Synthesis of Fluorene-based derivatives, Characterization of Optical properties and their Applications in Two-photon Fluorescence Imaging and Photocatalysis

2015

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SYNTHESIS OF FLUORENE-BASED DERIVATIVES, 
CHARACTERIZATION OF OPTICAL PROPERTIES AND THEIR 
APPLICATIONS IN TWO-PHOTON FLUORESCENCE IMAGING AND 
PHOTOCATALYSIS

by

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B.S University of Nairobi, 2005

A dissertation submitted in partial fulfillment of the requirements 
for the degree of Doctor of Philosophy 
in the Department of Chemistry 
in the College of Sciences 
at the University of Central Florida 
Orlando, Florida

Spring Term 
2015

Major Professor: Kevin D. Belfield
The two-photon absorption (2PA) phenomenon has attracted attention from various fields ranging from chemistry and biology to optics and engineering. Two of the common NLO applications in which organic materials have been used are three-dimensional (3D) fluorescence imaging and optical power limiting. Two-photon absorbing materials are, therefore, in great demand to meet the needs of emerging technologies. Organic molecules show great promise to meet this need as they can be customized through molecular engineering, and as the development of two-photon materials that suit practical application intensifies, so does research to meet this need. However, there remains some uncertainty in the particulars of design criteria for molecules with large 2PA cross sections at desired wavelengths, as such research to understand structure-property relationships is matter of significant importance. As a result, the full potential of 2PA materials has not been fully exploited. Several strategies to enhance the magnitude and tune the wavelength of 2PA have been reported for π-conjugated organic molecules. On this account, we have designed novel fluorophores using the fluorene moiety and modified it to tune the properties of the compounds.

Chapter 2 of this dissertation reports the successful application of fluorene-based compounds in photocatalysis; a process that involves the decomposition of organic compounds into environmentally friendly carbon dioxide and water attesting to the photostability of the fluorene moiety. A facile organic nanoparticle preparation method is reported in chapter 3 using the reprecipitation method, whose surface was then modified using a naturally occurring surfactant, Lecithin, and were then successfully used in fluorescence cell imaging. Chapter 4 reports the design and synthesis of a fluorene-based compound using an acceptor, s-indacene-1, 3,
5, 7(2H, 6H)-tetra one, or Janus Dione, a moiety that is relatively new and that has not been fully exploited despite its very attractive features. Owing to the hydrophobicity of this compound, notwithstanding its unprecedented 2PA cross section, it was not applicable in fluorescence cell imaging but provided the tenets for the design of related derivative. This limitation was circumvented in the concluding chapter by tuning the compound’s hydrophilicity. The hydrophilic Janus dione probe was then used as envisioned for cell imaging as the dual prerequisites for fluorescence imaging probes; large 2PA cross sections and high fluorescence quantum yields were met.
To Tyra and Abigail
ACKNOWLEDGMENTS

I wish to register my heartfelt gratitude to my research advisor, Prof. Kevin Belfield, who supported my research work without cease over the years. None of these projects would have seen the light of the day without his support and guidance. My deepest appreciation to my dissertation committee; Dr. Karin Chumbimuni-Torres, Dr. Shengli Zou, Dr. Pedro Patino and Dr. Zixi Cheng for setting aside time to serve in my committee despite their busy schedules. My sincere thanks to Dr. Alma Morales and Dr. Andrew Frazer who oriented me on working on these projects and for being very patient with me as I learnt the ropes of chemical synthesis.

I also place on record, my gratitude to Dr. Adam Woodward who worked with me on all the projects. He did all the photophysical characterization of the compounds, was kind enough to teach me some of the photophysical characterization techniques and put up with my pestering questions. Many thanks to Xinglei Liu, Simon Tang and Dr. Xiling Yue for helping with the cell imaging work and to Ebrahim Zadeh and Dr. Taihong Liu for their expert advice on synthesis techniques. A big thanks to Aldas Daugela for his collaboration with the organic nanoparticles project. My gratitude to Dr. Sheng Yao, Dr. Yuanwei Zhang and to the research group of Dr. David Mazyck at the University of Florida (UF), Department of Environmental Engineering, for making the photocatalysis project a success.

To my unfathomably supportive family, especially my mum and dad, please receive my heartfelt thanks for your unconditional love and support. Special thanks to my loving daughter Tyra and niece Abigail, my ardent supporters, who unceasingly cheered me on every step on the way and to whom I dedicate this work.
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<td>2PA</td>
<td>Two-photon absorption</td>
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<td>Three dimensional</td>
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<td>Absorption</td>
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<td>GM</td>
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CHAPTER 1: INTRODUCTION

1.1 Abstract

This chapter introduces the two photon absorption (2PA) phenomenon, its advantages over one photon absorption and its current applications. A background on photocatalysis; the processes involved and why the process is gaining popularity is detailed in section 1.2. The motivations behind the projects are also explained in each of the two sections.

1.2 Two-photon vs one-photon

Two-photon absorption (2PA) is a nonlinear process in which a molecule absorbs two photons near simultaneously, of energy hv (hv1 and hv2) to access an excited state of energy 2hv (hv1+hv2). The sum of the photon energies is equivalent to the energy difference between the initial and the final states of the transition. This results in a rate of the 2PA scaling quadratically unlike single-photon absorption, which scales linearly with intensity, I, of the incident radiation.

![Jablonski diagram](image)

**Figure 1:** Jablonski diagram showing the difference between one photon and two-photon absorption.

Seminal work done by Maria Goppert-Mayer in 1931 details the theoretical framework for two-photon absorption. The two-photon phenomena is short-lived (10^{-15} s) in the visible region,
which explains why it is classified as an instantaneous nonlinear process. In addition, if the second photon afforded is to complete the two-photon absorption event in the fleeting time, a large photon flux is required to increase the probability of this transition.  

With the advent and subsequent commercialization of high peak-power and tunable laser sources, 2PA has found applications in various fields. The main feature of this phenomena that makes it attractive is its probability to be greatly enhanced using high intensity radiation because the rate of 2PA is quadratically dependent on I, which guarantees that the process benefits from high spatial selectivity when a focused laser beam is used.  

This is shown schematically in Figure 2 where a fluorescent chromophore is excited with two beams; one-photon absorption (1PA) and 2PA result in a sharp contrast between the subsequent fluorescence; the 2PA fluorescence is localized in the focal volume whereas that of one photon extends all along the path of the focused light.

Figure 2: Comparison of the spatial selectivity between one-photon and two-photon absorption.
Consequent to the quadratic dependence of this process on the incident radiance, only in the focal volume does 2PA occur. Besides the transverse confinement of the process in the x and y directions, the confined volume is confined axially too. This is ascribed to the fact that the irradiance is in the vicinity of the focus falls off as the I proportional to $z^{-2}$, the propagation distance, which results in in 2PA decreasing as $z^{-4}$ with the distance from the focus and the excitation is confined in a small volume around the focus. As a result, there’s negligible 2PA below and above the focal plane. This generates a three- dimensional high spatial resolution as shown schematically in Figure 3. This is crucial in two photon induced fluorescence when used for three dimensional imaging in biological tissues. 3, 5, 6, 9-10

Absorption and scattering are minimal in the red and near-infrared (NIR) making it a spectral window for deep penetration into highly absorbing and turbid biological media. For two-photon microscopic studies of biological systems, the fundamental wavelength should be above the absorption from the molecules in the tissues and below the absorption due to overtones of H$_2$O. 10-11

Figure 3: Three dimensional spatial resolution of 2PA.3
An additional attractive feature of 2PA is that since neither of the two photons may access the real excited state on its own (provided the photon energies lie below the linear absorption edge of the sample), no one-photon process can occur. This implies that the light can penetrate into highly absorbing media without inducing any excitation.\textsuperscript{8,9}

The photophysical phenomena that come with 2PA are illustrated in Figure 4. Excitation occurs from the ground state $S_0$ to a two photon allowed state $S_2$ which can be followed by any of the following two processes; internal conversion to the first singlet state $S_1$ or intersystem crossing (ISC) to the triplet state $T_1$. Fluorescence can then occur as the system radioactively relaxes from $S_1$ to the ground state. A further process, excited-state absorption (ESA) which is photoinduced absorption from the excited state can take place from any of the excited states, $S_1$, $S_2$ or $T_1$ as shown in Figure 4.\textsuperscript{3,12}

![Jablonski diagram showing the two-photon process and subsequent processes.](image)

**Figure 4**: Jablonski diagram showing the two-photon process and subsequent processes.\textsuperscript{3}

A combination of the above phenomena has attracted attention from various fields ranging from chemistry, biology, options, and engineering, among others. Two of the commonly used applications are in three-dimensional (3D) fluorescence imaging and optical power limiting.
Imaging exploits the two-photon induced fluorescence whereas the optical limiting capitalizes on ISC and ESA. Besides physical mechanisms, 2PA can induce highly localized photo-induced chemical reactions which have led to its application in 3D micro fabrication, optical data storage and in photodynamic cancer therapy. Singlet oxygen sensitizers are good candidates for cytotoxicity and photodynamic therapy studies in biological tissues.

Materials exhibiting large 2PA cross sections ($\delta$) at desirable wavelengths are required for these applications because they are advantageous as greater degree of excitation can be attained or lower laser intensities can be used for pumping. This would result in more reliable and accessible 2PA applications since laser damage of molecules and materials in the vicinity could be reduced, less power and less expensive lasers can be employed. Organic molecules show great promise to meet this need as they can be tailored through molecular engineering and they can be designed to be target-specific. Unfortunately, most of the known organic molecules have relatively small 2PA cross section and the design criteria for molecules with large 2PA cross sections at desired wavelengths is not universally understood, thus these materials have not been fully developed or utilized.

1.3 Fluorene

The base structure used for all the compounds synthesized in this research work is the fluorene chromophore. A number of advantages come with this structure. The chromophore is aromatic due to the cyclic conjugated nature of the two benzenes in its structure with each benzene possessing six pie electrons that are localized around the ring (Figure 5)
As a result, this makes the chromophore highly polarizable and improves the chances of nonlinear interaction upon excitation. The fluorene core is also rigid improving its thermal and photochemical stability. This ensures that it can withstand the intense radiation which nonlinear excitation requires. Photodegradation work by Belfield et al. on a fluorene derivative showed minimal difference when the compound is excited using one photon and two photon conditions proving that fluorene derivatives are desirable for use in linear and nonlinear optical applications.\textsuperscript{3, 17, 20} Fluorene is highly fluorescent and a library of compounds investigated are reported to possess high to decent quantum yields and have successfully been used in two-photon fluorescence microscopy.\textsuperscript{11, 17-20}

Moreover, the fluorene chromophore can be functionalized at different sites; namely 2, 4, 7 and 9 as depicted in Figure 5. Different substituent groups can be appended onto the chromophore with the goal of modifying the compound’s structural properties. The compound’s solubility can be tuned at the 9 position, which is particularly important as highly concentrated solutions of the order $10^{-4}$ to $10^{-2}$ M are used during characterization of two-photon absorption.\textsuperscript{3, 19}

Functionalization at positions 2 and 7 and sometimes 4 proffers the greatest benefits. These positions enable the generation of different chemical archetypes by appending various functional groups to the fluorene chromophore. The groups that can be appended consist of
electron active moieties either electron withdrawing or electron donating, denoted donor (D) or acceptor (A) respectively. Different motifs can result when positions 2 and 7 are functionalized:

i) Donor-\(\pi\)– conjugated core-donor (D-\(\pi\)–D)

ii) Acceptor-\(\pi\)– conjugated core-Acceptor(A-\(\pi\)–A) or

iii) Donor-\(\pi\)– conjugated core- Acceptor (D-\(\pi\)–A)

The strength of the electron accepting groups and that of the electron donating groups can be adjusted to further tune the fluorene chromophore. This can involve adding additional fluorene chromophores units, or some other \(\pi\)–conjugated substructures like vinyl or styryl structures. This results in the conjugation being extended resulting in new motifs: A-\(\pi\)-\(\pi\)-A, D-\(\pi\)-\(\pi\)-D, D-\(\pi\)-\(\pi\)-\(\pi\)-A, D-\(\pi\)-\(\pi\)-\(\pi\)-A, etc. \(^3, 15, 17, 19-21\)

1.4 Photocatalysis

Volatile organic compounds (VOCs) and hazardous air pollutants (HAPs) are the main byproducts that are generated in the processing of forest products. Some VOCs are toxic and carcinogenic to human health for example benzene. \(^22-24, 26\) Methanol makes the biggest fraction of all components of gases emitted from pulp and paper mills. \(^25\) Thermal oxidation is the most commonly technique that is used to control these byproducts. The method is however expensive as it requires investment of capital and constant fuel supply to drive the process. \(^24-26\) There’s therefore a great demand for a more cost effective, more efficient and environmentally benign technology.

Photocatalysis comprises of reactions which use a catalyst activated by light. Photocatalytic oxidation of aqueous and gaseous contaminants was borne out of the successful
photocatalytic water splitting on TiO$_2$ in 1972. It has since been used successfully for the degradation of organic pollutants and has been found to be attractive, nonselective and a room temperature process. Among the reactions that employ photocatalysis include decomposition of organic compounds into environmentally friendly water and carbon dioxide resulting in interesting properties of surfaces covered with a photocatalyst: they are self-cleaning, antibacterial, and viricidal. They are thus attractive candidates for environmental applications like water purification and waste-water treatment.  

The process involves illumination of a semiconductor which results in the formation of photoexcited electrons and holes (in a vacant conduction band) that then reacts with contaminants adsorbed on the photocatalyst surface. The electronic configuration of semiconductors provides light-induced charges for redox processes; they are characterized by a filled valence band and an empty conduction band. The elementary steps involved are:

\[ \text{TiO}_2 + \text{hv} \rightarrow \text{h}^+ + \text{e}^- \]  

Where h$^+$ and e$^-$ are the oxidizing and reducing agents respectively.

An electron in an electron-filled valence band is excited by photoirradiation to a valence conduction band leaving a hole in the valence band. The electron-holes formed can either recombine or participate in redox reactions. The electrons and holes thus formed drive the oxidation and reduction reactions that follow on the photocatalytic surface. This reaction is thus regarded as the activation reaction of the photocatalytic process.

The oxidation and reduction reactions that follow can be expressed in the following reactions
Oxidation reaction: \[ h^+ + OH^- \rightarrow OH^- \] (2)

Reduction reaction: \[ e^- + O_{2\,ads} \rightarrow O_{2\,ads}^- \] (3)

When the organic compounds are completely transformed by the photocatalytic oxidation process, the hydroxyl radical derived from the oxidation reaction of the adsorbed water or adsorbed OH- is the main oxidant.

Net reaction: \[ OH^- + VOC + O_2 \rightarrow n\,CO_2 + m\,H_2O \] (4)

Methanol can be oxidized directly via photogenerated electron holes or by indirectly by hydroxyl radicals which are continuously formed from adsorbed water that reacts with the electron holes. The degradation pathway that then ensures results in the formation of formaldehyde, formic acid eventually forming benign carbon dioxide and water.\textsuperscript{24}

TiO\textsubscript{2} is the most commonly semiconductor used in environmental applications because of its physical and chemical stability, relatively cheaper, non-toxic and its resistance to corrosion. Light used should be of adequate intensity that exceeds the TiO\textsubscript{2} band gap energy. The crystal structure of TiO\textsubscript{2}; rutile and anatase have an absorption threshold that corresponds to 410 nm and 380 nm respectively. Therefore the absorption spectrum of anatase does not correspond to the solar spectrum as less than 5\% of solar spectrum falls in that region. If used for photocatalysis, it results in very low photoreaction rates limiting commercial application. Surface modification of the photocatalysts is the latest focus of research with the aim of improving the efficiency of the photocatalysts. Some of the strategies involve improving the charge separation by inhibiting the charge recombination, increasing the wavelength response by exciting in the visible region,
changing the selectivity or yield of a particular product. Noble metals e.g. Pt has been reported to be photocatalytic as are other metals like Au, Pd, Ru.

