Repeatable and adjustable on-demand sciatic nerve block with phototriggerable liposomes

Alina Y. Rwei, Jung-Jae Lee, Changyou Zhan, Qian Liu, Meryem T. Ok, Sahadev A. Shankarappa, Robert Langer, Daniel S. Kohane

Supporting Information Appendix

Materials and Methods

Synthesis of PdPC(OBu)$_8$. The synthesis of PdPC(OBu)$_8$ was based on the previously reported metal-insertion reaction (1). A mixture of 1,4,8,11,15,18,22,25-octabutoxy-29H,31H-phthalocyanine (Aldrich, Milwaukee, WI) and $PdCl_2$ (Aldrich, Milwaukee, WI) at a 1:3 molar ratio was dissolved in anhydrous dimethylformamide and purged with nitrogen for 30 min. The solution was stirred at 120^{o}C for 24 h. Purification was performed by the addition of excess H_2O. The filtered solid was vacuum-dried and characterized by UV-Vis absorption, λ_{max} 722 nm in toluene and 729 nm in ethanol. The yield was 71%.

Singlet Oxygen Detection. A singlet oxygen quencher, 9,10-dimethylanthracene (Sigma-Aldrich, Milwaukee, WI , DMA), was dissolved in dimethyl sulfoxide (DMSO) and injected into a liposomal solution to have a final DMA concentration of 5 μM. The concentration of DMSO in the liposomal solution did not exceed 1% vol/vol. The fluorescence emission at 430 nm (excitation: 375 nm) was monitored after irradiating the solution with a 730 nm laser at 50 mW/cm^2 for 1 min.

Liposome Characterization. Liposome size was determined with Beckman Coulter Multisizer 3. Liposomal TTX content was determined by ELISA (Reagen, Moorestown, NJ) after removing the lipid fraction using the Bligh and Dyer method (2). Liposomal sulforhodamine B content was determined by UV-Vis absorption (λ_{max} = 565 nm) after disrupting the liposomes with octyl β-D-glucopyranoside (OGP) (Sigma-Aldrich, Milwaukee, WI) (3). Liposomal PdPC(OBu)$_8$ content was determined by UV-Vis absorption at 729 nm after disrupting the liposomes in ethanol.

Spectrophotometric Determination of Lipid Peroxidation. Lipid peroxidation was evaluated by assaying the formation of conjugated dienes, which absorb UV light at wavelengths 230 – 235 nm in organic solvents (4, 5) and 238 nm in lipids (6). The phototriggered lipid peroxidation reaction was measured in both ethanol, where the lipid and PS were dispersed, and in PBS, where PS was encapsulated in the lipid bilayer (Lipo-PS). The molar ratio of the lipid and PS were the same in both studies (13:1 [DLPC: PdPC(OBu)$_8$]). The solution was irradiated at 730 nm, 50 mW/cm^2, and the absorption at 230 – 240 nm was monitored.

In Vitro Release of Fluorescent Dye. Self-quenching sulforhodamine B was used as a hydrophilic model dye. A calibration curve of fluorescent intensity with respect to sulforhodamine B concentration in PBS was made (Figure S7) and the linear range at low sulforhodamine B concentrations was determined (Figure S7B). To measure their phototriggered release, sulforhodamine B liposomes were diluted in PBS to a bulk dye concentration of 10 μg/mL and irradiated with a 730 nm laser (150 mW/cm^2) at 37^{o}C for

the reported duration. The fluorescent intensity (excitation/emission: 560/580 nm) was recorded. The release of dye from liposomes upon irradiation was quantified according to the following equation:

$$\text{Normalized Cumulative Release} = \frac{(F - F_0)}{(F_{break} - F_0)}$$

F = Fluorescence of solution upon irradiation

F_0 = Fluorescence of liposome solution prior to irradiation

F_{break} = Fluorescence of surfactant (octyl β-D-glucopyranoside)-disrupted liposome solution

To determine the oxygen dependency of the phototriggered release, a deoxygenated liposome solution was made by purging the liposome solution with nitrogen for 30 min, after which the solution was irradiated with a 730 nm laser at room temperature (50 mW/cm^2, 5 min). The solution was sealed during irradiation to prevent atmospheric oxygen diffusing into the nitrogen purged system. The fluorescent intensity was compared before and after irradiation.

TTX Stability Upon Irradiation with Photosensitizer. To measure the stability of Lipo-PS-TTX upon exposure to singlet oxygen, Lipo-PS-TTX was irradiated under 730 nm laser at 50 mW/cm^2 for 15 min. TTX was extracted using the Bligh and Dyer method. The extracted solution was quantified by ELISA.

In Vitro TTX Release and ELISA Quantification. Drug release studies were performed by placing TTX-loaded liposomes of 100 µL into a Slide-A-Lyzer MINI dialysis device (Thermo Scientific, Tewksbury, MA) with a 20,000 MW cut-off, further dialyzed with 14 mL PBS and incubated at 37^{o}C on a platform shaker (New Brunswick Innova 40, 150 rpm). At predetermined intervals, the dialysis solution was exchanged with fresh, prewarmed PBS. To measure the phototriggerability of the liposomes, irradiation was performed using a 730 nm laser (50 mW/cm^2) for 10 min at the 5-h and 9-h time points. The concentration of TTX was quantified by ELISA.

Cell Culture. Cell culture of C2C12 mouse myoblasts (American Type Culture Collection (ATCC) CRL-1772) and PC12 rat adrenal gland pheochromocytoma cells (ATCC, CRL-1772) was performed as reported (3). In brief, C2C12 cells were cultured in DMEM with 20% FBS and 1% Penicillin Streptomycin (Invitrogen). Cells were seeded onto a 24-well plate at 50,000 cells/mL and incubated for 10-14 days in DMEM with 2% horse serum and 1% Penicillin Streptomycin to differentiate into myotubules. PC12 cells were grown in DMEM with 12.5% horse serum, 2.5% FBS and 1% Penicillin Streptomycin. Cells were seeded onto a 24 well-plate, and 50 ng/mL nerve growth factor was added 24 h after seeding (Invitrogen).

