The Synthesis and Optical Properties of the Squaraine Scaffold and Ph Responsive Cyanine Dyes Containing Pyridine at the Meso-Carbon

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THE SYNTHESIS AND OPTICAL PROPERTIES OF THE SQUARAINE SCAFFOLD AND pH RESPONSIVE CYANINE DYES CONTAINING PYRIDINE AT THE MESO-CARBON.

by

WHEELER R. LOVETT

Under the Direction of Maged Henary, Ph.D.

ABSTRACT

This thesis introduces a brief review of the phenomena of fluorescent dyes, then a discussion on the synthesis and applications of water-soluble squaraine dyes. It then transitions to the discussion of the synthesis and characterization of hydrophilic and hydrophobic squaraine dye derivatives of indolenine as well as the synthesis of a selection of benzothiazole derived pentamethine cyanine dyes with a meso-pyridine substituent. The structural identification of these compounds was performed using various spectroscopic techniques. Experiments confirmed in the later portion of the thesis characterize the optical properties including solvatochromism and pH responsiveness.

INDEX WORDS: Hydrophilic dye, Hydrophobic dye, Fluorescent dye, Cyanine, Squaraine, pH probe, Pyridine dye, Meso substitution
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WHEELER R. LOVETT

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2020
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College of Arts and Sciences
Georgia State University
May 2020
DEDICATION

I would like to dedicate this work to my parents, Jeffery and Della Lovett, for their constant encouragement and consideration and to my partner, Chandler Cheek, for her invaluable support and inspiration.
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WATER SOLUBLE POLYMETHINE DYES AND THEIR APPLICATIONS

1.1 A Brief Natural History of Organic Dyes

Dyes are a diverse subsection of the universal chemical library that interact with light in a way that generates a strong sensation of color back to an observer. They are found in a variety of locations in the natural world and have been used throughout human history as a way of modifying and adding beauty to the materials around us. From a chemical perspective, these compounds can be split into two categories, Inorganic and Organic dyes. The root of these names deals with whether the material contains the presence of carbon-hydrogen bonds. Inorganic dyes such as the brilliant Prussian Blue of ferric ferrocyanide gain their colors from energy transitions available to the higher $d$-orbital electrons of the oxidized metal-chelate bonds. Organic dyes can have the same color generating ability as these higher electrons orbital metal complex that originates instead from a structural motif known in organic chemistry as a chromophore. This motif is generally composed of alternating double and single carbon bonds in a conjugated system with longer conjugation systems absorbing longer wavelengths of light.

Nature has evolved a multitude of natural organic dyes throughout the branches of flora and fauna with one of the most recognizable and abundant being the signature green of chlorophyll $a$. Chlorophyll $a$, is arguably the most prominent and important chromophore bearing compound in nature as it is the principle light scavenger employed by plants and photosynthetic microbes in order to drive photorespiration, the production of oxygen, and thus all advanced aerobic life on earth. The structure of chlorophyll $a$ shown in Figure 1 contains 10 pairs of alternating double and single bonds that absorbs both a blue photon of light at 430 nm and a red photon of light at 664 nm. Together, these two absorption bands gives a green color that is perceptible to our eyes and promotes electron transport through chloroplasts for photorespiration. This second function of photorespiration is by far the more significant as the average energy output that is harnessed by
this specific color bearing molecule is responsible for rest of aerobic life on the planet and is estimated to be 356 gigawatts every day. This energetically compares to a modern nuclear reactor’s output of roughly 1 gigawatt per day. Chlorophyll is so important physiologically to humans that our eyes have evolved more cone cells that can recognize this color than any other cone cells.

Another dye motif that has been expressed various times throughout the tree of life is the anthraquinone motif. The basic core is named anthracene-9,10-dione, and is described simply as a triple ring structure composed of two benzyl rings joined to each other’s respective 1’ and 2’ positions (See Figure 2). This core structure is electron rich and has 12 sp² orbitals aligned in the same plane, dispersing the molecule’s valence orbitals across the entire face of the polycyclic structure and allowing for the absorption of 377 nm wavelength light. With anthraquinone’s varied appearances in the tree of life, various biochemistry’s have added structural modifications to this core design that change the properties of the chromophore system. For example: substituting
hydrogens for electron donating groups can add to the electron density and change the energetics of the molecular orbitals. The perennial herb, *Rubia tinctorum*, produces the anthroquinone, alizarin, with two hydroxyl groups added to the core structure at C3 and C4 that absorbs a redshifted wavelength of 450 nm. Historically referred to as “rose madder”, it was originally processed from the harvested roots of the madder plant.\(^\text{10}\) Alizarin has been found coloring artifacts at the early Indus river site of Mohenjo-daro as well as been referenced for its properties in the writings of Hippocrates and Pliny the Elder.\(^\text{11}\) Alternatively in the cells of *Caloplaca marina*, a lichen that has colonized every continent on Earth, enzymes have produced a variation of anthroquinone with hydroxyl groups at C3 and C1, a methoxy group at C5, and a methyl group at C13. This variation has the common name parietin, absorbs light at 434 nm, and is believed to

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**Figure 2:** Anthraquinone expression at various places in the tree of life. Copyright Wikimedia commons 2009, 2012, 2017
have evolved to absorb damaging ultraviolet light that would harm the lichen as it grows at higher altitudes.\textsuperscript{12}

Dark red variations of anthraquinone are also historically harvested from arthropods. Chinese silks dyed red with kermesic acid (see Figure 3) derived from the \textit{Kermes} beetle family have been uncovered dating from the Han dynasty (200 B.C-200 A.D) and jars of crushed \textit{Kermes} shells have been found in jars in Neolithic cave art studios in France.\textsuperscript{13, 14} This is also seen in the further functionalization of anthracene-9,10-dione with a carboxylic acid group at C13, a glycosidal group at C5, hydroxyl groups at C3, C4, C6, C12, and a methyl group at C14 through the biochemistry of the small, red, cactus eating American cochineal, \textit{Dactylopius coccus} yields a dark crimson that when crushed treated with aluminum salts yields the dyestuff carmine.\textsuperscript{15} Kermes had colored the red robes of royalty for millennia with early evidence of its use at in Egyptian, Greek, and Roman culture before the Columbian Exchange and the import of the America cochineal led to its disuse. Carmine still finds use today as the red base for many organic lipsticks.\textsuperscript{16}

![Figure 3: A) structure of kermesic acid, B) female Kermes vermilio beetles, C) Chinese silk dyed with kermes. Copyright Wikimedia 2012, CNSM 2017](image-url)
Heterocycles, compounds in which an element other than carbon is included in a ring structure, also make appearances in the natural chromophore library. One of the most prominent groups is the anthocyanin family. These are composed of a phenyl ring that is substituted alpha to a positively charged oxygen in a chromenylium polycyclic heterocycle. They are traditionally water soluble due to the charged oxygen atom as well as the presence of hydroxyl and glycosidal groups. A prominent chemical of this family that is functionalized with five hydroxyl groups is cyanidin and is found in many dark blue and purple plants such as blackberries, raspberries, and grapes. The possession of these hydroxyl groups and other functional groups that are susceptible to chemical changes in certain pH conditions can allow for pH responsiveness from compounds in this family. Deprotonation of hydroxyl groups in high pH increases the electron donating capability by freeing a charged lone pair to add into the system. Inversely, the protonation of hydroxyl groups in low pH decreases the electron donation capability. The ability for hydroxyl groups to engage in hydrogen bonding with water also functionalizes traditionally non-polar unsaturated aromatic rings to become more water soluble. Another dye material sourced from weld, the small weed *Reseda luteola*, is based on a separate heterocycle, berberine. Berberine is a charged nitrogen heterocycle that is created when weld is crushed that has a bright yellow color. This dye is more commonly spread due throughout the world due to its spontaneous formation from the common plant alkaloid, reticuline, under cellular lysing and methylation conditions that triggers a pericyclic cascade to form the final structure shown in Figure 4C.

The bold, orange extract that gives color to a Tibetan Buddhist monk’s robes and currently adorns the Indian national flag has its chemical roots in a hydroxyl and glucuronic substituted xanthone heterocycle derivative known as euxanthic acid. The xanthone structure is similar to that of anthraquinone except that one of the bridging ketone carbons is replaced with an ether
oxygen. The processing and origins of this dye still has historical controversy as it has been observed to have produced by force feeding mango leaves to cows and collecting their nutrient poor urine. Reducing that urine and filtering off any precipitate to yield a dark orange powder that can be mixed with magnesium or calcium salts to form a stable pigment.\textsuperscript{20}

Figure 4: A) Cyanidin expressed in grapes, and B) crude euxanthic acid in Indian Yellow, C) berberine expressed in weld weed. Copyright Wikimedia 2009, 2012, 2008
One of the historical sources of blue dyes comes from the indigo plant, *Indigofera tinctoria*, native to the Indian subcontinent and described in the writings of the famous explorer and spice trader Marco Polo.\(^{21}\) This is structurally derived from an isolated glycosidic indole, indicant, that is hydrolyzed after extraction, then oxidized to indoxyl. This in turn forms a covalent adduct with another equivalent of itself to form indigotin, commonly known as the dyestuff indigo blue. This method of production is over 6000 years old based on traces of indigo blue on weaved textile samples found in pre-Columbian Peru.\(^{22}\) Indigo is an example of convergent evolution in chromophore biochemistry because a very similar structure is found in the invertebrate family of Muricidae, predatory sea snails native to the Mediterranean sea. This dyestuff was historically produced by pulverizing the hypobranchial gland of the mollusk to yield a dark purple color.\(^{23}\) This color was in fact from a blend of indigo blue and a red compound that turned out to be the

![Figure 5: Historical Dyes: a) structure of indigotin. b) bundle of indigo blue dyed cloth., c) structure of dibromoindigotin. d) detailed mosaic of Byzantine Emperor Justinian I adorned in tyrian purple robes. Copyright Wikimedia 2006, 2015](image-url)
brominated derivative of the indigo blue. Because of the demand for vibrant purple colors, large quantities of the Muricidae snails were required to extract tiny amounts of the material. Thus, purple became symbolic of wealth, power, and resource access continuing on to adorn the royalty and generalship of the Byzantine Empire. The chemicals that make up these colors are remarkably similar given that the biological systems responsible shared a closest ancestor over 500 million years ago. The blue compound found in the indigo plant is composed of two indole groups joined together into a short conjugated system (Figure 4A). This same structure is also evident in the mollusk only now it possesses two additional bromine atoms across from the electron donating nitrogen atoms of the compound (Figure 4C).

Human biochemistry produces our own natural dyes as well, though they are far more reserved than some of our distant cousins on the tree of life. The pigment that tints our skin as well as many organisms is known as melanin and is composed of a vast series of cross linkages between the amino acid tyrosine. These long polymers are primarily responsible for absorbing damaging high energy UV light but also give off a light tannish brown. The search for novel colors had led to the vast scouring and archiving of the natural world. Historically, these dye materials been highly sought after with entire economies, national histories, and lives changed in their pursuit. Adorning oneself in color has signified status across cultures for millennia and the search for these materials such as new green feathers to furnish an Aztec warrior’s plumed headdress or for the deep crimson that saturates the Queen of England’s royal cape has been the passion of thousands. However, as material science and industrial chemistry came into its own during the industrial revolution a new opportunity for never before seen dyes was presented. The development of the chemical industry in the 19th century was in part fueled by the newly developed dyestuffs that
chemists were finding far more economically advantageous to synthetically produce than naturally source.²⁶

1.2 Light and Color

Light, one of the great ethereal properties of the natural world, has mystified philosophers and scientists for millennia. Many of light’s properties were mathematically derived as early 3rd century B.C.E. in Euclid’s treatise on the linear properties of light, *Optica.*²⁷ The most defining moment in our modern understanding of light was Max Planck’s revolutionary realization in 1900 that the individual packets of light only have discrete energy associated with them. This means that every individual packet, or “photon”, has its own specific energy value and corresponding wavelength. This quantization of light is the basis of the term quantum mechanics. Each photon has a specific wavelength associated with the energy level that is inversely related to the energy of the photon, Plank’s constant, and the speed of light.²⁸ Organizing light by its wavelength gives the electromagnetic spectrum associates low energy light with long wavelengths and higher energy light with shorter wavelengths. The electromagnetic spectrum ranges in natural observation from

![Figure 6: The Electromagnetic Spectrum. Copyright NASA 2007](image-url)
the intense gamma ray bursts that originate from incredibly energetic nuclear events in neutron stars and black holes, to the ultra-low frequency radio transmissions detected from lightning strike induced electron transitions.⁹

The higher energy wavelengths such as gamma, x-ray, and even some of the shorter ultraviolet wavelengths have enough energy to knock electrons out of their shells around atoms and are therefore considered ionizing radiation. This is the type of radiation that is traditionally warned of as being dangerous due to its potential for destructive cellular interactions, primarily with the fragile and vital DNA strands found inside the nucleus. This can lead to partial breaks in the sequence, or double breaks that can cause even more damaging or miss-matched rejoining of improper sequences. This plays out pathologically with cell death as the nucleus is no longer able to function and is flagged by apoptosis signalizing pathways as having it’s DNA fidelity corrupted.³⁰ The ionizing action comes from the wavelength of light and its associated quantized energy corresponding to the potential energy of the associated electron transition from being bound in the HOMO (highest occupied molecular orbital) to being an unbound electron that is separated from its original atom. In this way, it can be said that ionizing radiation can break chemical bonds.

Longer wavelength light such as the ultra-violet (UV) and visible spectrum also correspond to electron excitations like ionizing radiation, but because of their lower energy they do not have the strength to fully delocalize an electron. Instead, these wavelengths correspond to electron transitions within a molecular orbital system. Even though any orbital system has a finite electron in its outer orbital, there are various unfilled electron orbitals that exist at higher energy states and further distances from the nucleus or molecular core. It is the transition of the electron in the HOMO (highest occupied molecular orbital) to either the LUMO (lowest unoccupied molecular orbital) or any number of higher electronic states that exists while still being in an atomic pair with
the orbital system. The wavelengths between 400 and 700 nm have been favored by evolution in humans as those are the only wavelengths that are detectable by our eyes and are referred to as the visible spectrum. While we are surrounded by all manners of electromagnetic radiation, this range most corresponds to the way that static matter interacts with the light of the sun. This allows us to detect and discern the vibrant blue of copper sulfate electronic transitions, the deep crimson of iron transitions in hemoglobin, and the pale green of calcium transitions in jade. In this way, the eye is analogous to a band filtered and qualitative spectrometer that for millions of years has been useful to the ancestors to discern matter from matter and thus evolutionarily advantageous.31

Less energetic wavelengths still interact with matter, but in a less dramatic manner. As the visible spectrum moves into the infrared spectrum, there is an overlap of light corresponding to the lower energy electronic transitions and light corresponding to the stretching of bond lengths. This region between the red end of the visible spectrum and the start of the next section, infrared is referred to as the near infrared region (NIR) or the far-red region. This region of radiation is of particular interest to the dye development field for its advantageous properties in biological imaging. The human body gives off thermal energy as infrared radiation. It also has various chromophores that absorbs light in the visible range as well, which gives rise to skin and blood color but does not operate in this NIR region which provides a window for biological imaging.

Matter’s interaction with the electromagnetic spectrum gives way to purely vibrational energy interactions as the energy lowers further and is unable to excite electrons out of a HOMO at all. This interaction is perceived by us as the radiation of heat, when a photon is converted into thermal energy that is kinetic motion at the nanometer scale. When summed together, kinetic energy can be comparatively high or low, from the gentle heating of the skin on a summer day, to the high intensity infrared pulse concurrent with a thermonuclear explosion that carbonizes organic
matter. As the wavelengths get even longer, the interaction between molecules and photons of microwave radiation is expressed as rotational motion. This is the primary basis of the microwave oven found in most kitchens. An emitter beams radiation with a wavelength of approximately 12 cm at a steady intensity at pieces of food in the microwave oven which causes rotation in the water molecules bound within the food. This rotational energy is dissipated throughout the media as thermal energy that heats up the food. Radio waves interact with matter in even more subtle ways, but have been harnessed by humans for amazing technical achievements. Radio waves have much larger wavelengths and interact primarily by the intrinsic oscillations in the electromagnetic field inherent in the wave. These oscillations can be detected by electronics and specifically designed antenna and then encoded to generate information. By changing the intensities of these oscillations, a song can be encoded and then beamed out from radio antennas, travel hundreds of miles and induce electronic oscillations in a car antenna that sends electrical signals to a speaker that in turn plays the voice of Billy Holiday. The longest wavelengths of this end of the spectrum are used primarily for military and public communication such as submarines and satellites. At these long wavelengths, the electromagnetic radiation can pass straight through high density objects such as concrete, deep water, and even through the earth itself.

1.3 Fluorescence

The electronic transitions from a HOMO to higher and more energetic orbitals can be visualized as a series

![Jablonski Diagram](https://commons.wikimedia.org/wiki/File:Jablonksi_Diagram.png)
of horizontal lines organized vertically by energy, much in the manner of traditional molecular orbital diagrams. These were coined by the Polish Spectroscopist, Alexander Jablonski to describe the phenomenon of fluorescence in 1933.\textsuperscript{36} Fluorescence describes the emission of a photon light from a material after the absorption of a shorter wavelength of light.\textsuperscript{37} This is electronically described as a transition from the ground state (S\textsubscript{0}) of the molecular system to a higher, singlet state (S\textsubscript{1}) where a singlet connotes that the electron in the higher orbital is unpaired. Fluorescence is expressed in various natural compounds but was first described in the 16\textsuperscript{th} century in solutions of extracts of the kidney wood tree, \textit{Ligum nephriticum} that would give off various iridescent colors based on the angle of observation. This fluorescent quality was also observed in other solutions of quinine, chlorophyll, and various minerals.\textsuperscript{38, 39} The biological compounds that produce this effect share a common structural motif of alternating pi systems which form a conjugated electron system. The molecular structure of the dye that provides the fluorescent property is called a fluorophore. Conjugation of pi bonds into a long aromatic system induces electron changes that encourage the absorption and fluorescence of light. The presence of alternating pi bonds lower the energy gap between HOMO and LUMO in the system as the HOMO is traditionally the bonding pi orbital stretched across the whole conjugate chain, while the LUMO is the antibonding pi orbital.

