Spectroscopy and Gas-Phase Binding of Modified Desferrioxamine B Indicator Displacement-Based Sensors

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The University of Southern Mississippi

SPECTROSCOPY AND GAS-PHASE BINDING OF MODIFIED DESFERRIOXAMINE B INDICATOR DISPLACEMENT-BASED SENSORS

by

William Scott Jones

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

December 2014
ABSTRACT

SPECTROSCOPY AND GAS-PHASE BINDING OF MODIFIED DESFERRIOXAMINE B: INDICATOR DISPLACEMENT-BASED SENSORS

by William Scott Jones

December 2014

A coupling reaction has been used to successfully synthesize two novel probes utilizing coumarin derivatives as fluorophores. The structure, spectroscopy, and thermodynamics of these probes were characterized and studied. These probes, along with a classical probe using a N-methyl-anthranilate fluorophore, are used to take advantage of the ability of siderophores to coordinate Fe$^{3+}$ ions which is coupled with the attractive phosphorescence properties of lanthanide ions. The use of lanthanide metals to create an indicator displacement assays is a novel application of these probes for the detection of Fe$^{3+}$ ions. The use of Tb$^{3+}$ ions as the indicator with a coumarin-based fluorophore showed the best detection which was both selective for the Fe$^{3+}$ ion over other metal ions and showed the ability to detect the Fe$^{3+}$ ion in water in concentrations as low as 3.9 µM. The work described with DFB also includes the thermodynamics for the DFB-iron complex in the gas-phase using ESI-MS. These ESI-MS techniques were applied to studying the fragmentation of a tripodal ligand with urea binding moieties that showed the ability to cyclize when fragmented. The fragmentation was found to cease due to a templating effect brought from an anion coordinated by the urea moieties of the probe.
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A Dissertation
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of The University of Southern Mississippi
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<th>Description</th>
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<tr>
<td>3-DAP</td>
<td>3-diethylaminophenol</td>
</tr>
<tr>
<td>4-DASA</td>
<td>4-diethylaminosalicylaldehyde</td>
</tr>
<tr>
<td>Acac</td>
<td>Acetylacetone</td>
</tr>
<tr>
<td>CHEF</td>
<td>Chelation Enhanced Fluorescence</td>
</tr>
<tr>
<td>CID</td>
<td>Collision Induced Dissociation</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic Voltammetry</td>
</tr>
<tr>
<td>DFB</td>
<td>Desferrioxamine B</td>
</tr>
<tr>
<td>DIPEA</td>
<td>Diisopropylethylamine</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDG</td>
<td>Electron Donating Group</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>ElectroSpray Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>EWG</td>
<td>Electron Withdrawing Group</td>
</tr>
<tr>
<td>Et₃N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>FEF</td>
<td>Fluorescence Enhancement Factor</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster Resonance Energy Transfer</td>
</tr>
<tr>
<td>GtC</td>
<td>Gigatons Carbon</td>
</tr>
<tr>
<td>HOBt</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma Mass Spectrometry</td>
</tr>
<tr>
<td>IDA</td>
<td>Indicator Displacement Assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>IRMPD</td>
<td>Infrared-Multiphoton Dissociation</td>
</tr>
<tr>
<td>ISC</td>
<td>Intersystem Crossing</td>
</tr>
<tr>
<td>ISR</td>
<td>Indicator-Spacer-Receptor</td>
</tr>
<tr>
<td>LoD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>LRET</td>
<td>Lanthanide Resonance Energy Transfer</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NBD</td>
<td>Nitrobenz-2-oxa-1,3-diazole</td>
</tr>
<tr>
<td>NMA</td>
<td>N-Methylanthranilate</td>
</tr>
<tr>
<td>Ormosil</td>
<td>Organically Modified Silane</td>
</tr>
<tr>
<td>P(O)Cl₃</td>
<td>Phosphoryl Chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PEBBLE</td>
<td>Probe Encapsulated by Biologically Localized Embedding</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced Electron Transfer</td>
</tr>
<tr>
<td>RA</td>
<td>Relative Abundance</td>
</tr>
<tr>
<td>SIM</td>
<td>Single Ion Monitoring</td>
</tr>
<tr>
<td>TBA</td>
<td>Tetrabutyl Ammonium</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethylorthosilicate</td>
</tr>
<tr>
<td>TMOS</td>
<td>Tetramethylorthosilicate</td>
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</tbody>
</table>
CHAPTER I

INTRODUCTION

Why Does Iron Matter?

Iron is an important transition metal found in both the +2 (ferrous) and +3 (ferric) oxidation states, referred to simply as iron when speaking of both. The ferric ion (Fe$^{3+}$) is a hard Lewis acid while the ferrous ion (Fe$^{2+}$) is a borderline Lewis acid. The ferric ion has a tendency to form favorable interactions with hard Lewis bases such as oxygen atoms, while the ferrous ion will form strong interactions with borderline Lewis bases such as nitrogen atoms. The geometries of Fe$^{3+}$ ions (Figure 1.1) range from trigonal planar (three ligands)\(^1\) as shown by the thiolate complex (1.1) to distorted square antiprismatic (eight ligands),\(^2\) complex 1.6. Even though all coordination environments exist for iron coordination, square planar (four ligands),\(^3\) complex 1.2, distorted trigonal bipyramidal (five ligands),\(^4\) complex 1.3, and pentagonal bipyramidal (seven ligands)\(^5\), complex 1.5, are known, the octahedral (six ligands),\(^6\) complex 1.4, and trigonal prismatic geometries are the most common. Both of these common geometries are dependent on the pH of the solution (vide infra). An interesting compound that shows all of these geometries is the Fe$^{n+}$ hydroxide species found in aqueous solution at pH 8.0 (e.g. Fe(OH)$_2$, Fe(OH)$_2^+$, Fe(OH)$_3$, etc.).\(^7,8\)
Labile Iron Pools

Iron is found in both unicellular and multicellular organisms as labile iron pools—that is iron available for metabolic processes. These pools form from a variety of pathways including recycling of iron from heme proteins. The cytosolic conditions that labile iron is found in is a reducing environment, whereby reactive oxygen species, such as peroxide, convert any free Fe$^{3+}$ ions to Fe$^{2+}$ ions by the classical Fenton reaction, producing Fe$^{2+}$ ions for cellular pathways such as the function of the mitochondria. Utilizing this labile iron requires a set of proteins and small molecules that include the plasma protein transferrin and the iron storage protein, ferritin. These proteins interact with both ferric and ferrous iron using amino acids, such as tyrosine and aspartate or
glutamate, which bear side chains containing hydroxyl or carboxylic acid moieties; both of which are hard bases that can coordinate to the iron center strongly. Additionally, both transferrin and ferritin utilize histidine in binding Fe$^{3+}$ ($K_d = 4.7 \times 10^{-20}$ M$^{-1}$)$^{13}$ and Fe$^{2+}$ ($K_d = 9.1 \times 10^{-8}$ M$^{-1}$)$^{14}$ ions, respectively. The side-chain of the histidine amino acid has an imidazole group whose nitrogen atom that is a borderline soft Lewis base, but allows for binding both Fe$^{2+}$ ions but also Fe$^{3+}$ ions, be it with less affinity.$^9$

Iron in the Ocean

The importance of iron in the ocean’s environment has only been understood within the past 30 years.$^{15-17}$ It is now believed that iron plays significant roles in the growth of phytoplankton, which by extension could have an impact on the ability of the ocean to sequester carbon dioxide. The late John Martin studied the effects of iron on the ocean as well as historical iron levels in the ocean, concluding, “that the enhanced supply of [iron] from the atmosphere stimulated photosynthesis, which led to the drawdown in atmospheric CO$_2$ levels during glacial maxima.”$^{17}$ This led to The Iron Hypothesis, as it was demonstrated that iron is a key nutrient and constrains the growth of microorganisms in many parts of the ocean that have plentiful nutrients (PO$_4^{3-}$, NO$_3^-$, SiO$_4^{4-}$) and sunlight, but lack iron.$^{15}$ The exact role of iron as utilized by microorganisms in the ocean is an area of interest and is still being studied extensively as part of the larger carbon cycle that describes the movement of carbon through the atmosphere, ocean, and land (Figure 1.2).$^{18}$ Atmospheric dust is the primary source of oceanic Fe$^{3+}$ ions, which is transported upon coordination by siderophores (vide infra) for uptake by microorganisms. Even though iron is a metal cation, it’s concentration profile in the water column is similar to classical nutrients, for example PO$_4^{3-}$ and NO$_3^-$, where surface concentrations are
enriched by alluvial deposition (0.35-0.6 nM) while the subsurface waters at 50-200 m are highly depleted (0.02 nM) where phytoplankton, which require Fe$^{3+}$ ions as a key nutrient, thrive. The concentration of iron rises sharply upon moving into deeper waters before becoming constant (0.3-0.7 nM) by a depth of 1000 m (Figure 1.3).\textsuperscript{15}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{iron_cycle.png}
\caption{The oceanic iron cycle showing the chelation of iron by biomolecules and reduction of Fe$^{3+}$ ions to Fe$^{2+}$ ions as the primary avenues of biological uptake.\textsuperscript{19}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{iron_profile.png}
\caption{Concentration curve of dissolved iron found in the ocean showing a nutrient-like depth profile. Reprinted by permission from Macmillan Publishers Ltd: Nature Geoscience, Boyd, Vol. 3, 675-82, copyright 2009.}
\end{figure}
The presence of iron species in sea water is a complex mixture of a variety of solid, colloidal, and dissolved forms. Free dissolved oxygen in the ocean makes for easy oxidation of Fe$^{2+}$ ions, meaning Fe$^{3+}$ ions are the primary oxidation state found in sea water.\(^{19}\) The remaining Fe$^{2+}$ ions are found primarily either in siderophore complexes or as siderite (FeCO$_3$) or [FeOH]$^+$\(^{20,21}\). For the ferric iron in the ocean the primary forms are hematite (Fe$_2$O$_3$), which precipitates, and Fe(OH)$_3$ which is in equilibrium with its dissolved, colloidal, and solid states. The predominant Fe(OH)$_3$ ($K_{sp} = 2.8 \times 10^{-39}$) species is due to the ocean pH of 8.2, but other forms of dissolved iron are present as Fe$^{3+}$ ions and the iron hydroxide species [Fe(OH)]$^{2+}$, [Fe(OH)$_2$]$^+$, and [Fe(OH)$_4$]$^-$.\(^8,21\) For the hydroxide and free Fe$^{3+}$ ion species, the percentage of dissolved iron that can be found in any given one of these species can be calculated by Equation 1.0 where $[H^+]$ is calculated from the pH of the solution and the activity coefficient, while $K_c$ are the calculated equilibrium constants for each species under the given conditions.\(^{22}\)

\[
\frac{[Fe(III)]_{Total}}{1 + K_cFe(OH)^{2+}\frac{[H^+]}{[H^+]}} + K_cFe(OH)^{2+}\frac{[H^+]}{[H^+]}} + K_cFe(OH)^{3+}\frac{[H^+]}{[H^+]}} + K_cFe(OH)^{4+}\frac{[H^+]}{[H^+]}} = [Fe^{3+}] \quad \text{Eq. 1.0}
\]

Therefore in an average sample of sea water at pH 8.2, the total dissolved iron concentration is 0.76 nM and the fraction of iron found as Fe$^{3+}$ ions is 2%\(^7\). This highlights the importance of organic ligands in concentrating Fe$^{3+}$ ions, which is the only form of iron that is useful to microorganisms.\(^{23}\) The average concentration of total dissolved iron ranges from 0.02-0.8 nM at depths where phytoplankton thrives at 50-200 m. This concentration is too low for microorganism growth; ideally the concentration should exceed 0.1 μM, magnitudes larger than actual iron availability.\(^{23}\)
As a consequence of the low solubility of iron and the small fraction of dissolved iron that is Fe\(^{3+}\) ions, nature has evolved to produce a unique class of ligands that selectively bind and transport the Fe\(^{3+}\) ions into the microorganism, with much early work in this field done by Raymond and Hider.\(^{24,25}\) These ligands are known as siderophores (from Greek meaning iron bearer), a class of compounds produced by plants, bacteria, and fungi on both land and water to concentrate usable iron in their environment.\(^{26}\) When siderophores bind iron, the resulting iron complex is able to transport iron across cell membranes, increasing iron’s bioavailability. The siderophore enterobactin, compound 1.7, is often cited as the strongest natural chelator of Fe\(^{3+}\) ions (log \(K\) of 52).\(^{6}\) Commercially, a few siderophores are available for purchase, such as desferrioxamine B (DFB), compound 1.8, produced by *Streptomyces pilosus* and commercially available under the name Desferal.

The drug Desferal is utilized in the treatment of a variety of diseases including iron overload, malaria, thalassemia, and sickle cell anemia.\(^{25,27,28}\) To treat malaria, DFB is used as an iron scavenger that is able to penetrate the parasitic malaria cells and strip the Fe\(^{3+}\) ion from the cell, thus decreasing the cells ability to grow, or destroying the cell. At the same time, DFB is unable to penetrate healthy red blood cells, making it the desired treatment. For thalassemia and sickle cell anemia, regular blood transfusions may be required as treatment.\(^{29}\) The transfusions lead to the destruction of red blood cells in the process and the saturation of transferrin by Fe\(^{3+}\) ion, which leads to an increase in non-transferrin-bound-iron in the body, increasing risks of organ damage and cancer due to free radical formation, as a consequence of Fenton chemistry with the free Fe\(^{3+}\) ion.\(^{10,30}\) Desferal can be administered orally, but is often given intravenously instead, as in the
cases of iron overload sickness, either from blood transfusions or other sources. This is due to the poor oral activity and lack of absorption of DFB by the gastrointestinal tract due to the mesylate salt form of DFB used.\textsuperscript{30,31} Strategies to overcome the low oral activity of DFB and design and mechanisms of modified siderophores will be discussed further below, as they relate to the design of an iron sensing system soluble and functional in aqueous systems at basic pH.

**Why Detect Iron?**

As discussed, iron plays important roles both in the human body as well as in the ecosystem, particularly the ocean. It has become well established that iron is an important nutrient which has its own cycle (\textit{cf.} Figure 1.2) of movement, utilization by microorganisms, and deposition back into the environment. The iron cycle is part of the larger carbon cycle within the oceanic sub-cycle that sequesters carbon dioxide from the atmosphere and ultimately deposits it into oceanic sediment. An extension of \textit{The Iron Hypothesis (vide supra)} that has been explored is that the link between algae growth and iron concentration. It was hypothesized that seeding iron into the ocean would aid in
increasing the ability of the ocean to sink CO\textsubscript{2} from the atmosphere\textsuperscript{18,32,33}. Sequestering CO\textsubscript{2} via iron seeding works by changing the equilibrium between the ocean and atmosphere via the contributions of marine biota to the carbon cycle. The iron cycle would be impacted by iron seeding directly. Thus increasing the amount of CO\textsubscript{2} stored by marine biota, via the carbon cycle, Figure 1.4, would in turn increase the amount of CO\textsubscript{2} sequestered to the intermediate (200-1000 m) and deep ocean (>1000 m) reservoirs of CO\textsubscript{2} by marine biota. As a consequence of the complexity of the oceanic ecosystem, it is important to know how long iron will remain in the water column and how far it will drift once seeded. The long term effect of iron seeding is currently unknown, meriting a need for better methods of detecting iron for studies of any seeding experiments carried out on a large geographic or temporal scale.

\textit{Figure 1.4.} The Carbon Cycle, showing the movement of carbon through the ecosystem from the bottom of the ocean to the atmosphere. Reservoir sizes are shown in gigatons carbon (GtC) and fluxes/rates are shown in GtC per year. Copyright: \textit{Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change Figure 7.3.}
As the role in controlling iron concentration in the ocean and in biological systems is dependent on the role of siderophores, this convenient starting point for molecular probe design to target Fe\textsuperscript{3+} ions needs to be addressed and will be the focus of the next section.

Modified Siderophores

Siderophores exist in many shapes and sizes to enhance the mobility of Fe\textsuperscript{3+} in the environment around an organism. The common attribute of siderophores are their binding moieties; catechols (enterobactin, 1.7), hydroxamic acids (desferrioxamine B, 1.8), and \(\alpha\)-hydroxy-carboxylic acids (achromobactin, 1.9), Figure 1.5.\textsuperscript{34} Each of these binding moieties feature two oxygen atoms that act as hard Lewis bases to form dative covalent bonds with the Fe\textsuperscript{3+} ion in a six-membered chelating ring. Each siderophore contains three of these bidentate binding sites, making siderophores hexadentate ligands that excel at chelating Fe\textsuperscript{3+} in either its preferred octahedral or trigonal prismatic geometry (\textit{vide supra}).

