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Peptide-Mediated Release of Folate-Targeted Liposome Contents from Endosomal Compartments[1]

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Abstract: An egg phosphatidylcholine-derived liposome preparation has been developed that achieves low pH-dependent cargo unloading in the absence of a pH-sensitive lipid component by use of the 30 amino acid residue peptide, EALA. This peptide adopts an amphiphilic α-helical conformation in a mildly acidic environment, and when encapsulated into neutral liposomes or covalently attached to one of its lipid components, the peptide can facilitate the release of liposome contents. Our liposomes have also been designed to be tumor cell specific by incorporation of a folate−PEG−PE lipid conjugate which allows the liposomes to enter receptor-bearing cells through the pathway of folate receptor-mediated endocytosis. In combination, these features allow for the cytoplasmic release of 20−25% of the encapsulated cargo of internalized liposomes within several hours of administration. To quantitate the extent of the cytoplasmic unloading of liposomal contents, a new methodology was developed that exploits the dramatic increase in quantum yield of propidium iodide upon binding DNA. As the internalized liposomes interact with the endosomal membranes and release their dye into the cytoplasm, a rapid increase in fluorescence is measured to yield quantitative data on the kinetics of intracellular unloading.

The development of liposomes as vehicles of intravenous drug delivery has received renewed attention in recent years.[2] Previous problems with liposome stability and premature removal by the reticuloendothelial system have been largely solved by optimization of liposome size and composition, and by protecting the liposome surface with gangliosides or polyoxyethylene-derivatized lipids.[3,4] It is now generally possible to design liposomes that can circulate for many hours before removal by the reticuloendothelial system[5,6] or phagocytosis by macrophages.[7,8]

Despite the above improvements, several obstacles still remain that limit the potential applications of liposomes as drug delivery

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(1) Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PEG, poly(ethylene glycol); PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; FDMEM, folate-deficient minimum essential media.

(2) (a) Litzinger, D. C.; Huang, L. *Biochim. Biophys. Acta* **1992**, *1113*, 201−227. (b) Chu, C. J.; Szoka, F. C. *J. Liposome Res.* **1994**, *4*, 361−395. (c) Lasic, D. D. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1685−1698.

(3) (a) Klibanov, A. L.; Maruyama, K.; Beckerleg, A. M.; Torchilin, V. P.; Huang, L. *Biochim. Biophys. Acta* **1991**, *1062*, 142−148. (b) Szoka, F.; Magnusson, K. E.; Wojcieszyn, J.; Hou, Y.; Derzko, Z.; Jacobson, K. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1685−1689. (c) Litzinger, D. C.; Huang, L. *Biochim. Biophys. Acta* **1992**, *1104*, 179−187. (d) Liu, D.; Huang, L. *Biochim. Biophys. Acta* **1990**, *1022*, 348−354.

(4) Bally, M. B.; Nayar, R.; Masin, D.; Hope, M. J.; Cullis, P. R.; Mayer, L. D. *Biochim. Biophys. Acta* **1990**, *1023*, 133−139.

(5) Poznansky, M. J.; Juliano, R. L. *Pharmacol. Rev.* **1984**, *36*, 277−336.

(6) Gibzon, A.; Catane, R.; Uziely, B.; Kaufman, B.; Safra, T.; Cohen, R.; Martin, F.; Huang, A.; Barenholz, Y. *Cancer Res.* **1994**, *54*, 987−992.

(7) (a) Ho, R. J. Y.; Rouse, B. T.; Huang, L. *J. Biol. Chem.* **1987**, *262*, 13973−13978. (b) Heath, T. D.; Montgomery, J. A.; Piper, J. R.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 1377−1381. (c) Matthay, K. M.; Heath, T. D.; Papahadjopoulos, D. *Cancer Res.* **1984**, *44*, 1880−1886.

(8) (a) Papahadjopoulos, D.; Allen, T. M.; Gabizon, A.; Mayhew, E.; Huang, S. K.; Lee, K. D.; Woodle, M. C.; Lasic, D. D.; Redemann, C.; Martin, F. J. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11460−11464. (b) Blume, G.; Cevc, G.; Crommelin, M. D. J. A.; Bakker-Woudenberg, I. A. J. M.; Kluft, C.; Storm, G. *Biochim. Biophys. Acta* **1993**, *1149*, 180−184. (c) Allen, T. M.; Agrawal, A. K.; Ahmad, I.; Hansen, C. B.; Zalipsky, S. *J. Liposome Res.* **1994**, *4*, 1−25. (d) Mayer, L. D.; Tai, L. C. L.; Ko, D. S. C.; Masin, D.; Ginsberg, R. S.; Cullis, P. R.; Bally, M. B. *Cancer Res.* **1989**, *49*, 5922−5930.

