ANALYSIS OF DNA INTERACTIONS AND PHOTOCLEAVAGE BY PHENYL MESO SUBSTITUTED CYANINE DYES IN THE NEAR-INFRARED RANGE

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ANALYSIS OF DNA INTERACTIONS AND PHOTOCLEAVAGE BY PHENYL MESO SUBSTITUTED CYANINE DYES IN THE NEAR-INFRARED RANGE

by

CHRISTINA M. FISCHER

Under the Direction of Kathryn B. Grant, Ph.D.

ABSTRACT

Cyanine dyes are attractive photosensitizers for photodynamic therapy due to their ease of structure modification and intense absorption in the near-infrared range. Photosensitizers that can bind to DNA and absorb at long enough wavelengths of light to deeply penetrate biological tissue are in high demand for treatment of cancer and other diseases. The following study analyzes the DNA interactions of three pentamethine cyanine dyes with very similar structures, all of which absorb light at wavelengths longer than 800 nm. The work described involves an extensive study of the photocleavage abilities and DNA binding characteristics of these dyes. Our lead compound was a bromophenyl meso substituted symmetrical quinoline cyanine dye. Spectroscopic data, gel electrophoresis experiments and other studies were used to provide evidence of DNA binding mode, ROS production, and of dye-sensitized DNA photocleavage at the unprecedented wavelength of 850 nm.

INDEX WORDS: Near-infrared, Photodynamic therapy, Cyanine dye, Reactive oxygen species, DNA, Photocleavage, Spectroscopy
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SUBSTITUTED CYANINE DYES IN THE NEAR-INFRARED RANGE

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CHRISTINA M. FISCHER

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ANALYSIS OF DNA INTERACTIONS AND PHOTOCLEAVAGE BY PHENYL \textit{MESO} SUBSTITUTED CYANINE DYES IN THE NEAR-INFRARED RANGE ABOVE

by

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Georgia State University
December 2017
DEDICATION

I dedicate this thesis to Elizabeth and Charlie; their constant, unfiltered sibling support was just what I needed to get through any situation. To my parents, thank you for your love and support, you both were always there no matter what good or bad decisions I made. And to Chris, your sarcastic comments and loving dedication throughout the entire process were more than I deserved, thank you and I love you. Also to my cat, Kitty Kat, because everyone needs a fluffy little stress reliever.
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1 INTRODUCTION

1.1 Photodynamic Therapy

The use of light therapy can be dated back over 4000 years to Egypt. Ancient Egyptians would treat vitiligo by exposing skin to UV light after the ingestion of plants that contained light-sensitive compounds.\(^1\)-\(^3\) Photodynamic therapy (PDT) was later coined as the name for this type of treatment. Some of the early recorded uses of PDT were from the late 19\(^{th}\) century when a combination of light and acridine dyes was found to kill unicellular organisms, as well as the use of eosins to treat skin cancer.\(^1\) Finally, in 1995, the FDA approved the first PDT agent for clinical use called Photofrin®, and since then, the focus of the field has been to find new and more effective compounds.\(^2\)

The way PDT works as a therapeutic method is by utilizing the light absorption properties of compounds, like Photofrin®, to create reactive species of oxygen that can, through various mechanisms, drive a cell to apoptosis or necrosis. This method of treatment has been used as a non-invasive, cost effective way to eliminate lesions and cancerous growths, as well as for treatment of age-related macular degeneration, coronary heart disease, and in various antimicrobial, antiviral and antibacterial applications.\(^1\)-\(^4\)

The compounds in question are referred to as photosensitizers (PS). For a PS to be useful in PDT treatment, it must be able to become excited to its triplet state and then transfer energy or electrons to surrounding ground state triplet oxygen (Figure 1-1).\(^5\) When these PS are injected into malignant cells and irradiated with light, they create reactive oxygen species (ROS), and these react with macromolecules in proximity, including DNA base pairs.
Figure 1.1: Simplified representation of a Jablonski diagram as a depiction of photochemical mechanisms involved in PDT.

1.1.1 Photosensitizers

There are a few conventional photosensitizers that have been FDA approved for medical use and a number of others currently in clinical trials. Two examples can be seen in Figure 1-2. Others include temoporfin, verteporfin, and methyl aminolevulinate (MAL).
Figure 1.2: Examples of FDA approved photosensitizers profimer sodium, Photofrin® (top), 8-aminolevulinic acid (ALA), Ameluz® (bottom)\textsuperscript{6-7}.

Out of these examples, Photofrin® was not only the first, but it is the most widely used PDT photosensitizer.\textsuperscript{4} It has been utilized for various cancers such as esophageal, bladder, stomach and some skin cancers. Even with these applications, it is still not an optimal PS. The drug itself is a mixture of dimeric and oligomeric forms, as well as unknown compounds, making its true composition unknown.\textsuperscript{4} Along with not being fully understood, the complex absorbs light at 630 nm.\textsuperscript{6} Photofrin® can also lead to prolonged skin sensitivity, causing patients to avoid direct sunlight for several weeks after treatment.\textsuperscript{8} ALA-PDT was approved by the FDA for use in actinic keratosis treatment and absorbs light at 635 nm. The optimum window for a PS to absorb light is between 700 nm and 1200 nm due to the depth of skin penetration reached by light in this range (Figure 1-3).\textsuperscript{6,9-10}
Wavelengths shorter than 700 nm of light cannot penetrate deeply enough into the tissue and are only useful for treating superficial surface diseases, making it necessary to find more red-shifted PS which absorb light that penetrates further. Most of the compounds currently on the market are derivatives of profimer sodium and therefore do not absorb in the biological window. Outside of the 700 nm to 1200 nm window of light is where proteins, water, and other vital macromolecules absorb. Therefore, irradiating outside this range can be dangerous to the healthy tissues.

Chromophores that can create ROS efficiently have a generally stable triplet excited state. Once in their triplet states, they can then follow the Type 1 reaction and transfer electrons to ground state triplet oxygen ($^3$O$_2$) to create superoxide anion radicals (O$_2^-$). These radicals can follow a Fenton type reaction to create H$_2$O$_2$, eventually leading to the production of hydroxyl radicals.

*Figure 1.3: Approximate wavelength penetration through biological tissues.*

![Approximate wavelength penetration through biological tissues](image)
radicals (‘OH), that are so reactive they have a diffusion distance of only 0.8 to 6.0 nm. In the triplet state, PS can also follow the Type 2 reaction pathway and transfer energy to ground state triplet oxygen to create singlet oxygen (¹O₂). For singlet oxygen to be produced, the triplet state of a PS must have a triplet state energy that is equal to or exceeds the excitation energy of ¹O₂, 95 kJmol⁻¹. For hydroxyl radical production, the PS must have a triplet state reduction potential greater than the ground state triplet oxygen conversion to superoxide anion radicals. Singlet oxygen and hydroxyl radicals are able to oxidize biological molecules, which, in living systems, can eventually lead to cell apoptosis or necrosis. The best way to get photosensitizers to lead a cell to apoptosis is to target mitochondrial DNA.

The structure of a good DNA photosensitizer is one that can bind to nucleic acids efficiently through one or more modes. These PS should have the ability to either intercalate into the DNA base pairs, which is most commonly seen with compounds containing planar aromatic ring systems, bind in the major or minor groove, and/or bind electrostatically to the external phosphate backbone. With the intense reactivity of the ROS created, a compound that is bound to DNA, with enough access to surrounding oxygen, can generate the reactive species close enough to the base pairs to interact and cut the DNA. Hydroxyl radicals in this proximity cut the DNA strands by abstracting hydrogen atoms from deoxyribose carbons. This abstraction leads to radical formation on the sugar allowing these carbons to react with surrounding molecules like oxygen, ultimately disrupting the sugar-base bonds creating breaks in the DNA strand. Singlet oxygen causes DNA strand breaks by abstracting an electron from guanine, ultimately disrupting the strand. Recently compounds have been found that can cut DNA when irradiated with light above 750 nm. For example, a catecholate Fe (III) complex was shown to
cleave DNA in 84% yield at 785 nm. The longest wavelength ever to be used to sensitize DNA cleavage is 830 nm.

1.1.2 DNA Photocleavage

When DNA base pairs are cut by ROS produced from light activating a PS, it is referred to as DNA photocleavage. Many of the experiments in this thesis use circular pUC19 plasmid DNA, 2,686 base pairs (bp) in length. When this DNA is intact, it is in a supercoiled compact form. If a single stranded cut is made by the reaction with ROS, then it becomes relaxed into its nicked form. If at the same spot, an additional cut on the opposite strand of the plasmid is made, a double stranded break creates the plasmid’s most relaxed form, linear. Each form of DNA moves at a different rate during agarose gel electrophoresis. This separation allows the DNA cleavage to be easily quantitated through analysis of these gels.

