Liposome−Cell Interactions in Vitro: Effect of Liposome Surface Charge on the Binding and Endocytosis of Conventional and Sterically Stabilized Liposomes†

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ABSTRACT: The cellular uptake of liposomes is generally believed to be mediated by adsorption of liposomes onto the cell surface and subsequent endocytosis. This report examines the effect of liposome surface charge on liposomal binding and endocytosis in two different cell lines: a human ovarian carcinoma cell line (HeLa) and a murine derived mononuclear macrophage cell line (J774). The large unilamellar liposomes were composed of 1,2-dioleolyl-*sn*-glycero-3-phosphatidylcholine with and without the addition of either a positively charged lipid, 1,2-dioleoyl-3-dimethylammonium propanediol (DODAP), or a negatively charged lipid, 1,2-dioleolyl-*sn*-glycero-3-phosphatidylserine. In some experiments 5 mol % of the anionic PEG_{2000}−PE or a neutral PEG lipid of the same molecular weight was added. HeLa cells were found to endocytose positively charged liposomes to a greater extent than either neutral or negatively charged liposomes. This preference was not lipid-specific since inclusion of a cationic cyanine dye, DiIC18-(3), to impart positive charge in place of DODAP resulted in a similar extent of endocytosis. In contrast the extent of liposome interaction with J774 cells was greater for both cationic and anionic liposomes than for neutral liposomes. The greater uptake of positively charged liposomes by HeLa cells was also observed with sterically stabilized liposomes (PEG liposomes). Although the overall amount of endocytosis for all the PEG liposomes examined was attenuated relative to conventional liposomes, the extent of endocytosis was greatest for positively charged PEG liposomes, whereas negatively charged PEG_{2000}−PE liposomes were hardly endocytosed by the HeLa cells. Incorporation of a neutral PEG lipid into liposomes permits the independent variation of liposome steric and electrostatic effects in a manner that may allow interactions with cells of the reticuloendothelial system to be minimized, yet permit strong interactions between liposomes and proliferating cells.

Liposomes have long been considered good candidates for efficient drug carrier and delivery systems. By virtue of their large aqueous interior and biocompatible lipid exterior, they offer a possible means of local delivery of therapeutic agents to the site of interest while reducing systemic toxicity. Recent advances in the formulation of conventional and sterically stabilized liposomes encapsulating chemotherapeutic drugs have achieved enhanced pharmaceutical efficacy over free drugs against certain tumor models. If liposomes in circulation are able to reach a tumor site they may release their contents in the vicinity of the cancer cells and/or be taken up by the cells. In the latter case, fusion of the liposome with endosomal membranes appears to be necessary for the effective delivery of the chemotherapeutic agent from the liposome to the cell. In the course of our studies of the photoactivated fusion of liposomes (*1*, *2*), we have now examined the uptake of liposomes with cells in tissue culture in order to more properly design photosensitive liposomes for interaction with cells.

An understanding of the mechanism of liposome uptake by cells and the method of drug delivery by liposomes is still emerging (*3*−*5*). The uptake of liposomes by resident as well as cultured macrophages has been studied previously (*6*−*8*). The effect of variations in liposome composition on the endocytosis by bone marrow macrophages was observed to be similar in vivo and in vitro (*9*). Cellular uptake of liposomes is generally believed to be mediated by adsorption of liposomes onto the cell surface and subsequent endocytosis. Early studies indicated that liposomes which bind to the surface are internalized through a coated pit-mediated pathway in some cells (*10*, *11*). Negatively charged liposomes containing phosphatidylserine (PS),[1] phosphatidylglycerol (PG), or phosphatidic acid (PA) were observed to be endocytosed faster and to a greater extent than neutral liposomes by phagocytotic (endocytotic) cells (*5*, *9*, *12*).

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DOPA, 1,2-dioleolyl-*sn*-glycero-3-phosphatidic acid; DOPC, 1,2-dioleolyl-*sn*-glycero-3-phosphatidylcholine; DOPE, 1,2-dioleolyl-*sn*-glycero-3-phosphatidylethanolamine; DOPS, 1,2-dioleolyl-*sn*-glycero-3-phosphatidylserine; DODAP, 1,2-dioleoyl-3-dimethylammonium propanediol; DOTMA, 2,3-dioctadecenyl-oxypropyl-*N*,*N*,*N*-trimethylammonium chloride; EDTA, ethylene-diaminetetraacetic acid tetrasodium salt; HPTS, 1-hydroxypyrene-3,6,8-trisulfonic acid; LUV, large unilamellar vesicles (liposomes); MEM, modified Eagle's medium; PA; *sn*-glycero-3-phosphatidic acid; PC, *sn*-glycero-3-phosphatidylcholine; PE, *sn*-glycero-3-phosphatidylethanolamine; PEG, poly(ethylene glycol); PG, *sn*-glycero-3-phosphatidylglycerol; PS, *sn*-glycero-3-phosphatidylserine; Rh-PE, rhodamine labeled PE; RES, reticuloendothelial system.

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Negative surface charge can be recognized by receptors found on a variety of cells, including macrophages (*3*, *9*, *12*, *13*).

While it is still not known what factors define the uptake of liposomes by various cells, the different extents of binding for a given liposome composition by different types of cells suggest that the binding itself may be the crucial step. This was proposed by Lee et al. (*12*) from comparative studies of the endocytosis of liposomes of varying compositions by two different cell lines, that is, CV1 cells, an African green monkey kidney cell line, and J774 cells, a murine macrophage cell line chosen to represent mononuclear phagocytes in the RES. The inclusion of 9 mol % PS, PG, or PA increased the extent CV1 cell uptake of PC/cholesterol liposomes by 20-fold, whereas 33 mol % of these anionic lipids was required to reach this same level of liposome uptake in J774 cells. The aqueous contents uptake was measured with the liposomally encapsulated fluorescent probe, HPTS, whose pH dependence was used to differentiate between liposomes at neutral pH and the ones in low-pH compartments (*14*). The bilayer probe Rh-PE was also employed to calculate the lipid associated with the cells (*7*). A further study by Lee et al. (*5*) indicated that liposome binding at the J774 cell surface controlled the overall rate of liposome/cell interaction. The number of binding sites and binding constants was greater for PS/Chol/PC liposomes than for Chol/PC liposomes, whereas the rate constants for endocytosis after binding were similar for each type of liposome. Moreover the number of high-affinity sites for charged liposomes was higher for J774 cells grown in monolayer than those grown in suspension. Surface-associated J774 cells are a better representative model of resident macrophages fixed in the sinusoids of the RES. These studies suggest that the choice of suitable liposome compositions may facilitate the delivery of encapsulated contents to a target cell in preference to uptake by macrophages of the RES.

