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Development of GTP-responsive liposomes by exchanging the metal-DPA binding site in a synthetic lipid switch

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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 egg) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Liposome extruder and polycarbonate membranes were obtained from Avestin (Ottawa, Canada) or Avanti Polar Lipids, Inc (Alabaster, AL). 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. Osmotic pressure was measured with Wescor Vapor Pressure Osmometer 5520. Fluorescence studies were performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. DLS and zeta potential analyses were carried out with a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at l = 633 nm. Plots were generated using Origin Pro 2022b. All error bars in plots indicate the standard errors of at least three experimental replicates.

Synthetic Procedures

Bis-DPA precursor **1** (10 mg, 0.0096 mmol) was synthesized according to a previously reported protocol,[1] and dissolved with $CHCl_3$/MeOH (4/1, v/v) in a 4 dr vial. Copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$, 3.3 mg, 0.0192 mmol) was then added and the reaction was stirred overnight. After completion, the solvent was removed by rotary evaporation to yield the brown Cu-chelated product **BCuDPAL** in a quantitative yield.

Liposome preparation and Assays

Preparation of liposomes for Nile red release assays

Stock solutions of 5 mM lipid switch **BCuDPAL** were prepared in $CHCl_3$/MeOH solution (1/1, v/v). 32.46 mM PC and 5 mM Nile red stock solutions were prepared in chloroform. All stock solutions were kept in a -20 ºC freezer after preparation. Proper volumes of each stock solution were pipetted into a 1 dr vial to reach a total lipid concentration of 2 mM with desired percentages of each lipid composition. Nile red was added as an extra 5 mol% of the total lipid content. The organic solvents were next evaporated under a nitrogen stream and the resulting lipid films were kept under vacuum for at least one hour. Next, the films were hydrated with proper volumes of 1×TBS buffer (pH=7.4, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) in a 60 ºC water bath for 1 hr. The vials were taken out and vortexed every 20 min. Next, ten freeze-thaw cycles were performed with a dry ice-acetone bath and 60 ºC water bath. Finally, the liposome solutions were extruded through a 200 nm polycarbonate membrane for 19 passes with an extruder purchased from either Avestin or Avanti. The resulting liposomes were stored at 4 ºC and were studied within a maximum of 48 hours.

Phosphorylated metabolite selectivity screen for liposome Nile red release

50 mM stock solutions of each phosphorylated metabolite were prepared by dissolving the appropriate salts (sodium phosphate dibasic heptahydrate (Pi, $Na_2HPO_4{\cdot}7H_2O$); sodium pyrophosphate dibasic (PPi, $Na_2H_2P_2O_7$); adenosine 5'-diphosphate sodium salt (ADP); adenosine 5'-triphosphate disodium salt, hydrate (ATP); D-fructose-6-phosphate disodium salt (FP); D-fructose-1,6-bisphosphate trisodium salt (FBP); sodium tripolyphosphate (TPi); cytidine-5'-triphosphate disodium salt (CTP); guanosine-5'-triphosphate trisodium salt (GTP); uridine 5′-triphosphate trisodium salt hydrate (UTP); D-*myo*-inositol-1,4,5-triphosphate sodium salt (IP_3)) with MilliQ water. Adenosine 5′-monophosphate monohydrate (AMP) was prepared as a 25 mM stock solution in MilliQ water due to solubility issues. For each study, 45 µL of liposome solution encapsulating Nile red was first added into a sub-micro quartz cuvette. After an initial scan, 5 µL of 50 mM stock solutions of the phosphorylated metabolite under study were added to the cuvette (final concentration = 5 mM, 11.25 µL for AMP), and the fluorescence intensities were recorded after incubation for 60 min (excitation wavelength = 552 nm; For PC liposomes, excitation slit = 5 nm, emission slit = 5 nm; For 10% **BCuDPAL**/PC liposomes, excitation slit = 10 nm, emission slit = 10 nm; For 15% **BCuDPAL**/PC liposomes, excitation slit = 20 nm, emission slit = 10 nm; For 20% **BCuDPAL**/PC liposomes, excitation slit = 10 nm, emission slit = 20 nm; For 25% **BCuDPAL**/PC liposomes, excitation slit = 20 nm, emission slit = 20 nm). Control experiments were run by adding 5 or 11.25 µL MilliQ water. When processing the data, the fluorescence intensities at 635 nm, 630 nm, 620 nm, 615 nm or 610 nm (based on the λmax) were selected for PC, 10%, 15%, 20%, and 25% **BCuDPAL**/PC liposomes, respectively, and converted to percentage of initial fluorescence before phosphorylated metabolite addition. Experiments were run at least three times with different batches of liposomes. Averaged data are reported with error bars denoting standard error.

