

Electronic Supplementary Information for:

Croconaine rotaxane for acid activated photothermal heating and ratiometric photoacoustic imaging of acidic pH

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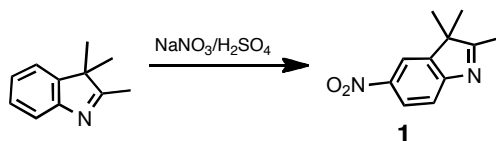
Experimental Section

Materials: 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), cardiogreen [Indocyanine Green (ICG)], IR780 iodide, and Cholesterol-PEG600 n=10-11 (Ch-PEG600) were obtained from Sigma Aldrich. 2,3,3-Trimethylindolenine (98%) and croconic acid (98%) were purchased from Alfa Aesar. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) were purchased from Avanti Polar Lipids (USA) and stored in a freezer at -20°C . *N*-(Carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine, ammonium salt (DSPE-PEG2000) was purchased from Corden Pharma (Switzerland). Commercially available solvents were used without further purification unless otherwise stated. HPLC grade solvents — purchased from Sigma-Aldrich and EMD Chemicals — were used for HPLC and all spectroscopic measurements. Water was de-ionized and microfiltered. Before opening, the chemicals were warmed to room temperature.

Synthesis and Characterization of Croconaine (Croc) and Croconaine Rotaxane (CrocRot):

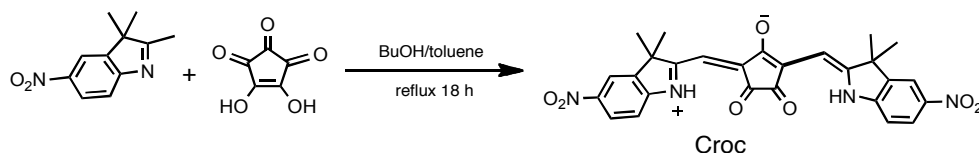
9,10-Bis(aminomethyl)anthracene^{S1} and 5-(*tert*-butyl)isophthaloyl dichloride^{S2} were synthesized using previously reported procedures. All reactions were performed under Ar atmosphere using dry solvents unless otherwise specified. Analytical thin layer chromatography (TLC) was performed on silica gel 60-F254 (Merck) plates and detected under UV lamp and/or by developing with KMnO_4 . Column chromatography was performed on silica gel 60 (SorbTech).

Compound 1: 2,3,3-Trimethylindolenine (2.0 mL, 12.5 mmol) was stirred in a salted ice water bath for 5 minutes. NaNO_3 (1.09 g, 13 mmol) was added in one portion. H_2SO_4 (31.5 mL, 591 mmol) was added drop wise over 10 minutes. During the addition of H_2SO_4 , the reaction mixture color changed from yellow to red. The resulting solution was stirred for 1 h at -5°C . The solution was neutralized slowly with solid NaOH, while maintaining a reaction temperature of -5°C . A red crystalline solid formed. This crude product was dissolved in 50 mL EtOAc and washed with DI H_2O (3×40 mL). The organic layer was isolated and dried over MgSO_4 , filtered, and concentrated under reduced pressure to yield compound **1** (1.47 g, 58%) as a dark red solid. No further purification was necessary. The structure and purity were confirmed by ^1H NMR and HRMS.^{S3}



Croconaine dye (Croc): Compound **1** (205.3 mg, 1 mmol) and croconic acid (69.5 mg, 490 μ mol) were dissolved in 1-butanol (8 mL) and toluene (8 mL). The reaction mixture was refluxed for 18 hours. Over this time, the mixture changed color from transparent red to dark brown-black. The reaction flask was placed in the -23°C freezer for 2 h. During this time, Croc precipitated out of solution. The dark grey solid was filtered and washed with ether (3×5 mL). To ensure that all volatile solvents were removed, the solid was placed under reduced pressure overnight. The resulting solid did not require further purification. Croc was isolated (146 mg, 58%) as a dark grey solid and the spectral data matched the literature values.^{S4} ^1H NMR (500 MHz, CDCl_3 , 25°C): δ 8.29-8.36 (m, 2H), 8.20-8.25 (m, 2H), 7.39-7.44 (m, 2H), 6.09-6.31 (m, 2H), 1.60-1.62 (m, 12H) ppm. The spectrum indicates two sets of signals due to cis and trans geometric isomers as discussed on the next page.

HRMS (ESI-TOF) (+ve) m/z : calculated for $\text{C}_{27}\text{H}_{23}\text{N}_4\text{O}_7$ $[\text{M}+\text{H}]^+$ 515.1561, found 515.1551.



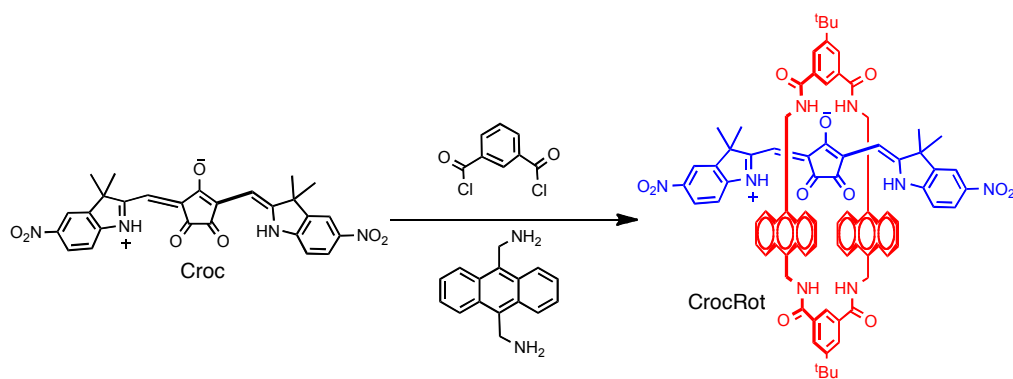
Croconaine Rotaxane (CrocRot): 5-(*tert*-Butyl)isophthaloyl dichloride (256.7 mg, 1.28 mmol) and 9,10-bis(aminomethyl)anthracene (203.9 mg, 1.28 mmol) were each dissolved in 80 mL anhydrous CHCl_3 . Each solution was brought into separate 100 mL syringes. Et_3N (1 mL) was added to the syringe containing 9,10-bis(aminomethyl)anthracene. The two solutions were simultaneously added dropwise to a stirred solution of Croc (48.7 mg, 0.32 mmol) in 40 mL CHCl_3 via mechanical syringe pump apparatus over 8 hours. After stirring overnight, the resulting dark green-black solution was concentrated under reduced pressure to yield a dark green solid. The crude product was purified via silica gel column chromatography (Column 1: 10-50% acetone in CHCl_3 , Column 2: 40% acetone in CHCl_3) to yield CrocRot (10%) as a dark green-black solid.

^1H NMR (600 MHz, CDCl_3 , 25°C): δ 13.31 (m, 2H), 9.09-9.62 (m, 2H), 8.44-8.71 (m, 4H), 8.33 (m, 2H), 8.11 (d, $J = 1.5$ Hz, 2H), 7.79-7.99 (m, 8H), 7.74-7.41 (m, 2H), 7.09-7.20 (m, 2H),

6.49-7.02 (m, 8H), 5.17-5.43 (m, 8H), 4.58-5.05 (m, 2H), 1.43-1.72 (m, 18H), 0.75-1.15 (m, 12H) ppm.

^{13}C NMR (150 MHz, CDCl_3 , 25°C): δ 186.2, 184.1, 176.4, 167.1, 166.3, 166.1, 153.8, 152.8, 145.4, 144.5, 142.0, 141.3, 133.5, 133.3, 133.2, 130.7, 130.3, 130.1, 129.7, 129.4, 129.3, 126.5, 125.7, 125.5, 124.9, 124.8, 118.5, 113.7, 95.3, 65.9, 50.4, 45.6, 37.9, 36.6, 31.5, 31.4, 31.2, 26.5, 15.3 ppm.

HRMS (ESI-TOF) (+ve) m/z : calculated for $\text{C}_{83}\text{H}_{75}\text{N}_8\text{O}_{11}$ $[\text{M}+\text{H}]^+$ 1359.5550, found 1359.5526; calculated $[\text{M}+\text{Na}]^+$ 1381.5369, found 1381.5386.



Structural Features of Croconaine Rotaxane (CrocRot): The ^1H NMR for CrocRot indicates two sets of signals because the encapsulated croconaine dye exists as two geometric isomers (Figure S5). Inspection of the spectral patterns (especially for the NH residues at 13.4 ppm) indicated that they are cis and trans isomers in $\sim 2:1$ ratio. This structural assignment agrees with literature conclusions based on computer modeling of analogous squaraine dye isomers.^{S5} Dye isomers that allow internal hydrogen bonding ($\text{NH}\cdots\text{O}$) are energetically more stable than dye isomers that lack this internal hydrogen bonding. Figure S6 compares ^1H NMR spectra for Croc, CrocRot, and empty macrocycle, and shows that rotaxane formation produces the expected downfield shifts for macrocycle proton signals C and NH, and upfield shifts for macrocycle protons E and F and dye proton 5. A ^1H - ^1H COSY experiment established coupling connectivity (Figure S7) and a ^1H - ^1H ROESY (Figure S8) showed spatial proximity of proton 4 to proton F, and proton 5 to proton C, confirming the interlocked rotaxane structure. Additional evidence for rotaxane formation is the characteristic 22 nm red-shift of the Croc λ_{max} (Figure S9a).^{S2} CrocRot exhibited a pH-dependent absorption change from its basic form ($\lambda_{\text{max}} \sim 684$ nm) to acidic form

($\lambda_{\text{max}} \sim 808$ nm) (Figure S9b). A comparison of the anthracene absorption and fluorescence bands for CrocRot and empty macrocycle showed strong quenching in CrocRot, consistent with rotaxane formation (Figure S10). The purity of CrocRot was confirmed by HPLC (Figure S11a). The interlocked molecule does not unthread in organic solvent (even in CH_3CN which is known to strongly promote dethreading of analogous squaraine pseudorotaxane structures^{S1}) or when it is located within a liposome membrane that has been repeatedly heated (Figure 2). NMR studies showed that mixing equal amounts of CrocRot and empty macrocycle in CDCl_3 for 1 week at 37 °C or 4 hours at 50 °C does not lead to any rotaxane formation by slippage. Thus, the steric barrier for threading or unthreading of CrocRot at 37 °C is very high.

Methods

The Croc dye used in this study has the following photophysical properties in CHCl_3 : (λ_{max})_{basic} 630 nm, (λ_{max})_{acidic} 780 nm, ϵ $1.5 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$, λ_{em} 820 nm, Φ_{F} 0.047.

The CrocRot used in this study has the following photophysical properties in CHCl_3 : (λ_{max})_{basic} 684 nm (λ_{max})_{acidic} 808 nm, ϵ $8.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, λ_{em} 848 nm, Φ_{F} 0.03.

Indocyanine green (ICG) was used as a standard for quantum yield measurements ($\Phi_{\text{F}} = 0.132$ in ethanol).

NMR Spectroscopy: ^1H , ^{13}C , ^1H - ^1H COSY, and ^1H - ^1H ROESY NMR spectra were recorded on Bruker AVANCE III HD 400, Bruker AVANCE III HD 500, and Varian DirectDrive 600 spectrometers at 298 K in deuterated solvents.

HRMS (ESI): High-resolution electrospray ionisation (ESI) mass spectrometry (MS) was performed using a Bruker micrOTOF II spectrometer.

High Performance Liquid Chromatography (HPLC): HPLC was performed on a Waters Alliance HPLC system using analytical HPLC column (Dionex Acclaim RSLC 120 C18 2.2 μm 2.1 x 100 mm). HPLC runs were performed by using a linear gradient (5-100%), A: H_2O with 0.1% formic acid and B: CH_3CN with 0.1% formic acid with a flow rate of 0.4 mL min^{-1} . CrocRot was dissolved in CH_3CN -MeOH mixture and filtered prior to injection.

pH Meter: Mettler Toledo pH meter (In Lab[®] 413 SG/2m IP67) is used to prepare buffer solutions at different pH. The pH values were calibrated at 25 °C with standard buffers of pH 10.00 ± 0.01 , 7.00 ± 0.01 , and 4.01 ± 0.01 .

