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Upconverting nanovesicles for the activation of ruthenium anti-cancer prodrugs with red light

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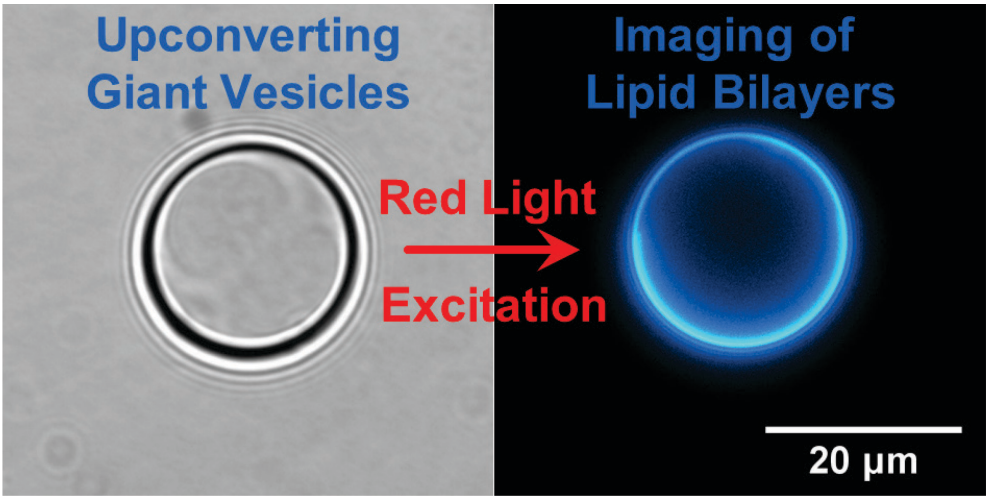
CHAPTER 5

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



Red-to-blue triplet-triplet annihilation upconversion was obtained in giant unilamellar vesicles. The upconverted light was homogeneously distributed across the membrane and could be utilized for the imaging of individual giant vesicles in three dimensions. These results show the great potential of TTA-UC for imaging applications under anoxic conditions.

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5.1 Introduction

Upconversion luminescence (bio)imaging offers great advantages over conventional imaging. The absence of auto-fluorescence results in high contrast images, while photons of low energy, *i.e.* within the phototherapeutic window (600 – 1000 nm), afford higher tissue penetration and negligible irradiation damage. For these reasons lanthanoid-based upconverting nanoparticles (UCNPs), for example, have attracted much interest.[1] However, UCNPs suffer from several disadvantages, such as the need for high excitation power, the low absorption cross section of lanthanoid ions, and low upconversion efficiency in aqueous solution (typically ≤0.5%).[1b] In contrast, triplet-triplet annihilation upconversion (TTA-UC) requires low excitation intensity (<100 $mW.cm^{-2}$), employs sensitizers having high extinction coefficients in the phototherapeutic window, and has achieved upconversion quantum yields up to 14% in aqueous solution.[1b, 2]

In TTA-UC, low-energy photons are converted into higher-energy photons by means of a photophysical mechanism involving a couple of molecular dyes called the sensitizer and annihilator (see Chapter 2, Figure 2.1 for a qualitative Jablonski diagram).[3] The sensitizer absorbs the low-energy light, undergoes intersystem crossing (ISC) to a triplet state, and transfers its energy to the annihilator molecule by triplet–triplet energy transfer. Further collision of two triplet annihilator molecules leads to triplet–triplet annihilation (TTA), whereby one annihilator molecule is promoted to the excited singlet state, whereas the other one falls back to the ground state. The singlet annihilator returns to the ground state by emission of a high-energy photon, thus realizing upconversion. Most molecular dyes used in TTA-UC are highly lipophilic and require supramolecular strategies to be used in aqueous solution.[4] For example, sub-micrometer sized TTA-UC particles have been proposed for *in vitro* or *in vivo* imaging.[1b, 4c, 4d] We now demonstrate that TTA-UC can also be used for the imaging of lipid membranes.