DLS analysis of liposomes before and after triggered release

Samples were prepared by diluting the liposomes before and after triggered release 10× with proper buffer. All measurements were taken at a scattering angle of 173º at 20 ºC. Bar graphs were generated by the average of at least three experiments with error bars denoting standard error.

Preparation of liposomes for calcein release assays

The same stock solutions of **BCuDPAL** and PC prepared for Nile red release assays were used. 50 mM calcein stock solutions were prepared by dissolving calcein with 25 mM Tris-HCl buffer and the pH was then adjusted to 7.4 by adding 1 N NaOH. The osmolality of the solution was determined to be 255 mOsm/kg. Proper volumes of each lipid stock solution were pipetted into a clean 1 dr vial to reach a total lipid content of 5 mM with desired percentages of each lipid composition. The organic solvents were next removed with a nitrogen stream and the resulting lipid films were further dried under vacuum for at least 1 hr. The films were next hydrated with 50 mM calcein solution at 60 ºC in a water bath for four sets of 15 min with

vortexing after each set. Next, the solutions were subjected to ten freeze-thaw cycles with a dry ice-acetone bath and a 60 ºC water bath, followed by extrusion through a 200 nm polycarbonate membrane for 21 passes with an extruder purchased from either Avestin or Avanti. Finally, the unencapsulated calcein dye was removed by size-exclusive chromatography. To do so, a micro-column was first packed with Sephadex G-50 (pre-saturated with isotonic TBS buffer). The fractions were next collected every ~1 mL from the column and the second fraction showing significant turbidity was collected. The presence of liposomes was further verified by treating liposomes with Triton X-100 detergent, where an increase in fluorescence intensity was observed.

Calcein release assays via GTP treatment (both titration and time-dependent/kinetic experiments)

An aliquot of 50 μL liposomes encapsulating calcein prepared through the procedure described above was added into a sub-micro quartz cuvette. After an initial scan, 1 μL of a 50 mM GTP stock solution in MilliQ water was added into the cuvette each time, and the fluorescence intensities were recorded (λ_{ex}=495 nm, λ_{em}=525 nm, Ex/Em slit=10/5 nm). After completion, the assay was calibrated by adding 1 μL of 20% Triton X-100 detergent to lyse the liposomes and induce 100% release. When processing the data, fluorescence intensities at 525 nm were selected and reported as a percentage of the fluorescence after Triton X-100 treatment for each sample. Experiments were run at least three times with different batches of liposomes. Averaged data are reported with error bars denoting standard errors.

For time-dependent/kinetic studies, liposomes were generated using the same procedure. An aliquot of 90 μL liposomes encapsulating calcein was added in a sub-micro quartz cuvette. After an initial scan, 10 μL of a 100 mM GTP stock solution in MilliQ water was added into the cuvette (final concentration = 10 mM) and the fluorescence intensities were recorded (λ_{ex}=495 nm, λ_{em}=525 nm, Ex/Em slit=10/5 nm) over time. After completion, the assay was calibrated by adding 1 μL of 20% Triton X-100 detergent to lyse the liposomes and induce 100% release. Experiments were run at least three times with different batches of liposomes. Averaged data were reported with error bars denoting standard errors. Kinetic experiments were run on a Cary Eclipse Fluorescence Spectrophotometer using kinetic mode. The excitation wavelength was set to 495 nm and the fluorescent intensities at 525 nm were constantly collected overtime.

Fluorescence microscopy studies for liposome aggregation after GTP treatment

Solutions (1 mM) of 0% and 20% **BCuDPAL** liposomes in PC labeled with 0.08% rhodamine L-α-phosphatidylethanolamine (Rd-PE) in 1×TBS buffer (pH=7.4, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) were prepared using the previously described thin-film hydration procedures.

An aliquot of the liposome solution (45 µL) was first added into a FluoroDish Cell Culture Dish (WPI Inc.) and allowed to settle for 15 min before imaging using a 63x 1.4NA oil objective on a Leica SP8 White Light Laser Confocal Microscope (Wetzlar, Germany). Rd-PE was excited using 561 nm laser line and the emission was collected between 566 - 620 nm with a HyD detector. Laser power was adjusted for 20% **BCuDPAL** liposomes due to quenching effect of copper on Rd-PE. In this study, 5 µL of 5 mM GTP stock solution was directly added to the settled liposome sample in dish (final concentration = 0.5 mM). Images were taken as a Z-stack (over 15 min) and the time-course videos were generated from the same experiment.