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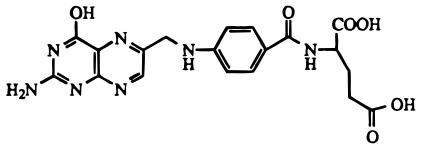
vehicles. First, a strategy must be developed to promote liposome departure from the capillary bed at appropriate sites of action. Further, novel ligands must be discovered that can facilitate docking and endocytosis of liposomes at their target cells. And finally, due to low unloading efficiencies of internalized liposomes,[2] strategies must be developed that will enable rapid release of hydrolytically sensitive contents from internalized liposomes before they are trafficked to lysosomes for destruction.

We have begun to explore solutions to the latter two obstacles using human cells in culture as a model system. In previous work we found that the vitamin folic acid (**1**) can be used to target liposomes to cancer cells, if the folate is tethered to the liposome via a long polyoxyethylene spacer.[9] In this study, we examine a possible solution to the last obstacle mentioned above, i.e. conversion of a highly stable circulating liposome into an unstable/fusogenic liposome following entry into endosomal compartments of the target cell. By taking advantage of the decrease in pH within the endosomal compartment,[10] we show here that encapsulation of a peptide with a pH-dependent conformational switch can facilitate release of liposomal contents from stable phosphatidylcholine liposomes following uptake by folate receptor-bearing cells. We further describe a novel assay that permits continuous monitoring of such liposome unloading in live cells *in vitro*. We believe this assay should allow rapid optimization of liposome compositions for maximal unloading efficiency.

AALAEALAEALAEALAEALAEALAAAAGGC(Acm)

(EALA)



(1)

Experimental Section

Materials. KB cells were obtained from the Purdue Cancer Center. Folic acid, propidium iodide, piperidine, and 1-methyl-2-pyrolidinone were purchased from Sigma Chemical Co. (St. Louis, MO). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). All amino acids and solid phase resin were purchased from Bachem Biosciences (King of Prussia, PA). All other chemicals and reagents were purchased from Aldrich (Milwaukee, WI) or Mallinckrodt (Paris, KY) unless otherwise specified.

Cell Culture. An attached monolayer of KB cells was continuously cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in folate-free medium supplemented with 10% heat-inactivated fetal calf serum. The folate contributed by the fetal calf serum is sufficient to reconstitute the entire medium with 2 to 3 nM folate, i.e. a value within the physiological range for human serum.[11] Prior to each experiment, cells were transferred to 33-mm culture dishes at 10^5 cells per dish and grown in culture medium for an additional 24 h to approximately 50% confluence.

Synthesis of the Folate−PEG−PE Construct. The synthesis of the folate−PEG−PE construct was accomplished as described by Lee[9a] with the following modifications. The product of the reaction of phosphatidylethanolamine (PE) and glutaric anhydride was precipitated in a 10-fold excess volume of cold acetone, and the resulting solid was dissolved in $CHCl_3$. The folate−PEG−amine was separated from unreacted folic acid and the side product, PEG−bis−folate, by precipitation in cold chloroform. The filtrate was dried *in vacuo*, dissolved in H_2O, and dialyzed first against 10 mM sodium bicarbonate (pH 9) followed by H_2O to remove dimethyl sulfoxide. The purified folate−PEG−amine was lyophilized, and the final folate−PEG−PE product was washed with cold acetone to remove unreacted precursors and resuspended in $CHCl_3/CH_3OH$ (3:1).

Design of the EALA Peptide. The EALA peptide sequence was modified from the GALA peptide that had been designed by Szoka and co-workers.[12] In the EALA design, the goal was to enhance the helix-forming potential of the peptide, and to allow for covalent attachment of the peptide to the lipid component, while maintaining features that had previously been found essential for pH-dependent amphiphilic α-helix formation.[12] To this end an extended C-terminal sequence was added consisting of 4 Ala, a residue with high helix forming potential,[13] and 2 Gly residues to introduce flexibility and distance between the peptide and lipid when covalently attached. The C-terminal residue, Cys with acetamidomethyl (Acm) side chain protection, was chosen to allow for covalent ligation of this peptide to PE.

Synthesis and Purification of the EALA Peptide. The EALA peptide was synthesized using a solid phase methodology on Wang's *p*-alkoxybenzyl alcohol resin[14] using a fluorenylmethoxycarbonyl (Fmoc)-based strategy.[15] The peptide was synthesized in a stepwise manner by the *N*-hydroxybenzotriazole method and was purified by preparative reverse phase HPLC. The peptide was characterized by fast atom bombardment mass spectrometry (FAB-MS, NBA matrix), $M + Na^+$ peak = 2791.2 (calculated 2791.15).