Giant Unilamellar Vesicles (GUVs) are classical tools in fluorescence imaging, as their large size (1–100 μm diameter) allows for direct observation of individual vesicles by optical microscopy techniques.[5] GUVs have for example been used for visualizing lipid rafts, membrane fusion, or ion transport.[6] In this study we functionalized PEGylated GUVs with palladium tetraphenyltetrabenzoporphyrin (**1**) as photosensitizer and perylene (**2**) as the annihilator (Figure 5.1a), and studied red-to-blue TTA-UC in the

membrane of the vesicles by optical microscopy. The aim of the study was to investigate the dye distributions across the membrane, the homogeneity of upconverted emission in the lipid bilayer, and the upconversion stability under imaging conditions. The growth of high-quality giant vesicles with a well-defined shape in physiologically relevant conditions, *i.e.*, at high ionic strengths, was until recently considered as a challenge, but a new method was recently developed by some of us that is compatible with such conditions (up to 320 mOsm.kg^{-1}).[7]

5.2 Results and discussion

Upconverting giant vesicles **GUV12** were thus prepared from a lipid mixture of 95 mol% phospholipid (either 1,2-dimyristoyl-sn-glycero-3-phosphocholine, *i.e.* DMPC, or 1,2-dioleoyl-sn-glycero-3-phosphocholine, *i.e.* DOPC), 4 mol% sodium N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG-2000), 0.5 mol% compound **2**, and 0.02 mol% compound **1**. The complete procedure is described in the experimental section. Briefly, the dye-containing lipid mixture in chloroform was deposited on a chemically cross-linked dextran–poly(ethylene glycol) hydrogel substrate, dried to form a lipid film, and then the film was re-hydrated with phosphate buffered saline (PBS) supplemented with 0.3 M sodium sulfite (Na_2SO_3) and 0.2 M sucrose at 293 – 308 K. Transferring the solution onto a microscopy slide allowed for bright field imaging on a custom-build microscope based on an inverted microscopy setup. The images (Figure 5.1c) confirmed that for both lipid compositions (DMPC or DOPC) free-floating single vesicles were obtained, together with clusters of smaller vesicles. The images also show that the self-assembled vesicles were giant (diameter 1 – 100 μm), unilamellar, and spherical. The fact that almost identical procedures can be employed for preparing GUVs from lipids having a marked difference in their gel-to-liquid transition temperature (T_m = −17.3 °C and 23.9 °C for pure DOPC and DMPC, respectively)[8], demonstrates the flexibility of the GUV preparation method. For comparison, much smaller LUVs (samples **LUV12**) with an average diameter of *ca*. 150 nm were prepared from the same lipid mixture but using a standard hydration-extrusion protocol (Figure S.IV.1).

