Supporting Information

pH-Triggered Echogenicity and Contents Release from Liposomes

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Table of Contents:

1. Experimental section:

1.1 Preparation of liposomes……………………………………………………………………S3

1.2 Preparation of doxorubicin loaded liposomes…………………………………………………S3

1.3 Size distribution analysis (Dynamic Light Scattering)……………………………….……..S4

1.4 Transmission electron microscopy………………………………………………………..S4

1.5 Atomic force microscopy……………………………………………………………....S5

1.6 Ultrasound imaging of liposomes…………………………………………………………S5

1.7 pH Triggered release…………………………………………………………………..…S6

1.8 Ultrasound enhanced pH triggered release………………………………………………..S6

1.9 Effect of temperature on ultrasound triggered release……………………………………….S7

1.10 Cell culture and liposomal uptake studies…………………………………………………S7

1.11 Cell viability studies…………………………………………………………………..S8

1.12 Migration assay………………………………………………………………….…S8

2. Results

2.1 Particle size distribution and Transmission electron microscopic images…………………S10

2.2 Fluorescence correction for carboxyfluorescein………………………………………....S10

2.3 pH triggered release profile of sodium bicarbonate POPC liposomes……………………..S12

2.4 pH triggered release from sodium bicarbonate POPC liposomes…………………………..S13

2.5 pH triggered release from ammonium bicarbonate DSPC liposomes………………………S13

2.6 Release from control POPC liposomes (without bicarbonate encapsulation) when incubated in different pH buffers for 2 hours……………………………………………………………S14

2.7 Liposomal uptake studies with MCF-7 cells………………………………………………...S15

1. Experimental Section:

1.1 Preparation of liposomes:

Liposomes were prepared with POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Avanti Polar Lipids) lipid solution using traditional lipid film formation, hydration and sonication method. The lipid solution (4 mL of 1 mg/mL) was placed in a rotary evaporator for approximately 10 minutes to allow for the lipid film to form, and then placed under a vacuum overnight to remove traces of chloroform. Subsequently, 4 mL of 100 mM carboxyfluorescein solution was added, along with ammonium bicarbonate (0.4 M) and allowed to hydrate for three hours. The lipid dispersion was then sonicated for ten minutes to form liposomes. The liposomes were then exposed to three freeze (-70 °C) and thaw (23 °C) cycles to ensure dye encapsulation inside the aqueous interior of the liposomes. After the freeze-thaw cycles, an extrusion apparatus (Avanti Polar Lipids) was used to extrude the liposomes through 800 nm, then 200 nm polycarbonate membrane filters. The liposomes were then placed in a Sephadex-G100 gel filtration column preconditioned with HEPES buffer (pH 7.4, osmolarity adjusted to liposomal levels) to separate unencapsulated contents from the liposomes. Liposome fractions were collected and used for subsequent studies.

For cellular uptake studies, the lipids used were POPC (99 mol%) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-5000] (ammonium salt; Avanti Polar

Lipids) (1 mol%). The same procedure as described in the previous paragraph was followed for preparing these liposomes.

1.2 Preparation of doxorubicin loaded liposomes:

Doxorubicin (Bridge Bioservices) was encapsulated by the traditional passive entrapment method. Briefly, the lipid film was hydrated with 400 mM ammonium bicarbonate and 0.2 mg of doxorubicin per mg of lipid for 3 hours. After bath sonication for 10 min and freeze thaw cycles liposomes were passed though Sephadex column to remove unencapsulated doxorubicin. Encapsulation efficiency was determined by recording the absorbance of doxorubicin at 475 nm before and after gel filtration. The doxorubicin content was determined by plotting the absorbance onto the calibration curve established at 475 nm.

1.3 Size distribution analysis (Dynamic light scattering):

Dynamic light scattering method (NanoZS 90 Zetasizer, Malvern Instrument) was used to study size distribution of the liposomes. For these experiments, 0.1 mg/mL of liposomal solution was taken in DTS 0012 polystyrene disposable sizing cuvette. The measurements were performed at a scattering angle of 90°. Samples were equilibrated for 60 seconds and ten readings were taken for a single sample at a constant temperature (25 °C). Each batch of liposomes was studied for size distribution and each experiment was repeated three times for consistency.