Cell Viability. To determine the cytotoxicity of the liposomes upon irradiation, liposomes with and without irradiation (730 nm, 50 mW/cm^2, 15 min) were exposed to cells by a 24-well Transwell® membrane (Costar 3495, pore size 0.4 µm). The Transwell® membranes were inserted into the cell culture well plates and 100 µL liposome samples were added. Cell viability was evaluated by the MTT assay 96 h after exposure to liposomes.

Table S1. Characterization of liposomes

Sample Name	Compound Concentration			Loading Efficiency			Size[+] (μm)
	TTX (μg/mL)	$PdPc(OBu)_8$ (μg/mL)	Sulforhodamine B (mg/mL)	TTX	$PdPc(OBu)_8$	Sulforhodamine B	
Lipo-PS-TTX	78.9 ± 17.1	221.1 ± 10.5	--	24% ± 5%	88% ± 4%	--	5.6 ± 3.9
Lipo-PS	--	224.9 ± 8.1	--	--	90% ± 3%	--	5.0 ± 3.5
Lipo-TTX	108.9 ± 18.5	--	--	33% ± 6%	--	--	5.0 ± 3.4
Lipo-PS-SRho	--	147.5 ± 9.9	8.5 ± 0.0	--	59% ± 4%	17% ± 0	5.4 ± 3.8

Data are means ± SD, n=4

[+]Median of volume-weighted diameter

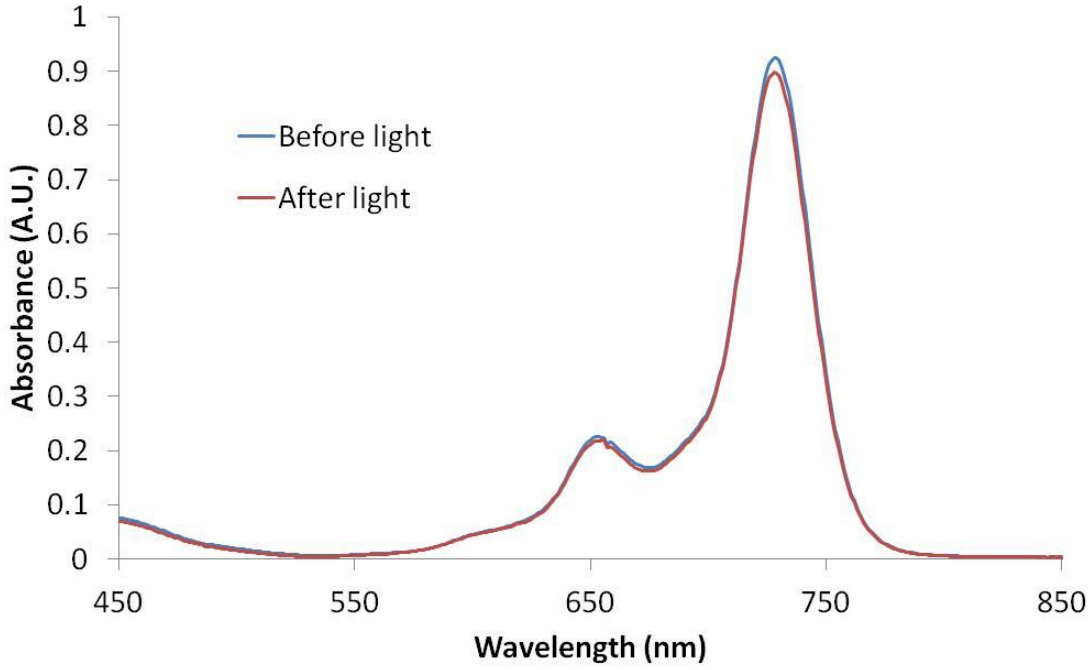

Figure S1. Photostability of $PdPC(OBu)_8$ in ethanol. Absorbance was measured before and after irradiation (730 nm, 50 mW/cm^2, 15 min).

