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

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CHAPTER 10

SUMMARY, CONCLUSIONS, AND OUTLOOK

10.1 TTA-UC nanovesicles in solution and in living cells

As described in Chapter 3, PEGylated DMPC liposomes were successfully prepared that were capable of generating blue photons by triplet-triplet annihilation upconversion of either green or red light with two distinct molecular chromophore pairs (Figure 10.1). The upconversion intensity in liposomes was found to be comparable to that in toluene solution at 20 °C and was linearly dependent on excitation intensity above only 0.05 W.cm⁻² (Chapter 5). To investigate the location where upconversion took place, red-to-blue upconverting giant vesicles (GUVs) were prepared and imaged with upconversion luminescence microscopy, as reported in Chapter 5. The results demonstrated that TTA-UC was occurring in the lipid bilayer of the giant vesicles, and the high quality and stability of the upconverted images enabled the 3D reconstruction of upconverting GUVs. Because TTA-UC in liposomes would have to be performed at human body temperature (37 °C), the temperature dependency of TTA-UC was investigated in a series of neutral PEGylated phospholipid liposomes, of which the results are reported in Chapter 6. It was found that for phospholipids with a gel-to-liquid phase transition, the TTA-UC intensity maximized near the main transition temperature (T_m). This result was explained by the fact that molecular diffusion of dyes, and thus TTA-UC efficiency, increases towards T_m , while thermal quenching of the photosensitizer as a function of temperature decreases the TTA-UC efficiency above T_m . The TTA-UC efficiency in DOPC, DLPC, and DMPC liposomes were very similar at 37 °C. Thus, TTA-UC in liposomes can be generally realized and the phospholipid can be freely chosen to further optimize the liposomal formulation in terms of medium stability, biocompatibility, clearance from the bloodstream, and surface functionalization.

Building further on these results, it was important to establish whether TTA-UC upconversion in vesicles can take place in living cells. Indeed, in Chapter 7 and Chapter 8 it is described that upconverting liposomes were endocytosed

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by cancer cells and that the red-to-blue upconversion luminescence could be imaged under hypoxic conditions. The liposomes were localized in endosomes and lysosomes, and were degraded by the cells within 24 h after uptake. However, the upconversion luminescence bleached rapidly (within a couple of seconds) and the intensity was rather low due to quenching by molecular oxygen in the cells. As described in Chapter 8, the oxygen sensitivity was improved by co-treating the cells with a biologically relevant concentration of glutathione and L-ascorbic acid, which greatly improved the upconversion luminescence inside the cells. Furthermore, the upconverting liposomes were found to be non-cytotoxic in the dark and under high-power red light irradiation; no PDT effect was observed, probably because of the low photosensitizer concentration in the membrane that results in very low singlet oxygen production.

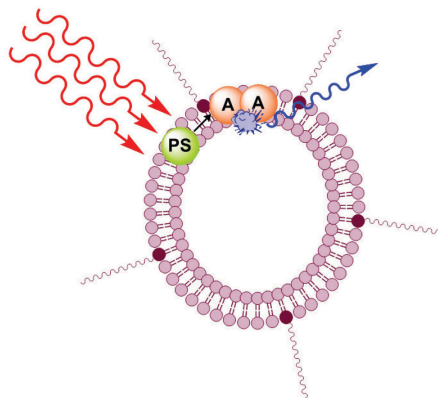


Figure 10.1. Red-to-blue TTA-UC in nanovesicles with a photosensitizer (PS) and annihilator (A) dye.