1.2 Cyanine Dyes

The following study analyzes the interactions between DNA and cyanine dyes. Cyanines are a specific class of fluorescent dyes known for their favorable photophysical properties, along with their high affinity for DNA. Their ease of synthesis allows for considerable modifications making them prime candidates for PS studies and optimization for use in PDT.

1.2.1 History

Cyanine dyes were first synthesized in 1856 and were named after their vibrant blue color that was exhibited by the first members of their class. They began to be used in the textile industry because of their bright colors and ease of synthesis and from there, they evolved to be used as fluorescent probes. Cyanine dyes can easily be modified to adjust their absorption and emission wavelengths in the visible and near infrared regions, which makes them useful in targeted bioimaging. More dyes are being found that can absorb in the near-infrared range,
giving them the ability to be activated by light even when embedded deeply in tissue. Analogs are also being synthesized that can interact with nucleic acids with very high affinity.\textsuperscript{21-22} A combination of various modifiable features makes these useful dye candidates for photodynamic therapy studies, especially in the therapeutic biological window.

1.2.2 Structure

There are several types of cyanine dye structures, and most are modeled around a basic backbone that can be seen in Figure 1-4. Most cyanines are made up of two nitrogen-containing heterocycles that can vary based on function. These two rings are connected by a polymethine bridge with an odd number of carbons.

![Figure 1.4: General cyanine dye structure.](image)

Changes to the heterocycles or the bridge can cause significant spectroscopic and functionality changes across dyes. Modifications, like having symmetric heterocycles versus asymmetric heterocycles and lengthening the bridge, can drastically change the absorption and emission spectra of the cyanines. These various changes can also increase the amount of reactive species that are produced upon irradiation.\textsuperscript{4, 9, 21, 23}

The cyanines of interest can alternate between a quinoline, indole, benzoxazole or benzothiazole ring system, which are only a handful of the total number of heterocycles that have been employed in cyanines (Figure 1-5).
Figure 1.5: General cyanine dye structure.

With a set ring system, an additional change made to cyanine dyes is the length of the polymethine bridge. The length of this bridge can affect the stability and absorption of the complex. The addition of methine carbons on the polymethine bridge can red shift the absorbance of the whole compound with every two carbon additions giving a red shift of around 100 nm. Although useful, increasing bridge length too much can lead to a significant decrease in dye stability.\(^{24-25}\) A third change that in most cases can lead to added stability is the addition of an electron withdrawing substituent to the bridge.\(^{26}\) This decreases the tendency of the dye to become oxidized in aqueous solutions. When a heavy atom, such as a halogen, is added to the bridge, this can increase the rate of inter-systems crossing (ISC) from the dye’s excited singlet state to its triplet state. With increased ISC, more reactive oxygen species can be produced giving better cell death yields.\(^4\) These changes influence how these complexes bind and interact with DNA, as well as how they absorb light and interact with the oxygen in solution.

One modification studied in the following project is the addition of a phenyl ring to the \textit{meso} position of the pentamethine bridge. The conjugation of the bridge itself acts to delocalize the cyanines positive charge on the rings. The addition of a phenyl ring adds aromaticity that acts to further delocalize the charge along the \(\pi\)-conjugated system, equalizing the electron density at each of the rings.\(^{27}\) The added phenyl group contributes to the conjugation within the molecule.
and this increase in conjugation contributes to the ideal π system, allowing further stabilization of the positive charge on the ring systems.\textsuperscript{27-28}

Structural changes, including the extension of the polymethine bridge, have led to the discovery of various degradation pathways of these cyanine dyes. A significant process involves the ROS needed to cleave DNA, reacting directly with the dye and causing photodegradation. The singlet oxygen (O\textsuperscript{1}) and superoxide anion radicals (O\textsuperscript{2}-) produced by the compounds are so reactive they have been found to interact with the polymethine bridge of the dye, cutting it into fragments.\textsuperscript{25} This can be visualized by a loss of visible color, or photobleaching, during light reactions.\textsuperscript{29} Various stability modifications to the dye structure can help prevent degradation, such as increasing rigidity and, as mentioned, by adding electron withdrawing groups to the polymethine bridge.\textsuperscript{25} The stability of these dyes can directly affect how they bind to various biomolecules.

1.2.3 DNA Interactions

Cyanine dyes have been studied intensely for their fluorescent properties.\textsuperscript{9} They have become an integral part of medicinal studies due to their ability to act as efficient fluorescent probes, as well as their tumor selectivity.\textsuperscript{9} One feature that has been found through these studies is that in the presence of biomolecules such as DNA and their subsequent binding to them, the fluorescence intensity significantly increases, signifying intense interactions between dye and biomolecule.\textsuperscript{14, 30}

There are a few DNA binding modes that have been predicted for dyes. Due to their planar ring systems, certain dyes have been known to bind in between adjacent base pairs in a double-stranded DNA (dsDNA) helix.\textsuperscript{31} This has been referred to as intercalation. When these molecules are intercalated, they can cause structural changes to the dsDNA, like extending and
unwinding the helix. When bound by intercalation, the dye itself can bind electrostatically to the base pairs and can add stability to the extended DNA helix through π-π stacking. The bound portions of dye overlap with the DNA base pairs, adding rigidity to the dye and creating an overall stable dye-DNA complex. Since binding of dyes depends on their structure, there are other binding modes in addition to intercalation. Another one, in particular, is groove binding. This binding can occur in the major or minor groove of the DNA helix; these molecules are bound and stabilized by electrostatic, hydrogen bonding, and hydrophobic interactions with the base pairs and the phosphate backbone. A third mode of binding involves a combination of both intercalation and groove binding where one of the heterocycles of the cyanine dye stacks in the base pairs while the other interacts with the groove. Out of all of these binding modes, pentamethine cyanines prefer minor groove binding over intercalation due to the elongated structure of the dye.

Self-aggregation of cyanine dyes is yet another phenomenon that is directly affected by not only the structure of the dyes but also the presence of biomolecules. One of the biggest reasons for hydrophobic dye aggregation is water. It is well known and well observed through UV-visible spectroscopy that dyes’ monomeric forms seem to create new aggregate structures in aqueous solutions. This aggregation can be attributed to the high dielectric constant of water and how it associates with the charges on the dye. Aggregation may also occur in the presence of polar organic solvents, such as dimethyl sulfoxide (DMSO). While on its own, the positive charge distributed along the dye would not allow for aggregation. When in the presence of water, the high dielectric constant reduces the repulsion between the similar charges on the dyes, allowing it to bind in various multimeric complexes. Aggregation can be controlled by utilizing the best solvents for stable dyes.
Cyanine dye aggregation can happen in response to the binding of biomolecules. Structures such as DNA can facilitate self-aggregation when the dye is bound within a DNA groove. The dye monomer can interact face-to-face with itself through π-stacking within the DNA groove making an H-aggregate, which can be visualized by a hypsochromic shift in the UV-visible spectra, giving the aggregate its name. J-aggregation forms if the monomers of the dye align themselves with some off-set within the groove, in a staircase manner. These types of self-aggregation cause distinct shifts in UV-visible spectra.\textsuperscript{13,35} Depending on the structure of the dye and the conjugation, it is possible to form either a J or H aggregate and in some cases both aggregation forms.

It is necessary to study the molecular interactions of these cyanine dyes with respect to their uses in biomedical applications. Their interactions with themselves and biomolecules like DNA can provide useful insights into how they can be used in photodynamic therapy.

1.3 Summary of Research

In the following study, the cyanine dyes synthesized in the lab of Professor Maged Henary by Matt Laramie and Eduardo Soriano were analyzed and compared for their abilities to interact with DNA (Figure 1-6). The variations in structures and how these variations affect the interactions will be discussed. Based on their structures, they are expected to bind to DNA in diverse ways as well as absorb light of different wavelengths. Their light absorption differences affect the complexes’ abilities to photocleave plasmid DNA. The dyes in question, have affected DNA cleavage at NIR range, specifically at light wavelengths above 800 nm. Due to the low energy photons absorbed by employed dyes, DNA photocleavage in this range is uncommon. The following experiments were performed to analyze the dyes as potential DNA photosensitizers and to discover the best dye structure for future photosensitizing studies. UV-
visible spectroscopy was used to determine light absorption, dye stability, and DNA interactions, with bromophenyl dye 1 having the greatest absorption of light in the NIR range and being considerably more stable in aqueous solutions as compared to chlorophenyl dye 2. Gel electrophoresis was used to resolve DNA damage DNA photocleavage studies conducted at various wavelengths. Circular dichroism and photocleavage inhibition assays using a known major groove binding agent, a minor groove binding agent, and an intercalating agent were used to study binding modes. Scavenger assays were conducted to determine the energy pathway that activated triplet oxygen to create ROS for damaging DNA. Brominated dye 3 was studied previously by Tayebeh Fatemipouya and was found to absorb light and cleave plasmid DNA at 808 nm and 830 nm. This dye will act as a reference compound for the remaining dyes under investigation in this thesis.

