Fluorescence Off-On Sensors for F-, K+, Fe3+, and Ca2+ Ions

2014

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FLUORESCENCE OFF-ON SENSORS FOR F⁻, K⁺, Fe³⁺, AND Ca²⁺ IONS

by

BINGLIN SUI
M.S. Southwest University, China, 2009

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

Fall Term
2014

Major Professor: Kevin D. Belfield
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ABSTRACT

Fluorescence spectroscopy has been considered to be one of the most important research techniques in modern analytical chemistry, biochemistry, and biophysics. At present, fluorescence is a dominant methodology widely used in a great number of research domains, including biotechnology, medical diagnostics, genetic analysis, DNA sequencing, flow cytometry, and forensic analysis, to name just a few. In the past decade, with the rapid development of fluorescence microscopy, there has been a considerable growth in applying fluorescence technique to cellular imaging. The distinguished merits of fluorescence techniques, such as high sensitivity, non-invasiveness, low cytotoxicity, low cost, and convenience, make it a promising tool to replace radioactive tracers for most biochemical measurements, avoiding the high expense and difficulties of handling radioactive tracers.

Among the wide range of applications of fluorescence technique, fluorescent sensing of various cations and anions is one of the most important and active areas. This dissertation is all about developing fluorescent sensors for physiologically significant ions, including F⁻, K⁺, Fe³⁺, and Ca²⁺. All of these sensors demonstrate fluorescence “turn-on” response upon interacting with their respective ions, which makes them much more appealing than those based on fluorescence quenching mechanisms.

In Chapter II, a novel highly selective fluorescence turn-on F⁻ sensor (FS), comprised of a fluorene platform serving as the chromophore, and two 1,2,3-triazolium
groups functioning as the signaling moieties, is described. The function of FS is established on the basis of deprotonation of the C-H bonds of 1,2,3-triazolium groups, which makes FS the first reported anion sensor based on the deprotonation of a C-H bond. Easy-to-prepare test strips were prepared for determining F⁻ in aqueous media, providing an inexpensive and convenient approach to estimate whether the concentration of F⁻ contained in drinking water is at a safe level.

Chapter III contains an optimized synthesis of a reported K⁺-selective group (TAC), and the development of two TAC-based fluorescence turn-on K⁺ sensors (KS1 and KS2). The synthetic route of TAC is shortened and its overall yield is enhanced from 3.6% to 19.5%. Both KS1 and KS2 exhibited excellent selectivity toward K⁺ over other physiological metal cations, high sensitivity for K⁺ sensing, and pH insensitivity in the physiological pH range. Confocal fluorescence microscopy experiments demonstrate that they are capable of sensing K⁺ within living cells. 2PA determination reveals that KS2 has a desirable 2PA cross section of 500 GM at 940 nm, which makes it a two-photon red-emitting fluorescent sensor for K⁺.

Chapter IV describes the development of a novel BODIPY-based fluorescence turn-on Fe³⁺ sensor (FeS). FeS is a conjugate of two moieties, a BODIPY platform serving as the fluorophore and a 1,10-diaza-18-crown-6 based cryptand acting as the Fe³⁺ recognition moiety. FeS displays good selectivity, high sensitivity, reversibility, and pH insensitivity toward Fe³⁺ sensing. Based on its excellent performance in determining Fe³⁺ and very low cytotoxicity, FeS was effectively applied to sensing Fe³⁺ in living cells.
In Chapter V, a new BODIPY-based fluorescence turn-on sensor (CaS) was designed and synthesized for selectively and sensitively determining Ca\(^{2+}\). CaS is comprised of two moieties, a BODIPY fluorophore and a Ca\(^{2+}\) complexing unit. CaS demonstrated selective fluorescence turn-on response towards Ca\(^{2+}\) over other biological metal cations. Moreover, CaS exhibited desirable sensitivity for Ca\(^{2+}\) detection, which makes it more suitable for extracellular Ca\(^{2+}\) determination. In addition, CaS was insensitive to the pH of the physiological environment, especially in the pH range of blood and serum. Therefore, CaS has potential to be applied to sensing Ca\(^{2+}\) ions in extracellular environments.

Chapter VI discusses potential future work of KS2 and CaS, following the results achieved in this dissertation. Based on the desirable performances of both sensors in sensing their respective ions, future work could largely be focused on their applications in cellular imaging.
To my parents
ACKNOWLEDGMENTS

First and foremost, I must give my sincerest thanks to my advisor, Dr. Kevin D. Belfield, without whom this dissertation would never have been possible. It was Dr. Belfield who opened the door and paved the way for me to achieve all the fruits during my PhD study. Today, I can still clearly remember the first conversation we had in his office on Aug. 15th, 2011. His words motivated me to try my best every minute in the past three years. They will keep motivating me in my future life. In the process of my research, there was full of Dr. Belfield’s enlightenment, guidance, support, encouragement, patience, and enthusiasm. For me, Dr. Belfield is not only a PhD advisor but also a career mentor.

I thank Dr. Aniket Bhattacharya, Dr. D. Howard Miles, Dr. Shengli Zou, and Dr. Andrew Frazer for being my committee members.

I also thank all the members of our research group for the friendly laboratory atmosphere they created, which made us a big harmonious family. Special thanks to Bosung Kim, Xiling Yue, and Simon Tang for the collaboration and help.
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<th>Definition</th>
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<tbody>
<tr>
<td>$^{1}$H</td>
<td>Hydrogen 1 isotope</td>
</tr>
<tr>
<td>1PA</td>
<td>One-photon absorption</td>
</tr>
<tr>
<td>2PA</td>
<td>Two-photon absorption</td>
</tr>
<tr>
<td>2PFM</td>
<td>Two-photon fluorescence microscopy</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>Carbon 13 isotope</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom ($10^{-10}$ m)</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AcO$^{-}$</td>
<td>Acetate ion</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>Ar</td>
<td>Argon</td>
</tr>
<tr>
<td>atm.</td>
<td>Atmospheric pressure</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-Bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BF$_4^{-}$</td>
<td>Tetrafluoroborate anion</td>
</tr>
</tbody>
</table>
BH$_3$/THF  Borane tetrahydrofuran complex
BODIPY  4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene
BrCH$_2$CH$_2$Br  1,2-Dibromoethane
$n$-Bu$_4$N$^+$  Tetrabutylammonium ion
$n$-BuLi  $n$-Butyllithium
t-BuOK  Potassium tert-butoxide
t-BuONa  Sodium tert-butoxide
CaCO$_3$  Calcium carbonate
calcd  Calculated
CaS  Calcium sensor
CD$_2$Cl$_2$  Deuterated dichloromethane
CDCl$_3$  Deuterated chloroform
CH$_2$Cl$_2$  Dichloromethane
CH$_3$CN  Acetonitrile
Cs$_2$CO$_3$  Cesium carbonate
CuAAC  Copper(I) catalyzed azide alkyne Huisgen cycloaddition
CuI  Copper(I) iodide
d  Days or doublet
DART  Direct analysis in real time
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>dd</td>
<td>Doublet-doublet</td>
</tr>
<tr>
<td>DDQ</td>
<td>2,3-Dichloro-5,6-dicyano-1,4-benzoquinone</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO-δ6</td>
<td>Deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DODC</td>
<td>3,6-Dioxaoctanedioic acid dichloride</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>dppf</td>
<td>1,1’-Bis(diphenylphosphino)-ferrocene</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Em</td>
<td>Emission</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental protection agency</td>
</tr>
<tr>
<td>equiv.</td>
<td>Equivalent</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Ex</td>
<td>Excitation</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>Iron(III) chloride hexahydrate</td>
</tr>
<tr>
<td>FeCl₃/C</td>
<td>A mixture of FeCl₃ and activated carbon</td>
</tr>
<tr>
<td>FeS</td>
<td>Iron sensor</td>
</tr>
<tr>
<td>FHF⁻</td>
<td>Bi-fluoride ion</td>
</tr>
<tr>
<td>FI</td>
<td>Fluorescence intensity</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FS</td>
<td>Fluoride sensor</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GM</td>
<td>Goppert-Mayer unit for the 2PA cross section (1 \times 10^{-50} \text{ cm}^4 \text{ s photon}^{-1} \text{-molecule}^{-1})</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H₂NNH₂·H₂O</td>
<td>Hydrazine monohydrate</td>
</tr>
<tr>
<td>HCT-116 cells</td>
<td>Epithelial colorectal carcinoma cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
</tr>
<tr>
<td>HR-MS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>ICT</td>
<td>Internal charge transfer</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>K$_2$CO$_3$</td>
<td>Potassium carbonate</td>
</tr>
<tr>
<td>KF</td>
<td>Potassium fluoride</td>
</tr>
<tr>
<td>KI</td>
<td>Potassium iodide</td>
</tr>
<tr>
<td>KS1</td>
<td>Potassium sensor 1</td>
</tr>
<tr>
<td>KS2</td>
<td>Potassium sensor 2</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Lit.</td>
<td>Literature</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
</tr>
<tr>
<td>M$^{-1}$</td>
<td>L/mol (liter per mole)</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>desorption/ionization</td>
<td></td>
</tr>
<tr>
<td>MCL</td>
<td>Maximum contaminant level</td>
</tr>
<tr>
<td>Me₃O·BF₄</td>
<td>Trimethyloxonium</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega Hertz</td>
</tr>
<tr>
<td>μM</td>
<td>Micromole per liter (10⁻⁶ moles per liter)</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter (10⁻³ L)</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mm²</td>
<td>Square millimeters</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimoles (10⁻³ moles)</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrum</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaH</td>
<td>Sodium hydride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NaI</td>
<td>Sodium iodide</td>
</tr>
<tr>
<td>NaN₃</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NH₄OAc</td>
<td>Ammonium acetate</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>NIS</td>
<td>N-iodosuccinimide</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer (10⁻⁹ m)</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBFI</td>
<td>Potassium-binding benzofuran isophthalate</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium on carbon</td>
</tr>
<tr>
<td>Pd(dba)₂</td>
<td>Bis(dibenzylideneacetone) palladium(0)</td>
</tr>
<tr>
<td>Pd(PPh₃)₂Cl₂</td>
<td>Bis(triphenylphosphine) palladium(II) dichloride</td>
</tr>
<tr>
<td>Pd(PPh₃)₄</td>
<td>Tetrakis(triphenylphosphine) palladium(0)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Photo-induced electron transfer</td>
</tr>
<tr>
<td>POCl₃</td>
<td>Phosphorus(V) oxychloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>$R^2$</td>
<td>R-squared value</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>s</td>
<td>Seconds or singlet</td>
</tr>
<tr>
<td>SOCl$_2$</td>
<td>Thionyl chloride</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>TAC</td>
<td>[2.2.3]-Triazacyclonane</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>TPEN</td>
<td>N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine</td>
</tr>
<tr>
<td>TsCl</td>
<td>$p$-Toluene sulfonyl chloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>vis</td>
<td>Visible</td>
</tr>
<tr>
<td>wt %</td>
<td>Weight percentage</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>δ</td>
<td>ppm or 2PA cross section</td>
</tr>
<tr>
<td>ε</td>
<td>Molar absorption coefficient</td>
</tr>
<tr>
<td>(\lambda_{\text{ex}})</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>(\lambda_{\text{max}})</td>
<td>Wavelength of maximum absorption/emission</td>
</tr>
</tbody>
</table>
CHAPTER I: BACKGROUND AND INTRODUCTION

I.1 Introduction to Fluorescence

As early as in 1565, the observation of fluorescence from a liquid solution was reported, in this instance by Nicolás Monardes, a Spanish physician and botanist, who found this phenomenon as a blue tinge of the infusion of a wood from Mexico and used it to attempt to treat kidney and urinary diseases, known as *Lignum nephriticum* (Latin for “kidney wood”).\(^1\)\(^-\)\(^4\) In the ensuing centuries, the unusual optical properties of the wood caught the interest of Kircher, Grimaldi, Boyle, Newton, Herschel, and many other scientists.\(^1\)\(^-\)\(^2\) Edward D. Clarke in 1819 and René Just Haüy in 1822 observed fluorescence in fluorites. David Brewster described the same phenomenon for chlorophyll in 1833. John Frederick William Herschel reported the first observation of fluorescence from a quinine solution in sunlight in 1845.\(^5\)\(^-\)\(^6\) In 1852, it was George Gabriel Stokes who first introduced the term “fluorescence” in his report and correctly identified fluorescence as an emission process.\(^7\) Therefore, this work marked a milestone in fluorescence research. In the first lines of the paper, Stokes mentioned that his research was motivated by Herschel’s previous report about the quinine solution.

In 1935, Alexander Jablonski illustrated the processes that occur between the absorption and emission of light using a diagram, now known as a Jablonski diagram (Figure I-1) named after Alexander Jablonski.\(^8\) Alexander Jablonski is regarded as the father of fluorescence spectroscopy because of his great accomplishments in this field. Jablonski diagrams provide a theoretical basis for the development of fluorescence and
are often used as the starting point for discussing light absorption and emission. As the mechanism of fluorescence was widely understood, fluorescence received more and more attention and started to be explored as a technique.

![Jablonski diagram](image)

**Figure I-1.** Jablonski diagram.

In the past three decades, there has been a dramatic growth in the application of fluorescence in the field of biological sciences. Fluorescence spectroscopy is considered to be one of the most important research tools in biochemistry and biophysics. At present, fluorescence is a dominant methodology widely used in a great number of research domains, including biotechnology, medical diagnostics, genetic analysis, DNA sequencing, flow cytometry, and forensic analysis, to name just a few. Due to the high sensitivity of fluorescence detection, there has been a remarkable growth in the use of fluorescence for cellular imaging, which renders fluorescence techniques promising tools to replace radioactive tracers for most biochemical measurements, avoiding the high expense and difficulties of handling radioactive tracers. Moreover, fluorescence imaging can also effectively reveal the localization of intracellular molecules.
During the past 20 years, two-photon excitation techniques have emerged and grown rapidly. Usually, fluorescence is generated by fluorophores absorbing a single photon with a wavelength within its conventional or linear absorption band. Pulsed lasers with femtosecond pulse widths are capable of exciting fluorophores through a two-photon absorption (2PA) process. 2PA is a simultaneous absorption of two photons of identical or different frequencies in order to excite a molecule from one state (usually the ground state) to a higher energy electronic state. The energy difference between the involved lower and upper states of the molecule is equal to the sum of the energies of the two photons. When the laser intensity is high enough, a fluorophore can simultaneously absorb two long-wavelength photons to reach the first singlet state (Figure I-2). This process occurs only at the focal point of the laser beam. Such lasers have become convenient to use and integrated with microscopes. Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging of living tissues at a very high depth, which can be up to two mm. Two-photon excitation microscopy can be a superior alternative to confocal microscopy due to its efficient light detection, reduced phototoxicity, and deeper tissue penetration.

Figure I-2. Simplified Jablonski diagram for two-photon excitation.
I.2 Fluorescence Sensing

Fluorescence sensing of chemical and biological analytes is an active research field.\textsuperscript{11-15} The efforts devoted on this subject were initially driven by the desire to reduce the use of radioactive tracers. Nowadays, the high sensitivity of fluorescence sensing techniques is another important reason for attracting so much attention. Fluorescence sensing also meets the need for rapid and low-cost determination methods for a wide range of chemical, biochemical, clinical, and environmental processes.

Fluorescence sensing requires a change in a spectral property, such as fluorescence intensity, emission spectrum, excitation spectrum, fluorescence lifetime, or anisotropy, as a response to an analyte. The most popular fluorescence sensing approach is the fluorescence intensity based strategy, that is, fluorescence intensity of the probe changes in response to an analyte. A great number of this type of sensors have been developed for pH, cations, anions, DNA, RNA, ATP, enzymes, amino acids, glucose, etc.\textsuperscript{16-22}

Typically, as illustrated in Scheme I-1, fluorescent probes are comprised of three different moieties: (1) a recognition moiety, responsible for the selective reaction with analyte; (2) a signaling unit, transducing the interaction between the recognition moiety and the analyte into fluorescence signal change; and (3) a suitable linker that connects the two former moieties.\textsuperscript{16,22-23} There are generally four types of reaction mechanisms between fluorescent probes and the corresponding analytes: (1) complexation; (2)
formation or cleavage of a covalent bond; (3) redox reaction; and (4) protonation-deprotonation.\textsuperscript{22}

Scheme I-1. Schematic illustration of the conceptual mechanism and components of fluorescent sensors.

I.3 Metal Cation Sensing

Extensive efforts have been devoted to the development of fluorescent probes for various metal cations. The research on metal cation sensing can be traced back to the discovery of crown ethers and their ability to form complexes with metal cations.\textsuperscript{24-26} After Pedersen first reported the cation-complexing property of crown ethers in 1967, a large amount of subsequent work was performed to create more complex structures to bind a variety of metal cations.\textsuperscript{27-33} Among various metal cations, alkali and alkaline earth metal cations, especially Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}, and Ca\textsuperscript{2+}, attracted most of the public attention because of their well-known biological significance.\textsuperscript{27-28,30-63} With the discovery of the important roles that transition metal cations play in a myriad of biological and environmental processes, more and more attention began to be focused on developing
sensors for transition metal cations, such as Cr$^{3+}$, Fe$^{3+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Pd$^{2+}$, Ag$^{+}$, Cd$^{2+}$, Hg$^{2+}$, and Pt$^{2+}$. Complexation is the most widely used strategy to develop fluorescent sensors for various metal cations by virtue of their strong binding affinities with electronegative heteroatoms such as N, O, S. There are some general principles for probes based on this strategy: (1) matchable ring/cavity size for a given metal cation, e.g., crown ethers with different ring sizes bind different alkali metal cations; (2) suitable ligands forming five- or six-membered ring complexes with metal cations, such as probes with an EGTA (ethylene glycol tetra-acetic acid) unit complexing Ca$^{2+}$; and (3) soft−hard acid−base principle, for instance, soft sulfur-containing receptors exhibit high affinities for soft metal cations such as Ag$^+$ and Hg$^{2+}$.

For transducing the complexation reaction into a fluorescence signal change, a photophysical process is necessarily involved. Fluorescence resonance energy transfer (FRET), internal charge transfer (ICT), or photo-induced electron transfer (PET) is generally incorporated to translate cation recognition into a spectroscopic signal.

FRET is a mechanism describing energy transfer between a pair of different fluorophores, a fluorescence donor and a fluorescence acceptor. In the FRET process, the donor chromophore in its excited state transfers its energy to a nearby ground state fluorescence acceptor through a non-radiative dipole-dipole coupling process. Then the excited acceptor fluorophore relaxes back to its ground state, emitting fluorescence. FRET is mainly determined by four factors: (1) the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor; (2) the
distance between the fluorescence donor and the acceptor; (3) the relative orientation of the donor and acceptor transition dipoles; and (4) the quantum yield of the donor. Scheme I-2 shows a FRET-based fluorescent probe (1) for Zn$^{2+}$, which is a combination of fluorescein acting as the fluorescence donor and rhodamine as the acceptor. The emission spectrum of fluorescein overlaps well with the absorption spectrum of rhodamine. In compound 1, the rhodamine unit is in a spirolactam form that displays almost no absorption in the visible region. Consequently, no FRET occurs between the fluorescein and the rodamine moieties. When 1 is excited at 485 nm (the maximum absorption wavelength of fluorescein), only green fluorescence emission at 518 nm can be observed, which is a characteristic of fluorescein. However, in the presence of Zn$^{2+}$, the rodamine moiety is turned into the ring-opened form, which exhibits an absorption peak at 560 nm. As a result, the FRET process occurs between the fluorescein and the rodamine moieties, and, thus, fluorescence emission at 590 nm emerges. It should be noted that the ring opening of rhodamine has proven to be an efficient way for developing various fluorescence off–on sensors for metal cations.

Scheme I-2. Response mechanism of a FRET probe (1) for Zn$^{2+}$. Ref 21, copyright 2013 American Chemical Society.
In probes based on the ICT process, an electron-donating group, which in most cases is an amino group, in the analyte recognition moiety is linked directly to a fluorophore, which acts as an electron-accepting unit. The lone pair electrons of the electron-donating group are in conjugation with the fluorophore. In such a “push-pull” case, excitation by light brings about a serious redistribution of electron density of the probe, i.e., ICT process from the electron donor to the fluorophore occurs.\textsuperscript{208-209} When binding with metal cations, both the energy gap between LUMO and HOMO of the molecule and the efficiency of ICT are changed, resulting in absorption and emission intensity changes as well as spectral shifts. Scheme I-3 shows a typical example of an ICT-based probe, Fura 2 (2).\textsuperscript{210-211} Upon complexing with Ca\textsuperscript{2+}, Fura 2 exhibits a remarkable blue-shifted fluorescence and increased fluorescence intensity.

**Scheme I-3.** Response mechanism of an ICT probe (2) for Ca\textsuperscript{2+}. Ref 21, copyright 2013 American Chemical Society.
In PET probes, the analyte recognition moiety (the receptor) is often connected to a fluorophore via a spacer, rendering the receptor and the fluorophore unconjugated, which essentially differs from ICT-based probes. The electronegative atom, usually a nitrogen atom, contained in the receptor moiety has high-energy lone pair electrons, which can transfer an electron to the fluorophore in the excited state, resulting in fluorescence quenching to the fluorophore. Upon complexing metal cations, the reduction potential of the receptor is enhanced and, thus, the HOMO of the receptor becomes lower in energy than that of the fluorophore. As a result, the PET process from the receptor to the fluorophore is restricted, and consequently fluorescence quenching of the fluorophore is reduced, leading to increased fluorescence intensity of the fluorophore.\textsuperscript{16,212-213} Therefore, the PET mechanism has potential to be explored to develop fluorescence “turn-on” sensors. Scheme I-4 depicts a typical example of a PET probe, FluoZin-3 (3).\textsuperscript{214} In the absence of Zn\textsuperscript{2+}, FluoZin-3 emits rather weak fluorescence because of the PET process from the receptor moiety to the fluorescein fluorophore. However, upon binding Zn\textsuperscript{2+} ions, fluorescence of the fluorophore can be considerably increased by prohibiting the PET process. FluoZin-3 has been applied to selective imaging of Zn\textsuperscript{2+} in living cells.\textsuperscript{214-215} Due to the advantages of fluorescence “turn-on” sensors in cell imaging applications, the PET mechanism has been the most frequently applied one and is attracting more and more attention in developing various fluorogenic probes for metal cations.
I.4 Anion Sensing

Anion sensing chemistry can be traced back to the late 1960s, around almost the same time when the preparation and cation coordination studies of crown ethers were first reported by Pedersen.\textsuperscript{24} In the 1970s, cation coordination chemistry received a great deal of attention and cation recognition research began to attract considerable interest. As a result, both the academic study and industrial applications of cation sensing have been well developed. By contrast, little attention had been paid to anion recognition chemistry until the beginning of 1990s.\textsuperscript{216-217} In the last twenty years, substantial strides have been made in the field of anion recognition and detection. The rapid growth of this subject is attributed to the recognized significance of anions, which was previously underappreciated. Anions are ubiquitous and have been proven to play manifold and indispensable roles in a great number of chemical, biological, and
environmental processes. For example, it is well known that DNA and ATP are both polyanions; and the over use of phosphate compounds leads to the eutrophication of rivers.