Encapsulation of EALA into Folate-Targeted Liposomes. Liposomes encapsulating the EALA peptide were prepared by the extrusion method.[16] Egg phosphatidylcholine (50 mg, PC) and 5 mg of folate−PEG−PE were dissolved in 2.0 mL of $CHCl_3$. The lipids were dried under reduced pressure to a thin film, and rehydrated overnight in 1.0 mL of 10 mg/mL propidium iodide in PBS for control liposomes, or with the addition of 2 mg of EALA in 1.0 mL of PBS for the evaluation of peptide mediated endosomal release. After 5 cycles of freezing and thawing, each liposome suspension was extruded 10 times through a 100 nm polycarbonate membrane (Nucleopore, Pleasanton, CA), yielding liposomes of approximately 120 nm diameter. The liposome encapsulated fluorescent probe and coencapsulated EALA were separated from free peptide and probe on a 1.5 cm × 20 cm Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) pre-equilibrated in PBS. The liposome fractions eluted in the void volume and were stored at 4 °C until use (within 1 week). Encapsulation of ∼10 μM peptide (total suspension volume assumed) was achieved, as determined by quantitative amino acid analysis. The concentration of propidium iodide in each liposome preparation was determined using a standard curve based on the fluorescence of known concentrations of propidium iodide (Ex = 510 nm, Em = 625 nm). Concentration of encapsulated propidium iodide was found to be ∼20 μM, as determined by fluorescence spectroscopy. Total lipid content in the same suspension was 15 mg/mL.

Synthesis of Folate-Targeted and EALA−PE Conjugated Liposomes. Liposomes with EALA covalently attached to their surfaces were prepared by the extrusion method as described above, except 50 mg of egg PC, 5 mg of folate−PEG−PE, and 2.5 mg of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidophenyl) butyrate] were employed in the preparation. To covalently attach the EALA peptide to the maleimide lipid, the Acm group on the C-terminal Cys

(9) (a) Lee, R. J.; Low, P. S. *J. Biol. Chem.* **1994**, *269*, 3198−3204. (b) Lee, R. J.; Low, P. S. *Biochim. Biophys. Acta.* **1995**, *1233*, 134−144.

(10) (a) Tycko, B.; Keith, C. H.; Maxfield, F. R. *J. Cell Biol.* **1983**, *97*, 1762−1776. (b) Tycko, B.; Maxfield, F. R. *Cell* **1982**, *28*, 643−651.

(11) Antony, A. C.; Kane, M. A.; Portillo, R. M.; Elwood, P. C.; Kolhouse, J. F. *J. Biol. Chem.* **1985**, *260*, 14911−14917.

(12) Subbarao, N. K.; Parente, R. A.; Szoka, F. C.; Nadasdi, L.; Pongracz, K. *Biochemistry* **1987**, *26*, 2964−2972.

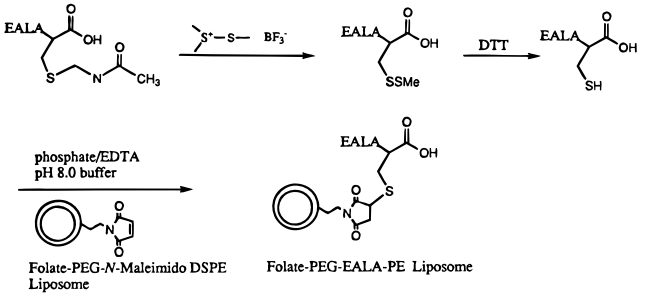
(13) Chou, P. Y.; Fasman, G. D. *Annu. Rev. Biochem.* **1978**, *47*, 251−276.

(14) Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328−1333.

(15) Atherton, E.; Sheppard, R. C. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1987; Vol. 9, pp 1−38.

(16) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55−65.

Scheme 1. Synthesis of EALA-Conjugated Liposomes



was removed with dimethyl(methylthio)sulfonium tetrafluoroborate to yield EALA-SSMe (Scheme 1).[17] Prior to reaction with maleimide lipid, the EALA−SSMe peptide was then reduced to the free thiol (EALA−SH) by treatment with a Reduce Imm (Pierce, Rockford, IL) reducing column. Column fractions containing EALA−SH were added directly to the purified liposome suspension, degassed, and allowed to shake for 2 h. Liposomes with covalently attached EALA were again separated from unreacted peptide on a 1.5 cm × 20 cm Sepharose CL-4B column pre-equilibrated with PBS, and stored at 4 °C until use (within 1 week). Covalent linking of 1.3−1.5 μM peptide was achieved, as determined by quantitative amino acid analysis.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded on a Jasco J600 spectropolarimeter at 37 °C. The spectra are an average of 3 scans from 200 to 260 nm. The helical content was calculated from the value of the mean residue ellipticity at 222 nm.[18] CD spectra were taken in several buffers to determine the pH-dependent conformational changes of this peptide: PBS, pH 7.4 (136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4); MES, pH 6.0 (20 mM MES and 135 mM NaCl); and citrate, pH 5.0 (20 mM citric acid and 135 mM NaCl). The concentration of stock peptide solutions was quantitated by amino acid analysis.