Figure 1.6: Structures of the cyanines dyes investigated following studies, synthesized by Professor Maged Henary’s lab at Georgia State University.
2 EXPERIMENT

2.1 Materials

All experiments were performed using deionized distilled water (ddH₂O). A solution of 10 mM sodium phosphate buffer pH 7.0 was made from monobasic and dibasic sodium phosphate salts obtained from Fisher Scientific (Fairlawn, NJ). Calf thymus DNA (CT-DNA) from Invitrogen (Cat. No. 15633-019, 10 mg/mL, average size ≥2000 bp) was used for all UV-visible spectroscopy experiments and all circular dichroism assays. Gel electrophoresis experiments were performed with pUC19 plasmid DNA. Standard protocols were utilized in the transformation of plasmid into competent E. coli cells (Stratagene, XL-1 blue) along with the cloning of said plasmid in bacterial cultures. The plasmid was purified using a QIAfilter Plasmid Mega Kit (Qiagen™, Cat. No. 12263). Ethidium bromide (EtBr), agarose, dimethyl sulfoxide (DMSO), pentamidine isethionate salt, sodium azide (≥99.9%), sodium benzoate (99%), catalase from bovine liver, and D₂O were purchased from Sigma-Aldrich (St. Louis, MO). Electrophoresis loading buffer was made from 15.0% (w/v) ficoll and 0.025% (w/v) bromophenol blue. Tris acetate EDTA (TAE) electrophoresis buffer was made from ethylenediaminetetraacetic acid disodium salt (EDTA) purchased from IBI Scientific (Peosta, IA) and tris(hydroxymethyl)aminomethane (tris base), purchased from Research Product International (Mt. Prospect, IL). Methyl green was obtained from GTI Laboratories Supplies (Houston, TX). Potassium iodide (99.99%+) was purchased from Aldrich Chem Co. (Milwaukee, WI). Cyanine dyes were synthesized by Matt Laramie and Eduardo Soriano under the direction of Professor Maged Henary (Georgia State University).
2.2 Instrumentation

UV-visible spectroscopy spectra were recorded on a PerkinElmer Lambda 35 spectrophotometer using UV WinLab version 6.0.3 in quartz cuvettes. Each spectrum was processed using Microsoft Office Excel. All photocleavage experiments were performed with light emitting diode (LED) lasers acquired from Laserlands or Lilly Electronics. These lasers had peak emission wavelengths and power outputs of 532 nm (100 mW), 808 nm (300 mW), 830 nm (350 mW) and 850 nm (100 mW). Set agarose gels were loaded into a Bio-Rad Laboratories gel electrophoresis box, and then a current of 100 mV was applied using a GIBCO BRL electrophoresis power supply from Life Technologies. All electrophoresed agarose gels were visualized with a transilluminator set at 302 nm (VWR Scientific, LM-20E) and then photographed with a Canon digital camera in a UVP PhotoDoc-It™ imaging system. Gels were quantitated using ImageQuant version 5.2 software. Circular dichroism (CD) and induced circular dichroism (ICD) spectra were obtained using a Jasco J-810 spectropolarimeter.

2.3 Methods

2.3.1 UV-Visible Spectroscopy

All samples were scanned in the same spectrophotometer using quartz cuvettes and a wavelength range of 1100 nm to 200 nm. For time course experiments, spectra were recorded every 5 min until the absorption spectra remained constant. In saturation experiments, small aliquots of CT-DNA were added to the prepared solution of dye, water, and 10 mM sodium phosphate buffer pH 7.0 until changes in dye absorption were no longer observed. Each sample was pre-equilibrated for 15 min prior to recording the spectrum, and after which, each spectrum was run twice back to back to ensure the dye was stable and reached equilibrium. The samples
contained 20 µM of dye in DMSO, in the presence of 10 mM sodium phosphate buffer pH 7.0 buffer with CT-DNA added after each run in varying volume increments until saturation.

In time course experiments, cuvettes contained either 10 µM of dye in DMSO without DNA or 10 mM sodium phosphate buffer (pH 7.0), 10 µM dye in the absence of 150 µM bp CT-DNA but in the presence of 10 mM sodium phosphate buffer, or 10 µM dye in the presence of buffer and 150 µM bp CT-DNA.

2.3.2 Gel Electrophoresis Preparation

All electrophoresis experiments were run using 1.5% agarose gels, prepared with 1.5 g of agarose dissolved in 100 mL 1XTAE buffer, pH 7.0. The agarose solution was then microwaved until homogenization, where no unwanted and partially melted agarose crystals were observed. A total of 10 µL of 5 µg/mL EtBr was added to the agarose which was then cast into a gel tray with a comb for 1 h until solidification. The gel was then moved into an electrophoresis box and submerged in TAE as running buffer which had been mixed with 20 µL of the EtBr. After the comb was removed, a total of 20 µL from each 30 µL reacted sample was loaded into the wells of the gel and electrophoresed for 1 h at 100 V. The EtBr-stained gel was then carefully analyzed using an ultraviolet transilluminator set at 302 nm.

2.3.3 Sample Preparation

Thirty µL samples containing ddH2O, 10 mM sodium phosphate buffer pH 7.0, 20 µM of dye, and 38 µM bp of pUC19 plasmid DNA were irradiated with a variety of lasers. Before irradiation, the samples were pre-equilibrated for 15 min in the dark. Irradiation runs were done in a thermometer fitted metal block that was kept in an ice bath at ~10 °C. A total of 3 µL of electrophoresis loading buffer was then added to each sample. To the pre-prepared 1.5% agarose
gel described above, 20 µL of each sample were loaded into individual wells and then electrophoresed for 1 h at 100 V.

2.3.4 **DNA Photocleavage Pre-equilibration Titration**

Reactions containing 25 µM dye 1 were prepared as described above and then irradiated with 808 nm, 830 nm, and 850 nm lasers for 10 min. The lasers were positioned, for this experiment and every subsequent assay, directly above each tube using either clamps or pre-formed styrofoam blocks fit to each laser, leaving less than 1 cm between the laser and the tube. The samples varied in the pre-equilibration time, the times including 0 s, 5 min, 10 min, 15 min, 20 min, 30 min, 60 min and 90 min. Reactions were carried out in a metal block in an ice bath and maintained at a temperature ~10 °C. After irradiation, 20 µL of each sample was loaded onto a pre-formed 1.5% agarose gel and electrophoresed for 1 hr at 100 V (Table 2-1). The optimum pre-equilibration time of 15 min was found for dye 1 and was applied to all other photocleavage assays across dyes for consistency.

<table>
<thead>
<tr>
<th>Time Pre-Equilibrated(min)</th>
<th>Dye</th>
<th>DNA</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>- +</td>
<td>+ +</td>
<td>- +</td>
</tr>
<tr>
<td>5</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>10</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>15</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>20</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>30</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>60</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>90</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

**Table 2.1: Example of sample addition to wells of agarose gel for pre-equilibration assay.**

2.3.5 **DNA Photocleavage Concentration Titration**

Samples of dye 1 were prepared as described above with the exception of the concentrations of dye, which were 5 µM, 10 µM, 15 µM, 20 µM, 25 µM, 30 µM, 40 µM and 50 µM. These samples, aside from a dark control containing 50 µM of dye 1, were irradiated using a 100 mW 850 nm laser for 10 min after a 15 min pre-equilibration time in the dark. Samples were
kept in the metal block in an ice bath at a temperature of ~10 °C. To a prepared 1.5% agarose gel, 20 μL of each sample were loaded into individual wells and electrophoresed for 1 h at 100 V (Table 2-2). A dye concentration of 20 μM was determined and used for all subsequent photocleavage experiments.

Table 2.2: Example of sample addition to wells of agarose gel for concentration titration assay.

