New Porphyrin Architectures for Biomedical Applications

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

From the green chlorophyll in plants and algae that we depend upon to transform sunlight into useful energy, to the red heme that carries oxygen to the cells in our bodies, porphyrins are the colors of life. It is not surprising then, that porphyrins have been actively interrogated as tools for diverse applications to improve biotechnology and medicine. With the goal of improving cancer therapy and diagnosis in mind, this thesis examines new modular porphyrin architectures. These constructs have interesting properties that extend beyond their originally intended use as phototherapeutic agents. In Chapter 1, a comprehensive background on porphyrin-based activatable photosensitizers is presented. In Chapter 2, porphyrin singlet oxygen and fluorescence quenching is examined in a model system with respect to Förster theory. Chapter 3 examines a new DNA responsive molecular beacon that was quenched using multiple quenching moieties and its application for nanoparticle aggregation. Chapter 4 describes extremely self-quenched nanovesicles formed from porphyrin-lipid conjugates that displayed a number of desirable properties for nanomedicine applications. Chapter 5 presents a brief discussion and some potential future directions of the research. It is my hope that the data presented in this thesis set the stage for new porphyrin-based approaches to make a translational impact in the battle against cancer and other diseases.
Acknowledgments

First, I must thank Dr. Gang Zheng for being a wonderful supervisor, confidant, friend, and scientific free spirit. From when I first set foot in your office, it has been remarkably exciting and enjoyable learning from you about science and everything else. Thanks to my committee members Dr. Warren Chan and Dr. Linda Penn for guidance and support over the past four years. I am indebted to everyone in the lab for making research so eye-opening and working in the lab so enjoyable (Andrew, Andy, Arash, Aron, Bernice, Ben, Chao, Cheng, Gigi, Ian, Jiyun, Juan, Ken, King, Lili, Liz, Mi, Mike, Mojdeh, Natalie, Qiaoya, Steven, TD, Tracy, Xin, Zhihong, and everyone else). To my family and friends, I love you deeply and none of this would have been possible without your support. And finally, thanks to Chab and Sunny, for all the past and future adventures, good times and precious memories.
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Abbreviations used

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’-dimonomethylether triethylene glycol aminophenyl)vinyl] benzene

DNA: Deoxyribonucleic acid

EDC: Ethyl-3-(3-dimethylaminopropyl)carbodiimide

Et-NBS: carboxybutylamino diethylaminobenzo phenothiazinium

EPR: Enhanced permeability and retention

EYPC: Egg yolk phosphatidylcholine

FAP: fibroblast activating protein

FMOC: Fluorenylmethyloxycarbonyl chloride

FRET: Förster resonance energy transfer

GFP: Green fluorescent protein

HDL: High-density lipoprotein
HOBt: 1-Hydroxybenzotriazole

HBTU: 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HPLC: High-performance liquid chromatography

IP3: Inositol triphosphate

LDL: Low-density lipoprotein

MMP: Matrix metalloproteinase

MRI: Magnetic resonance imaging

MTEGPV: (E,E)-2,5-dibromo-1,4-bis[2-(4'-dimonomethylether 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

PTT: Photothermal therapy

Q: Quencher
RF: Radio frequency

siRNA: Small interfering ribonucleic acid

TFA: Trifluoroacetic acid

TIS: Triisopropylsilane

TLC: Thin layer chromatography

TMPyP: Meso-tetra(methylpyridinium) porphyrin

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
Chapter 1
Activatable Photosensitizers for Imaging and Therapy

1.1 Acknowledgments
In large part, this chapter is a reformatted version of the manuscript entitled “Activatable Photosensitizers for Imaging and Therapy”, published in *Chemical Reviews*. Under the supervision of Gang Zheng, my contributions to the manuscript included writing most of the manuscript text, while receiving valuable contributions from Tracy Liu and Juan Chen.

1.2 Introduction

1.2.1 Photodynamic Therapy
PDT is a minimally invasive treatment that destroys target cells in the presence of oxygen when light irradiates a photosensitizer, generating large quantities of reactive oxygen species. These overcome enzymatic defense mechanisms against these species, and singlet oxygen in particular 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 due to its minimally invasive nature, selectivity, the ability to treat patients with repeated doses without initiating resistance or exceeding total dose limitations (as associated with radiotherapy), 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. Over the past decade, the clinical use of PDT has greatly increased. Current clinical applications of PDT include the treatment of solid tumours in skin (basal cell carcinomas), lung, esophagus, bladder, head and neck, brain, ocular melanoma, ovarian, prostate, renal cell, cervix, pancreas and bone carcinomas. Dysplasias, papillomas, rheumatoid arthritis, actinic
keratosis, cosmetics, psoriasis, neovascularization in age associated macular degeneration, endometrial ablation, port wine stains, atherosclerotic plaques, and prophylaxis of arterial restenosis have also been treated clinically using PDT.\textsuperscript{5,6} The use of PDT to treat bacterial and fungal infections has been explored for over 30 years.\textsuperscript{7} Table 1-1 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 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.

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<td>Approved: Palliative head and neck cancer.</td>
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<td>Metvix (5-aminolevulinic acid methyl ester)</td>
<td>Approved: Actinic keratoses, superficial basal-cell carcinoma and basal-cell carcinoma.</td>
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<tr>
<td>Le-Tex (lutetium texaphyrin)</td>
<td>Clinical trials: Prostate cancer and coronary artery disease.</td>
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<tr>
<td>NP66 (mono-L-aspartyl chlorin-e\textsubscript{a})</td>
<td>Approved: Early lung cancer.</td>
</tr>
<tr>
<td>Photochlor (Hexyl ether pyropheophorbide-a derivative)</td>
<td>Clinical trials: Lung carcinoma, basal cell carcinoma’s, Barrett’s esophagus.</td>
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<tr>
<td>Photolon (chlorine-e\textsubscript{a}-polivinylypyrrolidone)</td>
<td>Approved: Malignant skin and mucosa tumors, myopic maculopathy, central choroidal neovascularization.</td>
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<tr>
<td>Photosens (aluminum phthalocyanine)</td>
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<tr>
<td>Tookad (palladium-bacteriochlorophyrine-a)</td>
<td>Clinical trials: Prostate cancer.</td>
</tr>
<tr>
<td>Visudyne (benzoporphyrin derivative</td>
<td>Approved: Age-related macular degeneration, subfoveal choroidal, neovascularisation.</td>
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\textsuperscript{a: Approval status varies regionally. Consult references for further details.\textsuperscript{5,8-13}}

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 an appropriate 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 a process that has varying efficiency, depending on the photosensitizer. In the excited triplet state, the photosensitizer can generate reactive species through two mechanisms, Type I and Type II processes. 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. 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. 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 conducive to necrosis. PDT can cause acute local inflammation, inducing an immune response against cancer cells.

1.2.2 Imaging with Photosensitizers
Photosensitizers are not restricted solely to therapeutic generation of singlet oxygen and reactive oxygen species. 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 spectrum 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, 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. 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 non-target 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 off-target 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. Many aPS rely on the same mechanisms as fluorescent counterparts. For instance, a fluorophore-linker-quencher activation scheme could be nearly identical to a photosensitizer-linker-quencher activation scheme, with the only difference being in the fluorophore or quencher that is conjugated. 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. Radiolabelled photosensitizers and MRI contrast agent conjugated photosensitizers have been described and provide a multifunctional probe with the capabilities of two imaging modalities (fluorescence imaging and PET/MRI) as well as therapeutic function.\textsuperscript{17,18}

1.2.3 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.\textsuperscript{19,20} 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.\textsuperscript{21} 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.\textsuperscript{22} 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, personal medicine requires improved generalized methods to directly target these gene or gene products. As we will discuss, certain aPS can be activated by certain genes 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 (e.g. red-shifted for deeper tissue penetration).\textsuperscript{23} Clinical photosensitizer delivery to target tissues is currently a passive process. The limitations of passive delivery are that it 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 conjugation to targeting antibodies.\textsuperscript{24,25} 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.\textsuperscript{26} Lipoproteins can be loaded with many photosensitizers in their core and then be targeted to cells that express specific receptors.\textsuperscript{27,28} 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.\textsuperscript{29,30} 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.\textsuperscript{31} 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.\textsuperscript{32} Enhancement of photosensitizer targeting has been achieved through conjugation to various targeting peptides, including the RGD peptide and the VEGF targeting peptide.\textsuperscript{33,28} Like peptide-directed targeting, aptamer-based nucleic acid targeting is also a powerful targeting technique.\textsuperscript{34,35} Photosensitizer-aptamer conjugates have been developed that enhance photosensitizer delivery to cancer cells.\textsuperscript{36} While most targeting approaches attempt to bring the photosensitizer to the diseased cells directly, another approach is vascular photosensitizer targeting.\textsuperscript{37} 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.\textsuperscript{38,39} 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.\textsuperscript{40,41} aPS are a special class of photosensitizers that are turned on by a wide variety of molecular stimuli, resulting in increased generation of reactive oxygen species. 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 1-1, along with photosensitizer delivery to oxygenated tissues and light placement, molecular activation adds a third layer of specificity to PDT targeting of diseased cells. Molecular actiation refers to a physical or chemical change that increases the fluorescence and singlet oxygen yield of the photosensitizer. While photosensitizer and light delivery are indispensible for singlet oxygen generation, activatable

![Pyro-BHQ1 and Pyro-BHQ3 structures](image)
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 by photosensitizers activated in diseased cells does not significantly affect surrounding healthy tissue as singlet oxygen has a limited diffusion distance between 10 and 300 nm, according to different estimates.\textsuperscript{43-45} Therefore, only when the photosensitizer reaches the diseased tissue, when characteristics of the diseased tissue (e.g. overexpression of certain enzymes) 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.

1.3 Activatable photosensitizer design considerations

1.3.1 Activation strategy
Singlet oxygen generation and deactivation by conventional photosensitizers have been the focus of extensive research.\textsuperscript{46} An aPS must generate increased singlet oxygen 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 1-2, 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. Contact quenching and solvent effects can also influence the next deactivation point, internal conversion. Internal conversion is a non-radiative process in which the excited molecule returns to the ground state through heat release. Quenching through Förster Resonance Energy Transfer (FRET), photoinduced 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. 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. FRET deactivation of photosensitizers is useful for aPS design. FRET is a non-radiative 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 1-3.\textsuperscript{42} To better understand FRET deactivation of singlet oxygen, different quenchers with varying amount of spectral overlap with the fluorescence emission of a photosensitizer, pyropheophorbide-\textit{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 not only useful 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.\textsuperscript{42} 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 quencher for another covalently linked photosensitizer (Figure 1-4).\textsuperscript{50} Different metal complexes had varying
degrees of quenching efficiency on 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.

1.4 Photosensitizer selection
Selection of an appropriate photosensitizer is of paramount importance for aPS design. Factors which 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 due to their strong singlet oxygen quantum yields and well known chemistries. 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, the solubility of the compound, and may affect the activation kinetics of the drug.

Table 1-2 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 large extinction coefficients to the order of $10^5$ or $10^6$ M$^{-1}$cm$^{-1}$ of these photosensitizers renders 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 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.\cite{52} 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.

### 1.5 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 1-5. In this scheme, the photosensitizers require carboxylic acid functional groups. Many other diverse conjugation strategies have been demonstrated, including thiol,\(^{53}\) isothiocyanate,\(^{54}\) enyne metathesis,\(^{55}\) and click\(^{56}\) chemistries to conjugate photosensitizers to other molecules.
<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Abs. Peaks(s) (nm)</th>
<th>Extinction Coefficient(s) M⁻¹cm⁻¹</th>
<th>Em. Peak (nm)</th>
<th>ΦF</th>
<th>Φ Δ</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum phthalocyanine tetrasulfonate</td>
<td>676</td>
<td>169,000</td>
<td>684</td>
<td>0.51⁺ 0.38⁻</td>
<td>57-59</td>
<td></td>
</tr>
<tr>
<td>Bacteriochlorophyll-α</td>
<td>360 / 770</td>
<td>66,000 / 71,000</td>
<td>788</td>
<td>0.14 0.35⁻</td>
<td>57,60</td>
<td></td>
</tr>
<tr>
<td>Benzoporphyrin derivative (BDP-MA)</td>
<td>428 / 686</td>
<td>76,000 / 34,000</td>
<td>692</td>
<td>0.05 0.76</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Chlorin-e₆</td>
<td>402 / 663</td>
<td>150,000 / 59,000</td>
<td>667</td>
<td>0.19 0.65</td>
<td>57,62-64</td>
<td></td>
</tr>
<tr>
<td>Iodinated bodipy</td>
<td>534</td>
<td>110,000</td>
<td>548</td>
<td>0.02 ~ 1</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Methylene blue</td>
<td>665</td>
<td>91,000</td>
<td>685</td>
<td>0.02 0.55</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Porphycene</td>
<td>358 / 630</td>
<td>139,000 / 52,000</td>
<td>640ᵀ</td>
<td>0.44 0.34</td>
<td>57,66-68</td>
<td></td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>402 / 626</td>
<td>40,000 / 3000</td>
<td>633</td>
<td>0.16 0.56⁺</td>
<td>57,69,70</td>
<td></td>
</tr>
<tr>
<td>Pyropheophorbide-α analogs</td>
<td>410 / 665</td>
<td>97,000 / 46,000</td>
<td>672</td>
<td>0.43⁺ 0.45⁺</td>
<td>42,71</td>
<td></td>
</tr>
<tr>
<td>Rose bengal</td>
<td>560</td>
<td>90,000</td>
<td>575</td>
<td>0.11 0.68</td>
<td>72,57</td>
<td></td>
</tr>
<tr>
<td>Tetraphenylporphyrin</td>
<td>419 / 647</td>
<td>470,000 / 3400</td>
<td>653</td>
<td>0.11 0.63</td>
<td>73-75</td>
<td></td>
</tr>
<tr>
<td>Silicon Naphthalocyanine</td>
<td>347 / 781</td>
<td>134,000 / 327,000</td>
<td>780</td>
<td>0.17 0.33</td>
<td>57,76,77</td>
<td></td>
</tr>
<tr>
<td>Meso-tetra(4-methoxyphenyl) porphyrin</td>
<td>420 / 650</td>
<td>460,000 / 4800</td>
<td>658</td>
<td>0.14 0.65</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Lutetium texaphyrin</td>
<td>470 / 733</td>
<td>68,000 / 23,000</td>
<td>747</td>
<td>0.01 0.23⁺</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Zinc phthalocyanine</td>
<td>672</td>
<td>150,000</td>
<td>676</td>
<td>0.06 0.62</td>
<td>23,80</td>
<td></td>
</tr>
<tr>
<td>Zinc Tetraphenylporphyrin</td>
<td>423 / 586</td>
<td>540,000 / 3700</td>
<td>610</td>
<td>0.03 0.73</td>
<td>57,73,81</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Data recorded in the following solvents: a = Acetone, b = Benzene, c = Dichloromethane, e = Ethanol, f = Dimethyl Formamide, m = Methanol, t = Tetrahydrofuran, u = Toluene, w = Aqueous, x = In Triton X-100 Micelles. Some data extracted from graphs or similar chemical structures.
Using amide bond chemistry, development of modular photosensitizer building blocks is simple. Figure 1-6 demonstrates the generation of a photosensitizer phosphoramidite, the standard monomer for nucleic acid synthesis.\textsuperscript{82} 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 1-7 and makes use of two separate protecting groups. Initially a peptide is synthesized using standard methods, except an N terminal 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 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 HPLC.

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure1-4.png}
\caption{Figure 1-4. Metalloporphyrin quenching of photosensitizers}
\end{figure}

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.\textsuperscript{49}
Figure 1-5. 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 1-6. Generation of a 5’terminating pyropheophorbide-a phosphoramidite fit for automated DNA synthesis

Figure 1-7. Possible synthetic approach to the solid phase peptide conjugation a photosensitizer and a quencher
The photosensitizer is shown in red, the quencher in blue and the solid phase support in gray.
Figure 1-8. pH dependent change of methylene blue and toluidine blue singlet oxygen quantum yields.\textsuperscript{83,84}

Figure 1-9. Environment induced change in the MTEGPV singlet oxygen quantum yield
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.\textsuperscript{85}

Figure 1-10. pH activatable photosensitizer based on electron transfer
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.\textsuperscript{86}

Figure 1-11. Hydrophobically activatable photosensitizer with protein targeting moiety for chromophore assisted light inactivation
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.\textsuperscript{87}

Figure 1-12. 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.

Figure 1-13. Change in palladium-bacteriopheophorbid-a singlet oxygen quantum yield in solvents of different hydrophobicity.\textsuperscript{88}
1.6 Examples of activatable photosensitizers

1.6.1 Environment activated photosensitizers

The singlet oxygen production efficiency of photosensitizers is dependent on solvent properties including pH and hydrophobicity. As shown in Figure 1-8, 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.\(^{83}\) The structurally related photosensitizer toluidine blue undergoes a similar increase in singlet oxygen upon deprotonation.\(^{84}\) Solvent hydrophobicity plays a large role in determining photosensitizer efficiency. As shown in Figure 1-13, the photosensitizer palladium-bacteriopheophorbide-\(a\) undergoes an approximate two fold change in singlet oxygen quantum yield as it moves from a hydrophobic acetone solvent to a micelle environment in deuterated water.\(^{88}\) 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.\(^{89}\) Changes in hydrophobicity and pH can occur simultaneously, yielding dramatic effects. As shown in Figure 1-9, 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.\(^{85}\) 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 the 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 pKa that are only active in protonated form, pH-activatable photosensitizers were demonstrated to effectively kill cells (Figure 1-10). This approach was extended to develop photoinduced electron transfer quenchers that are only active in hydrophobic solvents with low dielectric constants. As shown in Figure 1-11, 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 1-3, 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.

<table>
<thead>
<tr>
<th>Environmental factor</th>
<th>Environment change</th>
<th>Photosensitizer</th>
<th>Fold activation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobicity</td>
<td>Solvent dielectrics ranging from CHCl₃ to CH₃CN</td>
<td>Iodinated bodipy derivatives</td>
<td>50</td>
<td>87</td>
</tr>
<tr>
<td>pH</td>
<td>Electron transfer quencher protonation</td>
<td>Iodinated bodipy derivatives</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>pH</td>
<td>pH 5 to 9</td>
<td>Methylene blue</td>
<td>5</td>
<td>83</td>
</tr>
<tr>
<td>pH</td>
<td>pH 5 to 9</td>
<td>Toluidine blue</td>
<td>5</td>
<td>84</td>
</tr>
<tr>
<td>Solvent effects</td>
<td>From detergent micelles to acetone</td>
<td>Palladium-bacteriopheophorbide-α</td>
<td>2</td>
<td>88</td>
</tr>
<tr>
<td>Solvent and pH effects</td>
<td>Protonation and change from D₂O to toluene</td>
<td>DMAPV and MTEGPV</td>
<td>5</td>
<td>85</td>
</tr>
</tbody>
</table>

Notes: a) An extensive summary of singlet oxygen changes in detergents and various solvents is reported elsewhere.⁸⁹
1.6.2 Enzyme activated photosensitizers

Enzymes are catalytic, diverse, central to all facets of cellular function, and therefore are 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 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 drugs that target disease-related proteases exist for cancers, hypertension, myocardial infarction, periodontis, AIDS, thrombosis, respiratory disease and pancreatitis.\textsuperscript{90} 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.\textsuperscript{91} 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,\textsuperscript{92} caspases,\textsuperscript{93} secretases,\textsuperscript{94} and matrix metalloproteases.\textsuperscript{95} The discovery of a genetically encoded photosensitizer, KillerRed, opens up the possibility to develop similar fluorescent protein based aPS.\textsuperscript{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. More recently, imaging and probe advances have progressed to permit in vivo protease imaging using near infrared probes. These smart probes are moving towards clinical trials and have been validated ex vivo in human specimens suffering from carotid endarterectomy, where cathepsin protease activity was detected.

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 1-12. 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 digestion, the photosensitizers are cleaved from the backbone, separate 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 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 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.99,100

The first example of an aPS geared towards pure PDT purposes used the short peptide approach with a specific amino acid sequence targeting the caspase 3 protease.101 As shown in Figure 1-14, 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. Caspasases are the executioners of apoptosis and are generally inactive in healthy cells. While it is generally undesirable for an aPS 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.102 As shown in Figure 1-15, 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 metallo proteinase family regulates normal development, but also plays a role in the pathogenesis of cancers. MMP-7 in particular is upregulated in several cancers since the enzymatic activity of breaking down the extracellular matrix can give cancer cells a competitive advantage. The MMP-7 aPS used an enzyme specific sequence, a pyropheophorbide-a photosensitizer and a BHQ3 quencher (Figure 1-19). 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. 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 1-16, 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 as it 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.\textsuperscript{104} FAP functions as an endopeptidase that cleaves between the proline and asparagine residues of α\textsubscript{2}-antiplasmin and peptide substrates.\textsuperscript{105} Thus, an FAP aPS was generated by designing a biolinker peptide containing proline and asparagine residues (Figure 1-19). \textit{In vitro} and \textit{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 1-17, 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.

