Supporting Information

Photo-initiated destruction of composite porphyrin-protein polymersomes

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Materials and Methods

Materials. Polyethylene oxide-polybutadiene (PEO_{30}-PBD_{46}, denoted OB29) diblock copolymer was purchased from Polymer Source (Montreal, Quebec, Canada). Horse spleen apoferritin (HSAF), horse spleen ferritin (HSF), density gradient medium (optiprep®, iodixanol solution), bovine serum albumin (BSA), equine skeletal myoglobin (Mb), Dulbecco's phosphate buffered saline (DPBS, + Mg^{2+}, + Ca^{2+}), and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (St. Louis, MO). Cy3 NHS-ester was purchased from GE Healthcare (Piscataway, NJ), BODIPY-FL iodoacetamide from Invitrogen (Carlsbad, CA), and Econo-Pac 10DG columns from Bio-Rad (Hercules, CA). DMSO (ACS grade), methylene chloride (HPLC grade) and sucrose were all purchased from Fisher Chemicals (Pittsburgh, PA). Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride (≥ 98% pure) was purchased from Calbiochem (San Diego, CA). 20,000 Da MWCO Slide-A-Lyzer dialysis cassettes were purchased from Pierce Biotechnology (Rockford, IL). The meso-to-meso ethyne-bridged (porphinato)zinc(II) dimer (PZn_2) was synthesized in the Therien laboratory, where its photophysical properties were previously characterized.[1] All materials were used as received.

Polymersome Assembly. Thin films of polymer were created on roughened Teflon surfaces by depositing 200 μL of a 1 mM solution of OB29 with or without porphyrin dye at a 5:1 molar ratio (polymer:dye) in methylene chloride. Films were dried in a vacuum oven for 12 h and then hydrated with 1 or 2 mL of a 290 mOsm sucrose + PBS buffer with or without protein at 65 °C for 24 h. Vesicle solutions were vortexed after heating.

Vesicle Separation. Vesicles were separated from free protein by diluting 500 μL polymer vesicle solution in 9.5 mL PBS + 1% BSA. This solution was then placed on a 2 mL cushion of sucrose buffer + density gradient medium in a centrifuge tube. The tube was spun at 20,000 rpm for 1 h, and the resulting band of 500 – 1000 μL concentrated solution of vesicles was removed. Samples were then dialyzed into 290 mOsm PBS within 20,000 Da MWCO dialysis cassettes for 12 h at 4 ºC.

Chemical Labeling of HSF, HSAF, BSA, and Mb. The concentration of HSAF, BSA, or Mb was determined by UV-Vis spectroscopy (ε_{280} = 480,000 $M^{-1}cm^{-1}$ for HSAF,[2] ε_{280} = 44,300 $M^{-1}cm^{-1}$ for BSA, and ε_{280} = 15,400 $M^{-1}cm^{-1}$ for Mb). BSA required several FPLC size-exclusion purification steps to eliminate protein aggregates and fragments. The concentration of HSF was determined by Bradford assay. The Cy3 dye was used to label surface lysines on the protein, whereas BODIPY-FL labeled surface cysteines. The protein labeling protocols provided by the manufacturers were followed for both Cy3 and BODIPY-FL conjugations, with the following modifications. For the Cy3 labeling: i) 10 mg/mL protein in 290 mOsm PBS was used, ii) the conjugation reaction was carried out for 2-3 h, and iii) the labeled protein was separated from free dye with 290 mOsm sucrose + PBS buffer. For the BODIPY-FL labeling: i) 500-fold molar excess (20-fold per ferritin subunit) of TCEP was used, ii) 400-fold molar excess (17-fold per ferritin subunit) of dye was used, and iii) the labeled protein was separated from free dye with 290 mOsm sucrose + PBS buffer. Absorbance measurements were made by UV-Vis spectroscopy with an Agilent 8453 spectrophotometer with temperature controller and magnetic stirrer (Agilent 89090A), using a quartz cuvette with 1-cm pathlength. The maximum absorbance of BODIPY-FL and Cy3 are at 502 nm and 550 nm, with molar extinction

coefficients of 76,000 $M^{-1}cm^{-1}$ and 150,000 $M^{-1}cm^{-1}$, respectively. Labeling efficiency for HSAF was quantified as described in the manufacturer protocols.

Fluorescence Recovery After Photobleaching (FRAP). FRAP experiments were carried out on an Olympus Fluoview FV1000 confocal microscope (Center Valley, PA), equipped with a UPLFLN 40x oil objective lens. The FV1000 comes equipped with a SIM scanner system, allowing for confocal imaging and laser stimulation to be carried out independently and virtually simultaneously. A 351-nm laser at 90% power was used to bleach small regions of polymersomes for 10 sec. The fluorescence intensities of the regions were tracked before, during, and after bleaching, using lasers of appropriate wavelengths (488 nm for BODIPY-FL, 543 nm for Cy3, and 633 nm for PZn_2) scanning at 2 μs/pixel. The measured intensities were plotted against time to generate fluorescence recovery curves.

