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Heterogeneous Liposome Membranes with pH-Triggered Permeability Enhance the *in Vitro* Antitumor Activity of Folate-Receptor Targeted Liposomal Doxorubicin

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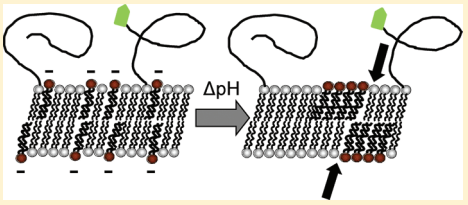
Supporting Information

ABSTRACT: The killing efficacy of doxorubicin from liposome-based delivery carriers has been shown to correlate strongly with its intracellular trafficking and, in particular, its fast and extensive release from the delivery carrier. However, previously explored pH-triggered mechanisms that were designed to become activated during liposome endocytosis have also been shown to interfere with the liposome stability *in vivo*. We have designed pH-triggered gel-phase liposomes with heterogeneous membranes for the delivery of doxorubicin. These liposomes are triggered to form "leaky" interfacial boundaries between gel−gel phase separated domains on the membrane bilayer with lowering pH. The pH-triggered mechanism does not compromise liposome stability *in vivo* and results in superior *in vitro* killing efficacy of delivered doxorubicin when liposomes are endocytosed by a clathrin-mediated pathway. In the present work, we evaluate the general applicability of these liposomes when targeted to the folate receptor (FR) of KB cancer cells *in vitro* and become endocytosed by a less acidic pathway: the caveolae pathway. FR-targeting liposomes exhibit almost 50% decrease in cell association for increase in liposome size from 120 to 280 nm in diameter after relatively short incubation times (up to 4 h). The fraction of internalized vesicles, however, is approximately 60% of the cell associated vesicles independent of their size. Our findings demonstrate that, for the same doxorubicin uptake per cancer cell, the killing effect of doxorubicin delivered by pH-triggered lipid vesicles is greater (IC_{50} = 0.032 mM for a 6 h incubation) than when delivered by a conventional non-pH-responsive composition (IC_{50} = 0.194 mM). These findings suggest higher bioexposure of cells to the therapeutic agent possibly via faster and more extensive release from the carrier. Animal studies of FR-targeting non-pH-responsive liposomal doxorubicin report stronger therapeutic potential for the targeted approach relative to nontargeted liposomes and to free doxorubicin. The findings of the present study suggest that the targeted pH-triggered liposomes could potentially further enhance the therapeutic outcomes of doxorubicin *in vivo*.



KEYWORDS: folate, folate receptor, folate receptor-targeting, liposomes, pH-sensitive liposomes, folate receptor-targeted liposomes, cancer, chemotherapy, doxorubicin, caveolae, receptor-mediated endocytosis

1. INTRODUCTION

Liposomes are particularly successful in the delivery of chemotherapeutics for cancer therapy with several compositions approved for diseases that include ovarian cancer, metastatic breast cancer and AIDS-related Kaposi's sarcoma.[1,2] The advantageous characteristics of liposomes include the high drug-to-carrier ratios, the natural basis of the constituent lipids, and the physical encapsulation of delivered therapeutics that negate the need of conjugation chemistries of the drug to the carrier.[3] These chemistries may not only impact the activity of conjugated therapeutic agents but may also delay the release of therapeutics in their active form, therefore, affecting their intracellular trafficking profiles.

The killing efficacy of delivered chemotherapeutics to malignant sites depends to a great extent on their intracellular trafficking.[4−6] In particular for doxorubicin, which is used in the present studies as a model therapeutic compound, fast and extensive release from liposome-based delivery carriers has been shown to correlate strongly with the observed killing efficacy.[5] Toward this goal, the use of pH-triggered mechanisms that become activated during the endosomal acidification—after cellular internalization of liposomes—has been extensively explored.[7]

The design of lipid membranes that are triggered to release their contents at acidic pH values aims to either introduce within the lipid membrane new interfaces at which the molecular packing among lipids is defective,[8] therefore, increasing the membrane permeability, or to create porelike or other structures spanning the membrane (see the informative review by Drummond et al.[7]). Several different strategies have been reported for the former approach and include formation of pH-triggered interfacial defects on the lipid membrane driven by either (a) lateral phase separation of lipid mixtures in which some components preferentially form structures other than a lamella[9,10] or (b) lateral phase separation on membranes composed only of lamellar forming gel−gel interfacial boundaries.[11,12]

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Our approach pertains to the formation of "leaky" interfacial boundaries between gel−gel phase separated domains. These interfaces within the bilayer contain extensive transient discontinuities at the molecular level due to defects in lipid packing.[11,12] Lipid bilayers, in the form of vesicles, are composed of two types of lipids: (i) a "domain" forming lipid with titratable anionic headgroups, such as phosphatidic acid (PA) or phosphatidyl serine (PS) (lipid A), and (ii) a nonionizable lipid, such as phopshatidyl choline (PC), with hydrocarbon tails of different length than those in lipid A (lipid B). At high pH values the lipid A headgroups are charged, repulsion between the headgroups makes the lipid energetically less likely to crystallize, and the membrane appears spatially homogeneous. As the pH value is lowered, the anionic A headgroups become protonated, reducing electrostatic repulsion while increasing hydrogen bonding between the newly protonated A headgroups. These conditions favor phase separation and formation of domains rich in newly protonated type A lipids. At the interfacial boundaries between the different lipid domains, the extent of transient packing defects among the acyl tails of lipids is controlled by the relative acyl tail lengths of lipid pairs and the lipid acyl tail dynamics (lipid fluidity). Different acyl tail lengths of lipid pairs combined with slow lipid acyl tail dynamics (gel-phase lipids) enhance the pH-dependent transient packing defects and, therefore, the pH-dependent bilayer membrane permeability, which translates into triggered release of encapsulated contents.[12]

The above lipid vesicles, unlike liposomes composed of inverted hexagonal phase lipids,[13−15] are shown to exhibit long circulation times, to demonstrate permeability responsiveness not affected by the extent of PEGylation, and to be autonomous;[11] i.e. liposomes do not require fusion with an adjacent membrane to become destabilized and release their contents. In addition, the mechanism of phase separation is reversible with pH, therefore, resulting in liposomes with the potential for "on demand" drug delivery.

