2000

Bioanalytical Applications in the Near-Infrared Region Using Fluorescent Covalent and Non-Covalent Probes With Near-Infrared Fluorescence Detection.

Clyde Vernon Owens Jr
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/7218
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

135

This reproduction is the best copy available.

UMI
BIOANALYTICAL APPLICATIONS IN THE NEAR-INFRARED REGION
USING FLUORESCENT COVALENT AND NON-COVALENT PROBES WITH
NEAR-INFRARED FLUORESCENCE DETECTION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Clyde Vernon Owens
B.S., Morehouse College, 1994
May 2000
Acknowledgements

Before I begin, the work of this dissertation or my whole travels through life would not have been possible without the blessings of my Heavenly Father, who has truly guided me as the poem “Footprints.”

Footprints

One night a man had a dream. He dreamed he was walking along the beach with the Lord. Across the sky flashed scenes from his life. For each scene, he noticed two sets of footprints in the sand; one belonging to him, and the other to the Lord.

When the last scene of his life flashed before him, he looked back at the footprints in the sand. He noticed that many times along the path of his life there was only one set of footprints. He also noticed that it happened at the very lowest and saddest times in his life.

This really bothered him and he questioned the Lord about it. “Lord, you said that once I decided to follow you, you’d walk with me all the way. But I have noticed that during the most troublesome times in my life, there is only one set of footprints. I don’t understand why when I needed you most you would leave me.”

The Lord replied, “My child, My precious child, I love you and I would never leave you. During your times of trial and suffering, when you see only one set of footprints, it was then that I carried you.”

- Author Unknown
To my Mother and Father: I would never be able to express my deepest love and appreciation for the support you've shown me my entire life. I could probably write another dissertation expressing my love and joy. There were numerous times that I just wanted to quit, but you both kept me grounded by reminding me to always keep the faith. As long as I live, I will always remember what you both taught me and that was; Remember Who You Are (Clyde Owens) Remember Whose You Are (Rev. and Mrs. C.V. Owens' Son) and Remember Whom You Belong To (GOD) I love you both!

To my darling sister Shelia Owens Jones: Just like Mom and Dad, you have played a vital role in my life to helping reach this major accomplishment. Thank you for the extra calls, cash on the side, and extreme laughter over the phone to help forget my problems. Even though I've grown up to be a man, I will always be your little baby brother.

To Romeo Owens (1994-1999): Man, I wish you were still here. I remember bringing you home as a little puppy. You were the cutest little thing bouncing around with so much energy. No matter how angry I would be when I came home, you were always so cheerful and excited wanting to play to cheer me up. You seem to bring so much joy to me as well as to my family and friends. You were my little child, my companion, and my friend. I knew your sole purpose in life was to help my get through graduate school and that you did. Someday if paths ever cross again, do me a favor and give me one of those special hugs like you use to. I miss you man!
To Benjamin Clark, Charles and Jemel Kitchen: You guys are the brothers that I've always turned to as my external family. We've known each other for more than 20 years, joking as kids what we planned to become as adults. Thanks to you guys, I'm now an old Ph.D. chemist. I hope that we continue to support each other in both the good and bad times.

To Dr. Anna Patrice Shanklin: What kind of man would I be if I did not acknowledge you? You were truly an inspiration in my life without realizing. Your ever smiling face seem to always enlighten me, as well as those you encountered in the Chemistry Department. Thank you the magnificent times we shared including the late night conversions to keep me focused. No matter how far or how near B.K., you will always be a special lady.

To Ms. Kate Shanklin: Thank you for the Sunday, Monday, Tuesday, Wednesday, Thursday, Friday, and Saturday delicious, Louisiana-spiced home cooked meals. While I was away from home, you treated me your son helping me to broaden my horizons. I learned more about proper etiquette and theatrics from you than I could have learned from any institution. You truly are a Flower of the South.

To the Soper Research Group (Former and Current): Dr. James Flanagan, Dr. Daryl Williams, Dr. Ben Legendre, Dr. Yolanda Davidson, Emanuel Wadell, Scott McWhorter, Sean Ford thank you for the brutal and not so brutal group meetings that we all endured together. I will forever cherish the parties, Hooters, group lunches, and football tailgates.
To Dr. Soper: Under your tutelage, I’ve learned more than just chemistry. I’ve learned how to become an independent and responsible adult. Your guidance provided me the understanding that hard work and true effort will always pay off in the end. Thank you to you and your wife for the house parties, tailgates, and numerous laughs.

To Dr. Hammer: Thank you from the bottom of my heart in helping me to become a stronger Organic Chemist. Thank you for your honesty and patience when listening to many of the crazy synthetic routes that I tried to derive. Also, thank you for believing in me that we could synthesize and purify these dyes.

To my Officiating Brothas Courtney Kirkland, Anthony Jordan, and John Heatly: Thank you fellas for showing me a new direction in life through basketball officiating. You guys stuck with me in the beginning and I cherish that we stick together in the end. I’ll see all three of you in the NBA Finals!

To Clinton Hunt and special expression to Roya Hughes: Thank you gentlemen for the memorable conversation during lunch. Lunches will never be the same wherever I go. Roya, thank you for being a big brotha, uncle, and most definitely a friend. Thank you for all your help during my 1212 teaching journey. Thank you for the positive criticism and advice. And most definitely, thank you for the memorable stories that you shared with me about your life. You know that I did not believe any of them.

To Michelle Ross: Thank you for the extra support that you provided when I just needed confidence to keep continuing this journey. I will always treasure our friendship forever.
# Table of Contents

Acknowledgements ........................................................................................................ii

Lists of Tables .............................................................................................................viii

Lists of Figures ..............................................................................................................ix

Lists of Schemes ..........................................................................................................xiv

Abstract ........................................................................................................................xvi

Chapter 1. Bioanalytical Applications Using Fluorescence Detection .....................1

1.1 Theory of Fluorescence .........................................................................................1
  1.1.1 Emission and Excitation Spectra ..................................................................4
  1.1.2 Fluorescent Quantum Yields and Lifetimes ..............................................7
  1.1.3 Variables Affecting Fluorescence ...............................................................7
  1.1.4 Propriationality of Fluorescence with Radiation .......................................9
  1.1.5 Instrumentation for Fluorescence ...............................................................10

1.2 Fluorescent Probes ...............................................................................................12
  1.2.1 Covalent Fluorescent Probes ......................................................................13
  1.2.2 Non-Covalent Fluorescent Probes ...............................................................14

1.3 Near-Infrared Fluorescence Detection ................................................................18
  1.3.1 Instrumentation ...........................................................................................19
  1.3.2 Near-Infrared Chromophores .....................................................................21

1.4 Research Goals ......................................................................................................23

1.5 References .............................................................................................................24

Chapter 2. Labeling Dyes for DNA Sequencing .......................................................26

2.1 Introduction .............................................................................................................26

2.2 Spectral Discrimination .......................................................................................27
  2.2.1 Visible Fluorescent Dyes ............................................................................27
  2.2.2 Near-IR Dyes for Sequencing ....................................................................31

2.3 Lifetime Methods ..................................................................................................32

2.4 Heavy Atom Modified Dyes ................................................................................36

2.5 Experimental Section .........................................................................................38
  2.5.1 Synthesis of Near-Infrared Heavy Atom Modified Dyes ..........................38
  2.5.2 Labeling and Purification of Sequencing Primers with Near-IR Heavy-Atom-Modified Dyes .................................................................38
  2.5.3 Spectroscopic Analysis ..............................................................................39

2.6 Results and Discussion ..........................................................................................41

2.7 Summary ...............................................................................................................53

2.8 References .............................................................................................................55

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
List of Tables

**Table 2.1** Chemical structures of some typical near-IR fluorescent dyes as well as their photophysical properties. The dyes contain an isothiocyanate to allow conjugation to amine containing molecules..........................33

**Table 2.2** Absorption and Emission Maxima, Extinction Coefficients and Fluorescence Quantum Yields for the Heavy Atom Modified Near-IR Fluorescent Dyes in Methanol..................................................43

**Table 2.3** Fluorescence Lifetimes of Near-IR Dyes in pure Methanol.................44

**Table 2.4** Fluorescence properties of the Heavy-Atom Modified Near-IR dyes, both free and conjugated to a 17mer sequencing primer, measured in a nonpolymerized acrylamide solution containing 40% formamide and 6%T/5%C. ................................................................................46

**Table 2.5** Electrophoretic mobilities of Heavy-Atom Modified Near-IR fluorescent dyes (7-11) in free solution. .................................................................54

**Table 3.1** CE analysis of the *Hae III* restriction digest of the native ΦX174 fragments and the Et-Br or TAG-stained fragments. Detection was accomplished using UV absorption at 254 nm. The restriction fragments were injected electrokinetically onto the CE column for 30 s at -5 kV.........................72
List of Figures

Figure 1.1. Jablonski diagram showing the various radiative and non-radiative pathways which occur following the absorption of a photon of light by a molecule..........................................................3

Figure 1.2. Excitation and Emission profile for Anthracene demonstrating Stokes shift and mirror image relationship..............................................................6

Figure 1.3. Schematic diagram of an instrument to measure fluorescence radiation....11

Figure 1.4. Fluorescent labeling probes. I, fluorescein isothiocyanate; II, tetramethylrhodamine isothiocyanate; III, rhodamine isothiocyanate.............14

Figure 1.5. Fluorescent probes that bind non-covalently to dsDNA. I, Ethidium bromide; II, Propidium Iodide; III, Thiazole-orange homodimers and oxazole-yellow homodimers..............................................15

Figure 1.6. Structure of some typical Near-IR Chromophores. I, Phthalocyanine; II, Napthalocyanine; III, Cyanine class family.................................................22

Figure 2.1. The fluorescent dyes used for single lane base-calling in DNA sequencing applications using spectral discrimination.............................................28

Figure 2.2. The chemical structures of Bodipy dyes used in DNA sequencing........30

Figure 2.3. Structure of fluorescent dyes MR-200, JA169, JA242, and CY5 utilized as fluorescent labels in DNA sequencing.................................................37

Figure 2.4. Structure of Near-IR heavy atom modified dyes (7-11).........................40

Figure 2.5. Absorption and emission spectra of the near-IR heavy atom-modified dyes dissolved in methanol at a concentration of 1 µM. In the case of the fluorescence spectra, the data were collected on a red-sensitive photon-counting spectrofluorometer using an excitation wavelength of 710 nm....42

Figure 2.6. Absorption spectra of the free dyes (A) and the dye-primer conjugates (B) measured in the nonpolymerized acrylamide solution containing 40% formamide. The absorption spectra were acquired using a dye concentration of 1 µM.................................................................48
Figure 2.7. Fluorescence decay profiles for dyes 8 and 11 conjugated to the M13mp18 sequencing primers measured in a nonpolymerized acrylamide gel solution containing 40% formamide and 1 x TBE along with the prompt peak (instrument response function). In this case, the dye concentration was set at 10 nM with 1 mW of laser power at 765 nm used for the excitation.

Figure 2.8. Capillary gel electropherogram of the heavy atom-modified near-IR dyes (8-11) conjugated to a 17mer M13mp18 sequencing primer. In this case, the dye-primer concentration was 1 nM, and the column consisted of a 6%T/5%C cross-linked polyacrylamide sieving matrix with 7M urea as the denaturant.

Figure 3.1. NIR LIF system for CE analysis of DNA restriction fragments. M1, M2, and M3 = mirrors; L = laser focusing lens; C = capillary tube; BD = beam dump; MO1 and MO2 microscope objective (see text); S = spatial filter; F = optical filters; SPAD = single photon avalanche diode; C/T = counter/timer; PC = computer. The LIF system was constructed in house by Dr. Bill Karr.

Figure 3.2. Absorption spectra of TAG in methanol (solid line), TRIS/acetate buffer with no DNA (dashed line), and a 100-fold molar excess of calf thymus DNA (dotted line). Dye concentration was 2 x 10^{-5} M.

Figure 3.3. Spectrofluorometric titration of TAG with calf thymus DNA. In all cases, the DNA concentration was measured in terms of the nucleotide bases and was varied from 5 x 10^{-6} M (yellow line), 5 x 10^{-5} M (red line), 1 x 10^{-5} M (blue line), 1 x 10^{-4} M (black line). The fluorescence was excited at 710 nm and the dye concentration used was 1 x 10^{-6} M. The insert shows the construction of a modified Benesi-Hildebrand indicated the binding constant of TAG to dsDNA was approximately 1 x 10^6 M^{-1}.

Figure 3.4. Electropherogram of ΦX 174 DNA restriction fragments with LIF detection. The dye concentration added to the running buffer was 1.0 μM and the running buffer was composed of 40 mM TRIS, 20 mM sodium acetate, 2.0 mM EDTA (pH 7.6) and 0.25% HPMC. The sample was electrokinetically injected for 6 s at -2.0 kV onto the column.

Figure 3.5. Electropherograms of ΦX 174 DNA restriction fragments obtained using (a) 4.0 μM, (b) 1.0 μM, and (c) 0.2 μM dye in the running buffer.
Figure 4.1. Preparation of labeling, water-soluble phthalocyanine dye placing the reactive group L in one “quadrant” of the macrocycle with one or more of the remaining quadrants to incorporate water-solublizing groups S

Figure 4.2. Structure of Water-Soluble Naphthalocyanine (4) and Phthalocyanine (7) dyes containing reactive isothiocyanate for labeling primary amines.

Figure 4.3. UV absorbance profile of Naphthalocyanine congeners (I, 2, II, III). The samples were evaluated after collection from HPLC during separation, so their concentrations were not determined. The solvent system of each profile consisted of a variation of the HPLC linear gradient (0.1 M triethylammonium acetate/20 mM sodium phosphate buffer (pH=10.1) and tetrahydrofuran over 30 min.

Figure 4.4. HPLC chromatogram of the Naphthalocyanine congeners (I, 2, II, III) obtained in the reaction of 4-nitrophthalonitrile and 6,7-dicyano-napthalenecarboxylic acid. Conditions: Hamilton PRP-1 analytical column (4.6 x 15 cm; flow rate 2 mL/min) and a gradient of 0.1 M triethylammonium acetate/20 mM sodium phosphate buffer (pH ~ 10.1) and tetrahydrofuran. The absorbance detector was set at 700 nm.

Figure 4.5. Structure of Naphthalocyanine congeners (I, 2, II, III) in the reaction of 4-nitrophthalonitrile and 6,7-dicyano-napthalenecarboxylic acid.

Figure 4.6. MALDI mass spectra of Naphthalocyanine congeners (I, 2, II, and III). The experiments were performed on a Perseptive Biosystems Inc. Voyager linear MALDI-TOF instrument with a N2 laser in both positive and negative-ion modes. Laser power set at the threshold level required to generate signal and each spectrum is an average of 32-40 scans. Each experiment was conducted three separate times at different locations on the sample spot to average results and ensure reproducibility.

Figure 4.7. HPLC chromatogram of the Phthalocyanine congeners (IV, 3, V, VI) obtained in the reaction of 3-nitrophthalonitrile and 4-(3,4-dicyanophenoxy) benzoic acid. Conditions: Hamilton PRP-1 analytical column (4.6 x 15 cm; flow rate 2 mL/min) and a gradient of 0.1 M triethylammonium acetate/20 mM sodium phosphate buffer (pH ~ 10.1) and tetrahydrofuran. The absorbance detector was monitored at 680 nm.

Figure 4.8. Structure of the Phthalocyanine congeners (IV, 3, V, VI) in the reaction of 3-nitrophthalonitrile and 4-(3,4-dicyanophenoxy) benzoic acid.
Figure 4.9. MALDI mass spectra of Phthalocyanine congeners (3, V, VI). The experiments were performed on a Perseptive Biosystems Inc. Voyager linear MALDI-TOF instrument with a N₂ laser in both positive and negative-ion modes. Laser power set at the threshold level required to generate signal and each spectrum is an average of 32-40 scans. Each experiment was conducted three separate times at different locations on the sample spot to average results and ensure reproducibility.

Figure 4.10. Reverse-phase HPLC chromatograms of M17 primer labeled with Naphthalocyanine and Phthalocyanine dye. Column: Reverse Phase-C₁₈ 50 x 4.6 mm i.d.; mobile phase: 0.1M TEAA, pH = 7.0 linear gradient 4 to 80 % acetonitrile in 20 min; flow rate: 1.7 mL/min. UV absorbance detection at 254 nm.

Figure 4.11. Absorbance (A) and emission (B) spectra of naphthalocyanine dye 4 (solid line) in 70:30 Methanol/DMF and dye-primer conjugate (open triangle) in 40 mM borate buffer/30% DMF. Dotted line (---) denotes native dye evaluated in 40 mM borate buffer. Dye concentration 2.5 x 10⁻⁶M.

Figure 4.12. Absorbance (A) and emission (B) spectra of phthalocyanine dye 7 (solid line) in 70:30 Methanol/DMF and dye-primer conjugate (open triangle) in 40 mM borate buffer/30% DMF. Dotted line (---) denotes native dye in 40 mM borate buffer. Dye concentration 2.5 x 10⁻⁶M.

Figure 4.13. LIF Electropherogram of Naphthalocyanine Dye. The separations were performed with a running buffer consisting of 40 mM borate (pH = 9.4) and 10% methanol. The cationic surfactant (0.5 mM dodecatriethylammonium bromide) was added to reverse the direction of the electroosmotic flow. Conditions: 40 cm 75 μm capillary column; 33 cm to the detection window; Field Strength 250 V/cm; Fluorescence detection at 750 nm.

Figure 4.14. LIF Electropherogram of Labeled Amino Acids with Naphthalocyanine Dye. The separations were performed with a running buffer consisting of 40 mM borate (pH = 9.4), 10% methanol, and 0.5 mM dodecatriethylammonium. Conditions: 40 cm 75 μm capillary column; 33 cm to the detection window; Field Strength 250 V/cm; Fluorescence detection at 750 nm.
Figure 4.15. UV Absorbance Electropherogram of Naphthalocyanine and Phthalocyanine Dye-Primer Conjugates. The separations were performed with a running buffer consisting of 40 mM borate (pH = 9.4), 10% methanol, and 0.5 mM dodecatriethylammonium. Conditions: 75 μm capillary column; Field Strength -250 V/cm; UV Absorbance detection at 254 nm…………………………………………………………………………………………117

Figure 4.16. Naphthalocyanine dye-conjugate UV Absorbance electrophorogram in a 3%T/3%C polyacrylamide gel. Conditions: 75 μm i.d.; total length of 40 cm and a detection window 33 cm from the injection end. The dye/oligonucleotide conjugate was electrokinetically injected The detection was performed using UV absorbance monitored at 254 nm…………………………………………………………………………………………118

Figure 4.17. Fluorescence Decay Profile for Napthalocyanine Native Dye and Labeled Primer in DMF and the instrument response function using time-correlated NIR Fluorescence spectrometer…………………………………………………………119
Lists of Schemes

**Scheme 3.1.** Synthetic scheme for Near-Infrared cationic dye Thiazole-Green, (TAG) ..................................................................................................................67

**Scheme 4.1.** Synthesis of Phthalonitrile 1a and Naphthalonitrile Precursors .........................................................................................................................88

**Scheme 4.2.** Synthesis of 12,24,36-Tricarboxy-(4-nitro-zincnaphthalocyanine) (2). ..................................................................................................................89

**Scheme 4.3.** Synthesis of 12,24,36-Tricarboxy-(4-amino-zincnaphthalocyanine) (3). ..................................................................................................................95

**Scheme 4.4.** Synthesis of 12,24,36-Tricarboxy-(4-isothiocyanatezincnaphthalocyanine) (4). ........................................................................................................96

**Scheme 4.5.** Synthesis of 11,19,27-Tricarboxyphenoxy-(4-nitro-zincphthalocyanine) (5). ..................................................................................................................97

**Scheme 4.6.** Synthesis of 11,19,27-Tricarboxyphenoxy-(4-amino-zincphthalocyanine) (6). ........................................................................................................102

**Scheme 4.7.** Synthesis of 11,19,27-Tricarboxyphenoxy-(4-isothiocyanate-zincphthalocyanine) (7). ........................................................................................................103

**Scheme 5.1.** Reaction of 5-nitro-2,3-napthalenedicarbonitrile and 5(6)-sulfonapthalic anhydride ........................................................................................................123

**Scheme 5.2.** 5-nitro-2,3-napthalenedicarbonitrile and 2,3-napthalenedicarbonitrile reacted producing the hydrophobic macrocycle followed by fuming sulfuric acid ........................................................................................................124

**Scheme 5.3.** The reaction of 5-nitro-2,3-napthalenedicarbonitrile \( \text{\textsuperscript{\textit{\textendash}}} \text{\textendash} \) and 6-diethoxyphosphoryl-2,3-dicyanonaphthalene with zinc acetate ........................................................................................................125

**Scheme 5.4.** The condensation reaction of 6,7-dicyano-2-napthalenecarboxylic acid and 5-amino-2,3-napthalenedicarbonitrile with lithium metal ........................................................................................................126

**Scheme 5.6.** Reaction of Zinc Naphthalocyanine using the Meerwein Procedure ........................................................................................................127
Scheme 5.6. The condensation reaction of 6,7-carboxy-2,3-dicarbonitrile and 5-nitro-2,3-dicarbonitrile with zinc acetate.................................128

Scheme 6.1. Synthesis of cationic naphthalocyanine for DNA intercalation..........134
Abstract

The research presented in this dissertation entails the preparation, characterization, and analytical application of a series of novel tricarbocyanine, phthalocyanine, and napthalocyanine dyes which can be used as fluorescent probes for bioanalytical assays. The first part of this research focuses on the synthesis of a nuclear staining tricarbocyanine dye used for low-level detection of DNA restriction fragments separated via capillary electrophoresis. The second part focuses on the synthetic, purification, and applications of several tricarbocyanine, phthalocyanine, and napthalocyanine dyes. The tricarbocyanine dyes were synthesized to contain a heavy atom-modification to enhance $k_{isc}$ through spin-orbit coupling and a reactive functional group to react with primary amines of various biomolecules. The heavy-atom modified tricarbocyanine dyes also possessed similar absorption and emission maxima as well as similar electrophoretic mobilities, but they have unique fluorescence lifetimes. Like the tricarbocyanine dyes, the phthalocyanine and napthalocyanine dyes were also synthesized to contain a reactive group for facile conjugation to various biomolecules. The methodology for these dyes will be used for base-calling in DNA sequencing involving the use of a single-lane CGE separation of the near-infrared (near-IR) dye-labeled DNA fragments with the terminal bases identified via fluorescence lifetime discrimination.
Chapter 1

Bioanalytical Applications Using Fluorescence Detection

1.1 Theory of Fluorescence

The discovery of analytical luminescence can be traced back as early as the sixteenth century, when the Spanish physician Nicolas Monardes observed a mysterious blue tinge in water stored in cups made from the wood “lignum nephriticum”. In 1833, Sir David Brewster recognized a red emission from green leaf extracts. Although he first attributed the color to some type of dispersive-scattering phenomenon, today we know this characteristic emission as fluorescence from chlorophyll. In 1845 Herschel recorded the first fluorescence emission spectrum of quinine. However, it was not until 1852 that George Stokes determined that the emission from quinine was at a longer wavelength than the excitation, a difference that bears Stokes’ name. His subsequent studies provided the foundation for the use of fluorescence as an analytical tool.

