Photochemistry

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# Activation of a Photodissociative Ruthenium Complex by Triplet–Triplet Annihilation Upconversion in Liposomes**

*Sven H. C. Askes, Azadeh Bahreman, and Sylvestre Bonnet**

***Abstract:*** *Liposomes capable of generating photons of blue light in situ by triplet–triplet annihilation upconversion of either green or red light, were prepared. The red-to-blue upconverting liposomes were capable of triggering the photodissociation of ruthenium polypyridyl complexes from PEGylated liposomes using a clinical grade photodynamic therapy laser source (630 nm).*

Light-sensitive ruthenium(II) polypyridyl compounds are classical tools in photochemistry that have recently been proposed as prodrugs for photoactivatable anticancer therapy (PACT).[1] As shown in classical photodynamic therapy (PDT), the use of light to treat cancer allows for spatially and temporally controlling the toxicity of an anticancer drug, which lowers side effects for cancer patients. Meanwhile, loading anticancer drugs into drug carriers such as liposomes helps targeting the compounds to tumor tissues. Especially sterically hindered liposomes, that is, those grafted with polyethylene glycol chains, have been recognized as versatile and biocompatible drug carriers for the treatment of various diseases because of their long lifetime in the blood circulation. With such PEGylated liposomes tumor uptake is enhanced because of the so-called enhanced permeability and retention (EPR) effect.[2] In PACT, activation of, for example, ruthenium-functionalized liposomes could be realized after cell uptake using visible light. However, most ruthenium(II) polypyridyl compounds require activation with blue light (400–500 nm), that is, outside the phototherapeutic window (600–1000 nm), in which light permeates mammalian tissues optimally. In this work, in situ upconversion of red to blue light is realized using triplet–triplet annihilation upconversion (TTA-UC), and combined with ruthenium-functionalized liposomes to trigger the activation of the ruthenium complex using a clinical grade PDT laser source.

In TTA-UC, low-energy photons are converted into higher-energy photons by means of a bimolecular mechanism involving a sensitizer and two annihilator molecules.[3] The sensitizer absorbs the low-energy light, undergoes intersystem crossing (ISC) to a triplet state, and transfers its energy to an annihilator molecule by triplet–triplet energy transfer (TTET). Collision of two triplet annihilator molecules leads to triplet–triplet annihilation (TTA), whereby one 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. TTA-UC with a range of molecule pairs has been realized in organic solvent,[3a,b,d] ionic liquid,[4] polymers,[3a,c,5] and various water-soluble nanoparticles.[6] Here, we demonstrate the first examples of TTA-UC in the lipid bilayer of neutral liposomes.

Two well-investigated TTA-UC couples were considered for incorporation in liposomes: platinum octaethylporphyrin (**1**) and 9,10-diphenylanthracene (**2**) on the one hand, and palladium tetraphenyltetrabenzoporphyrin (**3**) and perylene (**4**) on the other hand (Figure 1). Obviously, when included in liposomes these highly apolar molecules favor the lipophilic interior of the lipid bilayer. Liposomes made of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and containing 4 mol% of sodium *N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-MPEG-2000), the sensitizer **1** or **3**, and/or the annihilator **2** or **4**, were prepared by extrusion in DPBS buffer solution (Table 1). The diameters of the liposomes (130–170 nm) were measured by dynamic light scattering. UV/Vis absorption and luminescence spectra of liposomes containing either the sensitizer or the annihilator, that is, of samples L1, L2, L3, and L4, were comparable to that of the corresponding compounds in toluene solution (see Figure S1 in the Supporting Information). Thus, incorporation of any of the four molecules shown in Figure 1 into the DMPC bilayers did not change their spectroscopic properties.

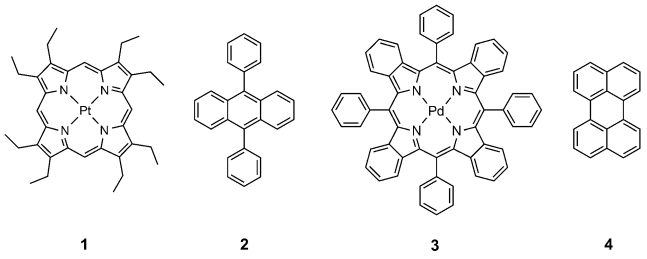


***Figure 1.*** Chemical structures of platinum octaethylporphyrin (**1**), 9,10-diphenylanthracene (**2**), palladium tetraphenyltetrabenzoporphyrin (**3**), and perylene (**4**).

