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Imaging the lipid bilayer of giant vesicles using red-to-blue light upconversion

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Supplementary information

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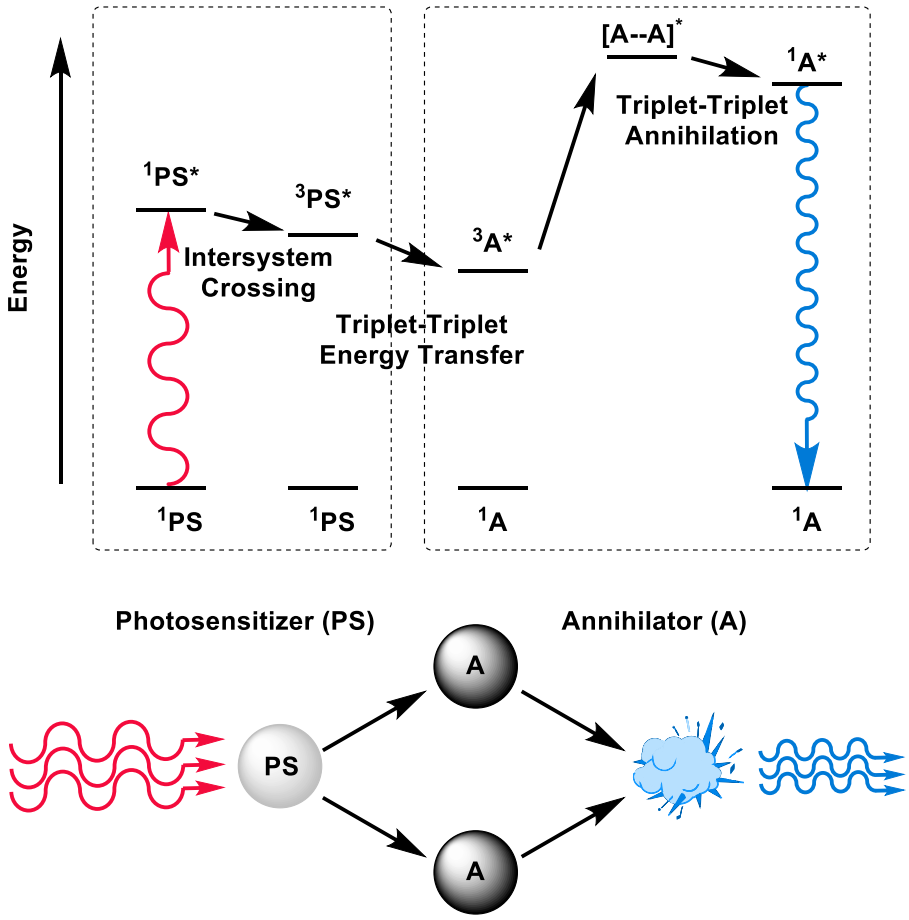

Figure S1. Jablonski diagram of the triplet-triplet annihilation upconversion scheme. Red light is absorbed by the photosensitizer (PS), which undergoes intersystem crossing to a triplet state. This triplet state can be transferred to a ground state annihilator (A) by triplet-triplet energy transfer. Two triplet excited state annihilator molecules can then perform triplet-triplet annihilation, thereby creating one ground state annihilator and one singlet excited state. The latter state returns to the ground state and emits a blue photon. Adapted from Singh-Rachford and Castellano.[1]

1. Materials

Palladium tetraphenyltetrabenzoporphyrin (**1**) was purchased from Frontier Scientific, Inc. (Logan, Utah, USA). Perylene (**2**) was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). Sodium N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG-2000), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from Lipoid GmbH (Ludwigshafen, Germany) and stored at -18 °C. Dulbecco's phosphate buffered saline (PBS) was purchased from Sigma Aldrich and had a formulation of 8 $g.L^{-1}$ NaCl, 0.2 $g.L^{-1}$ KCl, 0.2 $g.L^{-1}$ KH_2PO_4, and 1.15 $g.L^{-1}$ K_2HPO_4 with a pH of 7.1 – 7.5. All other chemicals were purchased from major chemical suppliers and used as received. Images and data were processed with Fiji ImageJ, Origin Pro, and Microsoft Excel software.