As with all sensing applications, selectivity is key. Even though siderophores have a high binding affinity for Fe\textsuperscript{3+} ions, they can also bind other trivalent metal ions effectively such as Al\textsuperscript{3+} ions. This has led to environmental issues such as natural siderophores chelating dangerous trivalent actinides stored in clay ditches and carrying them through the clay via microorganisms leading ultimately to the actinides being released into the water table below.\textsuperscript{35,36} The leaching by siderophores has been explored using lanthanide metals such as neodymium and europium as tracers.\textsuperscript{37} These lanthanide ions were chosen due to being similar to actinides in charge (+3) and coordination to
ligands, but not radioactive nor typically found in soil. This example of lanthanides as tracers in combination with siderophores highlights how the siderophores can be used in coordination of lanthanides. In the ocean, the only trivalent metal ion present in quantities larger than the $\text{Fe}^{3+}$ ion is the $\text{Al}^{3+}$ ion. The $\text{Al}^{3+}$ ion is able to coordinate to siderophores such as DFB ($\log K = 24.1$), with a weaker but significant affinity compared to the $\text{Fe}^{3+}$ ion interaction with DFB ($\log K = 30.7$).\textsuperscript{38,39} Due to the higher binding affinity with DFB, the concentration of $\text{Fe}^{3+}$ ions at the surface of the water due to alluvial deposition is maintained at a significantly lower level (as low as 0.02 nM) than that of $\text{Al}^{3+}$ ions (0.25-0.65 nM, average 0.33 nM at 120 m depth). This demonstrates the scavenging of $\text{Al}^{3+}$ is much less than that of $\text{Fe}^{3+}$, in part due to the greater selectivity of siderophores.\textsuperscript{6,40}

For the purpose of sensor design, siderophores lack a signaling unit and therefore must be functionalized appropriately. However, only a few siderophores are sold commercially, and due to the expense or intricate extraction they are costly to produce.\textsuperscript{27} Any modification of the siderophore must not interfere with binding metal ions. The siderophore DFB, \textbf{1.8}, is a hydroxamate siderophore, containing three hydroxamate groups and two amide groups along an aliphatic chain that bears a terminal amine and is commonly available as a mesylate salt. This terminal amine group is useful to tether to other functional groups by organic transformations. Examples include acid anhydrides, carboxylic acids, or aldehydes used to form amide and amine groups respectively (Chapter II).\textsuperscript{41}
Figure 1.5. Siderophores with binding moiety in box: 1.9 enterobactin (catechol, log $K = 52$), 1.8 desferrioxamine B (hydroxamic acid, log $K = 30.7$), and 1.9 vibrioferin ($\alpha$-hydroxycarboxylic acid, log $K = 24$).\textsuperscript{42-44}

The DFB molecule and its derivatives have been extensively used in iron chelation therapy leading to DFB (as the mesylate salt) being commercially available. Unfortunately, DFB mesylate has poor lipophilicity, improved by modification of its primary amine into a less polar substituent. The earliest modifications to DFB were performed by Lytton et al., who explored the addition of a nitrobenz-2-oxa-1,3-diazole (NBD) group at the primary amine of DFB (1.10), which would increase the siderophore’s ability to permeate cells as well as allow it to act as a sensor to detect the removal of Fe$^{3+}$ ions from the environment around it. The molecular probe (1.10) in
HEPES at pH 7.4 is shown to have a fluorescent emission in the presence of one equivalence of Fe$^{3+}$ ions. The fluorescence of 1.10 offers a useful feedback mechanism to demonstrate when the drug bound as much iron as possible, at which point the fluorescence emission increases, and no more is needed for treatment. A downside, to this system is the addition of the NBD group substantially increasing the cellular toxicity towards red blood cells. Loyevsky et al. approached the toxicity issue with a modified DFB utilizing an N-methyl-anthranilate (NMA) group attached to the primary amine of DFB (1.11). As with NBD, the NMA terminal group increases the lipophilicity of DFB and improves the drug’s ability to pass through a biomembrane. As with 1.10, compound 1.11 acts as a sensor for Fe$^{3+}$ ions, which opens up the potential to detect excess of compound 1.11 remaining in the serum of a patient undergoing treatment for malaria. The fluorescent compound 1.11 has a good emission band in the visible range (438 nm) in HEPES buffer at pH 7.4. A solution of 10 μM sees the signal decreases by 80% upon the addition of one equivalence of Fe$^{3+}$ ions. While compound 1.11 has shown action as both an anti-malarial treatment as well as a sensor for Fe$^{3+}$ ions, there is a need to tune the biomembrane permeation of DFB further. As previous work had provided only novel compounds, Ihnat et al. have approached the issue of tuning DFB by synthesizing a library of DFB compounds intended to increase its lipophilicity by addition of various fatty and aromatic groups (Compounds 1.12-1.13). To build this library, the primary amine of DFB was reacted with various acid chlorides and anhydride compounds. These modified DFB compounds demonstrated up to a 2000-fold decrease in solubility in water as well as establishing a 2-fold increase in octanol/buffer distribution with the addition of each methyl or methylene group, which translates to an increase in lipophilicity. This
library provided a useful starting point to Liu et al. who wished to further improve the mobility of DFB within the body while avoiding a rise in cell toxicity.\textsuperscript{46} The group chose adamantane-1-carboxylic acids (previously seen in clinical use for treatment of various illnesses) to use.\textsuperscript{47} A one-step synthesis was adapted from the work of Lau et al. in the bioconjugation of peptides to produce adamantane-1-carboxylic acid modified DFB (\textit{1.14}).\textsuperscript{48} The various adamantane-modified DFB compounds provided up to double the Fe\textsuperscript{3+} uptake as compared to unmodified DFB.

Since the first use of Desferal as a clinical drug, many approaches have been taken to modify its ability to treat a variety of diseases ranging from parasitic infections to genetic disorders.\textsuperscript{49} The unifying aspect of these modifications has generally been increasing the lipophilicity of Desferal and improving its mobility within the body while not impacting its uptake of Fe\textsuperscript{3+} ions. Still, there are a few clear examples of modified DFB being utilized as a sensor for Fe\textsuperscript{3+} ions alongside its use as a treatment for a disease. While until now the function of DFB as a molecular probe for Fe\textsuperscript{3+} ions has been a
secondary function, it is of interest to utilize this ability as the primary function. Previous modifications to DFB have provided a useful starting point, but have been focused on improving its ability as a drug. To improve the ability of DFB as a sensor, a strategy must be taken towards its modification that increases its fluorescence emissions and the quenching response to Fe$^{3+}$ ions demonstrated by its previously used fluorophores.

Fluorescent Sensing Systems

Fluorosensors have become a popular tool for the detection of the Fe$^{3+}$ ion. While fluorosensors come in a variety of forms, there are a few fundamental principles shared amongst them. For the purpose of this work, the discussion of fluorescence is geared towards mechanisms between host-guest systems while traditional fluorescence is omitted. Most systems rely on host–guest interactions where a ligand acts as the host (receptor unit) which is able to bind a guest (analyte) and thus modulate the emission of a fluorophore (indicating unit), when attached by a linkage (spacer), as in Figure 1.6.$^{50}$

![Fluorescent Sensing System Diagram](image)

*Figure 1.6. A standard indicator-spacer-receptor (ISR) arrangement.*

Fluorosensors are able to assume a myriad of shapes with many different receptor units, but are ultimately a receptor coupled to a fluorophore with a means for the fluorophore to be modulated by an analyte interacting with the receptor.$^{51}$ It is therefore straightforward to classify fluorosensors based on how the molecule modulates their
signal. They can be organized into four broad groups, each of which will receive a more detailed discussion (*vide infra*); a) on-OFF, b) off-ON, c) ratiometric, and d) chemodosimeter, Figure 1.7.

![Diagram of molecular sensing methods](image)

*Figure 1.7. Cartoon of molecular sensing methods.*

a) In on-OFF sensors, an analyte modulates a fluorophore by quenching

b) In off-ON sensors, an analyte modulates a fluorophore by enhancement or removal of quenching

c) In ratiometric sensors, an analyte creates a reversible shift in two emissions from a fluorophore or set of fluorophores

d) In chemodosimters, an analyte mediates an irreversible reaction that changes the state of the fluorophore

Fluorescent molecules have two broad mechanisms for quenching fluorophores: dynamic quenching, which modulates the indicator by collisions between it and the analyte in solution, and static quenching, which modulates the indicator by formation of a complex
with the analyte. Most molecular sensors rely on static quenching using a receptor as previously discussed, which removes the need for a highly efficient quenching interaction for the brief but repeated collisions of dynamic quenching. Quenching always refers to the conversion of a molecule’s electronic energy to heat.\textsuperscript{52} There are several mechanisms by which quenching can occur, shown in Figure 1.8.

\textbf{Figure 1.8.} Diagram of fluorescence mechanisms.

a) Intersystem Crossing (ISC) is a shift from the singlet to triplet state mediated by a heavy halogen or metal atom or triplet oxygen which leads to a radiationless relaxation.

b) Electron-Exchange Quenching (Dexter) interactions involve an excited electron moving from a donor’s LUMO to an acceptor while a ground-state electron from the acceptor’s HOMO moves to the donor.
c) Photoinduced Electron Transfer (PET) involves a charge transfer complex forming where an electron moves from the donor’s LUMO to the acceptor then relaxes before being transferred back to the donor.

d) Förster Resonance Energy Transfer (FRET) functions the same as LRET, but if the acceptor is not a fluorophore it will relax the excited electron without emitting.

The different quenching mechanisms offer the potential for any fluorescent molecule to be modulated by an analyte and used in a sensing system, with a wide variety finding use in detecting many different metals and organic molecules. Certain 3d transition metals are of interest as targets for fluorescence detection due to their ability to quench fluorophores. This quenching comes from the paramagnetic 3d metal species that are able to deactivate fluorescence through energy transfer. This energy transfer happens when the unfilled orbitals of the metal fall close to the HOMO levels of the fluorophore, allowing an efficient transfer, which can happen in several transition metals of interest such as Cu$^{2+}$ (d$^9$) and Fe$^{3+}$ (d$^5$). Some of the most common fluorophores used in detection of the Fe$^{3+}$ ion are rhodamine (1.15), fluorescein (1.16), and coumarin (1.17) dye molecules. Rhodamine and its derivatives are useful for their ability to form a spirolactam ring, such as in compound 1.18. This creates an easy way to modulate the emission of the system; rhodamine in its ring-closed state is both colorless and non-fluorescent, while the ring-open state absorbs strongly as well as giving an emission with a high quantum efficiency (often approaching unity). It is convenient to modify the N-position of the spirolactam ring with a moiety able to act as a ligand in an off-ON system, with certain analytes enabling the ringing open of the spirolactam (vide infra).
Fluorescein is a similar spirocycle to rhodamine that has found use as a tracer in environmental systems due to its low toxicity, as well as in applications for cellular imaging. Fluorescein is often modified in the meta or para position of its benzoic acid ring to accommodate a receptor to create a molecular probe for transition metals. This probe acts as an on-OFF sensor able to quench the emission of fluorescein by energy transfer to a paramagnetic transition metal ion. Lastly, coumarin is an easily modified bicyclic molecule that has found use in fields as diverse as textile dye to imaging cells. As a molecular probe the coumarin molecule is often modified in the 7-position. In particular the addition of a diethylamino functional group, compound 1.19, allows for the dye to absorb and emit in the visible spectrum (typical $\lambda_{\text{exc}} \approx 420$ nm, $\lambda_{\text{em}} \approx 460$ nm). Additionally, coumarin can be modified in the 3-position and 4-position to allow for a receptor to be attached, which is discussed in detail in Chapter II.

On-OFF Sensing Systems

Due to the various quenching mechanisms, on-OFF systems are very common for analytes such as Fe$^{3+}$ ions, which can quench through either energy or electron transfer.
Quierós et al. has synthesized a fluorescein-catechol indicator-receptor system (compound 1.20) to selectively detect Fe\(^{3+}\) ions over other metals tested (Al\(^{3+}\), Cu\(^{2+}\), Zn\(^{2+}\)), of which only Cu\(^{2+}\) produced a notable quenching effect, primarily due to the heavy metal effect. Additionally fluorescein has a high molar absorptivity (145,000 L·mol\(^{-1}\) cm\(^{-1}\)) which enables the molecular probe to detect analytes at lower concentrations. The probe, compound 1.20, was calculated to be coordinated to the Fe\(^{3+}\) ion in a 1:3 (M:L) ratio in MOPS buffer (pH = 7.4). The fluorescent signal of compound 1.20 quenched (\(\lambda_{\text{exc}} = 493\) nm, \(\lambda_{\text{em}} = 518\) nm) by 70% with 0.33 eq of Fe\(^{3+}\) and has a quantum yield of 0.55 (carboxyfluorescein was used as a standard). No binding constants were reported by the authors, only percentages of fluorescence.

Su et al. synthesized a fluorescein-based sensor where desferrioxamine B is tethered to the rhodamine moiety receptor (1.21) to detect Fe\(^{3+}\) ions at physiological pH (7.2). This molecular probe is able to detect Fe\(^{3+}\) ions at concentrations as low as 2 x 10\(^{-8}\) M while being selective for Fe\(^{3+}\) ions over other metal ions relevant to human physiology (Al\(^{3+}\), Cu\(^{2+}\), Ca\(^{2+}\), and Ni\(^{2+}\)), of which only Cu\(^{2+}\) produced notable quenching (65% original emission for Cu\(^{2+}\) compared to Fe\(^{3+}\) showing 45% of the original emission). The advantage of this system is the attachment of the siderophore to the molecular probe, taking advantage of the highly selective binding of Fe\(^{3+}\) ion by DFB. The molecular probe, compound 1.21, was synthesized as two isomers with the DFB attached either
meta or para to the carboxylic acid of fluorescein ($\lambda_{\text{exc}} = 460 \text{ nm}$, $\lambda_{\text{em}} = 524 \text{ nm}$). Much like unmodified fluorescein, the isomers of compound 1.21 are pH sensitive with an ideal pH of around 8.0, which places fluorescein into its highly fluorescent dianionic form.\textsuperscript{61} In methanolic solution, it was found that meta-1.21 (but not para-1.21) would efficiently quench in the presence of $\text{Fe}^{3+}$ ions. The difference in the spectroscopic response between the two isomers is due to the positioning of the amide linkage between the fluorescein and DFB. The para isomer of receptor 1.21 prohibits electron transfer between the highly conjugated $\pi$-system of fluorescein and the coordinated $\text{Fe}^{3+}$ ions. In the meta form of compound 1.21, the DFB (and $\text{Fe}^{3+}$ ion) resides at the bottom of the $\pi$-system and able to efficiently interact with the $\pi$-system which leads to quenching, as a consequence of PET. In this system the conjugated $\pi$-system in the meta-1.21 isomer allows DFB to act as a donor while fluorescein is the acceptor. In the absence of $\text{Fe}^{3+}$ ions, HOMO energy from the DFB is lower than the HOMO of fluorescein group, as shown in Figure 1.8. Upon the coordination of $\text{Fe}^{3+}$ ions to the DFB ligand, the HOMO level increases enough that it is able to donate an electron to the acceptor HOMO of fluorescein, thus resulting in quenching as fluorescein loses the energy via thermal decay rather than through an emission. The sensor, 1.21, showed $\text{Cu}^{2+}$ was also able to cause some quenching through the same PET mechanism as the $\text{Fe}^{3+}$ ion (see Figure 1.9), but as this sensing motif is intended for detecting labile iron, interference from $\text{Cu}^{2+}$ was argued to not be a major concern.\textsuperscript{38}
Figure 1.9. The energy levels of para-**1.21** with and without a coordinated Fe$^{3+}$ ion. Reprinted with permission from Su, B.-L.; Moniotte, N.; Nivarlet, N.; Tian, G.; Desmet, J. Pure Appl. Chem. 2010, 82, 2199.