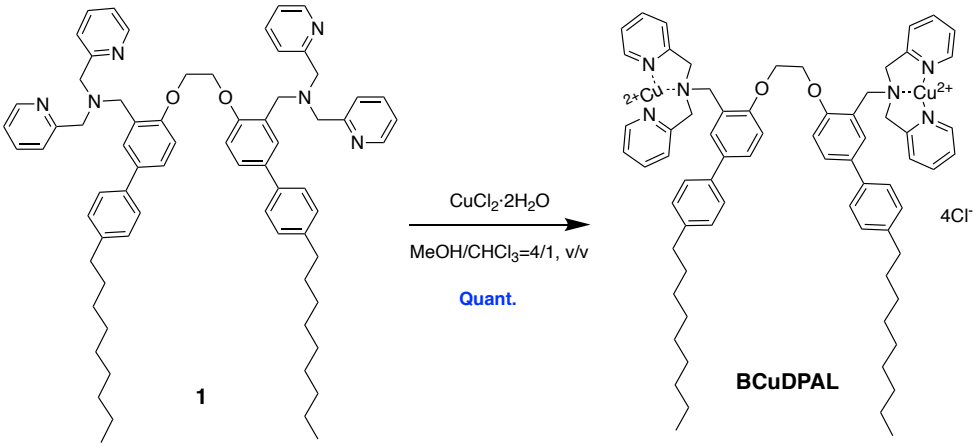
Zeta potential measurements of BCuDPAL liposomes before and after adding GTP

Zeta potential values were also measured with a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at l = 633 nm. Solutions (1 mM) of PC-based liposomes containing 0%, 10% and 20% **BCuDPAL** without dye encapsulation were prepared in 1 mM HEPES (pH=7.4, containing 30 mM NaCl). A 200 µL aliquot of liposomes was diluted with 800 µL buffer before addition of 10 µL of 50 mM GTP (conc of liposomes = 0.2 mM, conc of GTP = 0.5 mM). The solutions were next transferred into a DTS1070 folded capillary cell ready for measurement. All measurements were taken at 20 ºC. Data were generated with at least three replicates.

Reference:

1. Lou, J.; Schuster, J. A.; Barrera, F. N.; Best, M. D., ATP-responsive liposomes via screening of lipid switches designed to undergo conformational changes upon binding phosphorylated metabolites. *J. Am. Chem. Soc.* **2022,** *144* (8), 3746-3756.

Supplemental Figures:



Scheme S1. Synthetic route for **BCuDPAL** lipid switch. Intermediate **1** was treated with copper (II) chloride to generate the final lipid.

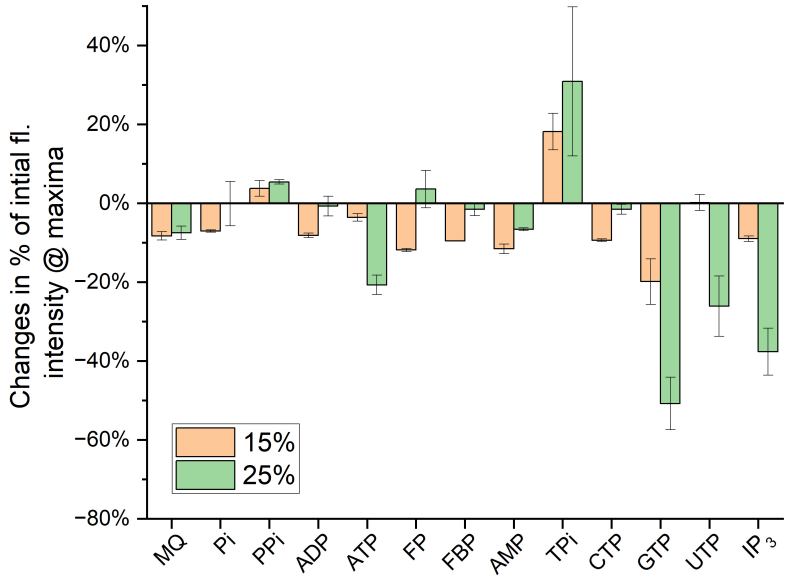


Figure S1. NR release selectivity screen by treating 15% or 25% **BCuDPAL**/PC liposomes with different phosphorylated metabolites. 15% **BCuDPAL**/PC liposomes showed slight decrease in NR fluorescence, while 25% **BCuDPAL**/PC liposomes showed significant fluorescence changes upon treatment with ATP, GTP, UTP, and IP_3. Error bars denote standard errors from at least three independent studies.

