Development of ROS-Triggered Masked Liposomes for Activated Cellular Delivery Using a Charge Balance Strategy

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General Experimental

Reagents and solvents were generally purchased from Sigma-Aldrich or Fisher Scientific and used without further purification. PC (L-α-phosphatidylcholine, mixed isomers from chicken eggs), 18:1 Liss Rhod PE (Rd-PE), 18:1 biotinyl cap PE (biotin-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (sodium salt)), Brain PS (L-α-phosphatidylserine (Brain, Porcine) (sodium salt)), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and PEG(2000)-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Liposome extruder and polycarbonate membranes were obtained from Avestin (Ottawa, Canada). Ultrapure water was purified via a Millipore water system (≥ 18 MW·cm triple water purification system). Small quantities (< 5 mg) were weighed on a Mettler Toledo XS105 dual range analytical balance. Fluorescence readings of microplate-based assays were recorded with a BioTek Synergy 2 Multi-Detection Microplate Reader with absorbance, fluorescence, polarization and time-resolved capabilities. Dynamic light scattering (DLS) and zeta potential (ZP) measurements were carried out with a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at l=633 nm. Fluorescence studies were performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Plots were generated using Origin Pro 2021b. All error bars in plots show the standard errors of at least three experimental replicates. Fluorometric analysis and AlamarBlue cell viability analysis were performed using Gen5 software and Cytation 1 cell imaging multimode microplate reader (BioTek Instruments, Winooski, VT) with an RFP filter (Ex 531/40, Em 593/40 nm) and DAPI filter (Ex 377/50, Em 447/60 nm). Lipid **1** was synthesized according to a previously reported protocol and was dissolved in a $CHCl_3$/Methanol (1:1, v:v) mixture to prepare a 5 mM stock solution.[1]

Liposome Preparation

Dynamic light scattering (DLS) of liposome size before and after H_2O_2 treatment

Stock solutions of 5 mM lipid **1** and 14.3 mM DOTAP were prepared in a mixture of methanol and chloroform, while 34.6 mM PC, 4mM PEG(2000)-PE and 10 Cholesterol stock solutions were prepared in chloroform. All stock solutions were stored in a -20 °C freezer after preparation. Proper volumes of each stock solution were pipetted into a small vial to obtain a mixture of 1 mM of total lipid content with the desired percentages of each lipid. The organic solvents were then evaporated under a nitrogen stream, and the resulting lipid film was hydrated with 1×TBS (pH=7.4, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) and placed in a 60 °C water bath for three sets of 20 min with vortexing after each set. After ten freeze-thaw cycles with a dry ice-acetone bath and a 60 °C water bath, the solutions were extruded through a 200 nm polycarbonate membrane for 11 passes with a LiposoFast extruder (Avestin, Inc.). The resulting liposomes were stored at 4 °C and were used up within two days.

Liposome solutions (1 mM) containing varying percentages of **1**/DOTAP/PC (0/0/100, 10/10/80, 20/20/80, 25/25/50, 25/20/55 and 30/20/50) were prepared. For liposomes after H_2O_2 treatment 2 μL of 2 mM H_2O_2 (final concentration of H_2O_2= 40 μM) was added into 100 μL liposomes and incubated for 14 hours before taking DLS readings. Samples were prepared by diluting the liposome solutions before or after H_2O_2 addition 10 times with 1×TBS buffer. All samples were determined at a scattering angle of 173° at 20 °C. The Z-Ave and PDI data reported are the averages of three tests with error bars indicating standard errors.

For stability studies over time, liposome solutions (1 mM) containing varying percentages of **1**/DOTAP/PC (0/0/100 and 20/20/60) and **1**/DOTAP/Chol/PEG(2000)-PE/PC (20/20/30/5/25) were prepared. Liposomes without or with H_2O_2 treatment were prepared by incubating 100 μL liposomes with 2 μL 1 × TBS or 2 μL of 2 mM H_2O_2 solution (final concentration of H_2O_2 = 40 μM) respectively. DLS readings were taken from day 0 to day 5. All samples were determined at a scattering angle of 173° at 20 °C. The Z-Ave and PDI data reported are the averages of three tests with error bars indicating standard errors.

Zeta potential (ZP) analysis of liposome surface charge before and after H_2O_2 treatment

Zeta potential values were also measured on the same instrument. Solutions (1 mM) of PC-based liposomes containing lipid **1** and DOTAP were prepared according to the formulations mentioned above in 1 mM HEPES (pH=7.4, containing 30 mM NaCl). For endpoint measurements, the liposomes after H_2O_2 treatment were prepared by adding 2 c of a 2 mM H_2O_2 solution into 100 μL of 1 mM liposomes (final concentration of H_2O_2 = 40 μM). After 14 hours incubation, a 100 μL aliquot of liposomes was diluted with 900 μL of buffer. The solutions were next transferred into a DTS1070 folded capillary cell ready for measurement. For time-dependent measurements, H_2O_2 was added to 1.2 mL liposomes to achieve the desired concentration. After a certain amount of time of incubation, 100 μL of liposomes were diluted with 900 μL buffer and subjected to ZP analysis. All measurements were taken at 20 °C. Data were generated with at least three replicates.

Preparation of liposomes for Nile red (NR) release assay

A stock solution of 5 mM Nile red was prepared in chloroform. Liposome solutions (1 mM) of PC-based liposomes containing lipid **1**, DOTAP, Cholesterol and PEG(2000)-PE were prepared in 1 × TBS (pH = 7.4, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) according to previously mentioned thin-film hydration method, where Nile red was added as an extra 5% of the total lipid content. Liposomes without or with H_2O_2 treatment were prepared by incubating 100 μL liposomes with 2 μL 1 × TBS or 2 μL of 2 mM H_2O_2 solution (final concentration of H_2O_2 = 40 μM) respectively. Fluorescence intensities of

the resulting solution were measured over time at room temperature (excitation wavelength = 552 nm, excitation slit = 5 nm, emission slit = 5 nm). When processing the data, intensities at 635 nm were selected and converted into percentage of initial fluorescence intensity before adding stimuli. Experiments were run at least 3 times with different batches of liposomes. Averaged data were reported with error bars denoting standard error.