1.4 Transmission electron microscopy:

The samples were observed using a JEOL JEM 2000 transmission electron microscope operating 100 kV and at low magnifications with the beam spread, which is not converged, to reduce the amount of electron beam interaction per unit area and hence beam damage to sample if it were to occur. The liposomal samples were dispersed to 1 mg/mL and dropped onto a 300 mesh Formvar coated copper grids previously coated with 0.01% poly-L-lysine and allowed to stand for one minute before wicking off with filter paper. Then it was allowed air dry for two minutes and after that it was negatively stained with 1% phosphotoungstic acid for 90 seconds and subsequently wicked with filter paper and then allowed to dry before being beamed.

1.5 Atomic force microscopy:

The liposomal sample was placed onto a mica sheet and air dried. For performing AFM imaging, a MultimodeTM atomic force microscope with Nanoscope III controller and J type piezo scanner (Veeco Metrology Group, Santa Barbara, CA) was used. Antimony (n) doped Si tip was used for obtaining images in Tapping ModeTM under laboratory conditions. Images of liposomes were taken before and after incubating in pH 6 buffer for an hour to study the effect of bubbles generated on the morphology of the liposomes. Images were captured, processed and labeled properly.

1.6 Ultrasound imaging of liposomes:

A Terason t3200 diagnostic ultrasound (MediCorp LLC) instrument was utilized to image liposomal solution incubated in different pH buffers. A layer of Aquasonic 100 (Parker Laboratories) ultrasound gel was applied on 15L4 linear ultrasound transducer (4-15 megahertz MediCorp LLC). The gel was placed over parafilm covering 96 well plates containing 200 µL of

liposomal solution in each well. The ultrasound scan properties were fixed at 0.7 mechanical index (MI) and 0.6 thermal index,(omni Mean activated, level C image map, level C persistence, high frequency, level three TeraVision, level 51 2D gain, level 60 dynamic range, 3 cm scan depth, and 22 Hz frame rate). The images were taken for liposomes (0.05 mg/mL) incubated in different pH buffers [7.4 (control), 7, 6, 5] at different time intervals and saved. Images were further analyzed using ImageJ software (version 1.45s, NIH, USA) to calculate mean and maximum grey scale values for each pH for a specific concentration of liposomes.

1.7 pH Triggered Release:

The release studies were carried out on a spectrophotometer (Spectramax M5, Molecular Devices) by exciting at 460 nm and monitoring the emission at 497 nm using a 96 well plate. In each well, 20 μL of the liposomal solution (0.02 mg/mL) was incubated with phosphate buffer saline solutions with pH adjusted to 7.4 (control), 6.0 and 5.0. The release was monitored for two hours and reading was taken at 30 second intervals. Each sample was taken in triplicates and each study was repeated three times in order to check the repeatability of the results. Release was calculated using formula:

$$\text{Release (\%)} = \frac{\text{Observed intensity - Initial intensity}}{\text{Final intensity - Initial intensity}} \times 100$$

1.8 Ultrasound enhanced pH triggered release:

For the release experiments, Sonitron 1000® (Richmar) ultrasound instrument was employed. Carboxyfluorescein encapsulated liposomes (0.02 mg/mL) were incubated in 48 well plate with

HEPES 25 mM buffers with different pH 7.4 (control), 6, and 5. Ultrasound probe tip was immersed into the solution and ultrasound was applied at different time points after incubation for different time intervals to check the release. Ultrasound parameters were optimized and employed to enhance contents release from liposomes (frequency 1 MHz, 100% Duty cycles, 2 W/cm^2, 5 minutes of application time). Release was monitored on Spectramax (Molecular devices) spectrofluorimeter (λ_{ex} = 460 nm; λ_{em} = 497 nm) after ultrasound application for of 2 hours. Percent release was calculated using formula mentioned in the previous section.