The design of anion receptors is much more challenging compared with that of cation receptors, which is an important reason for the tardy development of anion detection. Anions have lower charge density (charge to ionic radius ratio) because of their larger sizes in comparison to their isoelectronic cations (Table I-1). Consequently, electrostatic binding interaction between anions and their respective receptors is much less effective than it would be for smaller isoelectronic cations. In addition, numerous anions are sensitive to pH, being protonated by H\(^+\) and, therefore, losing the negative charge. Thus, the probes must have the ability to perform the sensing function within the pH range of their target anion. Moreover, solvents also play a significant role in anion recognition. Anions are solvated in solution and strong hydrogen bonds are formed between anions and hydroxylic solvents. Therefore, a useful anion receptor must compete efficiently with the solvation of the target anion and/or hydrogen bonds. For instance, a neutral anion receptor based solely on ion-dipole interactions can only bind anions in aprotic solvents, whereas charged receptors, which have stronger interactions with anions, may be able to recognize highly solvated, even hydrated anions in protic solvents.
Table I-1. Radii of isoelectronic anions and cations.

<table>
<thead>
<tr>
<th>Cation</th>
<th>$r$ [Å]</th>
<th>Anion</th>
<th>$r$ [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>1.16</td>
<td>F$^-$</td>
<td>1.19</td>
</tr>
<tr>
<td>K$^+$</td>
<td>1.52</td>
<td>Cl$^-$</td>
<td>1.67</td>
</tr>
<tr>
<td>Rb$^+$</td>
<td>1.66</td>
<td>Br$^-$</td>
<td>1.82</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>1.81</td>
<td>I$^-$</td>
<td>2.06</td>
</tr>
</tbody>
</table>

I.4.1 Anion recognition

Based on the different mechanisms of interactions between anions and their receptors, these interactions can be divided into several categories, including electrostatic interactions, complexation with metal ions, hydrogen binding, and combinations of some of these interactions working together. Polyamines are the first anion receptors produced to recognize anions through electrostatic interactions between anions and positively charged receptors. About 40 years ago, Schmidtchen prepared macrotricyclic quaternary ammonium compounds 4 and 5. Receptor 4, which has an internal diameter of 4.6 Å, was found to be able to hold iodide ion (diameter: 4.12 Å), while larger anions such as p-nitrophenolate can be encapsulated within the macrotricycle of receptor 5 due to the larger cavity size of 5. It is necessary to point out that receptors on the basis of electrostatic interactions generally suffer from low selectivity for a specific anion due to the similar binding affinities of a receptor for its analyte and other anions with similar structures.
Another type of anion receptor is based on the interaction between anions and organometallic compounds. Electron-deficient metal ion centers can bind to anions through bonding interactions caused by orbital overlaps. By exploiting this strategy, macrocyclic anion receptors containing metal atoms such as mercury, germanium, and tin have been produced. Among these metals, mercury is an especially advantageous candidate for use in the construction of artificial anion hosts because it is able to form remarkably stable carbon-metal bonds extending collinearly from the mercury atom. Compound 6, a 10-membered pentamercuramacrocycle, is one of the most elegant examples of mercury-based anion receptors. It can bind halide ions, forming a flat plate-like macro-ring.

Hydrogen bonding is another dependable approach for anion recognition. Compared with other approaches, hydrogen bonding has an important advantage. It allows for designing anion receptors with particular shapes that can potentially differentiate between anions with diverse geometries because hydrogen bonds are directional. Pascal reported the first anion receptor via hydrogen bonding in 1986.
Compound 7 is a purely amide-based receptor that shows a capacity of binding fluoride in DMSO. Subsequently, various N-H-containing structures have been exploited as good hydrogen bond donors for constructing receptors for anions, including amide, urea, thiourea, pyrrole, and indole.\textsuperscript{223-224,243-247} Compound 8 is capable of forming a complex with fluoride with a stability constant of 17200 M\(^{-1}\) in CH\(_2\)Cl\(_2\)\textsuperscript{248} and compounds 9 and 10 are able to bind very strongly with H\(_2\)PO\(_4^−\) ion in DMSO.\textsuperscript{249}

**Scheme I-6.** Structures of anion binding compounds 8, 9, and 10.

![Diagram of compounds 8, 9, and 10]

**I.4.2 Anion sensors**

As the development of anion receptors has been growing rapidly, considerable attention has recently been focused on developing anion sensors with the goal of improving chemical sensor technology. These sensors are designed to have the capability of selectively recognizing certain anions and sensing the anion recognition event by providing a macroscopic optical or electrochemical response.\textsuperscript{250-252} As schematically shown in Figure I-3, an effective and widely adopted approach for designing anion sensors is to couple an anion receptor to certain groups which have the ability to signal the anion binding process. The role that the signaling units play in the
process is a transducer that transduces an anion recognition event into an electrochemical or optical signal.

Electrochemical anion detection is a well-studied approach among all kinds of anion sensing techniques. Three main strategies have proven effective for electrochemically sensing anions: 1) potentiometric anion sensors (extracting an anion into a membrane through a non-electroactive anion receptor and determining the resulting potential of the membrane); 2) voltammetric/amperometric anion sensors (detecting the potential/current perturbation of a redox-active anion receptor upon anion binding); 3) chemically modified electrodes (attaching an anion receptor to an electrode through chemical reactions or electro-polymerization). For example, both compound 11 and compound 12 are electrochemical anion sensors based on Cp₂Co/Cp₂Co redox couple. Compound 11 is a good electrochemical sensor for AcO⁻ ion, whereas compound 12 is capable of sensing H₂PO₄⁻ ion much better than AcO⁻ ion.

**Scheme I-7.** Structures of compounds 11 and 12.
In addition to electrochemical anion sensing, optical anion sensing is another technique for anion detection. Optical anion sensors are compounds that are capable of exhibiting changes in either fluorescence or color in the presence of certain anions. Anion sensors displaying color changes are termed colorimetric anion sensors and those showing changes in fluorescence are named fluorescent anion sensors.

Using color changes as a signaling approach has been widely adopted since it does not require the use of a specific instrument as color changes can be directly observed by the naked eye. In this aspect, colorimetric sensors have apparent advantages over other types of molecular sensors. As a matter of fact, there are many examples of colorimetric sensors for anions, especially for fluoride ions.

It was reported by Sessler and co-workers that 2,3-dipyrrrol-2'-ylquinoxalines, such as compound 13, can be explored as a class of anion sensors that are able to detect fluoride ions in CH$_2$Cl$_2$ and DMSO.$^{256}$ In fact, compound 13 demonstrated a remarkable color variation from clear yellow ($\lambda_{\text{max}} = 455$ nm) to purple ($\lambda_{\text{max}} = 560$ nm) on addition of fluoride ions, which was in agreement with the large binding stability constant found between compound 13 and fluoride ions, whereas other anions did not provide any noteworthy color change.
The other branch of optical anion sensors is fluorescent anion sensors, which signal the anion recognition event by changing fluorescence properties. Compared with other types of chemical sensors, fluorescent sensors are advantageous for their high detection limit and high sensitivity, which has stimulated a great amount of interest in covalently attaching fluorophores in proximity to guest-recognition groups. Generally speaking, there are two modes of fluorescent sensors, fluorescence quenching ("on-off") mode and fluorescence enhancing ("off-on") mode. For fluorescent quenching sensors, the lack of high signal outputs seriously limit their application. Compound 14 was reported to be a good fluorescent sensor for phosphate ions. It provides multiple hydrogen bonding sites to generate a pseudo-tetrahedron cleft which can hold phosphate by forming a 1:1 complex. In comparison with other anions (F-, Cl-, Br-, SCN-, AcO-, NO₃-, ClO₄-, and HSO₄⁻), phosphate ions can be recognized by compound 14 in a highly selective manner. Upon the addition of phosphate ions, the fluorescent emission of compound 14 shifts from \( \lambda_{\text{max}} = 477 \) nm to \( \lambda_{\text{max}} = 377 \) nm.

Scheme I-8. Structures of compounds 13 and 14.
I.5 Click Chemistry

Click chemistry is a term describing chemical synthesis tailored to quickly and reliably generate substances by joining small units together. The term was first coined in 1998 and fully described in 2001 by K. Barry Sharpless. Within the click chemistry concept, one of the most popular reactions is the copper(I) catalyzed azide alkyne Huisgen cycloaddition (CuAAC) at room temperature, which was discovered independently and concurrently by Morten Meldal, Valery V. Fokin and K. Barry Sharpless. Scheme I-9 shows the reaction scheme of CuAAC reaction proposed by Valery V. Fokin and K. Barry Sharpless.


I.5.1 Application of click chemistry in anion recognition

With the emergence and fast development of the Cu(I)-catalyzed 1,3-dipolar cycloaddition of a terminal alkyne and an azide forming a 1,4-disubstituted 1,2,3-triazole ring, its application has been rapidly growing, particularly in the fields of synthetic,
medicinal, biological, and materials chemistry.\textsuperscript{263-270} After alkylation, the 1,2,3-triazole ring is transformed into a 1,2,3-triazolium group. The unique properties of the 1,2,3-triazole and 1,2,3-triazolium groups in terms of their abilities to participate in hydrogen bonding with anions has made click chemistry even more appealing. Compared with that of a 1,2,3-triazole ring, the hydrogen of a 1,2,3-triazolium group is much more acidic, and, consequently, has higher hydrogen bond donating ability due to the positive charge introduced in the process of the alkylation reaction. Compound 15 is a 1,2,3-triazolium-based anion receptor that shows remarkable ability to recognize H\textsubscript{2}PO\textsubscript{4} ions through hydrogen bonding interactions.\textsuperscript{271}

\textbf{Scheme I-10.} The structure of compound 15.
CHAPTER II: A FLUORESCENCE TURN-ON SENSOR FOR F⁻


II.1 Introduction

Among biologically important anions, fluoride ion has caught particular attention because of its duplicitous nature. On one hand, fluoride has beneficial effects in dental health and treatment for osteoporosis.²⁷²-²⁷⁶ For this reason, fluoride anion salts are commonly used in toothpaste, water fluoridation, topical and systemic fluoride therapy for preventing and reducing tooth decay, and various products associated with oral hygiene. The fluoridation of water is considered as "one of 10 great public health achievements of the 20th century" by the U.S. Centers for Disease Control and Prevention. On the other hand, pathological studies show that excessive fluoride intake can trigger adverse effects, such as dental and skeletal fluorosis,²⁷⁷ gastric and kidney disorders, and even cancers.²⁷⁸-²⁷⁹ The toxicity of fluoride ions involves the combination of fluoride with the calcium ions in blood, which are indispensable for the function of the nervous system, to form insoluble calcium fluoride, giving rise to hypocalcemia. Moreover, cellular studies show that excessive fluoride uptake can result in cell growth inhibition and even cell apoptosis.²⁸⁰ Therefore, the U.S. Environmental Protection Agency gives standards that an enforceable MCL (maximum contaminant level) for
fluoride in drinking water is 4.0 ppm to prevent osteofluorosis, and a non-enforceable secondary fluoride MCL is 2.0 ppm to protect against dental and skeletal fluorosis.

Owing to its significance for human health, the quantitative determination of the levels of fluoride is of growing interest. Ion chromatography and ion-selective electrode have been generally used in quantitative analysis of fluoride. However, these approaches involve disadvantages, for example, relatively complicated procedures and cumbersome equipment, high costs, extending operating time, etc. Therefore, optical sensors, especially fluorescent sensors, which are potential to be highly selective, sensitive, rapid, inexpensive, and convenient, have been designed for fluoride detection.\textsuperscript{281-296}

Compound 16, a simple anthracene based urea derivative, was reported to be the first highly selective fluorescent chemosensor for fluoride ions.\textsuperscript{289} As shown in Scheme II-1, the four N-H bonds of the two urea moieties are able to form a binding pocket, which can complex fluoride ions through hydrogen bonds. Compound 16 exhibits a selective fluorescent quenching effect only upon binding with fluoride ions in ACN-DMSO mixture. Afterwards, a variety of urea/thiourea containing fluorescent sensors have been developed for fluoride ion detection.\textsuperscript{283,290-292}
Another type of fluorescent fluoride sensors is designed on the basis of a well-known fact that fluoride ions have a high affinity to silicon. This unique chemical property has been widely applied in the development of various fluorescent sensors for fluoride ions.\(^{281-282,284,287-288,293-296}\) Compound 17 is an effective fluorescent sensor based on a BODIPY-coumarin platform for fluoride sensing.\(^{282}\) A triisopropylsilyl group is exploited as the potential reaction unit for fluoride ions because of the high reactivity of fluoride to silicon. Compound 17 exhibits an intense fluorescent emission band at 606 nm when excited at 420 nm. Upon reaction with fluoride ions, the triisopropylsilyl group is unmasked and compound 17 is converted into a deprotected product. Correspondingly, addition of fluoride ions results in a considerable decrease in the emission at 606 nm and simultaneous emergence of a blue-shifted fluorescent emission band at 472 nm. Therefore, compound 17 is a ratiometric fluorescent sensor for fluoride ions.
Besides silicon, fluoride has also a high affinity to boron. This phenomenon has already been explored to develop fluoride sensors too. Since the first report about fluorescent sensing of fluoride ions using boronic acid in 1998,\textsuperscript{297} many other fluorescent sensors based on the boron-fluoride interaction have been reported.\textsuperscript{298-310} Compared with boronic acids, the more stable triarylborons with high Lewis acidity have been proved to be more suitable for being explored as fluoride probes in aqueous or alcohol solvents because they have the ability to overcome the competitive binding of aqueous protons with fluoride ions.\textsuperscript{307-310} Moreover, diborane compounds have received more attention since they are more efficient at capturing fluoride ions than monoborane receptors.\textsuperscript{299,301-302,306-307} Compound 18 is bis-triarylboron based fluorescent sensor for fluoride ions.\textsuperscript{307} Compound 18 is highly fluorescent with a maximum emission wavelength at 414 nm. Upon the addition of fluoride ion, its fluorescence emission is dramatically quenched, which is due to the occupation of the empty p\textsubscript{π} orbital of boron by F\textsuperscript{−}.\textsuperscript{307} Therefore, Compound 18 is a “turn-off” sensor for fluoride ions.
Recently, a new fluorescence turn-on fluoride sensor, compound 19, is reported. The fluoride sensing function of compound 19 is established on the interaction between antimony and fluoride (Scheme II-4). Compound 19 is a stibonium salt, weakly fluorescent with an anthryl-based emission band at 427 nm. Upon binding to fluoride, its fluorescence emission intensity is highly increased, which makes it a good fluoride sensor even in aqueous environment.

**Scheme II-4.** The reaction of compound 19 with fluoride ion. Ref 309, copyright 2012 American Chemical Society.
As the most electronegative atom, fluoride has some unique chemical properties due to its small size and high charge density. Among various anions, fluoride ions have the highest affinity to protons and are capable of deprotonating hydrogen-containing polar moieties, such as O-H and N-H groups. These unique properties make fluoride sensors based on the deprotonation mechanism display high selectivity for F\(^-\) over other competitive anions, such as H\(_2\)PO\(_4\)-, CN\(^-\), and AcO\(^-\), which generally interfere harmfully in sensing fluoride with hydrogen-bonding based fluoride sensors.\(^{312-316}\) As a result, a variety of N-H containing groups such as sulfonamide\(^{313}\), imidazole\(^{315}\), urea\(^{314}\), and pyrrole\(^{316-317}\), have been exploited to construct deprotonation based fluoride sensors. As illustrated in Scheme II-5, the N-H group of compound 20, which is a 1,4-diketo-3,6-diphenylpyrrolo[3,4-c]pyrrole derivative, can be deprotonated by fluoride ions in CH\(_2\)Cl\(_2\). Upon the addition of fluoride, a drastic decrease of the fluorescent emission band at 563 nm and the appearance of a red-shifted emission band at 635 nm were observed.\(^{317}\)

**Scheme II-5.** The deprotonation of compound 20 by fluoride ion. Ref 315, copyright 2010 American Chemical Society.
On the basis of the foregoing background information, a novel fluorescence turn-on fluoride sensor (FS) is designed, taking advantage of the unique deprotonation ability of fluoride ions. As mentioned above, various fluoride sensors have been developed involving deprotonation of N-H groups.\textsuperscript{312-316} However, employing deprotonation of C-H bond for detecting fluoride ions is still a challenge owing to the low acidity of C-H bond. Although Cao et al. attempted to use the deprotonation of C-H bond by fluoride to explain the problem they met,\textsuperscript{318} they failed to provide any evidence to support it. To the best of our knowledge, no anion sensors have been reported by exploiting the deprotonation of C-H bond before FS.

As explained in I.5.1, the acidity of the hydrogen of a 1,2,3-triazolium group is relatively high compared with other types of C-H bond. Consequently, it is potential to be deprotonated by anions, especially fluoride ions, which makes it a promising candidate for being exploited as an anion recognizer through the deprotonation mechanism instead of hydrogen bonding. By adopting the recognition moiety-signaling unit combination strategy, as demonstrated in Scheme I-1, we designed a conjugate of 1,2,3-triazolium groups and a fluorene derivative which serves as the fluorescent signaling unit.

\textbf{II.2 Results and Discussion}

As shown in Scheme II-6, the synthesis of FS started with 3,5-dibromobenzyl alcohol. It reacted with trimethylsilylacetylene via a Sonogashira coupling reaction to form compound 21, followed by a desilylation reaction with KF to produce compound 22.
Then the hydroxyl group of compound 22 was converted to chlorine by thionyl chloride, providing compound 23. Compound 24 was prepared through phosphonylation of the chloromethyl group of compound 23 with NaI as the catalyst. Compounds 25, 26, and 27 were synthesized according to reported literature procedures with 2,7-dibromofluorene as the starting material. Compound 28 was obtained from a Horner-Wadsworth-Emmons reaction between compounds 24 and 27. Then it reacted with iodobenzene via a copper(I)-catalyzed alkyne-azide cycloaddition reaction to generate compound 29 in the presence of sodium ascorbate, N1,N2-dimethylethane-1,2-diamine, and NaN3 in a solvent mixture of DMSO:toluene:H2O (5:4:1). The methylation of compound 29 with 2.5 equiv. of Me3O·BF4 in anhydrous CH2Cl2 provided the final product FS in a yield of 35% after repeated recrystallization from chloroform.
Scheme II-6. Synthetic route of FS.\textsuperscript{a}

\[ 
\begin{align*}
\text{HO-Br} &\xrightarrow{a} \text{HO-Cl} & \text{21} \\
\text{HO-Br} &\xrightarrow{b} \text{Cl} & \text{22} \\
\text{Cl} &\xrightarrow{c} \text{EtOPO} & \text{23} \\
\text{EtOPO} &\xrightarrow{d} \text{Ph-N} & \text{24} \\
\text{Ph-N} &\xrightarrow{e} \text{Ph-N} & \text{25} \\
\text{Ph-N} &\xrightarrow{f} \text{Ph-N} & \text{26} \\
\text{Ph-N} &\xrightarrow{g} \text{Ph-N} & \text{27} \\
\text{Ph-N} &\xrightarrow{h} \text{Ph-N} & \text{28} \\
\text{Ph-N} &\xrightarrow{i} \text{Ph-N} & \text{29} \\
\text{Ph-N} &\xrightarrow{j} \text{FS} & \\
\end{align*}
\]

\textsuperscript{a}Reagents and conditions: (a) CuI, Pd(PPh\(_3\))\(_2\)Cl\(_2\), trimethylsilylacetylene, THF:triethylamine (4:1), reflux, 12 h; (b) KF, methanol, room temperature, 10 h; (c) SOCl\(_2\), triethylamine, CH\(_2\)Cl\(_2\), 0 °C, 30 min; then reflux, 2 h; (d) NaI, triethyl phosphate, acetone, 60 °C, 24 h; (e) 1-bromohexane, t-BuOK, DMF, 40 °C, 30 min; (f) diphenylamine, t-BuONa, Pd(dba)\(_2\), dppf, toluene, reflux, 16 h; (g) n-BuLi, THF, −78 °C, 40 min; then DMF, −78 °C, 1.5 h; (h) t-BuOK, THF, 0 °C, 30 min; then room temperature, 3 h; (i) iodobenzene, NaN\(_3\), Cul, sodium ascorbate, N\(^1\),N\(^2\)-dimethylene-1,2-diamine, toluene:DMSO:H\(_2\)O (4:5:1), room temperature, 3 h; (j) trimethylloxonium tetrafluoroborate, CH\(_2\)Cl\(_2\), room temperature, 3 d.

The photophysical and photochemical properties of FS were first examined with UV-vis spectroscopy. FS exhibited an absorption peak at 398 nm in DMSO. A series of
anions (F\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), CN\(^-\), SCN\(^-\), N\(_3\)^-\), NO\(_2\)^-\), NO\(_3\)^-\), ClO\(_4\)^-\), IO\(_4\)^-\), AcO\(^-\), HSO\(_4\)^-\), and H\(_2\)PO\(_4\)^-\), as n-Bu\(_4\)N\(^+\) salts) were added to the solution of FS to investigate its selectivity for certain anions. Figure II-1 shows the absorption spectra of FS before and after adding those anions. Upon the addition of fluoride ions, the absorption wavelength of FS blue-shifted from 398 nm to 385 nm and the absorption intensity increased. In contrast, the addition of other anions brought about no considerable change to the absorption spectra of FS. These results indicate that FS displays remarkable selectivity for fluoride ions over the other anions. Moreover, as illustrated in Figure II-2, with the increasing of the equiv. of fluoride ions, both the absorption intensity and the maximum absorption wavelength of the absorption spectra of FS were changed gradually.