Endosomal Release of Folate-Targeted Liposomes. To quantitate the intracellular release of contents from the EALA-containing liposomes, KB cells in FDMEM were incubated for 4 h at 37 °C with folate-targeted liposomes containing 5 μM propidium iodide. The cells were then washed and incubated with fresh FDMEM for the desired time, and then released from their culture dishes by incubation with 0.5 mL of non-enzymatic cell dissociation solution (Sigma) for 15 min. After gently resuspending in 1.5 mL of FDMEM, cell-associated fluorescence was measured on a Perkin Elmer MPF-44A fluorescence spectrophotometer (Ex = 540 nm, Em = 615 nm). Minor levels of light scattering and autofluorescence were subtracted from the measured propidium iodide signal. After each measurement, the cell suspension was sonicated in an ice−water bath for 15−20 min to determine the fluorescence of maximum propidium iodide release. The percent of propidium iodide release was calculated according to the following equation: % release = $(flu_t - flu_{initial}/ flu_{max} - flu_{initial})100$, where flu_t was the fluorescence at each time point, and flu_{max} was the fluorescence of maximum release at the same time point. To directly visualize these results, a second set of KB cells in FDMEM were incubated and washed in the same manner, and examined with an Olympus BH-2 fluorescence microscope.

Efficiency of propIdium Iodide Uptake into KB Cells. To quantitate the endocytosis efficiency of our folate-targeted liposomes, KB cells were incubated for 4 h, with 10 μM PI in various formulations: free PI, free PI, and 10 μM EALA, PC liposomes encapsulating PI, PC liposomes coencapsulating PI and EALA, folate-targeted liposomes encapsulating PI, folate-targeted liposomes coencapsulating PI and EALA. The later two formulations were also incubated with KB cells in the presence of 10 mM free folic acid. After the 4-h incubation, the cells were washed three times with either PBS or acid saline to remove membrane bound, but not endocytosed liposomes (150 mM NaCl adjusted to pH 3 with acetic acid). The cells were then solubilized by washing them three times with 1% TX-100. The cell-associated fluorescence was then measured on a Perkin Elmer MPF-44A fluorescence spectrophotometer (Ex = 540 nm, Em = 615 nm).

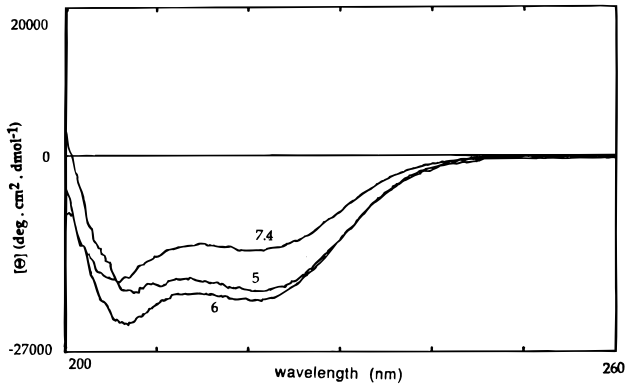


Figure 1. Circular dichroism spectra of EALA at pH 5.0, 6.0, and 7.4. The pH-dependent conformation of the EALA peptide was studied by introducing EALA to buffers of various pH's: PBS, pH 7.4; MES, pH 6.0; and citrate, pH 5.0 as described in the Methods section. Peptide concentration was ~40 μM.

Stability of Liposome Preparations. To determine the stability of the EALA containing liposome preparations, liposomes encapsulating PI and either coencapsulating EALA or containing lipid linked EALA were assayed for their efflux of PI under various conditions: 4 °C in PBS for 7, 14, and 30 days, and 37 °C in FDMEM for 4 and 24 h. At each time point, an aliquot of the liposome sample was fractionated on a 1.5 cm x 20 cm Sepharose CL-4B column in PBS. The fluorescence of each fraction was then measured on a Perkin Elmer MPF-44A fluorescence spectrophotometer (Ex = 510 nm, Em = 625 nm), and the percent of the total eluted fluorescence in the liposome peak was calculated.

Results

To evaluate the capability of the modified EALA peptide to undergo a pH-dependent conformational transition, the CD spectrum of the peptide at 3 separate pH measurements over the endosomal pH range was examined (Figure 1). Mean residue ellipticities, $[\theta]$, were measured and the value at 222 nm was used to estimate the percent α-helix.[18] Maximal helical content was found to be 38% at pH 6.0, and no additional increase in secondary structure was measured as pH was lowered further. The helical content at pH 7.4 and 5.0 was determined to be 25% and 37%, respectively. These data suggest that endosome uptake and mild acidification might be expected to trigger an increase in secondary structure in the EALA peptide.

