Electronic Supplementary Information (ESI) for

# Employing Bicontinuous-to-Micellar Transitions in Nanostructure Morphology for On-Demand Photo-Oxidation Responsive Cytosolic Delivery and off-on Cytotoxicity

Sharan Bobbala,†[a] Sean D. Allen,†[b] Sijia Yi,[c] Michael Vincent,[a] Molly Frey,[b] Nicholas B. Karabin,[a] and Evan A. Scott [abcd]*

[a]Department of Biomedical Engineering, Northwestern University, Evanston Illinois 60208, USA.

[b]Interdepartmental Biological Sciences Program, Northwestern University, Evanston, Illinois 60208, USA.

[c]Chemistry of Life Processes Institute, Northwestern University, Evanston, Illinois 60208, USA.

[d]Simpson Querrey Institute, Northwestern University, Chicago, Illinois 60611, USA.

* Corresponding Author: evan.scott@northwestern.edu

† These authors contributed equally to this work.

## Experimental Section

### Materials

Unless specified, all materials were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pheophorbide A and Camptothecin were obtained from Cayman Chemical (Ann Arbor, MI, USA). Self-Quenched BODIPY FL Conjugate of BSA (BODIPY-BSA) was acquired from BioVision, Inc (Milpitas, CA, USA).

### Synthesis of PEG-*b*-PPS Copolymer

$PEG_{17}$-*b*-$PPS_{75}$ was synthesized as described previously.[14] In brief, methoxy-PEG (Mn 750 g/mol) was modified with mesyl chloride to create methoxy-PEG mesylate. The mesylate leaving group was displaced by thioacetic acid to form methoxy-PEG thioacetate. Methoxy-PEG thioacetate (1 eq) was dissolved in 10 mL anhydrous dimethylformamide (DMF) in a schlenk flask under argon. 0.5 M sodium methoxide in methanol (1.1 eq) was added to produce a thiolate anion, and propylene sulfide (75 eq) was added. Ring opening polymerization driven by the thiolate anion as the initiator proceeded for 10 minutes before benzyl bromide (10 eq) was added to end-cap the polymer chains. The reaction proceeded for 4 hours before the DMF was removed by rotary evaporation at 60 °C. Crude product was precipitated in methanol to remove impurities. Methanol was decanted, and the product was dried in a vacuum desiccator.

### Formation of PEG-*b*-PPS BCNs

BCNs were formed by flash nanoprecipitation (FNP) technique using a hand-driven CIJ mixer as described previously.[3, 14] 10 mg of PEG-*b*-PPS polymer was dissolved in 500 μL of tetrahydrofuran (THF) and was impinged against 500 μL of Milli-Q water. After impingement the nascent BCNs were diluted in a 1.5 mL reservoir of Milli-Q water. THF was removed by vacuum desiccation overnight, resulting in a 5 mg/mL BCN formulation in water. In cases where hydrophobic cargo was loaded into BCNs, the cargo was dissolved along with the 10 mg of PEG-*b*-PPS polymer in the 500 μL of THF used for impingement. In cases where hydrophilic cargo was loaded into BCNs, the cargo was dissolved in the 500 μL of water used for impingement.

**Loading and determination of encapsulation efficiency**

The loading of hydrophilic molecules (Calcein, FITC-BSA, BODIPY-BSA) or hydrophobic molecules (Pheophorbide A or Camptothecin) in to BCNs was performed using FNP technique as stated above. Here, 500 μL of Calcien (1 mM) or fluorophore-BSA (2 mg/mL) in water was impinged against 500 μL of Pheophorbide A (0.2 mg/ml) or Camptothecin (0.6 mg/ml) in THF into an aqueous reservoir to form BCNs. After removal of THF *via* desiccation, BCNs were washed two times with distilled water by centrifugation (10,000 x g, 10 min) to remove all the unencapsulated payloads and further resuspended in PBS or water for analysis. The amount of Pheophorbide A was determined by calculating absorbance at 666 nm. Calcein, FITC/BODIPY-BSA, and Camptothecin loading was analyzed by measuring fluorescence intensities at 470/509, 495/525, and 359/434 (excitation/emission, nm), respectively.