Fluorescence is an analytical emission process which can be described as an excitation-deexcitation process involving photons. A molecule that has absorbed electromagnetic radiation is known to be in an excited electronic state. The molecule must now use some mechanism to aid in eliminating this excess energy. The competitive process of emitting a photon for deexcitation of an excited electronic state to the ground state results in fluorescence emission.

The fluorescence lifetime of an excited species, which is illustrated in Figure 1.1 by a Jablonski diagram, is brief because there are several pathways an excited atom or molecule can give up its excess energy and relax to its ground state. In this figure,
$S_0, \ldots, S_n$ and $T_1, \ldots, T_n$ represent the electronic energy levels of a molecule, where the state of the lowest energy, $S_0$, is the ground state. $S_1, \ldots, S_n$ and $T_1, \ldots, T_n$ are the excited singlet and triplet states, respectively.

Once a photon absorbs light, the relative number of molecules in the $v=0$ and $v=1$ vibrational states can be described by the Boltzmann distribution. The ratio of molecules ($R$, where $R = v_1/v_0$) in each of these states is given by:

$$R = e^{-\Delta E/kT} \quad (1.1)$$

where $\Delta E$ is the energy difference between the two levels, $k$ is the Boltzmann constant, and $T$ is the temperature in degrees Kelvin, K. At room temperature which is about 300 K, most molecules display a typical ratio value of 0.01, therefore, providing those molecules are in the lowest vibrational state $v=0$ of $S=0$ where the absorption of light mainly occurs. Through vibrational relaxation following light absorption, molecules can rapidly dissipate the excess energy as heat by collision with solvent molecules. Also, the molecules can pass from a low vibrational level of $S_2$ to an equally energetic vibrational level of the first excited singlet, $S_1$, through the process of internal conversion (IC). For many compounds, once the molecule reaches the first excited singlet, internal conversion to the ground state is a relatively slow process. Fluorescence occurs as a result of the molecule losing energy in the form of a photon of light followed by return to the ground state, $S_0$. Normally, fluorescence emission occurs very rapidly (about $10^{-9}$ to $10^{-7}$ sec) in order for many of the other molecules to compete effectively with other processes capable of removing excitational energy, such as internal conversion.
Figure 1.1 Jablonski diagram showing the various radiative and non-radiative pathways which occur following the absorption of a photon of light by a molecule.
While a molecule is in the excited state, one electron may reverse its electronic spin, and then transfer to an excited triplet state, T₁. This process of nonradiative transfer from the singlet to the triplet state is termed intersystem crossing (ISC). After transferal to the triplet state, the molecule can rapidly relax to the lowest vibrational level of the first excited triplet through a vibrational relaxation process. From T₁, the molecule can return to S₀ by emission of a photon. This emission is noted as phosphorescence, which is much longer lived than fluorescence (about 10⁻³ to 10 sec).

In terms of quantum mechanics, fluorescence can be described as the electric dipole transition from an excited singlet state to a lower singlet state, usually the ground state, through the release of energy in the form of a photon. Mathematically, the probability of fluorescence emission is proportional to \( R^2_{i\rightarrow f} \), where \( R^2_{i\rightarrow f} \) the transition moment integral between the excited state \( i \) and the relaxed state \( f \), is given by:

\[
R^2_{i\rightarrow f} = \int \Psi_f \hat{M} \Psi_i d\tau_e \int \Psi_f \Psi_i \tau_n
\]  

(1.2)

where \( \Psi_e \) represents the electronic wavefunction, \( \Psi_v \) is the vibrational wavefunction, \( \hat{M} \) is the electronic dipole moment operator and the Born-Oppenheimer principle of separability of electronic and vibrational wavefunctions has been invoked. According to the Franck-Condon factor, the electronic transitions of a molecule occur in a time frame in which the positions of the nuclei do not change, therefore, all electronic transitions are vertical. The Franck-Condon factors describing transition probabilities are usually equally probable for the absorption and emission processes.

1.1.1 Excitation and Emission Spectra. A molecule which fluoresces can be characterized by two representative spectra, noted as the excitation and emission spectra.
spectra. The excitation spectrum is a measure of the fluorescence intensity observed as a function of exciting wavelength at some fixed emission wavelength. The vibrational structure of an electronic spectrum corresponds to the vibrational nature of the excited state, $S_1$. When the excitation spectrum is compared to the absorption spectrum, the wavelength positions of each band in the absorption spectrum are usually the same as for the bands in the excitation spectrum. Excitation spectroscopy can provide absorption characteristics of a fluorescing compound, but at concentrations much lower than for absorption measurements.

The emission spectrum is a measure of the fluorescence intensity observed as a function of emitting wavelength at some fixed excitation wavelength. The bands in the emission spectrum correspond to vibrational energy levels of the ground state. The intensity of these bands are determined by the degree of overlap of the wavefunctions of the various levels of the ground ($S_0$) and excited states ($S_1$ and $S_2$). Molecules which fluoresce in condensed media show a shift of the emission spectra to longer wavelengths with respect to the absorption spectra due to the loss of energy between the excited and ground state. The difference in this energy is called the Stokes Shift (see Figure 1.2). The Stokes shift is fundamental to the sensitivity of fluorescence because it permits emission of photons to be detected against a low background, isolated from excitation photons. The emission spectra and absorption spectra will have an approximate mirror-image relationship if the vibrational spacing in the ground state ($S_0$) and the first excited singlet ($S_1$) are roughly equal.
Figure 1.2 Excitation and Emission profile for Anthracene demonstrating Stokes shift and mirror image relationship.\textsuperscript{3}
1.1.2 Fluorescent Quantum Yields and Lifetimes. The quantum yield, $\Phi_F$, or quantum efficiency is defined as the ratio of the number of photons emitted as fluorescence to the number of photons absorbed;\(^4\)

$$\Phi_F = \frac{k_r}{k_r + k_{nr}}$$  

(1.3)

where $k_r$ is the radiative rate of emission and $k_{nr}$ is the non-radiative rate (s\(^{-1}\)). The non-radiative rate is composed of several nonradiative rates, which can deactivate the excited state and is expressed as;

$$k_{nr} = k_i + k_{ec} + k_{ic} + k_{pd} + k_d$$  

(1.4)

where $k_i$ (intersystem crossing), $k_{ec}$ (external conversion), $k_{ic}$ (internal conversion), $k_{pd}$ (photodissociation), and $k_d$ (dissociation). The maximum value of the quantum yield is 1. When no fluorescence occurs, the quantum yield value has a minimum value of 0.

The fluorescent lifetime of the excited state can generally be defined by the expression;

$$I_t = I_0 e^{-\frac{t}{\tau}}$$  

(1.5)

where $I_0$ and $I_t$ are the intensity of emission of a species during irradiation and at time $t$ after such excitation. From equation 1.5, $\tau$ is seen to be the time required for luminescence to decay to 1 / $e$ of its initial value.

1.1.3 Variables Affecting Fluorescence. The molecular structure and chemical environment are two major influential factors in determining whether a substance will or will not fluoresce. These factors may also determine the intensity of emission when luminescence occurs. The most intense fluorescence is usually found in compounds
containing aromatic functional groups with low-energy $\pi - \pi^*$ transition levels. Compounds which contain aliphatic and alicyclic carbonyl structures or conjugated double-bond structures may also exhibit intrinsic fluorescence. However, the number of compounds is small when compared to those with the aromatic system.

Substituents on molecules also greatly influence fluorescence. Electron-donating substituents, such as $-\text{NH}_2$, $-\text{OH}$, $-\text{F}$, $-\text{OCH}_3$, $-\text{NHCH}_3$, and $-\text{N(CH}_3 \rangle$, often increase fluorescence because they tend to enhance the transition probability between the lowest excited singlet state ($S_1$) and the ground state ($S_0$). In contrast, electron withdrawing groups including $-\text{I}$, $-\text{Br}$, $-\text{Cl}$, $-\text{NO}_2$, and $-\text{COOH}$ partially or completely quench the fluorescence by attaching a low lying $n, \pi^*$ state. Thus, the rate of intersystem crossing is usually enhanced, resulting in enhancement of phosphorescence.

Solvents have a dramatic effect on fluorescence due to the dipole-dipole interaction between the solvent and the solute. These effects originate from the change in absorption accompanied by solvent relaxation. The effects of solvents on absorption reflect the dipole moment changes of solute induced by the solvent. When the solute is non-polar, the spectrum may shift to the red in polar or non-polar solvents. When the solute is polar, the spectrum may appear with a red or blue shift.

The temperature and pH also commonly affect the fluorescence of molecules. The quantum efficiency of fluorescence decreases with increasing temperature because the increased frequency of collisions at elevated temperatures improves the probability for deactivation by external conversion. The pH is important with molecules containing acidic or basic functional groups. Protonation of a fluorophore with electron donating
groups causes fluorescence to produce a blue shift, while deprotonation produces a red shift.

1.1.4 Proportionality of Fluorescence with Radiation. The quantitative relationship between the fluorescence power and concentration is derived from the following considerations. The fluorescence power is proportional to the number of molecules in the excited states, which is proportional to the radiant power absorbed by the sample. Thus;

\[ P_F = \Phi_F (P_o - P) \]  

(1.6)

where \( P_F \) is the radiant power or fluorescence intensity, \( \Phi_F \) is the quantum yield of fluorescence, \( P_o \) is the radiant power on the sample, and \( P \) is the radiant power emerging from the sample. \( P_o - P \) is therefore the radiant power absorbed by the sample. The quantum yield, \( \Phi_F \), is defined as the ratio of the number of photons emitted as fluorescence to the number of photons absorbed;³

\[ \Phi_F = \frac{\text{photons emitted as fluorescence}}{\text{photons absorbed}} \]  

(1.7)

Applying Beer's Law to equation 1.5, it can now be rewritten as;

\[ P_F = \Phi_F P_o (1 - e^{-\varepsilon bc}) \]  

(1.8)

and expanding equation 1.8 in a power series yields

\[ P_F = \Phi_F P_o \varepsilon bc \left( 1 - \frac{\varepsilon bc}{2!} + \frac{(2.3\varepsilon bc)^3}{3!} - \cdots \right) (n + 1) \]  

(1.9)

If \( \varepsilon bc \) is small, only the first term in the series is significant and equation 1.9 can be written as;³

\[ P_F = \Phi_F 2.303P_o \varepsilon bc \]  

(1.10)
1.1.5 Instrumentation for Fluorescence. A generalized fluorescence instrument shown in Figure 1.3 consists of (1) a source of radiation, (2) an excitation monochromator, (3) a sample cell, (4) an emission monochromator, (5) a photodetector, and (6) a data readout device. The radiation source may consist of either a xenon or mercury arc lamp. The xenon lamps are generally used in most spectrofluorometers because of the intense emitted radiation produced is continuous from the visible to the near-infrared region. The mercury arc lamps produce a very high intense radiation, but only at discrete wavelengths. In the early 1970s, lasers, such as Helium:Neon and Argon, were implemented as popular radiation sources because they provided an intense beam of monochromatic light which could be focused to a smaller spot producing a higher power incident beam.4

The incident beam is then sent through an excitation monochromator, which selects specific bands or wavelengths of radiation from the source and directs them through the sample in the sample cell. The sample cuvette, which is usually enclosed in a light tight compartment, provides sample containment and a uniform illumination surface. Illumination of the sample is generally performed in the 90° right-angle geometry, because none of the cuvette fluorescence or radiation reflected from other surfaces enters the emission monochromator.

The resultant fluorescence radiation, which has been displaced from the initial sample cuvette, is isolated by the emission monochromator and directed to the photodetector, which measures the power of the emitted radiation. Typical fluorescence signals are of very low intensity and large amplification is generated using a photomultiplier tube. Photomultiplier tubes are used because of their high gain and
Figure 1.3 Schematic diagram of an instrument to measure fluorescence radiation.
relatively broad spectral sensitivity to low radiation levels. The signal produced is transferred to a data readout device, such as a personal computer.

1.2 Fluorescent Chromophores

The use of fluorescent chromophores is a sensitive method to get information about the structure, function, and composition of many biomolecules. The chromophores, which are used for labeling antibodies, DNA, and lipids, are designed for high detection sensitivity. They have large extinction coefficients, high quantum yields, and are insensitive to pH and the polarity of their local molecular environments. Fluorescence can be sensitive in the ability of probe molecules to respond spectroscopically to subtle changes in their molecular environment. The dependence of absorption wavelength, extinction, emission wavelength, quantum yield provides a powerful method for understanding the behavior of the biomolecule. One of the key requirements for exploiting the sensitivity of probes of microenvironment is the careful design of the fluorophore structure so that it can be targeted to the site of interest in the biomolecule. Therefore, the skills of the organic chemists must be blended with those of the spectroscopist and molecular biologists to optimally exploit the potential of the fluorescent probes.

Ideal Properties of Fluorescent Probes

- **Large Extinction Coefficient at the Selected Region of Excitation.** The value of the extinction coefficient, $e$, should be at least as high as the standard fluorescein (60,000 L mol$^{-1}$ cm$^{-1}$) and ideally as high as the multichromophore phycobiliproteins ($2 \times 10^5$ L/mol$^{-1}$cm$^{-1}$) when used as a label for sensitive protein or nucleotide detection.
experiment. Large extinction coefficients demonstrate a highly sensitive means for detecting and determining absorbing species.

- **High Quantum Yield.** A high quantum yield or efficiency ensures that the excitation light employed in the analytical procedure is converted efficiently into detectable emission. In the solvent environment where the fluorescence measurement is to be evaluated, the quantum yield should be greater when the fluorescent probe is bound to the target molecule. Typical values for quantum yields range from 0 to 1.

- **Photostability.** Photostability is an important attribute for detecting a small number of probes, especially because photobleaching is dependent on the fluorophore’s environment. Under high intensity radiation, the fluorophores must have the photostability to withstand illumination before photobleaching of the excited molecule becomes the factor limiting detectability.

- **Minimal Perturbation by Probe.** The fluorescent probe should not perturb the function of the target molecule by reacting with key groups in active sites or by causing nonspecific binding of a labeled protein, or by causing steric perturbations because of its size.

1.2.1 **Covalent Fluorescent Probes.** The labeling reagent should be soluble in aqueous solutions and have the proper reactivity and selectivity. If too reactive, the tagging reagent may hydrolyze before binding to the target. Isothiocyanates, chlorotriazinyl derivatives, and hydroxysuccinimido esters are the most common functional groups that allow chromophores to be attached covalently to primary amino groups of the biomolecule. The fluorescence of the label should also be insensitive to solvent pH and polarity.
The most commonly used fluorescent tag is fluorescein isothiocyanate, which has been attached to antibodies, lectins, avidin hormones, biological macromolecules, and lipids (see Figure 1.4).\textsuperscript{7} Fluorescein demonstrates ideal properties such as its reasonably large extinction coefficient ($\varepsilon \sim 60,000 \text{ M}^{-1}\text{cm}^{-1}$), high quantum efficiency (>0.9), and good water solubility. However, there are cases where fluorescein has disadvantages. It is not particularly photostable, its absorption spectrum falls in a region where cell autofluorescence is produced, and its fluorescence is diminished as the pH drops below 8.

There have been other fluorescent probes (see Figure 1.4) synthesized to improve upon the properties of fluorescein and to provide additional chromophores with different absorbance and emission wavelengths.\textsuperscript{7} The rhodamine derivatives and phycobiliproteins are normally more stable than fluorescein and fluoresce in a region where less autofluorescence is produced.\textsuperscript{8} However, the rhodamine dyes demonstrate less water solubility than their counterparts making them particularly unattractive for labeling applications.

1.2.2 Non-covalent Fluorescent Probes. There has been extensive research investigating the interaction of non-covalent fluorescent probes with various target molecules. Non-covalent labeling of oligonucleotides with staining dyes has been used to detect polymerase chain reaction (PCR)-amplified fragments\textsuperscript{9-11} and restriction fragments\textsuperscript{12-14} after separation using analytical techniques such as capillary electrophoresis. These dyes consist of cationic, heteroaromatic fragments which intercalate between base pairs of the DNA double helix. The mono-intercalators like
Figure 1.4 Fluorescent labeling probes. I, fluorescein isothiocyanate; II, tetramethylrhodamine isothiocyanate; III, rhodamine isothiocyanate.
Figure 1.5 Fluorescent probes that bind non-covalently to dsDNA. I, Ethidium bromide; II, Propidium Iodide; III, Thiazole-orange homodimers and oxazole-yellow homodimers.
propidium iodide\textsuperscript{7} and ethidium bromide\textsuperscript{15,16} are the most commonly used general nucleic acid stains and are shown in \textbf{Figure 1.5}.

DNA binding agents tend to interact non-covalently with the host molecule through two general modes: (1) in a groove-bound fashion stabilized by a mixture of hydrophobic, electrostatic, and hydrogen-bonding interactions and (2) through intercalative association.\textsuperscript{17} Intercalation with DNA is understood as insertion of a planar chromophore between two stacked DNA base pairs.\textsuperscript{18} Through intercalation, there is an unwinding and lengthening of the double-stranded helix. These changes are a consequence of the untwisting of the base pair and helical backbone needed to accommodate the intercalator. Intercalation results in an ordered stacking of the bound species between base pairs at 3.4 Å separation. Upon intercalation, the average distance between two stacked base pairs increases from 3.4 Å to approximately 7-8 Å. The intercalator becomes rigidly held and orientated with the planar moiety perpendicular to the helical axis. The intercalating dyes usually bind at a stoichiometry of one dye per 4–5 base pairs of DNA. Once these dyes are bound to nucleic acids, their fluorescence is enhanced ~20- to 30-fold, their excitation maxima are usually shifted ~30–40 nm to the red and their emission maxima are usually shifted ~15 nm.\textsuperscript{19}

The dimeric dyes (see \textbf{Figure 1.5}) consist of two chromophores covalently linked by a polymethine cationic chain forming the dimeric molecule containing four positive charges. These dyes show intercalative binding like the monomers and impressively larger binding constants to dsDNA when compared to their monomeric counterparts. For example, the intrinsic DNA binding affinity constants of ethidium bromide and ethidium homodimer-1 are reported to be $1.5 \times 10^5$ and $2 \times 10^8$ M\textsuperscript{-1},

\hspace{1cm}

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
respectively.\textsuperscript{20} The extraordinary stability of dimeric dye/nucleic acid complexes ensures that the dye/DNA association remains stable even during electrophoresis.