![Figure 1-14. Caspase 3 specific activatable photosensitizer.](image)

Pyropheophorbide-\textit{a} is shown in red, the caspase 3 active amino acid linker sequence is shown in green and carotenoid quencher is shown in blue.\textsuperscript{101}

![Figure 1-15. Activatable photosensitizer with both apoptosis inducing and detecting capability.](image)

A moderately quenched aPS (6 fold increase upon activation) was incubated with HepG2 cells and treated with light. 1 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 without pretreatment there was no caspase activation.\textsuperscript{102} Copyright 2006 American Chemical Society.

![Figure 1-16. Viability of cells treated with an MMP-7 activatable photosensitizer.](image)

Cells were treated with light doses of 0, 1, 5 and 7.5 J/cm\textsuperscript{2} (in white, light gray, dark gray and black, respectively) and aPS. Positive cells showed a light dose and a specific aPS response.\textsuperscript{99} Copyright 2007 National Academy of Sciences, U.S.A.

![Figure 1-17. In vivo murine imaging of activatable photosensitizers.](image)

Intratumour injection of two xenografts expressing or not expressing the FAP enzyme.\textsuperscript{103} Only the fluorescence of the photosensitizer activated by FAP is visible in red. Copyright 2009 American Chemical Society.
Figure 1-18. Peptide zipper based control of an activatable photosensitizer

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 1-19. Modularity in activatable photosensitizer design

Simply by changing the amino acid cleavage sequence, different enzymes may be targeted. Pyropheophorbide-a (red) was linked to BHQ3 (blue) via the FAP or MMP7 enzyme specific sequences shown (green).

Figure 1-20. Representative segment of a chlorin-e₆ polymeric protease activatable photosensitizer

Chlorin-e₆ photosensitizers (red) were conjugated to a polylysine backbone. 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.

Figure 1-21. Representative segment of an amino acid sequence specific polylysine based activatable photosensitizer

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 1-22. β-lactamase activatable photosensitizer

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.
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 1-19), in vitro enzyme studies revealed the FAP aPS had much greater quenching efficiency compared to the MMP7 specific aPS. This can be attributed to 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 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.\textsuperscript{106} As illustrated in Figure 1-18, the zipper aPS consists of 5 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: 1) 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, 2) 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, 3) the polyanionic arm of the zipper prevents the probe from entering cells, by blocking the cell-penetrating function of the polycation, 4) the polycationic arm enhances cellular uptake of the photosensitizer after linker cleavage, and 5) 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 the 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 zipper aPS could lead to more selective and effective tumor destruction while eliminating collateral damage.

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.\textsuperscript{110} This aPS made use of chlorin-\textit{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-\textit{e}_6 aPS.\textsuperscript{107} 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 residues for enzymatic activation site and aPS water solubility. In the aPS shown in Figure 1-20, 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-\textit{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 \textit{in vivo} xenograft model and light treatment resulted in attenuated tumor growth.\textsuperscript{108} Like
MMPs, cysteine cathepsins are up-regulated and play an important role in a variety of cancers. Although cathepsin B is primarily a lysosomal enzyme, cancer cells are known to display extracellular cathepsin B activity as well which make it a good aPS target.

A thorough study in optimizing polylysine aPS parameters elucidated that while PEGylation is useful for enhanced aPS solubility, it is detrimental to self-quenching. PEGylated polylysine based aPS were determined to be 6 fold more fluorescent than non-PEGylated ones, showing 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 1-4 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 1-21, by attaching the photosensitizer to the polylysine backbone via conjugated peptides, arbitrary peptide sequences can be used for the aPS. In this example, free lysine

<table>
<thead>
<tr>
<th>PS/chain</th>
<th>Water Solubility (mM)</th>
<th>Quenching Factor</th>
<th>Fold activation</th>
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<td>&gt;10</td>
<td>1</td>
<td>1</td>
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<td>8.2</td>
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<td>6</td>
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<td>15</td>
<td>4.2</td>
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<tr>
<td>36</td>
<td>1</td>
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</table>

Table 1-4. Effects of photosensitizer substitution on polylysine based aPS.
groups were again capped with derivatized nicotinic acid to improve solubility and to prevent non specific 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.\textsuperscript{115}

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.\textsuperscript{3,116} 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.\textsuperscript{109} 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 1-22). The intact dimer had a 5 fold quenched fluorescence yield. Antibiotic resistant strains of \textit{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 cleaves the galactose sugar ring. An aPS was generated by fusing galactose to the thiazole orange photosensitizer (Figure 1-23).\textsuperscript{117} 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 1-5, 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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bioactive link</th>
<th>Photosensitizer</th>
<th>Quencher</th>
<th>Fold activation</th>
<th>Ref.</th>
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</thead>
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<tr>
<td>β-galactosidase</td>
<td>β-galactosidase</td>
<td>Thiazole Orange</td>
<td>DNA induced change</td>
<td>In cells only</td>
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<td>Pyropheophorbide-α</td>
<td>Caretenoid</td>
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<td>Caretenoid</td>
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<td>Caspase 3</td>
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<td>BHQ3</td>
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<td>102</td>
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<td>Self-quenching</td>
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<td>110</td>
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<tr>
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<td>BHQ3</td>
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<td>Thrombin</td>
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<td>Pheophorbide-α</td>
<td>Self-quenching</td>
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<td>115</td>
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<tr>
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<td>Polylsine</td>
<td>Pheophorbide-α</td>
<td>Self-quenching</td>
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<td>114</td>
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<td>Self-quenching</td>
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<td>107</td>
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<td>Trypsin</td>
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<td>Self-quenching</td>
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**1.6.3 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 remove 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.\(^{119-121}\) 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.\(^{122}\) 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 1-24, methylene blue has been shown to be quenched about 10 fold by guanine and cytosine rich oligonucleotides, but not those containing adenine and thymine.\(^{65}\) A cationic photosensitizers that can bind with DNA, meso-tetra(methylpyridinium) porphyrin (TMPyP), has also been shown to be quenched up to 2 fold by short oligonucleotides.\(^{123}\) 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.\(^{124}\) 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.\(^{125}\) 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 1-25). 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.

**Figure 1-23. β-galactosidase activatable photosensitizer**
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.

Figure 1-24. DNA binding induced decrease in methylene blue singlet oxygen generation
When incubated with GC rich DNA, methylene blue demonstrated a decreased singlet oxygen quantum yield.

**Figure 1-25. DNA binding induced increase in photosensitizer singlet oxygen generation**
Berberine displayed an increase in singlet oxygen quantum yield upon binding calf thymus DNA.

**Figure 1-26. Activatable photosensitizer based on target hybridization strand displacement**
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 1-27. Design of a molecular beacon activatable photosensitizer**
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.

**Figure 1-28. Aptamer mediated activatable photosensitizer**
Initially, a photosensitizer (red) is conjugated 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.
While aPS that are modulated by general DNA binding may serve some roles, to realize the benefits of nucleic acid sequence specific targeting, a functionalized photosensitizer design is required. As shown in Figure 1-26, one novel approach is to use a reverse hybridization strategy. 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 indispensable 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. 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, mRNA transport into the nucleus, and viral mRNA behavior of the poliovirus. Molecular beacon architecture has been extended to aPS (Figure 1-27). A pyropheophorbide-α 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. 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-α photosensitizer. Once in the cell, the aPS became activated. However, a scrambled sequence aPS showed much less activation, implying
specific beacon opening by cRAF 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. 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 display binding to a given target. Aptamers can be evolved to bind a wide variety of in vivo targets with high binding efficiency. In particular, aptamers
targeted to cancer-associated molecules have been developed for drug delivery and nanotechnology applications.\textsuperscript{137} As depicted in Figure 1-28, carbon nanotubes binding to a photosensitizer conjugated aptamer formed the basis of a novel aPS approach.\textsuperscript{128} 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 1-6 summarizes the reported nucleic acid based aPS.

<table>
<thead>
<tr>
<th>Activation mechanism</th>
<th>Target</th>
<th>Photosensitizer</th>
<th>Quencher</th>
<th>Fold activation</th>
<th>Ref.</th>
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<td>Methylene blue</td>
<td>GC rich binding induced environment change</td>
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<tr>
<td>DNA binding</td>
<td>DNA (multiple sixmers)</td>
<td>Meso-tetra(methylpyridinium) porphyrin</td>
<td>DNA binding induced environment change</td>
<td>~50 reduction</td>
<td>123</td>
</tr>
<tr>
<td>DNA binding</td>
<td>DNA in single cell</td>
<td>Meso-tetra(methylpyridinium) porphyrin</td>
<td>DNA binding induced environment change</td>
<td>Change only occurs in cells</td>
<td>124</td>
</tr>
<tr>
<td>DNA binding</td>
<td>DNA (calf thymus)</td>
<td>Berberine and palmatine</td>
<td>DNA binding induced environment change</td>
<td>From 0 to 0.02</td>
<td>126</td>
</tr>
<tr>
<td>Target strand displacement</td>
<td>CGC ACC ATA AAC CTT</td>
<td>Pyropheophorbide-a</td>
<td>BHQ3</td>
<td>&gt;20</td>
<td>82</td>
</tr>
<tr>
<td>Molecular beacon</td>
<td>cRAF1</td>
<td>Pyropheophorbide-a</td>
<td>Carotenoid</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Molecular beacon</td>
<td>GAPDH</td>
<td>Zinc phthalocyanines</td>
<td>Self-quenching</td>
<td>45</td>
<td>127</td>
</tr>
</tbody>
</table>

1.6.4 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 1-29). Electrostatic interaction between the anionic meso-tetra(4-sulfonatophenyl) porphyrin and aminoethanethiol surface-stabilized quantum dots gave rise to FRET-induced excitation 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 1-30). 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. While electrostatic attraction 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. Effective 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 were synthesized that displayed self-quenching. 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-\(\alpha\).\(^{141}\) Another approach to photosensitizer activation was to use two different control points, effectively functioning as a photosensitizer activation logic controller.\(^{136}\) This aPS (Figure 1-31) 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 greater than 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 1-7 summarizes the aPS that do not fit into the categories of environment, enzyme or nucleic acid activation.

<table>
<thead>
<tr>
<th>Activating mechanism</th>
<th>PS</th>
<th>Activation notes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrostatic assembly</td>
<td>Meso-tetra(4-sulfonatophenyl) porphyrin</td>
<td>Energy transfer occurred from quantum dots to photosensitizer</td>
<td>134</td>
</tr>
<tr>
<td>Electrostatic assembly</td>
<td>Hematoporphyrin</td>
<td>Anionic porphyrin and cationic conjugated polyelectrolyte. 30 fold increase for 2 photon excitation, 9 fold increase for normal excitation</td>
<td>138</td>
</tr>
<tr>
<td>Thiol-labile sulfonamide linkages</td>
<td>Meso-tetraphenyl porphyrins</td>
<td>Sulfonamide linkage could be cleaved by a variety of thiol molecules including cysteine, dithiothreitol and glutathione</td>
<td>139</td>
</tr>
<tr>
<td>Salt and pH controlled logic gate</td>
<td>Bodipy derivative</td>
<td>Photoinduced electron transfer quenching based logic switch displayed a 6 fold increase with high salt and protonation</td>
<td>136</td>
</tr>
<tr>
<td>Self-quenching nanoparticles in PLGA</td>
<td>Meso-tetraphenyl porpholactol</td>
<td>Timed release resulted in 8 fold fluorescence increase</td>
<td>140</td>
</tr>
<tr>
<td>Self-quenching by adsorption to human serum albumin</td>
<td>Pheophorbide-(\alpha)</td>
<td>Photosensitizer self-quenching caused 7 fold (\text{O}_2) decrease</td>
<td>141</td>
</tr>
</tbody>
</table>

### 1.7 Conclusion and outlook
At least two dozen activatable photosensitizers have been developed, 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 mechanism 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 towards clinical implementation. Activatable photosensitizers have progressed remarkably in a short period of time, but much work is required so they can fulfill their potential.

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Chapter 2

FRET Quenching of Photosensitizer of Singlet Oxygen Generation

2.1 Acknowledgments
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2.2 Abstract
The development of activatable photodynamic therapy (PDT) has demonstrated a need for effective photosensitizer quenchers. However, little is known quantitatively about Förster resonance energy transfer (FRET) quenching of photosensitizers, even though these quenchers are versatile and readily available. To characterize FRET deactivation of singlet oxygen generation, we attached various quenchers to the photosensitizer pyropheophorbide-α (Pyro) using a lysine linker. The linker did not induce major changes in the properties of the photosensitizer. Absorbance and emission wavelength maxima of the quenched constructs remained constant, suggesting that quenching by ground state complex formation was minimal. All quenchers sharing moderate spectral overlap with the fluorescence emission of Pyro (\(J \geq\))
5.1 x 10^{13} \text{M}^{-1}\text{cm}^{-1}\text{nm}^{4}) quenched over 90 percent of the singlet oxygen, while quenchers with weaker spectral overlap displayed minimal quenching. A self-quenched double Pyro construct exhibited intermediate quenching. Consistent with a FRET deactivation mechanism, extension of the linker to a 10 residue polyproline peptide resulted in only the quenchers with spectral overlap almost two orders of magnitude higher ($J \geq 3.7 \times 10^{15} \text{M}^{-1}\text{cm}^{-1}\text{nm}^{4}$) maintaining high quenching efficiency. Overall, there was good correlation (0.98) between fluorescence quenching and singlet oxygen quenching, implying that fluorescence intensity can be a convenient indicator for the singlet oxygen production status of activatable photosensitizers. Uniform singlet oxygen luminescence lifetimes of the compounds, along with minimal triplet state transient absorption were consistent with quenchers primarily deactivating the photosensitizer excited singlet state. In vitro, cells treated with well-quenched constructs demonstrated greatly reduced PDT induced toxicity, indicating FRET based quenchers can provide a level of quenching useful for future biological applications. The presented findings show that FRET based quenchers can potently decrease singlet oxygen production and therefore be used to facilitate the rational design of activatable photosensitizers.

2.3 Introduction
Photodynamic therapy (PDT) treats diseased regions of the body with the introduction of a photosensitizer followed by intense light irradiation onto a target area. The irradiation causes the photosensitizer to generate cytotoxic singlet oxygen that destroys cells through apoptosis or necrosis\(^2\). PDT has become a clinical treatment option for ophthalmic\(^3\) and cancer related diseases\(^4\). Although careful placement and delivery of the irradiation light confers a level of selectivity to PDT greater than most other disease treatments, PDT is still limited by damage it induces in adjacent healthy tissues. Furthermore, PDT is presently not an appropriate treatment
for conditions in which diseased cells are not confined to a small and homogenous region of the body.

**Figure 2-1: Solid phase synthesis scheme used to generate quenched photosensitizer.**
Solid phase synthesis scheme used to generate quenched photosensitizer. Lysine was first conjugated to Rink resin before labeling the N-terminus of lysine with the photosensitizer and the side chain amine with the quencher. Dabcyl is shown in this example.
To overcome limitations of PDT, approaches have been developed to further minimize damage the photosensitizer inflicts in non-target cells. Photosensitizers that are cleared within hours, and not days, have been developed to prevent cutaneous phototoxicity after light treatment. However, rapidly clearing agents do not permit greater light dosage to be used or prevent damage in tissue near the treatment site. Photoimmunotherapy relies on photosensitizer-conjugated antibodies to target cancer cells overexpressing certain cell surface markers. This approach holds promise, but it is unclear how effectively the extracellular antigen-antibody complexes can be internalized so that the singlet oxygen generated by the photosensitizers may attack intracellular targets. An alternate approach of growing interest is the use of activatable photosensitizers. These molecules usually contain a photosensitizer, a singlet oxygen quencher and an active linker to regulate the activation of the photosensitizer. In the inactive state, the photosensitizer remains in a quenched conformation, and generates only a small amount of singlet oxygen upon irradiation. Cleavage or extension of the linker by the target positions the photosensitizer further from the quencher and results in greater singlet oxygen generation. Bioactive linkers such as cleavable peptide substrates or nucleic acids capable of hybridizing to specific mRNAs have been shown to increase singlet oxygen production in response to biological targets. Other activatable photosensitizers have been developed that respond to pH and to nanoparticle delivery. Remarkably, in xenograft mouse models, protease activated photosensitizers have been used in vivo to achieve recession or reduced growth of tumors. The activatable photosensitizer concept has broad potential, since in theory many activatable optical probes could be converted for PDT by substituting the fluorophore for a photosensitizer. The benefits of activatable photosensitizers are negated if singlet oxygen reduction is not sufficient in the inactive conformation, resulting in killing of cells lacking the
target. Correct quencher selection is therefore an important step in designing an activatable photosensitizer.

Previous examination of fluorophore quenchers on nucleic acid molecular beacons showed that effective fluorescence quenching occurred via both Förster Resonance Energy Transfer (FRET) and through formation of ground state complexes\(^\text{14}\). However, ground state complex formation depends on sustained and direct contact between the photoactive molecule and the quencher, and thus it is difficult to rationally design beacons relying on this mechanism. On the other hand, FRET may be predicted by spectral overlap and is effective within a predetermined range of fluorophore and quencher distances. Unlike conventional fluorophores, effective photosensitizers display a high singlet oxygen yield. While there has been broad interest in quenching singlet oxygen generated by photosensitizers\(^\text{15}\), rather than FRET based quenchers, most studies have focused on direct quenching of the generated singlet oxygen\(^\text{16}\), collisional quenching of the photosensitizer singlet\(^\text{17}\) and triplet excited state\(^\text{18}\), and quenching by ground state formation\(^\text{19}\). The efficiency that metal ions, well known fluorophore quenchers\(^\text{20}\), can quench photosensitizers after covalent conjugation in a metal substituted porphyrin has been investigated\(^\text{21}\). Carbon nanotubes\(^\text{22}\) and fullerenes\(^\text{23}\) have also been explored as photosensitizer quenchers. It has been shown that photosensitizers themselves can act as FRET based quenchers of quantum dots\(^\text{24,25}\). This suggests the fluorescence properties of photosensitizers can be leveraged like conventional fluorophores to use FRET based quenchers to attenuate singlet oxygen production. To examine this hypothesis, we synthesized photosensitizers covalently attached to quenchers with varying degrees of spectral overlap and examined their photodynamic properties.
2.4 Materials and Methods

2.4.1 Synthesis and purification of quenched constructs

Synthesis of the quenched constructs was carried out as shown in Figure 2-1. Lysine was conjugated to Rink resin (Novabiochem) after Fmoc deprotection of the resin with 20%
piperidine in \textit{N,N}-dimethylformamide (DMF) for 30 minutes. The amount of activated amine was quantified by the cleaved Fmoc absorbance at 300 nm (\(\varepsilon = 7,800 \text{ M}^{-1}\text{cm}^{-1}\)). Resin was washed with DMF and the lysine linker was conjugated to the resin using a 5 fold molar excess of Fmoc-lysine-(MTT)-OH (Novabiochem), 1-hydroxybenzotriazole (HOBT) (Sigma) and \textit{O}-\((\text{benzotriazol-1-yl})-\textit{N,N,N',N'
,tetramethyluronium hexafluorophosphate} (\text{HBTU}) (Sigma) for 3 hours in DMF with 2\% \textit{N,N}-diisopropylethyl amine (DIPEA) at room temperature. After washing the resin with DMF, the MTT group on the lysine \(\varepsilon\)-amine was removed by incubation with 2\% trifluoroacetic acid (TFA) in dichloromethane for 20 minutes. The resin was washed with DMF and Pyro (purified from biomass as described previously\textsuperscript{26}) was conjugated to the lysine \(\varepsilon\)-amine using a 2 fold molar excess of HOBT and HBTU in 2\% DIPEA overnight at room temperature. Free Pyro was washed from the solid phase with DMF and the Fmoc-lysine(Pyro) resin was dried and stored at -20 °C.

To conjugate the various quenchers, the \(\text{N}\)-terminal Fmoc protecting group of the lysine was removed with 20\% piperidine in DMF for 30 minutes. After washing the resin with DMF, 5 fold excess quencher was incubated with the resin overnight with 2\% DIPEA at room temperature. 5 fold excess HOBT and HBTU was included for quenchers conjugated via a carboxylic acid functional group. After conjugation, the resin was washed with DMF and the constructs were cleaved from the resin using 95\% TFA and 5\% triisopropylsilane (TIS) for 1 hour followed by filtration, removal of the solvent by evaporation, and resuspension of the compound in a small amount of DMSO. The following quenchers were used: Blackberry Quencher (BBQ)-NHS (Berry Associates), Black Hole Quencher 1 (BHQ1)-NHS (Biosearch), Black Hole Quencher 3 (BHQ3)-NHS (Biosearch), Dabcyl (DAB)-COOH (Anaspec), Fmoc-tryptophan (TRP)-COOH (Novabiochem) and Disperse Blue 3-carboxylic acid (DB3)-COOH
(synthesized as previously described\textsuperscript{27}). Due to potential instability in 95\% TFA, QXL-680-NHS (Anaspec) was conjugated in solution phase after cleavage of the Fmoc-lysine(Pyro) from the resin. All constructs were purified by HPLC using a Zorbax 300SB-C8 column (Agilent) with 0.1\% TFA in water and acetonitrile as an eluant from using a preparatory HPLC (Waters). The purified constructs were then identified using the same column and method on an analytical HPLC with a Micromass ESI mass spectrometry (Waters). To examine the effects of an extended spacer, a C-terminal lysine and 10 residue polyproline peptide (Fmoc-PPPPPPPPP(Mtt)) was synthesized with an automatic peptide synthesizer (Protein Science).