Fluorescence-based microplate assay for vesicle interactions

Stock solution of 5 mM biotin-PE, 0.5 mM Rd-PE and 12mM PS were prepared in chloroform. Liposome solutions of 1 mM concentration were prepared from stock solutions of lipid **1**, DOTAP, PC, biotin-PE, Rd-PE, and PS using the previously mentioned thin-film hydration method with 1 × TBS (pH=7.4, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl). Liposome solutions (1.35 mL of 1 mM 5/1/94 PS/Biotin-PE/PC) were prepared in 1 × TBS buffer (pH=7.4) and diluted to 0.5 mM before use. A second batch of 1.35 mL of 1 mM **1**/DOTAP/PC/Rd-PE (0/0/99/1, 20/20/59/1 and 25/20/54/1) liposomes was prepared using the same method described above. Half of these liposomes were diluted to 0.5 mM with TBS buffer before use. Solutions of 20 mM H_2O_2 were prepared from 9.8 M H_2O_2 by dilution with 1 × TBS buffer. The wells on a streptavidin-coated microplate were washed with 1 × TBS buffer for 30 min under low vortex. After removing the wash buffer, each well was incubated with increasing concentrations of PS liposomes (100 µL total volume, diluted from 0.5 mM liposomes) for 1 hour at room temperature. Next, PS liposome solutions were removed, and the wells were further washed with 3 x 250 µL wash buffer. The microplate was placed under low vortex for three minutes during each wash. After the final wash, the PS-functionalized wells were treated with 0.5 mM liposomes containing 0-25% **1**, 0-20% DOTAP, 50-100% PC and 1% Rd-PE in the presence of H_2O_2. Control experiments without H_2O_2 addition were conducted at the same time by adding 100 µL of 0.5 mM diluted **1**-liposome solutions. For the wells with H_2O_2 treatment, 50 µL of 1 mM **1**-liposomes were first added, followed by the addition of 50 µL 20 mM H_2O_2 solutions (final H_2O_2 concentrations = 10 mM). After 5.5 hours of incubation, all the solutions were removed, and the wells were further washed with 3 x 250 µL wash buffer. The microplate was placed under low vortex for three minutes during the washes. Then, 100 µL 1 × TBS buffer was added to each well and the fluorescence readings were measured with the plate reader. The data are reported as the fluorescence intensities for each well (F) subtracting the initial background fluorescence intensities for each set of the experiment (F_0, wells without Rd-PE labeled liposome treatment) at 590 nm. Experiments were run at least three times with different batches of liposomes. Averaged data were reported with error bars denoting standard errors.[2]

Fluorescence microscopy analysis of cell delivery

Human malignant melanoma (A-375) cells were obtained from ATCC (Manassas, VA). Cells were grown in 100 mm dishes and then plated at 1×10^4 cells per well in a 96-well plate with clear flat bottom for cell delivery experiments. Cells were grown for 24 hours to reach 80% confluency in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% (v:v) fetal bovine serum (Gibco), and 1% of 50 U/mL penicillin/streptomycin (Gibco). Incubation was performed at 37 °C under 5% CO_2 with 95% humidity. Solutions of liposomes (2 mM) containing L-α-phosphatidylethanolamine (Rd-PE) for fluorescence tracking of varying percentages, such as, 0/0/99.92/0.08 (negative control), 20/20/59.92/0.08, or 0/20/79.92/0.08 (positive control) **1**/DOTAP/PC/Rd-PE in 1 × TBS buffer were prepared using the previously described thin-film hydration procedures. The 2 mM liposome solutions were incubated with 40 μM H_2O_2 for twelve hours at room temperature before being diluted with $DPBS^{++}$ buffer or media to 1 mM (final H_2O_2 concentration = 20 μM).

At 80% confluence, cells were treated with 100 μL per well of the corresponding 1 mM liposome solutions and incubated for 1 h at 37 °C or 4 °C (for analysis of liposomal entry by energy-dependent pathways). Cells were washed two times with 250 μL $DPBS^{++}$ pH 7.4, then fixed for 15 min at 37 °C by addition of 50 μL per well of 4% paraformaldehyde. Then, cells were washed two times with 250 μL $DPBS^{++}$ pH 7.4 and stained with NucBlueTM Fixed Cell ReadyProbesTM Reagent (DAPI, Fisher Scientific). Fluorometric analysis was performed using Gen5 software and Cytation 1 cell imaging multimode microplate reader (BioTek Instruments, Winooski, VT) with an RFP filter (Ex 531 / Em 593 nm) and DAPI filter (Ex 377 / Em 447 nm). Fluorescence microscopy images were acquired on the Cytation 1 Reader using 20X and 4X magnification. Averaged RFP fluorescence units for each sample were determined from images taken at 4X magnification. Replicates were performed using at least 3 batches of liposomes.[3]

Cell viability assay

Cell viability after liposome treatment was measured. Liposomes (2 mM) containing **1**/DOTAP/PC (0/0/100, 20/20/60 and 0/20/80) without any content encapsulation were prepared using the standard liposome preparation procedure described above and were incubated with or without 40 μM H_2O_2 for twelve hours at room temperature. The liposome solutions were diluted to 1 mM, 200 μM, 40 μM, 8 μM, 1.6 μM, or 0 μM liposomes using $DPBS^{++}$. A375 cells at were plated at 1×10^4 seeding density in a 96-well plate, including control samples. After 24 hours, the liposome samples were added and incubated for 1 hour at 37 °C. The cells were washed three times with 250 μL $DPBS^{++}$ pH 7.4, followed by the addition of 100 μL DMEM with 10% FBS and 1% Pen-Strep to each well and 10 μL of AlamarBlue reagent in light-free conditions. The cells were then incubated for 4

hours, and the cell viability was measured using Gen5 software and Cytation 1 cell imaging multimode microplate reader (BioTek Instruments, Winooski, VT) with red fluorescence filter (Ex 530/25, Em 590/35). Replicates were performed using at least 3 batches of liposomes.[4]

Supplemental Figures:

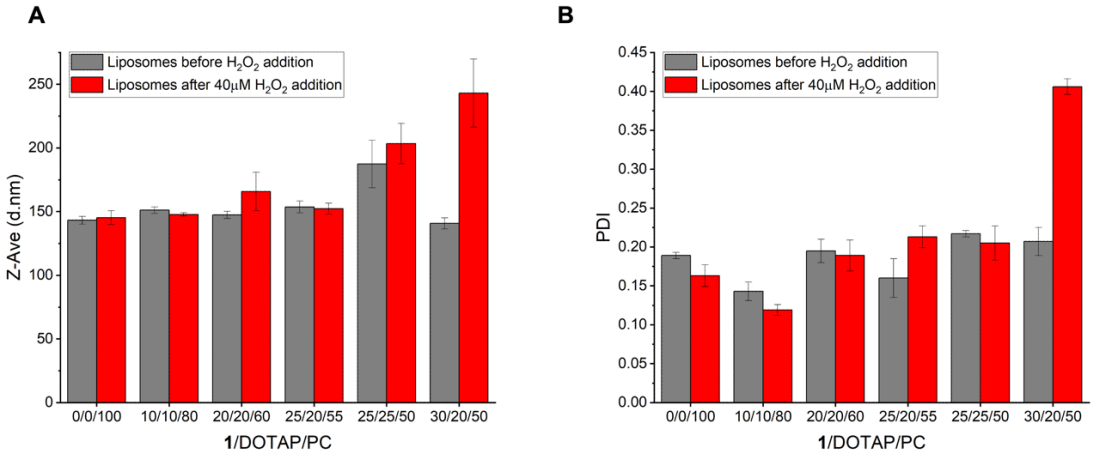

Figure S1. Bar graphs showing the DLS results for different formulations of **1**/DOTAP/PC (0/0/100, 10/10/80, 20/20/60, 25/20/55, 25/25/50, and 30/20/50) liposomes before and after 40 μM H_2O_2 incubation. **A.** Z-Ave changes for **1**/DOTAP/PC liposomes before and after H_2O_2 treatment for 14 hours. **B.** PDI changes for **1**/DOTAP/PC liposomes before and after H_2O_2 treatment for 14 hours. While some liposome formulations (0/0/100, 10/10/80, 20/20/60 and 25/20/55 **1**/DOTAP/PC) show the formation of uniform vesicles of expected sizes both before and after H_2O_2 treatment, other formulations (25/25/50 and 30/20/50 **1**/DOTAP/PC) resulted in vesicles that were either slightly larger than expected or did not exhibit uniform vesicle formation before or after H_2O_2 treatment.