Micropipette Aspiration. Micropipettes made of borosilicate glass tubing (Friedrich and Dimmock, Milville, NJ) were prepared using a needle/pipette puller (Model 720, David Kopf Instruments, Tujunga, CA) and microforged using a glass bead to give the tip a smooth and flat edge. The inner diameter of the pipettes ranged from 4.7 to 10.4 μm, and they were measured using microscopy and computer imaging software. Pipettes were filled with 290-295 mOsm PBS, then the surfaces were blocked with DPBS + 1% BSA for 2 min. The pipettes were then connected to an aspiration station mounted on the side of a Zeiss inverted microscope, equipped with a manometer, Validyne pressure transducer (models DP 15-32, and DP 103-14, Validyne Engineering Corp., Northridge, CA), digital pressure read-outs, micromanipulators (model WR-6, Narishige, Tokyo, Japan), and MellesGriot millimanipulators (course *x, y,* z control). Suction

pressure was applied via a syringe attached to the manometer. Vesicle-containing solutions in sucrose/PBS buffer (290-295 mOsm) were either diluted with an equal volume of 290-295 mOsm PBS or dialyzed overnight into PBS. Pipettes were used to select single vesicles. Pressure was increased stepwise in 5 cmH_2O increments, and the membrane was allowed one min after each pressure change to equilibrate. Experiments were imaged using DIC optics with a 40x objective and a Cohu black-and-white CCD camera (Cohu, Inc., San Diego, CA). ImageJ software was used to measure membrane extensions and vesicle diameters. Calculations of tension and area extension were performed using force balances discussed extensively by Evans and coworkers.[3]

Flow Cytometry. Polymer films were prepared and HSAF was labeled with BODIPY-FL as described. Films were hydrated with 1 mL sucrose + PBS buffer containing 0.5, 1.5, 3.0, 6.0, 10.0, or 15.0 mg/mL of HSAF. Samples were separated by ultracentrifugation as previously described and dialyzed overnight in PBS using 20 kDa MWCO Slide-A-Lyzer dialysis cassettes. A FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) was used to analyze samples. BODIPY-FL intensity was measured using the FL1 channel, and PZn_2 intensity was measured using the FL3 channel. Compensation was achieved using single positive fluorescent samples, and 25,000 events within a gate on vesicles larger than 1 μm diameter were counted for each experiment. Thresholding was set to zero, and all events were recorded during experimentation. Samples were protected from light during all steps of preparation. Analysis of flow cytometry data was performed using CellQuest/FlowJo software (BD Biosciences, Franklin Lakes, NJ/Tree Star, Inc, Ashland, OR).

Dynamic Light Scattering (DLS). Vesicles were prepared as described previously and sized by passing colloidal solutions twenty times through polycarbonate extrusion membranes with pore sizes of 5 μm, 1 μm, or 400 nm. Flow cytometry was performed on the samples, and size distributions of the remaining colloidal solutions were determined using DLS. Vesicles were mixed well in low-volume disposable cuvettes using a pipette. Three runs of 13-15 measurements on a Zetasizer Nano-S Instrument (Malvern Instruments, Southborough, MA) were performed. The results of these runs were averaged using accompanying DTS software (Malvern Instruments, Southborough, MA), and intensity transformations were used to determine particle size distribution. Distributions were normalized by dividing each intensity percentage by the highest value within the sample.

HSAF Release. Four OB29 films were deposited on roughened Teflon squares using the procedure described previously; three of these films contained PZn_2 porphyrin dimer at the 5:1 molar ratio (polymer:dye) used in all other experiments. Films were dried in a vacuum oven for multiple days. After drying, the films were hydrated with the same sucrose+PBS buffer used in previous experiments to make 4 samples: OB29 + 2 mL buffer containing HSAF at 10 mg/mL, OB29 + PZn_2 + 2 mL buffer, OB29+PZn_2 + 2 mL buffer containing HSAF at 10 mg/mL, OB29 + PZn_2 + 1 mL buffer containing HSAF at 10 mg/mL. 200 μL of PBS containing biocytin at 5 mg/mL were added to each sample during hydration. Samples were placed in a 65 ºC oven for 24 h and vortexed for 1 min while hot after heating. Vesicles prepared using this method are approximately 1 to 30 μm in diameter. Free HSAF was separated from samples by centrifugation (described previously). The concentration of polymer is approximately 1-2 mM using this method, and the number of vesicles per unit volume will vary significantly depending

on the size distribution of vesicles. The concentration of biocytin in the hydration solution is 1.3 mM for vesicles hydrated with 2 mL buffer and 2.6 mM for vesicles hydrated with 2 mL buffer. (It is estimated that 700 to 1000 pmol biocytin are encapsulated in 500 µL of vesicles prepared and separated according to this method.) 500 µL of each sample was then diluted in 9.5 mL of PBS containing 1% BSA by weight. A cushion of 80% sucrose buffer/20% optiprep buffer (by volume) was placed at the bottom of each diluted sample. Tubes were spun at 20,000 rpm for 60 min at 4 ºC, and approximately 1.5 mL concentrated polymersomes were removed from the interface of the two buffers. Samples were then placed in 10,000 Da MWCO dialysis cassettes and dialyzed in 4 L PBS. Buffer was changed 24 h into dialysis. The volumes changed during dialysis so that approximately 2-2.5 mL was removed from each dialysis cassette. Samples were analyzed using a UV-Vis spectrometer to evaluate relative concentrations based on the absorbance of the PZn_2. The 4 samples were then put in glass cuvettes with screw-top caps and stir bars. These samples were placed on a stir plate (which agitated the samples very slowly) in front of a mercury arc lamp for 4 h. After 4 h, samples were removed from in front of the arc lamp and analyzed again with the UV-Vis spectrometer. 1.5 mL of each sample was then placed in a 5000 Da MWCO AMICON centrifuge filter tube and spun at 2500x g for 10 min so that each tube contained 250 µL concentrated sample and approximately 1.25 mL filtrate. 100 µL of unseparated vesicles, concentrated sample, and filtrate from each of the 4 samples was added to wells of a black 96-well untreated plate; unseparated vesicles and concentrated sample results were similar (concentrated sample results reported in Figure 4). 25 µL of 10% Triton-X in DI water was added to 100 µL of both unseparated vesicles and concentrated samples to destroy the vesicles and release remaining biocytin. 100 µL of 2x green reagent (biotin quantitation, FluoReporter biotin quantitation assay kit for biotinylated proteins, Invitrogen, Carlsbad, CA)

was added to each well. The solution was allowed to react for 5 min, protected for light. The fluorescence was read at 485/550nm (excitation/emission) using a fluorometer. The reported fluorescence values are based on the average of 25 flashes. The excitation bandwidth was 9 nm, the emission bandwidth was 20 nm, and the integration time was 20 µs.