<table>
<thead>
<tr>
<th>Dye (μM)</th>
<th>50</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Light</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

2.3.6 DNA Photocleavage Time Course Assay

Samples were separately prepared and then irradiated with an 850 nm 100 mW laser at 5 s, 30 s, 60 s, 2 min, 3 min, 5 min, 10 min, 30 min, 60 min and 90 min time intervals. After each irradiation, the samples were removed from the light and kept in the dark. All reactions, including dark controls, were kept at ~10 °C. After irradiation, 20 μL of each sample were loaded onto the wells of a pre-formed 1.5% agarose gel and electrophoresed for 1 h at 100 V. The optimum reaction time of 10 min was found for dye 1 and then applied to all other photocleavage experiments across dyes for consistency.

2.3.7 Thermal Cleavage Assay

Samples containing 20 μM dyes 1 and dye 3 in the presence of 38 μM bp plasmid DNA in buffered pH 7.0 solutions were prepared and pre-equilibrated for 15 min. They were then either left in the dark at room temperature (rt) for 10 min, kept in a metal heat block at 37 °C for 10 min or kept in a metal heat block at 45 °C for 10 min. The samples were then loaded onto an agarose gel and electrophoresed for 1 h (Table 2-3).
Table 2.3: Example of sample addition to wells of agarose gel for thermal cleavage assay.

<table>
<thead>
<tr>
<th>Dye</th>
<th>1</th>
<th>3</th>
<th>-</th>
<th>1</th>
<th>3</th>
<th>-</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temp (˚C)</td>
<td>rt</td>
<td>rt</td>
<td>rt</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>45</td>
<td>45</td>
</tr>
</tbody>
</table>

2.3.8 Reactive Oxygen Species Scavenger Assay

Samples of 20 µM dye 1, 38 µM bp pUC19 plasmid DNA and varying concentrations of ROS scavengers in a 10 mM buffered pH 7.0 solution were prepared as described above. Each sample contained one of the following scavenger reagents: 100 mM sodium azide, 100 mM sodium benzoate, 100 mM EDTA, 20 U/µL of catalase, or 86% D₂O (v/v). Dye 1 was added to the solution followed by each scavenger. Then, each solution was pre-equilibrated for 15 min before a 10 min irradiation with a 100 mW 850 nm laser. All reactions were kept in a metal block in an ice bath at a temperature of ~10 ˚C. A dark control containing only dye 1 and plasmid as well as a light control containing only dye 1 and plasmid were included. The samples were then loaded onto an agarose gel and electrophoresed for 1 h (Table 2-4).

Table 2.4: Example of sample addition to wells of agarose gel for ROS scavenger experiment.

<table>
<thead>
<tr>
<th>Dye</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scavenger</td>
<td>-</td>
<td>-</td>
<td>Sodium Azide</td>
<td>Sodium Benzoate</td>
<td>EDTA</td>
<td>Catalase</td>
<td>D₂O</td>
</tr>
<tr>
<td>DNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Light</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Potassium iodide (KI) was also used as a scavenging agent. Samples of 20 µM of dye 1 and 38 µM bp pUC19 plasmid DNA were pre-equilibrated for 15 min in the presence of varying concentrations of KI (0.5 mM, 1 mM, 5 mM or 10 mM). Each sample was irradiated for 10 min with a 100 mW 850 nm laser. All reactions were kept in a metal heat block in an ice bath at a temperature ~10 ˚C. All samples were kept at this temperature in the dark when not being
irradiated. Twenty µL of each sample were then loaded into an agarose gel and electrophoresed for 1 hr.

2.3.9 **Circular Dichroism**

Using the optimized dye and CT-DNA concentrations from the UV-visible saturation studies, 1500 µL samples were prepared as follows. Samples contained: 50 µM bp of CT-DNA and 10 mM sodium phosphate buffer pH 7.0 in water; 20 µM of dye 1 and 10 mM sodium phosphate buffer pH 7.0 in water; or 20 µM of dye 1, 50 µM bp of CT-DNA, and 10 mM sodium phosphate buffer pH 7.0 in water. Each dye sample was pre-equilibrated for 15 min after which CD data were acquired. The spectra were recorded from 900 nm to 200 nm in a 3 mL quartz cuvette over 12 accumulations with a scan speed of 200 nm/min and a band width of 1 nm. Spectra were analyzed using Microsoft Excel 2013.

2.3.10 **Photocleavage Inhibition Assay to Determine DNA Binding Mode(s)**

Samples contained a 1:1 ratio of dye 1, 38 µM bp pUC19 plasmid, 10 mM sodium phosphate buffer pH 7.0 and an inhibitor, either pentamidine (P), ethidium bromide (E), or methyl green (M). Before irradiation, a final concentration of 50 µM of inhibitor was added to samples of plasmid pre-equilibrated with 50 µM dye 1 for 15 min. Additional samples were prepared in which 50 µM of dye 1 was added to a solution of 50 µM, inhibitor and plasmid that was pre-equilibrated for 15 min. Each sample was irradiated under a 100 mW, 850 nm laser for 10 min at ~10 °C. Dark controls containing only inhibitor, only dye, and only plasmid were included along with a light control containing only dye. The samples were then loaded onto an agarose gel and electrophoresed for 1 h (Table 2-5).
Table 2.5: Example of sample addition to wells of agarose gel for DNA binding inhibition. Pentamidine (P), Methyl Green (M), Ethidium Bromide (E), Dye (D). The Order indicates which solution was pre-equilibrated first.

<table>
<thead>
<tr>
<th>Dye</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>P</td>
<td>D+P</td>
<td>P+D</td>
<td>MG</td>
<td>D+M</td>
<td>M+D</td>
<td>EB</td>
</tr>
<tr>
<td>DNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Light</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

3 RESULTS

3.1 UV-Visible Spectroscopy

3.1.1 Dye Stability Assays

Before beginning analysis of the dye interactions with DNA, it was necessary to test the viability of each dye in the storage solvent, DMSO. Cyanine dyes tend to aggregate and slowly degrade in solution due to air and photooxidation, so it is important to understand how they react to various solutions over time. UV-visible spectroscopy was used to test the stability and aggregation properties of each dye. Either dye 1 or dye 2 was added to DMSO, and a spectrum was recorded every 5 min for 30 min. As seen in Figure 3-1, both dyes are stable and maintain their intensity for the entirety of the time course. Dye 1 presenting a $\lambda_{\text{max}}$ at 813 nm and an extinction coefficient of 89650 cm$^{-1}$ M$^{-1}$, and dye 2 absorbing at its maximum at 812 nm with an extinction coefficient of 44110 cm$^{-1}$ M$^{-1}$. The similarity in $\lambda_{\text{max}}$ between each dye is consistent with their similarity in structure. Dye 1 having a more intense absorption maximum could be due to stability and/or aggregation phenomena.
Figure 3.1: UV-visible absorption spectra of 10 µM dye 1 and dye 2, respectively, in DMSO. Dye was added to a DMSO solution, and a spectrum was recorded every 5 min for 30 min. Dyes are differentiated solely by their meso substitution.

Once the dyes were determined to be stable in DMSO, their spectra in an aqueous solution were analyzed. These dyes were being tested for their ability to function under biological conditions because stability in an aqueous environment is vital to the effectiveness of the dyes. The same time intervals were employed but in the presence of an aqueous solution containing a pH 7.0 sodium phosphate buffer to ensure biological pH. These resulting spectra can be seen in Figure 3-2.
Figure 3.2: UV-visible absorption spectra for 10 µM dye 1 and dye 2, respectively, in 10 mM sodium phosphate buffer pH 7.0. Dyes were added to aqueous solution and spectra were recorded every 5 min for 30 min.

While there is a hypochromic shift in comparison to the DMSO spectra in the presence of ddH₂O, both dyes remained fairly stable over the same time span. Dye 1 showed more stability than dye 2, again, perhaps due to the bromine in dye 1 compared to the chlorine atom in dye 2. The λ<sub>max</sub> of dye 1 and 2 blue shifted to 768 nm and 585 nm respectively, giving respective extinction coefficients of 33610 cm⁻¹ M⁻¹ and 22830 cm⁻¹ M⁻¹. It is important to note that even with this hypsochromic shift, dye 1 developed an additional intense shoulder that reaches above 900 nm, which could prove to be useful for deeper skin penetration of light based on the biological absorption window.

The dyes were then analyzed for their stability in the presence of water and CT-DNA. The dyes were individually added to pH 7.0 buffered solutions already containing CT-DNA and then were studied over time. When dye 1 is initially added to a solution containing DNA, the dye seems to be changing aggregation states. This process can be seen in Figure 3-3. The maximum absorption for each dye remained similar to the aqueous analysis at 764 nm and 587 nm.
Figure 3.3: UV-visible spectra of 10 µM dye 1 and dye 2, respectively, with 150 µM bp of CT-DNA in 10 mM sodium phosphate buffer pH 7.0. After the dyes were added to DNA solutions, spectra were recorded every 5 min up to 60 min for dye 1, and every 5 min up to 30 min for dye 2.