2. Methods

2.1. GUV preparation

All GUVs were prepared by lipid film re-hydration on dextran chemically cross-linked hydrogel substrates by a method described elsewhere.[2] The preparation of **GUV12** is described here as an example. Glass microscopy slides were first incubated with 1:1 vol MeOH : HCl (37%) for 30 min, then with 98% H_2SO_4 for 30 min, and then thiol-functionalized by incubating them for 1 h in a 2 wt% solution of (3-mercaptopropyl)triethoxysilane in dry toluene under a nitrogen atmosphere, and washing them three times with toluene. Directly after, a homogeneous film of Dex-PEG hydrogel was formed on this surface by drop-casting 600 µL of a 1:1 volume mixture of 2 wt.% maleimide-functionalized dextran, with a substitution degree of 3 maleimide groups per 100 glucopyranose residues of dextran (synthesis and characterization detailed in ref. 2), in water and 2 wt.% α,ω-PEG dithiol (1500 $g.mol^{-1}$) in water at room temperature. A homogenous hydrogel film was formed after 30 – 45 minutes at 40 °C. Then, 10 µL of lipid mixture stock solution in chloroform, containing 20 mM DMPC or DOPC, 0.8 mM DSPE-PEG-2K, 0.1 mM perylene (**2**), and 5 µM of compound **1**, was deposited on the hydrogel surface. The organic solvent was evaporated for 30 minutes under a gentle stream of air followed by a period of at least 30 minutes in a 30 °C vacuum oven. The lipid film was then hydrated with 400 µL phosphate buffered saline (PBS) supplemented with 0.2 M sucrose, and when wanted 0.3 M sodium sulfite, for 1 - 2 hours at room temperature (ca. 293 K) in case of DOPC GUVs, or at 308 K in case of DMPC GUVs. This receipt produced a solution containing free-floating vesicles that could be directly pipetted in a fluorescence cuvette for emission spectroscopy (see section 2.2). Alternatively, it was further used for the preparation of a microscopy experiment (section 2.3).

2.2. Emission spectroscopy on GUVs

For upconversion emission spectroscopy, approximately 700 µL of the above-mentioned solution of free-floating vesicles in buffer was transferred to a semi-micro cuvette and used as such in the setup detailed in section 2.5.

2.3. Preparation of a microscopy experiment with GUVs

For optical microscopy imaging, 300 µL of the solution containing free-floating vesicles in buffer (section 2.1) was transferred to an Eppendorf tube containing 700 µL phosphate buffered saline supplemented with 0.3 M sodium sulfite and 0.2 M glucose to allow the sucrose-loaded giant vesicles to sink to the bottom of the tube. After one hour, 200 µL of this GUV suspension was transferred to a visualization microscopy chamber that had previously been coated with bovine serum albumin (BSA). As a result of surface treatment with BSA and of the heavier weight of the sucrose-loaded vesicles, the giant vesicles were immobilized on the glass surface of the chamber, which allowed for imaging with minimal diffusion during image recording. The rest of the chamber was filled with 100 µL PBS supplemented with 0.3 M sodium sulfite and 0.2 M glucose. The vesicles were imaged within 24 hours.

2.4. LUV preparation and characterization

Upconverting LUVs, i.e. **LUV12** samples, were prepared as described before as a reference.[3] Aliquots of chloroform stock solutions containing the liposome constituents were added together in a flask to obtain a solution with 20 µmol DMPC, 0.8 µmol DSPE-MPEG-2000, 100 nmol perylene (**2**), and 5 nmol of compound **1**.

The organic solvent was removed by rotary evaporation and subsequently under high vacuum for at least 30 minutes to create a lipid film. 1.0 mL PBS buffer, optionally supplemented with 0.3 M Na_2SO_3, was added and the lipid film was hydrated by 5 cycles of freezing the flask in liquid nitrogen and thawing in warm water (50 °C). The resulting dispersion was extruded through a Whatman Nuclepore 0.2 µm polycarbonate filter at 40-50 °C at least 11 times using a mini-extruder from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). The number of extrusions was always odd to prevent any unextruded material ending up in the final liposome sample. The extrusion filter remained colourless after extrusion, suggesting complete inclusion of the sensitizer and annihilator in the lipid bilayer. Liposomes were stored in the dark at 4 °C and used within 7 days. The liposomes had an average diameter of ca. 150 nm and a polydispersity index of 0.1, as determined from dynamic light scattering measurements with a Malvern Instruments Zetasizer Nano-S machine, operating at a wavelength of 632 nm. Additionally, cryo transmission electron microscopy was performed on DMPC **LUV12** (see Figure S2) as described in Bahreman, A.; Limburg, B.; Siegler, M. A.; Koning, R.; Koster, A. J.; Bonnet, S. *Chem-Eur J* **2012**, *18*, 10271–10280.