Besides liposomes, red-to-blue TTA-UC was also realized in the membrane of polymersomes, which were self-assembled from polyisobutylene-polyethylene glycol block copolymers, as described in Chapter 9. Although red-to-blue upconversion was somewhat less efficient in polymersomes than in liposomes, the polymersomes were much more quickly internalized by living cells. The upconverting polymersomes were then successfully imaged in living cells, while the addition of glutathione and L-ascorbic acid greatly boosted their performance *in vitro*. Compared to liposomes, the strong rubbery membrane of polymersomes may be more resistant towards digestion in cells or in the digestive tract of mammals, which opens up possibilities in drug delivery. Altogether, TTA-UC in liposomes and polymersomes represents exciting opportunities for luminescence bio-

imaging, because auto-fluorescence and irradiation damage to the cells or tissue are effectively eliminated.

10.2 Overcoming the oxygen sensitivity of TTA-UC

The TTA-UC mechanism relies heavily on triplet-state chromophores, which are readily quenched by molecular oxygen. This quenching event usually leads to severely compromised TTA-UC efficiencies in air; an issue that is poorly resolved in literature. Indeed, in the initial work described in Chapter 3 it was realized that the upconversion was only observed when the liposome solutions were deoxygenated by bubbling with argon. To counter the issue of oxygen sensitivity in TTA-UC nanosystems, three strategies were pursued in this research (Figure 10.2). The first strategy was applying a potentially oxygen-impermeable coating to the nanoparticle. Chapter 7 describes how a nanometer-thick (organo)silica coating was applied to upconverting DMPC liposomes. Although these nanocomposites were readily taken up by cancer cells, unfortunately the silica coating did not decrease the quenching by oxygen (neither in solution nor in cells), most probably because of the porosity of the silica coating. However, upon drying of the silica-coated liposome dispersion in an excess of (organo)silica precursor, interesting lipid-silica nanocomposite materials were obtained that were capable of TTA-UC in air, thereby confirming that in certain conditions (organo)silica can act as oxygen barrier for TTA-UC systems. These results represent intriguing examples of the combination of phospholipids, water, and silica for the construction of tunable upconverting nanoparticles and materials.

In a second approach, it was realized that physical deoxygenation methods such as bubbling with argon could be replaced by adding a ground-state molecular oxygen scavenger to the liposomes, such as sodium sulfite, as described in Chapter 5. Sulfite depletes the oxygen dissolved in solution, thereby allowing efficient and stable TTA-UC to occur in air (Figure 10.2b). However, many ground-state oxygen scavengers are incompatible with biological systems (*e.g.* hydrazine), and they slowly deplete over time when oxygen leaks into the sample. To improve on this approach, the third strategy (Chapter 8) involved addition of a singlet-oxygen scavenger (“anti-oxidants”) such as L-ascorbic acid or glutathione. Instead of reacting with ground state oxygen, such anti-oxidants only react with excited-state singlet oxygen: Upon continuous irradiation of the TTA-UC system, the photosensitizer produces singlet oxygen, which is then scavenged by the anti-oxidant until all oxygen is

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consumed (Figure 10.2c). This strategy was very efficient in allowing intense and stable TTA-UC in air (>80% stability during the first hour of continuous irradiation). As described in Chapter 9, the role of anti-oxidant can also be fulfilled by histidine, trolox, or the anti-oxidants present in cell medium (e.g. bovine serum albumin or pyruvate). Thus, the anti-oxidant approach is a rather general and powerful strategy that can be applied to virtually any existing TTA-UC system.