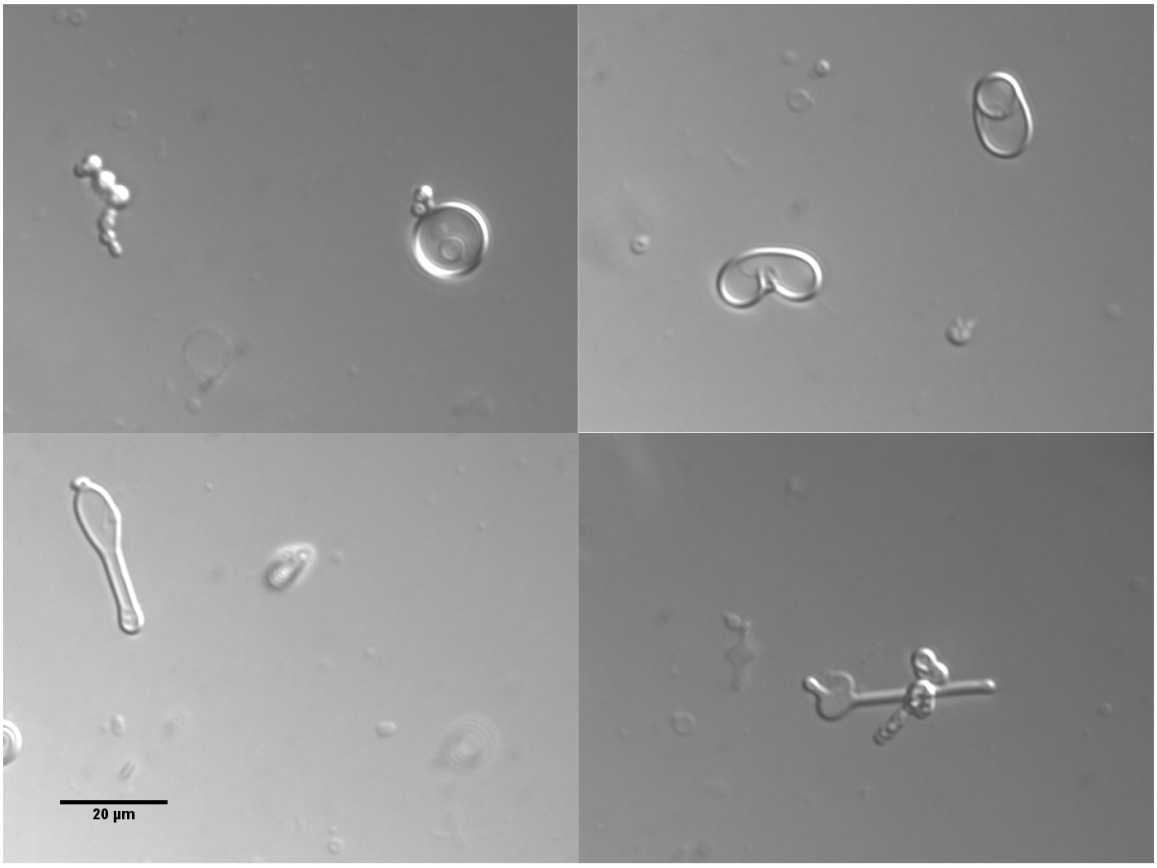


Figure S1: Ferritin-encapsulating polymer vesicles form in a variety of asymmetric shapes. These images were obtained by CLSM using DIC optics. All vesicles are loaded suspended in 290 mOsm solutions osmotically matched to the solutions within the aqueous core.