To highlight the spectroscopic binding characteristics for each dye at t = 0 min, Figure 3-4 represents an overlay of each dye with and without CT-DNA. For dye 1, there is a hypochromic shift and an increase in the intensity of the shoulder that reaches even further into the red. The appearance of a hypochromic shift has been found to indicate a strong interaction between the DNA bases and the bound dye. Dye 1 seems to be aggregating in the presence of CT-DNA, with a possible H-aggregate as the far left peak, decreasing over time while the possible monomeric and red shifted J-aggregate indicative peaks increasing with time. For dye 1 at 25 min, the possible H-aggregate form converts almost completely to the monomeric and J-aggregate forms, with the most red-shifted peak becoming the absorption maximum. Dye 1 at 25 min also seems to be reverting to its buffer only aggregation state, most likely due to the tendency for these dyes to self-aggregate on DNA. Dye 2 DNA binding is supported by a slight hyperchromicity and the appearance of a new peak at around 814 nm. (Dye 2 at 25 min was not included due to being super imposable to the spectrum at 0 min.)
Dye 1 may be reacting more readily with DNA due to the dye's intense absorption in its presence compared to dye 2. It is also predicted that dye 1 will react with DNA in the presence of long wavelengths, due to strong absorption above 800 nm of light by the dye-DNA complex.

Unfortunately, as time progressed, dye 2 increasingly became unstable while being stored in DMSO, losing its absorption intensity even when taken from its original frozen DMSO stock (data not shown). These dyes rely heavily on their light absorption properties for their use in PDT. Because of the gradual degradation of dye 2, over time DNA interaction studies became inconsistent and were deemed unreliable. The majority of the remaining DNA studies in this
thesis focused on the effects of dye 1 in comparison to previously studied pentamethine quinoline dye 3.

3.2 DNA Photocleavage Analysis Through Gel Electrophoresis

The use of dyes in PDT will be determined by their ability to cause cell apoptosis in the presence of light. One way to trigger apoptosis is by the destruction of DNA within the cell, especially mitochondrial DNA. Once the DNA is compromised, the cell can undergo this programmed death, which is deemed a preferable option to necrosis. Gel electrophoresis has been found to effectively study DNA damage. The naturally occurring form of pUC19 plasmid, supercoiled, shows up as the fastest migrating band in an agarose gel. In the event of a single-strand cut, the slowest migrating band, nicked, appears. And in the event of the double-stranded cut, a linear form of the plasmid shows up as the second slowest plasmid band in the agarose gel. Through gel electrophoresis, it is possible to evaluate the optimum light source, irradiation wavelength and time, as well as dye for future use in biological systems along with the mechanism through which the dye cleaves DNA.

3.2.1 Pre-Equilibration Titration

During analysis of the UV-visible spectrum of dye 1 in the presence of DNA, there is a noticeable equilibration time needed for the complex to bind to and stabilize to the CT-DNA (Figure 3-3). This was considered a possible concern for optimizing photocleavage experiments. To determine the best equilibration time in the presence of pUC19 plasmid DNA, a pre-equilibration titration was conducted and studied via electrophoresis. Dye 1 was added to solutions containing plasmid DNA and allowed to equilibrate in the dark for 5 min, 10 min, 15 min, 20 min, 30 min, 60 min and 90 min. After each equilibration time, the dyes were irradiated using and 850 nm lasers for 10 min. The results are shown in Figure 3-5.
Figure 3.5: Pre-equilibration titration of 25 µM dye 1 with 38 µM bp pUC19 in 10 mM sodium phosphate buffer pH 7.0. Samples equilibrated from 5 min to 90 min in the dark before irradiated at 850 nm for 10 min at ~10 °C.

The change in cleavage yields seen in Figure 3-5 was not as drastic as the changes in absorption shown in Figure 3-3. This could be attributed to the variation in the type of DNA used (calf thymus vs. pUC19), providing a variation in binding environment. It does seem that after 15 min the cleavage approximately remains the same with some variation in yield at the later time points. To ensure optimum cleavage yields, all dye 1 DNA reactions for remaining gels were, therefore, pre-equilibrated in the dark for 15 min before irradiation.

3.2.2 Concentration Titration

An additional key characteristic in an effective photosensitizer is the ability to achieve the desired photocleavage at low concentrations. A titration was conducted to find the optimal amount of dye needed. A concentration of 25 µM was initially employed based on the results of research with other cyanine dyes. Since the smallest changes in dye structure can drastically change the functions of a dye, this titration was necessary to ensure the best conditions for dye 1.

For the assay, solutions containing 38 µM bp of DNA were prepared with the following
concentrations of dye 1; 5 µM, 10 µM, 15 µM, 20 µM, 25 µM, 30 µM, 40 µM and 50 µM. Additionally, a light control containing only plasmid and a dark control containing 50 µM of dye 1 was included. The light reactions were irradiated with an 850 nm 100 mW laser for 10 min with a pre-equilibration time of 15 min. The results can be seen in Figure 3-6.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye 1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Conc. (µM)</td>
<td>50</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>30</td>
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<tr>
<td>Light</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>% nicked</td>
<td>2</td>
<td>4</td>
<td>11</td>
<td>29</td>
<td>37</td>
<td>45</td>
<td>51</td>
<td>56</td>
<td>75</td>
<td>74</td>
</tr>
</tbody>
</table>

Figure 3.6: Concentration titration of dye 1 with 38 µM bp pUC19 in 10 mM sodium phosphate buffer pH 7.0. Light samples irradiated at 850 nm for 10 min at ~10 °C. Dark sample were kept in the dark at ~10 °C.

Based on the figure above, the photocleavage yields remain roughly the same after 15 µM when comparing the appearance of the nicked band of the plasmid. At high concentrations of 40 and 50 µM, there is a noticeable cleavage of plasmid seen in lanes 9 and 10 by the near complete disappearance of the supercoiled form. Because it is preferable to work with low concentration of PDT drugs to minimize side effects, reactivity experiments were run at a dye concentration of 20 µM. Yet, it is interesting to note that the average DNA photocleavage obtained with 50 µM dye 1 was 76% ± 1.2% over three trials.
3.2.3 Time Course Assay

An important aspect of DNA photocleavage, with respect to PDT, is the ability of a dye to cleave DNA with the shortest possible irradiation time. A time course assay titration was used to determine the best irradiation time to be used for all cleavage experiments to come. Figure 3-7 represents a time analysis of dye 1 beginning with irradiation for 5 s and ending with 90 min of irradiation with an 850 nm laser at ~10 °C. This preliminary time course assay shows that after 10 min, the cleavage yield stabilizes, as seen by the nicked band of DNA, giving an optimum irradiation time of 10 min for remaining gel experiments.

![Figure 3.7: Time course assay for dye 1. Solutions of 20 µM dye 1 and 38 µM bp of pUC19 plasmid DNA in 10 mM sodium phosphate buffer pH 7.0, irradiated from 5 s up to 90 min under a 100 mW 850 nm laser. All reactions were kept at ~10 °C.](image)

Even though the maximum absorption for dye 1 was 764 nm, it is important to have efficient cleavage at the longest wavelength possible for biological applications. In this regard, dye 1 has shown promising cleavage at 850 nm. This can be attributed to the broad, over reaching absorption that spans this wavelength (Figure 3-4). The 850 nm laser was chosen over other NIR lasers in this study to take advantage of this rare occurrence. Additional time course experiments and absorption intensity comparisons with a range of lasers will now be discussed. With variations in laser wavelength, absorption, and laser power output, it is again important to
ensure that the optimum irradiation time is maintained across experiments. Towards this end, additional time course experiments were performed starting at 5 s and ending at 10 min for 850 (100 mW), 830 (350 mW) and 808 (300 mW) nm lasers. A 532 nm laser with a theoretical output of 100 mW was also tested. Because dye 1 has very limited absorption at 532 nm, the laser provides a desirable negative control. Very little photocleavage should be observed because of the low levels of light absorbed by the dye/DNA complex at this wavelength. Figure 3-8 includes representative gels for all 4 lasers. (Additional gels were included for all 4 lasers in Figures 0-1 to Figure 0-4.)

Figure 3.8: Time course assay of 20 µM dye 1 and 38 µM bp pUC19 in 10 mM sodium phosphate buffer pH 7.0 for 4 different lasers (left to right): 850 nm (100 mW), 830 nm (350 mW), 808 nm (300 mW) and 532 nm (100 mW). All samples irradiated for 10 min, excluding the dark controls. All samples were kept at ~10 °C throughout the experiment.