1.9 Effect of temperature on ultrasound triggered release:

Application of ultrasound leads to cavitation and thermal effects, and are often responsible for release from liposomes. To determine individual contribution of these two phenomena in release of contents, we carried out two different experiments. In one set up, we conducted release studies at room temperature (25 °C) and temperature of solution was noted before and after ultrasound application. In another experiment, we kept the plate on ice bath to decrease the temperature (below 10 °C) and noted the temperature before and after application of ultrasound. Although set up used to carry out this study allows reflection of ultrasound waves from air –water interface which gives rise to standing wave pattern, we see that the set-up is adequate for the present study for demonstration of proof of concept. Also, as mentioned in our previous publications, we noticed negligible (less than 1%) energy transfer to adjacent wells during stimulation indicating almost no interwell interferences[32, 33]. All experiments were performed thrice and in triplicates each time to ensure reproducibility of results and calculate standard deviations.

1.10 Cell culture and liposomal uptake studies:

For liposomal uptake studies, HeLa (cervical cancer) and PANC-1 (Pancreatic epithelioid carcinoma) was cultured in clear (without added Phenol red) RPMI media supplemented with 10 % fetal bovine serum and 1% antibiotics. Culture flasks were incubated at 37 °C in humidified atmosphere containing 5% CO_2. When 90% confluent; the cells were suspended using trypsin-versene reagent. The suspended cells were then cultured onto sterile 6 well culture plate until 90% confluent.

Once confluent, the media was removed and cells were gently washed with HBSS (HyClone®, Thermo Scientific, UT) 5-6 times to completely remove any media left. Subsequently, liposomes were suspended in HBBS (0.2 mg/mL) and were incubated with the cells for 30 minutes. HOESCHT 33342 dye (Enzo Life Sciences) in 1 in 1000 dilution was added to stain the nuclei of the cells. After specific time intervals, the liposomal solution was removed from wells and the cells were again washed with HBSS to remove any liposomes on the surface of cells. Cells were then observed under fluorescence microscope at different time points for liposomal uptake. All images were obtained with Olympus IX81® motorized inverted microscope, viewed using 20X and 40X objectives and captured using CellSens Standard software (version 1.6). A similar procedure was followed for doxorubicin-encapsulated liposomal uptake by HeLa cells.

1.11 Cell Viability Studies:

For cell viability studies, $5x10^5$ seeded cells onto the 400 nm pore sized transwell inserts (Thincert-6 well, Greiner bio-one) and bottom of 6 well plates. Once confluent cells in the upper compartment (Figure 11A-8) were exposed to different combinations treatments involving doxorubicin liposomes (targeted and non-targeted), bicarbonate liposomes without doxorubicin,

free doxorubicin and ultrasound. Cells were kept in incubator for 6 hours and then live-dead staining (Enzo life sciences) was performed. Cells in the upper and bottom compartment were incubated with live-dead stain for 15 minutes, washed thrice with HBSS and then observed under fluorescence microscope using filters (FITC for live cells and rhodamine for dead cells). Images were taken and merged using ImageJ software. Percent killing was calculated by measuring green (live) and red (dead) fluorescence using ImageJ software (http://rsbweb.nig.gov).

1.12 Migration Assay:

For migration assay $5x10^5$ cells/well in serum free media were seeded on the top of 8 µm pore sized cell culture inserts, with serum containing media in the lower chamber of 6 well plates. To determine %relative migration, cells were seeded onto membrane without any treatment. Once cells get attached (around 6-8 hrs), cells were exposed to ultrasound treatment and incubated overnight. After overnight incubation at 37°C, media was removed and cells were washed with HBSS thrice. Non-migrated cells on the filter were removed with sterile cotton swab and migrated cells were quantified by 10% of the total volume of Alamar blue. The assay measures the fluorescence of resorufin (red) formed by reduction of resazurin (blue) in the cytosol of viable cells (metabolically active). Percent relative migration was calculated using following formula:

% Relative migration = Fluorescence intensity of treated sample*100/Fluorescence intensity of control sample.