Although in liposome samples L1-2 and L3-4 both molecules of each upconverting couple were successfully inserted into the bilayer, it was initially uncertain whether their diffusion in the two dimensions of the bilayer would be

[*] S. H. C. Askes, A. Bahreman, Dr. S. Bonnet
Leiden Institute of Chemistry, Leiden University
Einsteinweg 55, 2333 CC Leiden (The Netherlands)
E-mail: bonnet@chem.leidenuniv.nl

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***Table 1:*** Overview of liposomal formulations used in this work. [DMPC], [PEG], [**1**], [**2**], [**3**], and [**4**], represent the millimolar bulk concentrations in DMPC, DSPE-MPEG-2000, **1**, **2**, **3**, and **4**, respectively, in DPBS buffer.

| Code | [DMPC] [mM] | [PEG] [mM] | [**1**] [μM] | [**2**] [μM] | [**3**] [μM] | [**4**] [μM] | [**5**] [mM] |
|---|---|---|---|---|---|---|---|
| L1-2 | 20 | 0.80 | 3.5 | 100 | – | – | – |
| L1 | 20 | 0.80 | 3.5 | – | – | – | – |
| L2 | 20 | 0.80 | – | 100 | – | – | – |
| L3-4 | 20 | 0.80 | – | – | 2.5 | 50 | – |
| L3 | 20 | 0.80 | – | – | 2.5 | – | – |
| L4 | 20 | 0.80 | – | – | – | 50 | – |
| L5 | 5.0 | 0.20 | – | – | – | – | 0.20 |

sufficient to allow TTA-UC to occur.[3a] After deoxygenation these samples were excited at either 532 or 630 nm, respectively, near the absorption maximum of the highest Q-band of **1** ($\lambda_{max} = 536$ nm) or **3** ($\lambda_{max} = 628$ nm), respectively. A bright blue luminescence was observed in both cases (Figure 2) after

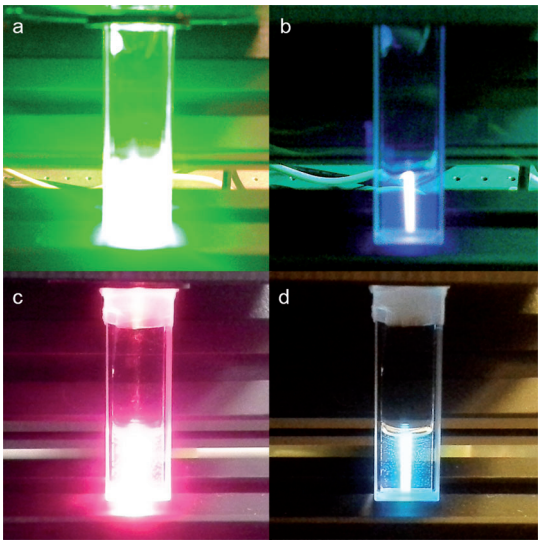


***Figure 2.*** Digital photographs of L1-2 and L3-4 under irradiation at 532 and 630 nm, respectively, with 27 mW excitation power (for both systems) in a beam of 2.6 mm in diameter (intensity: 0.51 $W cm^{-2}$). a) L1-2 without filter. b) L1-2 with a 533 nm notch filter and short pass filter for wavelengths shorter than 575 nm. c) L3-4 without filter. d) L3-4 with a 633 nm notch filter. The samples were deoxygenated and maintained at 298 K.

suppressing the scattered excitation light with notch and/or short pass filters. Under the same experimental conditions, no blue emission was observed for L1, L2, L3, or L4, thus proving that both components of each upconverting couple are necessary for the upconversion to occur. To the best of our knowledge, L1-2 and L3-4 are the first examples showing TTA-UC in liposomes. As both green-to-blue and red-to-blue upconversion was obtained, liposomes appear as a straightforward manner to solubilize TTA-UC couples in aqueous solution.