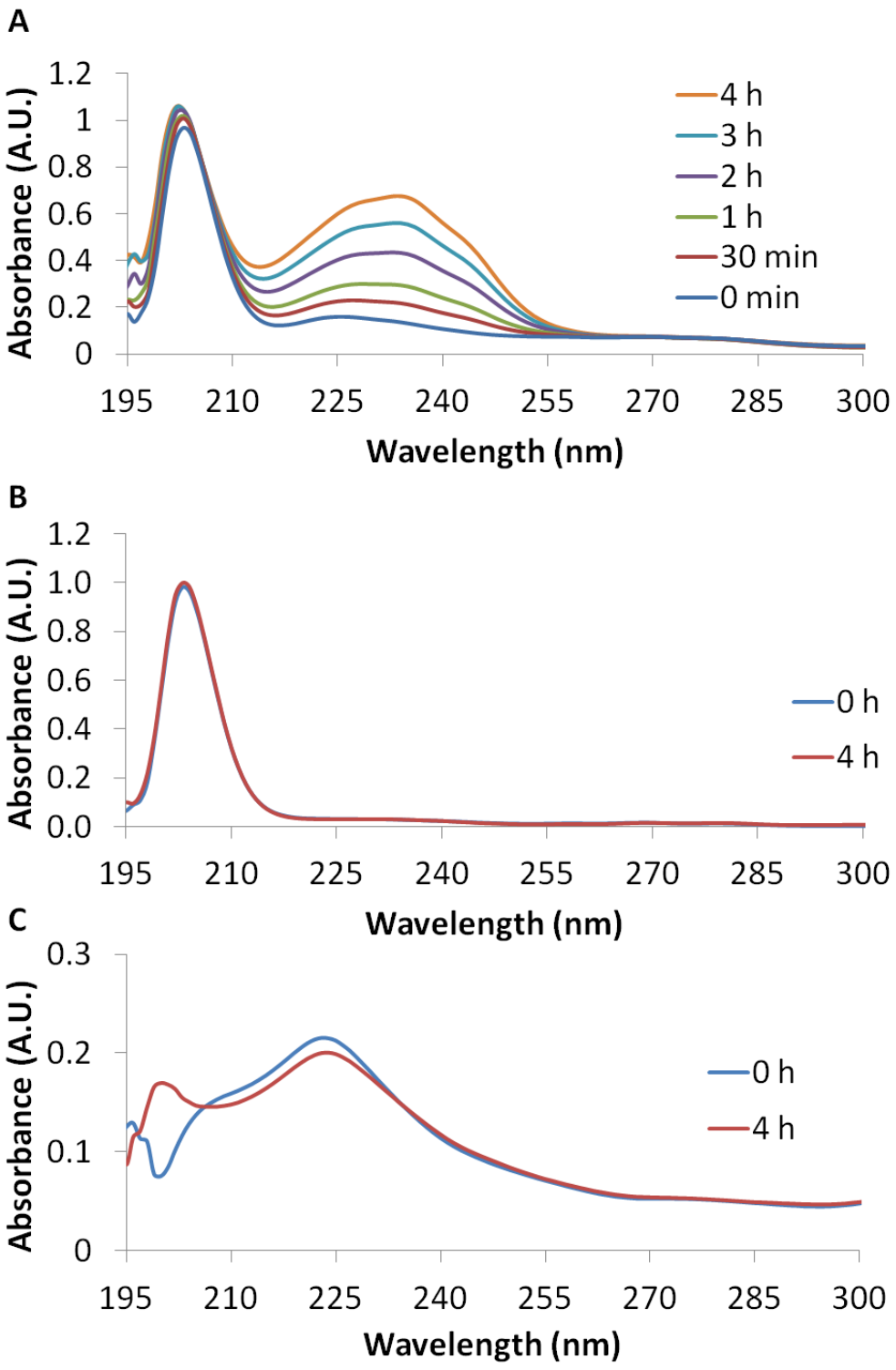

Figure S2. Effect of irradiation with a 730 nm laser at 50 mW/cm^2 on absorbance spectra of DLPC and/or PdPC(OBu)$_8$ (PS) at room temperature. (A) Effect of irradiation on an ethanolic mixture of 71 µM DLPC and 5.5 µM PS. (B) Effect of irradiation on DLPC in ethanol. (C) Effect of irradiation on PS in ethanol.

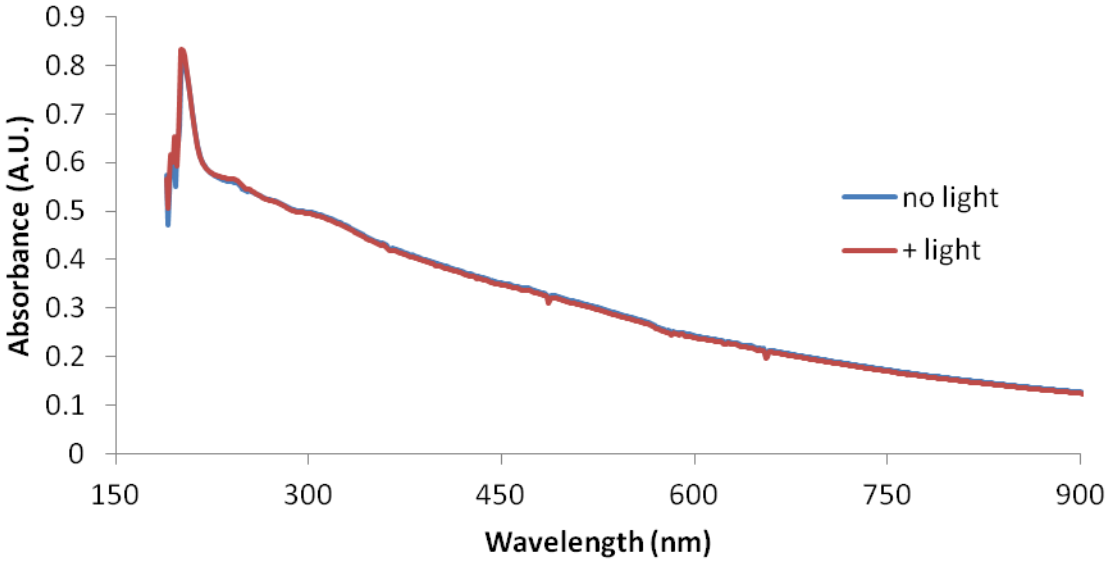

Figure S3. Absorption spectra of liposomes without $PdPC(OBu)_8$ (Lipo) in PBS at room temperature before and after 15 min irradiation with a 730 nm laser at 50 mW/cm^2