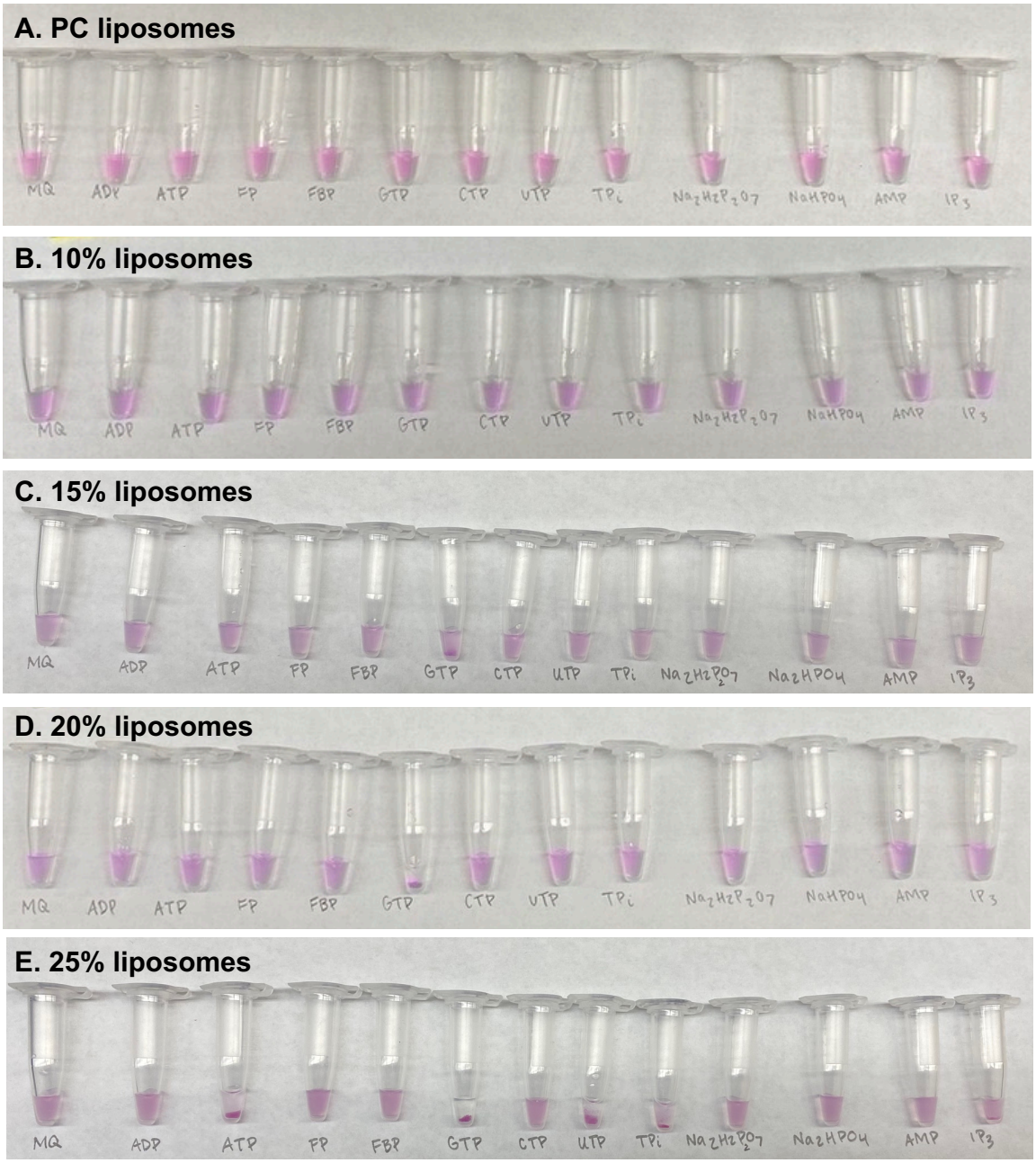


Figure S2. Images of phosphorylated and triphosphate metabolite NR release selectivity screen samples using PC (**A**), 10% **BCuDPAL** /PC (**B**), 15% **BCuDPAL** /PC (**C**), 20% **BCuDPAL** /PC (**D**) and 25% **BCuDPAL**/PC (**E**) liposomes. In the case in which NR release occurred, pink precipitate was observed at the bottom of the Eppendorf tube. From left to right: MQ, ADP, ATP, FP, FBP, GTP, CTP, UTP, TPi, PPi, Pi, AMP, IP_3.