As seen above, dye 1 not only has the ability to cleave pUC19 plasmid DNA but at very long wavelengths of light. Structure comparisons were then used to further understand the
abilities of this cyanine to interact with DNA. Dye 1 was synthesized in response to the success of a similar, previously studied, quinoline cyanine dye containing a single bromine atom at the *meso* position of the pentamethine bridge (dye 3). Dye 3 showed efficient photocleavage of DNA at 808 nm and 830 nm, but not at 850 nm.\(^{18}\) For dye 1, the phenyl group was added to the dye to increase overall aromaticity. This would be expected to enhance dye absorption, DNA binding, and photocleavage yields. The absorption spectra of dye 1 and dye 3 can be seen in Figure 3-9.

![Absorption spectra of dye 1 and dye 3 with 150 µM bp of CT-DNA in 10 mM sodium phosphate buffer pH 7.0. A spectrum was collected at t = 0 min and t = 30 min for each dye.](image)

*Figure 3.9: UV-visible spectra of 10 µM dye 1 and dye 3 with 150 µM bp of CT-DNA in 10 mM sodium phosphate buffer pH 7.0. A spectrum was collected at t = 0 min and t = 30 min for each dye.*

Based on the absorption intensity at 850 nm of each dye, dye 3 should be a less successful photocleaving agent at this wavelength. To test this theory, dye 3 was irradiated at 850 nm for 10 min at 10 °C, over the same time interval as Figure 3-8. The representative results for this gel can be seen in Figure 3-10. (Additional gels were included in Figure 0-5.)
Due to its low light absorption, dye 3 showed almost no cleavage when irradiated with the 850 nm laser under the same conditions as dye 1, supporting the theory that dye 1 would be a more efficient DNA photocleaving agent in the near-infrared range. Figure 3-9 shows that dye 1 more intensely absorbs at all tested laser wavelengths than dye 3. The amount of light absorbed by a dye should directly affect the amount of DNA photocleavage that is produced. The more light energy absorbed, the more energy or electrons the dye can transfer to surrounding oxygen atoms, creating increased DNA damage. The power output of the laser is also important. This trend can be seen in Table 3-1, that compares the percentage of nicked plasmid that appeared after 10 min irradiation at each wavelength to the power output and the absorption at the respective wavelengths. Dye 3 cleavage and absorption is also included as a reference.
Table 3.1: Comparison of the percentage of nicked plasmid generated after 10 min irradiation at 4 wavelengths. Absorption at each respective wavelength after 30 min is included.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Wavelength</th>
<th>Power Output</th>
<th>Abs</th>
<th>Average % N</th>
<th>StDev</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>808 nm</td>
<td>300 mW</td>
<td>0.3851</td>
<td>52%</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>830 nm</td>
<td>350 mW</td>
<td>0.3294</td>
<td>48%</td>
<td>2.36</td>
</tr>
<tr>
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As the wavelength is decreased from 850 to 808 nm, the percent of DNA photocleavage increases as dye absorption increases. The same can be said about the power output; the more powerful lasers produce increased photocleavage. The similarity in percent photocleavage between the 808 nm laser and the 830 nm laser could possibly be due to the difference in power output, with the 830 nm laser being stronger than the 808 nm laser. This trend is due to the increase in absorption with decreasing wavelength, as seen above. At 532 nm the absorption is minimal leading to low percent cleavage yields, making this laser an effective negative control.

3.2.4 Thermal Cleavage Assay

Limited damage to healthy cells is one key feature of PDT that makes it an appealing medical procedure. The way PS achieve this is by only becoming toxic in the presence of light. Choosing a PS that does not react with ground state oxygen unless irradiated at the preferred wavelength is vital in ensuring all healthy cells are kept intact. This includes finding PS that do not cleave DNA in the dark, or at increased temperatures. Photocleavage reactions performed with dye 1 were kept at a low temperature of 10 °C to ensure heat from the laser was not activating the complex and damaging the DNA. Testing the cleavage abilities of dye 1, as well as dye 3 for comparison, in the dark at varying temperatures is another experiment that can be used to eliminate the possibility unwanted dark DNA damage.
Figure 3.11: Thermal cleavage assay for 20 µM dye 1 and dye 3 with 38 µM bp pUC19 plasmid in 10 mM sodium phosphate buffer pH 7.0. Solutions were kept at room temperature (rt) in the dark, at 37 °C in a metal heat block or at 45 °C in a metal heat block for 10 min.

Dye 1 and dye 3 were pre-equilibrated with pUC19 plasmid DNA for 15 min and then were either kept at room temperature (rt) or placed in metal heat blocked heated to 37 °C or 45 °C, and left to react for 10 min. A DNA control was included for each temperature analysis as a reference. The results can be seen in Figure 3-11.

At all temperatures, supercoiled plasmid remained intact indicating that there was no thermal or dark cleavage for dye 1 or dye 3. This suggests that neither dye generates ROS unless irradiated with specific wavelengths of light and that photocleavage is due to light absorption and not the heat that is generated by the laser.

3.2.5 ROS Scavenger Assays

To determine the pathway in which reactive oxygen species were being generated, a series of reagents were selected us in DNA photocleavage reactions based on their ROS scavenging abilities. A known singlet oxygen scavenger, sodium azide, was used for its ability to access the effects of $^1\text{O}_2$. When paired with D$_2$O, a known singlet oxygen lifetime enhancer, both reagents can point to evidence of a type II ROS mechanism. To test for hydroxyl radicals,
sodium benzoate and potassium iodide (KI) were used for their abilities to readily react with hydroxyl radicals. EDTA was used for its metal chelating ability.\textsuperscript{38} By chelating trace levels of redox active metals in solution, EDTA can interrupt the Fenton-type reaction required for hydroxyl radical formation. Catalase was used to test for the involvement of H\textsubscript{2}O\textsubscript{2}, which reacts with Fe(III) or Cu(I) to generate 'OH. In the event of photocleavage inhibition from the hydroxyl radical scavengers, it can be concluded that a type I reaction mechanism is involved. The results from the scavenger reactions can be seen in Figure 3-12. The data were averaged over three trials. (The electrophoresis gels for Figure 3-12 are included in Figure 0-6 and Figure 0-7.)

\textbf{Figure 3.12: Reactive oxygen species scavenger assay presented as percent of photocleavage inhibition, with data averaged over three trials. The error is reported as standard deviation.}

A high sodium azide percent inhibition has been known to indicate singlet oxygen involvement. Singlet oxygen is generated by an energy transfer from the triplet state of the compound to molecular oxygen. As the wavelength is increased, energy generated is decreased making it less likely to produce singlet oxygen. However, this is supported by the inability of D\textsubscript{2}O to enhance the photocleavage reaction. There is also evidence of sodium azide acting as a
scavenger for hydroxyl radicals and superoxide anion radicals, which further points to the absence of singlet oxygen, adding support to hydroxyl radical production.\textsuperscript{39-41}

Potassium iodide, EDTA, and catalase all showed consistent photocleavage inhibition, further supporting the evidence of hydroxyl radical production. The ‘OH scavenger sodium benzoate did not produce the expected inhibition. This may be due to a shielding of the dye by DNA. If dye 1 is embedded deep in the DNA helix, like in the case of intercalation, large bulky reagents, such as sodium benzoate, would be less likely to scavenge the radicals being generated compared to sodium azide and KI which are smaller in size. The low % inhibition caused by catalase could be due to the large size or the fact that reactions were conducted at 10 °C, decreasing the reactivity of the enzyme.

From the presented data, it is believed that a type I reaction mechanism is taking place, in which dye 1 is transferring electrons from its triplet state to molecular ground state oxygen to produce superoxide anion radicals. These radicals may then be reacting with hydrogen peroxide in a Fenton-type reaction to produce hydroxyl radicals. Hydroxyl radicals are then likely abstracting hydrogens from deoxyribose sugars in DNA, causing single stranded DNA breaks to occur.

3.3 Binding Mode Determination

When dyes bind to DNA, be it in the grooves or through intercalation, they frequently alter DNA structure.\textsuperscript{42} Monitoring how the DNA changes structure upon the binding of a particular dye is an informative way to analyze the binding mode through which the dye and DNA interact. UV-visible spectra were therefore recorded to track the saturation point at which dye is completely bound to DNA, and CD spectra were recorded as a means of using the chiral characteristics of DNA to track the structure changes induced by binding of the dye.
3.3.1 Dye Saturation Assay

In order to record and then analyze CD spectra, it was first necessary to find the saturating DNA and dye concentrations at which all the dye would be bound to CT-DNA. Towards this end, a concentration titration assay was conducted using UV-visible spectroscopy. Increasing amounts of CT-DNA were added to a cuvette containing either dye 1 or dye 2 in 10 mM sodium phosphate buffer pH 7.0 until the UV-visible spectrum stopped changing. Results for dye 1 can be seen in Figure 3-13.