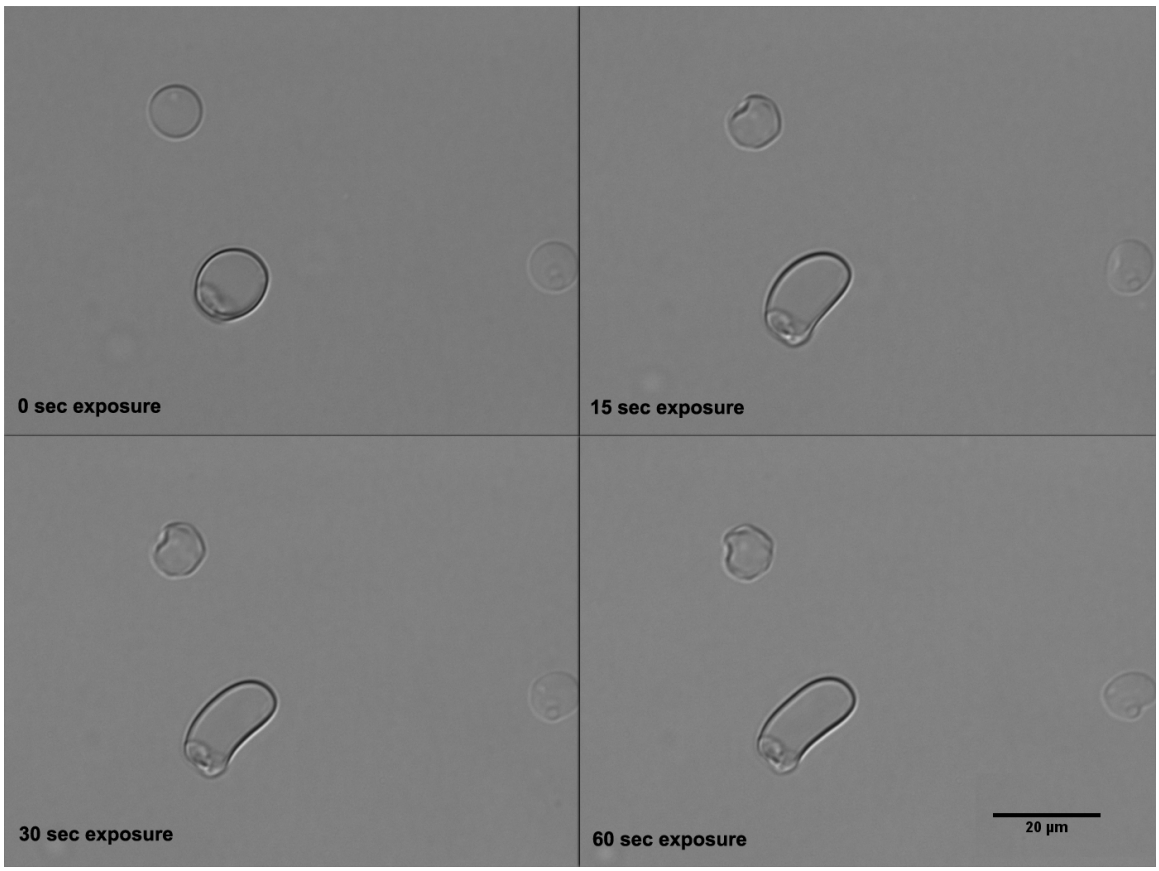


Figure S2: 9.9 mg/mL unlabeled HSAF + PZn_2-labeled vesicle. These images were obtained by CLSM using DIC optics. The timestamp refers to total exposure of these vesicles to a mercury arc-lamp. Both vesicles in these images deform during the periods in which they are exposed to light from the arc lamp.

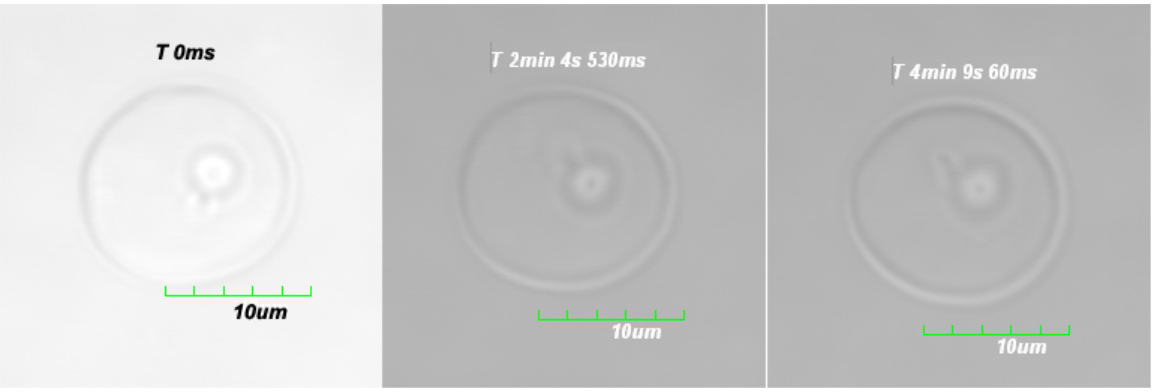


Figure S3: Vesicles produced with 1.5 mg/mL unlabeled HSAF, no PZn_2 (488 nm, 543 nm, 633 nm laser). No light induced destruction was observed.

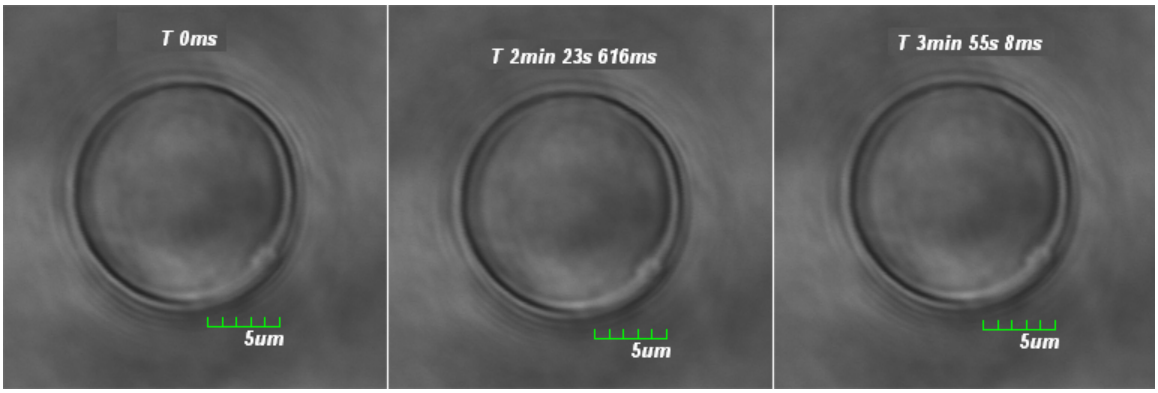


Figure S4: Vesicles produced with 1.5 mg/mL BODIPY-labeled HSAF, no PZn_2 (488 nm, 543 nm, 633 nm laser). No light induced destruction was observed.