This report compares the effect of liposome surface charge on liposomal binding and endocytosis by HeLa cells, a human ovarian carcinoma cell line chosen for its ubiquitous use in cancer research, and the murine macrophage J774 cells used previously as a model for mononuclear phagocytes of the RES (*12*). Prior studies indicate that endocytotic preference is likely to be cell-specific, as well as dependent on how the cells are grown (*5*, *12*). Since the present study compares the liposome/cell interaction for both J774 and HeLa cells, and the latter are adherent cells that will not grow in suspension, all experiments were performed with cells grown in monolayer. Moreover due to our interest in various photosensitve liposome compositions, the effect of positive as well as negative and neutral surface charge was examined. Interestingly, HeLa cells were found to endocytose positively charged liposomes to a greater extent than either neutral or negatively charged liposomes. It is particularly timely to examine the effect of positive surface charge on the endocytosis of liposomes, because several studies indicate that the transfection of mammalian cells via DNA/cationic lipid complexes is mediated by endocytosis (*15*−*18*).

In the 1970s it was shown that conventional liposomes used in vivo were rapidly cleared from the blood of intravenously injected rats in a dose-dependent and biphasic manner (*19*). Neutral and positively charged (with sterylamine) liposomes exhibited a slower rate of clearance than negatively charged vesicles (*20*). Liposomes with entrapped materials ended up in the fixed macrophages of the RES, mainly in the liver and spleen. Sterically stabilized liposomes were introduced to reduce liposome interaction and uptake by the RES. Liposomes with a sufficiently hydrophilic surface decreased the adsorption of proteins, opsonins, to the surface of the liposomal bilayer and thus curtailed interception by the RES. The current molecule of choice in the design of sterically stabilized liposomes is lipid-tethered poly(ethylene glycol), PEG (*9*, *21*−*24*). PEG coating of the liposome surface inhibits nonspecific adsorption of serum proteins and thereby prevents nonspecific recognition of liposomes by macrophages (*25*). Grafted PEG can also reduce the interaction of liposomes with macrophages. It was reported that 5 mol % of PEG_{5000}−PE on the surface of liposomes nearly inhibited the adhesion of liposomes to the surface of cells (*26*). The commercially available PEG lipid, a derivative of PE, is negatively charged and can therefore present an electrostatic as well as a steric barrier to interactions with systemic cells. As part of a broader investigation of steric and electrostatic effects on liposome/cell interactions, the endocytosis of cationic, neutral, and anionic PEG liposomes are compared in this report by incorporating either a commercially available anionic PEG−PE lipid or an uncharged PEG lipid, whose synthesis will be reported elsewhere.

MATERIALS AND METHODS

Materials. DOPC, DOPE, DOPS, DOPA, DODAP, PEG_x−PE (where x represents the PEG number average molecular weight), and Rh-PE were all purchased from Avanti Polar lipids (Birmingham, AL). HPTS and DiIC18-(3) were purchased from Molecular Probes (Junction City, OR). Other chemicals were purchased from Sigma Chemicals (St. Louis, MO). Water was distilled and then purified by a MilliQ filtration system (Millipore Corp., Bedford, MA.)

Liposome Preparation. Large unilamellar liposomes were prepared by freeze/thaw and extrusion as described previously (*27*). The LUV sizes were determined by quasi-elastic light scattering to be relatively monodisperse with an average diameter of 120 ± 10 nm (*28*).

Liposomes for endocytosis studies contained 0.2 mol % of Rh-PE. The fluorophore was added to weighed lipid film from a chloroform stock solution. The mixed lipid film was hydrated in HPTS buffer (35 mM HPTS, 10 mM glycine, 45 mM NaCl, pH 9.5) to a concentration of about 13 mM before the formation of LUV by extrusion. Encapsulated material was separated from unencapsulated material on Sephadex G-75 (Pharmacia) gel-filtration columns (1.6 × 20 cm) with glycine buffer (141 mM NaCl, 10 mM glycine, 0.1 mM EDTA, pH 9.5) as eluent. The buffers used were isoosmotic with the solutions of fluorophores, that is, 268 mosM/kg (Osmette S Osmometer, Precision instruments). Phospholipid concentration was determined colorimetrically using the method of Stewart (*29*).

Cell Culture. HeLa and J774 cells were purchased from the American Type Culture Collection (Rockville, MD). HeLa cells were maintained in monolayer in MEM, modified Eagle's medium, supplemented with 10% fetal bovine serum.

J774 cells were maintained in monolayer in DMEM, Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. Cells were routinely seeded at 2×10^6 cells in 75 cm^2 flasks (Costar), incubated at 37 °C and 5% CO_2 and passaged every 2 days. Cells were counted with a Coulter Counter (Coulter Electronics Inc.).

Liposome−Cell Incubation. HeLa cells were either seeded at 5×10^5 cells and grown in 25 cm^2 cell culture flasks (Costar) for 2 days for fluorescence spectroscopy experiments or seeded at 2×10^5 cells on chamber slides (4.2 cm^2/well) (Nunc) for 1 day for fluorescence microscopy experiments; cultures were incubated at 37 °C and 5% CO_2 in MEM media supplemented with 10% fetal bovine serum. J774 cells were seeded at 5×10^5 cells and grown in 25 cm^2 cell culture flasks for 2 days for spectroscopy experiments, and cultures were incubated at 37 °C and 5% CO_2 in DMEM media supplemented with 10% fetal bovine serum. In both cases the media was removed from cultures and the cells were washed three times in PBS/CMG buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.4 mM $CaCl_2$, 0.4 mM $MgCl_2$, 5 mM glucose, pH 7.4). Liposomes were diluted to 100 μM in 1 mL of PBS/CMG buffer, and the vested monolayers were incubated with the liposome suspension at 37 °C and 5% CO_2 for various times.