A tripodal hydroxyl-phenol-oxazoline system (**1.22**, **R** or **S**) has been synthesized by Kikkeri et al. as an artificial siderophore. This system mimics the binding of siderophores utilizing indicators that act as receptors as well.$^{62}$ The phenol-oxazoline binding moieties are able to coordinate Fe$^{3+}$ ion through the phenol hydroxyl group, as well as the oxazoline nitrogen atom to form a six-membered ring binding geometry around the Fe$^{3+}$ ions (Figure 1.10). The tripodal nature of the molecule also allows for Fe$^{3+}$ ions to be coordinated in an octahedral complex with a binding affinity as high as log $K = 34$ (methanol:water 80:20), comparable to that of naturally occurring siderophores (**vide supra**). The phenol-oxazoline groups in molecule **1.22** act as the
signaling group for the system, fluorescing due to an excited-state intramolecular proton transfer. In solution (methanol:water 80:20, acetate buffer pH 5.8) receptor 1.22 has a strong fluorescence band at 410 nm (λ_{exc} = 340 nm), which is quenched by 80% with an excess of Fe^{3+}. 
Another molecular probe which binds Fe\textsuperscript{3+} ions by an unusual polymeric structure as opposed to a 1:1 host–guest complex, was reported by Yao et al., compound 1.23, which demonstrates coordination to the Fe\textsuperscript{3+} ion by both the tris moiety of one molecule and the two oxygen atoms (one from the amide group and the other from the coumarin derivative) of a second molecule in a 2:1 complex.\textsuperscript{63} This decreases the fluorescence emission of compound 1.23 by 92% in the presence of 60 equivalences of the Fe\textsuperscript{3+} ion. The binding of Fe\textsuperscript{3+} ions to the carbonyls of coumarin and its amide linkage is able to quench the coumarin’s fluorescence through an electron or energy transfer. The hardness of the oxygen atoms as Lewis bases makes this sensor highly selective for Fe\textsuperscript{3+} ions, with other metals tested (Li\textsuperscript{+}, Na\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, Cr\textsuperscript{3+}, Mn\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, Ag\textsuperscript{+},
Cd\(^{2+}\), Pb\(^{2+}\), Hg\(^{2+}\)) showing a lack of quenching of the coumarin signal. The second binding moiety in the form of the tris terminal group allows for the self-assembly of a polymer 1D chain, resulting in multiple sensor units coordinating each Fe\(^{3+}\) ion, then increasing the selectivity of the sensor. The potential for this molecule to act as a polymer (Figure 1.11) was suggested by dimers identified through ESI-MS, though the monomer remains most abundant.

![Figure 1.11](image)

**Figure 1.11.** Polymerization of compound **1.23** in coordinating Fe\(^{3+}\) ions in a 2:1 M:L complex.

A rather interesting fluorophore was synthesized by Koner et al. who derivatized a 2-aminopyridine. This molecule was found to be able to bind Fe\(^{3+}\) ions through the pyridine moiety and Hg\(^{2+}\) ions at its amine moiety. Therefore, both Fe\(^{3+}\) and Hg\(^{2+}\) can be simultaneously detected, which is of interest due to the need to detect the former in various illnesses and the latter as a toxin.\(^{64}\) Compound **1.24** shows a distinct absorption band 246 nm and 335 nm assigned to the π-to- π* transitions at (ε = 23,680 L·mol\(^{-1}\)·cm\(^{-1}\) and 7,860 L·mol\(^{-1}\)·cm\(^{-1}\) respectively) in methanolic solution, which undergo a hyperchromic shift upon addition of two equivalences of Fe\(^{3+}\) ions (ε = 30,640 L·mol\(^{-1}\)·cm\(^{-1}\) and 13,440 L·mol\(^{-1}\)·cm\(^{-1}\) respectively) whereas two equivalences of Hg\(^{2+}\) causes a hypochromic shift in absorption (ε = 14,480 L·mol\(^{-1}\)·cm\(^{-1}\) and 4,690 L·mol\(^{-1}\)·cm\(^{-1}\) respectively). A fluorescence emission is seen at 410 nm (λ\(_{ex}\) = 330 nm), in methanolic
solution was shown to have a quantum yield of 0.15 (standard of 0.5M quinine in H$_2$SO$_4$) and quenched by 81% upon addition of two equivalences of Fe$^{3+}$ ions or by 55% in presence of two equivalences of Hg$^{2+}$ ions. No other metals or molecules studied (Li$^+$, Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, Cd$^{2+}$, Ag$^+$, Co$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, NH$_4^+$, Pb$^{2+}$, Al$^{3+}$, Co$^{3+}$) produced a significant quenching effect. As shown in Figure 1.12, the addition of the Fe$^{3+}$ ions causes a significant decrease (81%) in fluorescence while NaF has little effect directly, but when added after the Fe$^{3+}$ ions, regenerates the fluorescence signal to the original intensity. An additional two equivalences of Fe$^{3+}$ ions will then quench the emission again by ~70%.

Off-ON Sensing Systems

A disadvantage of the on-OFF system is the lack of sensitivity when targeting an analyte. This is due to the fluorescence signal eventually being masked by the background noise of the spectrometer, upon quenching. Furthermore, there are many quenching mechanisms (vide supra). An alternative approach is to design a system that increases the intensity of the fluorescence emission i.e., an off-ON system upon the coordination of the metal ion. The PET mechanism is often employed to first quench the emissions. Then upon the coordination of Fe$^{3+}$ to the sensor (1.25) to form the iron complex (1.26), the electron density is pulled away from the PET donor and thus no longer has an effect, allowing the indicator unit to emit. This off-ON mechanism has introduced new terminology into the field of sensor design. In this particular case it is known as chelation enhanced fluorescence (CHEF). The rhodamine molecule again is used in off-ON systems, such as the bis-rhodamine system prepared by Weerasinghe et
Compound 1.25 is colorless and non-fluorescent in buffer (0.01 M Tris-HCl at pH 7.0, 25:75 H2O:CH3CN) in absence of Fe3+ ions due to its ring-closed form. The addition of 40 equivalences of Fe3+ allows the spirolactam of the two rhodamine moieties to ring open, causing the appearance of a pink color as absorbance increases along with a 48-fold fluorescence enhancement (λ_{exc} = 510 nm, λ_{em} = 580 nm) and a quantum yield of 0.048 (rhodamine B as a standard). Additionally, compound 1.25 was found to have a detection limit of 5 x 10^{-5} M and is selective for Fe3+ ions, while no spectroscopic response was seen for most metals studied (Na+, K+, Ca2+, Mn2+, Fe2+, Ni2+, Zn2+, Cd2+, Hg2+, Pb2+). The one exception was the Cu2+ ion which produced a change in absorbance but no enhancement of fluorescence. This system additionally carries the ability to undergo two-photon fluorescence (λ_{exc} = 800 nm, λ_{em} = 585 nm) which doubles the emission response to a 96-fold fluorescence enhancement. This increased emission is partially due to absorption of visible light by the Fe3+ ion, whereas the ion does not absorb the infrared used in the two-photon fluorescence and partially due to the ringing open of the rhodamine moieties by the Fe3+ ion, which increases the two-photon cross section of the molecule.66
A single-rhodamine system (compound 1.27) has been synthesized by Yang et al. for imaging Fe³⁺ ions in living cells using a benzothiazole-rhodamine motif. Compound 1.27, is able to selectively detect Fe³⁺ ions over other metals tested (Li⁺, Na⁺, K⁺, Ba²⁺, Ca²⁺, Cd²⁺, Mg²⁺, Co²⁺, Mn²⁺, Zn²⁺, Pb²⁺, Ni²⁺, Hg²⁺, Ag⁺, Cr³⁺, Cu²⁺, Fe²⁺) in the range of 5 x 10^{-6} M to 2 x 10^{-5} M in methanol-water (45:55) solution. As with other rhodamine systems the addition of Fe³⁺ ions causes an increase in fluorescence (λ_{exc} = 550 nm, λ_{em} = 580 nm), in this example a quantum yield of 0.34 was calculated (against the standard rhodamine 6G) with an association constant of 4.52 x 10⁵ M⁻¹. The iron complex (1.28) shows an interesting feature in that the Fe³⁺ ions bind to the nitrogen atom of the benzothiazole group rather than the carbonyl moiety (Scheme 1.1). This can be explained by the lone pair of the nitrogen both being more favorable sterically as well as having a higher energy than the carbonyl lone pair, making compound 1.27 more energetically favorable to coordinate to Fe³⁺ ions.
Scheme 1.1. The binding of the $\text{Fe}^{3+}$ ion by 1.27 to form complex 1.28, showing a resonance stabilized coordination to the nitrogen of the benzothiazole moiety.

Improving upon the use of the benzothiazole receptor, Sikdar et al. synthesized a rhodamine-pyridine system (1.29) able to fluorometrically detect $\text{Fe}^{3+}$ ions in concentrations as low as $9 \times 10^{-8}$ M along with colorimetrically detecting $\text{Cu}^{2+}$ ions as many rhodamine-based systems are capable of due to the metal-catalyzed ring opening of the spirolactam system (Scheme 1.2). The pyridine ring is connected to a rhodamine 6G moiety via a hydrazone linkage that also binds to the metal ions, complex 1.30. The reaction is also reversible upon the addition of ethylenediaminetetraacetic acid (EDTA) to remove the $\text{Fe}^{3+}$ ion.

Scheme 1.2. The binding of $\text{Fe}^{3+}$ or $\text{Cu}^{2+}$ ions by 1.29 to form complex 1.30, reversible with EDTA and I⁻ ions.

As with other rhodamine systems, the addition of $\text{Fe}^{3+}$ ions ring opens the lactam and increases its fluorescence substantially in Tris-HCl (10mM, pH = 7.04, 50:50
water/CH$_3$CN) buffer to give an emission band at 552 nm with a quantum yield of 0.30 (standard used is unmodified rhodamine 6G). Compound 1.31 has shown to be selective in its fluorescence enhancement for Fe$^{3+}$ ions only with no other metals tested triggering a comparable enhancement (Li$^+$, Na$^+$, K$^+$, Ba$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Pb$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Ni$^{2+}$, Ag$^+$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$). Due to the effect of Cu$^{2+}$ on rhodamine-based sensors such as those previously shown above, Hu et al. synthesized a sensor that is entirely selective for Fe$^{3+}$ ions over other metals tested (Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, Ag$^+$, Ni$^{2+}$, Ce$^{3+}$, Co$^{2+}$). For compound 1.31, rhodamine-hydroxamate is modified with an acetyl group to give a binding moiety featuring hard Lewis bases in the form of oxygen atoms with the intent of producing a fluorescent sensor that is safe for bioimaging purposes. This improves the affinity for hard Lewis acids, such as Fe$^{3+}$ ions, over the affinity shown by previous rhodamine sensors that predominantly use borderline Lewis base atoms such as nitrogen atoms. As with other rhodamine receptors, in its ring-closed form receptor 1.31 is colorless and non-fluorescent. The addition of Fe$^{3+}$ ions in 50:50 CH$_3$CN:H$_2$O rings open the system giving an intense emission ($\lambda_{\text{exc}} = 530$ nm, $\lambda_{\text{em}} = 575$ nm) form a 1:1 binding that allows for detection of Fe$^{3+}$ ions with calculated limit of detection (LoD) = 7.0 x $10^{-8}$ M.
Ratiometric Sensing Systems

The on-OFF and off-ON mechanisms discussed previously show effects on emission from factors such as the concentration of receptor and the presence of various solvents and interfering ions. This poses an issue and requires careful standardization and control of conditions to avoid inaccuracies from changes in concentration. An elegant approach that eliminates the problems described above is a ratiometric system that compares the emission of two fluorophores or two emissions of a single fluorophore.\textsuperscript{70,71} While ratiometric sensors for metal ions have been known for many years, Jung et al. developed one of the earliest examples of a ratiometric sensor for the detection of Fe\textsuperscript{3+} ions in the form of a benzimidazole system.\textsuperscript{72} Compound 1.32 is a rigid structure where the imine groups limit the degrees of freedom of the benzimidazole arms along with participating in the coordination of Fe\textsuperscript{3+} ions. The four imine nitrogen atoms of compound 1.32 bind Fe\textsuperscript{3+} ions with their nitrogen lone pairs, similar to how porphyrins are able to interact with the Fe\textsuperscript{3+} ion. Addition of Fe\textsuperscript{3+} to the system in CH\textsubscript{3}CN:H\textsubscript{2}O (95:5, v/v) causes a shift in emissions (λ\textsubscript{exc} = 288 nm, λ\textsubscript{em} = 412 nm) with the 412 nm signal quenching while another red-shifted band appears at 475 nm. The ratio between these two emission bands allows for accurate (micromolar range) and selective detection of Fe\textsuperscript{3+} ions over other metals tested (Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Ba\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, Ag\textsuperscript{+}, Hg\textsuperscript{2+}). However, like other systems discussed Cu\textsuperscript{2+} ions show a quenching effect for the 412 nm band, but no shift or increase of the 475 nm band. Another ratiometric Fe\textsuperscript{3+} ion sensor was developed by Marenco et al. who used thiosemicarbazone groups, which are well-studied Fe\textsuperscript{3+} ion chelators utilized in cancer treatment, to act as a ratiometric probe.\textsuperscript{73} The molecular probe (1.33) shows two strong emission bands at 425 nm and 485
nm ($\lambda_{exc} = 370$ nm) in THF:H$_2$O (9:1, buffered to pH 7.4 with HEPES) that allows for a ratiometric analysis ($I_{485}/I_{425}$) of Fe$^{3+}$ ions in concentrations (as low as $1.7 \times 10^{-5}$ M) that are not seen with other metals studied (Li$^+$, Na$^+$, Mg$^{2+}$, K$^+$, Ca$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Sr$^{2+}$, Ag$^+$, Ba$^{2+}$, Pb$^{2+}$, Cu$^{2+}$). The addition of Fe$^{3+}$ ions to the system causes a 104% increase in the 425 nm band and 194% increase in the 485 nm band due to the coordination of Fe$^{3+}$ ions to receptor 1.33 interrupting the rapid isomerization of the imine bond that would normally lead to non-radiative relaxations.

Lin et al. proposed a phenanthroimidazole system able to function in CH$_3$CN:H$_2$O (50:50 v/v). For compound 1.34 the addition of Fe$^{3+}$ ions leads to its coordination into the bipyridine moiety, which triggers an increase in the emission at 440 nm and decrease in the 500 nm emission ($\lambda_{exc} = 352$ nm). The ratio between the two emissions ($I_{440}/I_{500}$) grows 9-fold upon addition of 30 equivalents of Fe$^{3+}$ ions and allows for detection of Fe$^{3+}$ ions in concentrations as low as $5.26 \times 10^{-6}$ M. For selectivity, receptor 1.34 has been shown to offer the aforementioned response only to Fe$^{3+}$ ions, while Mn$^{2+}$, Cd$^{2+}$, Fe$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, and Hg$^{2+}$ ions caused a quenching of the 500 nm emission with no enhancement of the 440 nm emission, while Cr$^{3+}$ ions enhanced the 440 nm emission but did not quench the 500 nm emission. Other metals (Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$) had no effect on either fluorescent emission. Wang et al. presents a benzimidazole-based sensor (1.35) for both Cr$^{3+}$ and Fe$^{3+}$ ions intended to improve upon previous designs by allowing for function in solvents DMSO:H$_2$O (1:99, v/v). In the presence of Fe$^{3+}$ ions, the emission of compound 1.35 ($\lambda_{exc} = 320$ nm) shows an increase at 380 nm and decrease at 443 nm. The ratio of the emissions ($I_{380}/I_{443}$) undergoes a 5-fold increase upon the addition of 50 equivalents of Fe$^{3+}$ ions, which allows for a detection limit of 2.0 x
This system was shown to be selective for Fe$^{3+}$ with no other metals having the same effect when tested (K$^+$, Ba$^{2+}$, Sr$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Hg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, La$^{3+}$, Cu$^{2+}$, Ag$^+$) save for Cr$^{3+}$. For the Cr$^{3+}$ ion, a larger enhancement of the 380 nm emission but not the 443 nm emission was found, making it distinguishable from the Fe$^{3+}$ ion. Binding is achieved through a motif similar to the previous benzimidazole system while the emission mechanism appears to be a PET mechanism as described previously.