All materials perceived as colorful are as such because they absorb or reflect light. This can be measured in terms of how much of a molar concentration of the substance effects the absorbance with molar absorptivity, $\varepsilon$, measured in absorption M\textsuperscript{-1} cm\textsuperscript{-1}. This is the basis of the Beer-Lambert relationship described in Equation 1 where $A$ is the measured absorbance, $\ell$ is the path length in centimeters, and $\varepsilon$ is the compounds’ molar absorptivity. The color that we detect

\textit{Equation 1:} \hspace{1cm} A = \ell \ast c \ast \varepsilon
from that substance is based on what wavelengths are missing from the full spectrum, but a molecule being fluorescent is dependent on how the energy is absorbed into the system relaxes back to the ground state. In non-fluorescent systems, the energy is diminished by non-radiative transitions; generally vibrational, translational, and rotational modes. Fluorescence occurs when the energy is released out radiatively as light as the system relaxes to the ground state. The energy of light emitted from fluorescence is lower than that of the incident light absorbed because a fraction of the energy is generally dispersed through non-radiative means as shown in Figure 7. Fluorescence is favored in systems that have high structural planarity and thus a more highly stabilized excited state as well as other motifs with minimal means of non-radiative relaxation pathways. Organic structural motives that encourage this sort of planarity generally consist of sterically restrained systems while motives that encourage non-radiative pathways include long polysaturated carbon chains that can experience a large number of vibrational modes. These various wiggles and wobbles that saturated carbon chains can bleed out whatever energy is absorbed by the chromophore and release the energy into their surroundings.
Figure 8: Scaffolds of various commercially available dyes
Organic chemists have spent decades developing compounds that fluoresce and have created a diverse library using this structural functional relationship, such as those seen in Table 1. All of these compounds share the same motif of an extended polymethylene chain originating with an electron donor and ending in an electron acceptor. The longer the chain, the longer the absorbed wavelength, this generalization is a way of visualizing the distribution of the HOMO across the molecule and estimating the wavelength of absorption. This is exemplified with indocyanine green (ICG) having a LUMO dispersed over 31 atoms and an absorption wavelength of 780 nm and coumarin 6 having a LUMO dispersed over 17 atoms and an absorption wavelength of 460 nm.\textsuperscript{41, 42} These dispersed molecular orbitals allow for the stabilization of the excited state enough to fluoresce. These compounds have Stokes’ shifts, the difference between their absorption and
emission, ranging from 14 to 85 nm. Since the ejected photon in fluorescence is of a lower energy than the photon absorbed, the ejected photon is of a redder wavelength.

Not every absorbed photon will result in the emission of light. Quantum mechanics prescribes different probabilities to the paths energy can take as it relaxes through molecular orbitals so the amount of light emitted from a molecule is in terms of percentage. This is traditionally called quantum yield and describes the percentage of fluorescent photons emitted from a system per photon of absorbed light. This, when paired with the molar absorptivity coefficient, can give a general description of the brightness and efficiency of the fluorophore. Table 1 shows the excitation and emission wavelengths, quantum yields, and fluorescence lifetimes, of a selection of commercially available fluorescent dyes that are used extensively in various fields extending from solar energy to oncology. Even quantum yields below 1% are considered bright enough for some research applications. Experimentally this can be calculated by comparing a novel fluorophore to the fluorescence intensity of a standard fluorophore when controlling for variables such as slit width and excitation wavelength. This relationship is described in Equation 2 between an experimental dye and a known standard, with $\Phi$ being the quantum yield, $\text{INT}$ is the area under the fluorescence spectrum, $A$ is the absorbance of the sample, and $n$ is the refractive index of the solvent used.

\[
\Phi = \Phi_R \frac{\text{INT}}{\text{INT}_R} \frac{1 - 10^{-A_R}}{1 - 10^{-A}} \frac{\eta^2}{\eta_R^2}
\]

Another important property is the fluorescent lifetime which refers to an estimation of how long a fluorophore remains in the excited state before it relaxes down. The longer a fluorophore sits in an excited state is a sign that the excited state is relatively stable and it has a higher likelihood of relaxing via a radiative pathway. These radiative events occur on the range of 0.1 to 50 nanoseconds, or roughly $10^{-8}$ to $10^{-10}$ seconds. As shown in Table 1, the dyes with the longest
fluorescence life time are two different rhodamine dyes, Rhodamine 6 and 101, which also have the highest quantum yields comparatively (4.08 ns, $\Phi = 95\%$ and 4.32 ns, $\Phi = 99\%$).\textsuperscript{43} Both of these structures consist of tricyclic compounds with high planarity and limited vibrational modes. This high planarity is especially true in Rhodamine 101 with the amino carbons vestigial to the central core bonded back to the aromatic core and restrained from motion. ICG and Cy3 are both dyes with short fluorescence lifetimes that also exhibit low quantum yields as they have longer aromatic systems but are less vibrational and rotationally constricted (0.52 ns, $\Phi = 0.3\%$ and 0.30 ns, $\Phi = 4.0\%$).\textsuperscript{43,44} The variation of fluorescence lifetime allows for a specialized mode of imaging aptly called fluorescence lifetime imaging microscopy (FLIM). FLIM is advantageous in that by measuring the intrinsic excited state lifetime, imaging can be resolved without the detrimental effects of photon scattering on image resolution.

1.4 The Polymethine Dye Family

The polymethine chain described previously is the most critical organic motif for fluorescence. This polymethine chain is composed of a series of alternating single and double bonds in a conjugated pi system, is the primary structural-functional motif used to design fluorescent compounds. Compounds with these capabilities can be further developed for a host of applications including but not limited to diagnostic imaging\textsuperscript{45-49}, solar panel design\textsuperscript{50}, photodynamic therapy (PDT)\textsuperscript{51}, and analytical probe design.\textsuperscript{52} There are a variety of scaffolds that belong to the polymethine dye family including phthalocyanines\textsuperscript{53-55}, porphyrins\textsuperscript{56-58}, BODIPY\textsuperscript{59-61}, rhodamines\textsuperscript{62-64}, cyanines\textsuperscript{65-67}, phenanthridines\textsuperscript{68-70}, acridines\textsuperscript{71,72}, and squaraines\textsuperscript{73-75}.

Phthalocyanines are a family of comparatively large fluorophores composed of 4 isoindole subunits linked by a polymethine carbon into macro-heterocycles. Because of their large number of electron donors and aromatic carbons these compounds absorb far into the NIR and their
condensed planar structure allows for increased chemical and thermal stability.\textsuperscript{76} The push-pull system of electrons originate on the nitrogen atoms and resonate across the entire molecule as well as encourages binding to a metal cation in the center of the macrocycle. These molecules have been functionalized and studied for years and have uses in solar cell development, LEDs, optoelectrical sensors, transistors, and switches as well as in photodynamic therapy.

Porphyrrins are similar to phthalocyanines in that they are both macro-heterocycles and different in that porphyrins are composed of conjugated pyrrole subunits instead of isoindole subunits. They are electronically similar as well with electrons originating from the nitrogen atoms coordinated to a metal atom and resonating throughout the macrocycle. Porphyrins are specifically of interest biologically in that they are one of the macro-heterocycle motifs that are seen conserved across almost the entire tree of life. As an organo-metallic chelator it is commonly seen bound to iron as the heme of hemoglobin in the blood. This motif has evolved to be present in various other systems such as in the oxidative core of the cytochrome p450 family of enzymes. It can also be bound to various other metals such as magnesium in chlorophyll, cobalt in vitamin B12, and nickel.
in the active site of methyl-coenzyme M reductase where it facilitates the production of methane in the guts of cows and other bacterial mats.\textsuperscript{77, 78} Interestingly, porphyrins include the mineral Abelsonite, a nickel based compound that forms orange linear crystals and is found exclusively in the state of Utah, U.S.\textsuperscript{79} Porphyrins are seen in similar applications to phthalocyanines such as in solar cell and photodynamic therapy as well as in chemo-sensor design, therapeutics, and diagnostics.\textsuperscript{80-83}

![Phenanthridine molecule](image)

**phenanthridine**

Phenanthridine dyes are composed of a nitrogen heterocycle with 3 aromatic rings. There is also an electron source and an empty orbital giving priming a traditional push pull system, in the example given these are primary amines.\textsuperscript{68, 69} These dyes are very widely seen in the biologist’s laboratory specifically with ethidium bromide, which has the same structure as the phenanthridine scaffold in Table 1 but with an ethyl group pendant to the heterocyclic nitrogen. This compound has gained its status thanks to a unique property; because of their planarity and size, phenanthridine derivatives have the ability to intercalate with DNA. That is to say that the dyes have the ability to slide in between the base pairs of the DNA secondary helix structure. Binding to DNA coincides with a vast increase in fluorescence as the dye is protected in its excited state by being sandwiched between two nucleic acids.\textsuperscript{84} While this has enabled countless DNA blots, it also has its risk as this intercalation can lead to translation and transcription errors within DNA that can ultimately lead to cancer. With these considerations in mind, they are still quite useful with applications in bioimaging, photo-redox catalysis, and anti-tumor therapy.\textsuperscript{85-87}
The acridine scaffold is an isomer of phenanthridine with all of the aromatic rings in a single line. In the given example it also has a push pull electron system fully across it originating from amino groups that lower the band gap for absorption. Acridine’s planarity and size allows for it to have the same intercalation behavior as phenanthridine and such the scaffold has been seen repeated in various applications geared towards its interaction with DNA. This includes imaging of DNA complexes and as well as more disruptive effects such as anti-cancer and anti-bacterial activity. The scaffold has also been modified for pH response and used to measure endo and exocytosis in neural synapses.

Another 3 ring heterocycle core is the rhodamine dye scaffold. These dyes have almost the same structure as acridine but with the ring heteroatom replaced by a cationic oxygen. The electronic distribution across the system works in almost the same way, only now the HOMO and LUMO extend across the benzoic group pendant to the bottom of the scaffold. The carboxylic acid group present on the motif adds the feature of pH response to the spectral qualities of the dye due to changes in the HOMO and LUMO of the rhodamine molecule when it becomes protonated.
Rhodamine dyes’ high stability, molecular brightness, and quantum yields make them fantastic tracer dyes in industrial, environmental, and biological conditions. They have also been made quite useful as analytical chemo-sensors, as well as biochemical reporters for flow cytometry and enzyme linked immunosorbent assay (ELISA).

Boron-dipyrromethene or BODIPY is another fluorescent dye scaffold composed of nitrogen heterocycles coordinated to an electron deficient atom like in the porphyrin and phthalocyanine macrocycles only this class of molecules are bonded to a boron that is further electron deficient and bound to two fluorine atoms. These dyes are characteristically known by a short stokes shift and a consistent quantum yield in a variety of solvents. The scaffold also has a ease of functionalization and has seen various aromatic substituents and chemically labile functional groups to shift bathochromic effects and provide a chemo sensor ability. The intense optical properties of this group of dyes and ease of functionalization has facilitated the development of a wide variety of applications including solar cell development, anti-bacterial activity, bioimaging, photodynamic therapy, ion sensing, and biomarker sensing.

The namesake of cyanine dyes comes from the English word “cyan” which itself has roots in the Greek word “kyanous” meaning “dark blue”. The cyanine scaffold takes a linear approach to aromaticity and is composed of a nitrogen donor and a nitrogen acceptor separated by an odd
number length polymethine chain. These scaffold parameters provide for a variety of points of manipulation, from the choice of the heterocycle to the length of the polymethine chain.\textsuperscript{96, 97} Because the length of the polymethine chain can be modulated, dyes with very similar functionalization and the same heterocycles can have varying chain lengths, and in response longer and longer absorption wavelengths. For example, the monomethine and trimethine variations of the dyes absorb in the visible spectrum, while the pentamethine dyes absorb around 700-800 nm and heptamethine dyes further into the NIR range.\textsuperscript{98} This wide range of functional wavelengths have seen these dyes used in chemosensing, optical and fluorescent imaging, multispectral optoacoustic tomography (MSOT), drug delivery, and biological tracing.\textsuperscript{99-103}

![Squaraine dye structure](image)

Squaraine dyes share a majority of similarities with cyanines with the exception of a distinct polymethine bridge composed of a squaric acid derived polymethine ring. A push pull electron system exists across the squaric acid bridge originating and ending at the nitrogen atoms on each heterocycle. The electron rich heterocycles provide additional electron donation into the electron poor squaric bridge and encourage the lowering of the HOMO LUMO band gap enough for these dyes traditionally operate within the Far Red range and exhibit high molecular brightness.\textsuperscript{73, 104-106} This electron deficient core also opens up the polymethine chain to nucleophilic attack and as such these dyes have comparatively lower stability. This deficiency has however opened the door for research into designing squaraine dyes that are protected with macro-structural considerations. This is particularly seen with the rotaxane motif interlaced over the linear
squaraine dye like a ring over a linear wire.\textsuperscript{107, 108} Because of their structural similarity, squaraine derivatives have been used in similar applications to that of cyanine with common literature appearances concerning biological fluorescent imaging, photodynamic therapy, photoacoustic imaging, chemo-sensing, and solar cell design.\textsuperscript{109-115}

Chromophores have very important relationships with mankind through their various expressions in nature and in the synthetic laboratory. The most important compound on the planet is arguably a chromophore responsible for the oxygenation of the atmosphere in chlorophyll a. Understanding the dyestuff sources and structures of natural chromophores can give context to their applications and uses as well as provide insight into novel synthetic compounds. Comparing the convergent biomolecules can help the reader appreciate the wide variety of biodiversity and human ingenuity in harnessing these materials. These natural dyes all have similarities to shown synthetic scaffolds with similar heterocycle use and push-pull electron systems which can provide understanding for further synthetic dye development.

1.5 Water-Soluble Squaraine Dyes and their Applications

Dyes that are able to operate within aqueous solutions are be useful for the majority of biomedical applications. The majority of commercial dyes produced however are hydrophobic and do not generally dissolve with water and do not operate at physiological pH. The only FDA approved imaging cyanine, indocyanine green (ICG), has its special status is in part to the solubilizing sulfonate groups that adorn the heterocyclic indolinium ring as well as the $N$-pendant groups of the molecule which can accurately map vasculature with its dark green hue with low toxicity and retention.\textsuperscript{116} The zwitterionic cyanine ZW-800 is very similar to ICG because it contains the same functional groups that encourage water solubility with its sulfonate group on the heterocycle as well as trimethyl ammonium groups pendant to the heterocycle nitrogen. This leads
to ZW-800 also showing a high biological clearance which has seen it’s rise in use for widespread biomedical applications, specifically targeting-molecule guided biological imaging.\textsuperscript{47}

When designing a dye to be water soluble there are various functional groups to consider that promote this characteristic in organic compounds. One of the primary drivers of solubility is the presence of an electronic dipole across a molecule. These dipoles can interact with the polar solvents to encourage solvation by intermolecular alignment of dipoles. If a formal charge is present in a structure, then it is likely to be asymmetrical and generate a dipole across the molecule. Formal charges have the largest surface charge density and therefore direct the majority of the dipole arrangement by heavily influencing the molecules polar surface area. Functional groups that are responsible for a formal charge include quaternary nitrogen atoms, deprotonated alcohols, deprotonated thiols, sulfonate groups, and deprotonated carboxylic acids.\textsuperscript{117} With polarity in mind, uncharged functional groups that unevenly disperse electron density throughout a molecule also contribute to intermolecular dipole-dipole interactions and more importantly, hydrogen bonding with the solvent environment. Alcohols, thiols, and ketones are the primary functional groups associated with encouraging hydrogen bonding and are thus associated with high aqueous solubility. The hydrogen bonding ketones is due to the lone electron pairs on ketone oxygens that can coordinate with electron-poor hydrogens. In alcohols and thiols this lone pair interaction can still occur, originating on the oxygen and sulfur respectively. In additional to this interaction, alcohols and thiols can contribute to intermolecular interactions with other lone pairs of solvent molecules.\textsuperscript{118}

One of the most promising water soluble squaraine applications is the imaging and breakdown of $\beta$-amyloid plaques in the brain. These plaques are amino acid fibrils that are common in the brains of Alzheimer’s patients which can bundle up and crowd the interstitial space
between neurons. This is believed to be partially responsible for the “murky” cognitive functions experienced by disease sufferers. A group of polyhydroxylated squaraine derived from two phenolic groups as the push-pull system are able to respond to the binding of amyloid plaques in vivo by decreasing in absorbance and fluorescence intensity as well as disrupting the macrostructures of the amyloid plaques and breaking them apart.\textsuperscript{119} Figure 9 shows that there is a loss of absorbance and fluorescence signal from the squaraine dyes as concentration of the amyloids in solution increase. Additionally, binding studies indicates that cysteine residues of the chain are responsible for the spectral change by binding into the squaric ring of the dyes and quenching them as well as altering the structure of the plaques. Interestingly, as outlined in Figure 9C, the natural fluorescence profile of thioflavin within amyloid in the presence of \textbf{A1, A2,} and \textbf{A3} dramatically decreases. This is representative of the tertiary structure of the polypeptide chain changing since previously favorable absorbance conditions for the visually active thioflavin are now altered as the polypeptides’ thioflavin is exposed to a separate environment. The researchers measured the association constants between the dye and plaque fibrils and found it to be spontaneous and on the range of $10^4$-$10^5$ M$^{-1}$. Tunneling electron microscopy (TEM) in Figure 9E-D of the amyloid fibrils when exposed to \textbf{A1} also confirmed the groups hypothesis with the visible disassembly and dispersion of the amyloid fibrils and the formation of spherical peptide particles as indicated by TEM. This provides an optimistic target group of molecules that can be both diagnostics with their “turn-off” spectral response to the amyloid fibrils as well as a therapeutic by causing the disruption and disassembly of the plaques, though the proper biological clearance with
minimal toxicity of the disrupted plaques would be a requirement for the compounds to be transitioned into a clinical setting to treat Alzheimer’s.