Absorption Spectroscopy: Absorption spectra were recorded on a PerkinElmer Lambda-25 spectrometer. All measurements were carried out in a micro quartz glass cell with a path length of 1 cm.

Fluorescence Spectroscopy: Fluorescence spectra were obtained at a Jobin Yvon Horiba FluoroMax-4 spectrofluorometer.

Photoacoustic Spectral Scanning and Imaging of Phantoms: Photoacoustic spectral scans and images were acquired using phantoms that were narrow vinyl tubes (5 mm external diameter) suspended in parallel orientation to the transducer face, near the bottom of a basin filled with water or a solution of 0.7% milk fat as a light scattering medium (Figure S20).^{S6} Photoacoustic imaging was performed on a Vevo[®] LAZR-2100 Photoacoustic-Ultrasonic imaging system (FUJIFILM VisualSonics Inc., Toronto, Canada) with a 24-mm-wide, 21-MHz linear acoustic array used for signal detection; the acoustic array is flanked by optical fiber arrays positioned such that their beams intersect at approximately 10 mm from the array face. A photoacoustic scan was created using a pulsed, tunable Nd:YAG laser (680 - 970 nm wavelength range) with a step size of 2 nm and pulse energies between 8 and 21 mJ. The phantoms contained solutions of Croc, CrocRot or ICG in ethanol, or alternatively CrocRot-SL in buffer at pH 7.4 or pH 5.0 (the typical dye concentration in these sample was ~15 μ M). Each scan was repeated 3 times and the scans are presented as mean photoacoustic signal intensities with error bars reflecting standard deviation. Ratiometric photoacoustic images (pH 5.0 and 7.5) were generated by applying a noise-elimination threshold (i.e., only signal two standard deviations above the noise floor was analyzed) to each single-wavelength image and then dividing pixel-wise an 812 nm thresholded image by a 690 nm thresholded image. The resulting ratiometric data was then color-coded with blue depicting a ratio <1, orange-red a ratio >5, and black indicating a signal-void pixel omitted from analysis due to thresholding.

Dynamic Light Scattering (DLS): Dynamic light scattering experiments were performed using a Malvern instrument (Malvern, UK, zeta sizer, Nano Series, Nano ZS, He-Ne laser wavelength at 633 nm) to examine the hydrodynamic diameters of the liposomes in buffer. All measurements were conducted at a backscattering angle 173° and a temperature of 25°C. Three runs were conducted per measurement and the average values were taken. A monodisperse population of the liposome was observed in buffer indicating a uniform liposome size (without any aggregation).

Transmission Electron Microscopy (TEM): TEM images were recorded on a FEI Titan 80-300 (USA) instrument with operating voltage of 300 kV and a Gatan 4x4k bottom-mount CCD camera. An aqueous solution of the CrocRot-SL (composition POPC:Ch-PEG600:DOTAP:CrocRot 84:10:3:3) was localized on a carbon coated copper grid (300 mesh) and the samples were stained with 2% (w/v) uranyl acetate.

Laser-Induced Heat Generation Studies: Croc and CrocRot loaded liposomes were added to appropriate amount of buffer at different pH in a 1 cm quartz cuvette equipped with a small magnetic stirrer. An Omega hypodermic thermocouple needle probe (HYPO-33-1-T-G-60-SMPW-M, made in USA) was placed in the solution and the temperature was recorded in a continuous fashion (1 sec intervals) using the associated USB converter; temperature changes were also recorded by infrared camera. A continuous diode laser (Thorlab, NJ, USA) beam was aligned to pass through the solution (above the magnetic stirring bar and avoiding close contact with the thermocouple) in an identical manner in every experiment. The laser wavelength was set at 808 nm and the power was controlled at 250 mW (optical power meter) during all of the experiments, with a beam diameter of 0.3 cm and laser power density 3.5 W/cm^2 .

Croc and CrocRot Loaded Liposomes for Cuvette Studies:

Preparation of Lipid/Croc and Lipid/CrocRot Film: POPC (10 mg mL^{-1}), DOTAP (10 mg mL^{-1}), Ch-PEG600 (10 mg mL^{-1}), Croc (1 mg mL^{-1}), and CrocRot (1 mg mL^{-1}) were dissolved in CHCl_3 . Appropriate quantities were mixed to get a defined Croc-SL (composition POPC:Ch-PEG600:DOTAP:Croc 84:10:3:3) or CrocRot-SL (composition POPC:Ch-PEG600:DOTAP:CrocRot 84:10:3:3) ratio. The solvent (CHCl_3) was removed by N_2 stream for 20 min at a temperature above the main phase transition temperature of the respective lipids and the residual solvent was removed under high vacuum for 2 h which produced a lipid/Croc or lipid/CrocRot film on the glass tube walls. The lipid films were rehydrated with an appropriate amount of buffer solution (20 mM HEPES, 150 mM NaCl, pH 7.4) yielding the desired stock solutions of lipid/Croc and lipid/CrocRot (total lipid+Croc or lipid+CrocRot concentration was 6.67 mM).

Multilamellar Vesicles (MLVs): After 1 h of incubation at $T > T_m$ (room temp), the hydrated lipid films were subjected to five freeze/thaw cycles (immerse the sample in liquid N_2 followed by 40°C at water bath) to detach the lipid film from the wall of the glass tube and also disperse

the vesicles. The dispersed multilamellar vesicles were incubated at $T > T_m$ (room temp) for 5 min and vortexed.

Large Unilamellar Vesicles (LUVs): The multilamellar vesicle (MLV) suspensions were extruded 31 times through a polycarbonate membrane (200 nm pore size, 19 mm diameter, Whatman Nuclepore Track-Etch Membrane Filtration Products) using a *Liposofast* extruder (*Avestin*, Ottawa, Canada) at $T > T_m$ (room temp) to produce unilamellar vesicle suspension. The unincorporated Croc dye and CrocRot was removed by gel filtration (size exclusion chromatography) using a column filled with *Sephadex*TM *G-25 Superfine* gel and cold HEPES buffer at pH 7.4 as eluent. All vesicles were stored on ice.

CrocRot-SL Biostability Studies: UV/vis scans showed that CrocRot-SL are highly stable in 2% FBS (w/w) in buffer at pH 7.4, 37°C (Figure S16a). Furthermore, there is no change in the UV/Vis absorption spectra of CrocRot-SL in the presence of potential biological interferents such as essential ions (10 mM for Na⁺, K⁺, 200 μM for Ca²⁺, Mg²⁺, Zn²⁺, Fe³⁺, Fe²⁺, Ba²⁺, and Cu²⁺, as their chloride salts), and bioactive small molecules such as glutathione (GSH, 5 mM), cysteine (Cys, 5 mM), and H₂O₂ (1 mM) under physiological conditions (pH 7.4, 37°C) (Figure S16b).

Cell Viability Assay: EMT-6 (ATCC CRL-2755) mouse mammary carcinoma cells were cultured in Waymouth's Media (Thermo Fischer) with 15% fetal bovine serum (FBS) and 1% streptomycin-penicillin and were incubated at 37°C, 5% CO₂. The cells were seeded into a new flask or 96-well plate after 3-4 days and grown to ~90% confluency. Different doses of CrocRot-SL at pH 7.4 were added to the EMT-6 cells and the well were incubated for 24 h. Cell viability was determined using the AlamarBlue fluorescence assay kit from Life Technologies. Healthy cells produce a fluorescence emission at 590 nm, and the emission intensity from healthy cells treated with culture medium was set as 100% cell vitality (Figure S16c).

In Vivo Photoacoustic Imaging and Scanning: The animal study was carried out in the Small Animal Imaging Facility at The University of Texas MD Anderson Cancer Center under a protocol approved by the Institutional Animal Care and Use Committee. Croc-IVSL (composition POPC:DSPE-PEG2000:DOTAP:CrocRot 79:15:3:3) were prepared as LUVs in 1 mM HEPES buffer (150 mM NaCl, pH 7.4) using the liposome preparation method described above. A single 500 μL dose of Croc-IVSL ([CrocRot] = 25 μM) was injected with a 25-gauge

needle into the peritoneal cavity of a living nude mouse (N = 2), which was kept anesthetized with 2.0% isoflurane with a 1.5 L/min medical air flow rate. Using the aforementioned Vevo[®] LAZR-2100 imaging system, the sagittal plane of the mouse peritoneal cavity was imaged before injection and 20 minutes after-injection using B-mode ultrasound and spectroscopic photoacoustic imaging between 680 and 970 nm with a step size of 2 nm. During imaging, the animal's body temperature was maintained with a heated imaging platform at 36°C.

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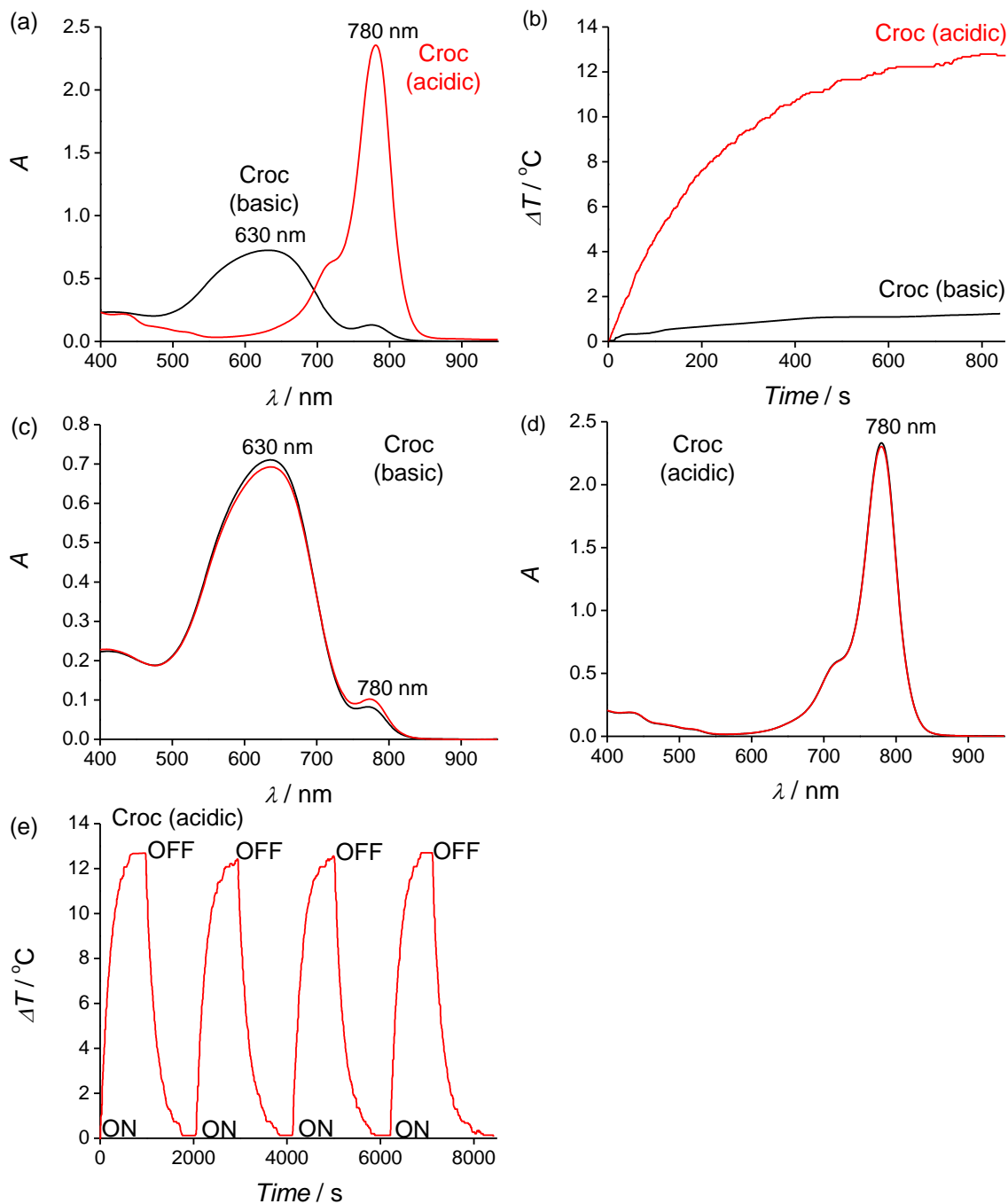
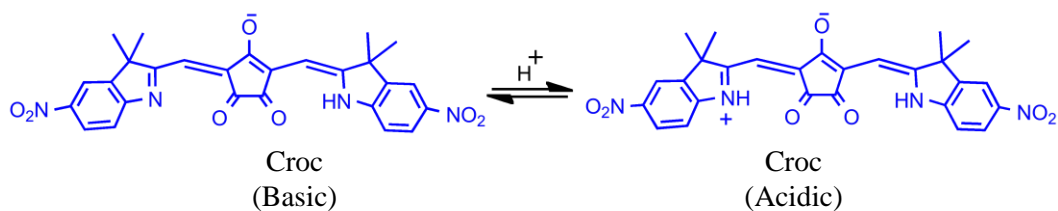


Figure S1. (a) UV/Vis absorption plots of acidic and basic Croc in ethanol (16 μM , 25 $^{\circ}\text{C}$). (b) Temperature change profiles during laser irradiation (808 nm) for acidic Croc (red), and basic Croc (black) in ethanol. UV/Vis absorption plots of basic Croc (c) and acidic Croc (d) before (black) and after (red) laser irradiation (15 min, 808 nm) in ethanol. (e) Multiple heating cycles (4 cycles) of acidic Croc with laser irradiation (808 nm).