Fluorescence Microscopy. The cells were washed three times in PBS/CMG and then viewed by epifluorescence with a Zeiss Axioplan Universal microscope (Carl Zeiss Inc., Thornwood, NY) using three filter sets. The first filter set allows excitation in the range of 395−440 nm with observation of the emission with a dichroic mirror and barrier filter at wavelengths greater than 470 nm. These conditions show the total fluorescence by HPTS (liposomes at both neutral and low pH). The second filter set allows excitation in the range of 450−490 nm and observation of emission at wavelengths greater than 510 nm, that is, HPTS liposomes mostly at neutral pH. The excitation with the third filter set at 510−560 nm with a long pass filter of 590 nm was suitable for rhodamine fluorescence. These files were saved as TIF files and imaged by Image-1 software (Universal Imaging Corp., West Chester, Pennsylvania).

Fluorescence Spectroscopy. Cells were washed three times in PBS/CMG buffer followed by treatment with 1.5 mL of PBS/EDTA buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 10 mM EDTA, pH 7.4) for 10 min at 37 °C; these conditions were sufficient for complete detachment of the cells. The resultant cell suspensions were diluted with 1.5 mL of PBS buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) in a fluorescence cuvette. The fluorescence spectra were obtained with a SPEX FluoroLog2 fluorometer (Spex Industries, Inc). Fluorescence emission for binding and endocytosis measurements using HPTS (λ_{ex} 380−480 nm) was obtained at 510 nm with continuous stirring at controlled (25 °C) temperature. The excitation intensities at λ_{ex} 403, 413, and 450 nm were measured. The addition of DiIC18(3) to liposomes did not interfere with the excitation spectra of HPTS. The intensity at 413 nm, the isosbestic point, is pH-independent and serves as a measure of total number of liposomes associated with the cells regardless of their location along the endocytotic pathway. The intensities at 403 and 450 nm are sensitive to pH, and the ratios of intensities at 403/413 nm and 450/413 nm were calculated

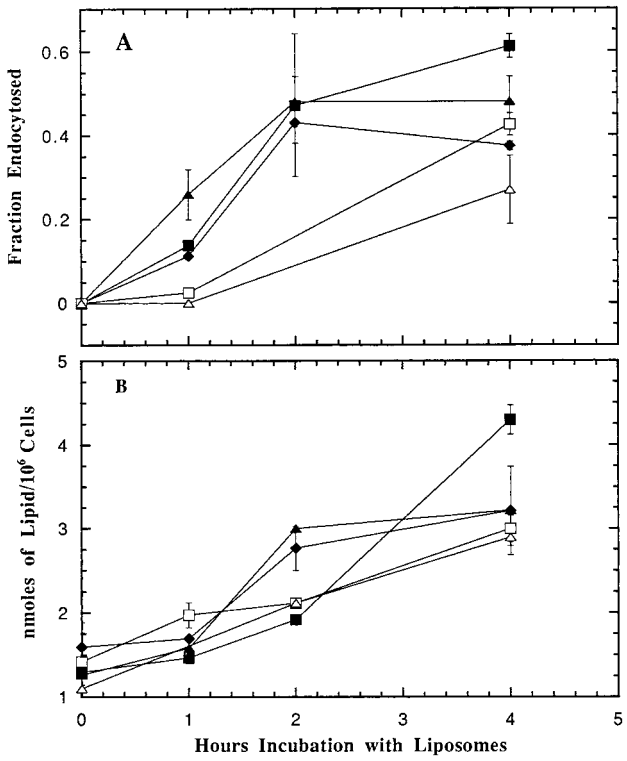


FIGURE 1: The effect of liposome charge on uptake and endocytosis by HeLa cells. Cells were treated with DOPC/DODAP (4:1) liposomes (■), DOPC/DODAP (20:1) (▲), DOPC (◆), DOPC/DOPS (11:1) (□), or DOPC/ DOPS (4:1) liposomes (△). Panel A: The fraction of liposomes endocytosed calculated by eq 1. Panel B: The effect of charge on total uptake of lipid; $N = 2$.

to estimate the fraction of liposomes endocytosed following the method of Daleke et al. (*14*) using the expression

$$\text{Fraction endocytosed} = (\text{ratio}_{\text{pH7.4}} - \text{ratio}_{\text{measured}})/(\text{ratio}_{\text{pH7.4}} - \text{ratio}_{\text{low}}) \quad (1)$$

The $\text{ratio}_{\text{pH7.4}}$ was obtained by measuring the ratio of intensities of fluorescence excitation at 450 and 413 nm for an aliquot of free HPTS in PBS at pH 7.4. The $\text{ratio}_{\text{low}}$ was determined by incubating cells with 100 μM liposomes for 2 h, washing the cells with PBS/CMG to remove unbound liposomes, and then incubating cells an additional 4 h in PBS/CMG to allow for the bound liposomes to be endocytosed. This is sufficient time to allow for liposomes to be found only in the endosomes of cells.[2] Experiments with HeLa cells were performed at least twice in duplicate, and the maximum deviation from the average value was 30% between experiments performed on different days.

The dye Rh-PE was used for simultaneous measurement of the liposomal uptake by cells. Fluorescence emission spectra (λ_{em} 570−615 nm) were obtained with excitation at 550 nm. A standard curve of rhodamine fluorescence was constructed by measuring the Rh-labeled liposome fluorescence as a function of concentration. The liposomal uptake is expressed in terms of nmol of lipid/10^6 cells. Lee et al. (*12*) showed that fluorescence resonance energy transfer from liposome-encapsulated HPTS to rhodamine in the liposomal bilayer was negligible.

[2] Private communication from D. Kirpotin, University of California, San Francisco.