Mercury is a highly toxic metal shown to severely damage nerve tissue in mammals and its early identification and tracking in residential water sources can prove invaluable in saving high-risk individuals from life-altering heavy metal poisoning. A squaraine based chemosensor was specifically designed to recognize mercury cations in aqueous solution. The general scaffold is based off a phenylamine electron donor-acceptor system with diethylcarbamodithioate functionalized chelating arms. The electron donation from these sulfur and nitrogen atoms allow for the specific binding of mercury over other metal cations such as silver, lead, cadmium, copper, etc. The dye on its own in aqueous solution exhibits a low spectral profile of absorbance at 548
nm due to its aggregation into tight packs. Chen et al designed compound B to be selective for mercury due to the specific size and lone pair localization of the azanediylbis(ethane-2,1-diyl) bis(diethylcarbamodithioate) chelating arms. These arms grab onto mercury and ignores other ions in solution which lessens the intensity of intramolecular dye interactions that keep the squaraine derivatives tightly bound into aggregates. This disruption of aggregate stacking with the newly mercury chelated dyes allows for the absorbance and fluorescence of light at 636 and 651 nm with a significant increase in fluorescence as outlined in Figure 10B. This “turn-on” indication is the most desired in chemosensor design as the positive response of a novel photon can provide more sensitivity with less false positives against a blank background signal.

Other water-soluble squaraine chemosensors have been developed based on a “turn-on” fluorescence response where the absorbance or fluorescence signal goes from off to on in response to a chemical stimulus. Specifically, a squaraine derivative designed to be activated from the nucleophillic attack of the unactive dye by thiophenol. Dye C is asymmetrical compound containing

![Chemical Structure](image)
a phenolic based moiety and a benz[c,d]indolium moiety. The benz[c,d]indolinium half contains the solubilizing functional groups with a polyethylene glycol (PEG) separated pentose sugar. The lone pair electrons in the PEG chain and present in the pentose sugar hydroxyl groups greatly add to solubilize the molecule as well as the alcohols on the phenolic moiety. The phenolic moiety is bound to a dinitrophenyl group by a sulfonic bridge. This bridge is susceptible to nucleic attack by thiols and upon cleavage enables the fluorophore to become active due to the creation of a new push-pull electron system. Sensor C was tested against various thiols to see if they would attack into the sulfonic bridge or not interact with the dye but was shown to be selective to thiophenol, a toxic aromatic thiol used in the chemical industry. This attacks the sulfur atom, triggering the release of a (2,4-dinitrophenyl)(phenyl)sulfide and sulfur dioxide as well as the newly hydroxylated dye which has increased absorbance and fluorescence as seen in Figure 11. The lone pair on this new hydroxyl group provides the “push” in the push-pull acceptor donor system and lowers the bandgap of the energetic system enough to be excited by visible light. A linear relationship of the fluorescence and absorbance changes in response to thiphenol was plotted and
showed a 0.058 degree relationship in absorbance change but an 8.90 degree relationship with absorbance. This high responsiveness enabled the imaging of thiophenol concentration with
detection limit of 10 nM.

A highly hydrophilic sulfonate functional group is used with high effectiveness as a nitrogen pendant functional group in two squaraine dyes derived by Belfield et al. The two developed compounds use the benzothiazole and benz[c,d]indolinium moieties as the electron source heterocycles for the dyes. The two dyes exhibited poor absorbance and fluorescence in aqueous solution with only a 3% quantum yield. However, their absorption and emission intensities as well as quantum yields were greatly increased in the presence of bovine serum albumin (BSA) as the two compounds have high affinity for the hydrophobic binding pocket II of BSA. The electrostatic forces of non-covalent binding to BSA and the protective effects the protein provides from the solvent environment enables a 10x increase in absorbance and fluorescence intensity for both dyes as displayed in Figure 12D. Binding to BSA also is shown to induce a bathochromic shift in the dyes as the hydrophobic binding pocket stabilizes the excited state of the dye with the standard absorptions of 641 and 655 nm for the benzothiazole derivative and benz[c,d]indolinium derivative respectively increased to 660 and 775 nm. This is seen also with the benzothiazole dye’s emission at 652 shifting to 669 nm and the benz[c,d]indolinium dye’s emission shifting to 684 nm, very close to the NIR region, making the dyes an advantageous set for biological imaging when bound into BSA conjugates. These dye-BSA conjugates when imaged in colorectal carcinoma cells showed a colocalization with the lysosomal dye, LysoTracker indicating the dye-BSA conjugates are taken up in the same lysosome based active transport mechanism that BSA is intercellularly absorbed as opposed to diffusion of the dye through the
Further water soluble squaraines with benzothiazole heterocycles have also been synthesized with the aim of creating photodynamic therapy drugs. Photodynamic therapy is when light can trigger a drug-like response. In this case the generation of singlet oxygen, a toxic species that induces cell death and when isolated to cancer cells either through appropriate biodistribution or through the selective application of activating light can significantly impact oncological outcomes. The lead dye was built around two iodine substituted benzothiazole...
heterocycles with a butyl carbon chain separated sulfonate group pendant to each nitrogen that provided solubility. Additionally, an asymmetrical dye built from the sulfonated benzothiazole and a $N$-ethyl benzothiazole and an asymmetrical dye built from the sulfonated benzothiazole and an anilinium moiety were prepared to test their effects on reactive oxygen species (ROS) generation. These dyes all formed H-aggregates in water that caused blue-shifted absorbance peaks at 598, 590, and 554 nm for the diiodated E1, monoiiodated E2, and asymmetric anilinium dye E3 with emission photons of 665, 658, and 668 nm at low quantum yields of 1%. The quantum yields for photon emission are low because a significant portion of the absorbed energy of the fluorophore transferred from the excited state of the dye into proximal triplet oxygen atoms. This energy is enough to encourage the transition of one of the valance electrons in oxygen to flip its spin and pair with its electron companion in a single orbital in the highly reactive single oxygen species. The diiodated E1 and monoiiodated E2 dyes showed the best conversion rates for triplet oxygen generation at 19% and 26% for the two. This can be due to the heavy atom effect originating in iodine that allows for complex geometric orbital transitions at lower energy level than in simpler s and p orbitals which are primarily involved in organic chemistry. These three dyes were all tested for their cytotoxic capabilities in Dalton’s lymphoma, a blood cancer that specifically targets T-cells, and showed 100% cancer cell death at 10 µM concentrations. The compounds also were imaged in various polar solvents to see the effects on their spectral profiles in Figure 13. They showed intense separation in spectral peaks when compared to water this is due to the large dipole moment across each dye allowing for pronounced solvent-dye interactions which can spectrally shift the absorption and emission of the dyes.
Sulfonated squaraine dyes have also been prepared exploring various squaric ring substitutions replacing a core ketone oxygen with a sulfur or a dicyano group. The non-substituted dye \( F_1 \) absorbs and emits at 632 and 642 nm respectively with a quantum yield of 6%. With a sulfur atom replacing an oxygen this increases to 636 and 648 nm for \( F_2 \) and with the dicyano group in \( F_3 \) this is even farther at to 667 and 685 nm. Though the non-substituted dye has the lowest absorption wavelength it possesses the highest molar absorptivity at 265,000 L cm\(^{-1}\) M\(^{-1}\)
1. F1 was also tested for its spectral effects when binding to BSA. This resulted in a 20 nm red shift in the absorption and a 6x increase in quantum yield as the binding of the hydrophilic dye lowered the energy requirement for excitation and stabilized the triplet state of the fluorophore as shown in Figure 14B.

Another set of dicyano ring-substituted squaraines were synthesized with multiple PEG functional groups to solubilize them in water for the selective targeting of DNA G-quadruplex macrostructures. These numerous oxygen lone pairs on the PEG group enable hydrogen bonding with water and encourage the dyes to dissolve though they are relatively bulky. The planarity of
the benzothiazole rings and the dicyano group on the squaric ring allows these dye derivatives to lay against the planar top surface of G-quadruplex structure. The G-quadruplex macrostructure is composed of 4 nucleic acids paired into a helical shaped based on their intermolecular interactions with all 4 nucleic acids and a cation chelated in the center of the macrostructure. These quaternary structures are of great importance in DNA signaling and are useful in understanding the DNA repair signaling cascade. The reduction of molecular motion when the dyes lay against the macrostructure translates to the increase in absorbance and emission of the dyes, G1 and G2. The prepared compounds, and specifically dye G2 with its PEGylated phenyl pendant groups, showed specificity for planar G-quadruplex surfaces and allowed for the redshift of absorption of the dye 20 nm and the increase of quantum yield to 70%. The spectral is change is due to the binding of the dye and nucleic acid structure which decreased the energetics of fluorescence and stabilized the excited state of the dye much in the same way that BSA is shown to improve spectral in similar dyes. These dyes are significant in that they are one of the most responsive and specific G4 quadruplex binding compounds available in the literature. Furthermore, the bound form of the dye is stabilized enough to enable two photon absorbance imaging (TPA) in which a photon of same wavelength of light can be absorbed twice, elevating the molecule to an even higher excitation
level and enabled imaging at 1275 nm.

A barbituric acid heterocycle act as the source of electron density for the polymethine push-pull system in dye H that are functionalized with a sulfonate group distanced by a alkyl carbon spacer for water solubility and with a N-hydroxysuccinimide-NHS ester as well for protein pairing. When combined with dicyclohexylcarbodiimide (DCC) in an anhydrous environment, this functional group allows for the protected binding of the dye to any targeting peptide or immunoglobulin molecule desired by a researcher. This compound absorbs at 786 nm and emits at 817 nm in PBS buffer solution and has sharp spectral peaks indicating low aggregation due to the compound’s

Figure 15: G4 quadruplex binding benzothiazole squaraine dyes G1 and G2. Figure taken without permission from reference 125. Copyright RSC 2018

The same binding affinity and increase in optical properties when bound to large biomolecules is seen again specifically in the hydrophilic compounds developed by Suzuki et al. A barbituric acid heterocycle act as the source of electron density for the polymethine push-pull system in dye H that are functionalized with a sulfonate group distanced by a alkyl carbon spacer for water solubility and with a N-hydroxysuccinimide-NHS ester as well for protein pairing. When combined with dicyclohexylcarbodiimide (DCC) in an anhydrous environment, this functional group allows for the protected binding of the dye to any targeting peptide or immunoglobulin molecule desired by a researcher. This compound absorbs at 786 nm and emits at 817 nm in PBS buffer solution and has sharp spectral peaks indicating low aggregation due to the compound’s
high solubility. The interactions with serum are similarly positive as other dyes in its class with a 25x increase in fluorescence when bound to BSA as shown in Figure 16. This increase in spectral qualities while bound is encouraging for this dye as a covalent linker dye as it can be expected to have increased fluorescent properties when in a bound state.

![Chemical structure of squaraine dye](image)

**Figure 16:** A) Absorbance and B) emission of barbituric derived squaraine, H, in PBS buffer with and without the presence of BSA. Figure used without permission from reference 126. Copyright JSAC 2008

Delcamp et al has previously synthesized squaraine compounds based on 2-methyl-2-phenylindoline and their work deals with the solvatochromic abilities and biological imaging abilities of their derivatives. These compounds using the same phenyl indolenine derivative functionalized with a sulfonate group separated from the aromatic heterocycle via an ether linked
butyl carbon chain. Not only does the sulfonate groups provide excellent solubility, the ether group linking them to the core ring is para from the indole adding into the system and add to the red-shift and increased optical properties of the dye. This compound absorbed light at 698 nm and emitted 716 nm in water with a molar absorptivity of 93,000 L cm$^{-1}$ M$^{-1}$ and a quantum yield of 30%. As shown in Figure 17, dye I was responsive to different polar solvents with absorbance values ranging 45 nm. This again is due to the high dipole moment across the dye allowing increased solvent interactions. Imaging with the dye in human embryotic kidney cells showed co-localization with Lyso-tracker, indicating that this hydrophilic compound is absorbed and sequestered in cells via their lysosomal network.$^{127}$

![Figure 17: Solvatochromic effects and bioimaging of sulfonated dye I. A) Solvatochromic effects on absorbance profile of dye I, B) Colocalization imaging of LysoTracker (green) and Dye I (red) in human HEK cells, C) Dye I (red) in human HEK cells. Figure used without permission from reference 126. Copyright ACS 2014.](image)

A dye with cationic functional groups that induce water solubility was developed to analyze
the aggregation behavior of squaraines in water.\textsuperscript{128} This dye has a pyridinium group separated by a butyl carbon chain pendant to a benzothiazole nitrogen of the compound. An aqueous solution of dye was prepared and resulted in the free monomer absorption peak of the dye at 643 nm as well as the H-aggregate of the dye, where all of the dyes stack with their aromatic faces perfectly aligned at the blue shifted and more energetic absorption band of 592 nm. To test the shift in aggregation the poly(acrylic acid) PAA polymer supported by sodium ions was used to induce J-aggregation where the molecules of dye stack in a brick-like fashion that is much less energetic and exists at the longer wavelength of 765 nm, a 122 nm bathochromic shift relating to a 0.35 eV lower system energy. To re-induce the H-aggregate a solution of CaCl\textsubscript{2} was added to bind the sodium from the PAA polymer and denature the polymer chain that was stabilizing the J-aggregate. This method can be used to create larger spectral variation of dyes in solution.

![Figure 18: In situ aggregation modulation of J. in water (blue), H-aggregate (magenta line), formation of J-aggregate with the addition of PAA-Na (red), release of dye and reformation of original spectrum (blue dash), reformation of H-aggregate (magenta dash), reformation of J-aggregate with the addition of PAA-nA (red dash). Figure used without permission from reference 127. Copyright ACS 2018.](image)

The literature of water-soluble squaraines provides a large insight into the capabilities of this class of small fluorophores as ion sensors, chemosensors, DNA sensors, photodynamic
sensitizers, and bioimaging agents and outlines some of the necessary chemical modifications to facilitate these structural-functional relationships. Operating within an aqueous environment allows for more practical applications of these molecules and is critical for the long-term functional and approval process of any biologically relevant squaraine to be used in trials. Further work in this field can be pursued by functionalizing other heterocycles for dye condensation with necessary solubilizing groups such as sulfonate, alcohols, carboxylic acids, and quaternary/cationic nitrogen atoms.

2 SYNTHESIS OF HYDROPHILIC AND HYDROPHOBIC SQUARAINES DYE DERIVATIVES

2.1 Rationale

The projects presented in the second chapter of this thesis are organized around a similar rationale used previously by our group to synthesize water-soluble imaging molecules. This lead compound, ZW-800, is one of the most promising compounds developed by Henary’s lab due to its high molecular brightness in the near infrared (NIR) region as well as the compound’s high solubility due to its zwitterionic charge state which enables the compound’s favorable and rapid biological clearance. This compound has been further developed and functionalized to investigate vascular leakage, blood brain barrier disruption and further intraoperative imaging applications. The principle for this research was to use the same zwitterionic property of the primary precursor salt used to develop ZW-800 as the precursor heterocycle for a series of dye condensations using the squaric acid polymethine scaffold as the bridge. The precursor salt is characterized by a negatively charged sulfonate group para to an indolinium nitrogen and a positively charged trimethyl ammonium nitrogen pendant to the same nitrogen via a propyl carbon chain. Since ZW-800 was shown to be cleared from biological systems in a manner untypical of polyaromatic dyes that tend to aggregate to the liver and intestines our rationale was to aim to see
similar hydrophilic behavior with squaraine analogues which already carry a zwitterionic ability. The work presented in this study aims continue using solubilizing approaches seen in ZW-800 and evaluating the effects of various other functional groups on solubility and molecular brightness. Additional halogenated, electron donating/withdrawing, and non-ring-substituted squaraine dyes were prepared with the same pendant quaternary nitrogen group as a reference to these substituents effects. A group of similar hydrophobic dyes were also prepared as a comparison.

![Figure 19: Nucleophillic bleaching of squaraine fluorophores](image)

Since one of the drawbacks of using squaraine as a polymethine dye scaffold is that the carbons alpha to the ketone in the squaric ring are very electron deficient due to the two ketone groups on the ring. These are especially susceptible to nucleophilic attack as demonstrated in Figure 19. An additional proposed hypothesis was that the presence of quaternary methyl nitrogen atoms that are freely able to move around the structure may allow for intramolecular interactions between the positively charged pendant group and the electron dense ketones on the squaric ring carbon. This internal-salt interaction between the negative and positive charges would then redirect the electron density on the central ring and influence the geometric distribution of the

![Figure 20: Proposed intramolecular interactions between positively charged quaternary nitrogen groups and negatively charged enolic oxygens](image)
HOMO and LUMO orbitals to improve stability. This withdrawal should also reduce the propensity of nucleophilic attack into the central ring.