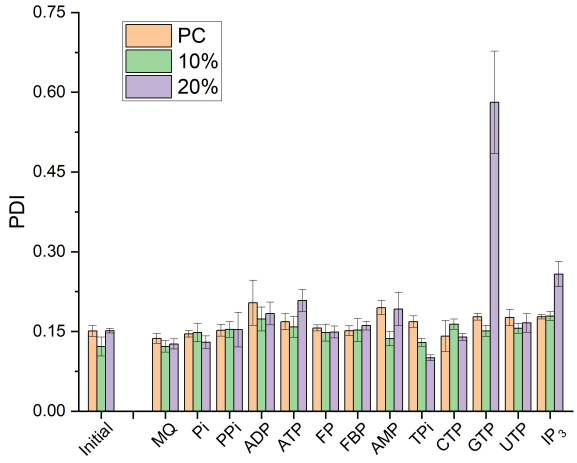


Figure S3. DLS analysis of the PDIs of 0%, 10%, and 20% **BCuDPAL**/PC liposomes before and after addition of different phosphorylated metabolites. Initial liposomes showed PDIs below 0.2, indicating uniform-size particles. Addition of GTP to 20% **BCuDPAL**/PC liposomes resulted in a dramatic increment in PDI, while all other formulations tested herein showed minimal changes. Error bars denote standard errors from at least three independent studies.

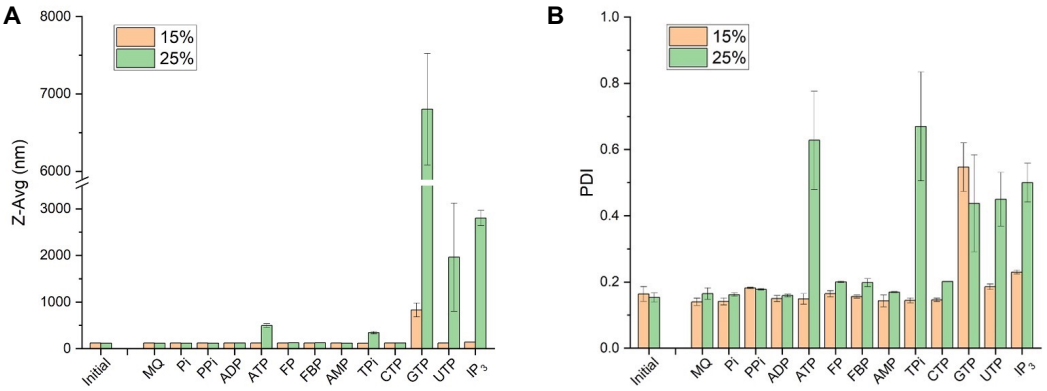


Figure S4. DLS analysis of 15% and 25% **BCuDPAL**/PC liposomes before and after metabolites, showing Z-average (**A**) and PDI (**B**). Initial liposomes showed uniform particle sizes of desired diameters. GTP addition to 15% **BCuDPAL**/PC liposomes resulted in slight increases in average particle sizes, while 25% **BCuDPAL**/PC liposomes showed particle size changes for ATP, TPi, GTP, UTP and IP_3 treatment. Error bars denote standard errors from at least three independent studies.