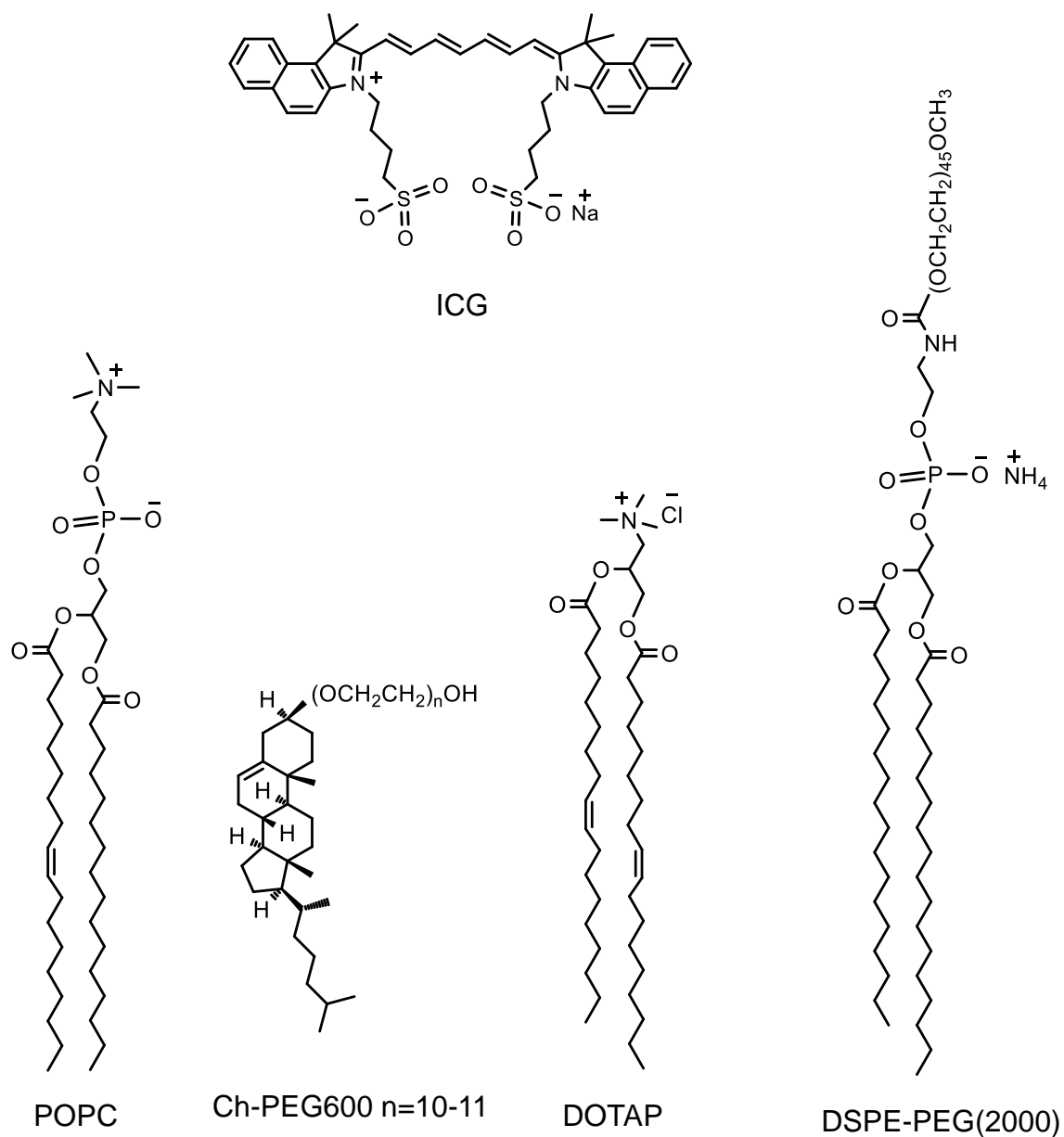


Figure S2. Molecular structures of indocyanine green (ICG), N-(Carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine, ammonium salt (DSPE-PEG2000), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), and Cholesterol-PEG600 (Ch-PEG600).

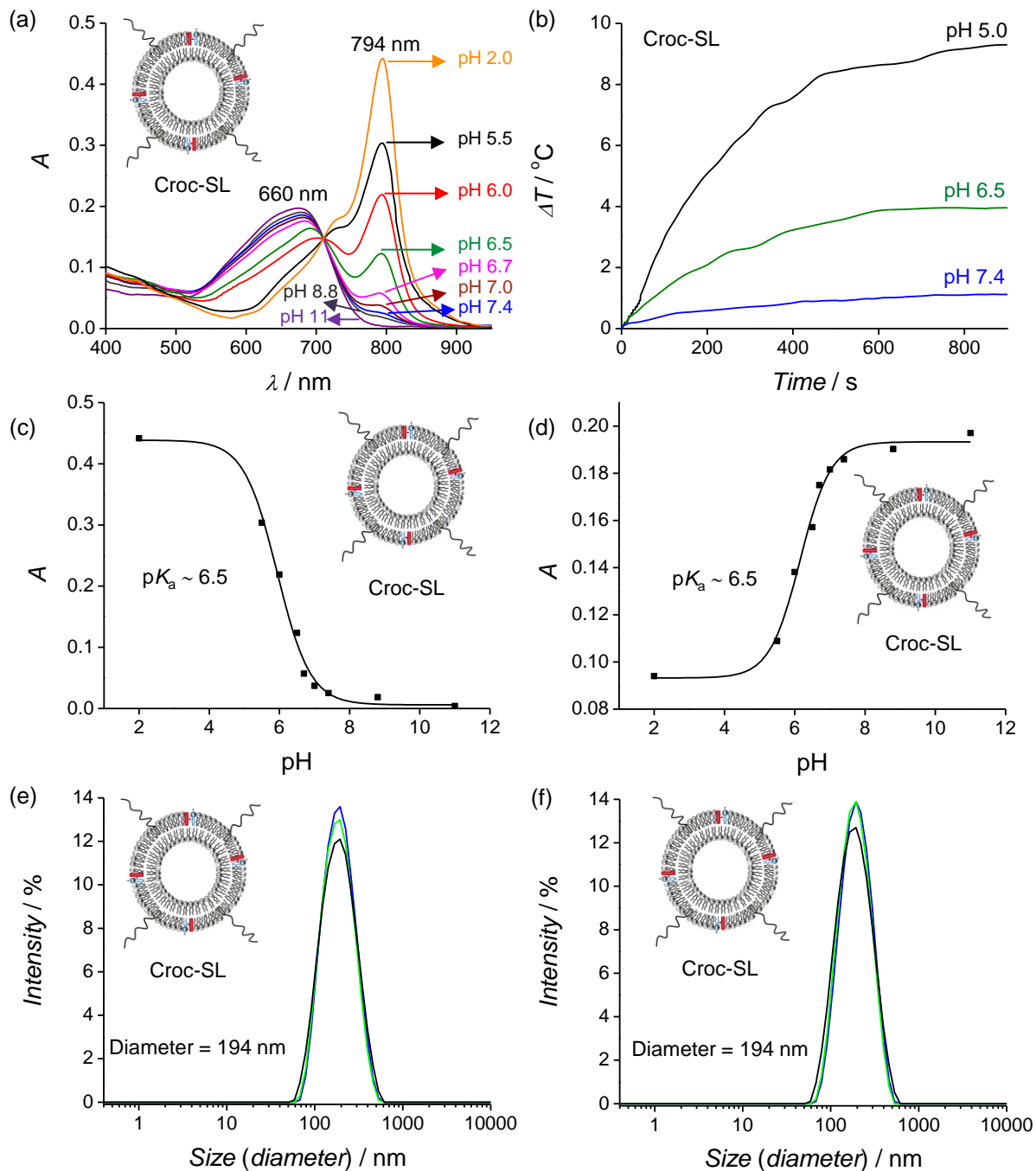


Figure S3. (a) UV/Vis absorption titration of Croc-SL in buffer at various pH values, [Croc] = 3 μ M. (b) Temperature changes during laser irradiation (808 nm) of Croc-SL at different pH values. (c) and (d) pH vs absorbance plots with sigmoidal curve fitting showing $pK_a \sim 6.5$. (e) and (f) DLS study of Croc-SL at pH 7.4 (blue), 6.5 (green), and 5.0 (black) in buffer before and after laser (15 min, 808 nm) irradiation respectively. Data indicates that the liposomes are stable after laser irradiation.

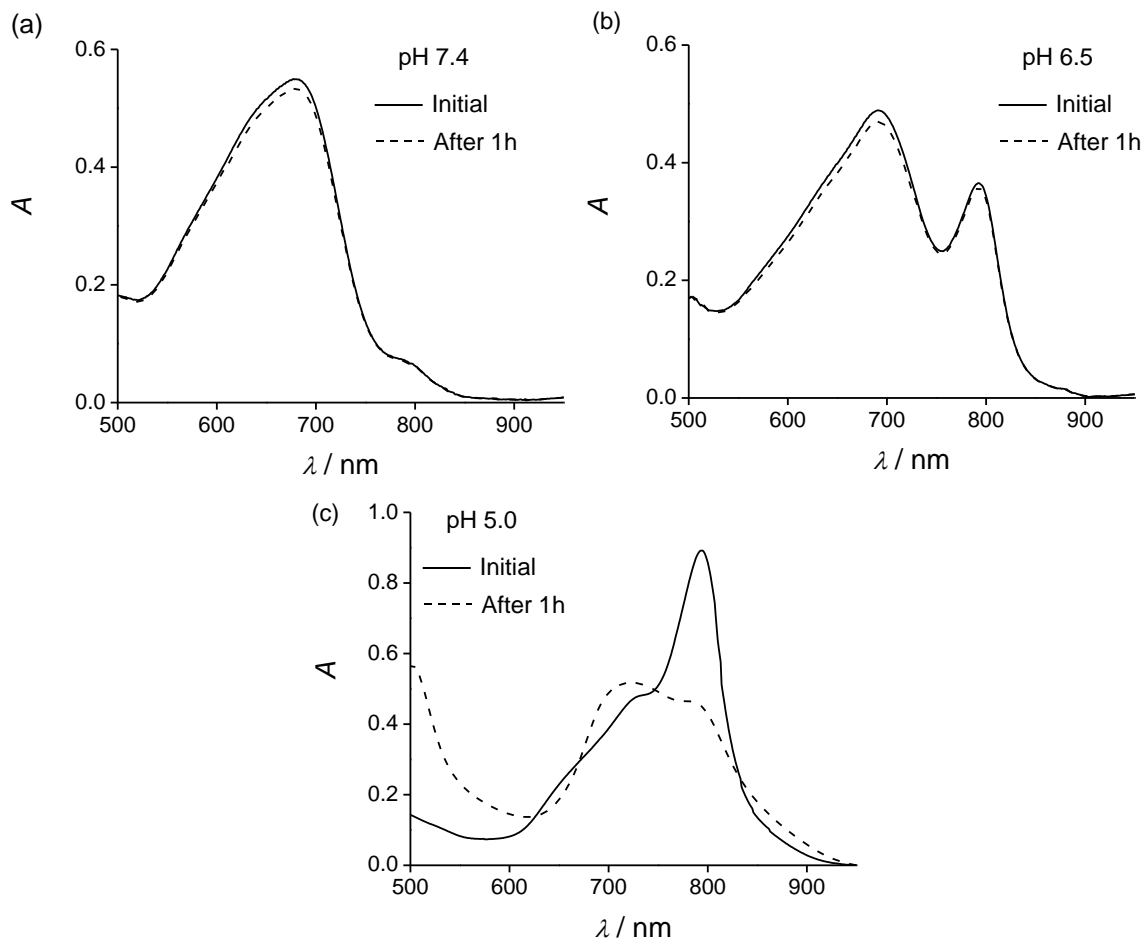


Figure S4. UV/Vis absorption plots of Croc-SL in buffer at pH (a) 7.4, (b) 6.5 and (c) 5.0. Spectra were acquired initially (solid lines) and after standing for 1 hour (dashed lines) at 4°C.

