Activatable Photosensitizers for Imaging and Therapy

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1. Introduction

1.1. Photodynamic Therapy

Photodynamic therapy (PDT) is a minimally invasive treatment that destroys target cells in the presence of oxygen when light irradiates a photosensitizer, generating highly reactive singlet oxygen.[1] Singlet oxygen then attacks cellular targets, causing destruction through direct cellular damage, vascular shutdown, and activation of an immune response against targeted cells. PDT has several advantages over conventional therapies because of its noninvasive nature, its selectivity, the ability to treat patients with repeated doses without initiating resistance or exceeding total dose limitations (as associated with radiotherapy), the fast healing process resulting in little or no scarring, the ability to treat patients in an outpatient setting, and the lack of associated side effects.[2] Over the past decade, the clinical use of PDT has greatly increased. Current clinical applications of PDT include the treatment of solid tumors in skin (basal cell carcinomas), lung, esophagus, bladder, head and neck, brain, ocular melanoma, ovarian, prostate, renal cell, cervix, pancreas, and bone carcinomas.[3,4] Dysplasias, papillomas, rheumatoid arthritis, actinic keratosis, cosmetics, psoriasis, neovasculaturization in age-associated macular degeneration, endometrial ablation, port wine stains, atherosclerotic plaques, and prophylaxis of arterial restenosis have also been treated clinically using PDT.[4,5] The use of PDT to treat bacterial and fungal infections has been in practice for over 30 years.[6] Table 1 is a partial list of the porphyrin-based photosensitizers that are currently approved for clinical applications or are in human trials. PDT has low systemic toxicity, it can

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selectively destroy target tissue, and it can be applied either alone or in combination with other therapeutic modalities such as chemotherapy, surgery, radiotherapy, or immunotherapy.

PDT requires three elements to generate singlet oxygen: a photosensitizer, appropriate light, and oxygen. When a photosensitizer in its ground state is exposed to light of a specific wavelength, it absorbs a photon and is promoted into an excited singlet state. The energy of the excited singlet state can be dissipated either by thermal decay or the emission of fluorescence. Alternatively, the excited singlet state can move to a lower-energy excited triplet state through intersystem crossing. In the excited triplet state, the photosensitizer can generate reactive species through two mechanisms, Type I and Type II processes.[1] In Type I PDT processes, the photosensitizer transfers an electron to various receptor molecules, giving rise to free radical production in forms that may include the superoxide anion, hydroxyl radical, or hydrogen peroxide.[7] In Type II processes, the excited triplet state photosensitizer interacts directly with molecular oxygen, producing reactive singlet oxygen. Type II PDT processes are the most relevant, and the generated singlet oxygen is responsible for the destruction of targeted tissue. Cellular death occurs due to sufficient oxidative stress as a result of singlet oxygen interaction with cellular components such as lipids, amino acid residues, and nucleic acids. The mechanism of cell death, be it apoptosis or necrosis, is dependent upon the localization of the photosensitizer within the cell and the amount of singlet oxygen generated.[8] Some evidence suggests a photosensitizer localized in the mitochondria or the endoplasmic reticulum is a better inducer of apoptosis, whereas a photosensitizer localized in the plasma membrane or in lysosomes is more condusive to necrosis.[2] PDT can cause acute local inflammation, inducing an immune response against cancer cells.[9]

1.2. Imaging with Photosensitizers

Photosensitizers are not restricted solely to therapeutic generation of singlet oxygen. Not only are many photosensitizers bright fluorophores, they tend to emit in the near-infrared (NIR) portion of the spectra that is useful for in vivo imaging. A fluorescently detectable photosensitizer is beneficial for aiding in defining and adjusting parameters during PDT treatment. If the malignant tissue retains the photosensitizer, the target site will light up to provide visible guidelines for therapy. The fluorescence spectra of a photosensitizer may differentiate normal and malignant regions, acting as an image-guidance tool. Fluorescent signatures may also be used as an optical biopsy, differentiating between benign and malignant disease and avoiding standard histological evaluation. In addition, evaluation of the success or failure of treatment may be monitored through the photosensitizer fluorescence (as target cells are destroyed, fluorescence signal decreases), which may be a useful dosimetric guide for real-time modification during therapy. Fluorescent photosensitizers can aid in determining photosensitizer localization and degree of photosensitizer uptake by diseased tissue.[2,7] These photosensitizer characteristics can be further exploited in photosensitizers that are only active in the presence of a target molecule upon which fluorescence and singlet oxygen production occur. While conventional photosensitizers often can serve as fluorophores suitable for in vivo studies, extraneous phototoxicity to nontarget tissues can occur in the course of imaging the photosensitizer localization. Photosensitizers that are not phototoxic outside activation or target sites would therefore be more useful imaging probes due to a reduction in nonspecific phototoxicity. Activatable photosensitizers (aPS, with this abbreviation denoting both singular and plural forms) are ideal imaging probes as molecular activation distinguishes target cells from normal cells. Activatable photosensitizers share similar activation mechanisms with activatable fluorophores, and there is a close relationship between these two imaging agents.[10] Many aPS rely on the same mechanisms as

Table 1. Clinical Indications Treated with Various Porphyrin-Based Photosensitizers

PS	indications and approval status[a]
ALA (5-aminolevulinic acid)	*Approved*: Actinic keratosis, basal cell carcinoma. *Clinical trials*: Bladder cancer, penile cancer, gliomas, acne vulgaris.
Foscan (*meta*-tetra(hydroxyphenyl) chlorin)	*Approved*: Palliative head and neck cancer.
Metvix (5-aminolevulinic acid methyl ester)	*Approved*: Actinic keratosis, superficial basal-cell carcinoma, and basal-cell carcinoma.
Lu-Tex (lutetium texaphyrin)	*Clinical trials*: Prostate cancer and coronary artery disease.
NPe6 (mono-L-aspartyl chlorin-e_6)	*Approved*: Early lung cancer.
Pc4 (silicon phthalocyanine)	*Clinical trials*: Cutaneous T-cell lymphoma, cutaneous skin cell lesions, sterilization of blood products.
Photochlor (Hexyl ether pyropheophorbide-*a* derivative)	*Clinical trials*: Lung carcinoma, basal cell carcinomas, Barrett's esophagus.
Photofrin (hematoporphyrin derivatives)	*Approved*: Advanced and early lung cancer, superficial gastric cancer, esophageal adenocarcinoma, cervical cancer and dysplasia, superficial bladder cancer, Barrett's esophagus. *Clinical trials*: Intraperitoneal cancer, cholangiocarcinoma, retenosis.
Photolon (chlorin-e_6-polyvinylpyrrolidone)	*Approved*: Malignant skin and mucosa tumors, myopic maculopathy, central choroidal neovascularization.
Photosens (aluminum phthalocyanine)	*Clinical trials*: Age-related macular degeneration.
Purlytin (tin ethyl etiopurpurin)	*Clinical trials*: Prostate cancer, metastatic breast cancer, Kaposi's sarcoma (in AIDS patients).
Tookad (palladium-bacteriopheophorbide)-*a*	*Clinical trials*: Prostate cancer.
Visudyne (benzoporphyrin derivative monoacid ring A)	*Approved*: Age-related macular degeneration, subfoveal choroidal, neovascularisation.

[a] Approval status varies regionally. Consult references for further details.[5,7,113−117]

fluorescent counterparts. It is, therefore, an interesting point that more aPS may be developed from borrowing designs from the better-known and larger pool of activatable fluorophores.

Another utility of photosensitizers is that they may be conjugated to agents from other imaging modalities. Radiolabeled photosensitizers and magnetic resonance imaging (MRI) contrast agent-conjugated photosensitizers have been described and provide a multifunctional probe with the capabilities of two imaging modalities (fluorescence imaging and positron emission tomography (PET)/MRI) as well as therapeutic function.[11,12]

1.3. Emergence of Activatable Photosensitizers as Smart Drugs

As the accessibility and throughput of genome sequencing and expression analysis rise to unprecedented levels, we are entering a new era of personalized medicine. At a fraction of the time and cost of the first human genome, individual and cancer genomes are now being regularly reported thanks to new revolutionary advances in DNA sequencing methods.[13,14] While next-generation sequencing is also proving its merit in analyzing quantitative mRNA transcriptomes, microarray analysis remains an affordable, accessible, mature, and robust choice for mRNA transcription profiling.[15] Progress in genetic research has yielded many disease signatures—lists of genes that tend to be up- or downregulated in affected tissues or individuals that can be used to better characterize and understand disease on a molecular basis.[16] Although knowledge of the genetic basis of disease is essential for directing further basic biochemical research, translating this wealth of new information into treatment approaches remains a separate challenge. Given that diagnostic methods have the capability to identify overexpressed genes associated with diseased conditions at the individual level, personalized medicine requires improved generalized methods to directly target these gene or gene products. PDT with aPS is an attractive therapeutic option since it can directly kill targeted cells without side effects in other parts of the body. Photosensitizers that are localized or activated at the target site can be used for a wide variety of molecular targets.

There are several approaches to improve the targeting of PDT agents, including use of the antibody or targeting-protein conjugation, small targeting ligand conjugation, and vascular targeting. These photosensitizers are generally known as the third generation of photosensitizers, which build on the second generation of photosensitizers that have improved optical properties for therapy.[17] Clinical photosensitizer delivery to target tissues is currently a passive process. The limitations of passive delivery are that it is not applicable for all types of cancers and other diseases and does not sufficiently inhibit photosensitizer accumulation in adjacent healthy tissues. Antibody-targeted PDT is an established technique that improves photosensitizer delivery through photosensitizer conjugation to targeting antibodies.[18,19] The antibodies then deliver the photosensitizer to specific antigens overexpressed on target cells. Despite promising results and decades of progress, antibody-targeted PDT has yet to see clinical implementation. One challenge is that the antibodies must have a low photosensitizer-to-antibody conjugation ratio to maintain targeting function. Since antibodies are hundreds of times larger than a photosensitizer, the requisite low conjugation efficiency limits the amount of photosensitizer that can be administered therapeutically. Furthermore, it may be difficult for the large photosensitizer-conjugated antibodies to enter the cell and generate singlet oxygen that can attack intracellular targets. Antibodies are not the only type of protein that can be used to target photosensitizers; for instance, transferrin has been used to deliver hematoporphyrin to cells by a receptor-mediated pathway.[20] Lipoproteins can be loaded with many photosensitizers in their core and then be targeted to cells that express specific receptors.[21,22] Small ligand conjugation to photosensitizers is another targeting technique that has shown a great amount of potential. Certain small ligands are uptaken by receptors that are overexpressed on cells in a variety of diseases. The folate receptor is overexpressed in many cancers. Conjugation of photosensitizers to the folate molecule improved photosensitizer uptake in target cells in vivo

through folate receptor mediated uptake.[23,24] Likewise, enhanced tumor glycolysis often occurs by overexpression of glucose transporters. Conjugation of photosensitizers to 2-deoxyglucose resulted in photosensitizer uptake in cancer cells via the glucose transporter.[25] Peptides are also useful as small targeting ligands. Dozens of peptide sequences have been shown to target various surface markers overexpressed in different types of cancers.[26] Enhancement of photosensitizer targeting has been achieved through conjugation to various targeting peptides, including the RGD peptide and the VEGF targeting peptide.[27,28] Like peptide-directed targeting, aptamer-based nucleic acid targeting is also a powerful targeting technique.[29,30] Photosensitizer−aptamer conjugates have been developed that enhance photosensitizer delivery to cancer cells.[31] While most targeting approaches attempt to bring the photosensitizer to the diseased cells directly, another approach is vascular photosensitizer targeting.[32] In this approach, a photosensitizer is targeted to the vasculature, including that which surrounds the target of treatment. For cancer treatment, light placement at the target site can effectively destroy the vasculature and endothelium around the tumor, resulting in tumor damage and starvation. This approach has shown clinical promise for treating prostate cancer and age-associated macular degeneration.[33,34] While the various targeting strategies attempt to restrict the placement of the photosensitizer, an activation strategy restricts the localization of photosensitizer activation, based on completely different mechanisms.

aPS hold potential to effectively target a wide range of genes or gene products that are specifically expressed in diseased cells.[35,36] aPS are a special class of photosensitizers that are turned on by a wide variety of molecular stimuli, resulting in increased cytotoxic singlet oxygen generation. Compared to other drugs, aPS have a unique advantage in targeted therapy because they can kill cells directly by singlet oxygen generation instead of by inhibition of gene expression or activity. For instance, even if a drug can inhibit an enzyme that is overexpressed in a certain disease, those cells may still be able to survive without that enzyme having full function. Even if an enzyme is an abundant and accurate biomarker, only if it is essential for cell survival will inhibition of enzyme function be able to directly destroy the diseased cells. Enzyme-activated photosensitizers do not rely on enzyme inhibition for function because they directly kill cells through singlet oxygen generation.