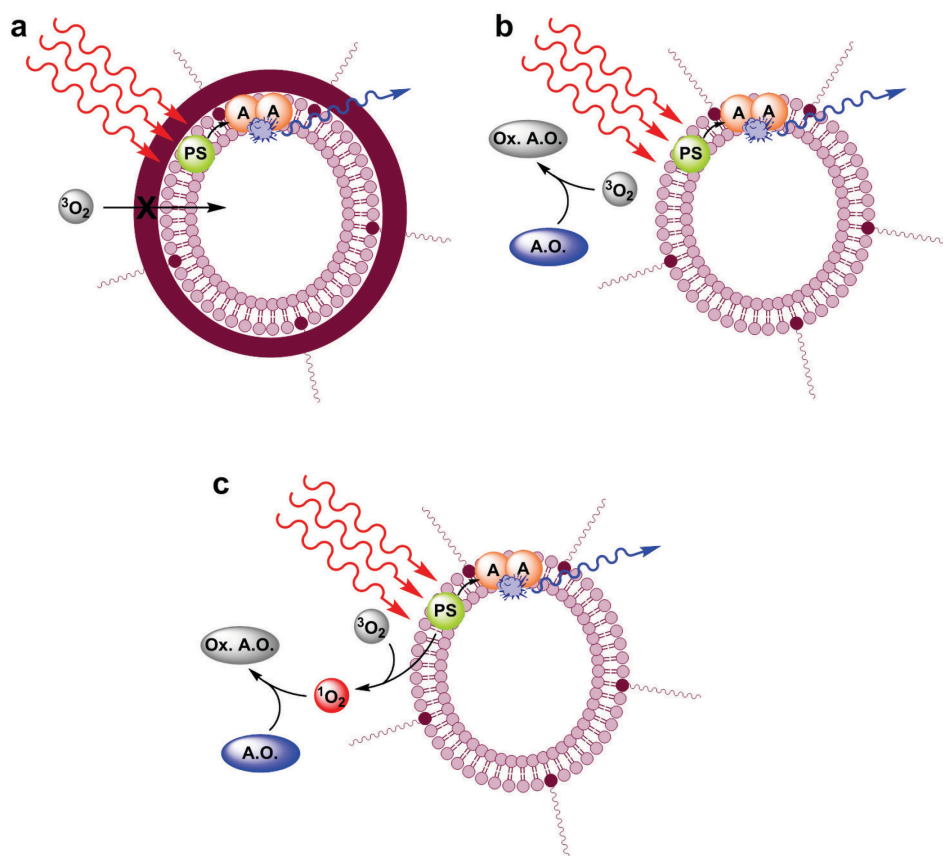


Figure 10.2. Cartoon illustrating the three different oxygen protection strategies pursued in this thesis. a) Coating the upconverting liposomes with an oxygen impermeable barrier. b) Chemical deoxygenation with a ground-state oxygen scavenger, i.e. anti-oxidant (A.O.), leading to an oxidized anti-oxidant (Ox. A.O.). c) Singlet-state oxygen scavenging: upon irradiation, the photosensitizer generates singlet oxygen, which then reacts with an anti-oxidant to form the oxidized anti-oxidant, thereby lowering the local oxygen concentration.

