removal by the reticuloendothelial system (8, 35–37). Both properties, *i.e.* pH sensitivity and prolonged circulation time, are highly desirable for the delivery of therapeutic macromolecules to cells, but, as our contents release experiments have shown, the measures to obtain one property appear to conflict with those to achieve the other. Nevertheless, experiments on the intracellular delivery of encapsulated calcein have indicated that even liposomes that show reduced pH sensitivity in biophysical assays can deliver their contents into cells in a pH-dependent manner.

Liposomes composed of CHEMS and DOPE were chosen for our studies, because in preliminary experiments they were found to be more stable in serum and had better miscibility with PEG-PE than other compositions examined. The intracytoplasmic delivery of molecules entrapped in these liposomes was assessed by a new method based on ratio flow cytometry. Rh-PE was incorporated into the liposome membrane at a mole fraction at which rhodamine fluorescence was independent of its concentration. The fluorescence intensity of this dye measured the total uptake of liposomes by cells. Calcein was encapsulated in the aqueous phase of liposomes at self-quenched concentrations, so that its release from the liposomes into the cytoplasm (and/or the endosome lumen) would significantly increase the fluorescence intensity. To express the quantity of released calcein per cell-associated liposome, the calcein/rhodamine fluorescence intensity ratio was calculated. The results obtained using this ratio correlated well with images observed by fluorescence microscopy, showing diffuse cytoplasmic calcein fluorescence when pH-sensitive liposomes were added to cells (Fig. 2). On the other hand, mainly punctate rhodamine

and calcein fluorescence were observed with control liposomes and with pH-sensitive liposomes in the presence of  $\text{NH}_4\text{Cl}$ .<sup>3</sup> Our results were consistent with previous observations suggesting that the release of calcein from pH-sensitive liposomes is inhibited by treatment of cells with  $\text{NH}_4\text{Cl}$  and other inhibitors of endosome acidification (31, 32, 34). This new ratio flow cytometry technique thus gave us a valuable tool to quantify the release of encapsulated water-soluble material from pH-sensitive liposomes inside cells.

Because of the fast progress of nucleic acid-based technologies in the treatment of diseases, the call for appropriate delivery vehicles becomes increasingly important. The ideal vehicle should avoid immediate uptake by the mononuclear phagocyte (reticuloendothelial) system and have prolonged circulation in blood, thus increasing the probability of reaching the desired targets. In addition, the vehicle should be able to deliver its contents efficiently into the cell cytoplasm, avoiding lysosomal degradation. Liposomes of various types are being considered as potential vehicles for the delivery of therapeutic nucleic acids (38–41). pH-sensitive liposomes that can circulate in the blood for long periods and that can deliver encapsulated macromolecules to target cells may be useful in this regard.

Several investigators have tried to construct serum-stable pH-sensitive liposomes (42–44). However, *in vivo* studies were tried only by one group, using the ganglioside  $\text{G}_{\text{M1}}$  to confer relatively prolonged residence in circulation to pH-sensitive liposomes composed of DOPE and dipalmitoyl-succinylglycerol (42). The incorporation of  $\text{G}_{\text{M1}}$  enhanced the retention of the liposome-associated radioactive tracer ( $^{125}\text{I}$ -tyraminylinulin) in the blood up to 2 h; but both control and  $\text{G}_{\text{M1}}$ -containing liposomes were almost completely cleared from circulation at later times (5 and 24 h). Attempts to confer steric stability to DOPE/cholesterol liposomes by adding PEG-PE were also unsuccessful (45). Here we report, to our knowledge, the first composition of liposomes with the combined properties of pH-sensitivity and prolonged circulation *in vivo*. Liposomes composed of CHEMS/DOPE/PEG-PE had  $t_{1/2}$  similar to that of our control sterically stabilized liposomes and to that of “Stealth” compositions described by others (28, 46). In contrast, CHEMS/DOPE liposomes were cleared rapidly from the circulation as detected by both the aqueous space marker and by the lipid marker.

Our pH-sensitive sterically stabilized liposomes delivered significantly higher amounts of an encapsulated water-soluble marker to the cells of the reticuloendothelial system, residing mainly in the liver and spleen, than any of the sterically stabilized liposomes described by others (28, 46) or control non-pH-sensitive liposomes used in our experiments (Table II). Very high amounts of  $^{111}\text{In}$  were also accumulated in the liver

<sup>3</sup> V. A. Slepshkin, S. Simões, and N. Düzgüneş, unpublished data.