To enable quantitation of the release of liposome contents from endosomal compartments, a fluorescent dye was sought whose spectral characteristics would change dramatically upon release from internalized liposomes. Propidium iodide (PI) was found to satisfy this criterion based on several unusual properties. First, PI was found to be impermeable to both living cells and liposomal membranes, assuring that cytoplasmic/nuclear PI fluorescence could only arise from dye specifically released at that location.[19] Second, a 60-fold increase in fluorescence intensity of PI was observed upon binding DNA (Figure 2), an event that occurs rapidly following entry of PI into a cell's

(17) Bishop, P.; Jones, C.; Chmielewski, J. *Tetrahedron Lett.* **1993**, *34*, 4469−4472.

(18) (a) Lyu, P. C.; Sherman, J. C.; Chen, A.; Kallenbach, N. R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5317−5320. (b) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108−4126.

(19) Taylor, D. L.; Wang, Y. L., Eds. *Methods of Cell Biology*; Academic Press: New York, 1989; Vol. 30, pp 417−448.

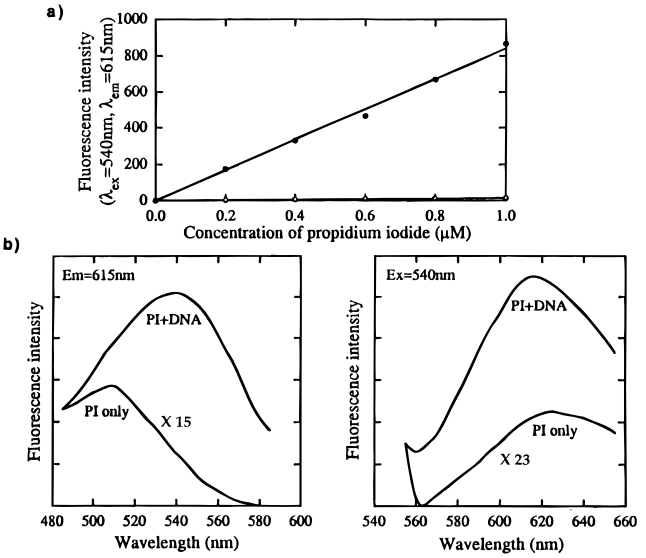


Figure 2. Comparison of propidium iodide fluorescence in the presence and absence of DNA. (a) Indicated concentrations of PI were mixed with aliquots of 0.5 mg/mL of DNA in 1 mL of PBS (pH 7.4) at room temperature. The fluorescence intensities of PI in the absence of DNA (○) and in the presence of DNA (●) were measured at $\lambda_{ex} = 540$ nm and $\lambda_{em} = 615$ nm. (b) The normalized fluorescence emission and excitation spectra of PI in the presence and absence of DNA. Left: Excitation maxima of PI = 510 nm, and PI + DNA = 540 nm. Right: Emission maxima of PI = 625 nm, and PI + DNA = 615 nm.

cytoplasm.[19] Third, no increase in PI fluorescence was observed upon addition of EALA (free peptide), or individual lipid components, confirming that the fluorescence increase is specifically a consequence of nucleic acid binding. Fourth, the excitation and emission maxima of PI shifted dramatically following DNA binding from $\lambda_{ex} = 510$ to 540 nm and from $\lambda_{em} = 625$ to 615 nm (Figure 2), a property seen upon binding RNA as well.[20] Finally, as shown in Figure 2, the fluorescence of PI bound to DNA is linearly related to dye concentration over a large concentration range, permitting a correlation of PI fluorescence with PI release into the cytoplasm. Thus, by selecting the appropriate excitation and emission wavelengths, the release of PI from internalized liposomes into a cell's cytoplasm where it has access to DNA and RNA should be readily quantifiable in using a fluorescence spectrophotometer.