We have previously reported the superior *in vitro* killing efficacy of delivered doxorubicin by these pH-triggered liposomes compared to DSPC/cholesterol-based PEGylated liposomes ("conventional" liposomal membrane composition)—similar to the FDA approved Doxil composition—when targeted to the HER2/*neu* receptor via liposome-conjugated trastuzumab.[11] Our findings suggest that the observed greater killing efficacy could be attributed to the faster doxorubicin release from pH-triggered liposomes during the acidification of the endocytic pathway of the clathrin-coated pit.[16,17]

In the present work, we evaluate the general applicability of these liposomes by utilization of a different type of endocytic uptake: the caveolae pathway that generally exhibits less acidic values.[18−23] This pathway has been reported—for the case of multivalent folate-labeled macromolecules—to be characterized by median pH values near pH 5.[24] Since the pH-triggered liposomes with gel-phase heterogeneous membranes were demonstrated to exhibit significant content release at this pH value,[11,12] the present study aims to evaluate their potential as folate-targeted liposomes to deliver doxorubicin to cancer cells expressing the folate receptor (FR). Previous studies have demonstrated that folate-labeled liposomes are internalized by KB cancer cells via the folate receptor.[18,19] The present study aims to compare the efficacy of pH-triggered liposomes relative to non-pH-sensitive folate-targeted PEGylated liposomal doxorubicin ("conventional" liposomal doxorubicin) that is reported to exhibit improved therapeutic effect in FR positive tumors in animal models compared to the nontargeted liposomal modality.[25−27]

The folate receptor is a promising target for improving the selectivity of targeted anticancer therapies since it is upregulated in several human malignancies including cancers of the ovary, lung, kidney, endometrium, breast, colon and others.[23] Healthy sites where FR is expressed include the apical surfaces of polarized epithelia and are not expected to be accessible to circulating targeted nanocarriers.[23] Some advantages of the folate−FR targeting strategy include the inexpensiveness of folate, its lack of immunogenicity when in monomeric form, and its stability in various solvents and toward denaturation.[28]

In this study we evaluate the effect of acidification during FR-mediated endocytosis on the killing efficacy of doxorubicin delivered by the FR-targeting pH-triggered liposomes and compare to FR-targeting DSPC/cholesterol-based non-pH-sensitive liposomal doxorubicin. We also briefly evaluate the role of liposome size on the extent of their selective targeting and internalization by FR expressing KB cancer cells.

2. MATERIALS AND METHODS

2.1. Materials. The lipids 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (21PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphate (monosodium salt) (DSPA), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Lissamine Rhodamine B sulfonyl) (ammonium salt) (DPPE-Rhodamine), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[folate (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-folate) were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphate buffered saline (PBS), Sephadex G-50, ethylene diamine tetraacetic acid (EDTA), calcein, Triton X-100, cholesterol, folate-free RPMI 1640, ammonium sulfate, methylthiazolyl-diphenyl-tetrazolium bromide (MTT) and doxorubicin hydrochloride were obtained from Sigma-Aldrich (Atlanta, GA). Heat inactivated fetal bovine serum (FBS) was purchased from Omega Scientific (Tarzana, CA). Eagle's minimum essential medium (EMEM) was purchased from ATCC (Manassas, VA).

2.2. Vesicle Preparation. The composition of pH-triggered lipid vesicles contains 21PC and DSPA lipids (at 9:1 mol ratio) with 5.0 mol % PEGylated lipid (5.0 mol % DSPE-PEG for nontargeted vesicles, and 4.8 mol % DSPE-PEG and 0.2 mol % DSPE-PEG-Folate for targeted vesicles) and 1.0 mol % DPPE-Rhodamine. Conventional lipid vesicles are composed of DSPC lipid and cholesterol (at 7:3 mol ratio) with 5.0 mol % PEGylated lipid (5.0 mol % DSPE-PEG for nontargeted vesicles, and 4.8 mol % DSPE-PEG and 0.2 mol % DSPE-PEG-Folate for targeted vesicles) and 1.0 mol % DPPE-Rhodamine. Lipids in chloroform (10 μmol total lipid) were combined in a 25 mL round-bottom flask. Chloroform was evaporated in a Buchi rotavapor R-200 (Buchi, Flawil, Switzerland) for 10 min at 50 °C followed by evaporation under N_2 stream for 5 min. The dried lipid film was hydrated with 1 mL of aqueous solution for 1.5 h at 50−55 °C. The lipid suspension was then extruded 19−21 times through two stacked polycarbonate membranes with a pore diameter of 100 or 400 nm (Avestin Inc., Ottawa, Canada). The extrusion was carried out in a water bath at a temperature about 10 °C higher than the highest T_m of the lipids in the vesicle composition. To separate unencapsulated contents, gravity was then used to pass vesicles through a Sephadex G-50 column eluted with PBS buffer (pH = 7.4, 300 mOsm) at room temperature.

2.3. Loading of Vesicles with Doxorubicin. Doxorubicin was loaded into preformed vesicles by the ammonium sulfate gradient technique.[29] Briefly, the dried lipid film was hydrated with 250 mM ammonium sulfate (pH 7.4). To establish an ammonium sulfate gradient upon vesicle extrusion, vesicles were passed through a Sephadex G-50 column eluted with PBS buffer. Vesicle suspensions were then incubated with doxorubicin (5 mM, dissolved in isosmolar 150 mM NaCl solution, pH 7.4, 300 mOsm) for 2 h at a temperature about 5−10 °C higher than the highest T_m of lipids in the vesicle composition in a 1-to-1 drug-to-lipid mole ratio. At the end of incubation, the vesicle suspension was eluted through a Sephadex G-50 column with PBS (pH 7.4, 300 mOsm) to separate vesicles from unencapsulated doxorubicin.