**Size and Morphological characterization**

The size of nanostructures was measured using Dynamic light scattering (DLS) on Zetasizer Nano-ZS (Malvern Instruments, UK). All DLS measurements were performed after 1 in 1000 dilution of samples with PBS.

Transmission electron microscopy (TEM) studies were performed using a 1.0% uranyl formate (UF) in water as negative stain. The stain was adjusted to pH 4.5 by adding 2 μl of 10 N KOH/1 mL of UF. 3 μl of nanoparticle sample (5 mg/mL polymer concentration) was applied to glow discharged carbon-coated copper grids (400-mesh). Samples were passed through two 30 μl volumes of water, and were subsequently negative-stained via passage through two 30 μl volumes of 1% UF. Samples were blotted with Whatman filter paper to remove excess stain. Roughly 0.5 μl stain remains on the grid following this procedure with an activity of $2.55 \times 10^{-5}$ μCi/grid. Images were acquired at 30,000X on a JOEL 1400 Transmission Electron Microscope operating at 120 kV.

Small angle X-ray scattering (SAXS) experiments were performed at the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) beamline at Argonne National Laboratory's Advanced Photon Source (Argonne, IL, USA) with 10 keV (wavelength λ = 1.24 Å) collimated X-rays. All the samples were measured in the q-range 0.001 to 0.5 $Å^{-1}$ and silver behenate was utilized to calibrate the q-range. The data reduction was made using PRIMUS 2.8.2 software, where the final scattering curve was obtained after subtraction of solvent buffer scattering. The micelle model fitting was performed using SasView.[15]

***In vitro* Singlet oxygen generation studies**

*In vitro* singlet oxygen generation was determined by chemical oxidation of 1,3-Diphenylisobenzofuran (DPBF). A stock solution (2 mM) of DPBF was prepared in dimethyl sulfoxide and then diluted to 100 μM using water for further analysis. A 1:1 ratio of DPBF and samples (6 μg/mL of free PhA or 6 μg/mL PhA loaded BCNs or Blank BCNs) were prepared. A 200 μL of this mixture was transferred in to a 96 well black plate and then irradiated immediately using Max-303 Xenon Light Source (385 to 740 nm, Asahi Spectra) at different light intensities (5 and 50 $mW/cm^2$) for 10 min. At predetermined time intervals DPBF absorbance was measured at 415 nm and singlet oxygen generation was then calculated as percentage reduction in DPBF absorbance as compared to the initial DPBF absorbance.

***In vitro* BCN degradation and Release of payloads**

*In vitro* degradation studies of PhA BCNs were performed under irradiation. Briefly, 200 μL of PhA BCNs (1 mg/ml) was transferred in to multiple wells of a 96 well black plate and then irradiated for 2.5 min at 50 $mW/cm^2$. The irradiated samples were immediately collected and centrifuged at 10,000 x g for 10 min. After centrifugation, the supernatants and pellets were collected separately and analyzed using DLS and TEM as described above.

*In vitro* release of calcein, FITC-BSA, ethyl eosin and PhA from BCNs was measured after irradiation. 200 μL of PBS containing PhA BCNs co-loaded with calcein or FITC-BSA, or PhA BCNs and ethyl eosin BCNs, were transferred in to multiple wells of a 96 well black plate and then irradiated at 50 $mW/cm^2$ for 1.25, 2.5, 5 and 10 minutes. Post irradiation samples were centrifuged at 10,000 x g for 10 min and supernatants were collected and further analyzed for amount released as described earlier. For PhA BCNs and ethyl eosin BCNs, % transmittance was measured using an M3 plate reader.

**Cell culture**

RAW 264.7 cells (murine macrophage cell line) were acquired from American Type Culture Collection (ATCC, Rockville, MD, USA) were employed for cell culture experiments. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 °C in the presence of air (95%) and $CO_2$ (5%).

**Cellular uptake studies**

RAW 264.7 cells ($2.5 \times 10^5$ cells/mL, 500 μL) were seeded in each well of a 48-well plate and adhered overnight. The adhered cells were added with 50 μL of free BODIPY-BSA (200 μg/mL) or BODIPY-BSA BCNs (PBS) and incubated for 4h. The polymer and BODIPY-BSA concentration in each well were calculated as 0.5 mg/mL and 20 μg/mL, respectively. After incubation, cells were washed two times with PBS, collected in to flow cytometry tubes and incubated with 50 μL Zombie Aqua (1:100) fixable cell viability dye (Biolegend, San Diego, CA) for 15 min at 4 °C. Then the cells were washed with 500 μL PBS, resuspended in cell staining buffer and analyzed using a BD Fortessa flow cytometer. The cellular uptake was measured as median fluorescence intensity (MFI) in the FITC channel, demonstrating the amount of BODIPY-BSA taken up by each cell.