The activation step adds a new element of control to PDT. Conventional PDT relies on light delivery and photosensitizer delivery to oxygenated tissue as discriminators of specificity. Compared to other disease treatments, directed light placement already confers excellent localized specificity to PDT, because distant body organs are unaffected and spared from singlet oxygen damage. As shown in Figure 1, along with photosensitizer delivery to oxygenated tissues and light placement, molecular activation adds a third layer of specificity to PDT targeting of diseased cells. While photosensitizer and light delivery are indispensible for singlet oxygen generation, activatable photosensitizers have varying amounts of background in their inactive state. Therefore, aPS may display some cellular toxicity if singlet oxygen production in the inactive state is not well-attenuated. However, since many aPS increase singlet oxygen production upon activation by a factor greater than 10-fold, these well-designed aPS do not display significant toxicity in their inactive state when irradiated. The singlet oxygen generated

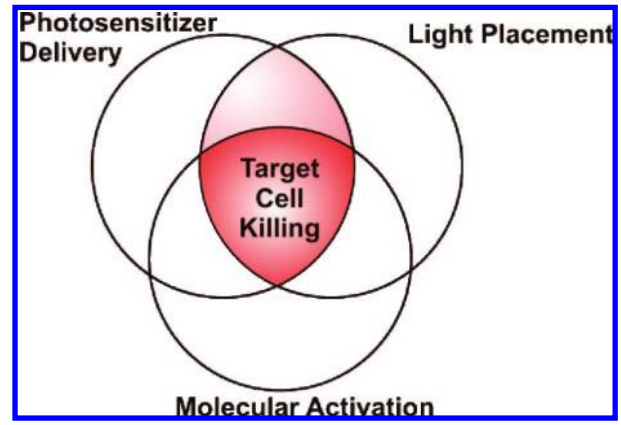

Figure 1. Venn diagram showing 3 layers of specificity that must intersect for activatable photosensitizer-based killing. The red shading indicates the intersection that will result in cell killing. White areas show intersections where no cell killing will occur. The lightly pink shaded area shows that cell killing will be impeded but may still occur if the activatable photosensitizer is not strongly attenuated in its inactive state.

by photosensitizers activated in diseased cells does not significantly affect surrounding healthy tissue because singlet oxygen has limited diffusion between 10 and 300 nm, according to different estimates.[37−39] Therefore, only when the photosensitizer reaches the diseased tissue, when the diseased tissue activates the photosensitizer, and when light is applied locally will the targeted cells be destroyed. Molecular activation permits the aPS to distinguish healthy from diseased cells, reducing damage to nearby healthy cells that otherwise might be destroyed during PDT with conventional photosensitizers.

2. Activatable Photosensitizer Design Considerations

2.1. Activation Strategy

Singlet oxygen generation and deactivation by conventional photosensitizers have been the focus of extensive research.[40] An aPS must increase singlet oxygen generation upon activation and irradiation. This is often accomplished by maintaining the photosensitizer in a quenched state prior to a molecular activation step that unquenches the photosensitizer. There are many possible approaches to maintaining continued aPS deactivation since there are many requisite steps that must occur prior to singlet oxygen-mediated cell killing. As expressed in Figure 2, the biophysical events that occur prior to generation of singlet oxygen are potential areas where quenching can be manipulated.

The earliest opportunity to generate an inactive photosensitizer is to prevent it from reaching a higher excited state. This can occur through alteration of the electron organization of the photosensitizer, which can be achieved through contact quenching. Contact quenching brings another molecule in continued contact with the photosensitizer in a manner that alters the excitation properties of the photosensitizer. Contact quenching is usually accompanied by an absorption shift and has been shown to be a potent quenching strategy that can function with a wide variety of fluorophores.[41] Contact quenching and solvent effects can also influence the next deactivation point, internal conversion. Internal conversion is a nonradiative process in which the excited molecule returns to the ground state through heat release. Quenching through Förster resonance energy transfer (FRET), photo-

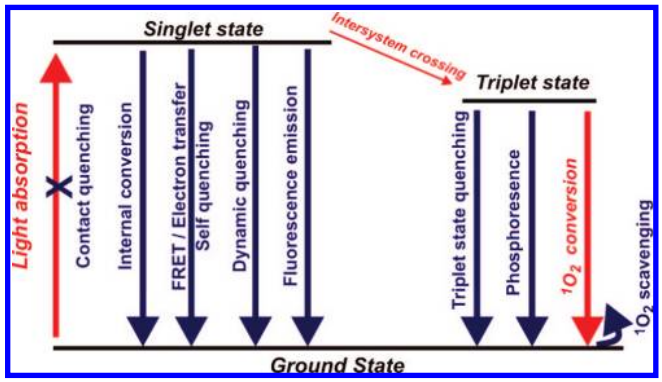

Figure 2. Simplified energy level diagram showing potential singlet oxygen deactivation pathways. Energy levels are shown in black. Typical pathway to singlet oxygen generation is shown in red. Various points of deactivation are indicated in blue. Photosensitizers can be designed to become deactivated and reactivated in response to specific molecular stimuli.

induced electron transfer, and self-quenching are potential quenching strategies that are particularly useful for efficient aPS design. After this point, the photosensitizer may be deactivated by dynamic quenching, in which another molecule physically collides with the photosensitizer and returns it to the ground state. Next, fluorescence emission will return a portion of the excited molecules to the ground state, as photosensitizers generally have some degree of fluorescence. The photosensitizers will then undergo intersystem crossing to the triplet state. In some cases, the heavy atom effect has been shown to be effective at increasing the efficiency of intersystem crossing.[42] After the photosensitizer enters a triplet state, a variety of quenchers can act on this long-lived triplet state before any phosphorescence is emitted. Finally, the essential step of singlet oxygen generation occurs when the photosensitizer triplet state is quenched by molecular oxygen, generating singlet oxygen. It is also possible to scavenge singlet oxygen after it has been generated. This concept has been demonstrated using a carotenoid and an activatable photosensitizer design.[43]

FRET deactivation of photosensitizers is useful for aPS design. FRET is a nonradiative energy transfer process in which the excited state photosensitizer donor transfers energy to a chromophore acceptor that shares absorptive spectral overlap with the photosensitizer fluorescence emission. The main advantage of FRET is that it is only effective when the photosensitizer and quencher are nanometers apart, and it is reliably predicted by the fluorescence and absorption spectra of the photosensitizer and quencher. The concept of FRET quenching is demonstrated in Figure 3.[44] To better understand FRET deactivation of singlet oxygen, different quenchers with varying amounts of spectral overlap with the fluorescence emission of a photosensitizer, pyropheophorbide-*a*, were covalently conjugated. When a short linker was used, quenchers effectively quenched the photosensitizer fluorescence and singlet oxygen generation regardless of the amount of spectral overlap the quenchers shared. However, when the linker was extended with a polyproline peptide, only the quenchers with greater spectral overlap could maintain effective quenching. Careful quencher selection requires not only that the quencher has sufficient FRET efficiency in the closed conformation, but the quencher also must not effectively quench the aPS in the activated conformation. The intrinsic fluorescence of photosensitizers is useful not only for fluorescence imaging purposes but also as a convenient monitor for singlet oxygen production. When constructs were generated with different quenchers that shared varying amounts of spectral overlap with the fluorescence emission of a single photosensitizer, a 0.99 correlation was observed between singlet oxygen quantum yields and fluorescence quantum yields.[44] Although fluorescence measurements cannot translate directly into singlet oxygen yields, this result shows that unquenching of aPS fluorescence will parallel changes in singlet oxygen production. This facilitates estimation of singlet oxygen production status since fluorescence is generally a more convenient parameter to measure than singlet oxygen. Another photosensitizer quenching study examined the effects of substituting different coordinated metals in one photosensitizer serving as a

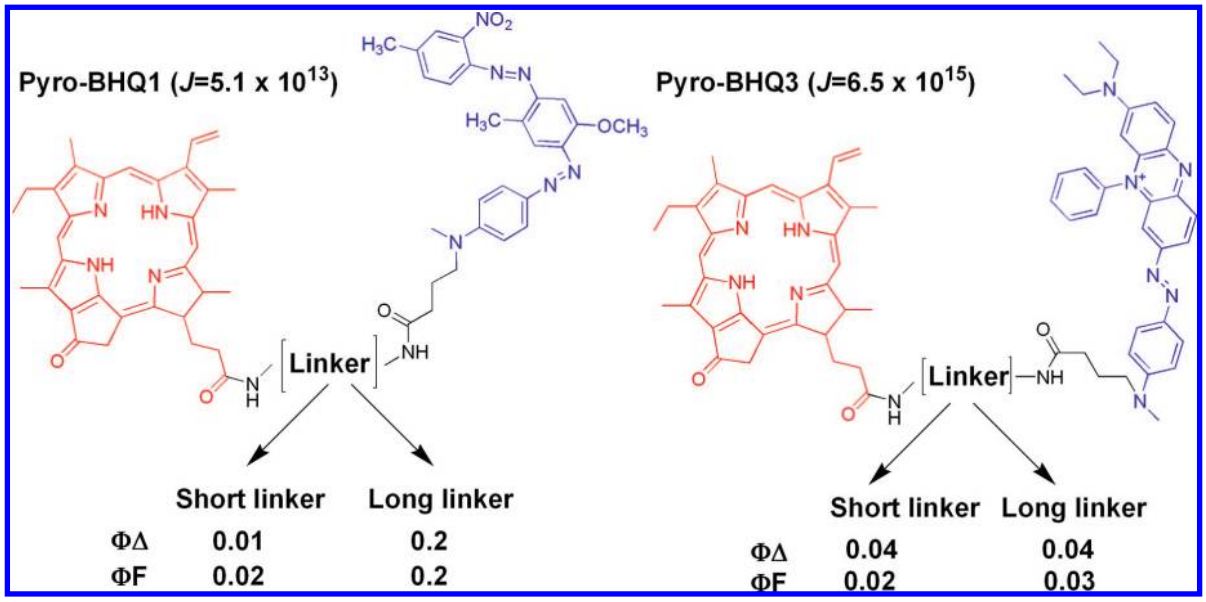

Figure 3. FRET control of singlet oxygen generation.[44] When the distance between photosensitizer and different quencher increased, only quenchers with large spectral overlap could maintain quenching efficiency. The red structures represent the pyropheophorbide-*a* photosensitizer, and the blue structures represent two different black hole quenchers. In methanol, extension of the linker from a lysine linker to a 10mer polyproline linker resulted in a loss of singlet oxygen and fluorescence quantum yield quenching efficiency for only the photosensitizer with smaller spectral overlap, J, which is shown in units of M^{-1} cm^{-1} nm^4.