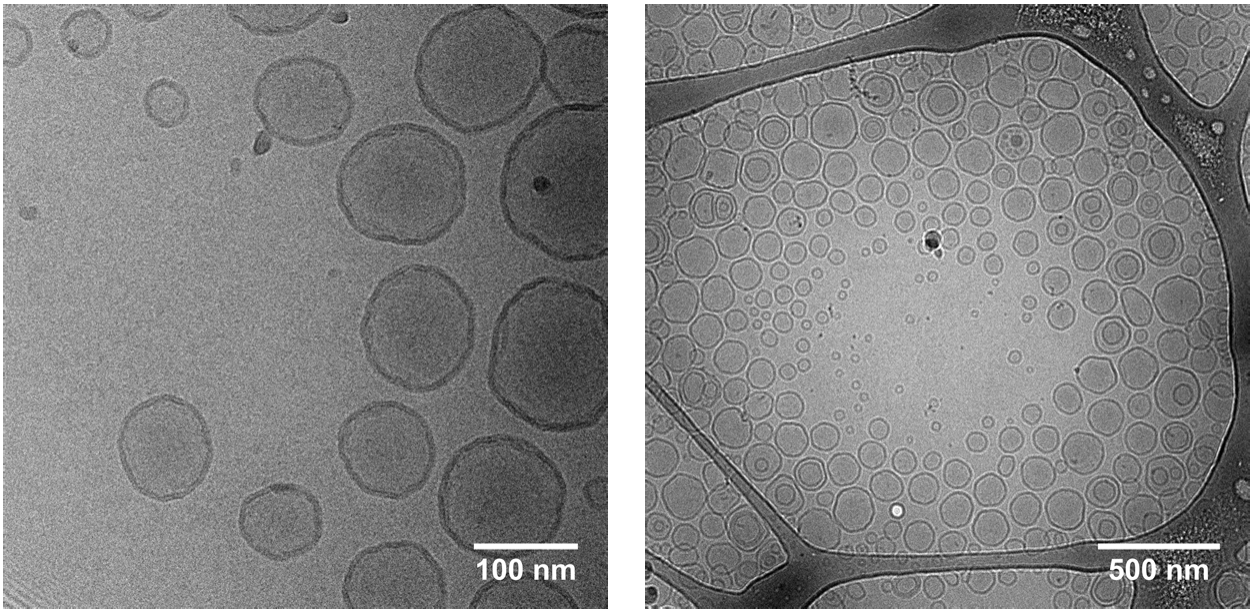

Figure S2. Cryo transmission electron micrographs of DMPC LUV12.

2.5. Upconversion emission spectroscopy

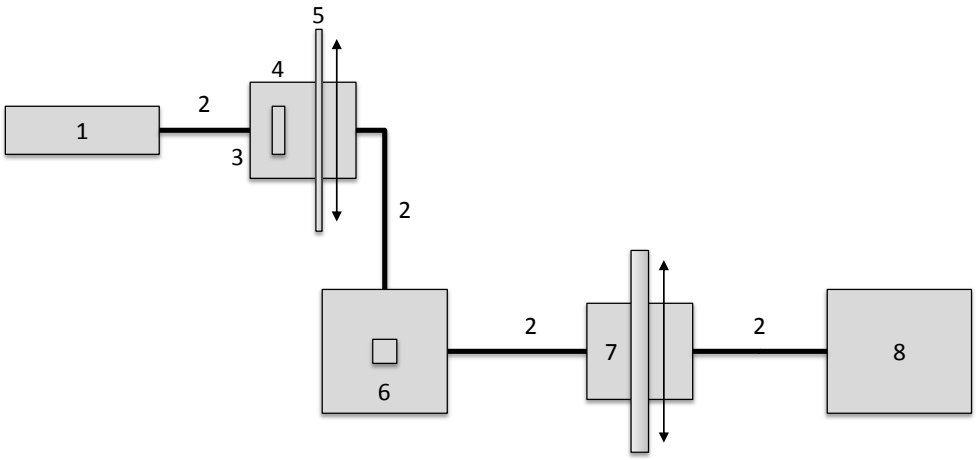

Figure S3. Setup used for emission measurements under red light irradiation. Legend: (1) 630 nm laser source, (2) optical fibers, (3) filter holder, (4) 630 nm band pass filter, (5) variable neutral density filter, (6) temperature controlled cuvette holder, (7) variable filter holder, and (8) CCD spectrometer.

Upconversion emission spectroscopy was performed in a custom-built setup (Figure S3). All optical parts were connected with FC-UVxxx-2 (xxx = 200, 400, 600) optical fibers from Avantes (Apeldoorn, The Netherlands), with a diameter of 200-600 µm, respectively, and that were suitable for the UV-Vis range (200-800 nm). For **LUV12** samples that were deoxygenated by argon bubbling: argon was bubbled through the sample (3.0 mL) with a rate of ~2 bubbles per second for at least 30 minutes in an external ice-cooled pear-shaped flask. After this period, bubbling was stopped while maintaining the argon flow, and the sample was warmed in a water bath of approximately 40 °C for 10 minutes. Then, the sample was transferred by means of cannulation with argon pressure to a 111-OS macro fluorescence cuvette from Hellma in a CUV-UV/VIS-TC temperature-controlled cuvette holder from Avantes, while keeping the sample under a constant flow of argon throughout the measurement. For **LUV12** samples that were deoxygenated by addition of sodium sulfite, 3.0 mL of the sample was simply transferred to the cuvette and emission spectra were recorded under air. Likewise, **GUV12** samples in sodium sulfite buffer (approximately 700 µL) were transferred to a 104F‑QS or 104F-OS semi-micro cuvette from Hellma.