In addition to their superior binding properties, dyes such as TOTO-1 and the other cyanine dimers are essentially nonfluorescent in the absence of nucleic acids and exhibit significant fluorescence enhancements upon DNA binding (100- to 1000-fold).\textsuperscript{21} Furthermore, the fluorescence quantum yields of the cyanine dimers bound to DNA are high (generally between 0.2 and 0.6), and their extinction coefficients are an order-of-magnitude greater than those of the ethidium homodimers.

1.3 Near-Infrared Fluorescence Detection

Traditionally, visible fluorescence detection has been used in bioanalytical applications.\textsuperscript{22} However, the use of visible excitation can produce large backgrounds, in the form of impurity fluorescence or scattering, from the sample matrix, reducing the signal-to-noise ratio. More of the disadvantages associated with visible laser-induced fluorescence detection (LIF) include the expensive and sophisticated instrumentation required for the detection. Ion lasers, such as Ar and Kr, are immense lasers that require extensive operator experience to operate as well as heavy demands on utilities. In addition, these type of lasers have short operational lifetimes and require replacement of expensive ion tubes.

Near-Infrared fluorescence (NIR) has recently been shown to be a viable alternative to visible fluorescence detection in many bioanalytical applications such as high performance liquid chromatography\textsuperscript{23,24}, the analysis of proteins \textsuperscript{25} and free solution capillary electrophoresis\textsuperscript{26}. The motivation for using fluorescence in the near-
IR includes smaller backgrounds observed during signal collection and the simpler instrumentation required for carrying out detection. In this region, background interference from other impurity molecules can be reduced or even eliminated because very few molecules contain intrinsic fluorescence. Since the amplitude of Raman or Rayleigh scattering is inversely proportional to the $4^{th}$ power of the excitation wavelength ($1/\lambda^4$), enhanced sensitivity can be achieved because of significant reductions in scattering effects. Also, the instrumentation required for near-IR fluorescence consists of inexpensive diode lasers and avalanche photodiodes. These components are solid-state allowing the detector to run an extended period of time requiring little maintenance or operator expertise.

Near-IR fluorescence can also be a very attractive detection strategy in gel sequencing because of the highly scattering medium that the separation is performed. Due to the intrinsically lower backgrounds that are expected compared to the visible, on-column detection can be performed without sacrificing detection sensitivity. NIR has been demonstrated in DNA sequencing applications using slab gel electrophoresis.\textsuperscript{27}

1.3.1 Near-Infrared Instrumentation. The instrumentation required for NIR fluorescence consists of four main components: the excitation source, the detector, the sample-holding apparatus, and the data acquisition device. The excitation source is a direct gap semiconductor laser diode with principle lasing lines greater than 700 nm.\textsuperscript{28} Semiconductor diode lasers are attractive excitation sources because they are very compact and inexpensive while supplying ample power (~100 mW) for ultrasensitive detection of analytes. In addition, diode lasers show a typical operational lifetime on
the order of 40,000 hrs compared to only approximately 2,000 hrs associated with ion lasers. Another attractive feature of diode lasers is that they can be operated off a simple battery, making them amenable to implementation in miniaturized and portable systems.

Diode lasers are simple semiconductors (p-n junction diodes) that are forward biased, causing a splitting of the Fermi level (measure of electron population distribution in the crystal lattice). Typically, the semiconductor is doped with a Group V or Group III element such as arsenic or gallium. Doping is a process where a tiny controlled amount of an impurity is introduced by diffusion into the heated silicon crystal. Electrons flow into the p-type region, while the holes flow into the n-type region. Recombination of the generated electron/hole pairs results in the spontaneous emission of light with the wavelength of the emitted light determined by the band gap, which is defined by the energy difference between the valence and conduction bands of the semiconductor material.

The single photon avalanche diode (SPAD) is an ideal detector for near-IR fluorescence. A SPAD consists of a semiconductor material, which is reversed-biased above its breakdown voltage. When a photon strikes the diode, it creates an electron/hole pair, which generates a cascading effect of electrons due to the high energy imparted to the primary electron or photogenerated carrier. These detectors offer good sensitivity and can operate very efficiently at room temperature. The photodiode detector does not require high voltage power sources in order to operate. Conventional photomultiplier tubes (PMT’s) require power sources in the 750-1000 V range.
The sample holding apparatus is designed to support the cuvette for measurements in solution or to support a capillary column for direct fluorescence measurements. Care must be taken in the design of the sample holding apparatus to aid in reducing excess scattered light from reaching the detector. The holding apparatus may also support a high numerical aperture microscope objective used in collecting the emission in a conventional 90° format.

The data acquisition device, which can be operated by a computer software program, converts the analog signal generated by the photodiode circuit into a readable format. The format is usually denoted by an emission profile of the analyte or by variable peak heights which correspond to the concentration of the sample analyte with the analog to digital converter and data acquisition software.

1.3.2 Near-Infrared Chromophores. The major type of chromophores that exhibit near-IR fluorescence belongs to the cyanine-class of dyes. Three of the more commonly used cyanine dyes are the carbocyanine, phthalo cyanine, and the naphthalocyanine dyes. These dyes have found numerous applications in the photography field, dye lasers and also, in photodynamic therapy. 32-34 However, the use of these dyes for fluorescence detection applications has only arisen recently due to the poor accessibility of dyes with functional groups to allow facile conjugation to target molecules.

The basic structures of these dyes are shown in Figure 1.6. The carbocyanines possess a conjugated polymethine chain, which links together two heteroaromatic
M = Al, Zn, Si, Sn, Ga

\[
\text{CIO}_4^-
\]

\[
\text{I} \quad \text{II} \quad \text{III}
\]

\[
\text{fragments. The characteristic spectroscopic feature of these dyes is the bathochromic shift in the absorbance and emission maxima they demonstrate with an increase in the length of the polymethine chain. For example, dicarbocyanines (n=2) show absorption maxima near 630 nm, while the tricarbocyanines (n=3) show absorption maxima near 780 nm. The carbocyanine dyes typically exhibit large extinction coefficients (200,000 M}^{-1}\text{cm}^{-1}\) and relatively large fluorescence quantum yields (0.15).

The phthalocyanine and naphthalocyanine dyes possess a conjugated ring structure which is linked together by four phthalo/naphthalo dicarbonitrile fragments.}
The characteristic spectroscopic feature of these dyes is the significant alteration in the fluorescence properties of the macrocycle following coordination of a metal to the core of the molecule. These dyes typically have greater fluorescence quantum yields and longer fluorescence lifetimes compared to the tricarbocyanine dyes. This stems from the fact that the ring structure of the macrocycle is much more rigid than the polymethine chain in the tricarbocyanines resulting in high rates of internal conversation significantly reducing the fluorescence quantum yield and fluorescence lifetime.35-37 The ability to tailor the spectroscopic properties of these dyes upon changes in the dye structure makes these dyes excellent candidates for bioanalytical applications.

1.4 Research Goals

The main focus of this research is to synthesize, characterize, and analytically apply new near-IR dyes for bioanalytical applications using laser-induced fluorescence detection. The synthesized near-IR chromophores will be used for single-lane, single fluorescence DNA sequencing utilizing near-IR fluorescence lifetime discrimination of labeled DNA fragments. To use this approach, the chromophores must have fluorescence lifetime values, which are significantly different to allow discrimination among the different probes, and possess similar absorption maxima in order to be excited by one laser source. The chromophores prepared in this research will be characterized by various analytical methodologies and applied to bioanalytical applications, such as staining dsDNA and amino acid analysis, as well as DNA sequencing.
1.5 References


2. Stokes, G. G. *Phil Trans* 1852, 142, 463.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Chapter 2
Labeling Dyes for DNA Sequencing

2.1 Introduction

Conventional methods for DNA sequencing employ laser-induced fluorescence detection of fluorescently labeled DNA strands. The use of fluorescent labels has become an integral component in sequencing applications due to the fact that it can provide on-line analysis; possesses limits of detection comparable to autoradiography; is easily integrated to micro-gel separation techniques and; can potentially allow the base-calling to be performed in a single separation lane using spectral discrimination.\textsuperscript{1,3,4} The important attributes associated with the dye set used for DNA sequencing should include the following: (1) The dyes must possess unique spectral properties to allow highly efficient base-calling during the fractionation step. When spectral discrimination is used for base-calling, it is advisable to have the emission bands well separated to alleviate cross-talk between detection channels. (2) The dye set must produce a uniform or constant perturbation on the electrophoretic mobility of the labeled oligonucleotide. If the mobility shift is dye-dependent, then post-run corrections must be implemented in order to avoid base shifts, producing errors during sequence reconstruction. (3) The dye set should allow facile excitation with a single laser source, and preferably, permit processing of the fluorescence on a single detection channel. The ability to use a single laser and detection channel will relieve some of the instrumental complexity associated with the sequencing device.
2.2. Spectral Discrimination

2.2.1. Visible Fluorescent Dyes  The commonly used approach for single lane base-calling in DNA sequencing applications using fluorescence is spectral discrimination, where a set of four spectrally distinct chromophores, which can be attached to either the sequencing primer or the dideoxynucleotide, are identified on the basis of unique emission maxima.¹ The dyes typically used are the fluorescein or rhodamine derivatives, which contain structural modifications to alter the absorption and emission maxima.¹,³,⁴ A set of commercial dyes shown in Figure 2.1 are available (6-FAM, JOE, TAMRA, and ROX) which nearly possess the aforementioned characteristics.⁵ These dyes can be excited with the 488- or 514-nm lines from an argon ion laser and then spectrally isolated using a series of optical filters onto appropriate photodetectors.³,⁶ While this dye set is fairly robust and works well with typical DNA cycle sequencing conditions, there are some difficulties in using this series. The major problems arise from the broad emission profiles, dye dependent mobility shifts and inefficient excitation of TAMARA and ROX with the 514.5 nm line from the argon ion laser, thus requiring the need for multiple excitation sources in some cases and multiple detection channels.

In order to eliminate the need for multiple excitation sources, the use of fluorescence-energy-transfer probes have been utilized in single lane DNA-sequencing applications.¹²-¹⁴ In this approach, a donor (FAM) is attached to the 5'-end of a sequencing primer, and an acceptor (FAM, JOE, TAMRA, ROX) is covalently bound to a modified thymidine residue eight or nine bases down the sequence. Since the primers
Figure 2.1. The fluorescent dyes used for single lane base-calling in DNA sequencing applications using spectral discrimination.
utilitize Förster resonance energy transfer, only a single excitation source is required (488nm), with the emission sorted onto appropriate detectors. Using these energy transfer dyes offers some advantages due to their improved detection sensitivities, namely eliminating the need for adjusting the concentrations of the dye primers and the need for smaller amounts of template in the sequence analysis. However, due to the structural requirements for efficient energy transfer and the need for two dyes per primer, this method will be difficult to adapt in primer walking strategies, and the use of dye-labeled dideoxynucleotides can be problematic. In addition, concerns with dye dependent mobility shifts are present.

Recently, a set of electrophoretically uniform fluorescent dyes for DNA sequencing has been reported and are shown in Figure 2.2. The dyes, which are BODIPY derivatives (4,4-difluoro-4-bora-3α, 4α-diaza-s-indacene-3-proprioninc acid), are attached to sequencing primers (5'-end) via a unique linker structure that produces excellent sequencing data without software correction for dye-dependent mobility shifts. In addition, the dye-primer set yields narrower spectral emission bandwidths compared to those of conventional dye-primer sets, resulting in smaller amounts of cross-talk between detection channels. However, as with the energy-transfer dyes, this approach will not easily be amenable to primer walking strategies or dye-labeled dideoxynucleotide DNA sequencing. Another particular shortcoming associated with this dye set is the chemical instabilities they display when subjected to extended heating at high temperatures during cycle sequencing.
Figure 2.2 The chemical structures of Bodipy dyes used in DNA sequencing
2.2.2 Near-Infrared Dyes for Sequencing

As stated in Chapter 1, the attractive feature associated with fluorescence in the near-IR (650-1100 nm) includes smaller backgrounds observed during signal collection and the rather simple instrumentation required for carrying out ultrasensitive detection. In most cases, the limit of detection for fluorescence measurements is determined primarily by the magnitude of the background produced from scattering or impurity fluorescence. This can be a significant factor in DNA sequencing since detection occurs within the gel matrix, such as urea or formamide, producing a major contribution of scattering photons. The lower background in the near-IR is due to the fact that there are very few molecules that intrinsically fluoresce within this region. Also, the $1/\lambda^4$ dependence of the Raman cross section provides a lower scattering contribution at these longer excitation wavelengths. The instrumentation required for near-IR fluorescence consists of inexpensive diode lasers and avalanche photodiodes, which are solid-state devices allowing the detector to run an extended period of time requiring little maintenance or operator expertise.

The basic structures of the dyes used for DNA sequencing in the near-IR are shown in Table 2.1. They are the tricarbocyanines dyes, which possess a conjugated polymethine chain that links together two heteroaromatic fragments. The characteristic spectroscopic feature of these dyes is the bathochromic shift in the absorbance and emission maxima they demonstrate with an increase in the length of the polymethine chain. For example, dicarbocyanines (n=2) show absorption maxima near 630 nm, while the tricarbocyanines (n=3) show absorption maxima near 780 nm. The carbocyanine dyes typically exhibit large extinction coefficients (200,000 M$^{-1}$cm$^{-1}$) and

31
favorably large fluorescent quantum yields (0.15) in aqueous solutions. The major disadvantage associated with these fluorophores are their poor water solubility. These dyes show a high propensity toward aggregation forming aggregates with poor fluorescence properties. This aggregation can be alleviated to a certain degree by inserting water soluble groups, such $\text{SO}_3^-$ or $\text{COO}^-$, within the molecular framework of the fluorophore. For conjugation, the near-IR fluorophore also must contain either an isothiocyanate or succinimidyl ester functional group to covalently attach to the sequencing primer or ddNTP.

Williams and co-workers reported a direct comparison between laser-induced fluorescence detection at 488 nm excitation and 780 nm excitation. In their study, a sequencing primer labeled with FAM or a near-IR dye were electrophoresed in a capillary gel column and the detection limits (LOD) for both were calculated. The results indicated that the limits of detection for the near-IR case were found to be $3.4 \times 10^{-20}$ moles, while for 488 nm excitation, the limit of detection was $1.5 \times 10^{-18}$ moles. The improvement in the LOD for the near-IR dye resulted from the significantly lower background observed in the near-IR region.

2.3 Lifetime Methods

As an alternative to spectral discrimination, various groups have suggested that fluorescence lifetimes can potentially serve as a viable method for base-calling in DNA sequencing applications. The principle advantages associated with lifetime discrimination for base-calling are the following: (1) Since the calculated lifetime is immune to concentration differences, dye labeled terminators can potentially be used as
Table 2.1. Chemical structures of some typical near-IR fluorescent dyes as well as their photophysical properties. The dyes contain an isothiocyanate to allow conjugation to amine containing molecules.

<table>
<thead>
<tr>
<th></th>
<th>Abs $\lambda_{\text{max}}$</th>
<th>Em $\lambda_{\text{max}}$</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRD 41</td>
<td>787</td>
<td>807</td>
<td>200,000</td>
<td>0.16</td>
</tr>
<tr>
<td>NN382</td>
<td>778</td>
<td>810</td>
<td>200,000</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
well as dye primers, with a wide choice in polymerase enzymes to suit the particular sequencing application; (2) lifetime values can be determined with higher precision than fluorescence intensities under appropriate conditions, improving the accuracy in base-calling; (3) lifetime determinations do not suffer from broad emission profiles associated with spectral discrimination; and (4) the fluorescence can potentially be processed on a single detection channel without the need for spectral sorting to multiple detection channels.

Many of these concerns associated with lifetime determinations for base-calling in DNA sequencing have been addressed using near-IR fluorescence. For example, several groups have demonstrated that semiconductor diode lasers, which can be operated in a pulsed mode and lase between 680 and 800 nm, in conjunction with a single-photon counting apparatus that is simple to operate, with performance characteristics comparable to those of visible devices, using mode-locked Nd:YAG lasers and microchannel plates. Soper and co-workers reported the use of lifetime identification methods in capillary electrophoresis (CE) in which a conventional time-correlated single photon counting instrument with a Ti:Sapphire laser system was used to acquire lifetimes on-line during free solution separation of free near-IR fluorescent dyes. The dyes were anionic (IR-144 and IR-125) and cationic (DTTCI, HITCI, IR-140, and IR-132) in nature and were separated in a running buffer consisting of 95% methanol and 5% distilled water set at a pH of 9.5. Methanol was incorporated to improve the fluorescence properties of the NIR dyes. Lifetimes were determined via the maximum likelihood estimator (MLE) method from decay profiles constructed over each electrophoretic band. It was determined that the migration order as determined by
fluorescence lifetimes agreed with the migration order evaluated from individual electrophoretic mobilities. Additionally the effects of neighboring components and background on the lifetime calculation were investigated. The inclusion of background and scattered photons into the calculation resulted in lifetimes biased towards shorter values. An inherent disadvantage of the MLE technique becomes evident with the inclusion of neighboring components (i.e., poorly resolved) into the calculation—the calculated value becomes a weighted average of the various components that constitute the decay.

In addition, a single NIR dye was labeled to a sequencing primer and a single base tract (C-terminated fragments) and was fractionated in a gel column with the lifetimes of the electrophoretic bands determined using the MLE algorithm. No fluorescence was observed from the gel matrix when utilizing NIR excitation, which improved the efficiency (precision) in the measurement. The standard deviation in the lifetime measurement was found to be approximately ±9 ps with decay profiles constructed from as few as 5,000 photocounts. The high precision resulted primarily from the fact that, in the near-IR, the low scattering cross sections and the minimal number of intrinsically fluorescent components produced low numbers of interfering photocounts in the decay.

Wolfrum and co-workers have demonstrated the use of a one-lane four dye lifetime approach to calling bases in DNA sequencing applications.22-24 In their work, a series of rhodamine derivatives (see Figure 2.3) were prepared which possessed absorption maxima at 630 nm and fluorescence lifetimes that varied between 1.6 and 3.7 ns. Using appropriate linker structures, dye dependent mobility shifts were
minimized, eliminating the need for post-electrophoresis processing. In a single lane, four-lifetime format with capillary electrophoresis for the separation platform, the researchers were able to demonstrate a read length of 660 bases with a probability of correct identification of the called bases greater than 90%.

Several problems do arise in considering lifetime discrimination for DNA sequencing, especially when utilizing the micro-separation techniques, such as capillary gel electrophoresis. The most pervasive problem is associated with the complex instrumentation required for lifetime determinations. For example, in time-domain techniques, a pulsed laser is required with a fast detector, typically a micro-channel plate photomultiplier tube, and sophisticated counting electronics. In addition, poor photon statistics (low number of photocounts) produced from low loading levels and the transient nature of the signal can produce poor precision in the measurement. Poor precision would also be compounded by the presence of large amounts of scattering and impurity photons included into the decay profile. Finally, complex algorithms are often required for extracting the lifetime from the decay profile making on-line determinations during electrophoresis difficult.

2.4 Heavy Atom Modified Dyes for DNA Sequencing

The heavy-atom modified dyes for DNA Sequencing are a set of unique fluorophores appropriate for single-lane base-calling for DNA sequencing using lifetime discrimination. The dyes developed for this type of application are near-IR tricarbocyanine dyes, which possess an intramolecular heavy-atom modification. The heavy-atom modification consisted of covalently inserting a halogen (I, Br, Cl, F) into the molecular framework of the dye. The intramolecular heavy atom results in
Figure 2.3. Structure of fluorescent dyes MR-200, JA169, JA242, and CY5 utilized as fluorescent labels in DNA sequencing.
perturbations in the singlet-state photophysics (fluorescence quantum yield, \( \Phi_f \), and fluorescence lifetime, \( \tau_f \)) due to enhanced intersystem crossing into the triplet state resulting from spin orbit coupling.\textsuperscript{25-27} The heavy atom, however does not alter the absorption or emission maximum of the base chromophore for these dyes and produces uniform electrophoretic mobilities in capillary gel conditions, negating the need for post-run corrections due to dye-dependent mobility shifts.

2.5. Experimental Section

2.5.1. Synthesis of Near-IR Heavy Atom-Modified Dyes. The near-IR heavy atom-modified dyes shown in Figure 2.4 were synthesized by Dr. James H. Flanagan, Jr.\textsuperscript{28-30}

2.5.2. Labeling and Purification of Sequencing Primers with Near-IR, Heavy-Atom-Modified Dyes. The M13mpl8 universal sequencing primers (17mer) containing a 6-carbon alkyl linker terminated with an amino group on the 5'-end were derivatized with the near-IR dyes according to procedures outlined by Li-COR.\textsuperscript{31} Briefly, 50 nmol of DNA was added to 25 \( \mu \)L of carbonate buffer (400 mM, pH 9.5), 25 \( \mu \)L of EDTA (2 mM), and 100 \( \mu \)L of the near-IR dye. After the reaction was allowed to proceed at room temperature for approximately 4 h, 10 \( \mu \)L of NaOAc and 480 \( \mu \)L of cold ethanol were added to the reaction mixture. The solution was centrifuged for 20 min at 10 °C and 15,000 rpm. The supernatant was discarded and the ethanol precipitation step repeated again. The DNA/dye conjugate was then dried, and 200 \( \mu \)L of water was added to the pellet. The DNA/dye conjugate was finally purified using preparatory HPLC under the following conditions: column, C8 (10 cm x 4.6 mm,
Brownlee); flow rate, 1.7 mL/min; mobile phase A, 0.1 M triethylammonium acetate, 4% CH$_3$CN, 96% H$_2$O; mobile phase B, 0.1 M triethylammonium acetate, 80% CH$_3$CN, 20% H$_2$O. The gradient conditions were 90/10 to 55/45 A/B over 5 min, 55/45 to 0/100 A/B over 20 min, hold at 0/100 A/B for 5 min. The collected fractions were pooled and taken to dryness using a centrifugal evaporator and stored in the dark at -20°C. The yield of dye-labeled primer was estimated to be 30%.