After peptide synthesis, Fmoc deprotection with 20\% piperidine in DMF and resin washing, Pyro was conjugated to N-terminal of NH\textsubscript{2}-PPPPPPPPP(Mtt) on the solid phase resin. The Pyro-labeled peptide was cleaved from the resin and the lysine MTT protecting group was removed with 95\% TFA and 5\% TIS. Quenchers were then conjugated as described above. Constructs were then precipitated with diethyl ether, purified by HPLC and the purity and identity were confirmed with HPLC-MS.

2.4.2 Photophysical properties and quantum yields

Fluorescence decay profiles were recorded using a nanosecond time correlated single photon counting system (IBH). 500 nM compound in methanol was placed in a quartz cell and excited with a NanoLED ($\lambda_{ex}$ = 370 nm), using an emission wavelength of 660 +/- 16 nm at room temperature. Data was collected until 5000 counts accumulated in the maximum channel. Data was fit using the IBH software to a single exponential decay function. Triplet lifetimes were obtained by flashing a solution containing 5 $\mu$M argon purged sample with a 308 nm laser. Spectra were collected 0.5 $\mu$s after the flash and represent the average of three measurements of
the same samples. For triplet decay, the absorbance at 410 nm was monitored after flash and fit to a single exponential using the OriginPro 8 software (OriginLab).

Absorption spectra of the quenchers alone and the quenched constructs were measured in methanol on a spectrophotometer (Varian). To determine quantum yields, sample concentration was adjusted so the maximum absorbance was approximately 0.05 and the exact absorbance was recorded. Fluorescence quantum yields were calculated using equation (1).

\[
\phi_{F,\text{SAMPLE}} = \phi_{F,\text{REF}} \times \frac{A_{\text{REF}}}{A_{\text{SAMPLE}}} \times \frac{F_{\text{SAMPLE}}}{F_{\text{REF}}} \tag{1}
\]

\( \phi_{F,\text{SAMPLE}} \) is the fluorescence quantum yield of the sample and \( \phi_{F,\text{REF}} \) is the reference fluorescence quantum yield of meso-tetraphenylporphyrin (TPP), taken as 0.13 in methanol\(^{28}\). \( A_{\text{SAMPLE}} \) is the absorbance at 410 nm of the quenched construct and \( A_{\text{REF}} \) is the absorbance at 410 nm of the TPP. \( F_{\text{SAMPLE}} \) and \( F_{\text{REF}} \) correspond to the background corrected emission spectra of the samples or TPP, integrated from 500 nm to 800 nm. Spectra were recorded on a Fluoromax fluorometer (Horiba Jobin Yvon) using 410 nm excitation and 3 nm excitation and emission slit widths. Because the quenchers displayed some absorbance at 410 nm, the fluorescence and singlet oxygen quantum yields of the quenched constructs were scaled by the following correction factors, based on the extinction coefficient at 410 nm of the quenchers compared to that of Pyro (97,000 M\(^{-1}\) cm\(^{-1}\)): BBQ: 1.07; BHQ1: 1.12; BHQ3: 1.12; DB3: 1.01; DAB: 1.19; QXL: 1.27. Where indicated, the Förster radius of the various quenchers was calculated using equation (2).

\[
R_0 = 0.0211(\kappa^2 n^4 Q_D J(\lambda))^{1/6} \tag{2}
\]

\( R_0 \) is the Förster radius, in nm. \( \kappa^2 \) represents the orientation factor, conventionally taken as 2/3. \( n \) is the refraction index of methanol, taken as 1.329 and \( Q_D \) is the fluorescence quantum yield.
of Pyro. The overlap integral, $J$, is described later in equation (6). Singlet oxygen quantum yields were calculated using equation (3).

$$
\phi_{\Delta,\text{SAMPLE}} = \phi_{\Delta,\text{REF}} \times \frac{A_{\text{REF}}}{A_{\text{Sample}}} \times \frac{^1O_2\text{Counts}_{\text{SAMPLE}}}{^1O_2\text{Counts}_{\text{REF}}}
$$

(3)

$\phi_{\Delta,\text{SAMPLE}}$ is the singlet oxygen quantum yield of the sample, $\phi_{\Delta,\text{REF}}$ is the singlet oxygen quantum yield of Pyro, taken as 0.52$^{29}$. $A_{\text{SAMPLE}}$ is the absorbance at 410 nm of the construct, while $A_{\text{REF}}$ is the Pyro absorbance at 410 nm. $^1O_2\text{Counts}_{\text{SAMPLE}}$ and $^1O_2\text{Counts}_{\text{REF}}$ are the background corrected singlet oxygen counts of the sample and reference, respectively. Quantum yields were calculated with the average of 3 to 4 sample preparations.

The singlet oxygen counts and lifetimes were assessed by direct measurement of singlet oxygen luminescence as described previously$^{30}$. In brief, the samples were irradiated with 523 nm light from a 10 ns pulsed frequency-doubled, Q-switched Nd:YLF laser operating at a repetition rate of 10 kHz and the luminescence signal was detected with a near-infrared sensitive photomultiplier tube (H9170-45, Hamamatsu). The luminescence emission spectra were sampled using five bandpass filters (10 nm width) centred at 1210, 1240, 1270, 1300, and 1330 nm. At each filter, the signal was acquired for 200 000 laser pulses and two cycles of the five filters were performed. The time-resolved luminescence signal was integrated from 0.5 to 40 microseconds for each filter at a time resolution of 100 ns. The singlet oxygen luminescence signal was calculated by subtracting the average of the integrated counts from the 1210, 1240, 1300 and 1330 nm filters from the integrated signal collected with the 1270 nm filter.

To determine the singlet oxygen luminescence lifetimes of the various samples, the time resolved data was fit with the following well-known 3 parameter equation using non-linear least squares fitting with OriginLab software.
\[
L_{270}(t) = A \frac{\tau_D}{\tau_T - \tau_D} \left[ \exp\left( -\frac{t}{\tau_T} \right) - \exp\left( -\frac{t}{\tau_D} \right) \right]
\]

(4)

\( L(t) \) is the observed luminescence signal as a function of time. \( A \) is a constant that incorporates the extinction coefficient and quantum yield of the photosensitizer as well as the irradiance, \( \tau_D \) is the singlet oxygen decay lifetime and \( \tau_T \) is the photosensitizer triplet state lifetime.

### 2.4.3 Photosensitizer bleaching

The samples were dissolved in a mixture of 50% methanol in water, at a concentration of 2.5 \( \mu \)M. Fluorescence and singlet oxygen were monitored while the samples were irradiated continuously with the 523 nm laser. Singlet oxygen luminescence was collected as described above. The fluorescence was collected with a SMA fiber optic collimator into a 400 micron diameter fiber optic which delivered the light to a spectrometer (USB2000, Ocean Optics, USA) with a 580 nm long-pass filter in front of the collimator. The fluorescence was integrated from 650 to 670 nm. The irradiance was 110 mWcm\(^{-2}\) and the total final radiant exposure was 116 Jcm\(^{-2}\). Experiments were performed with 3 separate samples.

### 2.4.4 Cell viability assay

KB-1 cells were seeded and grown for 2 days in a 96 well plate (2.5 x 10\(^4\) cells per well) before incubation with RPMI-1640 media containing 10% FBS and 1 \( \mu \)M construct in 0.5% DMSO and 0.2% TWEEN-80 (Sigma). After 16 hours incubation at 37\(^{\circ}\)C in a 5% CO\(_2\) incubator, the media was replaced with normal media and the cells were then treated with PDT with 3 different light fluences (1, 5 or 10 Jcm\(^{-2}\)) using a 667 nm laser with a 130 mWcm\(^{-2}\) fluence rate with 8, 40 and 80 second treatment times. 24 hours later, cell viability was assessed by
incubating 100 µL of 0.5 mg/mL MTT tracer, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (Invitrogen), with the cells for 1 hour. After removing the MTT, cells were resuspended in 200 µL of DMSO and the absorbance of the MTT was measured at 560 nm. Cell viability was calculated using equation (5).

\[ Viability = \frac{A_S - A_B}{A_N - A_B} \times 100\% \]  

(5)

A_B is the absorbance at 560 nm of the MTT in a blank well, A_N is the absorbance of cells at 560 nm without any drug or light treatment, and A_S is the absorbance at 560 nm of the well of interest. Averages and standard deviations were based on 7 experiments.

Figure 2-3: Properties of Pyro-Link.
A) Normalized excitation and emission spectra of 500 nM Pyro-Link (black, dashed lines) and Pyro (grey line) in methanol. B) Fluorescence lifetime of Pyro-Link. Grey marks indicate observed data and black line shows the single exponential fit. C) Triplet lifetime of Pyro-Link under argon. The grey line indicates observed absorbance at 410 nm (corresponding to recovery of the Soret band) and black curve shows the single exponential fit.

Figure 2-4: Normalized absorption spectra of quenchers and constructs.
A) Normalized absorption spectra of quenchers in methanol. The quenchers are labeled in the same order that the respective absorption maxima wavelength appears. The trace of DAB is shown in cyan, BHQ1 in green, BBQ in grey, DB3 in orange, BHQ3 in purple and QXL in red. The transparent grey rectangle corresponds to the wavelength range representing 90% of Pyrophephorh bile emission, centred around the fluorescence emission maximum. B) Normalized absorption spectra of the two indicated constructs in methanol.
Table 2-1: Properties of Pyro-linker and Pyro.

<table>
<thead>
<tr>
<th></th>
<th>Singlet lifetime</th>
<th>X²</th>
<th>Triplet lifetime</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyro-Link</td>
<td>6.4 ns</td>
<td>0.995</td>
<td>54.2 us</td>
<td>.998</td>
</tr>
<tr>
<td>Pyro</td>
<td>6.3 ns</td>
<td>0.978</td>
<td>51.5 us</td>
<td>.995</td>
</tr>
</tbody>
</table>

Table 2-2: Properties of quenchers

<table>
<thead>
<tr>
<th>Quencher</th>
<th>J (M⁻¹ cm⁻¹ nm⁴)</th>
<th>Abs max</th>
<th>ε (M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan (TRP)</td>
<td>3.8 x 10¹¹</td>
<td>280</td>
<td>9,000</td>
</tr>
<tr>
<td>Dabcyl (DAB)</td>
<td>1.2 x 10¹³</td>
<td>454</td>
<td>32,000</td>
</tr>
<tr>
<td>BHQ1</td>
<td>5.1 x 10¹³</td>
<td>521</td>
<td>38,000</td>
</tr>
<tr>
<td>DB3</td>
<td>5.0 x 10¹⁴</td>
<td>639</td>
<td>19,000</td>
</tr>
<tr>
<td>BBQ</td>
<td>3.7 x 10¹⁵</td>
<td>601</td>
<td>40,700</td>
</tr>
<tr>
<td>BHQ3</td>
<td>6.5 x 10¹⁵</td>
<td>654</td>
<td>42,700</td>
</tr>
<tr>
<td>QXL</td>
<td>2.2 x 10¹⁶</td>
<td>680</td>
<td>128,000</td>
</tr>
</tbody>
</table>

Figure 2-5: Structures of the various quenched constructs.
2.5 Results and Discussion

2.5.1 Generation of quenched constructs

To examine the quenching of pyropheophorbide-α (Pyro), a conjugation scheme was developed to hold the photosensitizer and quencher together with a lysine residue. A solid phase synthesis was used to simplify the purification process. The linker alone, TRP, DAB, Pyro, BHQ1, BBQ, BHQ3 and QXL-680 were conjugated to Pyro, generating Pyro-Link, Pyro-TRP, Pyro-DAB, Pyro-Pyro, Pyro-BHQ1, Pyro-BBQ, Pyro-BHQ3 and Pyro-QXL, respectively. Synthesis was carried out as described in the experimental section and Figure 2-1 shows the typical synthesis procedure for Pyro-DAB as an example. The final products were purified by preparatory HPLC, the purity of the compounds was confirmed by analytical HPLC and their correct identities were confirmed by mass spectrometry (Figure 2-2).

The basic photophysical properties of Pyro-Link were investigated and compared to Pyro alone. Figure 2-3A shows that Pyro and Pyro-Link had nearly indistinguishable fluorescence excitation and emission spectra, with the excitation spectra characterized by the Soret band at 410 nm and Q band at 663 nm, and the emission spectra displaying a 672 nm maxima with a minor shoulder at 725 nm. As shown in Figure 2-3B and Table 2-1, Pyro with and without the linker displayed a monoexponential fluorescence lifetime of 6.2 and 6.3 ns, respectively. The observed single exponential fluorescence lifetimes of Pyro and Pyro-Link were close to a previously reported modified Pyro lifetime of 6.5 ns. Triplet state lifetimes were measured using laser photolysis (Figure 2-3C) monitoring the recovery of the Soret band. The observed triplet lifetimes of Pyro and Pyro-Linker were 54.2 µs and 51.5 µs, respectively. These triplet lifetimes were longer, but comparable to a reported triplet lifetime of 35 µs for a modified
Pyro. The similar photophysical properties of Pyro and Pyro-Link show the linker did not induce major changes in the characteristics of the photosensitizer.

**Figure 2-6: Normalized absorption spectra of compounds**
Spectra were recorded in methanol. The x-axis shows the absorption wavelength in nm

**Figure 2-7: Pyro-Pyro displays intermediate quenching between Pyro-Link and Pyro-DB3**
A) Fluorescence emission spectra and B) singlet oxygen luminescence of Pyro-Link (black), Pyro-Pyro (medium gray), and Pyro-DB3 (light gray).
To determine if FRET can be used to effectively deactivate a photosensitizer, the quenching efficiency of several quenchers with varying degrees of spectral overlap with Pyro fluorescence emission was investigated. The quenchers used were Dabcyl-NHS (DAB), Black Hole Quencher 1-NHS (BHQ1), Disperse Blue 3-COOH (DB3), Blackberry quencher-NHS (BBQ), Black Hole Quencher 3-NHS (BHQ3) and QXL-680-NHS (QXL). They absorbed in different ranges between 450 nm to 750 nm, as shown in Figure 2-4A by the normalized absorption spectra of the quenchers. The quenchers shared varying degrees of overlap with Pyro fluorescence emission, which is represented by the shaded grey box covering 90% of the total Pyro fluorescence output. Figure 2-4B shows the absorption spectra of two constructs, Pyro-Link and Pyro-BBQ. FRET efficiency is related to the spectral overlap integral $J$, which depends on not only the wavelength range of overlap, but also the extinction coefficient of the acceptor, and may be calculated using equation (6).

$$J(\lambda) = \frac{\int_0^{\infty} F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int_0^{\infty} F_D(\lambda) d\lambda}$$

(6)

$F_D(\lambda)$ is the emission spectra of Pyro, $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor in M$^{-1}$cm$^{-1}$ and $\lambda$ is the wavelength in nm. As shown in Table 2, the quenchers covered a broad range of $J$ values. QXL, with an absorption peak well aligned with Pyro fluorescence emission and a large extinction coefficient of 128,000 M$^{-1}$cm$^{-1}$, has an overlap integral on the order of $10^{16}$ M$^{-1}$cm$^{-1}$nm$^4$. Conversely, TRP displayed only trace absorbance in the Pyro emission range and has an overlap integral almost 5 orders of magnitude less than QXL. The structures of the quenchers conjugate to Pyro are shown in Figure 2-5 (except for the QXL structure, which was not given by the manufacturer). The linker separated the Pyro and quencher by 5 carbon
atoms and 2 amide bonds. This spacer was chosen to emulate the close spacing of an activatable photosensitizer where the quencher and photosensitizer are held together by self-folding, as seen in nucleic acid beacons (a concept that can be extended to peptide based beacons\(^3\))\(^3\), or by hydrophobic interactions between the photosensitizer and quencher. All constructs were fairly hydrophobic and were soluble in methanol. DAB, BBQ, BHQ1, and BHQ3 contained N=N azo groups, BHQ1 and BBQ both contained nitro groups, and DB3 contained a quinine group. The absorbance spectra of the constructs are shown in Figure 2-6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abs Max</th>
<th>Em Max</th>
<th>(\Phi_t) (% quenching)</th>
<th>(\Phi_\Delta) (% (\Delta) quenching)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyro</td>
<td>411</td>
<td>672</td>
<td>0.49 (0.018) 2</td>
<td>0.52 (ref) -5</td>
</tr>
<tr>
<td>Pyro-Link</td>
<td>411</td>
<td>672</td>
<td>0.50 (0.032) 0</td>
<td>0.49 (0.034) 0</td>
</tr>
<tr>
<td>Pyro-TRP</td>
<td>412</td>
<td>672</td>
<td>0.49 (0.049) 1</td>
<td>0.53 (0.063) -5</td>
</tr>
<tr>
<td>Pyro-DAB</td>
<td>411</td>
<td>672</td>
<td>0.33 (0.049) 33</td>
<td>0.27 (0.051) 46</td>
</tr>
<tr>
<td>Pyro-Pyro</td>
<td>398</td>
<td>672</td>
<td>0.098 (0.029) 80</td>
<td>0.20 (0.0029) 60</td>
</tr>
<tr>
<td>Pyro-BHQ1</td>
<td>414</td>
<td>673</td>
<td>0.023 (0.002) 95</td>
<td>0.006 (0.002) 99</td>
</tr>
<tr>
<td>Pyro-DB3</td>
<td>411</td>
<td>674</td>
<td>0.025 (0.003) 95</td>
<td>0.034 (0.0032) 93</td>
</tr>
<tr>
<td>Pyro-BBQ</td>
<td>411</td>
<td>672</td>
<td>0.007 (0.0042) 99</td>
<td>0.013 (0.0048) 97</td>
</tr>
<tr>
<td>Pyro-BHQ3</td>
<td>410</td>
<td>673</td>
<td>0.023 (0.004) 95</td>
<td>0.036 (0.036) 93</td>
</tr>
<tr>
<td>Pyro-QXL</td>
<td>413</td>
<td>671</td>
<td>0.018 (0.015) 96</td>
<td>0.047 (0.019) 91</td>
</tr>
</tbody>
</table>

Table 2-3: Properties of quencher constructs
(standard deviations in brackets)
2.5.2 Photosensitizer Quenching

Deactivation of the photosensitizer excited singlet state should reduce the number of excited photosensitizer molecules available to enter the triplet state and consequently lower singlet oxygen generation. The deactivation of the singlet excited state of Pyro was evaluated by examining the fluorescence emission of the constructs. Figure 2-7A shows the fluorescence emission spectra in methanol of three constructs at 500 nM concentration: Pyro-Link, Pyro-Pyro, and Pyro-DB3. While the self-quenched Pyro-Pyro displayed an 80% reduction in fluorescence, the Pyro-DB3 quenched 95% of the Pyro emission. Quenching of singlet oxygen generation was assessed directly by near infrared luminescence of singlet oxygen. Figure 2-7B shows the 1270 nm peak of the singlet oxygen generated by the same constructs. A similar quenching trend was seen with Pyro-Pyro producing 60% less singlet oxygen than Pyro-Link and the Pyro-DB3 producing 93% less. The quenching summary for the entire set of constructs is shown in Table 2-3. Even though some quenched constructs displayed highly reduced emission intensity, the shape of the emission spectra was constant, with an emission peak maximum of 672 nm. The absorption maximum also remained constant near 411 nm for almost all the quenched constructs. The constant absorption, as well as the constant emission maxima of the constructs suggests that quenching by ground state complex formation was minimal, since ground state complexes display highly altered absorption properties. Pyro-Pyro was the only construct to show a significantly altered absorption profile. The blue-shifted 398 nm Pyro-Pyro absorption maxima is consistent with self-stacking or H-aggregates (hypsochromic) of
Pyro, a phenomenon observed previously with porphyrins that displayed the same wavelength shift upon aggregation\textsuperscript{34}. Activatable probes that rely on self-quenching have been used extensively for \textit{in vivo} imaging\textsuperscript{35}, and have advantages that they tend to have an easier synthesis protocol, and they also generate more signal per probe. However, these data indicate there may be a trade-off in the quenching efficiency in the inactive state compared to FRET quencher based activatable photosensitizers.