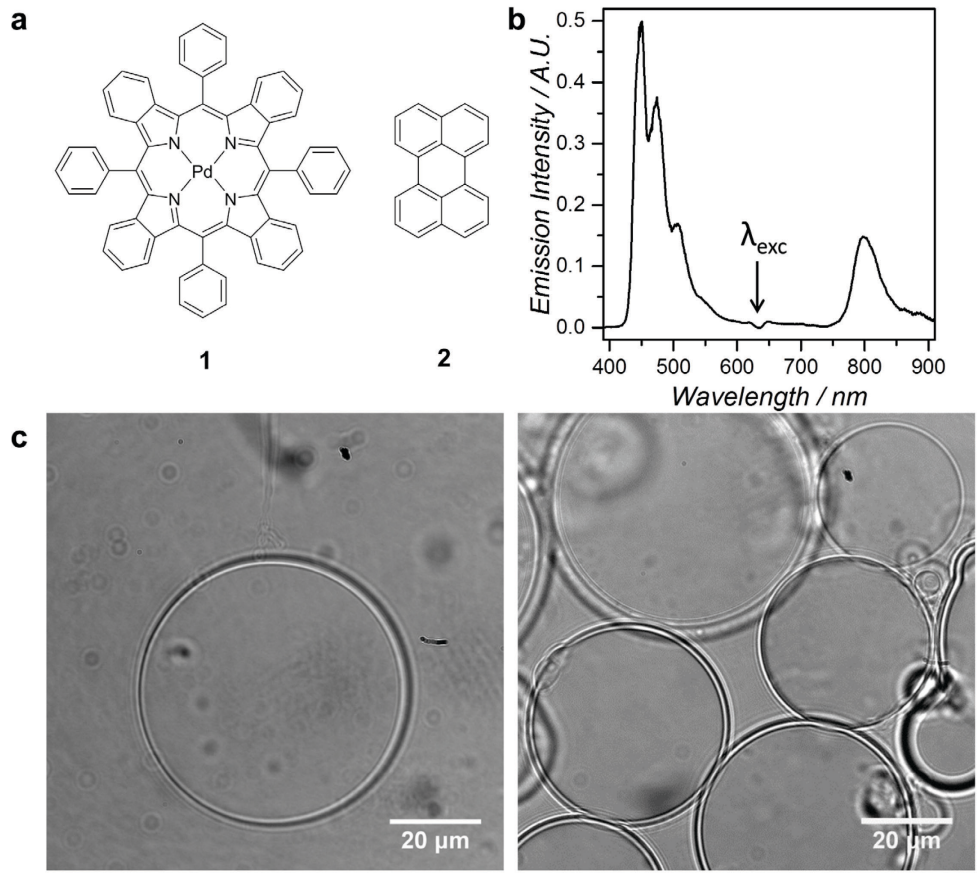

*Figure 5.1. a) Chemical structures of palladium tetraphenyltetrabenzoporphyrin (**1**) and perylene (**2**). b) Emission spectra of DOPC upconverting GUVs with 30 mW 630 nm excitation (0.24 W.cm^{-2} intensity) at 298 K in sulfite-supplemented (0.3 M) PBS buffer under air. c) Bright field micrographs of DOPC (left) and DMPC (right) upconverting giant vesicles at 298 K.*

Sodium sulfite was added in the buffer as an oxygen-scavenging agent. Since the triplet states involved in TTA-UC are readily quenched by molecular oxygen, it is common practice to deoxygenate samples before measuring upconverted emission. With LUVs de-oxygenation can be achieved by, for example, bubbling the solution with argon or N_2. In the case of GUVs imaging however, bubbling an inert gas through the solution would at least impair visualization of single GUVs during a long time period of time due to convection, or even lead to damaging of the giant vesicles, so that supplementing the buffer with an oxygen scavenger is highly preferred. In a preliminary experiment, upconversion emission spectra of **LUV12** samples deoxygenated by either argon bubbling for 30 minutes or by adding 0.3 M

sodium sulfite to the buffer, were compared (see Figure S.IV.2 and experimental section for details). When irradiated at 630 nm the emission spectrum of such LUVs at 298 K shows at 800 nm the phosphorescence band of **1**, and between 450 and 600 nm the blue singlet emission from **2** (Figure S.IV.2). The spectra from both deoxygenation methods were found to be very similar. It was thus concluded that Na_2SO_3 does not interfere with the photophysical processes at the origin of upconversion, and that sulfite might be used for scavenging dioxygen in a GUV-containing sample as well.

Indeed, even though addition of Na_2SO_3 significantly increased the ionic strength of the buffer (from 278 ± 1 mOsm.kg^{-1} for PBS buffer to 884 ± 11 mOsm.kg^{-1} when supplemented with 0.3 M sodium sulfite), as explained above sodium sulfite did not prevent the assembly of DMPC or DOPC **GUV12** using the hydrogel method. No differences in vesicle yield and morphology were observed in presence or absence of sodium sulfite in the buffer. This result demonstrates that the dextran–poly(ethylene glycol) hydrogel substrate is able to produce GUVs at high ionic strength, which is a significant advantage over alternative GUV preparation methods such as electroformation or gentle hydration, which often fail in such conditions. When irradiated at 630 nm under air, the emission spectrum of the DMPC or DOPC **GUV12** samples prepared in a sulfite-supplemented buffer was identical to the emission spectrum of the corresponding **LUV12** samples (Figure 5.1b and Figure S.IV.3), showing that the dyes **1** and **2** were indeed incorporated in the lipid bilayer.