2.5.3 Spectroscopic Analysis. The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin Elmer, Norwalk, CT). The uncorrected fluorescence spectra were collected on a Spex 3000 fluorometer (Spex, Edison, NJ). The spectrofluorometer contained a red-sensitive photomultiplier tube (R636, Hamamatsu Corp.) and emission gratings blazed for 750 nm. The fluorescence quantum yields were calculated relative to IR-125 in DMSO ($\Phi_f = 0.13$) according to the procedure outlined by Demas and Crosby.$^{32}$

Time-resolved fluorescence measurements were performed using a near-IR time-correlated single-photon counting instrument built in-house which included a mode-locked Ti:sapphire laser pumped by the all-lines output of an Ar ion laser (Coherent Lasers, San Jose, CA) and a passively quenched single-photon avalanche diode. The dye concentration used for lifetime determinations was $1 \times 10^{-8}$ M in the appropriate solvent system. The fluorescence lifetimes were calculated using a reiterative nonlinear least-squares algorithm written in-house, with decay profiles accumulated until approximately 10,000 photocounts were present in the channel with the maximum number of counts.
Figure 2.4 Structure of Near-IR heavy atom modified dyes (7-11)
Capillary zone electrophoresis was performed on a Waters Quanta 4000 CE System (Millipore, Marlborough, MA), with the output signals integrated on a Perkin-Elmer LCI-100 laboratory computing integrator (Norwalk, CT). Free solution separations were performed using a 75 µm i.d. capillary column (Polymicro Technologies, Phoenix, AZ) with a total length of 58 cm and a detection window 50 cm from the injection end. The running buffer consisted of 5 mM sodium borate buffer (pH 9.3) dissolved in 50:50 water/methanol. Dye concentrations of $5 \times 10^{-5}$ M dissolved in the running buffer were electrokinetically injected onto the column for 20 s with an applied voltage of 30 kV (517 V/cm). The separations were performed at an applied voltage of 25 kV (431 V/cm). The analytes were detected on-column using absorbance at 254 nm. Free solution mobilities were calculated relative to the mobility of riboflavin (neutral marker) in order to correct for the electroosmotic flow.

Capillary gel electrophoresis was performed in a 6%/5% polyacrylamide gel column (75,um i.d., J&W Scientific, Folsom, CA) with a total length of 33 cm and a detection window 26 cm from the injection end. A mixture of the dye (8-11)/oligonucleotide conjugates was electrokinetically injected onto the column for 3 s at an applied voltage of -5 kV, with separations performed in reverse mode at an applied voltage of -8.25 kV (250 V/cm). The detection was performed using laser-induced fluorescence, incorporating the system described above for lifetime measurements, except that the laser was operated in a continuous wave mode of operation.

2.6. Results and Discussion

The absorption and emission spectra of the heavy-atom modified near-IR dyes measured in methanol are shown in Figure 2.5. As can be seen from this figure, the
Figure 2.5. Absorption and emission spectra of the near-IR heavy atom-modified dyes dissolved in methanol at a concentration of 1 μM. In the case of the fluorescence spectra, the data were collected on a red-sensitive photon-counting spectrofluorometer using an excitation wavelength of 710 nm.
Table 2.2. Absorption and Emission Maxima, Extinction Coefficients and Fluorescence Quantum Yields for the Heavy Atom Modified Near-IR Fluorescent Dyes in Methanol

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{abs}(\text{nm})$</th>
<th>$\lambda_{em}(\text{nm})$</th>
<th>$\varepsilon (\text{M}^{-1} \text{cm}^{-1})$</th>
<th>$\Phi_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>778</td>
<td>802</td>
<td>181,000</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>765</td>
<td>794</td>
<td>230,000</td>
<td>0.07</td>
</tr>
<tr>
<td>8</td>
<td>766</td>
<td>796</td>
<td>216,000</td>
<td>0.15</td>
</tr>
<tr>
<td>9</td>
<td>768</td>
<td>798</td>
<td>254,000</td>
<td>0.14</td>
</tr>
<tr>
<td>10</td>
<td>768</td>
<td>797</td>
<td>239,000</td>
<td>0.14</td>
</tr>
<tr>
<td>11</td>
<td>768</td>
<td>797</td>
<td>221,000</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Introduction of the heavy atom modification onto these dyes produced only minor changes in the absorption and emission maxima. Table 2.2 lists the absorbance and emission maxima as well as the molar absorptivities and fluorescence quantum yields for dyes 1, 7-11. The addition of the intramolecular heavy atom produced only minor differences in the extinction coefficients, with all extinction coefficients $> 200,000 \text{ cm}^{-1} \text{M}^{-1}$, which are typical values for these types of tricarbocyanine dyes. In Table 2.2 are also shown the fluorescence quantum yields that were calculated for the dye series; as can be seen, the introduction of the heavy-atom modification had no appreciable effect on the $\Phi_r$ within the precision of the measurement, with the quantum yields for the heavy-atom dyes ranging between 0.14 and 0.15. In the case of dye 7, it can be seen that it had a smaller quantum yield (0.07) compared to those of the heavy-atom modified dyes (8-11). Also, for dye 1, where the chloro-substituent was attached directly to the chromophore, the fluorescence quantum yield was smaller than for those dyes where the heavy-atom modification was spatially removed from the base chromophore. In addition, its absorption and emission maxima were red-shifted from

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
those seen for dyes 7-11. If the intramolecular heavy atom affected only the rate of intersystem crossing, then it would be expected that the dyes containing a halogen would show fluorescence quantum yields less than those for dye 7, with the quantum yields decreasing in the order 8 < 9 < 10 < 11, with dye 1 showing the smallest quantum yield since the heavy atom (Cl) is attached directly to the chromophore and, in 8-11, it is spatially removed from the chromophore.

Table 2.3. Fluorescence Lifetimes of Near-IR Dyes in pure Methanol

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\tau_f$ (ps)</th>
<th>$\chi^2$</th>
<th>SD (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>529</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>873</td>
<td>1.1</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>947</td>
<td>1.3</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>912</td>
<td>1.2</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>880</td>
<td>1.1</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>843</td>
<td>1.2</td>
<td>8</td>
</tr>
</tbody>
</table>

The fluorescence lifetime data for these dyes in pure methanol are shown in Table 2.3, along with the $\chi^2$ values and the standard deviation in the measurements. Relative to dye 1, the insertion of the heavy atom directly onto the chromophore (1) perturbs the lifetime of the singlet state to a much larger degree than in those cases where the heavy atom was spatially removed from the chromophore (8-11). In the latter cases, spin-orbit coupling would be expected to occur predominately through space and not through bond (as in 1) and thus exert a smaller influence on the singlet state lifetime, consistent with our data. Interestingly, we found that $\tau_f$ increased with increasing molecular weight of the intramolecular heavy-atom modification, contrary to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
what is typically observed. We have recently carried out photophysical measurements of the triplet state using flash photolysis techniques to understand the observed trend. The results of these studies indicated that the heavy-atom modification does increase the efficiency of crossing into the triplet state, with the heavier atom showing a larger rate of intersystem crossing. However, the major non-radiative manifold in these dyes was not intersystem crossing but internal conversion, which is typically observed in these tricarbocyanine dyes. Both the intersystem crossing and internal conversion rates are affected by the presence of the heavy-atom modification, but to differing degrees, producing the observed trend in the lifetimes of these dyes. However, it should be pointed out that, for the present application, the order of the effect is inconsequential. The only important criterion is that the lifetimes can be discriminated with high precision to affect high accuracy in the base-calling. In the case of methanol, the average variation in this series of dyes was found to be 35 ps.

We next carried out a spectroscopic analysis of the heavy-atom modified near-IR dyes and the dye-primer conjugates in a DNA sequencing matrix. In this case, the absorption and fluorescence spectra were measured in a nonpolymerized acrylamide solution (6%T/5%C) containing 1× TBE and 40% formamide. The motivation for using formamide was to keep the organic content high, since the tricarbocyanine near-IR dyes have been shown to possess improved photophysical properties in high organic content solutions. While urea gels are commonly used for sequencing, formamide gels have been shown to offer some unique advantages, such as improved stability when operating under high electric field conditions.
Table 2.4. Fluorescence properties of the Heavy-Atom Modified Near-IR dyes, both free and conjugated to a 17mer sequencing primer, measured in a nonpolymerized acrylamide solution containing 40% formamide and 6%T/5%C.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Absorbance (nm)</th>
<th>Emission (nm)</th>
<th>Fluor. Lifetime (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>free</td>
<td>conjugate</td>
<td>free</td>
</tr>
<tr>
<td>8</td>
<td>764</td>
<td>773</td>
<td>792</td>
</tr>
<tr>
<td>9</td>
<td>764</td>
<td>773</td>
<td>793</td>
</tr>
<tr>
<td>10</td>
<td>765</td>
<td>774</td>
<td>793</td>
</tr>
<tr>
<td>11</td>
<td>766</td>
<td>774</td>
<td>794</td>
</tr>
</tbody>
</table>

The absorbance and fluorescence emission maxima are shown in Table 2.4 for both the free dyes and the dye-primer conjugates. The covalent attachment of the dye to the oligonucleotide resulted in only minor perturbations to the absorption and emission maxima. The absorption maxima for the native dyes were found to be ~765 nm, while the maxima for the dye-primer conjugates were ~773 nm, an 8-nm shift. We also observed aggregation effects in the ground-state absorption spectra for the free dyes in this matrix, while the dye-primer conjugate spectra showed only the monomeric forms (see Figure 2.6). The peak seen at 765 nm for the free dyes can be assigned to the monomer, since it was present in the case of methanol, where it is expected that little, if any, aggregation should occur. The broad peak centered at approximately 689 nm is most likely due to a dimer or other higher order aggregate that forms at this dye concentration (1 μM) in this particular solvent system but was absent in the case of methanol. It can also be seen from these data that the absorption of the monomeric...
form decreases in the order $8 < 9 < 10 < 11$. In the case of the dye-primer conjugates, there is an absence of this broad, blue-shifted band arising from the dimer or other higher order aggregate. The lack of aggregation for the dye-primer conjugates is likely due to the increased solvation expected for the conjugate due to the highly anionic nature of the oligonucleotide.

In the fluorescence spectra, the emission maxima for the dye-primer conjugates showed an approximate 6-nm shift compared to the native dyes in this solvent system. In addition, the emission intensities of the dye-primer conjugates were found to be somewhat higher than those associated with the free dyes in this gel matrix (data not shown). This effect was attributed to the increased absorption of the monomeric or fluorescing state of the dye due to the lack of aggregation effects observed for the conjugates at this dye concentration.

The fluorescence lifetimes for the heavy-atom-modified dyes and the dye-primer conjugates were next measured in this nonpolymerized acrylamide solution with the data listed in Table 2.4. In Figure 2.7 is shown a prompt peak and decay profiles for dyes 8 and 11 conjugated to the sequencing primer. All decays were found to be adequately fit to a single-exponential function with $\chi^2$ values which ranged from 1.1 to 1.7. As can be seen from the data in Tables 2.3 and 2.4 for the free dyes, the lifetimes were shorter in the nonpolymerized gel solution compared to those of methanol, but the general order in the lifetime values was preserved. In the case of methanol, dye 8 had a lifetime of 947 ps, while in the gel matrix it was found to be 760 ps.

The differences in lifetimes between the two solvent matrixes are consistent with our previous work, which has shown that the tricarbocyanines display solvent
Figure 2.6 Absorption spectra of the free dyes (A) and the dye-primer conjugates (B) measured in the nonpolymerized acrylamide solution containing 40% formamide. The absorption spectra were acquired using a dye concentration of 1 μM.
dependent photophysics, with generally poorer photophysics observed in more aqueous media. It was also noticed that the lifetime variation for the free dye series was found to be $24 \pm 8$ ps, while in the case of methanol, it was $35 \pm 3$ ps.

If these dyes are to be effectively used in a single-lane DNA sequencing format for base-calling, both the absolute magnitude and the variation in the lifetimes for the dye series are important. When the dyes possess short lifetimes, the criteria imposed on the instrument become critical: namely, a short instrument response function becomes necessary in order to adequately determine the lifetime. This will be particularly important under ultradilute conditions and short residence times, since it may be necessary to gate out scattered photons to perform the lifetime calculation over a time interval where the scattered photons contribute insignificantly. In the present case, our instrument showed a response function of 165 ps, significantly less than the lifetime of dye 11 (688 ps) measured in this nonpolymerized acrylamide solution. The variation or spread in the lifetimes for the dye series will determine the accuracy in the base-calling, since the bases are called by the lifetime calculated for each band in the electropherogram.

To evaluate the effects on the fluorescence lifetimes of these dyes by conjugating them to sequencing primers, the lifetimes of the dye-primer conjugates were determined in the nonpolymerized matrix containing formamide, the results of which are shown in Table 2.4. As can be seen from these data, the lifetime values between the free dyes and the dye-primer conjugates were found to differ, with the conjugates showing a longer lifetime than the free dye. For example, in the case of dye 8, the free dye showed
Figure 2.7 Fluorescence decay profiles for dyes 8 and 11 conjugated to the M13mp18 sequencing primers measured in a nonpolymerized acrylamide gel solution containing 40% formamide and 1 x TBE along with the prompt peak (instrument response function). In this case, the dye concentration was set at 10 nM with 1 mW of laser power at 765 nm used for the excitation.
a lifetime of 760 ps, while the conjugate of dye 8 possessed a lifetime of 889 ps. It was expected that the observed differences were not due to aggregation effects, since the dye concentration used (10 nM) was significantly less than those used to collect the absorption and emission spectra and little aggregation would be expected at this low dye concentration. Also, the average variation in the lifetimes for this dye series was found to be 51 ± 23 ps for the dye-primer conjugates, significantly larger than in the case of the free dyes. However, the variation between the dye-primer conjugates of 10 and 11 was only 24 ps, while those for the other pairs, 8/9 and 9/10, were 68 and 62 ps, respectively. Therefore, for base-calling applications, the photocounts necessary to achieve the required precision in the measurement will be set by the smallest variation within the series, which, in this case, is 24 ps. This means that at least 10,000 photocounts will be required per decay profile or electrophoretic band to achieve discrimination at 3σ.

We noticed that the observed lifetimes were sensitive to the amount of formamide present in the acrylamide solution. For example, when the amount of acrylamide and bisacrylamide was kept constant (6%T/5%C) and the percentage of formamide was reduced to 10%, the lifetimes were determined to be 621 ps for dye 8, 608 ps for dye 9, 534 ps for dye 10, and 513 ps for dye 11. In addition, the $\chi^2$ values were also higher (>2.0). Attempts to fit the data to double-exponential functions did not reduce the value of $\chi^2$ (i.e., improve the goodness of the fit). We also measured the lifetimes of these dyes in a nonpolymerized denaturing solution containing 7 M urea and found that these lifetimes were smaller than those found in the 40% formamide solutions. Dye 8 possessed a lifetime of 586 ps in 7 M urea, and dye 10 was found to
have a lifetime of 483 ps in this same medium. These data suggest that the lifetime values will be sensitive to the composition of the fractionating medium. However, even though the actual lifetime values of the heavy-atom-modified dyes did change with changes in the composition of the medium, the relative order of the lifetime values for each dye in the series was retained in any particular solvent matrix.

Effective use of these dyes in a DNA-sequencing protocol requires the electrophoretic mobilities ($\mu_{em}$) be uniform in order to minimize base-calling errors arising from dye-dependent mobility shifts. The apparent mobilities ($\mu_{app}$) of the heavy-atom-modified chromophores were calculated in free solution capillary zone electrophoresis using:

$$\mu_{app} = \frac{L_{eff}}{t_{m} E}$$ (3.1)

where $L_{eff}$ is the length of the capillary column from the injection to the detection window (cm), $t_{m}$ is the migration time of the analyte (s), and $E$ is the field strength (V/cm). The electrophoretic mobilities ($\mu_{em}$) were determined from the expression:

$$\mu_{em} = \mu_{app} - \mu_{eo}$$ (3.2)

where $\mu_{eo}$ is the electroosmotic flow. The calculated mobilities are shown in Table 2.5. As can be seen from these data, the heavy-atom-modified dyes (8-11) demonstrated rather uniform electrophoretic mobilities (within experimental error), with these values ranging between - 4.9 and - 5.2 x 10^{-5} \text{ cm}^2/(\text{V*s}). In the case of the near-IR dye not containing the heavy-atom modification (dye 7), it showed a mobility that was larger than those of the heavy-atom dyes, most likely a result of the smaller frictional contribution to the mobility produced by H compared to the heavy-atom modifications (I, Br, Cl, F) since all of these dyes possess similar charges.
The heavy-atom dyes 8-11 conjugated to the 17mer sequencing primer were then electrophoresed in a gel-filled capillary column to investigate mobility differences under these electrophoretic conditions. The resulting electropherogram is shown in Figure 2.8. As can be seen from this figure, a single peak was observed which migrated from the column in 1752 s. Shown in the inset of this figure is an expanded view of the resulting electrophoretic peak, which clearly shows only one peak, indicating that the heavy-atom-substituted dyes co-migrated; therefore, they possessed uniform mobilities under these gel conditions. Due to the lack of dye-dependent mobilities observed for this dye series, post-run corrections in the electropherogram will not be required during DNA sequencing applications, simplifying the base-calling and improving the accuracy in sequence reconstruction.

2.7. Summary

We have synthesized and characterized a series of chromophores that contain an intramolecular heavy atom (halogen) which does not perturb the absorbance or emission maximum of the base chromophore but induces a difference in \( \tau_r \) resulting from influences on both the intersystem crossing and internal conversion rates on the base chromophore. These dyes were conjugated to sequencing primers, and the conjugates were observed to have photophysical properties which differed from those of the free dyes in a formamide/acrylamide medium. Electrophoretic mobility studies in free solution carried out on these heavy-atom-modified near-IR dyes indicated that they possess uniform mobilities in free solution and, when conjugated to sequencing primers, comigrate under gel electrophoresis conditions.
Table 2.5. Electrophoretic mobilities of Heavy-Atom Modified Near-IR fluorescent dyes (7-11) in free solution

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\mu_{em}$ (cm$^2$/V*s) x 10$^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>-5.5 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>-5.1 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>-5.0 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>-4.9 ± 0.3</td>
</tr>
<tr>
<td>11</td>
<td>-5.2 ± 0.1</td>
</tr>
</tbody>
</table>

Figure 2.8. Capillary gel electropherogram of the heavy atom-modified near-IR dyes (8-11) conjugated to a 17mer M13mp18 sequencing primer. In this case, the dye-primer concentration was 1 nM, and the column consisted of a 6%T/5%C cross-linked polyacrylamide sieving matrix with 7M urea as the denaturant.
For DNA-sequencing applications, these dyes will serve as excellent labels using lifetime discrimination, since only one excitation source and one detection channel will be required to process the fluorescence data since they possess similar absorption and emission maxima. In addition, the uniform mobilities will eliminate the need for post-run corrections, typically necessary for multi-dye approaches. While performing a time-resolved measurement can be somewhat instrumentally prohibitive, the dyes we have prepared absorb light in the near-IR, allowing the use of solid-state pulsed-diode lasers to serve as excitation sources. In conjunction with avalanche photodiodes, a simple time-correlated single-photon-counting apparatus can be constructed to dynamically measure fluorescence lifetimes on-line during capillary gel electrophoresis. In addition, the use of near-IR fluorescence monitoring will significantly reduce matrix interferences, improving the precision in the lifetime measurement.