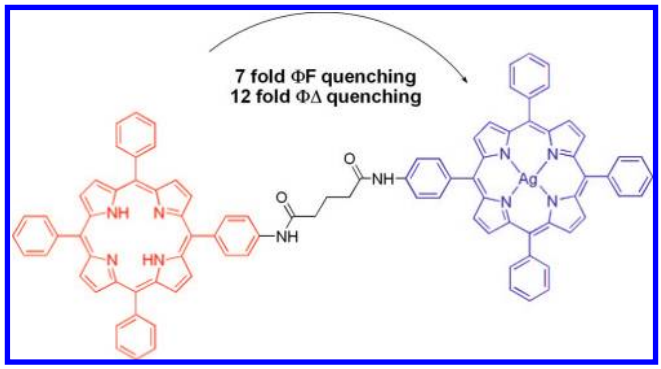

Figure 4. Metalloporphyrin quenching of photosensitizers.[45] Tetraphenylporphyrin (red) was linked to another metal-substituted tetraphenyl porphyrin (blue) that functioned as a quencher, and quantum yields were determined. The arrow represents energy transfer to the metal-substituted porphyrin resulting in quenching. Of the various metals tested, silver substitution resulted in the greatest quenching of fluorescence and singlet oxygen quantum yield.

quencher for another covalently linked photosensitizer (Figure 4).[45] Different metal complexes had varying degrees of quenching efficiency on the fluorescence and singlet oxygen quantum yields, with silver proving the most effective, being able to quench singlet oxygen generation over 10-fold. Self-quenching is another popular deactivation strategy. Self-quenching relies on two or more photosensitizers in close proximity that may aggregate and form ground-state complexes or may quench through energy transfer. Self-quenching approaches have an advantage that the activated photosensitizer will be brighter, simply because each aPS carries more photosensitizers. In many cases, self-quenching approaches have demonstrated effective singlet oxygen quenching; however, the degree of quenching is difficult to predict and must be determined empirically for each design. Since many photosensitizers are hydrophobic, use of multiple photosensitizers may affect the solubility of the aPS.

2.2. Photosensitizer Selection

Selection of an appropriate photosensitizer is of paramount importance for aPS design. Factors that may influence the selection include conjugation compatibility and yield, quencher compatibility, photosensitizer hydrophobicity, excitation profile, singlet oxygen quantum yield, fluorescence quantum yields, and photosensitizer dark toxicity. Porphyrin-based photosensitizers are often selected because of their strong singlet oxygen quantum yields and well-known chemistries.[46] Conjugation compatibility is essential for synthesizing the aPS. Besides requiring a suitable functional group to permit the conjugation, in some cases, multiple functional groups are present on a photosensitizer and conjugation will produce a mixed species of conjugated isomers. While an isomerically pure photosensitizer is ideal for purification and characterization, in some cases, mixed species are acceptable, especially with respect to large polymeric drugs. Photofrin, the most clinically used photosensitizer, is a mixture of multimeric photosensitizers, although this is generally considered a drawback. The choice of photosensitizer also depends on the product availability, cost, and required yield. Although some conjugatable photosensitizers are available commercially, many are not. Also, production of large-scale amounts of aPS sufficient for in vivo imaging or therapy requires a large starting amount of photosensitizer that may be prohibitively costly to obtain commercially. Therefore, in-house photosensitizer synthesis and derivatization may be beneficial in some instances. The physical properties of the photosensitizer are important considerations for aPS design. For instance, a very hydrophobic photosensitizer may interfere with the purification process of an aPS, may interfere with the solubility of the compound, and may affect the activation kinetics of the drug.

Table 2 displays photophysical properties for a variety of commonly used photosensitizers. Several possess reasonable fluorescence quantum yields ranging from 0.2 to 0.5. However, since fluorescence brightness is a product of extinction coefficient as well as fluorescence quantum yield, the strong extinction coefficients to the order of 10^5 or 10^6 M^{-1} cm^{-1} of these photosensitizers render them useful fluorescent probes. The amount of fluorescence and singlet oxygen generated by a photosensitizer is dependent on the photosensitizer extinction coefficient and the photosensitizer fluorescence and singlet oxygen quantum yields. A near unity singlet oxygen quantum yield will come at the expense of the fluorescence quantum yield, and photosensitizers of varying fluorescence and singlet oxygen quantum yields should be thus chosen depending on the desired emphasis

Table 2. Wavelengths, Extinction, and Quantum Yields of Selected Photosensitizers

photosensitizer	abs. peaks(s) (nm)	extinction coefficient (s M^{-1} cm^{-1})	em. peak (nm)	ΦF	ΦΔ	ref.
aluminum phthalocyanine tetrasulfonate[i]	676	169 000	684	0.51[d]	0.38[i]	118−120
bacteriochlorophyll-*a*[a]	360/770	66 000/71 000	788	0.14	0.35[b]	118, 121
benzoporphyrin derivative (BDP-MA)[f]	428/686	76 000/34 000	692	0.05	0.76	122
chlorin-e_6[d]	402/663	150 000/59 000[a]	667	0.19	0.65	118, 123−125
iodinated bodipy[f]	534	110 000	548	0.02	∼1	42
methylene blue[i]	665	91 000	685	0.02	0.55	93
porphycene[b]	358/630	139 000/52 000	640[g]	0.44	0.34	118, 126−128
protoporphyrin IX[f]	402/626	40 000/3 000	633	0.16	0.56[i]	118, 129, 130
pyropheophorbide-*a* analogues[f]	410/665	97 000/46 000	672	0.43[b]	0.45[b]	44, 131
rose bengal[d]	560	90 000	575	0.11	0.68	118, 132
tetraphenylporphyrin[b]	419/647	470 000/3 400	653	0.11	0.63	133−135
silicon naphthalocyanine[e]	347/781	134 000/327 000	780	0.17	0.33	118, 136, 137
meso-tetra(4-methoxyphenyl) porphyrin[g]	420/650	460 000/4 800[c]	658	0.14	0.65	138
lutetium texaphyrin[i]	470/733	68 000/23 000	747	0.01	0.23[f]	139
zinc phthalocyanine[b]	672	150 000	676	0.06	0.62	17, 140
zinc tetraphenylporphyrin[b]	423/586	540 000/3 700	610	0.03	0.73	118, 133, 141

Note: Data were recorded in the following solvents:
[a] Acetone. [b] Benzene. [c] Dichloromethane. [d] Ethanol. [e] Dimethyl Formamide. [f] Methanol. [g] Tetrahydrofuran. [h] Aqueous. [i] Triton X-100 micelles. Some data were extracted from graphs or similar chemical structures.

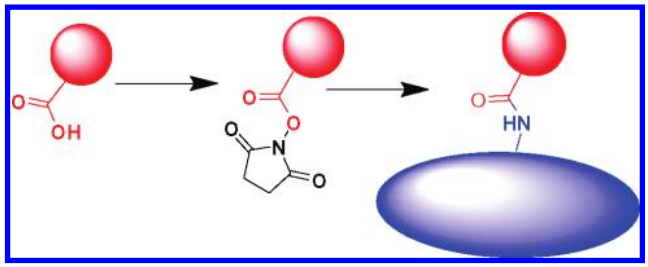

Figure 5. Typical amide bond chemistry of photosensitizer conjugation to an amine via NHS ester. The blue shape represents the target with a free amine, and the red sphere represents a photosensitizer with a free carboxylic acid group for conjugation.

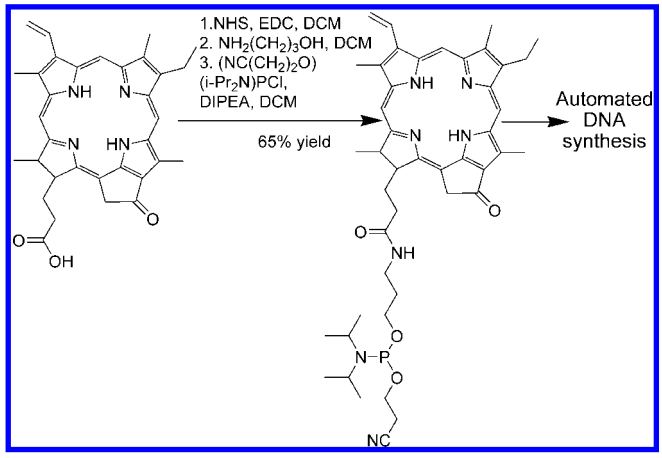

Figure 6. Generation of a 5′terminating pyropheophorbide-*a* phosphoramidite fit for automated DNA synthesis.[52]

or combination of imaging and therapy. Excitation and fluorescence emission are other important properties of photosensitizers. Since body tissue heavily absorbs light, selection of a photosensitizer that minimizes such interference by operating in the NIR window is imperative for effective in vivo therapeutic and imaging applications. Hemoglobin and myoglobin absorb heavily below 600 nm, while water absorbs heavily above 1000 nm, limiting the window to within this wavelength range.[47] Fortunately, even photosensitizers with shorter wavelengths do not necessarily need to be excluded for in vivo use due to progress in two-photon excitation, in which photosensitizers that are excited by a shorter wavelength can be excited with a wavelength twice as long that can more easily penetrate tissue. However, two-photon techniques confine the excitation light to small spatial areas, which may restrict certain therapeutic applications.

2.3. Photosensitizer Conjugation

Because aPS tend to be modular, consisting of a photosensitizer, a quencher, and a bioactive linker, conjugation of the photosensitizer is a requisite step in building the aPS. Generally, photosensitizer conjugation is dominated by standard NHS generation resulting in stable amide couplings, as seen in Figure 5. In this scheme, the photosensitizers require carboxylic acid functional groups. Many other diverse conjugation strategies have been demonstrated, including thiol,[48] isothiocyanate,[49] enyne metathesis,[50] and click[51] chemistries to conjugate photosensitizers to other molecules.

Using amide bond chemistry, development of modular photosensitizer building blocks is simple. Figure 6 demonstrates the generation of a photosensitizer phosphoramidite, the standard monomer for nucleic acid synthesis.[52] This allows the automated incorporation of photosensitizers during nucleic acid synthesis. Often it is desirable to attach a quencher and photosensitizer on opposing sides of an active biolinker. This can be accomplished in numerous ways, one of which is illustrated in Figure 7, and makes use of two separate protecting groups. Initially, a peptide is synthesized using standard methods, except an N terminal fluorenylmethyloxycarbonyl chloride (FMOC) protected residue and a C terminal lysine that will have the amine group used for conjugation are incorporated. Initially, the FMOC is removed using piperidine, exposing a free amine group. Then, a photosensitizer or quencher is conjugated to the exposed amine of the peptide on the solid-phase resin. After conjugation, the resin is washed extensively and the 4-methyltrityl protecting moiety is then removed using low-concentration trifluoroacetyl (TFA). The newly exposed amine may then be labeled by a quencher. Finally, the resin is washed again and the peptide is cleaved from the resin with 95% TFA. After conjugation, the peptide should be purified by high-performance liquid chromatography (HPLC).

3. Examples of Activatable Photosensitizers

3.1. Environment-Activated Photosensitizers

The singlet oxygen production efficiency of photosensitizers is dependent on solvent properties including pH and hydrophobicity. As shown in Figure 8, the common photosensitizer methylene blue displays a 5-fold change as the pH increases from pH 5 to pH 9 and the photosensitizer becomes deprotonated.[53] The structurally related photosensitizer toluidine blue undergoes a similar increase in singlet oxygen upon deprotonation.[54] Solvent hydrophobicity plays a large role in determining the photosensitizer efficiency. As shown in Figure 9, the photosensitizer palladium−bacteriopheophorbide-*a* undergoes an approximate 2-fold change in singlet oxygen quantum yield as it moves from a hydrophobic acetone solvent to a micelle environment in deuterated water.[55] Although not physiologically relevant, deuterated water increases singlet oxygen lifetime, increasing yields and facilitating measurements. More thorough studies have shown that solvent properties affect the photophysical properties of a wide range of photosensitizers.[56] Changes in hydrophobicity and pH can occur simultaneously, yielding dramatic effects. As shown in Figure 10, it has been demonstrated that the singlet oxygen quantum yield of (*E*,*E*)-2,5-dibromo-1,4-bis[2-(4′-dimonomethylether triethylene glycol aminophenyl)vinyl]benzene (MTEGPV) changed from 0.33 to 0.09 upon solvent change from toluene to deuterated water.[57] Singlet oxygen generation then became undetectable when the MTEGPV was protonated in deuterated water.