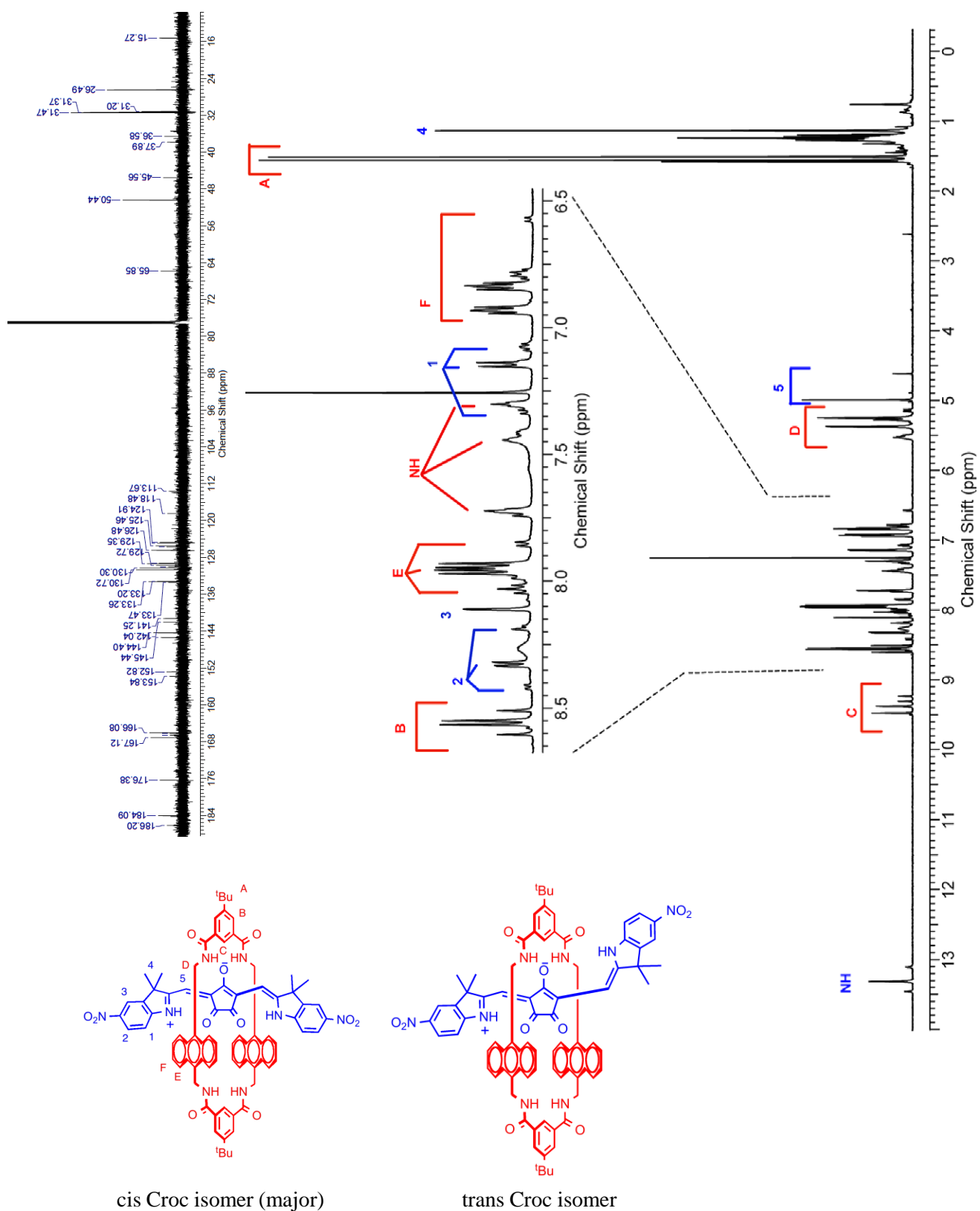


Figure S5. ¹H and ¹³C NMR spectra (CDCl₃, 600 or 125 MHz, 295 K) of CrocRot. The ratio of cis to trans encapsulated Croc isomers is ~2:1

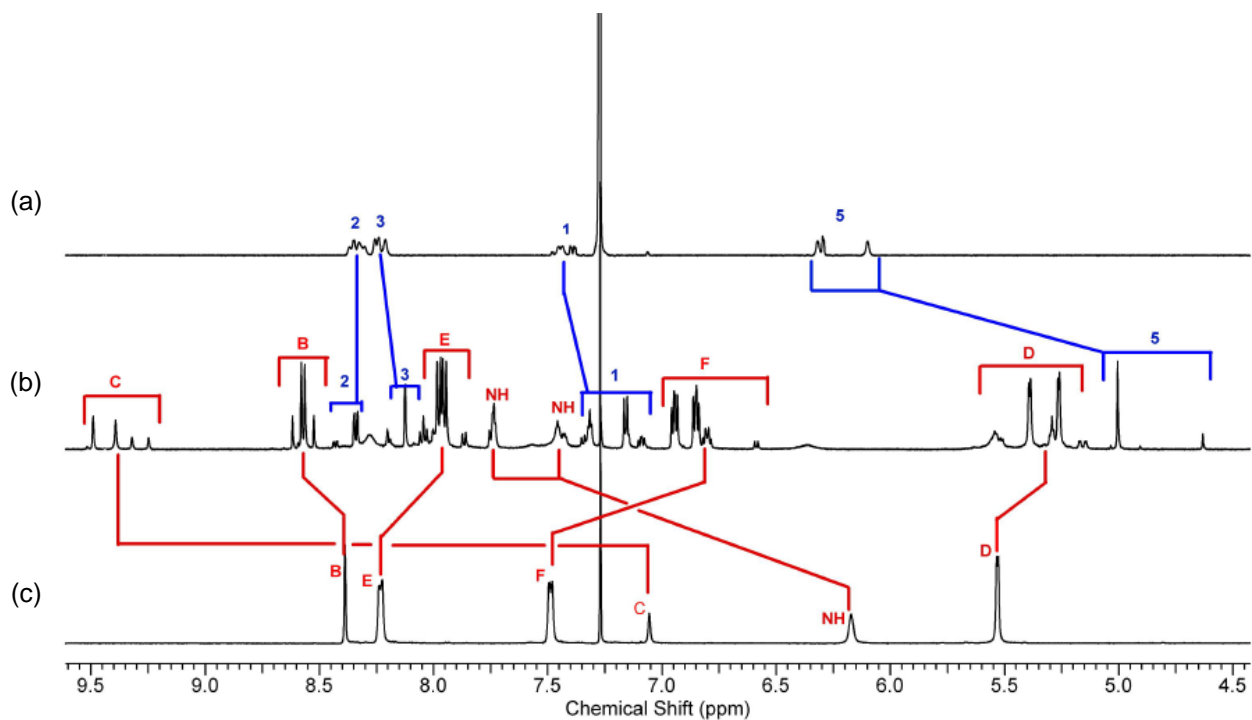
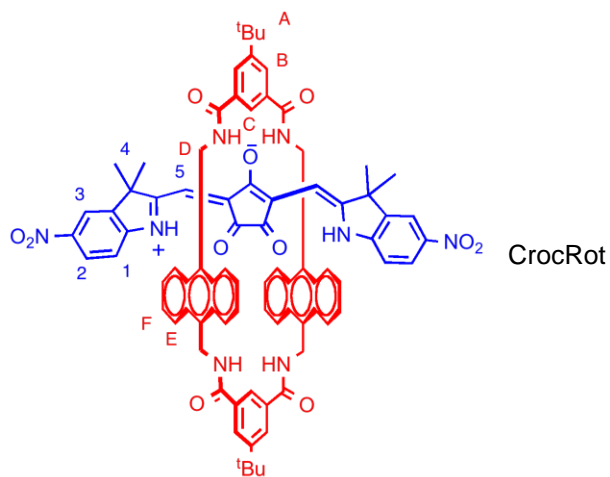


Figure S6 ¹H NMR spectra (CDCl₃, 600 MHz, 295 K) of (a) Croc dye, (b) CrocRot, and (c) empty macrocycle. Downfield shifts in macrocycle proton signals C and NH and upfield shifts in macrocycle protons E and F and dye proton 5 are observed, indicative of rotaxane formation.

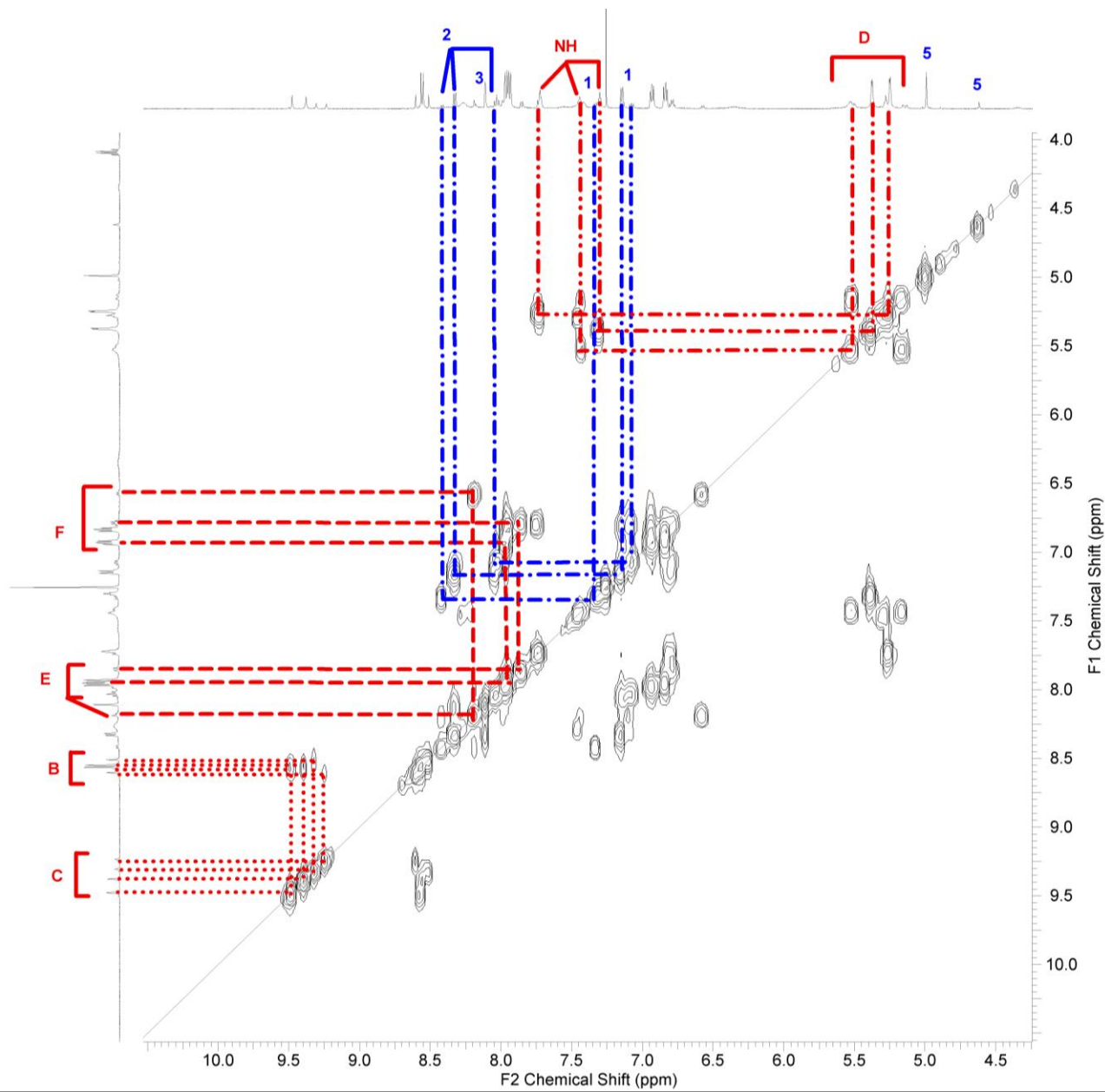


Figure S7. Selected region of ^1H - ^1H COSY NMR spectrum of CrocRot (CDCl_3 , 600 MHz, 298 K).