The sol-gel process is a wet-chemical technique (chemical solution deposition) used for the preparation of photocatalysts. Precursors consist of metal alkoxides and metal chlorides which undergo hydrolysis and polycondensation reactions. Alkoxides are commonly used in the sol-gel because they are soluble in water in the hydrolysis reaction where the hydroxyl group is attached to the silicon atom. The advantages of the sol-gel method are that it is easy to operate and inexpensive, the films are easily anchored on the substrate and it can be used for the deposition of the substrates with large surface area and complex surfaces. The process uses nanosemiconductor and UV light to convert organic compounds in indoor air into benign products; water vapor and carbon dioxide. Degussa P25 titania is used as a photocatalyst because it is commercially available, relatively inexpensive and has shown superior photocatalytic properties owing to its combination and close proximity to anatase and rutile phases. Silica is used in combination with titania because it is transparent, porous, and can support large weight percentages of titania. In addition, a synergy exists between titania and silica which is ascribed to the adsorptive nature of silica which increases the pollutant concentration near the titania surface.
CHAPTER 2: PHOTOCATALYTIC OXIDATION OF VOCS USING VISIBLE LIGHT FOR NEXT GENERATION LIFE SUPPORT

2.1 Project and Approach

The research group of Dr. David Mazyck at the University of Florida (UF) has developed a robust photocatalyst called Silica-Titania Composites (STC) that has shown promise for space and terrestrial life support applications. The STC consists of a porous silica adsorbent doped with TiO$_2$ (Figure 6).

![Figure 6: One STC pellet alongside one US quarter.](image)

Being an adsorbent that is also photocatalytic, the irradiated STC facilitates contact between the pollutant and the oxidizing radicals generated by the TiO$_2$. This reduces mass transfer kinetics and encourages complete mineralization of the pollutant. Further, the silica sorbent is continuously regenerated through the photocatalytic reactions, presenting a significant
advantage over traditional adsorbents that are generally discarded once exhausted. Other advantages of this technology include the following:

1) the TiO$_2$ is well dispersed and trapped in the silica gel matrix, which serves as a durable support for the nanosized photocatalyst;

2) the surface area of the STC can be in excess of 700 m$^2$/g, which enhances adsorption and concentrates the contaminant around the photocatalyst, making oxidation more efficient;

3) the STC pore size and pore size distribution can be modified to optimize performance;

4) the STC catalysts have a higher adsorption capacity and thus last longer than activated carbon for several applications$^{1-6}$;

5) the silica is semitransparent to UV thereby reducing irradiation requirements.

The STC technology has been successfully used for a number of applications (bench to full scale), including:

1) the removal of VOCs and hazardous air pollutants from gases emitted from pulp and paper mills$^{6-8}$;

2) the removal of synthetic organic compounds from gray water $^{9-14}$;

3) the removal of mercury from flue gas $^{1-3}$;

4) the removal of mercury from caustic exhaust at chloralkali facilities $^6$;
5) the purification of aircraft cabin air \(^{15}\);

6) the deactivation of pathogens \(^{16}\);

When compared in side by side studies, performance by the STC-based prototype reactors far exceeded that of commercially available air purification systems. Because of the tremendous benefits to using the STC material over conventional photocatalytic or adsorptive life support systems, the National Aeronautics and Space Administration (NASA) has funded several projects to develop an appropriate reactor to house the material. Dr. Mazyck’s team recently completed and delivered a 12 cfm reactor to NASA for the removal of VOCs from air. Nevertheless, the necessary irradiation provided by UV bulbs limits the reactor efficiency and system lifetime. Specifically, 254 nm bulbs utilize only about 40% of their input wattage, the remainder of which is dissipated in waste heat. Moreover, the bulbs have a lifetime of only about 9,000 hours. To date, this has been managed by developing a more efficient reactor using light emitting diodes as the UV source. This recent (2012) optimization improved energy use of the system by 25% and lessened concerns over using mercury containing UV bulbs. The next step for optimization of the technology is to sensitize the material to visible light (i.e., wavelength \(\lambda\) between 400 and 700 nm), and even NIR (700-800 nm) to permit the use of sunlight for space and terrestrial applications. The technology could therefore be applied for water or air treatment in areas with a limited power supply including remote military operations, disaster response, decontamination efforts, and environmental remediation.
2.2 Visible Light Sensitization of TiO\textsubscript{2}

Visible light sensitization of TiO\textsubscript{2} is accomplished by doping the catalyst with additives that promote electron transfer with lower photon energy requirement. As shown in Figure 7, when light of the appropriate wavelength is absorbed by a photocatalytic material, an electron (\(eV\)) is transferred from the valence band to the conduction band, leaving a positively charged hole behind (\(h^+\)) in the structure. The electrons and holes may either recombine or participate in oxidation/reduction reactions. For the transfer of electrons to occur successfully, the light must provide sufficient photonic energy (\(hv\)) to the electron for it to jump the valence/conduction band gap. Pure TiO\textsubscript{2} has a band gap energy of 3.2 eV. Therefore, to transfer electrons from the valence to the conduction band, TiO\textsubscript{2} must be irradiated with a wavelength shorter than 388 nm (254 and 365 nm are traditionally used), which excludes the visible light spectrum.
Considerable effort has been exhausted by many to sensitize TiO$_2$ to visible light. The general approach involves doping the structure with metals (e.g., platinum), oxygen vacancies in the crystal structure, or nonmetals (e.g., nitrogen$^{17}$) to decrease its band gap energy. More recently, some have used dyes to encourage electron transfer (“dye sensitized TiO$_2$”). In this method, visible light irradiation of dye molecules adsorbed to the TiO$_2$ surface cause the dye to transfer electrons to the TiO$_2$ conduction band, which then induce redox reactions. However, the reported dyes are photochemically unstable and undergo photo oxidation, greatly reducing efficiency of the system. We propose using a similar approach with the application of photostable dyes sensitizing at different wavelengths from visible to near infrared.
Dr. Kevin Belfield’s research group at the University of Central Florida has been actively engaged in the design of new photosensitizers, specifically formulated to anchor to the surface of TiO₂, iron oxide, and gold nanoparticles. The band gap of these photosensitizers is intentionally matched with TiO₂. TiO₂ nanoparticles will be modified with the photosensitizer through the photosensitizer’s carboxylic acid groups. Covalent attachment of photosensitizers onto the STC will offer an additional manifold for spectral absorption. These particular photosensitizers are comprised of stable aromatic chromophores that are resistant to photodecomposition. Thus, the TiO₂ photocatalyst will be active in the short wavelength visible (400 to 577 nm) to long wavelength visible (577 to 650 nm) producing reactive oxidants such as super oxide and other oxygen radical species.

2.3. Anchoring of the dyes to the TiO₂ and UV-Vis Characterization

2.3.1: Materials

- TiO₂ (P25), CAS-Reg. No. 13463-67-7 was purchased from Degussa Corporation.
- Ruthenium complex was purchased from Sigma-Aldrich.
- 3-(9-(2-(2-Methoxyethoxy)ethyl)-2,7-bis(4-(benzothiazol-2-y1)styryl)-fluoren-9-y1)propanoic Acid was synthesized as in the reference 20, (E)-2(Z)-3-(2-carbaxyethyl) benzo[d]thiazol-2(3H)-ylidene) methyl-4-((3-ethylbenzo[d]thiazol-3-imium-2yl)methylene)-3-oxocyclobut-1-enolate was synthesized as in the reference 21.
- The synthesis of 2-((7-(diphenylamine)-9, 9-dihexyl-9H-fluoren-2yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5, 6-dicarboxylic acid, 99-90, is described in Chapter 5
- Solvents: Dichloromethane, Ethanol, DMSO
The dyes were characterized by UV-vis absorption and then anchored onto TiO$_2$ by following the literature.\textsuperscript{22}

2.3.2: Cis-bis(isothiocyanato) bis(2, 2ˈ-bipyridyl-4, 4ˈ-dicarboxylato) ruthenium (II)-TiO$_2$ Complex, 99-26.

![Scheme 1: Anchoring Cis-Bis (isothiocyanato) bis (2, 2ˈ-bipyridyl-4, 4ˈ-dicarboxylato) ruthenium (II) onto TiO$_2$](image)

**Experimental**

The ruthenium complex; 0.5, 1, 1.5 and 2 % by weight was mixed with TiO$_2$ and then dissolved in 25 mL of ethanol. The mixture was stirred for 24 hrs. under subdued light. Ethanol was then removed under vacuum and two grams of each sample was prepared.
Characterization

UV-vis absorption was studied in ethanol and the spectrum displayed three absorption bands at 314, 397 and 535 nm as can be seen in the Figure 8.

Figure 8: UV-vis spectrum of 99-26 in ethanol
2.3.3: 3-(9-(2-(2-Methoxyethoxy)ethyl)-2, 7-bis (4-(benzothiazol-2-yl) styryl)-fluoren-9-yl) propanoic Acid -TiO₂ Complex, 99-43.

![Scheme 2: Anchoring 3-(9-(2-(2-methoxyethoxy)ethyl)-2, 7-bis (4-(benzothiazol-2-yl) styryl)-fluoren-9-yl) propanoic acid onto TiO₂.](image)

**Scheme 2:** Anchoring 3-(9-(2-(2-methoxyethoxy)ethyl)-2, 7-bis (4-(benzothiazol-2-yl) styryl)-fluoren-9-yl) propanoic acid onto TiO₂.

**Experimental**

3-(9-(2-(2-Methoxyethoxy)ethyl)-2, 7-bis (4-(benzothiazol-2-yl) styryl)-fluoren-9-yl)propanoic Acid, 0.5, 1, 1.5 and 2% by weight, was mixed with TiO₂ and then dissolved in dichloromethane (30mL) to form yellow solution. The mixture was then stirred at room temperature for 24 h. under subdued light. Dichloromethane was removed under vacuum and two grams of each sample was prepared.

**Characterization**

UV-vis absorption was studied in DMSO and the spectrum displayed two absorption bands at 412 and 433 nm as in *Figure 9*. 
Figure 9: UV-vis spectrum of 99-43 in DMSO.
2.3.4: (E)-2(Z)-3-(2-Carbaxyethyl) benzo[d]thiazol-2(3H)-ylidene) methyl-4-((3-ethylbenzo[d]thiazol-3-ium-2yl) methylene)-3-oxocyclobut-1-enolate-TiO₂ complex, 99-52.

Scheme 3: Anchoring (E)-2(Z)-3-(2-carbaxyethyl) benzo[d]thiazol-2(3H)-ylidene) methyl-4-((3-ethylbenzo[d]thiazol-3-ium-2yl) methylene)-3-oxocyclobut-1-enolate onto TiO₂

Experimental

TiO₂ was mixed with 0.5, 1, 1.5 and 2.0 wt% of the dye and the mixture dissolved in dichloromethane to form blue solution. The mixture was then stirred for 12 h under subdued light. The solvent was removed under vacuum to form a blue solid and one gram of each sample was prepared.

Characterization

Characterization of the dye was done by UV-vis absorption in DMSO and it displayed two absorption peaks at 347 and 656 nm as in the Figure 10.
Figure 10: UV-vis spectrum of 99-52 in DMSO.
2.3.5: 2-((7-(Diphenylamine)-9, 9-dihexyl-9H-fluoren-2yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5, 6-dicarboxylic acid-TiO$_2$ Complex, 99-90

Scheme 4: Anchoring 2-((7-(diphenlamino)-9, 9-dihexyl-9H-fluoren-2yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5, 6-dicarboxylic acid onto TiO$_2$.

Experimental

2-((7-(Diphenylamino)-9,9-dihexyl-9H-fluoren-2yl)methylene)-1,3-dioxo-2,3-dihydro-1H-indene-5,6-dicarboxylic acid, 0.5, 1, 1.5 and 2% by weight, was mixed with TiO$_2$ and then dissolved in dichloromethane (30mL) to form purple solution. The mixture was stirred at room temperature for 24 hrs. under subdued light. The solvent was removed under vacuum and two grams of each sample was prepared.

Characterization

UV-vis absorption was studied in DCM and the compound displayed an absorption maximum at 539nm as in Figure 11.
**Figure 11:** UV-vis spectrum of 99-90 in DCM.

**2.4: Synthesis of STC**

STC pellets were synthesized using an acid-catalyzed, solgel technique. TiO$_2$ was added to a tetraethylorthosilicate (TEOS, Fisher Scientific, reagent grade), water, and ethanol mixture with a volume ratio of 7:5:10. Nitric acid (1 N, Fisher Scientific, certified A.C.S.) while 3% hydrofluoric acid (Fisher Scientific, certified A.C.S.) was used to catalyze the reaction. The mixture was magnetically stirred for 30 min before gelation. The solution was transferred into assay plates and allowed to gel and age into cylindrical pellets for 48 h at room
temperature followed by 65 °C for another 48 h. The pellets were removed from the assay plates and transferred to Teflon containers covered with aluminum foil pierced with pin sized holes where they were dried at 103 °C for 18 h followed by 180 for 6 h.

The following dye-doped TiO₂ samples were used for this project. (Table 1). The numbers on the far left column represent the label for each dye and the percentage presents the doping dosage. TiO₂ was doped with various dyes at concentrations of 0.5, 1.0, and 1.5%. STC was synthesized with the dye-sensitized TiO₂ and surface characteristics were analyzed using a Quantachrome NOVA 2200e (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>0.5%</th>
<th>1.0%</th>
<th>1.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>99-26 (orange)</td>
<td>1.50 g</td>
<td>1.62 g</td>
<td>2.76 g</td>
</tr>
<tr>
<td>99-52 (blue)</td>
<td>0.81 g</td>
<td>0.83 g</td>
<td>0.72 g</td>
</tr>
<tr>
<td>99-43 (yellow)</td>
<td>1.80 g</td>
<td>1.83 g</td>
<td>1.86 g</td>
</tr>
<tr>
<td>99-90 (red)</td>
<td>1.79 g</td>
<td>1.60 g</td>
<td>1.37 g</td>
</tr>
</tbody>
</table>

**Table 1: Amounts of Doped STC**
Table 2: Surface characteristics of STC

<table>
<thead>
<tr>
<th></th>
<th>Surface Area (m²/g)</th>
<th>Pore Size (Å)</th>
<th>Pore Volume (cc/g)</th>
<th>BJH Volume (cc/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99-26 0.5%</td>
<td>801</td>
<td>27.36</td>
<td>0.6144</td>
<td>0.2175</td>
</tr>
<tr>
<td>99-26 1.0%</td>
<td>708</td>
<td>33.94</td>
<td>0.6010</td>
<td>0.3666</td>
</tr>
<tr>
<td>99-26 1.5%</td>
<td>778</td>
<td>34.91</td>
<td>0.6789</td>
<td>0.4490</td>
</tr>
<tr>
<td>99-52 0.5%</td>
<td>539</td>
<td>15.26</td>
<td>0.4108</td>
<td>0.1761</td>
</tr>
<tr>
<td>99-52 1.0%</td>
<td>654</td>
<td>15.16</td>
<td>0.4957</td>
<td>0.2164</td>
</tr>
<tr>
<td>99-52 1.5%</td>
<td>662</td>
<td>14.44</td>
<td>0.4774</td>
<td>0.1705</td>
</tr>
<tr>
<td>99-43 0.5%</td>
<td>558</td>
<td>15.89</td>
<td>0.4432</td>
<td>0.2057</td>
</tr>
<tr>
<td>99-43 1.0%</td>
<td>629</td>
<td>17.60</td>
<td>0.5531</td>
<td>0.2135</td>
</tr>
<tr>
<td>99-43 1.5%</td>
<td>446</td>
<td>19.44</td>
<td>0.4338</td>
<td>0.2760</td>
</tr>
<tr>
<td>99-90 0.5%</td>
<td>474</td>
<td>20.01</td>
<td>0.4742</td>
<td>0.3244</td>
</tr>
<tr>
<td>99-90 1.0%</td>
<td>671</td>
<td>16.65</td>
<td>0.5585</td>
<td>0.2989</td>
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<tr>
<td>99-90 1.5%</td>
<td>494</td>
<td>18.65</td>
<td>0.4604</td>
<td>0.2841</td>
</tr>
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</table>

2.4.1: Differences in STC

Varying amounts of doped TiO₂ samples contained various dyes, ranging from 0.7 grams to 1.8 grams. The STC recipe was adjusted based on the amount of available TiO₂. To keep consistency in the modification of the recipe, the limiting mass of STC was used for each experiment. This limiting mass was also used to produce the plain (no dye) STC. The lowest amount of STC produced from 1.7 grams of TiO₂ was approximately 5 grams of pellets. Ideally, 25 grams of STC is needed to fill up each reactor.

Most STC produced from doped TiO₂ resulted in extremely brittle pellets. Handling of the pellets over time caused them to crumble, decreasing the particles size and increasing the exposed surface area. Further time would be needed to identify the properties affecting the STC pellets. It is hypothesized that the dyes alter the
properties of titanium dioxide, mainly the density. The time to reach gelation varied between different batches of doped TiO$_2$, ranging from 5 to 9 h. The doped TiO$_2$ particles were not easily suspended in solution as compared to non-doped TiO$_2$. Therefore there was a non-uniform distribution of TiO$_2$ throughout the pellet during gelation; most of the titanium dioxide was concentrated at the bottom of each pellet. Additionally, TiO$_2$ doped with 2% dye were also provided, but 2 batches of 2% doped STC gelled much sooner than expected and had to be discarded. Therefore, only the 0.5-1.5% batches were used for experiments.

