Callaghan, S.; Senge, M. O. (2018):
The good, the bad, and the ugly – controlling singlet oxygen through design of photosensitizers and delivery systems for photodynamic therapy.
Photochemical & Photobiological Sciences 17, 1490–1514.
doi: 10.1039/C8PP00008E

Photochemical & Photobiological Sciences

PERSPECTIVE

The Good, the Bad, and the Ugly – Controlling Singlet Oxygen through Design of Photosensitizers and Delivery Systems for Photodynamic Therapy

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x
www.rsc.org/

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Singlet oxygen, although integral to photodynamic therapy, is notoriously uncontrollable, suffers from poor selectivity and has fast decomposition rates in biological media. Across the scientific community, there is a conscious effort to refine singlet oxygen interactions and initiate selective and controlled release to produce a consistent and reproducible therapeutic effect in target tissue. This perspective aims to provide an insight into the contemporary design principles behind photosensitizers and drug delivery systems that depend on a singlet oxygen response or controlled release. The discussion will be accompanied by *in vitro* and *in vivo* examples, in an attempt to highlight advancements in the field and future prospects for the more widespread application of photodynamic therapy.

1. Introduction

A good story has different protagonists such as the title roles portrayed by Clint Eastwood, Lee Van Cleef and Eli Wallach in Sergio Leone's epic.[1] Singlet oxygen plays the main protagonist (together with a supporting cast of other reactive oxygen species (ROS)) in the photodynamic therapy (PDT) story, a story which began at the turn of the 20th century with the development of photomedicine as we know it today. Finsen's seminal book entitled 'Phototherapy' published in 1899[2] and a series of works by von Tappeiner with Jesionek and Raab pioneered the field by refining the principles which underpin all conventional photomedical treatments.[3,4,5] In 1913, the photodynamic effect was then demonstrated in humans when Meyer-Betz injected himself with a compound derived from blood and upon exposure to light, experienced blistering and swelling of the skin.[6] Since these early advancements, photodynamic therapy has established itself as a safe and reliable treatment for various cancers,[7,8] dermatological conditions[9] and age-related macular degeneration,[10] and has also gained traction in other areas including, going back to its roots, antimicrobial PDT for the treatment of infectious diseases.[11] However, similar to Sergio Leone's classic 1966 'spaghetti western', it took some time until true critical acclaim and acceptance followed the initial success.[7,12] To some extent PDT still lags behind more conventional treatments of malignancies despite its established potential. In part, this is due to the problems associated with managing singlet oxygen, which – like many good actors – can play many roles, but can be difficult to control.

PDT relies on the interaction between light, a photosensitizer (PS) and ground state oxygen, which combine to deliver the therapeutic effect mediated by singlet oxygen or other ROS. Firstly, a photosensitizing drug is administered and selectively activated with light of an appropriate wavelength. Once triggered, the PS is promoted to an excited singlet state and then, *via* intersystem crossing (ISC), a longer-lived triplet state is occupied. From here ROS, including singlet oxygen, are produced and depending on the cellular localization of the PS and the degree of damage caused, necrosis and/or apoptosis is induced.[13]

PDT has many advantages over conventional treatments. It is more selective and thus less toxic to healthy tissue than chemotherapeutic drugs, has better cosmetic results and a lower risk of infection compared to surgery, and unlike radiotherapy, it does not cause side effects such as infertility. Moreover, PDT does not cause organ damage due to toxic drug accumulation, is less prone to induce resistance, and has dual functionality as it targets both the tumor vasculature and cancerous cells.[14] Finally, due to its unique mode of action PDT is immune to many conventional chemotherapeutic resistance mechanisms, a property that shows potential for combinational therapies.[14a] Despite this, PDT is not yet a front-line treatment for many cancers and drawbacks include limited tissue penetration, control of light dose, and photosensitivity. In any case, PDT is a clinically relevant therapy but requires novel approaches to optimize current treatments and advance the development of third or fourth generation PSs and effect a true change in treatment standards in the relevant areas.[15]

Researchers are attempting to provide viable improvements on the current regimes as they develop this so-called 'ideal PS'. The potential structural diversity occupies chemists who strive to

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introduce properties which would maximize functionality and provide an efficient and effective route from production to cell delivery. This is accompanied by formulation, nanodrug and theranostic materials science approaches,[16] and desired target properties including (Fig. 1):

- Water-solubility
- Optimal photophysical properties that compliment biological media and maximize ROS production
- Facile and reproducible syntheses
- Chemical purity
- Minimal side effects
- No aggregation (and excited state quenching)
- Straightforward administration
- Improved targeting and selectivity
- Swift excretion from the body but targeted distribution lasting long enough to provide the therapeutic effect
- Resistance to photobleaching.

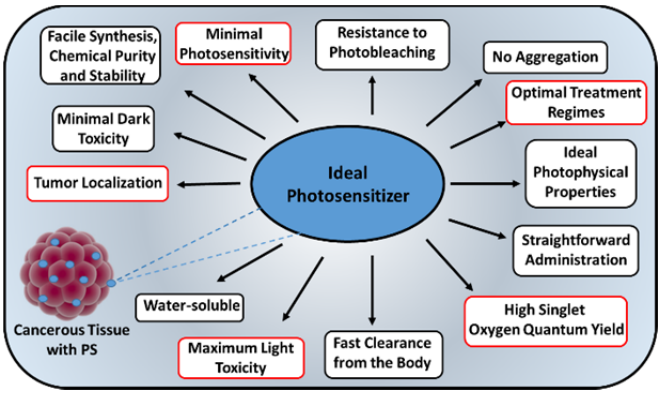

Figure 1 Properties of the ideal PS for application in oncology, with properties relevant to this perspective highlighted in red.

Other research is focused on utilizing singlet oxygen to improve PS design. As previously mentioned, singlet oxygen is the principal cytotoxic agent in PDT and is generated by energy transfer from the triplet state of a PS to ground state molecular oxygen.[12a,17] Its generation through photochemical processes and the associated pitfalls are also the reason why clinical applications critically depend on illumination protocols, dosimetry and instrumentation such as light sources, intensity, flux rate, etc.[18]

To obtain viable singlet oxygen yields a variety of methods are employed, including optimization of ISC and high PS loading in delivery vehicles. In many PSs, fluorescence competes with ISC but systems that produce fluorescence in response to singlet oxygen could be used to visualize oxidative stress in real time. The increased oxidizing ability of singlet oxygen in comparison to ground state oxygen is the dominant force behind the PDT effect and results from spin-paired electrons (Fig. 2). However, this enhanced reactivity is counterbalanced by limited selectivity and indiscriminate toxicity at the site of generation, which can cause damage to healthy cells. Photosensitivity is observed if the PS is triggered by light not pertaining to the treatment. An approach to circumvent this is the design of systems that are activated at the target site for an optimal therapeutic effect. This localization can be mediated by tumor activatable PSs or delivery systems.[19] Another major issue with singlet oxygen is its fast decomposition rates in biological media,[20] although newer studies have shown cases with longer lifetimes than previously believed.[21] Decay *via* first order kinetics occurs on a µs timescale resulting in the need for long treatment times. Thus, fractioning the light dose or designing systems that can produce singlet oxygen post illumination would significantly enhance current treatment regimens (Fig. 1).

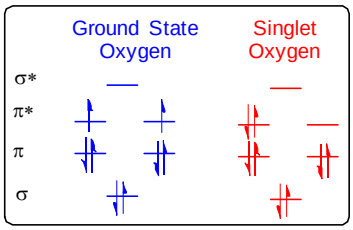

Figure 2 Electronic configuration of ground state (triplet) oxygen and singlet oxygen.

Designing PSs and delivery systems with the properties of singlet oxygen[22] in mind constitutes the basis of this perspective and we hope that the following will elicit further studies and translational work in this area. It is not intended as a comprehensive review on PDT or chemical strategies in PS design; wherever possible we have included references to leading reviews in the relevant areas. The studies highlighted in the present work attempt to harness the pitfalls of singlet oxygen to deliver an enhanced therapeutic effect by locally generating singlet oxygen using activatable PSs, slowly releasing singlet oxygen, or by using singlet oxygen responsive systems to effect a therapeutically advantageous change.

2. Activatable Singlet Oxygen Release

An advantage of PDT over standard cancer treatments is the light activation of specific tissue. However, in reality, the reliance on just this modality results in photosensitivity and toxicity to proximate healthy cells. To overcome this, systems have been designed with singlet oxygen generation that can be controlled on a molecular level resulting in local activation to afford a more refined therapeutic effect specific to target tissue. The local activation can be mediated by elevated tumor pH or intracellular thiols as well as decreased oxygen levels. The nucleic acid pairing mechanism and overexpression of proteins are also employed. There are many examples of activatable singlet oxygen release relevant to PDT, in fact, they are too numerous to mention in the constraints of this perspective,[23] thus, only a select few have been chosen to illustrate the main features.

The predominant approach to these so-called 'smart' PSs is activation of singlet oxygen generation by stimuli directly related to the cellular microenvironment of the target tissue through deactivation of quenching mechanisms.[24] These quenching mechanisms include (i) contact quenching, (ii) photo-induced

electron transfer (PET), and (iii) Förster resonance energy transfer (FRET).

For contact quenching mediated activation, the PS excited state properties are deactivated by direct interaction with other PS molecules but, once separated, singlet oxygen generation can resume. Contact quenching is achieved by high PS loading onto a scaffold and subsequent dispersion of the PS upon activation. Similar to this, the PET mechanism can deactivate a PS by using a covalently bound small molecule quencher until the stimulus disrupts the electron transfer and singlet oxygen generation recommences. Alternatively, quenching of the excited singlet state and inhibition of ISC can be achieved by FRET. FRET is the transfer of energy from a PS donor to a chromophoric acceptor tethered by a linker.[25] In this state, the PS is inactive, however, when the linker is cleaved the PS enters its activated state. FRET is strongly dependent on PS-quencher spectral overlap and distance (Fig. 3). The modulation of the triplet state of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY), including examples of FRET and PET dependent PSs, has been reviewed by Zhao *et al.*[26]

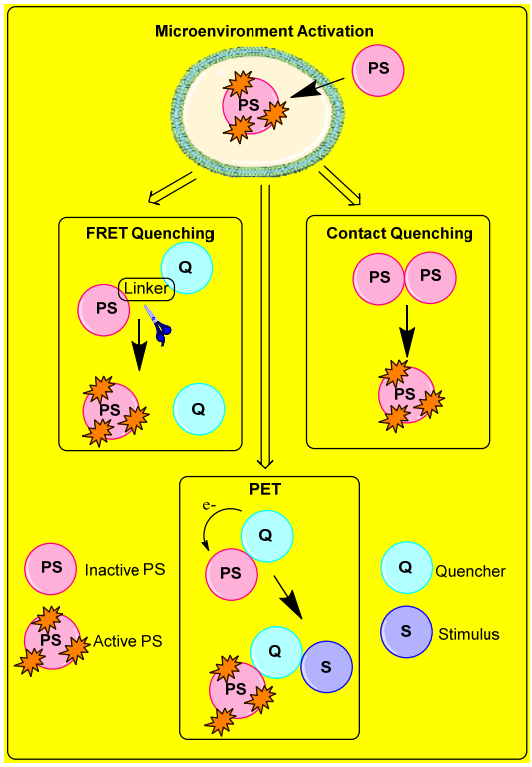

Figure 3 Schematic representation of common singlet oxygen activation mechanisms associated with microenvironment activation.