**MTT assay**

RAW 264.7 cells ($2 \times 10^5$ cells/mL, 100 μL) were plated in each well of a black 96-well plate and adhered overnight. The adhered cells were treated with free PhA (DMSO: PBS, 1:3) or PhA loaded BCNs (PBS) or CT BCNs or PhA-CT BCNs and incubated for 4h. After incubation, cells were immediately irradiated with at a light intensity of 50 mW/cm$^2$ for 1.25 and 2.5 minutes. Post irradiation, cells were incubated for predetermined time intervals and then added with MTT (5 mg/mL in PBS, 10 μL). After 4h MTT incubation with cells, formazan crystal deposition in each well was dissolved in DMSO (200 μL) and the absorbance was measured at 560 nm. All the samples were analyzed in quadruplicates.

The percentage cell viability was calculated as:

% cell viability = (OD of treated sample/ OD of untreated sample) * 100.

**Intracellular ROS generation**

2',7'–dichlorofluorescin diacetate (DCFDA) assay was utilized to measure intracellular ROS generation. Briefly, RAW 264.7 cells ($2 \times 10^5$ cells/mL, 100 μL) were seeded in each well of a black 96-well plate and adhered overnight. The adhered cells were incubated with free PhA (DMSO: PBS, 1:3) or PhA loaded BCNs (PBS) or left untreated. After 4 h incubation, cells were washed two times with PBS and incubated with

DCFDA (10 µM) for 45 min in the dark. Cells were then rinsed with PBS, irradiated (50 mW/cm$^2$, 2.5 min) and fluorescence was measured (ex/em, 485/535 nm) immediately using a microplate reader.

**Confocal microscopy**

RAW 264.7 cells (1 × $10^5$ cells/mL, 300 µL) were seeded in each well of an 8-well Chamber slide (Thermo Fischer Scientific) and adhered overnight. The adhered cells were incubated with FITC-BSA loaded BCNs or PhA co-loaded with FITC-BSA BCNs for 4h. After incubation, cells were washed two times with PBS and then added with 300 µL DMEM. Then the 8-well slides were irradiated at a light intensity of 50 mW/cm$^2$ for 2.5 min or kept under dark conditions. Post irradiated cells was then washed with PBS, added with 300 µL PBS and incubated with NucBlue™ Live ReadyProbes™ Reagent (nuclei stain, 1 drop) for 15 min in the dark. A similar procedure was followed for control slides that were kept under dark conditions. Wherever required, Invitrogen™ Lysotracker (Lysotracker Red™ DND-99 or Lysotracker Blue™ DND-22, 1 in 2000 dilution in DMEM) was incubated with cells for 45 min to stain lysosomes. The 8-well slides were then imaged within a humidified chamber using a 63× oil-immersion objective on a SP5 Leica Confocal Microscope using HyD detectors and lasers. Data analysis was performed using ImageJ Software.

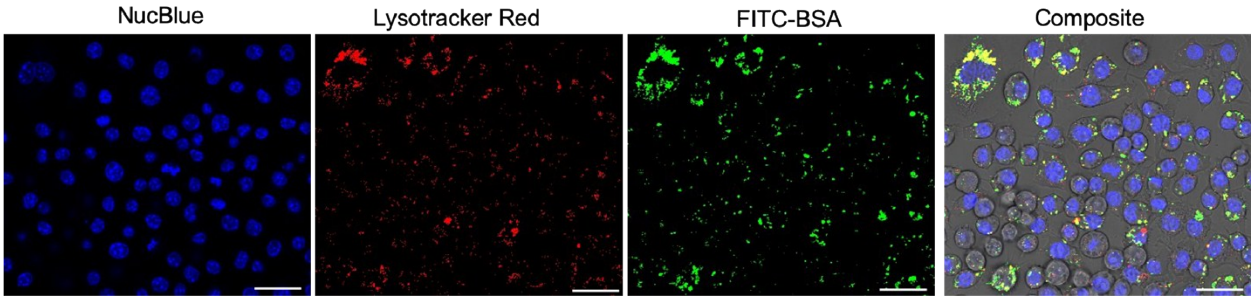


**Figure S1.** A representative confocal image of RAW 264.7 cells 44H after a 4H of incubation with FITC-BSA BCNs and subsequent washing. All cells were stained with NucBlue for nuclei and LysoTracker Red for lysosomes. Scale bar = 40 µm.