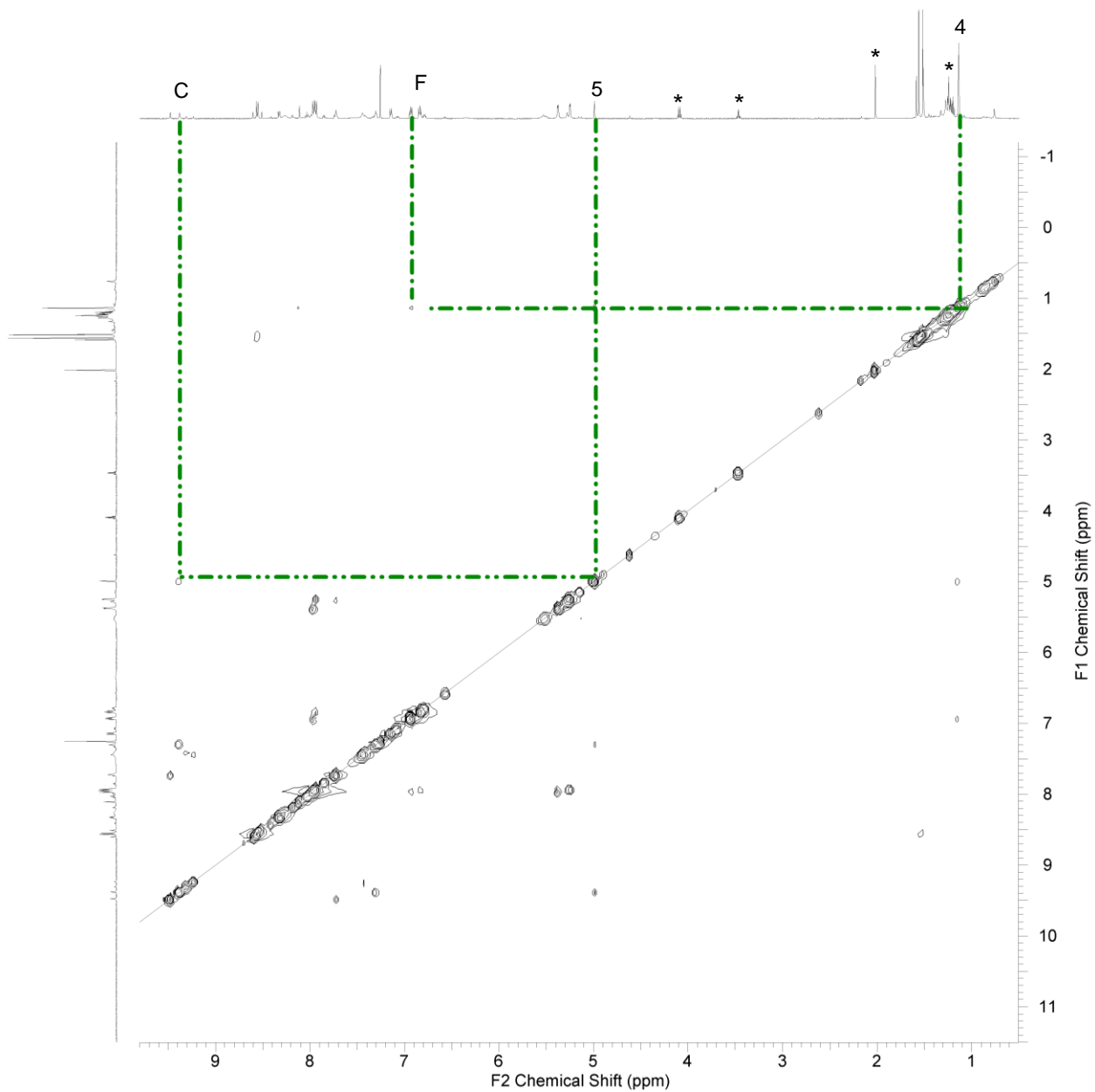


Figure S8. Selected region of ^1H - ^1H ROESY NMR spectrum of CrocRot (CDCl_3 , 600 MHz, 298 K). Signal 4 correlates to signal F and signal 5 correlates to signal C, confirming rotaxane formation. Asterisks indicate residual solvent peaks.

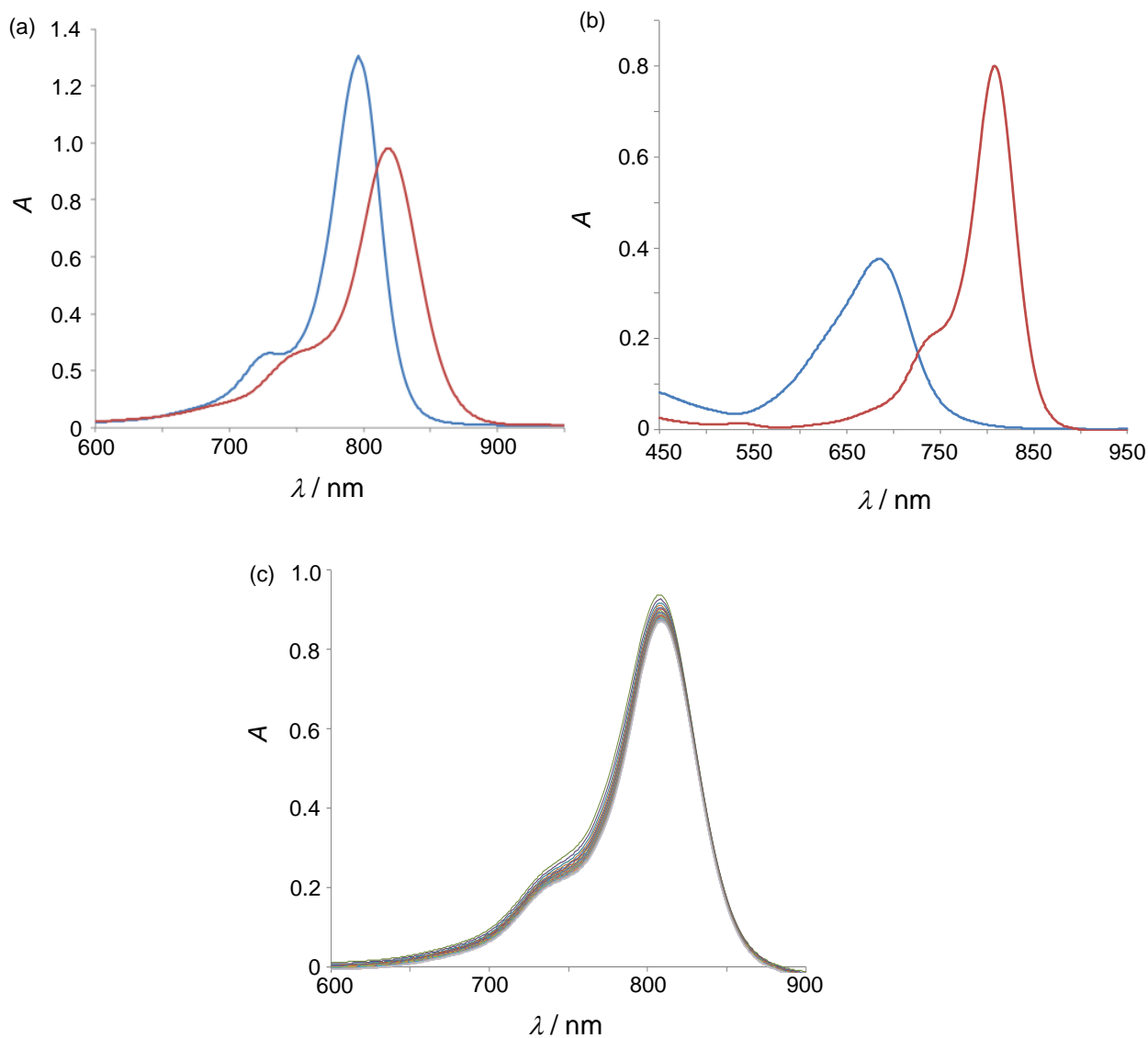


Figure S9. (a) UV/Vis absorption spectrum of free acidic Croc dye (blue) and acidic CrocRot (red) (CHCl_3 , $10.0 \mu\text{M}$, 25°C). The acidic CrocRot display a 22 nm bathochromic shift in the λ_{max} with respect to free acidic Croc dye. (b) Absorption spectrum of CrocRot (99:1 EtOH:DMSO, $10.0 \mu\text{M}$, 25°C) in acidic form (red) and basic form (blue). (c) Absorption spectrum of CrocRot (CH_3CN , $10.0 \mu\text{M}$, 25°C) in acidic form over 12 hours showing that dye does not dethread from surrounding macrocycle. CH_3CN was used for this experiment because this solvent is known to strongly promote dethrading of analogous squaraine psuedorotaxanes.^{S1}

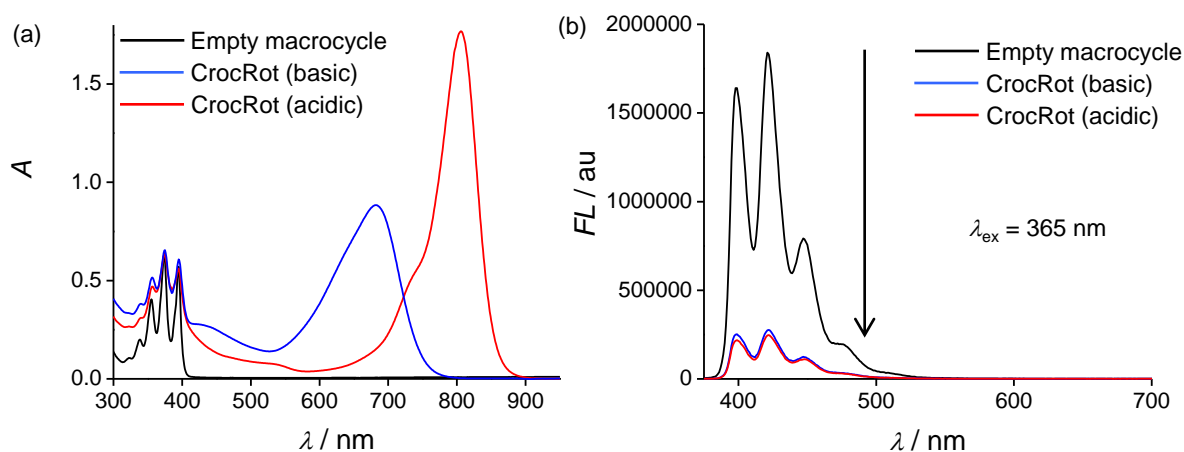


Figure S10. (a) UV/Vis absorption spectra of acidic CrocRot (red), basic CrocRot (blue), and empty macrocycle (black) (EtOH, 25°C). (b) Fluorescence spectra of acidic CrocRot (red), basic CrocRot (blue), and empty macrocycle (black) (EtOH, 25°C, $\lambda_{\text{ex}} = 365 \text{ nm}$). Compared to the empty macrocycle, there is significant quenching of the anthracene fluorescence with both forms of CrocRot, consistent with an interlocked rotaxane structure.