GUV12 samples were then visualized by emission microscopy at 298 K (Figure 5.2). When the vesicles were illuminated with violet light (405 nm), *i.e.* by direct excitation of perylene (**2**), fluorescence was clearly detected at the membrane (Figure 5.2b). To visualize upconversion, a 630 nm continuous wave PDT laser was coupled into the microscope and set at a power of a few milliwatts, resulting in the focal spot in an intensity of ~300 W.cm^{-2}. All wavelengths other than 450 − 575 nm were strictly blocked by a combination of notch and short-pass filters (Appendix IV). High-quality images were obtained that were superimposable to the bright field images and to the fluorescence images recorded under white and violet light irradiation, respectively (Figure 5.2a-c). Control samples were prepared in which the porphyrin sensitizer **1** was omitted from the formulation (**GUV2**). Images recorded in identical conditions were black, *i.e.*, no blue emission was observed (Figure S.IV.7). **GUV12** samples prepared in absence of sulfite

oxygen scavenger and observed under air did not give any observable emission either (Figure S.IV.8). Altogether, these observations prove that the blue images recorded under 630 nm irradiation of **GUV12** samples supplemented with sulfite comes from the TTA upconversion process and are not the result of sensitizer emission (at 800 nm) or of two-photon absorption. Overall, all data conclude that both dyes **1** and **2** co-localize in the membrane and result in TTA upconversion. At this scale of observation the upconverted emission is homogeneous across the membrane and no phase separation of the lipids or dyes was observed.

Under the red-light irradiation conditions initially used in the microscopy setup (630 nm at an intensity of 320 $W.cm^{-2}$), substantial bleaching of the upconverted emission of **GUV12** samples was observed even in presence of 0.3 M of sulfite. A plot of the averaged normalized pixel values as a function of red irradiation time shows that the upconverted emission is halved after less than 3 seconds (Figure 5.3). When the light intensity was lowered 60 times (*i.e.*, down to 5.2 $W.cm^{-2}$) clear upconversion images could still be recorded. In such conditions the bleaching rate was significantly lower (Figure 5.3), and the time necessary for halving the upconverted emission intensity of a pixel increased to approximately 15 seconds. The upconversion luminescence of **LUV-12** in a spectroscopy setup could be observed for less than 8 $mW.cm^{-2}$, with linear power dependency above 60 $mW.cm^{-2}$ (Figure S.IV.4). Overall, these findings show that high power is not a requirement for the upconversion imaging of **GUV-12**.