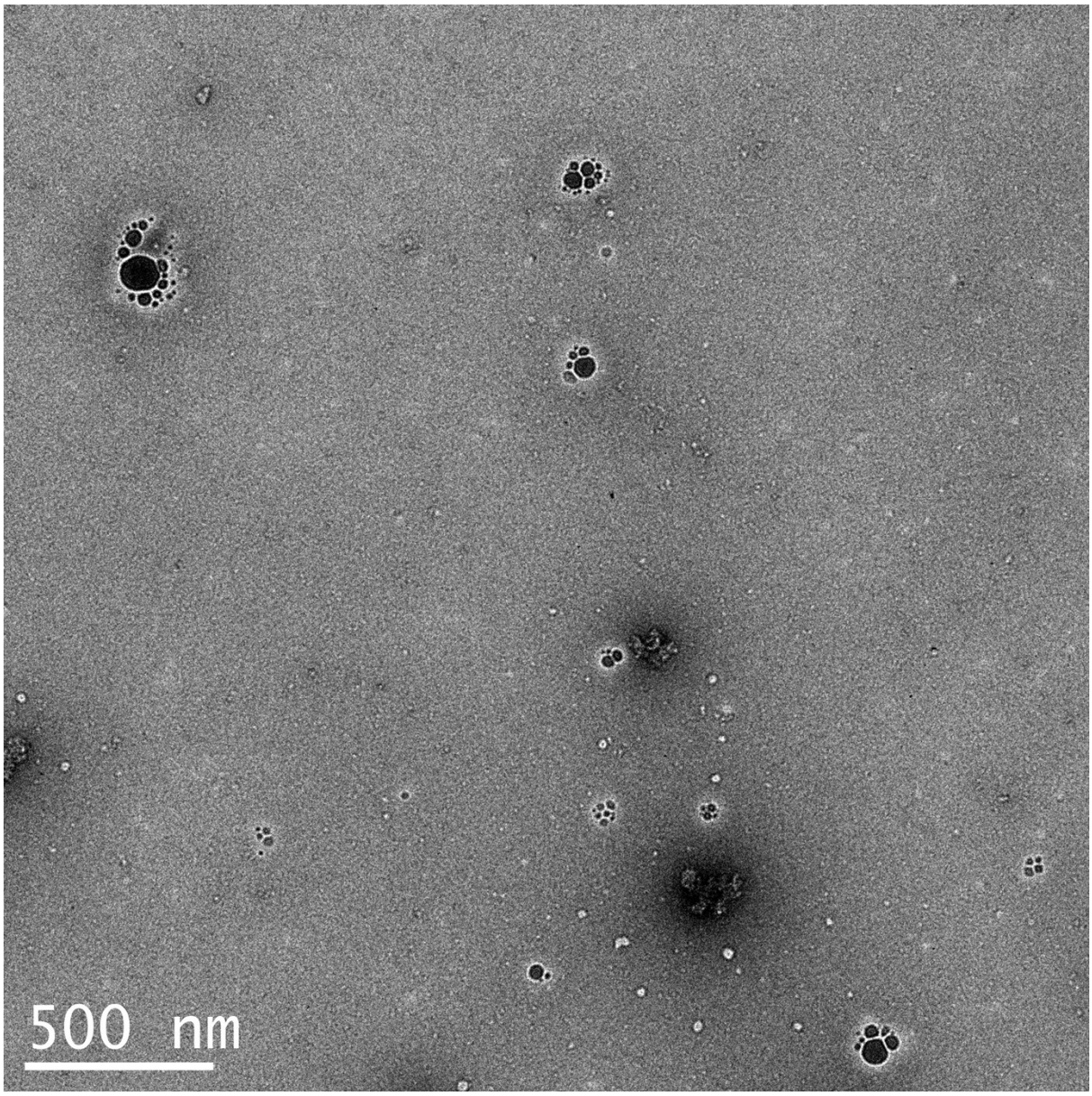


**Figure S2.** Low magnification negative-stain TEM micrograph BCNs. Scale bar = 500 nm.

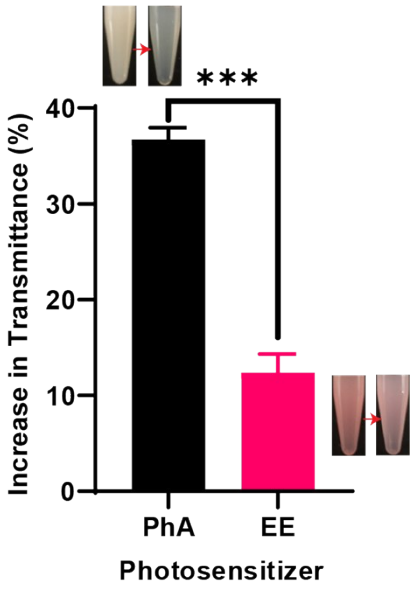


**Figure S3**. Change in turbidity of BCN solution after irradiation. A comparison between loaded PhA and ethyl eosin (EE) in their capacity to induce an increase in transmittance compared to non-irradiated controls. Also included are photographs of the solutions before and after irradiation (left to right). *n*=3, error bars = s.d., *** $p < 0.001$

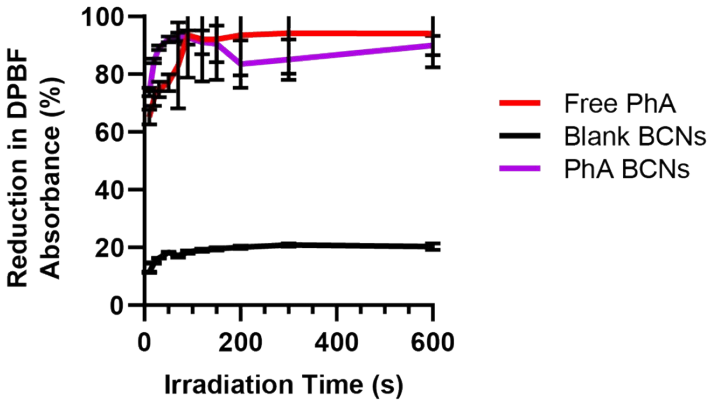