To characterize the propidium iodide-based liposome unloading assay, KB cells treated with PI-containing folate−PEG liposomes were examined by both fluorescence microscopy and spectroscopy. As shown in Figure 3b, liposomes coencapsulating EALA and PI displayed weak cytoplasmic fluorescence 6 h following incubation with KB cells (lower left micrograph). By 12-h incubation, the fluorescence intensity had increased significantly (lower middle), with most of the increase localizing to the nucleus and nucleoli. The intensity of the stained nucleoli continued to grow for the next 12 h (lower right), following which the changes were less obvious to the eye (data not shown). Nevertheless, quantitation of the fluorescence intensity by fluorescence spectroscopy of the washed cultured cells revealed

Table 1. The Efficiency of PI Endocytosis

PI formulation[a]	no. of PI molecules endocytosed by each cell ($\times 10^6$)[b]
PI	0.83 ± 0.16
PI + EALA	0.76 ± 0.09
PC liposome encapsulated PI	1.04 ± 0.25
PC liposomes coencapsulating PI and EALA	0.88 ± 0.10
folate−PEG liposomes encapsulating PI	63.92 ± 4.26
folate−PEG liposomes coencapsulating PI and EALA	65.11 ± 6.08
folate−PEG liposomes encapsulating PI competed by 1 mM folic acid	12.59 ± 1.84
folate−PEG liposomes coencapsulating PI and EALA competed by 1 mM folic acid	10.74 ± 0.93

[a] In each sample 10 nmol of PI was added to KB cells. [b] Each value represents an average of three parallel experiments ±SD.

that the liposomal contents continued to unload for at least the next 24 h (Figure 4, solid circles).

By visual inspection in the fluorescence microscope, KB cells treated with the EALA−PE containing liposomes appeared to follow a similar chronology of fluorescence changes, i.e. weak cytoplasmic staining probably due to RNA binding followed by nuclear staining (Figure 3a, upper micrographs). However, when evaluated more quantitatively by fluorescence spectroscopy (Figure 4, open triangles), significant differences were observed. Thus, the covalently attached peptide mediated a more rapid release of liposomal PI than the unattached encapsulated peptide, even though both preparations eventually approached similar equilibrium values. Further, the liposomal preparation lacking any form of EALA was clearly less efficient at cytoplasmic delivery than either EALA-supplemented preparation (Figure 4, open circles).

To determine the role of folate receptor-mediated endocytosis on the uptake of PI in the above liposome preparations, KB cells were incubated for 4 h with a variety of liposome preparations and liposome-free components, and uptake of PI was determined (Table1). Endocytosis of PI was only observed when folate−PEG was present in the liposomes, and could be inhibited in the presence of free folic acid. The amount of endocytosed PI with folate−PEG liposomes per cell is reasonable as each KB cell has only approximately 5×10^6 folate receptors, and the 4 h incubation is sufficient for approximately one round of endocytosis to occur prior to receptor recycling.[20] It is interesting to note that although EALA coencapsulation enhances PI unloading following endocytosis, it has no effect on the efficiency of liposome uptake.

If these egg phosphatidylcholine-based liposomes are to be used for use in *in vivo* drug delivery, it is essential to determine their stability in the presence of serum and, for storage purposes, for extended periods in PBS at 4 °C. Stability studies in PBS at 4 °C show essentially no leakage of encapsulated PI after 14 days, and less than 5% leakage after 1 month (Table 2). Likewise at 37 °C in the serum-containing buffer FDMEM, no leakage was observed with folate−PEG liposomes encapsulating PI either with or without coencapsulated EALA at 24 h; however, leakage was observed with the EALA covalently modified liposome preparations. Due to the stability of the encapsulated EALA liposomes in serum, we suggest that these liposomes may warrant further investigation for use in *in vivo* drug delivery.

Discussion

We have shown that neutral liposomes can be rendered pH-sensitive by incorporation of a pH-sensitive peptide in their

(20) Livache, T.; Fouque, B.; Teoule, R. *Anal. Biochem.* **1994**, *217*, 248−254.
(21) Wang, S.; Low, P. *Bioconjugate Chem.* **1995**, in press.
(22) Lasic, D. D.; Martin, F. J.; Gabizon, A.; Huang, S. K.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1991**, *1070*, 187−192.
(23) (a) Grit, M.; Crommelin, D. J. A. *Phys. Lipids* **1992**, *62*, 113−122. (b) Grit, M.; deSmit, J. H.; Struijke, A.; Crommelin, D. J. A. *Int. J. Pharm.* **1989**, *50*, 1−6. (c) Lasic, D. D. *Liposomes: From Physics to Applications*; Elsevier: Amsterdam, 1993.