2.1 pH and Other Environmental Conditions Activating Singlet Oxygen Generation

Second to light irradiation, environmental factors including pH and solvent polarity are conspicuous variables for the control of singlet oxygen release. For example, the effect of variable pH on thiazine dyes is well documented. Among them, the classic PS methylene blue is reported to have a five-fold increase in singlet oxygen production efficiency under basic conditions as opposed to in acidic media.[27] In another study, Ogilby and co-workers showed that protonation of PSs containing amine moieties results in a reduction of singlet oxygen quantum yields.[28] Similarly, the response of different phthalocyanines and tris(2,2'-bipyridyl)ruthenium(II) dichloride to polar solvents or micellar solutions has been described and the authors found that singlet oxygen yields were predominantly higher in dimethylformamide (DMF) when compared to micellular solutions.[29] To utilize these principles for PDT, these studies must be extended to biological conditions.[30] A typical example is the incorporation of PSs into biological membranes, which is dependent on the degree of hydrophobicity of the dye.[31] Here, depth of membrane incorporation, physical properties of the membrane, and orientation of the PS therein affect the photosensitizing efficiency.[32]

pH-responsive systems are of particular notability due to their physiological relevance, as cancerous tissue is known to be more acidic than normal tissue.[33] In 2005, O'Shea and co-workers showed, in a proof-of-concept study, that pH-dependence can be exploited to selectively activate singlet oxygen generation by modulating the PET process. They designed aza-BODIPYs (compound **1**) with pH-responsive amine units covalently attached at the 2 and 6 positions (Fig. 4). Their compounds displayed increased singlet oxygen production under acidic conditions (compound **2**) due to the deactivation of the PET mechanism and subsequent singlet oxygen generation from the aza-BODIPY unit. This was shown to be reversible upon the introduction of neutralizing equivalents of base. *In vitro* studies in a transformed fibroblast cell line demonstrated cytoplasmic localization and cytotoxicity; however, a clear correlation between cellular pH and cytotoxicity was not shown.[34]

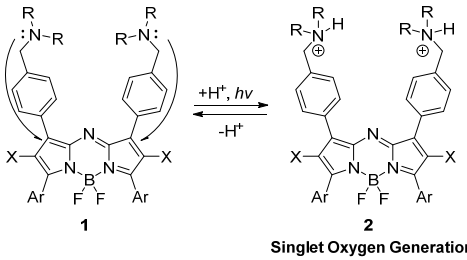

Figure 4 O'Shea's concept of a pH-responsive aza-BODIPY system.

Bu, Shi and co-workers later confirmed that cellular pH levels could activate the release of PS for singlet oxygen production.[35] Their systems used acidic H_2O_2, native to tumor tissue, to release up-conversion nanoprobes fashioned with PSs, from MnO_2 nanosheets *via* the reduction of MnO_2 to Mn^{2+}. Simultaneously, this reaction increased cellular oxygen levels to aid PDT. During the up-conversion process lower energy incident photons from NIR (near-infrared) light are absorbed and converted into a single higher energy photon for emission, which can activate surrounding PSs. Additionally, activation of the nanoprobes by X-rays could stimulate a radiotherapeutic effect. Solid tumors (4T1) in mice showed significantly inhibited tumor growth when treated with the functionalized nanosheets and NIR light. A synergistic effect was

also noted following treatment with X-rays, NIR light, and the nanosheets anchored with nanoprobes. The pH-dependent singlet oxygen generation and imaging from up-conversion luminescence were also confirmed *in vitro*.

Nanotechnology was also employed by other groups in the design of pH-sensitive PSs. Firstly, in 2013 Liu and co-workers described manganese-doped up-conversion nanoprobes, which were loaded with chlorin e_6 (Ce6) conjugated polymers using a layer by layer self-assembly strategy.[36] Under NIR excitation, Ce6 was activated to generate singlet oxygen. To elicit a pH response these nanostructures were coated with a charge-reversible polymer containing dimethylmaleic acid (DMMA) and polyethylene glycol (PEG). At a normal physiological pH of 7.4 the nanosurface was negatively charged but upon lowering to 6.8, in line with intratumoral pH, the amide bond between the amino group and DMMA was broken and the surface became positively charged, allowing easier cell uptake and longer retention in the acidic tumor microenvironment. Enhanced cellular internalization and PDT activity were shown in HeLa cells at a pH of 6.8 in comparison to at pH 7.4. The feasibility of this approach was evaluated *in vivo* using 4T1 murine breast cancer tumor-bearing Balb/c mice intratumorally injected with 0.4 mg of the nanoconstruct, which showed a significant increase in anti-tumor efficacy. Following intravenous injection of the nanoparticles, up-conversion luminescence was shown to be 6.4-fold more active compared to a control that could not be cleaved by the acidic conditions. Although promising, the authors did note that the majority of systemically injected nanoparticles accumulated in the liver and spleen and that nanoparticle uptake is insufficient to provide effective PDT.

A year later Ling *et al.* then designed a PS that also utilized charge switching.[37] Their self-proclaimed 'nanogrenades' were composed of iron oxide nanoparticles, with catechol anchors, which could self-assemble with Ce6 bearing pH-sensitive ligands. Under acidic conditions, imidazole units of the ligands were protonated leading to a switch from negative to positive charge and resulting particle swelling, payload delivery and cellular uptake. The decreased intracellular pH then ensured complete degradation of the construct by weakening hydrophobic interactions and causing the ionic monomers to repel each other (Fig. 5). Their approach was verified *in vitro* by demonstrating a pH-dependent T_1 magnetic resonance contrast effect using the iron oxide particles to image HCT116 tumors of approximately 3 mm in diameter, indicating that these compounds could be used in early tumor detection. In addition, high-resolution fluorescent imaging indicated tumor accumulation. The singlet oxygen generating properties are modulated in this system as Ce6 self-quenches in the construct but is activated under acidic conditions as the Ce6 units dissociate. The PDT effect was shown *in vivo* using both homogeneous HCT116 and heterogeneous CT26 tumor-bearing mice.

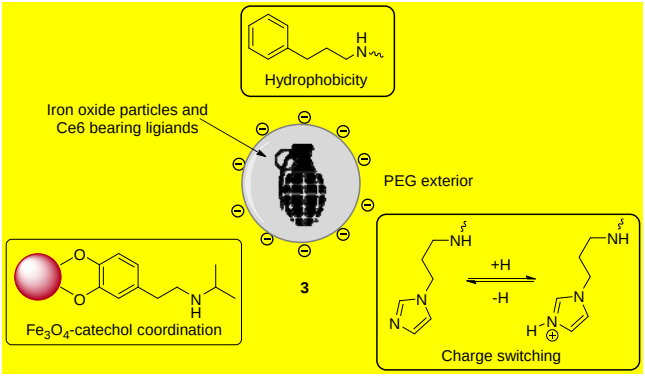

Figure 5 Schematic representation of key interactions utilized by 'nanogrenades'.

In a more recent study, Wong *et al.* used contact-quenching zinc(II)phthalocyanine dimers, which were linked through pH cleavable acetal groups, on alkyne-modified mesoporous silica nanoparticle scaffolds (Fig. 6, construct **4**) to demonstrate activatable singlet oxygen generation. When the pH is lowered in acidic tumor cells the dimers separate and activate to produce singlet oxygen and a fluorogenic response. MTT assays in HT29 cells indicated an IC_{50} value of 31 nM. Moreover, the local activation was confirmed in immunosuppressed nude mice bearing HT29 xenografts, as seen in Fig. 6, the fluorescence, due to tumor acid-mediated cleavage and release of the PS, increased over 10 h.[38]

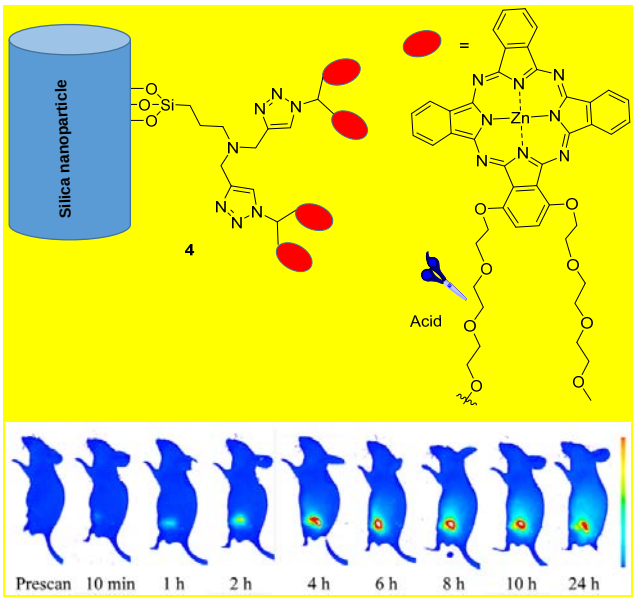

Figure 6 Top: Structure of silica nanoparticle scaffolds bearing quenched zinc(II)phthalocyanine dimers, construct **4**. Bottom: Full body fluorescence images of tumor-bearing nude mice injected with construct **4** (40 nmol/kg body weight) over 24 h. Reprinted (adapted) with permission from ref. 38. Copyright (2017) American Chemical Society.

Another environmental factor, particularly relevant to solid tumor-targeted PDT, is oxygen deficiency. In 2017, Piao *et al.* designed a seleno-rosamine-based PS with an azo moiety which prevents ISC to the triplet state *via* internal conversion. Upon reductive cleavage of this azo group under hypoxic conditions, ISC is restored and singlet oxygen is generated. Although the efficiency of PDT is dependent

on cellular oxygen levels, they demonstrated *in vitro* that mildly hypoxic tissue (5% oxygen concentration) showed specific tumor cell toxicity (Fig. 7, compound **5**).[39]

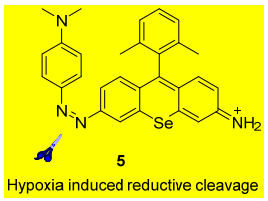

Figure 7 Example of PS activated under hypoxic conditions, compound **5.**

2.2 GSH-mediated Activatable Singlet Oxygen Generation

Other important environmental stimuli are intracellular thiols, glutathione (GSH) and cysteine (Cys). In addition to the increased levels of GSH in the cellular environment in comparison to the extracellular environment (1–11 mM vs. 0.002–0.010 mM),[40a] elevated GSH levels are also present in various breast, ovarian, head and neck, and lung cancers.[40b] This, in addition to their reducing properties, makes them ideal tools for activatable PSs.

Akkaya and co-workers developed systems activated by GSH that rely on the cleavage of a PS-quencher linker.[41] In these compounds a 2,4-dinitrobenzenesulfonyl (DNBS) unit is used as a cleavable linker and an electron sink to quench the excited state properties of the halogenated BODIPY. Thus, in the conjoined configuration, as shown for compound **6** in Fig. 8, singlet oxygen production was absent. Only after internalization and GSH-mediated cleavage did photosensitized singlet oxygen generation occur. This was evidenced using Annexin-V and Hoechst-33258 as fluorescence stains through visualization of apoptosis in HCT116 cells when they were treated with 40 nM of the PS and irradiated with a red LED. To further support their findings, fluorescent activated cell sorting showed elevated sub G-1 DNA levels following incubation with the active compound and red light, thus indicating that indeed GSH levels in cancerous tissue are sufficient to activate the PS and induce apoptosis in a controlled manner.

Zhao *et al.* also developed a similar BODIPY-based compound **7** (Fig. 8) which can be activated by GSH or Cys. In this system, cellular thiols cleave two bonds.[42] Firstly, a disulfide bond is broken to separate two chromophores, one of which is a halogenated BODIPY that can produce singlet oxygen after the DNBS quencher is also released by the thiols. The second chromophore is a fluorescent BODIPY and is also activated by the cleavage; thus, they showed that PDT and imaging can operate independently and simultaneously without competing. They reported an increase in singlet oxygen quantum yield from 17% to 72% and a fluorescence quantum yield increase form 1% to 18% following thiol activation.

In two other recent examples of GSH activatable systems, Zeng *et al*. reported bis(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dimers linked *via* an azo moiety, which once activated can participate in two-photon PDT,[43] and Hu *et al.* united the photodynamic effect with the action of a chemotherapeutic drug, mitomycin C, to achieve a synergistic effect.[44] Here, the chemotherapy drug also functions as the PET quencher and is linked *via* a GSH cleavable disulfide bond to the PS unit (Fig. 8, compound **8**). In the absence of light, the compound showed cytotoxicity in 4T1 cells, which was attributed to the chemotherapeutic drug being activated by cellular GSH. However, following light treatment the toxicity significantly increased to give an IC_{50} value of 3.7 µM, thus demonstrating a synergistic effect *in vitro*. This effect was also demonstrated *in vivo*. Compound **8** was formulated into PEG-based nanoparticles to increase biocompatibility and injected intratumorally into a mice model. The therapeutic effect was shown to be much greater for compound **8** nanoparticles in the presence of light in comparison to dark conditions where only the chemotherapeutic drug was active. Additionally, when the active compound was injected intravenously fluorescence was strongest in the tumor tissue, demonstrating GSH-mediated activation.