Although solvent and pH effects have long been factors recognized to affect singlet oxygen production, only recently have explicit efforts been made to exploit these properties for design of aPS. Rather than relying on the intrinsic properties of photosensitizer singlet oxygen generation with respect to solvents or pH, attempts have made use of controllable quenchers to modulate activation. In particular, photoinduced electron transfer quenching has been used to control aPS. By attaching photoinduced electron transfer-based quenchers with specific pK_a that are only active in protonated form, pH-activated photosensitizers were demonstrated to effectively kill cells (Figure 11).[58] This approach was extended to develop photoinduced electron transfer quenchers that are only active in hydrophobic solvents with

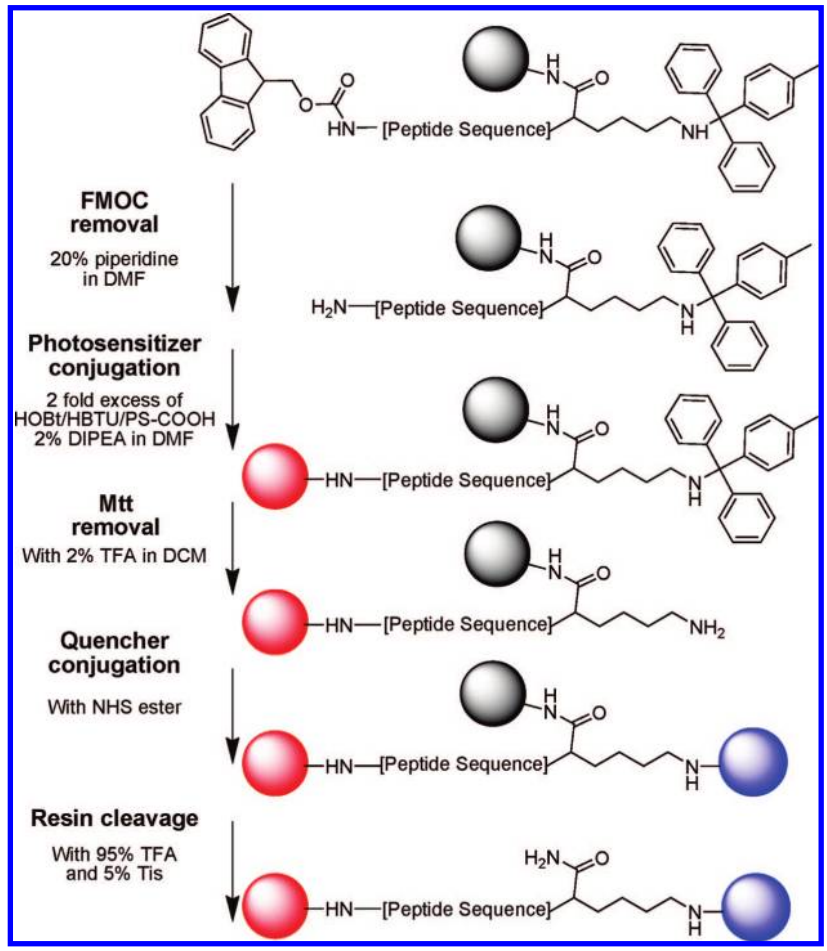

Figure 7. Possible synthetic approach to the solid-phase peptide conjugation of a photosensitizer and a quencher. The photosensitizer is shown in red, the quencher is shown in blue, and the solid-phase support is shown in gray.

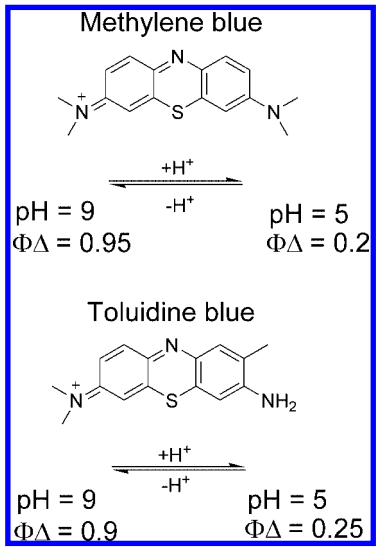

Figure 8. pH-dependent change of methylene blue and toluidine blue singlet oxygen quantum yields.[53,54]

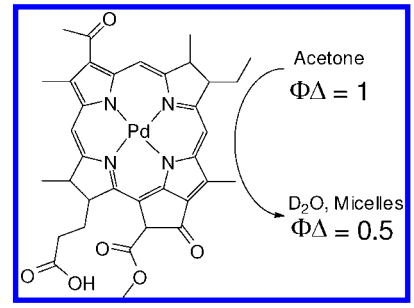

Figure 9. Change in palladium−bacteriopheophorbide-*a* singlet oxygen quantum yield in solvents of different hydrophobicity.[55]

low dielectric constants.[59] As shown in Figure 12, this aPS consisted of a photosensitizer, a modulatable photoinduced electron transfer quencher, and a protein-targeting ligand that directed the aPS to the IP3 receptor in cells. The photoinduced electron transfer quencher became inefficient upon binding in the hydrophobic pockets of cellular proteins. This approach demonstrated the specific inactivation of specific proteins in live cells.

Environmental activation is an important factor in controlling the singlet oxygen generation of photosensitizers. As shown in Table 3, there have been several examples of changes in singlet oxygen production induced by solvent pH and hydrophobicity. New aPS will find novel applications in which environmental effects can regulate singlet oxygen, in tandem with other targeting or activation methods.

3.2. Enzyme-Activated Photosensitizers

Enzymes are catalytic, diverse, and central to all facets of cellular function and are, therefore, excellent targets for aPS. Since enzyme overexpression is correlated with specific diseases in many cases, photosensitizer activation can be confined to the location of the active enzyme target, while in tissues not expressing the enzyme, the aPS remains inactive. A small amount of enzyme can continually catalyze

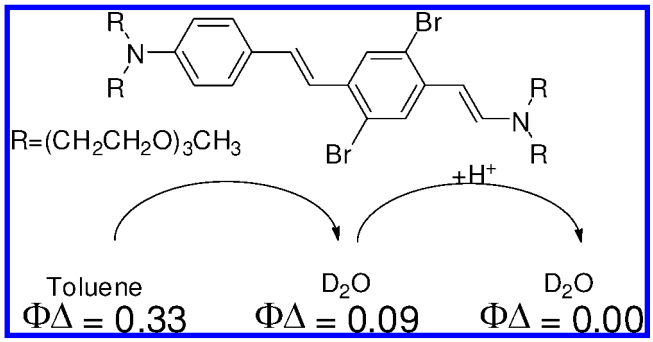

Figure 10. Environment-induced change in the MTEGPV singlet oxygen quantum yield.[57] Singlet oxygen production decreased as the photosensitizer moved from a hydrophobic environment into deuterated water. Then singlet oxygen production became undetectable as the photosensitizer was protonated in deuterated water.

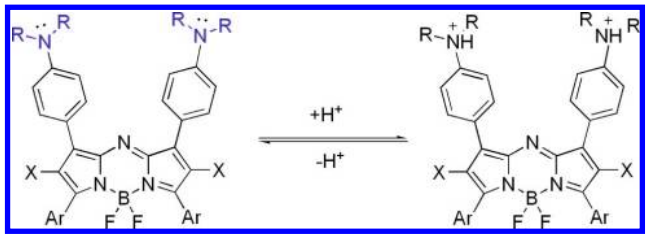

Figure 11. pH-activatable photosensitizer based on electron transfer.[58] An iodinated bodipy derivative increased singlet oxygen production status when the photoinduced electron transfer moieties (blue) became protonated (right). Refer to original reference for details on the X and Ar functional groups.

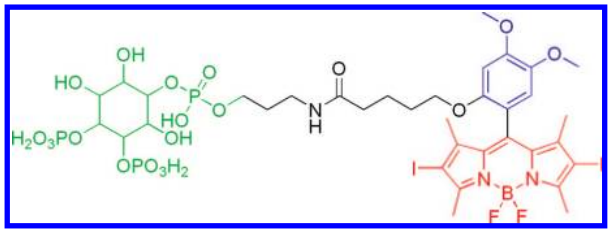

Figure 12. Hydrophobically activatable photosensitizer with protein targeting moiety for chromophore-assisted light inactivation.[59] Iodinated bodipy photosensitizer (red) was attached to a photoinduced electron transfer quencher (blue) with quenching efficiency dependent on solvent hydrophobicity. The inositol 1,4,5-triphosphate ligand (green) directed the photosensitizer to its protein target, where it was activated by binding in a hydrophobic pocket and could then specifically damage that protein through singlet oxygen generation.

photosensitizer activation, and therefore, one enzyme can activate a countless number of aPS, resulting in high signal amplification. Proteases, in particular, have been used as activators for aPS due to their well-characterized and catalytic activity. Proteases are expressed in a wide variety of diseases, and their importance to disease pathology makes them excellent therapeutic targets. Proteases are abundant and specific enough to several diseases that protease inhibitor therapeutics are often used as treatment. Clinically approved

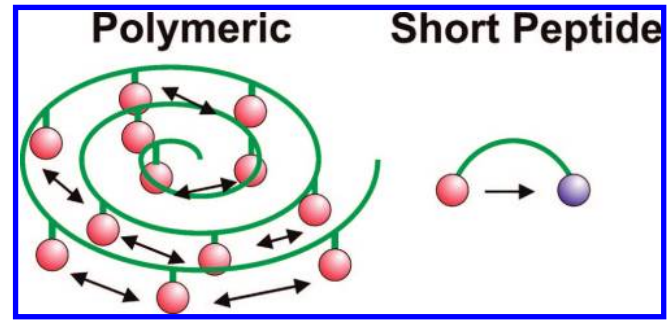

Figure 13. Different backbones for peptide-based activatable photosensitizers. The polymeric backbone (green) activatable photosensitizer comprises many photosensitizers (red) that exhibit self-quenching (represented by arrows). The short peptide-activatable photosensitizer comprises a photosensitizer (red) attached to a quencher (blue) via a peptide linker (green). Energy transfer from the photosensitizer to the quencher is represented by an arrow.

drugs that target disease-related proteases exist for cancers, hypertension, myocardial infarction, periodontis, AIDS, thrombosis, respiratory disease, and pancreatitis.[60] Proteases have also been used extensively in fluorescence imaging. Many genetically encoded fluorescent protease sensors have been extensively developed. A Factor Xa protease sensor was developed fusing two FRET-capable GFP variants linked with a Factor Xa peptide substrate.[61] The cleavage of the peptide linker between the two fluorescent proteins causes their dissociation and subsequent loss of FRET. A similar approach of using two fluorescent proteins fused by a specific linker sequence has been applied to detect other proteases including Botulinum toxin,[62] caspases,[63] secretases,[64] and matrix metalloproteases.[65] The discovery of a genetically encoded photosensitizer, KillerRed, opens up the possibility to develop similar fluorescent protein-based aPS.[66] Smaller amino acid peptide sequences that are cleaved by proteases can form the bioactive linker of aPS. An advantage of the peptide approach is that the accessibility and robustness of peptide synthesis facilitates obtaining the correct amino acid sequence in high yield. There has been a long history of using peptide-based fluorescence probes to image enzymatic activity in cells.[67] More recently, imaging and probe advances have progressed to permit in vivo protease imaging using near-infrared probes.[68] These smart probes are moving toward clinical trials and have been validated ex vivo in human specimens suffering from carotid endarterectomy, where cathepsin protease activity was detected.[69]

To date, peptide-based aPS generally have been based on either a polymer or short peptide sequence backbone. The two different backbones are shown schematically in Figure 13. The polymeric polylysine backbone can comprise hundreds of repeating lysine residues. Each lysine carries one amine group so the backbone holds potential to accommodate a high number of conjugated photosensitizers. At the correct conjugation density, the photosensitizers will self-quench due to their close proximity. Upon enzymatic