2.4.2 Experimental

The setup described in Figure 12 was used to test the oxidation of methanol in an air stream using STC doped with various dyes. 100 mL of deionized water in a bubbler was spiked with 0.922 mL of methanol. The temperature was maintained at 25 °C. The contaminated airstream was connected to a glass reactor containing 5 grams of STC mixed with 50 g of glass beads. 5 g of STC was the limiting amount produced based on the mass of received TiO$_2$. Ideally, 25 g of STC would be needed.

A 12-inch, T5 fluorescent tube bulb was placed at the center of the glass reactor. Illumination began once the air stream was turned on. After treatment, the airstream was drawn into a chilled impinger containing 60 mL of deionized water at a temperature of 4 °C. A 2 mL sample was collected from the chilled impinger at time intervals of 30, 60, 120, and 240 min after the start of illumination and analyzed using a Perkin Elmer gas chromatograph (GC). Standard curves were analyzed every week to verify the accuracy of the GC and discount the
possibility of errors from the analytical equipment.

Figure 12: Experimental Setup for the photocatalytic removal of methanol from air.

2.4.3: Initial Concentration

Various test runs were performed to determine a suitable initial concentration. Experimental runs (with no photocatalytic treatment) were performed with the suggested initial concentration of 0.2 ppm in air. The contaminated airstream was purged in DI water using a chilled impinger (4 °C) at a rate of 500 mL/min. A 2 mL water sample was collected and analyzed using the GC. A concentration of 0.2 ppm of methanol in air was not sufficient for a significant detectable level of methanol in the GC sample. The airstream concentration was increased incrementally and tested to determine a suitable initial concentration. It was determined that 1 ppm of methanol was sufficient for detection by the GC for the available
temperature, flow rate and instrumentation.

Five runs were performed using an empty reactor in order to measure the initial concentration of methanol in the chilled impinger. Samples were taken at 30, 60, 120, and 240 min and the average was taken to determine the initial concentration curve. Removal was determined by calculating the concentration after treatment divided by the concentration without treatment at the same time interval (C/C_0). Error bars represent the range of values.

2.4.4: Dark Reactions

Dark reactions were performed to measure the removal of methanol due to adsorption. Removal was negligible for all samples, ranging from no removal to 5% removal. The lack of adsorption may be attributed to the small amount of STC used in the reactor (5 g). It is suggested to repeat the dark experiments with enough STC to fill the reactor (approximately 25 g).

2.5 Results

Plain STC (undoped with dye) was tested under visible light and the results are presented in Figure 13. Since the reactor was not completely filled with STC, the larger error bars may be accounted for by the non-homogeneous bed structure between different runs. For loosely packed beds, with larger voids compared to densely packed beds, there is non-ideal collision between gas particles causing uneven mixing of the fluid. The gas tends to accumulate in the voids and preventing exposure with STC particles.

Most duplicate runs produced drastically higher removal than the first set of
experiments. In some cases, the first set of experiments resulted in a higher concentration of methanol compared to the initial concentration. It is hypothesized that excess functional groups from the dyes may be oxidized to methanol or other simple organic compounds with a similar peak than methanol when analyzed in the GC. These interfering compounds are oxidized during the first run and removal improves significantly for the second set of experiments when there are no other contaminants competing for oxidation. Additionally, the brittle pellets were disintegrating over time resulting in an increase of surface area, which may also account for the increased removal in the later experiments.

Figure 13: Methanol removal from air using regular STC $C_0 = 1$ ppm.

Figures 14 through 16 present the results for the STC doped with dye 99-26 (orange).
lowest concentration of 0.5% (Figure 14), removal was somewhat consistent compared to results for 1% and 1.5% doping (Figure 15 and Figure 16 respectively). 0.5% doping represents the lowest concentration of dye for this sample set, therefore the additional functional groups accompanied with the dye are not sufficient to interfere with the oxidation of methanol. Experiments with the 1% doped STC generated results higher than the initial concentration for all time intervals (Figure 15). It is hypothesized that 1.0% dye doping is sufficient to furnish excess functional groups that interfere with the reduction of methanol. These functional groups may be oxidized to methanol or a compound similar to methanol increasing the concentration detected by the GC for the first set of experiments. The duplicate experiments resulted in better removal of methanol, indicating that the excess functional groups had been oxidized in the first run and were no longer present to interfere with the photocatalytic oxidation of methanol. However, 1.5% doping of dye (Figure 16) did not result in detection of methanol above the initial concentration, even though a higher concentration of dye was present. It is important to note that the first set of experiments did result in much smaller removal than the duplicate experiments and the error between duplicates was the largest for the set, therefore there was some interference in removal for the first experiments. The non-homogenous bed structure may have also contributed to the large error bars as described earlier.
Figure 14: Methanol removal using STC doped with 0.5% 99-26.

Figure 15: Methanol removal using STC doped with 1% 99-26.
STC doped with 99-52 (blue) was the worst performing sample as seen in Figure 17 through Figure 19. These samples produced the largest error bars and highest methanol concentrations above the initial concentration. Only a small amount, 1.7 g, of TiO₂ was available to produce these pellets requiring in a drastic change in the recipe. The resulting pellets were extremely brittle. Transfers in and out of the reactor caused crumbling of the pellets, increasing the exposed surface area, which may have accounted for the higher removal for the duplicate runs. Figure 17 portrays results using STC with the lowest concentration of dye, 0.5 %, for this set. Removal of methanol was hindered for the first set of experiments resulting in concentrations above the initial concentration of methanol for all time intervals. The results for 1.0% concentration of dye were quite different, although the amount of dye present, and therefore functional groups, was
greater. The average removal was greater than 50% for all time intervals except for 30 minutes (Figure 18). Experiments using the 1.5% doped STC generated the worst data set for this group (Figure 19). The concentration of methanol measured for the first run was well over the initial concentration for all time intervals. However, when duplicate experiments were conducted, the concentration of methanol was under the detection limit of the GC. It is difficult to observe a trend with such large error bars, but it is hypothesized that the dye may be improving photocatalysis. The lower end of the error bars approaches the detection limit of the GC as the concentration of dye increases.

Hanging functional groups, not tightly doped into the STC matrix, contribute to increase in methanol detection by the GC for the first set of experiments. Once these functional groups were oxidized and removed, the efficiency of photocatalytic oxidation of methanol increases, powered by the dye embedded in the STC matrix.
**Figure 17:** Methanol removal from air using STC doped 0.5% 99-52.

**Figure 18:** Methanol removal from air using STC doped 1.0% 99-52.
**Figure 19:** Methanol removal from air using STC doped 1.5% 99-52.

STC doped with 99-43 (yellow) performed as expected, with increasing removal as the concentration of dye increased as seen in *Figure 20* through *Figure 22*. Experiments using 0.5% (*Figure 20*) resulted in the largest range of results for the group, although there was still a recognizable trend. Removal was quite high for 1.0% doped STC (*Figure 21*). All runs using a concentration of 1.5% resulted in methanol concentrations below the detection limit of the GC (*Figure 22*). There were no samples resulting in higher methanol concentrations than the initial concentration for the entire group. Based on these results, 99-43 is a suitable compound used for improving photocatalytic oxidation under visible light, with increasing removal as the concentration of dye increases.
**Figure 20:** Methanol removal from air using STC doped with 0.5% 99-43.

**Figure 21:** Methanol removal from air using STC doped with 1% 99-43.
The last sample, 99-90, also produced positive results. Removal was high for all samples, ranging in a C/Co of 0.5 to 0.99. STC doped with 0.5% dye demonstrated effective oxidation of methanol with little variation between duplicate runs (Figure 23). Discrepancy between different runs increased slightly for the 1.0% set, but results were still positive with a noticeable trend (Figure 24). The 1.5% set resulted in methanol concentrations at or below the detection limit of the GC for all time intervals (Figure 25). 99-90 is another dye appropriate for the efficient oxidation of methanol under visible light. Functional group interference was not observed and the initial concentration of methanol never increased about the initial concentration.

Figure 22: Methanol removal from air using STC doped with 1.5% 99-43.
**Figure 23:** Methanol removal from air using STC doped with 0.5% 99-90.

**Figure 24:** Methanol removal from air using STC doped with 1% 99-90.
Figure 25: Methanol removal from air using STC doped with 1.5% 99-90.

### 2.6 Conclusions

Positive conclusions were found for two samples based on the data above. Samples 99-43 and 99-90 produced good results with high methanol removal and consistency between most duplicate points. Experiments with samples 99-26 and 99-52 generated inconsistent results with large error bars and multiple samples above the initial concentration. It is hypothesized that these errors are due to excess functional groups from the dyes that undergo photocatalytic oxidation producing methanol or other simple organics detected via gas chromatography. Additionally, the STC recipe was modified since only a small amount of doped TiO₂ was available. There was not enough TiO₂ to produce “practice” batches in order to confirm that the change in recipe did not affect the final product. Many pellets produced were discolored and extremely brittle.
The following steps are recommended for future work:

- Provide larger amounts of doped TiO$_2$ in order to make several batches of STC. Additionally, excess STC should be available to completely fill the reactor creating a homogenous bed structure and preventing non-ideal mixing of the gas.

- Study the effect of the dyes on the physical and chemical properties of TiO$_2$ (changes in density, surface area, functional groups, pH).

- Expose the doped STC to light for an extended period of time before running experiments in order to remove interfering functional groups. Most experiments resulted in better removal for the duplicate runs once the STC pellets had already been exposed to one round of photocatalysis.

These preliminary results indicate that dye-sensitized TiO$_2$, particularly using fluorenyl-based dye sensitizers, may increase the oxidation of organic compounds under visible light. Additional tests must be conducted to understand the source of error and limit the variances between duplicate experiments. Ideally, at least triplicate experiments should be conducted to understand the source of error and reduce these errors.
CHAPTER 3: FACILE PREPARATION OF FLUORESCENT ORGANIC NANOPARTICLES AND THEIR APPLICATION IN CELL IMAGING

3.1 Abstract

This chapter reports a simple and convenient method for the preparation of water-dispersed fluorescent organic nanoparticles (FONs) that are based on 4H-pyran-4-ylidene chromophore by the reprecipitation method. The surface of the NPs was then modified using a naturally occurring surfactant, lecithin, to increase the fluorescence, colloidal stability and size of the nanoparticles. The FONs were then successfully used for cell imaging using the HCT-116 cell line.

3.2 Introduction

Organic nanoparticles are solid particles composed of organic compounds ranging in diameter from 10 nm to <1 µM. Fluorescent cellular probes with red and near infrared (R/NIR, >600 nm) emission are preferred for biological applications because of the low optical absorption and autofluorescence of biological media in this wavelength region. Most of the commonly used probes include organic dyes, fluorescent proteins, and fluorescent inorganic nanoparticles. Most of these, however, are intrinsically hydrophobic, susceptible to photobleaching, and toxic, thus limiting their application in bioimaging applications. Approaches to capture hydrophobic molecules in water-dispersed nanoparticles have been developed and they include the use of dye-doped silica NPs, liposomes, calcium phosphate NPs and polymeric micelles. The drawbacks with the strategies are that they are difficult to synthesize, time consuming, and their preparation may run into days. Fluorescent proteins are
relatively expensive, have low absorptivity, and possess low photobleaching thresholds. Inorganic nanoparticles or quantum dots are very popular because their luminescence properties can be tuned in the visible region by adjusting their size. They are however non-biodegradable and toxic to living organisms as most contain troublesome heavy metals. \cite{1-2,5,7-9}

Organic nanoparticles have received attention lately due to their attractive features; they are biodegradable, biocompatibility, flexible synthetic approaches and appropriate functionalities. \cite{8,10-13} Organic nanoparticles are more stable in presence of light and oxygen when compared to isolated molecules thus more suitable in applications that involve the use of light.\cite{5-6,8,11-12,14-15}

Fluorescent nanoparticles are showing promise for use in biomedical applications like imaging and in drug delivery \cite{1-2,16,17} In addition, the shifting of the luminescence to the visible red and to near IR is desirable for bioimaging purposes as it provides better imaging conditions and reduces scattering in tissues. Luminescent nanoparticles have to satisfy the following conditions: high brightness, photostable, colloidal stability (as their agglomeration in microparticles can be lethal \textit{in vivo}), and biocompatibility.\cite{10,13,16,18}

Fluorescent nanoparticles have been successfully prepared using fluorescent conjugated polymers, polydopamine nanoparticles, and aggregation induced emission or aggregation induced enhancement materials. Organic $\pi$-conjugated chromophores have been reported to form spherical-shaped nanoparticles by spontaneous aggregation in water \cite{1,7,19} Surfactants, e.g., pluronic have been successfully used to modify the surface of nanoparticles to impart water dispersibility, increase colloidal stability, biocompatibility and to improve the photoluminescent properties for use in bioimaging. \cite{1,2,19} Use of some of these surfactants however is limited in
biological applications due to the potential toxicity or environmental degradability of many synthetic surfactants e.g. CTAB or SDS. Research in the use of natural surfactants is gaining popularity, and recently Zhang et al. used a natural surfactant, lecithin to modify organic nanoparticles that were successfully used for cell imaging. Other techniques that have been employed to increase the colloidal stability of organic nanoparticles involve the use of bulky groups on the molecular units.

The 4H-pyran-4-ylidene structures have attracted a fair amount of attention because of their interesting optical properties. This moiety can function as an electron acceptor group with good photochemical stability. Substitution can take place at positions 2 and 6, generating a D-π-A or D-π-A-π-D structure. 4H-Pyran-4-ylidene derivatives are widely used in organic light-emitting diodes and, to some degree, in fluorescence bioimaging.

The strategy employed in the synthesis of fluorescent organic nanoparticles (FON) for this project is the reprecipitation method; a simple and convenient method for the preparation of water-dispersed FONs, and is based on the self-assembly of the active organic dye itself, yielding FONs that are dye-concentrated. The method entails dissolving an organic dye in a hydrophilic solvent (specifically THF for this work), and the solution is injected into water which acts as a poor medium (nonsolvent) for the organic compound resulting in precipitation owing to the insolubility in water to give a nanocrystal dispersion the FONs. The great disparity between the solubility of the compound in the two solvents and the good compatibility of the two solvents are the success of this method. The molecules are exposed to the poor solvent environment in a very short time thus inducing the nucleation and the growth of the molecules to
The surface of the organic nanoparticles was then modified using lecithin to accentuate their size, fluorescence, and colloidal stability.

3.3 Experimental

All reagents were purchased from commercial suppliers and used as received unless otherwise noted. THF was purified by distillation over sodium and benzophenone under nitrogen before use. CH$_3$CN was dried over calcium hydride. Reactions were done under a dry nitrogen atmosphere using standard vacuum line techniques. $^1$H and $^{13}$C spectra were carried on a Bruker Avance Spectrometer 400 MHz for $^1$H (referenced to TMS at $\delta=0.0$ ppm) and 101 MHz for $^{13}$C (referenced to CDCl$_3$ at $\delta=77.0$ ppm). High resolution mass spectrometry (HR-MS) analysis was performed in the Department of Chemistry, University of Florida, Gainesville, FL. Particle size of the FONs was analyzed by the light-scattering method using a Zetasizer NanoZS90 (Malvern Instruments).

3.3.1 Photophysical characterization

Linear absorption and fluorescence spectra were recorded using an Agilent 8453, and a PTI QuantaMaster spectrofluorimeter, respectively. The compounds were dissolved in spectroscopic grade solvents, and diluted to $\sim$ 10$^{-6}$ M in 10 mm quartz cuvettes for measurements. Fluorescence spectra were collected using a red-sensitive PMT and an InGaAs near-infrared detector, both corrected for the responsivities of the detector. Fluorescence quantum yields were calculated via a relative method, using 9, 10-diphenylantracene ($\Phi_f = 0.95$) as a reference. Excitation anisotropy measurements were performed in viscous solvents (e.g., silicone oil) to impede the molecular rotational relaxations.
Nonlinear two-photon absorption (2PA) measurements were collected using the open aperture Z-scan method at room temperature. Samples ($\approx 10^{-2}$ M) were prepared in DCM. This technique utilized a femtosecond laser system (Coherent Inc.), wherein the second harmonic output of a Verdi-10 CW Nd: YAG laser seeded a Mira 900 Ti: sapphire laser (repetition rate, $f = 76$ MHz; average power = 1.1 W; pulse duration, $\tau_p \approx 200$ fs), which in turn pumped a Legend Elite USP ($f = 1$kHz; energy $\approx 3.6$ mJ pulse$^{-1}$; $\tau_p \approx 100$ fs). This amplified output was directed into two optical parametric amplifiers (OPeRA Solo (OPA), Coherent Inc.), allowing tuning from 0.24 – 20 $\mu$m. The output of the first of these OPAs was used for the 2PA measurements.