![Figure II-1. UV-vis absorption spectra of FS (1.0 × 10\(^{-5}\) M) in DMSO upon addition of 200 equiv of various anions.](image)
In addition to UV-vis spectroscopy, fluorescent emission spectroscopy was also utilized to investigate the photophysical and photochemical properties of FS. Fluorescent emission spectroscopy is more effective and reliable than absorption spectroscopy for fluoride ions detection. The fluorescent emission properties of FS in the absence and presence of various anions were examined in DMSO. FS displayed a specific fluorescent emission response to fluoride ions, which was in good agreement with the results found in absorption spectroscopy experiments. Figures II-3A and II-3B show the pictures of FS before and after excitation at 365 nm using a hand-held UV lamp, respectively. The free FS is not fluorescent. Upon the addition of fluoride ions, a striking blue-green fluorescence was emitted. However, the addition of other anions...
brought about no fluorescent emission and the samples with these anions displayed no change in solution colors before and after excitation at 365 nm.

![Image](image_url)

**Figure II-3.** (A) A photograph of FS (6.0 μM) in DMSO with 20 equiv. of various anions. (B) Visual fluorescence responses of FS (6.0 μM) in DMSO with 20 equiv. of various anions under excitation at 365 nm using a hand-held UV lamp.

The fluorescence properties of FS in the absence and presence of various anions were further investigated with fluorescent emission spectroscopy. Figure II-4 shows fluorescence spectra of FS recorded with excitation at 385 nm in the presence of 20 equiv. of various anions. In agreement with the results obtained from visual fluorescence response experiments (Figure II-3B), FS with 20 equiv. of fluoride ions added displayed an intense fluorescent emission band at 498 nm with a high quantum yield up to 0.66. In contrast, no fluorescence signal was detected from the free FS and FS with 20 equiv. of other anions. All these results demonstrate that FS exhibits very high selectivity for fluoride ions over other anions and it is promising to be used as a specific fluorescent sensor for fluoride ions.
Figure II-4. Fluorescence emission spectra of FS (6.0 μM) in DMSO upon the addition of 20 equiv. of various anions (λ<sub>ex</sub> = 385 nm).

Figure II-5. Fluorescence emission Changes for FS (6.0 μM) upon the addition of 0-1000 equiv. of F<sup>-</sup> in DMSO (λ<sub>ex</sub> = 385 nm).
Following the fluoride selectivity experiments, the sensitivity of \( \text{FS} \) toward sensing fluoride ions was also examined with fluorescent emission spectroscopy. The fluorescence responses of \( \text{FS} \) induced by the addition of fluoride ions were determined with excitation at 385 nm in DMSO. As shown in Figure II-5, the fluorescent emission intensity increased gradually with the progressive addition of fluoride ions (from 0 to 1000 equiv.). The fluorescence intensity of \( \text{FS} \) as a function of the equiv. of fluoride ions is illustrated in Figure II-6A. The inset curve is an enlarged segment of Figure II-6A in correspondence to 0–20 equiv. of fluoride ions. As displayed in the inset, the addition of the first 4 equiv. of fluoride ions resulted in no fluorescent emission response in comparison to the free \( \text{FS} \). However, a remarkable fluorescence emission was recorded after 5 equiv. of fluoride ions was added. As the added amount of fluoride ions increased, the fluorescent emission intensity was enhanced with the concentration of fluoride ions. In detail, the fluorescence intensity increased dramatically in the range of 4-10 equiv. of fluoride ions. After that, the increasing rate of the fluorescent emission intensity was reduced. More significantly, it was found that the plot of log(FI) (fluorescence intensity) versus log\([\text{F}^-]\) (concentration of fluoride ions) is linear \( (R^2 = 0.9985) \) in the range of 4-10 equiv. of fluoride ions (Figure II-6B). These results indicate that \( \text{FS} \) is a sensitive and effective sensor that can be utilized to determine the concentration of fluoride ions.
Before exploring applications of FS for fluoride ions determination, the interactions between FS and fluoride ion were further investigated by NMR titration experiments. The NMR spectra of FS were recorded with different equiv. of fluoride ions in DMSO-$d_6$. Figure II-7 shows the partial $^1$H NMR fluoride ion titration spectra of FS in the presence of 0-20 equiv. of fluoride ions. Upon the addition of fluoride ions, the signal of triazolium CH protons (9.99 ppm) shifted downfield, gradually decreased, and eventually disappeared. As shown in Figure II-8, along with the changes of triazolium CH proton signal, a significant triplet signal (1:2:1) at 16.1 ppm with a coupling constant of $J \approx 120$ Hz emerged after 5 equiv. of fluoride ions was added. This is a characteristic $^1$H NMR signal of the bi-fluoride ion (FHF$^-$), which proves the formation of FHF$^-$\textsuperscript{315,322,323}. These results indicated that the CH group of the triazolium ring was deprotonated by fluoride.
Figure II-7. Partial $^1$H NMR (400 Hz) spectra of FS (5.0 mM) upon addition of F$^-$ in DMSO-$d_6$.

Figure II-8. The appearance of FHF$^-$ signal in $^1$H NMR (400 Hz) titration spectra of FS (5.0 mM) upon addition of F$^-$ in DMSO-$d_6$. 
The results obtained from $^1$H NMR were also confirmed by the changes recorded in $^{13}$C NMR spectra of FS as a result of the addition of fluoride ions (Figure II-9). Furthermore, the NMR results and the forgoing fact that the first fluorescence response of FS induced by fluoride ions was also detected right after the addition of 5 equiv. of fluoride ions suggest that the emergence of fluorescence emission originates from the deprotonation of the C-H bond.

**Figure II-9.** Partial $^{13}$C NMR (101 Hz) spectra of FS upon adding F$^-$ in DMSO-$d_6$.

Based on all the results demonstrated above, a plausible explanation about the interactions between FS and fluoride ions is proposed here. Two different kinds of interactions, H-bonded complexation and deprotonation, are involved in the titration process of FS with gradually increased equiv. of fluoride. The first addition of fluoride ions produces hydrogen-bonding complexes, whereas subsequently added fluoride
leads to deprotonation of the triazolium groups. In the deprotonation process, bi-fluoride ion FHF⁻ and two deprotonated species of FS, mono- and bis-deprotonated products, are generated. When 1 equiv. of fluoride ion is added, a complex is formed via hydrogen bonding. As a result, a slight decrease is induced in the ¹H NMR signal of the triazolium CH proton (Figure II-7). With following fluoride ions added, a considerable decrease and obvious downfield shift in the ¹H NMR signal of the CH proton of the triazolium ring is observed, which results from the even stronger hydrogen bonding interaction between fluoride ion and the proton of triazolium C-H bond. The results obtained from fluorescence titration experiments indicate that the hydrogen-bonding based species are not fluorescent since the initial addition of fluoride ions triggered no fluorescence response. However, as further more fluoride is added, deprotonated forms of FS are generated. Upon the addition of 4 equiv. of fluoride ions, the mono-deprotonated form of FS may be produced, which is not fluorescent. The formation of non-fluorescent mono-deprotonated FS explains why, no fluorescence response was detected (Figure II-6), but a very weak ¹H NMR signal of bi-fluoride ion FHF⁻ was recorded (Figure II-8) after the addition of up to 4 equiv. of fluoride. When more equivalents of fluoride ions are added, a neutral bis-deprotonated form of FS is produced, emitting strong fluorescence. It is necessary to be noted here that compound 29, the precursor of FS, is a highly fluorescent dye (quantum yield: 1.0). However FS, the methylation product of 29, bearing two positive charges, is non-fluorescent. This phenomenon is consistent with a well-known fact that cationic dyes have very weak fluorescence because of the greater bond length alternation character. Deprotonation
can effectively reduce this effect through removing the positive charge from the chromophore, resulting in fluorescence recovery. Therefore, after deprotonation of the triazolium groups, FS becomes neutral and fluorescent.

**Figure II-10.** Fluorescence response of test strips under irradiation at 365 nm using a hand-held UV lamp while determining fluoride ions at different concentrations in aqueous solution.

Encouraged by those promising results, filter paper based test strips of FS were prepared for facilitating F⁻ sensing in aqueous environment. As exhibited in Figure II-10, the fluorescence emission intensity of test strips increased with the concentration of fluoride ions in aqueous solution. The concentration of fluoride ion can be detected as low as ca. 10⁻⁴ M (1.9 ppm). U.S. EPA has two standards for the fluoride concentration in drinking water. The first standard, which is enforceable, is that maximum contaminant level (MCL) for fluoride is 4.0 ppm, and the secondary non-enforceable MCL is 2.0 ppm. Therefore, according to EPA’s standards, the test strips of FS are potential to be
explored to effectively determine whether the fluoride ion concentration in drinking water exceeds acceptable standards.

II.3 Conclusion

In summary, a novel highly selective fluorescence turn-on sensor was developed for fluoride anion detection. This new sensor is a combination of a fluorene platform, serving as the chromophore, and 1,2,3-triazolium groups, functioning as signaling moieties. The function of the sensor was established on the basis of deprotonation of the C-H bonds of 1,2,3-triazolium rings, which makes FS the first reported anion sensor based on the deprotonation of a C-H bond. In this work, fluoride-induced deprotonation of a C-H bond is evidenced and further applied to fluoride ion detection. FS exhibited specific fluorescence turn-on response toward fluoride anion, making it an excellent sensor for differentiating fluoride ions from other anions. Also, FS demonstrated high sensitivity for fluoride sensing. Furthermore, interactions between fluoride and the C-H bond of 1,2,3-triazolium group were examined by $^1$H NMR and fluorescence spectroscopy. On the basis of the results obtained from $^1$H NMR titration and fluorescence emission titration experiments, a reasonable explanation is suggested for the interactions between FS and fluoride anion. We anticipate that this interaction model will be explored to develop other sensing systems for selectively detecting various anions. Easy-to-prepare test strips based on FS were prepared for detecting the presence of fluoride ions in aqueous media. These test strips can be easily explored to determine fluoride anion at concentrations down to 1.9 ppm, which may provide the
basis inexpensively and conveniently to estimate whether the concentration of fluoride ions in drinking water is at a safe level.

II.4 Experimental

II.4.1 General

All reagents were purchased from commercial suppliers and all solvents were used as received without any further purification. All anions were used as their tetrabutylammonium salts. 2,7-Dibromo-9,9-dihexyl-9H-fluorene (25)\textsuperscript{319} and 7-bromo-9,9-dihexyl-N,N-diphenyl-9H-fluoren-2-amine (26)\textsuperscript{320} were synthesized following the exact procedures reported in their respective literatures. \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopic measurements were carried out using a Bruker AVANCE 400 MHz spectrometer in DMSO-\textit{d}_6 or CDCl\textsubscript{3} solution with tetramethysilane (TMS) as internal reference. UV-vis absorption spectra were measured using an Agilent 8453 spectrophotometer. Fluorescent emission spectra were recorded on a PTI QuantaMaster Spectrofluorometer.

II.4.2 Synthesis

3,5-Di(trimethylsilylethynyl)benzyl alcohol (21). To a mixture of 3,5-dibromobenzyl alcohol (2.66 g, 10.0 mmol), Cul (0.19 g, 1.0 mmol), and Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2} (0.35 g, 0.5 mmol) was added 60 mL degassed solvent mixture THF:triethylamine (4:1) followed by the dropwise addition of trimethylsilylacetylene (4.24 mL, 30.0 mmol) in an argon atmosphere. The reaction mixture was heated to reflux and stirred for 12 h. After cooling to room temperature, the solvent was removed under reduced pressure and the
residue was extracted with CH$_2$Cl$_2$/brine. The organic layer was dried over MgSO$_4$, then filtered and dried. The crude product was further purified by flash column chromatography on silica gel using CH$_2$Cl$_2$ as eluent to give 21 as a yellow liquid (2.88 g, 96%). $^1$H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.52 – 7.48 (m, 1H), 7.41 – 7.39 (m, 2H), 4.63 (d, J = 6.0 Hz, 2H), 1.71 (t, J = 6.0 Hz, 1H), 0.24 – 0.22 (m, 18H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ (ppm) 141.31, 134.61, 130.24, 123.74, 104.04, 95.23, 64.53, 0.02. HRMS (DART, m/z): calcd for C$_{17}$H$_{24}$OSi$_2$ ([M+H]$^+$) 301.1438; found 301.1428.

3,5-Diethynylbenzyl alcohol (22). A solution of 21 (2.70 g, 9.0 mmol) and KF (2.61 g, 45.0 mmol) in 150 mL methanol was stirred at room temperature for 10 h. Then the solvent was removed under reduced pressure and the residue was extracted with CH$_2$Cl$_2$/H$_2$O. The organic layer was dried over MgSO$_4$, filtered and concentrated in vacuo. Purification by flash column chromatography on silica gel using CH$_2$Cl$_2$ provided 22 as a yellow liquid (1.34 g, 95%). $^1$H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.54 – 7.52 (m, 1H), 7.50 – 7.45 (m, 2H), 4.67 (d, J = 6.0 Hz, 2H), 3.09 (s, 2H), 1.77 (t, J = 6.0 Hz, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$): δ (ppm) 141.58, 134.84, 130.81, 122.86, 82.59, 78.15, 64.39. HRMS (DART, m/z): calcd for C$_{11}$H$_8$O ([M+NH$_4$]$^+$) 174.0913; found 174.0919.

3,5-Diethynylbenzyl chloride (23). To a solution of 22 (1.25 g, 8.0 mmol) in 5 mL triethylamine and 25 mL anhydrous CH$_2$Cl$_2$ was added SOCl$_2$ (0.73 mL, 10.0 mmol) dropwise under argon atmosphere at 0 °C. After stirring for 0.5 h at 0 °C, the reaction mixture was heated to reflux and stirred for 2 h. Then it was poured into ice water and extracted with CH$_2$Cl$_2$. The organic layer was dried over MgSO$_4$, filtered and concentrated in vacuo. The crude mixture was purified by flash column chromatography.
on silica gel using CH$_2$Cl$_2$, giving the desired compound 23 as a white solid (0.73 g, yield 52\%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 7.56 (t, $J$ = 1.4 Hz, 1H), 7.49 (d, $J$ = 1.3 Hz, 2H), 4.52 (s, 2H), 3.11 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 138.21, 135.54, 132.50, 123.21, 82.15, 78.60, 44.99. HRMS (DART, m/z): calcd for C$_{11}$H$_7$Cl ([M+H]$^+$) 175.0313; found 175.0315.

**Diethyl 3,5-diethynylbenzylphosphonate (24).** To a mixture of 23 (0.70 g, 4.0 mmol) and NaI (0.75 g, 5.0 mmol) was added 20 mL acetone, followed by the addition of triethyl phosphite (1.04 mL, 6.0 mmol). The resulting mixture was heated to 60 °C and stirred for 24 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was extracted with ethyl acetate/H$_2$O. The organic layer was washed three times with distilled water and then once with brine. The organic layer was dried over MgSO$_4$, then filtered and dried. The crude product was further purified by flash column chromatography on silica gel using methanol/CH$_2$Cl$_2$ (1/100) as eluent to give 24 as a yellow liquid (0.88 g, 80%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 7.52 – 7.49 (m, 1H), 7.44 – 7.35 (m, 2H), 4.07 – 4.00 (m, 4H), 3.13 – 3.04 (m, 4H), 1.28 – 1.24 (m, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 134.29, 133.77, 132.75, 122.87, 82.44, 78.24, 62.44, 34.14, 32.77, 16.47. HRMS (DART, m/z): calcd for C$_{15}$H$_{17}$O$_3$P ([M+H]$^+$) 277.0988; found 277.0988.

**7-(Diphenylamino)-9,9-dihexyl-9H-fluorene-2-carbaldehyde (27).** To a solution of 26 (2.90 g, 5.0 mmol) in 20 mL anhydrous THF was added n-BuLi (3.0 mL of a 2.5 M solution in hexane, 7.5 mmol), within 15 min at −78 °C under argon atmosphere. The reaction mixture was stirred for 40 min before 1.0 mL of DMF was added dropwise.
After stirring for 1.5 h at −78 °C, the temperature was allowed to rise to 0 °C. The mixture was treated with dilute hydrochloric acid (5.0 mL, 2.0 M) at 0 °C. The organic phase was washed with water, aqueous NaHCO₃, and brine. The combined organic layer was dried over MgSO₄. After filtration and evaporation of the solvent, the crude product was purified by flash column chromatography on silica gel using ethyl acetate/hexanes (1/30) to give 27 as a yellow liquid (1.72 g, 65%). 

\[ \text{^1H NMR (400 MHz, CDCl}_3\text{): } \delta \text{ (ppm) 10.02 (s, 1H), 7.82 (dd, } J = 6.3, 1.7 \text{ Hz, 2H), 7.74 – 7.69 (m, 1H), 7.61 (d, } J = 8.3 \text{ Hz, 1H), 7.30 – 7.23 (m, 4H), 7.16 – 7.09 (m, 5H), 7.07 – 7.02 (m, 3H), 1.98 – 1.80 (m, } J = 18.8, 4H), 1.17 – 1.00 (m, 12H), 0.79 (t, } J = 7.2 \text{ Hz, 6H), 0.69 – 0.54 (m, 4H).} \]

\[ \text{^13C NMR (101 MHz, CDCl}_3\text{): } \delta \text{ (ppm) 192.45, 153.85, 151.46, 149.02, 147.81, 134.66, 134.20, 130.96, 129.45, 124.54, 123.27, 123.02, 121.85, 119.28, 118.40, 55.31, 40.19, 31.64, 29.68, 23.90, 22.67, 14.15}. \]

HRMS (DART, m/z): calcd for C₃₈H₄₃ON ([M+H]^+) 530.3414; found 530.3420.

\( (E)-7-(3,5-\text{Diethynylstyryl})-9,9-\text{dihexyl-N,N-diphenyl-9H-fluoren-2-amine (28)} \). To a mixture of 27 (1.32 g, 2.5 mmol) and 24 (0.83 g, 3.0 mmol) in 50 mL anhydrous THF at 0 °C was added dropwise 3.5 mL of 1.0 M t-BuOK (3.5 mmol) in THF in an argon atmosphere. The reaction mixture was stirred for 30 min at 0 °C, followed by 3 h at room temperature. Then it was quenched by adding distilled water and extracted with ethyl acetate. The organic layer was washed three times with distilled water and then once with brine. The organic layer was dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash column chromatography on silica gel using CH₂Cl₂/hexanes (1/7) as eluents provided 28 as a yellow solid (1.16 g, 71%). 

\[ \text{^1H NMR (400 MHz, CDCl}_3\text{):} \]
δ (ppm) 7.69 (d, J = 1.3 Hz, 2H), 7.65 – 7.58 (m, 2H), 7.55 (t, J = 1.3 Hz, 1H), 7.52 – 7.48 (m, 2H), 7.32 – 7.28 (m, 4H), 7.24 – 7.17 (m, 6H), 7.11 – 7.04 (m, 4H), 3.13 (s, 2H), 2.02 – 1.88 (m, 4H), 1.23 – 1.10 (m, 12H), 0.85 (t, J = 7.1 Hz, 6H), 0.78 – 0.74 (m, 4H).

13C NMR (101 MHz, CDCl3): δ (ppm) 152.61, 151.35, 148.07, 147.46, 141.48, 138.30, 135.89, 134.91, 134.16, 131.45, 130.30, 129.30, 126.04, 125.46, 124.00, 123.63, 122.96, 122.69, 120.96, 120.63, 119.50, 119.34, 82.76, 78.03, 55.13, 40.42, 31.64, 29.77, 23.91, 22.69, 14.18. HRMS (MALDI, m/z): calcd for C49H49N ([M+H]+) 651.3859; found 651.3853.

(E)-7-((3,5-Bis(1-phenyl-1H-1,2,3-triazol-4-yl)styryl)-9,9-dihexyl-N,N-diphenyl-9H-fluoren-2-amine (29). A mixture of degassed toluene:DMSO:H2O (4:5:1, 30 mL) was added to a mixture of 28 (0.98 g, 1.5 mmol), iodobenzene (1.22 g, 6.0 mmol), NaN3 (0.29 g, 4.5 mmol) and sodium ascorbate (59.4 mg, 0.3 mmol). Then CuI (0.11 g, 0.6 mmol) and N1,N2-dimethylethane-1,2-diamine (79.3 mg, 0.9 mmol) were added to the mixture in an argon atmosphere. The reaction mixture was stirred at room temperature and the progress of the reaction was followed by TLC. When compound 28 was completely consumed (about 2 hours), the reaction mixture was concentrated in vacuo to remove toluene and H2O. Then the residue was extracted with CH2Cl2/brine and washed with distilled water. The organic layer was dried over MgSO4, filtered and concentrated in vacuo. The crude mixture was purified by flash column chromatography on silica gel using methanol/CH2Cl2 (1/20), giving the desired compound 29 as a yellow solid (1.02 g, yield 76%). 1H NMR (400 MHz, CDCl3): δ (ppm) 8.38 (s, 2H), 8.32 (s, 1H), 8.16 (d, J = 1.3 Hz, 2H), 7.89 – 7.81 (m, 4H), 7.63 – 7.40 (m, 11H), 7.30 – 7.24 (m, 5H),
7.13 (d, J = 7.3 Hz, 5H), 7.02 (t, J = 7.4 Hz, 3H), 1.98 – 1.85 (m, 4H), 1.20 – 1.04 (m, 12H), 0.80 (t, J = 7.1 Hz, 6H), 0.71 (s, 4H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 152.68, 151.40, 148.13, 147.39, 141.30, 139.31, 137.22, 136.08, 135.32, 131.44, 131.00, 130.01, 129.32, 129.05, 126.72, 126.00, 123.99, 123.77, 122.66, 122.09, 121.02, 120.72, 119.53, 118.23, 55.17, 40.49, 31.69, 29.83, 23.97, 22.73, 14.20. HRMS (APCI, m/z): calcd for C₆₁H₅₉N₇ ([M+H]+) 890.4906; found 890.4905.