The sample in the cuvette holder was allowed to equilibrate at 298 K for 10 minutes. The sample was irradiated from the side with a 30 mW 630 nm laser light beam from a clinical grade Diomed 630 nm PDT laser (4 mm beam, 0.24 $W.cm^{-2}$). The 630 nm light was filtered through an FB630-10, 630 nm band pass filter (Thorlabs, Dachau/Munich, Germany) put between the laser and the sample. The excitation power was controlled using a NDL-25C-4 variable neutral density filter (Thorlabs), and measured using a S310C thermal sensor connected to a PM100USB power meter (Thorlabs). Emission spectra were recorded at a 90° angle with respect to the excitation source using a 2048L StarLine CCD spectrometer from Avantes. To visualize the spectrum from 550 nm to 900 nm, while blocking the red excitation light, a Thorlabs NF-633 notch filter was used in a variable filter holder. To visualize the spectrum from 400 nm to 550 nm, an OD4 575 nm short pass filter (Edmund Optics, York, United Kingdom, part no. 84-709) was used. All spectra were recorded with Avasoft software from Avantes and further processed with Microsoft Office Excel 2010 and Origin Pro software. The emission spectra obtained with the two filters were stitched together at 550 nm to obtain a continuous spectrum from 400 to 900 nm. No correction was needed to seamlessly connect the spectra (Figure S4).

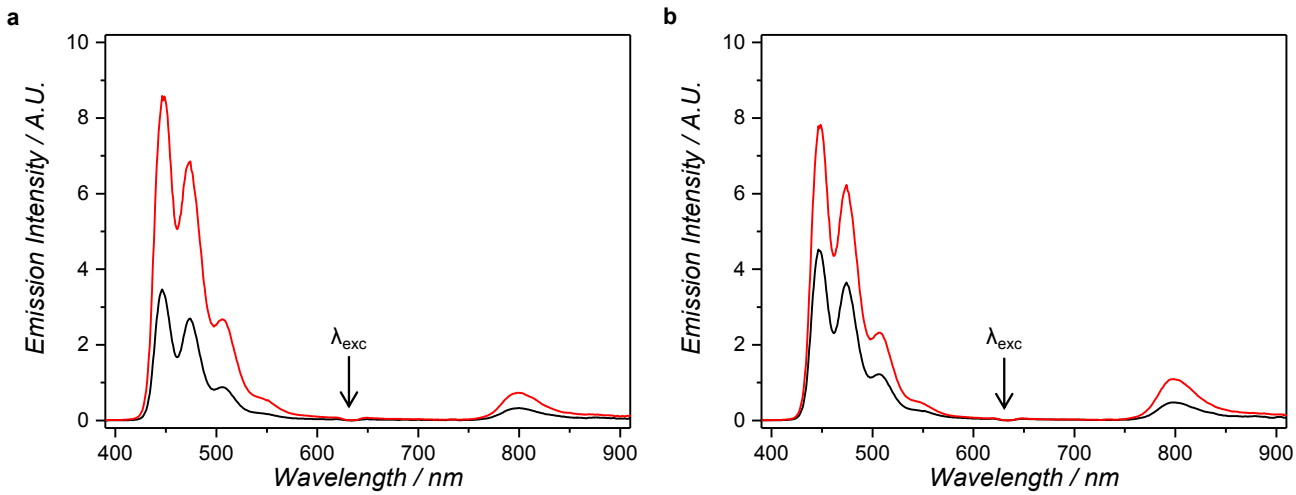

Figure S4. Emission spectra of DOPC (a) and DMPC (b) LUV12 samples ([lipid] = 1 mM, [DSPE-PEG-2000] = 0.04 mM, [2] = 5 µM, [1] = 0.25 µM) under 630 nm excitation at 298 K. The samples were either deoxygenated by bubbling argon for 30 min prior to measurement (black curves) or by addition of sodium sulfite at a concentration of 0.3 M to the buffer (red curves). Irradiation conditions: 3.0 mL sample volume in a macro fluorescence cuvette, with 30 mW 630 nm irradiation power (4 mm beam diameter, intensity 0.24 W.cm^{-2}). Bubbling of argon through the sample inevitably results in the formation of small bubbles on the walls of the measurement cuvette, resulting in scattering of light in both the excitation and the detection pathway. These bubbles are absent in the case of deoxygenation using the sodium sulfite oxygen scavenger, which explains why the observed intensities are higher for samples deoxygenated with sulfite.