Dabcyl, a common quencher that has relatively little ($J = 1.2 \times 10^{13} \text{M}^{-1}\text{cm}^{-1}\text{nm}^{4}$) spectral overlap with Pyro fluorescence emission, quenched 33% of the Pyro fluorescence emission and 46% of the singlet oxygen generation. While reducing half the singlet oxygen production may be useful for some cases, a higher level of singlet oxygen attenuation that minimizes background photosensitization is desirable for \textit{in vivo} applications. Quenchers with spectral overlap greater or equal to $J = 5.1 \times 10^{13} \text{M}^{-1}\text{cm}^{-1}\text{nm}^{4}$ were the most efficient, reducing upwards of 90% of the photosensitizer fluorescence and singlet oxygen production. Plotting the observed quantum yields as a function of Förster radius (Figure 2-8) shows the quenchers that had more overlap (and larger Förster radius) than Dabcyl were able to quench Pyro fluorescence and singlet oxygen yields with high efficiency. It was observed that after reaching a 90% threshold of fluorescence and singlet oxygen generation (achieved with BHQ1), there was no further a clear relationship between spectral overlap and quenching, potentially due to variability in dipole alignment.

There was strong correlation between fluorescence and singlet oxygen quenching for the constructs. The Pearson’s correlation coefficient between $\phi_F$ and $\phi_A$ for the values shown in Table 2-3 was 0.98, suggesting the two quantum yields were linked. Correlation between the fluorescence and singlet oxygen quantum yields is consistent with FRET induced singlet state
deactivation being the principal determinant of singlet oxygen generation inhibition for these constructs. However, differences between the extent of fluorescence and singlet oxygen quenching show that fluorescence and singlet oxygen are subject to different quenching processes and the latter may be affected by factors including triplet state quenching or singlet oxygen scavenging. Those may have been factors for BHQ1 and BBQ, two quenchers that displayed greater than expected singlet oxygen attenuation. Those two quenchers were the only ones to contain a nitro group, a well-known quencher\textsuperscript{36} which may have conferred additional singlet oxygen quenching capacity.

![Figure 2-8: Quantum yields of constructs plotted as a function of quencher Förster radius](image)

Fluorescence quantum yields are shown as diamonds, while singlet oxygen quantum yields are marked as triangles (measured in methanol).

Since the rigidity of the linker was unknown, it was difficult to ascribe how much of the singlet state deactivation process was due to FRET, and how much was due to collisional quenching. Although these measurements were performed in the nanomolar range and collisional quenching is usually observed at the millimolar scale during quencher titrations, the linker may increase the rate of contact between the Pyro and the quencher. Direct assessment of
meaningful Stern-Volmer constants by quencher titrations was not possible due to the heavy optical interference of micromolar amounts of quencher with fluorescence and singlet oxygen measurements. To assess the extent of collisional quenching, we examined the construct quenched by tryptophan, a well-known collisional quencher\textsuperscript{37-39}. This construct displayed negligible fluorescence and singlet oxygen quenching (2\% and -5\%, respectively), showing that collisional quenching was not a major deactivation pathway for Pyro-TRP and therefore was likely not a major factor for the deactivation of other constructs either.

![Figure 2-9: Structure and quantum yields of extended linker constructs](image)

**Figure 2-9: Structure and quantum yields of extended linker constructs**
A) Structure of the 10 residue polyproline linker. B) Quantum yields of constructs plotted as a function of quencher Förster radius of the 10 residue polyproline linked constructs. Fluorescence quantum yields are shown as diamonds, while singlet oxygen quantum yields are marked as triangles (measured in methanol). From smallest Förster radius to largest, the data shows the extended construct yields for DAB, BHQ1, DB3, BBQ, BHQ3 and QXL. Simulated values of fluorescence quenching are shown as dashed lines for two different distances as a function of Förster radius.

To confirm FRET was the primary deactivation mechanism, we synthesized and purified constructs with the single lysine linker extended by a 10 residue polyproline peptide (Figure
Polyproline was selected as the linker since it has frequently been used in FRET experiments as a spectroscopic ruler. Fluorescence and singlet oxygen yields were measured and are shown in Figure 2-9B, as a function of Förster radius. While BHQ1 and DB3 could efficiently quench Pyro singlet oxygen when separated by a single lysine linker, these quenchers did not maintain effective Pyro quenching upon extension of the linker and increased separation from the photosensitizer. However, the three remaining quenchers with larger Förster radii, BBQ, BHQ3 and QXL, maintained greater than 90% quenching of fluorescence and singlet oxygen. The simulated values of fluorescence quantum yield are shown for 2 different linker lengths using equation (7).

\[
\phi_{F,\text{simulated}} = \frac{r^6 \cdot \phi_{F,\text{unquenched}}}{r^6 + R_0^6 \cdot \phi_{F,\text{unquenched}}},
\]

\(\phi_{F,\text{simulated}}\) is the simulated quantum yield, \(\phi_{F,\text{unquenched}}\) is the quantum yield of Pyro, \(R_0\) is the variable Förster radius and \(r\) is the hypothetical distance between the quencher and fluorophore. The simulated traces show the quenched fluorescence quantum yield for \(r = 1.9\) nm and \(3.1\) nm.\(1.9\) nm corresponds to the expected length of the 10 residue proline linker in methanol, while \(3.1\) nm is the expected polyproline length in water, shown for reference. The actual separation was likely slightly greater than \(1.9\) nm, since the distance to the centers of the quencher and photosensitizer dipoles, along with the extra lysine residue used for conjugation contributed to the total separation in addition to the polyproline spacing. The observed quantum yields followed a similar pattern as the simulated quenched fluorescence quantum yields. However, as with the single lysine linker, the effective Förster radius of the quenchers appeared less than predicted since the observed quenching was lower than expected. Despite having ample spectral overlap, the quenchers with the greatest Förster radius could not completely quench Pyro, with
the maximum achieved quenching between 90% and 95%, while the predicted values were close to 100% for a quenchers with simulated Förster radius values greater than 5 nm and $r = 1.9$ nm. Non-ideal dipole alignment may have contributed to the lack of complete energy transfer.

The fluorescence and singlet oxygen quantum yields of the extended linker constructs had strong correlation (Pearson’s correlation coefficient between $\phi_F$ and $\phi_A = 0.99$), in line with an excited singlet state deactivation mechanism. Because activatable photosensitizers ultimately are intended for PDT, knowing how the singlet oxygen production responds to activation is essential. However, direct measurement of singlet oxygen is not always possible and often requires uncommon equipment and protocols. The data presented here suggest that measurement of the fluorescence of activatable photosensitizers is a good indicator for singlet oxygen status since the fluorescence response and singlet oxygen response was similar with quenchers of varying degrees of efficiency. Although not a substitute for direct singlet oxygen measurement, estimating singlet oxygen production status through fluorescence may allow activatable photosensitizers to be conveniently characterized with standard equipment such as fluorometers and fluorescence microscopes.

In some potential beacon designs, activation does not lead to complete separation of the photosensitizer and quencher. For instance, when a nucleic acid molecular beacon unfolds, the quencher and fluorophore still remain connected on the same molecule. In some circumstances, a quencher and photosensitizer predicted to have a very large Förster radius should be avoided, since it is possible that the activated and unfolded beacon still may be separated by a distance less than the quencher Förster radius. This could lead to quenching of the photosensitizer even in the activated state and would reduce the activated signal increase. Selection of
photosensitizer quenchers must therefore be planned carefully. A guideline based on the results observed here would be to assume a quencher Förster radius of 5 nm is required for linker separations of ~2 nm and a quencher Förster radius of 2.5 nm is required for shorter linker separations of less than ~1 nm.

2.5.3 Quenching occurs upstream of singlet oxygen generation

Excluding ground state complex formation, singlet oxygen quenching can be achieved by at least three pathways: Deactivating the photosensitizer singlet state; deactivating the photosensitizer triplet state; and/or by directly scavenging singlet oxygen. It has previously been shown that a carotenoid quencher can quench singlet oxygen generation by all the above three ways when covalently attached to Pyro\textsuperscript{43}. We examined whether or not FRET-based quenchers act as singlet oxygen scavengers. The singlet oxygen lifetimes of various constructs were measured in methanol. As shown in Figure 2-10A, all the constructs displayed a singlet oxygen luminescence lifetime of approximately 10 µs. The singlet oxygen lifetimes of some well quenched samples, such as Pyro-BBQ, were difficult to fit due to the very low singlet oxygen signal, resulting in large error bars in the measurement. Consistent with a singlet excited state deactivation model, and in contrast to the previously reported carotenoid quencher, the uniformity of the quenched construct lifetimes indicates that FRET based quenchers were not effective singlet oxygen scavengers. The triplet state difference spectra for Pyro-Link and a quenched construct, Pyro-QXL are shown in Figure 2-10B. It is apparent that the triplet state of Pyro lacks the Soret band absorption at 410 nm, resulting in the negative absorbance in the difference spectra. Pyro-Link had a triplet state difference absorption at the measurement time of 0.5 µs after the flash, showing that a portion of the constructs remained in the excited triplet state. However the QXL quenched construct did not display any meaningful difference spectra,
indicating the quenched construct had already returned to the singlet state by the time the spectra was recorded after the flash, consistent with FRET induced singlet state deactivation of the triplet state.

**Figure 2-10: Lifetime and triplet spectra**
A) Singlet oxygen luminescence lifetimes of the various compounds measured in methanol. Dark grey bars represent polyproline linked compounds and light grey bars indicate single lysine separated compounds. B) Triplet state difference spectra of 5 µM Pyro-Link and Pyro-QXL, recorded under argon purged methanol after flashing sample with 308 nm laser.

**Figure 2-11: Bleaching of quenched constructs**
2.5 μM samples were continually irradiated with the amount of radiant exposure indicated in the legend and fluorescence emission was monitored over time. Upper right inset shows the normalized fluorescence emission as a function of cumulative exposure for Pyro-Link (diamonds), Pyro-DB3 (crosses), Pyro-QXL (squares) and Pyro-Pyro (circles).

![Graph showing fluorescence emission vs cumulative exposure for different compounds](image)

**Figure 2-12: MTT viability assay**
Cells were treated with 1 μM of various compounds followed by PDT using the laser exposure indicated in the legend. Mock treated cells were incubated with 0.5% DMSO and 0.2% TWEEN-80 alone. Viability was assessed 24 hours after irradiation with the MTT assay.

### 2.5.4 Photobleaching

Photosensitizers may be subjected to extended light exposure. Continued irradiation of activatable photosensitizers could result in the photosensitizer or quencher losing efficiency over time due to photobleaching. Figure 2-11 illustrates that without quencher, Pyro underwent photobleaching upon extended irradiation, resulting in greater than 70% loss of fluorescence emission. On the other hand, the quenched constructs appeared to be resistant to photobleaching and extended irradiation actually resulted in an increase in photosensitizer fluorescence. The increased fluorescence production was likely due to singlet oxygen induced damage to the quencher or direct photobleaching of the quencher. Although this phenomenon would reduce specificity of the activatable photosensitizer during PDT, compared to Pyro, the fluorescence and corresponding singlet oxygen production was still an order of magnitude less for the quenched constructs investigated. For QXL, the total singlet oxygen production after 116 J cm$^{-2}$
remained less than 5% of that for Pyro, showing the quenched constructs would still deliver a high level of selectivity.

2.5.5 Suitability for cells

An advantage of activatable photosensitizers is that they can be delivered to both target and non-target cells and only the target cells will activate and dequench the photosensitizer. However, if the quenching is ineffective, a large number of non-target cells will also be killed, negating the benefits of the activatable PDT. To examine if the singlet oxygen production of the quenched constructs was sufficiently attenuated to prevent toxicity with PDT treatment, 1 µM compound was incubated with human nasopharyngeal carcinoma derived KB cells, PDT was performed and cell viability was assessed. Figure 2-12 shows cell viability after treatment with various compounds with an increasing laser irradiation dose. Mock treated cells showed a small loss in viability, although there was no response to increasing light dose, suggesting the DMSO and TWEEN delivery formulation had a mild inhibitory effect on cell viability. When incubated with the unquenched Pyro-Link construct, viability was reduced to 12% of the untreated control cells with the 1 Jcm$^{-2}$ light dose treatment. The 5 Jcm$^{-2}$ and 10 Jcm$^{-2}$ treatments killed almost all the cells, resulting in only 1.3% and 0.6% cell viability, respectively. Pyro-Pyro, which exhibited 60% singlet oxygen quenching efficiency was 80% less effective than Pyro-Link at killing cells at the 1 Jcm$^{-2}$ radiant exposure. The DB3 quenched construct, which had 93% quenched singlet oxygen yield, displayed even less toxicity than the self-quenched construct. Cells treated with Pyro-BHQ1, which displayed 99% singlet oxygen quenching, displayed no PDT light dose response. At the highest light dosage of 10 Jcm$^{-2}$, Pyro-Pyro, Pyro-DB3 and Pyro-BHQ1 displayed 9, 57 and 118-fold reduced toxicity, respectively, when compared with
Pyro-Link. The inhibited toxicity of the quenched photosensitizers shows the degree of quenching achieved is sufficient for maintaining a harmless inactive quenched state.\textsuperscript{44}

2.5.6 Conclusion

We have demonstrated that FRET based quenchers can be used to quench photosensitizer singlet oxygen generation. For effective quenching, the amount of required spectral overlap varies based on the conformation of the photosensitizer and quencher. For activatable photosensitizers where the quencher is located within a few nanometres to the photosensitizer, only moderate spectral overlap is required and using a quencher with increased spectral overlap may not further decrease fluorescence and singlet oxygen generation beyond 90\%. However, if the photosensitizer and quencher are positioned further away, as may be the case for peptide based activatable photosensitizers, the amount of spectral overlap required from the quencher increases as the linker length increases to maintain efficient singlet oxygen quenching. Based on the results observed, to ensure the FRET-based photosensitizer quencher is highly effective, a quencher Förster radius of 5 nm is required for linker separations of ~2 nm and a quencher Förster radius of 2.5 nm is required for shorter sub-nanometer linker separations. The quenching of fluorescence and singlet oxygen was similar, not only suggesting the primary deactivation pathway was from the photosensitizer singlet state, but also showing that fluorescence can be used as an indicator of singlet oxygen production status for activatable probes. Activatable photosensitizers hold potential for basic biochemical research and future therapeutics and the findings presented here show that FRET based quenchers can be used to facilitate their design.
2.6 References


Chapter 3

A New Photodynamic Molecular Beacon Design and its Application to Nanoparticle Aggregation

3.1 Acknowledgement
This chapter is a modified and reformatted merge of two separate manuscripts; “Facile Synthesis of Advanced Photodynamic Beacon Architectures”, published in Bioconjugate Chemistry by Lovell et al.\textsuperscript{1} and “Nanoparticle Aggregation Using Molecular Beacons” published in Angewandte Chemie by Lovell et al.\textsuperscript{2} Under the supervision of Gang Zheng, my contributions to these manuscripts included designing and carrying out most experiments, interpreting the data and writing the text of the manuscripts. Thanks to Juan Chen for assistance with synthethis, Elizabeth Huynh for fluorescence measurements, Mark Jarvi and Brian Wilson for assistance with singlet oxygen measurements, and Ken Ng and Hongling Jin for assistance with LDL and HDL purification.

3.2 Abstract
Nucleic acid photodynamic molecular beacons (PMBs) are a class of activatable photosensitizers that increase singlet oxygen generation upon binding a specific target sequence. Normally, PMBs are functionalized with multiple solution-phase labelling and purification steps. Here, we make use of a flexible solid-phase approach for completely automated synthesis of PMBs. This enabled the creation of a new type of molecular beacon that uses a linear superquencher architecture. The 3’ terminus was labeled with a photosensitizer by generating pyropheophorbide-labeled solid-phase support. The 5’ terminus was labeled with up to three consecutive addition cycles of a dark quencher phosphoramidite. These
photosensitizing and quenching moieties were stable in the harsh DNA synthesis environment and displayed increased hydrophobicity which facilitated purification by HPLC. Linear superquenchers exhibited highly efficient quenching. This fully automated synthesis method not only simplifies the synthesis and purification of PMBs, but also the creation of new activatable photosensitizer designs. DNA has been used in combination with nanoparticles for biodetection and nanostructure control. However, more tools are required to maintain development of these techniques. The superquenched DNA molecular beacons could be inserted into lipid nanoparticles. Unexpectedly, addition of the target nucleic acid then induced dramatic and irreversible aggregation, modulated by both nanoparticle concentration and the number of beacons per particle. This process discriminated single base mismatches. This novel phenomenon could find utility for DNA programmed nanoengineering.

3.3 Introduction
Activatable photosensitizers modulate singlet oxygen production in response to interactions with a variety of target biomolecules. Nucleic acid-activatable photosensitizers are based on hybridization to specific nucleic acid target sequences. Nucleic acid photodynamic molecular beacons (PMBs) synthesized with porphyrin or phthalocyanine photosensitizers have recently been developed. Molecular beacons are hairpin-loop structures that hold a quencher and fluorophore together, until the loop portion hybridizes to a target sequence, causing stem separation and unquenching. PMBs are an extension of prototypical molecular beacons that offer single base mismatch sensitivity and large fluorescence signal differences between the hybridized and non-hybridized states. Excluding molecular beacons, alternative strategies for DNA control of singlet oxygen have been also been proposed. Single walled nanotubes that bound and quenched a chlorin-e6 conjugated aptamer were displaced when the aptamer bound
its thrombin target, leading to photosensitizer activation. Another approach was based on the hybridization and displacement of pyropheophorbide-labeled oligonucleotides to another quenching DNA strand. These multi-component systems make use of separate quenching and photosensitizing molecules, which simplifies the synthesis and purification of each individual component but adds a layer of uncertainty of how the subunits behave in diverse hybridization conditions. Here, we report a new synthesis method for PMBs that facilitates their synthesis and purification, and also permits the development of novel photodynamic beacon architectures.

Although production of standard molecular beacons is routine, there have been few reports of synthesis and purification of PMBs. Normally, the nucleic acid portion is produced using standard DNA synthesis methods, then one beacon terminus is labeled with a photosensitizer and the other is labeled with a quenching (or self-quenching) moiety. There are several approaches to the conjugation of these functional groups. To simplify the labelling procedure, previous efforts resulted in the development of a porphyrin phosphoramidite. While this phosphoramidite is ideal for labelling the 5’ terminus of an oligonucleotide in the final addition cycle of a solid-phase synthesis, no subsequent additions are possible. The most common approach is the incorporation of amine-modified functional groups with different protecting groups that permit sequential deprotection and labelling of each terminus using an activated ester or acid-modified labelling moiety. In the case of self-quenched beacons, only a single type of conjugatable group is required since the same labelling reaction may be repeated at both termini. In some cases, it may be challenging to find a suitable solvent for dissolving the hydrophobic photosensitizer with the hydrophilic nucleic acid. If the oligonucleotide is conjugated at the 3’ prior to synthesis, purification problems can arise using reverse-phase HPLC, because short degradation sequences labeled with a hydrophobic photosensitizer or quencher can elute at the same time as the ion paired full length product. Solid phase synthesis
approaches have been described for dual terminal oligonucleotide labelling and are applied here to automate PMB synthesis and simplify purification.

Nucleic acids can be used as robust building blocks or scaffolds for new nanoarchitectures. The potential of nucleic acids has been demonstrated with DNA-based construction of a variety of shapes including, for example, an incredible range of two-dimensional patterns, three-dimensional crystals, and even containers with lids that can be opened. DNA scaffolds can be further functionalized with proteins to control enzyme cascades. Nucleic acids play a prominent role in integrated nanoparticle-biomolecule hybrid systems and can induce the assembly of other nanoparticles, such as colloidal gold, into large macroscopic and even crystalline materials. Other studies have demonstrated that gold nanoparticles themselves can induce the aggregation of proteins in solution. Alternatively, surface-enhanced Raman scattering (SERS) active nanoparticles can be used to detect protein aggregation. Here, we describe a different type of aggregation phenomenon based on lipid nanoparticles, rather than metallic ones; and intra-particle forces generated by molecular beacons (MBs), rather than linker based hybridization of nanoparticle networks. Irreversible aggregation of nanoparticles resulted from the opening of nanoparticle-inserted MBs.