(E)-4,4′-(5-(2-(7-(Diphenylamino)-9,9-dihexyl-9H-fluoren-2-yl)vinyl)-1,3-phenylene)bis(3-methyl-1-phenyl-1H-1,2,3-triazol-3-ium) bis(tetrafluoroborate) (FS). Dry and degassed CH₂Cl₂ (30 mL) was added to a mixture of 29 (0.71 g, 0.8 mmol) and trimethylxonium tetrafluoroborate (0.30 g, 2.0 mmol) in an argon atmosphere. The resulting mixture was stirred for 3 days at room temperature under argon. The reaction mixture was quenched by adding distilled water and extracted with CH₂Cl₂. The solvent was removed in vacuo and the residue was dissolved into 2 mL CH₂Cl₂. Then 20 mL diethyl ether was added to the solution and a yellow precipitate formed. After filtration, the crude product was recrystallized from chloroform 3 times to give FS as a yellow solid (0.31 g, 35%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 9.18 (s, 2H), 8.08 (d, J = 1.4 Hz, 2H), 7.98 (s, 1H), 7.86 (dd, J = 8.1, 1.7 Hz, 4H), 7.62 – 7.54 (m, 7H), 7.46 – 7.39 (m, 2H), 7.36 (d, J = 6.9 Hz, 1H), 7.30 – 7.21 (m, 5H), 7.17 – 7.13 (m, 6H), 7.06 – 6.99 (m, 3H), 4.46 (s, 6H), 2.05 – 1.84 (m, 4H), 1.19 – 1.05 (m, 12H), 0.79 (t, J = 7.0 Hz, 6H), 0.71 (s, 4H). ¹H NMR (400 MHz, DMSO-d₆): δ (ppm) 9.99 (s, 2H), 8.36 (d, J = 1.2 Hz, 2H), 8.12 – 8.10 (m, 5H), 7.88 – 7.75 (m, 8H), 7.69 – 7.63 (m, 3H), 7.55 (d, J = 16.0 Hz, 1H), 7.29 (t, J = 8.0 Hz, 4H), 7.12 (d, J = 2.0 Hz, 1H), 7.07 – 7.01 (m, 6H), 6.96 (dd, J =
8.0 Hz, 2.0 Hz, 1H), 4.59 (s, 6H), 1.93 (s, 4H), 1.10 – 1.02 (m, 12H), 0.75 (t, J = 7.2 Hz, 6H), 0.60 (s, 4H). \textsuperscript{13}C NMR (101 MHz, CDCl\textsubscript{3}): \(\delta\) (ppm) 152.87, 151.81, 148.05, 147.71, 143.01, 142.02, 141.09, 135.52, 134.67, 133.99, 132.11, 130.59, 129.37, 129.22, 128.87, 126.54, 125.91, 124.15, 123.87, 123.76, 123.40, 122.87, 121.86, 121.23, 120.71, 119.44, 119.31, 55.33, 40.17, 39.66, 31.78, 29.80, 24.12, 22.74, 14.21. HRMS (APCI, m/z): calcd for C\textsubscript{63}H\textsubscript{65}N\textsubscript{7}BF\textsubscript{4} ([M-BF\textsubscript{4}]\textsuperscript{+}) 1006.5342; found 1006.5336.

\textit{II.4.3 Preparation of test strips}

A well-cut filter paper (12 \times 3 mm\textsuperscript{2}) was immersed into a solution of FS in CH\textsubscript{2}Cl\textsubscript{2} (1.0 \times 10^{-3} M) for 5 s and then dried by exposure to air. For sensing fluoride ions in water, the test strip was dipped into a fluoride-containing aqueous solution for 3 min and then dried in the air.
CHAPTER III: FLUORESCENCE TURN-ON SENSORS FOR K⁺

III.1 Introduction

Potassium, the most abundant intracellular metal cation, makes up about 0.4% of the mass of human body. Intracellular and extracellular concentrations of K⁺ are about 150 and 4.5 mM for mammal cells, respectively.\(^{324-325}\) Potassium ions play diverse roles in a number of biological processes. They are not only involved in the regulation of blood pressure and intracellular concentrations of other ions, for example, chloride and calcium ions, which are transported across the plasma membrane, but also associated with the maintenance of proper pH balance and extracellular osmolarity.\(^{326-328}\) Moreover, the significance of potassium ions in heartbeat regulation has been discovered.\(^{329}\) Potassium also plays indispensable roles in cell proliferation, muscle contraction, epithelial fluid transport, and nerve transmission.\(^{330-334}\)

On the other hand, an unbalance in the potassium level in the human body can bring about certain human diseases such as renal disease, stroke, hypertension, seizures, and myasthenia.\(^{335-339}\) Furthermore, abnormal potassium fluctuations are a potential sign for the onset of several other diseases, including anorexia, bulimia, diabetes, alcoholism, heart disease, cancer, and AIDS.\(^{335-343}\) Hence, a delicate balance of potassium concentration is vital for human health.

However, the molecular mechanisms of potassium pathology and physiology are still inadequately examined to date. This situation is partly because of the lack of effective methods for accurately determining intracellular and extracellular potassium
concentration changes with good temporal and spatial fidelity. Therefore, selective and sensitive detection of potassium levels in human body fluids is of great importance, and developing an approach to achieve that is meaningful.

Continuous efforts have been devoted to extracellular and intracellular potassium detection due to the biological significance of potassium. Although various potassium sensors have been developed, none of them meet all the requirements for practical imaging applications. Common analytical methods, e.g., the potassium electrode, provide reliable results with high sensitivity. However, those techniques cannot be utilized for non-invasive study in biological systems. Also, they are not suitable for conducting experiments with small amount of cells, not to mention at the single cell level.

Compared with other methods, fluorescence techniques have some distinct advantages, including high sensitivity, non-invasiveness, and convenience, and thus have attracted growing attention. In 1989, Tsien et al. reported the first, and so far the best known, fluorescent potassium sensor, potassium-binding benzofuran isophthalate (PBFI), which is currently the only commercially available potassium probe. PBFI is comprised of a benzofuran derivative, serving as the fluorophore, and a diaza-18-crown-6 ether as the recognition moiety. PBFI is capable of sensing intracellular potassium level, whereas it is not suitable for extracellular potassium sensing because of the poor sensitivity that results from its insufficient potassium binding strength. Moreover, the selectivity of PBFI for K⁺ over Na⁺ is only modest, which highly limits its application in biological environments where K⁺ and Na⁺ always coexist.
Enlightened by the design of PBFI, further efforts were devoted to develop potassium probes with better properties. To increase the potassium binding strength, cryptands were synthesized and used as potassium recognition units. By coupling such a cryptand to a fluorophore, the potassium complexation of the cryptand is translated into a fluorescent signal change of the fluorophore. Masilamani et al. reported a fluorogenic potassium probe (30), which is a combination of a [222] cryptand and a coumarin fluorophore. Although the design of probe 30 successfully enhances the potassium binding strength, it still suffers from the interference from sodium. Moreover, this probe is pH-sensitive in physiological pH range, which is due to the protonation of the two aliphatic nitrogen atoms. Afterwards, Sammes et al. developed another [222] cryptand to overcome the pH interference problem.\textsuperscript{347} Instead of aliphatic nitrogens, probe 31 incorporated two aromatic nitrogens to reduce the pH sensitivity. While probe 31 is not sensitive to pH at physiological pHs, it still suffered from the sodium interference issue. In addition, probe 31 showed relatively small fluorescence signal change upon binding potassium ions.
On the basis of the advantages and disadvantages of the foregoing potassium probes, He et al. developed a [2.2.3]-triazacryptand (TAC) as a potassium recognition unit and connected it to an amino-naphthalimide fluorophore to produce probe 32. As a potassium sensor, 32 has some desirable properties: (1) high selectivity against sodium and other biological cations; (2) strong potassium binding strength; (3) large fluorescence signal change upon binding potassium ions; (4) no pH interference within physiological pH range. These advantages render it a better potassium sensor than the former ones and a good candidate for sensing extracellular potassium concentration changes in blood and serum.
Attracted by the excellent performance of TAC in recognizing potassium ions, Verkman et al. and Tian et al. subsequently developed several fluorescent potassium sensors based on different fluorophores, using TAC as the potassium-complexing moiety.\textsuperscript{36-37,39-40} All of these TAC-based potassium probes exhibit high K\textsuperscript{+}/Na\textsuperscript{+} selectivity and no pH interference. Probe 33, a conjugate of TAC and xanthylium, was the first fluorescence turn-on potassium sensor reported by Verkman.\textsuperscript{36} In the presence of 0-50 mM K\textsuperscript{+}, the fluorescence intensity of 33 (7 μM) increased up to 14-fold. Probe 33 was explored to monitor K\textsuperscript{+} waves in the brain cortex. However, its easy uptake by many cell types seriously limits its extracellular application. Tian et al. synthesized probe 34, which is a combination of TAC and a strong electron-withdrawing group, 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran.\textsuperscript{40} Probe 34 was capable of sensing K\textsuperscript{+} up to 1600 mM. In the presence of K\textsuperscript{+} with concentrations of 140 and 1400 mM, the fluorescence intensity of 34 was increased by 4- and 50-fold, respectively. The ability of sensing K\textsuperscript{+} over such a broad concentration range makes it the first fluorescent K\textsuperscript{+} sensor that is suitable for detecting highly concentrated K\textsuperscript{+} and selectively sensing intracellular K\textsuperscript{+} level. Confocal fluorescence microscopy experiments demonstrated that probe 34 could be utilized for detecting K\textsuperscript{+} within living cells.
All of the reported TAC-based potassium probes demonstrate that the TAC platform is the most desirable potassium recognition unit so far. However, there is a serious drawback of TAC. It is difficult to be reproduced because of its complicated multistep synthesis and low overall yield. Consequently, widespread application is limited. After carefully examining its synthetic route, we found that the synthesis of TAC is possible to be optimized. Hence, our first goal was to improve the synthesis of TAC, shortening its synthetic route and enhancing the overall yield. After that, TAC-based fluorescent potassium sensors were synthesized, characterized, and examined in solution and then cellular environment. Moreover, since there is no two-photon absorbing fluorescent potassium sensors reported so far (the merits of two-photon excitation microscopy are illustrated in section I.1), efforts were devoted to develop a new potassium sensor that can be excited by two-photon absorption.
III.2 Results and Discussion

The synthetic route of TAC reported by He et al. is shown in Scheme III-5. In the original synthetic route, compound 36 was synthesized in 2 steps with 2-nitrophenol as the starting material. Since compound 36 is commercially available, the work herein will not include its synthesis. In the reported synthesis, TAC was obtained in 7 steps with a 3.6% overall yield, starting from 5-methyl-2-nitrophenol, which was first reacted with 1,2-dibromoethane in DMF at 120 °C in the presence of K$_2$CO$_3$ to produce compound 35. Then 2 equiv. of 35 and 1 equiv. of compound 36 were refluxed in acetonitrile with KI as catalyst and K$_2$CO$_3$ as base to form compound 37. The nitro groups of compound 37 were reduced to amino groups by 2.2 atm hydrogen gas in DMF, using 5% Pd/C as the catalyst, affording compound 38. Subsequently, a 21-membered macrocycle compound 39 was produced through the reaction between compound 38 and DODC, which was obtained from reaction of 3,6-dioxaoctanedioic acid with oxalyl chloride, in CH$_2$Cl$_2$ with TEA as the base. In this reaction, two amide groups were synchronically formed, generating the 21-membered macrocycle. The newly formed amides were then reduced to amines by BH$_3$/THF, producing compound 40. Compound 41 was prepared through the same reaction that provided compound 39, with compound 40 instead of 38 reacting with DODC in CH$_2$Cl$_2$ and TEA. Another 21-membered macrocycle was formed in compound 41 with two amide groups that were also further reduced to amino groups by BH$_3$/THF to produce the desired product TAC.
Scheme III-5. Reported synthetic route of TAC.$^a$

After examining the procedures of each of the above steps, some modifications and optimizations were made to the synthesis of TAC. The improved synthetic route of TAC is shown in Scheme III-6. For the first step, the reaction temperature is the key point. By increasing the reaction temperature from the reported 120 °C to reflux in DMF, the yield of the reaction was increased from 50% to 81%. Considering the possibility that the starting material 5-methyl-2-nitrophenol may be oxidized by oxygen at high temperature, the reaction was performed under argon atmosphere. For the second step,
the literature procedure involved refluxing a mixture of 35 and 36 in acetonitrile in the presence of KI and K₂CO₃. Unfortunately, this approach is very inefficient. After refluxing for 16 h, mono-substituted aniline was produced as the major product and only trace desired product (bis-substituted aniline) was formed. Repeatedly adding compound 35 every 20 h 3 times resulted in a modest yield (around 50%) of compound 37. To achieve better reaction conditions, DMF was employed as the reaction medium rather than MeCN, and the reaction temperature was increased to 120 °C, but similar results were obtained.

Finally, a totally different reaction system was adopted to perform this reaction, using a mixture of 1,4-dioxane and water (2:3) as the reaction medium and CaCO₃ as base. As a result, the desired compound 37 was provided in a much higher yield (91%). For the step of reducing nitro groups of 37 to amino groups, hydrogen gas was used as the reductive reagent and compound 38 was obtained in a 97% yield. Our goal was also to find a more convenient approach, avoiding utilizing the volatile hydrogen gas. To achieve this goal, hydrazine monohydrate was employed as the reducing reagent. An equivalent yield (97%) of the product resulted when the reaction was catalyzed by Pd/C. Furthermore, an equally efficient and more economic catalyst FeCl₃/C (a mixture of FeCl₃ and activated carbon) also provided compound 38 in a good yield (96%).
Scheme III-6. Optimized synthetic route of TAC.\textsuperscript{a}

![Scheme III-6](attachment:scheme.png)

\textsuperscript{a}Reagents and conditions: (a) BrCH\(_2\)CH\(_2\)Br, K\(_2\)CO\(_3\), DMF, reflux, 2 h; (b) KI, CaCO\(_3\), water:dioxane (3:2), reflux, 36 h; (c) H\(_2\)NNH\(_2\)·H\(_2\)O, activated carbon, FeCl\(_3\)·6H\(_2\)O, THF:MeOH (1:1), reflux, 20 h; (d) 1,2-bis(2-iodoethoxy)ethane, K\(_2\)CO\(_3\), MeCN, reflux, 4 d; (e) 1,2-bis(2-iodoethoxy)ethane, CaCO\(_3\), water:dioxane (1:2), reflux, 4 d.

Macrocyclization reactions are extraordinarily important in the synthesis of TAC. In the reported synthetic route, both of the macrocyclization reactions were achieved by means of amide synthesis (steps d and f in Scheme III-5). Following each of these two steps, there was a reduction reaction, reducing the newly formed amides (compounds 39 and 41 in Scheme III-5) to amines (compound 40 and TAC), respectively. Here we attempted to synthesize compound 40 directly from compound 38 and TAC directly from 40, as displayed in Scheme III-6, skipping the synthesis of compounds 39 and 41.

Enlightened by the results obtained from synthesizing compound 37 in which the K\(_2\)CO\(_3\)/MeCN system worked well in producing mono-substituted anilines but poorly in forming bis-substituted anilines, these conditions were employed to prepare compound 40. In order to find an efficient way to synthesize 40, different conditions were screened,
including reactants, solvents, bases, and reaction temperatures. As shown in Table III-1, the K₂CO₃/MeCN system (entry 1) turned out to be the most efficient one among those examined. Compound 40 was realized through the reaction between 38 and 1,2-bis(2-iodoethoxy)ethane with K₂CO₃ as the base in MeCN under reflux. Although the yield (40%) of this reaction was not very high, it shortened the synthetic route of TAC and the yield was nearly equal to the overall yield (42%) of steps d (69%) and e (61%) in Scheme III-5. Therefore, to some extent, this new synthetic approach is more efficient.

The direct synthesis of TAC from 40 adopted the same approach that was applied to preparing 37 since it proved to be a good method for synthesizing bis-substituted anilines. By refluxing a mixture of 40, 1,2-bis(2-iodoethoxy)ethane, and CaCO₃ in 1,4-dioxane/water (2:1), TAC was produced in a good yield (69%), which is much higher than the combined overall yield (32%) of steps f (51%) and g (63%) in Scheme III-5.
Table III-1. Optimization of reaction conditions for the synthesis of 40.ª

<table>
<thead>
<tr>
<th>entry</th>
<th>A/B</th>
<th>Base</th>
<th>solvent</th>
<th>T</th>
<th>yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>K₂CO₃</td>
<td>MeCN</td>
<td>reflux</td>
<td>63ᵇ, 47ᶜ, 40ᵈ</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>K₂CO₃</td>
<td>MeCN</td>
<td>reflux</td>
<td>50ᵇ, 40ᶜ, 31ᵈ</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>TEA</td>
<td>MeCN</td>
<td>reflux</td>
<td>14ᶜ</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>TEA</td>
<td>MeCN</td>
<td>reflux</td>
<td>8ᶜ</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>TEA</td>
<td>toluene</td>
<td>reflux</td>
<td>35ᵇ, 29ᶜ</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>TEA</td>
<td>toluene</td>
<td>reflux</td>
<td>31ᵇ, 26ᶜ</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>K₂CO₃</td>
<td>DMF</td>
<td>120 °C</td>
<td>9ᶜ</td>
</tr>
<tr>
<td>8</td>
<td>B</td>
<td>K₂CO₃</td>
<td>DMF</td>
<td>120 °C</td>
<td>4ᶜ</td>
</tr>
</tbody>
</table>

ªReaction conditions: 38 (1.0 mmol), A/B (1.1 equiv.), base (3.0 equiv.), solvent (100 mL), 4 d, Ar. ᵇDetermined by ¹H NMR analysis.ᶜDetermined by LC-MS analysis.ᵈIsolated yield.

III.2.1 Potassium sensor 1 (KS1)

Having improved the synthesis of TAC, TAC-CHO was obtained following a literature procedure (Scheme III-7).³³ KS1 was then synthesized for K⁺ sensing in 10% yield through a condensation reaction of TAC-CHO with 2,4-dimethylpyrrole in CH₂Cl₂ in the presence of a catalytic amount of TFA at room temperature, followed by an
oxidation reaction with DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) and a subsequent chelation reaction with BF$_3$·OEt$_2$ in the presence of TEA at room temperature.

**Scheme III-7.** Synthetic route for KS1.$^a$

![Scheme III-7](image)

$^a$Reagents and conditions: (a) DMF, POCl$_3$, 0 °C to room temperature, overnight; then 70 °C, 1 h; (b) 2,4-dimethylpyrrole, TFA, CH$_2$Cl$_2$, room temperature, overnight; then DDQ, 4 h; then TEA, BF$_3$·OEt$_2$, overnight.

The fluorescence response of KS1 to K$^+$ ion was investigated in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v). Figure III-1 shows the fluorescence emission spectra of KS1 in the presence of various biologically-significant metal cations at their physiological concentrations. As expected, KS1 exhibited very high selectivity for K$^+$ over other metal cations. In the absence of cations, the free KS1 emitted a very weak fluorescence at 512 nm. Upon the addition of K$^+$ ions, the fluorescence intensity was dramatically increased, whereas the addition of any other metal cations induced little to no changes in the fluorescence intensity or spectrum of KS1.
Figure III-1. Fluorescence emission spectra of KS1 (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v) upon addition of various biological metal cations at their physiological concentrations: Li⁺ (1.0 mM), Na⁺ (150 mM), K⁺ (150 mM), Mg²⁺ (2.0 mM), Ca²⁺ (2.0 mM), Zn²⁺ (50 μM), Cu²⁺ (50 μM), Fe³⁺ (50 μM), Co²⁺ (50 μM), Ni²⁺ (50 μM). λₑₓ = 480 nm.

To further examine the efficiency of KS1 towards sensing K⁺ ions, fluorescence emission titration experiments were carried out in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v). Solutions were balanced with NaCl to maintain a constant ionic strength of 150 mM. As demonstrated in Figure III-2, gradual enhancement of the fluorescence intensity of KS1 was observed upon progressive addition of K⁺ ions. The fluorescence intensity was increased by 14-fold in the presence of 150 mM K⁺. Higher concentrations of K⁺ ions were not able to further increase the fluorescence intensity of KS1. Moreover, KS1 was capable of detecting 1 mM K⁺ with co-existence of a high concentration of Na⁺.

Since the extracellular and intracellular concentrations of potassium are ca. 4.5 mM and
150 mM$^{324,348}$ respectively, KS1 is potentially suitable for determining physiological K$^+$ levels.

![Figure III-2](image-url) Enhancements in fluorescence emission spectra of KS1 (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v) upon continuous addition of 150 mM K$^+$ ions. Ionic strength was maintained at 150 mM by addition of NaCl. $\lambda_{ex} = 480$ nm.

Moreover, the pH sensitivity of KS1 was determined in H$_2$O/MeCN (4:1, v/v) solutions with various pH values between 3.0 and 8.0. As shown in Figure III-3, no obvious changes of the fluorescence emission spectra of KS1 were observed in the pH range of 6.2-8.0. When the pH was reduced to 6.0, a small enhancement in the fluorescence intensity of KS1 was recorded. Further lower pH values induced stronger fluorescence emission until the pH of the solution reached 3.0. Significantly, these results indicate that KS1 is insensitive to pH in the physiological pH range (6.8-7.4).
Encouraged by the above results, KS1 was explored to detect the change of intracellular potassium. Figure III-4 shows images of cells taken at different time points (0, 30, 60, 90, 120 min) and the average fluorescence emission intensity of each image is shown in Figure III-5. U87MG cells were incubated with a 5 µM solution of KS1 in complete MEM medium for 10 min. As expected, cells were highly fluorescent as shown in images III-4A and III-4F, which is consistent with intracellular K\(^+\) levels high enough to trigger strong fluorescence of KS1.

It has been shown that the combination of nigericin, ouabain octahydrate, and bumetanide can effectively bring about K\(^+\) efflux of cells.\(^{40,349}\) To examine the ability of KS1 to respond to levels of K\(^+\) changes in living cells, a mixture of a solution of nigericin (5 µM), ouabain octahydrate (10 µM), and bumetanide (10 µM) in complete
MEM medium was pumped into cell chambers and images of living cells were taken at different times. With the K$^+$ depletion induced by nigericin, ouabain octahydrate, and bumetanide, the fluorescence intensity from cells gradually decreased to a very weak state (from image III-4F to III-4J). For the control experiment, after the same time, cells remained highly fluorescent (images III-4B to III-4E), exhibiting little visible change compared to their original state (image III-4A). These results indicate that KS1 is promising to be utilized to monitor intracellular K$^+$ levels of living cells.

**Figure III-4.** Images of live U87MG cells in MEM medium (A-E), and in an MEM medium solution of nigericin (5 µM), ouabain octahydrate (10 µM), and bumetanide (10 µM) (F-J), at 0 min (A, F), 30 min (B, G), 60 min (C, H), 90 min (D, I), and 120 min (E, J) after incubation with KS1 (5 µM).
Figure III-5. Average fluorescence intensity of cell images as a function of time. (A) control experiment (Images III-4A to III-4E); and (B) cells treated with nigericin, ouabain octahydrate, and bumetanide (Images III-4F to III-4J).