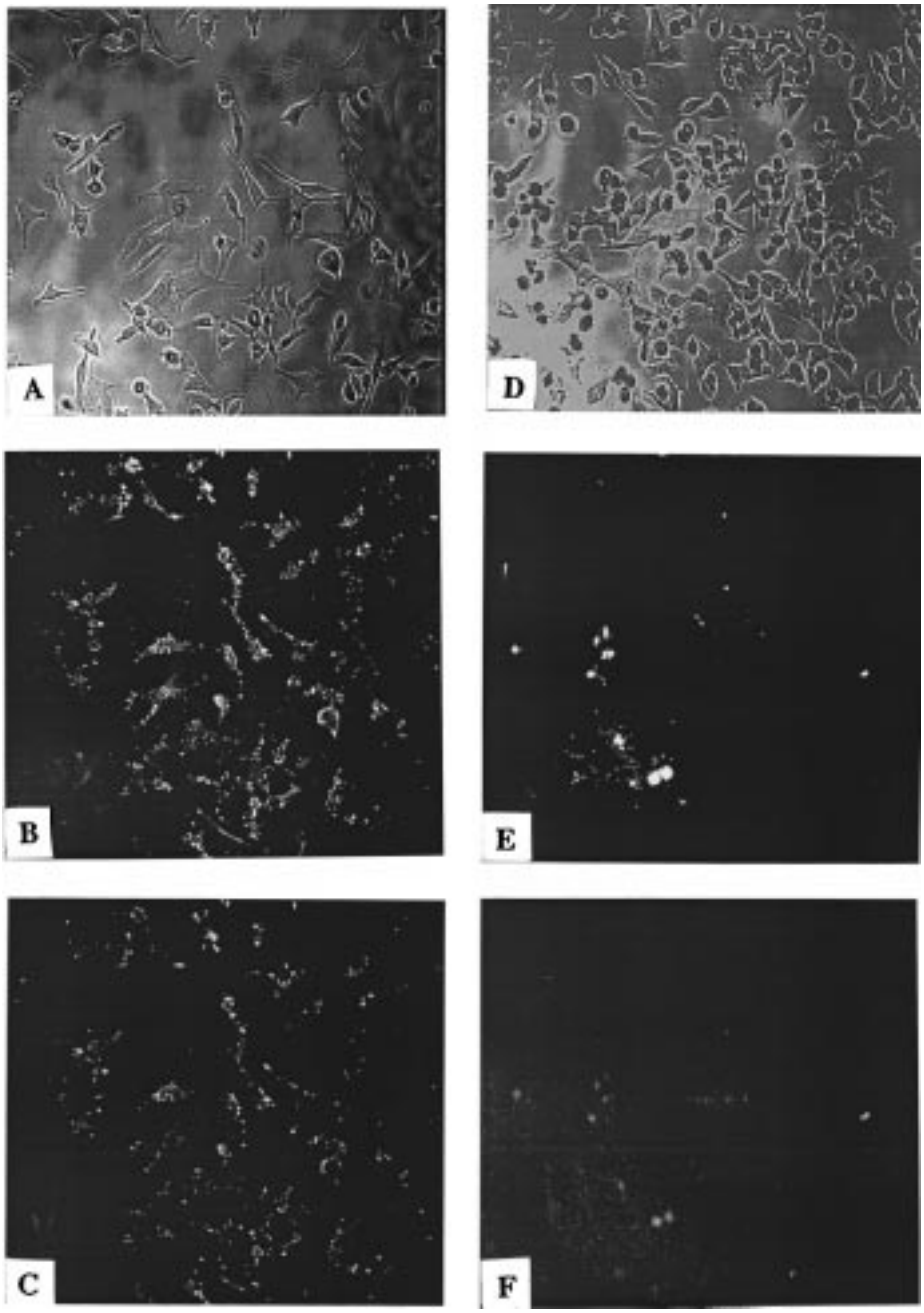


FIGURE 2: Fluorescence micrographs (200× magnification) showing the effect of charge on endocytosis by HeLa cells. Panels A, B, and C are micrographs of cells incubated for 4 h with DOPC/DODAP (20:1) liposomes. Panels D, E, and F are micrographs of cells incubated for 4 h with DOPC/DOPS (4:1) liposomes. Panels A and D are micrographs taken with transmitted light to show the location of the cells on the chamber slide. Panels B and E are the same cells viewed by epifluorescence with λ_{ex} 395−440 nm to show the location of HPTS in compartments of both high and low pH, that is, liposomes located both within the endosome and bound to the surface of the cells. Panels C and F are the same cells viewed with λ_{ex} 450−490 nm and correspond to the HPTS at high pH, those liposomes which are bound to the surface of the cells. HPTS emission in panels B and E that is not found in panels C and F corresponds to HTPS at low pH within the endosomes of the cells.

Stability of Liposomes. Liposomal stability to HPTS leakage over the course of the experiments was examined. Fluorescence emission measurements of liposomes containing HPTS (λ_{ex} 380−480 nm) were obtained at 510 nm with continuous stirring at controlled temperature (25 °C) just as for binding and endocytosis. The excitation intensities at λ_{ex} 403, 413, and 450 nm were measured. Liposomes were stirred for 4 h in dialysis tubing with MW cut off of 15 000 in PBS/CMG pH 7.4 buffer. No change in HPTS fluorescence was observed indicating minimal loss of the fluorophore over the time course of the experiment.

RESULTS AND DISCUSSION

The uptake (total cell association including binding and endocytosis) of liposomes by HeLa cells and J774 cells was compared. The liposomes were composed of DOPC with and without the addition of either a positively charged lipid (DODAP) or a negatively charged lipid (DOPS) as a given percentage of total phospholipid. In some experiments 5 mol % PEG_{2000}−PE or a neutral PEG lipid of the same molecular weight was added. The method used to measure the endocytotic capabilities of these cells was developed by

Papahadjopoulos and co-workers (*14*). Each liposome preparation was formed with encapsulated HPTS for analysis of endocytosis and with bilayer-bound Rh-PE for measurement of total cellular uptake. The amount of HPTS and Rh-PE associated with cells increased in parallel, that is, the ratio of cell associated fluorescent labels did not change significantly, over the course of the experiments. These data indicate that the liposomes continued to encapsulate HPTS during the incubation with cells. The membrane-impermeable dye, HPTS, responds to changes in pH. The calculation of the fraction of dye endocytosed assumes that HPTS is in one of two compartments, outside the cell at pH 7.4 or within endosomes or lysosomes at low pH. Thus measured pH values between a high of 7.4 and a low of 6.8 are an average of liposomes bound to the cell and those within the cell. These methods permit the measurement of binding and cellular endocytosis of liposomes using fluorescence spectroscopy and microscopy.

Effect of Surface Charge on Liposome Endocytosis by HeLa and J774 Cells. The effect of liposome surface charge on the amount of endocytosis by HeLa cells was examined. The liposomes studied included DOPC/DODAP (20:1 or 4:1), DOPC, or DOPC/DOPS (11:1 or 4:1). The amine headgroup of DODAP has a pK_a of 8.5. Earlier reports found that CV1 cells and J774 cells preferentially endocytosed liposomes with a negative surface charge, specifically CV1 cells showed significant liposome uptake when the liposomes carried 9 mol % negative charge, whereas endocytosis of liposomes by J774 cells was only significant when the liposomes had a higher anionic lipid content (*5*, *12*). We found that the interaction of liposomes with HeLa cells was also sensitive to liposome surface charge. Figure 1a shows the fraction of liposomes endocytosed by HeLa cells over a 4 h period as calculated using eq 1. The greatest extent of endocytosis was observed for liposomes composed in part from DODAP, which is positively charged at pH 7.4. The total number of liposomes associated with these cells over a 4 h incubation of liposomes with the cells (expressed as nanomoles of lipid associated with one million cells) is shown in Figure 1b. Since the process of liposomal uptake is a two-step process consisting of liposome binding to cell surface sites followed by endocytosis, it is not surprising that the trends in the two graphs are similar. However the overall extent of uptake observed is different, because a liposome population may bind to a cell but not be endocytosed.