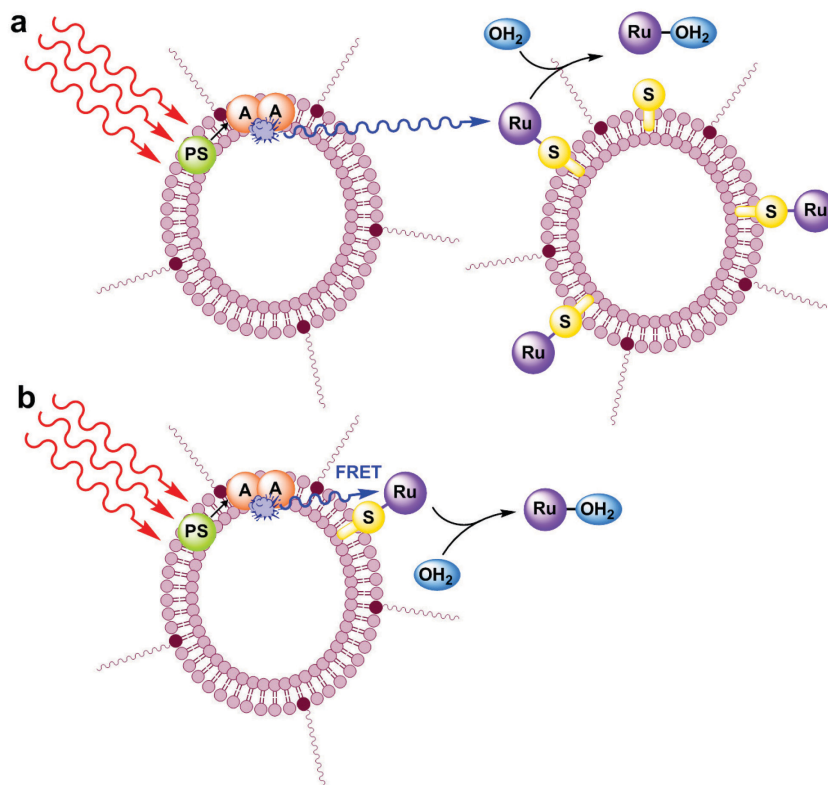


Figure 10.3. Cartoon illustrating the combination of TTA-UC in liposomes and light-activatable, membrane-anchored Ru-polypyridyl complexes. a) TTA-UC and Ru complex are physically separated on two different liposomes: the generated blue light from TTA-UC is transferred to the Ru complex via radiative energy transfer (Chapter 3). b) TTA-UC and Ru complex are located on the same liposome: the generated blue light from TTA-UC is transferred to the Ru complex via FRET (Chapter 4), after which the activated Ru-aqua complex dissociates from the membrane. PS: photosensitizer; A: annihilator; FRET: Förster Resonance Energy Transfer.

10.3 Activation of Ru(II) prodrugs by TTA-UC

The aim of this thesis was to activate Ru(II) prodrugs with red light by means of an upconverting drug carrier. This goal was first met in Chapter 3, where it was described how the photodissociation reaction of a Ru(II) polypyridyl complex from liposomes could be triggered by upconverting liposomes in a physical mixture of red-to-blue upconverting liposomes and Ru complex-doped liposomes. In this initial work, the blue upconverted light was transferred to the Ru-complex by radiative energy transfer, i.e. by emission of blue light and reabsorption of this light by the Ru-complex. When the upconverting dyes and the Ru-complex were doped in the same liposome membrane, as described in Chapter 4, it was found that the upconverted light

was transferred to the Ru-complex by Förster Resonance Energy Transfer (FRET) with more than 85% efficiency for moderate Ru-complex molar doping amounts (4 mol% with respect to the phospholipids). These studies supported the potential of Ru-prodrug activation by red-to-blue TTA-UC, but unfortunately the experiments did not succeed yet in air due to quenching of TTA-UC by oxygen. To allow the system to function in air, the photoreaction was successfully performed in presence of L-ascorbate and glutathione, as described in Chapter 8. It was also attempted to test liposomes doped with TTA-UC dyes and Ru-prodrugs *in vitro*. However, the Ru-complexes that were used exhibited high cytotoxicity without irradiation as well, and did not become significantly more toxic upon irradiation; for these reasons, activation by red-to-blue TTA-UC did not lead to a pronounced photochemotherapeutic effect, and the feasibility of the approach remains uncertain. At this point, more research efforts are necessary for the design of membrane-anchored Ru-prodrugs with high photo-indices and low toxicity in the dark before the feasibility of the activation-by-upconversion approach can be validated in biological systems.

10.4 General remarks

The results described in this thesis provide valuable insights for developing biological applications of TTA-UC. Liposomes and polymersomes were successfully used as multifunctional red-to-blue TTA-UC platform for bio-imaging and activation of light-activatable Ru prodrugs. It is clearly demonstrated that blue-light sensitive Ru-polypyridyl complexes, that normally do not respond well to light in the phototherapeutic window, can be activated by red light by means of TTA-UC. The biological evaluation of this activation-by-upconversion strategy requires more research attention to elucidate which parameters need optimization, such as nanoparticle design, TTA-UC stability, oxygen sensitivity and presence of anti-oxidants, light dose, amount of nanoparticle dopants (dyes and drug), and photo-index of the Ru prodrugs. We expect that the results of this thesis will lead to exciting applications in photoactivation chemotherapy that provide an alternative for photodynamic therapy in hypoxic tumors.