![UV-visible saturation assay of 20 μM dye 1 in 10 mM sodium phosphate buffer pH 7.0. CT-DNA was added in increments of 10 μM bp. Spectra were corrected for sample dilution.](image)

Figure 3.13: UV-visible saturation assay of 20 μM dye 1 in 10 mM sodium phosphate buffer pH 7.0. CT-DNA was added in increments of 10 μM bp. Spectra were corrected for sample dilution.

Apparent dye 1 saturation occurred quickly, with major changes in dye absorption occurring after the addition of only 10 μM bp of CT-DNA. The DNA and dye were allowed a 15 min pre-equilibration time before the spectra were recorded due to the UV-visible spectra shown in Figure 3-3. Each spectrum was recorded twice to insure they were superimposable in support
of complete equilibration. Figure 3-13 shows that saturation was achieved at a CT-DNA concentration of 50 µM bp DNA with 20 µM of dye 1.

In order to continue spectroscopic analysis of dye 2, a saturation assay was attempted. As seen in Figure 3-14, even in the presence of high DNA concentrations, a stable saturation point could not be reached. This assay was repeated with new dye stocks, varying titration concentrations of CT-DNA as well as varying concentrations of dye. Because no titration endpoint was reached, dye 2 was again deemed an unsuitable PDT photosensitizing candidate.

![Figure 3.14: UV-visible saturation assay of 20 µM dye 2 in 10 mM sodium phosphate buffer pH 7.0. CT-DNA was added in increasing increments, beginning with 100 µM bp until 10% volume was reached at 1063 µM bp CT-DNA. Spectra were corrected for sample dilution.](image)

**3.3.2 Circular Dichroism**

With the saturation data from Figure 3-13, it is possible to study the DNA binding characteristics of dye 1 by utilizing the chirality of CT-DNA. On its own, the DNA shows up in a circular dichroism spectrum with a negative band around 245 nm as well as a longer wavelength
band around 260 – 280 nm. The exact positions and intensities of these bands can vary based on the sequence and form of DNA.\textsuperscript{43} A decrease in the CD peak at 280 nm could provide evidence of intercalation. This decrease is due to the increase in the helical twist angle of the duplex DNA from the unwinding of the DNA caused by the binding of the ligand within base pairs. The binding of the dye to DNA can also create induced circular dichroism (ICD) peaks, in which an achiral ligand can present CD peaks in the DNA spectrum through electric transition moments brought about by the binding of the dye to DNA.\textsuperscript{35,44} These peaks usually correspond to maxima in the UV-visible absorption spectra and can provide insight into the possible binding modes.\textsuperscript{33} The CD spectra of samples either containing only CT-DNA, only dye 1 or a combination of CT-DNA and dye 1 can be seen in Figure 3-15. The UV-visible spectrum of 20 μM of dye 1 at the saturating concentration of 50 μM bp DNA is included in the Figure to show correlation. The UV-visible spectra for 20 μM of dye 1 at 150 μM bp CT-DNA at t = 0 min is also included to better emphasize the shoulder present at around 590 nm.
By looking at the CT-DNA CD peak at 280 nm, there is an apparent decrease in intensity when dye 1 is present. This suggests that the helical twist angle of the DNA is being increased by the dye, and points to a possible intercalative binding mode. At longer wavelengths, there is an intense ICD signal near the range of UV-visible absorption for this dye in the presence of CT-DNA. The first ICD peak crosses the axis at 590 nm and could possibly correspond to a shoulder in the UV-visible graph at around 580 nm. This shoulder can be best seen in Figure 3-13 and appears to decrease with increasing DNA concentration and increasing time. This first peak also appears to be an exciton peak, identifiable by a positive and negative bisignate peaks of similar shape and similar intensity. Exciton peaks have been found to be present for compounds that
polymerically bind to DNA. The multiple peaks that appear in the overlaid UV-visible spectrum at $t = 0$ min represent polymeric binding through self-aggregation, adding additional support to the CD spectra.

A second exciton peak is present, crossing at 650 nm. Polymeric binding has been reported as being indicative of groove binding due to the space needed for dye aggregation. It is possible that additional ICD peaks could appear above 750 nm, corresponding to the UV-visible $\lambda_{\text{max}}$, and at around 870 nm (Figure 3-13), but due to detector sensitivity, CD signal over 750 nm was not decipherable. The intensity of the ICD signal is also predicted to be indicative of groove binding over intercalation due to the variation in the electric transitions between the dye and the DNA base pairs, which are calculated to be less for intercalating agents than for groove binders. Since dye 1 presents an intense excitonic peak, greater than 10 M$^{-1}$cm$^{-1}$, some form of groove binding can be inferred. The number of ICD peaks as well as the shape and sign of the bisignates slightly vary from previously investigated cyanine CD results that suggest minor groove binding. These variations in the spectra could suggest the possibility of major groove binding properties. Although dye 1 is smaller than the major groove as a monomer, it is possible for polymeric forms of the dye to fit well. It is difficult to gain information on possible binding modes from the CD spectra in Figure 3-14. Difficulty in analysis could be attributed to the fact that in the major groove, the intensity of the peaks as well as the signs may vary greatly because of the increased possibility of polymeric binding allowed by the size of the major groove, strength of electronic couplings, and the binding sensitivity to the environment.

The alignment of the CD absorption with the UV-visible spectrum does suggest DNA interactions, and the nature of the peaks points to a combination of groove binding along with the possibility of intercalation. It is suggested that the exciton peaks cross at a wavelength similar to
the absorption maximum of the dye.\textsuperscript{35, 44} The literature reports that certain cyanine dyes have the ability to partially groove bind as well as intercalate, but just from the above spectrum it is difficult to know for sure within which groove the dye is bound. With the possibility of multiple binding modes, aggregation states, and detector sensitivity, it is difficult to conclusively deduce binding mode from the CD spectrum.\textsuperscript{21}

3.3.3 Gel Electrophoresis Binding Mode Inhibition Assay

Photocleavage inhibition assays using known minor groove, major groove, and intercalative binding compounds have shown to be useful indicators of DNA binding modes.\textsuperscript{46} For the following experiments pentamidine, a known minor groove binder, methyl green, a known major groove binder, and ethidium bromide, a known intercalator were employed. To deduce the binding mode of dye 1, each of these compounds was used to inhibit photocleavage by binding to plasmid DNA and preventing the dye from binding. Since these inhibitors do not absorb at the wavelength of irradiation (850 nm), even in the presence of DNA, there is no overlap in energy that would lead the inhibitors themselves to generate ROS, making these three compounds ideal reagents for inhibition studies (Figure 0-9).

For each of the three inhibitors, two samples were prepared. In the first sample, plasmid DNA was pre-equilibrated with cyanine dye 1 for 15 min, and then, before irradiation, the inhibitor was added to the solution. For the second sample, an inhibitor was added to a solution containing plasmid and pre-equilibrated for 15 min, and immediately before irradiation, dye 1 was added. Each sample was irradiated at 850 nm for 10 min at ~10 °C. Controls, including each inhibitor in the presence of plasmid and light, were incorporated. A representation gel is shown in Figure 3-15. (Additional gels were included in Figure 0-8.) The percent inhibition across all
samples can be seen in Figure 3-16 with values averaged over 3 trials, and error bars representing standard deviation.

Figure 3.16: Photocleavage inhibition study resolved by gel electrophoresis. Samples containing 50 µM dye 1 (D) with 38 µM bp pUC19 in 10 mM sodium phosphate buffer pH 7.0 and 50 µM of the following binding inhibition reagents: pentamidine (P), methyl green (M) or ethidium bromide (E) irradiated under an 850 nm 100 mW laser for 10 min at ~10 °C. Reagent name order indicates the order of addition to the sample.