after injection of regular pH-sensitive liposomes. In both cases when pH-sensitive liposomes were injected the amount of radioactivity associated with liver and spleen significantly exceeded the amount of  $^{111}\text{In}$  excreted with urine. In contrast, when non-pH-sensitive liposomes were used, the amount of water-soluble marker in urine was significantly higher than in liver and spleen (Table II). Taking into account that free  $^{111}\text{In}$  is immediately removed from the circulation through the kidneys, we suggest that this difference in tissue distribution reflects different mechanisms of intracellular delivery of aqueous contents by pH-sensitive liposomes in comparison to non-pH-sensitive ones. In the former case,  $^{111}\text{In}$  is likely to be released into the cytoplasm from the endosomes, thereby escaping the lysosomal pathway, which normally leads to the excretion of liposomal contents from the cells. It is also interesting to note that in the case of sterically stabilized pH-sensitive liposomes, much higher levels of radioactivity were associated with the spleen than when regular pH-sensitive liposomes or non-pH-sensitive sterically stabilized liposomes were used. A similar preferential distribution to the spleen was found by Litzinger and Huang (45) when they injected DOPE/cholesterol/PEG-PE liposomes into mice. One explanation for this tissue distribution of liposomes containing DOPE and PEG-PE may be that these liposomes can avoid the first barrier of the reticuloendothelial system localized in liver but are taken up by the secondary barrier macrophages localized in the spleen.

The precise mechanism of intracytoplasmic delivery by sterically stabilized pH-sensitive liposomes remains unclear, particularly since their sensitivity to acidic pH in buffer was greatly reduced compared with controls without PEG-PE. It is likely that the interaction of cell membrane proteins with CHEMS/DOPE/PEG-PE liposomes increases their pH sensitivity in endosomes. One possible mechanism for this enhanced pH sensitivity is the phase separation of the PEG-PE component or the dissociation of the PEG moiety, rendering the liposomes more responsive to acidic pH. An alternative mechanism is the aggregation of the liposomes within endosomes, caused by interaction with endosomal membrane proteins that may penetrate the steric barrier provided by PEG-PE. The low pH-induced destabilization of CHEMS/DOPE liposomes in buffer was shown previously to be dependent on aggregation (30). We have observed that the interaction of CHEMS/DOPE/PEG-PE liposomes with soluble CD4 receptor molecules caused the aggregation and subsequent dramatic increase in the pH sensitivity of these liposomes in buffer.<sup>3</sup>

The unique properties of sterically stabilized pH-sensitive liposomes may be utilized for the intracytoplasmic delivery of plasmids, antisense oligonucleotides, and ribozymes *in vivo* for the treatment of human immunodeficiency virus and other infections, in which the targets are blood cells or tissue macrophages. Experiments to test this possibility are currently in progress in our laboratories. The demonstration that subcutaneously or intraperitoneally administered sterically stabilized liposomes accumulate in lymph nodes (47) also suggests that pH-sensitive sterically stabilized liposomes can be utilized for the delivery of various anti-human immunodeficiency virus agents to infected lymph nodes.