The fluorescence micrographs shown in Figure 2 provide further evidence for the trends observed by emission spectroscopy. Two sets of HeLa cells are shown, one incubated for 4 h with DOPC/DODAP (20:1) liposomes (panels A, B, and C), and the other incubated for 4 h with DOPC/DOPS (4:1) liposomes (panels D, E, and F). Panels A and D are transmitted light micrographs that show the location of the cells on the chamber slide. The incubation of the HeLa cells with liposomes in PBS/CMG buffer for 4 h had no apparent effect on the cell morphology. The cells continued to appear normal, to spread out, and to adhere to the surface of the slide. Panels B and E show the same cells viewed by epifluorescence with λ_{ex} 395−440 nm, where the fluorescence is due to HPTS in compartments of both high and low pH; that is, liposomes located both within the endosome and bound to the surface of the cells. Panels C and F show the same cells viewed with λ_{ex} 450−490 nm which produced the HPTS fluorescence at high pH, that is, those liposomes which are bound to the surface of the cells. Thus, the HPTS emission seen in panels B and E but not in panels C and F corresponds to HPTS located in low-pH compartments of the cells. These microscopy data also indicate that HeLa cells both bind and endocytose positively charged liposomes more readily than those having a negative surface charge.

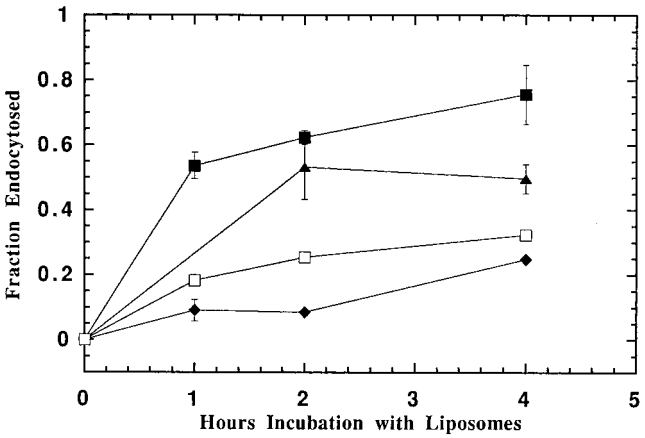


FIGURE 3: The effect of charge on endocytosis of liposomes by J774 cells. Cells were incubated in the presence of 100 μM lipid for the time indicated using DOPC/DODAP (2:1) liposomes (■), DOPC/DODAP (20:1) (▲), DOPC (◆), or DOPC/DOPS (2:1) liposomes (□); $N = 2$.

Recall that Lee et al. (*5*, *12*) found that the interaction of negatively charged lipsomes with J774 cells was greater than that of neutral liposomes. Figure 3 summarizes our studies of the interaction of positively charged (DODAP), neutral, and negatively charged (DOPS) liposomes with J774 cells. The data are presented as the calculated fraction endocytosed as a function of the incubation time of cells with 100 μM liposomes. These data indicate that J774 cells endocytose charged liposomes (either positive or negative) to a greater extent than uncharged DOPC liposomes. Moreover the positively charged DODAP liposomes were taken up to a greater extent than the DOPS-containing liposomes. The apparent effect of liposome surface charge on J774 cell/liposome interaction could be due to receptors found at the cell surface which mediate particle recognition and adhesion. It is known that cell surface receptors for the Fc domain of immunogloblins and the RGD tripeptide mediate recognition, adhesion, and phagocytosis of molecules for mammalian macrophages. RGD peptide/receptor interactions appear to be mediated by charge/charge interactions (*30*, *31*). Although the liposome binding sites in J774 cells have yet to be identified, it has been suggested that a membrane protein and Ca^{2+} are essential to liposome binding (*32*). The fibronectin receptor, the first RGD peptide receptor to be recognized, has a Ca^{2+} binding domain and requires Ca^{2+} for ligand-to-receptor interactions (*33*). Charged liposomes may interact with this receptor or similar receptors present in J774 cells.

Effect of Cationic Lipid Structure on Endocytosis of Liposomes by HeLa Cells. To further characterize the interaction of positively charged liposomes with HeLa cells,

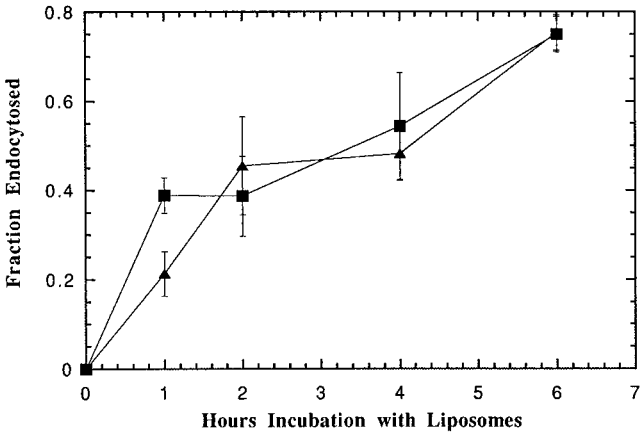
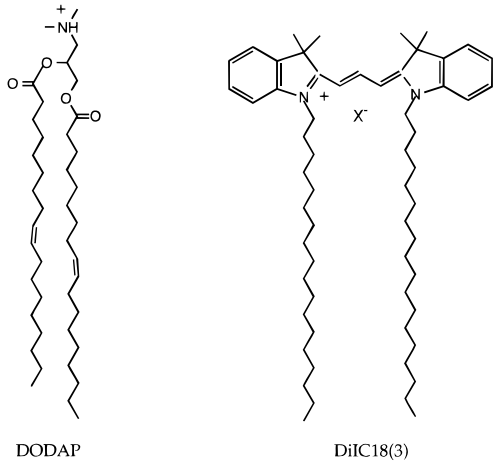