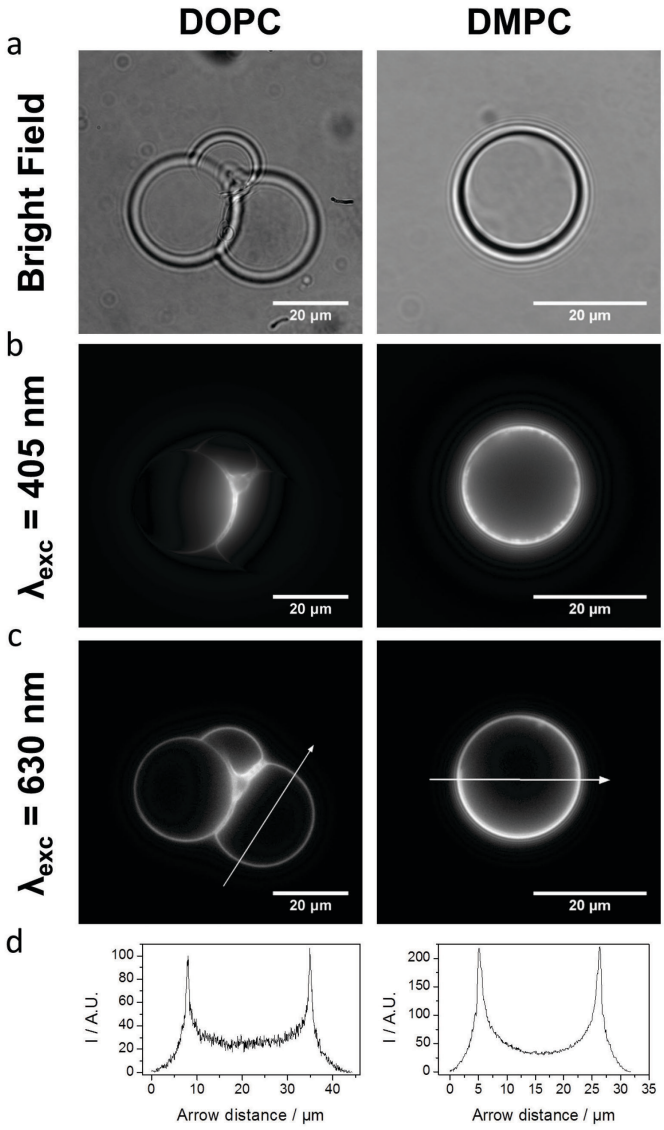

*Figure 5.2. Imaging of DOPC (left) and DMPC (right) upconverting giant vesicles (**GUV12**) with a) bright field, b) 405 nm excitation and 450-500 nm detection, and c) 630 nm excitation and 450-575 nm detection. d) Upconversion intensity profile plot following the arrows in the images directly above (c). At 630 nm: laser spot size diameter 39 µm, power 3.8 mW, intensity 320 W.cm^{-2}. At 405 nm: laser spot size diameter 22 µm (power 1 mW, intensity 60 W.cm^{-2}) for DOPC image or 39 µm (power 1 mW, intensity 300 W.cm^{-2}) for DMPC image. Images were acquired at 298 K in sulfite-supplemented (0.3 M) PBS buffer.*