The red-to-blue upconversion quantum yield was found to be high at human body temperature (1.5% at 37 °C) and maximized at much lower excitation intensities ($\sim 0.1 \text{ W.cm}^{-2}$) compared to lanthanoid-based upconversion nanoparticles (UCNPs), which typically achieve upconversion quantum yields

in water much lower than 0.5% at much greater excitation intensities ($>1 \text{ W.cm}^{-2}$). In contrast to TTA-UC nanovesicles, UCNPs also suffer from low absorption, poorly reproducible synthesis routes and poor stability in aqueous solution, cell growing medium, or serum. Naturally, TTA-UC systems also have disadvantages, such as the oxygen sensitivity and photostability. However, in recent years, it has been successfully shown that oxygen sensitivity is a hurdle that can be overcome with creative approaches and continuous research efforts.^[1] As a contribution to this, in this thesis it was successfully demonstrated that the oxygen sensitivity of TTA-UC nanovesicles can be greatly reduced (even *in vitro*!) when biocompatible anti-oxidants were added. Of course, still much work is required in designing and synthesizing new types of TTA-UC nanoparticle systems with much lower intrinsic oxygen sensitivity, higher temporal stability *in vitro*, higher upconversion efficiencies, while maximizing biocompatibility and biostability. Overall, in our opinion TTA-UC nanoparticles outperform UCNPs in many ways. Yet curiously, the field of biological TTA-UC research receives much less attention than the field of UCNPs: a quick search on SciFinder (August 2016) with keywords “triplet-triplet annihilation upconversion” produces 365 results (of which ~10 are biology-oriented), while “upconversion nanoparticles” produces 2318 results, of which most are aimed towards biological application of UCNPs. We expect that the results described in this thesis reinforce the applicability of TTA-UC nanoparticles in biology and hope that they will convince the scientific community that this research field deserves more attention.

10.5 Outlook

10.5.1 Oxygen sensitivity

Although substantial work in this thesis was dedicated towards the reduction of the oxygen sensitivity of TTA-UC nanoparticle systems in combination with PACT, a number of improvements can be made. In the future design and preparation of TTA-UC nanoparticle systems, the oxygen sensitivity must first be minimized in the design, and then characterized in air-equilibrated solutions and in biological systems. As outlined in this thesis, it has become apparent that oxygen sensitivity can be minimized by using anti-oxidants that scavenge ground state or singlet state oxygen. It may be also possible to use nanoparticles with built-in anti-oxidants. For example, in a vesicle design the aqueous interior may be filled with a high concentration of water-soluble anti-oxidants; alternatively the membrane can be functionalized with oxygen-

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scavenging moieties such as unsaturated carbon-carbon bonds or lipophilic anti-oxidants such as β -carotenes (Figure 10.4). Regardless of which anti-oxidant is used and at which location, it is important to evaluate their toxicity. It may also be important to separate anti-oxidants from TTA-UC dyes and Ru prodrugs, because:

- i. Some anti-oxidants or anti-oxidant products quench triplet state dyes, for example α -tocopherol or hydroquinone.^[2]
- ii. Anti-oxidants such as glutathione may cause photo-reduction of dyes and/or Ru prodrugs
- iii. An anti-oxidant such as glutathione or histidine may coordinate to heavy metal photosensitizers or to the activated Ru complex.
- iv. Oxidized anti-oxidant products may react further with upconversion dyes and degrade them, such as in the case of peroxidized carbon-carbon bonds.