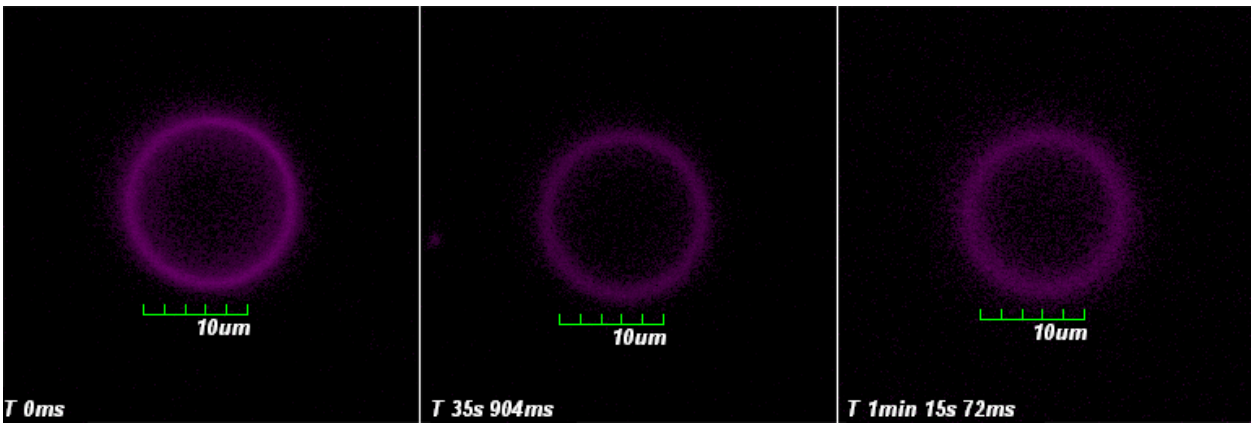


Figure S5: Vesicles produced with PZn_2 at 5:1 molar ratio (polymer:dye), no protein (488 nm, 543 nm, 633 nm laser). No light induced destruction was observed.

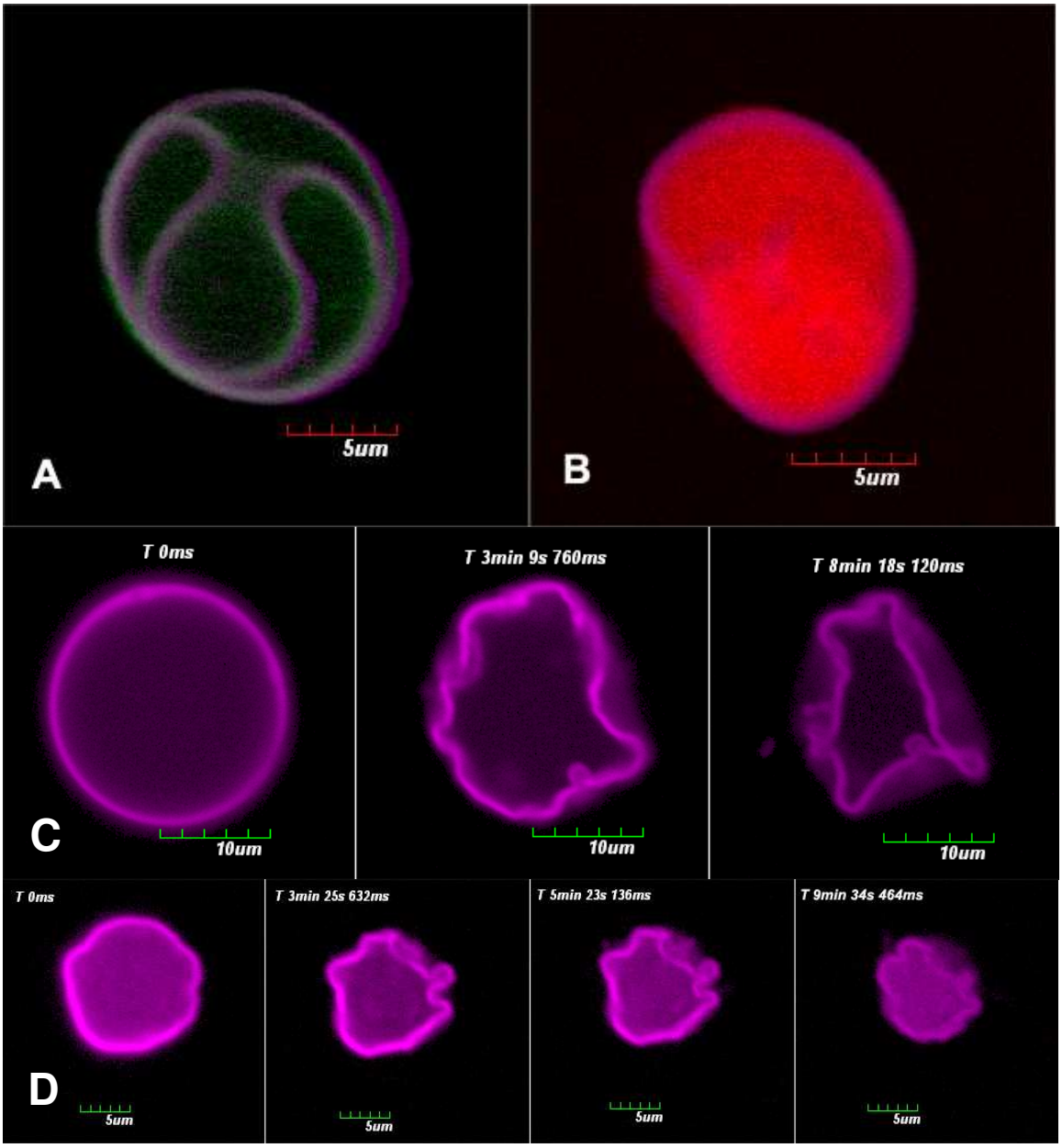


Figure S6. Overlay images of PZn_2 encapsulated in the membrane bilayer (purple) and either BODIPY-FL (green)-labeled (apo)ferritin or Cy3 (red)-labeled (apo)ferritin in the vesicle. (A) BODIPY-FL-labeled ferritin (1.5 mg/mL) localizes almost completely at the membrane. (B) Cy3-labeled ferritin (1.5 mg/mL) is diffuse throughout the aqueous core. (C) unlabeled HSAF (9.9 mg/mL) + PZn_2 vesicle. This vesicle was imaged using three lasers (488 nm, 543 nm, 633 nm) on the CLSM. The three images are stills (proceeding in time from left to right, as indicated by the timestamp) during which the vesicles were continuously scanned. The purple color denotes PZn_2. (D) myoglobin (10 mg/mL) + PZn_2 vesicle. This vesicle was imaged using three lasers (488 nm, 543 nm, 633 nm) on the CLSM. The three images are stills (proceeding in time from left to right, as indicated by the timestamp).