**Figure S4**. Generation of singlet oxygen assayed by percent decrease in DPBF absorbance for free solubilized PhA, blank BCNs, and PhA BCNs. *n* = 4, error bars = s.d. Irradiated at 50 mW/cm$^2$.

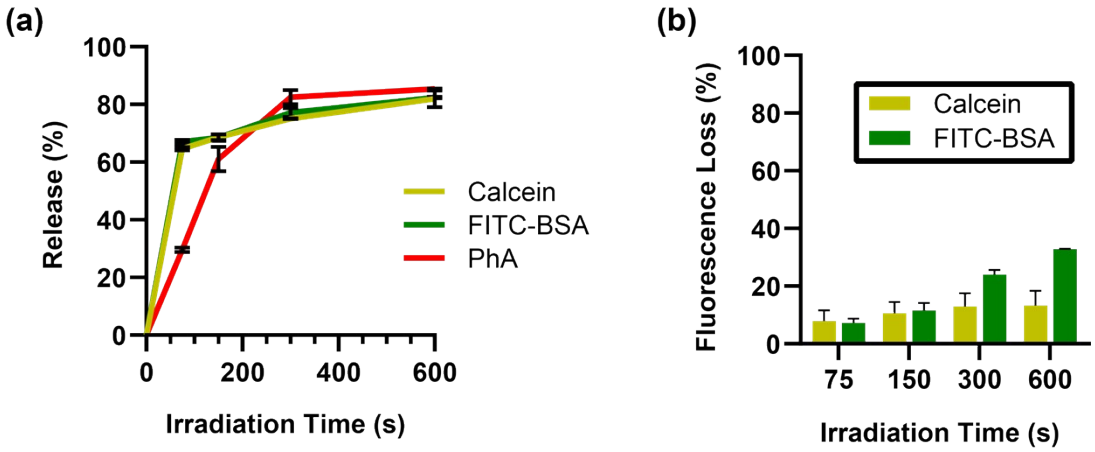


**Figure S5**. Photo-oxidation induced release of hydrophilic cargo. (a) Release of calcein, FITC-BSA, and PhA from BCNs after photo-irradiation for varying times (*n* = 4, error bars = s.d.) compared to non-irradiated samples. (b) Fluorescence loss represents the percent decrease in the fluorescence compared to non-irradiated samples. n = 4, error bars = s.d.