3.3.2 Cell imaging

Organic nanoparticles 1a and 1b were incubated with HCT-116 cells. The cells were seeded on poly-D-lysine coated coverslips at the concentration of $4 \times 10^4$ cells/well and incubated for 48 h. Dyes were diluted to 10 $\mu$M from stock solutions and added to cells. 1 h later, cells were washed with PBS and fixed with 4% formaldehyde solution. NaBH$_4$ solution was then applied twice at 1 mg/mL to eliminate autofluorescence. Coverslips were then mounted with ProLong$^\text{®}$ Gold antifade reagent (Invitrogen, USA). Images were taken with an Olympus IX-81 DSU microscope.

3.4 Synthesis

6-bromohexanoic acid was esterified by dissolving it in methanol in the presence of a catalytic amount of concentrated H$_2$SO$_4$ and refluxing the mixture overnight to yield methyl 6-bromohexanoate, 5 as in the reference literature.$^{27}$ Methyl 6-(4-formyl-2, 6-dimethoxyphenoxy) hexanoate, 6, was synthesized by adapting literature $^{28}$ and subsequent ester cleavage was done to yield 6-(4-formyl-2, 6-dimethoxyphenoxy) hexanoic acid, 7, as in literature.$^{29}$ 7-Bromo-9,9-
dihexyl-9H-fluorene-2-carbaldehyde, 2, was synthesized by lithiation of 2, 7-dibromo-9, 9-dihexyl-9H-fluorene with a stoichiometric amount of n-BuLi, then reaction with dimethylformamide (DMF), and the reaction mixture was then subjected to acid hydrolysis. Buchwald-Hartwig amination coupling was employed for the arylamination of 7-bromo-9,9-dihexyl-9H-fluorene-2-carbaldehyde using Pd catalyst, Cs₂CO₃ to yield 7-(diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde, 3 as previously reported.³⁰

**Scheme 5:** Synthesis of the Chromophore; 6-((4-(E)-2-(4-(dicyanomethylene))-6-((E)-2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl) vinyl)-4H-pyran-2-yl) vinyl)-2, 6-dimethoxyphenoxy) hexanoic acid, 1.
The two key steps that followed involved the Knoevenagel condensation between 7-(diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde, 3 and 2-(2,6-dimethyl-4H-pyran-4-ylidene)malononitrile to yield (E)-2-(2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)-6-methyl-4H-pyran-4-ylidene)malononitrile, 4. Reaction between the aforementioned product and 6-(4-formyl-2,6-dimethoxyphenoxy)hexanoic acid, 7 furnished 6-(4-((E)-2-(4-(dicyanomethylene)-6-((E)-2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)-4H-pyran-2-yl)vinyl)-2,6-dimethoxyphenoxy)hexanoic acid, 1.

To prepare the fluorescent organic nanoparticles, dye 1, was dissolved in THF and then added to water and aqueous solution of Lecithin by modifying the reference literatures to form hydrophilic fluorescent organic nanoparticles. 2,16


\(n\text{-BuLi (12.5mL, 1.6M in hexane) was added dropwise to a dry THF solution (50 mL) of 2, 7-dibromo-9, 9-dihexyl-9H-fluorene (10 g, 20.32 mmol) under nitrogen and at } -78^0\text{C. 2.4 mL of DMF was then added to the solution after stirring for 1 h. The solution was stirred for 2 h. and the solution then brought to room temperature and quenched with distilled H}_2\text{O till the pH was neutral. The solution was then extracted three times with toluene, dried with anhydrous MgSO}_4\text{ and the solvent removed under reduced pressure to give a yellow green solution. The crude product was purified by column chromatography using silica gel (hexanes:ethyl acetate 7:3) to yield a colorless oil (6.16 g, 69%).}^{1}\text{H NMR (400 MHz, Chloroform-}d\text{) }\delta 10.04 (s, 1H), 8.03 – 7.74 (m, 3H), 7.77 – 7.53 (m, 1H), 7.60 – 7.33 (m, 2H), 1.97 (qdd, } J = 13.5, 10.9, 5.5 \text{ Hz, 4H), 1.36 – 0.87 (m, 12H), 0.74 (t, } J = 7.1 \text{ Hz, 6H), 0.55 (ddt, } J = 17.9, 13.8, 6.5 \text{ Hz, 4H).}^{13}\text{C NMR}}\)
Synthesis of 7-(diphenylamino)-9, 9-dihexyl-9H-fluorene-2-carbaldehyde, 3.

A mixture of 7-bromo-9,9-dihexyl-9H-fluorene-2-carbaldehyde (5 g, 11.6 mmol), diphenylamine (2.88 g, 17.03 mmol), Pd(OAC)$_2$ (0.061 g, 0.27 mmol), P(tBu)$_3$ (0.121 g, 0.60 mmol) and Cs$_2$CO$_3$ (5.53 g 16.98 mmol) in dry toluene (40 mL) was degassed for 15 min. The mixture was then stirred and heated at 120 °C for 48 h in a screw capped vial. The reaction mixture was then cooled to room temperature and filtered to form a yellow-brownish oil which was purified using column chromatography (hexanes:CH$_2$Cl$_2$ 1:1) to yield yellow oil (4.86 g, 81%). $^1$H NMR (400 MHz, Chloroform-d) 0.42 – 0.72 (4 H, m), 0.70 – 0.91 (7 H, t, J 7.1), 0.89 – 1.21 (13 H, m), 1.85 – 1.92 (2 H, m), 1.91 – 1.98 (2 H, m), 7.00 – 7.11 (3 H, s), 7.10 – 7.23 (5 H, m), 7.22 – 7.38 (5 H, m), 7.57 – 7.69 (1 H, d, J 8.2), 7.68 – 7.77 (1 H, d, J 8.1), 7.77 – 7.91 (2 H, m), 10.00 – 10.09 (1 H, s). $^{13}$C NMR (101 MHz, CDCl$_3$): δ 192.65, 154.07, 151.68, 149.25, 148.03, 147.90, 134.88, 134.43, 131.18, 129.67, 124.76, 123.49, 123.25, 123.18, 122.07, 119.50, 118.62, 55.54, 40.42, 37.48, 33.13, 32.31, 31.87, 31.62, 30.42, 30.09, 30.05, 29.92, 29.75, 27.47, 24.14, 23.08, 23.05, 22.91, 20.12, 14.50, 14.39.

Synthesis of (E)-2-(2-(7-(diphenylamino)-9, 9-dihexyl-9H-fluoren-2-yl) vinyl)-6-methyl-4H-pyrano-4-ylidene) malononitrile, 4.

7-(Diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde (0.2 g, 0.37 mmol) was mixed with the pyran(0.255 g, 1.48 mmol) and dissolved in dry CH$_3$CN(100 mL). Piperidine(0.15 mL) was added and the reaction mixture refluxed for 72 h to yield reddish solution. The solvent was
removed under reduced pressure and the crude product purified on silica gel using hexanes/ethyl acetate 7:3 to yield brown solid (0.12 g, 48%). $^1$H NMR (400 MHz, Chloroform-d) δ 0.36 – 0.86 (10 H, m), 0.84 – 1.26 (12 H, m), 1.61 – 1.94 (4 H, tt, J 13.2, 6.8), 2.16 – 2.66 (3 H, s), 4.98 – 5.39 (1 H, d, J 0.7), 6.44 – 6.51 (1 H, d, J 1.5), 6.61 – 6.67 (1 H, m), 6.67 – 6.72 (0 H, s), 6.91 – 7.00 (3 H, td, J 8.4, 8.0, 1.7), 7.01 – 7.09 (4 H, m), 7.13 – 7.24 (6 H, m), 7.34 – 7.39 (1 H, s), 7.39 – 7.45 (1 H, d, J 8.5), 7.45 – 7.52 (1 H, m), 7.52 – 7.59 (1 H, d, J 7.9). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 162.31, 159.96, 156.74, 153.25, 151.91, 148.55, 148.14, 144.23, 139.29, 135.20, 132.82, 129.63, 127.88, 124.56, 123.53, 123.29, 122.20, 121.38, 119.96, 119.01, 116.94, 115.57, 107.17, 106.80, 59.24, 55.49, 40.58, 31.89, 29.98, 24.17, 22.93, 20.38, 14.42.

**Synthesis of methyl 6-bromohexanoate, 5.**

6-Bromohexanoic acid (0.3 g, 1.53 mmol) was dissolved in methanol and a catalytic amount of H$_2$SO$_4$ (0.03 mL) was added. The reaction mixture was refluxed overnight. Upon completion of the reaction, methanol was removed and the crude product washed with aqueous NaHCO$_3$, brine and extracted with diethyl ether to yield yellow oil (0.29 g, 91%) and the product used without any further purification. $^1$H NMR (400 MHz, Chloroform-d) δ 3.70 – 3.46 (m, 3H), 3.43 – 3.13 (m, 2H), 2.35 – 2.09 (m, 2H), 1.92 – 1.67 (m, 2H), 1.57 (ddt, J = 10.0, 7.4, 4.1 Hz, 2H), 1.39 (tdd, J = 8.4, 6.6, 2.5 Hz, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 174.14, 51.82, 34.10, 33.84, 33.76, 32.70, 27.95, 24.37, 24.21.

**Synthesis of methyl 6-(4-formyl-2, 6-dimethoxyphenoxy) hexanoate, 6.**

A two-necked pear shape flask was charged with 3, 5-dimethoxy-4-hydroxybenzaldehyde (1.0 g, 5.47 mmol), bromoalkanoic acid (1.25 g, 5.97 mmol), and DMF (17 mL). The flask was flushed
with nitrogen and the reaction mixture heated at 85 °C for 30 min. Anhydrous K₂CO₃ (1.33 g, 9.62 mmol) was added in portions and the reaction further heated at 85 °C overnight. The mixture was let to cool to room temperature and then poured into 100 mL of distilled water and the product extracted with CH₂Cl₂ to form yellow oil. The crude product was purified by column chromatography using hexanes/ethyl acetate 1:1 to give (1.3 g, 80%) of the product. ¹H NMR (300 MHz, Chloroform-d) δ 9.97 – 9.55 (m, 1H), 7.23 – 6.84 (m, 2H), 4.11 – 3.87 (m, 2H), 3.91 – 3.69 (m, 7H), 3.69 – 3.43 (m, 4H), 2.95 – 2.65 (m, 2H), 2.23 (t, J = 7.4 Hz, 2H), 1.82 – 1.09 (m, 7H) ¹³C NMR (101 MHz, CDCl₃) δ 13.90, 13.93, 17.85, 17.89, 20.23, 24.89, 24.94, 24.99, 25.64, 25.68, 27.15, 27.19, 27.57, 27.83, 27.96, 27.99, 30.01, 30.05, 30.42, 31.30, 34.30, 50.40, 51.64, 53.83, 55.64, 56.43, 56.51, 73.43, 73.52, 106.05, 106.99, 107.03, 124.93, 130.39, 131.90, 143.23, 143.84, 153.94, 153.99, 154.13, 154.17, 174.35, 176.40, 191.39.

Synthesis of 6-(4-formyl-2, 6-dimethoxyphenoxy) hexanoic acid, 7.

A mixture of methyl 6-(4-formyl-2, 6-dimethoxyphenoxy) hexanoate (0.15 g, 0.48 mmol) and (nBu₃Sn)₂O (0.57 g, 0.96 mmol) in benzene (2.5 mL) was heated at reflux and after 24 h. HCl (0.5 M, 2 mL) was added. The organic phase was washed with 5% NaHCO₃, extracted with ethyl acetate and dried with Na₂SO₄. The crude product was purified on silica gel using hexanes/ethyl acetate 7:3 to yield the product (0.1g, 71%). ¹H NMR (400 MHz, Chloroform-d) 1.29 – 1.86 (10 H, m), 2.01 – 2.46 (2 H, tt, J 7.8, 3.9), 3.66 – 3.89 (6 H, m), 3.89 – 4.16 (2 H, td, J 6.6, 3.8), 6.91 – 7.13 (2 H, m), 9.61 – 9.91 (1 H, m).¹³C NMR (101 MHz, CDCl₃) δ 191.48, 179.74, 174.48, 154.21, 143.33, 131.94, 131.91, 107.10, 73.66, 73.56, 56.54, 51.79, 34.84, 34.35, 30.13, 30.08, 28.26, 28.16, 28.05, 27.65, 27.33, 27.00, 26.59, 25.78, 25.72, 25.54, 24.98, 18.55, 18.48, 16.77, 15.06, 14.98, 13.96, 13.86.
Synthesis of 6-(4-((E)-2-(4-(dicyanomethylene)-6-((E)-2-(7-(diphenylamino)-9, 9-dihexyl-9H-fluoren-2-yl) vinyl)-4H-pyran-2-yl) vinyl)-2, 6-dimethoxyphenoxy) hexanoic acid, 1. (E)-2-(2-(7-(Diphenylamino)-9, 9-dihexyl-9H-fluoren-2-yl) vinyl)-6-methyl-4H-pyran-4-ylidene) malononitrile, 4 (0.12 g, 0.179 mmol) and 6-(4-formyl-2, 6-dimethoxyphenoxy) hexanoic acid, 7 (0.064 g, 0.215 mmol) were dissolved in distilled CH$_3$CN (20mL). Piperidine (0.1 mL) was then added. The reaction mixture refluxed at 130 0C for 5 d. The crude product was purified on silica gel using methanol/ethyl acetate 1:9 to yield red solid (45 mg, 26%). $^1$H NMR (400 MHz, Chloroform-d) 0.56 – 1.00 (10 H, m), 0.99 – 1.43 (18 H, m), 1.51 – 1.67 (3 H, qd, J 8.5, 7.3, 2.2), 1.67 – 1.79 (2 H, q, J 7.6), 1.76 – 1.89 (3 H, dt, J 14.0, 6.6), 1.85 – 2.01 (4 H, m), 2.31 – 2.53 (2 H, t, J 7.5), 3.87 – 4.02 (6 H, s), 4.00 – 4.18 (3 H, t, J 6.5), 6.71 – 6.79 (2 H, m), 6.77 – 6.83 (1 H, s), 6.81 – 6.88 (2 H, d, J 5.5), 7.01 – 7.10 (3 H, m), 7.08 – 7.21 (5 H, m), 7.23 – 7.34 (5 H, m), 7.43 – 7.50 (1 H, s), 7.48 – 7.61 (4 H, m), 7.58 – 7.64 (1 H, d, J 4.4), 7.60 – 7.70 (2 H, d, J 8.1). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 179.65, 159.17, 158.68, 156.18, 154.27, 153.24, 151.99, 148.62, 148.14, 144.31, 140.14, 139.22, 138.22, 135.15, 132.91, 130.42, 129.63, 128.19, 124.57, 123.53, 123.31, 121.91, 121.39, 119.94, 119.01, 118.31, 117.45, 115.68, 107.40, 107.16, 105.61, 73.65, 70.92, 59.65, 56.70, 55.53, 40.61, 34.28, 31.89, 30.14, 30.07, 29.97, 29.72, 25.79, 24.86, 24.18, 22.91, 14.48, 14.40 HRMS ( m/z) [M+H] $^+$ calcd for C$_{63}$H$_{67}$N$_3$O$_6$ 961.5030; Found 961.5046.
3.5 Results and Discussion

3.5.1 Photophysical Properties

Compound 1 was readily soluble in a variety of organic solvents and fluorescent in some specifically toluene, tetrahydrofuran and dichloromethane. The chromophore displayed an intense and broad absorption band in the near UV-visible region with two distinct peaks, the main one at 445 nm and the second at 314 nm. The compound displayed positive solvachromism in the emission spectra but negligible shift in the absorption spectra. (Figure 26) The large stoke shift in CHCl$_2$ is ascribed to strong solvent-solute-dipole-dipole interactions due to large dipole moments and orientational polarizability in the solvent. The fluorescence quantum yield of the chromophore in less polar solvents, e.g., toluene, was 0.31, THF 0.15 and decreased to 0.03 in more polar solvent, e.g., CHCl$_2$ (Table 3). The excitation anisotropy is correlated with the spectral position of the various electronic transitions and is used to estimate the position of 2PA allowed transitions. The electronic transitions locations were revealed to be between 300 and 550 nm.
Figure 26: Absorption (solid lines) and emission (dashed lines) spectra in toluene (black), DCM (red), excitation anisotropy in silicone oil (dark green), and two-photon absorption spectrum in DCM (blue points) for the chromophore 1.