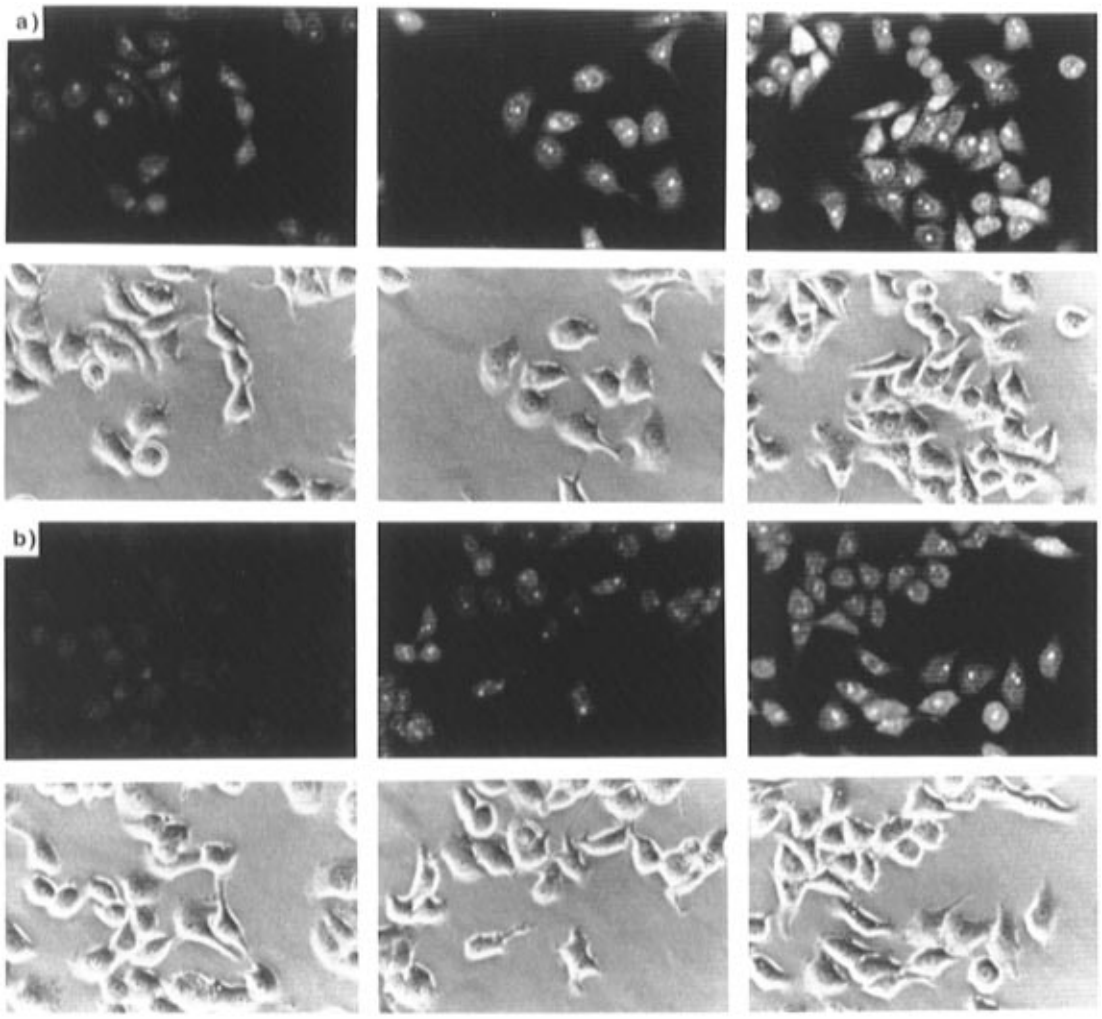


Figure 3. Endosomal release of propidium iodide from folate-targeted liposomes containing EALA. KB cells were incubated for 4 h with liposomes containing either (a) phospholipid-linked EALA or (b) coencapsulated free EALA, followed by transfer to fresh FDMEM and further incubated for the desired times. The cells were then viewed by fluorescence microscopy. left, 6 h total incubation; middle, 12 h total incubation; right, 24 h total incubation. Phase contrast images of the same field of cells are shown below each fluorescence image.

Table 2. The Stability of Folate-Targeted Liposomes[a]

	4 °C in PBS[b]			37 °C in FDMEM[b]	
liposome type	7 days	14 days	30 days	4 h	24 h
folate−PEG liposomes encapsulating PI	0	0	2.28 ± 0.61	0	0
folate−PEG liposomes coencapsulating PI and EALA	0	0	2.16 ± 0.88	0	0
folate−PEG liposomes with EALA−PE encapsulating PI	0	0	4.09 ± 0.92	1.83 ± 0.27	19.62 ± 3.20

[a] Each value represents an average of three parallel experiments ±SD. [b] Percentage of content leakage.

lumenal solutions. In cases where the encapsulated cargo might be destroyed upon delivery to lysosomes, such pH sensitivity could enhance therapeutic potency by facilitating the release of liposomal contents into the cytoplasm prior to trafficking to the degradative compartment. Because nucleic acids, proteins, carbohydrates, and many of their precursors are altered/destroyed by lysosomal enzymes, rapid escape from the endocytic pathway might be critical to the efficacy of liposome-encapsulated drugs.