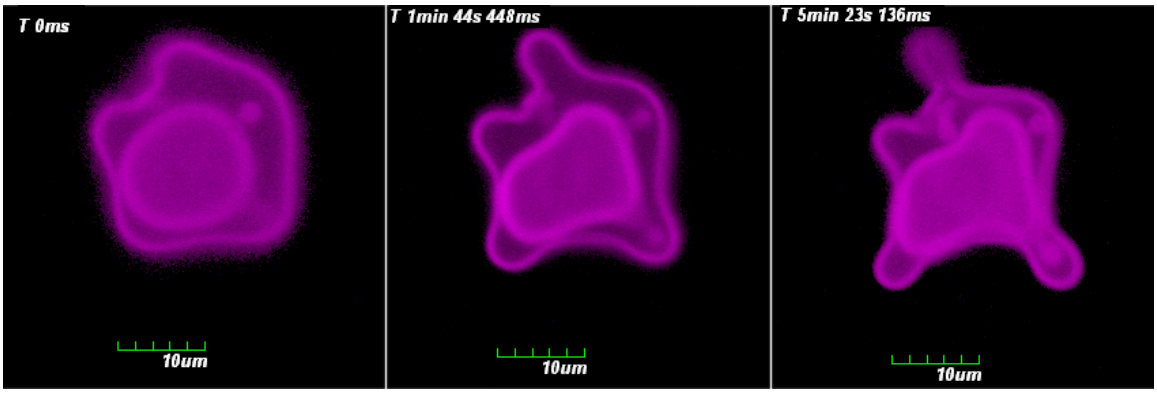
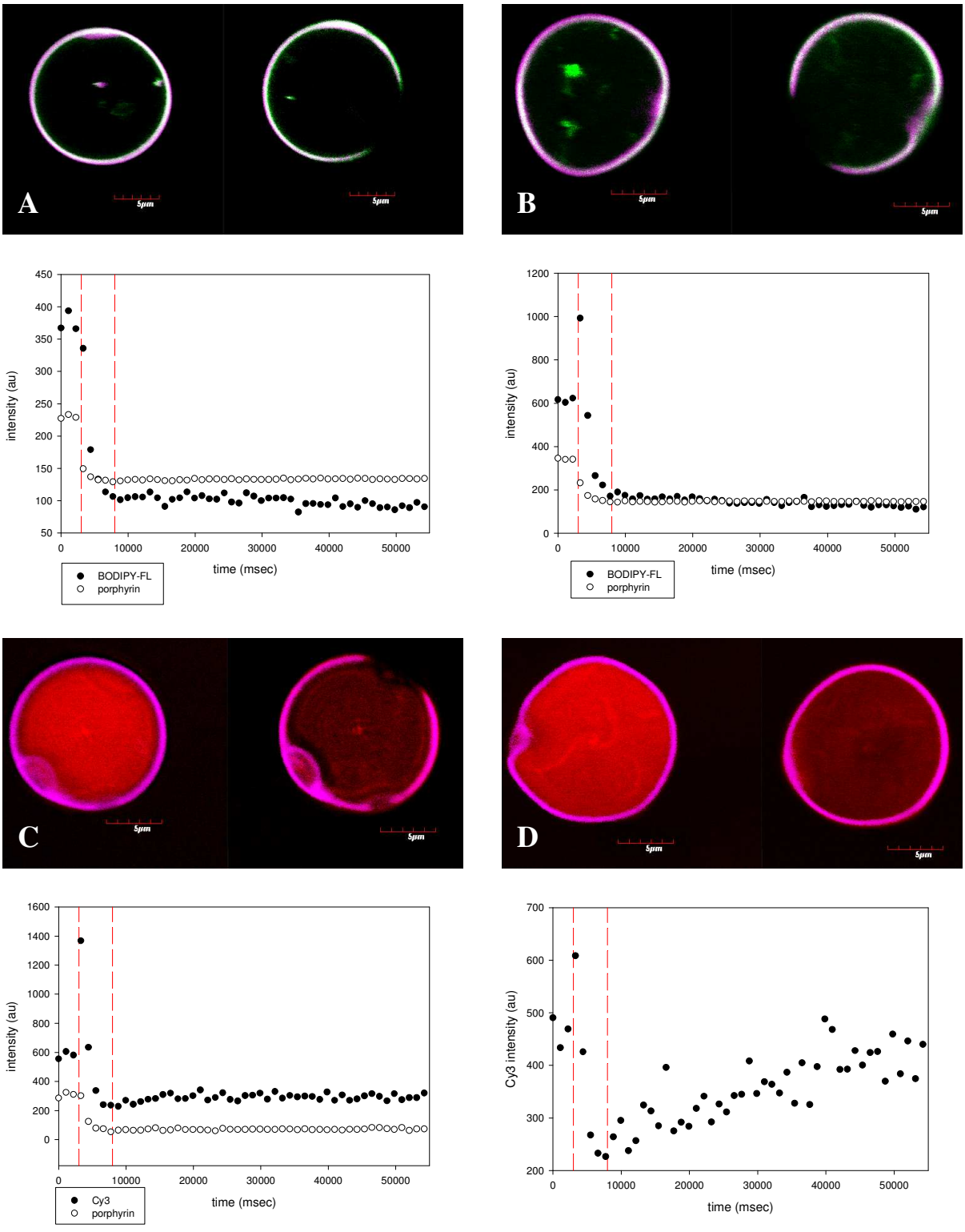


Figure S7: 9.9 mg/mL BSA + PZn_2-labeled vesicle. This vesicle was imaged using three lasers (488 nm, 543 nm, 633 nm) on the CLSM. The three images are stills (proceeding in time from left to right, as indicated by the timestamp). This vesicle represents the largest magnitude of shape change that we observed in BSA + PZn_2 vesicles.