The luminescence spectra of L1-2 and L3-4 were measured at 298 K in argon (Figure 3). Upon excitation at 532 nm,

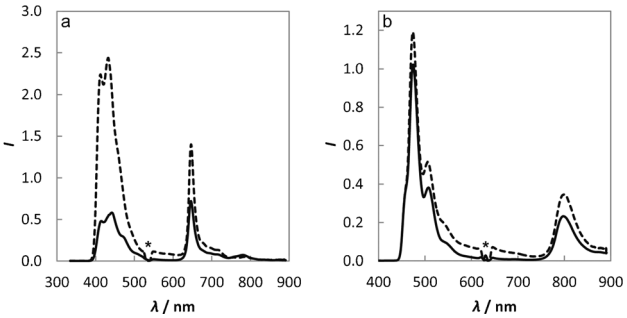


***Figure 3.*** a) Emission spectra of liposome sample L1-2 (solid line) and of a toluene solution containing **1** and **2** at the same bulk concentrations ([**1**] = 3.5 μM and [**2**] = 100 μM, dashed line). b) Emission spectra of the liposome sample L3-4 (solid line) and of a toluene solution containing **3** and **4** at the same bulk concentrations ([**3**] = 2.5 μM and [**4**] = 50 μM, dashed line). Asterisks indicate excitation (532 nm for L1-2 and 630 nm for L3-4). The samples were deoxygenated before measurement. Spectra acquired at 298 K, excitation power for both samples 27 mW, 2.6 mm diameter beam, intensity 0.51 $W cm^{-2}$.

L1-2 shows a structured upconversion band at 433 nm, corresponding to emission of **2** in toluene (Figure S1b). A second band was present as well; its emission maximum (646 nm) was consistent with the phosphorescence of **1** in toluene (Figure S1a). Similarly, L3-4 excitation at 630 nm leads to an upconversion band at 473 nm, and a second band at 800 nm (Figure 3b). The upconversion emission corresponds to emission of **4** in toluene (Figure S1d), apart from the first peak at 447 nm that was filtered by the 633 nm notch filter used for rejecting the scattered excitation (Figure S2). The peak at 800 nm in the emission spectrum of L3-4 corresponds to the phosphorescence of **3**, as observed in toluene (Figure S1c).

The luminescence spectra of both upconverting couples were measured in toluene using the same bulk concentrations for the sensitizer and annihilator as for L1-2 and L3-4. The upconversion intensity for couple **1**–**2** was found to be four times weaker in liposomes than in toluene at 298 K, and for couple **3**–**4** it was comparable for both sample types (Figure 3). Upon inserting the sensitizer and annihilator in the lipid bilayer two phenomena take place simultaneously. On the one hand, compartmentalization of the lipophilic molecules in the bilayer increases their local concentrations, which increases the probability of intermolecular collisions and therefore the rates of TTET and TTA. On the other hand, two-dimensional diffusion in a lipid bilayer is somewhat slower than in a nonviscous isotropic toluene solution, which may decrease TTA-UC efficiency in liposomes. Overall, our data show that the trade-off is excellent and allows efficient TTA-UC to occur in PEGylated DMPC liposomes (at 298 K).

Measurements of upconversion quantum yields ($\Phi_{uc}$) are usually done by relative actinometry.[3a] However, intense scattering in liposome samples would make any comparison with a reference compound in homogeneous solution challenging. For this reason, the upconversion quantum yields of L1-2 and L3-4 were measured using an absolute method, that is, an integrating sphere and a calibrated spectrometer (see the Supporting Information). The setup was similar to that


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used by Boyer et al. for determining the upconversion quantum yield of lanthanoid-based nanoparticles.[7] For L1-2, L3-4, and for their toluene analogs, $\Phi_{uc}$ was determined upon irradiation using a 10 mW continuous beam (Table 2).