Ge et al. proposes a rhodamine-coumarin system (1.36) that is able to selectively detect Fe$^{3+}$ ions over other metals (Na$^+$, K$^+$, Mg$^{2+}$, Ba$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$ Ag$^+$, Pb$^{2+}$, Hg$^{2+}$, Ni$^{2+}$, Cu$^{2+}$) in EtOH:HEPES 99:1 at pH 7.2. Chelation of Fe$^{3+}$ into this system to form 1.37, as shown in Scheme 1.3, both triggers a CHEF interaction with the rhodamine moiety as well as quenching the coumarin moiety ($\lambda_{exc} = 425$ nm, $\lambda_{em} = 460$ nm, 580 nm). By tracking the ratio between the emissions at 460 nm and 580 nm, the amount of Fe$^{3+}$ ions entering the system can be accurately tracked. The system shows a quantum efficiency of 0.38 in ethanol (rhodamine B as standard) and features a detection limit of $5.2 \times 10^{-7}$ M.
Chemodosimeter Systems

The previous sections have described various sensing systems for Fe$^{3+}$ ions that are all considered to be reversible by the addition of a competing ligand such as EDTA to remove metal ions from the system. An alternative strategy is a chemodosimeter which monitors analytes in a system via a generally irreversible reaction that modulates the fluorophore signal. This one-time use sensor often provides an off-ON system that avoids equilibrium effects that alter the sensor response. Both are features that are advantageous to the detection of metal ions. Strategies for chemodosimeters for Fe$^{3+}$ ions often involve reactions catalyzed by the metal ion itself. For this reason, the Schiff base functional group is of interest due to the ability of strong Lewis acids, such as the Fe$^{3+}$ ion, to catalyze the hydrolysis of imines, as in Scheme 1.4.\textsuperscript{77} Using this strategy, Lin et al. present an off-ON bis-coumarin system \textbf{(1.38)} that is able to detect Fe$^{3+}$ ions over other metals studied (Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Hg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Zn$^{2+}$), Figure 1.13.\textsuperscript{78} As in any other example, the Cu$^{2+}$ ion caused quenching of the sensor. In CH$_3$OH/H$_2$O (98:2, v/v), compound \textbf{1.38} is quenched by a Dexter Mechanism from the imine moiety which gives the compound a quantum yield of 0.004 (against quinine sulfate as a standard). The addition of Fe$^{3+}$ catalyzes the hydrolysis of the imine bonds to

\textit{Scheme 1.3.} Binding of Fe$^{3+}$ ions to compound \textbf{1.36} forms iron complex \textbf{1.37} and triggers CHEF in rhodamine while quenching the emission of coumarin.
give compound 1.39 (Scheme 1.5), and thus shows a 140-fold increase in emission ($\lambda_{\text{exc}} = 330 \text{ nm}$, $\lambda_{\text{em}} = 392 \text{ nm}$), Figure 1.13, with a quantum yield of 0.27. Each sample was reacted for 50 minutes before detection to show that the Fe$^{3+}$ ions had a catalytic effect compared to hydrolysis potentially induced by water in the solvent system alone as shown by the free column in Figure 1.14.

\[
\begin{align*}
\text{R} & \quad \text{N}^{+} \quad \text{R}' \\
& \quad \text{H}_2\text{N}^{+} \quad \text{R}'
\end{align*}
\]

\textbf{Scheme 1.4.} Metal-catalyzed hydrolysis of imine using Fe$^{3+}$ ions; i) 50:50 heptane:THF, FeCl$_3$ in aqueous solution added then stirred.

\[
\begin{align*}
\text{1.38} & \quad \text{Fe}^{3+} \\
& \quad \text{1.39}
\end{align*}
\]

\textbf{Scheme 1.5.} Fe$^{3+}$-triggered hydrolysis of imine bonds of compound 1.38 leading to formation of compound 1.39 and ethylenediamine.
Figure 1.13. Emission of 1.38 (5 µM) upon addition of Fe$^{3+}$ ions (0, 0.1, 1, 3, 5, 7, 9, 12, 15 eq.). $\lambda_{\text{exc}} = 330$ nm. Copyright © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Figure 1.14. Fluorescence Enhancement Factor (FEF) of compound 1.38 (5 µM) in presence of various metal ions (12 eq.). $\lambda_{\text{exc}} = 330$ nm, $\lambda_{\text{em}} = 392$ nm. Copyright © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

To improve the previous off-ON bis-coumarin system, Lin et al. synthesized a coumarin-based ratiometric chemodosimeter with a high ratio between the two emission
states ($\lambda_{em1} = 573$ nm, $\lambda_{em2} = 461$ nm) by modifying coumarin with the electron donating group (EDG) diethylamine and electron withdrawing group (EWG) diaminomaleonitrile.\textsuperscript{79} The receptor $\textbf{1.40}$ imine is catalytically hydrolyzed, Scheme 1.6, on the addition of Fe$^{3+}$ ions in MeOH:H$_2$O (99:1), which cleaves the diaminomaleonitrile group of coumarin to form compound $\textbf{1.41}$. To ensure the hydrolysis had gone to completion and to show that the Fe$^{3+}$ ion once again catalyzed the hydrolysis, samples were allowed to react for 35 minutes before being scanned. The catalytic hydrolysis leads to a large blue shift ($\lambda_{exc} = 389$ nm) from an emission of 573 nm to 461 nm with a ratio between the signals moving from 0.05 to 55 (Figure 1.15). Due to the nature of the Schiff base hydrolysis, receptor $\textbf{1.40}$ was found to be highly selective for Fe$^{3+}$ over other metals investigated (Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Hg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, Zn$^{2+}$). It was also irreversible with the addition of EDTA failing to alter the end emission.

\textit{Scheme 1.6.} Catalytic hydrolysis of compound $\textbf{1.40}$ to produce aldehyde $\textbf{1.41}$ and diaminomaleonitrile.
Figure 1.15. Emission ratios ($I_{461}/I_{573}$) for receptor 1.40 (5 μM) upon addition of various metal ions (20 equivalences). $\lambda_{\text{exc}} = 389$ nm. Reprinted with permission from Lin, W.; Yuan, L.; Cao, X. *Tetrahedron Lett.* 2008, 49, 6585. Copyright 2014 Elsevier.

In an attempt to design a CHEF-based sensor for Fe$^{3+}$ ions using squaramide hydroxamate, Lim et al. have shown a chemodosimeter (1.42) is able to detect Fe$^{3+}$ selectively over other metals tested (Na$^+$, K$^+$, Mg$^{2+}$, Al$^{3+}$, Ga$^{3+}$, Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Pb$^{2+}$). Compound 1.42 shows a squaramine that has been modified with a hydroxamate group to extend its binding moiety by one unit to improve coordination of Fe$^{3+}$ ions. In presence of Fe$^{3+}$ ions, a 9-fold increase in fluorescence emissions is seen ($\lambda_{\text{exc}} = 347$ nm, $\lambda_{\text{em}} = 451$ nm) in sodium acetate buffer (pH 4.4) with a detection limit of $1.8 \times 10^{-5}$ M. The emissions are not reversed by the addition of an excess of EDTA due to the Fe$^{3+}$ ion chemically oxidizing the amine linkage between coumarin and squaramine to an imine, which is then hydrolyzed leaving the coumarin aldehyde as a product.
Taking advantage of the Schiff base hydrolysis triggered by Fe$^{3+}$ ions along with the ability of Fe$^{3+}$ ions to ring open the lactam of rhodamine, Lee prepared a rhodamine 6G-based chemodosimeter. Compound 1.43 is able to detect the Fe$^{3+}$ ion over +1 and +2 metals (Na$^+$, K$^+$, Fe$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ag$^+$, Hg$^{2+}$, Ba$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, Zn$^{2+}$), while acting as an off-ON system ($\lambda_{\text{exc}} = 528$ nm, $\lambda_{\text{em}} = 551$ nm) in H$_2$O:CH$_3$CN (95:5, v/v). The sensor (1.43) modulates by a Schiff base hydrolysis triggered by the Fe$^{3+}$ ion to produce compound 1.44 as shown in Scheme 1.8. This allows for a detection limit down to 1 x 10$^{-6}$ M with a quantum yield of 0.6 (against rhodamine 6G).

Scheme 1.7. Fe$^{3+}$ triggered imine hydrolysis of compound 1.43 to cleave 2-hydroxy-5-nitrobenzaldehyde from rhodamine 6G (1.44) and opening the lactam ring.

To improve upon the sensors modulated by ring opening and Schiff base hydrolysis reaction, Jackson et al. attempted to modulate a sensor by a oxidation reaction converting a catechol group to 1,2-benzoquinone to give compound 1.45. Receptor 1.45
shows the lone pair of the aniline nitrogen has a resonance interaction with the
naphthalimide fluorophore. Upon addition of Fe$^{3+}$ in a 2:1 ratio to the sensor, the catechol
motif is oxidized to give compound 1.46, allowing it to engage in a resonance interaction
with the aniline lone pair (Scheme 1.9). It was found that compound 1.46 was able to
tautomerize to compound 1.47 which would then undergo a Schiff base hydrolysis to
form compound 1.48, altering the energetics of the fluorophore and turning the emission
back on. This allows for detection of Fe$^{3+}$ ions ($\lambda_{\text{exc}} = 400$ nm, $\lambda_{\text{em}} = 520$ nm) in
CH$_3$CN/H$_2$O (99:1, v/v) with a quantum yield of 0.74 (compared to quinine sulfate as
standard).\textsuperscript{83}

\begin{center}
\includegraphics[width=\textwidth]{scheme1.8.png}
\end{center}

\textit{Scheme 1.8.} FerriNaphth showing redox of Fe$^{3+}$ during coordination that converts
compound 1.45 to compound 1.46, which can tautomerize (1.47) and then hydrolyze
(1.48).

\textbf{Indicator Displacement Sensors}

All examples shown previously have followed the basic ISR form (\textit{vide supra}). An
alternative to this arrangement is an indicator-displacement assay (IDA), which avoids
issues by attaching an indicator covalently to a receptor. Instead IDA attaches the
indicator through a non-covalent interaction.\textsuperscript{84} Taking advantage of the reversible nature
of the indicator-receptor association, a competitive analyte can then displace the
indicator, modulating its emission. These systems can in theory fall into any of the
previously described methods of modulation. One of the first IDA was developed by
Anslyn, whereby the triethylbenzene-based probe (1.49) can bind a dye molecule (1.51)
within its cavity to be displaced upon the addition of carboxylates (1.50).\textsuperscript{85} While the
sensor has a good affinity for compound 1.51 allowing it to act as an indicator, its cavity
better responds to triphosphates such as compound 1.50 which demonstrates a log $K = 8$.
There have only been a handful of IDA systems developed for the detection of the Fe$^{3+}$
ion. I reported a system in 2010 (Chapter III) while another example was synthesized by
Venkateswarulu et al.\textsuperscript{86} This indicator (1.52) is quenched by complexation with Cu$^{2+}$ ions
to form a 1:2 (metal:ligand) complex. Certain trivalent metals (Fe$^{3+}$, Al$^{3+}$, Cr$^{3+}$) are able
to displace receptor 1.52 from Cu$^{2+}$, taking its place in coordination and also forming 1:2
(metal:ligand) complex with the indicator which produces a strong emission in an off-ON
process ($\lambda_{\text{exc}} = 365$ nm, $\lambda_{\text{em}} = 413$ nm). The indicator shows a quantum yield of 0.49
(quinine in 0.1 M H$_2$SO$_4$ as standard) in 8:2 v/v MeOH/H$_2$O (pH 7.2, HEPES buffer),
with a log $K$ for Cu$^{2+}$ of 6.04. For comparison, the metals Al$^{3+}$, Cr$^{3+}$, and Fe$^{3+}$ show a log
$K$ of 6.68, 7.09, and 6.68 respectively, driving the ability of this system to detect them by
displacement of Cu$^{2+}$ and allow it to be selective for trivalent metals over others tested
(Na$^+$, K$^+$, Mg$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Mn$^{2+}$, Ag$^+$, Hg$^{2+}$).
Lanthanide Indicators

The lanthanide metals are of interest for a variety of applications due to their 
[Xe]4f\(^n\)6s\(^2\) (n = 0-14) electronic configuration. Their 4f sub-shell is shielded by the 
5s\(^2\)5p\(^6\) sub-shells, which gives rise to some unique magnetic and spectroscopic properties. 
This includes the decrease in ionic radius as the 4f orbitals fill (known as the lanthanide 
contraction).\(^{87}\) Lanthanide ions, for example La\(^{3+}\), Lu\(^{3+}\), and Gd\(^{3+}\) are well known NMR 
shift agents for organic compounds (and proteins) as a way to help determine their 
structures.\(^{88-92}\) It is known that lanthanides can influence the chemical shifts of the proton 
atoms that are in close proximity to hard Lewis bases coordinated to the lanthanide metal 
center.\(^{90}\) As a consequence, many overlapping NMR signals can be resolved with proton 
shifts of up to one ppm. The Gd\(^{3+}\) ion has been used as a contrast agent for magnetic
resonance imaging (MRI) scans by increasing the contrast of flowing blood or revealing the presence of tumors and lesions.$^{93}$

The unique spectroscopic properties of lanthanides arise from the shielded 4f subshell only allowing for weak interactions with ligands, avoiding many quenching effects during $f\rightarrow f$ transitions. These $f\rightarrow f$ transitions are Laporte forbidden, due to the transition of electrons from one degenerate 4f orbital to another in a metal center with high multiplicity as determined by the number of unpaired electrons. Though shielded, the 4f orbitals still undergo slight perturbations from ligands in the first and second coordination sphere. Due to these disruptions of symmetry, electric dipole transitions are possible leading to many lanthanides exhibiting long-lived (0.2-1.5ms) with unique, and sharp emission bands in the visible range.$^{94}$ The molar absorptivity for the lanthanides is very low ($\varepsilon = 5\text{--}10 \text{ M}^{-1}\cdot\text{cm}^{-1}$) due to the Laporte forbidden nature of the $f\rightarrow f$ transitions.$^{95}$ Overcoming this low absorptivity requires an indirect method the lanthanide can be excited by, the antenna effect, which perturbs the symmetry of the 4f orbitals and breaks the degeneracy. The results of this effect are the emitting states for each lanthanide (Figure 1.16) which are arranged following Hund’s Third Rule, where in the Tb$^{3+}$ ion which has a subshell more than half full ($f^8$) the lowest emitting state holds the highest $J$ value ($J = 4$). In the Eu$^{3+}$ ion the subshell is less than half full ($f^6$) and the lowest energy emitting state holds the lowest $J$ value ($J = 0$). This antenna effect has been demonstrated through energy transfers from ligands in the triplet ($T_1$) state by Weissman (Figure 1.16).$^{96}$
Figure 1.16. Jablonski diagram showing ligand-centered excitation of a fluorophore to its singlet state followed by an intersystem crossing (ISC) into the emitting state of Tb$^{3+}$ or Eu$^{3+}$ as well as the vibrational overtones of H$_2$O that potentially quench lanthanide metals.

Classical Triplet-State Sensitization

In 1942, Weissman demonstrated that a number of organic ligands can sensitize Eu$^{3+}$, Tb$^{3+}$, and Sm$^{3+}$ ions when coordinated to the metal center.$^{96}$ Whereby an organic molecule containing a $\pi$-system can absorb a photon, which is excited to a singlet state ($S_1 \leftarrow S_0$). This process is either followed by a quenching mechanism by molecular fluorescence ($S_1 \rightarrow S_0$) or an intersystem crossing from the singlet state ($S_1$) to the triplet state ($T_1$). The triplet state is then able to undergo a spin-forbidden radioactive deactivation, known as phosphorescence ($T_1 \rightarrow S_0$), in later years (Figure 1.16). Crosby and Whan proposed an alternative mechanism of deactivation wherein the triplet state
(T₁) is able to undergo a non-radioactive energy transfer to the excited state of a lanthanide (T₁→Ln*). The lanthanide ion is then able to undergo a radioactive transition back to a 4f state which gives the characteristic emission of the metal (Ln*→Ln), Figure 1.16.