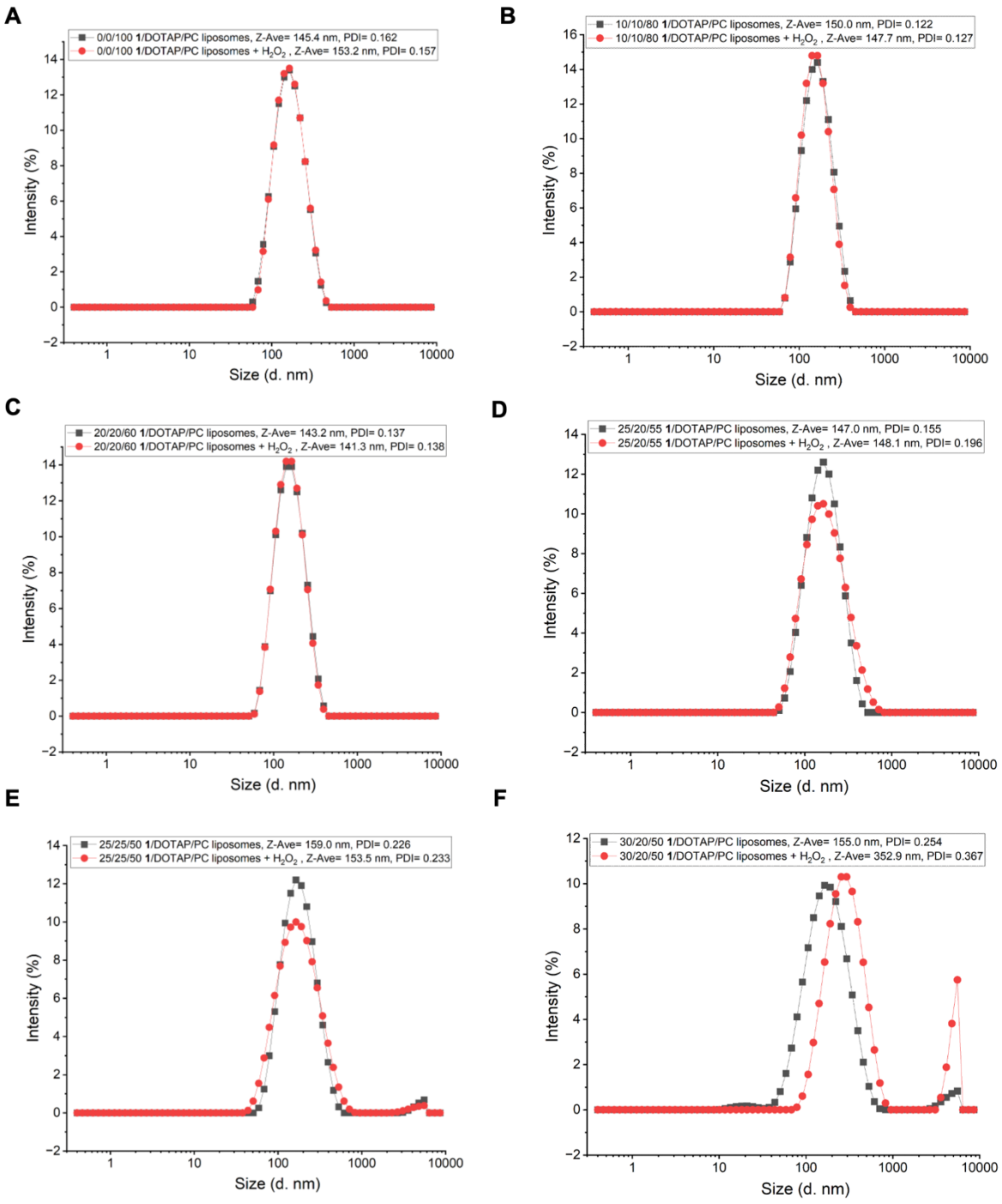

Figure S2. DLS raw distribution curves for **1**/DOTAP/PC liposomes **A.** 0/0/100 **B.** 10/10/80 **C.** 20/20/60 **D.** 25/20/55 **E.** 25/25/50 and **F.** 30/20/50 before and after 40 μM H_2O_2 treatment for 14 hours. While some liposome formulations (0/0/100, 10/10/80, 20/20/60 and 25/20/55 **1**/DOTAP/PC) show the formation of uniform vesicles both before and after H_2O_2 treatment, other formulations (25/25/50 and 30/20/50 **1**/DOTAP/PC) either

resulted in slightly larger than expected particles or did not show uniform vesicle formation before or after H_2O_2 treatment.

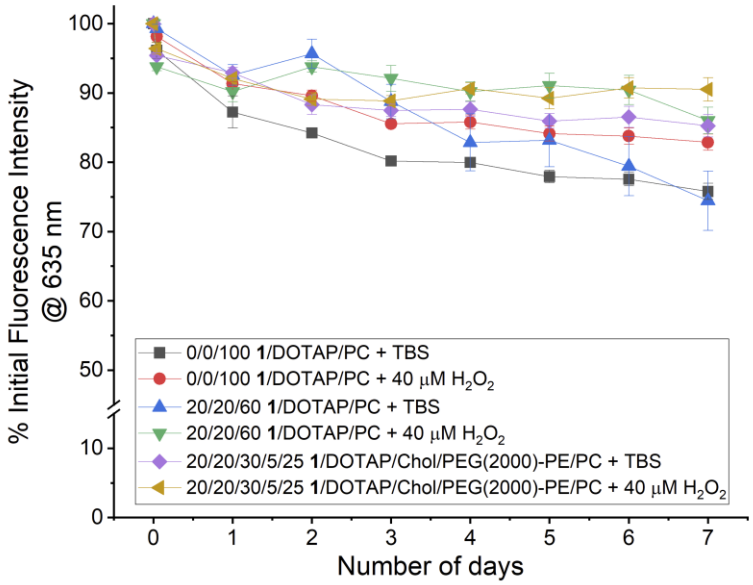

Figure S3. Nile red release profiles using **1**/DOTAP/PC (0/0/100 and 20/20/60) and **1**/DOTAP/Chol/PEG(2000)-PE/PC (20/20/30/5/25) liposomes of 1 mM concentration showed that significant cargo was not released with 40 μM H_2O_2 treatment. Control experiments in which 1 mM liposomes were treated with 1 × TBS did not show significant background release either. Error bars denote standard errors from at least three independent experiments.