Table 3. Environmentally Activated Photosensitizers

environmental factor	environment change	photosensitizer	fold activation	ref.
hydrophobicity	solvent dielectrics ranging from $CHCl_3$ to CH_3CN	iodinated bodipy derivatives	50	59
pH	electron transfer quencher protonation	iodinated bodipy derivatives	10	58
pH	pH 5−9	methylene blue	5	53
pH	pH 5−9	toluidine blue	5	54
solvent effects[a]	from detergent micelles to acetone	palladium−bacteriopheophorbide-*a*	2	55
solvent and pH effects	protonation and change from D_2O to toluene	DMAPV and MTEGPV	5	57

[a] An extensive summary of singlet oxygen changes in detergents and various solvents is reported elsewhere.[56]

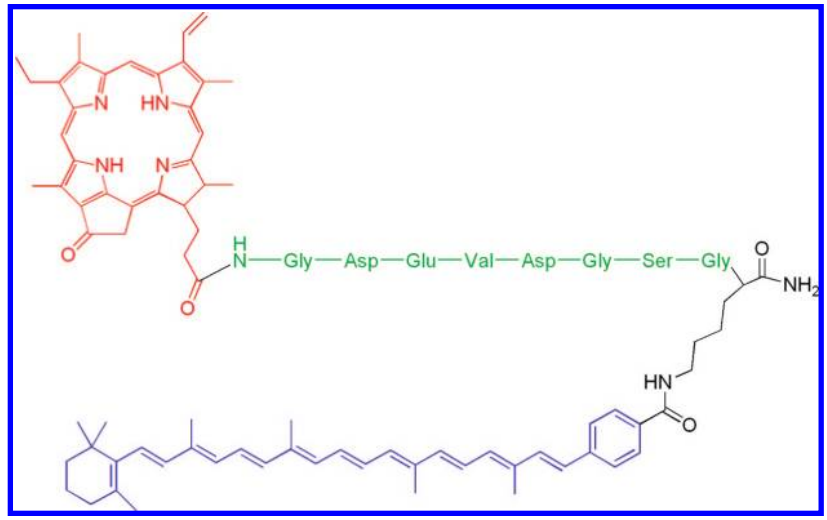

Figure 14. Caspase 3 specific activatable photosensitizer.[72] Pyropheophorbide-*a* is shown in red, the caspase 3 active amino acid linker sequence is shown in green, and the carotenoid quencher is shown in blue.

digestion, the photosensitizers are cleaved from the backbone, are separated from the other photosensitizers, and become unquenched. Since many photosensitizers, including the commonly used porphyrin and chlorin photosensitizers, are fairly hydrophobic, caution must be taken to ensure a sufficiently low ratio of photosensitizers to lysine residues to maintain solubility. On the other hand, at a low photosensitizer substitution ratio, self-quenching efficiency may be too low. Interspersed poly(ethylene glycol) (PEG) moieties have been used to improve solubility. Because many proteolytic enzymes cleave the peptide bond adjacent to lysine residues, conjugation of the photosensitizer to the lysine amine group may also have the undesired effect of eliminating the active sites on the aPS. This problem may be avoided by linking the photosensitizer to the backbone by an additional adapter peptide. The synthetic challenge is to optimize the amount of photosensitizer and other functional moiety substitution to obtain a product that is quenched but remains soluble and contains a sufficient number of recognizable active sites. It may not be possible to obtain a chemically pure polymeric aPS since the substitution patterns will not be identical. The short peptide linker-based aPS overcomes some of these limitations and can be chemically and isomerically pure. It comprises an amino acid sequence that tethers a single photosensitizer and quencher. Rather than utilizing a self-quenching mechanism, the quenching is typically based on a FRET-compatible dark quencher. The short peptide aPS is much smaller than a polylysine aPS and, thus, may be too small to accumulate in tumors from the enhanced permeability and retention (EPR) effect that occurs with particles that are larger in size. However, shorter aPS can enter target cells in other ways that are independent of the EPR effect. It has been shown that the hydrophobic photosensitizer moieties can deliver aPS across the plasma membrane.[70,71]

The first example of an aPS geared toward pure PDT purposes used the short peptide approach with a specific amino acid sequence targeting the caspase 3 protease.[72] As shown in Figure 14, the aPS consisted of a photosensitizer (pyropheophorbide-*a*), a bioactive linker of a specific amino acid sequence, and a quencher (carotenoid). Upon incubation with caspase 3, the peptide portion of the aPS was cleaved and singlet oxygen production increased 4-fold. Caspases are the executioners of apoptosis and are generally inactive in healthy cells. While it is generally undesirable for an aPS

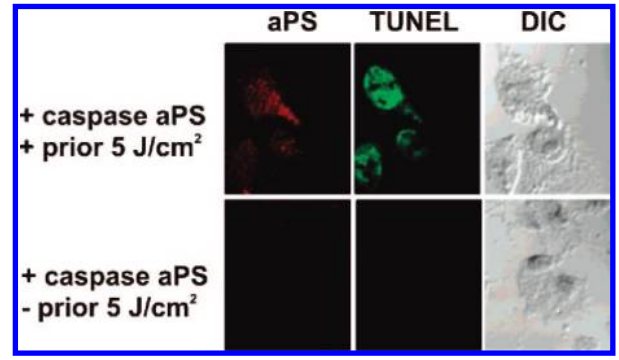

Figure 15. Activatable photosensitizer with both apoptosis-inducing and -detecting capability.[73] A moderately quenched aPS (6-fold increase upon activation) was incubated with HepG2 cells and treated with light. One hour later, cells were fixed and imaged. The top row shows fluorescence in both the photosensitizer and the TUNEL channels, indicating caspase activation. The bottom channel displays no fluorescence signal, showing that without pretreatment there was no caspase activation. Adapted with permission from ref 73. Copyright 2006 American Chemical Society.

to kill cells in the inactive state, for a caspase-specific aPS to be effective, it should generate some apoptotic activity leading to caspase activation, which would further activate the aPS. Because singlet oxygen generation is dependent on irradiation intensity, using a greater light dose may induce apoptosis and caspase activation even with a well-quenched photosensitizer. This concept was validated for a similarly constructed caspase 3 aPS that was composed of pyropheophorbide-*a*, a caspase 3-cleavable amino acid sequence, and a BHQ3 quencher.[73] As shown in Figure 15, pretreating the cells with the aPS and PDT resulted in both caspase activation and detection by the aPS, as confirmed by the apoptosis TUNEL assay in a different confocal channel. When cells were incubated with the aPS but not pretreated with irradiation, both the aPS and the apoptosis indicator were not detectable. While caspase-targeted aPS permit both induction and detection of apoptosis during PDT, a drawback is that they do not preferentially target a disease-associated biomarker. An aPS was developed that targeted matrix metalloproteinase 7 (MMP-7), which is associated with many cancers. The matrix metalloproteinase family regulates normal development but also plays a role in the pathogenesis of cancers. MMP-7 in particular is found

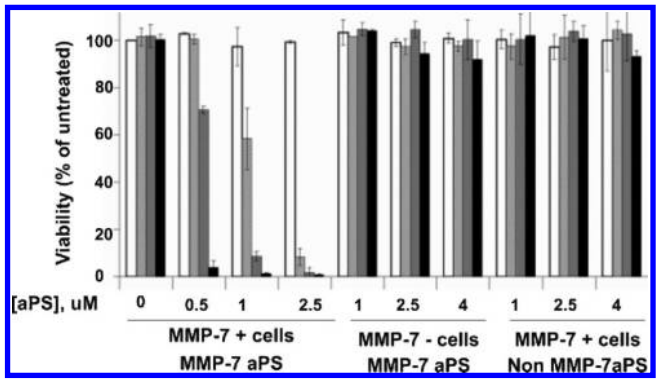

Figure 16. Viability of cells treated with an MMP-7-activatable photosensitizer.[70] Cells were treated with different light doses of 0, 1, 5, and 7.5 J/cm^2 (shown in white, light gray, dark gray, and black, respectively) and the indicated concentrations of activatable photosensitizer. Cell viability was determined in reference to untreated cells. Positive cells showed a light dose and a specific aPS dose response. Adapted with permission from ref 70. Copyright 2007 National Academy of Sciences, U.S.A.

upregulated in several cancers.[74] The MMP-7 aPS used an enzyme-specific sequence, a pyropheophorbide-*a* photosensitizer, and a BHQ3 quencher (Figure 18). Direct measurement of singlet oxygen showed that, upon incubation with MMP-7, the aPS increased singlet oxygen production 19-fold, a level that corresponded to the same production level of the quencher-free construct.[70] Controls demonstrated there was no beacon activation by MMP-7 in the presence of an MMP-7 inhibitor, nor by MMP-7 when the beacon amino acid sequence was modified. As shown in Figure 16, the MMP-7 aPS could effectively kill cells that expressed MMP-7 in a light dose and aPS dose dependent manner. Cells that did not express the enzyme were not affected by the aPS and light exposure. When the MMP-7 positive cells were treated with a non MMP-7 beacon, no reduction of cell viability was observed. This specific killing of MMP-7-expressing cells underscores the power of aPS in targeting diseased cells at the molecular level while protecting healthy cells from singlet oxygen induced damage. Another aPS target protease that has been investigated is fibroblast activating protein (FAP). FAP is cell surface glycoprotein serine protease overexpressed in tumor-associated fibroblasts. It has emerged as an important biomarker because it is found in 90% of human epithelial cancers, but it is not expressed in cancer cells themselves, healthy fibroblasts, or other normal tissues except during wound healing.[75] FAP functions as an endopeptidase that cleaves between the proline and asparagine residues of α_2-antiplasmin and peptide substrates.[76] Thus, an FAP aPS was generated by designing a biolinker peptide containing proline and asparagine residues (Figure 18). In vitro and in vivo studies confirmed the aPS could be activated specifically by the FAP enzyme and displayed a remarkable 200-fold increase in fluorescence. As shown in Figure 17, intratumor injections of the aPS into xenografts either expressing or not expressing FAP resulted in the FAP-positive tumors activating the aPS and resulting in high photosensitizer fluorescence.

As shown in Figure 18, an attractive feature of aPS is that a modular approach is possible, where only the biolinker is changed for different targets. However, even though the two aPS used the same pyropheophorbide-*a* and BHQ3 quenching pair (Figure 18), in vitro enzyme studies revealed the FAP aPS had much greater quenching efficiency compared to the MMP-7-specific aPS. This can be attributed to

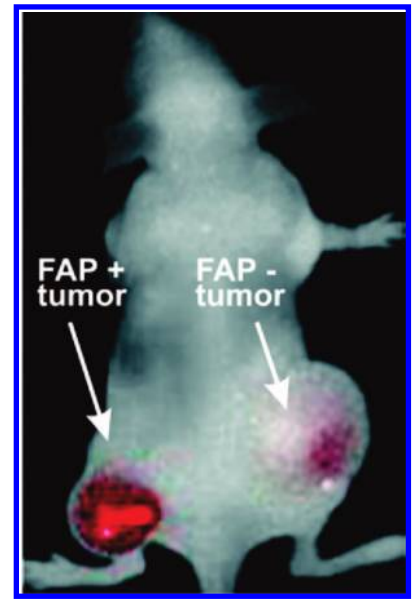

Figure 17. In vivo murine imaging of activatable photosensitizers. Intratumor injection of two xenografts expressing or not expressing the FAP enzyme. Only the fluorescence of the photosensitizer activated by FAP is visible in red. Adapted with permission from ref 75. Copyright 2009 American Chemical Society.