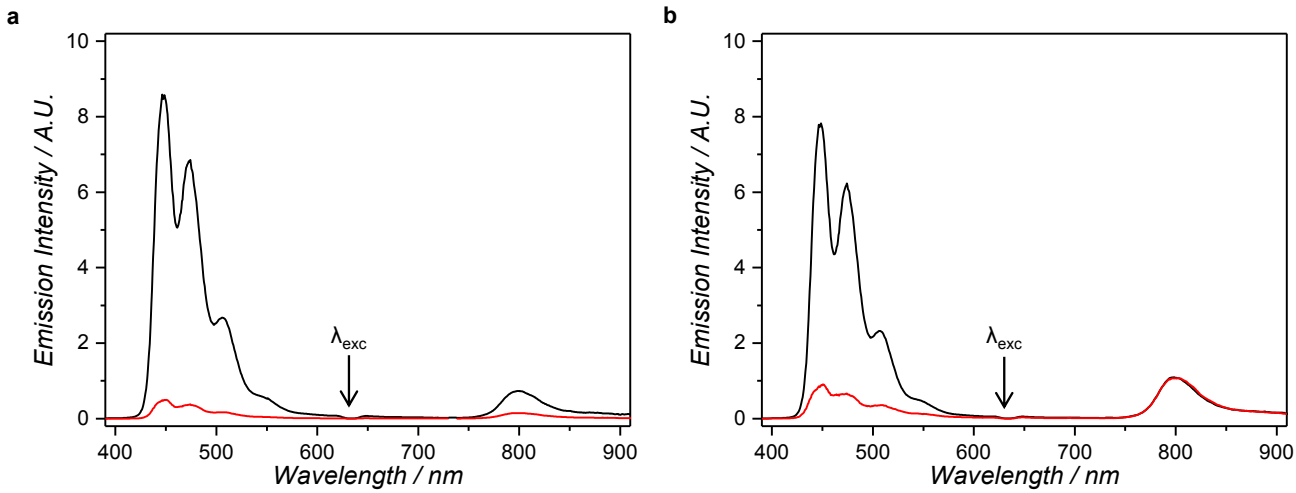

Figure S5. Emission spectra of DOPC (a) and DMPC (b) LUV12 (black curves) and GUV12 (red curves) with 30 mW 630 nm excitation (0.24 W.cm^2 intensity) at 298 K. In the case of LUVs, [DMPC] = 1 mM, [DSPE-PEG-2000] = 0.04 mM, [2] = 5 µM, [1] = 0.25 µM, whereas in the case of GUVs, the lipid concentration was not known, but the components in the membrane were introduced in the same molar ratio as for the LUV samples. In all cases, the buffer was deoxygenated by addition of sodium sulfite (0.3 M) and the spectra were measured under air.

2.6. Power dependency measurements

Luminescence emission spectra of DMPC and DOPC liposomes samples **LUV-12** were recorded at various excitation powers from 1 to 40 mW so that the excitation intensity (P) was 8 to 318 mW.cm^{-2} (4 mm laser beam diameter). The samples were placed in a Hellma 101-OS macro fluorescence cuvette (2.25 mL, [lipid] = 1.0 mM) and thermally equilibrated at 298 K before measurement in the same fluorescence setup as described in

section 2.5. In this case, the spectrum was visualized with only a Thorlabs NF-633 notch filter between the sample and the detector.

The recorded spectra were integrated from 420 to 575 nm to obtain the integrated upconversion luminescence intensity (I_{UC}), which was then plotted in a double logarithmic plot as a function of the excitation intensity (**Figure S6**). The low power (≤ 40 mW.cm^{-2}) and high power (≥ 120 mW.cm^{-2}) regimes were consistently fitted with slopes around 1 and 2, respectively, which shows the typical power dependency of TTA-UC.[1] The intersection of these straight lines represents the intensity threshold (I_{th}) at which the power dependency changes from quadratic to linear. I_{th} was found to be 50 and 59 mW.cm^{-2} for the upconversion in DMPC and DOPC **LUV-12**, respectively. Assuming no difference in power dependency between **LUV-12** and **GUV-12**, these results indicate that all microscopy images with red light excitation (P ≥ 5.2 W.cm^{-2}) were acquired in the linear power regime.