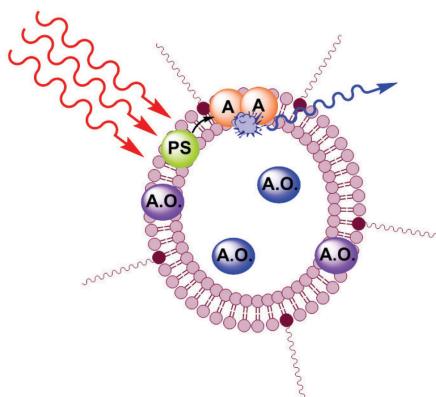
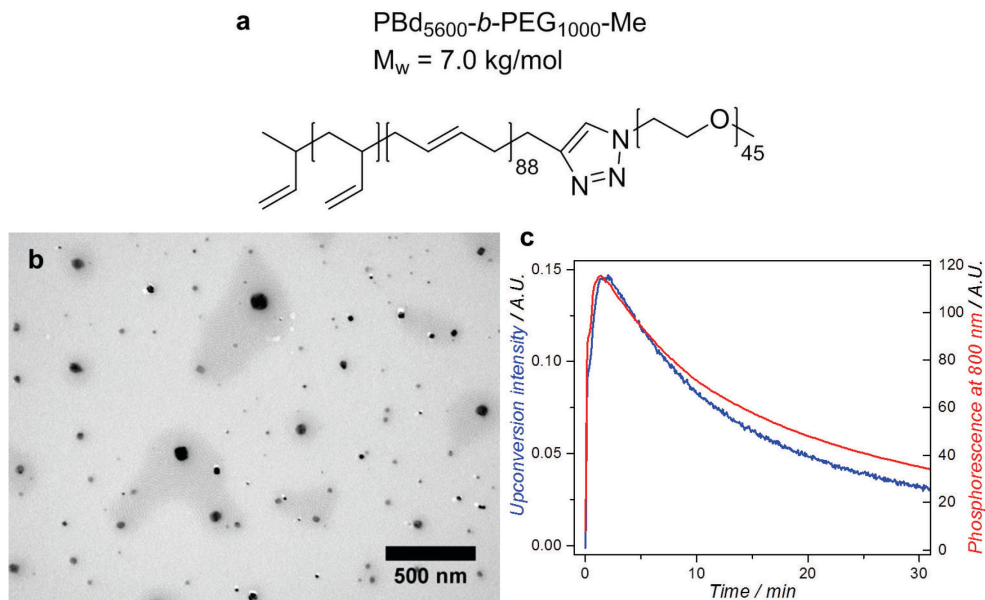


Figure 10.4. Upconversion polymersomes with built-in anti-oxidants, either in the hydrophilic interior or inside the hydrophobic membrane.

To illustrate this built-in anti-oxidant strategy, during the work described in this thesis a red-to-blue upconverting polymersome formulation was prepared with a polybutadiene-polyethylene glycol block copolymer (kindly provided by Prof. dr. Jan van Hest, Radboud University, Figure 10.5a) and investigated with emission spectroscopy without any further addition of anti-oxidants (Figure 10.5c). The polymer and TTA-UC dyes concentration (5 mg/mL, 5 μ M PdTPTBP, 100 μ M 2,5,8,11-tetra(*t*-butyl)perylene), and irradiation conditions (0.4 W.cm⁻² 630 nm excitation) were identical to those used for Figure 9.3d (Chapter 9). In this case the upconversion was observed

instantaneously and at lower polymer concentration (still observable at 1.6 mg/mL polymer), suggesting that the unsaturated bonds of polybutadiene were more effective in scavenging singlet oxygen than polyisobutylene. Curiously, in a preliminary experiment upconversion could not yet be realized in living cells.