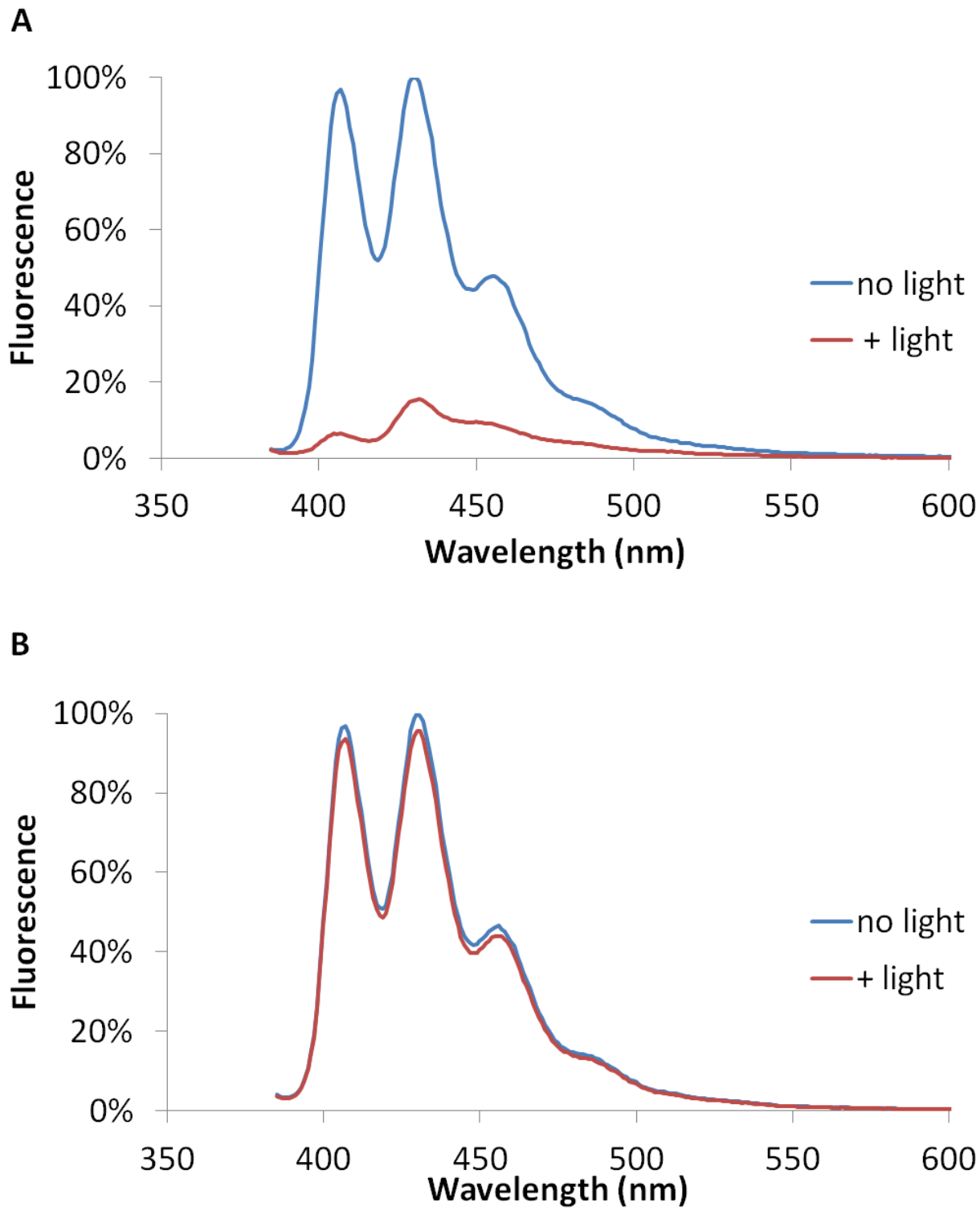

Figure S4. Fluorescence of a liposome-encapsulated singlet oxygen indicator, 9,10-dimethylanthracene, with and without irradiation of 730 nm laser at 50 mW/cm^2 for 1 min. (A) Lipo-PS (B) Lipo