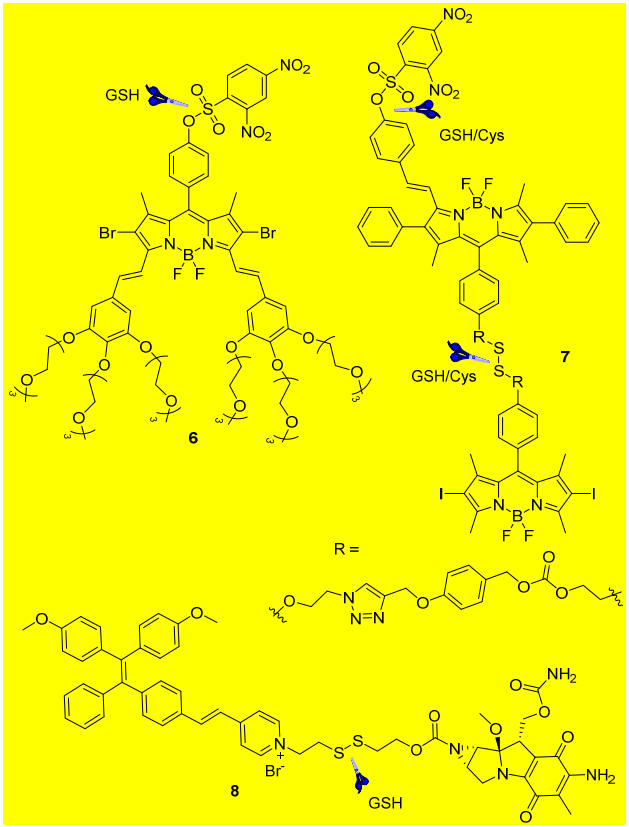

Figure 8 Examples of thiol activatable PSs.

In another example of this concept being applied to nanotechnology, Durand and co-workers developed PS disulfide-based bridged silsesquioxane nanoparticles. The constructs were both biocompatible and degradable at near-physiological conditions and in MCF-7 cancer cells. Moreover, *in vitro* two photon excited fluorescence and therapy resulted in cellular uptake and cell death between 40 and 60%, respectively.[45]

2.3 Dual-modality Activation of Singlet Oxygen Generation

Several examples of tumor activatable PSs have now been discussed but to take the idea a step further, dual modality systems, in which two stimuli are used to activate a PS, have been developed in the

context of pro-drug activation strategies.[46] It was anticipated that using two stimuli would increase tumor selectively as to generate singlet oxygen two independent events have to occur simultaneously, resulting in less damage to healthy tissue.

In an early study, Ozlem and Akkaya designed systems that were activated to generate singlet oxygen under elevated Na^+ and proton concentrations. The BODIPY-based PS **9** carried a crown ether to detect Na^+ ions and pyridine groups to detect changes in pH which directly affected PET efficiency (Fig. 9).[47] Interestingly, the highest relative singlet oxygen quantum yields were seen when both probes were stimulated simultaneously; however, this proof-of-concept study did not include *in vitro* or *in vivo* studies.

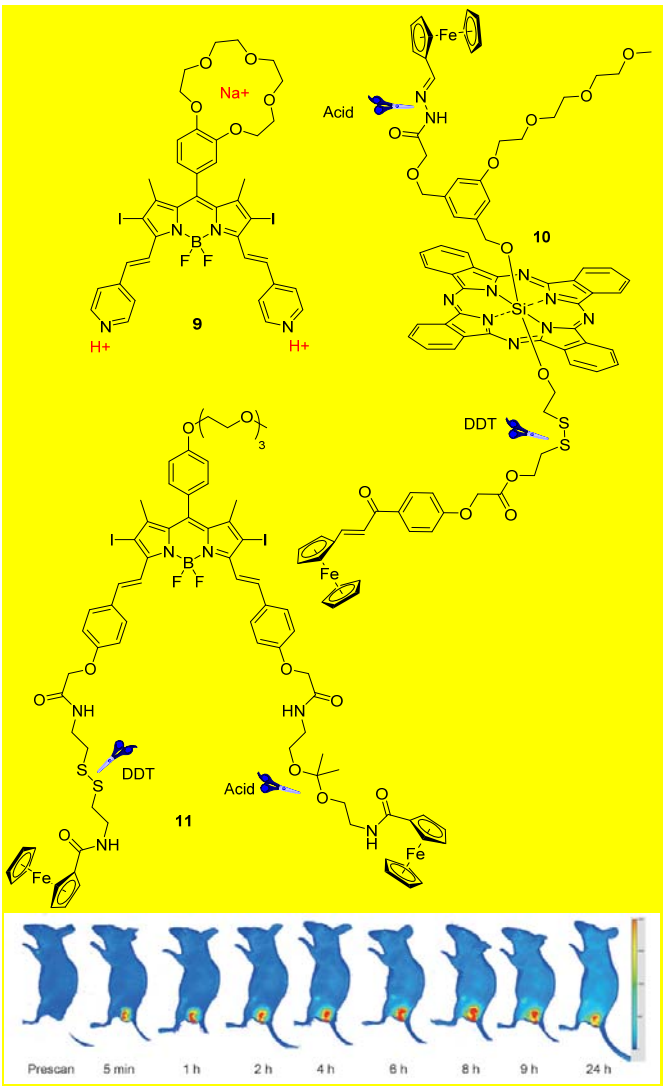

Figure 9 Top: Structures of compound **9**, a pH and Na^+ ion dual activatable PS and compounds **10** and **11**, pH and dithiothreitol dual activatable PSs. Bottom: Fluorescence images of tumor-bearing mice over 24 h after intratumoral injection of compound **11**, showing dual activation of PS. Reprinted (adapted) with permission from ref. 49. Copyright (2016) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Lau *et al*. later designed a biocompatible system. The bisferrocenyl silicon(IV) phthalocyanine **10** (Fig. 9) possessed disulfide and hydrazine linkers, which were cleaved by dithiothreitol (DTT) under acidic conditions to release the PS from ferrocenyl quenchers and activate population of the triplet state.[48] Their studies attempted to simulate an acidic and reducing tumor environment. They noted that the best singlet oxygen efficiencies were accessed at a pH of 6 with a DDT concentration of 10 μM. Their *in vitro* studies in MCF-7 breast cancer cell lines used DDT doses between 2-4 μM and ionophore nigericin, as a pH modulator, to control the conditions. The PDT effect was shown to be dependent on DDT dose but this could not be extended to pH because incubation of the cells with ionophore nigericin caused toxicity. In addition to this, following light irradiation the pH and DDT sensitive system (in a dose of 1 μmol/kg of body weight) was shown to inhibit tumor growth in nude mice, due to the dual activation of the PDT effect by tumor stimuli.

Two years later the same group published a similar study on pH and DDT sensitive systems using related BODIPY-based compounds, including compound **11**. Again, they reported that the compound shown displayed the greatest photoresponse when both stimuli were present. In a highlight from the study, increasing fluorescence was observed over 9 h *in vivo* when compound **11** was injected into a HT29 human colorectal carcinoma in mice (Fig. 9, bottom), showing the cleavage-mediated activation *in vivo* and potential for these systems as dual theranostic and PDT agents.[49] Other dual pH and temperature and multi-functional responsive PSs were developed, for example, by the groups of Kim, de la Escosura and Torres, respectively.[50]

2.4 Nucleic Acid or Protein-mediated Activation of Singlet Oxygen Generation

Arguably, a more sophisticated approach to molecular level biomolecule-mediated activation is singlet oxygen release using enzymes or nucleic acids.[46]

2.4.1 Nucleic Acids. Nucleic acids are considered in the design of activatable PSs as the base pairing mechanism has proven to be accurate, dependable and has been shown to modulate singlet oxygen generation.[51] For example, there is a direct correlation between the singlet oxygen quantum yields of methylene blue and simple conjugation to nucleic acids.[52] Additionally, increased singlet oxygen quantum yields are associated with berberine and palmatine upon binding to DNA.[53] This phenomenon is PS-dependent and the opposite effect is noted for other compounds.[54] In the context of PDT, there are three major strategies used to target specific nucleic acid sequences for application in PDT.

Firstly, in the reverse hybridization strategy, two complementary sequences are each conjugated to either a quencher or PS and following interaction with the target DNA, the PS sequence is displaced from the quencher, resulting in singlet oxygen generation through a nucleotide-templated photoreaction[55] (Fig. 10a). This was demonstrated by Ogilby, Gothelf and co-workers in 2006[56a] who used pyropheophorbide-a as the PS and black hole 3 as the quencher. They reported that 85% of singlet oxygen production was regained after interaction with the target DNA sequence.[56]

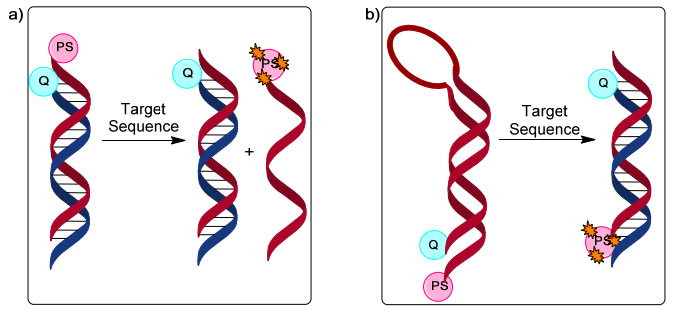

Figure 10 Diagram of a) reverse hybridization strategy and b) molecular beacon strategy.

Secondly, molecular beacons[57] also relying on controllable quenching were developed by Chen and co-workers. Interestingly, they were able to demonstrate the ability of their system to effect a PDT response *in vitro*.[58] In this system, a strand of DNA consisting of a hairpin loop specific to the target *c-raf-1* mRNA and hybridizing stem kept the PS, pyropheophorbide-a, and quencher, carotenoid, in close proximity (Fig. 10b). *C-raf-1* is associated with malignant progression of tumors.[59] Once in contact with the target, the sequence loses its hairpin and the PS and quencher are separated, restoring singlet oxygen production by 9-fold. PDT efficiencies were investigated in cells expressing *c-raf-1* and the authors noted a dose dependent response, for example with a 2 μM dose of active compound the cell viabilities were reduced to approximately 38%.[58] In a similar system, not geared towards PDT, Soper and co-workers relied on self-quenching of metallophthalocyanines, which gave 98% quenching in the inactive state.[60]

Finally, aptamers, oligonucleotides that bind to a specific target, have been investigated for application in phototherapy. In earlier work, positively charged carbon nanotubes were used to bind and efficiently quench (98%) a PS, Ce6 attached to a negatively charged thrombin aptamer. Upon interaction with its target sequence, the aptamer moves the PS away from the quencher, allowing for the generation of singlet oxygen by the PS. In this study, singlet oxygen sensor green was used to monitor singlet oxygen production and a 13-fold increase after irradiation for 10.5 min at 404 nm and the introduction of the aptamer target, thrombin, was found.[61] In another example of the Tan group's contribution to this field,[62] they developed up-conversion nanoparticles with an aptamer specific to cell membrane receptor protein tyrosine kinase 7.[63] To the aptamer was attached a guanine-rich sequence which formed G quadruplex structures and associated with a tetracationic 5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphyrin salt, to ensure it was quenched by the nanoparticles. Following excitation by NIR light, the up-conversion nanoparticles emitted visible light for imaging and activated the PS. Following light treatment at a concentration of 100 μg/mL, a moderate PDT effect was demonstrated in human T lymphoblast (CEM) cells, with cell viabilities of 43% reported. Although these systems are interesting from a design perspective, issues associated with introducing negatively charged DNA into *in vivo* models has hampered the development of the field.