2.4. Measurement of Doxorubicin Release from Vesicles. Vesicle suspensions containing 1.0 mol % DPPE-Rhodamine, at 0.125 mM final total lipid concentration (that was quantified based on Rhodamine fluorescence) were incubated in 10% serum supplemented media (RPMI 1640 not containing L-glutamine, phenol red, and sodium bicarbonate) at four pH values (7.4, 5.5, 5.0, 4.0) at 37 °C in the absence of CO_2. At different time points (5, 60, 120, 240, and 1440 min) samples were removed from the parent suspensions and were passed through a Sephadex G-50 column to separate the released doxorubicin. Vesicles in the eluted suspensions were then solubilized using Triton X-100 (1.67 mM final concentration) followed by incubation at 85 °C for 5 min. The fluorescence intensity of doxorubicin (excitation wavelength: 470 nm, emission wavelength: 492 nm) of the samples was measured by an SLM AMINCO 8000 spectrofluorometer (SLM Instruments, Inc.) and was compared to the intensity of solubilized samples removed from the parent suspensions without separation. The ratio of the two values was used to evaluate drug retention.

2.5. Vesicle Size Measurement by Dynamic Light Scattering. The size distribution of vesicles was measured using dynamic light scattering of vesicle suspensions. The N4 Plus autocorrelator (Beckman-Coulter) with a 632.8 nm He−Ne laser light was used for the measurements. Scattering was detected at four angles, 23.0, 30.2, 62.6, and 90.0°, for four measurements at each angle. The average vesicle size was determined to be the extrapolated value of the y-intercept at zero angle vs $\sin^2(\theta)$.[30]

2.6. Cell Line. The human carcinoma KB cell line was purchased from ATCC (Manassas, VA) and was propagated using EMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2. Prior to experiments, cells were grown for at least two passages in folate-free RPMI 1640 medium supplemented with 10% of heat inactivated FBS.

2.7. Cell Binding and Internalization Studies. KB cancer cells expressing folate receptors were trypsinized, centrifuged and resuspended in two aliquots in 10% serum supplemented media at a density of 11×10^6 cells/mL. Vesicle suspensions (containing targeted and nontargeted vesicles labeled with 1% mol DPPE-Rhodamine) were incubated with 2.75 mL of cell suspensions in a total volume of 3.75 mL and 0.6 mM total lipid at 37 °C and 5% CO_2. At different time points (5, 60, 120, 240, and 1440 min) two samples of 0.31 mL, containing 2.5 million cells per sample, were removed and washed twice with ice cold PBS. To quantify the cell associated vesicles, the fluorescence intensity of Rhodamine (excitation wavelength, 550 nm; emission wavelength, 590 nm) was measured in cell suspensions under continuous magnetic stirring using an SLM AMINCO 8000 spectrofluorometer (SLM Instruments, Inc.) and the intensities were compared to cell suspensions (of identical cell density) that had not been incubated with lipid vesicles, to correct for potential scattering effects. Measured intensities due to light scattering by cells accounted for less than 27% and 50% of the measured intensities for targeted and nontargeted vesicles, respectively. All reported values are corrected by subtraction of these values. Then, to remove the noninternalized vesicles, the same samples were incubated with a stripping buffer (50 mM Glycine, 100 mM NaCl, pH 2.8) for 5 min. After the end of incubation cell suspensions were centrifuged again, resuspended, and measured for fluorescence to quantify the fraction of internalized vesicles. For these studies, the ratio of 1-to-10 liposomes-to-folate receptors was kept constant assuming on average approximately three million folate receptors per KB cell.[31]

For the flow cytometry studies, lipid vesicle membranes were labeled with the fluorescent lipid NBD-PE (excitation, 465 nm; emission, 535 nm). Cells were incubated with various lipid vesicle compositions as described above. Fluorescence counting of cell suspensions (10,000 events) was measured using a Becton Dickinson FACS Calibur flow cytometer (San Jose, California) with a 488 nm argon laser and analyzed with the software CELL Quest (San Jose, California).

2.8. Cell Viability Assay. Cells were plated on 96-well plates at a density of 7,500 cells/well for the period of two doubling times (2×25 h) before the start of the experiment to ensure good adhesion of the cells to the surface. Folate-receptor targeted and nontargeted vesicles with and without encapsulated doxorubicin, as well as free doxorubicin, were added to the cells in a total volume of 300 μL per well at different concentrations and were incubated for 6 and 12 h. At the end of the incubation period, the cells were gently washed with sterile PBS, and incubated further with fresh growth media for the period of one doubling time. At the end of the incubation period, cells were washed again with sterile PBS and the MTT assay was used to evaluate cell viability.

2.9. Extent of Association of Delivered Doxorubicin with Cells. Depending on the dilution of doxorubicin-containing vesicles, targeted and nontargeted vesicles as well as free doxorubicin were added to cells that were plated on 96-well plates ([doxorubicin] > 100 μM), 12-well plates (0.010 μM < [doxorubicin] < 100 μM), and 6-well plates (0.010 μM < [doxorubicin]) at a density of 30,000, 0.6 million, and 1.2 million cells per well and in a total final volume of 0.25 mL, 3.0 mL, and 8.0 mL per well, respectively, for 6 and 12 h. The lipid-to-drug ratio was kept approximately constant across all vesicle compositions (0.4 μmol of doxorubicin per μmol of lipid). At the end of incubation, the incubation medium was removed and was analyzed for doxorubicin content as explained below. Cells were gently washed thrice with sterile PBS and were further incubated with trypsin (0.05 wt %/wt). Cells were removed from wells and were lysed by addition of 0.4 mL of distilled water and 0.1 mL of Triton X-100 (5 wt %/wt) followed by sonication for 15 min using a Branson 1510 water bath sonicator (Danbury, CT). To quantitate doxorubicin in the incubation medium and the cell lysate, 2.5 mL of acidified isopropanol (10 wt %/wt) was added to 0.5 mL of the sample.[32] Cell associated and nonassociated doxorubicin were measured fluorometrically (excitation, 470 nm; emission, 592 nm) using an SLM AMINCO 8000 spectrofluorometer (SLM Instruments, Inc.), and the intensities were compared to medium for cell incubation and to cell lysates (of identical cell density) that had not been incubated with any of the lipid structures with doxorubicin or with free doxorubicin to correct for potential scattering effects. Measured intensities due to light scattering by cells accounted for approximately 10% of