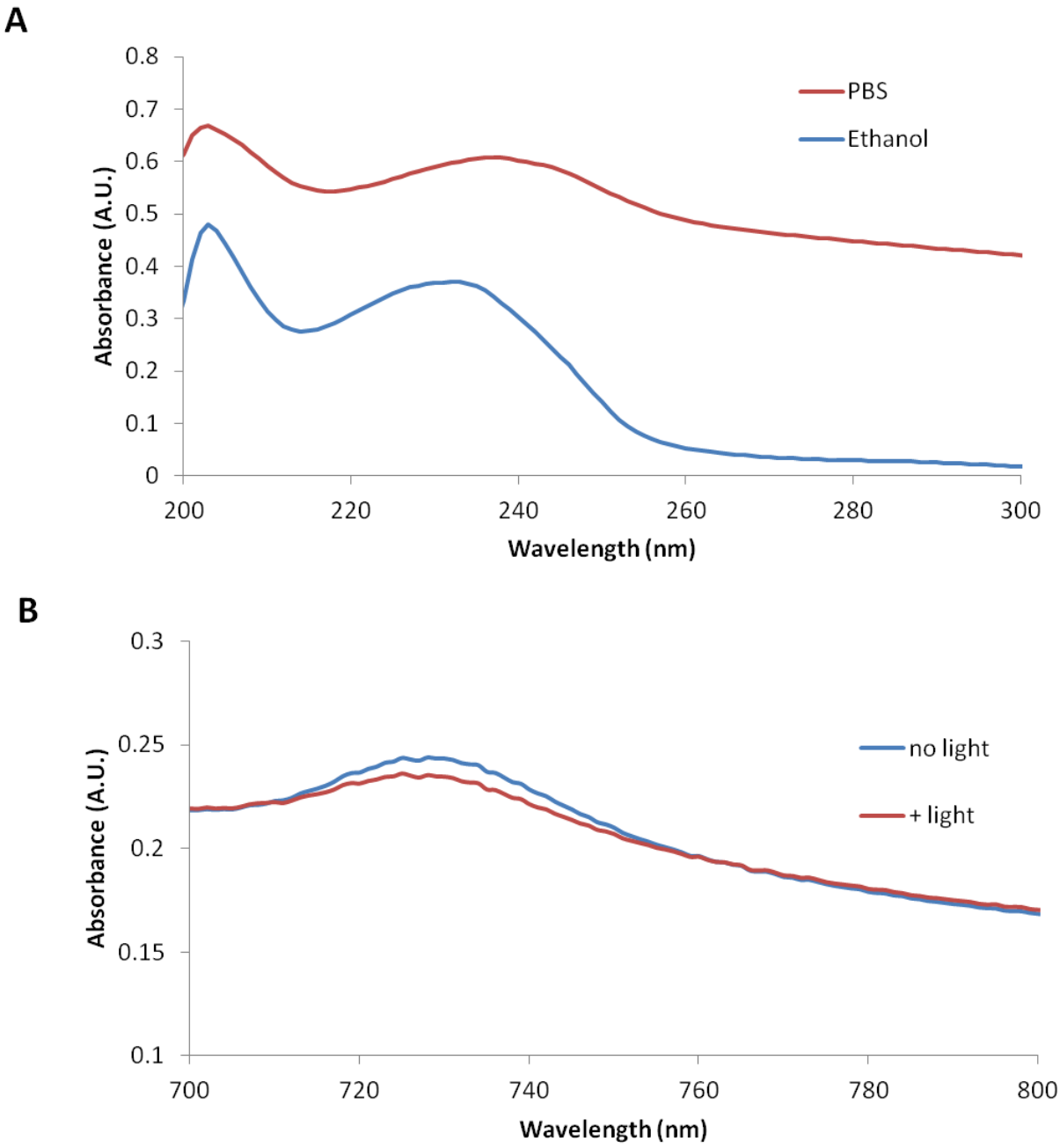

Figure S5. Lipo-PS absorption spectra at room temperature upon irradiation with a 730 nm laser (50 mW/cm^2) for 15 min. (A) Lipo-PS in PBS and ethanol after irradiation. In PBS, irradiated Lipo-PS showed an absorption peak at 238 nm; whereas in ethanol, the absorption peak was shifted to 233 nm. (B) Photostability of Lipo-PS

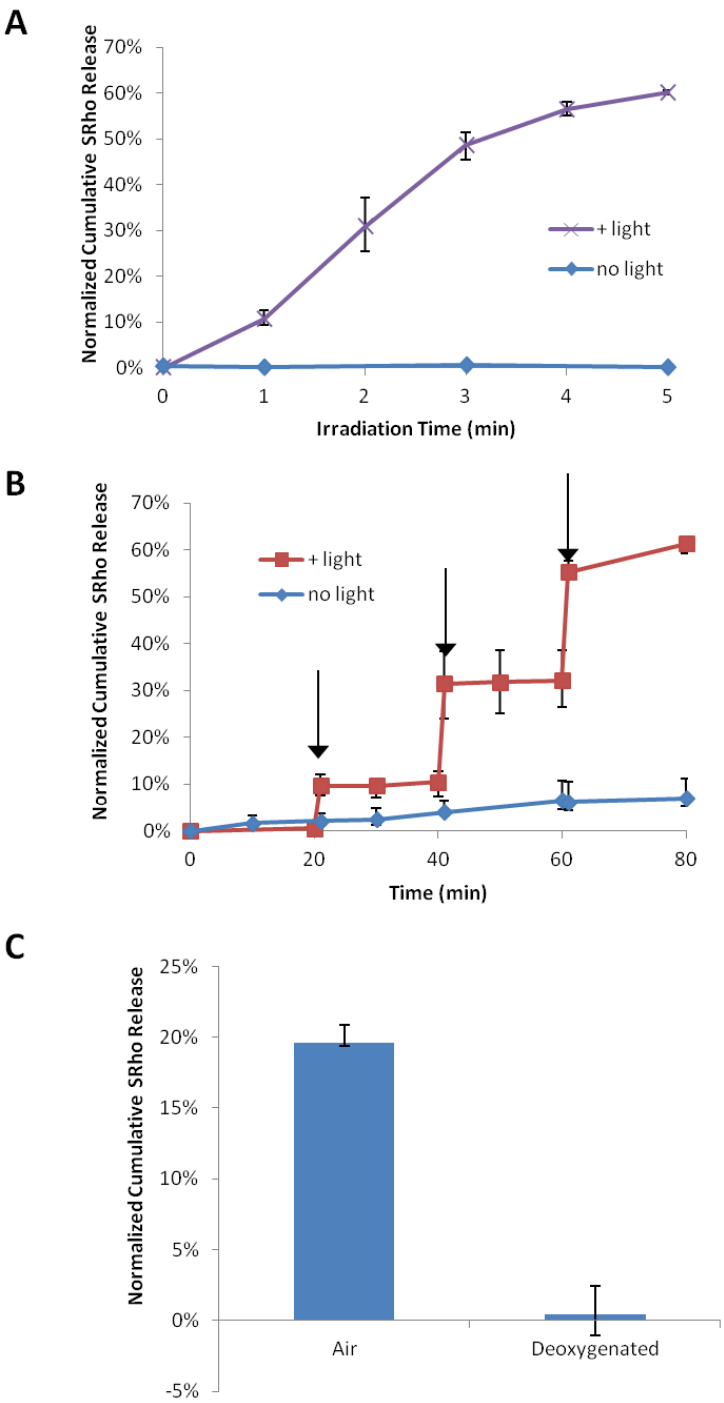

Figure S6. Phototriggered release of sulforhodamine B (SRho) from Lipo-PS-SRho. (A) Release of SRho from liposomes with irradiation (730 nm, 150 mW/cm^2) at 37 ^{o}C. (B) Release of SRho from liposomes that were irradiated (730 nm, 150 mW/cm^2, 1 min) at 20 min, 40 min and 60 min at 37 ^{o}C. (C) Fluorescent intensity of sulforhodamine-B liposomes in solution irradiated (730 nm, 50 mW/cm^2, 5 min) in air or in the absence of oxygen. The calculation of normalized cumulative release is detailed in *SI Appendix Materials and Methods*. Data are means ± SD, n=4.