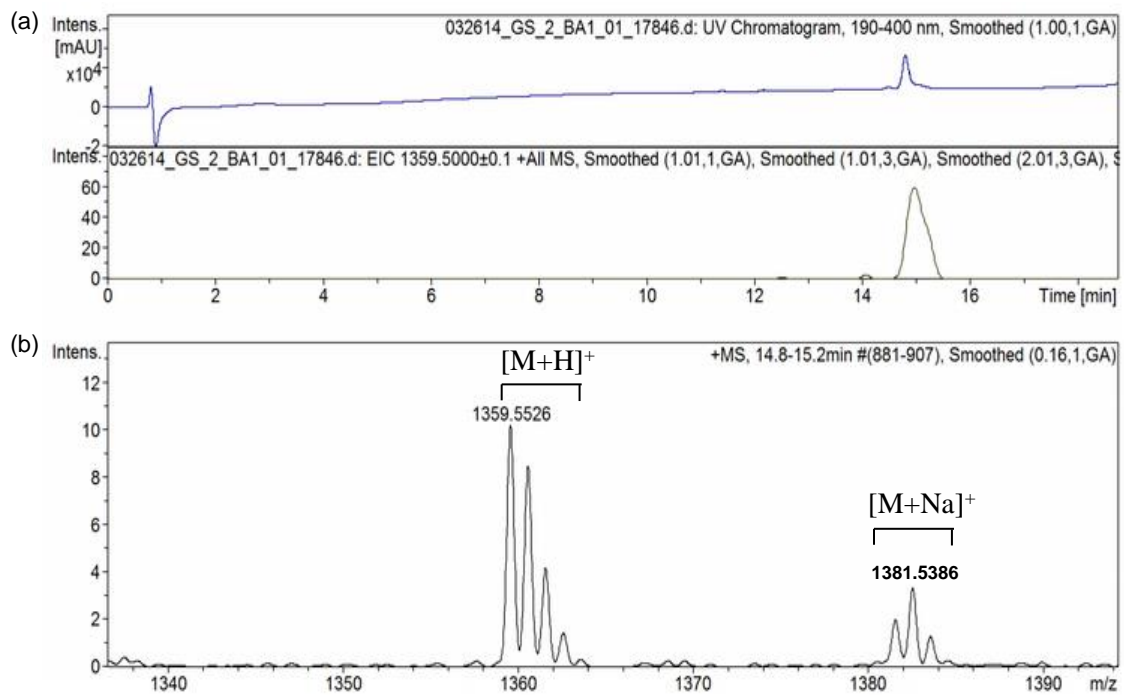


Figure S11. (a) HPLC trace of CrocRot. (b) HRMS (ESI) of CrocRot m/z $[M+H]^+$ 1359.5526 and $[M+Na]^+$ 1381.5386.

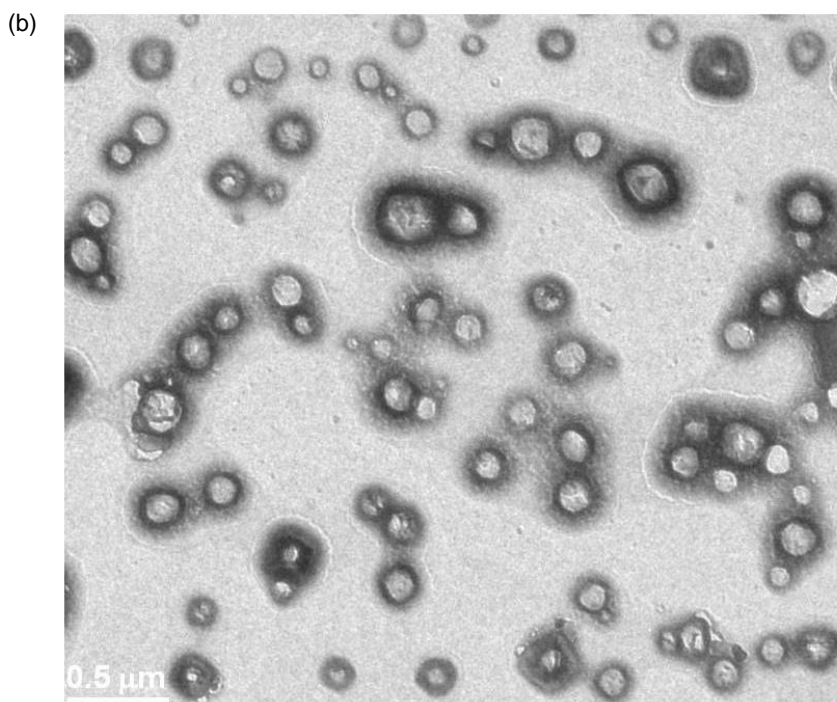
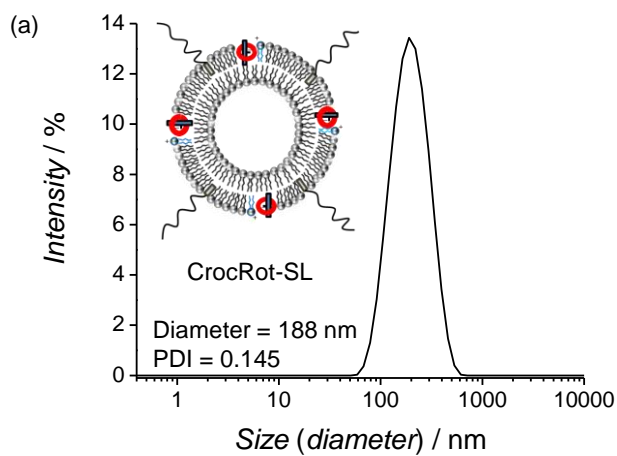


Figure S12. (a) DLS study of CrocRot-SL (hydrodynamic diameter 188 nm and PDI = 0.145). (b) TEM image of CrocRot-SL at pH 7.4, negative staining with 2% (w/v) uranyl acetate. Average diameter of the liposome is 179 nm.

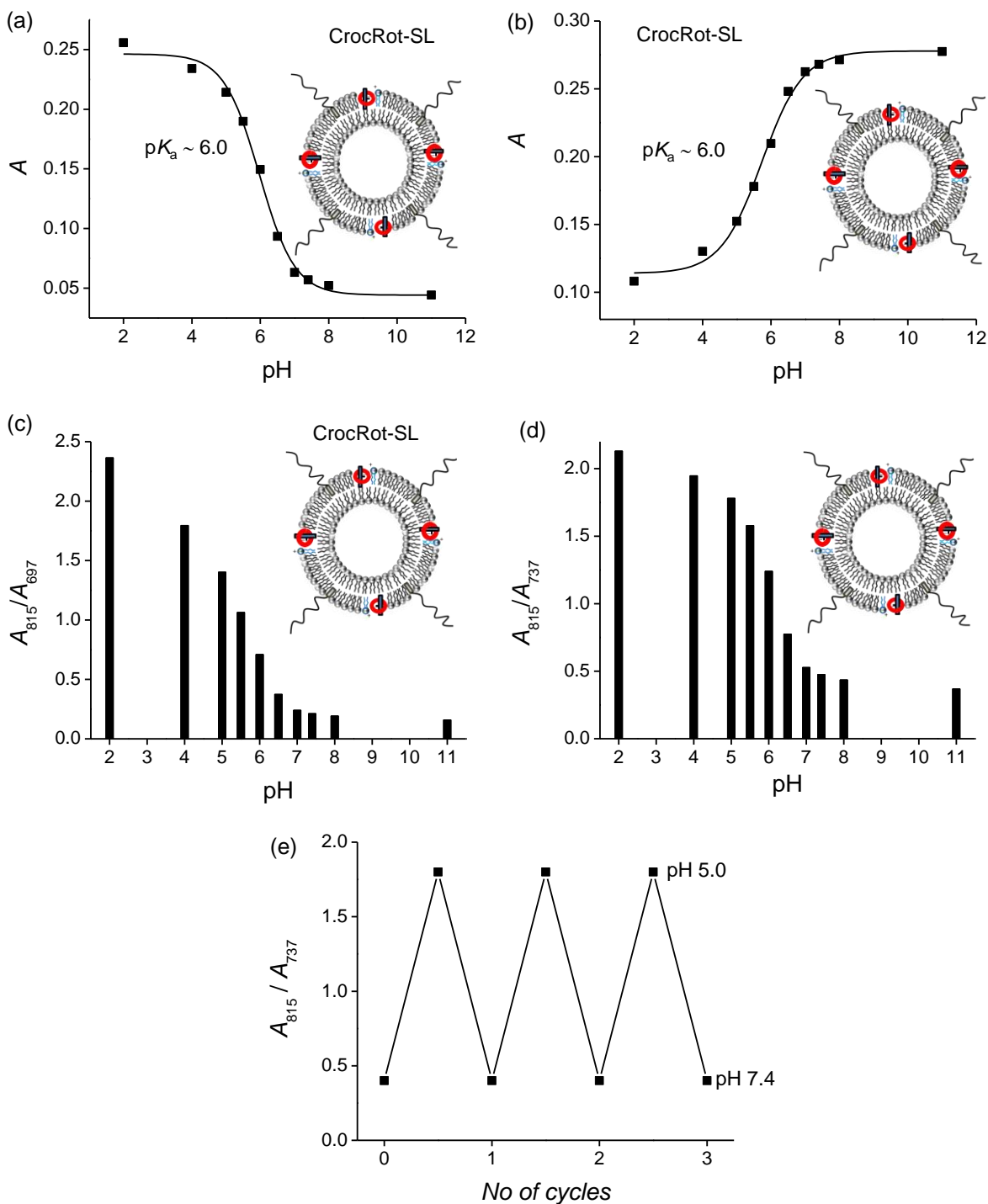


Figure S13. pK_a determination for CrocRot-SL. (a and b) pH vs absorbance plots with sigmoidal curve fitting giving $pK_a \sim 6.0$. The absorption peak ratio (c) 815/697 and (d) 815/737 for CrocRot-SL in buffer at various pH values. (e) Cycling of a single CrocRot-SL solution between pH 7.4 and 5.0, shows that the spectral switching is fully reversible and there is no spectral evidence for CrocRot unthreading.

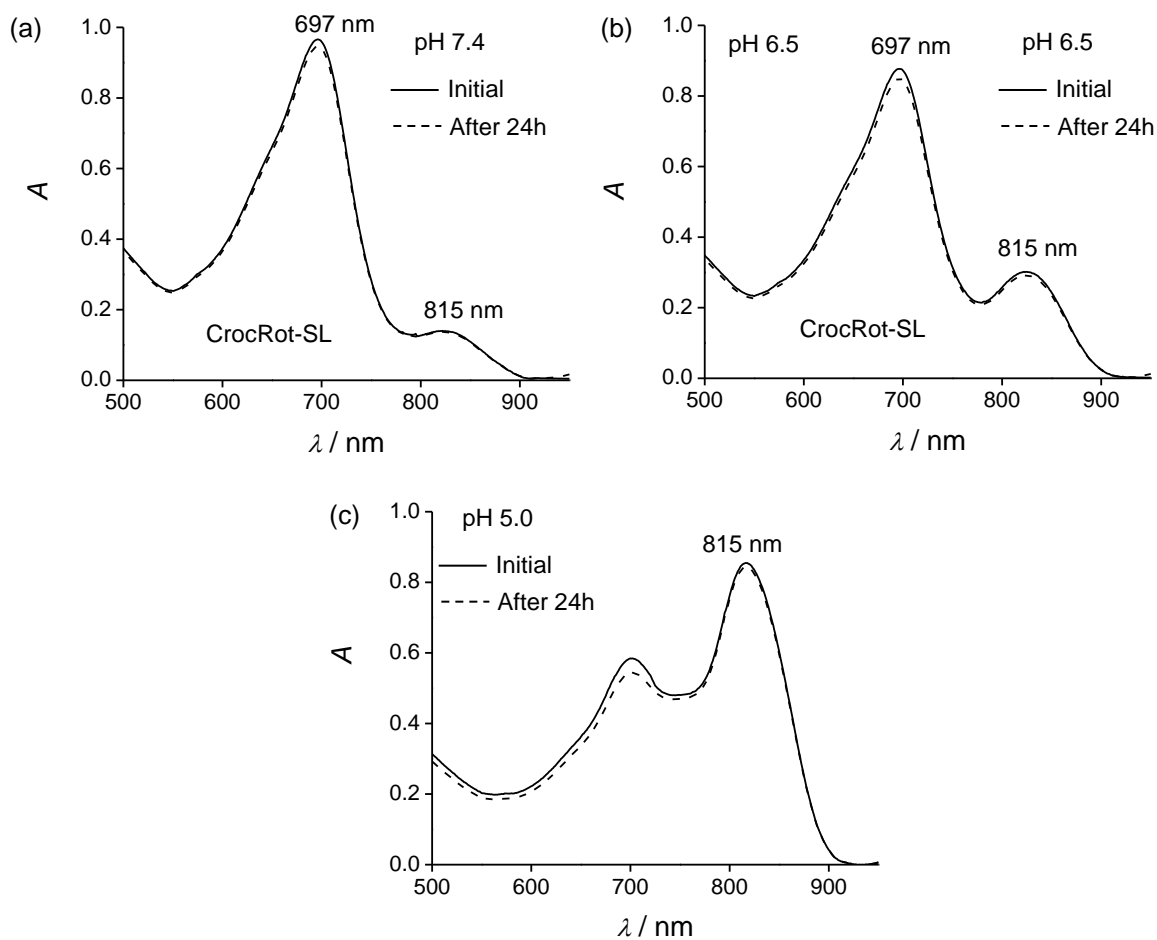


Figure S14. UV/Vis plots of CrocRot-SL in buffer at pH (a) 7.4, (b) 6.5 and (c) 5.0. Spectra were acquired initially (solid lines) and after standing for 24 hours (dashed lines) at 4°C. There is no spectral evidence for CrocRot band broadening or unthreading of the interlocked dye.