The classical antenna effect acts as a through-space interaction between the antenna and the lanthanide; therefore there is no overlap between atomic orbitals. Instead the two states interact via a columbiaic coupling between the dipoles of the antenna donor and the lanthanide acceptor in a manner similar to a Förster Resonance Energy Transfer (FRET). However, as a lanthanide ion is involved, this specific mechanism is often referred to as the Lanthanide Resonance Energy Transfer (LRET). The interaction between the dipoles in the antenna and lanthanide are controlled both by distance and orientation, equation 1.1. Whereby \( k_T(r) \) is the rate of energy transfer from a donor to an acceptor, \( \tau_D \) is the decay time of the donor in absence of the acceptor and \( R_0 \) is the Förster distance where the energy transfer from donor to acceptor is 50% efficient, and \( r \) is the donor-to-acceptor distance. The efficiency of energy transfer falls off at a rate of \( r^{-6} \).

\[
k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6 \quad \text{Eq. 1.1}
\]

Additionally, an orientation factor \( (k^2) \) also determines efficiency based on the directional overlap of the dipoles. A few other factors that must be accounted for such as back transfer of electrons from the T₁ state to the Ln* state (T₁→Ln*). This will occur if the energy gap between the donating triplet state and accepting lanthanide is sufficiently small (3000-6000 cm⁻¹). As the energy gap is a significant factor, this issue has been explored by Takalo et al., who extensively studied the triplet states of ligands compared to the quantum yield of complexes formed with Eu³⁺ and Tb³⁺. Ligands 1.53-1.55
are possible antennas proposed by Reinhoudt and Verhoeven, satisfying both the ability to coordinate to a lanthanide metal via their pyridine rings as well as having sufficient energy levels for LRET.\textsuperscript{103} The comparison of triplet energy of the antenna to quantum yield of the lanthanide complex can be compared to show that there is a very large energy gap between the Tb\textsuperscript{3+} ion's emitting state of \(5\)\(D_4\) (21,000 cm\(^{-1}\)) and the next highest energy state for it while the Eu\textsuperscript{3+} ion's emitting state of \(5\)\(D_0\) (17,500 cm\(^{-1}\)) has its higher energy states of \(5\)\(D_1\) and \(5\)\(D_2\) lying near it in energy. This means that the \(5\)\(D_1\) and \(5\)\(D_2\) energy states of the Eu\textsuperscript{3+} ion are also able to absorb energy through the LRET mechanism and then decay through non-radiative vibrational states to the \(5\)\(D_0\) emitting state, Figure 1.16. These higher energy states are also able to cause a non-radiative decay of the antenna triplet state through back transfer, complicating the sensitization of Eu\textsuperscript{3+} ions. To avoid this back transfer an energy gap of 3000cm\(^{-1}\) above the \(5\)\(D_4\) state of Tb\textsuperscript{3+} ion and 6000cm\(^{-1}\) above the \(5\)\(D_0\) state of Eu\textsuperscript{3+} ion is required.\textsuperscript{103}
Project Objectives

The goal of this project is to synthesize an effective sensor for Fe\(^{3+}\) ions under the conditions of:

- Simulated sea water; phosphate-buffered saline at pH 8.2
- At concentrations of Fe\(^{3+}\) ions found in the ocean (0.02-0.6 nM)
- Selective for Fe\(^{3+}\) ions over other metals present in the ocean
- Using IDA with Eu\(^{3+}\) or Tb\(^{3+}\) ions as the indicator

To reach these targets, a modified-DFB molecular probe from literature has been studied in a novel way in an IDA with Tb\(^{3+}\) ions, and two new modified-DFB probes based on coumarin fluorophores are presented. The coumarin fluorophores are targeted for their use as antennas for the Eu\(^{3+}\) and Tb\(^{3+}\) ions which we studied. The study of these probes begins in methanol and then moves to buffered systems. The structure and function of these sensors are studied by \(^1\)H-NMR, \(^13\)C-NMR, fluorescence spectroscopy, and mass spectrometry.
CHAPTER II
SYNTHESIS OF MOLECULAR PROBES

Coumarin is a well-studied benzopyrone compound first discovered in the tonka bean\textsuperscript{104} and used in many applications such as perfumes\textsuperscript{105}, dye lasers\textsuperscript{106}, fabric conditioners\textsuperscript{107}, and pharmacological compounds\textsuperscript{108}. The coumarin backbone (Figure 2.1), absorbs and emits light in the UV region, making it of little interest for fluorescence spectroscopy. However, derivatives of the coumarin scaffold have been found to be a very useful fluorescent probe. Typically modified at its 3, 4, and 7 positions, the 5 and 6 position can also be modified.

![Coumarin molecule with labeling scheme](image)

*Figure 2.1. Cartoon of the core coumarin molecule showing the labeling scheme.*

**Sensitizer Rationale**

Based on the rationale of design discussed above and in Chapter I, coumarin derivatives 1.41 and 2.1-2.3 are of interest. Outlined here is the synthesis, which starts out with a Knoevenagel cyclization in a one-step procedure from salicylaldehyde and malonate, which are both commercially available.\textsuperscript{109,110} The reason why there are many coumarin derivatives is due to the wide range of salicylaldehyde and malonate that have different functional group substituents at the three, four and seven positions of the coumarin scaffold (Figure 2.1).
Compound 2.1 has previously been prepared as an intermediate in the synthesis of a molecular probe for nerve agent detection via a PET mechanism\(^\text{110}\). Therefore it was a good starting point to tether the coumarin to DFB. Compound 2.1 has an aldehyde group at the three position, this to be reacted with a primary amine to form a Schiff base. Additionally the chloro group at the 4-position would allow for post-modification of the coumarin scaffold.

![Chemical structures](image)

The synthesis of 2.1 is achieved by reacting the commercially available 3-diethylaminophenol (3-DAP) with bis(2,4,6-trichlorophenyl) malonate in toluene to form the 4-hydroxy intermediate product, 2.4 (Scheme 2.1). A Vilsmeier-Haack formylation was then carried out to incorporate an aldehyde group in position three while the 4-hydroxy group is simultaneously converted to the chloro group in the same step by phosphoryl chloride (P(O)Cl\(_3\)) in DMF. This reaction produces the desired product, compound 2.1, the structure of which was confirmed by \(^1\text{H}-\text{NMR}\) and agreed with literature values\(^\text{110}\).
Scheme 2.1. Synthesis of modified coumarin 2.1 from 3-diethylaminophenol and bis(2,4,6-trichlorophenyl) malonate. Reagents and conditions: i) Toluene, reflux; ii) P(O)Cl₃/DMF, cooled in acetone-ice bath.\textsuperscript{110}

Preparation of Molecular Probe 1.11

The initial studies started off with a well-studied molecule discussed previously (Chapter I). Compound 1.11 is prepared by literature methods using a one-step synthesis between \textit{N}-methylisatoic anhydride (NMA) and DFB mesylate, Scheme 2.2.\textsuperscript{28}

Scheme 2.2. Synthesis of 1.11 from DFB mesylate and NMA. Reagents and conditions: i) Et₃N in DMF; ii) DFB, stir for 12 h at 60 °C.

This reaction takes place between the cyclic anhydride and the primary amine of DFB under basic conditions in DMF to produce an amide linkage and NMA fluorophore. While the literature method of purification allowed for the isolation of 1.11, the method using silica columns produced a low yield (<15%). Due to this, the purification procedure was modified so the compound was instead centrifuged in distilled water followed by recrystallized from methanol and diethyl ether (yield of 80%). Compound 1.11 was identified by \textsuperscript{1}H-NMR (\textit{d}_6-DMSO) and ESI-MS and agreed with previously published work.\textsuperscript{28} In NMR, 1.11 was identified by the loss of the primary amine of DFB at 7.60
ppm and the appearance of an amide shift at 8.24 ppm. ESI-MS showed a base peak at 694 m/z identified as \([1.11 + H]^+\) and was further studied by collision-induced dissociation (CID). Figure 2.2 shows fragmentation pattern of 1.11, which agrees with literature studies of DFB characterization by CID.\(^\text{111}\)

\[ \text{Figure 2.2. Fragmentation pattern of 1.11 showing dominant signals from base peak of 694 m/z. Conditions: Methanol, capillary temperature 110 °C, voltage +5.00 kV, charging agent: formic acid.}\]

The optical properties of 1.11, Figure 2.3, show \(\lambda_{\text{max}} = 340\) nm in methanol with a relatively low molar extinction coefficient of 3800 L·mol\(^{-1}\)·cm\(^{-1}\). A single emission band is visible at 420 nm for a Stoke’s shift of 80 nm, which shows a moderate quantum yield of \(\Phi = 0.55\) (against anthracene in cyclohexane).
Figure 2.3: Compound 1.11 in methanol. \( \lambda_{\text{max}} = 340 \text{ nm} \), \( l_{\text{max}} = 420 \text{ nm} \), Stoke’s shift = 80 nm, \( \varepsilon_{340} = 3800 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \), \( \Phi = 0.55 \) against anthracene (\( \Phi = 0.36 \) in cyclohexane).

With the molecular probe 1.11 in use testing spectroscopic methods (Chapter III), the linking of the coumarin derivative 2.1 to DFB was then explored.

**Attempted Preparation of 2.5 via Imine Condensation**

The first attempt was to couple the coumarin aldehyde, 2.1, to the amine group of a DFB ligand via a condensation reaction to form the imine linkage to form 2.5. Compound 2.1 and DFB were stirred in dry methanol under argon with 1.5 equivalences of triethylamine to act as a base and a small amount of magnesium sulfate to collect the water generated from the reaction (Scheme 2.3) hereby encouraging the reaction to be product favored by removing the water. The reaction was carried out at different time intervals (1, 6, 12, and 24 hours), which had little effect on the reaction results.
Scheme 2.3. Attempted synthesis of imine 2.5 followed by reductive amination. Reagents and conditions: i) Et$_3$N/DMF, DFB.

The reaction was also heated to 40°C and reacted for one or six hours to ensure the reagents remained soluble. All attempts to isolate the imine were carried out by recrystallization from methanol and ether due to reasons discussed previously. A brown powder was obtained after each attempt, which could not be identified by $^1$H-NMR, FT-IR, or ESI-MS as 2.5. The lack of the signature imine proton in the NMR supported that the reaction had not occurred. The ESI-MS was also inconclusive with no signals identifiable as calculated (823 m/z) which prohibited analysis by CID, further supporting the failure of the reaction. FT-IR additionally did not show a discernible C=N stretch for the imine. While the product could not be identified, it was also found that there was not any starting material remaining in the reaction; an aldehyde signal near 10 ppm could not be seen and the NMR spectra showed a variety of new unidentified signals, as did ESI-MS. Attempts were made to separate the compounds but this was to little avail. While the new unidentified signals pointed to unwanted side reactions, the possibility that the imine was too easily hydrolyzed to isolate was tested first.

Reductive amination was also attempted using a variety of reducing agents (NaBH$_4$, NaBH$_3$CN, zinc metal) to produce the amine of 2.5. An _in situ_ reductive amination was carried out by adding four equivalences of NaBH$_4$ to the imine reaction after it had stirred for the first six hours and the reaction was allowed to stir at room
temperature for an additional four hours, after which time an acid wash-up was carried out using hydrochloric acid. The reaction was purified by recrystallization as before to yield a brown solid that was characterized by $^1$H-NMR and ESI-MS. The spectra even after purification was similar to the imine reaction with a NMR spectrum that was difficult to interpret. The ESI-MS as well showed a spectrum with many unidentifiable signals. It was clear an unwanted reaction was taking place at this point. At first, it was anticipated that the NaBH$_4$ was too strong; therefore, we switched to a Borch Reaction that uses a milder reducing agent, NaBH$_3$CN, selective for C=N bonds. The Borch Reaction was carried out in the same way as the imine reaction with 2.1. The DFB was heated to 40 °C and stirred in methanol after which Et$_3$N (1.5 equivalences) was added as a base along with four equivalences of NaBH$_3$CN. The solution was then allowed to stir for 24 hours. The reaction was worked up using HCl and NaOH and then purified by recrystallization from methanol to yield a brown solid. This reaction was also unsuccessful with no identifiable results in the $^1$H-NMR or ESI-MS spectra, both looking similar to the NaBH$_4$ reaction. A last direct reductive amination was then attempted using zinc metal, an even milder reducing agent. This method is effective for forming secondary amines from aldehydes and primary amines. DFB and 2.1 were placed in an aqueous solution of 5% (w/v) KOH along with an excess of 15 equivalences of zinc metal powder with the reaction being stirred for 12 hours at room temperature. It was found that 2.1 was insufficiently soluble in aqueous solution which likely contributed to the failure of the reaction. Attempts to purify the product by recrystallization showed evidence that the starting material remained unchanged which was confirmed by $^1$H-NMR finding signals identical to those of each starting material, most notably the 2.1
aldehyde signal at 10.00 ppm and the DFB primary amine signal at 7.60 ppm. With three separate methods of reductive amination failing to produce results, the NMR of each sample was examined more closely, which found inconsistency in the side products produced in each reaction were limited to the aromatic region. From this it was determined the imine condensation was also producing multiple side reactions. It is anticipated that the β-unsaturated nature of the carbon at the four position of 2.1 influenced the reactivity. The carbon atom at the four position was substantially more susceptible to nucleophilic attacks due to its unsaturated nature as well as the chlorine leaving group attached to it, Scheme 2.4, reacting with both the primary amine of DFB as well as any of its three hydroxamic acid groups. It is anticipated that the triethylamine influenced the side reaction. Not only does triethylamine ensure the primary amine of DFB is deprotonated for the imine condensation, but triethylamine (pK\text{a} = 10.72) would also potentially deprotonate the hydroxamic acid moieties (pK\text{a} = 7.9-8.5) of DFB. Additionally, triethylamine would assist in the action of chlorine as a leaving group by forming triethylamine hydrochloride as it is commonly used for in preparation of esters and amides from acycle chlorides. To avoid side reactions due to the chloro group, synthesis of two new test compounds was attempted.
Scheme 2.4. Potential side reactions in the synthesis of 2.5. (a) Nucleophilic attack by the primary amine of DFB on the β-unsaturated carbon of 2.1. (b) Nucleophilic attack by the hydroxamic acid of DFB on the β-unsaturated carbon of 2.1.
Model Systems

It was evident that the chloro group was affecting the reactivity of the coumarin. Therefore I took a step back to look at aromatic systems with no substituents present. We chose to use aromatic aldehydes for this purpose. Benzaldehyde was of interest as this was the simplest aromatic aldehyde that was commercially available for synthesis of a Schiff base. 9-Anthraldehyde was also of interest as it is more comparable in size to the coumarin molecule. Additionally, we believed the anthracene carried the potential to act as a sensitizer for lanthanide metals (Chapter I). The imine condensation between benzaldehyde and DFB (2.6) produced a white powder in low yield (<15%). The $^1$H-NMR (DMSO-$d_6$) spectra showed a signal at 8.33 ppm assigned as the H-C=N singlet of the imine. ESI-MS was inconclusive with no signal found at the calculated 649 m/z, though a signal corresponding to the hydrolyzed fragment [DFB+H]$^+$ was observed at 560 m/z and confirmed by CID, showing the imine was easily cleaved in the spectrometer. Attempts to identify the imine via IR were unsuccessful as well. The presence of three hydroxamic acid and two amide groups in the DFB chain can at times be resolved as two signals with the hydroxamic acid C=O stretch at 1651 cm$^{-1}$ and the amide C=O stretch at 1600 cm$^{-1}$, but often due to intramolecular interactions these two signals show one broad signal centered at 1620 cm$^{-1}$, which could cover any apparent imine stretch. As this was a test reaction and showed only a low yield of compound 2.6, we did not pursue this reaction further by scaling it up for spectrophotometric studies as NMR and ESI-MS had confirmed its synthesis. Reductive amination of 2.6 using NaBH$_4$ was carried out with the above parameters with the product being worked up and recrystallized from methanol and diethyl ether. Analysis by $^1$H-NMR showed the imine
signal at 8.30 ppm had been lost but the signals for the aromatic benzyl ring as well were absent, suggesting that rather than forming 2.6, the imine had been hydrolyzed with only DFB precipitating from the solution. This was supported by the $^1$H-NMR of the sample matching that of the DFB starting material. While the reductive amination of 2.6 failed, the imine condensation was a success, which led to attempts to synthesize 2.7 via the imine condensation of 9-anthraldehyde with DFB. The reaction was carried out using the above parameters with the only change being replacing dry methanol as the solvent with dry 50:50 ethanol:methanol in an attempt to better dissolve 9-anthraldehyde. The solubility of 9-anthraldehyde posed a continuous problem with the reaction and is possibly the reason why no product could be identified. The reaction produced a yellow solid (<10% yield) that was characterized by $^1$H-NMR which showed an imine signal at 8.70 ppm with no residual signal for the aldehyde of 9-anthraldehyde at 11.50 ppm. IR showed no visible C=N imine stretch but also did not show a C=O stretching frequency for aldehyde; either could be hidden under the broad 1620 cm$^{-1}$ stretch of the aforementioned hydroxamic acid and amide signals. In the case of both 2.6 and 2.7 the imine was successfully synthesized, but in very low yield. Further investigation of these compounds was not attempted as the presence of the imine supports that the chloro group in coumarin 2.1 was the cause of the side reactions. Due to this, we needed to change our design strategy.
Modified Coumarin Scaffolds: Attempted Preparation of 2.10 by Coupling Compound 1.41 to DFB

It is evident that the 4-chloro group was playing a significant role and was hindering the successful synthesis of 2.5. The next approach was to remove the chloro group from the coumarin scaffold to eliminate any side reactions.