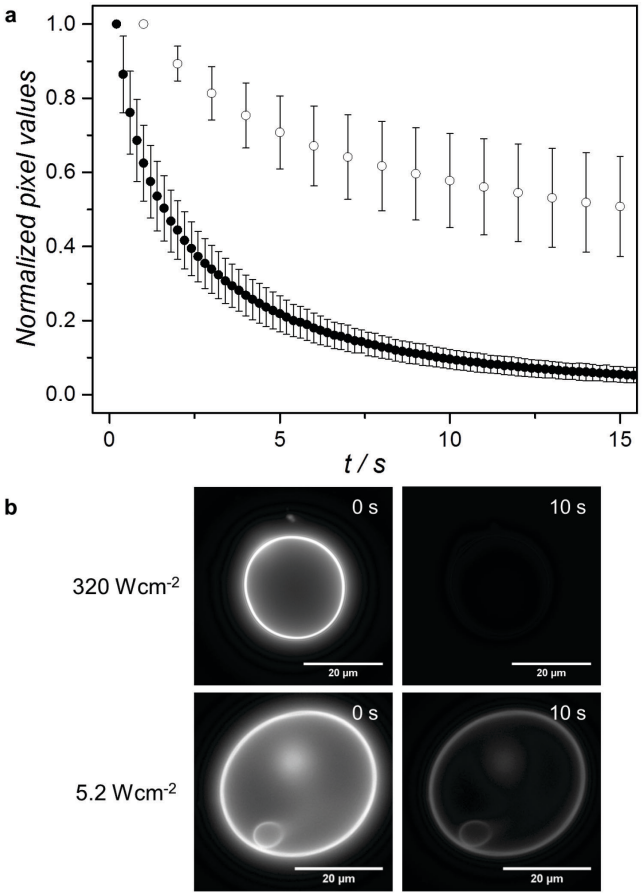

Figure 5.3 a) Averaged normalized pixel values as a function of red irradiation time during upconversion imaging of ***GUV12*** *samples in sulfite-supplemented PBS buffer (0.3 M). Conditions: 630 nm excitation at 320 W.cm^{-2} (black filled circles) or 5.2 W.cm^{-2} (empty circles), detection in the 450–575 nm region, T = 298 K. Snapshots were taken with an exposure time of 0.2 s (320 W.cm^{-2}) or 1.0 s (5.2 W.cm^{-2}). Error bars represent standard deviation based on six individual measurements. b) Upconversion emission microscopy images of* ***GUV12*** *samples at t = 0 s (left) and at t = 10 s (right) at an illumination intensity of 320 W.cm^{-2} (top) and 5.2 W.cm^{-2} (bottom). Excitation at 630 nm, detection at 450–575 nm.*

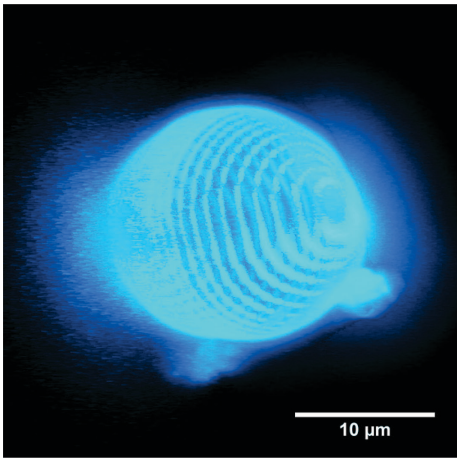

*Figure 5.4. 3D reconstructed image of a DMPC **GUV12** sample, rotated counter-clockwise by 50° about the y-axis. Each z-slice was imaged at 298 K with 630 nm excitation (320 W.cm^{-2}) and detection in the 450-575 nm region. The z-distance between slices was 1.0 µm. Video V1 (http://www.rsc.org/suppdata/c5/cc/c5cc02197a/c5cc02197a2.mpeg) exhibits a 360° rotational view of this image and of four other individual DMPC **GUV12**.*

In optimized conditions, we realized that the upconverted emission was intense enough to be utilized for reconstructing in 3D the membrane of the giant vesicles. Z-stack upconversion image acquisition was indeed performed on both DMPC and DOPC **GUV12** samples. The illumination intensity was deliberately chosen to be high (320 W.cm^{-2}) to make sure that z-stack image acquisition was short (200 ms exposure time per slice, *ca*. 45 slices per stack, total acquisition time < 10 s). In such conditions, the slight lateral motion of the GUVs did not significantly affect the imaging process. From these stacks, 3D reconstructions were made (*e.g.* Figure 5.4), of which a video was compiled (See Video V1 on-line at http://www.rsc.org/suppdata/c5/cc/c5cc02197a/c5cc02197a2.mpeg). This reconstruction demonstrates that the TTA-upconverted emission can be utilized for the three-dimensional reconstruction of an object that is 10 to 30 µm in size.

5.3 Conclusion

In conclusion, DOPC and DMPC giant vesicles capable of upconverting red light to blue light by means of triplet-triplet annihilation were prepared by lipid film hydration on a hydrogel substrate at high ionic strengths. The preparation method is facile and does not involve any specific equipment. Sodium sulfite added as an oxygen scavenger to the vesicle samples allows for observing upconversion even under air. According to optical microscopy, the upconverted emission allows for recording high quality images showing that

upconversion is homogeneously realized across the lipid bilayer. The quality and stability of the upconverted images enabled the 3D reconstruction of upconverting GUVs. These results show the great potential of TTA upconversion for imaging applications under anoxic conditions, and open a route towards cell membrane imaging with upconverted light.

5.4 Experimental section

5.4.1 General

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-phospho ethanolamine (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.

5.4.2 GUV preparation

All GUVs were prepared by lipid film re-hydration on dextran chemically cross-linked hydrogel substrates by a method described elsewhere.[7] 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 recipe produced a solution containing free-floating vesicles that could be directly pipetted in a fluorescence cuvette for emission spectroscopy (see section 5.4.3). Alternatively, it was further used for the preparation of a microscopy experiment (section 5.4.4).