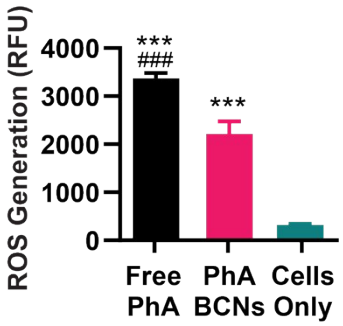


**Figure S6**. ROS generation upon irradiation of cells. Intracellular ROS generation measured by increase in fluorescence of DCFDA. RAW 264.7 cells were left untreated or were treated with free PhA or PhA BCNs and were irradiated for 2.5 minutes.

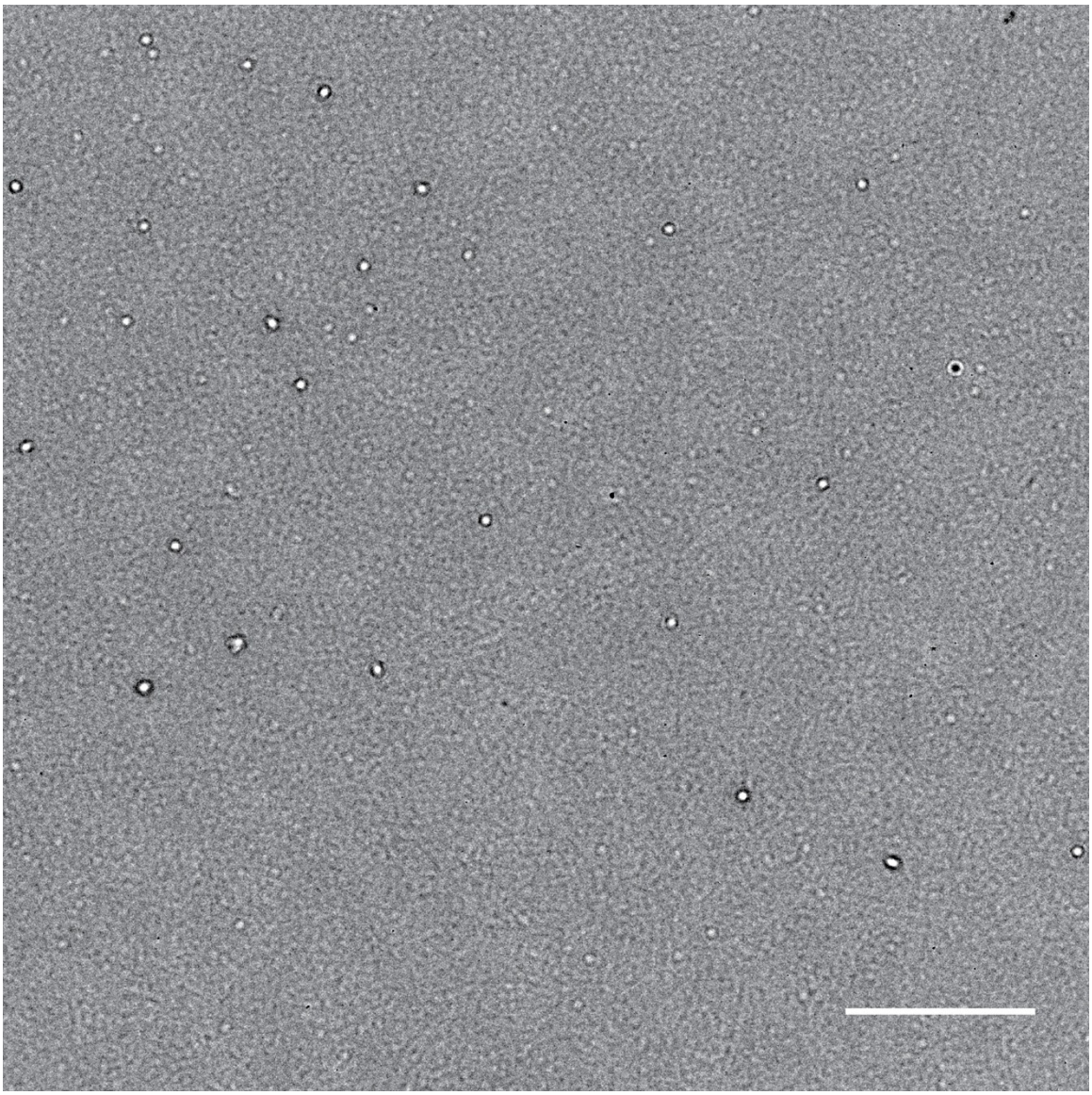

**Figure S7.** Low magnification negative-stain TEM micrograph of irradiated supernatant. Scale bar = 1 µm.

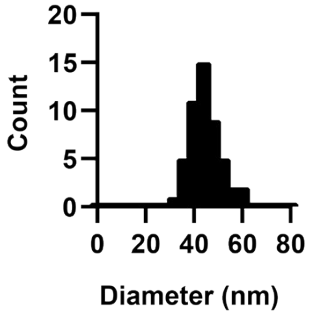