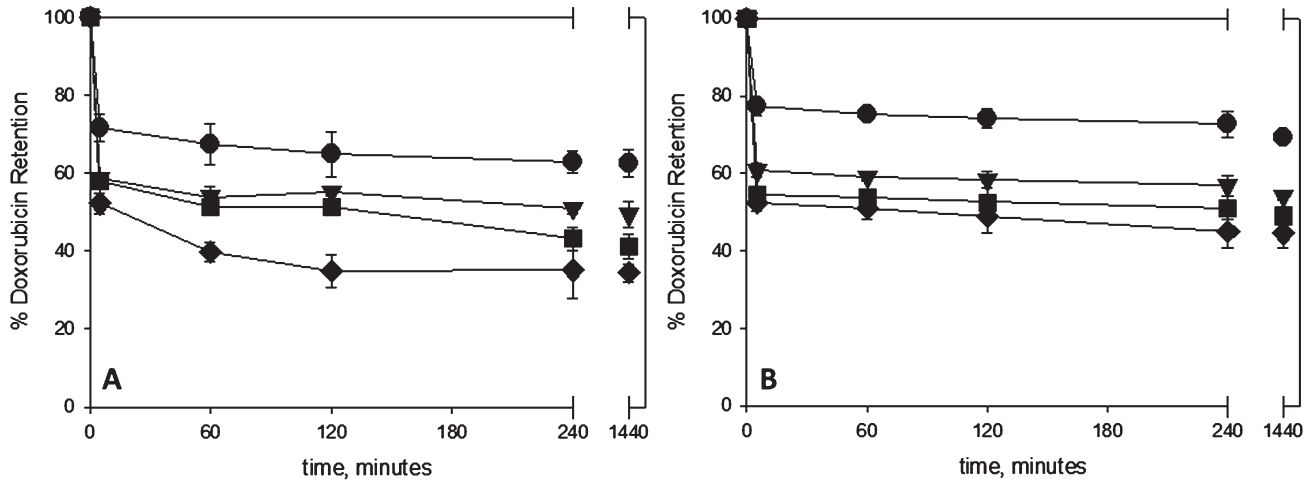

Figure 1. Retention of doxorubicin by pH-triggered lipid vesicles as a function of pH: (A) targeted vesicles; (B) nontargeted vesicles. (●) pH 7.4, (▼) pH 5.5, (■) pH 5.0, (◆) pH 4.0. Lines are guides to the eye. The error bars correspond to standard deviations of repeated measurements (two vesicle preparations, two samples per preparation).

the measured intensities. All reported values are corrected by subtraction of these background values.

2.10. Statistical Analysis. Results are reported as the arithmetic mean of n independent measurements ± the standard deviation. Student's t test was used to calculate significant differences in the behavior (content retention, binding reactivity, killing efficacy) between the pH-triggered and the DSPC/cholesterol-based vesicles. P values less than 0.05 are considered to be significant.

3. RESULTS

3.1. Vesicle Sizes. The size of PEGylated lipid vesicles was not affected by the presence of folate-conjugated lipids. Both lipid compositions exhibited comparable size distributions. The average diameter values of vesicle suspensions that were extruded through 100 nm pores were 121 ± 9 nm and 122 ± 11 nm for pH-triggered and for DSPC/cholesterol-based lipid vesicles, respectively (n = 14). The average diameter values of vesicle suspensions that were extruded through 400 nm pores were 285 ± 4 nm and 275 ± 5 nm for targeted and nontargeted pH-triggered lipid vesicles, respectively (n = 7).

3.2. Retention of Encapsulated Doxorubicin. The loading efficiencies of doxorubicin into preformed lipid vesicles was not affected by the presence of folate-functionalized lipids, but depended on the membrane composition. Lipid vesicles with pH-triggered membranes exhibited 46.8 ± 2.8% and 48.8 ± 2.0% loading yield in the presence and absence of folate-functionalized lipids, respectively (averaged over seven loading preparations). Using the identical loading protocol, loading efficiencies decreased to approximately 39.0 ± 11.9% and 37.4 ± 9.1% for DSPC/cholesterol-based lipid vesicles of the same extent of PEGylation in the presence and absence of folate-functionalized lipids, respectively.

Figures 1A and 1B show pH-dependent retention of encapsulated doxorubicin by targeted and nontargeted pH-triggered lipid vesicles, respectively. At pH 7.4, corresponding to the physiological pH during circulation in the bloodstream, similar extents (P > 0.05) of 63% and 69% of encapsulated doxorubicin are stably retained by targeted and nontargeted vesicles, respectively, after a 24 h exposure to serum supplemented medium at 37 °C. This is less than but comparable to doxorubicin retention by DSPC/cholesterol-based lipid vesicles that exhibit values of approximately 80% at the same conditions (P > 0.05), Figures 2A and 2B).

Within minutes after lowering pH to 5.5, approximately 18% and 21% of the encapsulated doxorubicin is rapidly released from pH-triggered targeted and nontargeted vesicles, respectively (Figures 1A and 1B, triangles). The observed increase in membrane permeability has been previously studied, and been attributed to the increased formation of lipid heterogeneities within the liposomal membrane[12] (see Figure S1 in Supporting Information). As expected, DSPC/cholesterol-based vesicles exhibit stable retention of doxorubicin that is independent of pH. The presence of folate does not affect the extent of retention of doxorubicin from either composition (all P < 0.05 for pH-triggered and DSPC/cholesterol-based vesicles at all time points for pH values ≤5.5).