FIGURE 4: The effect of the headgroup of cationic lipids on the endocytosis of liposomes by HeLa cells. HeLa cells were treated with DOPC/DODAP (20:1) liposomes (■) or DOPC/DiIC18(3) (20:1) liposomes (▲) for the time indicated at 100 μM lipid in PBS/CMG buffer; $N = 2$.

the cationic lipid was changed from the dimethylammonium lipid, DODAP, to the cyanine dye, DiIC18(3), a green dye



that is commonly used for membrane labeling. The data presented in Figure 4 indicate that the HeLa cell/liposome interaction is similar whether the liposomes were composed of DOPC/DODAP (20:1) or DOPC/DiIC18(3) (20:1). DODAP has a localized charge at the protonated nitrogen, whereas the cyanine dye charge is delocalized over the conjugated dye structure. These data suggest that HeLa cells effectively endocytose cationic liposomes on the basis of charge rather than the structure of the cationic lipid. This is consistent with nonspecific binding rather than requiring a specific receptor. However, the uptake of liposomes was significantly greater than the uptake of solutes, for example, free HPTS, indicating that fluid-phase pinocytosis is only a minor factor to the total uptake of liposomes (data not shown).

Effect of Weak Bases on the pH of Cell-Associated HPTS. If a weak base such as ammonium chloride or chloroquine is added to cells, it will concentrate in the acidic organelles of the cells, for example, endosomes, and the distribution will depend on the relative pH values inside and outside the acidic organelles (*34*). Entry of liposomally encapsulated HPTS into acidic cellular compartments, presumably the endosome, causes a sharp decrease in the fluorescence due to excitation at λ_{ex} 450 nm while the fluorescence from

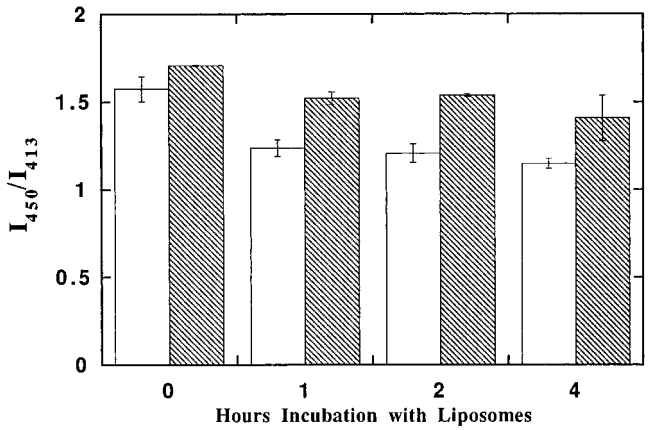


FIGURE 5: The effect of ammonium chloride on the pH of cell-associated HPTS. Cells were incubated with DOPC/DODAP (20:1) liposomes (100 μM) for the time indicated. Excitation spectra were obtained before (open bar) and after (hatched bar) the addition of 30 μL of 50 mM NH_4Cl to a stirred cuvette: λ_{ex} 380−480 nm, λ_{em} 510 nm. The data are plotted as the ratio of HPTS fluorescence at excitation wavelengths 450 and 413 nm as a function of the incubation time of cells; $N = 2$.

excitation at the isosbestic point, λ_{ex} 413 nm, remains unchanged. The ratio of 450 nm/413 nm permits normalization of fluorescence intensities to the total amount of HPTS associated with the cells (*14*). The addition of either chloroquine (100 μM) or ammonium chloride (50 mM) effectively raised the average pH of the liposomally encapsulated HPTS associated with HeLa cells. This was demonstrated by plotting the ratio of the fluorescence obtained by excitation at 450 and 413 nm versus the time of incubation. The excitation spectrum for HPTS was determined before a 30 μL aliquot of weak base was added to the cells in a stirred suspension, and then the spectrum was remeasured following two minutes of incubation with the base. Figure 5 shows the effect of adding ammonium chloride. A similar response was observed after adding chloroquine to the cell suspension. In both cases the pH of the acidic compartment was raised by the introduction of the weak base, indicating that a significant portion of the HPTS was located inside the endosome of the cells (*35*). The effect could be reversed by washing the cells with buffer without added weak base.

Effects of Liposome Surface Charge on the Endocytosis of PEG Liposomes. The preferential uptake of positively charged conventional liposomes by HeLa cells in culture led to a consideration of the possible effects of surface charge on the interaction of sterically stabilized liposomes with cells. Generally the incorporation of a few mole percent of PEG_{2000}−PE significantly increases the circulation time of liposomes in vivo. This important phenomenon is usually attributed to the steric effect of the bilayer-tethered polymer. The conformation of PEG_x−PE could be either random coil (mushroom) or more extended (brush) depending on the magnitude of x and the mole fraction of the PEG_x−PE in the bilayer membrane. If liposome stabilization, that is, enhanced circulation time, is strictly due to steric factors, then the negative surface charge imparted to the liposomes by the PEG−PE should have little effect on the interaction of liposomes and cells. An initial examination of this hypothesis was undertaken by comparing the liposome HeLa cell interaction of PEG_{2000}−PE liposomes (negative surface