Table 3: Absorption and emission of 1 in Tol, THF and DCM, stoke shifts and quantum yield.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$\lambda_{\text{max}}^{\text{abs}}$ $^a$</th>
<th>$\lambda_{\text{max}}^{\text{em}}$ $^a$</th>
<th>$\Delta \lambda$ $^b$</th>
<th>$\Phi_f$ $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>/nm</td>
<td>/nm</td>
<td>/nm</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Toluene</td>
<td>449</td>
<td>591</td>
<td>142</td>
<td>0.31</td>
</tr>
<tr>
<td>1</td>
<td>DCM</td>
<td>445</td>
<td>767</td>
<td>322</td>
<td>0.03</td>
</tr>
<tr>
<td>1</td>
<td>THF</td>
<td>445</td>
<td>703</td>
<td>258</td>
<td>0.15</td>
</tr>
</tbody>
</table>

$^a$ Absorption and emission maxima ±1 nm; $^b$ Stokes shift ±2 nm; $^c$ fluorescence quantum yields ±10%

Two photon absorption band was observed in the 700 to 1100 nm range with the maxima slightly above 800 nm, close to the linear absorption maximum at ca. 445 nm. The 2PA maxima
is around 1000 nm of 150-200 GM rising from a region (700-900 nm) undulating around 100 GM. The shorter wavelength absorption allowed measurements at shorter wavelengths (<700 nm), where the determined cross sections rise to >500 GM, though a peak could not be resolved due to encroaching linear absorption (Figure 26).

3.5.2 Fluorescent Organic Nanoparticles (FONs) Preparation

![Scheme 6: Schematic representation of the preparation of hydrophilic Fluorescent Organic Nanoparticles (FONs), 1a without lecithin, 1b, modified with Lecithin.](image-url)
THF dye solution (0.1 mL, 1 mM) was added dropwise into 9.9 mL of deionized H₂O to form a yellow solution. The solution was stirred vigorously for 20 min. To prepare the surfactant modified FONs, Lecithin (2.5 mg) was dissolved in 99 mL of distilled water. THF dye solution (1 mL, 1 mM) was added dropwise while the solution was stirred vigorously. Orange solution formed was then stirred for 20 min. This is depicted in Scheme 6.

3.5.3 Nanoparticle size

Dynamic light scattering (DLS) was used to determine the unimodal distribution of the FONs. In the absence of lecithin, the average size of the FONs was around 30 nm (Figure 27, a) and on modifying with Lecithin, the size almost doubled (Figure 27, b) supporting the fact that there was an interaction between the chromophore and the surfactant. The fluorescent nanoparticles are of good water dispensability, and little aggregation was observed even after one month from their day of preparation.

![Figure 27: Size of the FONs (1a) Without Lecithin (1b) Modified with Lecithin](image)

3.5.4 Fluorescence Spectra

The UV absorption spectra of the FONs dispersed in water indicated a maximum absorption peak located at ~450 nm indicating that the encapsulation of the chromophore in organic nanoparticles had minimal effect on the $\lambda_{\text{max}}^{\text{abs}}$. There is minimal difference between the
two sets of nanoparticles as evident from the absorption spectrum save for a rise seen at short wavelength in the spectrum which is attributable to the presence of the lecithin in solution which produces light scattering effect suggesting the presence of nanoparticles.\(^{15}\)\(\text{Figure 28}\) There’s a notable difference in the emission spectra with the FONs with lecithin generating a broader spectrum and increasing the fluorescence quantum yield \(\Phi_f\) from 3\% to 5\%. \(\text{Table 4}\)

The maximum emission wavelength is red-shifted to 648 nm and 662 nm respectively generating a large stoke shift in both cases. These fluorescent properties are greatly beneficial for application in cell imaging applications. The formation of the nanoparticles with lecithin is attributed to the strong interaction between the hydrophobic part of the chromophore and the hydrophobic segments of lecithin as the hydrophilic segment of lecithin were on the sphere rendering the nanoparticles water dispersible.

**Table 4: Absorption and emission, Stoke’s shift and Quantum yield for the FONs, without lecithin 1a and with lecithin 1b**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>(\lambda_{max}^{abs}) (^{a})/nm</th>
<th>(\lambda_{max}^{em}) (^{a})/nm</th>
<th>(\Delta\lambda) (^{b})/nm</th>
<th>(\Phi_f) (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>H(_2)O</td>
<td>449</td>
<td>648</td>
<td>199</td>
<td>0.03</td>
</tr>
<tr>
<td>1b</td>
<td>H(_2)O</td>
<td>450</td>
<td>662</td>
<td>212</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^{a}\) Absorption and emission maxima ±1 nm; \(^{b}\) Stokes shift ±2 nm; \(^{c}\) fluorescence quantum yields ±10\%
Figure 28: Absorption (solid lines) and emission (dashed lines) spectra for 3 in organic nanoparticles in water without, 1a (black), and with, 1b (red) lecithin added.

3.6. Cell imaging applications of the Nanoparticles

The cell imaging application of the nanoparticles was explored by incubating the FONs with HCT-116 cells. Images of the cells incubated with 10 μM of the nanoparticles for 48 hours are shown in Figure 29. Confocal laser scanning microscopy (CLSM) images of the colloidal solutions without lecithin and with addition of lecithin gives visual images of the FONs.
Both set of nanoparticles showed good cellular uptake as fluorescence was observed in the cells. The images indicated that the cells kept their normal morphology indicating their good biocompatibility. Organic nanoparticles showed good internalization into cells (Figure 29). With the presence of lecithin, more nanoparticles were observed inside cytoplasm, indicating the assistant function of lecithin for cell endocytosis. These results further give credence to the enhancement of fluorescence by lecithin and the potential of these nanoparticles for application in cell imaging using this naturally occurring surfactant.

3.7. Conclusion

We report a facile preparation method for fluorescent organic nanoparticles that are biocompatible and whose fluorescence, size and colloidal stability was enhanced with the addition of a naturally occurring surfactant, lecithin. Taken together, these properties make the FONs good candidates for cell imaging.
CHAPTER 4: PHOTOPHYSICAL AND COMPUTATIONAL ANALYSIS OF A SYMMETRICAL FLUORENE-BASED JANUS DIONE DERIVATIVE

4.1 Abstract

Molecular symmetry plays an important role in a molecule’s physical characteristics and spectra. However, results can sometimes be counterintuitive due to unpredictable properties of a molecule. Photophysical characterization of a centrosymmetrical indacene-1, 3, 5, 7(2H, 6H)-tetraone, or Janus dione, derivative revealed a two-photon absorption maximum closely associated with the main linear absorption band (a typically two-photon forbidden transition for molecules of high symmetry). Though observed previously in similar compounds, little explanation has been offered for this anomaly. Our investigation of a symmetrical Janus dione, including linear and nonlinear photophysical characterization along with TD-DFT calculations, provides insight regarding the origin of this phenomenon.

4.2 Introduction

The two-photon absorption (2PA) phenomenon has attracted attention from various fields ranging from chemistry and biology to optics and engineering. 2PA is a nonlinear process that involves the unusual capability of a molecule to absorb two photons simultaneously in order to populate an energy level equal to the sum of the energies of the two photons absorbed.\textsuperscript{1-7} Various organic chromophores are used in optical communication, information processing, frequency doubling, and integrated optics.\textsuperscript{3-5,6-8} Organic nonlinear optical (NLO) materials have many advantages over inorganic materials, some of which include large NLO coefficients, greater ease
of synthetic design, and lower cost.\textsuperscript{3,5,7-9} Two of the common NLO applications in which organic materials have been used are three-dimensional (3D) fluorescence imaging and optical power limiting.\textsuperscript{10-11} Two-photon absorbing materials are, therefore, in great demand to meet the needs of emerging technologies.

The main drawback of multiphoton excitation is that high excitation powers have to be used due to the low probability of the nonlinear absorption process ($10^{-51}$ to $10^{-46}$ cm$^4$ s) while compared to the linear absorption cross section ($10^{-19}$ to $10^{-16}$ cm$^2$), which often results in bleaching in the focal plane. The bleaching can be significantly reduced by use of more efficient two-photon absorbing chromophores.\textsuperscript{12-14} Commercially available fluorophores typically have very low 2PA cross sections (<20 GM), with the highest values around 200 GM, hence are used at high concentrations to generate a detectable fluorescence signal.\textsuperscript{13}

Organic molecules show great promise to meet this need as they can be customized through molecular engineering, and as the development of two-photon materials that suit practical application intensifies, so does research to meet this need.\textsuperscript{3,8,15-16} However, there remains some uncertainty in the particulars of design criteria for molecules with large 2PA cross sections at desired wavelengths, as such research to understand structure-property relationships is matter of significant importance. As a result, the full potential of 2PA materials has not been fully exploited. Several strategies to enhance the magnitude and tune the wavelength of 2PA have been reported for $\pi$-conjugated organic molecules.\textsuperscript{7,13-14,17}

One-dimensional quadrupolar systems have an inversion center where two peripheral donors (or acceptors) are connected to a central donor or acceptor, respectively, displaying high 2PA cross sections via quadrupolar intramolecular charge transfer. Very large 2PA values can be
obtained with D-A-A-D or A-D-D-A systems having strong D or A termini. Results show that the 2PA cross section is large in either of the configurations, irrespective of the direction of the flow of the electrons: from the ends to the center of the molecule or from the center to the ends. This enhancement in the 2PA cross section is attributed to intramolecular charge transfer from the terminal donor groups to the \( \pi \)-bridge. The magnitude of the charge transfer can be controlled by modifying the molecular structure in such a way as to affect the amount of charge transfer. In particular the 2PA cross section can be increased by lengthening the conjugation of the \( \pi \)-system since the charge is transferred over a longer distance.

It has also been established that modulating the degree of ground-state polarization or the degree of charge separation in the ground state of these molecules can exert significant influence over the molecular polarizability and hyperpolarizabilities. The charge separation in the ground state can be controlled primarily by either modifying the chemical structure, i.e., altering the strength of the donating and accepting substituents and/or extending the conjugation of the molecule. It is also well documented that 2PA is governed by two factors, namely transition dipole moment and transition frequencies (energies) of the molecule. The structure-property relationships are, thus, varied to optimize the transition dipole moments and frequencies involved in 2PA. Symmetric charge transfer and change in quadrupole moment is important for molecules with small ground-state quadrupole moments. Changing the structural features of the molecules that could enhance the quadrupole moment upon excitation is beneficial in enhancing the transition dipole moments upon excitation and magnitude of 2PA.

Increasing the conjugation length and extent of symmetrical charge transfer from the end of the molecule to the middle or vice versa results in a large increase in 2PA and in the shift of the
2PA to longer wavelength.\textsuperscript{11} Coplanarity of the conjugation bridge also plays a role in ensuring effective conjugation and polarizability of the molecules.\textsuperscript{7,13,22} Substantial activity has involved acceptors that are strongly electron deficient and that minimize chromophore aggregation in addition to possessing $\pi$-electron bridges that provide a good compromise between facilitating charge transfer, preventing aggregation, while maintaining reasonable thermal and photochemical stability.\textsuperscript{7}

On the basis of this observation, we designed a novel push-pull fluorophore in which two terminal electron donating groups end groups are connected to a strong central acceptor group, s-indacene-1,3,5,7(2H,6H)-tetraone, also known as Janus dione.\textsuperscript{24-28} Our hypothesis for a larger 2PA cross section is supported by the precedent literature that reported large 2PA cross sections using this acceptor and work that demonstrated that tuning of aryl amines raises the 2PA.\textsuperscript{3,5,8,18} The combination of large 2PA cross section and high fluorescence quantum yield exhibited by this molecule should facilitate a variety of applications in biology, medicine, photonics, optical limiting, and material science among others.\textsuperscript{7,9,14,19-21,29}

4.3 Experimental

4.3.1 Synthetic procedure

All reagents were purchased from commercial suppliers and used as received unless otherwise noted. THF was purified by distillation over sodium and benzophenone under nitrogen before use. CH$_3$CN was dried over calcium hydride. 7-(Diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde was prepared as previously reported.\textsuperscript{30} Reactions were conducted under a dry N$_2$ atmosphere using standard vacuum line techniques. $^1$H and $^{13}$C spectra were recorded in CDCl$_3$ solutions with a Bruker Avance III Spectrometer at 400 MHz for $^1$H (referenced to TMS at $\delta = 0.0$
ppm) and 101 MHz for $^{13}$C (referenced to CDCl$_3$ at $\delta = 77.0$ ppm). High resolution mass spectrometry (HR-MS) analysis was performed in the Department of Chemistry, University of Florida, Gainesville, FL. Column chromatographic purification was performed with silica gel as the stationary phase.

**Synthesis of 3, 7-dioxy-1, 5-dioxo-1, 5-dihydro-s-indacene-2, 6-dicarboxylic acid diethyl ester bis (triethylammonium) salt, 9.**

A mixture of pyromellitic anhydride (20 g, 91 mmol), ethyl acetoacetate (35 mL, 275 mmol), and Et$_3$N (112 mL, 1101 mmol) was heated in acetic anhydride (300 mL) at 100 °C for 2 h. The mixture was then cooled to room temperature and kept overnight at 0-5 °C, to form a brownish precipitate which was washed with acetic anhydride and ether to yield 9 as orange solid (15.2 g, 29 %). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.88 – 7.39 (m, 2H), 4.38 – 4.01 (m, 4H), 3.43 – 3.02 (m, 13H), 1.52 – 1.04 (m, 25H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ 191.53, 166.72, 143.20, 111.70, 98.68, 77.82, 77.71, 77.51, 77.19, 58.56, 46.48, 15.23, 8.90.

**Synthesis of s-indacene-1, 3, 5, 7(2H, 6H)-tetraone, 11.**

9 (5 g, 8.7 mmol) was dissolved in water (500 mL), forming dark orange solution that was then cooled in ice followed by addition of concentrated H$_2$SO$_4$ acid (5 mL). An orange precipitate formed, was filtered off, washed with EtOH, and air dried to afford 10. The product was used in the next reaction without further purification by dissolving 10 (2.7 g, 7.56 mmol) in dry CH$_3$CN (200 mL) to form deep red solution that was refluxed for 3 h. The solution was then cooled to room temperature to form greyish precipitate that was filtered off and recrystallized in CH$_3$CN to
form 11 in 81% yield. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.55 (s, 2H), 3.46 (s, 4H). $^{13}$C NMR (101 MHz, d$_6$-DMSO): δ 197.72, 148.23, 113.27, 46.39. HRMS (ESI-TOF): m/z [M+H]$^+$ calcd for C$_{12}$H$_6$O$_4$ 215.0344, found 215.0340.