Naturally, the whole area of singlet oxygen-nucleic acid interplay is more complex than can be sketched here. Nucleic acids themselves are targets of ROS attack, either directly or through control of their expression,[64] they can bind PSs,[65] be delivered and released through photochemical internalization,[66] and more.

2.4.2 Proteins. In another approach to activatable PSs, enzyme overexpression associated with many cancers and their catalytic functionality can be harnessed to facilitate targeted PDT. Specifically, enzymes can be used to mediate the cleavage of specific short chained peptides or to catalyze chemical transformations to bring about the activation of singlet oxygen generation.

In 2004, Zheng and co-workers detailed a construct consisting of a pyropheophorbide (PS) and carotenoid (quencher) linked by a self-folding caspase-3-specific peptide sequence (as illustrated for compound **12**, Fig. 11).[67] In this proof-of-concept study, they showed that the apoptosis-associated caspase-3 can activate singlet oxygen production by cleaving the specific peptide sequence. They reported that the PS-peptide-quencher unit had an 8-fold lower singlet oxygen yield compared to the PS and peptide sequence alone. Following the addition of caspase-3, there was a 4-fold increase in singlet oxygen production. Two years later they were able to demonstrate this concept *in vitro* with moderate increases in fluorescence which can be used to identify cells undergoing apoptosis after PDT treatment.[68] These early caspase-3 reliant systems, although a major stepping stone in the field, had their share of limitations. Since caspase-3 is associated with apoptosis it is mainly inactive to normal tissue and thus not present to activate the PS in cells which are healthy, this includes tumor tissue which is not undergoing cell death. In an attempt to overcome this, the same group developed singlet oxygen unquenched activatable caspase-3 systems.[69]

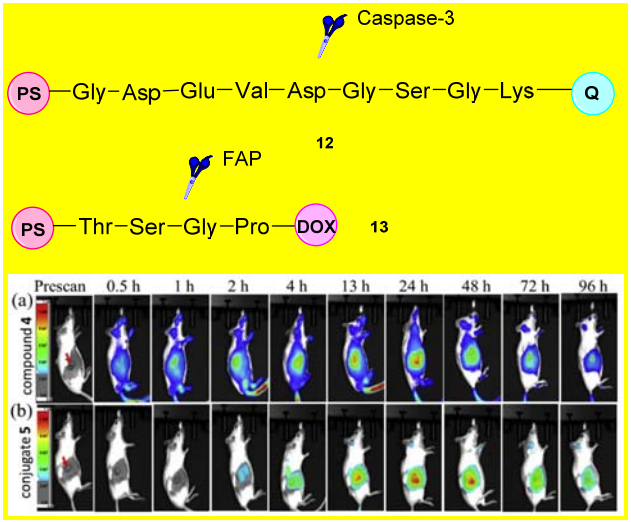

Figure 11 Top: Structures of enzyme-activated PSs (PS = pyropheophorbide a, Q = carotenoid). Bottom: *In vivo* fluorescence images of mice bearing H22 hepatocellular tumor before and after intravenous injection of (a) free PS control and (b) compound **12**. Reprinted (adapted) with permission from ref. 68. Copyright (2017) Elsevier, Paris.

In addition to the other issues, caspase-3 is not a tumor-specific target, thus, in the interim years, other proteases were utilized for cancer-specific singlet oxygen release. They included matrix

metalloproteinase-7,[70] cathepsin B,[71] and fibroblast activation protein (FAP),[72] which are all up-regulated in a variety of cancers. Some of these systems were limited by secondary structures interfering with the PS-quencher distances and thus these principles were utilized in more elaborate zipper molecular beacons.[73]

In a recent example similar to the work on GSH activatable systems (*vide infra*), Huang and co-workers married the therapeutic effects of PDT and chemotherapeutic drugs in a tumor-specific FAP responsive compound **13**.[74] The compounds consisted of a zinc(II)phthalocyanine fastened to a doxorubicin (DOX) prodrug *via* a cleavable tetrapeptide. In the tethered, inactive state the PS was quenched by aggregation-induced contact quenching and by quenching induced by the prodrug *via* a possible electron transfer process. The authors demonstrated in HepG2 cells that after incubation with FAP and illumination, compound **13** effected cytotoxicity to afford an IC_{50} value of 0.13 μM. More interestingly, nude mice bearing H22 murine hepatocellular tumors injected intravenously with compound **13** (2 μM/kg of body weight) showed fluorescence only in the tumor region, whereas a control of free PS with a peptide sequence showed full-body fluorescence, indicating that this compound can be selectively activated in target tissue (Fig. 11).[74]

As well as proteases, other enzymes have been targeted including β-galactosidase, a common gene expression marker, which can be easily introduced into cells. Nagano's work in this field aimed to develop small molecule-based PSs. They showed in 2010 that a xanthene-based PS could be selectively activated by hydrolysis mediated by β-galactosidase.[75a] A preceding paper discussed a similar system based on thiazole orange which showed a cytotoxic response to a HEK293 *lacZ*(+) cell line based on β-galactosidase action.[75b] More recently, Urano and co-workers furthered their earlier work by developing a construct based on a selenium-substituted rhodol scaffold fashioned with β-galactoside, compound **14**, which undergoes decyclization in the presence of the enzyme, and, in this form, its singlet oxygen production is dramatically increased (Fig. 12). By using *ex vivo* PDT studies on a larval *Drosophila* wing disk model they showed that photosensitization takes place in the posterior region, the only area where β-galactosidase is present, thus indicating that a small-molecule PS can be specifically activated by β-galactosidase. They also showed using dead/living stains that extensive cell toxicity was present following treatment with compound **15** but when using compound **14** there was only cell death in the posterior region, consolidating a β-galactosidase-mediated PDT mechanism.[76] A similar system targeting γ-glutamyl hydroxymethyl selenorhodamine green has also recently been described.[77]

Finally, in an example from anti-bacterial PDT, indicating that this approach can be used for a wide variety of medical treatments,[11,78] Hasan and co-workers showed that β-lactam-resistant and methicillin-resistant *Staphylococcus aureus* are susceptible to their β-lactamase enzyme activated PS, compound **16** (Fig. 12). In the quenched form, two PSs are connected *via* a β-lactam cleavable linker and once in the target bacteria the antibiotic resistance mechanism is hijacked to release and activate the PS by using the overexpressed β-lactamase to cleave the linker.[79]

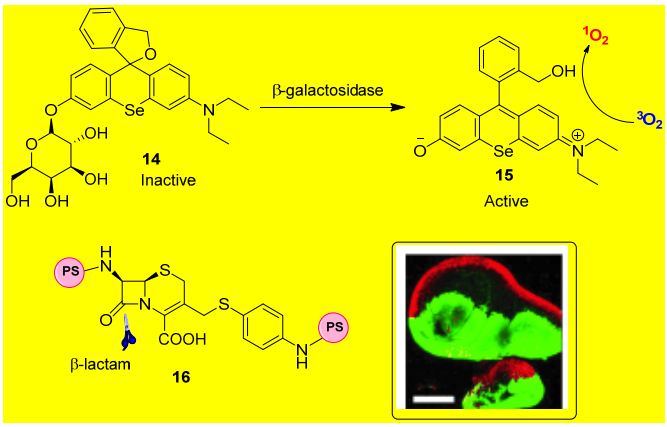

Figure 12 Structures of enzyme-activated PSs. Inset: Live/dead fluorescence staining of wing disks 3 h after treatment with **14**. CalceinAM (live cells, green) and EthD-1 (dead cells, red). Reprinted (adapted) with permission from ref. 76. Copyright (2016) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.5 Other Approaches to Activatable Singlet Oxygen Generation

2.5.1 Supramolecular Chemistry and Activatable PSs. Another approach to activatable PSs is to design assemblies that have photophysical properties which differ from their monomeric counterparts. If this modulation is related to singlet oxygen generation these systems can be used in PDT. One family in particular, the cucurbit[n]urils, has previously been used to demonstrate alterations in dye properties upon self-assembly; these properties include fluorescence,[80a] singlet oxygen generation,[80b] and cellular uptake.[80c]

In a recent study Wang *et al.* described a self-assembly system that demonstrated such an activatable PDT effect *in vivo*. The construct compromised of the PS toluidine blue conjugated to biotin, compound **17**, as a tumor targeting ligand, which can self-assemble with cucurbit[8]uril in aqueous media (Fig. 13, compound **18**). In the assembled state the PS is a dimer and its photophysical properties are quenched. Upon the introduction of a competitive binder such as *N*-terminal aromatic peptides, found in the intracellular environment, the PS is released, resulting in singlet oxygen generation. Additionally, fluorescence was shown to recover after 20 s following the introduction of a Phe-Gly-Gly tripeptide. Moreover, the cucurbit[8]uril protects the PS from enzymatic reduction, enhancing *in vivo* stability. Squamous cell carcinoma tumor-bearing nude mice were used to demonstrate this concept. Following intravenous injection of the active compound (2 mg/per kg bodyweight), fluorescence was used to access biodistribution and retention, with the highest fluorescence observed 4 h post-injection. In comparison, free toluidine blue conjugated to biotin displayed decreased tumor accumulation, indicating that the cucurbit[8]uril prevents PS degradation. The assembly also displayed better PDT efficiency compared to the control after three treatments with light (660 nm, 1 W/cm^2, 5 min).[81]

The following year a publication by Robinson-Duggon *et al.* also used cucurbit[n]urils as the basis for their self-assembly molecular

switch. They showed that when toluidine blue O was bound to cucurbit[7]uril singlet oxygen generation was enhanced due to triplet state stabilization in comparison to free toluidine blue O but singlet oxygen production was suppressed in the cucurbit[8]uril complex. This process exhibited reversibility by relying on competitive binding. The presented singlet oxygen generating switch has potential as a PDT agent but needs to be consolidated by an *in vivo* or *in vitro* demonstration.[82]

Another self-assembly process that can give differing and advantageous photophysical properties is aggregation. This concept is widely known for aggregation-induced emission, where fluorescence is increased by a variety of mechanisms including, most notably, restriction of molecular motion or charge-transfer states[83] but recently aggregation-induced singlet oxygen generation was reported by Kim *et al.*[84] The highlight of their study was two electron-poor BODIPYs fashioned with electron-rich anthrylphenylene methylanthrylphenylene groups (Fig. 13, compounds **19** and **20**), which form aggregates with red-shifted and broader absorption spectra in aqueous media. Following selective excitation, two color or red emission was observed due to the formation of a charge transfer state. They reported that this charge transfer complex decays *via* ISC thus producing singlet oxygen. The two BODIPYs showed similar cytotoxicity upon treatment of A529 cells and irradiation, with cell viabilities of 25% after 10 min and 2% after 20 min.

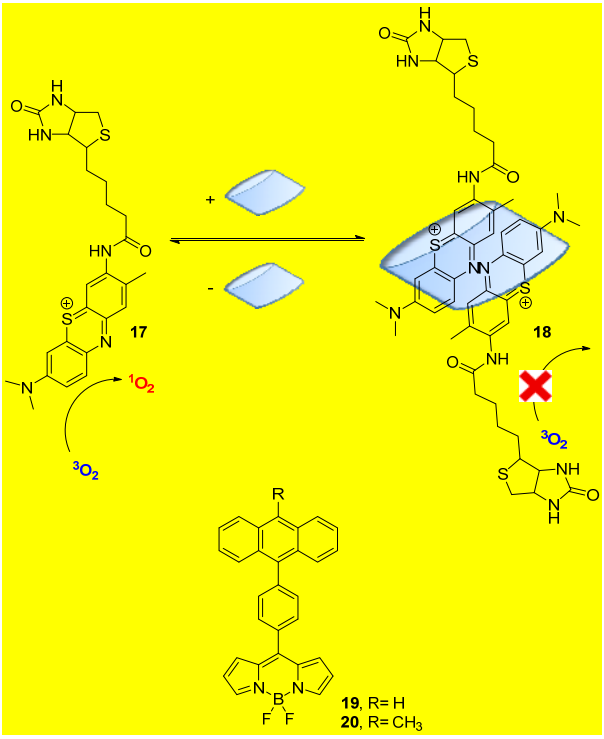

Figure 13 Schematic representation of cucurbit[8]uril complex with toluidine blue derivative, compounds **17** and **18**. Structures of aggregation-induced singlet oxygen generating compounds **19** and **20**.