This new approach also had an additional benefit as the synthesis could avoid going through the coumarin intermediate 2.4; therefore, removing a step from the reaction. In this approach, 4-diethyaminosalicylaldehyde (4-DASA), and diethyl malonate were utilized, with a catalytic amount of piperidine, Scheme 2.5.117-119 This reaction was placed in ethanol which was refluxed for 24 hours, monitored by TLC, and then purified by silica column using 30:70 ethyl acetate:hexane as an eluent and/or further purified by recrystallization from methanol and diethyl ether. The ethyl ester of the product 2.8 is then thermally decarboxylated by refluxing in 12 M HCl at 110 °C for 24 hours followed by the addition of NaOH (45% w/v) to raise the pH to 4-5 to give 2.9. A Vilsmeier-Haack reaction using P(O)Cl₃ and DMF stirred at 50 °C for 24 hours incorporates an aldehyde group onto the coumarin in the three position to produce the final product, 1.41 in a 20% yield. The synthesis of 1.41 was confirmed by ¹H-NMR with the spectra showing an aldehyde signal at 10.15 ppm while the aromatic and diethylamine signal agreed with literature values.120 ESI-MS was also used to identify the product but could not confirm 1.41 likely due to the lack of easily ionized functional groups that would allow for charging.
Scheme 2.5. Synthesis of modified coumarin \textbf{1.41} from 4-diethylaminosalicylaldehyde and diethyl malonate. Reagents and conditions: i) Piperidine (catalytic amount), ethanol, reflux; ii) 10\% (w/v) NaOH, reflux 3 h; iii) P(O)Cl\(_3\)/DMF, cooled in acetone-ice bath.

Once it was confirmed that \textbf{1.41} had been synthesized, attempts were made to attach it to the DFB molecule via an imine linkage as with \textbf{2.1}. The same procedure from Scheme 2.3 was followed with \textbf{1.41} and DFB being placed into methanol with triethylamine, then stirred for 24 hours to produce \textbf{2.10}. The product was analyzed by \(^1\)H-NMR which while cleaner than the spectra of \textbf{2.9}, no imine signal could be confirmed around 8 ppm. The aldehyde signal at 10.15 ppm for \textbf{1.41} was absent suggesting the imine may have formed and then hydrolyzed triggering an unknown side reaction.

Reductive aminations (\textit{vide supra}) were attempted to produce the reduced version of \textbf{2.10} with an amine linkage which would avoid the potential of hydrolysis of the imine linker. Both NaBH\(_4\) and NaBH\(_3\)CN were utilized as reducing agents in both indirect and direct reductive aminations using conditions described previously. An amine signal at 3.5-4.5 ppm could not be confirmed while many new signals had appeared and the 10.15 ppm aldehyde signal of \textbf{1.41} was absent once more. With the issues encountered by the imine condensation and subsequent reduction to an amine, the coupling of a coumarin to DFB
via an amide linkage formed between a carboxylic acid on the coumarin, and the primary amine of DFB was then attempted.

![Chemical Structure](image)

**Preparation of Compound 2.2 from 2.8**

It also became apparent that the Schiff base approach was not a viable synthetic strategy either, due to the fickle nature of the imine group. Again we switched to a different approach by removing the aldehyde group and replacing it with a carboxylic acid moiety. The carboxylic acid group could then be coupled to the DFB via a peptide coupling reaction to form an amide group, a more stable moiety. A coumarin derivative with a carboxylic acid in place of the aldehyde of 2.1 was now required. This could be conveniently synthesized from one of the intermediates of 2.1 (Scheme 2.6). The 3-ethyl ester coumarin, 2.8, was used as a starting point by hydrolyzing the ester to a carboxylic acid to produce compound 2.2. This synthesis was carried out by refluxing 2.8 in NaOH (10% w/v) for 30 minutes before cooling to 0 °C and acidifying to pH 2 using 6 M HCl. The reaction was purified by silica column for an overall yield of 15% for 2.2. The low yield is likely due to the need to balance hydrolyzing the ester without decarboxylating the coumarin.
Scheme 2.6. Synthesis of modified coumarin 2.2 from 2.8. Reagents and conditions: i) 10% (w/v) NaOH and refluxing for 30 m, cooling to 0 °C and acidification to pH 2 using 6 M HCl.

Once purified, 2.2 was analyzed with $^1$H-NMR (DMSO-$d_6$) and ESI-MS. The NMR spectra showed a loss of the ester methyl signal at 1.40 ppm and methylene signal at 3.50 ppm. ESI-MS showed 2.2 in methanol with no charging agent to be present primarily in its dimer form charged by sodium [2M + Na]$^+$ at 545 m/z with an adduct coordinating water and charged by sodium [M + H$_2$O + Na]$^+$ at 347 m/z in 15% relative abundance and the sodium adduct [M + Na]$^+$ at 284 m/z in 5% relative abundance. CID of 2.2 showed fragmentation primarily through rearrangement that lead to the loss of CO$_2$ from the pyrone ring of each coumarin in the dimer while a secondary pathway came from the loss of CO from one coumarin and CO$_2$ from the other (Figure 2.4). Throughout this ionization process the dimer is stable suggesting the carboxylic acids of 2.2 are not being lost to fragmentation, presumably due to the stable hydrogen bonding interaction. Possible mechanisms for this rearrangement are shown by Figure 2.4, based on charge-remote fragmentation mechanisms shown previously to effect the fragmentation of coumarins modified with 7-amino groups.$^{121}$ In this charge-remote fragmentation mechanism, sodium charges the coumarin dimer by interacting with its 7-diethylamino group efficiently.$^{122}$ To test this mechanism coumarin-3-carboxylic acid, 2.3, was tested under the same conditions as 2.2. The ESI-MS of 2.3 showed a dominant signal at 213 m/z for [M + Na]$^+$ and a signal at less than 10% relative abundance for [2M + Na]$^+$,
which gives the opposite of the signal intensities for 2.2. Fragmentation of the [2M + Na]\(^+\) signal does show a similar fragmentation mechanism, suggesting that while the charge-remote fragmentation, or a similar mechanism, is possible without the 7-diethylamino group, it is not favorable. Similarly the formation of the coumarin dimer is not favorable in absence of the 7-diethylamino group. It is thus reasonable to assume that the Na\(^+\) ion will coordinate to the acid groups, preventing dimerization. Both the stability of the dimer and the lower collision cross-section presented by the carboxylic acids compared to the pyrone ring of coumarin support this mechanism.\(^{123}\)
Figure 2.4. Potential fragmentation patterns of 2.2 and corresponding signals. (a) Shows spectra from ESI-MS in positive mode from sample of 2.2 (5 µM) in methanol, capillary temperature 110 °C, capillary voltage 5 kV. (b) Shows charged dimer of 2.2 producing signal 518 m/z through loss of carbon monoxide from the pyrone ring followed by the loss of carbon dioxide to give the 475 m/z signal. (c) Shows sequential loss of carbon dioxide from the pyrone rings of each coumarin in the dimer to produce the 501 m/z signal followed by the 457 m/z signal.
Attempted Synthesis of Compound 2.12 by Coupling DFB with 2.2

With the successful synthesis of 2.2 complete, the next step was to couple it to DFB via an amide linkage to synthesize 2.12. The reactivity of carboxylic acid derivatives such as acid chlorides and acid anhydrides was considered, starting with the most reactive group—an acid chloride. The acid chloride, 2.11, was generated by reacting 2.2 with \( \text{SOCl}_2 \) under argon in a flame dried flask. Excess \( \text{SOCl}_2 \) was removed by vacuum distillation, the acid chloride then used immediately without isolation by dissolving it in ether then adding the acid chloride dropwise to a solution of DFB in base, Scheme 2.7. The solution was allowed to stir for 12 hours before having the brown solid formed, filtered, and washed with ether.

![Scheme 2.7. Attempted synthesis of modified DFB 2.12 from 2.11. Reagents and conditions: i) 0.5 M NaOH, stir 12 h.](image)

The solid material produced by the reaction was analyzed by \( ^1\text{H}-\text{NMR} \), which showed no identifiable amide signal around 8 ppm, but did show groupings of aromatic signals that suggest the hydroxamic acids of the DFB may have been acylated by the acid chloride to give side products.\(^{45}\) The acylation of DFB’s hydroxamic acids would give an ester, which was determined to have formed by FT-IR. The FT-IR spectra showed a strong signal at 1678 cm\(^{-1}\) corresponding to the \( \text{C}=\text{O} \) stretch of an ester along with two more signals at 1268 cm\(^{-1}\) and 1250 cm\(^{-1}\) corresponding to the \( \text{C}-\text{O} \) and \( \text{N}-\text{O} \) stretches.
respectively. The hydroxamic acid signals of DFB normally found as part of a broad stretch at 1620 cm\(^{-1}\) were absent in this spectra. Based on the indication of an unwanted reaction between the hydroxamic acids of DFB and the acid chloride apparent, steps were necessary to avoid this reaction.

**Attempted Blocker Preparation**

Since we removed all reactive functional groups from the coumarin scaffold, we turned our attention to the DFB molecule. The most obvious method to stop the reaction of the hydroxamic acid groups of DFB was to use the Fe\(^{3+}\) ion as a *de facto* protecting group. The terminal amine of DFB would be free to couple with the coumarin carboxylic acid (2.2). Once the DFB and coumarin were coupled, the Fe\(^{3+}\) ion could then be removed by precipitation as Fe(OH)\(_3\) before filtration, yielding the desired receptor. To protect the hydroxamic acid groups of DFB, FeCl\(_3\)·6H\(_2\)O was used, added into the solution of DFB before the acid chloride. To remove the Fe\(^{3+}\) ion from the DFB, 45% (w/v) NaOH was used to raise the pH of the solution to 14 which would precipitate Fe(OH)\(_3\) to be filtered using a 0.45 µm nylon syringe filter. The solution was then acidified with 6 M HCl back to pH 2 before filtering off an orange solid. Analysis by \(^1\)H-NMR showed that the Fe\(^{3+}\) ion had not been removed fully from the product, which made NMR impossible due to extensive broadening from the paramagnetic Fe\(^{3+}\) ion. Further testing with different filters failed to remove the Fe\(^{3+}\) ion fully, always leaving too much contamination for NMR. Due to this issue, the blocking of the hydroxamic acid groups using the Fe\(^{3+}\) ion was found to be ineffective. An alternative way to avoid acylation was to use a less reactive acid anhydride. Compound 2.2 was converted into an acid anhydride (Scheme 2.8) by placing a solution of the carboxylic acid in dichloromethane:dioxane
(1:1) into a solution of sodium carbonate in thionyl chloride. The mixture was refluxed for two hours and tracked by TLC until no starting material remained. After cooling, the solution was washed with dichloromethane and dried before being analyzed by $^1$H-NMR to show the acid anhydride, if formed, had returned to its carboxylic acid state based on the presence of a carboxylic acid signal at 8.50 ppm.


In an attempt to avoid the decomposition of the acid anhydride, the reaction was attempted and immediately added a solution of DFB in DMF with triethylamine. The reaction was allowed to stir for 24 hours at 50 °C before being filtered and analyzed. $^1$H-NMR showed only starting material with the presence of the coumarin carboxylic acid signal at 8.50 ppm and the primary amine of DFB at 7.60 ppm. With the acid anhydride reaction unsuccessful, the carboxylic acid coumarin, 2.2, was used without modification to couple to the primary amine of DFB via a peptide coupling.

Preparation of 2.12 by Peptide Coupling DFB with Compound 2.2

The peptide synthesis chosen is a method commonly used and thus less likely to cause side reactions that complicate the synthesis. In this reaction, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is used to activate the carboxylic acid along with hydroxybenzotriazole (HOBt) which converts the active carboxylic acid to an active ester. The active ester will then react with the amino group of DFB to form the amide,
To conjugate the carboxylic acid and amine, DFB and 2.2 are dissolved in DMF along with EDC and HOBT (Scheme 2.9). To this mixture diisopropylethylamine (DIPEA) is added as a base to control the pH of the solution to keep it in the efficient range of pH 4-6. The mixture was stirred for 48 hours and then had the DMF removed by vacuum distillation, the oil was then purified by recrystallization from diethyl ether and methanol.

Scheme 2.9. Synthesis of modified DFB 2.12. Reagents and conditions: i) EDC/HOBT in DMF followed by DIPEA, stir for 24 h.

Compound 2.12 was isolated as a yellow powder recovered in 95% yield with careful recrystallization. The product was analyzed by \(^1\)H-NMR, ESI-MS and spectroscopy. The NMR showed a new signal identified as the N-H proton of the amide linker was found at 8.67 ppm. ESI-MS found a signal at 804 \(m/z\) corresponding to \([M + H]^+\) ion as well as a signal at 826 \(m/z\) corresponding to \([M + Na]^+\). As 2.12 was confirmed to be fluorescent, its absorbance and fluorescence properties were analyzed using UV-Vis and fluorescence spectroscopy (Figure 2.5), showing 2.12 to have spectroscopic properties comparable to literature values for the coumarin alone. Receptor 2.12 showed excellent and efficient fluorescence in the visible region which led to further analysis as a fluorescent sensor for the Fe\(^{3+}\) ion and for use in an indicator displacement assay for detecting the Fe\(^{3+}\) ion (\textit{vide infra}). While 2.12 showed some promise in acting as a sensitizing antenna for the Eu\(^{3+}\) ion, it was of interest to produce a modified DFB
system with a higher emission energy than the Eu$^{3+}$ ion’s ideal absorption wavelength of 425 nm (Chapter I).

![Graph](image)

**Figure 2.5.** Compound 2.12 in 100% MeOH. $\lambda_{\text{max}} = 420$ nm, $I_{\text{max}} = 460$ nm, Stoke’s Shift = 40 nm, $\varepsilon_{420} = 43,900$ L·mol$^{-1}$·cm$^{-1}$ and $\Phi = 0.73$ against fluorescein ($\Phi = 0.79$ in ethanol).

To do this, commercially available coumarin-3-carboxylic acid was chosen, doing away with the 7-diethylamino group that substantially red shifted the coumarin’s absorption and emission. The peptide synthesis used for 2.12 was used as well for the new compound, 2.14. The reaction was found to be efficient, producing 2.14 which appeared as an off-white powder in an 80% yield with fluorescence properties substantially blue shifted compared to those of 2.12, as predicted (Figure 2.6). The $^1$H-NMR spectrum confirmed the loss of the primary amine of DFB at 7.60 ppm. A new signal for the N-H of the amide was found at 8.67 ppm. ESI-MS did not show the predicted signal at 733 m/z corresponding to [M + H]$^+$ ion or a signal at 755 m/z.
corresponding to [M + Na]⁺, nor any other identifiable signals. This is likely evidence for the charging of DFB derivatives 1.11 and 2.12 being charged by the protonation of their terminal secondary and tertiary amines respectively. As 2.14 lacks such an amine, it shows no charging in positive mode or in negative mode.

![DFB derivative](image)

**Figure 2.6.** Compound 2.14 in methanol. λ\text{max} = 340 nm, I\text{max} = 400 nm, Stoke’s Shift = 60 nm, \( \varepsilon_{340} = 6000 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \) and \( \Phi = 0.57 \) against anthracene (\( \Phi = 0.36 \) in cyclohexane).

**Synthesis Summary**

Our first molecular probe was 1.11, which was synthesized from literature methods. To extend our selection of probes and explore the coupling of coumarin dyes to DFB, we utilized 7-diethylamino-4-chlorocoumarin. The chloro group at the 4-position of
coumarin proved to cause side reactions that made coupling it to DFB via imine or amide unviable. To explore both imines and amines as linking groups between DFB and a fluorophore, we tested reactions for these using benzaldehyde and anthraldehyde. From these reactions, we determined that the imine is too fickle to utilize in the synthesis—both for an imine linker and for reduction to an amine—of modified DFB probes and thus shifted our attention to peptide coupling reactions. We successfully synthesized 2.12 using 7-diethylamino-coumarin-3-carboxylic acid. To offer variety in the photophysics of the coumarin-modified DFB probes we also successfully synthesized 2.14 using coumarin-3-carboxylic acid.