**Synthesis of (2Z,6Z)-2,6-bis((7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)methylene)s-indacene-1,3,5,7(2H,6H)- tetraone, 8.**

A mixture of 7-(diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde (0.75 g, 1.42 mmol) and S-indacene-1,3,5(2H,6H)-tetraone (11, 0.13 g, 0.62 mmol) was dissolved in 30 mL glacial acetic acid and refluxed for 48 h. After cooling the reaction mixture to room temperature, the product was extracted three times each with CH$_2$Cl$_2$ and H$_2$O, then dried with anhydrous MgSO$_4$. The product was purified by recrystallization in CH$_2$Cl$_2$/acetone to give 8 as dark blue solid (0.61 g, 80%). $^1$H NMR (400 MHz, CDCl$_3$): δ 0.60 – 0.76 (d, $J = 20.0$ Hz, 7H), 0.76 – 0.85 (t, $J = 7.0$ Hz, 11H), 1.00 – 1.22 (m, 22H), 1.21 – 1.34 (m, 5H), 1.83 – 1.97 (m, 4H), 1.98 – 2.13 (m, 4H), 6.98 – 7.14 (m, 7H), 7.13 – 7.20 (m, 7H), 7.21 – 7.36 (m, 12H), 7.60 – 7.70 (d, $J = 8.3$ Hz, 2H), 7.71 – 7.80 (d, $J = 8.0$ Hz, 2H), 8.01 – 8.16 (s, 2H), 8.51 – 8.58 (m, 3H), 8.57 – 8.69 (m, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ 189.41, 189.33, 187.49, 154.57, 151.25, 150.32, 149.46, 148.26, 147.49, 147.31, 147.05, 144.55, 135.67, 133.97, 131.24, 129.36, 129.24, 127.12, 124.71, 123.44, 122.49, 122.10, 119.36, 117.86, 117.71, 99.98, 55.24, 53.42, 40.00, 31.50, 31.46, 29.71, 29.58, 23.86, 22.55, 14.04, 14.03. HRMS (ESI-TOF): m/z [M+H]$^+$ calcd for C$_{88}$H$_{88}$N$_2$O$_4$ 1237.6822 found 1237.6824
4.3.2 Photophysical Characterization

Absorption spectra were recorded for solutions (≈ 10⁻⁶ mol dm⁻³) of 8 in a range of solvents of differing polarity, as permitted by solubility, in a 1 cm quartz cuvette using an Agilent 8453 spectrophotometer. Fluorescence measurements (steady state, excitation anisotropy, and lifetime) were collected using a PTI QuantaMaster fitted with a xenon lamp and red sensitive PMT for steady state measurements, and a nitrogen-pumped dye laser and gated PMT for time-resolved measurements. Fluorescence quantum yields were measured via a relative method, using 9, 10-diphenylanthracene (DPA) as a standard.³¹ Photodecomposition quantum yields were measured via one-photon excitation using a green laser diode (λ_ex ~ 532 nm; I₀(λ) ≈ 90 mW cm⁻²) and recording absorption spectra at predefined time increments. This data was then incorporated into Equation 5, where D (λ, 0), D(λ,t_ir) are the initial and final optical density of the solution, ε(λ) is the extinction coefficient (dm⁻³ mol⁻¹ cm⁻¹), t is irradiation time (s) and λ is excitation wavelength (cm), Nₐ is Avogadro’s number, t_ir is total irradiation time, I₀(λ) is the spectral distribution of the excitation irradiance.³²

$$\Phi_{ph} = \frac{[D(\lambda, 0) - D(\lambda, t_{ir})]N_A}{10^2 \varepsilon(\lambda) \int_{\lambda} \int_{0}^{t_{ir}} I_0(\lambda) [1 - 10^{D(\lambda, t)}]d\lambda dt} \quad (5)$$

Degenerate 2PA spectra were collected using an open aperture z-scan set-up, with a Coherent Inc. Legend amplified system seeded by a Coherent Inc. Mira Ti:sapphire laser pumping an Opera solo OPA, as previously described.³³-³⁴
4.3.3 Quantum Chemical Calculations

The initial structure was prepared with the GaussView5 program, before a preliminary optimization of the coordinates with an AM1 calculation. This was then fully optimized using the B3LYP density functional theory (DFT) method in the GAUSSIAN09 software package, using the D95* basis set to define the atoms. Time dependent DFT (TD-DFT) calculations were then performed on the optimized structures with the M06 DFT method. The hexyl chains on the 9-position of the fluorene moieties were truncated to methyl groups for both molecules as they have no bearing on the photophysical properties of the chromophore, and to reduce calculation times.

4.4 Results and Discussion

4.4.1 Synthesis

The molecular strategy was to extend the conjugation of the \( \pi \)-bridge by utilizing S-indacene-1, 3, 5(2H, 6H)-tetraone (11) as an acceptor. The key step in the synthesis was the Knoevenagel condensation of aldehyde 3 with the activated methylene groups of the tetraone by refluxing in glacial acetic acid. The synthesis of Janus dione 11 is depicted in Scheme 7. Buchwald-Hartwig amination coupling was employed for the arylamination of 7-bromo-9, 9-dihexyl-9H-fluorene-2-carbaldehyde using Pd catalyst, \( \text{Cs}_2\text{CO}_3 \) to yield 3, as previously reported.
Scheme 7: Synthesis of (2Z,6Z)-2,6-bis((7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)methylene)s-indacene-1,3,5,7(2H,6H)-tetraone, 8.

4.4.2 Photophysical Characterization

The absorption spectra of 8 in solvents of differing polarities (Figure 30) show the presence of a main absorption band centered at ~585 nm, and two smaller bands at ~400 nm and ~300 nm with roughly half the intensity of the main band. Fluorescence was only observed in toluene solution and was intense with a quantum yield of 0.70. The absence of fluorescence in more polar solvents
suggests that solvent rearrangement to accommodate the excited state dipole consumes enough excitation energy such that radiationless relaxation is the only viable alternative. The photodecomposition quantum yields determined for 8 were consistent with those of other fluorenyl based fluorescent probes previously investigated (Table 4).\textsuperscript{37-38}

![Absorption (solid lines) and emission spectra (dashed lines) for 8 in toluene (black), DCM (red), and THF (blue).](image)

**Figure 30:** Absorption (solid lines) and emission spectra (dashed lines) for 8 in toluene (black), DCM (red), and THF (blue).

**Table 4:** Photophysical data for 8 in solvents of varying polarity.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{\text{abs, max}}^{a}$/nm</th>
<th>$\lambda_{\text{em, max}}^{a}$/nm</th>
<th>$\Delta\lambda^{b}$/nm</th>
<th>$\varepsilon^{c}$/10$^3$ M$^{-1}$ cm$^{-1}$</th>
<th>$\Phi_{f}^{d}$/</th>
<th>$\tau^{e}$/ns</th>
<th>$\Phi_{\text{ph}}^{f}$/10$^{-6}$</th>
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<tr>
<td>Tol</td>
<td>585</td>
<td>655</td>
<td>70</td>
<td>83.9</td>
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<tr>
<td>DCM</td>
<td>591</td>
<td>-\textsuperscript{f}</td>
<td>-\textsuperscript{f}</td>
<td>72.1</td>
<td>-\textsuperscript{f}</td>
<td>-\textsuperscript{f}</td>
<td>0.99</td>
</tr>
<tr>
<td>THF</td>
<td>574</td>
<td>-\textsuperscript{f}</td>
<td>-\textsuperscript{f}</td>
<td>65.2</td>
<td>-\textsuperscript{f}</td>
<td>-\textsuperscript{f}</td>
<td>8.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Absorption and emission maxima ±1 nm; \textsuperscript{b} Stokes shift ±2 nm; \textsuperscript{c} extinction coefficients ±5%; \textsuperscript{d} fluorescence quantum yields ±10%; \textsuperscript{e} fluorescence lifetimes ±10%; \textsuperscript{f} not determined.
The anisotropy trace recorded for 8 lacks a plateau in the main absorption band, instead displayed an increase, consistent with multiple transitions are represented by the band, as well as suggesting a two-photon allowed transition (Figure 31).\textsuperscript{38}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure31.png}
\caption{Absorption (black) and emission (blue) spectra in toluene, and excitation anisotropy (dark green) in silicone oil for 8.}
\end{figure}

The two-photon absorption measurements for 8 were performed in toluene solution (Figure 31). The peak of this spectrum shows reasonable agreement with the linear absorption spectrum, albeit with a slight hypsochromic shift from it. This result is counterintuitive due to the symmetrical structure of 8: the 2PA maximum would have been expected to occur at a shorter wavelength, in a similar fashion to a squaraine dye.\textsuperscript{39}
Figure 32: Absorption (black) and emission (red) spectra in toluene, excitation anisotropy in silicone oil (dark green), and two-photon absorption in toluene (dark red points) for 8.

4.4.3 Computational Calculations

Nominal change was observed in the conformation upon optimization of 8 (Figure 33, center). The central part of the molecule exhibits a very high level of planarity, with the diphenylamino moieties giving the structure most of its 3D depth. Transitioning from the HOMO to LUMO (Figure 33), the electron density transfers away from the peripheral diphenylamino donors to Janus dione acceptor moiety at the center. The HOMO and HOMO-1 have minor differences in shape, but differ in the phase on one side of the molecule. With this, and the appearance of the other orbitals, each can be identified as gerade or ungerade (Figure 33).
Figure 33: Optimized structure (center) and frontier molecular orbitals labeled as gerade (g) or ungerade (u) for 8.

Time-dependent density functional theory (TD-DFT) calculations indicate a single transition responsible for the main absorption band: HOMO $\rightarrow$ LUMO (Figure 34). However, at a slightly shorter wavelength, a transition was observed between HOMO-1 and LUMO, although the oscillator strength is very low (nearly 20,000 times smaller), confirming that the compound is
restricted by the Laporte selection rule given its symmetric nature. Transitions between HOMO-1 and LUMO+1, and HOMO and LUMO+2 are seen to be formally forbidden as well due to the symmetry of the molecule (Table 5).

![Graph](image.png)

**Figure 34:** Calculated (blue) and experimental (in toluene, black) linear absorption spectra, oscillator strengths (bars), and calculated 2PA spectrum (dashed blue) for 8.

**Table 5:** Calculated molecular orbital transitions for 8.

<table>
<thead>
<tr>
<th>Wavelength /nm</th>
<th>Oscillator strength</th>
<th>Transition</th>
<th>Relative proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>579</td>
<td>1.8856</td>
<td>HOMO-1 → LUMO+2</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOMO → LUMO</td>
<td>0.93</td>
</tr>
<tr>
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</tr>
<tr>
<td>475</td>
<td>0.0124</td>
<td>HOMO-2 → LUMO+1</td>
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<tr>
<td></td>
<td></td>
<td>HOMO → LUMO+1</td>
<td>0.95</td>
</tr>
<tr>
<td>463</td>
<td>0.0000</td>
<td>HOMO-1 → LUMO+1</td>
<td>0.98</td>
</tr>
<tr>
<td>442</td>
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<td>0.96</td>
</tr>
<tr>
<td>441</td>
<td>0.8035</td>
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<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOMO-1 → LUMO+2</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOMO → LUMO</td>
<td>0.06</td>
</tr>
</tbody>
</table>
It can be noted that the calculated 2PA spectrum presented in Figure 34 displays the same hypsochromic shift from the calculated linear absorption that is seen with the experimental data (Figure 32); this 2PA maximum corresponds to the one-photon forbidden transition HOMO-1 → LUMO, as predicted by expansion of the Laporte selection rule, and indicating that a two-photon transition is between these states.

4.5 Conclusion

Through a condensation reaction with two equivalents of a fluorenyl aldehyde and tetraone, a novel Janus dione-based two-photon absorbing dye was synthesized. This compound exhibited intense fluorescence ($\Phi_F = 0.7$) in non-polar solvents. Though the measured 2PA spectrum presented unexpected results, the use of TD-DFT calculations revealed the nature and origin of the transition: the degeneracy of HOMO and HOMO-1 and their opposing symmetry permitted two-photon excitation close to twice the wavelength of the main linear absorption band.
CHAPTER 5: HYDROPHILIC DYSES BASED ON 1, 3-INDANDIONE-5, 6-DICARBOXYLIC ACID AND THEIR APPLICATION IN CELL IMAGING

5.1 Abstract

Two fluorene-based dyes were synthesized using 1, 3-indandione-5, 6-dicarboxylic acid as the acceptor. 2-((7-(Diphenylamino)-9, 9-dihexyl-9H-fluoren-2-yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5, 6-dicarboxylic acid, used for photocatalytic oxidation of methanol as reported earlier in Chapter 2, had solubility enhanced by introducing the polyethylene glycol group at the fluorenyl 9-position to yield 2-((7-(diphenylamino)-9, 9-polyethyleneglycol-550-monomethylether-9H-fluoren-2-yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5, 6-dicarboxylic acid. Through the interaction of the latter with the biomacromolecule bovine serum albumin (BSA), the dye’s fluorescence was enhanced. Capitalizing on this, the dye was incubated with HCT 116 cells for cell imaging using fluorescence microscopy.

5.2 Introduction

Serum albumin (ALB) is the major protein in blood plasma and is used to bind and transport various ligands to specific sites due to its ability to bind to its two major binding sites; namely site I and site II. Both binding sites are structurally selective with noncovalent binding occurring at site I and a combination of hydrophobic and electrostatic interactions dominate at site II. Bovine serum albumin (BSA) is one of the most widely used serum albumins and is used as a model protein because of its structural homology with human serum albumin (HSB). Low levels of ALB may indicate liver and kidney malfunctions or malnourishments due to poor diet. It’s therefore important to monitor levels in the blood plasma and other biological fluids to
gauge a patient’s health. As such, the development of analytical tools to detect BSA in biological samples has attracted a lot of attention. \(^6\) BSA has found applications in protein morphology studies and in drug delivery as the serum albumin conjugates enhance solubility of hydrophobic drugs in plasma. Nanoparticles made of albumin have found application as carriers to encapsulate hydrophobic drug and have been used in the treatment of cancer.\(^1\)

There are numerous hydrophobic cavities inside BSA with a hydrophilic surface and is negatively charged at neutral pH. As a result, the polarity in the bulk solution and that in the hydrophobic core of the BSA are significantly different. Organic dyes bind to the hydrophobic domain of BSA, resulting in a change of the polarity of the environment surrounding the dye changes upon addition of BSA. Consequently, shifts in the absorption and emission bands of a polarity-sensitive dye may be induced by the variation of the microenvironment’s polarity. In turn, the photophysical properties of the dye changes with increasing BSA concentration. Owing to a protein’s crucial roles in biological systems, development of probes that can noncovalently bind and exhibit fluorescent response to individual proteins is gaining popularity.\(^6\) Several fluorescent probes have been developed and used for the detection of proteins in solution, e.g., cyanine and squaraine dyes in which an increase in the intensity and lifetime due to the change of environment as a result of protein microencapsulation is observed.\(^1\)

The prerequisites for a fluorescent probe are that it should bind to a component in the biological environment and be soluble in aqueous solution. Alternatively, fluorescent labels may noncovalently bind with biomolecules using a combination of forces; hydrophobic, electrostatic and/or hydrogen bonding interactions, resulting in the formation of a stabilized complex. The noncovalent interactions are however weaker than the chemical bond linkage but they occur at a
faster rate and at physiological pH range. With a large noncovalent binding constant and when
the reaction stoichiometry is known, purification can be circumvented. In addition, noncovalent
fluorescent labels are normally encapsulated by the biomolecules shielding them from quenchers
that are abundant in biological environments, a stability advantage.1

For biological imaging, probes with large two-photon absorption cross sections are
required. These probes should absorb at long wavelength to reduce light scattering and to
simplify study of thicker samples, enabling deeper penetration depth. Commercially available
fluorophores that are specific to certain organelles have very low two photon cross sections (<20
GM) with the highest values in the neighborhood of 200 GM and are used at high concentrations
to generate a detectable fluorescence signal.7 In vivo imaging with fluorescent probes is severely
restricted because conventional fluorescent probes normally absorb in the UV-vis region where
absorption by biomolecules is high, leading to autofluorescence and photodamage. In addition,
visible light is easily scattered and cannot penetrate deeply into tissues. The strong
autofluorescence causes high background noise resulting in low signal to noise ratios often
making them unsuitable for in vivo imaging.7-8 In contrast, light in the NIR region is poorly
absorbed by biomolecules and can penetrate deeper in tissues, increasing the signal-to-noise
ratios obtained, rendering the probes useful for in vivo imaging.8-15

Two-photon excitation has advantages over conventional confocal microscopy for
imaging and for manipulating cellular functions; 2PA occurs at the focal point of the microscope
objective where the laser intensity is maximum so the dye bleaching and fluorescence
surrounding the focused point is avoided. The excitation source is generally a NIR short pulse
laser, which takes the advantage of the transparency window of biological tissues thus reducing
phototoxicity compared to using UV excitation. The nonlinearity of interaction with light is advantageous when working with biological samples because out of focus photobleaching and photodamage are reduced besides the greater penetration depth that comes with the use of multiphoton excitation. The main drawback of multiphoton excitation, however, is that high excitation powers have to be used due to the low probability of the nonlinear absorption process (10^{-51} to 10^{-46} cm^{-4} s) compared to the linear absorption cross section (10^{-19} to 10^{-16} cm^{2}) that results in bleaching in the focal plane. The bleaching can be significantly reduced by use of more efficient nonlinear optical fluorophores. To reduce the excitation power used for cell imaging there’s great need for new organic dyes that allow the use of low two-photon excitation power in the milliwatt or sub-milliwatt range.

This work extends on compounds we synthesized earlier using s-indacene-1,3,5,7(2H,6H)-tetra one or Janus dione as an acceptor in Chapter 4, which proved nonideal for application in cell imaging owing to their insolubility in aqueous solutions besides being non fluorescent despite possessing record 2PA cross sections. The high 2PA cross section and acceptable quantum yields potentiate the application of these probes as labels in bioimaging and as nonlinearly activated optical sensors.