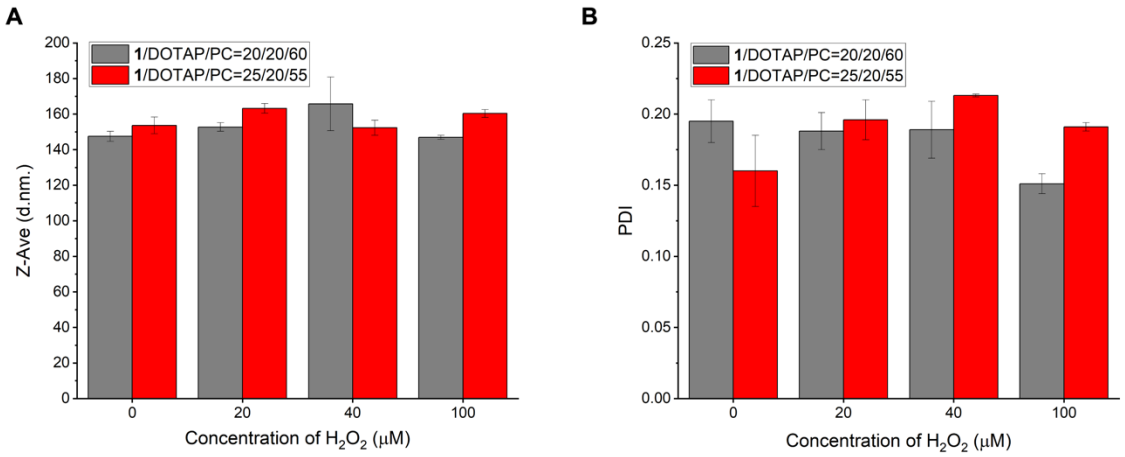

Figure S4. Bar graphs showing the DLS results for different formulations of **1**/DOTAP/PC (20/20/60 and 25/20/55) liposomes before and after treatment with different concentrations of H_2O_2 (20 μM, 40 μM, and 100 μM). **A.** Z-Average changes for

1/DOTAP/PC liposomes before and after incubation with different concentrations of H_2O_2. **B.** PDI changes for **1**/DOTAP/PC liposomes before and after incubation with different concentrations of H_2O_2. Minimal changes were observed for liposome sizes before and after H_2O_2 incubation.

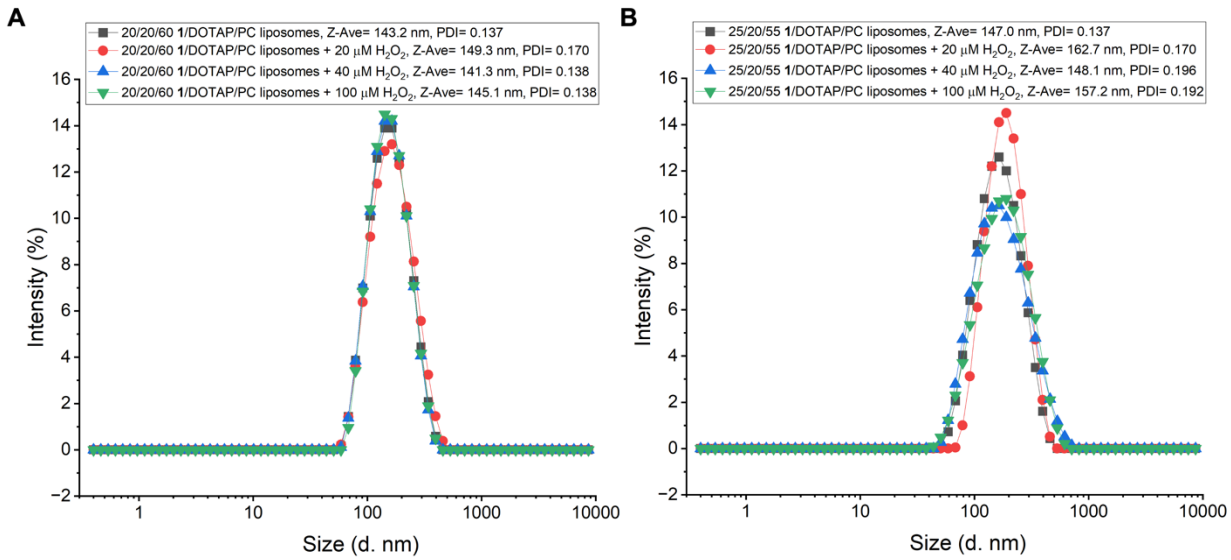

Figure S5. DLS raw distributions for lipid **1**/DOTAP/PC liposomes **A.** 20/20/60 **B.** 25/20/55 before and after incubation with different concentrations of H_2O_2 (20 μM, 40 μM, and 100 μM). Significant size changes were not observed before and after incubation with different concentrations of H_2O_2.

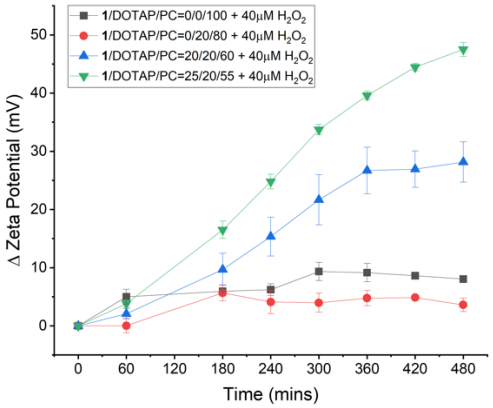

Figure S6. Absolute ZP differences for different liposomes at varying time points after H_2O_2 addition. While the negative (**1**/DOTAP/PC = 0/0/100) and positive controls (**1**/DOTAP/PC = 0/20/80) did not show a significant change in ZP values, liposomes

containing lipid **1** (**1**/DOTAP/PC = 20/20/60 and 25/20/55) showed a significant increase in ZP.