2.2 Synthesis of Hydrophilic and Hydrophobic Squaraine Dyes

The synthesis of the first group of dyes is composed of three steps, the formation of the indole heterocycle 1-3 and 11, the alkylation of these heterocycles, and the condensation of a dye. This begins with the preparation of indoles 1-3 from the condensation of each corresponding phenylhydrazine precursor with 3-methylbutan-2-one in glacial acetic acid during reflux. This is a variation of the Fischer indole synthesis which involves the insertion of the primary amine of the phenylhydrazine into the carbonyl group of the desired ketone, its isomerization to the resulting enamine, and further pericyclic rearrangement into an imine before forming the indole through the loss of ammonia. This is isolated through recrystallization of the formed solid indole product and the evaporation of excess unreacted precursors. The reagents for the alkylation step are conserved for the hydrophilic dyes as the primary functional group of interest is the cationic trimethyl-ammonium group separated by a propyl group spacer. Therefore when undergoing
alkylation for all of these compounds, 3-bromo-\(N,N,N\)-trimethylpropan-1-aminium and the precursor indole 1-3 and 11 were individually placed in a seal tube with toluene and heated at 130 °C for 72 h in which time the lone electron pair of the aromatic indole nitrogen attacks and displaces the propanammonium bromine in an \(S_N2\) reaction. As this reaction progressed, the resulting indolinium salts 4-6 were generated as dark reddish to pink solids that were recrystallized from hot toluene. The final condensation reaction was performed with a 1:1 blend of toluene and butanol in the reaction vessel and with an attached Dean-Stark distillation column filled with toluene. The Dean-Stark distillation column works in principle by removing water from the reaction vessel. As water is formed as a byproduct of the condensation, it evaporates as a toluene/water azeotrope before collecting in the bottom stem of the Dean-Stark tube. This gradually displaces more dry toluene into the vessel and pushes the reaction forward via Le’Chatlier’s principle. This reaction is then performed at 120 °C to push the reaction forward and to reach the boiling temperature of toluene. An electrophilic attack into squaric acid itself is not as favorable, so this reaction is performed at high temperatures and in the presence of butanol due to butanol’s favorable ability to replace the hydroxyl groups in squaric acid, thus priming the carbons of the squaric ring to be even more electrophilic and reactive. The indolinium salts 4-6 were condensed with squaric acid at reflux over time periods ranging 8 to 24 h in order to form the hydrophilic dyes 7-9. These reactions were monitored for completion by thin layer chromatography (TLC) and UV-Vis spectroscopy. These dyes were then recrystallized from methanol and diethyl ether before purification via column chromatography with a blend of 5-15% methanol in diethyl ether.
The procedure for the synthesis of dye 13 has some slight changes to accommodate for the slightly differing chemistry of the dioxymethyl functional group. This salt, 12, has been previously prepared by our group using a microwave assisted synthesis methodology and shown to be an electron donor that red shifts the absorption of synthesized pentamethine cyanine dye derivatives.\textsuperscript{135} A more electrophilic ketone is needed to synthesize the dioxyindole derivative therefore 3-methylbutan-2-one was treated with atomic bromine in acetic acid at 0 °C and allowed to reach room temperature to form a halide bond on the most substituted alpha carbon for compound 10. This was then reacted with benzo[\textit{d}][1,3]dioxol-5-amine in dimethylformamide and potassium carbonate to yield an enamine intermediate. This was precipitated using cold aqueous saline, filtered, and then dried. This crude was then treated with p-toluenesulfonic acid in toluene to push forward the pericyclic rearrangement to the indole 11. The nitrogen alkylation reaction was performed in the same manner as dyes 7-9 with 3-bromo-\(N,N,N\)-trimethylpropan-1-ammonium and indole 11 reacted in a seal tube with toluene at pressure at 130 °C for 72 h. As the reaction progressed, a dark brown precipitate was formed which was then washed with hot toluene and recrystallized from methanol and diethyl ether to yield indolinium salt 12. Squaric
acid and salt 12 were then condensed in a round bottom flask with 1:1 (v/v) butanol and toluene with an attached Dean-Stark distillation column and monitored for progress using TLC and UV-Vis spectroscopy. The crude dye was then recrystallized from methanol and diethyl ether before purified via column chromatography with 10% methanol in diethyl ether.

The hydrophobic dyes in this series were prepared with the same overall scheme as the previous four dyes and were synthesized starting from the indoles 1 and 3. The hydrophobic salts were prepared by refluxing an indole with an alkyl halide in acetonitrile for 72 h. Each of these were isolated by washing the crude with acetonitrile before recrystallization with acetone. The isolated salts 14-16 were then reacted with squaric acid for 16 hours at 120 °C in an equal molar parts by volume mixture of butanol and toluene to form dyes 17-19. This reaction was performed in a round bottom flask with an attached Dean-Stark apparatus and condenser and seen in Figure 21. The preparation of these dyes was monitored by TLC and UV-Vis spectroscopy and isolated by extracting the crude dye with ethyl acetate then purifying the dyes with column chromatography with a eluent system of 5% methanol in dichloromethane. All of the prepared dyes 7-9, 13, 17-19

Scheme 3: Synthesis of hydrophobic squaraine dyes 17-19
were characterized via melting points, $^1$H NMR and $^{13}$C NMR, then assayed for their absorbent and fluorescent properties.

2.3 Optical Measurements

2.3.1 Molar Absorptivity

The absorption spectra of each dye were recorded in a Varian Spectrophotometer interfaced (Varian Inc. Palo Alto, Ca.). This was linked to Cary WinUV Scan Application v3.00 at increasing concentrations (µM) in UV-Vis cuvettes with a pathlength of 10 mm. For the measurement of molar absorptivity, a stock solution was prepared for each dye by adding a mass of each compound on an analytical balance to in order to make an approximate 1.0 mM solution of the dye. These stock solutions were prepared in DMSO to improve solubility then used to obtain the molar absorptivity in ethanol. Each sample was vortexed for 1 min and sonicated for 30 min before its absorbance was recorded.

2.3.2 Fluorescence Data

Each fluorescence spectra for the dyes were obtained using a Shimadzu RF-5301 Spectrofluorophotometer (Shimadzu Corporation Analytical Instruments Division, Duisburg, F.R. Germany). This instrument was interfaced with a PC and operated using the RF-5301PC software. The parameters used for each experiment were thus: slit width of excitation set to 5 nm, slit width of emission set to 5 nm, sensitivity set to high, gain set to medium, wavelength speed set to medium, a light source consistent with a 150 W Xenon lamp bulb, and an excitation wavelength set to 10 nm blue shifted from the absorbance of each respective dye sample. Disposable polystyrene fluorescence cuvettes with a 10 mm pathlength were used for each measurement. Each fluorescence measurement was recorded using a solution with an absorption value less than 0.1 A.U. to avoid inner filter effects. This means that several of the samples are diluted by a constant
value to ensure proper data acquisition. The data was analyzed and all corresponding calculations were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA.)

2.3.3 Solvatochromism Data

The environmental solvent effects on absorbance and fluorescence of each dye were recorded using a standard concentration of the dye in various polar organic solvents. This concentration was set at 20 µM to have optimal absorbance signal, then diluted 20x for recording the fluorescence signal. The samples were measured in 10 mm pathlength disposable polystyrene fluorescence cuvettes. The absorbance for each solvent was recorded using the Varian Spectrophotometer interfaced with Cary WinUV Scan Application v3.00 on a PC and the fluorescence was recorded using a Shimadzu RF-5301 Spectrofluorophotometer interfaced with RF-5301PC on a PC. Each sample was sonicated for 30 min and vortexed for 1 min before their spectra was recorded.

2.3.4 Computational Methods

The LUMO and HOMO orbitals were calculated using Hartree Fock Density Functional Theory (HF-DFT) self-consistent fields (SCF) algorithms. This was performed using a Geometric Direct Minimization, a 6-31G* basis set, and B3LYP methodology via Spartan18. These values were calculated using simulations of the dyes in a vacuum.

2.4 Results and Discussion

2.4.1 Representative Characterization

Compound 7 was chosen as a representative of the prepared group for complete chemical characterization and analysis. The 1H NMR spectra of 7 was recorded in DMSO-\(d_6\) as shown in Figure 22. The structure of the dye has been separated into three primary groups defined by their color, each based on their shared characteristics and chemical shift. Additionally, since these dyes
are symmetrical across the two ketones in their squaric ring, each dye can be classified in terms of each respective half-dye. The first region of interest, H\textsubscript{a}, is composed of four aromatic protons (multiplied by 2 for a total of 8 on the dye) that take the shape of three multiplets (ppm = 7.51, 7.37, 7.20). These hydrogens are color coded as blue and give the farthest down field signal due to the high electron density and thus de-shielding effect of the aromatic indole ring.

![Figure 22: Analysis of \textsuperscript{1}H NMR for dye 7 in DMSO-d\textsubscript{6}](image)

The second signal of interest is the "characteristic signal" of the compound in that it is indicative of the final product and is represented by the color green in Figure 22. This downfield singlet between the aromatic hydrogens and more upfield alkyl hydrogens represents the hydrogens associated with the polymethine bridge. For the squaraine dye scaffold this shows up as single proton (multiplied by 2 for a total of 2 on the dye) at 5.86 ppm. This new aromatic signal along with the loss of three protons is the primary sign of the conversion of the iodolium salt into the squaraine dye. The next two signals, H\textsubscript{c} and H\textsubscript{e}, correspond to two sets of alkyl hydrogens each alpha to a nitrogen as parts of the carbon spacer on each pendant group and are represented as red.
Hc is the most downfield set at 4.20 ppm as they are located next to the nitrogen that experiences the greatest electron density and is component of the push-pull fluorophore system. Hc is the next most downfield group at 3.67 ppm as they are alpha to the quaternary trimethyl-ammonium nitrogen. Each of these signals should theoretically exist as triplets as when using the n+1 rule but do to the low resolution of the spectrum they only resolve to a single peak. The red singlet at Hf, when integrated, corresponds to the 9 methyl hydrogens of the trimethyl-ammonium groups (multiplied by 2 for a total of 18 on the dye) at 3.16 ppm. The most shielded set of hydrogens on the pendant group are at Hd and exist as a crude quintet at 2.18 ppm that integrates to 2 hydrogens (multiplied by 2 for a total of 4 on the dye). The most down field group of hydrogens on the molecule in Hg exist as a singlet integrated to 6 hydrogens (multiplied by 2 for a total of 12 on the dye). All of these signals together sum to a total of 62 hydrogens (halved to 31). The J-coupling constant is used to identify what peaks in a ¹H NMR spectra are engaging with splitting with each other and can help add to the evidence pile that helps decipher the spectrum. All of the prepared compounds had poor solubility in even the most polar available NMR inactive solvents and do not have a high level of shimming. Therefore the resolution of each ¹H NMR is not high enough to determine the specific J-constants.
Dye 7 is composed of 38 carbons, 20 of which are marked as blue in Figure 23 and correspond to deshielded aromatic carbons and 18 of which are marked as red in and correspond to shielded aliphatic carbons. Since there is a plane of symmetry across the molecule through the two ketones of the center squaraine ring, the signals from the dye should be expected to be halved but with an additional carbon due to unequal electron density through the plane of symmetry. This is seen in the recorded spectrum with 18 carbon signals measured through the instrument. The most downfield signal is 180.5 ppm and corresponds to a carbon alpha to the push-pull originating nitrogen atoms (C8 and C22) and the most upfield signal is 17.2 ppm and corresponds to the two methyl carbons of the indole (C10, C11, C31, and C32). Of the total signals, three belong to sp3 saturated carbons, three belong to sp3 aliphatic carbons, five belong to sp2 saturated carbons, five sp2 ipso carbons, and one sp3 ipso carbon.

Figure 23: Analysis of $^{13}$C NMR spectrum for dye 7 in DMSO-$d_6$.
2.4.2 1H NMR D$_2$O Exchange

An interesting $^1$H NMR behavior expressed itself after failed solubility for NMR analysis of dyes 9 and 13. Both of these compounds required sonication with D$_2$O as an solvent instead of DMSO-$d_6$ due to the two dyes much more hydrophilic and increased zwitterionic character. When performed in D$_2$O, no bridge signal, H$_b$, would appear at 5.8 to 6.0 ppm. At the same time, no alkyl signal integrating to 3 hydrogens would appear either. This signals the consumption of the indolinium salt, but not the formation of the polymethine bridge as shown in Figure 24 below. Further puzzling was the reaction’s monitored progression was also positive as a characteristic absorption band appeared and the starting material was being consumed according to TLC and that a fluorophore corresponding to the proposed structure was being synthesized.

![Figure 24: Disappearance of polymethine bridge $^1$H NMR signal with D$_2$O of dye 7 after 38h](image)

This spectral behavior was further studied by taking dye 7, a hydrophilic dye that was able to dissolve for NMR analysis in DMSO-$d_6$, and treating the sample with D$_2$O to observe the effects over time. After 24 hours of dark incubation at room temperature there was an 85% loss in signal from the bridge proton, this completely disappeared after an additional 24 hours (see Figure 24). The hypothesis for this loss of signal is that an exchange is occurring between the bridge hydrogen...
and a deuterium atom from heavy water, the mechanism of which is proposed in Figure 25. As a susceptible electrophile, the dye is attacked by the oxygen lone pairs from heavy water and quenched to form an alcohol and disrupt the conjugated aromatic system. This reaction is reversible however and when leaving, the heavy alcohol group has two options for what hydrogen atom it will source to become a proper leaving group. If this progresses in the top path shown in Figure 25, there is no discernable change in the dye spectra as there is still a proton in place on the bridge, but if the bottom path is taken, the proton signal is reduced by half as a heavy deuterium atom is no longer $^1$H NMR active. As there are millions of millions of heavy water atoms inserted to the sample even in a single drop, as this exchange is allowed to occur over time, eventually the numerically favored product of two deuterium atoms substituted on the central squaraine bridge. Since deuterium is not $^1$H NMR active due to its lack of paramagnetic electrons in its valence, there is not signal on the spectrum. The presence of the resulting dye can be inferred by comparing the $^1$H NMR spectrum of the precursor heterocycle and looking for the disappearance of an alkyl region singlet that corresponds to the nucleophilic center of the precursor that is altered to become the polymethine bridge.

![Figure 25: Proposed mechanism of deuterium exchange with squaraine dyes](image)

### 2.4.3 Optical Properties

UV-Vis absorption and fluorescence was recorded for each dye in ethanol in order to
determine molar absorptivity and quantum yield as shown in Table 2. ICG was used as a calibration dye to ensure proper data collection for the synthesized squaraine derivatives and was measured within 1% of error in both molar absorptivity and quantum yield when compared to previous literature measurements. Molar absorptivity was calculated by taking the slope of the linear relationship between the absorption and the concentration of each dye at in a standard series of solutions. Each dye was recorded at an approximate molarity of 1 μM to ensure that the absorbance recorded was below 1 absorbance unit (A.U) so that the Beer-Lamber relationship would hold true for the measurement of the compound. All of the compound shared similar molar absorptivity values ranging from 134,910 to 163,816 L mol⁻¹ cm⁻¹. Each compound’s quantum yield was calculated using standard methodology and shown to be within similar ranges of 12% to 22%.

Table 2: Optical properties of prepared squaraine dyes 7-9, 13, 17-19 and ICG in ethanol

<table>
<thead>
<tr>
<th>Dye</th>
<th>λ_{abs} (nm)</th>
<th>λ_{em} (nm)</th>
<th>Stokes Shift (nm)</th>
<th>ε (L mol⁻¹ cm⁻¹)</th>
<th>φ_f (%)</th>
<th>Molecular Brightness (ε * φ_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>631</td>
<td>642</td>
<td>11</td>
<td>223,548</td>
<td>13</td>
<td>2906124</td>
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<td>8</td>
<td>669</td>
<td>681</td>
<td>12</td>
<td>220,765</td>
<td>22</td>
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<tr>
<td>9</td>
<td>639</td>
<td>648</td>
<td>9</td>
<td>227,544</td>
<td>19</td>
<td>4323336</td>
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<tr>
<td>13</td>
<td>638</td>
<td>651</td>
<td>13</td>
<td>179,772</td>
<td>22</td>
<td>3954984</td>
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<tr>
<td>17</td>
<td>632</td>
<td>648</td>
<td>16</td>
<td>163,154</td>
<td>14</td>
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<tr>
<td>18</td>
<td>634</td>
<td>645</td>
<td>11</td>
<td>121,011</td>
<td>12</td>
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<tr>
<td>19</td>
<td>639</td>
<td>649</td>
<td>10</td>
<td>115,365</td>
<td>12</td>
<td>1384380</td>
</tr>
<tr>
<td>ICG</td>
<td>787</td>
<td>818</td>
<td>31</td>
<td>192,877</td>
<td>13</td>
<td>2507401</td>
</tr>
</tbody>
</table>

The average Stokes’ shift for these dyes were characteristic with an average of 12 nm. The greatest Stokes shift was seen in the hydrophobic butyl dye 17 and the lowest shift was seen in the target dye, 9. The Stokes’ value directly relates to the energy difference between the photon absorbed and the photon emitted from the fluorophore and thusly the portion of energy that is transformed non-radiatively within the fluorophore as it relaxes. In dye 17 this portion of energy absorbed by the fluorophore that is not released back out in a photon of light is 0.048 eV, this is
lower than in 9 with 0.027 eV being absorbed. These energy levels are comparable to the average vibrational bond energetics of most molecules at room temperature. The average Stokes’ shift of the hydrophilic dyes is 11 nm while the average shift of the hydrophobic dyes is 13 nm, meaning on average the amount of absorbed energy is 15% less in the hydrophilic dyes.