PDT using self-assembled systems is an emerging field but the relative synthetic accessibility of the systems mentioned in the above paragraphs in comparison to their linker-dependent analogues described earlier is advantageous for the further development of these activatable PSs.

2.5.2 Photochromic Switches and Activatable PSs. Photochromic switches utilize light of a specific wavelength to reversibly convert from one state to another and have been applied to a variety of fields.[85,86] These molecular machines made their debut in PDT in 2014 when Hou *et al.* described a diarylethene based photochromic switch capable of turning on and off singlet oxygen generation by modulating charge transfer to ground state oxygen.[87] The photochromic switching properties of diarylethenes are well documented[86] but in their PDT system zinc tetraphenylporphyrin (TPP) was excited with 420 nm light in the presence of an open diarylethene and singlet oxygen generation could occur as there was efficient energy transfer from the excited state of the PS to ground state oxygen. However, when treated with UV light the diarylethene was converted to the closed form and energy transfer from the excited PS to the closed form of the diarylethene was more efficient than to ground state oxygen, thus stopping singlet oxygen generation (Fig. 14, compounds **21**–**23**). By alternating the irradiation between UV and visible light the switch was operational.

In the following year, Royal and co-workers described another system based on a diarylethene, namely a dimethyldihydropyrene–cyclophanediene couple, which could generate singlet oxygen without employing an external PS.[88] Similar to the previous system the closed form can be converted into the opened form under visible light irradiation and back converted under UV light treatment. They found that in the presence of oxygen and treatment with 660 nm light the closed form can act as PS and generate singlet oxygen, which can be stored on the open form and released upon heating at 35 °C for several hours. This process will be discussed in detail in the next section but they demonstrated the quantitative formation of the trapped state.

Both of the previously mentioned systems suffered from poor biocompatibility that hindered the development of the field. This was overcome by the incorporation of these photochromic switches into a metal organic framework as detailed by Zhou and co-workers.[89] They built a singlet oxygen-generating porous coordination network, which contained 1,2-bis(2-methyl-5-(pyridin-4-yl)thiophen-3-yl)cyclo-pent-1-ene as the photochromic switch and tetrakis(4-carboxyphenyl)-porphyrin as the PS. Using the decay of 1,3-diphenylisobenzofuran (DPBF) absorption effective singlet oxygen generation was demonstrated. Following visible light irradiation, DPBF decay was negligible but in the open form DPBF diminished over 150 s when using 50 mm of DPBF in 2.8 mL of acetonitrile and 1.42 mmol of the metal organic framework. Additionally, the switching ability was also shown by recording the differences in emission spectra. They followed the initial publication with *in vitro* studies using a similar Zr-metal organic framework nanoplatform incorporating a PS and photochromic switch.[90] They demonstrated the stark contrast in PDT efficiency between the off and on switch in B16 melanoma cells (Fig. 14).

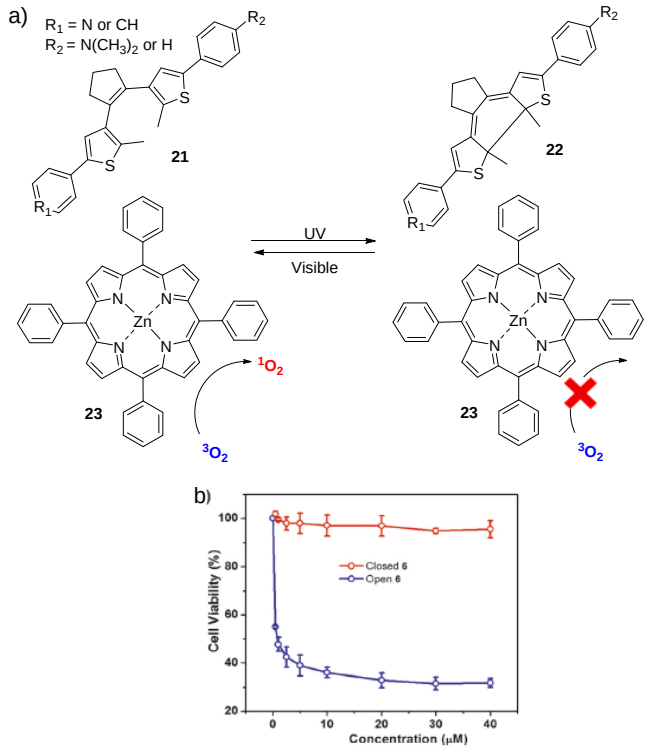

Figure 14 a) Illustrative example of a diarylethenes based photochromic switching system. b) Cell viability studies using B16 cells after PDT treatment (420 nm, 30 min irradiation) at various concentrations of photoswitching system (TPP and diarylethene-based switch in Zr-metal organic framework), showing the switchable PDT effect. Reprinted with permission from ref. 90. Copyright (2016) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

3. Delayed Release Singlet Oxygen Systems

A major roadblock to the widespread clinical application of PDT is a lack of patient compliance with treatment regimens due to the pain associated with intense and prolonged light exposure. In addition to this, blistering and itching caused by photosensitivity continues post-treatment.[8,91] On the other hand, long treatment times and strong light are required to give an effectual therapeutic response because singlet oxygen has a limited half-life in biological media.[92] Singlet oxygen decay is not easily quantified and indirect experiments are complicated by quenching or side photoreactions. However, it is accepted that the process is dependent on both temperature and solvent effects and is in the μs range for water. In a recent publication, Ogilby and co-workers used time-resolved phosphorescence experiments, a direct method, to report the lifetime of singlet oxygen as 3.45 μs at 25 °C in water.[92c] A practical solution to these issues is rest periods between successive light exposures, with the added advantage of allowing tissue oxygen levels to recover from the PDT. However, as singlet oxygen generation only occurs in the presence of light, the therapeutic effect is fragmented.[18] Thus, designing systems that could generate, store and slowly release singlet oxygen in cells after irradiation has been terminated, would be of great benefit to an enhanced PDT effect in the clinical setting.[93]

3.1 Chemically 'Storing' and Releasing Singlet Oxygen

Like many of the intricate systems being investigated in modern scientific fields, the underlying principle of slow release singlet oxygen or reversible binding of oxygen[94] has historic origins and was first described by Dufraisse, Moureu and Dean in 1926.[95] In this pioneering research, the authors synthesized rubene (naphthacene, **24**) and noticed a color change in an aerated solution following irradiation with light (Fig. 15). They isolated a compound containing a peroxide that decomposed upon heating to between 110 °C and 150 °C. This decomposition yielded the parent hydrocarbon and an oxygen species. In fact, what occurred was a reversible [4+2] cycloaddition (Diels-Alder type reaction) involving oxygen and the aromatic system, compound **24** to form an endoperoxide, compound **25**.[96] Crucially, in 1967 Wasserman and Scheffer proved that the released oxygen species is in the singlet state.[97]

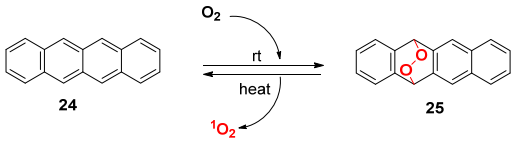

Figure 15 The reversible Diels-Alder reaction between rubene and oxygen, first demonstrated by Dufraisse, Moureu and Dean in 1926.

Since this early work, other aromatic molecules capable of this transformation have been identified, including naphthalene and anthracene derivatives, each having their own distinct decay properties.[93,94] The endoperoxide half-life is dependent on temperature and stability. Factors affecting endoperoxide stability include the size of the aromatic system, steric effects, solvent effects and substitution patterns. In the next chapter of this story, systems that incorporate these endoperoxide forming units onto a PS are described. These constructs allow for the generation, storage and trapping of singlet oxygen, in the same molecule. There are numerous examples[88,98] but the idea was first demonstrated by Rihter *et al.* in 1993 with 1,6,10,15,19,24,28,33-octabutoxynaphthalocyanine (Fig. 16, compound **26**). Irradiation of PS **26** in benzene generated singlet oxygen from the triplet excited state of the phthalocyanine core, which was subsequently added to the aromatic periphery, resulting in the formation of the endoperoxide bearing compound **27**. Decomposition of compound **27** to the parent phthalocyanine, **26**, occurred thermally or photochemically under visible light.[99] It should also be noted that homolysis of the endoperoxide bond may occur which leads to rearrangement reactions and decomposition of the aromatic unit, preventing the reversible cycloadditions.[94] However, in a recent study by our own group, we showed that these rearrangement reactions could generate a fluorgenic response that compliments the PDT effect. We reported non-halogenated, meso-substituted BODIPYs, including compound **28**, that were capable of singlet oxygen generation *via* charge separated states and a photo-induced electron transfer process (Fig. 17). A portion of the generated singlet oxygen performed a cycloaddition across the anthracene unit, as seen for the other slow release systems. The subsequent

rearrangement reaction yielded fluorogenic photoproducts. Thus, these systems can generate singlet oxygen without relying on optimizing ISC and induce apoptosis with LD_{50} values of 4 mM. Additionally, the singlet oxygen production can be detected by responsive fluorescence leading to the visualization of oxidative stress *in vitro* (Fig. 17).[100]

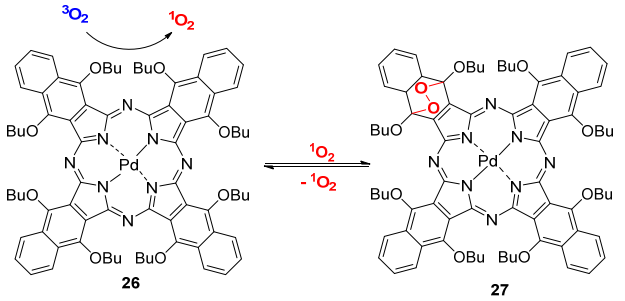

Figure 16 Structure of a naphthalocyanine system capable of singlet oxygen generation, trapping and storage.

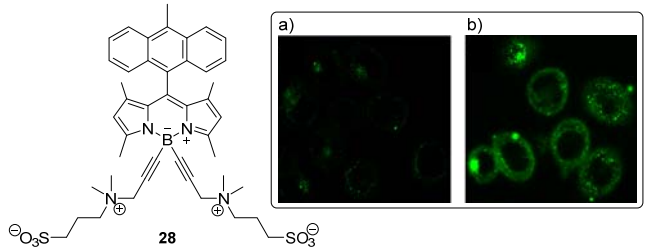

Figure 17 Left: Structure of a BODIPY-anthracene dyad capable of a singlet oxygen-mediated fluorescence response. Right: Confocal microscopy images of cells incubated with 1 μM of compound **28** for (a) 2.5 min and (b) 5 min after irradiation (400–700 nm, 23.8 mW cm^{-2}). Reprinted with permission from ref. 100. Copyright (2017) American Chemical Society.