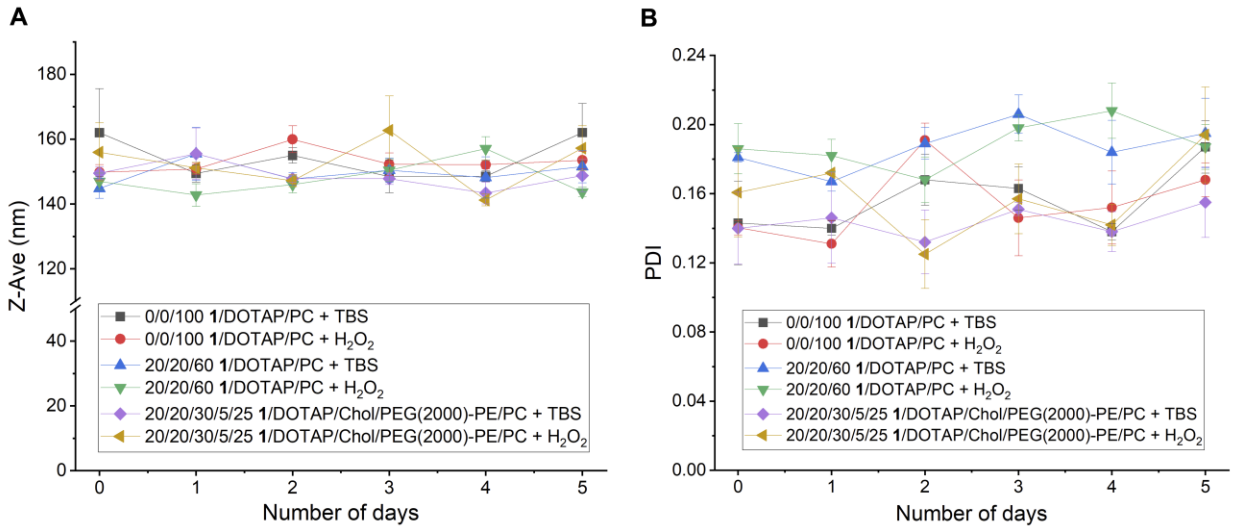

Figure S7. DLS size change results for different formulations of **1**/DOTAP/PC (0/0/100 and 20/20/60) and **1**/DOTAP/Chol/PEG(2000)-PE/PC (20/20/30/5/25) liposomes without and with 40 μM H_2O_2 incubation over 5 days. **A.** Z-Average changes for **1**/DOTAP/PC (0/0/100 and 20/20/60) and **1**/DOTAP/Chol/PEG(2000)-PE/PC (20/20/30/5/25) liposomes without and with 40 μM H_2O_2 incubation. **B.** PDI changes for **1**/DOTAP/PC (0/0/100 and 20/20/60) and **1**/DOTAP/Chol/PEG(2000)-PE/PC (20/20/30/5/25) liposomes without and with 40 μM H_2O_2 incubation. Minimal changes were observed for liposome sizes over a 5-day period indicating liposome stability.

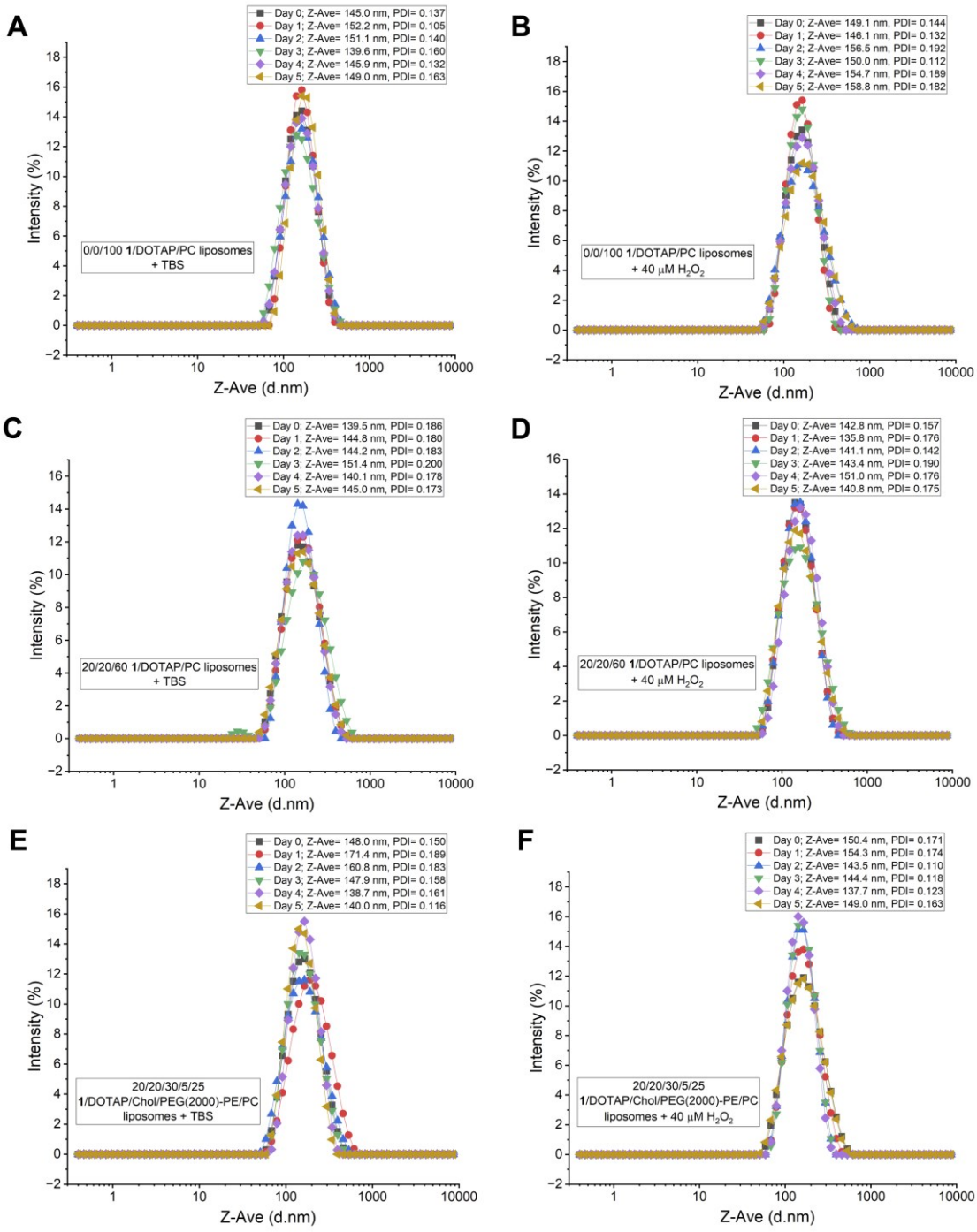

Figure S8. DLS raw distribution curves for **1**/DOTAP/PC and **1**/DOTAP/Chol/PEG(2000)-PE/PC liposomes without and with H_2O_2 treatment over 5 days. 0/0/100 **1**/DOTAP/PC liposomes with **A.** without and **B.** with 40 μM H_2O_2 treatment, 20/20/60 **1**/DOTAP/PC liposomes with **C.** without and **D.** with 40 μM H_2O_2 treatment, 20/20/30/5/25 **1**/DOTAP/Chol/PEG(2000)-PE/PC liposomes with **E**. without and **F.** with 40 μM H_2O_2

treatment over 5 days. All liposome formulations show minimal changes in Z-Average and PDI over 5-days period.