biolinker secondary structure and chemical characteristics that can greatly affect quenching efficiency. Since the quenching is dependent on the distance and the amount of contact between the photosensitizer and the quencher, the biolinker and the solvent influence aPS quenching and activation efficiency. One approach to achieve reliably high quenching is to use a polyanion and polycation peptide zipper mechanism.[77] As illustrated in Figure 19, the zipper aPS consists of five functional modules: a protease-cleavable peptide linker; a polycation and a polyanion attached to each end of the linker, forming a zipper structure via electrostatic attraction; and a photosensitizer and a quencher, conjugated to the opposite end of the polycation and polyanion, respectively. The zipper mechanism provides several advantages: (i) the formation of the polycation/polyanion zipper through electrostatic attraction improves the silencing of the beacon by bringing the photosensitizer and quencher into closer contact, (ii) a hairpin conformation of the substrate sequence occurs as a result of the zipper, improving the accessibility and cleavage rate of the enzyme-specific linker, (iii) the polyanionic arm of the zipper prevents the probe from entering cells, by blocking the cell-penetrating function of the polycation, (iv) the polycationic arm enhances cellular uptake of the photosensitizer after linker cleavage, and (v) quenching is no longer dependent upon the natural folding of the peptide linker, since the zipper is solely responsible for silencing the aPS activity. In the presence of a target protease, the peptide linker is specifically cleaved, causing the quencher-conjugated polyanion to dissociate from the photosensitizer-attached polycation, resulting in unquenching and polycation-enhanced photosensitizer delivery to target cells. The challenge of the zipper aPS is to balance maximal quenching efficiency with optimal two-step activation (protease cleavage and zipper dissociation), while enhancing target cell uptake. The zipper concept is a general approach to improve the functionality of a wide range of aPS through simple switching of substrate sequences. The increased selectivity, fluorescent production, and targeted uptake of a

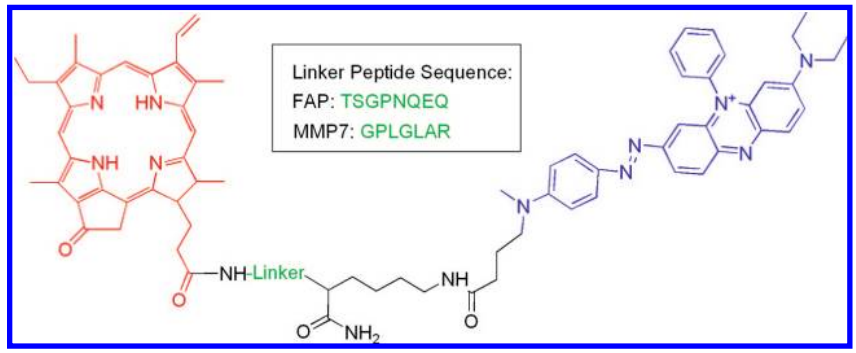

Figure 18. Modularity in activatable photosensitizer design. Simply by changing the amino acid cleavage sequence, different enzymes may be targeted.[70,75] Pyropheophorbide-*a* (red) was linked to BHQ3 (blue) via the FAP or MMP-7 enzyme-specific sequences shown (green).

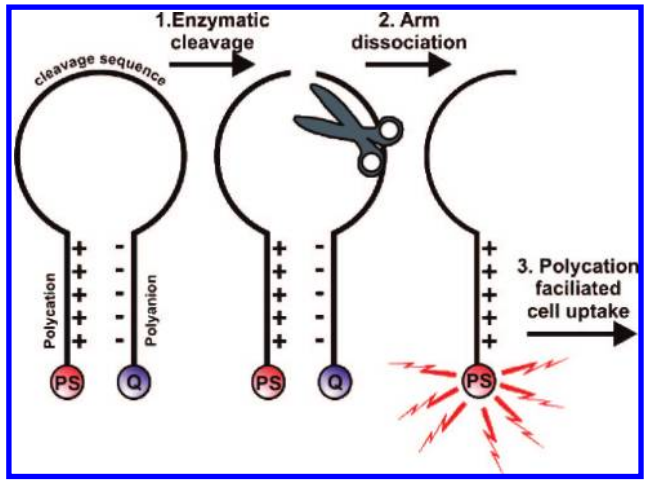

Figure 19. Peptide zipper-based control of an activatable photosensitizer.[77] To eliminate quenching efficiency variability due to sequence-specific effects, polycation and polyanion arms were used to hold the photosensitizer (red) and quencher (blue) close together through ionic interaction. Upon enzymatic cleavage of the target sequence, the photosensitizer and quencher dissociate, leading to increased singlet oxygen production. The photosensitizer remains tethered to the polycation arm, which was shown to increase cellular uptake.

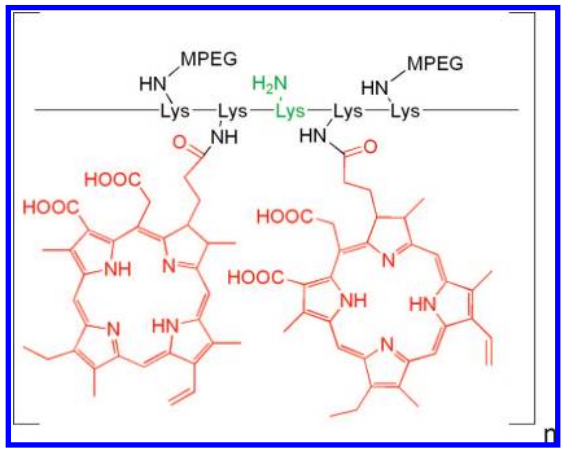

Figure 20. Representative segment of a chlorin-e_6 polymeric protease activatable photosensitizer.[79,80] Chorin-e_6 photosensitzizers (red) were conjugated to a polylysine backbone. Methoxy polyethylene glycol (MPEG) was also conjugated to improve photosensitizer solubility. Enzymatic cleavage at free lysine residues (green) resulted in a loss of self-quenching and increased singlet oxygen generation.

zipper aPS could lead to more effective tumor destruction while eliminating collateral damage.

Table 4. Effects of Photosensitizer Substitution on Polylysine-Based aPS

pyropheophorbide-*a* substitution on a 25 kDa polylysine backbone[84]				chlorin substitution on a 30% PEGylated, 48 kDa polylysine backbone[79]	
PS/chain	water solubility (mM)	quenching factor	fold activation	PS/chain	fold activation
1	>10	1	1	1	1
6	>10	35	6	6	2
12	8.2	46	19	15	4.2
18	1.3	131	12	36	1
24	0.2	146	8		
30	0.01	628	6		

Several studies have used polymeric aPS that are activated by enzymes. The first reported peptide-based polymeric aPS deserves special recognition as the first description of an enzyme-based aPS.[78] This aPS made use of chlorin-e_6 self-quenching with a methacrylamide backbone, and the photosensitizer was activated with cathepsin B. A similar approach was used to generate a polylysine-based chlorin-e_6 aPS.[79] The polylysine class of aPS contains a multitude of lysine active sites, an amino acid that forms a cleavage location for several different proteases. The reactive amine groups also provide accommodation for conjugation to multiple photosensitizers, increasing the payload of the aPS. However, a balance is required to maintain unmodified lysine

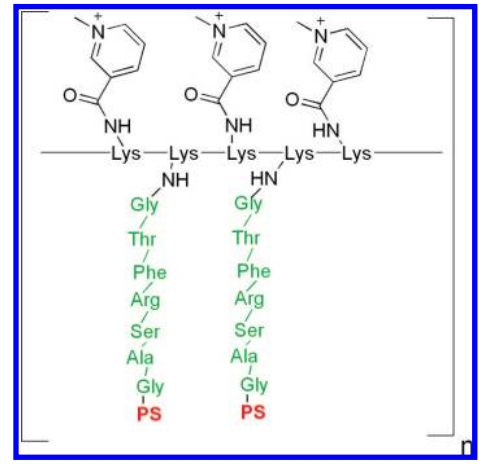

Figure 21. Representative segment of an amino acid sequence-specific polylysine-based activatable photosensitizer.[142] By attaching photosensitizer-conjugated peptides (green) to a polylysine backbone, specific amino acid sequences may be used for activation targets. To avoid nonspecific cleavage at lysine residues, free lysines were capped with a derivatized nicotinic acid with a cationic quaternary amine group.

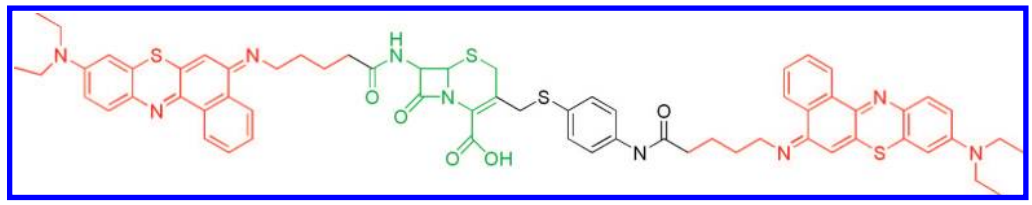

Figure 22. β-lactamase activatable photosensitizer.[87] Two EtNBD photosensitizer moieties (red) were linked via a beta lactam ring (green) and demonstrated self-quenching. Upon ring cleavage by beta lactamase, the photosensitizers became unquenched and increased singlet oxygen production.

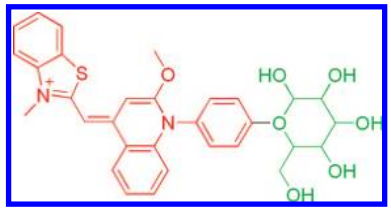

Figure 23. β-galactosidase activatable photosensitizer.[88] The photosensitizer thiazole orange (red) was conjugated to galactose (green). In cells, cleavage of the galactose by β-galactosidase allowed better DNA binding of the photosensitizer and an increase in singlet oxygen generation.

residues for enzymatic activation site and aPS water solubility. In the aPS shown in Figure 20, the aPS was optimized with a variable number of substituted photosensitizers, along with 5 kDa PEG moieties attached to 30% of the lysine residues. Different chlorin-e_6 substitution ratios led to an optimal ratio of 15 photosensitizers per aPS. Upon incubation with trypsin, the aPS increased fluorescence and singlet oxygen production 4.2- and 5.4-fold, respectively. Using cathepsin B as a target, this same construct was used in an in vivo xenograft model and light treatment resulted in attenuated tumor growth.[80] Like MMPs, cysteine cathepsins are upregulated and play an important role in a variety of cancers.[81] Although cathepsin B is primarily a lysosomal enzyme, cancer cells are known to display extracellular cathepsin B activity as well, which makes it a good aPS target.[82,83]

Thorough study in optimizing polylysine aPS parameters elucidated that, while PEGylatation is useful for enhanced aPS solubility, it is detrimental to self-quenching.[84] PEGylated polylysine-based aPS were determined to be 6-fold more fluorescent than non-PEGylated ones, showing that PEGylation may interfere with the photosensitizer interactions that give rise to self-quenching. As a PEG replacement, a derivatized nicotinic acid with a cationic quaternary amine group was found to effectively improve aPS water solubility, without interfering with quenching efficiency. Table 4 illustrates the balance that must be found when using polylysine-based aPS. When the substitution ratio is too high, enzyme activity and water solubility suffer, whereas a low substitution ratio leads to a low quenching factor.