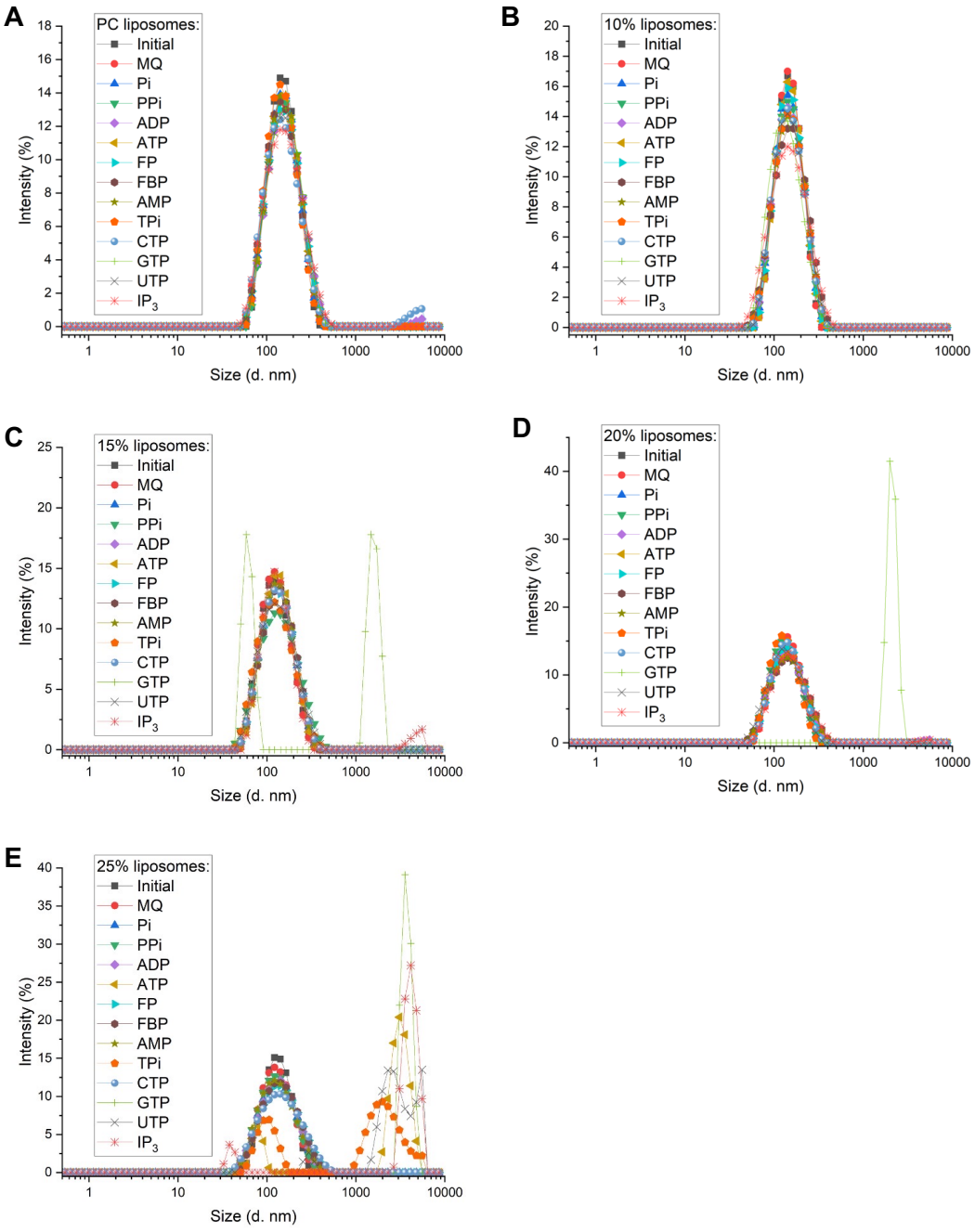


Figure S5. Representative raw distribution curves for 0% (**A**), 10% (**B**), 15% (**C**), 20% (**D**), and 25% (**E**) **BCuDPAL**/PC liposomes before and after treatment with various phosphorylated metabolites.

Table S1. Z-average and PDI values for the representative curves presented in Figure S5.

	PC		10%		15%		20%		25%	
	Z-avg (nm)	**PDI**	**Z-avg (nm)**	**PDI**	**Z-avg (nm)**	**PDI**	**Z-avg (nm)**	**PDI**	**Z-avg (nm)**	**PDI**
Initial	140.6	0.127	138.2	0.086	116.0	0.129	129.7	0.141	118.4	0.140
MQ	138.6	0.123	136.9	0.094	119.1	0.151	130.4	0.111	119.4	0.148
Pi	137.8	0.147	137.5	0.110	120.5	0.131	129.8	0.125	117.7	0.167
PPi	147.4	0.140	137.9	0.114	120.7	0.180	123.2	0.107	116.2	0.176
ADP	149.7	0.182	137.8	0.120	120.1	0.141	129.9	0.174	121.0	0.163
ATP	143.6	0.133	139.0	0.111	121.1	0.133	140.7	0.158	642.1	1.000
FP	142.2	0.144	137.8	0.112	120.1	0.155	126.8	0.136	125.6	0.196
FBP	139.6	0.133	139.1	0.135	121.3	0.151	132.7	0.144	124.4	0.186
AMP	148.9	0.178	137.2	0.117	122.1	0.125	130.3	0.148	117.1	0.168
TPi	140.9	0.164	135.8	0.126	114.0	0.152	120.2	0.109	412.9	0.818
CTP	145.0	0.195	137.6	0.148	122.5	0.151	129.0	0.127	124.5	0.202
GTP	145.9	0.161	126.6	0.163	891.5	0.819	4603	0.487	5663	0.428
UTP	145.3	0.180	137.5	0.144	120.9	0.177	118.6	0.126	3398	0.401
IP_3	141.7	0.169	132.2	0.157	130.1	0.236	139.8	0.187	2821	0.455