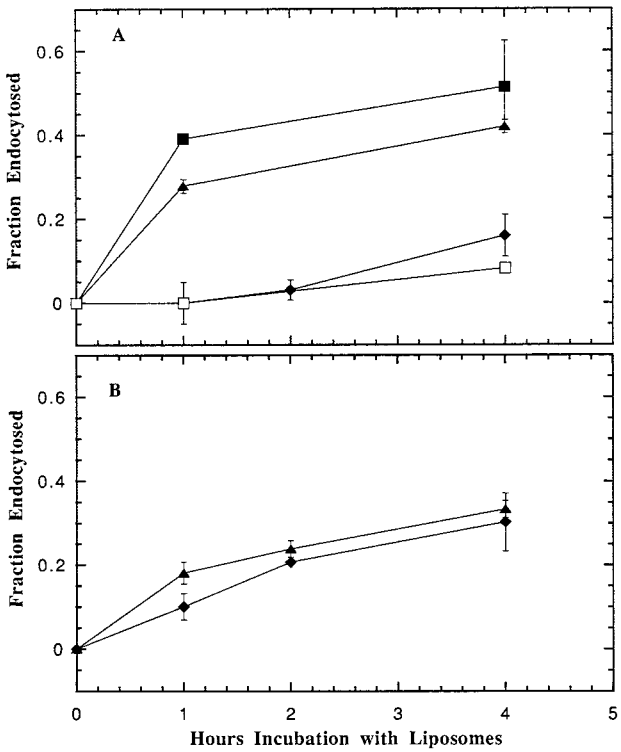


FIGURE 6: Effects of PEG lipids on the endocytosis of liposomes (100 μM) by HeLa and J774 cells. Panel A: HeLa cells were incubated with liposomes composed of DOPC/DODAP/neutral PEG_{2000} (20:1:1) (■), DOPC/neutral PEG_{2000} (20:1) (▲), DOPC/DOPA/neutral PEG_{2000} (19:1:1) (□), or DOPC/PEG_{2000}−PE (20:1) (◆) liposomes. Panel B: J774 cells were incubated with DOPC/neutral PEG_{2000} (20:1) (▲) or DOPC/PEG_{2000}−PE (20:1) (◆) liposomes; $N = 2$.

charge) with neutral PEG liposomes, of similar molecular weight, using an uncharged PEG_{2000} lipid designed in our laboratory. The experimental results for the endocytosis of liposomes composed of either DOPC/DODAP/neutral PEG (20:1:1), DOPC/neutral PEG (20:1), or DOPC/PEG−PE (20:1) are shown in Figure 6A. The cellular uptake of positively charged PEG liposomes was greater than that of the neutral PEG liposomes, whereas the negatively charged PEG−PE liposomes were hardly endocytosed by the HeLa cells. These data parallel the effects of liposome surface charge in conventional liposomes shown in Figures 1 and 2. Furthermore the liposome/HeLa cell interaction of liposomes composed of DOPC/neutral PEG plus an equimolar amount of dioleoyl phosphatidic acid (DOPA) (molar ratio 19:1:1) is similar to that of the DOPC/PEG−PE (20:1) (Figure 6A). The interaction of cells and liposomes that have comparable surface charges and tethered PEG_{2000} chains appears to occur in a similar manner whether or not the charge is localized on the PEG lipid. Consequently a potentially interesting attribute of the neutral PEG lipid is the flexibility it pemits in liposome formulation, because the steric and electrostatic characteristics can be independently varied.

The interaction of anionic and neutral PEG LUV with J774 cells is summarized in Figure 6B. Interestingly the J774 cells do not endocytose either liposome population to the same extent as observed in the liposome/HeLa cell experiments. Only a small difference was found in the limited liposome endocytosis by J774 cells of neutral PEG LUV and negatively charged PEG LUV. The incorporation of PEG−PE into liposomes is reported to inhibit the adhesion of liposomes to macrophages (*26*, *36*).

Table 1: Nanomoles of Lipid Associated with One Million Cells at 37 °C as a Function of Liposome Composition and Cell Line[a]

liposome composition[b]	%	charge	cell line	nmol of lipid/10^6 cells
PC/DAP (4:1)	20	+	HeLa	4.3 ± 0.2
PC/DAP (20:1)	4.8	+	HeLa	3.2 ± 0.5
PC	0		HeLa	3.2 ± 0.2
PC/PS (11:1)	8.2	−	HeLa	3.2 ± 0.2
PC/PS (4:1)	20	−	HeLa	2.9 ± 0.1
PC/DAP/neu PEG (20:1:1)	4.5	+	HeLa	3.3 ± 0.1
PC/neu PEG (20:1)	0		HeLa	2.2 ± 0.1
PC/neu PEG/PA (19:1:1)	4.8	−	HeLa	1.8 ± 0.1
PC/PEG−PE (20:1)	4.8	−	HeLa	2.1 ± 0.2
PC/DAP (2:1)	33	+	J774	3.2 ± 0.5
PC/DAP (4:1)	20	+	J774	3.9 ± 0.1
PC/DAP (20:1)	4.8	+	J774	3.1 ± 0.5
PC	0		J774	1.6 ± 0.1
PC/PS (2:1)	33	−	J774	3.0 ± 0.3
PC/neu PEG (20:1)	0		J774	2.2 ± 0.2
PC/PEG−PE (20:1)	4.8	−	J774	2.1 ± 0.2

[a] The calculated amount of the total lipid associated with one million cells after 4 h of incubation of liposomes and cells at 37 °C. [b] The molar ratio of the lipids is shown in parentheses. Lipids: PC, 1,2-dioleoylPC; PS, 1,2-dioleoylPS; PA, 1,2-dioleoylPA; DAP, 1,2-dioleoyl-3-dimethylammonium propanediol; neu PEG, neutral lipid with attached PEG_{2000}; PEG−PE, PE with attached PEG_{2000}.

Table 1 summarizes the data obtained for the interaction of liposomes of various compositions with either HeLa or J774 cells. The total lipid associated with cells was calculated from the Rh-PE emission. These data show a somewhat greater total interaction of positively charged conventional liposomes with the HeLa cells than observed with either neutral or negatively charged liposomes, for example, 4.3 nM lipid/million cells for DOPC/DODAP (4:1) compared to 2.9 nM lipid/million cells for DOPC/DOPS (4:1). This coupled with the greater fraction of endocytosis (obtained by the HPTS assay) of positively charged liposomes resulted in a 3-fold increase in the amount of DOPC/DODAP (4:1) liposomes found in the low-pH compartments of HeLa cells compared to the DOPC/DOPS (4:1) liposomes. In contrast, the extent of J774 uptake of liposomes into low pH compartments was quite similar for both cationic and anionic conventional liposomes. Similar trends for each cell line were observed for the PEG liposomes. The extent of liposome uptake into low-pH cellular compartments was only substantial for the cationic PEG liposomes and HeLa cells. If the PEG liposomes were neutral or anionic, the amount of lipid associated with cells in low-pH compartments was much less.