Several structural effects of the various functional groups on the squaraine derivatives were responsible for resulting spectral effects. The sulfonate group found in compound 8 is an electron withdrawing group that pulls electron density in the conjugated system back towards the electron poor sulfur atoms that are para to each of the indole nitrogen atoms. This physical extension of the HOMO-LUMO system into the sulfonate group is seen spectrally as a 38 nm stokes shift. When compared to the non-substituted dye 7, the bromine addition to compound 9 is a mixed bag electronically speaking in that it is a large, electronegative atom with full p electron orbitals that give it an electron withdrawing effect. However, the presence of those p electron orbitals allows

Figure 26: Absorption and emission profiles of synthesized squaraine dyes in ethanol at 20 uM
the bromine atom to be considered electron donating based on resonant properties. This blend of electronic properties is seen spectrally as an 8 nm stokes shift in dye 9 compared to dye 7 and a 5 nm stokes shift in dye 19 compared to dye 18. The dioxyethyl group in dye 13 shares many of the same characteristics as bromine in that it is electronegative and can act as a withdrawing group and also possesses lone pair electrons that can resonate throughout the conjugated system and act as a donating group. The electronic pathways available for the dioxyethyl group include both the para and the ortho positions on the indole benzene ring and therefore can work to electronically counteract each other. These properties are as expected as the additional electronic space for the from each functional group molecular orbitals allow for the lowering of the band gap of excitation between the HOMO and LUMO for each of the squaraine derivatives. These electronic shifts also lead to the stabilization of the excited states of the dye as seen in the reported quantum yields increasing up to 69% when compared to the non-substituted hydrophilic dye 7. The hydrophobic series of dyes all have a similar quantum yield that averages to 0.13. This combined with their relatively low molar absorptivity in ethanol means that of the group of squaraines prepared, compounds 7 and 8 have the lowest reported brightness. Combining the molar absorptivity and the quantum yield generates the sum unit, molecular brightness with is a combined metric for overall efficacy for a fluorophore.

Each compound was also tested for it’s dark-stability and photo-stability in ethanol and compared to ICG as shown in Appendix 1. For dark-stability each compound was left in solution in the dark for a week and compared to its initial absorption to determine how much of the compound is degraded. ICG decomposed 20% after 7 days but the synthesized derivatives all showed less than 6% in decomposition. For photo-stability the dyes were dissolved in ethanol and exposed to constant UV-irradiation and measured over a period of 24 h. ICG showed complete
decomposition over this period of time but compounds 7-9, 13, 17-19 all showed only a maximum of 20% decomposition. This indicates that the compounds are both more shelf and imaging stable than ICG.

### 2.4.4 Computational Studies

Computational work was performed on these compounds to provide insight into the electronics of the synthesized derivatives. The quantitative structure-activity relationship (QSAR) values of area, volume, polar surface area (PSA), and dipole moment are quantitative parameters that can help contextualize the synthesized squaraine dyes within the greater library of hydrophilic polymethine dyes. The molecular orbitals calculated through Spartan18 are based on calculated, optimized geometries and represent the electron band gap responsible for the absorption of visible light. All of these values are listed in Table 3 below. Spartan18 was also able to generate 3D modeled surfaces representing the electron density of each squaraine dye, their highest occupied molecular orbitals (HOMO), and their lowest unoccupied molecular orbitals (LUMO) which are visualized in Figures 27 and 28. These studies were performed to lend insight into the electronics of the synthesized dyes. The PSA and calculated dipole moments can explain the degree of solvent interactions by each compound. The calculated molar orbital energies provide insight into the ground energetics of the molecule in a vacuum without any solvent effects. All differences between calculated and experimental energies are due to these solvent-molecule interactions.

The modeled orbitals that were computer generated for dye 7 show the HOMO existing in its resting ground state, a singlet, with the majority of the space of the orbital primarily across the polymethine bridge with some depositing back onto the indole aromatic ring. When excited into the LUMO, this contrasts to the HOMO with a greater density projected across the polymethine bridge and decreased orbital presence back from the indole ring. This indicates that the electron
deficient core provides a lower-energy pathway during electron excitation than in the standard polymethine chain and is partially responsible for the -6.24 eV potential of the LUMO and its -2.21 eV band gap. When looking at the electrostatic potential surface of the dye, this same electron orbital character is evident, with the valence molecular orbital residing on the ketone oxygens and the indole rings towards the two push-pull nitrogen atoms. This distribution generated an overall polar surface area of 25.12 Å² primarily localized around the indole rings and ketone oxygens. The trimethyl-ammonium arms are contrasted as quite electron poor but their cationic charge still likely encourages intermolecular stabilization of the excited and polarized state. The calculated dipole across the system was 12.87 debye or 2.679 eÅ which directly relates to the polarization across the dye and its intramolecular interactions with a solvent environment. The dipole of the fluorophore is most prominent in the excited state due to the triplet orientation of the systems

Table 3: Spartan18 computed physical properties of dyes 7-9, 13, 17-19

<table>
<thead>
<tr>
<th></th>
<th>Area (Å²)</th>
<th>Volume (Å³)</th>
<th>PSA (Å²)</th>
<th>Dipole (debye)</th>
<th>HOMO (eV)</th>
<th>LUMO (eV)</th>
<th>ΔE (eV)</th>
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</thead>
<tbody>
<tr>
<td>dye 7</td>
<td>688.84</td>
<td>678.18</td>
<td>25.12</td>
<td>12.87</td>
<td>-8.45</td>
<td>-6.24</td>
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<tr>
<td>dye 8</td>
<td>731.22</td>
<td>715.12</td>
<td>28.85</td>
<td>6.22</td>
<td>-8.55</td>
<td>-6.35</td>
<td>-2.20</td>
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<tr>
<td>dye 9</td>
<td>772.77</td>
<td>750.29</td>
<td>127.36</td>
<td>20.17</td>
<td>-4.51</td>
<td>-2.47</td>
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<tr>
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<td>726.01</td>
<td>59.70</td>
<td>27.34</td>
<td>-8.03</td>
<td>-6.00</td>
<td>-2.03</td>
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<tr>
<td>dye 17</td>
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<td>569.62</td>
<td>25.77</td>
<td>6.92</td>
<td>-4.41</td>
<td>-2.19</td>
<td>-2.22</td>
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<tr>
<td>dye 18</td>
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<td>701.18</td>
<td>28.83</td>
<td>1.17</td>
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<tr>
<td>dye 19</td>
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<td>737.35</td>
<td>25.84</td>
<td>2.60</td>
<td>-4.66</td>
<td>-2.97</td>
<td>-1.69</td>
</tr>
</tbody>
</table>

LUMO electron further contributing to a electronic potential differential across the molecule. The various polar orientation of solvent molecules surrounding the dye is decided by dipole of the dye
and thus having a large dipole will have more solvent interactions with it’s environment when

The HOMO and LUMO maps for each molecule show a consistent trend for all of the synthesized derivatives in their geometries. The different bonding and anti-bonding portions that combine to form the entire HOMO or LUMO are represented in red and blue in Figures 27 and 28 and show that the ground state and the excited state of the molecule exist primarily within the same geometric region for each compound. One of the specific changes is the movement of the molecular orbital around the central squaraine ring’s electron negative oxygen atoms away from the center of the molecule as the orbitals are redirected back through the polymethine chain

Figure 27: Molecular orbitals and electrostatic potential of dye 7 as calculated and modeled by Spartan18 excited.

The HOMO and LUMO maps for each molecule show a consistent trend for all of the synthesized derivatives in their geometries. The different bonding and anti-bonding portions that combine to form the entire HOMO or LUMO are represented in red and blue in Figures 27 and 28 and show that the ground state and the excited state of the molecule exist primarily within the same geometric region for each compound. One of the specific changes is the movement of the molecular orbital around the central squaraine ring’s electron negative oxygen atoms away from the center of the molecule as the orbitals are redirected back through the polymethine chain
towards the indolinium nitrogen atoms. The consistency in modeling between all of the compounds

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Figure 28: HOMO, LUMO, and electron density of dyes 8, 9, 13, 17-19
is indicative that the system’s molecular orbitals are accurately representative by the calculations presented.

The polar surface area (PSA) of each compound is a topological surface that represents the summation of all of the polar atoms (N, O, S) across the molecule and is used in medicinal chemistry as an index relatable to cellular permeability. This is color coded in Figure 28 as showing the more electron rich and thus more polar regions of the molecule as red and with the more electron poor regions as blue. The synthesized compounds show high polar dispersity between the base compounds, 7 and 18, and the electron rich functional groups in compounds 8, 9, 13, and 19. With molecules designed to penetrate the blood-brain-barrier (BBB) and disperse through the nervous system, this value generally needs to be lower than 90 Å² to ensure proper penetration. The average PSA of the dyes 7, 8, 17-19 was 26.89 Å² with dyes 9 and 13 being exceptions based on the large contribution to polar surface area from their sulfonate and dioxymethyl group. The generated surfaces for electron density in Figures 27 and 28 show that the majority of density for the dyes lies along the ketone oxygens on the squaraine ring, followed by contributions from functional groups on the indole groups.

The interactions that the prepared compounds have with their solvent environment are heavily influenced by their dipole moment across the molecule. The dipole moment (measured in debye’s or electron angstroms/eÅ) can change in response to its orientation’s response to electromagnetic radiation. This also related to the way that solvents interact with the molecule by modeling the general orientation of electron donor and acceptor groups within a solvent molecule. The higher the dipole moment of a compound, the more force it can exude on polar solvent molecules in its immediate environment. The molecules with the largest dipole moments are dyes 9 and 13, which also have the largest electron density and polar surface area. The dyes with the
lowest dipole moments, 17-19 are hydrophobic dyes that should have fewer solvent interactions than their hydrophilic counter parts with an on average 79% lower dipole moment.

The average energy gap of each molecule was -2.09 eV which energetically equates to a photon with a wavelength of 594 nm. This 46 nm lower than the observed average absorbance wavelengths for each dyes. This extra lowering of -0.15 eV is due to the environmental solvatic effects that often act by encouraging the stability of the molecular orbitals and allowing for easier transitions. This energy gaps of the functional dyes are heavily influenced by the electronically rich groups and have lower band gap energies compared to the non-substituted 7, with 8, 9, and 13 having lowered the energies by 0.01, 0.17, and 0.18 eV respectively. This was again seen in the difference between dye 18 and its brominated variation 19 with a 24% decrease in the calculated band gap of the dye. This is due to stabilization of the energetic excitation system which we interpret to be due to the internal salt behavior of the trimethyl-ammonium dyes present on the molecule.

These calculated parameters show that the water-soluble compounds 7-9, and 13 have the most possibility for solvent interactions and would have their energies most affected by polarity. This differences in the calculated energy values and the experimental spectral energy profiles come from two sperate interactions. The first is the way that solvent molecules interact with the molecule to drain energy from the system in a negative effect. The second is the ways that solvent molecules can stabilize the excited state of the fluorophore and encourage emission. These two factors together provide an interplay that lends more to the full picture of the energetics of the molecules.

2.4.5 Solvatochromism

The degree of solubility of the lead dyes in aqueous solution led towards the study of solvatic effects on the absorption and emission of each squaraine dye. Each dye was prepared in a
20 µM solution of the dye for maximum absorbance, then diluted 20x for recording emission. The same concentration of dye gives different spectral intensities as well as different absorbance and emission values. The solvents were chosen based on the most commonly used organic polar solvents as well as water and range from 10.2 to 2.2 with their polarity index. This also includes a series of five alcohols with increasing carbons lengths starting with methanol for more incremental modulation of solvent polarity.

These dyes experienced a significant solvatochromic response with high recorded Stokes’ shifts. This increase in the Stokes’ shift means that a lower energy particle of light is being released when dissolved in pentanol as when compared to ethanol. The leftover energy absorbed by the chromophore that is not radiated and is of a much higher energy level than in lower polarity solvents. For dye 17 this translates to a band gap lowered by 0.06 eV when transitioning the dye from ethanol to pentanol due to dye 17 experiencing the largest range in Stokes’s shift with 37 nm in pentanol compared to 16 nm in ethanol. This change in band gap energy is directly related to decrease in adverse solvent interactions between polar solvents and the polar excited state and

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| Polarity | 10.2 | 7.2 | 6.4 | 6.8 |

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| Polarity | 6.6 | 5.2 | 4.3 | 3.9 | 2.2 |

Table 4: Solvatochromic properties of dyes 7-9, 13, 17-19 at 20 µM in various organic solvents.
thusly a more stabilized excited state and lower energy level. Of these dyes, all registered an absorption and emission peaks in the spectrometer, dimethyl formamide and acetonitrile deteriorated the polystyrene cuvettes used to collect spectroscopic data and thus their spectra is
not included in the body of the text.

Figure 29: Solvatochromic effects on absorbance and emission of squaraine derivatives 7, 8, & 13
Figure 30: Solvatochromic effects on absorbance and emission of hydrophobic squaraine derivatives 17 and 19
All of the synthesized derivatives shared similar changes in their absorbance and emission profiles, compounds 9 and 18 showed the clearest spectral separations and thus their profiles will be discussed in more depth.

![Absorbance and Emission Profiles](image)

Figure 31: Solvatochromic effects on zwitterionic dye 9

For dye 9 the most red-shifted absorbance was with DMSO at 652 nm. The Stokes’ shift for dye 9 in DMSO was also relatively low, indicating that the interactions between DMSO and dye 9 were favorable enough to lower the overall energy of the fluorophore and not interfere adversely with the excited state as to non-radiatively dispel energy from the fluorophore. There was, however, a stark drop in absorbance when compared to water as well as in increase in the Stokes’ shift. This is due to water forming a much tighter solvation shell around the highly charged dye with a 40% higher polarity than DMSO. Pentanol and butanol provided the greatest absorption and emission intensities as they have the most limited solvent-dye interaction among the
hydrophilic set of dyes. This behavior was conserved across the other hydrophilic dyes 7, 8, and 13 as seen in the Figures 29 and 30.

![Absorption and Emission of Dye 18 in Various Polar Solvents](image)

**Figure 32: Solvatochromic effects on hydrophobic dye 18**

Dye 18 has with large propylphenyl paddles pendant to the indole nitrogen atoms that encourage hydrophobicity. When put to the same solvatochromic comparison as the hydrophilic dyes, dye 18 performs similarly. Its most red-shifted absorbance occurs in water at 658 nm but the compound has an almost negligible emission at 668 nm due to the poor solubility favoring dye aggregation which quenches fluorescence. As polarity decreases, absorption increases along with a general trend shifting the absorption and emission towards the NIR region. The lowest Stokes’ shift occurs in butanol at with a just 0.02 eV, less than 10%, of the absorbed energy being released non radiatively. Similar effects were seen with dyes 17 and 19 based on their shared aliphatic character as documented in the attached appendices.

### 2.5 Conclusion

A set of seven squaraine derived polymethine dyes were synthesized with red-shifted absorbance and fluorescence wavelengths. All of the derivatives were produced in high yields and purity using a conserved synthesis scheme. The compounds all absorbed and fluoresced in the far-
red end of the visible spectrum with similar molar absorptivity and quantum yields. Each of the synthesized derivatives showed improved photo- and dark-stability when compared to ICG. The hydrophilic and hydrophobic derivatives all exhibited positive solvent interactions with high polarity solvents and were able to bathochromically shift in response to different solvent environments as well as have reductions and enhancements in their spectral intensities. These electronic properties as well as their solvent-spectral relationship were further explored with computational work and modeling of the polarity and molecular orbitals of the derivatives. Future work with these compounds would involve measuring the fluorescence lifetime of each compound in order to further gain insight into their excited states. Additionally, these compounds provide great opportunities for testing their bioimaging capabilities to continue pursuing their similarities to ZW-800.

2.6 Experimental

Seven squaraine derivatives were synthesized using reagents that are commercially available from Fisher Scientific, Matrix Scientific, and Sigma Aldrich. All precursors were used without advanced purification. All solvents used were HPLC grade from Sigma Aldrich. The $^1$H NM and $^{13}$C NMR spectra were recorded on a Bruker Advance (400 MHz) spectrometer with DMSO-$d_6$, D$_2$O, or CDCl$_3$ containing tetramethylsilane (TMS) as an internal calibration standard. The different reagents for each reaction are discussed further below with primarily Indocyanine green (ICG) being purchased (Sigma-Aldrich, MO, USA) for its use as a reference standard for molar absorptivity, quantum yield, and stability studies. Hydrochloric acid (37% w/w, Fisher, Pittsburg, PA), sodium carbonate, sodium chloride, DMSO (99% HPLC grade, Matrix Scientific, Elgin, SC), dimethylformamide (99% HPLC), acetonitrile (99% HPLC), methanol (99% HPLC),
ethanol (97%), isopropanol (97%), butanol (99% HPLC), and pentanol (99% HPLC) were all obtained commercially for use in spectroscopic assays.