Finally, in a recent example of decay property studies, pyridylanthracenes were investigated by Fudickar and Linker.[101] The authors synthesized the 9,10 endoperoxides of compounds **29–31**. They found that the *para* and *meta* isomers decomposed quantitatively at 90 °C in under 24 h to yield the parent hydrocarbon, however, the *ortho* isomer had to be heated to 135 °C to achieve the same effect. Additionally, the formation of a rearrangement product, as well as the parent hydrocarbon in a 62% yield, was noted. Their results were explained by steric effects. For the *meta* and *para* isomers, upon heating, the steric influence exerted by the two aromatic CH hydrogens (Fig. 18, red) on the adjacent CO bond of the endoperoxide caused easier homolysis. In contrast, the presence of the nitrogen atom in the *ortho* position did not allow for the same interaction and cleavage of the endoperoxide OO bond could compete, leading to the rearrangement product, illustrating how subtle steric and solvent effects can modulate the endoperoxide decomposition.[102]

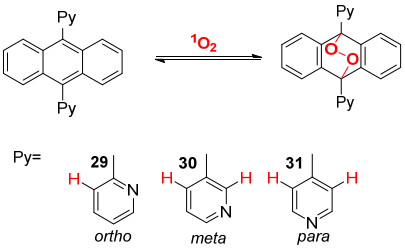

Figure 18 Compounds **29–31**: *Ortho*-, *meta*- and *para*-pyridylanthracenes synthesized by Fudickar and Linker for endoperoxide decomposition studies.

3.2 *In Vitro* Slow Release Singlet Oxygen Studies

As previously mentioned, in the context of PDT, compounds capable of these [4+2] reversible cycloadditions can be utilized to trap and store singlet oxygen and then thermally release it under dark conditions. However, in order to study these compounds *in vitro*, to assess their potential as PS for PDT, two design parameters had to be adjusted, namely (i) the introduction of water-soluble moieties or incorporation of delivery systems and (ii) structural modifications to ensure endoperoxide decomposition and subsequent release of singlet oxygen occurred at medically relevant temperatures.

Pyridone units have become an 'oxygen storage unit of choice' for photomedicine related slow release singlet oxygen because in optimized systems they meet these design requirements and their inherent water-solubility facilitates *in vitro* studies. Firstly, they have been used in photomedicine for the regeneration of anoxic tissue, a major contributor to necrosis during grafting and wound healing.[103] Benz *et al.* studied a library of substituted pyridone based endoperoxides and found that at 37 °C in water they had varying half-lives and retro Diels-Alder yields. The endoperoxides were formed by treating the parent pyridone with 5,10,15,20-tetraphenylporphyrin and irradiation with a sodium lamp at 0 °C. A highlight of the study was a methylated pyridone endoperoxide with a half-life of 8.5 h and retro Diels-Alder yield of 78%, proving that they can undergo singlet oxygen release at body temperature. They used this compound (10 μg/mL) to carry out *in vitro* studies and, under optimized conditions (using 100 μg/mL of vitamin C as a singlet oxygen quencher to mitigate the toxic effects of singlet oxygen), observed growth in oxygen-deficient cells. In fact, 3T3 fibroblast and rat smooth muscle cells showed increased growth rates under anoxic conditions for one day and for four days, respectively.[103]

In 2013, Changtong *et al.* reported 2-pyridone moieties which were conjugated to a porphyrin. This single construct was capable of singlet oxygen generation photosensitized by the porphyrin, storage by the pyridone units and subsequent thermal release. They showed that endoperoxide decay between 25 °C and 50 °C was reversible and followed first order kinetics. Interestingly, the endoperoxide could be generated at room temperature and singlet oxygen release continued for several hours post irradiation.[98b]

Although the previous work highlighted these pyridone-based compounds as potential PDT agents they were limited by poor water-solubility. In 2016, Akkaya and co-workers validated the

concept *in vitro* using halogenated distyryl-substituted BODIPY fashioned with these 2-pyridone moieties that were delivered to the cells using micelles.[104] BODIPY **32** was transformed into the endoperoxide using light irradiation in the presence of methylene blue (Fig. 19). The efficiency of the native BODIPY and the endoperoxide bearing analogue were compared in cytotoxicity studies. HeLa cells were treated with varying (9 nM–2.5 μM) concentrations of each compound and irradiated with red light for 10 min every hour for 24 h. These light-dark cycles were followed by 24 h of incubation in the dark. Significantly more cytotoxicity was observed for the endoperoxide bearing BODIPY as opposed to the parent pyridone functionalized BODIPY, compound **32**. The release of singlet oxygen from the endoperoxide using DPBF as a singlet oxygen trap was studied as well. Irradiation of the parent halogenated BODIPY in a DMSO solution resulted in singlet oxygen generation and a decrease in DPBF absorbance. This was followed by a dark stage in which DPBF absorbance proceeded to decrease. The pattern continued over several light-dark cycles, in essence showing that singlet oxygen is produced from the PS but also released from the endoperoxides under dark conditions making these compounds applicable to fractional PDT.

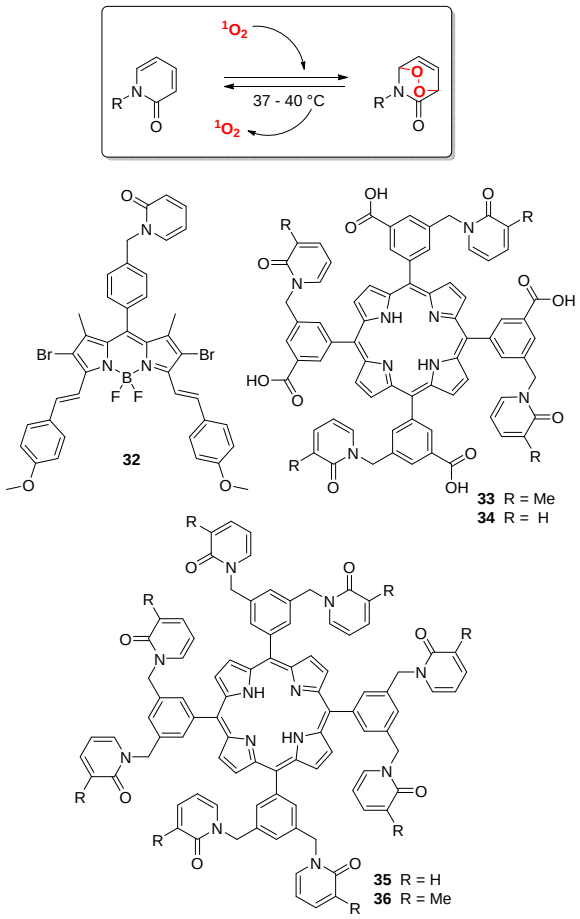

Figure 19 Structures of pyridone-based slow release PDT PSs.

Their work was followed by another *in vitro* study on singlet oxygen generation, trapping and delayed release. Therein,[105] we reported water-soluble porphyrins based on octa- and tetra-pyridone substituted porphyrins (compounds **33–36**). Our PS designs relied on the inherent solubility of the pyridone units and water-soluble carboxylic acid moieties. In addition to this, we investigated how the substitution pattern impacted cytotoxicity efficiencies. The PDT effect and delayed singlet oxygen generation were demonstrated in HT-29 cells that were irradiated with visible light in the presence of the PS and then returned to the incubator for 24 h, where MTT assays were conducted. At a concentration of 200 μM, an 80% reduction in cell viability of the octa-methylpyridone substituted porphyrin **36**, in comparison to 23% for the unsubstituted analogue **35**, was shown. This observation indicated that the influence of the substitution pattern has an effect on endoperoxide stability and a resultant slower release of singlet oxygen post-irradiation, which translated to a more effective therapeutic response. The effect was not as pronounced for the tetrasubstituted compounds **33** and **34**.

In addition to the pyridone approach, thermolysis of endoperoxide gold nanoparticles is an option for delayed singlet oxygen release *in vitro*. In earlier work, gold nanoparticles functionalized with anthracene derivatives bearing endoperoxides were shown to release singlet oxygen for up to several weeks upon photothermal or magnetic activation.[106]

Later Kolemen *et al*. demonstrated that singlet oxygen release could be mediated by NIR light *in vitro*.[107] In their work, they described an anthracene-based endoperoxide linked to a gold nanorod (Fig. 20, compound **37**), with each nanorod carrying 6.5 × 10^9 anthracene derivatives. They showed that when a DMSO solution of the nanoconstruct and a singlet oxygen scavenger, in this case, DPBF, was either irradiated with a laser at 830 nm or gradually heated from 25 °C to 100 °C there was a decrease in DPBF absorption due to singlet oxygen release from the endoperoxide. In contrast, a control of only the gold nanorods gave no response. To demonstrate this *in vitro* HeLa cells were treated with the functionalized nanorod and incubated for 24 h. The cells were then exposed to Cyto-ID, to detect oxidative stress and 808 nm NIR light (2.0 W/cm^2) for 10 min. After 24 h, a reduction in cell viability was confirmed by MTT assays. Although it was demonstrated that chemical generation of singlet oxygen can cause apoptosis *in vitro* using light-induced thermolysis, optimizations of the light doses would make these more appropriate for the clinical setting.

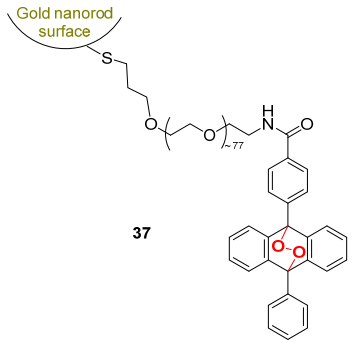

Figure 20 Structure of photothermally activated slow release PDT PS.

4. Singlet Oxygen Responsive Systems

Classic PDT is characterized by the fierce reactivity of singlet oxygen damaging target tissue in an uncontrollable and erratic manner but, at low concentration, ROS modulate a variety of important biological functions including signal transduction pathways and protein structures.[108] Similarly, singlet oxygen and ROS can be exploited to effect a targeted chemical change through cleavage of sensitive units or modulating a chemical transformation. An example of the latter was described by Cosa and co-workers.[109] They showed that a BODIPY based PS conjugated to a singlet oxygen trapping unit could be activated by ROS to produce singlet oxygen with a 40-fold amplification *via* the oxidation of the trapping unit, which terminated the PET process and restored effective ISC. They demonstrated that ROS stressed Gram-negative *Escherichia coli* had a greater PDT response in comparison to the regular cells. Additionally, when singlet oxygen generation is initiated by the interaction between a PS and light the systems response can be activated on demand, these include the activation of a PS by ceasing quenching effects or the release of cargo from a delivery system. If the response is the release of more PSs or other active entities a cascade event is established. The major advantage of these systems is their ability to be locally activated within a specific timeframe, often leading to a large-scale release of PS.

4.1 Singlet Oxygen Sensitive Linkers

The driving force behind the development of singlet oxygen sensitive linkers is the optimization of older cleavable linkers, which rely on high energy UV-light.[110] The issue being that UV-light itself can inflict cellular death independently of the PDT effect. In addition, it suffers from poor tissue penetration, which limits PDT treatments to exposed surfaces.[111] Thus, designing systems that can be cleaved with light in the visible or near IR region is advantageous. There have been numerous reports of ROS sensitive systems including selenium-, boron-, and thioketal-based linkers[112] but for the purpose of this perspective only newer systems that directly interact with singlet oxygen will be considered.

4.1.1 Vinyl Heteroatom-based Linkers. As discussed, singlet oxygen can operate as an activating agent by cleaving a responsive linker. In an early example, Breslow and co-workers described cyclodextrin dimers that were joined by a vinyl dithioester-based linkage.[113] The cyclodextrin moieties solubilize phthalocyanine-based PSs by using the chelating effect to coordinate them in their hydrophobic interior while presenting their hydrophilic exterior to the biological media (*e.g.*, Fig. 21, compound **38**). Following illumination with light from a halogen lamp (50 W), singlet oxygen generated by the PSs interacts with the vinyl dithioester-based linkage and separation of the cyclodextrin monomers occurs. Mechanistically, singlet oxygen adds to an electron-rich alkene to form the dioxetane ring intermediate, which then decomposes to two aldehydes, compound **40**. The introduction of heteroatoms adjacent to the alkene moiety increases the relative reactivity of the linkage. Separation of the monomers results in the termination of the chelating effect and binding of the fragment products to the cyclodextrin. This in turn causes precipitation of the PS in water, which can be directed to a small area by light. Progress of the reaction was monitored by NMR and indicated the disappearance of alkene and appearance of aldehyde protons, proving that cleavage of the double bond underpinned the photochemical process. They suggested that this principle could be used to locally concentrate PSs for PDT.