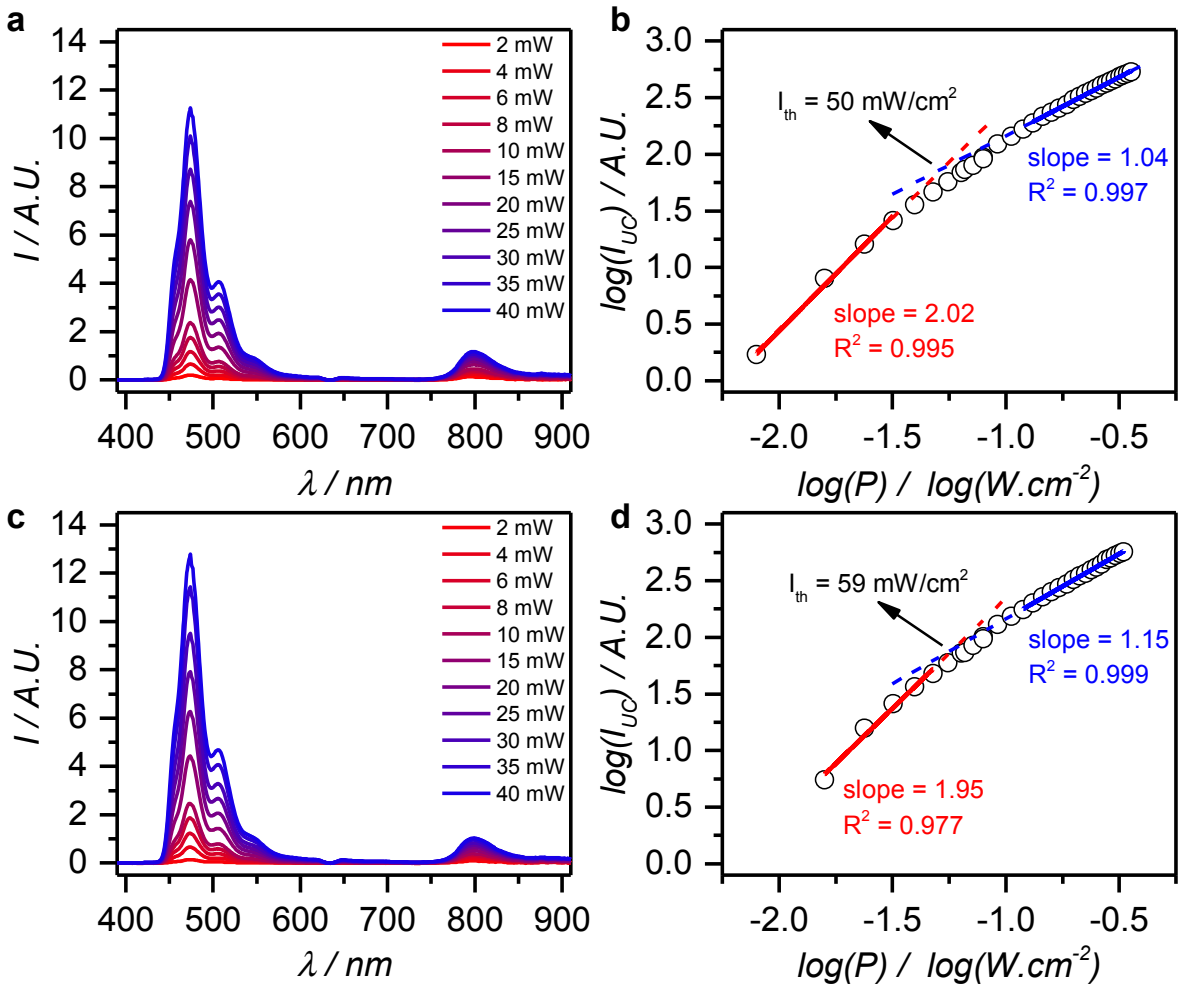

Figure S6. Luminescence emission spectra of DMPC LUV-12 (a) and DOPC LUV-12 (c) at various excitation intensities. Double logarithmic plot of the upconversion luminescence intensity (I_{UC}) of DMPC LUV-12 (b) and DOPC LUV-12 (d), integrated from 420 to 575 nm, as a function of the excitation intensity P (in W.cm^2). The low power regime was fitted with straight lines with slopes 2.02 (R^2 = 0.995) and 1.95 (R^2 = 0.977) for DMPC and DOPC LUV-12, respectively (red solid lines), and the high power regime was fitted with straight lines with slopes 1.04 (R^2 = 0.997) and 1.15 for DMPC and DOPC LUV-12, respectively (blue solid lines). From the intersection of the extrapolated fits (red and blue dashed lines), the intensity threshold (I_{th}) was found to be 50 mW.cm^{-2} for DMPC LUV-12 and 59 mW.cm^{-2} for DOPC LUV-12. Irradiation conditions: [lipid] = 1.0 mM, T = 298 K, laser beam diameter 4 mm.