**3.4 Materials and Methods**

**3.4.1 Generation of pyropheophorbide control pore glass**
All chemicals were obtained from Sigma (Canada) unless noted otherwise. Pyropheophorbide control pore glass (CPG) was generated from amine-modified CPG (3’ Amino-Modifier C7 500 CPG, 20-2957-10, Glen Research, USA). In a typical labeling reaction, 200 mg of CPG was suspended in N,N-dimethylformamide (DMF) containing 20% piperidine for 20 min, changing the solution every 5 min, to remove the amine-protecting FMOC group. The amount of
activated amine was quantified by the removed FMOC absorbance at 300 nm using an extinction coefficient of 7800 M$^{-1}$cm$^{-1}$. The CPG was then washed with DMF to remove all traces of piperidine. A 3-fold excess of pyropheophorbide, HOBt and HBTU was then added to the CPG in 2 mL DMF with 2% N,N-diisopropylethylamine. The reaction was placed under argon and left on a rotator overnight at room temperature. The CPG was then washed extensively with DMF, the solvent was removed and the CPG was dried under vacuum. To pack the CPG columns for solid phase synthesis, 400 nmol synthesis scale columns (Biosset, Russia) were sealed on one end with porous steel, and connected to a vacuum line via a small filter to prevent CPG from entering the vacuum. The pyropheophorbide CPG was then slowly aspirated into the solid phase column until the depth was approximately two thirds full (as judged by a narrow pipette tip). The other end was sealed with porous steel and the columns were stored at -20°C.

3.4.2 Solid phase synthesis and purification of the photodynamic molecular beacon
Solid phase synthesis was performed on an ASM-800 DNA synthesizer (Biosset). Phosphoramidites and DNA synthesis reagents were obtained from Glen Research (dA-CE (10-1000-C5), Ac-dC-CE (10-1015-C5), dmf-dG-CE (10-1029-C5), dT-CE (10-1030-C5), 3% TCA/DCM (40-4140-61), ETT (30-3140-52), oxidizing solution (40-4230-57), capping A (40-4110-57), and capping B (40-4220-57). The sequence synthesized was 5’-GTGAGATTCTTCCAGATTGTTTTTTT-TCTCAC-3’ and the program was configured according to the manufacturer recommended protocol. The quenchers were added by modifying the synthesis program to add 0, 1, 2, or 3 additions of BlackBerry Quencher-650-dT CEP (BL1010, Berry Associates, USA), which was dissolved in small amount of anhydrous acetonitrile along with
some molecular sieves and was connected to the DNA synthesizer. After synthesis, the CPG was removed from the column and deprotected and cleaved with AMA (ammonium hydroxide:methyl amine, 1:1 ratio) for 15 min at 55 °C. The solvent was evaporated in a speed vacuum and the beacons were then stored at -20° C until being dissolved in water and subjected to reverse-phase HPLC purification using a Zorbax C18, 4.6x250 mm column and a 0.4 mL/min 10% to 100% acetonitrile in 0.1 M TEAA gradient over 45 min with the column heated to 60 °C. After purification, the solvent was evaporated and the beacons were resuspended in water. The beacon absorption profiles were verified in water, concentrations were adjusted to 20 µM and the beacons were stored at -20° C. The identities of the beacons were verified by MALDI-TOF mass spectrometry (Trilink Biotechnologies, USA) as follows:

0-Q: Calculated: 10174.0; found: 10176.1. 1-Q: Calculated: 11174.1; found: 11174.6; 2-Q: Calculated: 12174.1; found: 12174.9; 3-Q: Calculated: 13174.2; found: 13175.9

3.4.3 Fluorescence and singlet oxygen measurements
Kinetic fluorescence measurements were made at room temperature in 1 mL of phosphate buffered saline solution with magnetic stirring (150 mM NaCl, 10 mM phosphate, pH 7.4). The beacon target was ordered from Sigma-Genosys (Canada) and consisted of the sequence 5’-GTGAGAAAAAACAATCTGGAAGAAAAAC-3’. The fluorescence was monitored using a Fluoromax fluorometer (Horiba Jobin Yvon) with stirring with 420 nm excitation wavelength (5 nm slits) and 680 nm emission wavelength (5 nm slits). After the PBS background was recorded, 50 nM beacon was added and the signal recorded. At 50 s, a 10-fold excess of beacon target was added and the fluorescence monitoring was continued. To determine the quenching of the beacons relative to the no-quencher (0-Q) beacon, the emission spectra of the 50 nM beacon samples was recorded with 420 nm excitation and 600-750 nm
emission. All spectra were integrated and a PBS blank was subtracted. Time-resolved singlet oxygen luminescence was measured by irradiating the samples with a 524 nm laser from a 10 ns pulsed, frequency-doubled Q-switched Nd:YLF laser with a 10 kHz repetition rate. Different wavelengths were recorded using multiple bandpass filters (1240, 1270, 1300, and 1330 nm) and the singlet oxygen luminescence signal was calculated by subtracting the signal adjacent to the main 1270 nm peak. Signals were integrated from 0.6-50 µs after the laser pulse. The photodynamic beacons were measured at a concentration of 400 nM in a 25% H2O/75% D2O buffered saline solution and a 10-fold excess of target was added to activate the beacon. Measurements took 20 seconds and were repeated in triplicate.

3.4.4 Preparation of LDL nanoparticles.
Expired human plasma was obtained from the local blood transfusion services. LDL was isolated by sequential ultracentrifugation of human plasma as previously described (Havel et al., J. Clin. Invest. 1955, 34, 1345). Centrifugation procedures were conducted at 4°C unless otherwise specified. Briefly, 300 mL of human plasma (within 48 hours of expiry) was dialyzed in saline overnight supplemented with 2 mM EDTA at 4°C. Subsequently, dialyzed plasma (d=1.006 g/mL) was divided equally into polycarbonate tubes suitable for ultracentrifugation. Each tube was layered with 3-4 mL of 1.006 g/mL layer buffer comprised of KBr and NaCl. The tubes were centrifuged at 40,000 RPM for 18 hr using a Ti 70.1 fixed-angle rotor (Beckman Coulter). Upon completion of the first spin, the supernatant containing the fatty layer was removed and the lower portion of the tube was collected. The pooled infranatant was adjusted to the density of 1.066 g/mL with a KBr/NaCl layering buffering and centrifuged again at 45,000 RPM for 18 hr. The supernatant from this spin was collected and stored at 4°C until further use. LDL concentration was quantified by using the modified Lowry protein assay (Sigma) using an absorption wavelength of 670 nm. To convert concentration into units of
nanoparticle molarity, a molecular weight of $5.5 \times 10^5$ Da was assumed for ApoB (the ApoB100 form, the sole protein component of LDL).

### 3.4.5 Nucleo-Lipoprotein Nanoparticle Preparation and Characterization

Pyropheophorbide labeled control pore glass was used for attaching the photosensitizer on the 3’ terminus of the beacon. The beacon was synthesized using standard phosphoramidite chemistry and the sequence used was: 5’ gtgaga-ttctccagattttttttctcac 3’ (with the stem-loop-stem boundaries marked with dashes). Blackberry quencher dT phosphoramidite (Berry & Associates) was then attached with 0 to 3 subsequent additions to generate the 0, 1, 2 or 3 quencher beacons. The beacon was purified with reverse phase HPLC, identified using mass spectrometry and quantified using absorption at 260 nm. For the electrophoretic mobility shift assay, 15 pmols of beacon (0, 1, 2 or 3 quencher) were incubated 0, 0.1, 0.25, 0.5, 1.5 or 2 pmols of LDL in PBS for 1 hour at room temperature. After incubation, glycerol loading buffer was added and the nanoparticles were subjected to electrophoresis using a 0.8% agarose gel with TAE buffer and a 100 V electric field for 30 minutes. 0.002% GelRed stain (Biotium) was included in the gel to visualize the molecular beacon mobility with a GelDoc UV scanner (Bio-Rad). For transmission electron microscopy, 10 uL of nanoparticles were placed onto a glow discharged copper grid. The solution was then rapidly blot-dried, washed with water twice and then once with 1% uranyl acetate. Images were then acquired on an H-7000 electron microscope (Hitachi).

### 3.4.6 Characterization of DNA Induced Nanoparticle Aggregation

Unless otherwise noted, precipitation assays used 1 µM LDL with 6 µM 3Q beacon, incubated for one hour in PBS in a total volume of 6 µL. 5 fold molar excess of target was added and
aggregation proceeded over another hour. The standard target sequence (from Sigma) used was: 5′ tgtgagaaaaaaaacatatctggaagaaaaaaac 3′. To determine soluble ApoB protein concentration after aggregation, insoluble ApoB was removed from the sample by centrifugation at 14,000 g for 10 minutes and soluble protein in the supernatant was quantified using the FluoroProfile (Sigma) protein quantification kit as per the manufacturer directions. For dynamic light scattering measurements, the aggregated solution was transferred to 400 µL of PBS and measurements were taken in a NanoSizer dynamic light scattering instrument (Malvern). For liposomes, a 5 mg film of 6:4 DSPC:Cholesterol (Avanti Polar Lipids) was hydrated with 1 mL PBS with 5 freeze thaw cycles. Liposomes were then extruded 10 times through a 100 nm polycarbonate membrane (Avanti Polar Lipids). 1 µL of the liposomes was incubated with 30 pmol 3Q beacon for 1 hour then 10 fold molar excess beacon was added.

To assess the various single base mismatch targets, the following sequences were used as the target in 5 fold molar excess to beacon when incubating with the 3Q inserted nucleo-lipoprotein nanoparticle (mismatch underlined):

16mer match target: 5′ tgtgacaaaaaacaat 3′
16mer mismatch target: 5′ tgtgagaaaaaacaat 3′
15mer match target: 5′ tgtgagaaaaaaacat 3′
15mer mismatch target: 5′ tgtgagaaaaaaacatat 3′
14mer match target: 5′ tgtgagaaaaaaaacaat 3′
14mer mismatch target: 5′ tgtgagaaaaaaaacaat 3′
13mer match target: 5′ tgtgagaaaaaaaacat 3′
13mer mismatch target: 5′ tgtgagaaaaaaaacatat 3′
12mer match target: 5′ tgtgagaaaaaaaacat 3′
12mer mismatch target: 5′ tgtgagaaaaaaaacatat 3′
Fluorescence of the beacon in the nanoparticle was monitored on a Fluoromax-5 fluorometer (Horiba Jobin Yvon) using 420 nm excitation and 680 nm emission (5 nm slit widths) in 1 mL of PBS with stirring. At the indicated time, a 5 fold excess of target was added.

To assess the effect of the position of the mismatches, the following sequences were used as the target for incubating examining the various mismatches using the 13mer target (mismatch underlined):

1´: 5´ ctgagaaaaaac 3´
3´: 5´ gccagaaaaaac 3´
5´: 5´ gtgaagaaaaac 3´
7´: 5´ gtgagacaaaaac 3´
9´: 5´ gtgagaaacaaac 3´
11´: 5´ gtgagaaaaaac 3´
13´: 5´ gtgagaaaaag 3´

3.5 Results and Discussion

3.5.1 Generation of superquencher molecular beacons
To generate a complete solid phase synthesis approach for PMBs, we first labeled controlled pore glass (CPG) with a photosensitizer. Pyropheophorbide was a suitable choice, since this fluorescent photosensitizer was stable under the harsh conditions of multiple DNA synthesis cycles and oligonucleotide deprotection. The photosensitizer was conjugated to the CPG using a simple dehydration reaction after removing the CPG FMOC group (Figure 3-1A). After conjugation and washing, the highly static charged CPG displayed a dark green color (Figure
A solid phase packing scheme was developed using a vacuum line, in which empty columns were two-thirds filled to ensure reproducible synthesizer reagent flow-through during the synthesis (Figure 3-1C). For the quenching moiety on the opposing 5’ terminus, we employed a commercially available phosphoramidite quencher, BlackBerry dT (BBQ-dT). This is non-terminating and so could be used for multiple additions. As an example of a useful application of this solid phase synthesis approach, we generated a new class of PMBs that feature the first linear superquencher.

![Figure 3-1: Solid phase support for automated PMB synthesis.](image)

A) Generation of pyropheophorbide solid phase CPG support. B) Photograph of pyropheophorbide CPG. C) Packing method for achieving reproducible solid phase synthesis.
Superquenchers are assemblies of multiple quenchers that efficiently quench molecular beacon fluorescence when the beacon is in the inactive state \(^{22}\). These have not been previously explored as singlet oxygen quenchers. Standard superquenchers use a trebler phosphoramidite that branches the oligonucleotide, permitting multiple quencher additions in the next addition cycle (Figure 3-2A, left). While this approach is effective, it requires the use of an additional specialty phosphoramidite (the trebler phosphoramidite, in addition to the quencher phosphoramidite). Instead, we used multiple additions of the BlackBerry quencher phosphoramidite at the 5’ terminus to achieve the first “linear superquencher” (Figure 3-2A, right) that has multiple quencher moieties attached sequentially (via phosphodiester bonds) in series rather than in parallel. The PMB included pyropheophorbide on the 3’ terminus, the oligonucleotide sequence indicated in the figure (which is a shared stem beacon \(^{23}\) with one stem complimentary to a portion the target sequence) and 0, 1, 2 or 3 BlackBerry quenchers.
linked on the 5’ terminus. After synthesis, the PMBs were deprotected with methylamine: ammonium hydroxide (1:1) for 15 min at 55° C and were then purified by reverse-phase HPLC. This rapid protocol resulted in PMBs that could be synthesized and purified in a single day. The PMB identities were confirmed with mass spectroscopy. Figure 3-2B shows the reverse-phase HPLC elution profiles of the beacons. As expected, each additional quencher increased the hydrophobicity and retention times of the beacon. One advantage of the increased retention time was that the linear superquencher could be distinguished from pyropheophorbide-labeled degradation products that tend to elute near the full-length beacon with no quencher or a single quencher (note that Figure 3-2 shows traces for the purified beacons). The addition of 2 or 3 hydrophobic quenching moieties shifted the elution time so that HPLC purification of the full-length product was straightforward. The absorption profile of the 0,1,2 or 3-quencher beacons shown in Figure 2C confirmed that the oligonucleotide (260 nm) and pyropheophorbide (410 nm and 680 nm) ratios remained constant, while each additional quenching moiety contributed to the broad peak from 500 to 750 nm). The separate spectra of pyropheophorbide and Blackberry quencher are shown in Figure 3-3.

The fluorescence response of the beacons was characterized to confirm the beacon activation. As shown in Figure 3-4A, all beacons rapidly hybridized to the target nucleic acid, resulting in increased fluorescence. While the single and double quencher were activated 30- and 40-fold, respectively, the triple quencher had a higher activation of approximately 90-fold. This activation is similar to the 100-fold activation observed using gold as a potent molecular beacon quencher 24, yet it is not as effective as the 300-fold activation observed with standard branched superquenchers 22. However, when the fluorescence intensity of the closed beacon was compared to the 0-Q beacon that lacked any quencher (as opposed to comparing the closed beacon to the target-activated beacon), 300-fold quenching was indeed observed (Figure 3-4B).
This shows that the quenching of the triple quencher was extremely effective, but there was some residual intramolecular quenching in the activated state likely due to the extremely hydrophobic nature of the photosensitizer and quenchers. The singlet oxygen generation of the beacons was next examined. As shown in Figure 3-4C, in the closed state, the beacon with only 1 quencher generated lower singlet oxygen luminescence than that of the buffer alone. However, when the target was added, a clear singlet oxygen luminescence peak appeared at 1270 nm as expected. Maximum singlet oxygen activation occurred within 80 seconds of target addition (Figure 3-4D). Because the singlet oxygen luminescence of the closed-state beacon was below the instrument detection limits with even the 1-Q beacon, we could not accurately determine the fold activation of the 2-Q or 3-Q PMBs. It would be expected to be similar to the fold activation of fluorescence, since the degree of fluorescence quenching is highly correlated to the degree of singlet oxygen quenching.25

![Normalized absorption spectra of pyropheophorbide acid (A) and Blackberry (BBQ) quencher (B). Spectra were measured in methanol at room temperature.](image)

**Figure 3-3: Absorption of photosensitizer and quencher.** Normalized absorption spectra of pyropheophorbide acid (A) and Blackberry (BBQ) quencher (B). Spectra were measured in methanol at room temperature.
Figure 3-4. Characterization of superquenched beacons
A) 50 nM PMB fluorescence response to addition of a 10-fold excess of target DNA in buffered saline. F is the beacon fluorescence and \( F_{\text{INIT}} \) is the beacon fluorescence at the initial time point. B) Beacon fluorescence quenching compared to the 0-Q beacon. C) Direct measurement of luminescence in the 1-Q beacon. Triangles: beacon + target; Circles: buffer alone; diamonds: beacon alone. D) Singlet oxygen luminescence activation kinetics of the 1-Q beacon measured in buffered saline supplemented with 75% D\(_2\)O.

3.5.2 Application to nanoparticle aggregation

We synthesized a MB functionalized with pyropheophorbide (Pyro) along with 0, 1, 2, or 3 BlackBerry quencher (BBQ) moieties as previously described. The MB comprised a 6 base stem and a 19 base loop (Figure 3-5a). The 5’ stem of the MB was also complementary to the target sequence since such “shared stem” MBs have favorable thermodynamic profiles.\(^{26}\) Both Pyro and BBQ are hydrophobic, and each additional quenching moiety further enhanced the
MB hydrophobicity. We hypothesized that these increasingly hydrophobic MBs might insert into lipid nanoparticles such as low density lipoprotein (LDL). As endogenous nanocarriers, lipoprotein nanoparticles are promising platforms for delivery of contrast agents and drugs due to their small size, biocompatibility and capacity to carry a range of cargo and even other small nanoparticles.27,28

LDL nanoparticle concentration was determined by examining ApoB protein content, since each LDL is stabilized by only one ApoB protein. As shown in Figure 5b, upon incubation of the hydrophobically modified MBs with purified human LDL, hybrid nucleo-lipoprotein nanoparticles were generated and could be assessed using a gel shift assay. Schematic representations of MB insertion into LDL are shown in Figure 3-5c. After the negatively charged beacons inserted into the LDL, the electrophoretic mobility changed. When the beacon lacking any quenchers (0Q MB) was incubated with increasing amounts of LDL, it did not insert effectively into the nanoparticle (Figure 3-5b, note the asterisks representing the uninserted MB). A similar pattern was observed for the single quencher MB (1Q MB) although at the 7.5:1 beacons:nanoparticle incubation ratio, approximately half the total amount of beacon inserted stably into the nanoparticles. When the 2Q MB was used, the majority of the beacon inserted into the LDL at the 15:1 beacons:nanoparticle incubation ratio. Finally, when the 3Q MB was used, up to 30 beacons could be inserted into each LDL nanoparticle. Thus, the 3Q MB could most effectively insert into the lipid nanoparticles. To ensure beacons were completely inserted into the nanoparticles without requiring further purification, the 3Q MB was used in subsequent experiments with a low beacon:nanoparticle ratio of 6 beacons per nanoparticle. MBs did not drastically alter the size and shape of the LDL nanoparticle, as
revealed by transmission electron microscopy comparing unmodified LDL to LDL with the 3Q MB inserted (Figure 3-5d).

Figure 3-5: MB insertion into nanoparticles.

a) Structure of MB modified with Pyro (red) and multiple BlackBerry quencher (BBQ) units (blue). b) Gel shift assay demonstrates multiple BBQ units enhance MB insertion into nanoparticles. 15 pmols of MB with the indicated number of BBQ units were incubated with increasing amounts of LDL then subjected to agarose gel electrophoresis. Asterisks indicate the migration of the unbound beacon while shifted bands correspond to nanoparticles containing the inserted beacon. c) Schematic illustration of the 4 different types of MBs with Pyro (P) and BBQ (Q) inserted into LDL nanoparticles. d) Transmission electron micrographs of negative stained LDL and LDL with the 3 quencher beacon inserted.
Figure 3-6: Nanoparticle aggregation induced by target DNA.

a) Photographs of visible aggregation induced by target DNA. LDL was incubated with or without 6 MBs per particle. A 10 fold molar excess of target DNA was added and incubated for 30 minutes. A piece of dust is seen in the control tube following target addition. b) Protein aggregation of LDL nanoparticles as a function of the number of MBs per nanoparticle. Error bars +/- S.D. (n=3). c) Dynamic light scattering shows aggregation is modulated by both nanoparticle concentration and number of beacons per particle (B/P). Large Z-averages indicate aggregation (instrument detection limit was 3 microns). Error bars +/- S.D. (n=3). d) Aggregation of liposomes detected by dynamic light scattering. Liposomes (6:4 DSPC:Chol) were incubated with the 3Q beacon and target as indicated.