Figure 3.16 and 3-17 shows that methyl green pre-bound to DNA (lane 10 in Figure 3-16) provides the most photocleavage inhibition (62%). Methyl green is a known major groove binding agent, suggesting that the process in which dye 1 cleaves DNA involves binding to the major groove. There is also increased inhibition with pre-bound of ethidium bromide (42%), the known intercalative reagent, suggesting that at least part of dye 1 could be bound between base pairs. The lowest levels of inhibitor were exhibited by the minor groove binder pentamidine (Figure 3-16 and 3-17). When comparing the binding affinities of each reagent, ethidium bromide has the highest affinity to DNA at $1.00 \times 10^7$ M$^{-1} \text{bp}$, pentamidine binds with an affinity of $6.07 \times 10^5$ M$^{-1} \text{bp}$, and methyl green binds the weakest at $8.80 \times 10^4$ M$^{-1} \text{bp}$. Just based on the binding affinities to DNA, ethidium bromide, and pentamidine should bind more readily to the DNA causing dye inhibition, suggesting that the inhibition by methyl green compared to the
other two reagents can be attributed to dye 1’s inability to bind to DNA in the presence of methyl green.

**Figure 3.17:** Percent inhibition of DNA photocleavage. Averages and standard deviations are taken over 3 trials. The order of reagent name indicates the order of addition to the reaction. Pentamidine (P), methyl green (M), ethidium bromide (E).

## 4 CONCLUSIONS

Throughout the overall study, dye 1, a symmetrical quinoline pentamethine cyanine dye with a bromophenyl meso substitution on the methine bridge, proved to be the most efficient photocleaving agent. In preliminary studies dye 3, a quinoline containing pentamethine cyanine dye with a meso substituted bromine atom on the methine bridge showed promise for near-infrared photocleavage at 808 nm, and 830 nm. Since the current PS agents for PDT do not absorb at long enough wavelengths to treat deep tissue diseases, it is important to find new PS that can photocleave DNA at even longer wavelengths. The addition of a phenyl ring at the meso substituted position while conserving the heavy atom effect of the bromine atom, enabled dye 1 to absorb more light than dye 3 at longer wavelengths and photocleave DNA more efficiently.
Initial UV-visible spectroscopy data showed that both dye 1 and chlorophenyl substituted dye 2 were stable in the presence of CT-DNA, with dye 1 providing the more intense absorption with a molar extinction coefficient of 33810 cm⁻¹ M⁻¹ at 764 nm. Dye 1 also provided the most absorption over 800 nm, including increased absorption around 850 nm of light upon the addition of DNA. Dye 2 was less successful in preliminary studies. The variation in halide between dye 1 and dye 2 drastically changed the stability of the dye in solution and powder forms. The DMSO stock solutions of dye 2 degraded noticeably over time, making it difficult to obtain reproducible cleavage data.

During photocleavage experiments, 20 µM of dye 1 was found to convert supercoiled plasmid to its nicked form in up to 40% yield at 850 nm. The photocleavage was also tested at 808 nm and 830 nm of light, and these cleavage yields were parallel to the amount of light absorbed by DNA bound dye 1 at the irradiation wavelength and laser output. According to the UV-visible spectra of dye 1, as the wavelength decreases the absorbance intensity increases. The same trend appeared in the DNA photocleavage experiments. As the absorbance intensity increases, the photocleavage yields increased suggesting that dye 1 can cleave plasmid DNA across wavelengths above 808 nm of light. When the concentration of dye 1 was increased from 20 µM to 50 µM, DNA was cleaved in 76% ± 1.2% yield.

Binding studies done on dye 1 gave evidence of major groove binding to DNA. In the presence of CT-DNA, dye 1 presents intense excitonic ICD signals indicative of polymeric groove binding consistent with its UV-visible absorption spectrum. Dye 1 was also found to unwind DNA, seen in the shorter wavelength region of the CD DNA spectra, indicative of possible intercalative binding. Indirect binding studies were necessary to confirm which binding modes were involved. Through a gel photocleavage inhibition assay, methyl green followed by
ethidium bromide successfully inhibited the DNA photocleavage abilities of dye 1, indicating that some part of dye 1 must be within the major groove for the dye to effectively bind. Binding in the major groove is different from the majority of previously studied cyanine dyes which prefer to bind within the minor groove but has been previously reported for other pentamethine cyanine dyes.\textsuperscript{50} There is also the possibility of some intercalative binding, which has been seen through Armitage’s work on cyanine dyes.\textsuperscript{21} The effectiveness of dye 1’s photocleavage abilities is additional support for groove binding. A molecule bound in the groove is not as shielded from the surrounding solvent, giving it more access to the molecular oxygen needed to form ROS, unlike intercalated compounds which are well protected from \( ^3\text{O}_2 \) by the DNA base pairs.\textsuperscript{31}

Binding studies add additional support to the reactive oxygen species scavenging assays. A type I ROS mechanism was proposed. Major groove and intercalative binding might keep the dye far enough out of solution that bulky scavenger molecules, such as sodium benzoate, would have difficulty reaching the ROS being generated, due to the hydroxyl radical’s fast diffusion rate. Sodium azide, catalase, EDTA, and KI all showed significant photocleavage inhibition, leading to the conclusion that hydroxyl radicals are causing the DNA strand breaks visualized throughout the study.

The ease of synthesis and intense absorption in the visible to near-infrared light ranges make cyanine dyes promising DNA photosensitizing agents. With dye 1 being the first photosensitizing agent to photocleave DNA at a wavelength longer than 830 nm, it can act as an important lead compound in future drug design. Dye 1 could prove a significant stepping stone for future photodynamic therapy studies, as well as being significant to the field of photochemistry. Knowing now that there are dyes that have enough triplet state energy when irradiated at the low energy and deeply penetrating wavelength of 850 nm, to photocleave
plasmid DNA could impact future studies outside of and within the field of photodynamic therapy.
REFERENCES

APPENDIX

Figure 1 Time course assay of 20 µM dye 1 and 38 µM bp pUC19 over 2 trials irradiated at 850 nm (100 mW) with trial 3 (bottom) being a triplicate of 10 min only. All samples were kept at ~10 °C in 10 mM sodium phosphate buffer pH 7.0.

Figure 2 Time course assay of 20 µM dye 1 and 38 µM bp pUC19 irradiated at 830 nm (350 mW) (top). Trials 2 and 3 (bottom) were run at 10 min only. All samples were kept at ~10 °C in 10 mM sodium phosphate buffer pH 7.0.
Figure 3 Time course assay of 20 µM dye 1 and 38 µM bp pUC19 irradiated at 808 nm (300 mW) (top). Trials 2 and 3 (bottom) were run at 10 min only. All samples were kept at ~10 °C in 10 mM sodium phosphate buffer pH 7.0.

Figure 4 Time course assay of 20 µM dye 1 and 38 µM bp pUC19 over 2 trials irradiated at 532 nm (100 mW) with trial 3 (bottom) a triplicate of 10 min only. All samples were kept at ~10 °C in sodium phosphate buffer pH 7.0.
Figure 5 Time course assay of 20 µM dye 3 and 38 µM bp pUC19 in 10 mM sodium phosphate buffer pH 7.0 over 2 trials irradiated at 850 nm (100 mW) for 10 min. All samples were kept at ~10 °C.

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Figure 6 Reactive oxygen species scavenger assay gel electrophoresis trials 1 - 3. Each sample was run with 20 µM of dye 1, 38 µM bp of pUC19 plasmid DNA and various amounts of scavenging agents: 100 mM sodium azide (SA), 100 mM sodium benzoate (SB), 100 mM EDTA, 20 U/µL catalase (cat), D₂O, 86% (v/v). All light samples were irradiated at 850 nm (100 mW) for 10 min. The samples were kept at ~10 °C in 10 mM sodium phosphate buffer pH 7.0.

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Figure 7 Potassium iodide (KI) ROS scavenger gel electrophoresis trials 1-3 of 20 µM dye 1 and 38 µM bp pUC19 plasmid DNA in 10 mM sodium phosphate buffer pH 7.0. A concentration titration was run using various mM concentrations of KI (top). Trial 3 of the most effective concentration, 10 mM, is included (bottom). All samples were irradiated with an 850 nm laser (100 mW) for 10 min and kept at ~10 °C.

Figure 8 Photocleavage inhibition study using gel electrophoresis trials 1-4. Samples containing 50 µM dye 1 (D) with 38 µM bp pUC19 in 10 mM sodium phosphate buffer pH 7.0 and 50 µM of the following binding inhibition reagents: pentamidine (P), methyl green (M) or ethidium
bromide (E) irradiated under an 850 nm (100 mW) laser for 10 min at ~10 °C. Reagent name order indicates the order of addition to the sample.

Figure 9 UV-visible spectroscopy of each inhibition reagent in the presence and absence of 100 µM bp of CT-DNA in 10 mM sodium phosphate buffer pH 7.0. A concentration of 50 µM of each reagent was used (left to right): pentamidine, ethidium bromide, and methyl green. (The spectra were recorded by Kanchan Basnet.)