III.2.2 Potassium sensor 2 (KS2)

After verifying the effectiveness of the TAC group as a K⁺ recognition unit and obtaining desirable properties of KS1 as a promising K⁺ sensor for sensing intracellular potassium, a hydrophilic TAC-based fluorescent sensor was designed and synthesized for K⁺ sensing. This new potassium sensor (KS2) was also established on a BODIPY platform, but its design strategy is different from that of KS1. First, two long polyethylene glycol chains (PEG) were introduced to the BODIPY core to increase the water solubility of the dye. Second, the TAC group was conjugated to the BODIPY core via a double bond to extend the conjugation extent of the dye and increase its two-
photon absorption (2PA) cross section to make it a two-photon excitable fluorescent potassium sensor.

The synthetic route for **KS2** is shown in Scheme III-8. The synthesis of **KS2** began with PEG 550, which was used to prepare compound 42 according to a literature procedure.\(^\text{350}\) Compound 42 was then treated with 2,4-dihydroxybenzaldehyde in the presence of potassium carbonate at 130 °C, producing compound 43 in DMF. Compound 44 was synthesized via a condensation of 43 with 2,4-dimethylpyrrole in CH\(_2\)Cl\(_2\) in the presence of a catalytic amount of TFA at room temperature, followed by an oxidation reaction with DDQ and subsequent chelation reaction with BF\(_3\)·OEt\(_2\) in the presence of TEA at room temperature. Iodination of 44 with NIS (N-iodosuccinimide) in the dark at room temperature in CH\(_2\)Cl\(_2\) afforded 45 as red liquid. A Sonogashira coupling reaction was performed between 45 and phenylacetylene with Pd(PPh\(_3\))\(_4\) and CuI as catalysts at 45 °C in THF:TEA (4:1 v/v), affording 46 as a purple liquid. The target product (**KS2**) was obtained through a Knoevenagel condensation reaction between compound 46 and TAC-CHO in the presence of piperidine and glacial acetic acid in n-butanol:toluene (1:1 v/v).
Scheme III-8. Synthetic route for KS2.\textsuperscript{a}

\begin{align*}
\text{OH} & \quad \xrightarrow{\text{a}} \quad \text{TsCl, NaOH, THF, H}_2\text{O, 0 }^\circ\text{C} \quad \xrightarrow{\text{b}} \quad \text{K}_2\text{CO}_3, \text{DMF, 130 }^\circ\text{C, overnight} \quad \xrightarrow{\text{c}} \quad \text{2,4-dimethylpyrrole, TFA, CH}_2\text{Cl}_2, \text{room temperature, overnight; then DDQ, 4 h; then TEA, BF}_3\text{OEt}_2, \text{overnight} \quad \xrightarrow{\text{d}} \quad \text{NIS, CH}_2\text{Cl}_2, \text{in the dark, room temperature, 5 h} \quad \xrightarrow{\text{e}} \quad \text{phenylacetylene, CuI, Pd(PPh}_3)_4, \text{THF:TEA (4:1), in the dark, 45 }^\circ\text{C, 16 h} \quad \xrightarrow{\text{f}} \quad \text{TAC-CHO, piperidine, glacial acetic acid, n-butanol:toluene (1:1), reflux, overnight.}
\end{align*}

\textsuperscript{a}Reagents and conditions: (a) TsCl, NaOH, THF, H\textsubscript{2}O, 0 °C; (b) K\textsubscript{2}CO\textsubscript{3}, DMF, 130 °C, overnight; (c) 2,4-dimethylpyrrole, TFA, CH\textsubscript{2}Cl\textsubscript{2}, room temperature, overnight; then DDQ, 4 h; then TEA, BF\textsubscript{3}OEt\textsubscript{2}, overnight; (d) NIS, CH\textsubscript{2}Cl\textsubscript{2}, in the dark, room temperature, 5 h; (e) phenylacetylene, CuI, Pd(PPh\textsubscript{3})\textsubscript{4}, THF:TEA (4:1), in the dark, 45 °C, 16 h; (f) TAC-CHO, piperidine, glacial acetic acid, n-butanol:toluene (1:1), reflux, overnight.
The K⁺ sensing ability of KS2 was examined in 5 mM HEPES buffer (pH 7.2)/MeCN (3:1, v/v). The fluorescence emission spectra of KS2, in the presence of various biological metal cations at their physiological concentrations, are shown in Figure III-6. The free KS2 emitted almost no fluorescence in the absence of cations. Upon the addition of K⁺, a striking red fluorescence with a maximum emission wavelength at 644 nm was immediately observed. However, the addition of other metal cations brought about no considerable change in the fluorescence spectra of KS2. Therefore, similar to KS1, KS2 presented very high selectivity for K⁺ ion over other biologically-relevant cations.

![Fluorescence emission spectra of KS2](image)

**Figure III-6.** Fluorescence emission spectra of KS2 (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (3:1, v/v) upon addition of various biologically-important metal cations at their physiological concentrations: Li⁺ (1.0 mM), Na⁺ (150 mM), K⁺ (150 mM), Mg²⁺ (2.0 mM), Ca²⁺ (2.0 mM), Zn²⁺ (50 μM), Cu²⁺ (50 μM), Fe³⁺ (50 μM), Co²⁺ (50 μM), Ni²⁺ (50 μM). λₑₓ = 600 nm.
K⁺ titration experiments were carried out to investigate the efficiency of KS2 towards K⁺ sensing in 5 mM HEPES buffer (pH 7.2)/MeCN (3:1, v/v) solutions. To maintain a constant ionic strength of 150 mM, sample solutions were balanced with NaCl. UV-vis absorption spectroscopy was first used to conduct titration experiments. As displayed in Figure III-7, the solution of KS2 without adding K⁺ ions exhibited an absorption peak at 633 nm. Upon the progressive addition of K⁺ ions, the absorption maximum wavelength of KS2 gradually blue-shifted to 617 nm. At the same time, the absorption intensity of the sample was moderately increased. The behavior of KS2 in absorption titration experiments was different from that of KS1, which demonstrated no change in the absorption spectra upon the addition of K⁺ ions.

![Figure III-7](image)

**Figure III-7.** Absorption spectra of KS2 (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (3:1, v/v) upon progressive addition of K⁺.
To further examine the $\text{K}^+$ sensing ability of $\text{KS2}$, fluorescence emission titration experiments were performed in 5 mM HEPES buffer (pH 7.2)/MeCN (3:1, v/v). Solutions were balanced with NaCl to maintain a constant ionic strength. Figure III-8 shows the fluorescence emission spectra of $\text{KS2}$ in the absence and presence of different concentrations of $\text{K}^+$ ions. With increased concentration of $\text{K}^+$, the fluorescence emission intensity was enhanced accordingly until the concentration of $\text{K}^+$ reached 20 mM. In the presence of 20 mM $\text{K}^+$, the fluorescence intensity of $\text{KS2}$ was increased by 29-fold, further addition of $\text{K}^+$ ions resulted in no additional change in the fluorescence spectra of $\text{KS2}$. In addition, $\text{KS2}$ was capable of detecting 0.1 mM $\text{K}^+$ with co-existence of a high concentration of Na$^+$. 

Figure III-8. Fluorescence emission spectra of $\text{KS2}$ (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (3:1, v/v) upon progressive addition of $\text{K}^+$. $\lambda_{\text{ex}} = 600$ nm.
Also, the pH sensitivity of KS2 was investigated at various pH between 3.0 and 8.0 in H₂O/MeCN (3:1, v/v) solutions. Figure III-9 shows the fluorescence emission spectra of KS2 at pH 3.0, 4.0, 5.0, 5.8, 6.0, 6.5, 7.0, and 8.0. In the solutions of KS2 with pH values between 6.0 and 8.0, no considerable fluorescence emission signal was detected. However, when the pH of a sample was lower than 6.0, a characteristic fluorescence signal of KS2 was recorded. As the pH decreased, the fluorescence emission intensity of KS2 became stronger until the pH of the solution reached 3.0. These results suggest that KS2, similar to KS1, is not sensitive to pH in physiological ranges.

**Figure III-9.** Fluorescence emission spectra of KS2 (10 μM) in H₂O/MeCN (3:1, v/v) as a function of pH. λₑₓ = 600 nm.
Moreover, 2PA experiments were carried out to determine the 2PA cross section of KS2. The open-aperture Z-scan method\textsuperscript{351} was used for direct 2PA cross section measurements over a broad spectral region, 940-1260 nm, with a single laser beam as the irradiation light source. Figure III-10 shows the one-photon and two-photon absorption spectra of KS2 in DCM. The maximum absorption wavelength in the 1PA spectrum is 637 nm. For 2PA, KS2 exhibited a maximum 2PA cross section ($\delta_{2PA}$) of $\approx 500$ GM [1 GM (Göpper Meyer) = $10^{-50}$ cm$^4$ s$^{-1}$ photon$^{-1}$] at 940 nm. Compared to the data of reported for 2PA fluorescent probes for other metal cations (no 2PA fluorescent probe for K$^+$ has been reported so far), the high 2PA cross section, long NIR absorption wavelength, and red-emitting fluorescence of KS2 make it an excellent prospective 2PA fluorescent K$^+$ sensor.

![Figure III-10. One-photon absorption (red) and 2PA (green circles) spectra of KS2.](image)

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III.3 Conclusion

In summary, the synthesis of TAC was successfully optimized. The synthetic route was shortened, and the overall yield was enhanced from 3.6% to 19.5%. Two BODIPY-based fluorescent K⁺-selective sensors, KS1 and KS2, were synthesized and characterized. Both exhibited excellent selectivity toward K⁺ ion over other physiological metal cations and high sensitivity for K⁺ sensing. In comparison to KS2, KS1 was able to determine K⁺ at higher concentration. In contrast to that of KS1, the absorption profile of KS2 can also be affected by the addition of K⁺ ions. Moreover, both dyes were pH insensitive in the physiological pH range. KS1 and KS2 were investigated to cell imaging. Confocal microscopy experiments demonstrated that they were both capable of sensing K⁺ within living cells. Furthermore, 2PA determination revealed that KS2 possessed a desirable 2PA cross section of 500 GM at 940 nm. Therefore, KS2 is a promising two-photon red-emitting fluorescent sensor for K⁺ ions.

III.4 Experimental

III.4.1 General

All reagents and solvents were purchased from commercial suppliers and used without further purification. ¹H and ¹³C NMR spectra were carried out on a Bruker AVANCE spectrometer (400 MHz). LC-MS analysis was recorded on an Agilent 1260 Infinity – 6230 TOF LC/MS. HR-MS analyses were performed at the Department of Chemistry, University of Florida. Flash column chromatography was performed on a
CombiFlash® Rf+ automated flash chromatography using RediSep Rf Gold® normal-phase HP silica columns. UV-vis absorption spectra were recorded on an Agilent 8453 spectrophotometer. Fluorescence emission spectra were measured using an FLS980 fluorescence spectrometer. Fluorescence microscopy images were recorded on an Olympus IX-81 DSU microscope equipped with a Hamamatsu EM-CCD C9100 digital camera using a FITC filter cube (Ex: 477/50; DM: 507; Em: 536/40) and a 20x (Olympus LUCplanFLN 20x, N.A. = 0.45) objective lens. Two-photon absorption measurements were performed using a Coherent Legend Elite system (amplified Ti:Sapphire system) and an optical parametric generator/amplifier (Coherent OPerA Solo), providing laser pulses of 100 fs (FWHM) duration with 1 kHz repetition rate. A single laser beam from the first OPA was used for direct 2PA cross section measurements by the open-aperture Z-scan method. The 2PA measurements were performed in 1 mm spectrofluorometric quartz cuvette with concentration of $3 \times 10^{-3} \text{ M} \leq C \leq 4 \times 10^{-3} \text{ M}$ at room temperature.

III.4.2 Synthesis

*Synthesis of compound 35.* To a mixture of 5-methyl-2-nitrophenol (7.66 g, 50 mmol), 1,2-dibromoethane (47.0 g, 250 mmol), $\text{K}_2\text{CO}_3$ (13.8 g, 100 mmol) was added 50 mL degassed anhydrous DMF under argon atmosphere. The reaction mixture was heated to reflux and stirred for 2 h. After cooling to room temperature, the solvent was removed under reduced pressure. Then the residue was added 100 mL $\text{CH}_2\text{Cl}_2$ and filtered. The filtrate was washed with 1N NaOH (100 mL × 3) and brine (100 mL × 2). The organic layer was dried over MgSO$_4$, then filtered and condensed to give a brown
liquid, which crystalized after cooling to room temperature to provide 35 as a yellow solid (10.5 g, 81%). The NMR data are in accord with the literature values.  

*Synthesis of compound 37.* A mixture of CaCO$_3$ (2.0 g, 20 mmol) and KI (3.32 g, 20 mmol) in 30 mL distilled water was heated to reflux under argon atmosphere. Then a solution of 35 (7.80 g, 30 mmol) and 36 (1.67 g, 10 mmol) in 20 mL degased 1,4-dioxane was added. The resulting reaction mixture was stirred under reflux for 16 h. More 35 (2.60 g, 10 mmol) was added and the reaction mixture was refluxed for another 20 h. After cooling to room temperature, the mixture was filtered and the filtrate was condensed to 20 mL. Then the filtrate was extracted with CH$_2$Cl$_2$ (20 mL × 3). The organic layer was dried over MgSO$_4$, filtered and concentrated *in vacuo.* Purification by flash column chromatography using CH$_2$Cl$_2$ provided 37 as yellow solid (4.76 g, 91%). The NMR data are in accord with the literature values.  

*Synthesis of compound 38.* To a solution of 37 (4.73 g, 9.0 mmol) in 25 mL anhydrous THF was added activated carbon (0.27 g) and a pre-dissolved 25 mL methanol solution of FeCl$_3$·6H$_2$O (0.49 g, 1.8 mmol) under argon atmosphere. The resulting reaction mixture was heated to reflux and then hydrazine monohydrate (9.0 g, 180 mmol) was added dropwise. After refluxing for 20 h, the reaction mixture was cooled to room temperature and filtered. The residue was washed with 10 mL THF and the combined filtrate was condensed. Then the crude mixture was dissolved into 50 mL CH$_2$Cl$_2$ and washed with brine (50 mL × 2). The organic layer was dried over MgSO$_4$, filtered and condensed to give 38 as a light yellow liquid (4.03 g, yield 96%). The NMR data were in accord with the literature values.
Synthesis of compound 40. A mixture of K₂CO₃ (0.42 g, 3.0 mmol) and 50 mL degased acetonitrile was heated to reflux under argon atmosphere. Then a solution of 38 (0.47 g, 1.0 mmol) and 1,2-bis(2-iodothoxy)ethane (0.41 g, 1.1 mmol) in 50 mL degased acetonitrile was added dropwise over 4 h. The resulting reaction mixture was stirred under reflux and the reaction progress was monitored by LC-MS. After four days, the LC-MS results indicated that the reaction was complete. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was extracted with CH₂Cl₂/H₂O and washed three times with brine. The organic layer was dried over MgSO₄, then filtered and condensed. The crude product was further purified by flash column chromatography using 2.5% methanol/CH₂Cl₂ as eluent to give 40 as an off-white solid (0.23 g, 40%). The NMR data are in accord with the literature values.

Synthesis of TAC. A mixture of CaCO₃ (0.30 g, 3.0 mmol) and 50 mL distilled water was heated to reflux under argon atmosphere followed by the addition of 50 mL degased 1,4-dioxane. Then a solution of 40 (0.58 g, 1.0 mmol) and 1,2-bis(2-iodothoxy)ethane (0.37 g, 1.0 mmol) in 50 mL degased 1,4-dioxane was added dropwise over 4 h. The resulting reaction mixture was stirred under reflux and the reaction progress was monitored by LC-MS. 4 days later, the LC-MS results indicated that the reaction completed. After cooling to room temperature, the mixture was filtered and the filtrate was condensed to 50 mL. Then the filtrate was extracted with CH₂Cl₂ (50 mL × 3). The organic layer was dried over MgSO₄, filtered and concentrated in vacuo. Purification by flash column chromatography using 3.0% methanol/CH₂Cl₂ provided TAC as a colorless foamy solid (0.48 g, 69%). The NMR data are in accord with the literature values.
Synthesis of KS1. To a solution of TAC-CHO (0.36 g, 0.5 mmol) and 2,4-dimethylpyrrole (0.11 g, 1.1 mmol) in CH$_2$Cl$_2$ (50 mL) was added 2 drops of trifluoroacetic acid. The mixture was stirred at room temperature overnight, then a mixture of 2,3-dichloro-5,6-dicyano-p-benzoquinone (0.14 g, 0.6 mmol) in CH$_2$Cl$_2$ (10 mL) was added. The reaction mixture was stirred continuously for another 4 h. After the addition of 3 mL triethylamine, 3 mL BF$_3$·OEt$_2$ was added dropwise to the mixture. The mixture was kept stirring at room temperature overnight, then filtered through a celite pad. The residue was washed with 20 mL CH$_2$Cl$_2$ and the combined filtrate was rotary evaporated to dryness. The residue was dissolved in 30 mL CH$_2$Cl$_2$ and the solution was washed with 30 mL 5% aqueous NaHCO$_3$ solution followed with water (30 mL × 2). The organic layer was dried over anhydrous MgSO$_4$, filtered and then evaporated in vacuo. The crude product was purified by flash column chromatography using 2.0% methanol/CH$_2$Cl$_2$ as the eluent to give the desired KS1 as a red solid (0.048 g, 10%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 7.25 (d, $J = 8.0$ Hz, 1H), 6.87 (d, $J = 7.7$ Hz, 2H), 6.78 (d, $J = 1.7$ Hz, 1H), 6.74 (dd, $J = 8.0$, 1.7 Hz, 1H), 6.66 (d, $J = 7.7$ Hz, 2H), 6.54 (s, 2H), 5.97 (s, 2H), 4.15 – 3.28 (m, 39H), 2.54 (s, 6H), 2.24 (s, 6H), 1.45 (s, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 155.39, 153.28, 152.56, 143.11, 141.88, 139.08, 138.33, 132.63, 131.75, 128.32, 121.97, 121.54, 121.38, 121.20, 113.94, 113.52, 71.37, 71.23, 70.17, 68.28, 66.96, 59.25, 53.98, 52.97, 21.21, 14.70, 14.45. HRMS (MALDI-TOF, m/z): calcd for C$_{52}$H$_{68}$N$_5$O$_8$BF$_2$ ([M+H]$^+$) 940.5210; found 940.5233.

Synthesis of compound 42. Sodium hydroxide (7.8 g, 196.0 mmol) in 40 mL water and PEG 550 (38.0 g, 70.0 mmol) in 40 mL THF were mixed and cooled in an ice-
water bath with stirring and p-toluenesulfonyl chloride (24.0 g, 126.0 mmol) in 40 mL THF was added dropwise over 2 h. The reaction mixture was stirred for an additional 2 h at 5 °C, poured into 100 mL ice-water and extracted with CH₂Cl₂ (50 mL × 2). The combined organic extracts were washed with water (50 mL × 2) and brine (50 mL × 2), dried over MgSO₄ and the solvent was removed in vacuo to yield 42 as a colorless liquid (40.7 g, 99%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.78 (d, J = 8.6 Hz, 2H), 7.33 (d, J = 8.6 Hz, 2H), 4.17 – 4.11 (m, 2H), 3.69 – 3.50 (m, 48H), 3.36 (s, 3H), 2.43 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 144.87, 133.13, 129.92, 128.08, 72.03, 70.84, 70.70, 70.67, 70.61, 69.34, 68.77, 59.13, 21.74.

Synthesis of compound 43. To a mixture of 42 (17.6 g, 25 mmol), 2,4-dihydroxybenzaldehyde (1.38 g, 10 mmol), K₂CO₃ (3.04 g, 22 mmol) was added 50 mL degased anhydrous DMF under argon atmosphere. The reaction mixture was heated to 130 °C and stirred overnight. After cooling to room temperature, the solvent was removed under reduced pressure. Then the residue was added 100 mL CH₂Cl₂ and filtered. The filtrate was washed with brine (100 mL × 2). The organic layer was dried over MgSO₄, then filtered and condensed under vacuum. The crude product was further purified by flash column chromatography using 3.0% methanol/CH₂Cl₂ as eluent to give compound 43 as a colorless liquid (10.7 g, 89%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 10.32 (s, 1H), 7.79 (d, J = 8.7 Hz, 1H), 6.55 (dd, J = 8.7, 2.2 Hz, 1H), 6.48 (d, J = 2.2 Hz, 1H), 4.18 (dd, J = 9.8, 5.8 Hz, 4H), 3.91 – 3.84 (m, 4H), 3.76 – 3.57 (m, 87H), 3.54 (dd, J = 6.1, 3.3 Hz, 4H), 3.37 (s, 6H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 188.26, 165.28,
Synthesis of compound 44. To a solution of 43 (6.01 g, 5.0 mmol) and 2,4-dimethylpyrrole (1.05 g, 11.0 mmol) in 500 mL CH₂Cl₂ was added 5 drops of trifluoroacetic acid. The mixture was stirred at room temperature overnight, then a mixture of 2,3-dichloro-5,6-dicyano-p-benzoquinone (1.36 g, 6.0 mmol) in 100 mL CH₂Cl₂ was added. The reaction mixture was stirred continuously for another 4 h. After the addition of 30 mL triethylamine, 30 mL BF₃·OEt₂ was added dropwise to the mixture. The mixture was kept stirring at room temperature overnight, then filtered through a celite pad. The residue was washed with 200 mL CH₂Cl₂ and the combined filtrate was rotary evaporated to dryness. The residue was dissolved in 300 mL CH₂Cl₂ and the solution was washed with 300 mL 5% aqueous NaHCO₃ solution followed with water (300 mL × 2). The organic layer was dried over anhydrous MgSO₄, filtered and then evaporated in vacuo. The crude product was purified by flash column chromatography using 2.5% methanol/CH₂Cl₂ as the eluent to give 44 as a dark red liquid (1.53 g, 22%).