When the target nucleic acid was added to the 3Q MB-inserted nanoparticles, visible aggregation occurred unexpectedly (Figure 3-6a). The aggregates could be pelleted by centrifugation, and were further assessed for ApoB content. The insoluble aggregates included the ApoB protein, and a concentration dependent aggregation pattern was observed in which full nanoparticle precipitation could be achieved with 6 beacons inserted per particle (Figure 3-6b). We next made use of dynamic light scattering to assess aggregation of the nanoparticles (Figure 3-6c). The upper detection limit for the light scattering instrumentation was 3 microns. Standard LDL is approximately 25 nm in diameter. A limitation of this assay was that when
there was a small amount of aggregation, light scattering could not accurately distinguish between a small population of large aggregates or a homogenous population of smaller aggregates. Therefore, this assay was used to verify the aggregation state, and not for insight into the shape and size distributions of the aggregates. At nanoparticle concentrations of 400 nM and 100 nM, aggregation was observed with both 3 and 6 beacons per particle upon target addition. At 50 nM nanoparticle concentration, only the nanoparticle with 6 beacons per particle displayed full aggregation, and the nanoparticle with 3 MBs per particle exhibited less aggregation. At 15 nM nanoparticle concentration, nanoparticles with 6 beacons per particle displayed diminished aggregation, and those with 3 beacons per particle had minimal aggregation compared to 50 nM nanoparticle concentration. Therefore, the nanoparticle aggregation process was modulated both by the number of MBs per particle and the nanoparticle concentration at target addition. Since the aggregates contained the ApoB protein (Figure 3-5b), we next examined whether or not a proteinaceous component was essential for the aggregation phenomena. When DSPC:Cholesterol (6:4) liposomes were incubated with the 3Q MB, only a small fraction of beacon binding was observed (data not shown). Despite the incomplete beacon binding, addition of the target nucleic acid to liposomes incubated with the beacon specifically induced aggregation (Figure 3-6d). Thus, neither protein aggregation nor a protein component was essential for nanoparticle aggregation, suggesting that aggregation is based on the interactions between lipids and structural rearrangements resulting from the hydrophobically modified MB opening.
We next examined whether the MB driven aggregation could discriminate single base mismatches (Figure 3-7a). When a 16 base target was used, aggregation occurred even when a mismatch was introduced. When a 15mer target was used, aggregation was less efficient for the single base mismatch target. When 14mer and 13mer targets were used, aggregation was observed for only the correct target sequence. When the target length was reduced to a 12mer, even the correct target sequence induced diminished aggregation. These results were consistent with a process dependent on MB opening. This was further supported by the observation that
the 13mer single mismatch target could not effectively open the MB, but the matched 13mer target could, leading to fluorescence unquenching (Figure 3-7b). The location of the mismatch within the target sequence was also considered by examining single base mismatches at every other position in the 13mer target. Since the target was a shared stem target, the last 6 bases of the target hybridize with the 5´ terminus of the shared stem sequence beacon sequence, and the first 8 bases of the targets target the loop portion of the beacon. With one exception, single mismatched targets could not induce aggregation, regardless of the mismatch position (Figure 3-7c). When the 13´ position was mismatched, partial aggregation was observed. This base hybridized with the very first base of 5´ beacon terminal, suggesting this position is important for the aggregation.

In summary, the solid-phase approach was effective for generating PMBs that are more convenient to synthesize and purify. By using pyropheophorbide modified CPG, the entire PMB could be synthesized in one run on a standard DNA synthesizer using only one specialty quenching phosphoramidite. Purification was also facilitated by the change in HPLC retention time induced by the presence of multiple quenchers. This synthetic approach permitted the generation of a modified and powerful singlet oxygen superquencher, in which the quenchers were assembled linearly rather than in a branched configuration. The 3-Q PMB displayed favourable characteristics for purification and low-background activation. Fully automated DNA synthesis methods facilitate effective nucleic acid activatable photosensitizer design and implementation. MBs inserted into lipoprotein and liposome lipid nanoparticles and selectively induced irreversible nanoparticle aggregation through target nucleic acid recognition. In the presence of the target, the process was modulated by two controllable variables: nanoparticle concentration and the number of beacon per particle. This phenomenon is a promising new
technique for DNA-nanoparticle manipulations. Further work will examine beacon and target sequences with varying lengths and G-C contents, and will explore directed aggregation using payload bearing nanoparticles to surfaces decorated with target DNA.

3.6 References


Chapter 4

Self-quenched, Self-assembled Porphyrin-Lipid Nanovesicles for Multimodal Imaging and Therapy

4.4 Acknowledgement

This chapter is a revised and reformatted version of the manuscript entitled “Porphysome nanovesicles generated by porphyrin bilayers for use as multimodal biophotonic contrast agents”, published in Nature Materials by Lovell et al.1 Under the supervision of Gang Zheng, my contribution to this work included designing and carrying out most experiments, analyzing and interpreting the data and writing the manuscript text. Thanks to Warren Chan for assistance in planning experiments, Chulhong Kim and Lihong Wang for photoacoustic experiments, Cheng Jin for animal experiments, Elizabeth Huynh for formulation characterization, John Rubinstein for electron microscopy experiments, Honglin Jin for toxicity experiments and Andrew Cao for assistance in preparing the porphysome starting materials.

4.5 Abstract

Optically active nanomaterials promise to advance a diverse range of biophotonic techniques through nanoscale optical effects and integration of multiple imaging and therapeutic modalities. Here, we report the development of porphysomes, nanovesicles formed from self-assembled porphyrin bilayers that generated large, tunable extinction coefficients, structure-dependent fluorescence self-quenching, and unique photothermal and photoacoustic properties. Compared to standard activatable photosensitizers described in Chapter 1, porphysomes displayed much higher fluorescence self-quenching. Porphysomes facilitated sensitive
visualization of lymphatic systems using photoacoustic tomography. Near-infrared fluorescence generation could be restored upon dissociation, creating opportunities for low-background fluorescence imaging. As organic nanoparticles, porphysomes were enzymatically biodegradable and induced minimal acute toxicity in mice with intravenous doses of 1000 mg/kg. Like liposomes, the large aqueous core of porphysomes could be passively or actively loaded. Following systemic administration, porphysomes accumulated in tumors of xenograft-bearing mice and laser irradiation induced photothermal tumor ablation. The nanoscale optical properties and biocompatibility of porphysomes demonstrate the multimodal potential of organic nanoparticles for biophotonic imaging and therapy.

4.6 Introduction

Therapeutic and diagnostic techniques benefiting from components that heavily absorb light include fluorescent and colorimetric detection\(^2,3\), photothermal and photodynamic therapy\(^4-6\), photoacoustic tomography (also known as optoacoustic tomography)\(^7-10\), optical frequency domain imaging\(^11\), and multimodal techniques\(^12\), amongst others. Since inorganic nanoparticles often interact strongly with light, they can be used as agents for these techniques. For instance, quantum dots are valuable fluorescent probes and have extinction coefficients in the range of \(10^5\) to \(10^6\) M\(^{-1}\)cm\(^{-1}\).\(^13\) Gold nanoparticles are useful for colorimetric detection, photothermal and photoacoustic techniques owing to their much higher (on the order of \(10^9\) to \(10^{11}\) M\(^{-1}\)cm\(^{-1}\)) extinction coefficients.\(^14\) Despite recent progress\(^15\), optically active inorganic nanoparticles have not yet achieved broad clinical implementation, possibly stemming from drug loading that is typically limited to the nanoparticle surface and concerns regarding long-term safety\(^16-19\). In
contrast, organic nanoparticles (including liposomes, lipoproteins, micelles, nanospheres and polymersomes) have found many human therapeutic applications as a result of robust biocompatibility and drug delivery capacity\(^9\). However, as these organic nanoparticles generally do not intrinsically absorb light in the near infrared, they have been of limited use for biophotonics. While supramolecular assemblies can be formed entirely by porphyrin conjugates, intensely light-absorbing organic small molecules, these constructs have not been thoroughly explored as biophotonic tools owing to a lack of stability, solubility or biological utility\(^20\). Here we introduce “porphysomes”, organic nanoparticles self-assembled from phospholipid-porphyrin conjugates that exhibit liposome-like structure and loading capacity, high absorption of near-infrared light, structure-dependent fluorescence quenching, excellent biocompatibility, and have promise for diverse biophotonic applications.

4.7 Materials and Methods

4.7.1 Synthesis of pyropheophorbide-lipid

In a standard reaction, 100 nmol of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (Avanti Polar Lipids), 50 nmol pyropheophorbide (prepared from *Spirulina Pacifica* algae as described previously; Zheng *et al.*, Bioconj. Chem., 2002, 13-392), 50 nmol of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma), 25 nmol of 4-(dimethylamino) pyridine (Sigma) and 50 µL of N,N-diisopropylethylamine (Sigma) were combined in 10 mL of anhydrous dichloromethane. The reaction mixture was stirred at room temperature under argon in the dark for 48 hours. The solvent was evaporated and the residue was subjected to thin layer chromatography purification (20 x 20 cm pre-coated silica gel plate with fluorescent indicator, 1.5 mm in thickness). Chloroform-methanol-glacial acetic acid-water 65:25:8:2 (volume ratio)
was used as the solvent. The major band with $R_f=0.4$ was isolated from the plate and eluted giving a final yield of 45%. Recently, we found that improved purification could be achieved by using diol modified silica (Sorbtech) and eluting the product with 8% methanol in DCM after washing out impurities with 2% and 5% methanol in DCM. The pyropheophorbide-lipid was then dried under nitrogen and stored under argon at -20°C in 1 µmol aliquots. Purity (>95%) and identity (acyl-migrated regioisomer product) were confirmed with HPLC and mass spectrometry (Waters MicroMass HPLC; Phenomenex Jupiter C4 column, 0.4 mL/min flow from 25% to 95% acetonitrile over 30 minutes followed by a 10 minutes hold in 0.1 % trifluoroacetic acid, compound eluted at 32 minutes. Expected mass: 1012.3; observed mass: 1013.1).

Figure 4-1: Synthesis scheme of pyropheophorbide-lipid
4.7.2 Synthesis of bacteriochlorophyll-lipid

In a standard reaction, 100 nmol of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, 50 nmol of bacteriochlorophyll acid (prepared as described previously; Kozyrev et al., J. Org. Chem., 2006, 71-1949) 50 nmol of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 25 nmol 4-(dimethylamino) pyridine and 50 µL of N,N-diisopropylethylamine were combined in to 10 mL of anhydrous dichloromethane. The reaction mixture was stirred at room temperature under argon in dark for 48 hours. The solvent was evaporated and the residue was subjected to thin layer chromatography plate purification (20 x 20 cm pre-coated silica gel TLC plate with fluorescence indicator, 1.5 mm in thickness). Chloroform-methanol-glacial acetic acid-water 65:25:8:2 (volume ratio) was used as the developing system. The final product was obtained in 38% yield with R_f=0.4. The final product spontaneously oxidized to yield an oxidized derivative of bacteriochlorophyll-lipid, which was verified by mass spectrometry and the expected structure is shown in Scheme 2. After purification, the lipid was aliquoted, dried and stored under argon at -20° C. The purity (>95%) and identity (acyl-migrated regioisomer product) were confirmed by analytical HPLC and mass spectrometry. (Same protocol as pyropheophorbide-lipid. Compound eluted at 32 minutes. Expected mass: 1104.3; observed mass: 1104.8).
4.7.3 Generation of metallic pyropheophorbide-lipid

To generate porphyrin-lipid conjugates with a chelated metal, 10 fold excess free zinc acetate (Bioshop Canada) was incubated with pyro-lipid in methanol for 1 hour at room temperature under argon. The same protocol was repeated with copper acetate and palladium acetate. Free metal was removed with 5 butanol/water extractions. The metal porphyrin-lipid was then aliquoted, dried and stored under argon at -20°C. The stable metal incorporation, purity (>95%) and identity of the porphyrin lipids were confirmed by HPLC and mass spectrometry (elution time 31 min; expected mass: 1075.6; observed mass: 1075.0).
4.7.4 Formation of porphysomes

Porphyrin-lipid films were prepared in 12 mm x 75 mm borosilicate test tubes (Fisher Scientific) by combining 95 molar % porphyrin-lipid with 5 molar % distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol) (PEG-2000-PE, Avanti Polar Lipids) in chloroform. For folate conjugated porphysomes, 4 molar % PEG-2000-PE was supplemented with 1 molar % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-folate(polyethylene glycol) (Folate-PEG-PE, Avanti Polar Lipids) in chloroform. Films were dried under a stream of nitrogen gas and further dried under vacuum for 1 hour. The lipid film was stored at -20° C under argon until hydration with phosphate PBS (150 mM NaCl, 10 mM phosphate, pH 7.4) and was then subjected to five freeze-thaw cycles, by freezing the test tube in liquid nitrogen and thawing it in water heated to 65° C. The porphosome suspension was extruded 15 times using a Mini-Extruder (Avanti Polar Lipids) through a 100 nm pore size polycarbonate membrane (Avanti Polar Lipids) at 65° C. Porphysomes were usually formed at 1 or 0.5 mg/mL combined porphyrin-lipid and PEG-lipid concentration. Final porphosome concentration was
assessed after their extrusion by measuring the absorption of a dilute sample in methanol (Bioshop Canada) and using extinction coefficients of 97,000 M⁻¹cm⁻¹ at 410 nm for pyropheophorbide-lipid and 37,000 M⁻¹cm⁻¹ at 750 nm for bacteriochlorophyll-lipid and assuming 83,000 porphyrin-lipids per 100 nm porphysome containing 95% porphyrin-lipid²¹. Usually 1-2 µL of porphysomes were diluted in 1 mL of methanol for the measurement. Porphysomes were stored at 4° C under argon until use. For the large scale porphysomes used for in vivo toxicity assessment, porphyrin-lipid was combined with PEG-lipid in a 50 mL round bottom flask and the organic solvent was evaporated under reduced pressure. The flask was then hydrated with approximately 10 mL of PBS (for ~75 mg lipid) and the solution was subjected to 5 freeze-thaw cycles. Porphysomes were then formed by sonicating the flask at 55° C for 1 hour. Porphysomes were then filtered through a 0.2 µm filter (Acrodisc filter, Pall) and concentrated with a centrifugal conical tube concentrator with 100 kDa membrane pore size (Millipore). Final size (125 nm) was assessed by dynamic light scattering and concentration was determined by absorption. To form small 30 nm porphysomes, a pure porphyrin-lipid film was generated with 0.1 mg porphyrin-lipid and dried under nitrogen and vacuum. The film was rehydrated with 200 µL of water and was sonicated for 10 minutes at 55° C.

4.7.5 Characterization of size and shape of porphysomes

Liposome and porphysome size was measured using a Malvern Nanosizer ZS90 (Malvern Instruments). Liposome and porphysome solutions were diluted to 6 µg/mL in PBS and three measurements were performed with 15 runs each and the results averaged. Electron microscopy specimens were prepared by incubating 0.05 mg/mL pyropheophorbide porphysomes (5% PEG-lipid, 95% pyro-lipid) on glow discharged carbon coated grids for 2 minutes, rinsing three times with milli-Q water and staining with 2% uranyl acetate. Samples were then visualized
with a Tecnai F20 electron microscope (FEI Company) operating at 200 kV and images were recorded with a Tietz F114 CCD (TVIPS).

4.7.6 Characterization of porphysome self-quenching

Porphysomes and liposomes were formed by first creating separate stock solutions of porphyrin-lipid, egg yolk phosphatidylcholine (EYPC) and cholesterol in chloroform. Free pyropheophorbide was dissolved in methanol. These constituents were combined at the indicated molar ratios (with a constant EYPC:CHOL ratio, and increasing amounts of pyro-lipid or free pyropheophorbide) in separate test tubes. The organic solvent was then evaporated under a nitrogen stream and trace organic solvent was removed by drying the films under vacuum. The separate lipid films containing all the indicated components were then hydrated with PBS, freeze-thawed and extruded as described above. Emission spectra were recorded with a Fluoromax fluorometer (Horiba Jobin Yvon) using 2 nm slit widths. Porphysome solutions were diluted to 0.02 µg/mL in PBS and those containing free pyropheophorbide or pyropheophorbide-lipid were excited at 420 nm and emission was measured and integrated from 600 nm to 750 nm. Background subtraction of an equal concentration of 100 nm egg phosphatidyl choline:cholesterol (3:2) liposomes was performed. NBD liposomes were formed in the same manner as porphysomes, but by replacing the porphyrin-lipid with 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-sn-glycero-3-phosphocholine (Avanti Polar Lipids). NBD liposomes were excited at 470 nm and emission was measured and integrated from 500 nm to 600 nm. The fluorescence self-quenching $F_{DET}/F_0$ of each sample was determined by ratio of the integrated fluorescence emission in the presence or absence of 0.5% Triton X-100 (Bioshop) over four measurements from separate preparations.
4.7.7 Resonance light scattering of porphysomes

Pyropheophorbide porphysomes and gold nanorods (40 nm length by 15 nm width, estimated $\varepsilon_{680} = 3.5 \times 10^9$ M$^{-1}$ cm$^{-1}$, based on Orendorff and Murphy, 2006, J. Phys Chem. B., 110-3990) kindly provided by the Kumacheva lab, University of Toronto) were adjusted to the same absorbance at 680 nm of 0.067 in PBS. Excitation and emission were then set to the same wavelength using 1 nm slit widths and scanned from 400 nm to 700 nm. After blank subtraction, the resonance scatter of the two samples was divided. Similar results were obtained with commercial 650 nm wavelength nanorods (Nanopartz).

4.7.8 Differential scanning calorimetry

Differential scanning calorimetry was performed on 5 mg/mL samples of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), hydrogenated soy phosphatidyl choline (HSPC) and pyropheophorbide porphysomes using a 6100 Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation). Samples were prepared by forming 5 mg lipid films, rehydrating in 1 mL of PBS and sonicating at 60°C for 15 minutes. Samples were degassed in a vacuum for 30 minutes prior to measurement and scanned at a rate of 1°C/min. PBS was used as the reference and one heating and cooling cycle as the baseline. For each sample, three cooling and heating scans from 5°C to 95°C were performed and the results averaged to determine the phase transition temperature of the lipid.

4.7.9 Photothermal properties of porphysomes

5 µL drops were placed on a piece of parafilm. All solutions were measured in PBS, with liposomes and porphysomes normalized to 0.5 mg/mL concentration. Porphysomes and gold nanorods were also normalized to an optical density at 680 nm of 0.8. Samples were irradiated with a 673 nm diode laser with 150 mW output and the temperature equilibrated within 60
seconds. Surface temperature was then imaged using a temperature calibrated infrared camera (Mikroshot).

4.7.10 Characterization of photoacoustic properties of porphysomes

Photoacoustic measurements were carried out using a Ti:Saphire tunable laser setup with a ultrasound transducer as previously described (see Cho et al., J. Phys. Chem., 2009, 113-9023). The light fluence was less than 7 mJ/cm² for photoacoustic measurement, within the ANSI limit. The axial and transverse resolutions of the system were 150 μm and 590 μm, respectively. By measuring the arrival times of generated photoacoustic signals, one-dimensional depth-resolved images (called A-lines) were acquired. Additional raster scanning along two transverse directions provided the three-dimensional images. The acquired volumetric data was processed in a form: a maximum amplitude projection - a projection of the maximum photoacoustic signal along each A-line onto the corresponding plane. Measurements were carried out at 760 nm using bacteriochlorophyll porphysomes in PBS solution. For structural dependent studies, the photoacoustic signal of porphysomes was compared to porphysomes that had been lysed with 0.5% Triton X-100.

Animal experiments were performed in compliance with Washington University guidelines. *In vivo* lymphatic mapping with porphysomes was performed using Sprague-Dawley rats (~200 g) and a 100μL injection of 9 nM bacteriochlorophyll porphysomes on the left forepaw. The region of interest was shaved prior to injection and photoacoustic measurements. After 2.5 hours, animals were sacrificed and first draining lymph node photoacoustic signal was confirmed *ex-vivo* (data not shown). Data shown is representative of 3 experiments.
4.7.11 Fluorescence activation of porphysomes with KB cells

KB cells were cultured in folate negative RPMI 1640 media (Invitrogen) with 10% FBS and seeded in an 8 well glass chamber (Lab-tek Chamber Coverglass, Nunc) with 30,000 cells in 200 µL media per well two days prior to imaging. Cells were incubated with pyropheophobide porphysomes (30 pM porphysome concentration) for 3 hours at 37° C in the media without serum and imaged with confocal microscopy (Olympus FluoView 1000) using 633 nm laser excitation. The porphysome containing media was not removed prior to imaging and 0.5 µL of 5 mg/mL Hoechst 33258 stain (Sigma) was added to visualize cell nuclei using 405 nm laser excitation. Data shown is representative of over 10 experiments, and specific folate mediated uptake was also confirmed by flow cytometry (data not shown). For colocalization studies, cells were also incubated with Alexa 488 transferrin (Invitrogen) or lysotracker (Invitrogen), as well as Hoechst 33258 prior to live-cell confocal microscopy. Cell viability was assessed by incubating porphysomes overnight with KB cells in media lacking serum. 20 µL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen, 5 mg/mL) was then added to each well and the plate was incubated with cells 1 for hour. Media was replaced with 150 µL of 70% isopropanol in 0.1 M HCl, shaken for 20 minutes and absorbance was measured at 570 nm to determine viability relative to an untreated control. Animal experiments were performed in compliance with University Health Network guidelines. 3x10^6 KB cells were inoculated subcutaneously in nude mice and the xenograft grew for 2-3 weeks. Mice (weighing approximately 30 g) were then injected via tail vein with bacteriochlorophyll porphysomes (7.5 pmol). Imaging was performed using a Maestro imaging system (CRI) using a 710 to 760 nm bandpass excitation filter and an 800 nm longpass emission filter with 2 second exposure time. Data shown is representative of 3 experiments.
4.7.12 Biodegradation of porphysomes

Pyropheophorbide porphysomes (with pyropheophorbide-lipid concentration of 400 µM) were incubated with 200 U lipase (from *Rhizomucor miehei*, Sigma) for 24 hours at 37°C in PBS containing 0.5% Triton X-100 and 10 mM CaCl₂. The solution was then subjected to HPLC-MS analysis as described for porphyrin-lipid purification and absorption was analyzed at 400 nm. Following previously described methods, 100 µM pyropheophorbide was then incubated in 0.25% Triton X-100 with 25 units of horseradish peroxidase (type II, Sigma), 250 µM of hydrogen peroxide and 500 µM 2,4-dichlorophenol (Sigma), and absorption loss at 700 nm was monitored. After 1 hour, another 250 µM hydrogen peroxide was added and the reaction was monitored for another hour.