Results:

2.1 Particle size distribution and Transmission electron microscopic images:

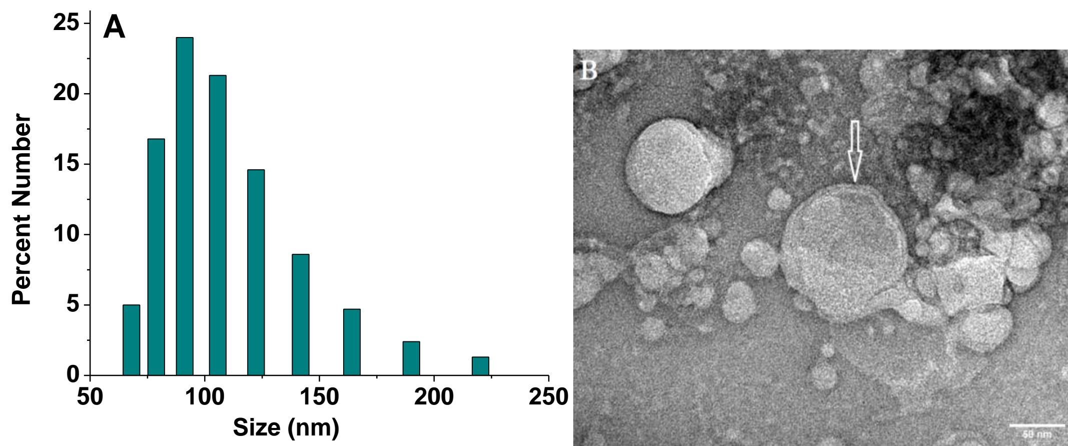

Figure S1. (**A**) Representative particle size distribution (by number) of the liposomes using dynamic light scattering instrument. (**B**) Transmission electron microscopic image (JEOL JEM 2100 LaB_6 200 kV) of pH tunable echogenic POPC liposomes using negative staining by 1% phosphotungstic acid. The beam is spread and not converged, to reduce the amount of electron beam interaction per unit area and to minimize beam damage to liposomal sample. The white arrow (Panel **B**) indicates the unilamellar bilayer structure of liposome.

Fluorescence correction for carboxyfluorescein:

Fluorescence emission intensity of carboxyfluorescein decreases with decrease in pH. To compensate for this decrease, we obtained a calibration curve for carboxyfluorescein emission intensity by exciting at the isosbestic absorption (460 nm) and monitoring emission at the isosbestic point at 497 nm.

$I_tpH = rpH \cdot I_tmpH$ …….. Equation (1)

Where I_tpH is the corrected fluorescence intensity, rpH is the correction factor for the effect of pH and I_t,mpH is the measured fluorescence intensity at a particular pH and time (t) of interest.

To calculate correction factor for the effect of pH (rpH), calibration curves were generated for the fluorescence intensity of free carboxyfluorescein at pH 7.4, 6 and 5. Concentrations of carboxyfluorescein were taken from desired range for all the release experiments (0.5 µM to 5 µM). Correction factor (rpH) = Slope at pH 7.4 / Slope at particular pH