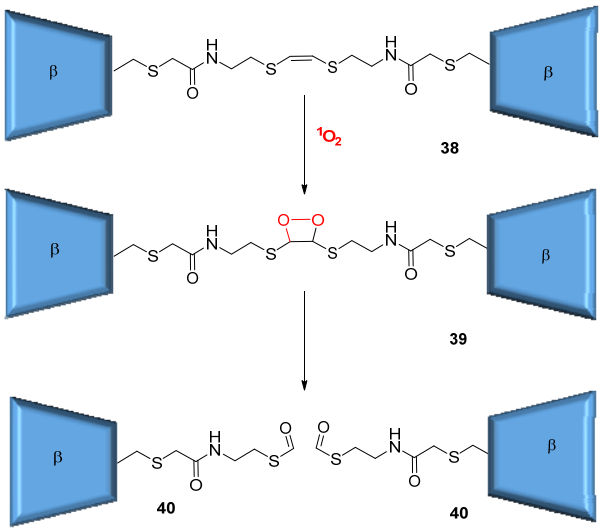

Figure 21 Diagram of cyclodextrin PS loaded dimers showing the mechanism of singlet oxygen-mediated alkene cleavage.

In a 2008 proof-of-concept study, Jiang and Dolphin showed that targeted release of a prodrug under visible light irradiation is possible. In their system, a PS and prodrug were joined by an olefin linkage, similar to the previously described system, but they used oxygen as the heteroatom in their di-*O*-vinyl ether linkage.[114] They prepared a library using combinations of tetraphenylporphyrin monoacid and verteporfin as the PS and ester or amide drug mimics, ibuprofen or naproxen derivatives as the pro-drugs, in both the *E* and *Z* forms. The compounds were irradiated with visible light at room temperature and an internal standard was used to prove that the highly efficient cleavage was solvent independent. The involvement of singlet oxygen in the cleavage process was confirmed using a tetraphenylporphyrin-drug mimic complex. In deuterated acetone photocleavage of the complex occurred in 8 min at a concentration of 4 nM, however, in the presence of a scavenger, 1,4-diazabicyclo[2.2.2]octane (DABCO) the reaction time was increased to 60 min at a concentration of 5 mM.

In the following years, the site-specific delivery of a PS was ingeniously developed by Greer's group using an optical fiber to supply light for the cleavage of the singlet oxygen sensitive linker, which resulted in the detachment of the PS from the optical fiber tip.[115] The concept was demonstrated *in vitro* together with the Hasan group.[116] In one study, their optimized system consisted of a pheophorbide PS and surface-bound nanofluorosilane, which prevents the PS from associating with the surface and induces quenching (Fig. 22, compound **41**). In their cytotoxicity studies,

ovarian cancer cell films were irradiated for 1 h and cell viability decreased by approximately 60% with the control (a non-functionalized tip) displaying a 4% reduction. Recently, the Greer group conducted an investigation into the advantages of using fluorinated tips for PDT, showing its greater resistance to biofouling.[117] These works are indicative of how the molecular engineering of singlet oxygen generating molecules can bridge the realm to chirurgical applications, *e.g.*, in the form of molecular scalpels.[118]

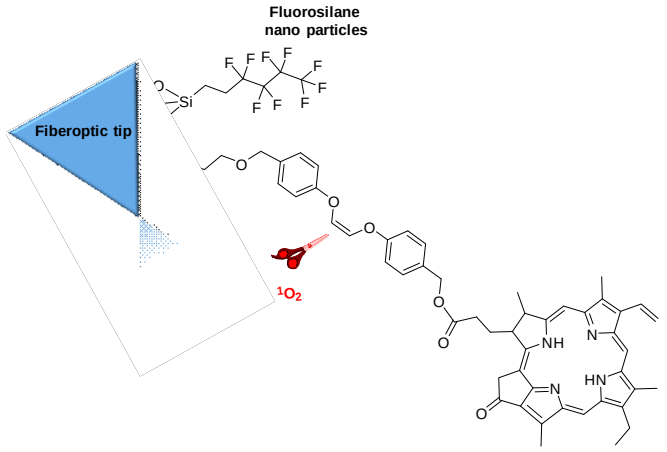

Figure 22 Fiberoptic tip with cleavable PS unit.

To expand the applicability of these systems, drug delivery constructs have been developed which use these photocleavable vinyl heteroatom linkages as the underlying principle to activate drug release. Over the past few years, silica and polymer-based drug delivery systems have been developed.

Although other publications also demonstrate the use of mesoporous silica nanoparticles (MSNP) and these linkers in delivery systems,[119] the concept can be exemplified by a study from Yang *et al*.[120] In their work they incorporated Ce6, as a singlet oxygen generator, into the silica matrix of their MS nanorods. The nanorods were loaded with either a chemotherapeutic drug, DOX, or small nanoparticles such as *cis*-Pt(IV) pro-drug conjugated to a dendrimer. The pores of the nanorods were blocked with singlet oxygen responsive bis(alkylthio)alkene bearing bovine serum albumin. PEG was then conjugated to the surface to increase water-solubility and biostability. To assess the PDT effect, 4T1 cells murine breast cancer cell were incubated with DOX-loaded nanorods for 12 h and then irradiated with 660 nm light for 1 h (5 mW/cm^2). MTT assays after 24 h indicated that there was a significant decrease in cell viability compared to the uncleavable analogue. Additionally, using unloaded nanorod showed only a marginal viability decrease, indicating that the combinational PDT and chemotherapy was more effective than PDT alone. The unloaded nanorods were intravenously injected into 4T1 tumor-bearing mice and fluorescence imaging of the PS was used to visualize localization. As shown in Fig. 23, the optimum tumor accumulation of the nanorod occurred after 8 h. When using the DOX-loaded nanorod, *in vivo* experiments showed slowed tumor growth, with a prominent synergistic effect.

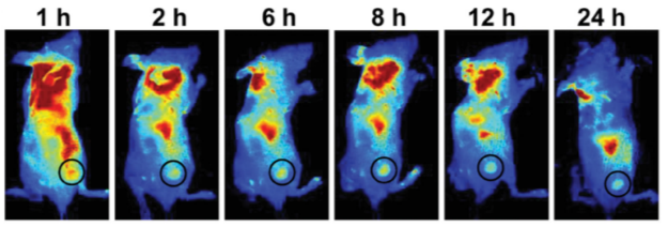

Figure 23 *In vivo* fluorescence images of 4T1 tumor-bearing mice intravenously injection with DOX-loaded nanorod with photocleavable linkage and PEG units. Reprinted (adapted) with permission from ref. 120. Copyright (2016) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

In another study, which employed combination therapy to enhance the therapeutic effect, micelles comprising of hydrophilic PEG units linked to a hydrophobic, biodegradable polycaprolactone block, *via* a vinyldithioether linker, were used. Following light irradiation and cleavage, the nanostructure's integrity is disrupted and the loaded drug is released. Micelles co-loaded with DOX and Ce6 showed the greatest cytotoxicity *in vitro* with cell viability less than 40% at a concentration of 0.25 μg/mL.[121]

This year, Liu and co-workers employed a co-loaded nanoparticle to effect a singlet oxygen-mediated therapeutic response. The nanoparticle consisting of a coordinating polymer based on hafnium ions and bis-(alkylthio)alkene moieties as singlet oxygen sensitive linkers and was loaded with both DOX and Ce6. The exterior of the nanoparticle was fashioned with a water-soluble layer using DOPA, DPPC and PEG units (Fig. 24). The constructs were effectively activated by 600 nm light, which induced singlet oxygen generation by the PS and subsequent release of more PS and DOX. In 4T1 cells the Ce6 and DOX-loaded nanosystems proved more effective at cellular toxicity in comparison to singly loaded structures. Interestingly, the Ce6-loaded system was also superior to free Ce6, indicating that the delivery system enhances cell uptake. This effect was also demonstrated *in vivo*.[122]

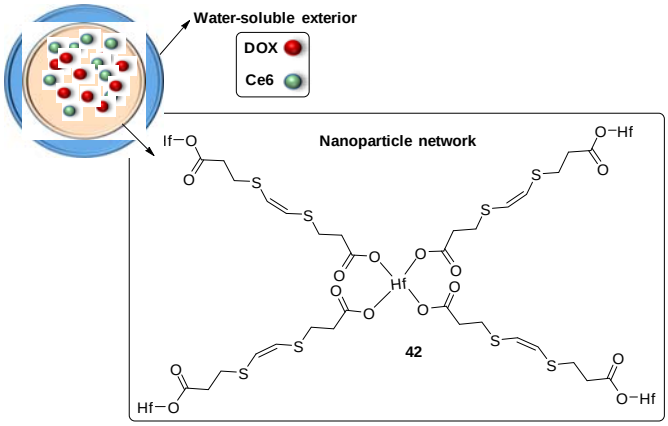

Figure 24 Illustration of PS- and DOX-loaded singlet oxygen responsive delivery systems.

4.1.2 Aminoacrylate-based Linkers. Considering the limited synthetic accessibility of vinyl diethers and dithioethers another class of singlet oxygen responsive linkers emerged. These newer

linkers, described by Bio *et al.*, are based on aminoacrylates and were coined 'photo-unclick chemistry' products.[123] The cleavage reaction follows a similar mechanism to the previous work, where singlet oxygen adds to a double bond, followed by ring opening and possible conversion to the corresponding alcohol. A library of compounds incorporating thio-acrylates, oxy-acrylates, amino-acrylthioates and aminoacrylamides was synthesized with minimal synthetic effort and yields between 80-90% and the aminoacrylate linker was identified as the optimum system for photochemical cleavage. To prove the applicability of these systems, especially their efficiency in water, they designed a FRET pair connected by the aminoacrylate linker (see Fig. 25, compound **43**). In the inactive pair, rhodamine B is quenched by a dithiaporphyrin PS but post-irradiation, at 690 nm by a diode laser at 200 mW cm^{-2}, 100% cleavage was achieved after 10 min and emission increased 8-fold. This proof-of-concept study did not include singlet oxygen or PDT investigations but did lay the foundations for the application of these linkers in PDT.

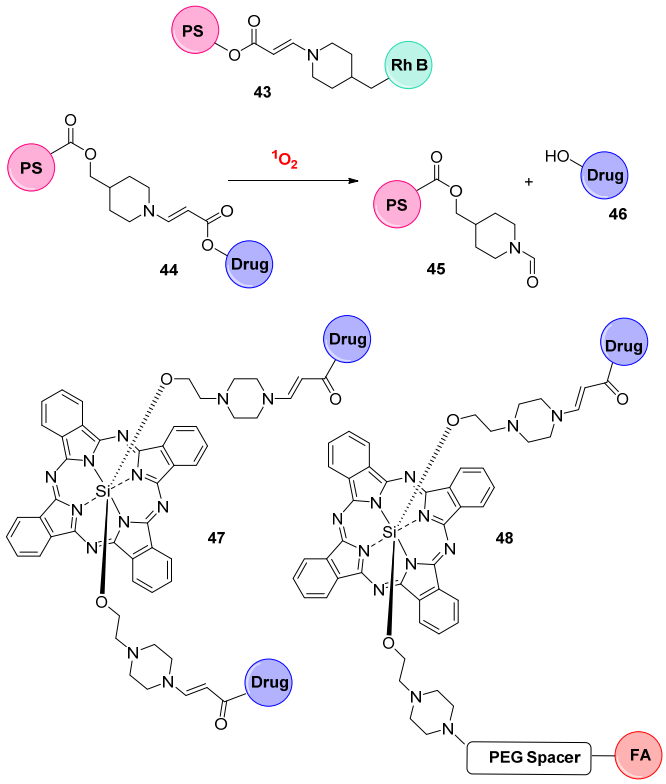

Figure 25 Structures incorporating aminoacrylate-based linkers.