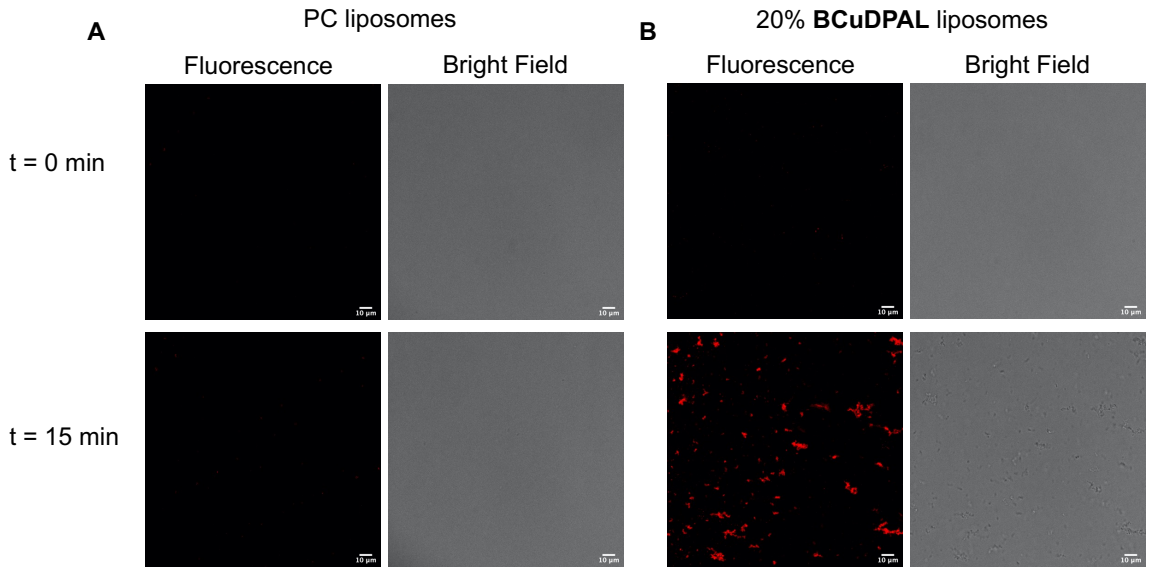


Figure S6. Confocal fluorescence microscopy images for 0% (**A**) or 20% **BCuDPAL** (**B**) liposomes labeled with Rd-PE before and after 15 minute incubation with 0.5 mM GTP. GTP addition resulted in **BCuDPAL** liposome aggregation that ultimately formed larger fluorescent particles, while PC liposomes did not show aggregation over time after GTP incubation. Scale bars denote 10 μm.

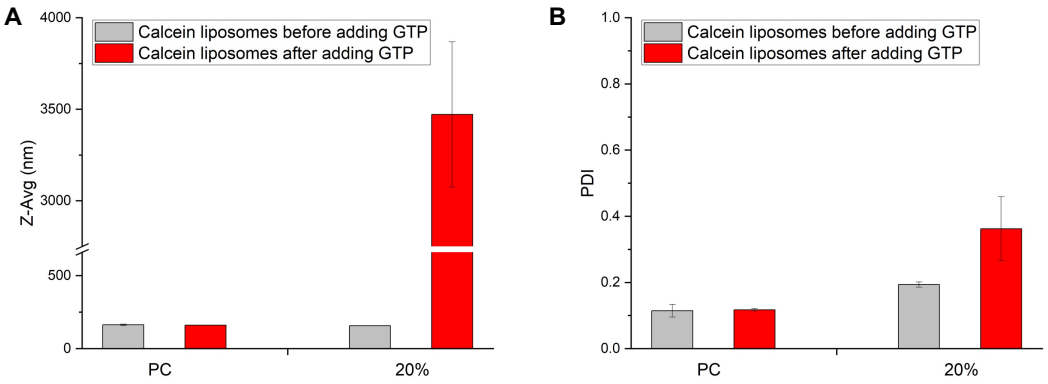


Figure S7. DLS results showing size (**A**) and PDI (**B**) changes for calcein-encapsulated PC liposomes containing 0% or 20% **BCuDPAL** before and after GTP treatment. Grey bars indicate uniformly sized liposomes were formed pre-GTP treatment. The dramatic size and PDI changes were observed after GTP addition for 20% **BCuDPAL** liposomes. Error bars indicate standard errors from at least three studies.