4.7.13 Toxicity, biodistribution and blood clearance of porphysomes

Animal experiments were performed in accordance with University Health Network guidelines. 6 week male BALB/c mice were obtained from Charles River. Blood was sampled from the saphenous vein approximately 6 hours before porphysome or saline injection. Blood was subjected to the Mammalian Liver Profile tests (Abaxis), and MASCOT hematology profiling (Drew Scientific) according to manufacturer protocol. The total bilirubin value for the Liver Profile test was excluded since several readings gave errors. Mice were injected via tail vein with porphysomes (1000 mg/kg) or an equal volume of PBS. Over a two week period, mice were observed for behavioral changes and weight was monitored. Mice were then sacrificed, after cardiac puncture to obtain blood for analysis. Mice carcasses were placed in a 10% formalin solution and sent to Ontario Veterinary College (Guelph, Ontario) for histopathology analysis. Tissues examined included: trachea, esophagus, thyroid gland, thymus, heart, lungs,
liver, kidneys, spleen, small intestine, cecum, colon, urinary bladder, prostate, seminal vesicles, testes, epididymus, skin, femur, bone marrow, skeletal muscle, head, eyes, ears, and brain.

For biodistribution, female nude mice (~23 g) bearing KB tumors were injected with porphysomes (with 5% PEG-lipid; with or without 30 molar % cholesterol) containing 100 nmol pyro-lipid (n=5 in each group). Mice were sacrificed 24 hours post-injection and organs were collected. 30 mg of tissue were weighed and homogenized in 1 mL PBS on ice for 2 minutes. Triton X-100 was added to a final concentration of 1 %, and the mixtures were vortexed for 2 minutes and then centrifuged at 13,200 rpm for 15 minutes (5415D Microcentrifuge, Eppendorf). Fluorescence of the supernatant was then measured (excitation, 410 nm; emission, 675 nm; slit width, 5nm), and the % of injected dose per gram of tissue was calculated based on a standard curve to calibrate pyro-lipid concentration.

For blood clearance, female nude mice (~20 g) were injected with regular porphysomes (95% pyro-lipid, 5% PEG-lipid) or cholesterol porphysomes (65% pyro-lipid, 5% PEG-lipid, 30% cholesterol) via tail vein based on an injection dose of 100 nmol pyro-lipid (n=4 for each group). Blood was sampled from the saphenous vein using a 25 gauge needle to puncture the vein and heparinized capillary tubes (Fisher) to collect the blood up to 72 hours post-injection, and centrifuged at 3000 rpm (5415D Microcentrifuge, Eppendorf) for 10 minutes to isolate plasma. The porphysome concentrations were measured based on the fluorescence (excitation, 410 nm; emission, 675 nm; slit width, 5nm). The logarithm values of plasma concentrations were plotted as a function of time, showing that it is a one compartment model. GraphPad Prism was used for data analysis for the best-fit line and half-life.
4.7.14 Fluorophore and drug loading of porphysomes

To encapsulate 5(6)carboxyfluorescein (Anaspec), a 1 mg porphysome film with or without 30 molar % cholesterol was hydrated with 250 mM carboxyfluorescein, 10 mM Tris pH 8 (pH was adjusted with sodium hydroxide). After freeze-thaw and extrusion, free carboxyfluorescein was removed by gel filtration using a PD-10 column (GE Healthcare) equilibrated with PBS. 300 µL fractions were collected and a 20 µL aliquot of each fraction was added to a 300 µL solution of 0.5% Triton X-100 and 10 mM Tris pH 8. Fluorescence of the fractions was then analyzed with a SpectraMax fluorometer (Molecular Devices) by measuring the porphyrin fluorescence with 415 nm excitation and 685 nm emission, and measuring the carboxyfluorescein fluorescence with 485 nm excitation and 525 nm emission. Relative carboxyfluorescein incorporation was determined by first summing the total carboxyfluorescein fluorescence in the excluded, porphysome-containing fractions. Fluorescence measurements of the different types of porphysomes were performed at the same time. Carboxyfluorescein incorporation was then determined (relative to the non-cholesterol porphysomes) by dividing the carboxyfluorescein fluorescence of the cholesterol-containing porphysomes by the non-cholesterol containing porphysomes. To incorporate doxorubicin, a 0.45 mg/mL (0.78 mM) solution of doxorubicin hydrochloride (Sigma Aldrich) with 0.078 mM NaOH was loaded into porphysomes with or without 50 molar % cholesterol. A 1 mg film was hydrated with 1 ml 155mM ammonium sulfate pH 5.5 and subject to freeze-thaw cycles and extrusion. Free ammonium sulfate was removed by gel filtration using a PD-10 column equilibrated with PBS and the porphysome containing fractions were collected in 2 mL. A 500 µL aliquot was incubated with doxorubicin (25% of the pyro-lipid concentration) for 2 hours at 37° C. Following incubation, free doxorubicin was removed by gel filtration using a PD-10 column equilibrated with PBS. 95
300µl fractions were collected and for each fraction, a 20µL aliquot was added to 280 µL 0.5% Triton X-100. Porphyrin and doxorubicin fluorescence in each fraction were then measured with a SpectraMax fluorometer (Molecular Devices) using wavelengths of 420 nm excitation and 680 nm emission for the porphyrin and 485 nm excitation and 595 nm emission for doxorubicin. Doxorubicin incorporation was determined by dividing the sum of the doxorubicin fluorescence in the excluded, porphysome-containing fractions by the sum of doxorubicin fluorescence from all collected fractions.

4.7.15 Photothermal therapy using porphysomes

KB tumors were generated in female nude mice by injecting $2 \times 10^6$ cells into the right flank of female nude mice (~23 g). When tumor volumes reached 4-5 mm, 42 mg/kg of porphysomes containing 30 molar % cholesterol were injected via tail vein. 24 hours later, mice were anesthetized with 2% (v/v) isofluorane and tumors were irradiated with a 658 nm laser (Orion, Laserglow Technologies). Laser output at 660 nm was measured as 750 mW and the spot size was 5 mm by 8 mm. Tumor temperatures were recorded with an infrared camera (Mikroshot). For one week following treatment, all mice received enrofloxacin (0.25 mg/mL) in their drinking water. Tumor volume was measured daily and mice were sacrificed once tumor size reached 10 mm.
Figure 4-4: Porphysomes are optically active nanovesicles formed from porphyrin bilayers

a, Schematic representation of a pyropheophorbide-lipid porphysome. The phospholipid headgroup (red) and porphyrin (blue) are highlighted in the subunit (left) and assembled nanovesicle (right). b, Electron micrographs of negatively stained porphysomes (5% PEG-lipid, 95% pyropheophorbide-lipid). c, Absorbance of the porphyrin-lipid subunits incorporated in porphysomes formed from pyropheophorbide (blue), zinc-pyropheophorbide (orange), and bacteriochlorophyll (red) in PBS. d, Resonance light scattering spectra ratio between gold nanorods and pyropheophorbide porphysomes. Nanorod and porphysome concentration was adjusted to have equal optical density at 680 nm. e, Dynamic light scattering size profiles of indicated porphysomes recorded in PBS.
Figure 4-5: Generation of 30 nm porphysomes
Dynamic light scattering measurements show that pyropheophorbide-lipid that was rehydrated and sonicated in water (red) generated small, 30 nm porphysomes. Porphysomes that were created through extrusion through a 100 nm polycarbonate membrane were larger in size (blue).

Figure 4-6: Optical extinction of porphyrin-lipid subunits in organic and aqueous solvent
The absorbance is shown for the indicated porphyrin-lipid in methanol (black). For reference, the absorbance of porphysomes (composed of the porphyrin-lipid incorporated into 100 nm porphysomes measured in PBS) is also shown in gray.
4.8 Results and Discussion

Porphysomes were formed by supramolecular self-assembly. The porphysome subunits consisted of porphyrin-lipid conjugates generated by an alkylation reaction between lysophosphatidylcholine and pyropheophorbide, a chlorophyll-derived porphyrin analog. This hydrophobic chromophore was positioned in place of an alkyl sidechain, maintaining an amphipathic structure (Figure 4-4a). This conjugate could be self-assembled in aqueous buffer with extrusion to form porphysomes. 5 molar % polyethylene glycol (PEG) lipid was included in the formulation to enhance in vivo pharmacokinetics. Transmission electron microscopy showed these porphysomes were spherical vesicles 100 nm in diameter (Figure 4-4b). At higher magnifications, the porphysome structure was revealed as two layers of higher density material separated by a 2 nm gap, corresponding to two separate monolayers of porphyrin. Pyropheophorbide porphysomes exhibited two absorption peaks at 400 nm and in the near infrared window at 680 nm (Figure 4-4c). Further red-shifted porphysomes (760 nm) were produced by using subunits generated from another type of porphyrin, a bacteriochlorophyll analog that was synthesized in the same manner as pyropheophorbide-lipid. Alternatively, a protocol was developed to insert metal ions into the porphyrin-lipid structure, resulting in shifted optical density bands (440 nm and 670 nm) and demonstrating the unique phenomenon that porphysomes can form metal-chelating bilayers. These different types of porphysomes could be useful in scenarios where specific operating wavelengths are required (e.g. to match a given laser excitation source). To verify the absorbance spectra corresponded to light absorption rather than scattering, we compared porphysomes to wavelength-matched gold nanorods (with 680 nm extinction peaks) using resonance scattering. Porphysomes displayed up to 100 times less resonance light scatter at the optical density wavelength peak at which the
samples were normalized (Figure 4-4d). The monodisperse 100 nm sizes exhibited by various types of porphysomes (Figure 4-4e) are in a suitable range to take advantage of the enhanced permeability and retention effect for passive accumulation in tumors\textsuperscript{25,26}. Flexibility in size control was demonstrated as sonication of porphyrin-lipid in water produced smaller 30 nm nanoparticles (Figure 4-5), which could be useful for applications requiring smaller nanoparticle sizes. Geometric calculations for vesicles 100 nm in diameter composed of subunits with phosphatidylcholine headgroups suggest there are approximately 8x10\textsuperscript{4} porphyrin conjugates per porphysome\textsuperscript{21}. Based on pyropheophorbide absorbance (accounting for differences in the absorbance of the intact porphysome measured in PBS and the dissociated porphyrin-lipid obtained by diluting 1-2 µL of porphysomes in 1 mL of methanol, as shown in Figure 4-6, we estimate a pyropheophorbide porphysome extinction coefficient, ε\textsubscript{680}, of 2.9 x 10\textsuperscript{9} M\textsuperscript{-1}cm\textsuperscript{-1}. This large, near-infrared extinction coefficient is a reflection of the dense porphyrin packing in the bilayer that generates the unique nanoscale optical behavior of porphysomes.

To understand the implications of such a high number of porphyrin-lipid conjugates in a 100 nm diameter nanovesicle, fluorescence self-quenching was examined. As increasing amounts of porphyrin-lipid were included in the formulations of standard liposomes (3:2 molar ratio of egg yolk phosphatidylcholine:cholesterol), self-quenching increased up to 1200 fold when porphysomes were formed completely by porphyrin-lipid subunits (Figure 4-7a). This is much greater than typical porphyrin quenching\textsuperscript{27} and suggests an energetically favorable supramolecular structure in which the porphyrin-lipid orientation facilitates extensive porphyrin interaction and quenching. Because PEG-lipid was added to enhance \textit{in vivo} pharmacokinetics, its potential to modulate porphysome self-quenching was assessed. While incorporating 5 molar
% distearoylphosphatidylcholine (the lipid portion of the PEG-lipid) did not affect quenching, 5 molar % PEG-lipid modestly enhanced self-quenching to over 1500 fold (Figure 4-7b). This increase was due to the stabilizing effect of PEG, consistent with observations that porphysomes containing PEG maintained their size and monodispersity for at least 9 months, whereas those without PEG aggregated rapidly. To assess whether any nanostructure composed of dye-lipid subunits would be sufficient to generate extreme self-quenching, vesicles formed from NBD-lipid (a non-ionic dye conjugated to a lipid in a manner similar to porphyrin-lipid) were examined. NBD-lipid could not form monodisperse 100 nm vesicles (data not shown) and self-quenching was only 20 fold, highlighting the role of porphyrin interaction in defining porphysome structure and nanoscale properties. Differential scanning calorimetry revealed the porphyrin-lipid had no apparent transition temperature, suggesting that porphyrin stacking is distinct from the typical acyl chain interactions that drive normal lipid transitions in liposomes (Figure 4-8). To determine if quenching was solely a characteristic of porphyrin confinement in a bilayer, the behavior of free porphyrin in liposomes was examined. The maximum amount of free pyropheophorbide that could be incorporated into liposomes was only 15 molar %, since manual extrusion became physically impossible beyond this amount. Porphysomes displayed 5 times more self-quenching at corresponding levels of porphyrin-lipid incorporation (Figure 4-7), demonstrating again that the porphyrin bilayer structure is essential for extensive self-quenching. Porphyrin-loaded liposomes have been described for biological applications, but can only accommodate a small molar fraction of porphyrin and cannot prevent porphyrin redistribution to serum proteins. Other porphyrin vesicles and diblock copolymers have been described that incorporate porphyrin subunits, but lower porphyrin density resulted in lesser extinction coefficients and an absence of significant fluorescence self-quenching.
Figure 4-7: Porphysomes demonstrate extensive and structurally-driven self-quenching.

a. Porphysome quenching as a function of molar % pyropheophorbide-lipid (mean +/- SD from 4 experiments). \( F_0 \) corresponds to the fluorescence of the porphysomes in PBS and \( F_{DET} \) is the fluorescence after disruption of the porphysomes using 0.5% Triton X-100. Nanovesicles were formed from films containing the indicated molar % porphyrin-lipid and the remainder egg yolk phosphatidylcholine:cholesterol (3:2). b. Self-quenching of various nanovesicle formulations (mean +/- SD from 4 experiments). *The maximum free porphyrin that could be loaded in liposomes before manual extrusion became physically impossible was 15 molar %.

Figure 4-8: Porphyrin-lipid lacks a conventional transition temperature

Differential scanning calorimetry revealed that while hydrogenated soy phosphatidyl choline and dimiristoyl phosphatidyl choline have clear transition temperatures, pyropheophorbide-lipid does not. The calorimetry was performed in PBS with a lipid concentration of 5 mg/mL.
Figure 4-9 Multimodal optical utility of porphysomes

a. Photothermal transduction. Solutions were irradiated with a 673 nm laser and imaged with a thermal camera. b. Ratio of photoacoustic amplitudes measured for porphysomes and methylene blue +/- 0.5% Triton X-100 (mean +/- SEM from 10 measurements). c. Photoacoustic images of tubing containing porphysomes and PBS measured +/- 0.5% Triton X-100. d. Dual modality for photoacoustic contrast and activatable fluorescence. d-i: Lymphatic mapping. Rats were imaged using photoacoustic tomography before and after intradermal injection of porphysomes (2.3 pmol). Secondary lymph vessels (cyan), lymph node (red), inflowing lymph vessel (yellow) and 5 mm scale bar are indicated. d-ii: Fluorescence activation after I.V. injection of porphysomes (7.5 pmols) in a KB xenograft-bearing mouse. e. Triggered fluorescence activation upon folate receptor mediated uptake in KB cells. Porphysomes were incubated for 3 hours with KB cells and porphyrin-lipid (yellow) and nuclei (blue) were visualized with confocal microscopy.
As porphysomes are highly self-quenched, energy that is normally released to fluorescence and singlet oxygen generation (pyropheophorbide has a combined fluorescence and singlet oxygen quantum yield approaching unity) is dissipated elsewhere. As seen in Figure 4-9a, upon exposure to laser irradiation, energy was released thermally, with an efficiency comparable to gold nanorods (photothermally active inorganic nanoparticles), whereas laser irradiation of standard liposomes generated no significant increase in solution temperature. As photoacoustic signal generation is related to thermal expansion, porphysomes also generated strong photoacoustic signals, proportional to concentration and detectable as low as 25 picomolar although detection in this range was slightly nonlinear (Figure 4-10). Although photoacoustic signal is correlated to absorption, when detergent was added to disrupt the porphysome structure (actually generating an increase in absorption), photoacoustic signal decreased up to 6 fold (Figure 4-9b). The detergent had no effect on the photoacoustic signal of the clinically-used contrast agent methylene blue, suggesting the structurally based self-quenching of porphysomes is requisite for nanoscale photoacoustic properties. This basic phenomenon of photoacoustic signal attenuation upon detergent-induced porphysome dissociation is demonstrated in the photoacoustic images in Figure 4-9c.

![Figure 4-10:Photoacoustic detection of porphysomes](image)

**Figure 4-10:** Photoacoustic transduction of bacteriochlorophyll porphysomes measured in PBS as a function of porphysome concentration (mean +/- SEM from 20 measurements).
Figure 4-11: Distinct spectral responses of blood and porphysomes *in vivo*

Normalized photoacoustic response for the indicated portion of the image shown in the inset. The *in vivo* porphysomes that accumulated in the lymph node have the same spectral response as porphysomes in solution placed in tubing and measured *in vitro*.

Secondary and tertiary lymph nodes became detectable in a rat with intradermal injection of 2.3 pmol of porphysomes. Yellow arrow indicates inflowing lymph vessel. Red and blue arrows indicate the first draining and subsequent lymph nodes, respectively. 5 mm scale bar is indicated.

Figure 4-12: Photoacoustic mapping of multiple lymph nodes using porphysomes
We next examined the unique quality that porphysomes are intrinsically suited for both photoacoustic tomography and fluorescence imaging \textit{in vivo}. Photoacoustic techniques are gaining recognition and have recently been used to non-invasively detect circulating cancer cells in blood vessels\textsuperscript{31}, as well as in sentinel lymph nodes\textsuperscript{32}. When porphysomes were injected intradermally in rats, the local lymphatic network became clearly detectable within 15 minutes as porphysomes drained to the lymph vessels and nodes (Figure 4-9d-i). Porphysomes displayed a strong photoacoustic signal permitting the visualization of the first draining lymph node (red), the inflowing lymph vessel (yellow) and surrounding lymph vessels (cyan). The presence of porphysomes in these lymphatic vessels was directly confirmed by the distinct spectral signature of porphysomes in comparison to that of blood (Figure 4-11). Other lymph nodes could be traced over time (Figure 4-12). By using a 6.5 ns pulse width, 10 Hz laser, photoacoustic measurements did not generate sufficient heating to damage surrounding tissues. Next, to investigate whether porphysomes were suited for \textit{in vivo} fluorescence imaging, they were injected intravenously into mice bearing KB cell xenografts. 15 minutes after injection, there was low overall fluorescence signal, demonstrating the self-quenching of porphysomes \textit{in vivo} (Figure 4-9d-ii, left). After 2 days, high tumor fluorescence was observed as porphysomes accumulated in the tumor and became unquenched (Figure 4-9d-ii, right), potentially through an enhanced permeability and retention effect or receptor mediated endocytosis (the porphysomes used for fluorescence imaging included 1 molar % of folate-PEG-lipid). The concept of porphyrosome quenching \textit{in vivo} was more dramatically illustrated when we injected detergent disrupted porphysomes into mice and observed much higher initial fluorescence (Figure 4-13). Thus, based on unique self-assembled and nanoscale properties, porphysomes are intrinsically multimodal for both photoacoustic tomography and low background fluorescence imaging.
Figure 4-13: Low fluorescence background of porphysomes

a, Porphysome and saline solutions prior to and after detergent addition (10% Cremophore EL). b, Fluorescence imaging demonstrating the fluorescence signal that appeared in Fig 3c-ii after injection of porphysomes (left) or injection of the same concentration of porphysomes that had been disrupted with Cremophore EL prior to injection (right). Note the mouse shown on the right moved slightly between the white light and fluorescence imaging.
Figure 4-14: Colocalization of porphysomes in early endosomes and lysosomes

**a**, KB cells were co-incubated with porphysomes containing 1 molar % folate-PEG-lipid, and Alexa 488 transferrin for 3 hours prior to live cell confocal microscopy. Channels are colored as indicated. **b**, KB cells were incubated with porphysomes containing 1 molar % folate-PEG-lipid for 3 hours, then with lysotracker for 30 minutes prior to confocal imaging. Channels are colored as indicated.
To examine the behavior of porphysomes upon uptake by cancer cells, folate receptor targeted porphysomes were produced by including 1 molar % folate-PEG-lipid. The folate receptor is overexpressed in a variety of cancers and effectively internalizes liposomes conjugated to folate. When KB cells (which over-express the folate receptor) were incubated with folate-porphysomes, specific uptake was observed by confocal microscopy and could be inhibited by free folate (Figure 4-9e). Since intact porphysomes in the incubation media were essentially non-fluorescent, confocal imaging was performed without a need to change the media. Control experiments revealed that the porphyrin-lipid ended up in endosomes and lysosomes, based on partial colocalization with transferrin and lysotraker (Figure 4-14).