2.8. References


55


35. Flanagan, J. H.; Legendre, B. L.; Soper, S. A. "manuscript in preparation,"


Chapter 3

High Resolution Separation of DNA Restriction Fragments Using Capillary Electrophoresis and Laser-Induced Fluorescence Detection

3.1 Introduction

Capillary electrophoresis (CE), in conjunction with entangled polymer solutions as a sieving matrix, has been shown to be an attractive fractionating method for double-stranded DNAs (dsDNAs) due to the high efficiencies and resolution that are attainable and the speed associated with the separation technique.\(^{1-3}\) However, due to the low mass-loading levels associated with capillary electrophoresis, stringent requirements associated with the detection protocol are necessary in order to detect modest concentration levels of dsDNAs. While native UV detection at 254 nm can be implemented, it suffers from poor sensitivity due to the short optical pathlength associated with capillary electrophoresis. Therefore, laser-induced fluorescence (LIF) with intercalating dyes has proven to be the method of choice in critical applications requiring low levels of detection.\(^{4-8}\)

LIF detection has typically been undertaken using intercalating dyes which possess absorption and emission maxima in the visible region of the electromagnetic spectrum (450-630 nm). The detection is affected by monitoring the perturbation in the spectroscopic property of the dye when in the bound state. Cationic dyes, which have planar aromatic or hetero-aromatic rings and exhibit enhancements in their fluorescence emission upon complexation with dsDNA, include the mono-intercalating dyes such as ethidium bromide (EtBr)\(^9\), thiazole orange (TO)\(^10\), oxazole yellow \(^{10}(YO)\), and the dimeric forms of these dyes.\(^{11-13}\) Ethidium bromide (EtBr) shows a fluorescence
enhancement of 20 when it interacts with dsDNA and the dye has also been found to improve resolution in the CE analysis of dsDNA as compared to separation of the native dsDNAs.\textsuperscript{14} The dimeric dyes consist of two chromophores covalently linked through a polycationic chain and show larger binding constants to dsDNA when compared to their monomeric counterparts. Because the fluorescence quantum yields of the dimeric form of the dyes are significantly improved in the bound-state compared to those in free solution, the background fluorescence from free dye is very low which makes these dyes excellent probes for the low-level quantification of dsDNA.

In capillary electrophoresis applications, the monomeric dyes are typically added to the running buffers due to their smaller binding constants and the dsDNA does not need to be pre-stained prior to the electrophoresis. Because the binding constants to dsDNA for the dimeric forms of the dyes are higher, the DNA is typically prestained with the dye and it is unnecessary to add the dye to the running buffer during the electrophoretic separation. The sensitivity of detection using the dimers is improved compared to the monomers, but it suffers from poor resolution due to the multiple binding modes of the dye.\textsuperscript{15,16}

The difficulty with utilizing visible LIF detection is that the excitation source is a gas-ion laser, for example an Ar-ion or He-Ne laser, which has a limited operational lifetime and, in some cases, can be expensive and difficult to operate. In addition, visible excitation can encounter interferences in the form of scattering and fluorescence impurities, which can degrade the limit of detection, especially when utilizing bis-intercalating dyes.
An attractive alternative to visible fluorescence is the use of near-IR fluorescence detection. Near-IR fluorescence has recently been demonstrated to be a viable detection strategy in liquid chromatography,\textsuperscript{17-19} free solution capillary electrophoresis\textsuperscript{20-24} and capillary gel electrophoresis for DNA sequencing applications.\textsuperscript{25} One of the principle advantages of near-IR fluorescence is that simple diode lasers can be used as the excitation source for the LIF detector, increasing operational lifetime and reducing the cost of setting up such a detection system. In addition, near-IR fluorescence typically displays fewer interferences from the background matrix resulting from impurity fluorescence and a smaller scattering contribution, improving the limit of detection.\textsuperscript{25}

In this work, we wish to report on the use of near-IR laser induced (LIF) fluorescence detection of restriction fragments separated by CE with an entangled polymer solution and staining using an intercalating dye which shows absorption and emission properties in the near-IR. The staining dye, thiazole green (TAG) was prepared by extending the methine chain in the common mono-intercalator, TO-PRO 3, resulting in a shift in the absorption maxima into the near-IR.\textsuperscript{26} The LIF system consisted of a GaAlAs diode laser operating at 750 nm and a single photon avalanche diode. Both of these devices are solid-state and easily operated, producing an LIF detection system that has a potentially long operational lifetime and is very inexpensive, but displays detection sensitivities for dsDNAs comparable to the visible systems which use gas-ion lasers.
3.2 Experimental Section

Malonaldehyde bis(dimethyl acetal), iodomethane, and aniline were obtained from Aldrich Chemical Co., Milwaukee, Wi. 2-methylbenzanthiazole, lcpidine, and 1,3-diiodopropane were purchased from Lacanster, Windham, NH. Spectroscopic grade methanol, ethanol, chloroform were received from Mallinckrodt, Paris, NY. Anhydrous sodium acetate, ethyl ether, and acetic anhydride were also purchased from Mallinckrodt. Concentrated hydrochloric acid, acetic acid, and ethyl acetate were obtained from EM Science., Gibbstown, New Jersey. Tris-HCl, g-(methoxyacyrloxy)propyltrimethoxysilane (MAPS), and ethidium bromide were obtained from Amresco (Solon, OH). Hydroxypropylmethylcellulose, (MW = 86,000), anhydrous ethylenediaminetetraacetic acid (EDTA), and N, N, N', N' tetramethylethylendiamine (TEMED) were obtained from Sigma Chemical Company, (St. Louis, MO). Methanol, TAE buffer [(40 mM Tris) and 20 mM NaC2H3O2·3H2O were obtained from EM Science (Cherry Hill, N. J.), 2 mM EDTA], pH = 7.61, \( \Phi X174/HaeIII \) digest was obtained from Life Technologies (Gaithersburg, MD), bare silica capillary was obtained from Polymicro Technologies, Inc. (Phoenix, Arizona), 18 m\( \Omega \) water was obtained from a purification apparatus in our department.

3.2.1 LIF Detector. The near-IR LIF detection system was constructed in-house by Dr. Bill Karr and is schematically shown in Figure 3.1. It consisted of a 7 mW, 750 nm diode laser (GaAlAs, Melles Griot, Irvine, CA) and controlled by a diode laser driver (Model 06 DLD 201, Melles Griot). The laser head contained an anamorphic prism pair to produce a circular beam that was single mode and also a thermoelectric cooler to maintain the diode temperature to prevent mode hopping due to
temperature fluctuations. The laser beam was focused onto the capillary detection window with a planoconvex lens (25 mm diameter, 25.0 mm focal length, Edmund Scientific, Barrington, NJ). The capillary was affixed horizontally (parallel to the optical bench) onto a home-made plexiglas capillary holder, which was mounted on top of an X-Y micropositioner for positioning the capillary with respect to the laser beam and collection optics. The fluorescence was collected at right angles using a 40X epifluorescence microscope objective (Melles Griot) with a numerical aperture of 0.65. The fluorescence was imaged onto a slit serving as a spatial filter to reduce the amount of scattered photons generated at the air/glass and glass/liquid interfaces of the capillary from reaching the detector. The fluorescence was further isolated from the scattering photons by a 780 nm bandpass filter (Oriel, Stratford, CT) and a 780 nm long pass filter (Edmund Scientific, Barrington, NJ). The fluorescence was then focused onto the photoactive area of the detector with a 20X microscope objective (Melles Griot). The detector was a SPAD (Model SPCM-AQ-141, EG&G Optoelectronics Canada, Vaudreuil, Canada) with a 200 μm diameter photoactive area and possessed a dark and maximum light count rate, before saturation, of approximately 30 and 1.6 x 10^6 counts/sec, respectively. In order to increase the linear dynamic range, the SPAD was operated in an actively quenched mode. The LIF signals were acquired on a personal computer (Gateway 2000, Model P5-120) using a 16-bit counter/timer board (Model CYRCTM 05, CyberResearch Inc., Brandford, CT). The high voltage power supply was obtained from Spellman (High Voltage Electronics Co, Plainview, NY).

3.2.2 Capillary Electrophoresis Experiment. Electrophoretic separations of native, ethidium bromide, and TAG-stained ΦX174 Hae III digested DNA was
Figure 3.1. Near-IR LIF system for CE analysis of DNA restriction fragments. M1, M2, and M3, mirrors; L, laser focusing lens; C, capillary tube; BD, beam dump; MO1 and MO2, microscope objectives; S, spatial filter; F, optical filters; SPAD, single-photon avalanche diode; C/T, counter/timer; PC, computer. The LIF system was constructed in-house by Dr. Bill Karr.
evaluated by Dr. Yolanda Davidson using a Waters Quanta 4000 electrophoresis apparatus with UV detection at 254 nm (Millipore, Marlborough, MA). The electrophoresis system was operated in the reverse polarity mode (negative potential at the injection end of the capillary) and was coupled to an LCI-100 laboratory computing integrator (Perkin-Elmer, Norwalk, CT) for displaying the data.

Restriction digest samples (ΦX174/HaeIII) were diluted (1:4) using 18 MΩ, milliQ water prior to electrophoresis and injected directly onto the CE capillary using electrokinetic injection. The sieving matrix consisted of a 0.25% HPMC solution, which was made by adding the appropriate amount of polymer to the running buffer (40 mM TRIS, 20 mM acetate, 2 mM EDTA, pH = 7.61). The sieving buffer was placed in a water bath and stirred while heating to completely dissolve the polymer. Prior to electrophoresis, the sieving buffer was filtered twice with 0.45 µm filter paper to remove particulates and undissolved polymer.

Fused silica capillaries (75 µm i.d., 375 o.d.) were purchased from Polymicro Technologies, Inc., Phoenix, Arizona) and were coated with a linear acrylamide (1% T) following published procedures. Briefly, the capillary was first rinsed with 1.0 M NaOH, then 1.0 M HCl, each followed with copious rinsing with doubly-distilled H₂O. The capillary was rinsed overnight with a 50/50 MAPS/MeOH solution after which the column was dried in an oven for several hours at 80°C. A 1%T linear acrylamide solution was made in 1 x TBE (TRIS, borate, EDTA) from a 40%T/0%C stock solution. The solution was thoroughly degassed by water aspiration for approximately 2.5 hours. Twenty-five microliters of fresh 10% ammonium persulfate (APS) in H₂O was added to the degassed acrylamide solution, followed by 5 µL of TEMED. The capillary was
quickly filled with the acrylamide solution and allowed to rest horizontally until complete polymerization occurred after which high pressure was used to remove excess linear acrylamide. The total capillary length used for both conventional and LIF CE was 39 cm (31 cm from injection to detection).

The capillary and cathodic and anodic reservoirs were filled with the running buffer solution which contained the Tris/Acetate/EDTA buffer, 0.25% (w/v) hydroxypropylmethyl cellulose and the appropriate concentration of the staining dye. Before injection of sample, the column was pre-conditioned by applying a field strength of 175 V/cm for 15 minutes. Non-stained dsDNA samples were injected directly into the capillary electrokinetically using a -50 V/cm field strength for 6 s for the LIF system (30 s for conventional CE). The electrophoresis was carried out using a field strength of 178 V/cm.

3.2.3 Synthesis of Near-IR Cyanine Dye. The near-IR nuclear staining dye, TAG, was prepared following modifications of previously described procedures. The synthetic steps are outlined in Scheme 3.1. 2-methylbenzothiazole (0.067 mol) was added to 3 equivalents of methyl iodide and refluxed for 18 hrs in ethanol at 72°C to form 2. The iodoalkyl derivative 3 was prepared by adding 0.034 mL of lepidine to 5 equivalents of 1,3-diiodopropane (0.176 mol) and refluxed in toluene at 110°C for 18 hrs. The yellow-brown precipitate was filtered with extensive washing using ethyl ether and collected. Malonaldehyde dianil hydrochloride was prepared from malonaldehyde bis (dimethyl acetal) and aniline. The intermediate was produced by refluxing 2 (0.0121 mol) and 4 (0.100 mol) in a 1:1 mixture of acetic acid and acetic anhydride. The reaction progress was monitored via UV absorption and judged complete when the
286 nm band nearly vanished. Reaction of 3 (0.64 mol) and 5 (0.0059 mol) in ethanol with sodium acetate after 30 mins of refluxing produced 6 (thiazole-green, TAG). This reaction was also monitored by UV/vis absorption, which indicated the presence of the symmetrical (665 nm) and unsymmetrical (735 nm) dye. TAG was purified by flash chromatography using a chloroform:methanol solvent (9:1, v/v). The NMR and mass spectral analysis revealed the identity and purity of 6.

The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin Elmer, Norwalk, CT). The uncorrected fluorescence spectra were collected on a Spex 3000 fluorometer (Spex, Edison, NJ). The spectrofluorometer contained a red-sensitive photomultiplier tube (R636, Hamamatsu Corp.) and emission gratings blazed for 750 nm. The fluorescence quantum yields were calculated relative to IR-125 in DMSO ($\Phi_f = 0.13$) according to the procedure outlined by Demas and Crosby.32

3.3 Results and Discussion

In Figure 3.2 is shown the absorption spectra of TAG in methanol, TAE buffer and TAE with a 100-fold molar excess (in nucleotide bases) of calf thymus DNA. In methanol, the dye gave a single band with an absorption maximum at 735 nm. This band could be assigned to the monomer, since it is expected that the dye shows little tendency to aggregate in this solvent.28 In TAE buffer, the spectrum shows two broad bands at approximately 705 nm and 533 nm, superimposed on a diffuse background. These spectral properties are indicative of extensive dye aggregation, forming dimers and other higher order aggregates, which is a typical phenomenon for the extended cyanine dyes.28,29 In the presence of double-stranded calf thymus DNA, the monomer
band is partially restored ($\lambda_{\text{max}} = 740$ nm) and another prominent band appears at 650 nm, which could arise from a dimer form of the dye.

In Figure 3.3 is shown a series of fluorescence spectra which were obtained at various concentrations of calf thymus DNA (dye concentration = 1 $\mu$M). As can be seen, drastic enhancements in the fluorescence were observed upon addition of calf thymus DNA. Construction of a modified Benesi-Hildebrand plot\(^3\) for this data indicated that the binding constant of TAG to dsDNA was approximately $1 \times 10^6$ M\(^{-1}\).

The binding constant was calculated from the equation;

$$\frac{1}{F - F_0} = \frac{1}{K_f G Q [\text{TAG}]_0 [\text{DNA}]} + \frac{1}{G Q [\text{TAG}]_0}$$  \hspace{1cm} (3.1)

where $K_f$ is the complex binding constant, $F$ is the fluorescence signal at various [TAG/DNA] concentrations, and $Q$ is quantum yield of Thiazole-Green [TAG]. The constant, $G$, is derived from the fluorescence signal and concentration of the complex, and $F_0$ is the fluorescence intensity at the emission maxima of TAG in water. From Eq. 3.1, a plot of $1/F-F_0$ vs $1/[\text{DNA}]$ should be linear with the slope equal to $1/K_f G Q [\text{TAG}]$.

Integration of the emission spectra in the buffer only and in a 100-fold molar excess (in nucleotide bases) of dsDNA indicated that the fluorescence enhancement ratio of bound to free dye was 102.

In order to make a comparison of the separation efficiency of native, EtBr and TAG-stained DNA, the Hae III restriction fragments of $\Phi$X174 were analyzed using CE with UV detection at 254 nm. In Table 3.1 is shown the plate numbers (N), total zone variance ($\mu^2_{\text{tot}}$) and the apparent electrophoretic mobilities ($\mu_{\text{app}}$) of these restriction fragments under different staining conditions.
The number of theoretical plates were calculated using the equation;

\[ N = 5.54 \times \left( \frac{t_m}{w_{0.5}} \right)^2 \]  

where \( t_m \) is the migration time and \( w_{0.5} \) is the peak width at half peak height.

The electrophoretic mobilities (\( \mu_{ep} \)) were calculated using the equation;

\[ \mu_{ep} = \frac{L L_o}{V} \left( \frac{1}{t_m} - \frac{1}{t_{EOF}} \right) \]  

where \( L \) is the total length of the capillary in centimeters, \( L_o \) is the effective length (i.e., to the detection window) of the capillary in centimeters, \( V \) is the separation voltage in volts, \( t_m \) is the migration time of the analytes in minutes, and \( t_{EOF} \) is the migration time of the neutral standard in minutes. Generally, the TAG-stained restriction fragments produced higher plate numbers than the EtBr stained DNA or the non-stained DNA. Also seen in this data is that the apparent electrophoretic mobilities of the TAG-stained DNA fragments are smaller than those associated with the EtBr-stained fragments. This result is supported by the binding constant measured for the TAG/DNA complex, which indicated a value larger than that associated with EtBr to dsDNA. Therefore, the increased stability of the dye/DNA complex would result in a lower mobility due to reductions in the negative charge of the biopolymer and increases in the frictional coefficient resulting from elongation of the double helix produced from intercalation and the increased molecular mass associated with the complex. It should be noted that in all cases, we were unable to resolve the 271/281 bp fragments in this restriction digest under these CE conditions and using this CE system.

Figure 3.4 shows the CE separation of the Hae II digest of \( \phi X174 \) in a polyacrylamide-coated capillary using near-IR LIF detection. From the apparent
Figure 3.2 Absorption spectra of TAG in methanol (solid line), TRIS/acetate buffer with no DNA (dashed line), and a 100-fold molar excess, in nucleotide bases, of calf thymus DNA (dotted line). The dye concentration was $2 \times 10^{-5}$ M.
Figure 3.3 Spectrofluorometric titration of TAG with calf thymus DNA. In all cases, the DNA concentration was measured in terms of the nucleotide bases and was varied from $5 \times 10^{-6}$M (yellow line), $5 \times 10^{-5}$M (red line), $1 \times 10^{-5}$M (blue line), $1 \times 10^{-4}$M (black line). The fluorescence was excited at 710 nm and the dye concentration used was $1 \times 10^{-6}$M. The insert shows the construction of a modified Benesi-Hildebrand indicated the binding constant of TAG to dsDNA was approximately $1 \times 10^{6}$ M$^{-1}$.
Table 3.1  CE analysis of the Hae III restriction digest of the native ΦX174 fragments and the Et-Br or TAG-stained fragments. Detection was accomplished using UV absorption at 254 nm. The restriction fragments were injected electrokinetically onto the CE column for 30 s at -5 KV.

<table>
<thead>
<tr>
<th>Native DNA</th>
<th>Ethidium Bromide</th>
<th>Thiazole-Green</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu_{app} ) (cm(^2) V(^{-1}) s(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>( N \times 10^m )</td>
<td>( c \times 10^n )</td>
</tr>
<tr>
<td>72</td>
<td>4.77</td>
<td>4.26</td>
</tr>
<tr>
<td>118</td>
<td>3.84</td>
<td>4.16</td>
</tr>
<tr>
<td>194</td>
<td>3.16</td>
<td>3.98</td>
</tr>
<tr>
<td>234</td>
<td>4.60</td>
<td>3.88</td>
</tr>
<tr>
<td>271</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>281</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>310</td>
<td>6.00</td>
<td>3.70</td>
</tr>
<tr>
<td>603</td>
<td>4.44</td>
<td>3.22</td>
</tr>
<tr>
<td>872</td>
<td>4.49</td>
<td>2.95</td>
</tr>
<tr>
<td>1,078</td>
<td>5.27</td>
<td>2.82</td>
</tr>
<tr>
<td>1,353</td>
<td>5.43</td>
<td>2.70</td>
</tr>
</tbody>
</table>

\(^a\) Detection was accomplished using UV absorption at 254 nm. The restriction fragments were injected electrokinetically onto the CE column for 30 sec at -5 kV. \(^b\) Plate numbers were calculated assuming a Gaussian peak from the FWHM of the electrophoretic peak and the migration time. \(^c\) Plate numbers determined for the LIF system. The 281 fragment comigrated with the 271 fragment (noted by dashed line).
electrophoresis conditions was estimated to be 20 fg (SNR = 3), comparable to the
visible LIF detection of this same digest stained with TOTO.4

From the electropherogram depicted in Figure 3.4, it is seen that the 271/281-bp
fragments are nearly baseline resolved, with a resolution determined to be 1.3,
significantly better than that observed for the conventional CE results where the native,
EtBr and TAG-stained 271/281 bp fragments co-migrated. Since the capillary length
and conditions were similar in both cases and the sieving buffer was identical, the
enhanced resolution in the LIF system most likely results from improvements in the
efficiency of the separation and not differences in selectivity. This supposition is
supported by our data, which showed that the plate numbers for the 603 bp fragment
when stained with TAG, was found to be 4.13 x 10^5 plates/m for the conventional CE
case, while for the LIF system, the plate numbers for this same fragment was 1.06 x 10^6
plates/m. From these plate numbers, the total zone variance (σ^2_tot) was calculated
using: 30

\[ \sigma^2_{\text{tot}} = \frac{L^2}{N} \]  \hspace{1cm} (3.4)

where L is the column length and N is the number of theoretical plates. In the case of
conventional CE, σ^2_tot (603 bp fragment) was found to be 3.5 x 10^{-3} cm^2 and for the LIF
system, σ^2_tot was 6.1 x 10^{-4} cm^2. Since the total zone variance has contributions from
diffusion, finite injection and detection volumes, parabolic temperature profile,
siphoning, conductivity differences and solute-wall interactions, it is expected that the
major differences observed in the total zone variance in this particular case resulted
from differences in the injection [σ^2_{inj} = l^2_{inj}/12 where l_{inj} is the length of the sample

73
Figure 3.4 Electropherogram of \( \Phi X174 \) DNA restriction fragments with LIF detection. The dye concentration added to the running buffer was 1 \( \mu \)M, and the running buffer was composed of 40 mM TRIS, 20 mM sodium acetate, 2 mM EDTA (pH 7.6), and 0.25% HPMC. The sample was electrokinetically injected for 6 s at -2 kV onto the column. The electric field strength used for the separation was 175 V/cm.
plug] and detection volumes \( \sigma_{\text{det}}^2 = \frac{l_{\text{det}}^2}{12} \) where \( l_d \) is the length of the detector cell], since all other CE conditions were similar in both cases. The significant improvement in efficiency most likely results from the fact that the zone variances arising from the finite injection volume and detection volume are smaller in the LIF case compared to the conventional case since, in the LIF system, the beam length was significantly smaller and the amount of sample inserted onto the column was reduced. The need for extended injection conditions in the case of UV detection resulted from the need to obtain sufficient signal strengths for analysis by inserting more material onto the column.