3.3. Binding and Internalization Profiles. Figures 3A and 3B show that folate-receptor targeted vesicles with similar sizes (approximately 120 nm in diameter), with identical extent of PEGylation and folate grafting densities, exhibit comparable binding and internalization behaviors independent of the underlying membrane compositions (all P > 0.05 during the first 4 h of incubation; only at the 24 h time point significant difference in cell association, P < 0.05, is observed for the two FR-targeting vesicle compositions). Parallel cell binding studies evaluated by flow cytometry confirm the role of FR-targeting in increasing selective cell association of lipid vesicles (see Figure S2 in Supporting Information). Targeted pH-triggered vesicles exhibit 5.3 ± 0.4% and 3.2 ± 0.2% binding and internalization extents after 4 h of incubation, respectively (Figure 3A). At the same time point, targeted DSPC/cholesterol-based vesicles (Figure 3B) exhibit 6.4 ± 0.6% and 4.9 ± 0.6% binding and internalization extents, respectively. In both cases, the extent of bound nontargeted vesicles was less than 1% of the total available lipid vesicles for incubation times up to 4 h. Conversely, however, pH-triggered lipid vesicles of a larger average size (285 ± 4 nm and 274 ± 5 nm, in diameter, for targeted and nontargeted vesicles, respectively) exhibit significantly lower binding to KB cells (3.5 ± 0.4% of total) accompanied by internalization of only 2.2 ± 0.3% of total targeted vesicles after 4 h of incubation (Figure 3C). After 24 h of incubation all folate-targeted vesicles

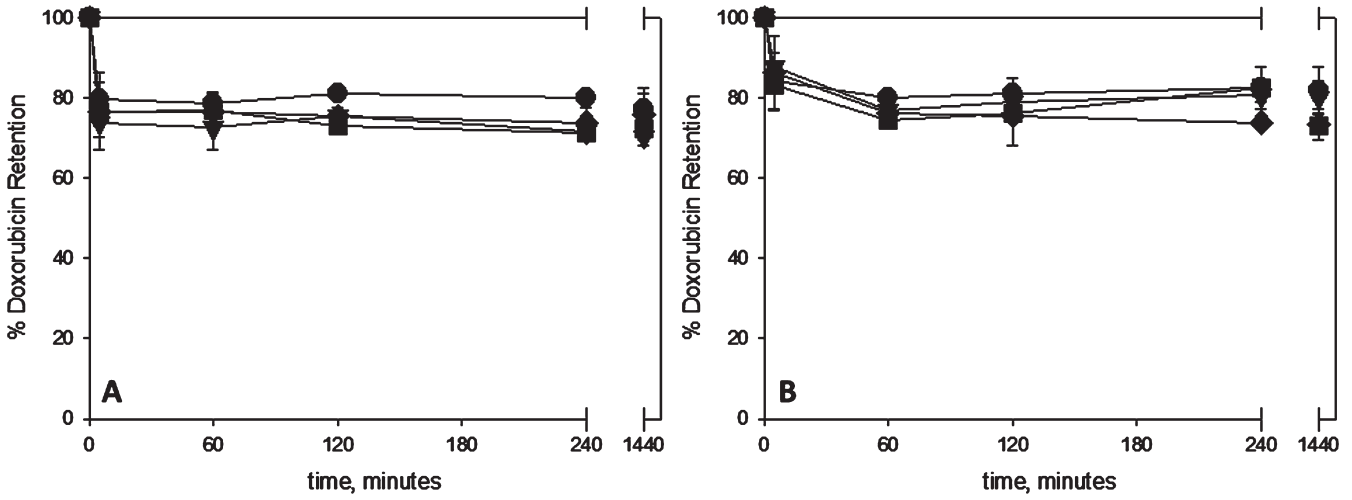

Figure 2. Retention of doxorubicin by DSPC/cholesterol-based lipid vesicles as a function of pH: (A) targeted vesicles; (B) nontargeted vesicles. (●) pH 7.4, (▼) pH 5.5, (■) pH 5.0, (◆) pH 4.0. Lines are guides to the eye. The error bars correspond to standard deviations of repeated measurements (two vesicle preparations, two samples per preparation).

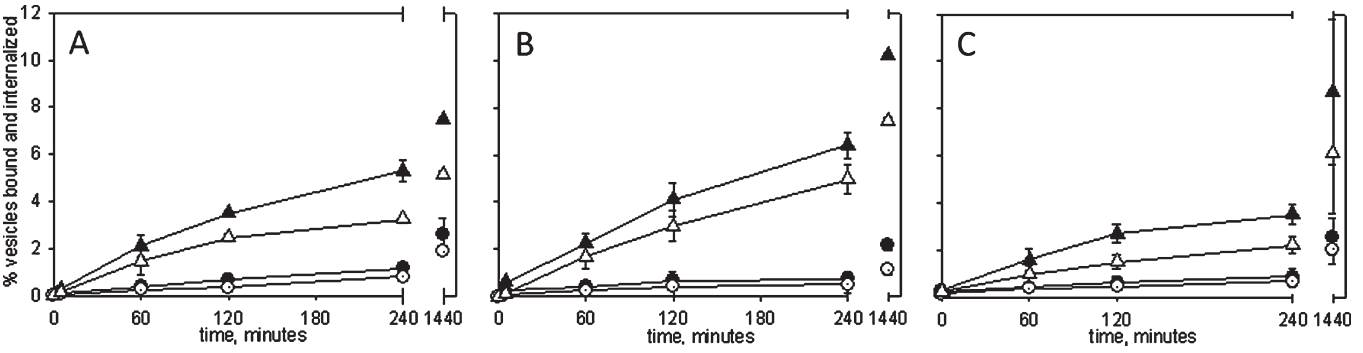

Figure 3. Extent of binding (filled symbols) and internalization (open symbols) of targeted and nontargeted vesicles to KB cancer cells: (A) pH-triggered vesicles (approximately 120 nm in diameter); (B) DSPC/cholesterol-based vesicles (approximately 120 nm in diameter); (C) pH-triggered vesicles of larger size (approximately 280 nm in diameter). (▲) cell bound FR-targeting vesicles, (Δ) cell internalized FR-targeting vesicles, (●) cell bound nontargeted vesicles, (○) cell internalized nontargeted vesicles. Lines are guides to the eye. The error bars correspond to standard deviations of repeated measurements (two vesicle preparations, two preparations of cell suspensions, two samples per preparation).

exhibit approximately comparably high association with cancer cells (ranging from 7 to 10% of total lipid).