5.3 Experimental Section

5.3.1 Synthetic Procedures

All reagents were purchased from commercial suppliers and used as received unless otherwise noted. THF was purified by distillation over sodium and benzophenone under nitrogen before use. CH₃CN was dried over calcium hydride. Reactions were conducted under a dry nitrogen atmosphere using standard vacuum line techniques. ^1^H and ^1³^C spectra were recorded in CDCl₃.
solution with a Bruker Avance III Spectrometer 400 MHz for $^1$H (referenced to TMS at $\delta=0.0$ ppm) and 101 MHZ for $^{13}$C (referenced to CDCl$_3$ at $\delta=77.0$ ppm). High resolution mass spectrometry (HR-MS) analysis was performed in the Department of Chemistry, University of Florida, Gainesville, FL.

5.3.2 Photophysical characterization

Linear absorption spectra for the compounds were recorded in range of solvents with differing polarity in 1 cm quartz cuvettes using an Agilent 8453 spectrophotometer. Emission spectra were obtained on an Edinburgh Instruments FLS 980, utilizing both a red sensitive and a liquid nitrogen cooled NIR PMT, with signal corrected for detector responsivity and the solvent background. The solutions of the compounds used for these measurements were on the order $10^{-6}$ M concentration. Relative fluorescence quantum yields were determined against the standard 9, 10-diphenylanthracene (DPA).

Degenerate 2PA spectra were obtained using a homebuilt Z-scan apparatus using a Coherent Inc. Legend amplified system seeded by a Coherent Inc. Mira Ti:sapphire laser pumping an Opera solo OPA, detailed previously, for excitation. Samples were prepared in solutions with concentrations on the order of $10^{-2}$–$10^{-3}$ M.

5.3.3 BSA titration

A series of BSA phosphate buffer solutions with different concentration (0–10 μM) were prepared. Compound 13 was dissolved in DMSO first due to its limited solubility in PBS buffer. The DMSO stock solution was then slowly added to 3 mL BSA-buffer to obtain a mixture with 20 μM$^{-1}$ containing DMSO less than 1% (The influence of the presence of DMSO is negligible).
5.3.4 Determination of BSA bonding constants

A mixture of 10 μM BSA with various concentration (0~18 μM) in phosphate buffer was placed in a quartz cuvette. The fluorescence spectra were measured with the excitation at 280 nm. The maximum emission intensities at 335 nm were recorded and the binding constant calculated using the Scatchard equation.  

\[ \frac{r}{c_f} = nK_a - rK_a \]  

(6)

Where \( K_a \) is the binding constant, \( n \) is the number of binding sites per BSA molecule, \( r \) is the moles of compound bound per mole BSA (\( r/c_B \)), \( c_f \) is the concentration of free dye in the solution which is calculated by \( c_t-c_b \) (\( c_t \) is the total concentration of compound that was added to the mixture, \( c_b \) is the amount of dye bound to protein). \( c_b \) values were obtained from the intercept of the plot ( \( \frac{1}{F} \) vs \( \frac{1}{c_t} \) ), where \( F \) is the fluorescence intensity difference in the presence and absence of compound 13. The binding constant, \( K_a \), was obtained from the plot’s slope of \( \frac{1}{c_f} \) vs \( \frac{1}{c_f} \).

5.3.5 Binding-site selectivity

Dansylproline (DP) and dansylamide (DNSA) was used for investigating the binding-site selectivity of compound 13 to BSA, because DP and DNSA specifically bind to the site- I and site- II on BSA respectively. Due to poor solubility of DP and DNSA in PBS buffer, the stock solution containing 1 μM DP and 1 μM DNSA was made using DMSO solution. A series of solutions were made by fixed concentration (5 μM) of BSA and DP (0.1 mM) or DNSA (0.05 mM) in phosphate buffer. Upon the addition of probe 13 (0~24 μM), the change of absorption and
emission spectra were recorded ($\lambda_{ab} = 510 $ nm and $\lambda_{em} = 551$ nm for DNSA; $\lambda_{ab} = 511$ nm and $\lambda_{em} = 555$nm for DP).

5.3.6 Cytotoxicity Assay

Before applying the probe for cell imaging, the probe’s cytotoxicity with HCT 116 cells (America Type Culture Collection, Manassas, VA) was investigated by an MTS assay. HCT-116 cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin at 37 °C, under 5% CO$_2$ environment. Then HCT 116 cells were placed in 96-well and incubated until there were no fewer than $6 \times 10^4$ cells per well. Next the cells were incubated with 13 at different concentration (40 μM, 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.62 μM) for additional 20 h. Next, 20μL of CellTiter 96 Aqueous one reagent was added to each well and incubated for 4 h. The respective absorbance values at 490 nm were read on a SpectraMax M5 plate reader (Molecular Devices) to determine the relative amount of formazan produced. All absorbance values were subtracted from the blank volume from a cell-free control.

5.3.7 Fluorescence Bioimaging

HCT 116 cells were placed onto poly-D-lysine-coated glass coverslips in 24 well plates, and incubated for 48 h before incubation with the dye. The cells were then fixed with 3.7% formaldehyde in PBS at room temperature for 15 min and washed (3X) with PBS buffer solution. NaBH$_4$ (1μg/mL) solution in PBS were added to each well for 15 min and then washed (3X) with PBS buffer solution. HCT 116 cells were further treated with Triton X (Invitrogen, Carlsbad, CA, USA) for 10 min and washed (3X) with PBS. Next, the cells were incubated with the
probe at different concentration (10 μM, 20 μM, 30 μM, 40 μM) for 30 min. After washing three times with PBS, they were incubated with Hoechst (Invitrogen, Carlsbad, CA, USA, 1 μg/mL) for additional 15 min. Fluorescence microscopy images were recorded on an Olympus IX-81 confocal microscope equipped with an EM-CCD C9100 digital camera. Filters used for excitation and emission wavelength were depending on the dye; Fluor (Ex 377/50, DM 409, Em 520/400) for Hoechst nuclear stain, and Cy5 (Ex 604/40, DM 660, Em 672/712) for probe 13.

5.4 Synthesis

The compounds synthesized consist of push pull chromophores with an amino functionality as the donor and 1, 3-indandione-5, 6-dicarboxylic acid as the acceptor. The synthesis of the acceptor, 1, 3-indandione-5, 6-dicarboxylic acid and the subsequent condensations with the aforementioned acceptor is depicted in Scheme 8. The two compounds both contain the fluorene core but with a modification at the 9-position. Poly (ethylene glycol) moieties were introduced in compound 13, a strategy normally employed to prepare water soluble polymers and therapeutic agents as it induces hydrophillicity. Poly (ethylene glycol) monomethyl ether tosylate was prepared by tosylation of the poly (ethylene glycol) monomethyl ether at the alcohol terminus with tosyl chloride and the fluorene was alkylated with the resultant product. The synthesis of this compound is to be published elsewhere.
Scheme 8: Synthesis of 2-((7-(diphenylamino)-9, 9-dihexyl-9H-fluoren-2-yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5,6-dicarboxylic acid, 12 and 2-((7-(diphenylamino) - 9, 9-polyethyleneglycol-550-monomethylether -9H -fluoren-2-yl) methylene)-1, 3-dioxo-2, 3-dihydro-1H-indene-5, 6-dicarboxylic acid, 13.

The key step in the synthesis of the compounds was the Knoevenagel condensation of the aldehyde derivatives with the methylene groups of the 1, 3-indandione-5, 6-dicarboxylic acid by
refluxing in glacial acetic acid to yield the chromophores 12 and 13. 34-35 1, 3-Indandione-5, 6-dicarboxylic acid was synthesized as reported. 36

Synthesis of 7-Oxo-1,3,5-trioxo-3,5-dihydro-1H-1H-2oxa-s-indacene-6-carboxylic Acid Ethyl Ester Triethylammonium salt, (14).

Pyromellitic anhydride (20 g, 91.75 mmol), ethyl acetoacetate (11.93 g, 91.75 mmol), Et₃N (77 mL) were dissolved in acetic anhydride (300 mL) to form a dark solution which was then heated at 70 °C for 30 min. The reaction mixture was then cooled at room temperature and then left in the refrigerator overnight to form a precipitate which was then filtered, washed with acetic anhydride and Et₂O to form yellow orange crystals of 14 (4.2 g, 12%). ¹H NMR (400 MHz, Chloroform-d) δ 7.93 (s, 2H), 4.16 (s, 2H), 3.24 (s, 7H), 1.28 (s, 13H). ¹³C NMR (101 MHz, Acetone) 188.72, 168.34, 166.61, 141.48, 136.34, 120.35, 99.50, 57.94, 46.47, 14.57, 8.33.

Synthesis of 1, 3-Indandione-5, 6-dicarboxylic Acid, (16).

To a solution of 7-oxo-1,3,5-trioxo-3,5-dihydro-1H-2-oxa-s-indacene-6-carboxylic acid ethyl ester triethylammonium salt,14 (4.1 g,10.53 mmol) in water (200 mL) at 0 °C was added 16.8 mL concentrated H₂SO₄ dropwise and the solution stirred for 1 h, resulting in yellow precipitate, which was filtered and washed thoroughly with EtOH and dried to yield 15 (2.22 g, 69%). The compound was used in the next reaction without further purification as in the reference paper. A suspension of 2.1g of 15 in anhydrous CH₃CN (100 mL) was refluxed for 45 min to form a yellow solution and the solvent removed under reduced pressure. The product was recrystallized in CH₃CN to afford the product as off-white solid (0.82 g, 51%): ¹H NMR (400 MHz, Acetone-d₆) δ 8.14 (s, 2H), 3.34 (s, 2H). ¹³C NMR (101 MHz, Acetone) δ 205.80, 196.44, 166.88, 144.87,
139.07, 123.29, 45.47 HRMS (ESI-TOF): m/z [M+H]^+ calcd for C_{11}H_{26}O_{6} 234.0164, found 234.0090.

**Synthesis of 2-((7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)methylene)-1,3-dioxo-2,3-dihydro-1H-indene-5,6-dicarboxylic acid, (12).**

1,3-Indandione-5,6-dicarboxylic acid (91 mg, 0.39 mmol) and the 7-(diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde (0.2 g, 0.37 mmol) were mixed in a two-necked flask and dissolved in glacial acetic acid (10 mL) to produce yellow solution. The reaction mixture was refluxed for 24 h, forming purple solution. The reaction mixture was cooled and extracted with dichloromethane and brine, resulting in dark solid. The crude product was purified by precipitation in hexane while dissolved in dichloromethane. Further purification was done by recrystallization in hexane/dichloromethane to yield 12 as dark solid (0.21 g, 78%). ^1H NMR δ H (400 MHz, acetone-d$_6$) 0.09 – 0.81 (11 H, m), 0.74 – 1.34 (13 H, m), 1.83 – 1.95 (2 H, td, J 11.9, 4.8), 1.99 – 2.10 (2 H, td, J 12.9, 5.6), 6.84 – 6.98 (1 H, dq, J 7.3, 2.1), 6.98 – 7.10 (5 H, s), 7.08 – 7.17 (1 H, d, J 2.6), 7.16 – 7.37 (4 H, td, J 8.2, 2.5), 7.64 – 7.79 (1 H, m), 7.78 – 7.89 (1 H, t, J 5.9), 7.87 – 8.03 (1 H, d, J 4.0), 8.18 – 8.38 (2 H, m), 8.42 – 8.57 (1 H, d, J 7.4), 8.64 – 8.81 (1 H, d, J 3.7). ^13C NMR (101 MHz, acetone) δ 188.96, 188.00, 154.42, 151.29, 149.63, 148.34, 147.96, 147.62, 143.63, 141.13, 139.88, 135.37, 134.73, 131.80, 129.76, 129.14, 127.86, 124.80, 124.40, 124.27, 123.75, 122.94, 122.51, 119.66, 118.29, 55.45, 40.09, 31.63, 24.06, 22.57, 13.70. HRMS (ESI-TOF): m/z [M+H]^+ calcd for C_{49}H_{47}NO_{6} 745.3403 found 745.3337.
Synthesis of $2\text{-}((7\text{-(diphenylamino)})\text{-}9, 9\text{-}\text{polyethyleneglycol-550-monomethylether}\text{-}9\text{H}\text{-}\text{fluoren-2-yl})\text{ methylene)}\text{-}1, 3\text{-dioxo-2, 3\text{-dihydro-1H-indene-5, 6-dicarboxylic acid}}$, (13)

1,3-Indandione-5,6-dicarboxylic acid (12 mg, 0.05 mmol) and 7-(diphenylamino)-9, 9-polyethyleneglycol-550-monomethylether-9H-2-carbaldehyde (60 mg, 0.047 mmol) were mixed in a two-necked flask and dissolved in glacial acetic acid (10mL), forming yellow solution. The reaction mixture was refluxed for 24 h, affording purple solution. The reaction mixture was cooled and extracted with dichloromethane and brine to produce dark solid. The crude product was purified by precipitation in hexane while dissolved in dichloromethane. Further purification was accomplished by recrystallization in hexane/dichloromethane to yield 13 as dark solid (48mg, 69%). $^1\text{H NMR} \delta_H (400 \text{MHz, acetone-d6}) 3.16\text{-}3.21 (6 \text{H, m}), 3.26\text{-}3.89 (86 \text{H, m}), 6.53\text{-}7.13 (9 \text{H, dt, } J 24.6, 8.8), 7.13\text{-}7.56 (7 \text{H, m}), 7.51\text{-}8.11 (2 \text{H, m}), 8.08\text{-}8.31 (1 \text{H, s}).^1\text{C NMR} (101 \text{MHz, acetone}) \delta 192.18, 174.31, 167.37, 153.29, 148.02, 147.90, 131.80, 130.13, 129.97, 129.89, 125.01, 124.57, 123.94, 123.71, 119.85, 108.23, 100.31, 72.85, 72.02, 70.51, 70.09, 67.25, 61.26, 58.26, 51.89, 39.39, 33.74, 32.05, 25.11, 22.74, 20.25, 13.78. HRMS-MALDI-DTL: High resolution MS shows a series of ions centered around m/z 1513 with fragments related to the various ethoxylate oligomers that differ by $\Delta$ m/z 44, an ethoxy unit.

5.5 Results and Discussion

5.5.1 Photophysical Characterization

Absorption spectra recorded for 12 show an intense, well resolved band centered at $\sim$510-540 nm (Figure 35), shifting slightly as the solvent was varied (Table 6). At shorter wavelength,
at least two peaks are seen at ~310 nm and ~355 nm, the former linked to isolated excitation of
the diphenylamine moiety as previously noted.\textsuperscript{37} The emission recorded for a toluene solution
upon excitation of the main absorption band has marked symmetry with the absorption profile
\textit{(Figure 35)}, covering ~100 nm (full width, half maximum).

\textbf{Figure 35}: Absorption (black) and emission (red) spectra of \textit{12} in toluene.
The photophysical properties of 13 were investigated in different solvents, including ethanol (EtOH), tetrahydrofuran (THF), acetone (ACT), acetonitrile (ACN), dioxane (DIX), and dimethyl sulfoxide (DMSO), at room temperature. This range of solvents was selected because some fluorene derivatives are sensitive to the environmental polarity.\(^1\) The linear absorption, emission spectra and photophysical parameters of 13 are showed in Figure 36 and Table 7 respectively. In comparison to the absorption spectra for 12, 13 displays a different ratio of the two peaks at ~310 nm and ~520 nm.

The absorption spectra of compound 13 did not change much with change in polarity of the solvents; the maximum wavelength of the emission spectra, however, changed from 674 nm in DIX to 786 nm in ACN. The compound exhibited a strong solvatochromic behavior with a maximum Stokes shift greater than ~280 nm in ACN.
Figure 36: Normalized absorption and emission spectra for 13 in (a) THF, (b) EtOH, (c) DMSO, (d) DIX, (e) ACT, (f) ACN

Table 7: Photophysical properties for compound 13.

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<td>1.86</td>
<td>-</td>
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</tr>
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$P(\varepsilon)$

|        | 0.294 | 0.688 | 0.868 | 0.887 | 0.924 | 0.938 |

$^a$ Absorption and emission maxima ±1 nm; $^b$ Stokes shift ±2 nm; $^c$ fluorescence lifetimes ±10%; $^d$ polarity factor calculated as $P(\varepsilon) = (\varepsilon-1)/(\varepsilon+2)$, where $\varepsilon$ is the dielectric constant.
The degenerate two-photon absorption spectrum for 12 in toluene shows a maximum that overlays with the linear absorption obtained in the same solvent (Figure 37). This result is to be expected given the asymmetrical structure of the chromophore.\textsuperscript{38} Unfortunately the presence of one-photon absorption at wavelengths shorter than 840 nm prevented the examination of two-photon absorption in this region.