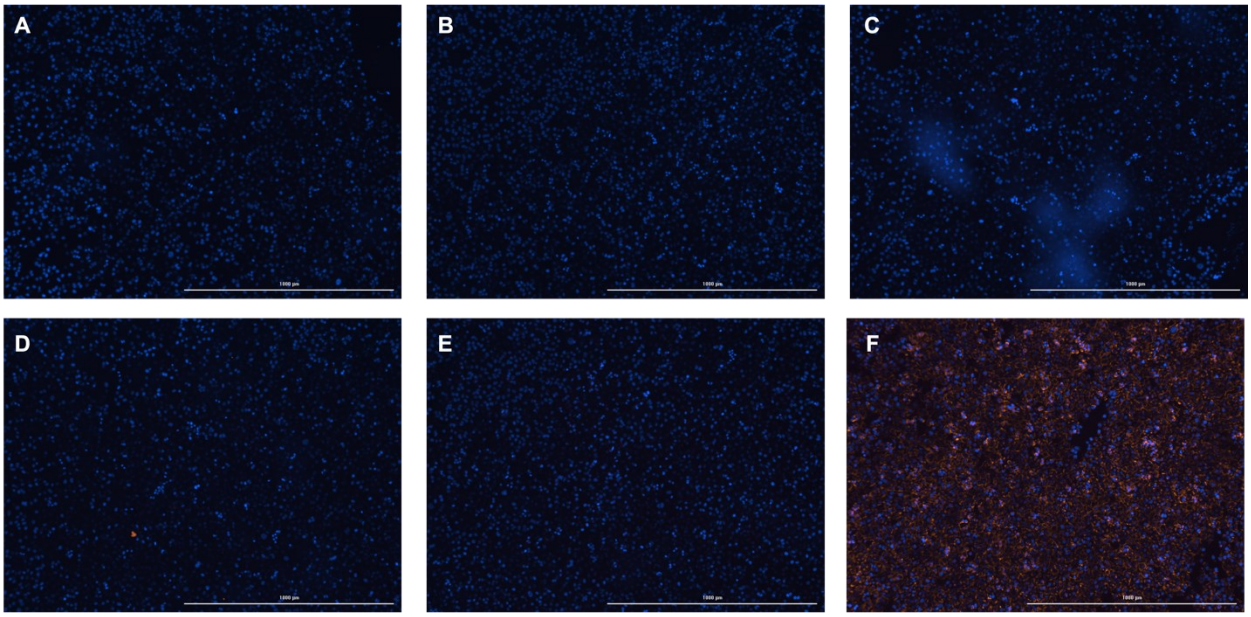

Figure S9. Fluorescence images showing enhanced liposomal association with A375 cells only when **1**/DOTAP/PC/Rd-PE liposomes were treated with H_2O_2 in $DPBS^{++}$ buffer. Representative 4X fluorescence images of cells incubated with $DPBS^{++}$ buffer only (**A**), 40 μM H_2O_2 (**B**)**,** 0/0/99.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment, and 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**E**) and with (**F**) 40 μM H_2O_2 pre-treatment. Blue: DAPI; Red: Rd-PE; Scale bar: 1000 μm.

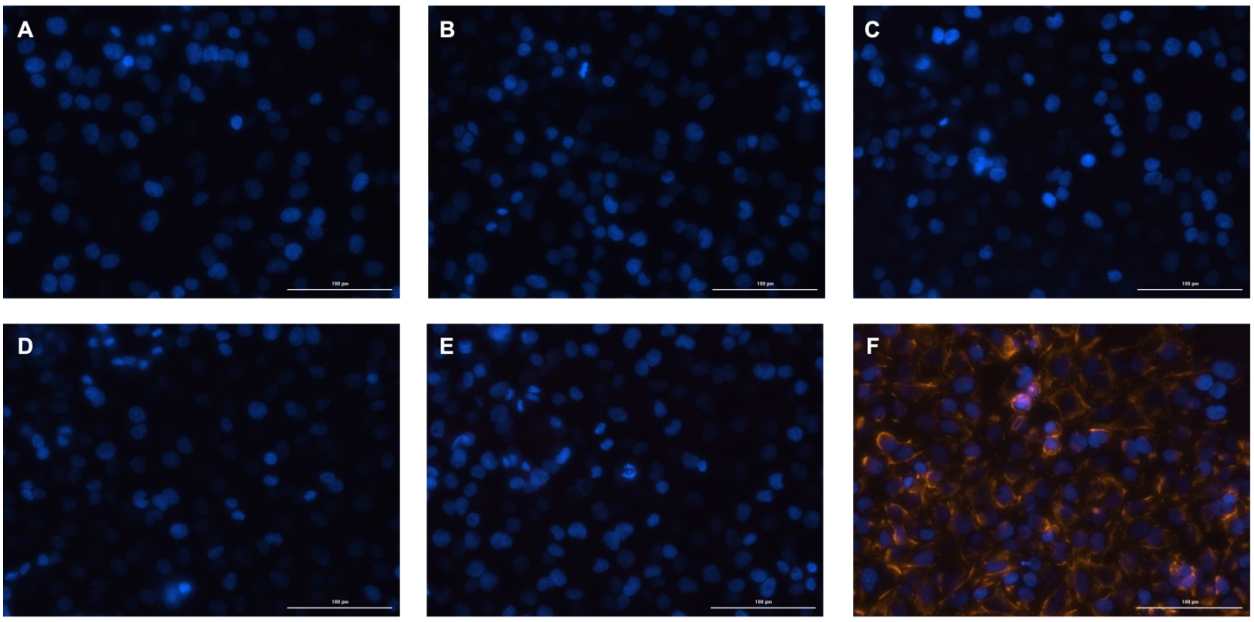

Figure S10. Fluorescence images showing enhanced liposomal association with A375 cells only when **1**/DOTAP/PC/Rd-PE liposomes were treated with H_2O_2 in $DPBS^{++}$. Representative 20X fluorescence images of cells incubated with $DPBS^{++}$ buffer only (**A**), 40 μM H_2O_2 (**B**)**,** 0/0/99.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment, and 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**E**) and with (**F**) 40 μM H_2O_2 pre-treatment. Blue: DAPI; Red: Rd-PE; Scale bar: 100 μm.