3.4. Killing Efficacy of Folate-Receptor Targeted Liposomal Doxorubicin. The killing efficacy *in vitro* of liposomal doxorubicin targeted to the folate-receptor of KB cancer cells is greater when delivered by pH-triggered vesicles than by DSPC/cholesterol-based vesicles independent of the incubation time. Figure 4A shows that after 6 h of incubation with KB cells the targeted pH-triggered vesicles (IC_{50} = 0.032 mM, filled triangles) are 6-fold more potent ($P < 0.05$) than the targeted DSPC/cholesterol-based vesicles (IC_{50} = 0.194 mM, filled squares). After 12 h of incubation (Figure 4B), the targeted pH-triggered vesicles (IC_{50} = 0.009 mM, filled triangles) are still more potent ($P < 0.05$) than the DSPC/cholesterol-based vesicles (IC_{50} = 0.022 mM, filled squares) but at a lower extent (2.5-fold). At both incubation times studied, free doxorubicin exhibited the lowest IC_{50} values (0.004 and 0.001 mM at 6 and 12 h, respectively). The FR-modification significantly enhanced the killing efficacy of both types of vesicles (for all comparisons, $P < 0.05$). For pH-triggered vesicles, targeting improved the killing efficacy by 2.75-fold (IC_{50} = 0.088 mM) and 3.6-fold (IC_{50} = 0.033 mM) after 6 and 12 h of incubation, respectively. For DSPC/cholesterol-based vesicles, targeting improved the killing efficacy by 1.65-fold (IC_{50} = 0.321 mM) and 3-fold (IC_{50} = 0.067 mM) after 6 and 12 h of incubation, respectively. Figures 4A and 4B show that folate-receptor targeted vesicles not containing doxorubicin are not toxic to cancer cells (open symbols).

Table 1 and Table 2 show the extent of cell associated doxorubicin, for 6 and 12 h of incubation, respectively, that was evaluated in parallel to the cell viability studies in an attempt to compare more accurately the killing efficacies of pH-triggered vesicles and DSPC/cholesterol-based vesicles. Cell-associated doxorubicin increases for all types of vesicles and for free doxorubicin with increase in the doxorubicin concentration per well and with the length of the incubation time, as expected. Folate-receptor targeted vesicles with encapsulated doxorubicin of both compositions exhibit higher doxorubicin uptake (columns A and C) than the corresponding nontargeted vesicles (columns B and D). Both forms of pH-triggered vesicles show higher uptake of doxorubicin than DSPC/cholesterol-based

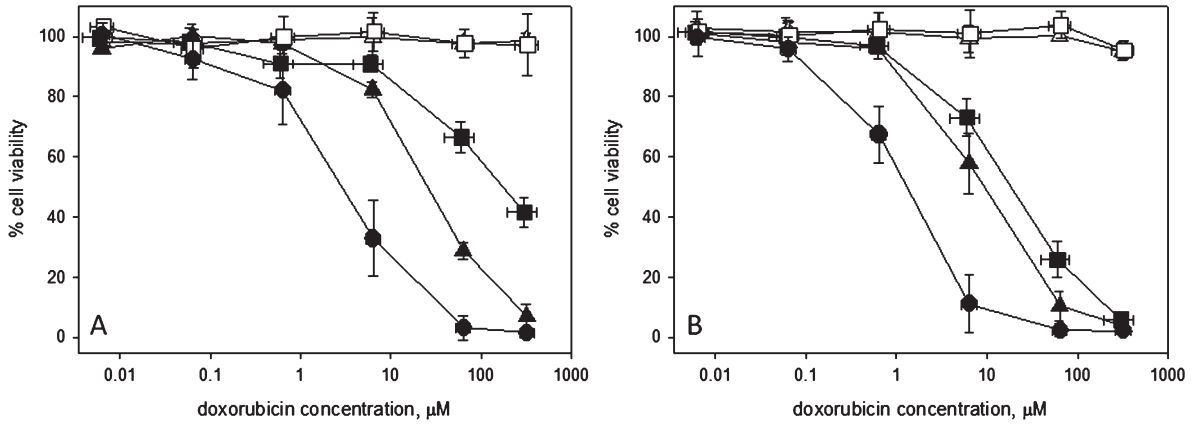

Figure 4. Viability of KB cancer cells overexpressing folate receptors after treatment with different modalities following (A) 6 h and (B) 12 h of incubation. (▲) Liposomal doxorubicin encapsulated in FR-targeting pH-triggered vesicles; (■) liposomal doxorubicin encapsulated in FRtargeting DSPC/cholesterol-based vesicles; (●) free doxorubicin; (Δ) FR-targeting pH-triggered vesicles not containing doxorubicin; (□) FR-targeting DSPC/cholesterol-based vesicles not containing doxorubicin. Lines are guides to the eye. The error bars correspond to standard deviations of repeated measurements (three vesicle preparations, three samples per preparation).

Table 1. Doxorubicin Uptake per 50,000 KB Cancer Cells after 6 h of Incubation (Two Vesicle Preparations, Two Samples per Preparation)

	amt of cell-associated doxorubicin (ng) per 50,000 cancer cells				
[doxorubicin]/well, μM	A. folate receptor targeted pH triggerable vesicles	B. nontargeted pH triggerable vesicles	C. folate receptor targeted DSPC based vesicles	D. nontargeted DSPC based vesicles	E. free doxorubicin
616.3	1665.30 ± 190.50	1078.70 ± 40.60	980.00 ± 28.80	920.00 ± 34.40	3724.00 ± 284.40
309.7	290.90 ± 28.20	224.00 ± 12.60	225.60 ± 33.10	164.70 ± 47.60	696.70 ± 81.30
61.6	162.10 ± 33.20	113.10 ± 36.50	52.40 ± 17.10	37.30 ± 12.50	332.60 ± 102.00
6.2	7.00 ± 1.70	4.9 0 ± 3.50	5.40 ± 0.15	4.30 ± 0.17	24.90 ± 6.30
0.62	0.57 ± 0.23	0.40 ± 0.10	0.70 ± 0.04	0.51 ± 0.07	1.50 ± 0.50
0.06	0.36 ± 0.01	0.30 ± 0.81	0.07 ± 0.01	0.02 ± 0.007	0.31 ± 0.00
0.01	0.07 ± 0.05	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.70	0.11 ± 0.03