**Synthetic Procedures**

**General.** All $^1$H and $^{13}$C-NMR spectra were taken at ambient temperature using a Bruker Ultrashield 400 spectrometer with DMSO-$d_6$ as the solvent and tetramethylsilane (0 ppm) as an internal standard. Multiplicities in $^1$H-NMR were reported as (br) broad, (d) doublet, (dd) doublet of doublets, (m) multiplet, (q) quadruplet, and (s) singlet. UV-Vis samples were analyzed using a Beckman-Coulter DU-800 spectrophotometer. Molar absorptivities calculated by recording the absorbance of a series of diluted solutions with the data then being graphed to give the molar absorptivity as the slope of the graph. Fluorescence experiments utilized a PTI QuantaMaster 40 with a 75 W xenon lamp and XenoFlash source for steady-state experiments and time-gated experiments, respectively. Infrared spectroscopy studies were performed using a Nicolet Nexus 470 FT-IR.

7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid ethyl ester (2.2).

4-Diethylaminesalicylaldehyde (3.00 g, 1.55 x $10^{-2}$ mol) was dissolved in EtOH (190 proof, 20 mL) to which diethyl malonate (2.80 mL 1.84 x $10^{-2}$ mol) and piperidine
(500 µL, 5 µmol) were added to the ethanolic solution, which was refluxed for 18 h. The reaction was allowed to cool before extracting with CHCl₃ (50 mL) and washed with brine (3 x 50 mL). The organic layer was then dried using anhydrous magnesium sulfate, filtered and reduced under reduced pressure vacuum to produce an amber oil (3.19 g, 1.10 x 10⁻² mol) for a 71% yield. ¹H-NMR (400 MHz, DMSO-d₆) δ_H: 8.4 (1H, s, Ar), 7.4 (1H, d, Ar), 6.6 (1H, d, Ar), 6.5 (1H, s, Ar), 4.4 (2H, q, CH₂), 3.4 (4H, q, CH₂), 1.4 (3H, t, CH₃), 1.2 (6H, t, CH₃).

7-(diethylamino)-2H-chromen-2-one (2.9). Compound 2.8 (5.69 g, 1.97 x 10⁻² mol) was taken and decarboxylated by the addition of 12N HCl (25 mL) followed by refluxing overnight. The green solution produced was then placed into an ice bath to cool and saturated sodium acetate (50 mL) was added. To this solution 45% (w/v) NaOH was added dropwise until a pH of 4-5 was reached, causing a brown precipitate to form. The product was extracted with CHCl₃ (50 mL) and washed with ddH₂O (3 x 50 mL) before being reduced to dryness under vacuum to give a brown solid (3.10 g, 1.07 x 10⁻² mol) for a 54.5% yield. ¹H-NMR (400 MHz, DMSO-d₆) δ_H: 7.5 (1H, d, Ar), 7.2 (1H, d, Ar), 6.5 (1H, d, Ar), 6.4 (1H, s, Ar), 6.0 (1H, d, Ar), 3.4 (4H, q, CH₂), 1.4 (3H, t, CH₃), 1.2 (6H, t, CH₃).

7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde (1.41). A formylation reaction is carried out by reacting DMF (2 mL) with phosphoryl chloride (POCl₃) (2 mL) under argon for 30 minutes at 50 °C. Compound 2.9 (3.10 g, 1.43 x 10⁻² mol) was placed into solution in anhydrous DMF (10 mL) then added dropwise to a POCl₃ solution. The mixture was allowed to react at 60 °C overnight while stirring. Reaction was extracted with CHCl₃ and reduced under vacuum before being purified using a silica column with
ethyl acetate:hexane (50:50) as the eluent. Purified material was reduced under vacuum then recrystallized from methanol and ether to provide an orange crystalline solid (1.34 g, 6.21 x 10^{-3} mol) giving a 43.4% yield. $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta_H$: 10.2 (1H, s, CHO), 8.3 (1H, 2, Ar), 7.5 (1H, d, Ar), 6.7 (1H, d, Ar), 6.5 (1H, s, Ar), 3.5 (4H, q, CH$_2$), 1.3 (6H, t, CH$_3$). $^{13}$C-NMR (400 MHz, DMSO-$d_6$) $\delta$ 197.6, 161.2, 154.0, 133.5, 111.0, 108.2, 96.9, 45.0, 12.8.

7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (2.2). Compound 2.8 (3.10 g, 1.07 x 10^{-2} mol) was added to 10% (w/v) NaOH (60 mL) and allowed to reflux for 30 minutes before cooling in ice bath. Then added 12 N HCl dropwise to adjust the pH to 2 followed by ddH$_2$O (50 mL) and filtered a brown solid from solution. Product was purified by silica column using ethyl acetate:hexane (70:30) as the eluent to produce an orange crystalline solid (655 mg, 2.05 x 10^{-3} mol) giving a 19.2% yield. $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta_H$: 8.4 (1H, s, Ar), 7.7 (1H, d, Ar), 6.8 (1H, d, Ar), 6.6 (1H, s, Ar), 3.5 (4H, q, CH$_2$), 1.1 (6H, t, CH$_3$). $^{13}$C-NMR (400 MHz, DMSO-$d_6$) $\delta$ 190.6, 163.4, 153.8, 134.0, 111.2, 104.4, 95.9, 44.1, 12.4. MS (ESI, 110 °C, +5kV, MeOH charged with 1% formic acid) [2M + H]$^+$ 545 m/z (Base Peak), [M+ H$_2$O + Na]$^+$ 347 m/z (15 % RA), [M + Na]$^+$ 284 m/z (5% RA). Spectroscopy: $\lambda_{max} = 420$ nm, $I_{max} = 460$ nm, $\varepsilon_{420} = 43,900$ L·mol$^{-1}$·cm$^{-1}$, $\Phi = 0.73$ against fluorescein ($\Phi = 0.79$ in ethanol).

7-(diethylamino)-4-hydroxy-2H-chromen-2-one (2.4). From literature methods,$^{110}$ 3-diethylaminophenol (1.65 g, 1 x 10^{-2} mol) and bis(2,4,6-trichlorophenyl) malonate (4.63 g, 1 x 10^{-2} mol) were added to a solution of dry toluene (10 mL) then refluxed while stirring for two hours. The reaction was cooled to room temperature and filtered, the product washed with toluene (10 mL) and dried under vacuum. A grey solid was
produced (1.28 g, 5.50 x 10^{-3} mol) for a 55% yield. $^1$H-NMR: See literature. $^{110}$ $^{13}$C-NMR: See literature. $^{110}$

4-chloro-7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde (2.1). From literature methods $^{110}$ 2.4 (1.05 g, 4.5 x 10^{-3} mol) was placed into solution in anhydrous DMF (3.2 mL), that was heated to 100 °C until all solid was dissolved. The solution was then chilled in an ice bath and de-gassed with argon, which caused light gray crystals to form. To the solution POCl$_3$ (1.1 mL, 1.82 g) was added dropwise over a 15 minute period. The reaction was allowed to stir for one hour then extracted with CH$_2$Cl$_2$ (100 mL). The organic layer of the extraction was washed with a saturated NaHCO$_3$ (3 x 50 mL) and then brine (50 mL). After being reduced by vacuum distillation, the dark oil produced was purified using column chromatography (silica, 50:50 hexanes:ethyl acetate). An orange solid was recovered (565 mg, 2.03 x 10^{-3} mol) in a 45% yield. $^1$H-NMR: See literature. $^{110}$ $^{13}$C-NMR: See literature. $^{110}$

DFB Condensed With N-Methylisatoic Anhydride, 1.11. DFB (350 mg, 5.3 x 10^{-4} mol) and NMA (99 mg, 5.6 x 10^{-4} mol) were placed into solution in anhydrous DMF (10 mL) along with a 1.5 eq. excess of Et$_3$N. This solution was heated to 40 °C and stirred for 6 hours before being allowed to cool. The solution was washed and centrifuged with ddH$_2$O (3 x 30 mL) followed by ether (3 x 30 mL) to produce a white powder (295 mg, 4.2 x 10^{-4} mol) in an 80% yield. $^1$H-NMR: See literature. $^{13}$C-NMR: See literature. MS (ESI, 110 °C, +5kV, MeOH charged with 1% formic acid) [M + H]$^+$ 694 m/z (Base Peak), [M + Na]$^+$ 716 m/z (35 % RA). Spectroscopy: $\lambda_{max} = 340$ nm, $I_{max} = 420$ nm, $\varepsilon_{340} = 3800$ L·mol$^{-1}$·cm$^{-1}$, $\Phi = 0.55$ against anthracene ($\Phi = 0.36$ in cyclohexane).
General Procedure for Imines. Synthesis for 2.5-2.7 and 2.10 was carried out by first dissolving 350 mg (5.3 x 10^{-4} mol) of DFB in 2 mL of dried methanol (MeOH) along with a small amount of magnesium sulfate (MgSO_4) to remove water formed by imine condensation. To this solution a 1.5 eq excess of Et_3N was added and allowed to stir in for 15 minutes before 1.05 eq of the aldehyde being condensed with DFB is added. Once all reactants had dissolved the solution was heated to 40 °C and stirred overnight. Reaction was from heat and hot filtered to remove MgSO_4 from solution, then reduced MeOH under vacuum before recrystallizing with ether.

DFB Condensed With Benzaldehyde (2.6). Product from imine condensation appeared as white crystalline solid (40 mg, 6.4 x 10^{-5} mol) that was recrystallized further from methanol and hexane to give a 12% yield. ^1H-NMR (300 MHz, DMSO-d6) δ_H: 9.6 (2H, m, NH), 8.3 (1H, s, Ar), 7.8 (1H, s, NCH), 7.7 (2H, m, Ar), 7.6 (3H, s, OH), 7.4 (2H, d, Ar), 3.5 (6H, t, CH_2), 3.0 (4H, q, CH_2), 2.6 (4H, t, CH_2), 2.5 (4H, m, CH_2), 2.3 (4H, t, CH_2), 1.9 (3H, s, CH_3), 1.2-1.6 (18H, m, CH_2). MS (ESI, 110 °C, +5kV, MeOH charged with 1% formic acid) [DFB + H]^+ 560 m/z.

DFB Condensed With Anthracene-9-Carbaldehyde (2.7). Imine condensation gave yellow powder (31 mg, 4.2 x 10^{-5} mol) Product did not recrystallize and was placed into CH_2Cl_2 (50 mL) to be extracted using brine. The organic layer was dried under vacuum to produce a pale yellow solid in an 8% yield. ^1H-NMR (300 MHz, DMSO-d6) δ_H: 9.6 (2H, m, NH), 8.7 (1H, s, Ar), 8.6 (2H, d, Ar), 8.1 (2H, d, Ar), 7.9 (1H, s, NCH), 7.7 (3H, s, OH), 7.6 (4H, q, Ar), 3.5 (6H, t, CH_2), 3.0 (4H, q, CH_2), 2.6 (4H, t, CH_2), 2.5 (4H, m, CH_2), 2.3 (4H, t, CH_2), 1.9 (3H, s, CH_3), 1.2-1.6 (18H, m, CH_2).
Reductive Amination of 2.7 with Zinc. Placed DFB (200 mg, 3 x 10^{-4} mol) up in 5% (w/v) KOH (10 mL) and heated to 40 °C to ensure DFB dissolved. To the DFB solution of zinc metal (200 mg, 4.5 x 10^{-3} mol) was added to give a large excess. A solution of 9-anthraldehyde (62 mg, 3 x 10^{-4} mol) in MeOH (3 mL) was created and added to the DFB solution dropwise over the period of an hour before heat was increased so solution would reflux for 24 hours. Zinc was filtered from reaction and a liquid-liquid extraction was performed with the organic layer being dried under vacuum. 1H-NMR (400 MHz, DMSO-d6) δH: 9.6 (2H, m, NH), 8.9 (3H, t, Ar), 8.2 (2H, d, Ar), 7.8 (2H, d, Ar), 7.7 (3H, s, OH), 7.6 (2H, d, Ar), 3.5 (6H, t, CH₂), 3.0 (4H, q, CH₂), 2.6 (4H, t, CH₂), 2.5 (4H, m, CH₂), 2.3 (4H, t, CH₂), 2.0 (3H, s, CH₃), 1.2-1.5 (18H, m, CH₂).

General Procedure for Acid Chlorides. Synthesis of compound 2.12 was attempted by first generating the acid chloride of the appropriate sensitizer. Acid chlorides were prepared by reacting under argon in a flame dried flask the appropriate carboxylic acid (4 x 10^{-4} mol) with SOCl₂ (5 mL) so that the SOCl₂ was in great excess. The reaction was stirred for 15 minutes then dried under vacuum and washed with ether to remove the excess SOCl₂, leaving the acid chloride as a dark colored solid. Immediately chloroform or THF (1.5 mL) was added to dissolve the acid chloride.

General Procedure for Amides from Carboxylic Acids. Compounds 2.12 and 2.14 were synthesized by placing DFB (300 mg, 4.56 x 10^{-4} mol) up in freshly distilled DMF (10 mL) along with a sensitizer in excess (5.00 x 10^{-4} mol), EDC (131.1 mg, 6.84 x 10^{-4} mol) and HOBt (105.4 mg, 6.84 x 10^{-4} mol) before allowing the solution to stir until all reactants were dissolved. At this point DIPEA (158.2 uL, 9.08 x 10^{-4} mol) was added and
the reaction was stirred for 48 hours. DMF was removed under vacuum and the resultant oil was washed twice with water and twice with ether before drying once more.

DFB Coupled to 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid, (2.12).

Produced yellow-brown oil that turned to yellow oil after removal of DMF. Yellow oil was dissolved in MeOH and hot filtered before being reduced under vacuum and recrystallized with ether. Product was recovered by filtration as a yellow powder (348.2 mg, 4.3 x 10⁻⁴ mol) in a 95% yield. ¹H-NMR (400 MHz, DMSO-d₆) δ: 9.6 (2H, m, NH), 8.7 (1H, s, Ar), 7.8 (3H, s, OH), 7.7 (1H, d, Ar), 6.8 (1H, d, Ar), 6.6 (1H, s, Ar), 3.5 (10H, m, CH₂), 3.0 (4H, q, CH₂), 2.6 (4H, t, CH₂), 2.5 (4H, m, CH₂), 2.3 (4H, t, CH₂), 2.0 (3H, s, CH₃), 1.2-1.5 (18H, m, CH₂), 1.1 (6H, t, CH₃). ¹³C-NMR (400 MHz, DMSO-d₆) δ 172.0, 171.4, 170.6, 163.4, 153.8, 111.8, 96.1, 49.0, 48.1, 47.8, 44.1, 30.5, 29.4, 29.3, 28.0, 27.5, 26.9, 24.1, 23.8, 21.0, 12.6. MS (ESI, 110 °C, +5kV, MeOH charged with 1% formic acid) [M + H]+ 803 m/z (Base Peak), [M + Na]+ 826 m/z (22 % RA).

Spectroscopy: λₓₓₙₙₓ = 420 nm, Iₓₓₙₙₓ = 460 nm, ε₄₂₀ = 43,900 L·mol⁻¹·cm⁻¹, Φ = 0.73 against fluorescein (Φ = 0.79 in ethanol).

DFB Coupled to 2-oxo-2H-chromene-3-carboxylic acid, CUB-DFB (2.14).

Compound produced a translucent yellow oil after removal of DMF. Oil was placed up in MeOH and hot filtered before being reduced under vacuum and recrystallized with ether. Product was recovered by filtration as an off-white powder and dried to yield 300.6 mg product (82% yield). ¹H-NMR (400 MHz, DMSO-d₆) δ: 9.6 (2H, m, NH), 8.9 (1H, s, Ar), 8.7 (1H, t, NH), 8.0 (1H, d, Ar), 7.8 (1H, t, Ar), 7.7 (3H, s, OH), 7.5 (2H, m, Ar), 3.5 (6H, t, CH₂), 3.0 (4H, q, CH₂), 2.6 (4H, t, CH₂), 2.5 (4H, m, CH₂), 2.3 (4H, t, CH₂), 2.0 (3H, s, CH₃), 1.2-1.5 (18H, m, CH₂). ¹³C-NMR (400 MHz, DMSO-d₆) δ 172.0, 171.4,
170.6, 161.6, 161.0, 154.2, 147.9, 134.5, 131.8, 125.9, 119.8, 119.2, 117.8, 49.0, 48.1, 47.8, 30.5, 29.4, 29.3, 28.0, 27.5, 26.9, 24.1, 23.8, 21.0. Spectroscopy: $\lambda_{max} = 340$ nm, $I_{max} = 400$ nm, $\varepsilon_{340} = 6000$ L·mol$^{-1}$·cm$^{-1}$, $\Phi = 0.57$ against anthracene ($\Phi = 0.36$ in cyclohexane).
CHAPTER III
SPECTROSCOPIC ANALYSIS

After the synthesis of receptors 1.11, 2.12, and 2.14 the next stage was to investigate their spectroscopic properties. The mechanisms of fluorescence have already been discussed in Chapter I. Our initial studies were carried out in organic solvent first then moved into aqueous solutions. The goal of these studies was to confirm that the molecular probes work in artificial sea water ultimately, leading to a device for monitoring Fe$^{3+}$ levels in the ocean.