We have modeled our peptide after the GALA peptide of Szoka and colleagues.[12] However, to facilitate the coil-to-helix transition at less acidic pH, we have added additional helix-forming amino acids. As revealed in Figure 1, the strategy was successful, yielding a peptide that achieved its maximal helical content at pH 6 rather than pH 5. When KB cells are incubated with folate bearing liposomes, they are first internalized via the non-coated pit (caveolae) pathway, but are quickly transferred to endosomal compartments.[24] Since endosomal pH gradually decreases as the endosome migrates and fuses with the lysosome,[10] an earlier destabilization of the internalized liposome could conceivably improve drug potency.

We have synthesized two liposome constructs and have compared their capacity to facilitate liposome unloading with the conjugated or entrapped EALA peptide. Our data reveal a major difference only at early time points, with both preparations approaching a maximum of ~25% release of contents at long incubation times in cell culture studies. While the headgroup-conjugated peptide may exhibit an early kinetic advantage, it may simultaneously introduce new obstacles into the liposome delivery technology, since any particle decorated with multiple copies of a foreign peptide will undoubtedly be highly antigenic.

(24) Turek, J. J.; Leamon, C. P.; Low, P. S. *J. Cell. Sci.* **1993**, *106*, 423−430.

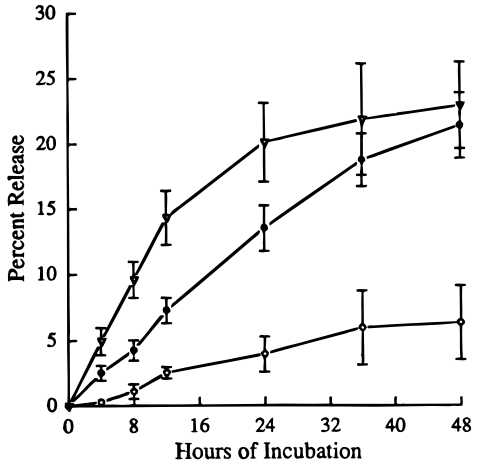


Figure 4. Kinetics of release of liposome-encapsulated PI following endocytosis by KB cells. KB cells were incubated with liposomes containing PI and free EALA (●), liposomes containing PI and covalently attached EALA (▽), or liposomes containing only PI (○). At the indicated times, cells were washed, dislodged from the culture dishes, and examined in the fluorescence spectrophotometer at λ_{ex} = 540 nm and λ_{em} = 615 nm. To evaluate maximum potential PI release, the same sample of cells was then sonicated to disrupt membrane barriers and re-examined for fluorescence intensity. The percent endosomal release (Y axis) was then calculated from these two measurements as described in the Materials and Methods section. The values presented represent an average of three parallel experiments ±SD.

Encapsulation of the peptide within the liposome's lumen, coupled with the folate−PEG−PE coating[8] of the liposome, may largely eliminate this problem, although removal of cell-targeted liposomes from circulation by macrophages may still be problematic.[25]

A second advantage of employing an encapsulated peptide to endow the liposome with pH sensitivity is that this method introduces no unnatural lipids into the formulation. Thus, modified lipids can be antigenic or directly toxic to interacting cells.[22] Further, chemically altered lipids could conceivably be difficult to metabolize and therefore accumulate in the body. Also, free fatty acids and other modified single chain lipids are often rapidly removed by serum albumin and other plasma components,[23] robbing the liposomes of their pH-sensitive components before they can reach their target cells. Although the folate−PEG−PE lipid conjugate cannot be rigorously classified as a natural lipid component, the phosphatidylethanolamine to which the folic acid has been tethered has not been chemically altered. Taken together, the inclusion of only natural lipids, which may be degraded through normal cellular processes, must be considered an attractive alternative to the use of modified lipids.

Finally, while the specific peptide and lipid components we have employed to facilitate liposome unloading may not be optimal, we believe the assay we have developed should greatly accelerate the design of an optimal unloading liposome. Thus, measurable PI fluorescence is detected in the cell only when the liposomes have successfully released their contents into the cytoplasm. Then, upon binding to RNA or following diffusion into the nucleus and association with DNA, a dramatic increase in fluorescence is observed. Because many assays of this type can be conducted on cultured cells in a single afternoon, the process of identifying and refining an optimal liposome composition should proceed rapidly. Future work will focus on the mechanism by which EALA facilitates the travel of PI across the liposomal and endosomal membranes.

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(25) (a) Claassen, E.; Van Rooijen, N. *Biochim. Biophys. Acta* **1984**, *802*, 424−434. (b) Van Pooijen, N.; Van Nieuwmegen, R. *Cell Tissue Res.* **1984**, *238*, 355−358. (c) Van Rooijen, N.; Kors, N. V. D.; Ende, M.; Dijkstra, C. D. *Cell Tissue Res.* **1990**, *260*, 215−222.