A
B
C
D
5μm
intensity (au)
time (msec)
BODIPY-FL
porphyrin
Cy3
Cy3 intensity (au)

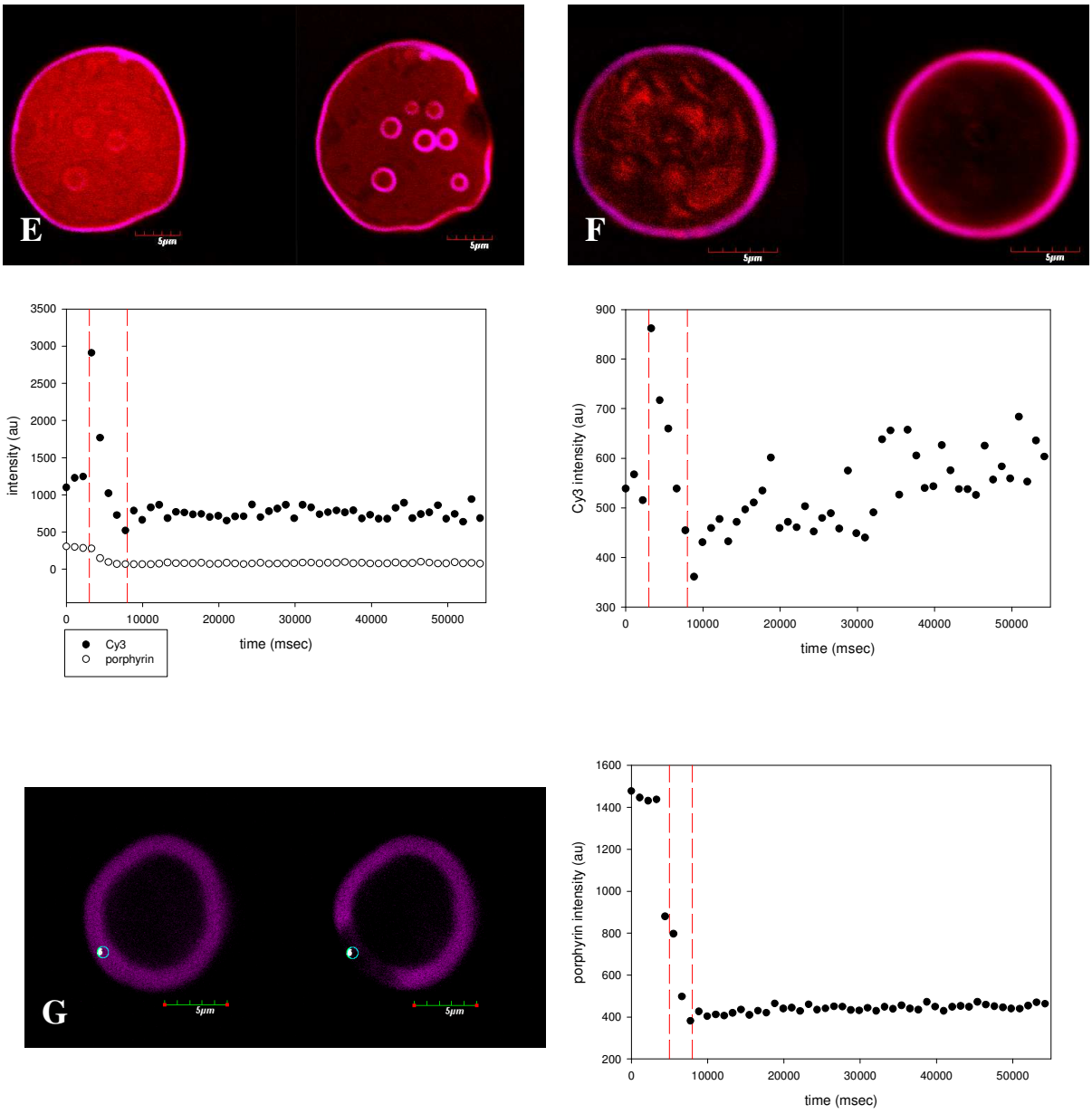


Figure S8: FRAP results: pre-FRAP experiment and post-FRAP experiment image and corresponding fluorescent intensity versus time plots. All samples incorporate PZn_2 in the membrane bilayer at 5:1 polymer:dye molar ratio and ferritin at 1.5 mg/mL in hydration solution. The samples tested incorporated HSF or HSAF labeled with BODIPY-FL or Cy3. (a) BODIPY-FL-apoferritin, membrane bleach (b) BODIPY-FL-ferritin, membrane bleach (c) Cy3-apoferritin, membrane bleach (d) Cy3-apoferritin, aqueous core bleach (e) Cy3-ferritin, membrane bleach (f) Cy3-ferritin, aqueous core bleach (g) control (no ferritin), membrane bleach. Bleaching occurred during the time period within the vertical lines.