Table 2. Doxorubicin Uptake per 50,000 KB Cancer Cells after 12 h of Incubation (Two Vesicle Preparations, Two Samples per Preparation)

	amt of cell-associated doxorubicin (ng) per 50,000 cancer cells				
[doxorubicin]/well, μM	A. folate receptor targeted pH triggerable vesicles	B. nontargeted pH triggerable vesicles	C. folate receptor targeted DSPC based vesicles	D. nontargeted DSPC based vesicles	E. free doxorubicin
616.30	2498.00 ± 478.90	1512.00 ± 26.70	2262.00 ± 91.30	1340.00 ± 169.60	11,971.00 ± 81.00
309.70	1128.80 ± 42.60	382.20 ± 137.10	844.80 ± 36.20	216.90 ± 53.20	1488.00 ± 476.50
61.60	568.50 ± 38.10	337.00 ± 25.70	358.80 ± 90.60	102.90 ± 28.50	1020.10 ± 178.50
6.20	27.30 ± 2.40	13.00 ± 3.40	25.20 ± 1.90	13.90 ± 1.70	83.60 ± 10.10
0.62	4.21 ± 0.80	2.60 ± 1.00	11.00 ± 0.50	0.92 ± 0.11	5.50 ± 0.51
0.06	1.28 ± 0.05	0.70 ± 0.26	0.26 ± 0.01	0.26 ± 0.01	1.20 ± 0.04
0.01	0.16 ± 0.05	0.11 ± 0.00	0.10 ± 0.01	0.10 ± 0.03	0.34 ± 0.03

vesicles. This is probably due to the greater release of doxorubicin from pH-triggered vesicles (as suggested by Figures 1 and 2) into the cell surrounding medium which then passively diffuses into cells. For both incubation times and all concentrations used, doxorubicin in its free form exhibits the greatest cell uptake compared to all other modalities possibly due to its high diffusivity across plasma membranes at physiological pH.[33,34]

4. DISCUSSION

The next generation of drug delivery carriers for cancer therapy is designed to exhibit active targeted selectivity combined with optimized intracellular trafficking of the delivered therapeutic agent.[35,36] The folate receptor (FR) is a promising cellular target, and several small folate−drug conjugates are already in the process of clinical evaluation.[37] Nanometer sized carriers

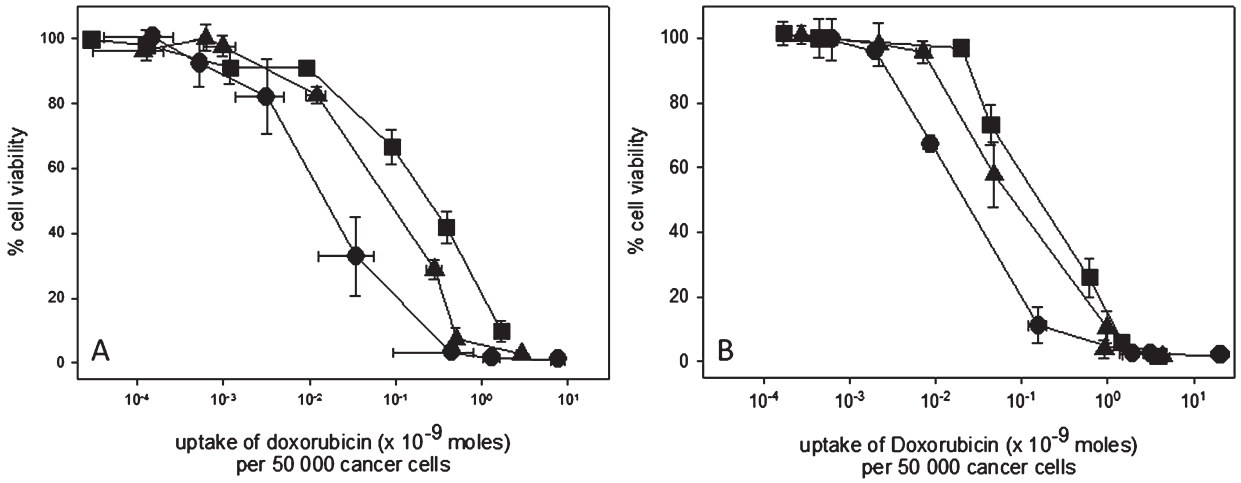

Figure 5. Correlation between the toxicity on KB cancer cells and the cell-associated doxorubicin per cancer cell following (A) 6 h and (B) 12 h of incubation. (▲) Liposomal doxorubicin encapsulated in FR-targeting pH-triggered vesicles; (■) liposomal doxorubicin encapsulated in FR-targeting DSPC/cholesterol-based vesicles; (●) free doxorubicin. Lines are guides to the eye. The error bars correspond to standard deviations of repeated measurements (two vesicle preparations, two samples per preparation).

targeted to the folate receptor and, in particular liposomes, are also being evaluated in preclinical studies.[25−27,38]

For FR-targeting liposomes, to improve the intracellular trafficking of therapeutics, several pH-activated mechanisms have been explored with the aim to induce fast release of the drug from the carrier during caveolae-mediated endocytosis.[23] Release mechanisms are required to become activated at pH values not as acidic as in the case of endocytosis mediated by clathrin-coated pits.[23] For the case of liposomes, these strategies include the coencapsulation into liposomes of pH-sensitive peptides that become fusogenic with lowering pH values.[39] Alternatively, to accelerate drug release from liposomes in acidic pH, hydrolysis-susceptible lipids are included in the liposome membrane. These lipids are transformed into lysolipids with lowering pH locally altering, therefore, the bilayer membrane curvature[40] or are hydrolyzed into lipid structures (such as DOPE) forming inverted hexagonal phases that would also locally introduce areas of different local bending propensities, affecting, therefore, the membrane structure and permeability.[41]

Here, we study a pH-sensitive approach that involves only lamellar-forming lipids. This approach does not require the chemical synthesis of a new component (all constituents are readily available from commercial sources), and does not require the liposomal coencapsulation of pH-sensitive membrane-active agents such as polymers or peptides that could potentially complicate the preparation method. In addition, the pH-triggered lipid vesicles studied were previously demonstrated to exhibit blood circulation times similar to the FDA approved liposomal composition of Doxil (a DSPC/cholesterol-based PEGylated lipid membrane).[11]

Our findings show fast and significant release of doxorubicin from pH-triggered liposomes at pH 5.5 that corresponds to the early phase of acidification of the caveolae pathway.[24] Doxorubicin is a weak base and exhibits decreasing permeability across cellular membranes (passive diffusion) with decreasing pH.[33,34] Therefore, at the pH value of 5.5 the permeability of doxorubicin across cell membranes is certainly reduced. In this study, enhancer molecules to improve doxorubicin's permeability across the endosomal membrane were not included, but recent studies report the particularly promising potential of endosomolytic enhancers such as polyHis[42] to overcome this particular obstacle in intracellular trafficking.