**General synthesis of squaraine dyes 7-9, 13, 17-19.**

A solution of squaric acid (2 mmol) and a hydrophilic indolinium salt (4.01 mmol) were added to a two neck round bottom flask equipped with a magnetic stir bar. To this a 30 mL solution of equal parts by volume toluene and butanol was added. The flask was then equipped with a Dean-stark apparatus and condenser in the primary neck and a chemically resistant rubber septum on the secondary neck. The round bottom flask was set in a heated oil bath and brought to 120 °C. Samples of the reaction mix were taken every 15 min to monitored via TLC for the loss of squaric acid and via UV-vis spectroscopy for the formation of a dye peak ~650 nm and the loss of a half-dye peak ~450-550 nm. Once the reaction was completed around an average of 16 hours, the heat was turned off and the still hot round bottom flask was transferred to a rotary evaporator device to remove as much of the high boiling solvent while it was still close to its boiling point. For the hydrophilic dyes, 7-9, and 13 the resulting crude blue reside was then re-dissolved in minimal methanol (1-3 mL) before being recrystallized in ethyl acetate. This would precipitate out a blue powder that was then re-dissolved in methanol and recrystallized again in ethyl ether twice before allowing to dry. The resulting blue solid was then purified over silica column chromatography with a gradient solvent system of methanol and dichloromethane. For the hydrophobic dyes, 17-19, the resulting crude was dissolved in minimal dimethyl sulfoxide (1 mL) aided by heating and sonication. This was then slowly added dropwise into a liter solution of ice water to precipitate a dark purple solid. The water was then decanted off and the resulting purple solid filtered and washed with cold ice water. The crude solid was then recrystallized from hot acetone before being purified over silica chromatography with a gradient solvent system of methanol and ethyl acetate.
(E)-4-((3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-iurn-2-yl)methylene)-2-(((E)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate. (7). Yield 86%, m.p. >300 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) $\sigma$: 1.707 (12H), 2.182 (4H), 3.162 (18H), 3.665 (4H), 4.199 (4H), 5.863 (2H), 7.208 (2H), 7.382 (2H), 7.508 (4H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\sigma$: 14.63, 17.20, 20.49, 21.99, 25.65, 26.52, 48.83, 52.36, 54.31, 61.77, 62.47, 86.26, 110.39, 122.32, 123.55, 123.88, 128.00, 141.33, 141.91, 169.07, 179.60, 180.53.

(E)-4-((5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-iurn-2-yl)methylene)-2-(((E)-5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate bromide. (8). Yield 83%, m.p. >300 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) $\sigma$: 1.7089 (12H), 2.1416 (4H), 3.1073 (18H), 3.5579 (4H), 4.1507 (4H), 5.8596 (2H), 7.4516 (2H), 7.5469 (2H), 7.8103 (2H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\sigma$: 13.57, 13.61, 23.85, 25.69, 26.50, 27.30, 44.06, 46.20, 47.55, 49.07, 52.40, 57.40, 57.67, 62.42, 86.83, 112.44, 116.16, 125.57, 130.73, 141.30, 143.79, 168.81, 180.57, 194.70.

2-((E)-(3-(((E)-3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-2-oxido-4-oxocyclobut-2-en-1-ylidene)methyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate. (9). Yield 87%, m.p. 249-252 °C; $^1$H NMR (400 MHz, D$_2$O) $\sigma$: 1.5084 (12H), 2.3210 (4H), 3.0526 (9H), 3.5227 (4H), 4.4360 (4H), 7.8697 (6H); $^{13}$C NMR (100 MHz, D$_2$O) $\sigma$: 21.41, 22.20, 44.76, 48.94, 53.17, 54.99, 62.39, 115.47, 121.01, 127.12, 141.80, 142.15, 147.42, 195.84, 200.00.

(E)-2-((E)-(7,7-dimethyl-5-(3-(trimethylammonio)propyl)-5,7-dihydro-6H-[1,3]dioxolo[4,5-f]indol-6-ylidene)methyl)-4-((7,7-dimethyl-5-(3-(trimethylammonio)propyl)-7H-[1,3]dioxolo[4,5-f]indol-5-ium-6-yl)methylene)-3-oxocyclobut-1-en-1-olate bromide. (13).
Yield 83%, m.p. 251-255 °C; $^1$H NMR (400 MHz, D$_2$O/DMSO-d$_6$) $\delta$: 1.4818 (12H), 2.3064 (4H), 3.0961 (18H), 3.5712 (4H), 4.3918 (4H), 5.9957 (2H) $^1$C NMR (100 MHz, D$_2$O/DMSO-d$_6$) $\delta$: 21.46, 22.25, 44.63, 48.95, 53.21, 54.54, 62.32, 97.23, 103.15, 104.12, 134.60, 137.17, 148.58, 149.57, 196.34.

$(E)$-4-((1-butyl-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-2-(((E)-1-butyl-3,3-dimethylindolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate. (17). Yield 92%, m.p. 258-259 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 1.0102 (s, 12H), 1.8034 (m, 18H), 5.9884 (2H), 7.0057 (2H), 7.1665 (2H), 7.3313 (4H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$: 1.11, 13.95, 20.46, 27.14, 29.19, 43.60, 49.35, 86.66, 109.45, 122.36, 123.74, 127.82, 142.31, 142.57, 170.17, 179.29, 182.54.

$(E)$-4-((3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-ium-2-yl)methylene)-2-(((E)-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate. (18). Yield 86%, m.p. 198-199 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 0.955 (qi, 4H), 1.630 (12H), 2.258 (4H), 2.825 (4H), 4.525 (2H), 7.276 (10H) 7.487 (8H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$: 1.07, 14.22, 22.81, 24.50, 29.19, 24.50, 47.77 54.69, 115.15, 123.29, 126.76, 128.40, 128.57, 128.73, 128.87, 129.49, 130.12, 139.57, 140.86, 141.66, 196.12.

$(E)$-4-((5-bromo-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-ium-2-yl)methylene)-2-(((E)-5-bromo-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate. (19). Yield 82%, m.p. 195-197 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 0.9633 (4H), 1.0390 (12H), 1.9705 (4H), 4.5938 (4H), 5.9437 (2H), 7.2931 (8H), 7.6176 (10H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$: 13.90, 13.96, 14.04, 16.83, 20.42, 20.48, 23.50, 27.08, 29.13, 30.28, 43.85, 49.47, 50.33, 55.03, 87.13, 110.47, 117.18, 122.95, 124.26, 127.95, 129.36, 130.35, 137.26, 139.28, 141.09, 143.22, 169.92, 195.57.
3 SYNTHESIS OF CARBOCYANINE DYE DERIVATIVES CONTAINING PYRIDINIUM MOIETY AT THE MESO-POSITION

3.1 Introduction and Rationale

The carbocyanine dye scaffold was originally discovered in the United Kingdom in 1856 by Williams et al after the reaction of a solution of quinoline and 4-methyl-quinoline with pentyl iodide and ammonia, generating a brilliant blue solid. This compound was the first of a new dye scaffold which generally consists of an electron donor and an electron acceptor nitrogen pair separated by a polymethine carbon chain. These cyanine structures can be simple and have open amine groups such as in streptocyanine subfamily. Cyanines can begin to get more complicated with the introduction of nitrogen bearing heterocyclic rings as the electron donors and acceptors such as in the asymmetrical hemicyanine and the symmetrical standard cyanine as shown in Figure 33.

![Cyanine dye classifications](image)

Figure 33: Cyanine dye classifications

Much of the classification of cyanine dyes is based on the odd-numbered length of polymethine chain separating the nitrogen atoms (mono-, tri-, penta-, hepta-) and the choice of heterocycles on the ends of the chain. The longer the polymethine chain, the longer the absorption and emission of the dyes. This chain length along with the choice of heterocycle traditionally decides the nomenclature of the dye. This heterocycle has a significant effect on the optical profiles of the dyes when compared to standard streptocyanines with electron rich scaffolds such as pyridinium, 4-pyrylum, 4-thiopyrylum, indolinium, benzothiazolium, and benz[c,d]indolinium
each contributing to red-shifts in the absorption and emission spectra.\textsuperscript{47, 66, 137-140} These structures are presented below in Figure 34.

![Figure 34: Commonly used heterocycles for cyanine condensation](image)

The benzothiazolium salt is particularly of interest due to its planarity and orbital hybridization around the sulfur atom contributing to red-shifting compounds towards to the NIR range. Compounds using this heterocycle were first synthesized in later 18\textsuperscript{th} century via the condensation of 2-methylbenzothiazole and \textit{N}-pentyl-benzothiazolium iodide in a solution of ammonia.\textsuperscript{141} Benzothiazole derived dyes have been used in various biological imaging due to their planarity promoting effectiveness in binding to DNA complexes.\textsuperscript{142} These compounds have also been used to bypass the blood-brain barrier and image amyloid plaques in Alzheimer’s disease models.\textsuperscript{143}

One of the sensing applications of fluorescent dyes is pH responsiveness, known as a change in the spectroscopic qualities of a dye in solution in response to the concentration of free protons.\textsuperscript{103, 144-146} The benzothiazole heterocycle has previously been deployed in pH sensitive azo dyes in a scaffold composed of two benzothiazole heterocycles bridged by a nitrogen-nitrogen double bond.\textsuperscript{147} Another synthesized example is composed of an amine substituted benzothiazole heterocycle moiety bonded to an aniline moiety via an azo nitrogen double bond.\textsuperscript{148} Uncharged asymmetrical dyes have also been prepared from benzothiazole and quinoline heterocycles bonded into a monomethine dye.\textsuperscript{149} These compounds do not exhibit fluorescence at pH 10, but at high enough proton concentrations are induced into a push-pull system from a protonated nitrogen to a
non-protonated nitrogen. This induces a 3.5x increase in absorbance as the band-gap of the fluorophore is lowered enough to absorb visible light ~500 nm. The merocyanine class of dyes, a poly-substituted cyanine derivative with more than two push-pull nitrogen atoms, has also been synthesized with the benzothiazole heterocycle as well as with a phenolic moiety pendant to the polymethine chain that enables a pH response. Unlike the uncharged monomethine dye, which engages in increased absorbance and fluorescence in low pH, this benzothiazole derived merocyanine experiences a decrease in absorbance and fluorescence as well as a blue shift in absorbance and fluorescence.\(^{150}\) In the class of traditionally symmetrical benzothiazole dyes, pH responsiveness has been reported in tri- and pentamethine dyes with carboxylic acid groups pendant to each nitrogen.\(^{151}\) In low pH environments this protonates and results in a decrease in absorbance and fluorescence signal but no bathochromic shift due to the protonation mechanism not directly involving the aromatic system of the fluorophore. This type of pH responsive compounds have also been seen in traditional symmetrical cyanines derived from the heterocycle indolium as well.\(^{152}\)

Dyes designed to measure pH usually have a functional group that is pH responsive as seen in the amine, phenol, and carboxylic acid groups shown above. A group of uncharged dyes were developed to become changed in response to low pH, possess a sulfonate groups pendant to the heterocycles, and the additional feature of a substitution of a hydrogen in the meso position of the polymethine chain with a bromine or chlorine atom.\(^{153}\) This meso-functionality is not related to the dye’s pH response, but the meso-position has been valuable in other research ventures that have utilized pH responsive substitutions on the meso-position of the polymethine chain. This is beneficial because it can allow for the direct conjugation of the pH responsive moiety into the existing fluorophore system. The functionalization of a heptamethine cyanine with a pH responsive
pyrazole unit has been reported to blue-shift with decreasing pH as well as decrease fluorescence and absorbance intensity.\textsuperscript{154} This meso substitution is able to be accomplished by choosing a polymethine chain precursor that can maintain its functionality through a Vilsmeir formulation to form the electron rich polymethine reagent \textsuperscript{155} Addition functionalization at the meso-carbon of the polymethine chain has allowed for the attachment of targeting ligands, the binding of the dye to biological substrates, and functionalization with anti-bacterial properties.\textsuperscript{156}

The basis for this project was the development of a chemosensor that would be able to respond to low pH, give a high molecular brightness, and work within the far-right end of the electromagnetic spectrum. Research into meso-substitution with pyridyl and nitrobenzene groups in benzothiazole has been performed by Král et al with a small family of symmetrical dyes with meso-substituted dyes.\textsuperscript{157} The extend of this paper though did not address the capability for these meso-functionalized compounds to operate as pH probes. Therefore, a class of benzothiazole carbocyanine derived dyes with a meso-pyridyl group were designed, synthesized, and their optical properties were measured. This includes the pH dependent absorbance and fluorescence spectrum as well as computational modeling of the molecule’s electron orbital and electrostatic forces. The

![Protonation mechanism and proposed alternate resonance structures of meso-pyridine pentamethine derivative](image)

Figure 35: Protonation mechanism and proposed alternate resonance structures of meso-pyridine pentamethine derivative 25
primary hypothesis behind the scope of this work was that the pyridine moiety nitrogen would be a pH responsive functional group that also would be directly conjugated into the fluorophore of the molecule and would change the molecular orbital distribution and absorption band gap of the dye in response to the nitrogen lone pair binding with a free proton as shown in Figure 35.

This hypothesis included that the protonation of pyridyl nitrogen will cause the molecular orbitals of the dye to extend up into the pyridyl group and congregate around the newly acquired proton. In Figure 35, the proposed resonant pathways of the first prepared dye, 25, are shown. The initial push-pull system of resonance Path A is shown originating on the benzothiazole system’s free nitrogen lone pair, extends from the heterocycle moiety on the right, pushes across the polymethine chain, and terminates on the cationic nitrogen of the opposite benzothiazole. For the protonated version, Path B, the origin of the “push” in the push-pull resonance is still the benzothiazole nitrogen’s free lone pair, then pushes into the polymethine chain and up into the pyridyl group before depositing as a lone pair on the newly protonated pyridyl group nitrogen.

Equation 3:

\[ E = \frac{n^2\pi^2\hbar^2}{2ml^2} \]

The energetics of this fluorophore can be modeled in the simple linear model of an electron, the particle in a box model (PIB) shown in Equation 3. In this model the linear bond length that the electron travels is L, the mass of the electron is m, Planck’s constant is h, the number of particles is n, and the resulting energy of that particle system is E. From this simple conjugated orbital representation, the only variable that is not constant is the path length that the electron takes in both the HOMO and LUMO orbitals. Since the length is inversely related to the system’s energy, the shorter path length that HOMO and LUMO electrons resonate across in a fluorophore, the higher the energy required to excite that fluorophore. Due to the Plank relationship, higher energies
have shorter wavelengths. In this way, the PIB model can be applied to simply describe the energetics of simple conjugated systems.\textsuperscript{158} The protonated versions of the compounds discussed show a lower absorbance value and thus must be at a higher energy than the non-protonated forms. The protonated pathway should have a smaller molecular orbital extended across it, than the non-protonated pathway and this new molecular orbital extended across this pyridine group should be of a higher energetic level than the non-protonated form of the dye. This is perceptible as a blue shift in the absorbance spectrum.

3.2 Synthesis of pentamethine carbocyanine dyes containing pyridine moiety at the meso carbon.

As presented in Scheme 4, the synthetic procedure for dyes 25-28 is composed of three separate steps. The first step involves the alkylation of the benzothiazole nitrogen using an alkyl iodide. For compounds 20 and 21 this is methyl iodide, for compound 22 this is ethyl iodide, and for compound 23 this is iodopropylbenzene. This reaction is performed in a solution of acetonitrile with a slight excess of the alkylating agent to ensure the reaction is pushed to full consumption of the heterocycle. Separately, 3-methyl-pyridine was treated to a Vilsmeir formulation to create the appropriate substituted pentamethine bridge. This reaction consists of electronically priming

\[ \text{Scheme 4: Synthesis of meso-pyridyl pentacyanine dyes 25-28} \]
dimethylformamide with the oxidizer phosphorus oxychloride to promote a nucleophilic attack from a deprotonated 3-methyl-pyridine. This generates the dialdehyde intermediate, 24, which is isolated by precipitating the product in ice water and filtering. This linker, 24, was then used in its crude form to yield the cyanine dye 25-28.

The condensation of dyes 25-28 required a high boiling solvent to achieve high enough temperature levels for the reaction to progress. Therefore, the reaction was carried out in butanol at 110 °C for 10-20 h. Triethylamine was used as a base to deprotonate the salts 20-23 which were then allowed to react in nucleophilic attack into the linker, 24, which is then deprotonated the meso position of the chain to facilitate full aromaticity. This reaction was performed with 2:1 equivalents of the desired salt compared to the linker with a slight excess of salt to ensure full reaction progression. This reaction was monitored by thin-layer chromatography (TLC) to ensure the consumption of linker 24 as well as UV-Vis spectroscopy to confirm that the half-condensed dye is pushed to completed dyes which absorbs near 640 nm. Finally, the compounds were worked up and dried under reduced pressure. Each compound was isolated with good yield > 92%.

3.3 Experimental

3.3.1 Chemical and methods

The four meso-pyridine pentamethine dye derivatives were synthesized using reagents that are commercially available from Fisher Scientific, Matrix Scientific, and Sigma Aldrich. All precursors were used without advanced purification. All solvents used were HPLC grade from Sigma Aldrich. The $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker (300 MHz) spectrometer with CDCl$_3$ containing tetramethylsilane (TMS) as an internal calibration standard. The different reagents for each reaction are discussed further below with primarily Indocyanine green (ICG) being purchased (Sigma-Aldrich, MO, USA) for its use as a reference standard for
molar absorptivity, quantum yield, and stability studies. Hydrochloric acid (37% w/w, Fisher, Pittsburg, PA), sodium carbonate, sodium chloride, DMSO (99% HPLC grade, Matrix Scientific, Elgin, SC), dimethylformamide (99% HPLC), acetonitrile (99% HPLC), methanol (99% HPLC), ethanol (97%), isopropanol (97%), butanol (99% HPLC), and pentanol (99% HPLC) were all obtained commercially for use in spectroscopic assays.