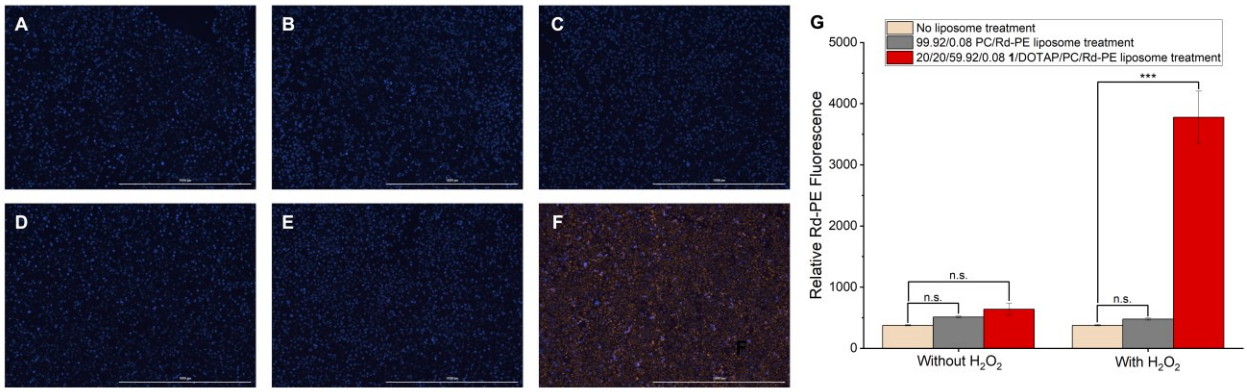

Figure S11. Fluorescence images showing enhanced liposomal association with A375 cells only when **1**/DOTAP/PC/Rd-PE liposomes were treated with H_2O_2 in media. Representative 4X fluorescence images of cells incubated with media only (**A**), 40 μM H_2O_2 (**B**)**,** 0/0/99.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment, and 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**E**) and with (**F**) 40 μM H_2O_2 pre-treatment. Blue: DAPI; Red: Rd-PE; Scale bar: 1000 μm. (**G**) Quantified relative Rd-PE fluorescence of cells treated with no liposomes, 99.92/0.08 PC/Rd-PE liposomes and 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes with and

without H_2O_2 treatment. Statistical analysis (*t* test) showed significant increases in fluorescence were observed only for cells treated with 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes after 40 μM H_2O_2 pre-treatment, where n.s. ($p > 0.05$), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). Error bars indicate standard error from at least three independent trials.

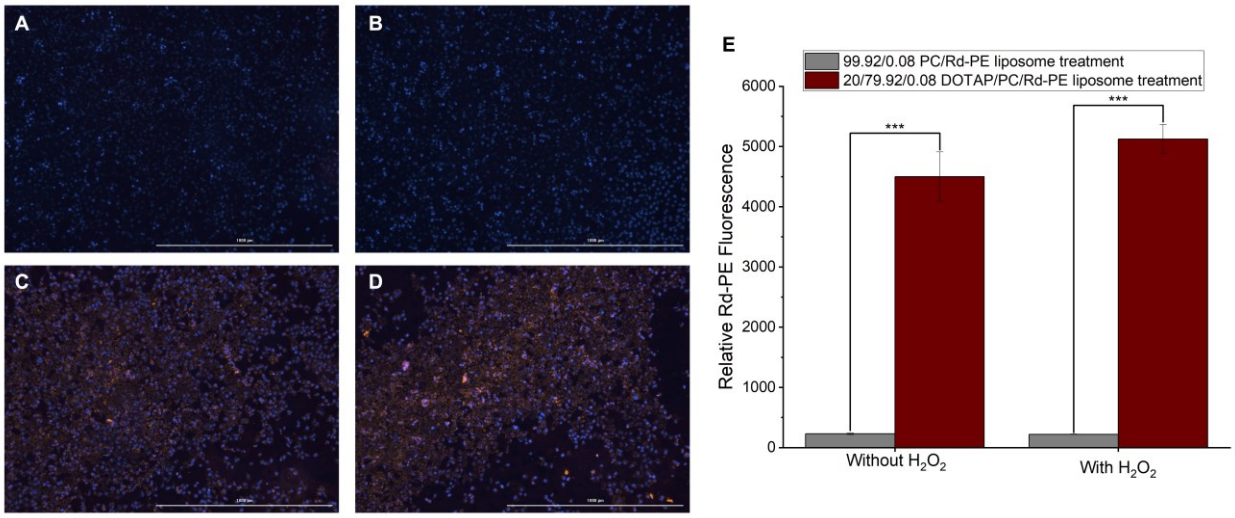

Figure S12. Fluorescence images showing liposomal association with A375 cells when DOTAP/PC/Rd-PE liposomes were treated with or without H_2O_2 in $DPBS^{++}$. Representative 4X fluorescence images of cells incubated in $DPBS^{++}$ with 0/99.92/0.08 DOTAP/PC/Rd-PE liposomes without (**A**) and with (**B**) 40 μM H_2O_2 pre-treatment, 20/79.92/0.08 DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment. Blue: DAPI; Red: Rd-PE; Scale bar: 1000 μm. (**E**) Quantified relative Rd-PE fluorescence of cells treated with 99.92/0.08 PC/Rd-PE liposomes and 20/79.92/0.08 DOTAP/PC/Rd-PE liposomes with and without H_2O_2 treatment. Statistical analysis (*t* test) showed significant increases in fluorescence were observed for cells treated with 20/79.92/0.08 DOTAP/PC/Rd-PE liposomes with or without H_2O_2 treatment, where n.s. ($p > 0.05$), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). Error bars indicate standard error from at least three independent trials.

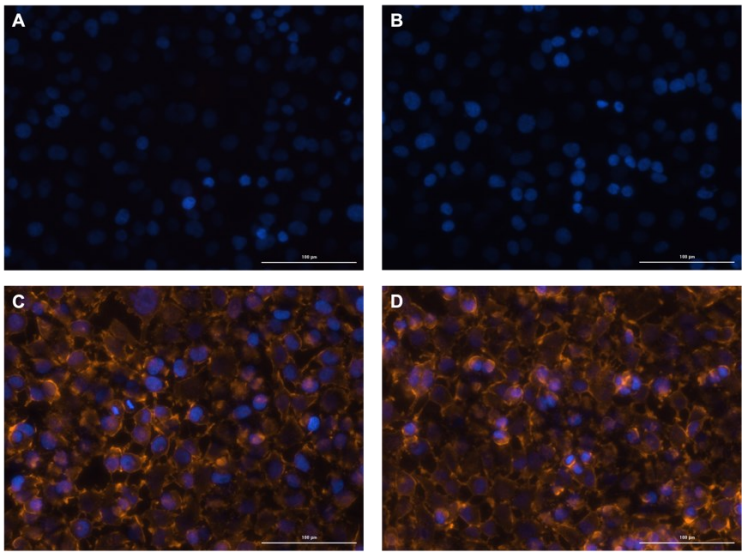

Figure S13. Fluorescence images showing comparable liposomal association with A375 cells when control DOTAP/PC/Rd-PE liposomes were either treated or not treated with H_2O_2 in DPBS^{++}. Representative 20X fluorescence images of cells incubated in DPBS^{++} with 0/99.92/0.08 DOTAP/PC/Rd-PE liposomes without (**A**) and with (**B**) 40 μM H_2O_2 pre-treatment, 20/79.92/0.08 DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment. Blue: DAPI; Red: Rd-PE; Scale bar: 100 μm.