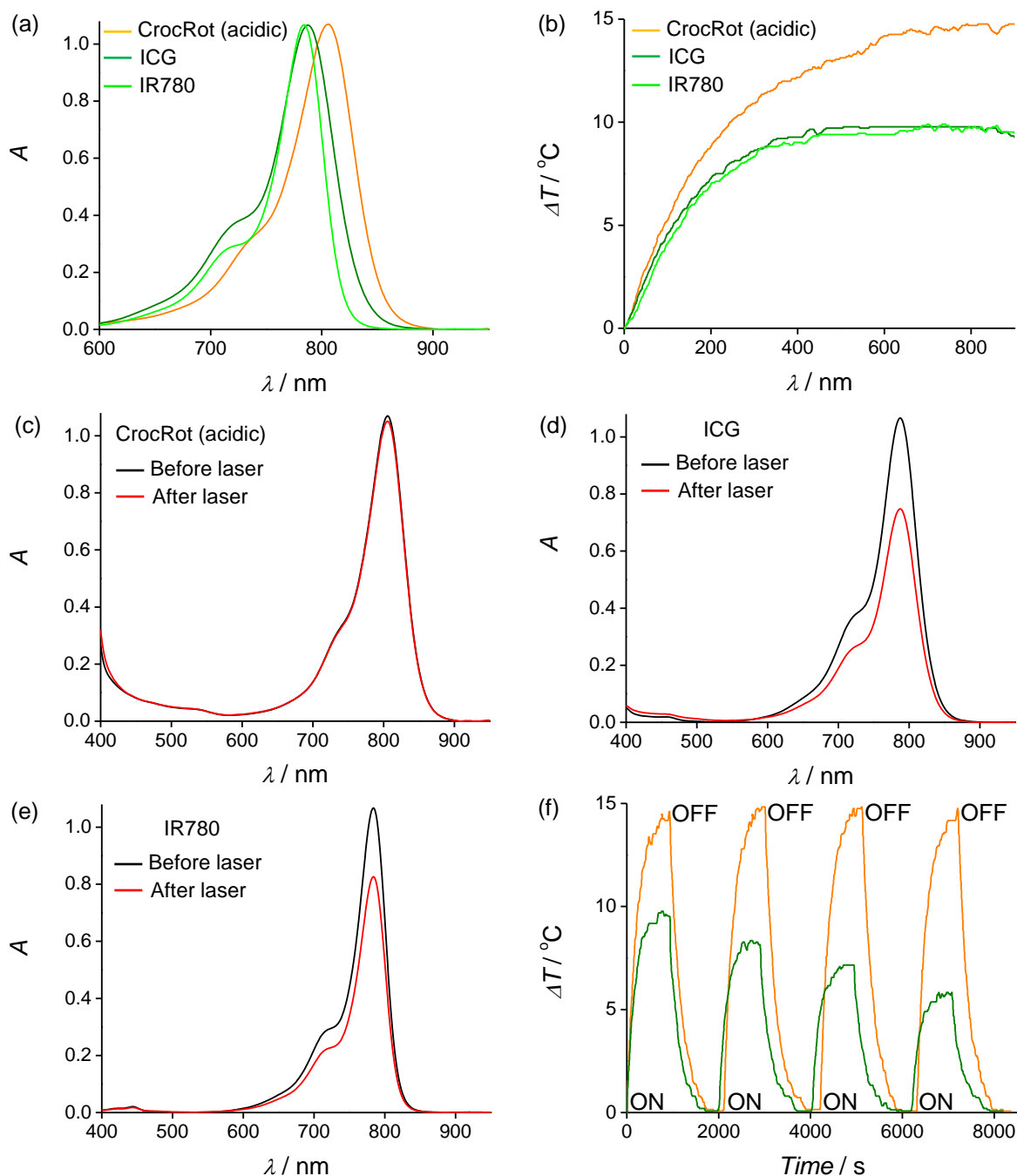


Figure S15. Comparisons showing high photostability and heating effectiveness of CrocRot relative to two commercial near-infrared dyes ICG and IR780. (a) UV/Vis absorption spectra of CrocRot (acidic, orange), ICG (dark green), and IR780 (light green) in EtOH. (b) Temperature change profiles during laser irradiation of CrocRot (acidic, orange), ICG (dark green), and IR780 (light green) in EtOH with same absorbance. (c-e) UV/Vis absorption of CrocRot (acidic), ICG, and IR780 in EtOH before (black) and after (red) laser (15 min, 808 nm) irradiation. (f) Multiple heating cycles of CrocRot (acidic, orange) and ICG (dark green) due to periodic laser irradiation (808 nm).

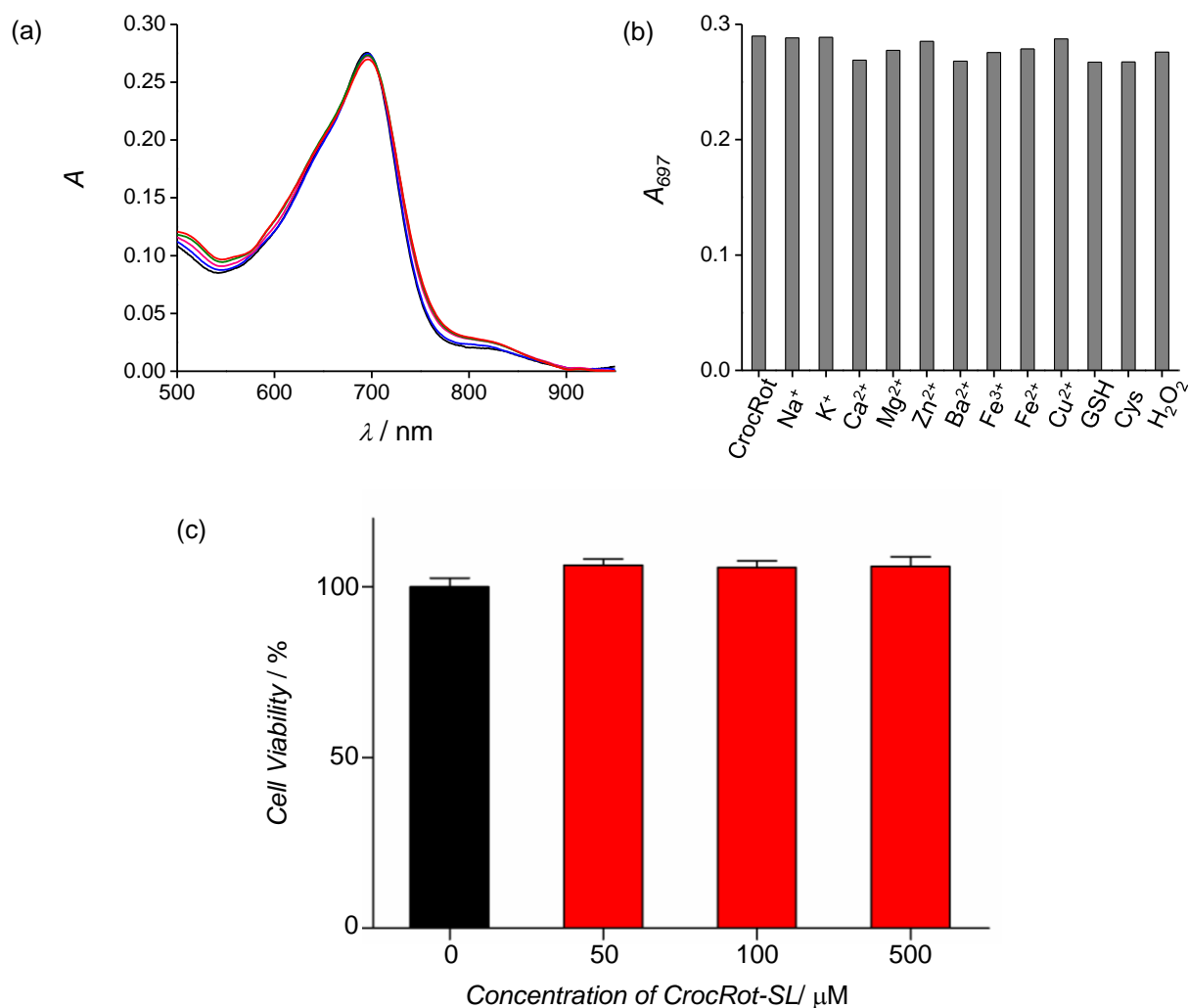


Figure S16. (a) No change in UV/Vis absorption plot of CrocRot-SL in 2% FBS (w/w) in buffer at physiological pH 7.4 over 24 hour at 37°C. (b) No change in UV/Vis response of CrocRot-SL in the presence of ions (10 mM for Na⁺, K⁺, and 200 μ M for Ca²⁺, Mg²⁺, Zn²⁺, Ba²⁺, Fe³⁺, Fe²⁺, and Cu²⁺), and bioactive small molecules [5 mM for glutathione (GSH), cysteine (Cys), and 1 mM for H₂O₂] in buffer at pH 7.4, 37°C. (c) Cell viability of EMT-6 mouse mammary carcinoma cells incubated for 24 hours with different doses of CrocRot doped stealth liposomes (CrocRot-SL) composed of POPC:Chol-PEG600:DOTAP:CrocRot 84:10:3:3 at pH 7.4, 37°C. The CrocRot-SL concentration corresponds to total amount of material in the liposomes which includes 3% CrocRot dye.

(a)



(b)

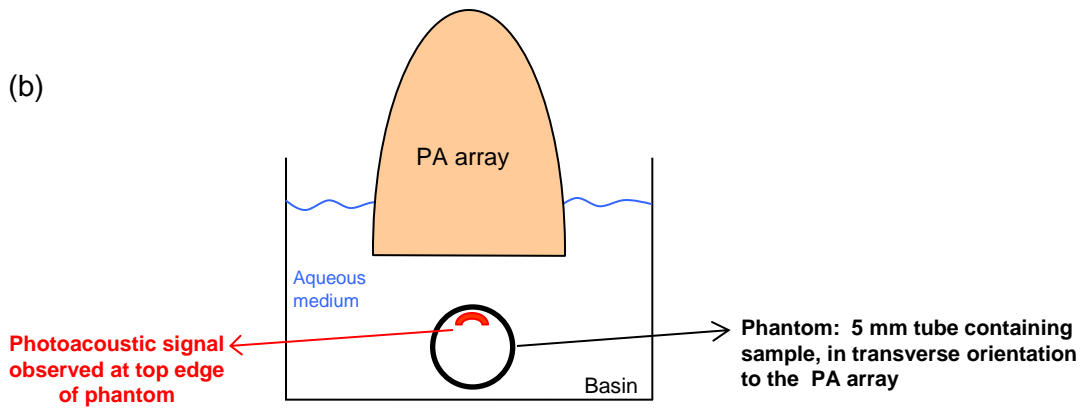


Figure S17. (a) Photograph of the photoacoustic imaging apparatus, showing the photoacoustic array partially submerged in a basin containing aqueous medium, with six phantoms (vinyl tubes) located near the bottom. (b) Schematic of the apparatus showing one phantom in transverse orientation to the transducer, and the photoacoustic signal producing the images in Figure 4b and S19.