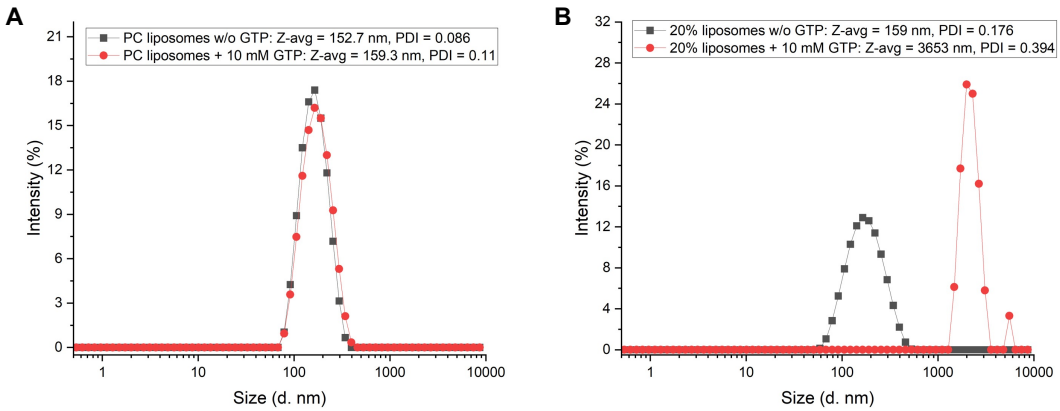


Figure S8. Representative raw distribution curves for PC (**A**) and 20% **BCuDPAL**/PC (**B**) liposomes before and after GTP treatment.

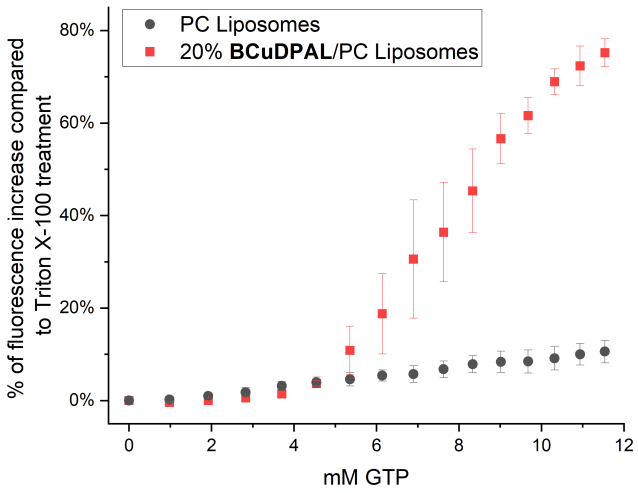


Figure S9. Calcein release profiles for 0% and 20% **BCuDPAL**/PC liposomes upon GTP treatment. Titration curves indicate a dose-dependent increase in calcein fluorescence for 20% **BCuDPAL**/PC liposomes upon GTP treatment, while PC liposomes only showed minimal background leakage.

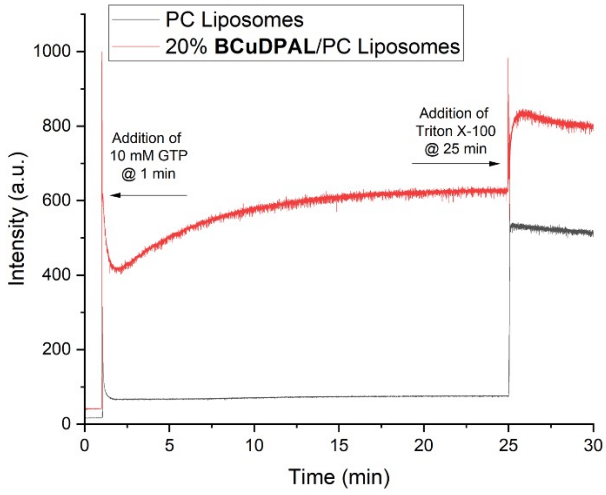


Figure S10. Representative calcein release kinetic curve using 0% and 20% **BCuDPAL**/PC liposomes. Immediate increases in fluorescence were observed after adding 10 mM GTP. Triton X-100 was added at the end of the experiments to calibrate the assay and induce 100% release.

Video S1. Time-course fluorescence microscopy video for 20% **BCuDPAL**/PC liposomes labeled with 0.08% Rd-PE shows the gradual formation of larger particles after GTP addition.

Video S2. Bright field of time-course fluorescence microscopy video for 20% **BCuDPAL**/PC liposomes labeled with 0.08% Rd-PE shows the gradual formation of larger particles after GTP addition.