One limitation of standard polylysine aPS is that using lysine as the linker restricts enzyme specificity to proteases that cleave at lysine residues. To extend the specificity of polylysine aPS, an approach was taken that attached short peptides to the polylysine backbone. As shown in Figure 21, by attaching the photosensitizer to the polylysine backbone via conjugated peptides, arbitrary peptide sequences can be used for the aPS.[142] In this example, free lysine groups were again capped with derivatized nicotinic acid to improve solubility and to prevent nonspecific aPS activation. The sequence used was specific for trypsin and chymotrypsin. Tryptic digest resulted in a 34-fold increase in aPS fluorescence. However, when the single arginine active site was replaced with an unnatural D-amino acid, no enzyme activation was observed. This approach was recently extended to thrombin activation.[85]

While nearly all enzyme-based aPS have been based on proteases, there are some recent and noteworthy exceptions. PDT has been explored as a viable antimicrobial therapy.[6,86] However, improving treatment discrimination against various bacteria remains a challenge. To generate a smarter antimicrobial approach, Zheng et al. targeted beta-lactamase, which is responsible for ampicillin resistance, as an activating enzyme.[87] This is a powerful strategy, since bacteria not producing this resistance enzyme will be destroyed by a standard ampicillin antibiotic treatment, but the bacteria that are resistant will then be susceptible to PDT using the aPS. The aPS was generated by fusing two 5-ethylamino-9-diethylaminobenzo(a)phenothiazinium (Et-NBS) derivatives together via a beta lactam ring (Figure 22). The intact dimer had a 5-fold quenched fluorescence yield. Antibiotic resistant strains of *S. aureus* were effectively destroyed using this approach. Another enzyme that has been used as an activator is beta-galactosidase, a widely used reporter enzyme that

Table 5. Enzyme-Activated Photosensitizers

enzyme	bioactive link	photosensitizer	quencher	fold activation	ref.
β-galactosidase	β-galactosidase	thiazole orange	DNA-induced change	cells only	88
β-lactamase	β-Lactam ring	EtNBS	self-quenching	5	87
Caspase 3	GDEVDGSG peptide	pyropheophorbide-*a*	caretenoid	3	43
Caspase 3	GDEVDGSG peptide	pyropheophorbide-*a*	caretenoid	4	72
Caspase 3	GDEVDGSG peptide	pyropheophorbide-*a*	BHQ3	6	73
Cathepsin B	GFLG peptide, methacrylamide polymer	chlorin-e_6	self-quenching	5	78
Cathepsin B	PEGylated polylysine	chlorin-e_6	self-quenching	6	80
FAP	TSGPNQEQ peptide	pyropheophorbide-*a*	BHQ3	200	75
MMP7	GPLGLAR peptide	pyropheophorbide-*a*	BHQ3	19	70
thrombin	GFPIPRSGGGGG peptide, modified polylysine backbone	pheophorbide-*a*	self-quenching	114	85
trypsin	polylysine	pheophorbide-*a*	self-quenching	19	84
trypsin	PEGylated polylysine	chlorin-e_6	self-quenching	5	79
trypsin	TPRSA peptide, modified polylysine backbone	pheophrobide-*a*	self-quenching	34	142

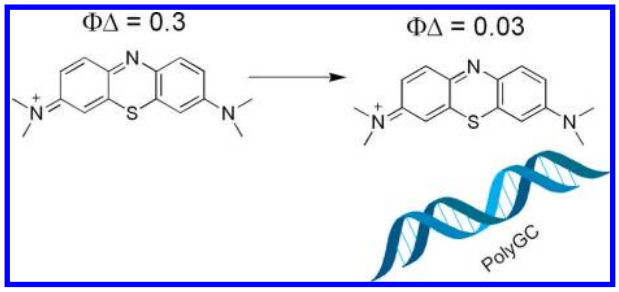

Figure 24. DNA binding-induced decrease in methylene blue singlet oxygen generation.[93] When incubated with GC-rich DNA, methylene blue demonstrated a decreased singlet oxygen quantum yield.

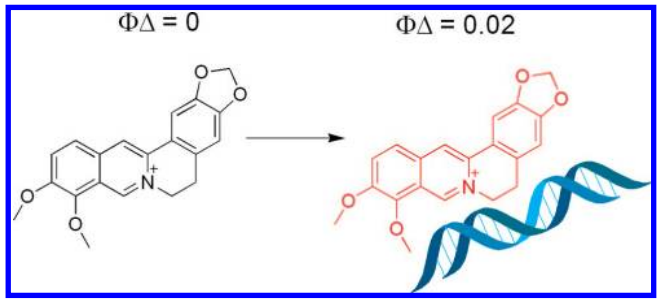

Figure 25. DNA binding-induced increase in photosensitizer singlet oxygen generation.[97] Berberine displayed an increase in singlet oxygen quantum yield upon binding calf thymus DNA.

cleaves the galactose sugar ring. An aPS was generated by fusing galactose to the thiazole orange photosensitizer (Figure 23).[88] Thiazole orange strongly increases fluorescence when bound to nucleic acids. By introducing the galactose moiety, DNA binding was hindered in cells, preventing full activation of the photosensitizer. When this aPS was incubated with cells expressing beta-galactosidase, the thiazole orange was liberated and the aPS could effectively bind nucleic acids and increase fluorescence and singlet oxygen production.

The diverse reactions that enzymes catalyze make them good targets for aPS. Proteases in particular have a role in a wide variety of diseases and have been chosen as aPS targets. As seen in Table 5, many enzyme-activated aPS have been developed. Most exhibit strong enzymatic activation that make them suitable for further study. It is noteworthy that many of these aPS have been validated in tissue culture cells and several have been validated in vivo in xenograft models.

3.3. Nucleic Acid-Activated Photosensitizers

Nucleic acids have demonstrated utility for regulating the activation of photosensitizers. Robust synthesis and well-characterized base pairing permits reliable and precise control over nucleic acid-based aPS. The potential implications of such aPS are compelling. Because gene mutations or altered gene expression lie at the heart of almost all diseases, nucleic acid aPS could form the basis of PDT that removes unwanted cells expressing specific genes and discriminating even single-base mismatches. Cellular delivery of the highly charged nucleic acid-conjugated aPS will be a barrier to in vivo testing and clinical implementation. However, this is the same challenge faced by antisense and siRNA therapeutics, areas where improvements in cellular nucleic acid delivery are rapidly being developed.[89−91] These developments will be applicable to nucleic acid-based aPS. For cell and in vivo work, normal nucleic acids are prone to degradation, and therefore, chemically modified backbones and bases are required.[92] Nucleic acid aPS can regulate singlet oxygen in a wide variety of manners.

Several reports have shown that photosensitizers can increase or decrease singlet oxygen production simply upon direct binding to nucleic acids. As demonstrated in Figure 24, methylene blue has been shown to be quenched about 10-fold by guanine- and cytosine-rich oligonucleotides but not by those containing adenine and thymine.[93] A cationic photosensitizer that can bind with DNA, meso-tetra(methylpyridinium) porphyrin (TMPyP), has also been shown to be quenched up to 2-fold by short oligonucleotides.[94] Upon DNA binding, TMPyP undergoes a characteristic Soret band red shift and displays a reduced fluorescence and triplet yield. These properties have been used to assess the DNA binding status of TMPyP during PDT.[95] Upon TMPyP incubation and light treatment, the fluorescence of TMPyP increased, along with the amount of singlet oxygen it generated, suggesting that TMPyP dissociated from the DNA in the nucleus of the cell upon photodamage. While the singlet oxygen yield of methylene blue and TMPyP is quenched upon DNA binding, other photosensitizers behave in the opposite manner. Berberine and palmatine, two isoquinoline alkaloids, possess a low fluorescence in water and drastically increase their fluorescence upon addition of DNA.[96] Subsequent photoirradiation generates singlet oxygen sufficient to induce DNA degradation. Further studies using direct singlet oxygen luminescence measurements showed that these two photosensitizers have negligible singlet oxygen quantum yields that increased to approximately 0.02 upon DNA binding (Figure 25).[97] However, even in the DNA bound state, this is a relatively low singlet oxygen quantum yield that may require higher light doses to achieve sufficient singlet oxygen generation.

While aPS that are modulated by general DNA binding may serve some roles, to realize the benefits of nucleic acid

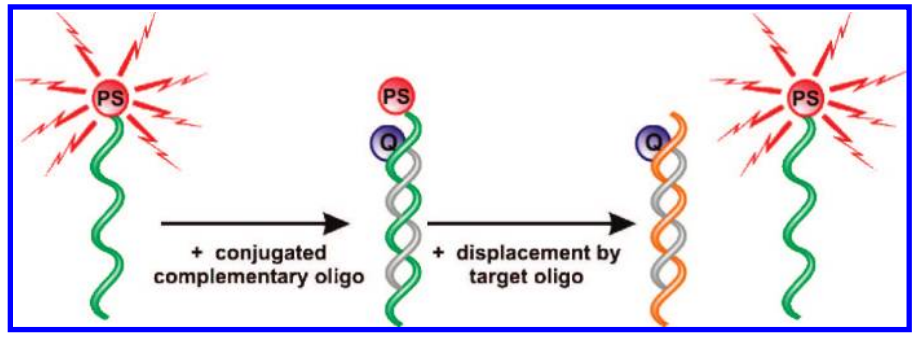

Figure 26. Activatable photosensitizer based on target hybridization strand displacement.[52] A photosensitizer (red) covalently attached to a nucleic acid (green) is hybridized to a complementary quencher-conjugated strand (blue). Upon exposure to the target nucleic acid (orange), the photosensitizer attached strand is displaced, leading to unquenching and singlet oxygen generation.

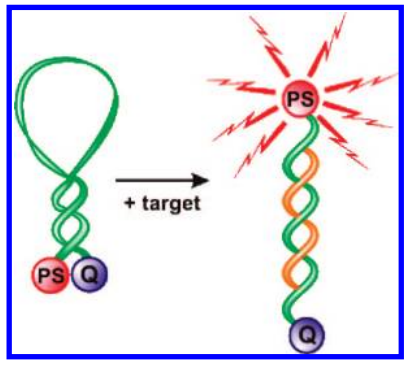

Figure 27. Design of a molecular beacon activatable photosensitizer.[71,103] A complementary stem portion maintains the photosensitizer (red) and quencher (blue) close together until a nucleic acid target (orange) binds to the loop portion of the beacon. Upon target hybridization, quenching efficiency decreases and singlet oxygen production increases.

sequence-specific targeting, a functionalized photosensitizer design is required. As shown in Figure 26, one novel approach is to use a reverse hybridization strategy.[52] A photosensitizer is linked to an oligonucleotide sequence sharing the same sequence as the target. Upon addition of a quencher-conjugated complementary oligonucleotide, the two strands hybridize, forcing the photosensitizer and quencher into close contact and attenuating the singlet oxygen signal. This quenched hybrid comprises the aPS. Upon interaction with the target nucleic acid, the photosensitizer-linked strand is displaced, resulting in photosensitizer unquenching and singlet oxygen generation. To ensure efficient displacement of the photosensitizer strand, a longer quencher strand and target strand may be used, facilitating the formation of the activated state even at an equimolar target-to-aPS ratio.

Since their inception, molecular beacons have proven indispensible for a wide range of applications. Conventional molecular beacons have a stem-loop structure with a quencher and fluorophore that are held together closely by the hybridizing stem structure.[98,99] Target oligonucleotide binding to the loop portion then forces the stem apart, leading to beacon activation. Several reports have shown that molecular beacons are capable of imaging mRNA inside living cells, including mRNA distribution in oocytes,[100] mRNA transport into the nucleus,[101] and viral mRNA behavior of the poliovirus.[102] Molecular beacon architecture has been extended to aPS (Figure 27). A pyropheophorbide-*a* photosensitizer was held in place next to a carotenoid quencher by a 6-base stem with a loop portion specific for the cRaf-1 oncogene.[71] This aPS used a modified 2′-*O*-methyl backbone to avoid degradation. Upon incubation with cRaf-1 expressing cells, aPS entry into the cells was observed and was dependent on the presence of the hydrophobic pyropheophorbide-*a* photosensitizer. Once in the cell, the aPS became activated. However, a scrambled sequence aPS showed much less activation, implying specific beacon opening by cRAF-1 mRNA. PDT was also performed and showed that the aPS was capable of destroying the target cells. Another molecular beacon aPS was developed that relied on self-quenching, rather than a dark quencher.[103] To achieve quenching, the beacon held two of zinc phthalocyanine photosensitizers, conjugated to the 5′ and 3′ termini of the beacon together via a 5-base stem. This construct demonstrated good quenching and activation, with the target inducing a 45-fold increase in aPS fluorescence.