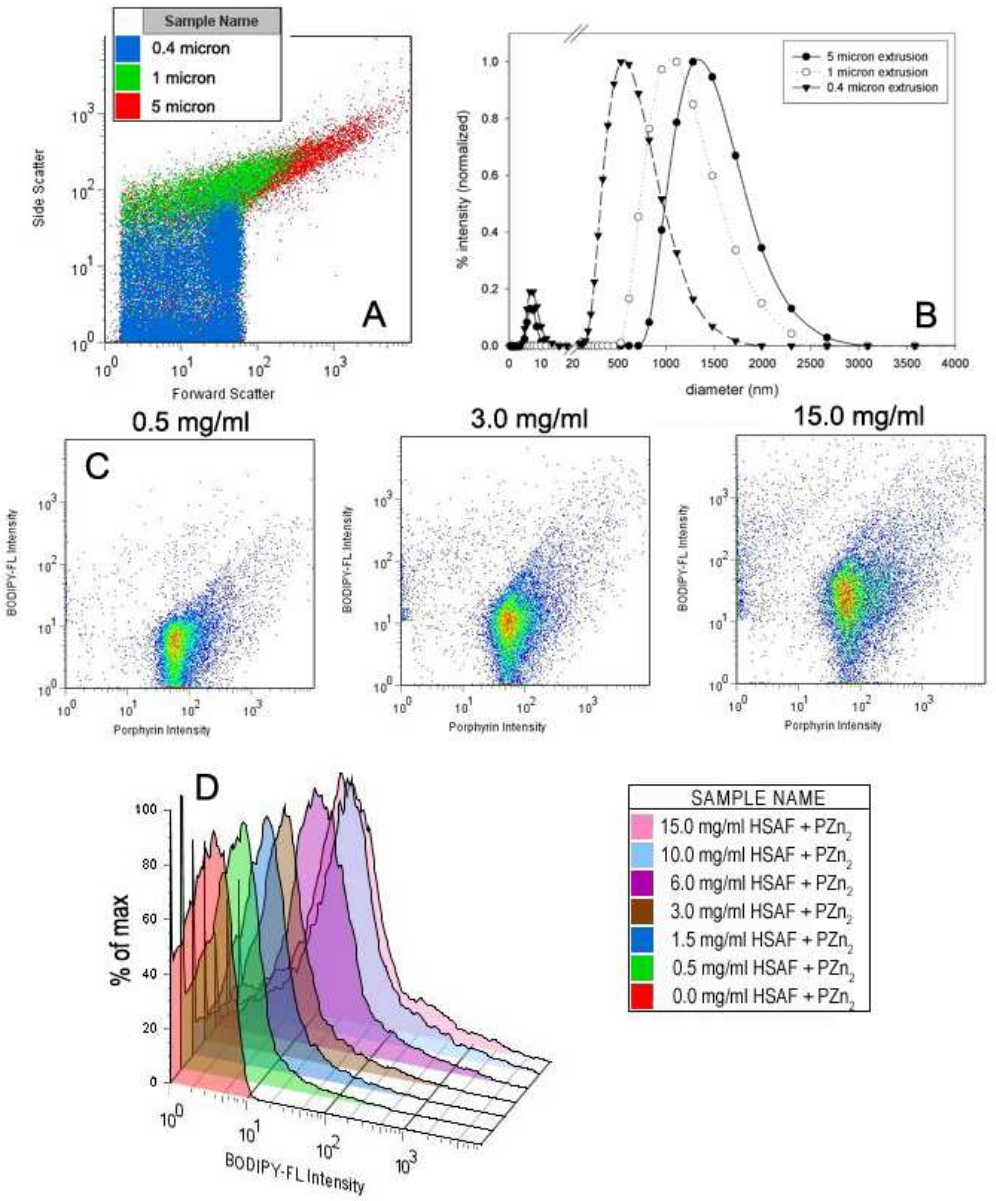


Figure S9: Forward scatter vs. side scatter for three populations of vesicles (A). Dynamic light scattering (DLS) shows size distribution for these populations (B). The blue, square-shaped region on the dot plot is excluded from calculations because it is impossible to distinguish sub-500-nm vesicles from free protein, amorphous polymer aggregates, and other debris. Three dot plots of porphyrin intensity (membrane surface area) versus BODIPY-FL fluorescence (corresponding to ferritin concentration) show that the average concentration of ferritin encapsulated within a population of vesicles increases with increasing concentration of ferritin in the hydration solution. The scatter also increases, which indicates a heterogeneous distribution of ferritin in a sample of similar size vesicles (C). A histogram of BODIPY-FL intensities for the different samples supports this finding, and the lack of a shift in the location of the peak from 10.0 mg/mL to 15.0 mg/mL ferritin indicates that there may be a limit in the amount of ferritin that a population of vesicles can incorporate (D).

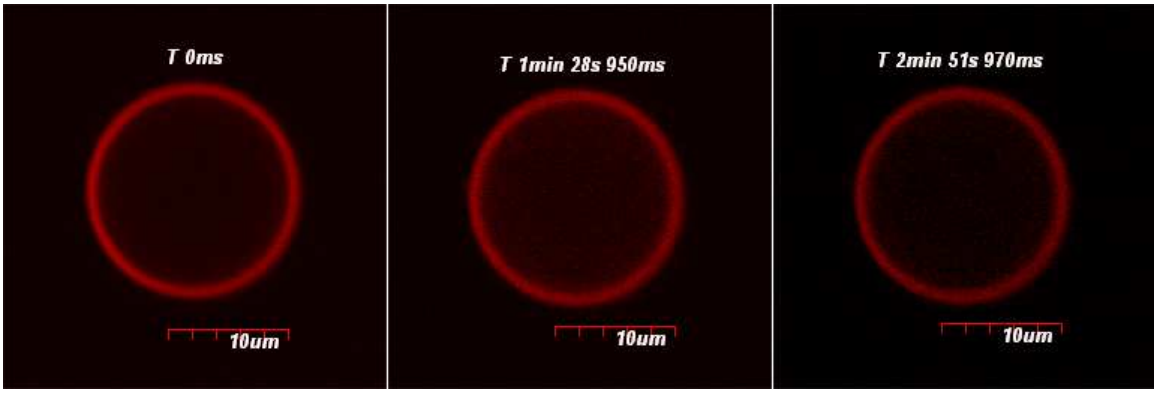


Figure S10: 1.5 mg/mL unlabeled apoferritin + Nile Red-labeled vesicle. This vesicle was imaged using three lasers (488 nm, 543 nm, 633 nm) on the CLSM. The three images are stills (proceeding in time from left to right, as indicated by the timestamp). We observed no changes in vesicle structure in this or any other vesicles in this sample.

References

(1) Duncan, T. V.; Susumu, K.; Sinks, L. E.; Therien, M. J. *J. Am. Chem. Soc.* **2006**, *128*, 9000-9001.
(2) Barnes, C. M.; Theil, E. C.; Raymond, K. N. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5195-5200.
(3) Evans, E. A. *Biophys. J.* **1973**, *13*, 941-954.