*Figure 10.5. Upconverting polymersomes based on polybutadiene-block-monomethylpolyethylene glycol copolymer (PBd-*b*-PEG-Me), prepared according to Georgieva et al.^[3] with [PBd-*b*-PEG-Me] = 9.8 mg/mL, [PdTPTBP] = 10 μM, and [2,5,8,11-tetra(*t*-butyl)perylene] = 0.20 mM. a) Molecular structure of PBd₅₆₀₀-*b*-PEG₁₀₀₀-Me block copolymer with PBd M_w ~5600 g/mol and PEG M_w 1000 g/mol, received from Prof. dr. Jan van Hest (Radboud University). b) TEM micrographs of the polymersomes. Hydrodynamic particle size according to DLS: 120 nm, 0.1 polydispersity index. c) Time traces of upconversion emission at 486 nm (blue, left axis) and phosphorescence of PdTPTBP at 800 nm (red, right axis) during 630 nm irradiation (50 mW, 0.4 W.cm⁻², 4 mm excitation path length) of upconverting PBd-*b*-PEG-Me polymersomes at 4.9 mg.mL⁻¹ concentration. Conditions: 600 μL sample in a non-stirred semi-micro cuvette at 20 °C. No oxygen scavenger was added. Directly comparable with data from Chapter 9, see Figure 9.3d.*

An alternative strategy to improve the oxygen sensitivity of TTA-UC is the design of supramolecular assemblies that feature tight networks of annihilator molecules. Such networks support fast diffusion of triplet excitons and greatly enhance the rates of TTET and TTA. In this way, TTA-UC is competitive with quenching by molecular oxygen and becomes appreciably efficient in air. A number of studies by the group of Kimizuka represent sophisticated examples of this strategy, which are expected to greatly impact the field of TTA-UC and might lead to highly efficient, oxygen-independent TTA-UC nanoparticles.^[4]

10.5.2 Biocompatibility of TTA-UC dyes

In the research described in Chapter 8, it was found that the annihilator dye, 2,5,8,11-tetra(*t*-butyl)perylene, accumulated in lipid droplets in A549 cells after digestion of the liposomes by the cell. This suggested that this dye could not be digested by the cell, and it raises concern on the long-term toxicity and mutagenicity. Thus, for real-world biological TTA-UC applications it is necessary to evaluate the mutagenicity and carcinogenicity of TTA-UC dyes.

10.5.3 Shifting the activation wavelength further to the NIR

In this thesis it was chosen to use red-to-blue TTA-UC to shift the prodrug activation wavelength into the phototherapeutic window (630 nm). However, the highest tissue transparency while minimizing absorption by water is achieved in the NIR region between 700 and 900 nm.^[5] In order to further shift the wavelength from 630 nm towards this region, other photosensitizer-annihilator pairs may be used that can be excited with 700 – 850 nm and emit in the green, yellow, or red.^[6] Since it was already demonstrated in Chapter 3 that liposomes can accommodate a diverse selection of lipophilic TTA-UC dyes, we expect that incorporation of a NIR sensitive photosensitizer and visible-range emitting annihilator in the liposomes is straightforward. However, it must be kept in mind that in order to further use the upconversion emission to achieve prodrug activation, the emission wavelength must overlap well with the prodrug absorption. Therefore, in the case of Ru polypyridyl complexes, NIR-to-green TTA-UC would be best suited for this approach.^[6e]

10.5.4 Nanoparticle-bound Ru-prodrugs

More research is necessary for the design of nanoparticle-anchored photoactivatable Ru prodrugs with high toxicity when irradiated and low toxicity in the dark. In the research described in Chapter 8, it was found that irradiation did not affect the cytotoxicity of certain Ru-prodrugs anchored to DMPC liposomes, while a relatively high toxicity was found in the dark. It was hypothesized that the degradation of Ru-doped liposomes was the cause of the observed dark toxicity. Thus, two key steps are needed to find a good PACT candidate to combine with a TTA-UC activation route: (i) it must be established that the activated complex is toxic once it is released inside the cell, and (ii) it is important to affirm that the complex is anchored and remains anchored to the nanoparticle throughout the entire treatment procedure. In this context, it may be beneficial if Ru-complexes are doped in more rigid nanoparticles such as polymersomes or silica nanoparticles. However, our

results are rather preliminary, and substantially more research must be performed in this area.