***Table 2:*** Upconversion quantum yield ($\Phi_{uc}$) in liposomes and toluene at 293 K.

| TTA-UC Couple | $\Phi_{uc}$ [%] in PEGylated DMPC liposomes | in toluene |
|---|---|---|
| **1**–**2**[a] | 2.3 (L1-2) | 5.1[b] |
| **3**–**4**[c] | 0.5 (L3-4) | 1.2[d] |

[a] 532 nm, 10 mW excitation power, 1.5 mm diameter beam (intensity 0.57 $W cm^{-2}$). [b] [**1**] = 3.5 μM, [**2**] = 100 μM. [c] 630 nm, 10 mW excitation power, 2.5 mm diameter beam (intensity 0.20 $W cm^{-2}$). [d] [**3**] = 2.5 μM, [**4**] = 50 μM.

At 293 K $\Phi_{uc}$ in PEGylated DMPC liposomes was found roughly half of that in toluene for both couples, with values of 2.3 and 0.5% for L1-2 and L3-4, respectively, versus 5.1 and 1.2% in toluene. To the best of our knowledge, this is the first time that the quantum yield of TTA-UC has been determined using an absolute method.

The TTA-UC process is diffusion-controlled, and therefore depends on temperature. For this reason, luminescence spectra were measured for L1-2 and L3-4 at 288, 293, and 298 K (Figure 4a and c). Upon warming, the sensitizer phosphorescence decreased for both samples, while the upconversion emission increased markedly. In contrast, for toluene samples at the same bulk concentrations both the upconversion and phosphorescence intensities slightly decreased with increasing temperatures (Figure S3) as a result of faster nonradiative decay. The liposome samples were subjected to three warming–cooling cycles while their luminescence was continuously monitored (Figure 4b and d). The temperature dependence of the upconversion was found to be reversible, which advocates for a reversible, physical cause rather than an irreversible chemical evolution (such as aggregation or photoreactions). As the variation of the ratio upconversion versus the phosphorescence occurs at a temperature that fits the gel-to-fluid phase transition temperature ($T_m$) of the DMPC membranes (296.9 K), we interpret this variation as a consequence of the much increased translational diffusion coefficient ($D_T$) of membrane-embedded molecules above $T_m$, compared to that at temperatures below $T_m$.[8] TTET and TTA are both expected to be much more frequent in the liquid phase of the membrane, that is, above $T_m$, which would lead to an increase in the probability of upconversion (an intermolecular process) at the cost of phosphorescence (a monomolecular process). Similar observations were made for TTA-UC in rubbery polymer matrixes by Singh-Rachford and co-workers.[5e]

To prove that in situ upconverted blue photons may be used to activate light-activatable prodrugs using red light, ruthenium-functionalized liposomes were mixed with the upconverting liposomes L3-4 (Figure 5b). The ruthenium complex $[Ru(tpy)(bpy)(SRR')]^{2+}$ ($\mathbf{5}^{2+}$, see Figure 5a and the Supporting Information) was selected because it has a single