**Figure S8**. Distribution of micelle diameters measured from TEM micrographs of irradiated supernatant. n = 100 measurements, three separate micrographs.

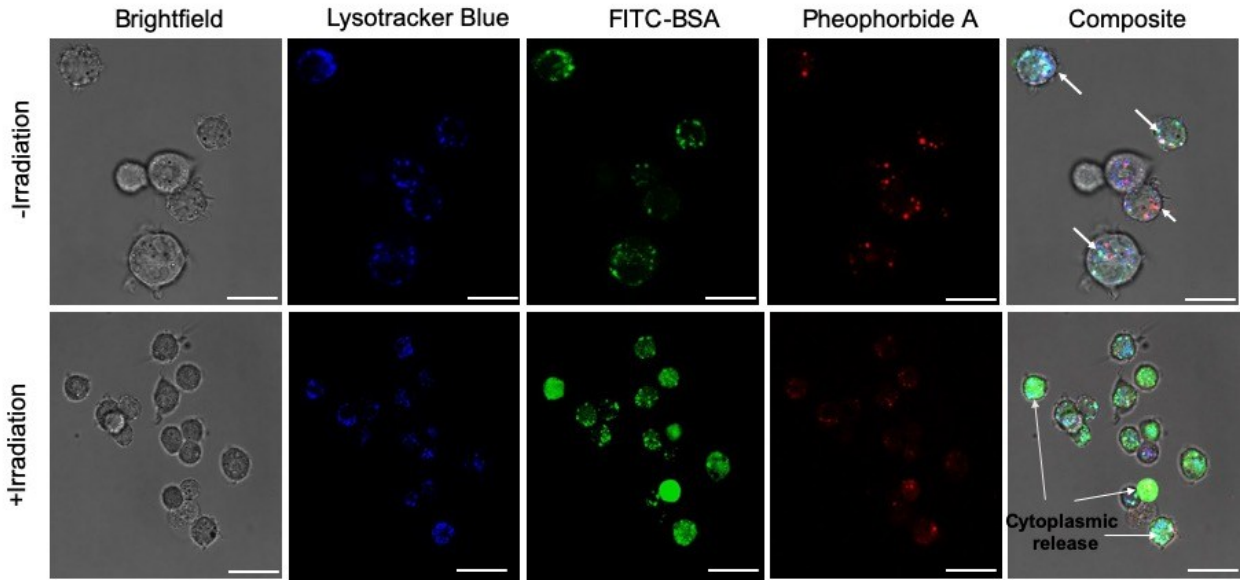


**Figure S9.** Confocal images of Pheophorbide A-BCNs loaded with FITC-BSA, internalized by RAW 264.7 cells, with or without irradiation. All cells were stained with Lysotracker Blue for lysosomes. White arrows in the top row represent colocalization signal of lysotracker with FITC-BSA and Pheophorbide A. Scale bar is 25 μm.