We next investigated the effect of dye concentration placed in the running buffer on CE detectability and efficiency for TAG-stained DNA restriction fragments. The results of this investigation for three different dye concentrations are shown in Figure 3.5 (0.2 \( \mu \)M, 1.0 \( \mu \)M and 4.0 \( \mu \)M). The fluorescence intensity of the DNA fragments decreased significantly when the dye concentration in the buffer was decreased to 0.2 \( \mu \)M, due to less dye/DNA complexes formed based on thermodynamic considerations (see Figure 3.5c). In addition, it was found that the peaks broaden as well when the dye concentration was reduced to 0.2 \( \mu \)M as compared to the case when 1 \( \mu \)M dye was used \((N = 1.38 \times 10^6 \text{ plates/m for 0.2 } \mu \text{M dye and } N = 2.49 \times 10^6 \text{ plates/m for 1.0 } \mu \text{M dye for the 603 bp fragment}). Increasing the dye concentration in the running buffer to 4.0 \( \mu \)M deteriorated the peak shapes of all DNA fragments, causing significant peak broadening and tailing (see Figure 3.5a, \( N = 6.80 \times 10^5 \text{ plates/m for the 603 bp fragment}). However, the peak heights were nearly equivalent (slightly higher and lower for 72-310 and 603-1353 bp fragments, respectively), indicating that the peaks were broadened.
under these conditions, as apparent from the reduced plate numbers for the 4 μM dye case. Schwartz et al.\textsuperscript{1} has postulated that the excess dye possibly binds to ssDNAs as well. If this binding process becomes thermodynamically preferable, the presence of excess dye could destabilize the DNA double helix. As a result, it is likely to result in broad peaks with longer migration times, consistent with our data.

![Electropherogram](image)

**Figure 3.5** Electropherogram of ΦX174 DNA restriction fragments obtained using (a) 4.0, (b) 1.0, and (c) 0.2 μM dye in the running buffer.
3.4 Summary

We have prepared a near-IR nuclear staining dye which forms stable complexes to dsDNA and exhibits a fluorescence enhancement ratio of bound to free dye of approximately 102, making it an ideal probe for the detection of DNA restriction fragments or other dsDNAs. In addition, this dye has an absorption maximum near the principle lasing line of a GaAlAs diode laser, allowing the construction of an LIF detector for capillary electrophoresis which consists of all solid-state components, reducing cost and simplifying operation. The detection limit of the dsDNA fragments was determined to be 20 fg of DNA per electrophoretic band, comparable to visible LIF systems. In addition, TAG-stained dsDNA fragments were found to have improved efficiency compared to EtBr-stained fragments, most likely resulting from the higher affinity of TAG to dsDNA compared to EtBr. The attractiveness of near-IR LIF detection also arises from the fact that the diode laser and avalanche diode can easily be miniaturized and integrated directly onto a microfabricated CE system.

3.5 References


78


Chapter 4

Phthalocyanine and Naphthalocyanine Dyes as Fluorescent Probes for Labeling Biomolecules

4.1 Introduction

During the past few years, fluorescent probes, which can be readily conjugated to target molecules, have become very important for a variety of uses in industry and medicine. Today, most bioanalytical applications use molecules that fluoresce in the visible region (400-650 nm) of the electromagnetic spectrum. Unfortunately, the spectroscopic properties of these fluorophores are susceptible to biological interferences in the sample matrix, limiting the sensitivity of the measurement. An attractive alternative to visible fluorescence is the use of near-IR fluorescence detection (650-1000 nm), which has recently been demonstrated to be a viable detection strategy in liquid chromatography, free solution capillary electrophoresis, and capillary gel electrophoresis for DNA sequencing applications. Near-infrared (NIR) fluorescence offers some attractive alternatives to UV or visible fluorescence detection because of the minimal background interferences that are observed. The minimal background interferences result from the limited number of components that show intrinsic fluorescence in this region of the electromagnetic spectrum. In addition, scattering background levels can also be lower because of the $\lambda^{-4}$ dependence of the Raman cross section on the excitation wavelength. Also, NIR fluorescence allows the use of inexpensive semiconducting devices, such as diode lasers and detectors.
The major requirements for any fluorogenic labels are; possess a high quantum yield, demonstrate large Stokes shift, and have chemical and photochemical stability.\textsuperscript{12} Fluorogenic labels should also contain a selective and reactive functional group (ex., isothiocyanate or isocyanate) for facile conjugation to the biomolecule. Also, for bioanalytical applications, the dyes should contain certain charged or polar functional groups to provide water solubility.

Recently there have been reports on near-IR dyes that covalently and non-covalently label biomolecules such as amino acids, proteins, nucleotides, and DNA primers.\textsuperscript{13,14} The dyes used are the symmetrical tricarbocyanine dyes, which consist of heteroaromatic structures linked by a polymethine chain containing conjugated carbon/carbon double bonds. We and others have developed novel functionalized tricarbocyanine dyes as NIR fluorescent probes for biomolecules.\textsuperscript{15} These dyes possessed large molar absorptivities (~2 x 10\textsuperscript{5} cm\textsuperscript{-1} M\textsuperscript{-1}), and excellent solubility in aqueous solution provided by the negatively charged sulfopropyl groups attached to the heteroaromatic rings. The major limitations associated with tricarbocyanines are their poor chemical and photochemical stabilities and low quantum yields in aqueous environments, therefore limiting their utility in ultra-sensitive applications.\textsuperscript{16}

An alternative to cyanine NIR fluorophores are the phthalocyanine (\textit{phth}; also know as tetraazaporphyrins or porphyrizines) family of compounds which are chemically and photochemically robust and have large extinction coefficients (> 10\textsuperscript{5} cm\textsuperscript{-1} M\textsuperscript{-1}) as well as large quantum yields.\textsuperscript{17} Additionally, the wavelength of absorbance and fluorescence can be adjusted by variation of substituents around the ring or by
variation of the metal center (Zn, Sn, Al, Si, etc.). For example, annulation of benzene rings onto the phth core to produce naphthalocyanine (nphth) dyes have $\lambda_{\text{max}}$ red-shifted 50-100 nm.\textsuperscript{18,19} In general phth dyes are very hydrophobic and essentially insoluble in water. Phthalocyanine compounds were first synthesized in 1928 by Bradbrook and Linstead during the preparation of phthalimide from phthalic anhydride. Functionalization of the periphery of the dye with charged groups (-SO$_3^-$, -CO$_2^-$, etc.) or attachment of a polar functionality to the metal center (PEG-Osi) does provide water-soluble phth, though general routes to water-soluble phth dyes with a labeling function are not known.

One way to approach the preparation of labeling water-soluble phth is to put the reactive group L in one “quadrant” of the phth (see Figure 4.1) and then use one or more of the remaining quadrants to incorporate water-solubilizing groups S. Phth and nphth dyes are generally prepared by cyclo-tetramerization of aromatic 1,2-dinitriles (phthalonitriles or naphthalonitriles) under either basic or Lewis acidic conditions.\textsuperscript{16,20,22} The simplest route to prepare non-symmetrically substituted phth dyes as discussed above would require the use of one phthalonitrile that contains the (latent) labeling group L and another containing the solubilizing group S in a molar ratio of 1:3. This mixed “condensation” route is commonly used to prepare non-symmetric phth and nphth dyes, but as yet has not been applied to dyes containing a mixture of labeling and water-solubilizing groups we desire.\textsuperscript{21} Herein, we report the synthesis, photophysical characterization, and labeling reactions of two new water-soluble near-IR
dyes nphth 4 and phth 7, both which contain a reactive isothiocyanate functional group used for labeling biomolecules (see Figure 4.2).

**Figure 4.1.** Preparation of labeling, water-soluble phthalocyanine dye placing the reactive group $L$ in one "quadrant" of the macrocycle with one or more of the remaining quadrants to incorporate water-solubilizing groups $S$.

**Figure 4.2.** Structure of Water-Soluble Naphthalocyanine (4) and Phthalocyanine (7) Dyes containing reactive isothiocyanate for labeling primary amines.
4.2 Experimental Procedures

2,3-naphthalenedicarbonitrile, 3-nitrophthalonitrile, 4-nitrophthalonitrile, 1,1’-thiocarbonyldi-2(1H)-pyridone, palladium 10% on activated carbon, sodium thiosulfate, fumaronitrile, sodium iodide, carbon tetrachloride, 3,4-dimethylbenzoic acid and zinc acetate dihydrate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Hydrochloric acid, and sulfuric acid were obtained from Fisher Scientific (Houston, TX). Potassium nitrate, sodium carbonate, sodium hydroxide, and urea were obtained from Sigma Chemical Co (St. Louis, Mo). All chemicals were used as received.

4.2.1 Purification and Spectroscopic Characterization Analysis. The mixture of the water-soluble macrocycles were assayed by a Rainin HPLC system using a Hamilton PRP-1 analytical column (4.6 x 15 cm; flow rate 2 mL/min Hamilton, Reno, NV) and a gradient of 0.1 M triethylammonium acetate/ 20 mM sodium phosphate buffer (pH ~ 10.1) and tetrahydrofuran. The Hamilton PRP-1 column was chosen because of its stability at high pH values compared to conventional C_8 or C_{18} columns. The HPLC Shimadzu absorbance detector was set and at 680 or 700 nm to monitor elution of the target molecule. For typical analytical analysis, 1 mg of dye was dissolved in 2 mL of 20 mM sodium phosphate buffer. A 20 μL aliquot was injected onto the column, which had been equilibrated with 100% 20 mM sodium phosphate buffer at 2 mL min^{-1}. Elution was carried out by a stepwise gradient, which was changed from 0 to 100% tetrahydrofuran over a period of 25 min. During this period, the tetracarboxylate, tricarboxylate, dicarboxylate, and monocarboxylated fractions shown in Figure 4.5 and Figure 4.8 eluted in order.
Large-scale separations were carried out on a 21.5 mm x 25 cm Hamilton PRP-1 preparatory column (Hamilton Reno, NV) packed with a polymeric reverse phase C₁₈ which was first equilibrated for 10 minutes with 20 mM sodium phosphate buffer (pH = 10.1) at 5 mL min⁻¹. The water-soluble dye mixture (less than 1g) was dissolved in 10 mL of buffer and injected onto the preparatory column. The HPLC stepwise gradient was slightly modified by extending the gradient times for the solvents to compensate for our increased flow capacity of 5 mL min⁻¹. During the gradient period, the tetracarboxylate, tricarboxylate, dicarboxylate, and monocarboxylated fractions eluted in the same order as in the analytical separation. Dyes were recovered from collected fractions, and the solvents removed using a rotary evaporator.

The absorption spectra were acquired on a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer. The fluorescence spectra were obtained on a SPEX Fluorolog spectrofluorometer (SPEX, Edison, NJ) equipped with a 75 W Xenon lamp. The emission gratings were blazed for 750 nm and the photomultiplier tube was a Hammamatsu R636 red-sensitive photomultiplier tube.

Capillary zone electrophoresis of the dye/amino acid conjugates was performed on a Beckman Pace 5000 CE system (Pasadena, CA). Free solution separations were performed using a 75 µm i.d. capillary column (Supelco, Bellefonte, PA) with a total length of 40 cm and a detection window of 33 cm from the injection end. The running buffer consisted of a 40 mM borate (pH = 9.4) and 10% methanol. A cationic surfactant (0.5 mM dodecatriethylammonium bromide, DTAB) was added to reverse the direction of the electroosmotic flow. Dye concentrations of 10 x 10⁻⁵ M dissolved in water were electrokinetically injected onto the column for 20 seconds with an applied voltage of -8
kV (135 V/cm). The separations were performed at an applied voltage of -15 kV (300 V/cm). The analytes were detected using absorbance at 254 nm.

Capillary gel electrophoresis was performed in a 3%T/3%C crosslinked polyacrylamide gel column 75 μm i.d. (J&W Scientific, Folsom Ca) with a total length of 40 cm and a detection window 33 cm from the injection end. The dye/oligonucleotide conjugate was electrokinetically injected onto the column for 30 seconds at an applied voltage of -15 kV, with separations performed in reverse mode at an applied voltage of -25 kV. The detection was performed using UV absorbance monitored at 254 nm.

4.2.2 General Synthesis of Zinc-Phthalocyanines and Zinc-Naphthalocyanine. The dinitrile derivatives 1α and 1c (see Scheme 4.1) were synthesized from previously published literature procedures. The phthalocyanine 5-7 derivatives were synthesized analogous to the naphthalocyanine 2-4 derivatives as stated below.

- 4-(3,4-dicyanophenoxy) benzoic acid (1α). 4-hydroxybenzoic acid (2.3 g; 17 mmol) and 4-nitrophthalonitrile (2 g; 11 mmol) were added to the suspension of potassium carbonate (3.5 g; 25 mmol) in DMSO (20 mL) and stirred at room temperature for 24 hours. The resulting suspension was diluted with water (200 mL) and the pH was adjusted to 1 with 6N HCl. The light brown precipitate was isolated by filtration and washed with 1N HCl. The crude product was purified by recrystallization from methanol. Yield 2.91 g (95%). IR (KBr): ν = 3107 cm⁻¹, 2230 cm⁻¹(CN), 1675 cm⁻¹(C=O). GC/MS: m/z 264 [M⁺]. H NMR (DMSO): δ = 13.05 (s, 1H), 8.15 (d, 1H), 8.02 (m, 2H), 7.91 (d, 1H), 7.52 (d, 1H), 7.25 (m, 2H).
- 3,4-bis(dibromomethyl)benzoic acid (1b). \[23\] 3,4-dimethylbenzoic acid (10 g; 66 mmol) in CC\(_4\) (50 mL) and N-bromosuccinimide (47 g; 267 mmol) was added with 0.40 g of benzoyl peroxide. The reaction was refluxed for 18 hours and irradiated with a UV lamp. The precipitate was filtered off and washed with water to remove excess succinimide. The orange precipitate was recrystallized from chloroform/acetone and dried under vacuum. Yield 25 g (80%). MP = 101° C. IR (KBr): \(\nu = 3076\) cm\(^{-1}\), 3020 cm\(^{-1}\), 1690 cm\(^{-1}\)(C=O). H NMR (DMSO): \(\delta = 13.5\) (br, 1H), 8.35 (s, 1H), 8.05 (d, 1H), 7.95 (d, 1H), 7.8 (s, 1H), 7.75 (s, 1H). MALDI-MS \(m/z\) 466 (M\(^+\)).

- 6,7-dicyano-2-napthalenecarboxylic acid (1c). Fumaronitrile (0.78 g; 10 mmol) 3,4-dibromomethylbenzoic acid (4.66 g; 10 mmol), and sodium iodide (8.34 g; 50 mmol) were added in DMF (50 mL). The reaction was refluxed for 12 hrs at 80° C. The dark reaction mixture was added to a stirred solution of sodium thiosulfate (9.1 g, 50 mmol) in water (300 mL). The yellow precipitate was filtered, recrystallized in toluene and dried under vacuum. Product yield 1.37 g (62%). MP = 210° C. IR (KBr): \(\nu = 3033\) cm\(^{-1}\), 2236 cm\(^{-1}\) (CN), 1690 cm\(^{-1}\) (C=O). H NMR (DMSO): \(\delta = 13.5\) (br, 1H), 9.05 (s, 1H), 8.95 (d, 1H) 8.80 (s, 1H), 8.25 (s, 1H), 8.00 (d, 1H). MALDI-MS \(m/z\) 221 (M\(^+\)).

- 12,24,36-Tricarboxy-(4-nitro-zincnaphthalocyanine) (2). In Scheme 4.2, using a condensation reaction, 6,7-dicyano-2-napthalenecarboxylic acid (0.474 g; 1.69 mmol) and 4-nitrophthalonitrile (0.135 g; 0.78 mmol) were combined with 0.100 g of zinc acetate dihydrate. The reaction mixture was ground together into a fine homogeneous powder and heated at 200° C for 20 minutes. The solid black cake produced was monitored by UV absorbance for the appearance of the near-IR peak.
Scheme 4.1 Synthesis of Phthalonitrile 1a and Naphthalonitrile Precursors 1c.
Reactions with the metal salts at 200-210°C proceeded unusually fast and further heating resulted in a decrease in the yield of the metal complex. Lowering the temperature slowed the reaction down, but did not increase the product yield. The dark green precipitate was dispersed in boiling 1M HCl, cooled to room temperature, and collected. The precipitate was redissolved in 1M NaOH, and filtered to remove any insoluble impurities. The filtrate was neutralized with 1M HCl, and rotary evaporated on vacuum to yield a green solid. The product, however, contained mixtures of at least four of components known as congeners (see Figure 4.5). Congeners can be defined as compounds that contain relatively the same number of atoms but differ in structural arrangement and properties, which are known as congeners.

Scheme 4.2 Synthesis of 12,24,36-Tricarboxy-(4-nitro-zincnaphthalocyanine) (2).
As stated in the purification procedure, an analytical method was developed injecting 1 mg of water-soluble dye onto a polymeric C\textsubscript{18} column equilibrated with 0.1 M TEAA/20 mM sodium phosphate buffer (pH=10.1). The linear gradient was performed using 0 to 100% THF. The components eluted in numerical order, i.e. tetra, tri, di, and mono. The characterization of the product congeners (I, 2, II, III) were identified by their high performance liquid chromatography profiles, retention times (Figure 4.4), UV Absorbance spectra (Figure 4.3), and mass spectral ions (Figure 4.6).

Tetracarboxylate (4 CO\textsubscript{2}) nphth (I): HPLC \( t_r = 5.68 \) min; \( \lambda_{\text{max}} \) (DMSO) = 768 nm; MALDI (m/z) 974 (M-H\textsubscript{2}O + acetate + Na). Tricarboxylate/mono-nitro (3 CO\textsubscript{2}$/ 1 NO\textsubscript{2}) nphth (2): HPLC \( t_r = 7.08 \) min; \( \lambda_{\text{max}} \) (DMSO) = 765 nm, 727 nm; MALDI (m/z) 905 (M + H). Di-nitro/dicarboxylate (2 CO\textsubscript{2}$/ 2 NO\textsubscript{2}) nphth (II): HPLC \( t_r = 16.03 \) min; \( \lambda_{\text{max}} \) (DMSO) = 720 nm, 689 nm; MALDI (m/z) 873 (M - H\textsubscript{2}O + acetate + Na). Tri-nitro/monocarboxylate (1 CO\textsubscript{2}$/ 3 NO\textsubscript{2}) nphth (III): HPLC \( t_r = 18.82 \) min; \( \lambda_{\text{max}} \) (DMSO) = 684 nm; MALDI (m/z) 809 (M + H), 761 (M-COOH). The tricarboxylate/mono-nitro was the desired component collected for final use because of the greater water solubility (3 CO\textsubscript{2}$) and one nitro functional group. This compound upon conversion of the nitro to the amine, and then isothiocyanate, would allow for only one site to react with our target biomolecule. Yield 650 mg (33%). IR spectrum of sample with KBr: \( \nu = 1645 \text{ cm}^{-1} \) (C=O), 1525 cm\textsuperscript{-1} (NO\textsubscript{2}), 1350 cm\textsuperscript{-1} (NO\textsubscript{2}). MALDI-MS calculated for C\textsubscript{47}H\textsubscript{21}N\textsubscript{9}O\textsubscript{6}Zn = 904, found m/z 905 (M+H). (\( \lambda_{\text{max}} \) DMSO = 765 nm, 727 nm)
Figure 4.3 UV absorbance profile of naphthalocyanine congeners (I, 2, II, III). The samples were evaluated after collection from HPLC during separation, so their concentrations were not determined. The solvent system of each profile consisted of a variation of the HPLC linear gradient (0.1 M triethylammonium acetate/20 mM sodium phosphate buffer (pH=10.1) and tetrahydrofuran over 30 min.
Figure 4.4. HPLC chromatogram of the Naphthalocyanine congeners (I, 2, II, III) obtained in the reaction of 4-nitrophthalonitrile and 6,7-dicyano-naphthalene-carboxylic acid. Conditions: Hamilton PRP-1 analytical column (4.6 x 15 cm; flow rate 2 mL/min) and a gradient of 0.1 M triethylammonium acetate/20 mM sodium phosphate buffer (pH ~ 10.1) and tetrahydrofuran. The absorbance detector was set at 700 nm.
Figure 4.5. Structure of Naphthalocyanine congeners (I, 2, II, III) obtained in the reaction of 4-nitrophthalonitrile and 6,7-dicyano-naphthalene-carboxylic acid.
Figure 4.6. MALDI mass spectra of Naphthalocyanine congeners (I, 2, II, and III). The experiments were performed on a Perseptive Biosystems Inc. Voyager linear MALDI-TOF instrument with a N$_2$ laser in both positive and negative-ion modes. Laser power set at the threshold level required to generate signal and each spectrum is an average of 32-40 scans. Each experiment was conducted three separate times at different locations on the sample spot to average results and ensure reproducibility.
• **Synthesis of 12,24,36-Tricarboxy-(4-amino-zincnaphthalocyanine) (3).** In a large pressure flask, 12,24,36-Tricarboxy-(4-nitro-zincnaphthalocyanine) 2 (0.400 g; 0.44 mmol) was dissolved in water (20 ml) and added to 30 mg of 10% palladium on charcoal (see Scheme 4.3). The reaction flask was pressurized to 40 psi under hydrogen and allowed to shake on a low pressure reaction apparatus for 3 hours at room temperature. The reaction product was filtered to remove the catalyst and rotary evaporated to remove the solvent. The product was filtered and the dark green precipitate was dried under vacuum. Yield 210 mg (54%). Elemental analysis calculated for C₄₇H₂₃N₉O₆ZnNa₃*8 H₂O; (Found): C, 51.9 (51.31); H, 3.6 (3.48); N, 11.6 (11.25). MALDI-MS calculated for C₄₇H₂₃N₉O₆Zn = 873, found m/z 874 (M+H). (λₑᵤₙ DMSO = 750 nm).