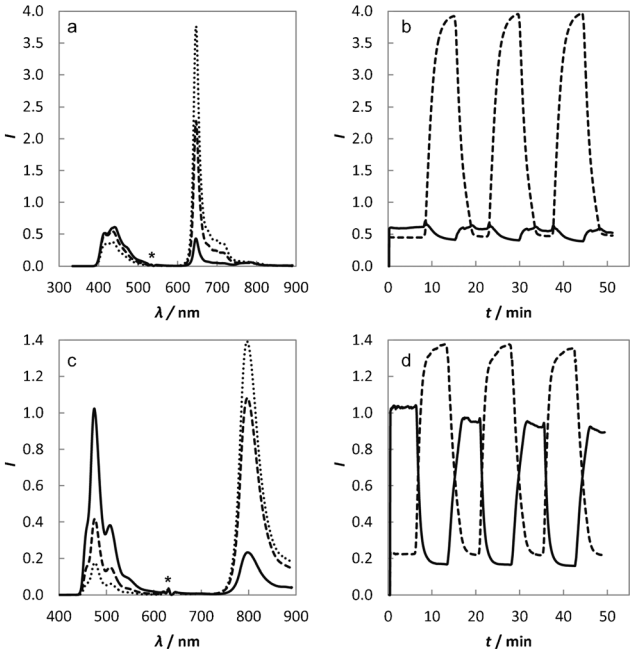


***Figure 4.*** a) Luminescence spectrum of L1-2 at 288 K (dotted), 293 K (dashed), and at 298 K (solid). b) Time dependency of the upconversion at 436 nm (solid) and of the phosphorescence at 646 nm (dashed) of L1-2 during three warming and cooling cycles from 288 to 298 K and from 298 to 288 K. c) Luminescence spectrum of L3-4 at 288 (dotted), 293 (dashed), and 298 K (solid). d) Time dependency of the upconversion at 473 nm (solid) and of the phosphorescence at 800 nm (dashed) of L3-4 during three warming and cooling cycles from 288 to 298 K and from 298 to 288 K. Asterisks indicate excitation wavelengths (532 nm for L1-2 and 630 nm for L3-4). The samples were deoxygenated before measurement. Excitation power for both samples: 27 mW, 2.6 mm diameter beam, intensity 0.51 $W cm^{-2}$.

light-sensitive Ru–S bond. This kind of photoactivatable ruthenium compound shows stability in the dark but hydrolyzes to the aqua species $[Ru(tpy)(bpy)(H_2O)]^{2+}$ ($\mathbf{6}^{2+}$) upon irradiation with blue light into its metal-to-ligand charge-transfer state.[9] A thioether-cholesterol ligand (SRR′) can be used to anchor the complex to lipid bilayers, as has been demonstrated in our group.[9a,c] PEGylated DMPC liposomes bearing 3.7 mol% of complex $\mathbf{5}^{2+}$ were prepared (sample L5, Table 1) and added in a 1:1 volumetric ratio to the red-to-blue upconverting liposome sample L3-4. Both types of liposomes being grafted with sterically hindering polyethylene glycol (PEG) tails, fusion of the liposomes does not occur, and only radiative energy transfer between the upconverting liposomes and the ruthenium-functionalized liposomes should take place (Figure 5b).[10]

The liposome mixture was deoxygenated and irradiated at 298 K for 2 h with the 630 nm laser light from a clinical grade Diomed PDT laser set at 120 mW power. The photoreaction was monitored by UV/Vis spectroscopy at fixed intervals during irradiation (Figure 6). Although the absorbance of **4** dominates the spectrum, the characteristic band of the hydrolyzed photoproduct ($\mathbf{6}^{2+}$) could clearly be seen, rising between 450 and 550 nm as a function of irradiation time. The isosbestic point at 457 nm showed that a single photochemical process was taking place. Monitoring the absorbance at

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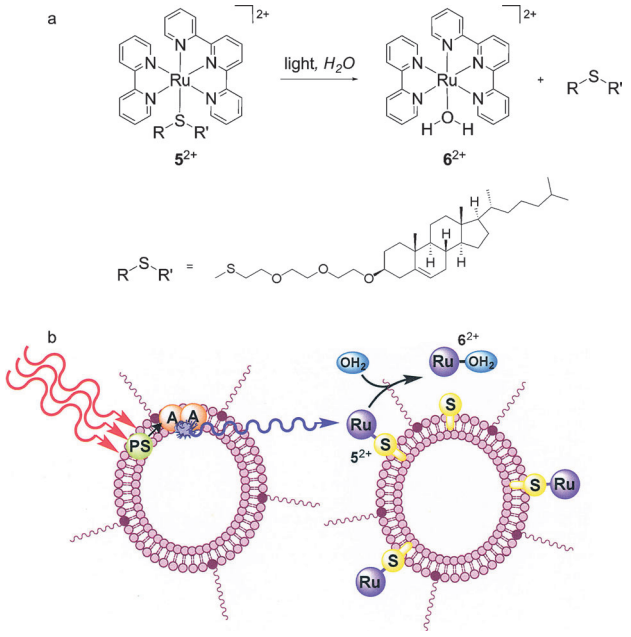


***Figure 5.*** a) Chemical structures of $\mathbf{5}^{2+}$ and $\mathbf{6}^{2+}$ and the conversion of $\mathbf{5}^{2+}$ into $\mathbf{6}^{2+}$. b) The TTA-UC process in the lipid bilayer, using a photosensitizer (PS) and an annihilator (A). Radiative energy transfer from the annihilator to complex $\mathbf{5}^{2+}$, denoted by a blue arrow, triggers light-induced hydrolysis of $\mathbf{5}^{2+}$ to release $\mathbf{6}^{2+}$ in solution.