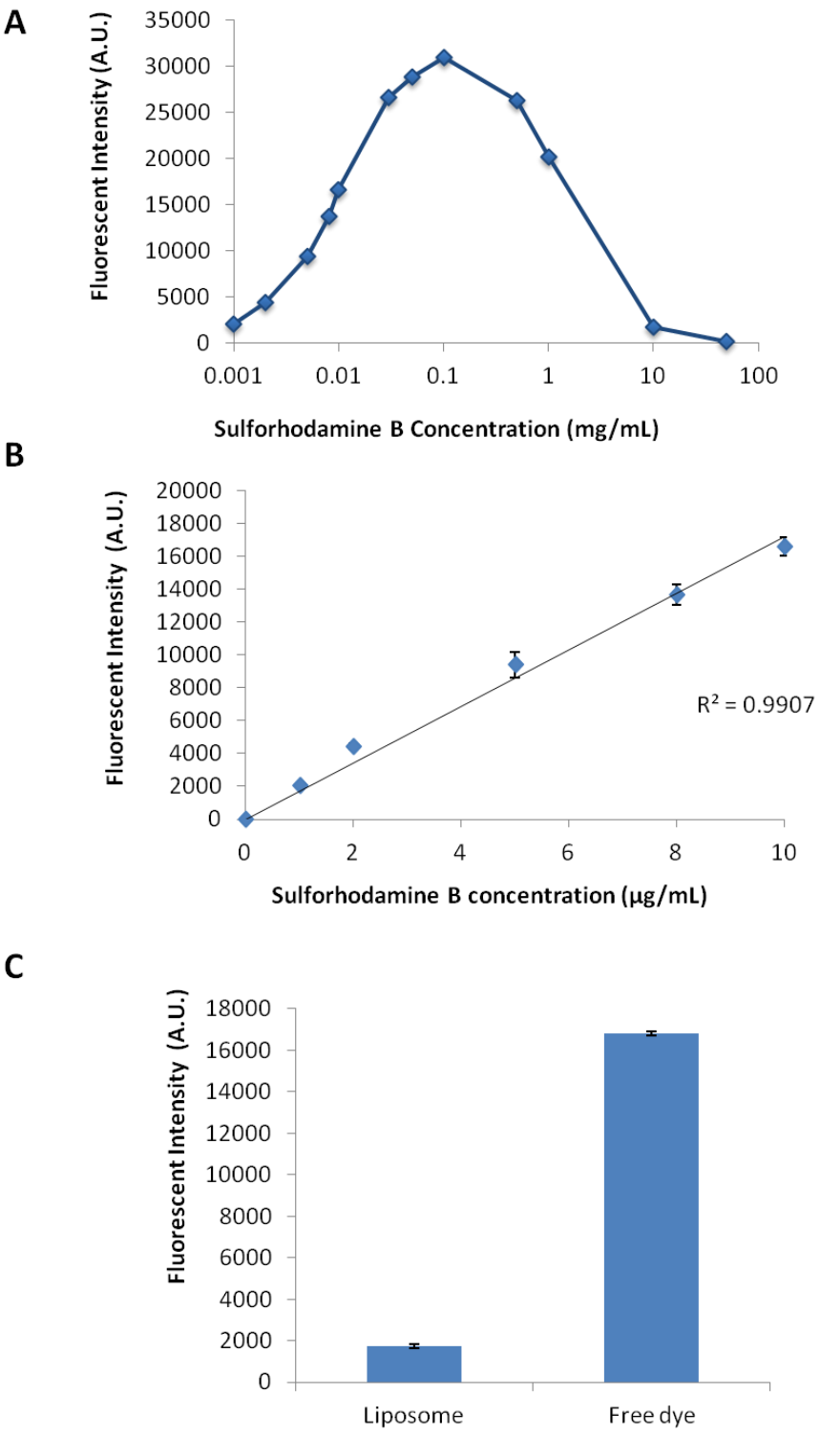

Figure S7. Fluorescent properties of sulforhodamine B. (A) Fluorescent spectrum (excitation: 560 nm / emission: 580 nm) of sulforhodamine B in PBS solution. (B) Linear range of fluorescence of dilute sulforhodamine B in PBS. (C) Fluorescence of 10 µg/mL sulforhodamine B in liposomes or in free solution. Data are means ± SD, n=4.