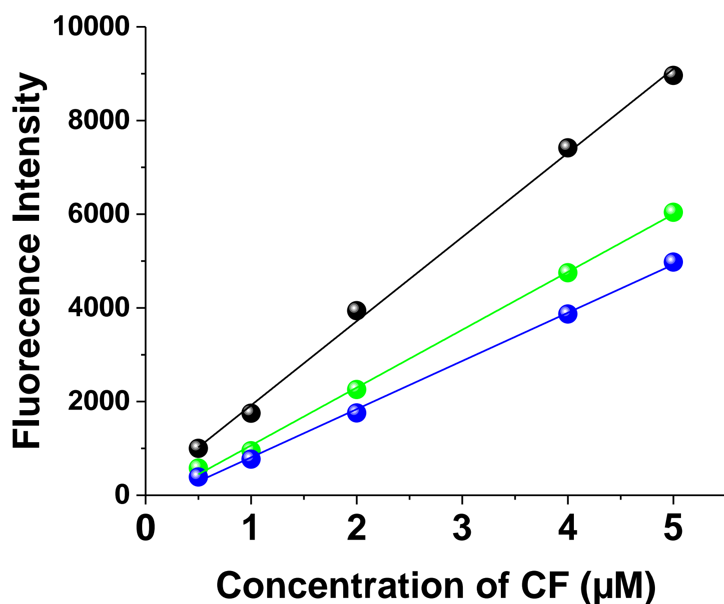

Figure S2. Calibration graphs for intensity of carboxyfluorescein (CF) as a function of pH. Black: pH 7.4; blue: pH 6; green: pH 5.

Corrections factors were found be 1.45 and 1.74 for pH 6, and 5 respectively. Corrected fluorescence emission intensity at particular pH was calculated each time by using equation 1.

2.2 pH triggered release profile of sodium bicarbonate encapsulating POPC liposomes:

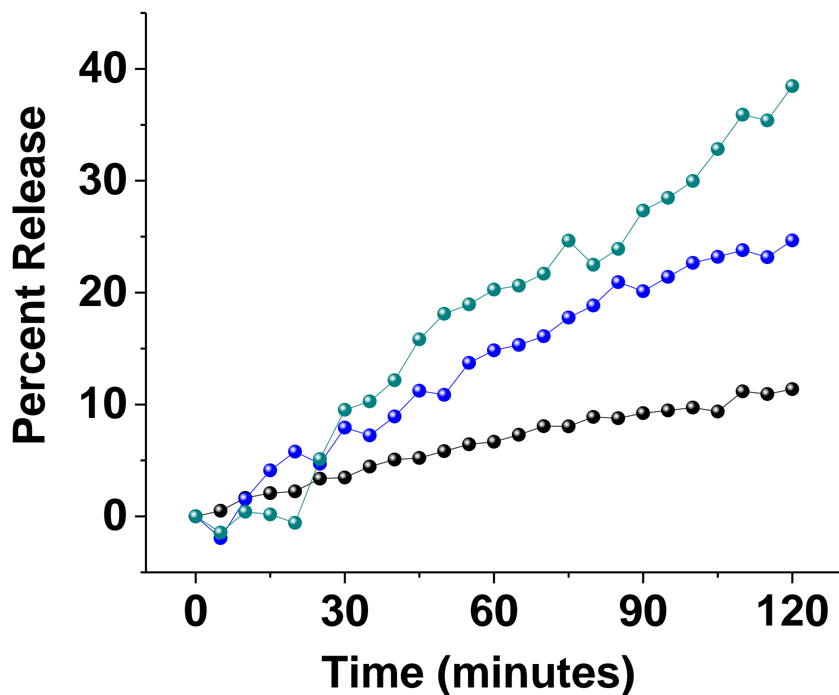

Figure S3. Release profiles of carboxyfluorescein from 400 mM sodium bicarbonate encapsulated liposomes, incubated in HEPES buffer pH 7.4 (black spheres), pH 6 (blue spheres), and pH 5 (dark cyan spheres). The lines are generated by connecting the observed data points.

2.3 pH triggered release from sodium bicarbonate encapsulating POPC liposomes:

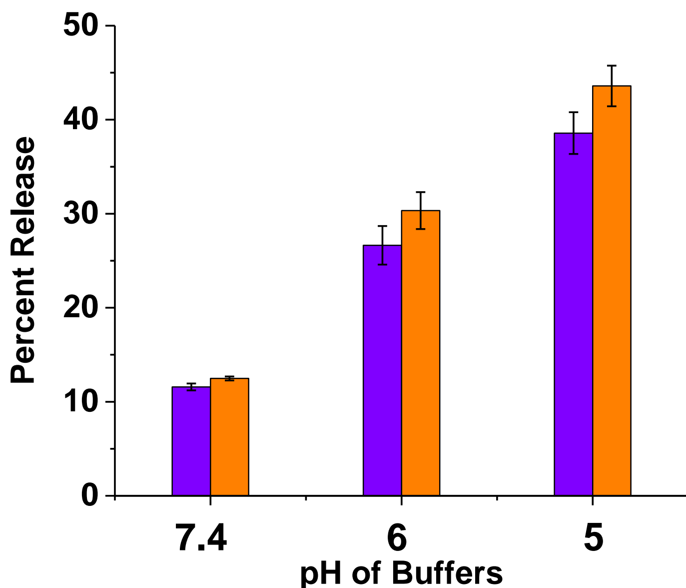

Figure S4. Release from pH tunable echogenic liposomes encapsulating 400 mM sodium bicarbonate after incubation in 25 mM HEPES buffer for 2 hours (violet) and 3 hours (orange) (n = 3).

2.4 pH triggered release from ammonium bicarbonate encapsulating DSPC liposomes:

Table 1. Percent release of carboxyfluorescein from 400 mM ammonium bicarbonate and 400 mM sodium bicarbonate encapsulated DSPC liposomes after incubation for 2 hours (Avg ± SD; n = 3)

Type of Precursor (400 mM)	**pH 7.4**	**pH 6**	**pH 5**
Ammonium bicarbonate	< 1	< 5	5 ± 1
Sodium bicarbonate	< 1	< 5	< 5

2.5 Release from control POPC liposomes (without bicarbonate encapsulation) when incubated in different pH buffers for 2 hours:

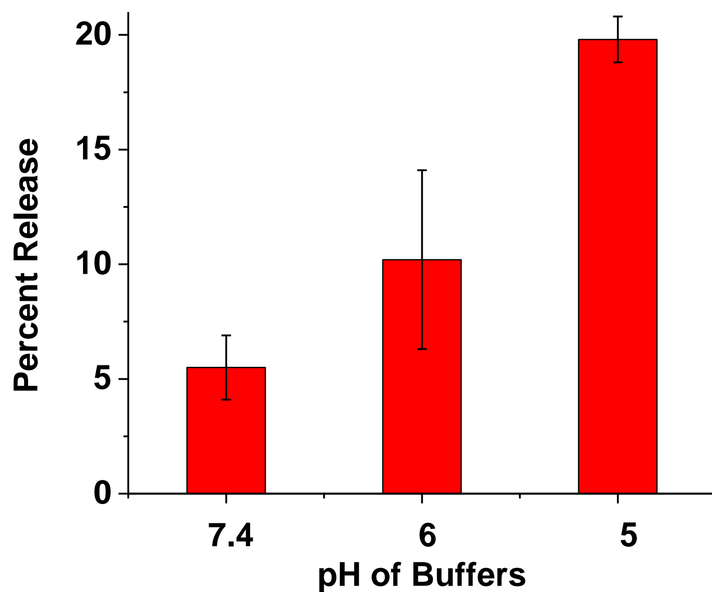

Figure S4: Release of carboxyfluorescein from POPC liposomes (without bicarbonate encapsulation) when incubated in different pH buffers for 2 hours (n = 3).