The interaction of liposomes with cells is a complex series of events that includes binding and subsequent endocytosis. Two markers for cellular uptake of liposomes were used in this study. To monitor the acidification of liposomes that were presumably inside the endosome, the pH-sensitive HPTS was encapsulated in the liposomes. The intensity of the excitation spectrum at 413 nm, the pH-insensitive isosbestic point, measures the concentration of cell-associated HPTS. However, any HPTS leakage from the liposomes prior to endocytosis would cause apparently low values for liposomal endocytosis. While our data did not show preferential leakage from charged liposomes over the course of the experiment, any unaccounted leakage would reduce

the amount of endocytosis observed. The second liposome label, Rh-PE, is a bilayer probe that monitors the cellular uptake of liposomes. If Rh-PE transferred from the surface of the liposome to the cell membrane during liposome/cell binding in the absence of endocytosis, then the Rh label in the cell membrane would yield apparently high values for liposomal uptake. This appears unlikely to have occurred in the experiments reported here, because the Rh emission observed by microscopy was always localized in cellular regions that also showed HPTS emission, that is, in a manner expected for bound liposomes. Even though HPTS leakage and Rh-PE transfer do not appear to be significant, it is important to note that the absolute values reported in Table 1 may be influenced to a small extent by these processes.

General Discussion. The data obtained in this study and reported in Figures 1 and 2 indicate that HeLa cells preferentially take up positively charged liposomes. The experiments with the weak bases, ammonium chloride (Figure 4) or chloroquine, show that the cellular uptake of these liposomes has the characteristics associated with endocytosis. Furthermore the cellular uptake of cationic liposomes does not appear to be headgroup-specific since the replacement of ammonium DODAP with a cationic dye having diffuse positive charge does not significantly alter the extent of uptake by HeLa cells (Figure 5). In the case of HeLa cells this implies that the preference for charge is not due to a receptor specific for a lipid headgroup, as suggested by Lee et al. (*12*) for CV1 cells, but is instead a more general mode of charge-sensitive binding and endocytosis. Moreover a similar effect of liposomal charge on the extent of HeLa cell endocytosis was observed with PEG liposomes (Figure 6). In these experiments the PEG group presents a steric barrier to close approach of the lipid headgroups to other surfaces. Since the charge associated with DODAP and DOPA is localized at the lipid headgroup, the cellular uptake of charged PEG liposomes is unlikely to be mediated by specific recognition of the lipid headgroups.

The earlier studies of Lee et al. (*12*) showed that CV1 cells preferentially endocytosed anionic liposomes with at least 9 mol % negative charge. The CV1 cells were used to mimic systemic cells of the body in order to ascertain whether liposome endocytosis by systemic cells might differ from that of RES cells. Thus they compared the CV1 cells with J774 cells and discovered that J774 cells ineffectively endocytosed liposomes unless they were composed of a substantial mole fraction of anionic lipid. These results suggested that it might be possible to minimize liposome interaction with RES cells by appropriate choice of the liposome composition. The present comparison of liposome/cell interactions of J774 cells and HeLa cells provides further insight into the cell-specific nature of cellular endocytosis of liposomes. Our observations of the interaction of anionic and neutral liposomes with J774 cells parallel the findings of Lee et al. (*12*). Note that only a qualitative comparison can be made between the two studies because the liposomes used here did not contain cholesterol. Cholesterol-free liposomes were selected because the photosensitive liposomes used in our other studies have not included cholesterol (*1*, *2*). The preferential uptake of cationic liposomes by HeLa cells is perhaps not surprising in view of earlier reports that cationic liposome/DNA complexes, which are widely used to mediate transfection of mammalian cells, enter cells by the general mode of receptor-mediated endocytosis (*15*−*18*). The observation that the cellular endocytosis of cationic liposome (sans the DNA) is sensitive to the mole fraction of cationic lipid may prove useful in the proper design of cationic lipid/DNA complexes for efficient cellular uptake. It has already been invaluable in our design of photosensitive liposomes for studies of the photoinduced destabilization of liposomes that have been endocytosed by specific cell lines (unpublished observations).

The fraction of positively charged lipid in a liposome is likely to be critical to its effective delivery and cellular uptake. Senior et al. (*37*) reported that the interaction of positively charged liposomes with plasma depended on the concentration of positive charge bearing lipid, the charge density, and to a lesser extent the structure of the cationic lipid. Significant increases in turbidity, due to liposome/plasma interactions, were observed when the mole fraction of DOTMA in the liposomes was changed from 0.1 to 0.2. These observations suggest that liposomes with at least 5−10 mol % of cationic lipid could be used to minimize plasma interactions and still facilitate cellular uptake by proliferative cells. An earlier report found that liposomes with a high proportion of cationic lipid are toxic to cells in culture (*38*). In contrast, over the course of the studies reported here, we did not observe any sign of cell death during any of the incubations with liposomes summarized in Table 1.

The effect of PEG−PE coating on the surface of liposomes is to prevent the rapid clearance of liposomes from the blood in the body by the RES. Liposomes with a sufficiently hydrophilic surface were found to decrease the adsorption of proteins, opsonins, to the surface of the liposome and to curtail recognition by macrophages of the RES (*9*, *25*, *36*). Macrophages participate in phagocytosis of particles mostly via the Fc receptors found on their surfaces. The method of endocytosis of liposomes cannot be of this type and therefore must bind to the cell surface and be endocytosed by a separate, nonspecific charge-mediated mechanism (*14*). Surface-associated PEG−PE groups may inhibit even the nonspecific endocytosis of liposomes since Du et al. (*26*) showed that 5 mol % of PEG_{5000}−PE inhibits adsorption of liposomes to the surface of cells. The data shown in Figure 6 supports this view and adds the further insight that PEG_{2000}−PE presents a steric barrier that is intrinsically associated with a negative surface charge. However by use of a neutral PEG lipid the steric barrier and the liposome charge can be independently varied. The HeLa cell endocytosis of liposomes was quite similar for liposomes with 5 mol % of anionic PEG−PE or with 5 mol % of neutral PEG lipid and the anionic lipid, DOPA. Therefore the charge need not be localized on the PEG lipid, but only associated with the liposome bilayer. The data in Figure 6 suggest some important opportunities in liposome design and raise some interesting questions. One question that is not addresed in the present study is whether the apparent effect of liposome charge on cellular uptake is a bilayer surface charge effect or is due to the total charge of the liposome and its contents. Since the liposomes for these studies were prepared with the same concentration of encapsulated HPTS, the current data do not provide a comparative test of the effect of surface and total charge. Returning to the comparison of PEG liposome uptake by J774 and HeLa cells, we note that the proliferative cells, that is, HeLa cells, readily distinquish

between positively charged and negatively charged PEG liposomes, whereas the J774 cells do not. Since these cells do not readily endocytose PEG liposomes of either charge, these observations suggest the possibility of designing PEG liposomes that have minimal interaction with cells of the RES, yet strongly interact with HeLa and other rapidly growing cells.

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