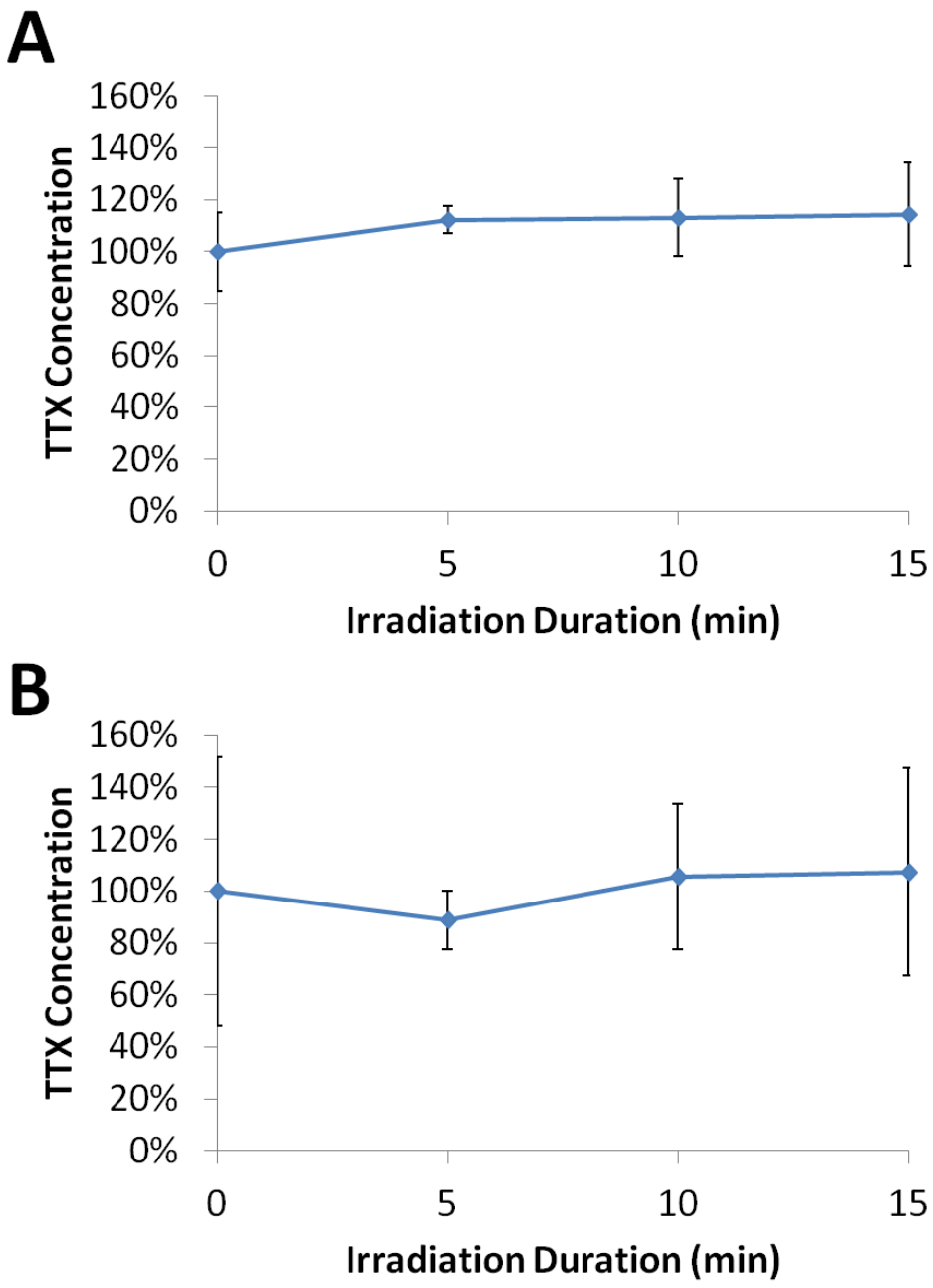

Figure S8. TTX stability upon irradiation with a 730 nm laser at 50 mW/cm^2 in the presence of PS, measured by ELISA. (A) Irradiation of TTX and PS in ethanolic solution at the same molar ratio as in Lipo-PS-TTX. (B) Lipo-PS-TTX.

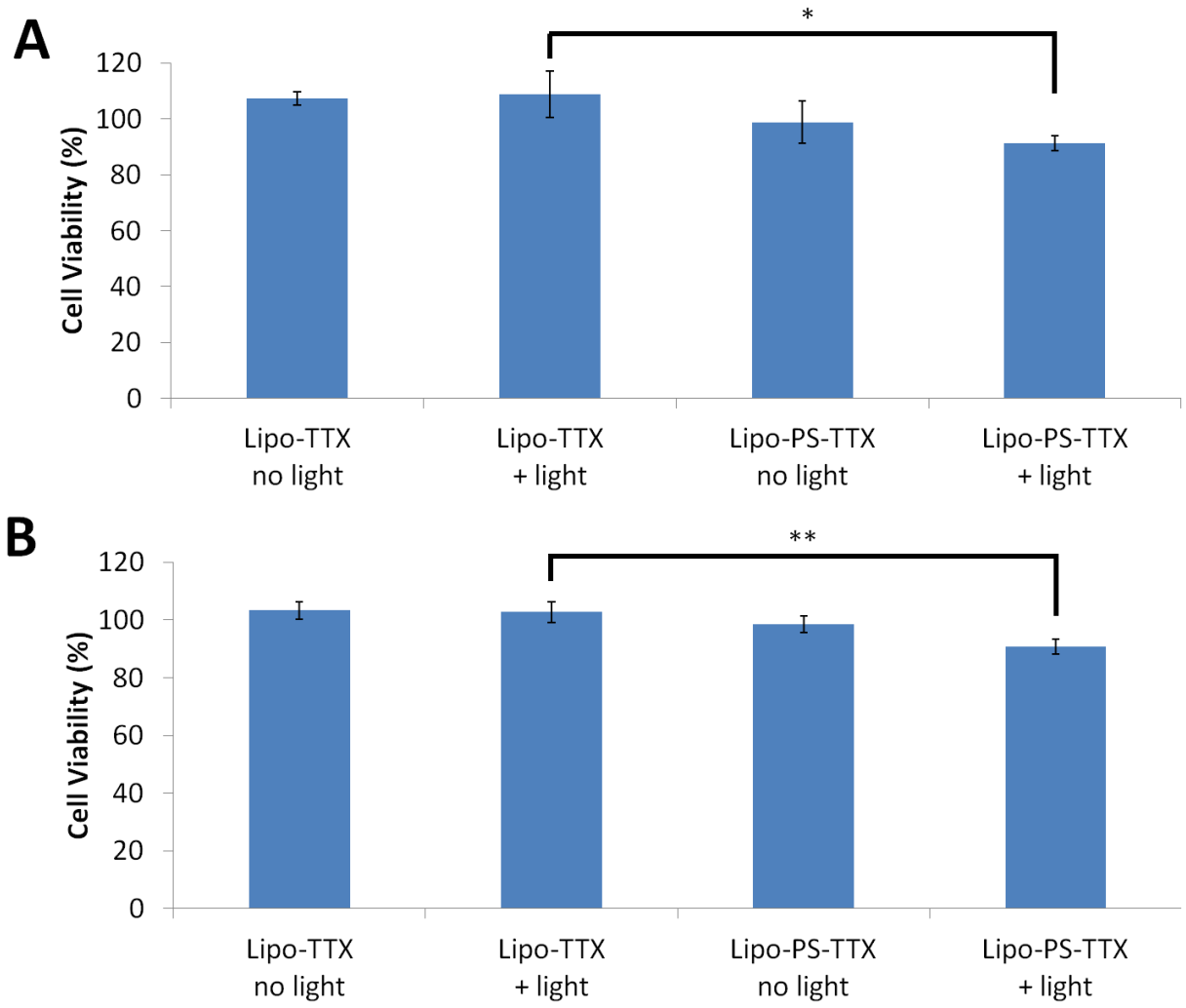

Figure S9. Cytotoxicity of TTX-loaded liposomes. (A) Viability of C2C12 cell incubated for 96 hours with test materials. (B) Viability of PC12 cell incubated with test materials. Light: irradiation at 730 nm (50 mW/cm^2, 15 min) prior to incubation. Lipo-TTX: TTX-loaded liposomes without PdPC(OBu)$_8$; Lipo-PS-TTX: TTX-loaded PdPC(OBu)$_8$ liposomes. Data are means ± SD, n=4. * P = 0.01; ** P = 0.0125. P-values < 0.0125 were considered statistically significant.