**General Synthesis of Benzothiazolium Salts 20-23**

The benzothiazole precursor used was 1,5-dimethylbenzothiazole for 20, 5-fluoro-1-methylbenzothiazole for 21, and 1-methylbenzothiazole for 22 and 23. 4 mmol of each benzothiazole derivative were individually added to a round bottom flask with 25 mL of DMF and 4.1 mmol of an alkylating agent. For compounds 20 and 21 this was methyl iodide, for compound 22 this was ethyl iodide, and for compound 24 this was (3-bromopropyl)benzene. The resulting solution was allowed to react at 60 °C for 24 h as the resulting benzothiazolium salt precipitated out of solution. The formed salts were then washed with cold diethyl ether before being dissolved in minimal methanol and recrystallized from diethyl ether.

**General Synthesis of Vilsmeir Linker 24**

10 mmol of dimethylformamide was added to a cold 3-neck round bottom flask equipped with rubber septa on the vestigial arms and a condenser column on the center. This was cooled to 0 °C in an ice bath then 10 mmol of phosphorous oxychloride was added dropwise over 15 min. The resulting Vilsmeir product was known to be formed when the solution turned a pale yellow. This was slowly allowed to reach room temperature while 5 mmol of 3-methylpyridine was added dropwise over 30 min. The solution was then heated to reflux for 4 hours to ensure the full reaction of all the 3-methylpyridine before being cooled back to room temperature. The crude slurry was then poured over ice water to precipitate the final product 24. This was filtered and washed with
cold ice water before being dried under reduced pressure. 24 was then used for dye condensation without further purification as based on previous procedures.159

**General Synthesis of Dye Derivatives 25-28**

A mixture of benzothiazolium salt (4 mmol) and linker (2 mmol) 24 were added to a 50 mL solution of butanol in a two-neck round-bottom flask equipped with a magnetic stir bar, a condenser running vertically, and a rubber septum in the last neck. To this, triethylamine (4.5 mmol) was added dropwise as the solution was brought to 110 °C over an oil bath. As the reaction progressed a dark blue color was formed in situ. A drop of the reaction mix was then removed via syringe and analyzed with TLC for the conversion of linker 24, and with UV-Vis spectroscopy for the increase of a dye peak (~640 nm) and the decrease of a half dye peak (~450-550 nm). Upon reaction completion, the round bottom flask was removed from the heat and quickly transferred to a rotary evaporation unit to remove the high boiling solvent under vacuum while it was still hot. The resulting crude residue was then dissolved in a minimal amount of methanol (1-3 mL) and precipitated from ethyl acetate or diethyl ether. This recrystallization was performed thrice before the resulting blue solid was purified over silica chromatography with a gradient solution of methanol and dichloromethane 1-10% (v/v).

**2-((1E,3Z,5Z)-5-(3,5-dimethylbenzo[d]thiazol-2(3H)-ylidene)-3-(pyridin-4-yl)penta-1,3-dien-1-yl)-3,5-dimethylbenzo[d]thiazol-3-ium iodide (25).** Yield 92%, m.p. 249-251 °C; MS-ESI+ m/z calc: 468.7 exp: (M)+ 468.2; 1H NMR (300 MHz, DMSO-d6) δ: 3.87 (s, 6H), 4.37 (s, 6H), 6.20 (d, 2H, J = 5.3 Hz), 7.31 (d, 2H, J = 14.1 Hz), 7.64 (s, 2H, J = 6.0 Hz), 7.95 (d, 4H, J = 6.0 Hz), 8.00 (d, 2H, J = 14.1 Hz), 8.93 (d, 2H, J = 5.3 Hz).

**5-fluoro-2-((1E,3Z,5Z)-5-(5-fluoro-3-methylbenzo[d]thiazol-2(3H)-ylidene)-3-(pyridin-4-yl)penta-1,3-dien-1-yl)-3-methylbenzo[d]thiazol-3-ium iodide.** (26). Yield 89%, m.p. 225-229
82

°C; MS-ESI+ m/z calc: 468.66 exp: (M)+ 468.3; \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\): 1.25 (t, 6H, \(J = 6.5\) Hz), 1.46 (qa, 4H, \(J = 6.5\) Hz), 4.38 (d, 2H, \(J = 6.5\) Hz), 6.25 (d, 2H, \(J = 7.1\) Hz), 7.48 (m, 2H, \(J = 14.2\) Hz), 7.62 (m, 2H, \(J = 5.8\) Hz), 7.81 (m, 4H, \(J = 7.1\) Hz). 8.11, (d, 2H, \(J = 14.2\) Hz), 8.94 (d, 2H, \(J = 5.8\) Hz).

\((E)-4-(5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yldiene)-2-(((E)-5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate bromide. \(27\)). Yield 93%, m.p. 223-226 °C; MS-ESI+ m/z calc: 476.6. exp: (M)+ 476.1; \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\): 1.91 (s, 6H), 6.09 (d, 2H, \(J = 7.4\) Hz), 7.36 (t, 2H, \(J = 14.2\) Hz), 7.76 (d, 2H, \(J = 5.9\) Hz), 8.03, (d, 4H, \(J = 7.4\) Hz), 8.11, (d, 2H, \(J = 14.2\) Hz), 8.88 (d, 2H, \(J = 5.9\) Hz).

3-(3-phenylpropyl)-2-((1\(E,3Z,5Z\))-5-(3-(3-phenylpropyl)benzo[\(d\]thiazol-2(3\(H\))-ylidene)-3-(pyridin-4-yl)penta-1,3-dien-1-yl)benzo[\(d\]thiazol-3-ium iodide. \(28\)). Yield 90%, m.p. 216-218 °C; MS-ESI+, m/z calc: 648.9 exp: (M+H)+ 324.7; \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\): 1.91 (qa, 4H, \(J = 7.2\) Hz), 2.66 (t, 4H, \(J = 7.2\) Hz), 4.32 (t, 4H, \(J = 7.2\) Hz), 7.15 (m, 10H, \(J = 8.1\) Hz), 7.25 (d, 2H, \(J = 8.1\) Hz), 7.41 (m, 4H, \(J = 14.2\) Hz), 7.62 (m, 4H, \(J = 5.8\) Hz), 7.71 (d, 2H, \(J = 8.1\) Hz), 8.09 (d, 2H, \(J = 14.1\) Hz), 8.93 (d, 2H, \(J = 5.8\) Hz).

3.4 Optical Properties

3.4.1 Molar Absorptivity

Molar absorptivity was recorded for each synthesized dye using a Varian Spectrophotometer (Varian Inc. Palo Alto, Ca.). This was paired to Cary WinUV Scan Application v3.00 on a PC to visualize and export data. Each sample was recorded at increasing molar concentrations (\(\mu\)M) in ethanol using standard polystyrene cuvettes with a path length of 1 cm. Each sample was prepared from a stock solution of the dye dissolved in dimethyl sulfoxide
(DMSO) and measured on an analytical balance to approximate a 1.0 mM solution of the dye. The stock solutions were chosen to be prepared from DMSO due to the solvent’s high solubility for both polar and polyaromatic substances and for the solvent’s ability to dissolve into ethanol effectively. Before recording their spectra, each sample was sonicated for 30 min then vortexed for 1 min to ensure complete dissolution and dispersant.

3.4.2 *Fluorescence Data*

The fluorescence emission for each synthesized dye was recorded with a Shimadzu RF-5301 Spectrofluorophotometer (Shimadzu Corporation Analytical Instruments Division, Duisburg, F.R. Germany). This was analyzed through a PC operating the RF-5301 acquisition software to visualize and record data. The experimental parameters for each fluorophore emission spectral acquisition was as follows: slit width of excitation set to 5 nm, slit width of emission set to 5 nm, sensitivity set to high, gain set to medium, wavelength speed set to medium, a light source consistent with a 150 W Xenon lamp bulb, and an excitation wavelength set to 10 nm blue shifted from the absorbance of each respective dye sample. Four-faced, polystyrene fluorescence cuvettes with pathlengths of 1 cm were used to each sample. Each sample was immediately taken from having an absorbance profile recorded and diluted 20x to ensure an absorption value of < 0.1 A.U. This is to minimize any inner filter effects from the concentration being too high. The cuvettes were then sonicated for 30 min and vortexed for an additional minute to ensure full dissolution and dispersion. The data was analyzed, visualized and all corresponding calculations were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA.)

3.4.3 *pH Data*

All meso-pyridyl compounds were tested for their absorbance and emission response to pH. Each sample was prepared in a polystyrene fluorescence cuvette with a path length of 1 cm.
These were then adjusted to a specific pH by combining a stock solution of 1.0 M HCl in ethanol or 1M Na₂CO₃, pure ethanol, and the requisite volume of stock solution of each dye to reach a total of 2.500 mL and 20 µM concentration of the dye. Each sample was sonicated for 30 min then vortexed for 1 min before spectral acquisition. pH resolved absorbance profiles were recorded in a Varian Spectrophotometer. This was linked to Cary WinUV Scan Application v3.00 on a PC to visualize and export data. pH resolved emission profiles for each dye was recorded in a Shimadzu RF-5301 Spectrofluorophotometer. This was measured through a PC operating the RF-5301 acquisition software to visualize and record data. The exported data was visualized and used to calculated response parameters using Microsoft Excel.

3.4.4 Computational Methods

HOMO and LUMO orbital geometries and energies as well as electrostatic potential surfaces were calculated using a Hartree-Fock Density Functional Theory (HF-DFT) self-consistent fields (SCF) algorithms. These calculations were performed using a Geometric Direct Minimization and a 6-31G* basis set and B3LYP methodology via Spartan18. Additionally, QSAR parameters such as polar surface area (PSA) and LogP were also calculated using the same software. The data acquired from this software was then further analyzed using Microsoft Excel.

3.5 Results and Discussion

3.5.1 ¹H NMR Characterization of Compound 25

Compound 25 was chosen as a representative dye for full ¹H NMR characterization and analysis. The ¹H NMR spectra of 25 was recorded in DMSO-d₆ and shown in Figure 36 below. For ease of analysis, the different ¹H NMR active proton signals are distinguished by color based on their structural position and electronic shielding. This dye possesses a plane of symmetry across the central meso-position that causes the shown signals in a spectrum to appear halved. The first
signal, $H_a$, is coded with a blue color and exists in the aliphatic chemical shift region at 3.87 ppm. This corresponds to the methyl group functionalized on the benzothiazole 6-membered ring. The most electron dense aliphatic signal would belong to $H_b$ and be pendant to the benzothiazole nitrogen.

The two next most shifted signal correspond to the protons $H_c$ and $H_d$ that exist on the polymethine chain. These spectrally are the two doublets at 6.20 and 7.31 ppm. This is decided based on their distance from the closet electron donor source. $H_d$ is closer to the nitrogen in the push-pull chain than $H_c$, therefore $H_c$ would have a lower chemical shift than $H_d$. They appear as a doublet due to splitting interactions between the two adjacent hydrogens. The next aromatic signals are from the benzothiazole ring system benzene core. The first signal proton at $H_e$ exists at 7.64 ppm as a singlet due to no adjacent hydrogens. The other two protons on the benzothiazole ring are coupled into a single signal shown as a larger signal at 7.95 ppm. The last two hydrogens in the dye are on the electron dense pyridyl group and appear as a doublet at 8.00 and 8.93 ppm with the most electron shielded signal belonging to the hydrogen alpha to the pyridyl nitrogen, $H_h$.
3.5.2 Optical Properties of Dyes 25-28

The optical properties associated with each of the meso-pyridine pentamethine dye derivatives are presented in Table 5 below. All of the prepared dyes when dissolved in ethanol gave an average absorption wavelength near 650 nm with a range of 5 nm and an average emission wavelength of 669 nm for an average Stokes shift of 19 nm. All of these dyes operate in the far-red range of the visible light spectrum. This causes each derivative to have a brilliant blue color due to the complementary color of the absorbed photon of red being blue. This is on average with other benzothiazole pentamethine dyes who operate in the same wavelength range.\textsuperscript{160}

Table 5: Optical properties of meso-pyridyl pentamethine dyes 25-28

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>Stokes Shift (nm)</th>
<th>$\varepsilon$ (L mol$^{-1}$ cm$^{-1}$)</th>
<th>$\phi_f$ (%)</th>
<th>Molecular Brightness ($\varepsilon * \phi_f$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>650</td>
<td>670</td>
<td>20</td>
<td>154,215</td>
<td>15</td>
<td>2313225</td>
</tr>
<tr>
<td>26</td>
<td>652</td>
<td>670</td>
<td>18</td>
<td>134,910</td>
<td>17</td>
<td>2293470</td>
</tr>
<tr>
<td>27</td>
<td>647</td>
<td>666</td>
<td>19</td>
<td>163,816</td>
<td>13</td>
<td>2129608</td>
</tr>
<tr>
<td>28</td>
<td>651</td>
<td>671</td>
<td>20</td>
<td>156,560</td>
<td>9</td>
<td>1409040</td>
</tr>
</tbody>
</table>
Figure 37 displays the normalized absorption and emission spectra for each compound and visualizes the Stokes’ shift between the photon absorbed and the photon emitted by the fluorophore system. The energy loss between the absorbed and emitted photons is due to non-radiative intermolecular processes which usually consists of bond vibrations and wiggles. Using the Plank relationship, the wavelength of absorption and emission can be converted into energy in electron volts with the average Stokes’ shift of the prepared compounds 25-28 of 0.07 eV. This can also be represented as inverse centimeters at 16,120 cm\(^{-1}\) which would be interpreted on an IR spectrum as the vibrational motion of aromatic C=C and C=N bonds. Compounds 25 and 26 can provide even further insight into the energetics of these non-radiative transitions by comparing the structural differences between the two. Compound 25 has a methyl group functionalized to C5 of the benzothiazole ring and compound 26 has two fluorine atoms in the place of these methyl groups. The fluorine atom in 26 decreases the stokes shift in part due to the decreased total number.
of bonds present in the molecule. The fewer C-H bonds means that there is not as many available bond vibrational modes and therefore less opportunities to radiate off energy.

The molar absorptivity of a compound gives an understanding of the relative number of photons absorbed per molar equivalent of the compound. For compounds 25-28, this is on average 152,375 L mol\(^{-1}\) cm\(^{-1}\) with the highest molar absorptivity in compound 27, at 163,816 L mol\(^{-1}\) cm\(^{-1}\) and the lowest in the fluorinated dye, 26 at 134,910 L mol\(^{-1}\) cm\(^{-1}\). When comparing dyes 25 and 26’s molar absorptivity, the fluorine atom in dye 26 seems to lower the absorptivity of the compound by 7%, indicating that some of the electronic influence of the halogen decreases the fluorophore’s ability to absorb light efficiently. Dyes 27 and 28 have similar but elevated \(\varepsilon\) values that can be attributed to their more hydrophobic pendant arm groups. These dyes each experience increased aggregation which stabilizes and encourages the excitation of the fluorophore.

The extra electron donation from the meso-pyridyl group causes an on average 10 nm red shift in the compounds as well as providing the dyes to operate as a chemosensor as will discussed. Some drawbacks coincide with the addition of the group in regards to certain optical properties of the dye when compared to previously prepared compounds in our group.\(^{46}\) The quantum yields of these dyes were on average 14% and resulted in an average molecular brightness of 20,363 L mol\(^{-1}\) cm\(^{-1}\). This is lower than the typically seen quantum yields of benzothiazoles without a meso-substitution and can be attributed to increased vibrational or rotational modes relaxation modes or unstable excited state molecular geometries due to the added bulky, electron rich, meso-substituent. Dye 26 has a marginally higher quantum yield than its counterpart, 25, that can be attributed to the increased electron density stabilizing the excited state of the fluorophore.
3.5.3 Photo- and Dark-stability

Compounds 25-28 all show markedly improved photo-stability and dark-stability when compared to FDA approve imaging compound ICG in ethanol.\textsuperscript{161} As shown in Figure 38, ICG decomposes by 20% percent after 7 days in the dark while the new compounds 25-28 have an average decomposition of 7%. The propylphenyl substituted dye 28 showed the highest degree of stability with only a 4% loss in absorbance intensity over a week in ethanol solution while dye 25 is comparable to ICG’s performance. The trend of increasing dark-stability is consistent with the increasing hydrophobicity, indicating that the increased dark-stability may be due to formation of hydrophobic aggregates in a polar solvent. Aggregations show less propensity to decomposition as there are less molecules of the dye exposed to the possible reactive solvent solution or any other quenching molecules such as atomic oxygen.