![Scheme 4.3 Synthesis of 12,24,36-Tricarboxy-(4-amino-zincnaphthalocyanine) (3).](image)
• Synthesis of 12,24,36-Tricarboxy-(4-isothiocyanate-zinc-naphthalocyanine) (4). 12,24,36-Tricarboxy-(4-amino-zinc-naphthalocyanine) 3 (300 mg; 0.34 mmol) was added to 250 mg of 1-1'-thiocarbonyldi-2(1H)-pyridone and dissolved in 3 mL of DMF for 2 hours (see Scheme 4.4). The DMF was rotavaporized and the dark green product was dried under vacuum. Yield 60 mg (28%). Elemental analysis calculated for C_{48}H_{21}N_{9}SO_{6}Zn*7H_{2}O; (Found): C, 51.8, (51.76); H, 3.2 (2.86); N, 11.3 (11.29). MALDI-MS calculated for C_{48}H_{21}N_{9}SO_{6}Zn = 916, found m/z 917 (M+H). (λ_{max} DMSO =756 nm, 723 nm).

Scheme 4.4 Synthesis of 12,24,36-Tricarboxy-(4-isothiocyanate-zinc-naphthalocyanine) (4).
Synthesis of 11,19,27-Tricarboxyphenoxy-(4-nitro-zincphthalocyanine) (5).

In Scheme 4.5, a condensation reaction using a similar procedure described for the nphth to prepare the water-soluble phth is described. 4-(3,4-dicyanophenoxy) benzoic acid (0.425 g; 1.60 mmol) and 3-nitrophthalonitrile (0.135 g; 0.81 mmol) were combined with 0.100 g of zinc acetate dihydrate. The reaction mixture was ground together into a fine homogeneous powder and heated at 200°C for 20 minutes. The dark green precipitate was dispersed in boiling 1M HCl, cooled to room temperature, and collected. The precipitate was redissolved in 1M NaOH, and filtered to remove any insoluble impurities. The filtrate was neutralized with 1M HCl, and rotary evaporated on vacuum to yield a green solid. The product yield, however, contained four mixtures of isomers or congeners.

Scheme 4.5 Synthesis of 11,19,27-Tricarboxyphenoxy-(4-nitro-zincphthalocyanine) (5).
As stated in the purification procedure for the nphth nitro-substituent, an analytical method was developed, which involved injecting 1 mg of water-soluble dye into a polymeric C_{18} column equilibrated with 0.1 M TEAA/20 mM sodium phosphate buffer (pH=10.1). The linear gradient was performed using 0 to 100% THF. The components eluted in the order, i.e. tetra, tri, di, and mono-carboxylic acid. The characterization of the product congeners (IV, 3, V, VI) were identified by their high performance liquid chromatography profiles, retention times (Figure 4.7), UV Absorbance spectra, and mass spectral ions (Figure 4.9). Tetracarboxylate (4CO_2) pth (IV): HPLC \(t_r = 9.22\) min; \(\lambda_{\text{max}}\) (DMSO) = 677 nm. Tricarboxylate/mono-nitro (3 CO_2/ 1 NO_2) pth (3): HPLC \(t_r = 11.11\) min; \(\lambda_{\text{max}}\) (DMSO) = 672 nm; MALDI (m/z) 961 (M – 3Na). Di-nitro/dicarboxylate (2 CO_2/ 2 NO_2) pth (V): HPLC \(t_r = 12.13\) min; MALDI (m/z) 904 (M - acetate - Na). Tri-nitro/monocarboxylate (1 CO_2/ 3 NO_2) pth (VI): HPLC \(t_r = 17.07\) min; MALDI (m/z) 811 (M – acetate + Na). The tricarboxylate/mono-nitro was the desired component obtained because of the greater water solubility (3 CO_2) and one nitro functional group. Yield 650 mg (33%). Elemental analysis calculated for C_{53}H_{27}N_{9}O_{11}ZnNa_{5}*9 H_2O: (Found): C, 48.9 (48.26); H, 3.5 (3.06); N, 9.8 (9.37). MALDI-MS calculated for C_{53}H_{27}N_{9}O_{11}Zn = 1029, found m/z 1028 (M+H). (\(\lambda_{\text{max}}\) DMSO = 672 nm).

- **Synthesis of 11,19,27-Tricarboxyphenoxy-(4-amino-zincphthalocyanine) (6).**
From Scheme 4.6, a large pressure flask with 11,19,27-Tricarboxyphenoxy-(4-nitro-zincphthalocyanine) 5 (0.500 g; 0.48 mmol) was dissolved in water (20 ml) and added
Figure 4.7. HPLC chromatogram of the Phthalocyanine congeners (IV, 3, V, VI) obtained in the reaction of 3-nitrophthalonitrile and 4-(3,4-dicyanophenoxy) benzoic acid. Conditions: Hamilton PRP-1 analytical column (4.6 x 15 cm; flow rate 2 mL/min) and a gradient of 0.1 M triethylammonium acetate/ 20 mM sodium phosphate buffer (pH ~ 10.1) and tetrahydrofuran. The absorbance detector was monitored at 680 nm.
Figure 4.8. Structure of the Phthalocyanine congeners (IV, 3, V, VI) obtained in the reaction of 3-nitrophthalonitrile and 4-(3,4-dicyanophenoxy) benzoic acid.
Figure 4.9. MALDI mass spectra of Phthalocyanine congeners (3, V, VI). The experiments were performed on a Perseptive Biosystems Inc. Voyager linear MALDI-TOF instrument with a N2 laser in both positive and negative-ion modes. Laser power set at the threshold level required to generate signal and each spectrum is an average of 32-40 scans. Each experiment was conducted three separate times at different locations on the sample spot to average results and ensure reproducibility.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
to 30 mg of 10% palladium on charcoal. The reaction flask was pressurized to 40 psi under hydrogen and allowed to shake on a low pressure reaction apparatus for 3 hours at room temperature. The reaction product was filtered to remove the catalyst and rotary evaporated to remove the solvent. The product was filtered and the dark green precipitate dried under vacuum. Yield 275 mg (54%). Elemental analysis calculated for C_{53}H_{29}N_9O_9ZnNa_3\cdot10\text{ H}_2\text{O}; (Found): C, 50.9 (50.66); H, 3.4 (3.1); N, 8.91 (8.8). MALDI-MS calculated for C_{53}H_{29}N_9O_9Zn = 999, found m/z 1000 (M+H). (\lambda_{max} 702 nm)

Scheme 4.6 Synthesis of 11,19,27-Tricarboxyphenoxy-(4-amino-zincphthalocyanine) (6).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Synthetic scheme of 11,19,27-Tricarboxyphenoxy-(4-isothiocyanate-zinc phthalocyanine) (7). 11,19,27-Tricarboxyphenoxy-(4-amino-zincphthalocyanine) 6 (350 mg; 0.35 mmol) was added with 250 mg of 1-L-thiocarbonyldi-2(1H)-pyridone and dissolved in 3 mL of DMF for 2 hours. The DMF was rotavaporized and the dark green product was dried under vacuum. Yield 86 mg (27%). Elemental analysis calculated for C_{54}H_{24}N_{9}SO_{3}Zn * 3H_{2}O; (Found): C, 59.5 (59.58); H, 3.01 (2.99); N, 11.15 (11.18) MALDI-MS calculated for C_{54}H_{24}N_{9}SO_{3}Zn = 1039, found m/z 1040 (M+H). (λ_{max}=686)

Scheme 4.7 Synthetic scheme of 11,19,27-Tricarboxyphenoxy-(4-isothiocyanate zincphthalocyanine) (7).
4.2.3 Labeling and Purification of Sequencing Primers with Nphth and Phth Functionalized Dyes. A M13 universal sequencing primer (17 mer) containing a 6-carbon alkyl linker terminated with an amino group on the 5’-end were derivatized with the dyes according to procedures outlined by Li-COR. Briefly, 50 nmol of DNA was added to 25 μl of 40 mM carbonate buffer (pH=9.1), 25 μl of 2mM EDTA, and 100 μl of the near-IR dye (5 nM) dissolved in DMF. After the reaction was allowed to proceed at room temperature for approximately 4 hours, 10 μl of 3M sodium carbonate and 480 μl of cold EtOH were added to the reaction mixture. The solution was centrifuged for 20 min at 10°C. The supernatant was discarded and the ethanol precipitation step repeated again. The DNA/dye conjugate was then dried and 200 μl of water was added to the pellet. The DNA/dye conjugate was finally purified using preparatory HPLC under the following conditions: Column, C18; flow rate, 1.7 mL/min; mobile phase A, 0.1M Triethylammonium acetate, 4% Acetonitrile, 96% water; mobile phase B, 0.1M Triethylammonium acetate, 80% Acetonitrile, 20% water. The gradient conditions were 90/10 to 55/45 A/B over 5 min, 55/45 to 0/100 A/B over 20 min, hold 0/100 A/B for 5 min. The collected fractions were pooled and taken to dryness using a centrifugal evaporator and stored in the dark at 0°C.

4.2.4 Labeling of Amino Acids with Nphth and Phth Functionalized Dyes. For the derivatization reaction, 500 μL of a 0.1 M solution of the amino acid was dissolved in borate buffer (0.2 M, pH=9.2) and reacted with 100 μL of a 1.2 x 10^{-3} M solution of each dye dissolved in DMF. Each amino acid was conjugated separately at
room temperature overnight. Prior to CE analysis, the amino acid reaction mixtures were pooled and serially diluted to a concentration of $1 \times 10^{-9}$ M.

4.3 Results and Discussion

Several different schemes were considered for preparing our labeling phth and nphth dyes. One issue that predicated the selected scheme was which functionality to use for labeling. In our case, we selected a functional group to target primary amines for applications in DNA analysis. A reactive group like an isothiocyanate or succinimide ester would not survive even the mildest conditions for phth assembly, so the reactive group would have to be incorporated later from a precursor that was stable to the reaction conditions and also easily and quantitatively converted to the reactive group. Isothiocyanates are easily prepared from amines with thiophosgene equivalents, however, attempted condensation of amino phthalonitriles was not successful. Thus, we chose to use the nitro group as an intermediate precursor for the amine.

Sulfonates are the most commonly used water-solubilizing group and they can either be incorporated in the phthalonitrile precursor or added post-synthetically by aromatic sulfonation reactions. In our hands, post-assembly sulfonation of nitro-substituted phth or nphth was unsuccessful due to decomposition of the macrocycle from harsh acidic conditions. Co-assembly of nitro-phthalonitriles and sulfonaphthalonitriles did produce near-IR products, but reduction of the nitro amine was unsuccessful.

Finally, we chose to incorporate Zn into the center of the phth as this is both synthetically convenient and provides highly fluorescent dyes. Some metals like copper, tin, or cobalt substituted in the central core yielded chromophores with low
fluorescence quantum yields in organic solvents. Co-assembly of the nitrophthalonitrile with the carboxynaphthalonitrile provided the most successful synthetic route for preparing our non-symmetrical macrocycle. A possible reasoning for the successful approach may have been that the 6,7-dicyano-2-napthalenecarboxylic acid intermediate was more labile and easily susceptible to reactivity with the nitro group in the condensation reaction because of its lower melting point (< 210°C).

Although the synthetic procedures for the dyes are very simple and straightforward, the purification of the dyes was extremely difficult because of the side products or constitutional isomers produced in the reaction. Previous authors have examined the separation of sulphonated metallophanthocyanine isomers using the mixed condensation and direct sulphonation procedures.25 The HPLC analysis for the direct sulphonation method using fuming sulfuric acid demonstrated poor resolution and numerous background impurities from the harsh acidic conditions used during synthesis. Therefore, the mixed condensation method was chosen as our systematic route by reason of being less problematic and providing better resolution during HPLC separation.

It was important in the condensation synthetic route to choose an appropriate 3:1 stoichiometric ratio of the two phthalonitrile coupling partners. In general, the condensation of two different dinitriles, A and B, with an appropriate metal would produce; nphth or phth containing four A or B units (AAAA or BBBB), three A units and one B unit (AAAB), three B units and one A unit (BBBA), and two A units and two B units (two isomers: ABAB and ABBB). For example, in the solventless reaction of 6,7-dicyano-2-napthalenecarboxylic with nitrophthalonitrile promoted by zinc acetate, the
molar ratio (3:1) of the two reactants is key to producing as much of the desired product containing only one nitro group, however, this was not the case as seen in the chromatograms shown in Figure 4.4 or Figure 4.7. From the HPLC chromatogram, the relative peak areas of the di-nitro and tri-nitro compounds (II, III, and V, VI) were greater than the peak areas of the tetracarboxy and tri-carboxyl compounds (I, II, 2, and 3). This may be apparent because the nitrophthalonitrile precursor has a lower melting point and will be more reactive than the carboxynapthalene precursor during assembly.

To aid in characterization, synthesis of the individual 6,7-dicyano-2-napthalenecarboxylic acid precursor and substituted nitrophthalonitrile precursor were reacted separately with zinc acetate and monitored by UV absorbance (see Figure 4.3). The water-soluble tetra-carboxylate zinc naphthalocyanine compound was determined to have an absorption maximum of 765 nm, which corresponded to the absorption maximum of the first fraction eluting from the HPLC (tᵣ = 5.68 min). The hydrophobic tri-nitro zinc naphthalocyanine compound was determined to have an absorbance maximum of 684 nm, which also corresponded to the fourth eluting component (tᵣ = 18.82 min) shown in the chromatogram Figure 4.4.

The synthetic steps for the water-soluble nphth and phth dyes are outlined in Schemes 4.2-4.7. 6,7-dicyano-2-napthalenecarboxylic acid (3 equiv) or 4-(3,4-dicyanophenoxy) benzoic acid (3 equiv) was reacted under neat conditions with the substituted nitrophthalonitrile (1 equiv) to produce compound 2 or 5. The reaction mixture also produced a mixture of tetra- through mono-nitro macrocycle congeners, which were extensively purified by HPLC. The purified product was brought to dryness under reduced pressure and the isolated water-soluble nitro compound (2 or 5)
was reduced to the amino functional group (3 or 6), by hydrogenation over palladium/charcoal. An original attempt for reduction of the nitro group was examined using Sn(II) chloride in hydrochloric acid. The reaction attempt was unsuccessful due to decomposition of the macrocycle and possible exchange of the zinc metal with tin chloride. For conversion of the amino functional group to the reactive isothiocyanate (4 or 7), 1-1'-thiocarbonyldi-2(1H)-pyridone was used over the preferred thiophosgene because of the higher stability of the compound at room temperature and higher product yields obtained.

After purification by reverse-phase chromatography, the analysis and structural characterization by mass spectrometry (MALDI-MS negative mode) of the substituted derivatives exhibited the characteristic molecular fragmentation pattern of the [M$^+$ - COOH] along the naphthalocyanine macrocycle for compound 2 and the phenoxy carboxy [M$^+$ - C$_6$H$_4$O] groups for compound 5. The infrared spectra with KBr demonstrated very strong lines at 1645 cm$^{-1}$ (C=O), 1525 cm$^{-1}$ (NO$_2$), 1350 cm$^{-1}$ (NO$_2$), However, the infrared spectra of the amino and isothiocyanate groups were severely suppressed making characterization difficult. We believe this maybe attributed to several IR bands arising from the metal and metal-ligand vibrations. The $^1$H NMR spectrum provided little information due to substantial aggregation of the samples in the solvents required for NMR, thereby broadening the signal peaks.

The HPLC chromatograms shown in Figures 4.10 depicts the conjugation reactions of a DNA 17mer oligonucleotide with the naphthalocyanine and phthalocyanine dyes containing the reactive isothiocyanate functional group. Both dyes demonstrate excellent conjugation efficiency toward the highly charged

108
Figure 4.10 Reverse-phase HPLC chromatograms of M17 primer labeled with Naphthalocyanine and Phthalocyanine dye. Column: Reverse Phase-C$_{18}$ 50 x 4.6 mm i.d.; mobile phase: 0.1M TEAA, pH = 7.0 linear gradient 4 to 80 % acetonitrile in 20 min; flow rate: 1.7 mL/min. UV absorbance detection at 254 nm.
oligonucleotide. Also, the addition of water-soluble groups along the chromophore may have increased the conjugation efficiency due to extensive solvation of both the analyte and dye. In the chromatogram, the three components in the naphthalocyanine conjugation mixture (unbound DNA, dye-DNA conjugate, and free dye) were completely separated using the appropriate HPLC conditions. The conjugate and the free dye eluted at 10 and 14 minutes, respectively. In the chromatogram for the phthalocyanine conjugation mixture, it is apparent that the conjugation efficiency for the phthalocyanine dye is slightly better when compared to the naphthalocyanine dye because of the disappearance of both unbound DNA and free dye peaks. This may be due to the increased distance of the water-soluble carboxylate groups away from the macrocycle, which possibly decreased steric or electrostatic interaction between the dye and oligonucleotide. Also, the extinction coefficients for the free dyes at 260 nm were very low compared to 680 and 730 nm, thereby reducing the absorbance signal in the chromatogram, even though excess dye was used in both conjugation reactions.

In Figure 4.11, the absorbance spectra of the naphthalocyanine native dye was found to display two absorption bands at ~ 755 nm and ~ 725 respectively, while the maximim for the dye-primer conjugate was found to be ~ 735 nm. The extinction coefficient (1.82 x 10^5 cm^-1 M^-1) and quantum yield (0.20) for the dye were measured in 70:30 CH$_3$OH/DMF. The split absorbance maxima in the electronic spectra of the zinc naphthalocyanine free dye are indicative of the asymmetrical character of this type of compound. When one of the four naptho groups along the macrocycle are replaced by a benzo group, it would be expected that only one major absorption band be observed.
The presence of a symmetric, single absorption band suggests that both the molecular asymmetry and the substituents exert a synergistic effect on the absorption properties of the macrocycle derivatives seen in Figure 4.3. Instead, we observed two bands in the naphthalocyanine absorption region. This observation is in agreement with the two-fold orbital degeneracy of the excited electronic state of the metallomacrocycle when the group of symmetry changes from $D_{4h}$ (symmetrical zinc naphthalocyanine) to $C_{2v}$ (compound 4).26

There were aggregation effects also observed in the UV absorbance spectra for the native dye when evaluated in 40 mM borate buffer (pH = 9.4). We attribute these additional absorbance peaks to be contributions from dimers, trimers, and other higher order aggregates in the solvent system. However, the dye-primer conjugate spectrum primarily displayed only the monomeric form as is evident from the similarity of the electronic spectra for the conjugate and free dye in CH$_3$OH/DMF.