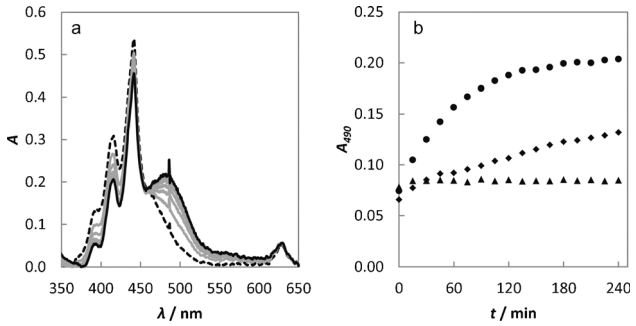


***Figure 6.*** a) Absorption spectra, after baseline correction, of a 1:1 vol% mixture of liposome samples L3-4 and L5 (Table 1) during red-light irradiation (630 nm). Dashed line: spectrum at $t=0$; black solid line: spectrum at $t=240$ minutes; gray lines: spectra measured every 30 minutes. b) Plot of the absorbance at 490 nm during red-light irradiation (630 nm) of a 1:1 vol% mixture of L3-4 and L5 (dots), of a 1:1 vol% mixture of L4 and L5 (diamonds), and absorbance at 490 nm of a 1:1 vol% mixture of L4 and L5 left in the dark (triangles). Irradiation conditions: power 120 mW, beam diameter 2.6 mm, intensity 2.3 $W cm^{-2}$, $T=298$ K, sample volume 1 mL.

490 nm allowed for quantitatively measuring the build-up of $\mathbf{6}^{2+}$ as a function of irradiation time, which reached a plateau after 3 h of irradiation (Figure 6b). As a control experiment, a mixture of liposomes L4 and L5 was irradiated under the same experimental conditions as above. In liposomes L4 the absence of sensitizer prevents upconversion from occurring, and the red photons can only excite the ruthenium complex by direct absorption in the tail of the $^1$MLCT band. The extinction coefficient of $\mathbf{5}^{2+}$ being very low at 630 nm ($\varepsilon \leq 900\,M^{-1}cm^{-1}$), even under a strong photon flux the photoconversion to $\mathbf{6}^{2+}$ was much slower than in the presence of L3-4 (Figure 6b), that is, the upconverting liposomes achieve efficient sensitization of the photosubstitution reaction. A second control experiment showed that no photodissociation occurred in the absence of light. Overall, these data are the first evidence that blue photons produced in situ by upconversion of PDT-compatible red photons, can be used to enhance the photodissociation rate of polypyridyl ruthenium complexes.

In conclusion, triplet–triplet annihilation upconversion was for the first time realized in PEGylated liposomes and characterized by absolute quantum yield measurement. Red-to-blue upconverting liposomes L3-4, when mixed with ruthenium-functionalized, PEGylated liposomes L5 and irradiated with a clinical grade PDT laser at 630 nm, were able to trigger the hydrolysis of the Ru–S bond using radiative energy transfer and to release complex $\mathbf{6}^{2+}$. The upconverting liposomes transform two low-energy photons, which penetrate far in biological tissues but are poorly absorbed by the ruthenium complex, into one blue photon that does not need to travel into tissues and can directly promote the complex into its photoreactive excited state. Metal–ligand photodissociation mediated by upconverted light represents exciting perspectives for photoactivatable chemotherapy in oxygen-poor tissues such as hypoxic tumors. Obviously, the oxygen sensitivity of TTA-UC in liposomes needs to be addressed before concluding on the practical application of such systems in vivo. However, the high quantum yield of TTA-UC in liposomes and the excellent molar absorptivity of porphyrin sensitizers, for example, compared to lanthanoid-based upconverting nanoparticles, may offer fascinating applications in bioimaging, photoactivatable chemotherapy, and other applications where the in situ generation of blue light is required.

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