\textbf{Figure 37:} Two-photon absorption spectrum for 12 in toluene (mauve points) and 13 in water overlaid on the linear absorption (black line) and emission (red line) spectra of 12 in toluene.

As the augmentation of the fluorene moiety to make the hydrophilic dye 13 has no impact on the conjugated system of the chromophore, the 2PA was only repeated in part to confirm the similarity. The data obtained for this shows that 13 has a similar profile to 12, although the
maximum cross section is somewhat smaller (Figure 37). This difference is likely due to a similar solvent effect as is seen in the linear absorption spectrum.

5.5.2 BSA titration and the determination of BSA bonding constants

The interaction between compound 13 and BSA was investigated by BSA titration. The absorption and fluorescence spectra of a mixture of 20 μM solution and various concentration of BSA in the phosphate buffer are showed in Figure 38. With the addition of BSA, the intensity of absorption spectra increased which was then followed by a slight decrease (Figure 38, a). The emission wavelength is bound to have a blue shift along with the intensity enhancement if the polarity of the microenvironment is reduced. 22 As expected, the probe had a blue shift from 764 nm to 696 nm (Figure 38, b), and the intensity 25 times stronger than the initial solution when the concentration of BSA reached 10 μM.
**Figure 38**: BSA titration experiment spectra: (a) absorption, (b) emission, and (c) the ratio of fluorescence intensity in different BSA concentration.
**Figure 39:** Determination of BSA binding constant, (a) the quenching of BSA fluorescence intensity, (b) the Scatchard plot for binding constant.

The BSA fluorescence intensity was quenched (*Figure 39, a*) with the addition of the probe, which is evidence that the probe has good affinity to BSA. The Scatchard method was used for the determination of binding constant, $K_a$.\textsuperscript{23-25} The Scatchard plot is shown in (*Figure 39, b*), and the binding constant was calculated to be $8.79 \times 10^4 \text{M}^{-1}$. This result indicated the probe could be used in bioimaging study.

**5.5.3 Binding-site selectivity**

There are two selective binding sites on BSA, namely, site I and site II. The hydrophobic forces drive organic compounds binding to site I, and a combination of hydrophobic, electrostatic forces and hydrogen bonding keeps them on site II.\textsuperscript{1-5,39-40} With addition of the probe to the BSA-DP complex, the absorption intensity increased (*Figure 40, a*) along with a decrease of the emission (*Figure 40, b*), and 48% of DP was replaced by 24 μM solution. (the concentration ratio of DP to
probe used was 2:1). Besides, there was also a distinctly absorption enhancement (Figure 40, c) and fluorescence decrease (Figure 40, d) for BSA-DNSA complex by adding the compound and 46% DNSA had been effectively replaced at 19 μM approximately (the concentration ratio of DNSA to 13 was 5:1). The competitive binding of the compound to BSA further proved that it has high affinity to BSA and reacted with BSA by non-selective binding. More work however needs to be done to elucidate the binding mechanism.
**Figure 40:** The changes of (a, c) absorption and (b, d) emission spectra for BSA-DP complex and BSA-DNSA respectively in competitive binding experiment, (e) the quenching of fluorescence by adding probe 13.

### 5.5.4 Cytotoxicity Assay

The cell viability assay results (*Figure 41*) showed that the percentage of viable cells remained above 80% when a dye concentration of up to 40 μM, and above 90% after incubation with 20 μM of probe 13. These results indicated that a dye concentration (30 μM) when used for cell imaging
would have negligible influence on HCT 116 cells and the compound’s potential for use in vivo study.

![Cell viability assay of HCT 116 cells incubated with 30 μM probe 13.](image)

**Figure 41:** Cell viability assay of HCT 116 cells incubated with 30 μM probe 13.

### 5.5.5 Fluorescence Bioimaging

HCT 116 cells were incubated with compound 13 for 20–60 min and only auto-fluorescence inside the cells was observed. This indicated there was no or only a small amount of uptake of the probe by living cells. The cells were permeabilized by Triton-X after fixation, followed by incubation with probe 13 for an additional 30 min. Hoechst was used to indicate the position of nucleus. **Figure 42** shows the fluorescence images of HCT 116 cells after incubation. The bright fields around the nucleus illustrate that the probe could bind to some specific organelles or proteins inside the cell selectively. However, more details about the binding position could not be ascertained due to the limitation of commercially available organelle-trackers for fixed cells.
Figure 42: The fluorescence images of HCT 116 cells incubated with (a) 30 μM probe 13 (30 min), (b) 30 μM probe 13 (30 min) and 15 μM Hoechst (15 min), and (c) merged image of DIC. Scale bar is 10 μm.

5.6 Conclusions

In summary, two fluorene-based dyes were synthesized using 1, 3-indandione-5, 6-dicarboxylic acid as the acceptor. Through the interaction of the hydrophilic moiety with BSA, the dye’s fluorescence was enhanced which is ascribed to the change of environment with the protein. No selectivity was observed for the BSA’s sites when experiments were conducted using BSA-DP and BSA-DNSA complexes calling for more work to be done to elucidate the binding mechanism. Goaded by the fluorescence enhancement of the probe when bound to BSA, the dye was incubated with HCT 116 cells for cell imaging using fluorescence microscopy. Results
revealed that the probe bound to some specific organelles or proteins inside the cell, setting the stage for future research to ascertain potential binding selectivity.
CHAPTER 6: FUTURE WORK

This work builds on Chapter 4 where the compound’s conjugation was extended. \((2Z, 6Z)\)-2,6-Bis(4-((E)-2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)benzylidene)s-indacene-1,3,5,7(2H,6H)-tetraone has an additional styryl group attached to the fluorene possessing an extended conjugated length. At the time of compiling this work, the compound’s photophysical characterization was ongoing.

6.1 Synthesis

The synthesis of \((2Z,6Z)\)-2,6-bis(4-((E)-2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)benzylidene)s-indacene-1,3,5,7(2H,6H)-tetraone was achieved by first employing Buchwald-Hartwig amination coupling for the arylamination of 2, 7-dibromo-9, 9-dihexyl-9H-fluorene using Pd(dba)_2, dppf as catalysts and tBuONa as base to yield 7-bromo-9,9-dihexyl-N,N-diphenyl-9H-fluoren-2-amine.\(^1\) Heck coupling reaction of vinylbenzaldehyde and 7-bromo-9,9-dihexyl-N,N-diphenyl-9H-fluoren-2-amine using palladium acetate/tri-o-tollyphosphine as catalyst and triethylamine as a base yielded the aldehyde derivative \(21.\)\(^2\) 4-vinylbenzaldehyde was obtained from Wittig reaction of telephaldehyde with methyltriphenylphosphonium iodide and potassium carbonate.\(^3\) Knoevenagel condensation of the aldehyde derivative with the methylene groups of the tetraone furnished \(18.\)\(^4\)\(^-\)\(^6\) These reactions are depicted in scheme 9.
Scheme 9: Synthesis of (2Z,6Z)-2,6-bis(4-((E)-2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)benzylidene)s-indacene-1,3,5,7(2H,6H)-tetraone, 18.


2, 7-Dibromo-9, 9-dihexyl-9H-fluorene (5 g, 10.16 mmol), diphenylamine (0.86 g, 5.1 mmol), Pd (dba)$_2$ (0.030 g, 0.052 mmol), dppf (0.030 g, 0.055 mmol), tBuONa (0.66 g, 6.86 mmol) were dissolved in dry toluene (50mL) under nitrogen to form dark brown solution. The reaction
mixture was refluxed for 24 h after which it was cooled to room temperature, filtered and extracted with CH$_2$Cl$_2$/brine. The reaction mixture was dried with anhydrous Na$_2$SO$_4$ to yield yellow green oil. The crude product was purified on silica using dichloromethane and hexane 3:7 to yield cream-colored solid (2.74g, 93%). $^1$H NMR $\delta$ H (400 MHz, Chloroform-d) 0.35 – 0.70 (5 H, m), 0.67 – 0.93 (7 H, t, $J$ 7.1), 0.91 – 1.24 (14 H, m), 1.78 – 2.06 (5 H, m), 6.90 – 6.98 (1 H, m), 7.07 – 7.16 (3 H, m), 7.23 – 7.33 (3 H, m), 7.39 – 7.50 (5 H, m), 7.50 – 7.57 (2 H, d, $J$ 8.6).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 14.41, 14.45, 22.98, 24.05, 24.12, 29.98, 30.05, 31.86, 31.91, 40.56, 40.60, 55.74, 56.08, 119.50, 120.55, 120.80, 120.86, 121.52, 121.88, 123.02, 123.87, 124.29, 126.37, 126.57, 127.30, 129.59, 130.29, 130.55, 135.47, 139.46, 140.35, 147.94, 148.29, 152.14, 152.94, 153.27.

**Synthesis of 4-vinylbenzaldehyde,20.**

In a two neck flask and under nitrogen, terephaldehyde (5 g, 37.28 mmol), methyltriphenylphosphonium iodide (15 g, 37.10 mmol), and potassium carbonate (5.15 g, 37.27 mmol), were dissolved in dioxane (50 mL). H$_2$O (1.25 mL) was added under vigorous stirring to form yellow solution. The reaction mixture was refluxed for 5 h and then cooled to room temperature. The mixture was filtered and the solvent removed under reduced pressure to yield a yellow solid. The product was extracted using hexane and the crude product purified on silica gel using dichloromethane to form low boiling clear liquid (4.1 g, 83%). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 9.90 (s, 1H), 8.08 – 7.59 (m, 2H), 7.46 (d, $J$ = 8.1 Hz, 2H), 6.68 (dd, $J$ = 17.6, 11.0 Hz, 1H), 5.83 (dd, $J$ = 17.6, 0.8 Hz, 1H), 5.35 (dd, $J$ = 10.9, 0.7 Hz, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 117.75, 127.05, 130.38, 136.20, 143.74, 191.98.
Synthesis of (E)-4-(2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-yl)vinyl)benzaldehyde, 21.

4-Vinylbenzaldehyde (0.6 g, 4.54 mmol), 7-bromo-9,9-dihexyl-N,N-diphenyl-9H-fluoren-2-amine (2 g, 0.345 mmol), Pd(OAc)$_2$ (20 mg, 0.01 mmol), tri-o-tolyphosphine (49 mg, 0.16 mmol) and Et$_3$N (7 mL) were mixed in a two-necked flask and dissolved in DMF(10 mL) to form dark solution. The solution was degassed for 10 min and then heated at 100 °C for 16 h. The reaction mixture was then cooled to room temperature, filtered and extracted with brine and dichloromethane. Anhydrous Na$_2$SO$_4$ was used to dry the product. The crude product was purified on silica gel using dichloromethane and hexanes (1:1) to yield yellow oil (1.39 g, 64%). $^1$H NMR δ (400 MHz, Chloroform-d) 0.46 – 0.62 (7 H, m), 0.61 – 0.80 (10 H, td, J 7.2, 2.6), 0.83 – 1.14 (12 H, m), 1.77 – 1.91 (4 H, m), 6.90 – 7.01 (1 H, m), 7.00 – 7.08 (2 H, m), 7.07 – 7.15 (1 H, m), 7.14 – 7.24 (4 H, m), 7.24 – 7.34 (1 H, s), 7.34 – 7.43 (4 H, m), 7.43 – 7.54 (3 H, m), 7.56 – 7.69 (5 H, m), 7.76 – 7.86 (3 H, m), 9.88 – 9.97 (1 H, s). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 191.92, 153.61, 151.35, 148.23, 144.14, 143.89, 141.04, 139.91, 136.21, 135.57, 132.99, 130.61, 130.41, 129.51, 127.15, 127.05, 126.55, 126.51, 124.24, 122.94, 121.70, 121.51, 121.46, 120.45, 55.74, 55.38, 40.68, 31.85, 31.81, 30.04, 29.97, 24.03, 22.91, 14.37, 14.33. HRMS (ESI-TOF): m/z calcd for C$_{46}$H$_{39}$NO 631.3814, found 631.3796.

Synthesis of (2Z,6Z)-2,6-bis(4-((E)-2-(7-(diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)benzylidene)s-indacene-1,3,5,7(2H,6H)-tetraone, 18.

(E)-4-(2-(7-(Diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)benzaldehyde (0.6 g, 0.95 mmol) and S-indacene-1,3,5(2H,6H)-tetraone (0.092 g, 0.43 mmol) were dissolved in glacial acetic acid(10 mL) in a two neck flask to form green yellow solution. The reaction mixture was
refluxed for 24 h, forming purplish solution that was then cooled to room temperature, affording precipitate. The precipitate was filtered, washed with acetic acid, and air dried. The crude product was purified by precipitation in hexanes while dissolved in dichloromethane and then recrystallized in hexanes/dichloromethane mixture to yield purplish solid (0.44 g, 71%). \(^1\)H NMR \(\delta_H (400 \text{ MHz, Chloroform-d}) 0.41 – 0.81 (21 \text{ H, m}), 0.79 – 1.21 (26 \text{ H, m}), 1.74 – 1.99 (8 \text{ H, d, } J 38.1), 6.78 – 7.00 (5 \text{ H, t, } J 6.9), 6.99 – 7.12 (8 \text{ H, d, } J 8.0), 7.12 – 7.24 (9 \text{ H, d, } J 7.4), 7.23 – 7.34 (4 \text{ H, s}), 7.33 – 7.51 (8 \text{ H, m}), 7.50 – 7.67 (4 \text{ H, d, } J 7.9), 7.74 – 7.92 (2 \text{ H, s}), 8.30 – 8.53 (4 \text{ H, d, } J 8.3). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta 0.38, 14.40, 14.44, 14.50, 22.97, 23.08, 24.15, 24.21, 27.47, 30.04, 30.08, 30.11, 31.31, 31.89, 31.93, 32.31, 40.67, 55.43, 55.80, 118.53, 119.43, 119.82, 120.55, 121.03, 121.63, 123.04, 123.76, 124.34, 126.59, 126.80, 126.90, 127.16, 127.25, 129.58, 130.46, 132.56, 134.92, 136.16, 141.31, 142.44, 145.11, 147.94, 148.27, 149.22, 151.47, 151.73, 153.02, 153.75, 187.61. HRMS (ESI-TOF): m/z [M+H]\(^+\) calcd for C\(_{104}\)H\(_{100}\)N\(_2\)O\(_4\) 1441.7761, found 1441.7753.
APPENDIX A: $^1$H AND $^{13}$C NMR SPECTRA OF NEW MOLECULES IN CHAPTER 3
$^1$H NMR spectrum of 2
$^1$H NMR spectrum of 2
$^1$H NMR spectrum of 3
$^1$H NMR spectrum of 3
$^1$H NMR spectrum of 4
$^1$H NMR spectrum of 4
$^1$H NMR spectrum of 5

Br\[\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOCH}_3\]

$\delta$ ppm: 1.34, 1.57, 1.86, 2.46, 3.04
$^{13}$C NMR spectrum of 5
1H NMR spectrum of 6.
$^{13}\text{C}$ NMR spectrum of 6
$^1$H NMR spectrum of 7
$^{13}$C NMR spectrum of 7
$^1$H NMR spectrum of 1
$^{13}$C NMR spectrum of 1
APPENDIX B: $^1$H AND $^{13}$C NMR SPECTRA OF NEW MOLECULES IN CHAPTER 4
$^1$H NMR spectrum of 9
$^{13}$C NMR spectrum of 9
$^1$H NMR spectrum of 11
$^{13}$C NMR spectrum of 11
$^1$H NMR spectrum of 8
13C NMR spectrum of 8
APPENDIX C: $^1$H AND $^{13}$C NMR SPECTRA OF NEW MOLECULES IN CHAPTER 5
$^1$H NMR spectrum of 14
$^{13}$C NMR spectrum of 14
$^1$H NMR spectrum of 16
$^{13}$C NMR spectrum of 16
$^1$H NMR spectrum of 12
$^{13}$C NMR spectrum of 12
$^1$H NMR spectrum of 13
$^{13}$C NMR spectrum of 13
HRMS-MALDI-DTL spectrum of 13
APPENDIX D: $^1$H AND $^{13}$C NMR SPECTRA OF NEW MOLECULES IN CHAPTER 6
$^1$H NMR spectrum of 19
$^{13}$C NMR spectrum of 19
$^1$H NMR spectrum of 20
$^{13}$C NMR spectrum of 20
$^1$H NMR spectrum of 21
$^{13}$C NMR spectrum of 21
$^1$H NMR spectrum of 18
$^{13}$C NMR spectrum of 18
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