An approach to photosensitizer activation that makes use of aptamers has been developed. Aptamers are nucleic acids that bind to a given target. Aptamers can be evolved to bind a wide variety of in vivo targets with high efficiency.[29] In particular, aptamers targeted to cancer-associated molecules have been developed for drug delivery and nanotechnology applications.[104] As depicted in Figure 28, carbon nanotube binding to a photosensitizer-conjugated aptamer formed the basis of a novel aPS approach.[105] Besides binding the aptamer via ionic interactions, the carbon nanotubes could also effectively quench the photosensitizer fluorescence. Upon addition of the aptamer target, the aptamer dissociated from the carbon nanotubes and bound the target, moving the photosensitizer away from the quencher. This concept was used with a thrombin binding aptamer, although in theory it could be extended to any aptamer with sufficient affinity for its target.

Nucleic acid-controlled activation of photosensitizers has shown to be specific and capable of binding to any given nucleic acid sequence. Table 6 summarizes the reported nucleic acid-based aPS.

3.4. Other Activation Mechanisms

Besides activation from environmental effects, enzymes, and nucleic acids, other notable generalized approaches have been used to generate aPS. Electrostatic assembly and cleavable bond formation, self-quenching, and multiple checkpoint controlled activation have been described as photosensitizer-activation mechanisms.

Rather than using FRET to quench photosensitizer singlet oxygen generation, electrostatic assembly has been used to induce FRET from a quantum dot to a photosensitizer, resulting in singlet oxygen production (Figure 29).[106] Electrostatic interaction between the anionic meso-tetra(4-sulfonatophenyl) porphyrin and aminoethanethiol surface-stabilized quantum dots gave rise to FRET-induced excitation

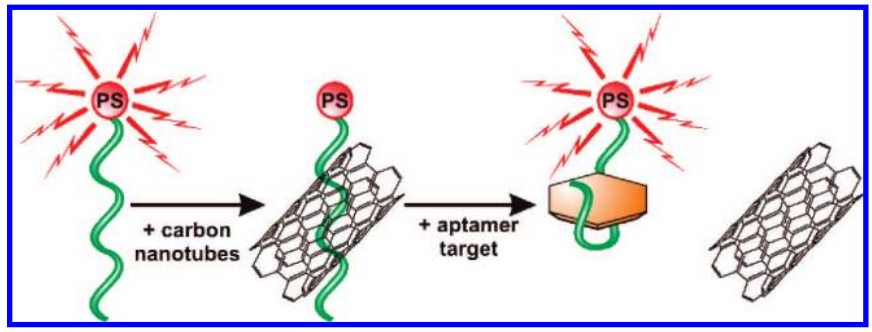

Figure 28. Aptamer-mediated activatable photosensitizer.[105] Initially, a photosensitizer (red) conjugated to an aptamer (green). The aptamer then binds a positively charged carbon nanotube, which also quenches photosensitizer singlet oxygen generation. Upon exposure to the target ligand (orange), the aptamer binds its target and separates from the carbon nanotubes, resulting in singlet oxygen generation.

Table 6. Nucleic Acid-Activated Photosensitizers

activation mechanism	target	photosensitizer	quencher	fold activation	ref.
aptamer target binding	thrombin aptamer	chlorin-e_6	carbon nanotubes	12	105
DNA binding	GC-rich oligos	methylene blue	GC-rich binding induced environment change	~90% reduction	93
DNA binding	DNA (multiple sixmers)	meso-tetra(methylpyridinium) porphyrin	DNA binding-induced environment change	~50% reduction	94
DNA binding	DNA in single cell	meso-tetra(methylpyridinium) porphyrin	DNA binding-induced environment change	change only occurs in cells	95
DNA binding	DNA (calf thymus)	berberine and palmatine	DNA binding-induced environment change	from 0 to 0.02	97
target strand displacement	CGC ACC ATA AAC CTT	pyropheophorbide-*a*	BHQ3	>20	52
molecular beacon	cRAF-1	pyropheophorbide-*a*	carotenoid	9	71
molecular beacon	GAPDH	zinc phthalocyanines	self-quenching	45	103

of the photosensitizer. This resulted in a photosensitizer singlet oxygen quantum yield of 0.41 when the quantum dot was excited. Using a similar approach, it has been shown that X-ray excitation can activate photosensitizers tethered to lanthanum fluoride nanoparticles via FRET (Figure 30).[107] X-ray activation of photosensitizers is particularly exciting since this approach has the potential to apply PDT deep into the body at any tissue depth. In another example of electrostatic interaction-based aPS, electrostatic assembly was shown to transfer energy from a cationic conjugated polyelectrolyte to a negatively charged hematoporphyrin.[108] While electrostatic assembly can regulate aPS, cleavable covalent bonds may be more robust in physiological environments. To this end, quenchers were attached to meso-tetraphenyl porphyrins via thiol-labile sulfonamide bonds.[109] Effective

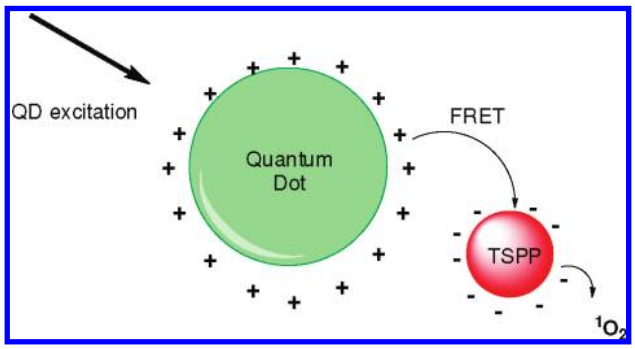

Figure 29. Electrostatic activatable photosensitizer.[106] Positively charged 2-aminoethanethiol surface-stabilized quantum dots (green) interacted with the negatively charged photosensitizer meso-tetra(4-sulfonatophenyl) porphyrin (red), resulting in FRET activation of the photosensitizer and singlet oxygen generation upon quantum dot excitation with light.

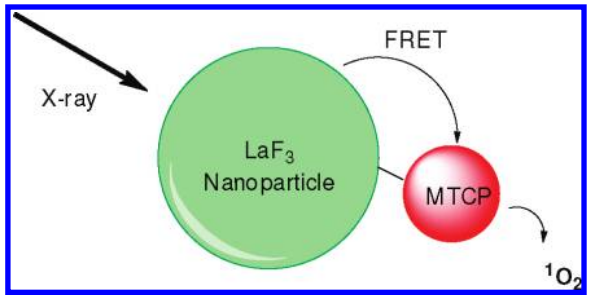

Figure 30. X-ray activation of a photosensitizer.[107] Lanthanum fluoride nanoparticles (green), which are luminescent upon X-ray excitation, were tethered to meso-tetra(4-carboxyphenyl) porphyrin photosensitizers (red). Upon X-ray excitation, energy transfer from the nanoparticles to the photosensitizer resulted in singlet oxygen production.

activation through bond cleavage was achieved with a variety of thiol compounds. Engineered sulfur bonds add another useful option to the aPS design toolbox.

Self-quenching of photosensitizers has proven useful for both enzyme and nucleic acid aPS, and it has also shown to be an independently useful mechanism. Monodisperse poly-lactic-*co*-glycolic acid polymeric nanoparticles containing the meso-tetraphenyl porpholactol photosensitizer that displayed self-quenching were synthesized.[110] These polymers displayed an 8-fold increase in fluorescence upon incubation with a lipid-containing solution. In vivo, these particles were used to treat tumors and resulted in a dramatic arrest in tumor growth. A similar self-quenching phenomena was observed when human serum albumin was adsorbed with pheophorbide-*a*.[111] Another approach to photosensitizer activation was to use two different control points, effectively functioning as a photosensitizer activation logic controller.[112] This aPS (Figure 31) was designed to respond to two important physiological parameters—salt and pH, but only when both the hydrogen ion and salt concentration were high. In this case, iodinated bodipy was attached to crown ether for salt-induced photoinduced electron transfer, as well as pyridyl groups for conferring pH sensitivity. This aPS was shown to undergo a >6-fold increase in singlet oxygen at low pH and high salt concentration, but no increase in low pH alone and only partial increase in high salt alone. Table 7 summarizes the aPS that do not fit into the categories of environment, enzyme, or nucleic acid activation.

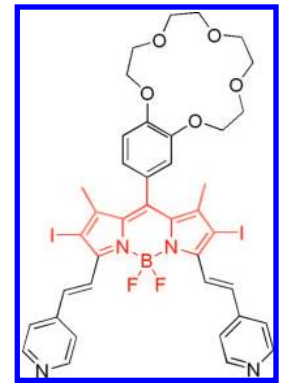

Figure 31. Logic gate functionalized activatable photosensitizer.[112] Similar to a logical AND gate, this photosensitizer only generated high amounts of singlet oxygen when it was exposed to both high salt and high [H^+] conditions together (but not either separately). The red portion shows the iodinated bodipy photosensitizer portion of the aPS.

Table 7. Photosensitizers Activated through Other Mechanisms

activating mechanism	PS	activation notes	ref.
electrostatic assembly	meso-tetra(4-sulfonatophenyl) porphyrin	Energy transfer occurred from quantum dots to photosensitizer.	106
electrostatic assembly	hematoporphyrin	Anionic porphyrin and cationic conjugated polyelectrolyte. Thirty-fold increase for 2-photon excitation, 9-fold increase for normal excitation	108
thiol-labile sulfonamide linkages	meso-tetraphenyl porphyrins	Sulfonamide linkage could be cleaved by a variety of thiol molecules including cysteine, dithiothreitol, and glutathoine.	109
salt and pH-controlled logic gate	bodipy derivative	Photoinduced electron transfer quenching-based logic switch displayed a 6-fold increase with high salt and protonation.	112
self-quenching in PLGA nanoparticles	meso-tetraphenyl porpholactol	Timed release resulted in 8-fold fluorescence increase.	110
self-quenching by adsorption to human serum albumin	pheophorbide-*a*	Photosensitizer self-quenching caused 7-fold $^1O_2$ decrease	111

4. Conclusion and Outlook

Over two dozen activatable photosensitizers have been developed, with most being described in the past 5 years. Activatable photosensitizers can potently and specifically kill diseased cells that differ from normal cells with respect to their environment, enzyme expression, or nucleic acid expression. The intrinsic fluorescence of activatable photosensitizers not only allows for convenient estimation of singlet oxygen production status but also permits useful in vivo imaging. The coming years will be an exciting time for aPS development. Certainly, new photosensitizer activation mechanisms will be discovered. Conversions of fluorescence imaging probes to activatable photosensitizers can occur from a wide pool of activatable fluorophores. Most importantly, it is imperative that these new photosensitizers are tested and validated in vitro and in vivo as PDT agents so we can move toward clinical implementation. Activatable photosensitizers have progressed remarkably in a short period of time, but much work is required so they can fulfill their potential.

5. Abbreviations

aPS	activatable photosensitizer(s)
ALA	5-aminolevulinic acid
BHQ	black hole quencher
BDP-MA	benzoporphyrin derivative monoacid ring A
DCM	dichloromethane
DIC	differential interference contrast
DIPEA	diisopropylethyl amine
DMAPV	(*E*,*E*)-2,5-dibromo-1,4-bis[2-(4′-dimonomethyl-ether triethylene glycol aminophenyl)vinyl] benzene
DNA	deoxyribonucleic acid
EDC	ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et-NBS	carboxybutylamino diethylaminobenzo phenothiazinium
EPR	enhanced permeability and retention
FAP	fibroblast activating protein
FMOC	fluorenylmethyloxycarbonyl chloride
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
HOBt	1-hydroxybenzotriazole
HBTU	2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	high-performance liquid chromatography
IP3	inositol triphosphate
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MTEGPV	(*E*,*E*)-2,5-dibromo-1,4-bis[2-(4′-dimonomethyl-ether triethylene glycol aminophenyl)vinyl] benzene
MTCP	meso-tetra(4-carboxyphenyl) porphyrin
Mtt	4-methyltrityl
mRNA	messenger ribonucleic acid
NHS	*N*-hydroxysuccinimide
NIR	near infrared
PEG	polyethylene glycol
PET	positron emission tomography
PDT	photodynamic therapy
PS	photosensitizer
Q	quencher
siRNA	small interfering ribonucleic acid
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TMPyP	meso-tetra(methylpyridinium) porphyrin
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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