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## REFERENCES

- Bangham, A. D., and Horne, R. W. (1964) *J. Mol. Biol.* **8**, 660–668
- Chonn, A., and Cullis, P. R. (1995) *Curr. Opin. Biotechnol.* **6**, 698–708
- Allen, T. M. (1994) *Trends Pharmacol. Sci.* **15**, 215–220
- Lasic, D. D., and Papahadjopoulos, D. (1995) *Science* **267**, 1275–1276
- Allen, T. M. (1994) *Adv. Drug. Delivery Rev.* **13**, 285–309
- Lasic, D., and Martin, F., (eds) (1995) *Stealth Liposomes*, CRC Press, Inc., Boca Raton, FL
- Woodle, M. C., and Lasic, D. D. (1992) *Biochim. Biophys. Acta* **1113**, 171–199
- Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K.-D., Woodle, M. C., Lasic, D. D., Redemann, C., and Martin, F. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 11460–11464
- Allen, T. M., Mehra, T., Hansen, C., and Chin, Y. C. (1992) *Cancer Res.* **52**, 2431–2439
- Vaage, J., Donovan, D., Loftus, T., Abra, R., Working, P., and Huang, A. (1994) *Cancer* **73**, 2366–2371
- Torchilin, V. P., Zhou, F., and Huang, L. (1993) *J. Liposome Res.* **3**, 201–255
- Düzgüneş, N., Straubinger, R. M., Baldwin, P. A., and Papahadjopoulos, D. (1991) in *Membrane Fusion* (Wilschut, J. and Hoekstra, D., eds), pp. 713–730, Marcel Dekker, Inc., New York
- Collins, D. (1995) in *Liposomes as Tools in Basic Research and Industry* (Philippot, J. R. and Schuber, F., eds), pp. 201–214, CRC Press, Inc., Boca Raton, FL
- Liu, D., Zhou, F., and Huang, L. (1989) *Biochem. Biophys. Res. Commun.* **162**, 326–333
- Slepushkin, V. A., and Düzgüneş, N. (1995) *Biophys. J.* **68**, A216
- Allen, T. M., Hansen, C., Martin, F., Redemann, C., and Yau-Young, A. (1991) *Biochim. Biophys. Acta* **1066**, 29–36
- Szoka, F., and Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 4194–4198
- New, R. R. C., (1990) in *Liposomes. A Practical Approach* (New, R. R. C., ed) pp. 71–74, Oxford University Press, New York
- MacDonald, R. C., MacDonald, R. I., Menco, B. P. M., Takeshita, K., Subbarao, N. K., and Hu, L.-R. (1991) *Biochim. Biophys. Acta* **1061**, 297–303
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Allen, T. M., and Cleland, L. G. (1980) *Biochim. Biophys. Acta* **597**, 418–426
- Liu, D., and Huang, L. (1989) *Biochemistry* **28**, 7700–7707
- Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. (1982) *Cancer Res.* **42**, 1530–1536
- Konopka, K., Pretzer, E., Plowman, B., and Düzgüneş, N. (1993) *Virology* **193**, 877–887
- Maxfield, F. R. (1982) *J. Cell Biol.* **95**, 676–681
- Stewart, C. C. (1990) in *Flow Cytometry* (Darzynkiewicz, Z., and Crissman, H. A., eds) pp. 427–450, Academic Press, San Diego
- Lee, K.-D., Nir, S., and Papahadjopoulos, D. (1993) *Biochemistry* **32**, 889–899
- Woodle, M. C., Matthay, K. K., Newman, M. S., Hidayat, J. E., Collins, L. R., Redemann, C., Martin, F. J., and Papahadjopoulos, D. (1992) *Biochim. Biophys. Acta* **1105**, 193–200
- Grant, C. W. M., Karlik, S., and Florio, E. (1989) *Magn. Reson. Med.* **11**, 236–243
- Ellens, H., Bentz, J., and Szoka, F. C. (1984) *Biochemistry* **23**, 1532–1538
- Straubinger, R. M., Düzgüneş, N., and Papahadjopoulos, D. (1985) *FEBS Lett.* **179**, 148–154
- Connor, J., and Huang, L. (1985) *J. Cell Biol.* **101**, 582–589
- Straubinger, R. M. (1993) *Methods Enzymol.* **221**, 361–376
- Chu, C. J., Dijkstra, J., Lai, M. Z., Hong, K., and Szoka, F. C. (1990) *Pharmacol. Res.* **7**, 824–834
- Senior, J., Delgado, C., Fisher, D., Tilcock, C., and Gregoriadis, G. (1991) *Biochim. Biophys. Acta* **1062**, 77–82
- Blume, G., and Cevc, G. (1990) *Biochim. Biophys. Acta* **1029**, 91–97
- Klibanov, A. L., Maruyama, K., Torchilin, V. P., and Huang, L. (1990) *FEBS Lett.* **268**, 235–237
- Lurquin, P. F. (1993) in *Liposome Technology* (Gregoriadis, G., ed) 2nd Ed., Vol. 2, pp. 129–139, CRC Press, Inc., Boca Raton, FL
- Leserman, L. (1995) in *Liposomes as Tools in Basic Research and Industry* (Philippot, J. R., and Schuber, F., eds) pp. 215–223, CRC Press, Inc., Boca Raton, FL
- Singhal, A., and Huang, L. (1994) in *Gene Therapeutics: Methods and Application of Direct Gene Transfer* (Wolff, J. A., ed) pp. 118–142, Birkhäuser Boston, Inc., Cambridge, MA
- Smith, J. G., Walzem, R. L., and German, J. B. (1993) *Biochim. Biophys. Acta* **1154**, 327–340
- Liu, D., and Huang, L. (1990) *Biochim. Biophys. Acta* **1022**, 348–354
- Kono, K., Zenitani, K.-I., and Takagishi, T. (1994) *Biochim. Biophys. Acta* **1193**, 1–9
- Viani, P., Cervato, G., Gatti, P., and Cestaro, B. (1993) *Biochim. Biophys. Acta* **1147**, 73–80
- Litzinger, D. C., and Huang, L. (1992) *Biochim. Biophys. Acta* **1127**, 249–254
- Bakker-Woudenberg, I. A. J. M., Lokerse, A. F., ten Kate, M. T., and Storm, G. (1992) *Biochim. Biophys. Acta* **1138**, 318–326
- Allen, T. M., Hansen, C. B., and Guo, L. S. S. (1993) *Biochim. Biophys. Acta* **1150**, 9–16