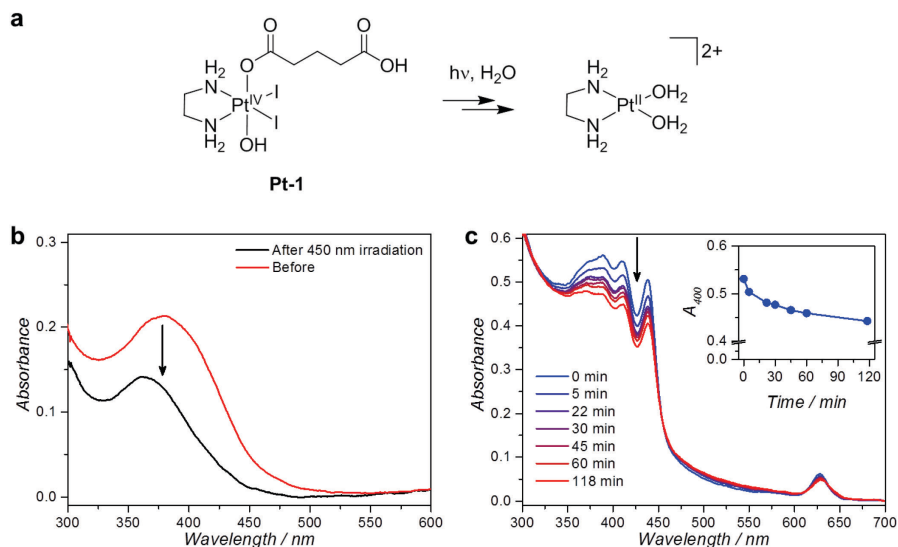


Figure 10.6. Photoactivation of Pt(IV) prodrugs with upconverting liposomes. a) Molecular structure of complex **Pt-1**, received from Prof. dr. Bednarski (Universität Greifswald) and the proposed conversion to the aquated Pt(II) species upon irradiation in water.^[7] b) Absorption spectroscopy before and after irradiation of **Pt-1** in PBS (0.13 mM) with 450 nm (arbitrary power). c) Absorption spectroscopy during red light irradiation (120 mW 630 nm; 1 W.cm⁻² intensity) of a mixture of **Pt-1** (0.25 mM) and upconverting liposomes (1.25 mM DMPC, 0.25 mM DSPE-mPEG-2000, 0.6 μM PdTPBP, 6 μM perylene) at 37 °C; the inset shows the absorbance at 400 nm as a function of irradiation time. The sample was deoxygenated prior to irradiation by bubbling argon for 30 min.

10.5.5 Activation-by-upconversion of other prodrugs

The general applicability of TTA-UC for the activation of other prodrugs than Ru-compounds deserves more attention in future work. During the work described in this thesis an experiment was conducted in collaboration with Prof. dr. Bednarski (Universität Greifswald) to examine whether compound **Pt-1** could be activated with red-to-blue upconverting liposomes (Figure 10.6). In water, in absence of upconverting liposomes, it is proposed that upon UV to blue light irradiation **Pt-1** converts to the aquated Pt(II) species,^[7] which can be observed by UV-vis absorption spectroscopy as a decrease of the LMCT absorption band (Figure 10.6b). Upon 630 nm irradiation of a mixture of upconverting liposomes and **Pt-1**, the same decrease in LMCT band was observed, suggesting successful conversion of **Pt-1** to the aquated Pt(II) species. Thus, upconverting liposomes may also be suitable for red-light

activation of Pt(IV) compounds. This preliminary experiment demonstrates that the strategy of combining PACT drugs with TTA-UC in nanoparticles can be easily extended to other (inorganic) prodrugs such as Pt(IV) compounds, CO or NO releasing molecules, and photocleavable coumarin derivatives.

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