**¹H NMR (400 MHz, CDCl₃):** δ (ppm) 6.98 (d, J = 8.2 Hz, 1H), 6.62 – 6.52 (m, 2H), 5.93 (s, 2H), 4.17 – 4.12 (m, 2H), 4.05 – 4.01 (m, 2H), 3.89 – 3.86 (m, 2H), 3.75 – 3.50 (m, 125H), 3.42 – 3.34 (m, 10H), 2.51 (s, 6H), 1.47 (s, 6H). **¹³C NMR (101 MHz, CDCl₃):** δ (ppm) 161.09, 156.72, 154.74, 142.85, 139.12, 132.12, 130.00, 120.78, 116.66, 106.32, 100.49, 72.46, 72.00, 71.06, 70.94, 70.59, 70.55, 70.43, 70.28, 69.80, 69.15, 69.09, 67.67, 61.65, 59.12, 14.64, 14.17.
**Synthesis of compound 45.** To a solution of compound 44 (1.42 g, 1.0 mmol) in 10 mL anhydrous CH$_2$Cl$_2$ was added N-iodosuccinimide (0.45 g, 2.0 mmol), and the resulting mixture was stirred for 5 h in the dark at room temperature. Then the reaction mixture was diluted with 20 mL CH$_2$Cl$_2$ and washed with brine (50 mL × 2). The organic layer was dried over MgSO$_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography using 3.0% methanol/CH$_2$Cl$_2$ as the eluent to give 45 as a red liquid (1.19 g, 71%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 6.93 (d, $J$ = 8.1 Hz, 1H), 6.64 – 6.56 (m, 2H), 4.19 – 4.15 (m, 2H), 4.06 – 4.02 (m, 2H), 3.90 – 3.88 (m, 2H), 3.73 – 3.52 (m, 132H), 3.40 – 3.31 (m, 10H), 2.60 (s, 6H), 1.50 (s, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 161.53, 156.68, 156.02, 145.10, 139.17, 132.01, 129.82, 116.26, 106.56, 100.66, 85.17, 72.01, 71.08, 70.97, 70.63, 70.47, 69.77, 69.06, 67.77, 59.13, 16.62, 16.05.

**Synthesis of compound 46.** To a mixture of compound 45 (1.67 g, 1.0 mmol), phenylacetylene (0.31 g, 3.0 mmol), Cul (0.038 g, 0.2 mmol), and Pd(PPh$_3$)$_4$ (0.12 g, 0.1 mmol) was added 10 mL degassed solvent mixture THF:triethylamine (4:1) under argon atmosphere. The resulting mixture was heated to 45 °C and stirred for 16 h in the dark. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was extracted with CH$_2$Cl$_2$/brine. The organic layer was dried over MgSO$_4$, then filtered and condensed. The crude mixture was purified by flash column chromatography using 5.0% methanol/CH$_2$Cl$_2$ to provide 46 as a purple liquid (1.25 g, 77%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 7.44 (m, 4H), 7.34 – 7.27 (m, 6H), 6.99 (d, $J$ = 8.2 Hz, 1H), 6.63 (m, 2H), 4.20 – 4.16 (m, 2H), 4.06 (t, $J$ = 4.6 Hz, 2H), 3.92
– 3.88 (m, 2H), 3.77 – 3.48 (m, 106H), 3.43 – 3.35 (m, 10H), 2.68 (s, 6H), 1.64 (s, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 161.37, 157.69, 156.64, 143.73, 140.12, 131.85, 131.30, 129.81, 128.37, 128.10, 123.44, 115.93, 115.66, 106.39, 100.56, 96.31, 81.77, 72.60, 71.93, 71.07, 70.89, 70.57, 70.37, 69.70, 69.01, 67.67, 59.03, 13.66, 13.09.

_Synthesis of KS$_2$. To a solution of 46 (0.16 g, 0.1 mmol) and TAC-CHO (0.072 g, 0.1 mmol) in 10 mL solvent mixture n-butanol:toluene (1:1) was added 0.24 mL piperidine and 0.20 mL glacial acetic acid. The resulting mixture was refluxed, and the water formed during the reaction was removed azeotropically by heating overnight in a Dean-Stark apparatus. Then the solvents were removed under vacuum, and the residue was purified by flash column chromatography using 10% methanol/CH$_2$Cl$_2$. The desired product KS$_2$ was obtained as green gum (0.024 g, 10%). $^1$H NMR (400 MHz, CD$_2$Cl$_2$): $\delta$ (ppm) 8.35 (m, 1H), 7.53 – 7.47 (m, 4H), 7.34 (m, 6H), 7.18 – 7.06 (m, 5H), 6.86 (d, $J$ = 7.9 Hz, 2H), 6.73 – 6.62 (m, 6H), 4.26 – 4.03 (m, 16H), 3.77 – 3.05 (m, 197H), 2.72 (s, 3H), 2.22 (s, 6H), 1.72 (d, 6H). $^{13}$C NMR (101 MHz, CD$_2$Cl$_2$): $\delta$ (ppm) 162.00, 157.38, 153.70, 153.34, 152.72, 151.77, 145.88, 138.88, 137.75, 135.76, 133.18, 131.71, 131.67, 131.45, 131.40, 130.64, 129.06, 128.91, 128.73, 128.62, 125.18, 124.11, 123.96, 122.87, 122.57, 121.99, 121.48, 116.18, 114.55, 113.74, 112.68, 107.05, 100.94, 98.47, 96.91, 84.99, 84.41, 82.45, 72.40, 71.58, 71.45, 71.32, 71.01, 70.88, 70.51, 70.10, 69.77, 69.53, 69.26, 68.49, 68.28, 67.70, 67.15, 59.13, 21.25, 14.27, 13.40, 13.20.
III.4.3 Cell culture for imaging

U87MG cells were seeded on poly-D-lysine functionalized 40 mm coverslips in Petri dishes and incubated in complete MEM medium for 2 d. **KS1** was first dissolved in DMSO to make a stock solution of 2.5 mM, and then diluted with complete MEM medium into 5 μM. Then cells were incubated with 5 μM of **KS1** for 10 min. The coverslips were then washed with DPBS and mounted onto a bioptics live cell imaging chamber. For the potassium depletion experiment, a mixture solution of nigericin (5 μM), ouabain octahydrate (10 μM), and bumetanide (10 μM) in complete MEM medium was pumped into cell chambers. For the control experiment, only complete MEM medium was pumped into cell chambers. Cells were kept at 37 °C and imaged with using an Olympus IX-81 DSU microscope every 30 min.
CHAPTER IV: A FLUORESCENCE TURN-ON SENSOR FOR Fe$^{3+}$

IV.1 Introduction

As the most common element on the earth by mass and the most abundant transition metal in human body, iron is omnipresent in the universe, from the core of the earth to cells of living organisms.\textsuperscript{352} As an essential element for life, iron plays indispensable roles in a wide range of chemical and biological processes at the cellular level and holds a central position in virtually all organisms.\textsuperscript{353-354} Both its deficiency and overloading can lead to a variety of diseases.\textsuperscript{355-356} With the identification of iron as a body constituent in the past century, the importance of iron for mammalian health became clear. The relationship between adequate iron ingest and prevention of certain diseases was well realized.\textsuperscript{357} Iron performs a major role in electron transfer, oxygen uptake, oxygen metabolism, enzymatic catalysis, and transcriptional regulation.\textsuperscript{358-366} On the other hand, iron is capable of promoting oxidation of proteins, lipids, and other cellular components. Therefore, iron will be harmful if its level exceeds the capacity of organisms to securely consume it. It has been reported that excessive Fe$^{3+}$ ions within human body are associated with increased incidence of dysfunction of organs, such as pancreas, liver, and heart, and development of certain diseases including hepatitis, hemochromatosis, and cancers.\textsuperscript{367-370} Moreover, some recent research has suggested that Fe$^{3+}$ ions are also involved in the underlying mechanisms of many neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease.\textsuperscript{371-374} Therefore, the determination of this biologically significant ion in physiological
environment has important consequences and the development of an efficient and reliable approach for the analysis of Fe\(^{3+}\) in biological samples is of much importance and urgency in biological and environmental concerns.

Owing to the advantages of fluorescence technique in comparison to other analytical methods, as introduced in Section I.2, considerable efforts have been devoted to developing fluorescent probes for Fe\(^{3+}\). However, the fluorescent indication of these Fe\(^{3+}\) probes is mostly signaled by fluorescence quenching due to the paramagnetic nature of Fe\(^{3+}\) ion,\(^{375-379}\) which seriously limits the biological applications of the probes. Probe 47 is an example of such probes.\(^{379}\) In the absence of Fe\(^{3+}\) ion, probe 47 emits strong fluorescence with a maximum wavelength at 732 nm. Upon the addition of Fe\(^{3+}\), the fluorescence of 47 is dramatically quenched. In the presence of 100 equiv. of Fe\(^{3+}\) ions, almost no fluorescence could be observed from probe 47.

**Scheme IV-1.** Structures of compounds 47 and 48.

In the past decade, a new type of rhodamine-based fluorescence “turn-on” sensors for Fe\(^{3+}\) ion was developed.\(^{74,380-382}\) The “off-on” fluorescence responses of these dyes are generated from Fe\(^{3+}\)-induced transformation from the spirocyclic form,
which is not fluorescent, to the highly fluorescent ring-open form of the rhodamine platform. Probe 48 is a rhodamine 6G based “off-on” fluorescent probe for detecting Fe$^{3+}$.

In the absence of Fe$^{3+}$, probe 48 displays very weak fluorescence, whereas upon the addition of Fe$^{3+}$ to 48 in an aqueous solution, Fe$^{3+}$-catalyzed hydrolysis of rhodamine 6G Schiff base occurs, which brings about a strong green fluorescence emission band at 551 nm, corresponding to the spirolactam ring-opened 48. Probe 48 shows high sensitivity and selectivity towards Fe$^{3+}$ sensing. However, it is not suitable for being explored to sense the dynamic changes of Fe$^{3+}$ ion concentration due to the irreversible nature of the catalytic hydrolysis reaction.

**Scheme IV-2.** Structures of compounds 49 and 50.

Recently, an anthracene-bearing bisdiene macrocycle (49) was reported as a “off-on” fluorescent Fe$^{3+}$ sensor. Probe 49 exhibits reversible fluorescent response when interacting with Fe$^{3+}$ ions. In an aqueous solution of 49 without Fe$^{3+}$, very weak fluorescence can be observed. After 4 equiv. of Fe$^{3+}$ is added, the Fe$^{3+}$-triggered
fluorescence emission enhancement factor is around 5.6-fold according to the emission at 425 nm. After that, the fluorescent emission of probe 49 stabilizes gradually. Upon adding N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), which is a strong transition metal chelator, the fluorescent emission spectrum of 49 complexed with Fe$^{3+}$ is recovered back to its original state.

It’s worth to note here that Bricks et al. synthesized a size-restricted dithiaaza-oxa macrocycle as a recognition unit for Fe$^{3+}$.

Probe 50 was obtained through connecting the dithiaaza-oxa macrocycle to a BODIPY fluorophore via a phenyl linker. The fluorescence response of probe 50 towards Fe$^{3+}$ is established on a PET process. As a fluorescence “off-on” probe for Fe$^{3+}$, 50 displays a moderately selective fluorescence response to Fe$^{3+}$ ion over the other tested metal cations. Although its sensitivity for Fe$^{3+}$ sensing is not very high, the design of probe 50 provides an effective prototype for developing PET-based fluorescent Fe$^{3+}$ sensors via connecting a Fe$^{3+}$-selective macrocycle to a fluorophore.

Although various fluorescent sensors were designed and synthesized for Fe$^{3+}$ detection, so far, none of them is able to meet all the requirements for biological application. Therefore, fluorescence “turn-on” sensors, which have high selectivity, sensitivity, and reversibility, and are biocompatible enough to be used in physiological environment, are still in great need.

The goal of this work here is to attempt to develop a fluorescent Fe$^{3+}$ sensor possessing those above-mentioned required properties. A novel 1,10-diaza-18-crown-6 based cryptand will be synthesized as the Fe$^{3+}$ recognition unit. Then the cryptand will
be conjugated to a BODIPY fluorophore to produce a fluorescent Fe$^{3+}$-selective sensor (FeS) which is potential to be used in biological imaging.

**IV.2 Results and Discussion**

FeS was prepared in 6 steps, beginning with 2-(2-Methoxyethoxy)aniline and 1,10-diaza-18-crown-6 (Scheme IV-3). 2-(2-Methoxyethoxy)aniline reacted with 2-chloroethanol in the presence of CaCO$_3$ and KI under reflux in water to provide compound 51. Then compound 51 was formylated by POCl$_3$ via a Vilsmeier-Haack reaction in DMF to produce compound 52. At the same time, the hydroxyl groups of compound 51 were chlorinated into chloro groups by POCl$_3$. Compound 53 was obtained from the reaction between 1,10-diaza-18-crown-6 and o-acetylsalicyloyl chloride with TEA as the base in CH$_2$Cl$_2$. Then the acetyl groups of compound 53 were hydrolyzed by refluxing with a mixture of NaHCO$_3$ and NH$_4$OAc in aqueous methanol (1:1) and compound 54 was produced. Compound 55 was prepared through a macrocyclization reaction between compounds 52 and 54 in the presence of KI and Cs$_2$CO$_3$ in a highly diluted DMF solution at 105 °C. The desired final product FeS in a 13% yield was achieved through a trifluoroacetic acid catalyzed condensation reaction of compound 55 with 2,4-dimethylpyrrole, followed by an oxidation reaction with DDQ and then a chelation reaction with BF$_3$·OEt$_2$ in the presence of TEA.
Scheme IV-3. Synthetic route of FeS.\textsuperscript{a}

The selectivity of FeS for Fe\textsuperscript{3+} was first investigated through UV-vis spectroscopy by adding various metal cations, including Li\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}, Rb\textsuperscript{+}, Cs\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+}, Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, Ag\textsuperscript{+}, Cd\textsuperscript{2+}, Pb\textsuperscript{2+}, Nd\textsuperscript{3+}, Sm\textsuperscript{3+}, and Er\textsuperscript{3+}, to a solution of FeS in H\textsubscript{2}O-CH\textsubscript{3}CN (9:1, v/v). As shown in Figure IV-1, the free FeS displayed an
absorption band at 499 nm. Upon adding Fe$^{3+}$ ions, the absorption intensity of FeS decreased, while no considerable change was induced to the absorption spectra by the addition of other cations except Cr$^{3+}$ and Hg$^{2+}$ ions. The addition of Cr$^{3+}$ ions resulted in a slight decrease to the absorption intensity of FeS, which however was much weaker than that resulted from Fe$^{3+}$ ions. Besides Fe$^{3+}$ and Cr$^{3+}$ ions, Hg$^{2+}$ also brought about some changes to the absorption spectra of FeS, including a small red-shift to its maximum absorption wavelength and at the same time a little reduction to its absorption intensity. These results indicate that FeS shows selectivity for detecting Fe$^{3+}$ over other metal cations.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{absorption_spectra.png}
\caption{UV-vis absorption spectra of FeS (7 µM) in H$_2$O-CH$_3$CN (9:1, v/v) upon addition of 100 equiv. of various metal cations.}
\end{figure}
In addition, as displayed in Figure IV-2, upon the addition of the first 4 equiv. of Fe$^{3+}$ ions, the absorption intensity of FeS decreased gradually. After that, further addition of Fe$^{3+}$ resulted in no more change to the absorption spectra of FeS.

![Absorbance vs Wavelength](image)

**Figure IV-2.** UV-vis spectral changes of FeS (7 µM) in H$_2$O-CH$_3$CN (9:1, v/v) observed upon addition of 1-700 equiv. of Fe$^{3+}$ ions.

Since fluorescent emission spectroscopy is a more useful tool than absorption spectroscopy in sensing ions, the selectivity of FeS towards Fe$^{3+}$ detection was further investigated with fluorescent emission spectroscopy. The fluorescent properties of FeS in the presence of the above-mentioned metal cations were studied in a H$_2$O-CH$_3$CN (9:1, v/v) solution. In good agreement with the findings observed in absorption experiments, FeS exhibited emission responses only to Fe$^{3+}$, Cr$^{3+}$, and Hg$^{2+}$ ions.
Figure IV-3 shows visual fluorescence responses of FeS with excitation at 365 nm using a hand-held UV lamp. The free FeS emitted very weak fluorescence in the absence of metal cations. However, different fluorescence responses were induced by the addition of various metal cations. When Fe$^{3+}$ ions were introduced to a solution of FeS, a striking yellow-green fluorescence emerged very fast, which is a characteristic fluorescent emission of BODIPY dye. Weaker fluorescent emission of FeS was observed in the presence of Cr$^{3+}$ and Hg$^{2+}$ ions. The addition of other metal cations of interest induced no apparent fluorescence change.

**Figure IV-3.** Visual fluorescence responses of FeS (7 µM) in H$_2$O-CH$_3$CN (9:1, v/v) upon addition of 100 equiv. of various metal cations with excitation at 365 nm using a hand-held UV lamp.

Figure IV-4 shows detailed changes in the fluorescent emission spectra of FeS resulted from the addition of various metal cations. Corresponding to the results of visual fluorescence response shown in Figure IV-3, FeS with Fe$^{3+}$ exhibited an intense emission band at 512 nm. The fluorescence emission intensity was enhanced by 28-fold in the presence of 100 equiv. of Fe$^{3+}$ ions. In contrast, the addition of 100 equiv. of Cr$^{3+}$ and Hg$^{2+}$ ions brought about only 4-fold and 3-fold enhancement in the fluorescence emission intensity of FeS, respectively. In agreement with the forgoing fact that Hg$^{2+}$ ions resulted in a small red-shift to the maximum absorption wavelength of FeS, the
addition of Hg\(^{2+}\) ions also shifted the maximum fluorescence emission wavelength of FeS from 512 nm to 522 nm besides increasing the fluorescence intensity of FeS. Little to no changes in fluorescence emission spectra of FeS was recorded upon the addition of 100 equiv. of other metal cations. These results demonstrated the desirable selectivity of FeS to Fe\(^{3+}\) ion.

**Figure IV-4.** Fluorescence emission spectra of FeS (7 µM) in H\(_2\)O-CH\(_3\)CN (9:1, v/v) upon addition of 100 equiv. of various metal cations. Inset: Emission intensity of fluorescence emission triggered by different metal cations. 1, blank; 2, Li\(^+\); 3, Na\(^+\); 4, K\(^+\); 5, Rb\(^+\); 6, Cs\(^+\); 7, Mg\(^{2+}\); 8, Ca\(^{2+}\); 9, Sr\(^{2+}\); 10, Ba\(^{2+}\); 11, Cr\(^{3+}\); 12, Mn\(^{2+}\); 13, Fe\(^{2+}\); 14, Fe\(^{3+}\); 15, Co\(^{2+}\); 16, Ni\(^{2+}\); 17, Cu\(^{2+}\); 18, Zn\(^{2+}\); 19, Ag\(^+\); 20, Cd\(^{2+}\); 21, Hg\(^{2+}\); 22, Pb\(^{2+}\); 23, Nd\(^{3+}\); 24, Sm\(^{3+}\); 25, Er\(^{3+}\). \(\lambda_{ex} = 480\) nm.

To study the sensitivity of FeS toward Fe\(^{3+}\) ion sensing, fluorescence responses of the interaction of FeS with Fe\(^{3+}\) were examined through fluorescence emission titration experiments in a H\(_2\)O-CH\(_3\)CN (9:1, v/v) solution. As shown in Figure IV-5, upon
progressive addition of 1-700 equiv. of Fe$^{3+}$ ions, the fluorescence intensity of FeS was gradually increased.

![Figure IV-5. Enhancements in fluorescence emission spectra of FeS (7 µM) in H$_2$O-CH$_3$CN (9:1, v/v) upon continuous addition of 1-700 equiv. of Fe$^{3+}$ ions. $\lambda_{ex} = 480$ nm.](image)

Figure IV-5. Enhancements in fluorescence emission spectra of FeS (7 µM) in H$_2$O-CH$_3$CN (9:1, v/v) upon continuous addition of 1-700 equiv. of Fe$^{3+}$ ions. $\lambda_{ex} = 480$ nm.

Figure IV-6A exhibits the fluorescent intensity of FeS as a function of the equiv. of added Fe$^{3+}$ ions. The initial addition of 20 equiv. of Fe$^{3+}$ ions brought about a dramatic enhancement in the fluorescence emission intensity of FeS. Following that, the rate of Fe$^{3+}$-induced fluorescence increase slowed down as more Fe$^{3+}$ ions added to the solution of FeS in the subsequent titration procedure till 300 equiv. of Fe$^{3+}$ ions was added. Then the fluorescent emission intensity gradually stabilized. More importantly,
as displayed in Figure IV-6B, in the range of 1-20 equiv. of added Fe$^{3+}$ ions, a linear relationship was found between the fluorescence intensity enhancement ($F_l-F_{l0}$, $F_l$: fluorescence intensity) of FeS and the equiv. of Fe$^{3+}$ ions ($R^2 = 0.9977$). Therefore, FeS is potential to be a quantitative sensor for measuring the concentration of Fe$^{3+}$ ion.

**Figure IV-6.** (A) Fluorescence emission intensity of FeS as a function of the equiv. of added Fe$^{3+}$. (B) Plot of $F_l-F_{l0}$ ($F_l$: fluorescence intensity) versus the equiv. of Fe$^{3+}$ ion in the range of 1-20 equiv. of Fe$^{3+}$ ions.

Besides selectivity and sensitivity, the reversibility of FeS was also investigated because it is an important property for an ion sensor. TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine), a strong Fe$^{3+}$ chelator, was used to free FeS from its Fe$^{3+}$-complexed form. As shown in Figure IV-7, when 20 equiv. of Fe$^{3+}$ ions was added to FeS in H$_2$O-CH$_3$CN (9:1, v/v), a remarkable fluorescent emission increase was observed. Then TPEN was added to the solution and it instantly decreased the fluorescence intensity of the solution. Upon the addition of 60 equiv. of TPEN (3 equiv. for Fe$^{3+}$), the fluorescence emission spectrum of FeS with 20 equiv. of Fe$^{3+}$ ions was
recovered back to the original spectrum of free FeS. These results indicate the good reversibility of FeS for Fe$^{3+}$ ion detection.

![Fluorescence spectra](image)

**Figure IV-7.** Fluorescence response reversibility of FeS (7 µM) in H$_2$O-CH$_3$CN (9:1, v/v). Fluorescence emission spectra of free FeS, FeS with 20 equiv. of Fe$^{3+}$, and FeS with 20 equiv. of Fe$^{3+}$ and 60 equiv. of TPEN. $\lambda_{ex} = 480$ nm.