2.7. Microscopy imaging

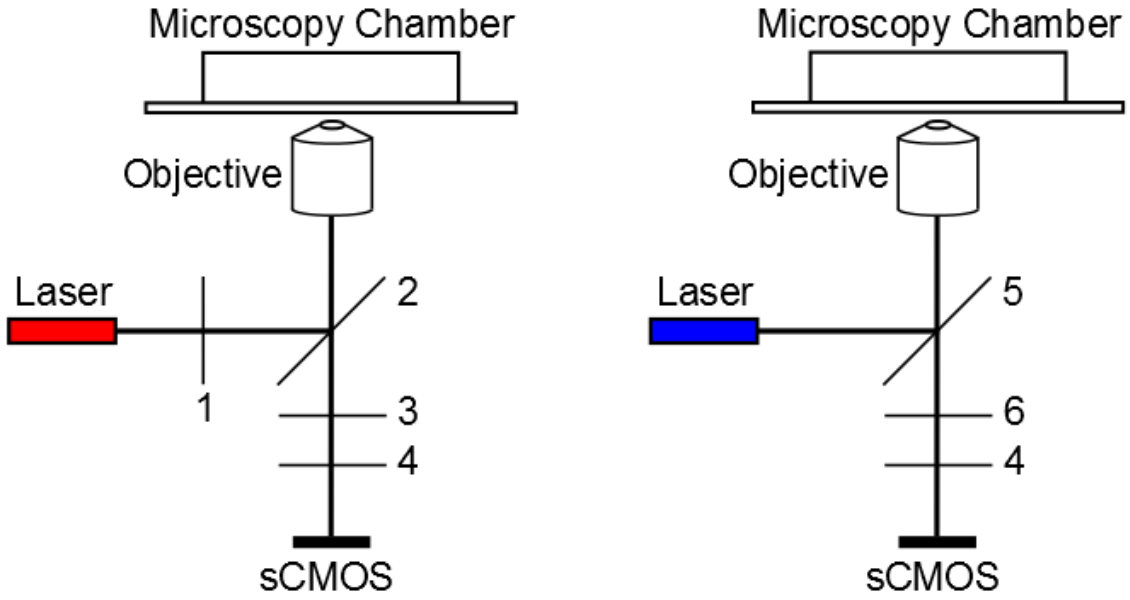

Figure S7. Microscopy setups used for imaging GUVs with 630 nm (left) and 405 nm (right) excitation. Legend: (1) Thorlabs FB630-10 band pass filter, (2) Chroma ZT405/532/635rpc dichroic beam splitter, (3) Edmund Optics 575 nm OD4 short pass filter, 4) Thorlabs NF633-25 notch filter, (5) Chroma ZT405/514/561rpc dichroic beam splitter, (6) Chroma ZET442/514/568 emission filter.

Bright field and (upconversion) emission imaging was performed with a customized Zeiss Axiovert S100 TV Inverted Microscope setup (Figure S7), fitted with a Zeiss 100x Plan Apochromat 1.4 NA oil objective and an Orca Flash 4.0 V2 sCMOS camera from Hamamatsu, which together produced images with 65 nm pixel size. For direct perylene excitation, a CrystaLaser 50 mW 405 nm Solid State laser was used, combined with a ZT405/514/561rpc dichroic beam splitter (Chroma Technology Corporation) and ZET442/514/568m emission filter (Chroma Technology Corporation) (see Figure S8 for the transmission spectra of this set). For upconversion emission microscopy, a Diomed clinical grade 630 nm continuous wave PDT laser was used as excitation source. The light was filtered through a FB630-10 630 nm band pass filter (Thorlabs) put between the laser and the Chroma ZT405/532/635rpc dichroic beam splitter. To block everything except upconversion emission, a NF633-25 633 nm notch filter (Thorlabs) and a 575 nm short pass filter (Edmund Optics, part no. #84-709) were placed between the sample and the camera, resulting in OD >13 at 630 nm and OD>4 around 800 nm (i.e. at the phosphorescence emission of compound **1**). The transmission curves of the filters and dichroic mirror are displayed in Figure S9. The output power of the 630 nm laser was typically 3.8 mW (39 µm spot size, 320 $W.cm^{-2}$) at the sample. The typical camera exposure time was 200 ms, unless otherwise specified.

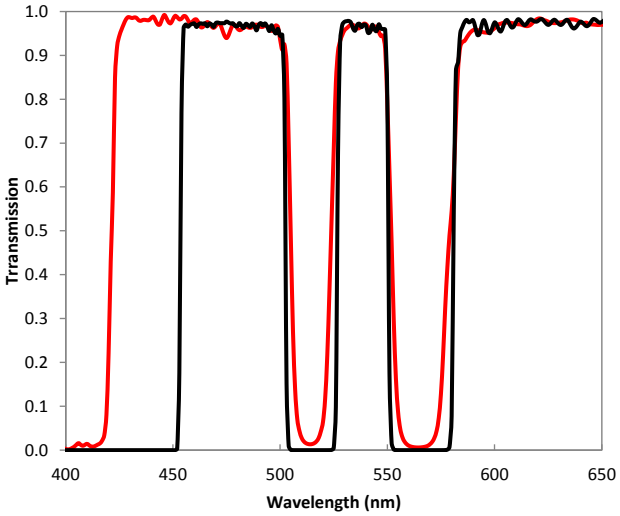

Figure S8. Transmission curves of the filter and dichroic beam splitter that were used for emission microscopy with violet light (405 nm), consisting of a Chroma ZT405/514/561rpc dichroic beam splitter (red) and a Chroma ZET442/514/568m emission filter (black).