In the emission profile, the naphthalocyanine native dye maximum was found to be ~ 765 nm while the dye-primer conjugate demonstrated a 15 nm red shift to 780 nm. In Figure 4.12, the absorption maxima for the phthalocyanine dye was found to be ~ 683 nm and the maxima for the dye-primer conjugate was determined to be ~ 694 nm, an 11 nm shift. The extinction coefficient ($1.9 \times 10^5$ cm$^{-1}$M$^{-1}$) and quantum yield (0.26) for the dye were measured in 70:30 CH$_3$OH/DMF. The emission profile for the free dye and dye primer where 692 and 700 nm, respectively. The phthalocyanine dyes have a characteristic lower spectroscopic range compared to the naphthalocyanines because of their modification in structure due to the absence of the benzene annealation along the phthalocyanine macrocycle.
Figure 4.11. Absorbance (A) and emission (B) spectra of naphthalocyanine dye 4 (solid line) in 70:30 Methanol/DMF and dye-primer conjugate (open triangle) in 40 mM borate buffer/30% DMF. Dotted line (—) denotes native dye evaluated in 40mM borate buffer. Dye concentration 2.5 x 10⁻⁶M.
Figure 4.12. Absorbance (A) and emission (B) spectra of phthalocyanine native dye 7 (solid line) in 70:30 Methanol/DMF and dye-primer conjugate (open triangle) evaluated in 40mM borate buffer/30% DMF. Dotted line (---) denotes native dye in 40mM borate buffer. Dye concentration $2.5 \times 10^{-6}$M.
Figure 4.13 shows the LIF electropherogram of the native LIF naphthalocyanine dye evaluated using capillary electrophoresis (reverse polarity) with a running buffer consisting 40 mM borate (pH = 9.4) and 10% methanol. The cationic surfactant [0.5 mM dodecatriethylammonium bromide (DTAB)] was added to reverse the direction of the electroosmotic flow. In general, methanol was added in the running buffer to aid in increasing the net fluorescence signal of the naphthalocyanine dye to aid detection. The migration time for the free dye was determined to be 22.5 minutes with an efficiency of $7.5 \times 10^4$ plates, m$^{-1}$. In order to investigate the labeling efficiencies of the naphthalocyanine dye, two amino acids, aspartic acid and phenylalanine, were conjugated to the naphthalocyanine dye and evaluated using the same electrophoretic conditions used for the free dye (see Figure 4.14). The aspartic acid conjugate migrated first when compared to phenylalanine and free dye, because of the additional negative charge of the carboxylate group on the amino acid residue. The theoretical plate numbers were determined to be $5.14 \times 10^5$ plates, m$^{-1}$ for aspartic acid and $3.1 \times 10^5$ plates, m$^{-1}$ for phenylalanine.

The water-soluble native dyes (4 and 7) conjugated to the universal 17mer sequencing primer were electrophoresed in free solution using UV absorbance detection shown in Figure 4.15. For the nphth and phth dyes, single peaks were observed which migrated through the column in 5.71 and 9.36 minutes, respectively (data not shown). The dye-primer conjugates were also observed to have single peaks and migrated from the column in 17.6 and 23.4 minutes, respectively. The presence of only a single peak in the electropherogram was an indication of the isomeric purity of the labeling dye. The difference in the migration times for the naphthalocyanine and phthalocyanine free
dyes and dye-conjugates were as a result of using a longer column in the latter experiment. In order to investigate the separation of an oligonucleotide in a gel matrix, electrophoresis of the naphthalocyanine labeled primer was evaluated in a 3%T/3%C polyacrylamide gel using UV absorbance detection. The naphthalocyanine labeled primer migrates as a single peak with a migration time of 10.5 minutes.

![Figure 4.13. LIF Electropherogram of Naphthalocyanine Dye.](image)

The separations were performed with a running buffer consisting of 40 mM borate (pH = 9.4) and 10% methanol. The cationic surfactant (0.5 mM dodecatriethylammonium bromide) was added to reverse the direction of the electroosmotic flow. Conditions: 40 cm 75 μm capillary column; 33 cm to the detection window; Field Strength 250 V/cm; Fluorescence detection at 750 nm.
Figure 4.14  LIF Electropherogram of Labeled Amino Acids with Naphthalocyanine Dye. The separations were performed with a running buffer consisting of 40 mM borate (pH = 9.4), 10% methanol, and 0.5 mM dodecatriethylammonium. Conditions: 40 cm 75 μm capillary column; 33 cm to the detection window; Field Strength 250 V/cm; Fluorescence detection at 750 nm.
Figure 4.15. UV Absorbance Electropherogram of Naphthalocyanine and Phthalocyanine Dye-Primer Conjugates. The separations were performed with a running buffer consisting of 40 mM borate (pH = 9.4), 10% methanol, and 0.5 mM dodecatriethylammonium. Conditions: 75 µm capillary column; Field Strength -250 V/cm; UV Absorbance detection at 254 nm.
Figure 4.16  Naphthalocyanine dye-conjugate UV Absorbance electrophorogram performed in a 3%T/3%C polyacrylamide gel. Conditions: 75 µm i.d.; total length of 40 cm and a detection window 33 cm from the injection end. The dye/oligonucleotide conjugate was electrokinetically injected. The detection was performed using UV absorbance monitored at 254 nm.
Time-correlated single-photon counting (TCSPC) measurements were also carried out to assess the fluorescence lifetimes of both the native and dye/primer conjugate. Figure 4.17 shows an exponential decay profile for the naphthalocyanine free dye and labeled primer in DMF along with the instrument response function. In all cases examined, the decays were adequately fit to a single-exponential function. Attempts to fit the data to a double-exponential did not increase the goodness of the fit, as indicated by the value of $\chi^2$. The fluorescent lifetime for the naphthalocyanine free dye was determined to be 2.28 ns with a $\chi^2$ value of 2.95, while the lifetime for the primer was slightly higher, 3.12 ns, with a $\chi^2$ value of 2.45.

![Figure 4.17. Fluorescence Decay Profile for Naphthalocyanine Native Dye and Labeled Primer in DMF and the instrument response function using time-correlated NIR Fluorescence spectrometer.](Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.)
4.4 Conclusions

In summary, we have developed two novel near-IR fluorescent fluorophores, 4 and 7, that demonstrate excellent water solubility and dissolved rapidly in aqueous buffer making them easier to use as labeling reagents. Both dyes showed absorbance and emission spectroscopic properties in the near-IR which aided in demonstrating very low interference making this particular region especially advantageous. The water solubility for the dyes was achieved from the negatively charged carboxylate groups attached along the metallomacrocyle.

4.5 References


24. Li-Cor, Private Communication.


Chapter 5

Unsuccessful Synthetic Approaches for Water-Soluble Macrocycles with a Reactive Functional Group

5.1 Introduction

In this chapter, we will discuss the synthetic methods that were unsuccessful in our approach for the synthesis of water-soluble naphthalocyanines containing a reactive isothiocyanate functional group. In many of the synthetic routes, we were successful in achieving most of the intermediates (ex: non-water-soluble tetranitro-/tetra amino-macrocycle and/or tetra-sulphonated/tetra-carboxylated macrocycle) before the isothiocyanate derivative. However, major problems arose when the two corresponding substituents (nitro vs. SO₃⁻) were reacted together resulting in an unsuccessful completion to the next step (conversion of nitro to amine) or decomposition of the macrocycle. It was believed that many of these substituted dicarbonitriles exhibited variably different reactivities resulting in the inability to prepare the desired product.

5.2 Unsuccessful Approaches

5.2.1 Approach One  In Scheme 5.1, 5-Nitro-2,3-naphthalenedicarbonitrile and 5(6)-Sulfonaphthalic Anhydride in a 3:1 molar ratio were reacted with zinc acetate successfully forming the macrocycle with a λₘₐₓ = 750 nm in DMSO.¹ ³ ⁴ Reduction of the nitro group to the amine using Sn (II) and hydrochloric acid resulted in decomposition of the compound.⁵ The decomposition was confirmed by monitoring the UV absorbance of the reaction mixture. Decomposition may have resulted from increasing the temperature and time of duration for the reaction. Also, it may have been
possible that the Sn (II) metal may have interchanged with the zinc metal in the central core of the macrocycle. Sodium sulfide was chosen as an alternative route for the reduction of the nitro group, however decomposition of the compound resulted as the final product. The pungent odor of sodium sulfide also made it extremely difficult to use as a reducing agent. For future experiments, the hydrogenation/catalyst method may be another alternative route to obtain the amino functional group.

Scheme 5.1. Reaction of 5-nitro-2,3-naphthalenedicarbonitrile and 5(6)-sulfonaphthalic anhydride.

5.2.2 Approach Two 5-nitro-2,3-naphthalenedicarbonitrile and 2,3 naphthalenedicarbonitrile were reacted in the appropriate molar ratio with zinc acetate producing the hydrophobic macrocycle shown in Scheme 5.2. This route was chosen
to use the sulphonation method which describes using fuming sulfuric acid for adding $\text{SO}_3^-$ groups to the peripheral edge of the macrocycle. The harsh acidic conditions used in the sulphonation method were unsuccessful resulting in macrocycle decomposition as monitored by UV absorbance of the reaction mixture. By varying the percentage of fuming sulfuric acid (10%-30%) and temperature ($0^\circ\text{C}-25^\circ\text{C}$), decomposition of the macrocycle was still observed. The nitro functional group was too labile and unstable to withstand the apparent harsh acidic conditions used in the sulphonation process.

Scheme 5.2. 5-nitro-2,3-napthalenedicarbonitrile and 2,3-napthalenedicarbonitrile reacted producing the hydrophobic macrocycle followed by fuming sulfuric acid.
5.2.3. Approach Three  In Scheme 5.3, this synthetic route consisted of reacting 5-nitro-2,3-napthalenedicarbonitrile and 6-diethoxyphosphoryl-2,3-dicyanonapthalene with zinc acetate.\(^{2,3,8,9}\) Complexation of the dinitriles failed in the absence of solvent (probably due to the high melting point of the phosphonate precursor, \(> 260^\circ\) C) or with the use of high boiling solvents, such as sulpholane and quinoline. The use of urea, which normally facilitates complexation of dinitriles, was not used in this reaction because of its possible interaction with the phosphonate moiety. An additional reaction was performed with the phosphonate precursor and zinc acetate to produce the tetra-phosphonate zinc naphthalocyanine, which resulted in decomposition of the macrocycle as found by disappearance of the near-IR absorption band.

![Scheme 5.3](image)

**Scheme 5.3.** The reaction of 5-nitro-2,3-napthalenedicarbonitrile and 6-diethoxyphosphoryl-2,3-dicyanonapthalene with zinc acetate.
5.2.4. Approach Four  As an alternative for preparing water-soluble naphthalocyanines, the lithium alkoxyde-catalyzed statistical condensation method was next attempted.\textsuperscript{10} By treating the naphthalonitrile with a lithium alkoxide, this will induce the cyclic tetramerisation reaction to the macrocycle as the lithiated derivative. An acid is used to convert the macrocycle into the metal free analogue allowing replacement with another metal. In Scheme 5.4, 6,7-dicyano-2-napthalene carboxylic acid and 5-amino-2,3-napthalenedicarbonitrile were reacted with lithium producing a hunter-green solid that absorbed in the near-IR.\textsuperscript{3,11} After removal of the lithium metal with acetic acid, the near-IR absorbance peak disappeared after the addition of zinc acetate proving this route to be unsuccessful. An original attempt was considered using the lithiated macrocycle instead of replacing the metal center. However, luminescent fluorescence measurements indicated that the compound did not fluoresce in organic or aqueous solutions.

\textbf{Scheme 5.4.} The condensation reaction of 6,7-dicyano-2-napthalene carboxylic acid and 5-amino-2,3-napthalenedicarbonitrile with lithium metal.
5.2.5. **Approach Five** A procedure developed by Meerwein *et al.* is another approach used for synthesizing water-soluble naphthalocyanines, and involves converting aromatic amines into sulfonyl chlorides. Their method involved the treatment of the diazonium chloride in concentrated hydrochloric acid with sulfur dioxide and copper (II) ion as a catalyst. The reference from which this approach was taken slightly modified their procedure to accommodate the specific properties of their compounds. In this approach, our design was to convert the tetra-amino zinc naphthalocyanine into a compound that was diazotized at only three amino sites leaving one amine group after protection with a t-boc group. However, this reaction proved unsuccessful by decomposition of the macrocycle from conversion during the diazonium reaction process.

![Chemical structure and reaction scheme](image)

**Scheme 5.6** Reaction of Zinc Naphthalocyanine using the Meerwein Procedure.

127
5.2.6. **Approach Six** In this approach, the condensation reaction of 6,7-carboxy-2,3-dicarbonitrile and 5-nitro-2,3-dicarbonitrile produced the naphthalocyanine macrocycle with an absorbance max of 760 nm in DMSO.\textsuperscript{3,11} The compound was easily isolated from the non-water soluble tetra-nitro naphthalocyanine component based upon the dye’s acidic and basic properties. The problem encountered in this approach involved the reduction using hydrogenation/palladium catalysts. Before the hydrogenation reaction, the compound was converted to its acidic form because of the dye’s greater solubility in dimethylformamide. After several attempts, the characteristic hunter-green color changed to a dark brown during the reaction resulting in decomposition of the macrocycle. One method that we did not attempt which proved to be successful for our original dyes (4 and 7, see Chapter 4) was to dissolve the compound in a basic solution before the hydrogenation reaction.

![Scheme 5.6. The condensation reaction of 6,7-carboxy-2,3-dicarbonitrile and 5-nitro-2,3-dicarbonitrile with zinc acetate.](image)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
5.3 Summary

In summary, it was very important that we consider the failed synthetic approaches used in the final development of the water-soluble naphthalocyanine dye containing a reactive functional group for bioconjugation. If this research is continued, the reader should have a greater understanding and become more versatile in the preparation of these compounds.

5.4 References


(6) Achar, B. F., G.; Parker, J. Polyhedron 1987, 6, 1463-1467.


Chapter 6

Conclusions and Future Work

6.1 Introduction

The main goal of this research has been to synthesize and characterize a novel set of fluorescent tricarbocyanine, phthalocyanine, and naphthalocyanine dye sets which demonstrate absorption and emission properties in the near-infrared region of the electromagnetic spectrum (650-1100 nm). These near-infrared chromophores have been developed as fluorescent tags and labels for different bioanalytical applications using laser-induced fluorescence detection.

In Chapter 1, the theories and principles of fluorescence were discussed with an emphasis on using the Near-Infrared region (650-1100 nm) as an advantage for fluorescence detection compared to visible detection. The motivation for using fluorescence in the NIR includes smaller backgrounds observed during signal collection and the simpler instrumentation required for carrying out detection. In this region, background interference from other impurity molecules can be reduced or even eliminated because very few molecules demonstrate intrinsic fluorescence. Also, the instrumentation required for NIR fluorescence consists of inexpensive diode lasers and avalanche photodiodes. Finally, the goals of my research were outlined including the synthesis and characterization of fluorescent probes for bioanalytical applications.

In Chapter 2, a series of near-IR fluorescent dyes were prepared which contained an intramolecular heavy atom for altering the fluorescence lifetimes to produce a set of probes appropriate for base-calling in a single-lane DNA sequencing
format. The heavy-atom modification consisted of an intramolecular halogen situated on a remote section of the chromophore in order to minimize the perturbation on the lifetimes and fluorescence quantum yields. In addition, the dye series possessed an isothiocyanate functional group to allow facile attachment to sequencing primers. The unconjugated dyes showed similar absorption and emission maxima ($\lambda_{\text{abs}} = 765$-768 nm; $\lambda_{\text{em}} = 794$-798 nm) as well as fluorescence quantum yields that were invariant, within experimental error, to the identity of the heavy atom. However, the lifetimes of these dyes were found to vary with the identity of the halogen substitution (I, $\tau_f = 947$ ps; F, $\tau_f = 843$ ps, measured in methanol), with an average variation within the dye series of 35 ps. The spectroscopic properties of the free dyes and the dyes conjugated to sequencing primers on the 5'-end of the oligonucleotide were determined in a DNA-sequencing matrix. The results indicated slight differences in the fluorescence properties of the free dyes compared to those of the dye/primer conjugates in this formamide matrix. Inspection of the ground-state absorption spectra showed significant aggregation for the free dyes in this solution, but the conjugated dyes exhibited no sign of aggregation due to the highly anionic nature of the oligonucleotide. The fluorescence lifetimes of the dye/primer conjugates demonstrated lifetimes which ranged from 735 to 889 ps, with an average variation of 51 ps, an adequate difference to allow facile discrimination of these dyes in DNA-sequencing. When the dye/primer conjugates were electrophoresed in a cross-linked polyacrylamide gel electrophoresis capillary column, they co-migrated, indicating that in single-lane sequencing applications when utilizing these dyes, no post-run correction would be required to correct for dye-dependent mobility shifts.
In Chapter 3, the near-IR dye thiazole green (TAG) was used as a monomeric nuclear staining dye for the low-level detection of DNA restriction fragments separated via high-performance capillary electrophoresis with near-IR laser-induced fluorescence detection. The absorption maximum and emission maximum where 735/765 nm respectively and in the presence of dsDNAs showed a fluorescence enhancement ratio of approximately 102. The high-resolution separation of the *HaeIII* restriction digest of *ΦX174* was carried out using capillary electrophoresis on the native, ethidium bromide-stained, and TAG-stained DNA fragments. The TAG-stained DNA fragments resulted in higher plate numbers compared to the native and EtBr- stained restriction fragments as well as enhanced resolution. Using a running buffer composed of an entangled polymer and 1 µM TAG with no prestaining of the dsDNA prior to the electrophoresis, the limit of detection was found to be 20 fg (SNR = 3) of DNA per electrophoretic band. In addition, using the LIF system, the 271/281 bp fragments were nearly baseline resolved, with plate numbers exceeding $1 \times 10^6$ plates/m.

In Chapter 4, we described the synthesis and properties of two novel asymmetric phthalocyanine and naphthalocyanine dyes to be used as near-IR fluorescent labels for biological targets. These dyes were synthesized to contain an isothiocyanate functional group, which reacts selectively with amino groups of many biomolecules to form a stable thiourea linkage. One problem with these dyes is their limited solubility in an aqueous environment. Therefore, we attached polar carboxylate groups along the macrocycle which increased its polarity and negative formal charge making the dye more hydrophillic. In order to demonstrate the usefulness of near-IR fluorescence using these dyes, a DNA oligonucleotide and some amino acids were
derivatized with the highly anionic naphthalocyanine and phthalocyanine labeling dyes. The conjugates and amino acids were separated using capillary electrophoresis in a running buffer comprised of methanol and a cationic surfactant added to reverse the electroosmotic flow. The fluorescence signal strength and resolution were found to be improved with the addition of methanol in the running buffer.

In Chapter 5, it was very important that we summarized the failed synthetic approaches used in the development of the water-soluble naphthalocyanine dye containing a reactive functional group for bioconjugation. If this research is continued, the reader should have a greater understanding and become more versatile in the preparation of these compounds.

6.2 Future Directions

For future directions, more work should be focused on the development of additional near-IR DNA intercalators as fluorescent probes for bioanalytical assay. As stated in Chapter 1, increasing the number of positive charges on the intercalator will increase the dyes binding affinity and fluorescence enhancement ratio to double-stranded DNA. One possible idea to incorporate this observation would be to develop a naphthalocyanine dye that contains four positive charges along the periphery of the macrocycle (see Scheme 6.1). This cationic naphthalocyanine dye should have similar properties to the dimeric dyes, which consist of two chromophores covalently linked by a polymethine cationic chain.

The synthetic route would consist of diazotizing 5-amino-2,3-napthalenedicarbonitrile at 0°C converting to 5-iodo-2,3-napthalenedicarbonitrile. The precursor, 5-iodo-2,3-napthalenedicarbonitrile, is reacted with zinc acetate at 250°C.
producing the tetra-iodo zinc naphthalocyanine macrocycle. The tetra-iodo naphthalocyanine macrocycle is then reacted with trimethylamine gas at room temperature to produce the cationic macrocycle.

**Scheme 6.1** Synthesis of cationic naphthalocyanine for DNA intercalation.
NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

135

This reproduction is the best copy available.

UMI
Clyde V. Owens, Jr was born in Gaffney, South Carolina, on September 5, 1972. He is the youngest of two children born to Dr. and Mrs. C.V. Owens, Sr. He attended Nations Ford and James Mason Smith Elementary Schools from 1978-1987. He graduated from Olympic Sr. High School in Charlotte, North Carolina, in 1990. There he was Student Body President, Men's Varsity Basketball Co-Captain and drummer on the high school marching band.

In the fall of 1990, he matriculated into Morehouse College in Atlanta, Georgia. He received a bachelor of science degree in chemistry in the Spring 1994. In the fall of 1994, he enrolled in the graduate program in the Department of Chemistry at Louisiana State University in Baton Rouge, Louisiana. At Louisiana State University, he is currently a candidate for the degree of Philosophy in analytical chemistry. Upon completion of his degree, he plans to begin his career as a post-doctoral Researcher at Oak Ridge National Laboratory in Oak Ridge, Tennessee.
Clyde Vernon Owens

Chemistry

Title of Dissertation: Bioanalytical Applications in the Near-Infrared Region Using Fluorescent Covalent and Non-Covalent Probes With Near-Infrared Fluorescence Detection

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

E. S. Field

Robert M. Hammer

Robert J. Wade

Patrick A. Rimbach

Date of Examination:

February 29, 2000