Furthermore, the pH sensitivity of FeS was also investigated in H$_2$O-MeCN (9:1, v/v) solutions with different pH values between 2.0 and 9.0. As displayed in Figure IV-8, no apparent change of the fluorescence emission spectra of FeS was observed in the pH range of 3.2-9.0. When the pH of the sample was reduced to 3.0, a mild enhancement in the fluorescence intensity of FeS was recorded. Further decreased pH values brought about stronger fluorescence emission. These results indicate that FeS is pH insensitive in the physiological pH range (6.8-7.4).
Figure IV-8. Fluorescence emission spectra of FeS (7 µM) in H$_2$O-MeCN (9:1, v/v) at different pH. $\lambda_{ex} = 480$ nm.

Encouraged by the above results, FeS was applied to sensing Fe$^{3+}$ ion in living cells. HCT-116 cells were used to conduct these experiments. Figure IV-9 shows the viability of HCT-116 cells after incubation with different concentrations of FeS. No obvious toxicity was found from cells incubated with up to 25 µM of FeS, which means FeS is potentially suitable for sensing Fe$^{3+}$ ions in living cells.
Figure IV-9. Viability of HCT-116 cells incubated with different concentrations of FeS.

Cell imaging experiments were performed to determine the sensing ability of FeS for Fe$^{3+}$ ions in living cells. Based on the results obtained from cell viability experiments, FeS was used at a concentration of 20 µM in cell imaging experiments. Fluorescence images and phase contrast images of HCT-116 cells incubated with FeS are shown in Figure IV-10. First, HCT-116 cells were incubated with a 20 µM solution of FeS in MEM medium for 10 min. Then a 30 µM solution of Fe$^{3+}$ in MEM medium was injected into the chambers containing HCT-116 cells and cells were immediately imaged (image IV-10a). The same MEM medium without Fe$^{3+}$ ions was used in the control experiment (image IV-10d). Then cells were imaged every 10 min. 60 min later, a much stronger fluorescence signal was recorded for cells incubated with Fe$^{3+}$ solution (image IV-10b).
However, cells treated with only MEM medium in the control experiment exhibited no obvious change in images (image IV-10e).

**Figure IV-10.** Images of live HCT-116 cells in a 30 µM Fe$^{3+}$ solution in MEM medium (a-c) and in only MEM medium (d-f) at 0 min (a, d) and 60 min (b, e) after incubated with FeS (20 µM). Images c and f are the phase contrast images of b and e, respectively.

Figure IV-11 shows the average fluorescence emission intensity of images of HCT-116 cells taken at different time points. The fluorescence signal change of cells is quantitatively demonstrated by comparing the fluorescence intensity data of cell images taken at different time. As displayed by curve B in Figure IV-11, cells incubated with Fe$^{3+}$ solution emitted gradually increased fluorescence as the incubation time getting longer. In contrast, fluorescence emission intensity of HCT-116 cells without Fe$^{3+}$ incubation stayed fairly constant (curve A in Figure IV-11). Besides, the phase contrast images (images IV-10c and IV-10f) showed that those cells were still in good conditions.
These results demonstrate that FeS is capable of sensing Fe$^{3+}$ ion in living cells without causing considerable damages to cells.

![Graph](image)

**Figure IV-11.** Average fluorescence intensity of cell images at different time points. (A) Control experiment; (B) Fe$^{3+}$ sensing experiment.

### IV.3 Conclusion

In summary, a novel BODIPY-based selective, sensitive and reversible fluorescence turn-on sensor (FeS) for Fe$^{3+}$ ion detection was synthesized and characterized. FeS is a conjugate of two moieties, a BODIPY platform serving as the fluorophore and a 1,10-diaza-18-crown-6 based cryptand acting as the Fe$^{3+}$ recognition moiety. FeS exhibited selective fluorescence turn-on response toward Fe$^{3+}$ over other
metal cations of interest, including Cr$^{3+}$ and Hg$^{2+}$ ions, which are the most common interfering metal cations for Fe$^{3+}$ detection. Also, FeS demonstrated high sensitivity for Fe$^{3+}$ sensing. A linear relationship was found between the fluorescence intensity enhancement and the equiv. of added Fe$^{3+}$ ion. Moreover, the turn-on fluorescence response of FeS to Fe$^{3+}$ ion was reversible. Treatment of Fe$^{3+}$-loaded FeS with TPEN restores the fluorescence emission back to baseline levels. In addition, FeS is insensitive to the pH of the physiological environment. Based on its excellent performance in Fe$^{3+}$ sensing and very low cytotoxicity, FeS was successfully applied to live cell imaging of Fe$^{3+}$.

**IV.4 Experimental**

**IV.4.1 General**

All reagents and solvents were purchased from commercial suppliers and used without further purification. All metal cations were purchased as their chloride, sulfate or nitrate salts and used as received. $^1$H and $^{13}$C NMR spectra were carried out on a Bruker AVANCE spectrometer (400 MHz). HR-MS analyses were performed at the Department of Chemistry, University of Florida. Fluorescence emission spectra were measured using an FLS980 fluorescence spectrometer. Fluorescence microscopy images were recorded on an Olympus IX-81 DSU microscope equipped with a Hamamatsu EM-CCD C9100 digital camera using a FITC filter cube (Ex: 477/50; DM: 507; Em: 536/40) and a 20x (Olympus LUCplanFLN 20x, N.A. = 0.45) objective lens.
IV.4.2 Synthesis

Synthesis of compound 51. A mixture of 2-methoxyethoxyaniline (16.7 g, 100 mmol), 2-chloroethanol (40.3 g, 500 mmol), KI (33.2 g, 200 mmol) and CaCO₃ (20.0 g, 200 mmol) in water (150 mL) was refluxed overnight under argon atmosphere. After cooling to room temperature, the resulting mixture was filtered and the filtrate was extracted with ethyl acetate (150 mL × 3). The combined organic phase was dried over MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel using ethyl acetate/methanol (99/1) to give compound 51 as colorless liquid (30.1 g, 90%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.22 (dd, J = 7.8, 1.7 Hz, 1H), 7.13 (td, J = 8.1, 1.7 Hz, 1H), 7.00 (td, J = 7.8, 1.4 Hz, 1H), 6.92 (dd, J = 8.1, 1.4 Hz, 1H), 4.15 – 4.10 (m, 2H), 3.78 – 3.74 (m, 2H), 3.61 (s, 2H), 3.45 (s, 3H), 3.20 – 3.13 (m, 4H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 155.38, 139.27, 126.18, 125.62, 122.35, 113.37, 70.76, 67.87, 59.70, 59.02, 58.08.

Synthesis of compound 52. To a solution of 51 (2.55 g, 10 mmol) in dry DMF (20 mL) was added POCl₃ (7.77 g, 50 mmol) dropwise under argon atmosphere at 0 °C. The resulting mixture was warmed to room temperature and then slowly heated to 60 °C. After stirring at 60 °C overnight, the reaction mixture was cooled to room temperature and then slowly poured into ice water. The excess POCl₃ was quenched by the addition of Na₂CO₃ powder. The mixture was extracted with CH₂Cl₂ (100 mL × 2) and the combined extracts were concentrated. The residue was then dissolved in ethyl acetate (100 mL) and the solution was washed with brine (100 mL × 2), dried over MgSO₄, and evaporated. The crude mixture was purified by flash column chromatography on silica
gel using hexane/ethyl acetate (4/1), giving the desired compound 52 as light yellow liquid (1.60 g, 50%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 3.44 (s, 3H), 3.64 – 3.73 (m, 8H), 3.76 – 3.80 (m, 2H), 4.16 – 4.22 (m, 2H), 6.94 (d, $J = 8.2$, 1H), 7.35 – 7.43 (m, 2H), 9.80 (s, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 41.44, 55.04, 59.12, 67.96, 70.79, 111.36, 117.17, 126.69, 130.11, 144.00, 150.69, 190.67. HRMS (ESI, m/z): calcd for C$_{14}$H$_{19}$Cl$_2$NO$_3$ ([M+H]$^+$) 320.0815; found 320.0830.

**Synthesis of compound 53.** To a solution of 1,10-diaza-18-crown-6 (2.62 g, 10 mmol) in 5 mL triethylamine and 40 mL anhydrous CH$_2$Cl$_2$ was added o-acetylsalicyloyl chloride (4.97 g, 25 mmol) in one portion at room temperature. A white suspension formed rapidly. The reaction mixture was stirred for 2 h at room temperature and then was diluted with CH$_2$Cl$_2$ (60 mL). The solution was transferred to a separation funnel and washed with brine (100 mL × 2), dried over MgSO$_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel using CH$_2$Cl$_2$/methanol (50/1) as the eluent to give 53 as yellow liquid (4.94 g, 84%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 2.25 (d, $J = 8.5$, 6H), 3.44 – 3.87 (m, 24H), 7.13 – 7.20 (m, 2H), 7.21 – 7.26 (m, 2H), 7.27 – 7.32 (m, 2H), 7.36 – 7.43 (m, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 20.99, 45.91, 49.49, 49.57, 69.57, 70.20, 70.65, 70.72, 123.15, 126.06, 127.67, 130.04, 130.18, 146.92, 168.44, 169.00. HRMS (ESI, m/z): calcd for C$_{30}$H$_{38}$N$_2$O$_{10}$ ([M+H]$^+$) 587.2599; found 587.2605.

**Synthesis of compound 54.** 80 ml aqueous methanol (1:1) was added to a mixture of compound 53 (4.11 g, 7 mmol), ammonium acetate (2.16 g, 28 mmol) and NaHCO$_3$ (2.35 g, 28 mmol). The resulting mixture was heated to reflux and stirred
overnight. After cooling to room temperature, the reaction mixture was diluted with 40 mL water and then filtered. The white residue was washed with H$_2$O and dried under vacuum, providing the desired compound 54 as white solid (3.24 g, 92%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 3.64 (s, 8H), 3.79 (t, $J = 2.7$, 16H), 6.81 – 6.86 (m, 2H), 6.97 (d, $J = 8.0$, 2H), 7.28 – 7.33 (m, 4H), 9.22 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 69.66, 70.74, 118.00, 118.59, 118.82, 127.86, 132.28, 157.90, 172.14. HRMS (ESI, m/z): calcd for C$_{26}$H$_{34}$N$_2$O$_8$ ([M+H]$^+$) 503.2388; found 503.2407.

**Synthesis of compound 55.** A solution of compound 52 (0.32 g, 1.0 mmol) and KI (0.37 g, 2.2 mmol) in 100 ml dry DMF was heated to 60 °C and stirred for 2 h at that temperature to make solution 1. A mixture of compound 54 (0.50 g, 1.0 mmol) and Cs$_2$CO$_3$ (0.98 g, 3.0 mmol) in 100 ml dry DMF was degased by argon for 30 min and then heated to 105 °C. After stirring for 1.5 h, the resulting mixture was added solution 1 dropwise over 4 h under argon atmosphere. The reaction mixture was stirred at 105 °C for 6 d. After cooling to room temperature, DMF was removed under reduced pressure. The residue was added 20 mL water and extracted with CH$_2$Cl$_2$ (20 mL × 2). The combined extracts were washed three times with water and then once with brine. The organic layer was dried over MgSO$_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography on silica gel using ethyl acetate/methanol (20/1) provided 55 as yellow solid (0.15 g, 20%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 3.34 – 4.21 (m, 39H), 6.90 (d, $J = 8.1$, 2H), 6.95 – 7.03 (m, 3H), 7.16 – 7.21 (m, 2H), 7.27 – 7.32 (m, 2H), 7.35 – 7.41 (m, 2H), 9.77 (d, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 46.58, 49.22, 54.33, 59.02, 59.12, 67.68, 69.04, 69.59, 70.84, 70.91, 71.17, 71.32, 110.33,
Synthesis of FeS. To a solution of compound 55 (0.38 g, 0.5 mmol) and 2,4-dimethylpyrrole (0.11 g, 1.1 mmol) in degased CH₂Cl₂ (50 mL) was added 2 drops of trifluoroacetic acid under argon atmosphere. The mixture was stirred at ambient temperature overnight, then a mixture of 2,3-dichloro-5,6-dicyano-p-benzoquinone (0.14 g, 0.6 mmol) in CH₂Cl₂ (10 mL) was added. The reaction mixture was stirred continuously for another 4 h. After the addition of 3 mL triethylamine, 3 mL BF₃·OEt₂ was added dropwise to the mixture. The mixture was kept stirring at ambient temperature overnight, then filtered through a celite pad. The residue was washed with 20 mL CH₂Cl₂ and the combined filtrate was rotary evaporated to dryness. The residue was dissolved in 30 mL CH₂Cl₂ and the solution was washed with 30 mL 5% aqueous NaHCO₃ solution followed with water (30 mL × 2). The organic portion was dried over anhydrous MgSO₄, filtered and then evaporated in vacuo. The crude product was purified by flash column chromatography on silica gel using CH₂Cl₂/methanol (25/1) as the eluent to give FeS as red solid (0.063 g, 13%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 1.48 (s, 6H), 2.55 (s, 6H), 2.95 – 4.34 (m, 39H), 5.98 (s, 2H), 6.76 – 7.02 (m, 6H), 7.08 – 7.24 (m, 3H), 7.26 – 7.31 (m, 2H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 14.46, 14.57, 14.73, 46.64, 49.25, 49.64, 54.45, 59.12, 59.18, 67.79, 68.38, 68.98, 69.66, 69.95, 70.19, 70.82, 70.88, 70.99, 71.14, 71.24, 71.37, 112.40, 112.62, 112.97, 114.05, 119.75, 121.03, 121.18, 121.28, 121.51, 121.59, 126.83, 127.25, 127.62, 127.78, 128.63,
HRMS (ESI, m/z): calcd for C_{52}H_{64}N_{10}O_{10}BF_{2} ([M+H]^+) 968.4796; found 968.4832.

**IV.4.3 Cell culture for imaging**

Epithelial colorectal carcinoma cells (HCT-116) were cultured in RPMI cell medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C and 5% CO₂. Then cells were seeded onto a 40 mm glass slide, which was later mounted onto a bioptics live cell imaging chamber after cells were incubated with a 20 µM solution of FeS in MEM medium for 10 min. For the Fe³⁺ sensing experiment, a 30 µM Fe³⁺ solution in MEM medium was pumped into cell chambers. For the control experiment, only MEM medium was pumped into cell chambers. Cells were kept at 37 °C and imaged with Olympus IX-81 DSU microscope every 10 min.
CHAPTER V: A FLUORESCENCE TURN-ON SENSOR FOR Ca$^{2+}$

V.1 Introduction

Calcium, a major component in the mineralization of teeth, shells, and bones, is the most abundant metal by mass in many animals. Approximately 99% of calcium in human body is stored in teeth and bones. Calcium is also the fifth-most-abundant element in the earth’s crust by mass, and the fifth-most-abundant ion by both mass and molarity in seawater.

Calcium is an essential mineral for living organisms and an important component of a healthy diet. The National Osteoporosis Foundation states, "Calcium plays an important role in building stronger, denser bones early in life and keeping bones strong and healthy later in life." Except the roughly 99% calcium stored in teeth and bones, the rest of calcium in human body has some other important functions. In cell physiology, calcium ions are important second messengers used in signal transduction.$^{60}$ Their movement in and out of cytoplasm works as a signal for many cellular processes.$^{385}$ Calcium plays a pivotal role in muscle contraction, which was discovered as early as in 1882. Also, calcium ions are important for maintaining the potential difference across excitable cell membranes. Moreover, calcium ions are required as a cofactor for many enzymes to function regularly, for example, the enzymes of blood-clotting cascade.

Calcium is of such importance to the physiology and biochemistry of animals that its levels are tightly maintained within specific limits, with bones as the storage site.$^{386}$ Both calcium deficiency and calcium overloading can bring about harmful
consequences. Low concentrations of extracellular calcium may lead to hypokalemic tetany. Long-term calcium deficiency can result in poor blood coagulation, rickets, and even osteoporosis. On the other hand, calcium overloading may cause damage to cells, leading to apoptosis or death by necrosis. Furthermore, long-term calcium overloading is associated with hyperkalemia, decreased neuromuscular excitability, impaired kidney function, cardiac arrhythmias, and decreased absorption of other minerals. It has also been reported that high calcium intake is in relation to prostate cancer.\textsuperscript{387}

Due to the vital role of calcium for human health, developing fluorescent calcium probes for physiological applications has been the focus of chemical and biological researchers for a quite long time. In this regard, Tsien et al. made significant contributions. In 1980, Tsien reported the synthesis of 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), which is a well-known recognition unit for calcium ions.\textsuperscript{388} By conjugating BAPTA to different chromophores, Tsien et al. synthesized various fluorescent calcium probes.\textsuperscript{28,210} The first series of fluorescent probes for calcium reported by Tsien et al. include Stil-1, Stil-2, Indo-1, Fura-1, Fura-2, and Fura-3, which are all UV light-excited probes.\textsuperscript{210} Among these fluorescent probes, Indo-1 and Fura-2 are the most preferred because of their relatively large fluorescence signal changes upon binding calcium ions. However, their absorption in the UV region seriously limits their applications in cellular environment. To solve this problem, Tsien et al. afterwards developed some other fluorescent calcium probes that can be excited by visible light, including Rhod-1, Rhod-2, Fluo-1, Fluo-2, and Fluo-3.\textsuperscript{28} In these probes, a BAPTA group is typically linked to a rhodamine or fluorescein fluorophore. The
maximum absorption and emission wavelengths of these probes are all in the visible region. These probes exhibit high selectivity and sensitivity towards calcium ion sensing. They are capable of detecting calcium at a concentration as low as 100 nM. As known, the intracellular and extracellular levels of calcium ion are around 100 nM and 1.0 mM, respectively. Therefore, these probes are potential for sensing intracellular calcium ions.

**Scheme V-1.** Structures of Stil-1, Stil-2, Indo-1, Fura-1, Fura-2, Fura-3, Rhod-1, Rhod-2.
However, the salt forms of these probes are not able to permeate the cell membrane, which prevents them from being up-taken by cells. As a consequence, it is necessary to use some techniques such as microinjection and electrophoration to put the probes into cells, which highly limits their application in cellular environment because of the inconvenience of those techniques. To address this problem, Tsien et al. developed a non-disruptive approach for loading these calcium probes into cells.\textsuperscript{27} The BAPTA group of the probes is made temporarily membrane permeable by masking its four carboxylates with esterifying groups, producing the so-called acetoxymethyl esters (AM esters). The AM ester form of the calcium probes can easily get into cells and then hydrolyses inside the cells with intracellular esterases as the catalyst, regenerating and trapping the original probes in the cells.

Following Tsien’s work, a variety of new BAPTA-based fluorescent probes for calcium ion determination were reported over the past decade.\textsuperscript{43-44,52,55-56,59-61,389-390} Some of these probes have been used to image calcium ions \textit{in vivo} and monitor the change of intracellular levels of calcium ions in various cells, consequently leading to considerable improvements in understanding the functions of calcium ion in physiology.
Compound 56 is a fluorescence “turn-on” calcium probe prepared by attaching a BAPTA group to a BODIPY fluorophore at the meso-position. Upon binding with calcium ions, the probe displays a 250-fold increase in fluorescence emission intensity. This probe is suitable for monitoring the intracellular calcium level change with high spatiotemporal resolution due to its high sensitivity for calcium detection. Compound 57 is another BODIPY-based fluorescent probe for calcium ions, using BAPTA as the recognition moiety. This probe exhibits desirable spectroscopic properties. In the presence of calcium ions, probe 57 shows very intense NIR fluorescence emission at 670 nm. It has been explored in real-time imaging of intracellular calcium ions. Compound 58 is also an NIR fluorescence “turn-on” sensor for calcium ions. This probe incorporates a Si-rhodamine platform as the fluorophore and a BAPTA unit as the calcium chelator. Upon complexation with calcium ions, probe 58 demonstrated over 1000-fold fluorescence emission intensity enhancement. The most important advantages of probe 58 in comparison to other existing fluorescent calcium probes, including its high signal-to-background ratio and NIR fluorescence characteristic, make it a much more suitable sensor for fluorescence imaging of calcium ions in neuronal systems.

Due to the attractive properties of two-photon excitation microscopy, as introduced in Section I.1, some 2PA fluorescent calcium probes have been developed in recent years by connecting the BAPTA group to certain fluorophores with high 2PA cross sections. Compound 59 is a 2PA fluorescent probe for calcium ions. The maximum 2PA wavelength of 59 is 780 nm and the cross section is 110 GM.
complexation of probe 59 with calcium ions brings about a 44-fold increase in its fluorescent emission intensity. This probe is capable of selectively detecting dynamic levels of intracellular free calcium ions in living cells and monitoring the Ca$^{2+}$ waves at a depth of about 150 mm in living tissues through two-photon excitation microscopy.

**Scheme V-3.** Structures of compounds 56, 57, 58, and 59.

Although the fluorescent calcium probes developed by Tsien et al. are often used as the gold standard and are currently the most widely used sensors in intracellular calcium detection due to their commercial availability, other progress has been made in this field to date. All these above-mentioned calcium probes (compounds 56-59) and other newly developed ones have exhibited preferable properties. They are promising for applications in the areas of biological and physiological calcium study.
However, these probes are only suitable for sensing intracellular levels of calcium ions because of their high bonding strength with calcium. Therefore, fluorescent calcium probes with weaker binding affinity to calcium ions are still in great need. Such probes should be capable of determining calcium ions at extracellular concentrations in whole blood, serum, and plasma. Herein, the goal of this work is to design and synthesize a fluorescent calcium sensor that will be suitable for sensing calcium levels in extracellular environments.