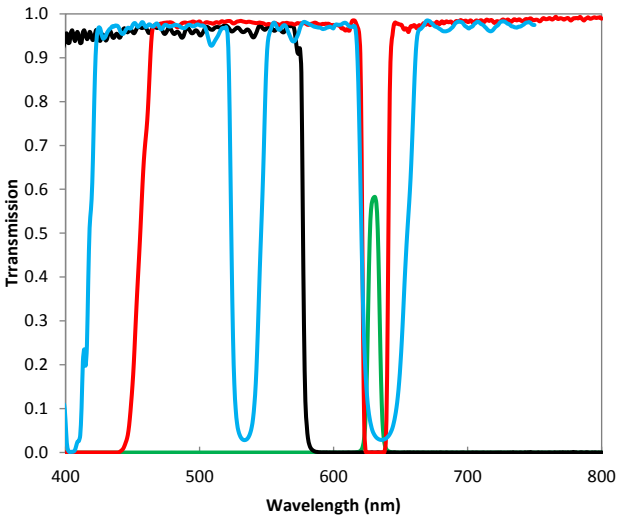

Figure S9. Transmission curves of the filters and dichroic beam splitter that were used for emission microscopy with red light (630 nm), consisting of a Thorlabs NF633-25 notch filter (red) and an Edmund Optics 575 nm OD4 short pass filter (black), a Thorlabs FB630-10 band pass filter (green), and a Chroma ZT405/532/635rpc dichroic mirror (blue).

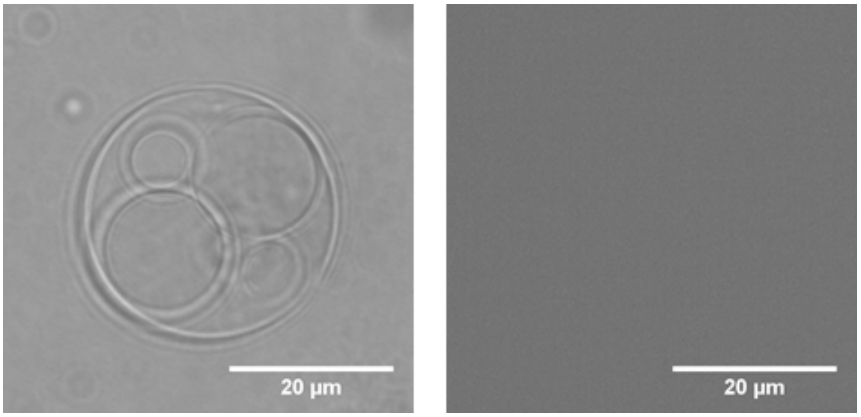

Figure S10. Bright field (left) and upconversion emission (right) photographs of DOPC GUV2, i.e. GUVs similar to GUV12 but deprived of the photosensitizer 1, in buffer without sodium sulfite and under air atmosphere.

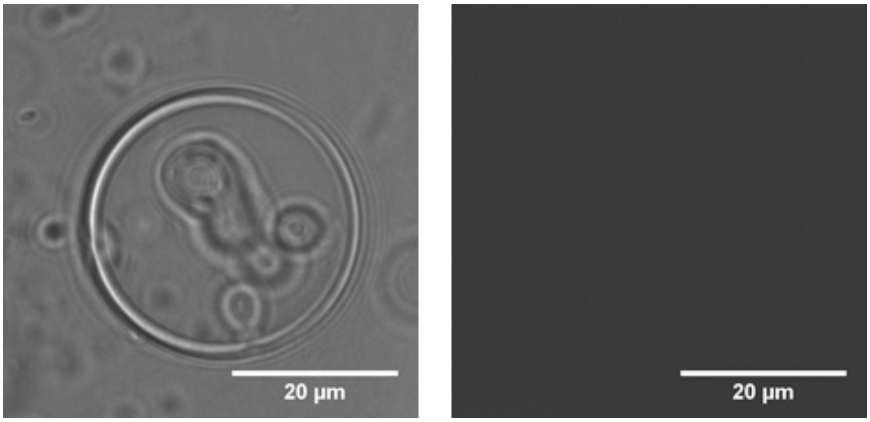

Figure S11. Bright field (left) and upconversion emission (right) photographs of DOPC GUV12 in air atmosphere in buffer without sodium sulfite.

2.8. Determination of bleaching curves

Giant vesicles were first located in bright field mode and were subsequently irradiated for 60 seconds at 630 nm with either 5.2 $W.cm^{-2}$ (62 µW, laser spot size diameter 39 µm) or 320 $W.cm^{-2}$ (3.8 mW, laser spot size diameter 39 µm) illumination intensity while acquiring an image every 1.0 or 0.2 s, respectively. For each image, the pixel values (A.U.) of the brightest half of all the pixels was averaged and normalized to one. Six individual vesicles were measured per time point. The mean and standard deviation are plotted versus time (s) in order to obtain a bleaching curve.

3. References

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2. N. Lopez Mora, J. S. Hansen, Y. Gao, A. A. Ronald, R. Kieltyka, N. Malmstadt and A. Kros, *Chem. Commun.*, 2014, **50**, 1953-1955.
3. S. H. C. Askes, A. Bahreman and S. Bonnet, *Angew. Chem., Int. Ed.*, 2014, **53**, 1029-1033.