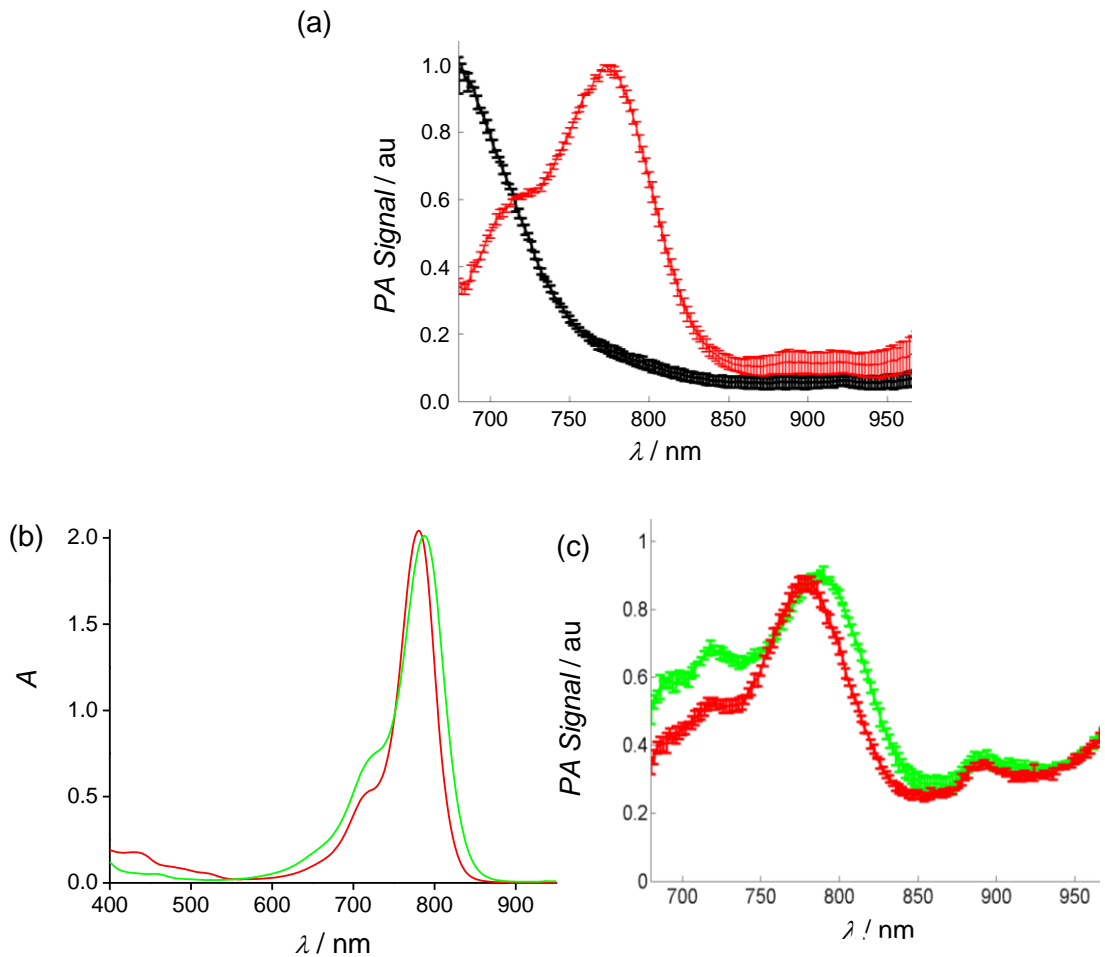


Figure S18. (a) Photoacoustic spectra of acidic Croc (red) and basic Croc (black) in ethanol. Mean ($N = 3$) and SD are shown. (b) UV/Vis absorption spectra of acidic Croc (red) and ICG (green) in ethanol. (c) Photoacoustic spectra of acidic Croc (red) and ICG (green). Mean ($N = 3$) and SD shown.

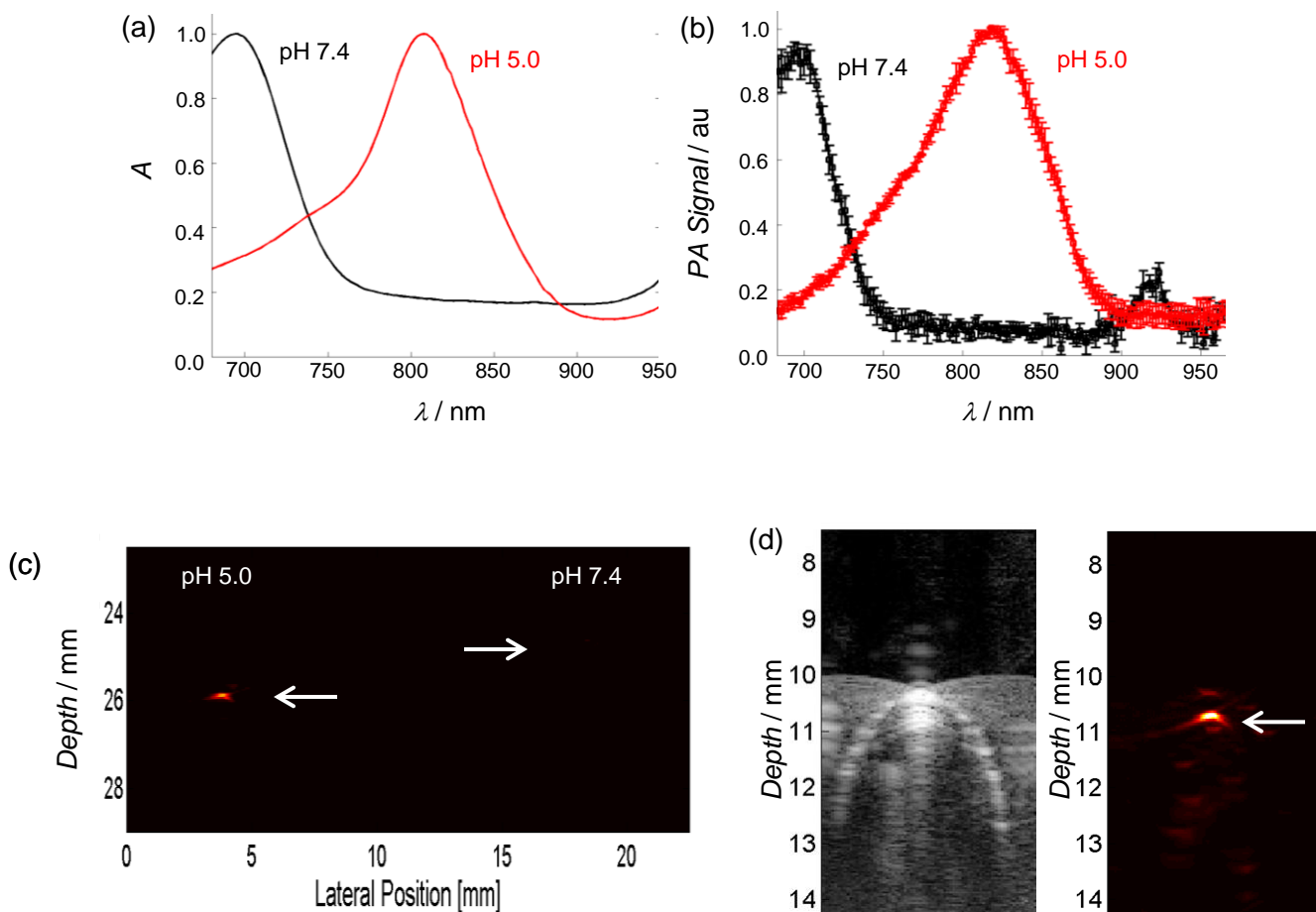


Figure S19. (a) UV/Vis absorption spectra of CrocRot-SL at pH 7.4 (black) and pH 5.0 (red). (b) Photoacoustic spectra of CrocRot-SL at pH 7.4 (black) and pH 5.0 (red), mean ($N = 3$) and SD shown. Note that samples are 4 days old. (c) Photoacoustic images (ex: 812 nm) of two phantoms (5 mm tubes) containing CrocRot-SL at pH 5.0 or pH 7.4, proximally located at 25-26 mm depth in scattering media. (d) Ultrasound (left) and photoacoustic image (right, ex: 812 nm) of a phantom, containing CrocRot-SL at pH 5.0, located at 11 mm depth in scattering media. These images also show reverberation noise. The white arrows indicate location of the photoacoustic signal at the top edge of the phantom closest to the transducer.

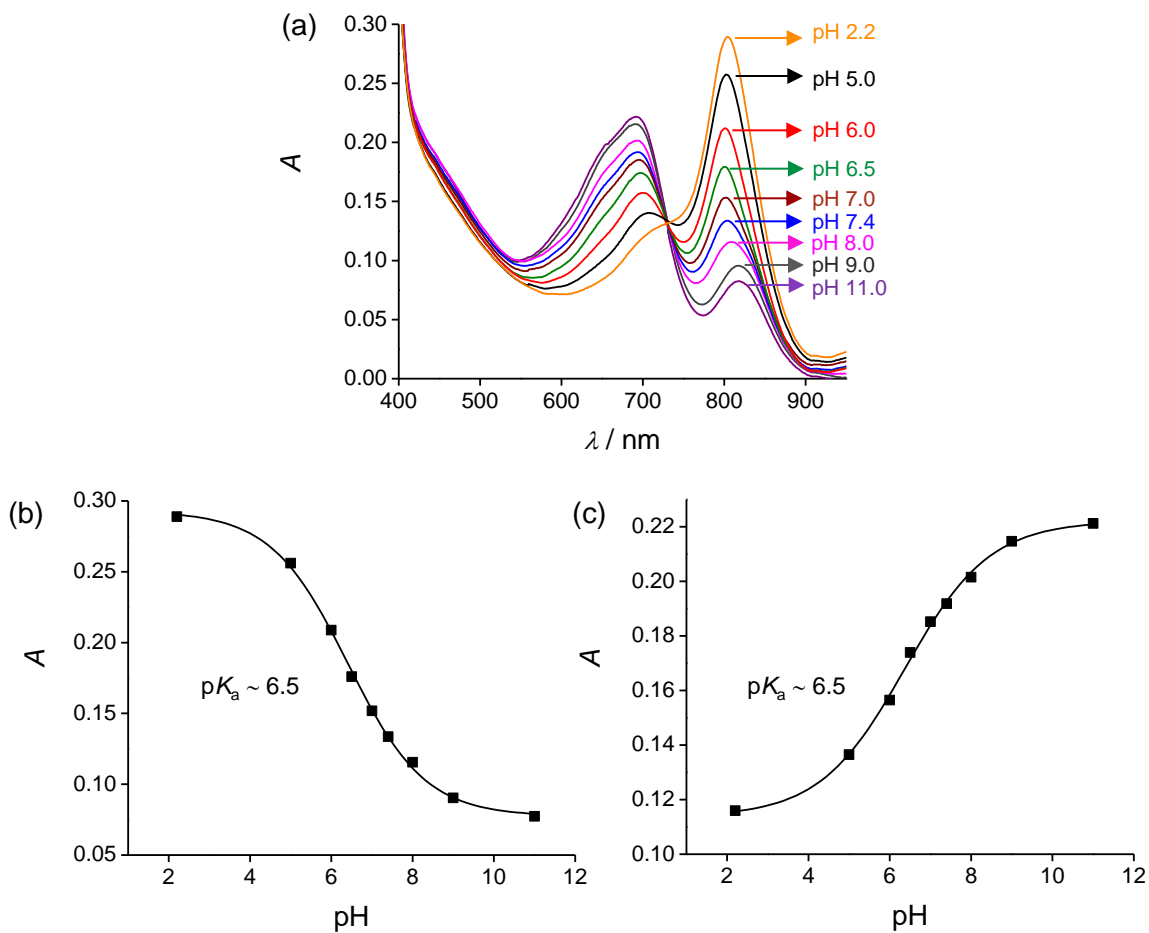


Figure S20. (a) UV/Vis absorption titration of CrocRot-IVSL (composition: POPC:DSPE-PEG2000:DOTAP:CrocRot 79:15:3:3) in buffer at various pH values, [CrocRot] = 3 μ M. (b) and (c) pH vs absorbance plots with sigmoidal curve fitting showing $pK_a \sim 6.5$.