5.4.3 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 5.4.6

5.4.4 Preparation of a microscopy experiment with GUVs

For optical microscopy imaging, 300 µL of the solution containing free-floating vesicles in buffer (section 5.4.2) 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 greater density 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.

5.4.5 LUV preparation and characterization

Upconverting LUVs, *i.e.* **LUV12** samples, were prepared as described before as a reference.[4f] 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 colorless after extrusion, suggesting complete inclusion of the sensitizer and annihilator in the lipid bilayer. LUVs were stored in the dark at 4 °C and used within 7 days. The LUVs 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 S.IV.1) as described before.[9]

5.4.6 Upconversion emission spectroscopy

Upconversion emission spectroscopy was performed in a custom-built setup (Figure 5.5). 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.

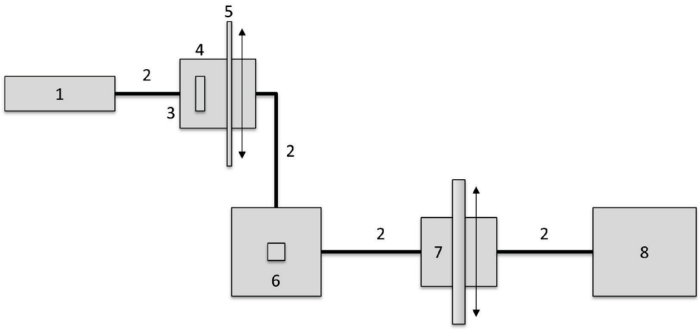

Figure 5.5. Setup used for emission measurements under red light irradiation. Legend: (1) 630 nm laser source, (2) optical fibers, (3) filter holder, (4) 630 nm band pass filter, (5) variable neutral density filter, (6) temperature controlled cuvette holder, (7) variable filter holder, and (8) CCD spectrometer.

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.

5.4.7 Power dependency measurements

Luminescence emission spectra of DMPC and DOPC **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 5.4.6. In this case, the spectrum was visualized with only a Thorlabs NF-633 notch filter in 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 S.IV.4). 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.

5.4.8 Microscopy imaging

Bright field and (upconversion) emission imaging was performed with a customized Zeiss Axiovert S100 TV Inverted Microscope setup (Figure 5.6), 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 S.IV.5 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 S.IV.5 and Figure S.IV.6. 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.

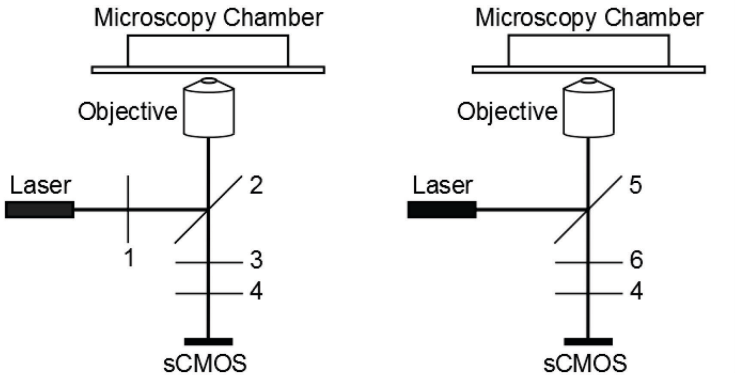

Figure 5.6. Microscopy setups used for imaging GUVs with 630 nm (left) and 405 nm (right) excitation. Legend: (1) Thorlabs FB630-10 band pass filter, (2) Chroma ZT405/532/635rpc dichroic beam splitter, (3) Edmund Optics 575 nm OD4 short pass filter, 4) Thorlabs NF633-25 notch filter, (5) Chroma ZT405/514/561rpc dichroic beam splitter, (6) Chroma ZET442/514/568 emission filter.

5.4.9 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.

5.5 References

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