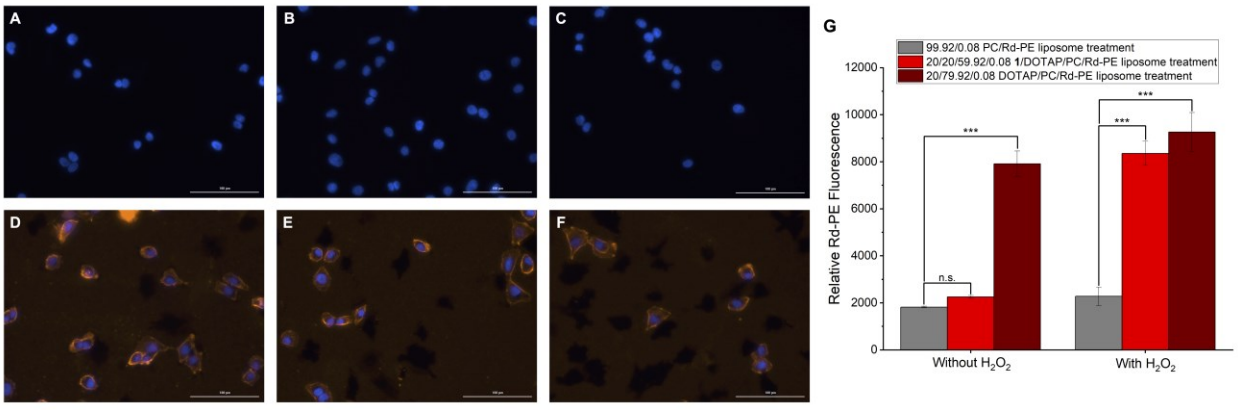

Figure S14. Fluorescence images showing enhanced liposomal association with A375 cells only when **1**/DOTAP/PC/Rd-PE liposomes were treated with H_2O_2 at 4 °C in DPBS++. Representative 20X fluorescence images of cells incubated with 0/0/99.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**A**) and with (**B**) 40 μM H_2O_2 pre-treatment, 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment, and 0/20/79.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**E**) and with (**F**) 40 μM H_2O_2 pre-treatment Blue: DAPI; Red: Rd-PE; Scale bar: 100 μm. (**G**) Quantified relative Rd-PE fluorescence of cells treated with 99.92/0.08 PC/Rd-PE liposomes, 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE and 20/79.92/0.08 DOTAP/PC/Rd-PE liposomes

with and without H_2O_2 treatment. Statistical analysis (*t* test) showed significant increases in fluorescence were observed only for cells treated with 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes after 40 μM H_2O_2 pre-treatment, where n.s. ($p > 0.05$), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). Error bars indicate standard error from at least three independent trials.

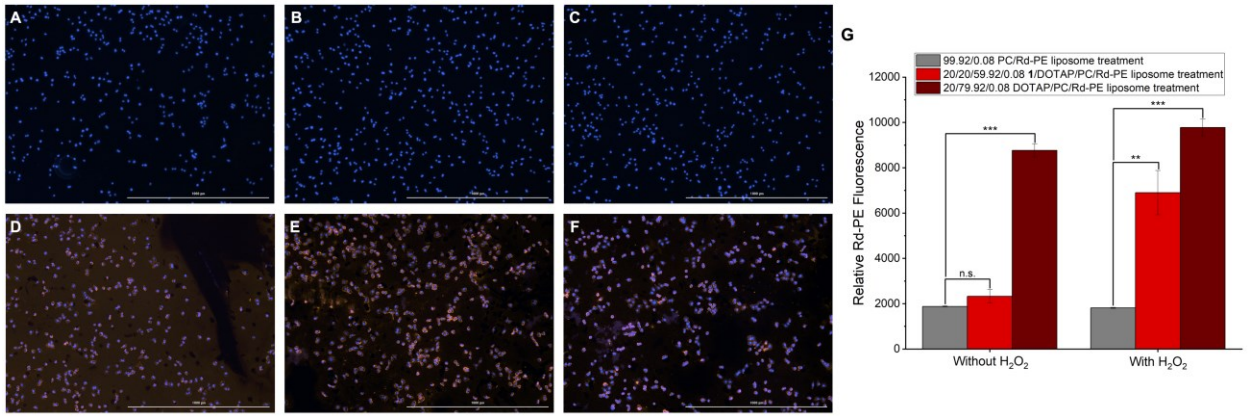

Figure S15. Fluorescence images showing enhanced liposomal association with A375 cells only when **1**/DOTAP/PC/Rd-PE liposomes were treated with H_2O_2 at 4 °C in media. Representative 4X fluorescence images of cells incubated with 0/0/99.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**A**) and with (**B**) 40 μM H_2O_2 pre-treatment, 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**C**) and with (**D**) 40 μM H_2O_2 pre-treatment, and 0/20/79.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes without (**E**) and with (**F**) 40 μM H_2O_2 pre-treatment Blue: DAPI; Red: Rd-PE; Scale bar: 1000 μm. (**G**) Quantified relative Rd-PE fluorescence of cells treated with 99.92/0.08 PC/Rd-PE liposomes, 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE and 20/79.92/0.08 DOTAP/PC/Rd-PE liposomes with and without H_2O_2 treatment. Statistical analysis (*t* test) showed significant increases in fluorescence were observed only for cells treated with 20/20/59.92/0.08 **1**/DOTAP/PC/Rd-PE liposomes after 40 μM H_2O_2 pre-treatment, where n.s. ($p > 0.05$), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). Error bars indicate standard error from at least three independent trials.

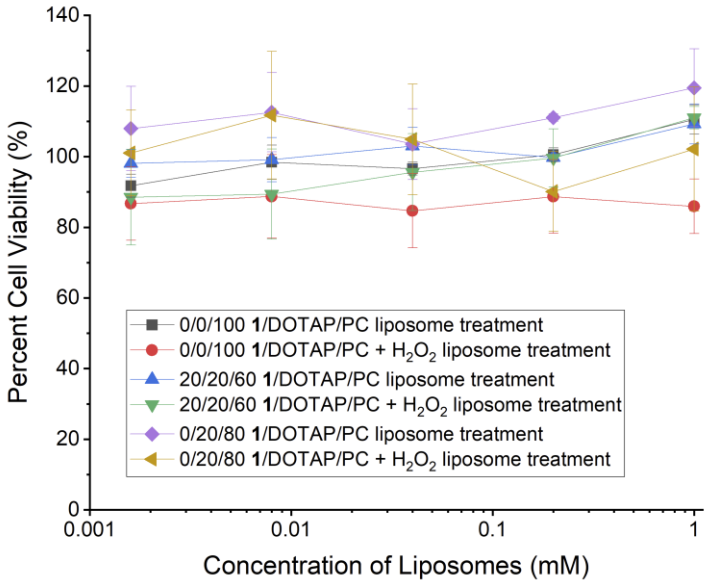

Figure S16. AlamarBlue assay to assess cell viability of A375 cells after treatment with different concentrations (0.0016, 0.008, 0.04, 0.2, and 1 mM) of 0/0/100, 20/20/60 and 0/20/80 **1**/DOTAP/PC liposome without and with 40 μM H_2O_2 treatment. Viability of 100% corresponds with cells treated with buffer only. The AlamarBlue assay indicates liposomes containing lipid **1** do not affect cell viability with or without H_2O_2 pre-treatment. Error bars represent standard errors of at least three replicates.

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