2.6 Uptake studies with MCF-7 cells and POPC liposomes:

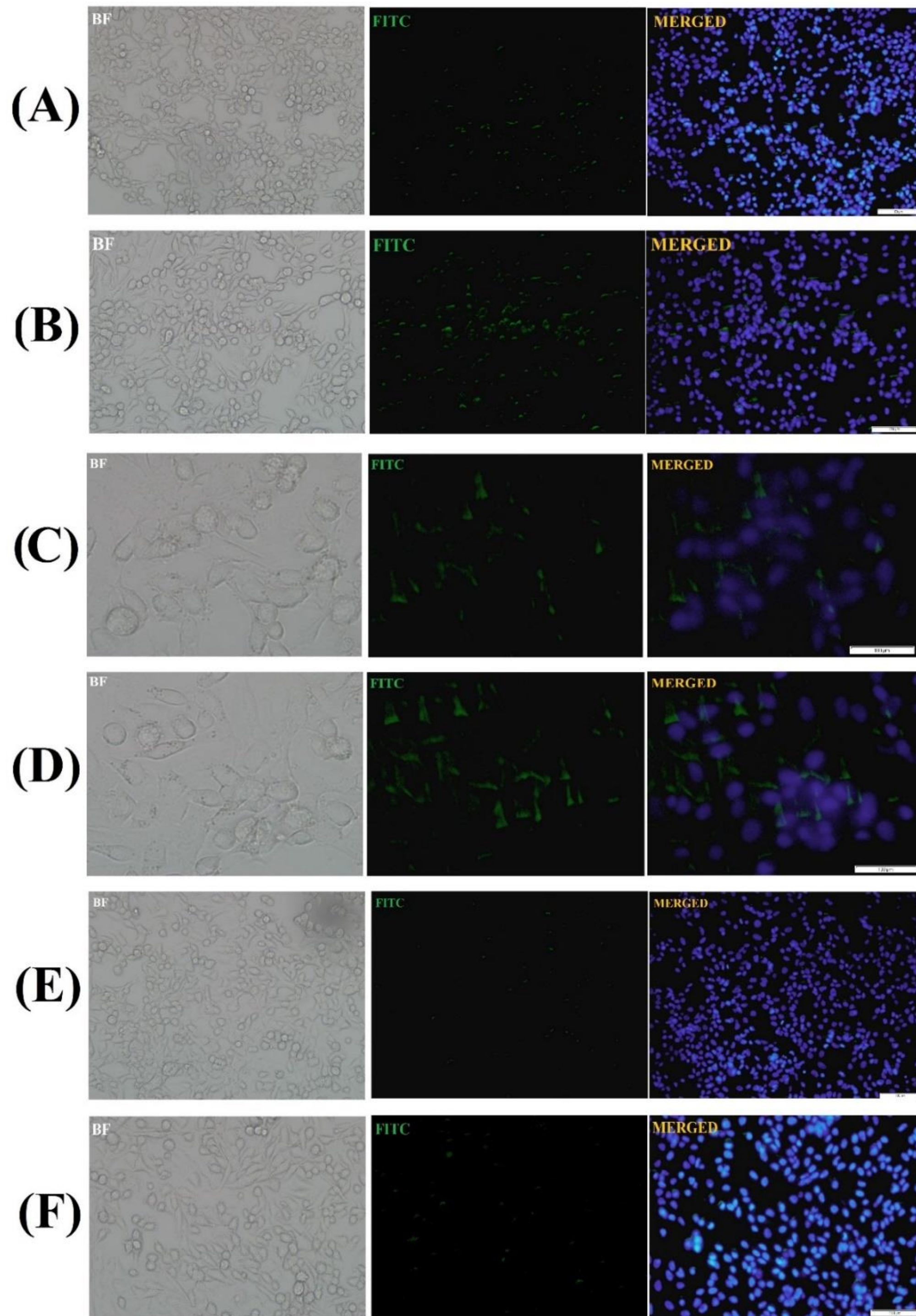

Figure S5. Fluorescence microscopic images for the uptake of pH-tunabl,e echogenic liposomes encapsulating carboxyfluorescein by folate receptor overexpressing MCF-7 cancer cells as a

function of incubation time. Images taken with different filters: bright (BF), FITC and DAPI. DAPI and FITC images were merged using ImageJ software. (**A**) Incubation time: 10 minutes (magnification: 20X); (**B**) Incubation time: 20 minutes (magnification: 20X); (**C**) Incubation time: 10 minutes (magnification: 40X); (**D**) Incubation time: 20 minutes (magnification: 40X); (**E**) Non-targeted pH tunable echogenic liposomes; Incubation time: 10 minutes (magnification: 20X); (**F**) Non-targeted pH tunable echogenic liposomes; Incubation time: 20 minutes (magnification: 20X).