In this study, we confirm that the FR-modification on liposomes significantly enhances their killing efficacy independent of the type of vesicle studied. Our studies *in vitro* confirm that selective targeting of the folate receptor enhances the amount of internalized liposomes and, therefore, of the intracellular localization of their therapeutic contents. In addition, we demonstrate that targeted liposomes with pH-triggered membrane permeability to encapsulated doxorubicin result in more effective killing of FR-expressing KB cancer cells compared to a targeted non-pH-sensitive liposomal doxorubicin composition similar to the FDA approved Doxil (conventional composition). Comparison of the difference in killing efficacy between the targeted and nontargeted forms of each lipid vesicle composition (to eliminate nonspecific cell killing effects due to leaked doxorubicin from vesicles during their incubation with cells) quantitatively demonstrates the greater efficacy of carriers with pH-triggered membrane permeability (see Figure S3 in the Supporting Information). The improved killing efficacy of pH-triggered vesicles may be potentially attributed to the faster intracellular trafficking of doxorubicin and, in particular, to its faster release from internalized liposomes with pH-sensitive permeability. Although doxorubicin's intracellular trafficking was not directly measured in these studies, the correlation between cell viabilities from Figures 4A and 4B and the extent of cell-associated doxorubicin shown in Tables 1 and 2 support this suggestion. In particular, Figure 5A (after 6 h of incubation) shows that, for any measured amount of cellular doxorubicin uptake ranging from 10^{-2} to 10 nmol per 50,000 cells, the pH-triggered liposomes (triangles) exhibit a killing effect improved by at least ten percentile units compared to liposomal doxorubicin delivered by the conventional DSPC/cholesterol-based lipid vesicles (squares). At the longer incubation time of 12 h (Figure 5B), improvement in killing efficacy by pH-triggered vesicles is also observed but at a lower extent. In other words, the bioexposure of cells to the therapeutic agent is probably higher when pH-triggered liposomes are employed which potentially release their

therapeutic cargo intracellularly faster than the conventional liposomes.

The effect of liposome size on the extent of their cell association through the folate receptor was briefly evaluated in these studies for the pH-triggered lipid vesicles. The cell associated fraction of FR-targeting vesicles was decreased by almost 50% for a liposome size increase from 120 to 280 nm in diameter after relatively short incubation times (up to 4 h *in vitro*). Since folate receptors reportedly occur in clusters on the cell membranes,[43] larger vesicles may be sterically hindering the receptors in the same cluster from binding more vesicles, therefore, decreasing the availability of receptors for binding. This suggested mechanism may possibly explain the observed decrease in the extent of larger bound vesicles. The fraction of internalized vesicles is approximately 60% of the cell associated vesicles for both small and large vesicles. Our findings are not in disagreement with the reported 50−66 nm size limit of endocytosed nanoparticles by caveolae,[18,43] because liposome suspensions are characterized by a distribution of sizes around the measured mean value. It is possible that lipid vesicles with sizes below the above "cutoff" values are actually internalized by cells.

At longer incubation times (24 h *in vitro*) no statistically significant effect of the size of lipid vesicles was observed on the extent of their specific cell association. It is suggested, therefore, that the relatively larger liposomes may have the potential to be used intraperitoneally for peritoneally disseminated disease—such as ovarian cancer overexpressing folate receptors[44]—since residence times of nanocarriers in the peritoneal cavity have been shown to increase with the carrier size.[45]

All *in vitro* studies demonstrate the greater killing efficacy of free doxorubicin compared to all liposomal constructs on the basis of the same doxorubicin concentration in the extracellular space. Free doxorubicin *in vivo*, however, does not result in greater control of tumor growth or enhancement of survival rates.[46] This is partly because doxorubicin exhibits fast clearance from the bloodstream resulting in low accumulation at vascularized tumors.[47] Nanometer sized carriers, on the contrary, have the advantage of the EPR effect[48] that results in their relatively enhanced accumulation and retention at the tumor site. Administration of greater doses of free doxorubicin to improve the uptake at the tumor sites may possibly be limited by side effects such as cardiomyopathy.[49]

Recently, reports on animal studies of FR-targeting liposomal doxorubicin based on the Doxil composition have demonstrated the strong therapeutic potential of the targeted approach relative to nontargeted Doxil and to free doxorubicin.[25,27] The findings of the present study suggest that pH-triggered liposomes targeted to the folate receptor could potentially further enhance the improved therapeutic outcomes of liposomal doxorubicin *in vivo*. Previous studies on these pH-triggered liposomes report that for a relatively wide range of extents of PEGylation (up to 13 mol %) the pH-responsive character of the bilayer is preserved.[11] However, extents of PEGylation lower than 1 mol % would possibly accelerate the clearance rates of these liposomes due to the exposed negative charge on the liposome surface.[3]

Lipid vesicles have several advantages that make them attractive carriers for cancer therapy. In addition to enhanced accumulation and retention at the tumor sites relative to healthy organs, to their ability to selectively target malignant cells and to their potential for reduced toxicities to healthy organs, lipid vesicles can be engineered to increase cell bioexposure by a pH-triggered mechanism to release encapsulated contents within malignant cells. This work shows that folate-receptor targeted vesicles that are designed to release the encapsulated doxorubicin in response to a decrease in local pH by forming domains with "leaky" interfaces have the potential to result in improved therapeutic effects compared to targeted conventional liposomal doxorubicin. These structures may ultimately be applied to improve the quality of life of patients with vascularized tumors and to possibly offer longer life expectancies.

■ ASSOCIATED CONTENT

Supporting Information. Information on the following studies: differential scanning calorimetry of pH-triggered lipid bilayers vs pH, flow cytometry to evaluate the selective cell association of FR-targeting liposomes, and calculation of the difference in killing efficacy for each lipid membrane composition. This material is available free of charge via the Internet at http://pubs.acs.org.

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