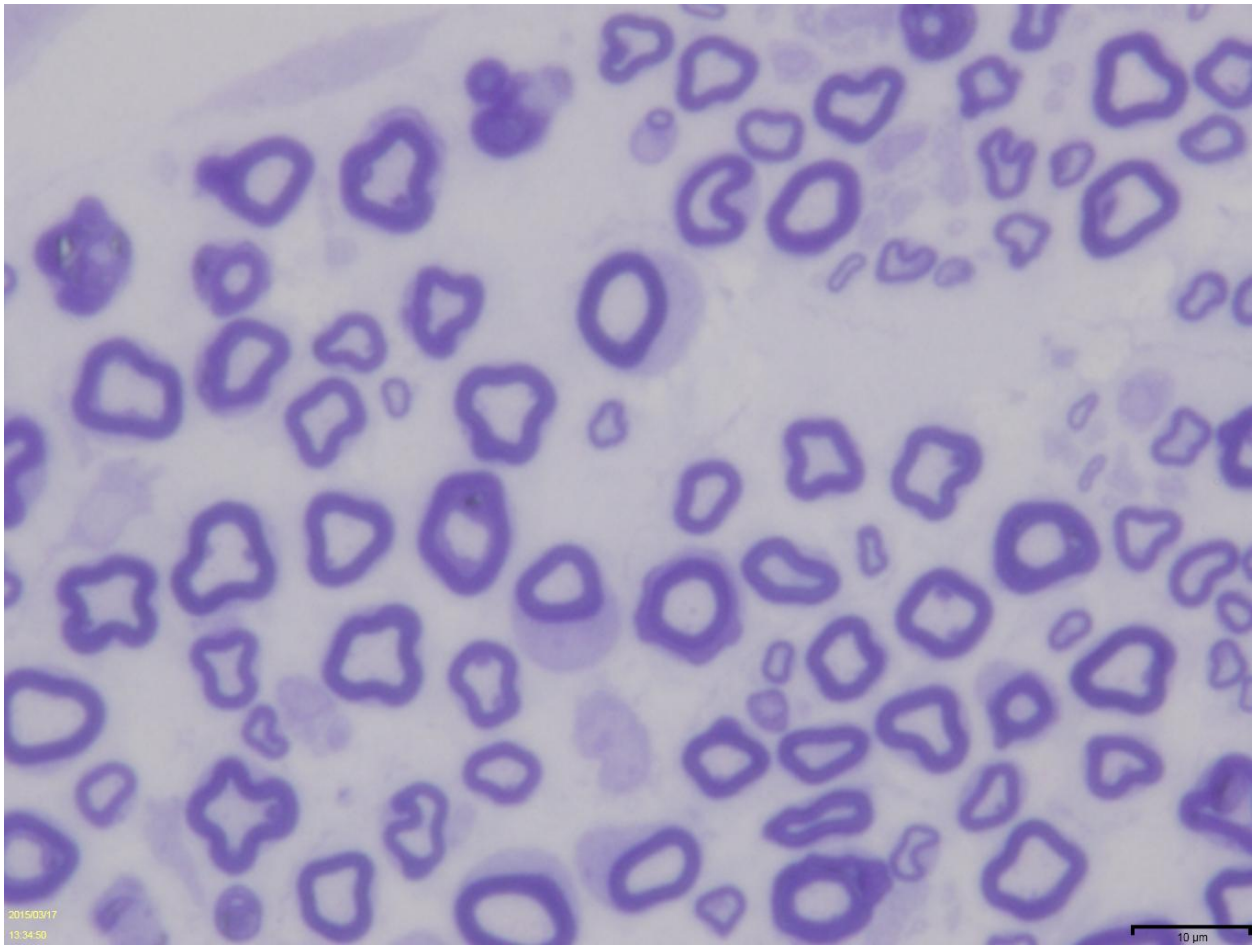

Figure S10. Representative photomicrograph of a toluidine blue-stained section of the sciatic nerve from a rat injected with Lipo-PS-TTX and irradiated as described in the text. The axons within the fasciculi are not as tightly packed as in the sections in Figure 5, indicating perineural edema. The scale bar represents 10 µm.

References

1. Soldatova AV*, et al.* (2010) Near-infrared-emitting phthalocyanines. A combined experimental and density functional theory study of the structural, optical, and photophysical properties of Pd(II) and Pt(II) α-butoxyphthalocyanines. *Inorg Chem* 50(3):1135-1149.
2. Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37(8):911-917.
3. Epstein-Barash H*, et al.* (2009) Prolonged duration local anesthesia with minimal toxicity. *Proc Natl Acad Sci USA* 106:7125-7130.
4. Kiwi J & Nadtochenko V (2004) New evidence for TiO2 photocatalysis during bilayer lipid peroxidation. *J Phys Chem B* 108(45):17675-17684.
5. Halliwell B & Chirico S (1993) Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr* 57(5):715S-724S.
6. Ohkawa H, Ohishi N, & Yagi K (1978) Reaction of linoleic acid hydroperoxide with thiobarbituric acid. *J Lipid Res* 19(8):1053-1057.