We next assessed factors relevant to potential clinical applications of porphysomes. To bypass the unknown, long-term side effects of inorganic nanoparticle accumulation in body organs, luminescent silica nanoparticles have been developed that decompose in aqueous solution over a period of hours. Porphysomes are stable for months when stored in aqueous solutions, but they were prone to enzymatic degradation (Figure 4-15a). Upon incubation with detergent and lipase, the phospholipid structure was cleaved, with the major aromatic product being pyropheophorbide, which was the starting material in the synthetic reaction generating the porphyrin-lipid. Like chlorophyll, pyropheophorbide is known to be enzymatically cleaved into colorless pyrroles when incubated with peroxidase and hydrogen peroxide. We verified this degradation by monitoring the loss of porphyrin absorption and confirmed that pyropheophorbide could be efficiently degraded by peroxidase. To our knowledge, this is the first example of an enzymatically biodegradable, intrinsically optical active nanoparticle. We next performed a preliminary study to assess the potential toxicity of porphysomes. When mice were treated with a high dose of porphysomes (1000 mg/kg), they remained healthy over a two week period, as demonstrated by a lack of major behavior changes or weight loss (Figure
At the two week time point, mice were sacrificed and blood tests were performed (Figure 4-15c). Liver function tests indicated mice hepatic function was generally normal, with the exception of somewhat elevated levels of bile acids and alanine transferase (less than 2 times the upper range of normal). Red blood cell counts and attributes were unaffected by the large dose of porphyrin-lipid, which did not interfere with the physiological regulation of endogenous porphyrin (heme). Unaffected white blood cell counts imply that porphysomes were not immunogenic at the two week time point, even at the high doses given to mice. Post-mortem histopathological examination of the liver, spleen and kidneys indicated these organs were in good condition and were not impacted by the high intravenous porphysome dose (Figure 4-15d).

The large aqueous core of the porphysome, contained within the porphyrin bilayer, has potential for cargo loading. When porphysomes (containing 5% PEG-lipid) were hydrated using a 250 mM carboxyfluorescein solution and extruded, only a limited amount of carboxyfluorescein was stably entrapped in the porphysomes as determined by gel filtration (Figure 4-16a, left). As cholesterol is known to enhance loading of compounds within phosphatidylcholine-based liposomes\textsuperscript{35}, we included 30 molar % cholesterol into the formulation and repeated the passive carboxyfluorescein loading. The cholesterol containing porphysomes were able to load ~20 times more carboxyfluorescein compared to the porphysomes lacking cholesterol (Figure 4-16a, right). At this high loading concentration, carboxyfluorescein itself was self-quenched in the porphysome (Figure 4-16b, left). Further, the porphysome remained fluorescently self-quenched (Figure 4-16b, right), suggesting that most of the light absorbed by the porphyrin bilayer was converted to heat. As expected, passive loading of carboxyfluorescein only entrapped a small fraction of the total fluorophore in the hydration solution.
**Figure 4-15** Porphysomes are enzymatically biodegradable and well tolerated *in vivo*

a, Enzymatic degradation of porphysomes. Porphysomes were lysed with 1% Triton X-100 and incubated with lipase in PBS. Degradation was probed using HPLC-MS analysis. Purified pyrophophorbide was incubated with peroxidase and degradation was verified by monitoring the loss of absorbance at 680 nm. b, Mouse mass change after intravenous administration of 1000 mg/kg porphysomes or PBS (mean +/- SD, n=3). c, Blood test parameters for mice with intravenous administration of porphysomes or PBS (mean +/- SD, n=3). Since some test values for gamma globulin transferase results were given as less than 5 U/L, all values less than 5 U/L are reported as 5 U/L. d, Representative hematoxylin and eosin stained sections of indicated organs from mice 2 weeks after I.V. injection of 1000 mg/kg porphysomes or PBS.
Figure 4-16: Active and passive loading of porphysomes

a, Passive loading of carboxyfluorescein (C.F.). Porphysomes composed without (Porph.) or with 30 mol. % cholesterol (Chol. Porph.) were extruded with 250 mM C.F. and gel filtration was performed. Fluorescence of Pyro (blue) and C.F. (green) was measured in 0.5% Triton X-100 to avoid quenching. b, Fluorescence quenching of Chol. Porph. (blue) loaded with C.F (green). Spectra were taken prior (dashed) and after (solid) addition of detergent and normalized to maximum fluorescence. c, Active loading of doxorubicin (Dox.). Fluorescence of gel filtration fractions (*collected when porphysomes began to elute) of porphysomes without or with 50 mol. % cholesterol. Fluorescence of pyro (blue) and Dox. (green) was measured with detergent. d, Fluorescence quenching of pyro in Chol. Porph. loaded with Dox. Normalized spectra were measured prior (solid) and after (dashed) addition of detergent. e, Size distributions of porphysomes loaded with C.F. (black) or doxorubicin (gray).
Biodistribution and blood clearance of porphysomes

**a,** Biodistribution of porphysomes (95% pyro-lipid, 5% PEG-lipid) or cholesterol porphysomes (30% cholesterol, 5% PEG-lipid, 65% pyro-lipid) 24 hours following I.V. injection of porphysomes containing a total of 100 nmol pyro-lipid. Mean values are indicated on the graph and standard deviations are shown for the liver and spleen samples of regular porphysomes in brackets (mean +/- SD from 5 mice in per group).

**b,** Blood clearance of porphysomes. Mice were injected intravenously with porphysomes with a total of 100 nmol pyro-lipid. 30 µL blood was collected from the saphenous vein at the indicated time points and pyro-lipid concentration was assessed by fluorescence measurements (mean +/- SD from 4 mice per group).

**Figure 4-17:** Biodistribution and blood clearance of porphysomes
Figure 4-18: Porphysomes as photothermal therapy agents

**a.** Photothermal therapy setup showing laser and tumor-bearing mouse. **b.** Representative thermal response in KB tumor-bearing mice injected I.V. 24 hours prior with 42 mg/kg porphysomes or PBS. Thermal image was obtained after 60 seconds of laser irradiation (1.9 W/cm²). **c.** Maximum tumor temperature during 60 second laser irradiation (mean +/- SD for 5 mice per group). **d.** Photographs showing therapeutic response to photothermal therapy using porphysomes. **e.** Survival plot of tumor-bearing mice treated with the indicated conditions. Mice were sacrificed when tumors reached 10 mm size (n=5 for each group).
One of the most powerful drug loading techniques is active loading, which uses pH or ion gradients to concentrate amphipathic weakly basic molecules into liposomes\textsuperscript{36} and polymersomes\textsuperscript{37}. The importance of this loading technique is reflected by Doxil®, the first clinically implemented nanoparticle\textsuperscript{38}, which is a liposomal formulation of actively loaded doxorubicin. We applied the ammonium sulfate gradient method\textsuperscript{36} with a doxorubicin to pyropheophorbide-lipid molar ratio of 1:5 to actively load doxorubicin into porphysomes. Without addition of cholesterol, some loading of doxorubicin was observed by gel filtration, but the fraction of the total doxorubicin incorporated from the solution was approximately 10% (Figure 4-16c, left). However, when 50 molar % cholesterol was added to the porphysome formulation, strong active loading was achieved and porphysomes loaded 90% of all free doxorubicin in solution into the porphysome core (Figure 4-16c, right). These porphysomes also maintained a self-quenching porphyrin bilayer (Figure 4-16d). Both actively and passively loaded porphysomes displayed monodisperse sizes between 150 nm and 200 nm (Figure 4-16e).

Photothermal therapy is an emerging technique that uses contrast agents that convert light into heat at target sites. Inorganic nanoparticles including gold nanoshells\textsuperscript{15}, gold nanorods\textsuperscript{39}, gold nanocages\textsuperscript{40} and graphene\textsuperscript{41} have been used to destroy tumors using photothermal therapy. To demonstrate the biophotonic therapeutic potential of an organic nanoparticle, we performed preliminary experiments using porphysomes as agents for photothermal therapy. We used porphysomes containing 30 molar % cholesterol since they demonstrated favorable biodistribution following systemic administration with more accumulation in the tumor (3% injected dose per gram) and less accumulation in the liver and spleen than standard porphysomes (Figure 4-17a). Cholesterol porphysomes also had a 35% longer serum half-life of 8.5 hours (Figure 4-17b). A 658 nm laser outputting 750 mW (with a power density of 1.9
W/cm²) was used to irradiate the KB tumors in xenograft bearing mice following porphysome administration (Figure 4-18a). 24 hours prior to treatment, mice were injected intravenously with 42 mg/kg porphysomes or a PBS control. The tumor was then irradiated with the laser for 1 minute and temperature was monitored using a thermal camera (Figure 4-18b). The tumor temperature in the porphysome group rapidly reached 60°C, whereas the tumors in mice injected with PBS were limited to 40°C (Figure 4-18c). Following treatment, mice in the porphysome and laser treated group developed eschars on the tumors, whereas the laser alone group and the porphysomes alone group did not. After 2 weeks the eschars healed and the tumors in the treated group were destroyed (Figure 4-18d). Unlike the tumors in mice treated with porphysomes and laser treatment, tumors in mice that received laser treatment alone or porphysome injection alone continued to grow rapidly and all the mice in those groups had to be sacrificed within 21 days (Figure 4-18e). This photothermal experiment corresponded to a treatment with a therapeutic index of at least 25, given the safety of porphysomes at 1 g/kg intravenous doses. We believe that porphysomes could impact a range of clinical applications, potentially exploiting synergistic, multimodal optical imaging and therapeutic approaches. However, to achieve clinical relevance, the rapid attenuation of light in biological tissues must be dealt with by leveraging improving light delivery methods or targeting diseases that affect organs that are more accessible to light.42

Like liposomes, porphysomes are self-assembled from simple monomers, are efficient nanocarriers, are enzymatically biodegradable and are highly biocompatible. A small molar percentage of lipid conjugated to targeting moieties, such as antibodies, aptamers, proteins or small targeting molecules could be easily incorporated to potentially direct porphysomes to a range of different target cells. Like optically active inorganic nanoparticles, porphysomes have
large, tunable extinction coefficients and are effective agents for photothermal and photoacoustic applications. Porphysomes display unique nanoscale optical properties and are intrinsically suited for multimodal imaging and therapeutic applications.

### 4.9 References


Chapter 5
Discussion and Outlook

5.1 Discussion

5.1.1 New approaches to photodynamic therapy
This thesis presented research pertaining to porphyrin-based optical cancer theranostics, with an underlying focus on novel approaches to both photodynamic and photothermal therapy. While photodynamic therapy has been established for decades, and is approved for a wide range of oncological and non-oncological indications\(^1\), its clinical impact with respect to cancer appears to be somewhat limited. It is difficult to assess the true impact of photodynamic therapy without an analysis of hospital standard of care data, but based on discussions with physicians and scientists, it appears PDT has a limited role in cancer therapy, at least in Ontario. Evidently, PDT does not offer strong enough advantages over other standards of care, such as surgical resection, localized techniques (e.g. radiofrequency-, ultrasound- or cryo- ablation), targeted radiation, or chemotherapy to gain widespread use. Given that safe, systemically delivered porphyrin photosensitizing agents with intrinsic imaging (fluorescence) and therapy (PDT) capability can be used in conjunction with specific light delivery to target tissues, why then is PDT not more successful? Light propagation in tissue is rapidly attenuated, which makes tumor treatment challenging. This can be overcome by irradiation of the tumor from different angles and depths using interstitial light delivery through optical fibers\(^2\), even though this adds a layer of complexity to the treatment. Patient discomfort following treatment due to skin photosensitization for a period of weeks is another drawback, although newer photosensitizers have reduced this time significantly.\(^3\) The lack of a clear optimal dosing regime further complicates treatment, and much work has been done to improve PDT dosimetry, since there
are several variables that affect response including photosensitizer injection amount, laser intensity and laser irradiation time. Ultimately, the goal of such dosimetry planning is to destroy the tumor tissue with singlet oxygen, while leaving healthy tissues undamaged. While tumors take up photosensitizers with some selectivity, some photosensitizer also accumulates in surrounding tissues. Thus, a limiting factor of PDT is that light directed at the tumor site damages adjacent healthy tissues. This prevents the use of greater photosensitizer amounts and stronger light doses and potentially introduces risk of incomplete PDT responses. As outlined in Chapter 1, activatable photosensitizers hold promise to drastically reduce the damage incurred in healthy tissue, while localizing PDT damage to the tumor. This has potential to be a transformative approach, since if photosensitizer activity is limited to the tumor, larger light doses and photosensitizer doses can be used to enhance tumor destruction, while at the same time leaving adjacent healthy tissue undamaged. Chapter 2 presented a biophysical study demonstrating that activatable photosensitizers can readily be designed and validated using conventional strategies already applied to the generation of fluorescence sensors. The activatable photosensitizer approach is powerful because theoretically, cells can be destroyed based on their specific characteristics. Gang Zheng has been the worldwide leader of this approach, initially developing a caspase protease activatable photosensitizer in 2004. During the course of this thesis, we extended this strategy to create the first nucleic acid triggered activatable photosensitizer that generated singlet oxygen in response to specific target sequences. This approach is, to my knowledge, the only method to directly destroy cells based on their genetic expression (as opposed to a phenotype caused by that expression). Anti-sense or siRNA technology has attracted enormous attention because it offers the potential to inhibit specific gene expression, but that in itself does not necessarily destroy the cell. Chapter 3 demonstrated that new architectures that we developed confer useful properties to molecular
beacons. By attaching multiple quenchers to a porphyrin-based solid phase support, beacons could more easily be synthesized and purified. The additional quenchers also conferred superior quenching to the photodynamic molecular beacon. Because these multiple quencher beacons were substantially more hydrophobic, they readily inserted into lipidic nanoparticles. When the target nucleic acid was added to the nanoparticle-inserted beacons, unexpected aggregation was observed along with the beacon opening. This phenomenon was characterized and could be programmed to recognize single base mismatches. This has potential implications that extend beyond photodynamic therapy into analytical or drug release applications.

5.1.2 Opportunities for photothermal therapy
While activatable photosensitizers hold promise to improve PDT efficacy, successful clinical applications must meet several requirements. PDT is based on chemical reactions produced by singlet oxygen and requires three components; a photosensitizer, light and oxygen. Considering that regions of hypoxia are frequently prevalent in solid tumors\(^7\), the lack of a homogenously well-oxygenated target tissue may decrease the reliability of PDT. Photothermal therapy (PTT) is an emerging modality that differs in many regards from photodynamic therapy, despite that both use laser light for treatment. Contrast-agent mediated PTT requires only two factors: the contrast agent and laser light. The damage is caused by physical heating of the target tissue, as opposed to a singlet oxygen based chemical reaction. Contrast-agent free laser ablation is currently used for some indications such as liver cancer\(^8,9\). This differs from PDT, which would be completely harmless to target tissues without the accumulation of a photosensitizer. In Chapter 2, we noted that pyropheophorbide can effectively fluoreesentcly self-quench when in close proximity. This rapid conversion to ground state results in energy dissipated as heat. We discovered porphysomes during the course of examining photosensitizer self-quenching within
liposome bilayers. Whereas free photosensitizers could not be loaded in liposomes beyond 15 molar percent, porphysomes could be formed entirely from porphyrin-lipid subunits. We observed that porphysomes displayed incredibly high self-quenching, a finding that prompted us to originally name these nanovesicles “quenchosomes”. The absorbed light energy was being dissipated as heat and therefore porphysomes were further explored as PTT agents. Porphysomes offer several key advantages over other potential contrast agents for PTT such as small organic molecules or inorganic nanoparticles. Small organic molecules suffer from rapid clearance time and a non-optimal size for passive accumulation in tumors. Inorganic nanoparticles such as gold nanoshells have been a triumph for translational nanomedicine, with early stage human PTT clinical trials underway. However, long-term safety issues exist in terms of biocompatibility and biodegradation. Porphysomes circumvent these problems by being of an appropriate size to benefit from the enhanced permeability and retention effect. Porphysomes are organic in nature and thus fully biocompatible and biodegradable. As they are self-assembled from a small molecule, porphysomes are expected to eventually degrade in vivo. In addition, they displayed unique nanoscale biophotonic properties such as photoacoustic and fluorescence “switching” in intact and dissociated forms.

5.2 Future Direction
Many interesting avenues of research became apparent during the course of these studies for both activatable photosensitizers, as well as porphysomes and the porphyrin-lipid monomer. With respect to DNA based activatable photosensitizers, further translational work is required to validate these beacons in cells. The hydrophobic nature of the superquenched beacons will likely permit enhanced entry into cells. However, a nuclease resistant backbone is required to prevent rapid degradation in serum. Other delivery options, such as using lipidic nanoparticles
such as HDL or LDL could also be investigated independently as novel delivery vehicles. Regarding the aggregation of lipidic nanoparticles, this approach could potentially be used for magnetic resonance imaging, where lipids chelated with paramagnetic ions will display a change in relaxivity rates in the aggregated states. Thus, in theory a gene-specific indicator of specific mRNA expression could be possible. Many steps are required before such a tool is feasible, including developing a strategy to deliver the lipidic nanoparticles, intact, into the cytosol of cells. There are more directions to explore for activatable photosensitizers, including simply creating a larger library of enzyme-activated photosensitizers by replacing the fluorophore of conventional enzyme or metabolite sensing fluorescent probes with a photosensitizer. Different types of quenchers and photosensitizers could be investigated as activatable photosensitizers. Although the field is less than 10 years old, it has seen a tremendous amount of recent activity. Ultimately, the utility of these probes needs to be established in animal models. If the fundamental advantage of activatable photosensitizers is that they become activated in the tumor and light treatment causes no harm to adjacent healthy tissues, that must be demonstrated.

As outlined in Chapter 4, porphysomes hold potential for multimodal imaging and therapy applications. The intrinsic nature of porphysome imaging modalities extends beyond optical modalities like fluorescence and photoacoustic imaging. By chelating a radionuclide (such as copper-64) PET imaging is possible. By chelating a paramagnetic ion such as manganese, magnetic resonance imaging is possible. While such imaging modalities can be packaged onto a nanoparticle through the addition of multiple exogenous functional groups, porphysomes have this compatibility built in. From a regulatory viewpoint, this simplified composition is advantageous and makes the clinical possibilities of multimodal imaging and therapy more
viable. These versatile imaging modalities could lead to paradigm shifts where, for example, imaging is used to assess porphysome uptake in tumors. This could be used directly as an optical, PET based or MR based diagnostic technique, or in a combination of multiple imaging modalities. More intriguingly, this could be used specifically to guide PTT using MR and PET. Complementary imaging and therapy could increase the probability of success since the accumulation of the contrast agent in the tumor can be verified to guide light treatment and assess treatment areas. This approach offers a theoretical upside over other focal therapies (e.g. RF ablation) because it provides information where the tumors are located that have accumulated contrast agent and can be successfully and selectively treated. One drawback of contrast agent-mediated PTT is that relatively high concentrations of contrast agent are required to achieve tumor specific heating, assuming the contrast agent accumulates in the tumor. An alternate approach that porphysomes could enable is the heat-catalyzed conversion of prodrugs loaded in the porphysome into active drugs for localized therapy. This would require the development of prodrugs modified with heat labile protecting groups. The advantage of such an approach is that the intrinsic loading capability of porphysomes could be used, without requiring large injected doses compared to those required for PTT. The extended possibilities for porphyrin-lipid are considerable. We have already observed that porphyrin-lipid can be used to form lipoprotein nanoparticles and ultrasound microbubbles. In the past 30 years, phospholipids and liposomes have been used in countless biotechnological and medical applications\textsuperscript{13}; porphyrin-lipid and porphysomes could be substituted instead, and in the process add nanoscale biophotonic character with intrinsic capability for metal chelation to those previously established materials and assays.
In conclusion, we have engineered a range of porphyrin-based molecules and assemblies with unique physical properties that might prove useful for applications in biomedical research. Future work should focus on applied studies required for clinical translational of these constructs.

5.3 References