In the series of publications that followed, the You group verified their findings *in vitro* and *in vivo* and the combinational PDT and targeted chemotherapy effect was investigated. They prepared a combretastatin A-4, a tubulin binder and dithiaporphyrin aminoacrylate linked construct **44**, which was activated *in vitro* with a 690 nm diode laser, as described before.[124] Post cleavage, the drug was released with a 6-fold increase in the IC_{50} values. Additionally, bystander toxicity of neighboring cells was noted and attributed to the chemotherapeutic drug, not PDT as the half-life of singlet oxygen is too short to diffuse into other cells. Optical imaging was used to visualize the effect *in vivo* and the results indicated that toxicity of the cleavable system was much stronger ($P < 0.05$) than an uncleavable analogue, indicating that photocleavage is paramount to the therapeutic effect.

This study was complemented with compounds containing other chemotherapeutic drugs, SN-38 or coumarin and noted that a construct containing coumarin released 99% of the drug in MCF-7 cells after irradiation for 30 min.[125] In 2014, they designed a phthalocyanine-based PS that could coordinate two combretastatin A-4 units *via* the cleavable linker.[126] The phthalocyanine is both a singlet oxygen generator and fluorescent agent and thus it was used to conduct optical imaging in mice, following administration in micelles. The result of the *in vivo* tests showed increased anti-tumor activity for compound **47** in comparison to their earlier constructs with only one drug linked. After 24 h illumination, the tumors were significantly reduced. Constructs bearing paclitaxel as the photochemical drug were also investigated.[127]

In an attempt to further enhance the therapeutic effects of these systems tumor-specific targeting units were incorporated into the compounds. Building on the design of compound **47**, a folic acid (FA) group was used to guide the compound to tumor tissue, relying on the folic acid receptor overexpression in cancer cells. The other arm bound to the PS was appended with the chemotherapeutic drug, combretastatin A-4 phosphate (see Fig. 25, compound **48**). The best response to FR-positive colon 26 tumor cells was from the compounds with the longest PEG spacers as they showed increased water-solubility and uptake. In addition, cell viability studies in colon 26 tumor cells showed significant toxicity after a 30 min treatment with laser light (690 nm) at 5.6 mW/cm^2. The IC_{50} values were reported to be between 16.5 nM and 48.5 nM and correlated directly with the length of the spacer. The constructs containing FA were more potent than the control without FA, indicating that the effects were FA mediated. The enhanced efficiency was in agreement with *in vivo* studies and visualized by optical imaging.[128]

These aminoacrylate-based linkers were also used in drug delivery systems by Palmans, Meijer and co-workers in 2015.[129] Their single chain polymeric nanoparticles had PSs attached to the hydrophobic interior to induce water-solubility. In addition, prodrugs were conjugated *via* photolabile aminoacrylates linkers. Following irradiation of the compounds with a halogen lamp, the prodrug, coumarin, was cleaved and the PS was activated. An increase in fluorescence over 200 min was observed for these compounds but for analogues without porphyrins to generate singlet oxygen there was a minimal change in fluorescence. Using the same linkers, Martínez-Carmona *et al.* capped MSNPs with porphyrin-based PSs and showed that 18 h after 30 min of irradiation 99% of the internal cargo was released.[130]

4.1.3 Dialkyloxyanthracene as Singlet Oxygen Sensitive Linker. We have already discussed a singlet oxygen responsive system that is mediated by endoperoxide formation across an aromatic unit,[95,96,97] namely the BODIPY-anthracene dyads described by Filatov *et al.*, which respond to singlet oxygen to yield fluorogenic photoproducts.[100] This fluorogenic response aids PDT by allowing

simultaneous imaging of affected tissue. In another example of an endoperoxide forming aromatic compound with potential applications in PDT, a $^1O_2$-sensitive linker is cleaved to effect a response, Fig. 26, compound **49**.[131] In their publication, Mokhir and co-workers detailed a library of phosphoramidites which contained the linker and other molecular fragments such as nucleosides, fluorogenic agents and a cholesteryl derivative. In the presence of singlet oxygen, the linker is cleaved firstly by endoperoxide formation *via* a cycloaddition followed by decomposition *via* a proton-catalyzed reaction with the release of the attached units. They applied this concept in the design of a singlet oxygen probe for live mammalian cells. The attached fluorophores are quenched when the linker is intact and activated upon cleavage. They reported that the fluorescence intensity of the probe is related to the concentration of singlet oxygen within the cells and responsive to both photogenerated singlet oxygen and that resulting from the decomposition of the endoperoxide, 3,30-(1,4-naphthylidene)dipropionate. In a similar manner, red-light-activated "caged" oligodeoxyribonucleotides were also detailed. Although this linker was not applied directly to a PDT agent it does show potential for a dual functional PDT agent similar to those described before or as an agent to detect effective PDT therapy in living cells.

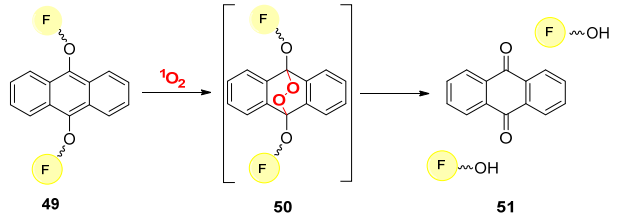

Figure 26 Singlet oxygen responsive linker with intermediate endoperoxide formation.

4.2 Singlet Oxygen Responsive Imidazole to Urea Transformations

Singlet oxygen interactions can also bring about rearrangement reactions that initiate a molecular or structural change. Imidazole has previously been employed as a singlet oxygen scavenger to monitor singlet oxygen production. Following interaction with the substrate *via* a cycloaddition, a rearrangement occurs, which affords urea as the product.[132]

This transformation was recently used to construct a nanoparticle delivery system for Ce6.[133] In this system, imidazole-based monomers were cross-linked by coordination with Zn^{2+} ions to form the micelles which were loaded with Ce6. Upon light irradiation, the generated singlet oxygen mediated the transformation to urea, which cannot coordinate with Zn^{2+} ions (Fig. 27). Additionally, the urea monomers are more hydrophilic than their imidazole counterparts and can incorporate water in an extensive hydrogen bonding network, resulting in the expansion of the nanoparticle and triggered release of the PS. The PS release causes an immediate enhancement of the PDT effect through increased singlet oxygen levels. Light-dependent drug release and cellular uptake *via* endocytosis were demonstrated *in vitro*. In addition, 4T1 cell cytotoxicity studies indicated that although free Ce6 showed the highest toxicity, responsive micellular delivery system consistently performed better over various concentrations when compared to the non-singlet oxygen responsive control, for example at 2 μg/mL the percentage cell viabilities were 60% versus 80% respectively. An *in vivo* study with mice bearing the same cancer line demonstrated that the imidazole-based micelles were more efficient than free Ce6 and the control micelle with significantly protracted tumor growth inhibition; this was attributed to the high loading capacity of the PS and light-dependent targeted Ce6 release.

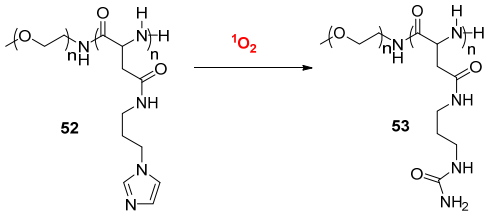

Figure 27 Monomer of singlet oxygen responsive imidazole-based micelles.

5. Conclusions and Outlook

To pay homage to the title film, similarities may be drawn between singlet oxygen and the mysteriousness of Blondie, the complexity of Tuco and the unforgiving ruthlessness of Angel Eyes. From this plot summary, it is evident that these different characteristics can be harnessed to operate in various roles to afford functionally and mechanistically different PDT agents, to varying success. Firstly, the concept of activatable PSs has been around for two decades and has proven itself, both *in vivo* and *in vitro*. Unfortunately, like many other PS designs, the synthetic complexity of some of these systems has resulted in a bottleneck in the translational pipeline to clinical application. In contrast, the application of slow release singlet oxygen systems in PDT has only recently gained attention and more effort is required to make this a viable option by demonstrating the concept *in vivo*. Finally, singlet oxygen responsive constructs display considerable potential especially as delivery systems with many successful studies *in vivo*.

A skilled actor, like singlet oxygen, has the ability to take on a variety of roles (indeed we might not need three different actors as S. Leone did; singlet oxygen is ambivalent enough in its acting abilities that a suitable design script and director at the molecular level can bring out the best of it) gracing several genres and thus its performance is not just limited to classic PDT PSs. In fact, other clinical applications of these agents are feasible including carrier systems for hypoxic tissue[134] and biosensors. In a recent example of a novel sensing strategy Trashin *et al.* used a perfluorinated Zn phthalocyanine to generate singlet oxygen that subsequently oxidized analytes, the products of which can be detected electrochemically.[135] Combination therapy has also come to the foreground of the PDT stage but with it questions arising from co-administration need to be further addressed and understood.[136]

In addition to the concepts described herein, there are several other emerging scripts that deal with the controlled generation and/or use of ROS and singlet oxygen. In all eventuality most of the examples highlighted here will never enter clinical practice and be

lost to the editing room floor. However, to identify the next 'blockbuster' PDT drug casting calls will have to continue with a turnover of new approaches to PSs and delivery system design, emerging applications in green chemistry or the security field to counteract CBRN agents, and more,[137] each with singlet oxygen playing the starring role. For example, continuing efforts into the modulation of the photochemical properties of PSs show great prospects and can be exemplified by Hosseini and co-workers' recent description of the correlation between axial ligands of phosphorus(V)porphyrins and singlet oxygen production.[138] Their work demonstrated that in the absence of an axial ligand the photosensitizing properties of the porphyrins were effective but when axial ligands were introduced charge transfer processes were activated resulting in quenching of fluorescence and singlet oxygen generation.

Another of these newer concepts we expect to feature predominately in the future of controlled singlet oxygen production is optogenetics. In this approach, proteins are genetically encoded to produce ROS, following interaction with light. The advantages of which are cell delivery using a DNA fragment coded to express the protein in a predetermined cellular location, thus offering subcellular control of cytotoxicity. An early member of the optogenetics family was the superoxide radical anion generating KillerRed.[139] Although KillerRed displayed potential for application in PDT,[140] a system with a higher singlet oxygen quantum yield would be more effectual.

Since then, other encodable proteins have been engineered to produce singlet oxygen more selectively, with reported increasing quantum yields (Φ_Δ). These include MiniSOG (Φ_Δ = 0.03),[141] TagRFP (Φ_Δ = 0.004),[142] Pp2FbFP L30M (Φ_Δ = 0.09)[143] and singlet oxygen photosensitizing protein (SOPP) (Φ_Δ = 0.25).[144] Excitingly, in a recent study by Ogilby and co-workers, SOPP has been optimized by modulating the protein scaffold to produce singlet oxygen with a quantum yield of 0.60.[145] Coupling the established success of optogenetics in many areas such as chromophore-assisted light inactivation and mutation studies[146] and the rapid advancement of the field in the last few years, future therapeutic applications can be envisioned, setting the stage for an exciting new chapter in the PDT story.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 764837 and was supported by grants from Science Foundation Ireland (IvP 13/IA/1894) and the Irish Research Council (GOIPG/2016/1250).

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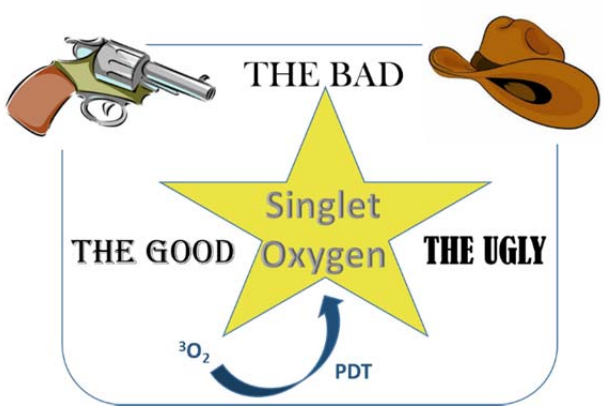
ToC graphic



ToC text entry

To afford effective photosensitizers and delivery systems for photodynamic therapy their design phase must consider all aspects of singlet oxygen. This perspective highlights that progression of this field is dependent on the control and modulation of its star, singlet oxygen.