V.2 Results and Discussion

As shown in Scheme V-4, synthesis of the calcium sensor (CaS) started with 2-(2-methoxyethoxy)aniline that was reacted with 2-chloroethanol in the presence of CaCO$_3$ and KI under reflux in water to provide compound 51. Then 51 was treated with NaH in THF at room temperature for 1 h, followed by the dropwise addition of t-butyl bromoacetate at 0 °C. The resulting reaction mixture was stirred at room temperature overnight to produce compound 60 as a colorless liquid. Compound 61 was obtained through a Vilsmeier–Haack reaction of compound 60 in the presence of POCl$_3$ in DMF. Compound 62 was prepared via a condensation reaction of compound 61 with 2,4-dimethylpyrrole in CH$_2$Cl$_2$ in the presence of a catalytic amount of TFA at room temperature, followed by an oxidation reaction with DDQ and a subsequent chelation reaction with BF$_3$·OEt$_2$ in the presence of TEA at room temperature. Then a TFA catalyzed hydrolysis reaction was performed to compound 62 at room temperature in CH$_2$Cl$_2$ to achieve the desired final product CaS as a red solid.
Scheme V-4. Synthetic route of CaS.\(^a\)

\[
\begin{align*}
\text{NH}_2 \text{O} \text{O}^- & \quad \xrightarrow{a} \quad \text{HO} \text{N} \text{O} \text{O}^- \quad \xrightarrow{b} \quad \text{N} \text{O} \text{O}^+ \\
& \quad \xrightarrow{c} \quad \text{O} \text{O}^+ \\
& \quad \xrightarrow{d} \quad \text{N} \text{B} \text{F}^- \text{B} \text{F}^- \\
\text{CaS} 
\end{align*}
\]

\(^a\)Reagents and conditions: (a) 2-chlorethanol, CaCO\(_3\), KI, water, reflux, overnight; (b) NaH, THF, room temperature, 1 h, then 0 °C, t-butyl bromoacetate, then room temperature, overnight; (c) DMF, POCl\(_3\), 0 °C to 60 °C, overnight; (d) 2,4-dimethylpyrrole, TFA, CH\(_2\)Cl\(_2\), room temperature, overnight; then DDQ, 4 h; then TEA, BF\(_3\)·OEt\(_2\), overnight; (e) TFA:CH\(_2\)Cl\(_2\) (1:3), room temperature, 1 h.

After CaS was synthesized, its selectivity as a sensor for Ca\(^{2+}\) was first studied in 30 mM HEPES buffer (pH 7.4). Figure V-1 shows the fluorescent emission spectra of CaS in the presence of various biological metal cations at their physiological concentrations. Without metal cations added, the free CaS emitted only weak fluorescence at 511 nm. Upon the addition of Ca\(^{2+}\) ions, a dramatically enhanced fluorescence emission signal was observed. Besides Ca\(^{2+}\), the presence of Zn\(^{2+}\) also brought about a mild increase in the fluorescence intensity of CaS, which was much
weaker than that induced by Ca$^{2+}$. Little to no changes in the fluorescence spectra of CaS was recorded upon the addition of other metal cations. These results demonstrated the desirable selectivity of CaS towards Ca$^{2+}$ determination. In addition, the same phenomena were also found in widely used BAPTA-based fluorescent Ca$^{2+}$ probes, which also suffer from the interference of Zn$^{2+}$.\textsuperscript{28,52,390}

![Fluorescence emission spectra of CaS (5 \mu M) in 30 mM HEPES buffer (pH 7.4) upon addition of various biological metal cations at their physiological concentrations: Li$^+$ (1.0 mM), Na$^+$ (150 mM), K$^+$ (150 mM), Mg$^{2+}$ (2.0 mM), Ca$^{2+}$ (1.0 mM), Zn$^{2+}$ (50 \mu M), Cu$^{2+}$ (50 \mu M), Fe$^{3+}$ (50 \mu M), Co$^{2+}$ (50 \mu M), Ni$^{2+}$ (50 \mu M). $\lambda_{ex} = 480$ nm.]

**Figure V-1.** Fluorescence emission spectra of CaS (5 \mu M) in 30 mM HEPES buffer (pH 7.4) upon addition of various biological metal cations at their physiological concentrations: Li$^+$ (1.0 mM), Na$^+$ (150 mM), K$^+$ (150 mM), Mg$^{2+}$ (2.0 mM), Ca$^{2+}$ (1.0 mM), Zn$^{2+}$ (50 \mu M), Cu$^{2+}$ (50 \mu M), Fe$^{3+}$ (50 \mu M), Co$^{2+}$ (50 \mu M), Ni$^{2+}$ (50 \mu M). $\lambda_{ex} = 480$ nm.

To investigate the efficiency of CaS towards Ca$^{2+}$ sensing, titration experiments were performed in 30 mM HEPES buffer (pH 7.4) solutions. Initially, UV-vis absorption spectroscopy was utilized to conduct titration experiments. As displayed in Figure V-2, in the absence of Ca$^{2+}$ ions, the solution of CaS exhibited an absorption peak at 498 nm.
Upon the progressive addition of Ca$^{2+}$ ions, the absorption intensity of the sample was slightly decreased.

![Absorption Spectra](image)

**Figure V-2.** Absorption spectra of CaS (5 μM) in 30 mM HEPES buffer (pH 7.4) upon continuous addition of 7.0 mM Ca$^{2+}$ ions.

As fluorescence emission spectroscopy is a more effective and reliable approach than absorption spectroscopy for titration experiments, the Ca$^{2+}$ sensing efficiency of CaS was further examined by fluorescence emission titration experiments in 30 mM HEPES buffer (pH 7.4). As demonstrated in Figure V-3, a gradual increase in the fluorescence intensity of CaS was observed upon continuous addition of Ca$^{2+}$ ions. The fluorescence intensity was enhanced by 18-fold in the presence of 7.0 mM Ca$^{2+}$. The addition of more Ca$^{2+}$ ions was not able to further enhance the fluorescence intensity of CaS. Furthermore, CaS was capable of detecting 0.1 mM Ca$^{2+}$, which indicated good sensitivity. Given that the average concentration of ionized calcium is around 1.0 mM in
blood, serum, and plasma, \textbf{CaS} is potentially suitable for determining \( \text{Ca}^{2+} \) levels in those fluids.

\textbf{Figure V-3.} Enhancement in fluorescence emission spectra of \textbf{CaS} (5 \( \mu \text{M} \)) in 30 mM HEPES buffer (pH 7.4) upon continuous addition of 7.0 mM \( \text{Ca}^{2+} \). \( \lambda_{\text{ex}} = 480 \text{ nm} \).

In addition, the sensitivity of \textbf{CaS} to pH was investigated in aqueous solutions within a pH range between 4.0 and 8.0. As displayed in Figure V-4, when the pH of the solution was over 6.8, no apparent change was observed in the fluorescence emission spectra of \textbf{CaS}. However, when the pH value was as low as 6.6, fluorescence intensity of \textbf{CaS} was moderately increased. Even lower pH values resulted in stronger fluorescence emission until the pH of the solution reached 4.0. These results demonstrate that \textbf{CaS} is pH insensitive in the physiological pH range, especially in blood and serum where the pH is buffered at 7.4.
Figure V-4. Fluorescence emission spectra of CaS (5 μM) in water at different pH. $\lambda_{ex} = 480$ nm.

V.3 Conclusion

In summary, a novel BODIPY-based fluorescence turn-on sensor (CaS) has been designed and synthesized for selectively and sensitively determining Ca$^{2+}$. CaS is comprised of two moieties, a BODIPY fluorophore and a Ca$^{2+}$ complexing unit. CaS demonstrates selective fluorescence off-on response towards Ca$^{2+}$ over other metal cations of interest. Although Zn$^{2+}$ can also trigger a fluorescence response, the increase in fluorescence intensity induced by Zn$^{2+}$ is much weaker than that by Ca$^{2+}$. Moreover, CaS demonstrated desirable sensitivity for Ca$^{2+}$ detection, which makes it more suitable for extracellular Ca$^{2+}$ determination than other known probes. In addition, CaS is insensitive to the pH of the physiological environment, especially to that in blood and
serum. Therefore, based on the advantages of CaS, it holds great potential for sensing Ca$^{2+}$ ions in extracellular environments.

**V.4 Experimental**

**V.4.1 General**

All reagents and solvents were purchased from commercial suppliers and used without further purification. All metal cations were purchased as their chloride, sulfate or nitrate salts and used as received. $^1$H and $^{13}$C NMR spectra were carried out on a Bruker AVANCE spectrometer (400 MHz). HR-MS analyses were performed at the Department of Chemistry, University of Florida. Flash column chromatography was performed on a CombiFlash® Rf+ automated flash chromatography using RediSep Rf Gold® normal-phase HP silica columns. UV-vis absorption spectra were recorded on an Agilent 8453 spectrophotometer. Fluorescence emission spectra were measured using an FLS980 fluorescence spectrometer.

**V.4.2 Synthesis**

*Synthesis of compound 60.* To a solution of 51 (5.11 g, 20 mmol) in 100 mL anhydrous THF was added 60% NaH in mineral oil (0.84 g, 21 mmol) in one portion under argon atmosphere at room temperature. The reaction mixture was stirred for 1 h at room temperature and then was cooled to 0 °C. A solution of tert-butyl bromoacetate (4.68 g, 24 mmol) in 5 mL anhydrous THF was added dropwise at 0 °C. The resulting mixture was warmed to room temperature and stirred for 4 h. Then the reaction was quenched by 100 mL water and transferred to a separation funnel. The water layer was
extracted with ethyl acetate (50 mL × 2). The combined organic layer was washed with brine (100 mL × 2), dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by flash column chromatography using hexane/ethyl acetate (3/1) as the eluent to give 60 as colorless liquid (1.94 g, 20%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.04 (dd, J = 7.5, 1.9 Hz, 1H), 6.94 – 6.83 (m, 3H), 4.12 (dd, J = 5.5, 4.4 Hz, 2H), 3.92 (s, 4H), 3.74 (dd, J = 5.5, 4.4 Hz, 2H), 3.61 (t, J = 6.2 Hz, 4H), 3.47 (t, J = 6.2 Hz, 4H), 3.42 (s, 3H), 1.45 (s, 18H).

¹³C NMR (101 MHz, CDCl₃): δ (ppm) 169.87, 152.77, 139.69, 122.79, 122.19, 121.47, 114.28, 81.48, 71.30, 69.99, 69.09, 67.89, 59.13, 52.74, 28.23. HRMS (ESI, m/z): calcd for C₂₅H₄₁NO₈ ([M+H]⁺) 484.2905; found 484.2916.

**Synthesis of compound 61.** To a solution of 60 (4.84 g, 10 mmol) in dry DMF (20 mL) was added POCl₃ (7.77 g, 50 mmol) dropwise under argon atmosphere at 0 °C. The resulting mixture was warmed to room temperature and then slowly heated to 60 °C. After stirring at 60 °C overnight, the reaction mixture was cooled to room temperature and then slowly poured into ice water. The excess POCl₃ was quenched by the addition of NaOH. The mixture was extracted with CH₂Cl₂ (100 mL × 2) and the combined extracts were concentrated. The residue was then dissolved in ethyl acetate (100 mL) and the solution was washed with brine (100 mL × 2), dried over MgSO₄, and evaporated. The crude mixture was purified by flash column chromatography using hexane/ethyl acetate (3/1), giving the desired compound 61 as light yellow liquid (2.08 g, 41%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 9.75 (s, 1H), 7.36 (dd, J = 8.3, 1.8 Hz, 1H), 7.32 (d, J = 1.8 Hz, 1H), 7.02 (d, J = 8.3 Hz, 1H), 4.15 (dd, J = 5.4, 3.8 Hz, 2H), 3.94 (s, 4H), 3.76 – 3.67 (m, 10H), 3.41 (s, 3H), 1.45 (s, 18H). ¹³C NMR (101 MHz, CDCl₃): δ
(ppm) 190.66, 169.71, 150.22, 145.96, 128.90, 127.11, 117.34, 111.35, 81.68, 70.91, 70.10, 69.11, 67.83, 58.99, 52.55, 28.23. HRMS (ESI, m/z): calcd for \( \text{C}_{26}\text{H}_{41}\text{NO}_9 \) \([\text{M}+\text{H}]^+\) 512.2854; found 512.2852.

*Synthesis of compound 62.* To a solution of compound 61 (0.26 g, 0.5 mmol) and 2,4-dimethylpyrrole (0.11 g, 1.1 mmol) in degased CH\(_2\)Cl\(_2\) (50 mL) was added 2 drops of trifluoroacetic acid under argon atmosphere. The mixture was stirred at ambient temperature overnight, then a mixture of 2,3-dichloro-5,6-dicyano-p-benzoquinone (0.14 g, 0.6 mmol) in CH\(_2\)Cl\(_2\) (10 mL) was added. The reaction mixture was stirred continuously for another 4 h. After the addition of 3 mL triethylamine, 3 mL BF\(_3\)-OEt\(_2\) was added dropwise to the mixture. The mixture was kept stirring at ambient temperature overnight, then filtered through a celite pad. The residue was washed with 20 mL CH\(_2\)Cl\(_2\) and the combined filtrate was rotary evaporated to dryness. The residue was dissolved in 30 mL CH\(_2\)Cl\(_2\) and the solution was washed with 30 mL 5% aqueous NaHCO\(_3\) solution followed with water (30 mL × 2). The organic portion was dried over anhydrous MgSO\(_4\), filtered and then evaporated *in vacuo*. The crude product was purified by flash column chromatography using hexane/ethyl acetate (4/1) as the eluent to give 62 as red gum (0.054 g, 15%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) (ppm) 7.14 (d, \( J = 8.1 \) Hz, 1H), 6.82 – 6.72 (m, 2H), 5.97 (s, 2H), 4.09 – 4.05 (m, 2H), 3.92 (s, 4H), 3.74 – 3.71 (m, 2H), 3.66 (t, \( J = 6.0 \) Hz, 4H), 3.55 (t, \( J = 6.0 \) Hz, 4H), 3.40 (s, 3H), 2.54 (s, 6H), 1.49 – 1.45 (m, 24H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)): \( \delta \) (ppm) 169.76, 155.35, 152.79, 143.22, 142.04, 140.48, 131.82, 128.32, 121.76, 121.17, 121.06, 113.59, 81.62, 71.15,
69.91, 69.07, 68.21, 59.13, 52.52, 28.25, 14.71, 14.50. HRMS (ESI, m/z): calcd for C\textsubscript{38}H\textsubscript{54}N\textsubscript{3}O\textsubscript{8}BF\textsubscript{2} ([M+H]\textsuperscript{+}) 729.4081; found 729.4089.

**Synthesis of CaS.** A solution of compound 62 (0.37 g, 0.5 mmol) in 5 mL solvent mixture TFA/CH\textsubscript{2}Cl\textsubscript{2} (1:3) was stirred for 1 h at room temperature. Then the reaction mixture was condensed to remove solvents under vacuum. The residue was purified by flash column chromatography using CH\textsubscript{2}Cl\textsubscript{2}/methanol (5/1) as the eluent to give CaS as red gum (0.039 g, 13%). \textsuperscript{1}H NMR (400 MHz, CD\textsubscript{3}CN): \( \delta \) (ppm) 7.39 (d, \( J = 8.0 \) Hz, 1H), 7.02 (d, \( J = 1.8 \) Hz, 1H), 6.91 (dd, \( J = 8.0, 1.8 \) Hz, 1H), 6.10 (s, 2H), 4.14 (dd, \( J = 5.4, 3.7 \) Hz, 2H), 3.99 (s, 4H), 3.69 – 3.67 (m, 2H), 3.59 – 3.53 (m, 8H), 3.32 (s, 3H), 2.48 (s, 6H), 1.49 (s, 6H). \textsuperscript{13}C NMR (101 MHz, CD\textsubscript{3}CN): \( \delta \) (ppm) 173.78, 156.52, 154.63, 144.52, 142.24, 134.07, 132.11, 126.10, 122.35, 121.77, 118.37, 114.57, 71.33, 69.29, 69.04, 68.29, 59.01, 54.65, 14.75, 14.66. HRMS (ESI, m/z): calcd for C\textsubscript{30}H\textsubscript{38}N\textsubscript{3}O\textsubscript{8}BF\textsubscript{2} ([M+H]\textsuperscript{+}) 617.2829; found 617.2831.
CHAPTER VI: FUTURE WORK

The investigation of some of the sensors, such as KS2 and CaS, can be continued in more depth. Considering their advantageous behavior in sensing their respective metal cations, additional investigations may be warranted.

VI.1 Potassium sensor KS2

For KS2, its selectivity towards probing potassium over other biological metal cations has been proven to be high. The fluorescence emission titration experiments indicate that it also has good sensitivity in sensing potassium. Moreover, it is insensitive to physiological pHs. Also, it has desirable two-photon absorption cross section. Combining all the merits that KS2 has in sensing potassium, it is highly potential to be a two-photon absorption, red-emitting, fluorescence turn-on sensor for physiological potassium determination.

The toxicity of KS2 in cells should be evaluated. After that, cells will be incubated with KS2 and cell images will be taken at different concentrations of cellular potassium. Both one-photon and two-photon excitation microscopy will be used to image cells.

VI.2 Calcium sensor CaS

For CaS, the following study will also be focused on biological applications. Similar to the future plan for KS2, the toxicity of CaS will first be examined. Given that CaS is a carboxylic salt, it should be difficult for it to pass through cell membranes.
Considering the potency that CaS has already exhibited for sensing calcium in extracellular environments, fluorescence emission microscopy will be used to determine extracellular levels of calcium. CaS may also be used to image calcium in tissues.
APPENDIX A: $^1$H AND $^{13}$C NMR SPECTRA OF NEW COMPOUNDS IN CHAPTER II
Figure A1. $^1$H NMR spectrum (400 MHz) of 21 in CDCl$_3$. 
Figure A2. $^{13}$C NMR spectrum (101 MHz) of 21 in CDCl$_3$. 
Figure A3. $^1$H NMR spectrum (400 MHz) of 22 in CDCl$_3$. 
Figure A4. $^{13}$C NMR spectrum (101 MHz) of 22 in CDCl$_3$. 

![Chemical Structure](image)
Figure A5. $^1$H NMR spectrum (400 MHz) of 23 in CDCl$_3$. 
Figure A6. $^{13}$C NMR spectrum (101 MHz) of 23 in CDCl$_3$. 
Figure A7. $^1$H NMR spectrum (400 MHz) of 24 in CDCl$_3$. 
Figure A8. $^{13}$C NMR spectrum (101 MHz) of 24 in CDCl$_3$. 
Figure A9. $^1$H NMR spectrum (400 MHz) of 27 in CDCl$_3$. 
Figure A10. $^{13}$C NMR spectrum (101 MHz) of 27 in CDCl$_3$. 
Figure A11. $^1$H NMR spectrum (400 MHz) of 28 in CDCl$_3$. 
Figure A12. $^{13}$C NMR spectrum (101 MHz) of 28 in CDCl$_3$. 
**Figure A13.** $^1$H NMR spectrum (400 MHz) of 29 in CDCl$_3$. 
Figure A14. $^{13}\text{C}$ NMR spectrum (101 MHz) of 29 in CDCl$_3$. 
Figure A15. $^1$H NMR spectrum (400 MHz) of FS in CDCl$_3$. 
Figure A16. $^1$H NMR spectrum (400 MHz) of FS in DMSO-$d_6$. 
Figure A17. $^{13}$C NMR spectrum (101 MHz) of FS in CDCl$_3$. 
APPENDIX B: $^1$H AND $^{13}$C NMR SPECTRA OF NEW COMPOUNDS IN CHAPTER III
Figure B1. $^1$H NMR spectrum (400 MHz) of KS1 in CDCl$_3$. 
Figure B2. $^{13}$C NMR spectrum (101 MHz) of KS1 in CDCl$_3$. 
Figure B3. $^1$H NMR spectrum (400 MHz) of 42 in CDCl$_3$. 
**Figure B4.** $^1^3$C NMR spectrum (101 MHz) of 42 in CDCl$_3$. 
Figure B5. $^{1}$H NMR spectrum (400 MHz) of 43 in CDCl$_3$. 
Figure B6. $^{13}$C NMR spectrum (101 MHz) of 43 in CDCl$_3$. 
Figure B7. $^1$H NMR spectrum (400 MHz) of 44 in CDCl$_3$. 
Figure B8. $^{13}$C NMR spectrum (101 MHz) of 44 in CDCl$_3$. 
**Figure B9.** $^1$H NMR spectrum (400 MHz) of 45 in CDCl$_3$. 
Figure B10. $^{13}$C NMR spectrum (101 MHz) of 45 in CDCl$_3$. 
Figure B11. $^1$H NMR spectrum (400 MHz) of 46 in CDCl$_3$. 
Figure B12. $^{13}$C NMR spectrum (101 MHz) of 46 in CDCl$_3$. 
Figure B13. $^1$H NMR spectrum (400 MHz) of KS2 in CD$_2$Cl$_2$. 
Figure B14. $^{13}$C NMR spectrum (101 MHz) of KS2 in CD$_2$Cl$_2$. 
APPENDIX C: $^1$H AND $^{13}$C NMR SPECTRA OF NEW COMPOUNDS IN CHAPTER IV
Figure C1. $^1$H NMR spectrum (400 MHz) of 51 in CDCl$_3$. 
Figure C2. $^{13}$C NMR spectrum (101 MHz) of 51 in CDCl$_3$. 
Figure C3. $^1$H NMR spectrum (400 MHz) of 52 in CDCl$_3$. 
Figure C4. $^{13}$C NMR spectrum (101 MHz) of 52 in CDCl$_3$. 
Figure C5. $^1$H NMR spectrum (400 MHz) of 53 in CDCl$_3$. 
Figure C6. $^{13}$C NMR spectrum (101 MHz) of 53 in CDCl$_3$. 
Figure C7. $^1$H NMR spectrum (400 MHz) of 54 in CDCl$_3$. 
Figure C8. $^{13}$C NMR spectrum (101 MHz) of 54 in CDCl$_3$. 
Figure C9. $^1$H NMR spectrum (400 MHz) of 55 in CDCl$_3$. 
Figure C10. $^{13}$C NMR spectrum (101 MHz) of 55 in CDCl$_3$. 
Figure C11. $^1$H NMR spectrum (400 MHz) of FeS in CDCl$_3$. 
Figure C12. $^{13}$C NMR spectrum (101 MHz) of FeS in CDCl$_3$. 
APPENDIX D: $^1$H AND $^{13}$C NMR SPECTRA OF NEW COMPOUNDS IN CHAPTER V
Figure D1. $^1$H NMR spectrum (400 MHz) of 60 in CDCl$_3$. 
Figure D2. $^{13}$C NMR spectrum (101 MHz) of 60 in CDCl$_3$. 
Figure D3. $^1$H NMR spectrum (400 MHz) of 61 in CDCl$_3$. 
Figure D4. $^{13}$C NMR spectrum (101 MHz) of 61 in CDCl$_3$. 
Figure D5. $^1$H NMR spectrum (400 MHz) of 62 in CDCl$_3$. 
Figure D6. $^{13}$C NMR spectrum (101 MHz) of 62 in CDCl$_3$. 
Figure D7. $^1$H NMR spectrum (400 MHz) of CaS in CD$_3$CN.
Figure D8. $^{13}$C NMR spectrum (101 MHz) of CaS in CD$_3$CN.
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