When in ethanol and exposed to constant UV-irradiation, ICG decomposes fully after 24 h. Comparatively, derivatives 25-28 all show increased photostability averaging 18%. The derivatives with the more hydrophobic substituents, 27 and 28, showed the highest photo-stability as well as the highest dark-stability with roughly a 15% and 10% respective reduction in the two dyes absorbance intensity after 24 h of irradiation. These two parameters are indicative of the compounds practical utilization ability. Dark-stability is directly proportional to the compounds’ shelf-life and photo-stability is related to the usable timescales in which the derivatives can operates as fluorophores before becoming too decomposed to be accurate.
3.5.4 pH Response

The capabilities of each dye were tested as a turn-off pH probe by measuring the reduction in signal that protonation induces in absorption and emission between pH 5 and 6. The pKa’s calculated for each dye through a titration study averaged to be 5.32 as shown in Appendix 2. This operating range should therefore give the optimal feedback for incremental pH changes. The highest pKa is present in the fluorinated dye, 26, and the lowest pKa corresponds to the propyl phenyl substituted dye, 28. The higher pKa could be due to the extra contribution of the fluorines’ full electron shells donating into the group. The lower pKa could be primarily due to poorer solvation and thus aggregation of the more hydrophobic dye, 28. The change of absorbance and emission intensity in response to varying pH was linearized for each derivative to yield a response coefficient that is useful in comparing the compounds as pH chemosensors. All of the prepared Table 6: pH responsive properties of dyes 25-28

<table>
<thead>
<tr>
<th>Dye</th>
<th>pKa</th>
<th>ABS Bathochromic Shift</th>
<th>ABS Reduction</th>
<th>ABS Response Coefficient</th>
<th>EMS Bathochromic Shift</th>
<th>EMS Reduction</th>
<th>EMS Response Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>5.27</td>
<td>8 nm</td>
<td>18%</td>
<td>0.1712</td>
<td>7 nm</td>
<td>36%</td>
<td>0.3710</td>
</tr>
<tr>
<td>26</td>
<td>5.49</td>
<td>9 nm</td>
<td>18%</td>
<td>0.1844</td>
<td>8 nm</td>
<td>34%</td>
<td>0.3661</td>
</tr>
<tr>
<td>27</td>
<td>5.32</td>
<td>12 nm</td>
<td>24%</td>
<td>0.2535</td>
<td>9 nm</td>
<td>37%</td>
<td>0.4307</td>
</tr>
<tr>
<td>28</td>
<td>5.19</td>
<td>13 nm</td>
<td>32%</td>
<td>0.4179</td>
<td>5 nm</td>
<td>57%</td>
<td>0.7105</td>
</tr>
</tbody>
</table>
compounds showed a similar trend in pH responses due with consistent linearization and high $R^2$ of the absorbance slope regardless of the functional groups present in the dye. The binding of a proton results in a blue-shift of the absorption spectra of 8 nm and an approximately 20% decrease in the absorbance maxima of compound 25 as shown in Figure 39. This spectral change in response to a chemical reaction (the binding of a free proton) allows the calculation of the pKa of that reaction. The pKa calculated for dye 25 was found to be 5.27 which is only marginally higher than that of protonated pyridinium and similar to other protonated aromatic amines.

The dye’s responsiveness can be calculated by acquiring a higher pH resolved set of spectra, normalizing the data, and linearly plotting the spectra against pH. The lead compound 25 for example has a slope value of this linear plot for absorbance at 0.1712. This is even higher for emission at 0.3710 due to a greater relative decrease. This is most likely due to decreased stability of the excited state due to the redistributed electron density towards the pyridyl group as this group is more exposed to the solvent environment and more likely to dissipate energy non radiatively than if the energy had spatially remained in the polymethine and benzothiazole core.
Figure 39: Spectroscopic pH response of dye 25 in ethanol at 20 μM
Figure 40: Spectroscopic pH response of dye 26 in ethanol at 20 μM
Figure 41: Spectroscopic pH response of dye 27 in ethanol at 20 µM
Figure 42: Spectroscopic pH response of dye 28 in ethanol at 20 uM
The combination of a blue shift in the absorbance and emission of the dyes as well as a reduction in the intensity of the absorbance and emission is due to the fluorophore of the protonated system being at a lower energy level, but having a less stabilized excited transition state. This blue shift behavior appears consistently in all of the dyes as shown in Figures 39-42. A blue shift of as much as 13 nm corresponds to the entirety of the systems band gap lowered by 0.04 eV. The absorbance intensity of the dyes was lowered roughly 25% and is representative of a proportionate amount of dyes in solution that are no longer entering an exited state compared to a neutral pH. The loss of nearly 60% of the fluorescence intensity of the dye at low pH is also due to a fraction of the dye not entering an exited state, but the 35% difference between the absorbance and emission is due to the excited state not having the same stability as the protonated form of the dye.

The higher the linear response coefficient, the better the dye is as a chemosensor. Dyes 25 and 26 perform similar with absorption and emission response coefficients only 0.013 and 0.005 units apart, but dyes 27 and 28 are starkly better. Dyes 27 and 28 are the more hydrophobic dyes and give linear absorption coefficients of 0.2535 and 0.4179 respectively, a 50% and 150% increase in responsivity. Emission is even more responsive with values of 0.3710 and 0.3661 for dyes 25 and 26 and 0.4307 and 0.7105 for dyes 27 and 28. This provides optimistic results for these compounds to be pursued in further applications due to operating in the lowered pH region of tumor tissues.162-164

3.5.5 Computational Studies

Physical parameters for the synthesized dyes were calculated to illuminate the inner electronic workings of each fluorophore system and are displayed in Table 7. Quantitative Structural Activity Relationship (QSAR) parameters such as molecular surface area and volume, polar surface area (PSA), dipole moment, and LogP/LogD are all numerical descriptors used by
various algorithms to model and match different drug like molecules to possible pharmacodynamic and pharmacokinetic abilities and related compounds. These values greatly help to contextualize the compounds within the greater library of synthesized drug-like dyes and insight into how they would act in vivo though a larger value set is needed for an accurate pharmacokinetic evaluation. Understanding these quantitative parameters, molecular geometries, and orbital energies provide insight to the electronic effects of protonation and solvent environment. These values are all describing the molecules floating in a vacuum and all discrepancies between the experimental and calculated values can be attributed to solvent environment effects.

All of the prepared compounds show a consistent trend in QSAR parameters when exposed to acid. There was an across the board increase in the polar surface area and dipole moment in all derivatives as well as a consistent decrease in the LogP/LogD of each dye when protonated. The color coded structures in Figures 43 and 44 correspond to the geometric locations of the HOMO and LUMO modeled in red and blue and as a red to blue heat map corresponding to the electron potential density surface of the dyes. The modeled HOMO and LUMO surfaces for compound 25 show a distribution along the polymethine bridge and that deposits back onto the benzothiazole rings. The valence molecular orbitals do not seem to spatially interact with the pyridine group at the meso-position, but the electron donating effects of this substituent are still seen with a redshifted benzothiazole dye of 10 nm when compared to the standard methyl pentamethine benzothiazole dye with dye 25’s absorption at 650 nm. When dye 25 is protonated there is no noticeable change in the HOMO with the same distribution across the benzothiazole rings and polymethine chain. There is, however, a significant change in the molecular orbital geometries of the protonated dyes’ LUMO with a majority of density pulling towards the pyridine group’s newly acquired proton. Note that while there is a primary density on the pyridine group, there is still
traces of the molecular orbital extending down into the polymethine chain and back towards the benzothiazole heterocycles. This speaks to our hypothesis that the capture of a proton will effectively shift the electronic resonance towards that acquired proton and shorten the overall lengths of the molecular orbitals.

The surface area and volumes of dyes 25-27 are all within the ranges of viable cell permeability while the large propylphenyl arms of dye 28 raise the volume of the compounds close to 700 Å². The average polar surface area for each dye is 8.38 Å² and increases on average 3.65 Å² upon protonation as the newly cationic pyridinium nitrogen accounts for more polar space on the

<table>
<thead>
<tr>
<th></th>
<th>Area (Å²)</th>
<th>Volume (Å³)</th>
<th>PSA (Å²)</th>
<th>Dipole (debye)</th>
<th>logP</th>
<th>HOMO (eV)</th>
<th>LUMO (eV)</th>
<th>ΔE (eV)</th>
</tr>
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<tbody>
<tr>
<td>dye 25</td>
<td>506.82</td>
<td>487.65</td>
<td>8.483</td>
<td>6.35</td>
<td>4.25</td>
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<td>-5.21</td>
<td>-2.28</td>
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<tr>
<td>dye 25H+</td>
<td>506.41</td>
<td>490.73</td>
<td>12.145</td>
<td>12.72</td>
<td>3.88</td>
<td>-9.86</td>
<td>-8.16</td>
<td>-1.70</td>
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<tr>
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<td>467.17</td>
<td>456.59</td>
<td>8.484</td>
<td>4.68</td>
<td>2.82</td>
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<td>-5.40</td>
<td>-2.34</td>
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<td>dye 26H+</td>
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<td>461.91</td>
<td>12.122</td>
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<td>-8.29</td>
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<td>dye 27</td>
<td>499.39</td>
<td>488.08</td>
<td>8.246</td>
<td>6.24</td>
<td>4.57</td>
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<td>692.2</td>
<td>8.289</td>
<td>23.81</td>
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<td>-7.49</td>
<td>-5.20</td>
<td>-2.29</td>
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<td>dye 28H+</td>
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<td>696.6</td>
<td>11.909</td>
<td>24.13</td>
<td>6.79</td>
<td>-9.8</td>
<td>-8.09</td>
<td>-1.71</td>
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Figure 43: HOMO, LUMO, and electronic density map of dyes 25 and 26 and their protonated equivalents
<table>
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<tr>
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<th>27H+</th>
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<td>HOMO</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>LUMO</td>
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<td><img src="image4.png" alt="Image" /></td>
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<tr>
<td>Electron Density</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
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</table>

<table>
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<th>28</th>
<th>28H+</th>
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</thead>
<tbody>
<tr>
<td>HOMO</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>LUMO</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
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<tr>
<td>Electron Density</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 44: HOMO, LUMO, and electronic density map of dyes 27 and 28 and their protonated equivalents
molecule. The PSA of the dyes relates to what portion of the molecule will have the most intermolecular solvent interactions as well as the dipole moment. The calculated dipole moment is primarily used to understand the relationship between the dye, their solvent environments, and oscillating electromagnetic fields. The fluorine atoms present in dye 26 lowers the dipole moment compared to dye 25 due to the electron density pulling the overall polarity towards the opposite of the molecule as the pyridine group. The protonation of each dye causes an average shift in the dipole moment by 5.04 debye though this ranges high as 7.55 debye in dye 26 and 0.32 debye in dye 28. Dye 26 experiences this high shift because the electron contribution of the fluorine atoms are easily overcome by the newly shifted electron density towards the newly acquired proton in the pyridinium group.

LogP and LogD are both partition coefficients that are useful metrics for understanding the general solubility of a compound. LogP is used for non-charged compounds while LogD describes charged molecules. These factors also directly correlate to how permeable a drug-like molecule would be in a biological system with drugs needing to be highly hydrophilic and have a high calculated LogP/LogD in order to have high pharmacokinetic distribution but still possessing the proper aliphatic binding capabilities necessary for requisite receptor pockets. The lowest LogP belongs to dye 26 due to its high electron density and the ability for fluorine to engage in polar bonding. The highest LogP belongs to the bulky dye 28 with its large aliphatic arms. On average, the protonation of these dyes causes a loss of 0.3 LogD which means that the protonated forms of the dyes are more soluble than the non-protonated forms. This should be expected due to the additional charge of the protonated pyridinium promoting solvation from polar groups.

The molecular orbitals for each dye have similar energetic conditions with average HOMO orbitals existing at -7.57 eV and average LUMO orbitals existing 2.30 eV lower at -5.27 eV. When
these dyes are protonated, the average HOMO energy rises to -9.95 eV for an average of a 2.38 eV difference in the energetic values. The LUMO energy of the derivatives also experience an increase in energy as the average bandgap level rises from 5.27 eV to 8.19 eV for an average of 2.92 eV as the newly protonated geometries raise the energetics of the system when the electrons are drawn up into the pyridine group. For the unprotonated dyes the average calculated band gap energy was -2.30 eV which is equal to that of a photon of 539 nm. The 111 nm/0.4 eV difference between this theoretical photon and the photon observed experimentally is due to the solvent effects of the dye’s environment lowering the band gap energy roughly 0.4 eV. In the protonated dye this band gap energy is lowered 0.54 eV to an average -1.76 eV as the band gap is shortened and supported by a solvation shell.

3.6 Conclusion

Four meso-pyridyl benzothiazole pentamethine dyes were synthesized with high-yields and verified using ¹H NMR. All of the synthesized derivatives absorbed between 645nm and 660 nm within the far-red range and had high molar absorptivity and quantum yield ranging from 9% to 17%. These compounds were also shown to have much higher dark- and photo-stability when compared to the leading FDA approved fluorophore, indocyanine green, with only a 4% loss of compounds in the dark after 7 days and a 10% loss under UV-irradiation after 24 h. The synthesized derivatives were also responsive to pH changes with a resulting blue shift in the absorbance and emission spectra as well as a decrease in the absorption and emission spectral intensities. All of the compounds shared the same trend in response to acid but the hydrophobic compound 28 showed the highest response coefficient and the greatest change in absorbance values. Computational studies performed on the dyes confirmed the changes in the HOMO and LUMO geometries and energies that correspond to our proposed alternative resonance pathways.
and energetics. Calculations in Spartan18 showed that protonation of the dyes increased LogD/LogP as well as increased polar surface area, both parameters that would heavily contribute to the changes in the spectra of the protonated compounds. Continued exploration of dyes with the meso-pyridine moiety would provide insight into the expansion of pentamethine pH responsive dyes their future applications.
REFERENCES


Iodinated Aminosquaraines as Potential Sensitizers for Photodynamic Therapy. Molecules 2019, 24 (5).


165. Kiprianow, P., Chemisches Zentralblatt *Zhurnal Obschei Khimil*, 1940 1940, 10, 613-617.
APPENDIXES

Appendix A

Chapter 2: Melting points, $^1$H NMR, $^{13}$C NMR, ESI-MS, absorbance plots, emission plots, solvatochromism plots, stability plots, biodistribution data, and computational analysis of presented squaraine compounds.

<table>
<thead>
<tr>
<th>ID Name</th>
<th>Descriptive Name</th>
<th>Melting Point Range (°C)</th>
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<tbody>
<tr>
<td>ICG</td>
<td>indocyanine green</td>
<td>237 - 238</td>
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<tr>
<td>7</td>
<td>SQ-QN</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>8</td>
<td>SQ-BQ</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>9</td>
<td>SQ-SO</td>
<td>249 - 252</td>
</tr>
<tr>
<td>13</td>
<td>SQ-DQ</td>
<td>251 - 255</td>
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<tr>
<td>17</td>
<td>SQ-BU</td>
<td>258 - 259</td>
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<tr>
<td>18</td>
<td>SQ-PP</td>
<td>198 - 199</td>
</tr>
<tr>
<td>19</td>
<td>SQ-BP</td>
<td>195 - 197</td>
</tr>
</tbody>
</table>
Absorbance in ethanol to determine $\varepsilon$ for dye 7

$$y = 223548x - 0.0522$$

$R^2 = 0.9992$

Absorbance of dye 7 in ethanol

Emission of dye 7 in ethanol at 20 $\mu$M
Absorbance in ethanol to determine $\varepsilon$ for dye 8

$y = 220765x - 0.012$

$R^2 = 0.9971$

Absorbance of dye 8 in ethanol

Emission of dye 8 in ethanol at 10 $\mu$M
Absorbance in ethanol to determine $\varepsilon$ for dye 9

$y = 227544x - 0.0171$
$R^2 = 0.9915$

Absorbance of dye 9 in ethanol

Emission of dye 9 in ethanol at 20 $\mu$M
118

Absorbance in ethanol to determine $\varepsilon$ for dye 13

$$y = 179772x - 0.0541$$
$$R^2 = 0.9932$$

Absorbance of dye 13 in ethanol

Emission of dye 13 in ethanol at 20 $\mu$M
Absorbance in ethanol to determine $\varepsilon$ for dye 18

\[ y = 121011x + 0.0279 \]
\[ R^2 = 0.9963 \]

Absorbance of dye 18 in ethanol

Emission of dye 18 in ethanol at 44 $\mu$M
Absorbance in ethanol to determine $\varepsilon$ for dye 19

$$y = 115365x - 0.0144$$

$R^2 = 0.9983$

---

Absorbance of dye 19 in ethanol

Emission of dye 19 in ethanol at 40 µM
D$_2$O exchange +24h

7
D₂O + 72h
Current Data Parameters

NAME             SQPP
EXPNO             1
PROCNO            1

F2 - Acquisition Parameters

Date_          20200315
Time              19.55 h
INSTRUM           spect
PROBHD   Z108618_0320
PULPROG            zg30
TD                65536
SOLVENT           CDCl3
NS                   64
DS                    2
SWH            8012.820 Hz
FIDRES         0.244532 Hz
AQ            4.0894465 sec
RG                   32
DW               62.400 usec
DE                 6.50 usec
TE                296.0 K
D1           1.00000000 sec
TD0                   1
SFO1        400.1424709 MHz
NUC1                 1H
P1                13.55 usec
PLW1        16.00000000 W

F2 - Processing parameters

SI                65536
SF          400.1400008 MHz
WDW                  EM
SSB      0
LB                 0.30 Hz
GB       0
PC                 1.00

18

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Appendix B

Chapter 3: Melting points, HNMR, 13CNMR, ESI-MS, absorbance plots, emission plots, pH response plots, stability plots, and computational analysis of presented meso-pyridinium compounds.

<table>
<thead>
<tr>
<th>ID Name</th>
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<td>MP-MM</td>
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<td>MP-ET</td>
<td>223 - 226</td>
</tr>
<tr>
<td>28</td>
<td>MP-PP</td>
<td>216 - 218</td>
</tr>
</tbody>
</table>
Absorbance of dye 26 in ethanol

Absorbance in ethanol to determine ε for dye 26

\[ y = 135720x - 0.054 \]
\[ R^2 = 0.9989 \]

Emission of dye 21 in ethanol at 25 μM
Absorbance in ethanol in order to determine $\varepsilon$ for dye 27

$y = 163816x + 0.0015$

$R^2 = 0.996$

Emission of dye 27 in ethanol at 25 µM
28