Data Acquisition Methods

In a normal steady-state fluorescence experiment, a fluorescence spectrum is obtained by scanning across a series of wavelengths using the emission monochromator of the instrument, Figure 3.1a. Using this method a titration can be performed by scanning across the series of wavelengths after each addition of an analyte to a solution of the molecular probe of interest, which gives a series of spectra. From this titration data a binding isotherm can be generated by either choosing a wavelength in the spectra to use or by integrating under the curve of the spectra. Systems with multiple emission bands yield useful information from the data obtained as the individual bands have the potential to be affected differently depending on how the guest effects the electronics of the system (see Chapter I for more details). Systems with a single band, such as coumarin, do not yield useful information from the shape of the spectrum as it decreases.

An alternative to scanning across the spectrum is to lock both the excitation and emission monochromators to the $\lambda_{\text{max}}$ and $I_{\text{max}}$ of the sample and scan over a period of time. This is known as a time-based spectra, which can contain, for example, a set of 240...
data points taken over a period of 60 seconds (Figure 3.1b). Each spectrum can then be averaged to give a more consistent measure of emission. The spectra taken through this method will yield consistent readings over time if the system under observation is at equilibrium. Therefore it is important that equilibrium is established before the scan is taken. Additionally, as shown in Figure 3.1a, fluorescence spectra for titrations are scaled by taking the signal at each point \((I)\) and dividing it by the initial intensity \((I_0)\). This scaling method was chosen based on the general trend of quenching amongst the titrations of this chapter, and is used unless otherwise noted.

*Figure 3.1.* Molecular probes upon the addition of Fe\(^{3+}\) ions (chloride salt). (a) 2.12, Ex = 420 nm, Slits = 0.60 mm; (b) 2.14, Ex = 330 nm, Em = 400 nm, Slits = 1.25 mm.

Another advantage of time-based experiments is its speed. This allows more discrete steps in a titration to be recorded in the same time period as a conventional wavelength scan. Once the system has reached equilibrium after each addition in a
titration (approximately 5 minutes), the time-based method is a very attractive method to construct binding isotherms and it is the technique employed in these studies.

**Binding of Fe$^{3+}$ Ions to 1.11, 2.12, and 2.14**

The coordination of Fe$^{3+}$ ions to siderophores is very complex. The stoichiometry of these systems is often reported as 1:1, however this is only true under acidic conditions and other stoichiometries exist across the entire pH range (vide infra). In the case of siderophores such as DFB the 1:1 complex (FeL) is only exclusively found under acidic conditions (pH = 1-3), Equations 3.1 and 3.2.

$$\text{Fe}^{3+} + L^{2-} \rightleftharpoons \text{FeL} \quad \text{Eq. 3.1}$$

$$K_1 = \frac{[\text{FeL}^+]}{[\text{Fe}^{3+}][L^{2-}]} \quad \text{Eq. 3.2}$$

As the pH increases to pH 6.4 and above, other species become prominent, Equations 3.3 and 3.4, and shown in Figure 3.2.$^{124}$

$$2\text{FeL}^+ + L^{2-} \rightleftharpoons \text{Fe}_2L_3 \quad \text{Eq. 3.3}$$

$$K_2 = \frac{[\text{Fe}_2L_3]}{[\text{FeL}^+]^2[L^{2-}]} \quad \text{Eq. 3.4}$$

The Equation 3.3 shows the stepwise formation of the 2:3 complex from the 1:1 complex. Since $K$ tends to vary greatly in magnitude, values given in this document are given as log $K$. A theoretical structure of the 1:1 and 2:3 complexes of Fe$^{3+}$ ions bound to 2.14 is shown in Figure 3.2. The 1:1 complex shows the interaction of the three hydroxamic acid groups of the DFB ligand chain in coordinating to the Fe$^{3+}$ ion as three binding motifs.

Two predictions of how the 2:3 complex could form are shown, the first a stepwise formation where the free amides of the two DFB chains coordinate to the Fe$^{3+}$ ion while the second shows a rearrangement of the 1:1 complex involved to coordinate via one of
its hydroxamic acid moieties while the carbonyls of the coumarin fluorophore move in to coordinate the Fe\(^{3+}\) ion. The second complex is less likely to form due to the stabilization of the 1:1 complex of \textbf{2.14} by the chelate effect. While there is precedence that the carbonyls of the amide and ester in the coumarin moiety could interact with the Fe\(^{3+}\) ion, we cannot state conclusively this plays a role in the formation of the 2:3 complex and this complex is only a prediction. These two different species play an important role when investigating the interactions between the metal and ligand and must be kept in mind when looking at the interaction between the two.
Figure 3.2. Cartoon of the 1:1 and 2:3 complex of 2.14 and the Fe$^{3+}$ ion.
When a one μM solution of 1.11 in 100% methanol is titrated with Fe$^{3+}$ ions, the emission of the fluorophore is quenched via an energy transfer mechanism, due to the paramagnetic nature of the Fe$^{3+}$ ions (Chapter I). The binding isotherm for 1.11 derived from this titration, Figure 3.4 shows a weak isotherm with only 15% of the original emission intensity quenched by the addition of 10 equivalences of Fe$^{3+}$ ions. The isotherm was fit by a least-squares method using HyperQuad. The data for 1.11 is shown in Table 3.1. The fitting shows equal amounts of the 1:1 and 2:3 species present in the solution. The mixture of species found here could be either due to the slightly acidic nature of methanol or due to the low concentration the experiment was carried out at, which will be explored further (vide infra). The limit of detection (LoD) for 1.11 was determined by the titration of 1.11 with Fe$^{3+}$ ions in triplicate, with the decrease in signal from the fluorophore of 1.11 measured. The LoD was then calculated by linear regression using the titration data to determine the minimal amount of Fe$^{3+}$ ions detectable by 1.11 at 1 μM concentration. The LoD calculations (Table 3.1) showed 112 nM as the lowest concentration of Fe$^{3+}$ ions possible for this probe to detect. This falls short of the needs for detecting Fe$^{3+}$ ions in the ocean where concentrations for the ion range from 0.02-0.54 nM, which in part drove our interest in coumarin-based probes.15

To better understand the stoichiometry of the Fe$^{3+}$ ion to 1.11, a Job’s plot of 1.11 at 1 μM in methanol was obtained, Figure 3.3. The result of this experiment shows a lack of consistency in the line and a mol fraction Fe$^{3+}$ of 0.84. While the mole fraction of a 1:1 complex is 0.5 and would be 0.4 for a 2:3 complex, the calculation for mole fraction is given in Figure 3.3 where the number of metal ions present in the complex and L is the number of ligands present. The mol fraction of 0.8 indicates a stoichiometry of 4:1 while
a mol fraction of 0.9 indicates a stoichiometry of 9:1, neither of which has precedent in regards to the Fe\(^{3+}\) ion coordinating to a siderophore ligand. As it is known there is an approximately equal amount of each species in this solution, it follows that this mole fraction of 0.84 would instead indicate the addition of the mole fractions for the 1:1 and 2:3 complex (0.5 and 0.4, respectively) to produce a fraction equal to 0.9. Deviations from the mole fraction of 0.9 are possibly due to the equilibrium between the 1:1 and 2:3 complexes given in Equation 3.5. This issue demonstrates it would be preferential to explore conditions where a single species is dominant to give a clearer understanding of the binding between the modified DFB probes presented here and the Fe\(^{3+}\) ion (vide infra).

*Figure 3.3.* Job’s Plot of **1.11** with FeCl\(_3\) in 100% methanol. Starting concentration for **1.11** 1 \(\mu\)M. \(\lambda_{ex} = 340\) nm, \(\lambda_{em} = 420\) nm, Slits 0.50 mm.
A one μM solution of 2.12 in 100% methanol is titrated with Fe\(^{3+}\) ions for a titration which produces the isotherm in Figure 3.4. The isotherm shows a more obvious curvature than 1.11 at the same concentration and the fluorophore is quenched by 85% by the addition of 10 equivalences of Fe\(^{3+}\) ions. Limit of detection for this probe under these conditions was shown to be 140 nM, higher than that of the previously discussed 1.11.

Fitting the isotherm in HyperQuad\(^{125}\), the data for 2.12, Table 3.1, shows a 60:40 split between the 1:1 and 2:3 species compared to the equal amounts of each found with 1.11. Additionally the log \(K_1\) and log \(K_2\) of 2.12 are shown to be higher than that of 1.11 by nearly a magnitude.

For 2.14 at one μM in 100% methanol titrated with Fe\(^{3+}\) ions, an isotherm is generated (Figure 3.4). The binding isotherm for 2.14 shows a clear curvature and falls off quickly at around 1.5 equivalences of Fe\(^{3+}\) ions, with 85% of the fluorophore’s emission quenched upon the addition of 10 μM of Fe\(^{3+}\) ions, similar to the response from 2.12. Limit of detection for this probe was found to be 87 nM (Table 3.1), the lowest of the three sensors discussed here, but still two magnitudes above the needed sensitivity for Fe\(^{3+}\) ions in the ocean. Fitting of 2.14 in HyperQuad is shown in Table 3.1. The speciation for 2.14 was found to be 50:50 between 1:1 and 2:3 species, showing that each of these compounds exists as a mixture of species in methanol at this concentration (Figure 3.5c). The log \(K_1\) and log \(K_2\) of 2.14 is found to be near that of 2.12 as would be expected from the compounds which differ only by the addition of a 7-diethylamino group to 2.12, but does show a dominance of 1:1 complex (Figure 3.5b). Compared to the coumarin-based probes, the probe 1.11 shows significantly weaker binding, possibly due to interactions of the carbonyl of coumarin with the Fe\(^{3+}\) ion, effectively quenching the
fluorophore more efficiently due to having a closer proximity to the metal ion (cf. 
Scheme 1.1). The less efficient quenching of the \textbf{1.11} complex also shows the free 
ligand to remain as the primary form of the probe (Figure 3.5a).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3_4}
\caption{Titrations with FeCl\textsubscript{3} in 100\% methanol of molecular probes at 1 \, \mu\text{M} 
concentration, \textbf{1.11} (\(\lambda_{\text{ex}} = 340 \, \text{nm}, \lambda_{\text{em}} = 420 \, \text{nm}, \text{Slits} \, 0.85 \, \text{mm})), \textbf{2.12} (\lambda_{\text{ex}} = 420 \, \text{nm}, \lambda_{\text{em}} 
= 460 \, \text{nm}, \text{Slits} \, 0.65 \, \text{mm})), \textbf{2.14} (\lambda_{\text{ex}} = 330 \, \text{nm}, \lambda_{\text{em}} = 400 \, \text{nm}, \text{Slits} \, 1.25 \, \text{mm}).}
\end{figure}
Table 3.1

Thermodynamic data for modified DFB compounds, conditions described in Figure 3.4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>log $K_1$</th>
<th>log $K_2$</th>
<th>LoD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.11</td>
<td>4.60</td>
<td>20.96</td>
<td>112</td>
</tr>
<tr>
<td>2.12</td>
<td>5.61</td>
<td>23.20</td>
<td>140</td>
</tr>
<tr>
<td>2.14</td>
<td>5.43</td>
<td>23.58</td>
<td>87</td>
</tr>
</tbody>
</table>

(a)

(b)
Figure 3.5. Fitting of the data used in Figure 3.4 in HypSpec for 1.11 (a), 2.12 (b), and 2.14 (c). Speciation shown for free ligand (red), 1:1 complex (blue), and 2:3 complex (green). Data being modeled shown by blue diamonds; red crosses show HypSpec model.

For each of these compounds the stepwise stability constants are found to be substantially different from the typical literature value given for DFB of log $K_2 = 30.5$
taken at one mM at pH 2. This difference has to do with concentration, but also differences of solvents used and pH as previously discussed. The decision to use one µM concentrations of the molecular probes for these studies was made based on what would give the lowest potential detection limit, which for the probes 1.11, 2.12, and 2.14 was found to be higher than intended. As our goal for detection limit was not met, we chose to look more deeply into the role of concentration with these probes.

Preliminary Concentration Studies

As we now have a good understanding of the speciation of modified DFB probes, the effect of concentration on the binding of Fe\textsuperscript{3+} ions was explored. On the molecular level the concentration of a sensor in solution can substantially alter the amount of complex formed due to thermodynamic factors, as well as alter the speciation under conditions where more than one species is formed. To determine the effect of concentration on thermodynamics, 2.14 was titrated with Fe\textsuperscript{3+} ions at different concentrations (1, 10, and 100 µM) in methanol (Figure 3.6). The isotherm given by these titrations was then modeled in HypSpec to determine the stepwise association constants, Table 3.2. To keep the isotherms on the same scale the x-axis is shown as [Fe\textsuperscript{3+}]/[2.14] to give equivalences of Fe\textsuperscript{3+} ions rather than concentration of the Fe\textsuperscript{3+} ion. Nearly nine magnitudes of difference are found in the stepwise equilibrium constant of the host–guest complex over only two magnitudes of difference in concentration. The solution of 2.14 at one µM when titrated with Fe\textsuperscript{3+} ions shows a shallow isotherm relative to the higher concentrations and as discussed in the previous section shows an equal distribution between the 1:1 and 2:3 complex species.
Raising the concentration by a factor of ten, the 10 μM sample shows a much steeper decrease in emission with 91% of the fluorophore’s emissions quenched by 10 μM of Fe$^{3+}$ ions, giving a steep isotherm. The isotherm for this sample was modeled in HypSpec to show a log $K_2 = 27.96$ giving a clear increase over the equilibrium constant for the one μM sample that comes within three magnitudes of the literature value.$^{44}$ Additionally at 10 μM, the species present is predominantly the 2:3 complex, with less than 5% of the 1:1 complex found by modeling. As this experiment showed the complex to be primarily of the 2:3 complex, a Job’s plot analysis was performed upon the solution (Figure 3.5), giving a mole fraction of 0.43. A stoichiometry of 2:3 is calculated to have a mole fraction of 0.42, with the error coming from the small amount of 1:1 complex remaining.

At the highest concentration of 100 μM the isotherm shows 99% of the initial emission quenched by 10 μM of Fe$^{3+}$ ions, Figure 3.6. When modeled in HypSpec the isotherm shows a log $K_2 = 32.2$, stronger than the reported literature value of log $K_2 = 30.5$. The speciation of the solution is predominantly the 2:3 complex which is 60% of the present species, while the 1:1 complex is 30% of the solution, demonstrating the effect of increased concentration of ligand on the stoichiometry of complexes found.
Figure 3.6. 2.14 at concentration 10 µM in 100% methanol titrated with FeCl₃. λ_{ex} = 330 nm, λ_{em} = 400 nm, slits = 0.60 mm.
Figure 3.7. Titration of \textbf{2.14} with FeCl$_3$ at varying concentrations in 100% methanol. $\lambda_{\text{ex}} = 330$ nm, $\lambda_{\text{em}} = 400$ nm. Slits = 0.85 mm (100 μM), 1.25 mm (10 μM), and 2.00 mm (1 μM).

It is evident that the concentration range of 10-100 μM is most favorable in methanol for producing binding constants for these samples. Additionally, the inner filter effect must also be considered, meaning that the light being absorbed by a sample must have passed through completely as otherwise erroneous changes might occur in the spectrum. The inner filter effect is directly related to the absorbance of light at the excitation wavelength for the compound, as given by Equation 3.8 where $F_{\text{att}}$ is the attenuated fluorescence and $A$ is the absorbance of the sample at the excitation wavelength.$^{52}$

$$F_{\text{att}} = 10^{-0.5A} \quad \text{Eq. 3.8}$$

For each titration shown in Figure 3.6, the absorbance of the sample was measured before being titrated. Using Equation 3.8, the data in Table 3.2 was calculated
showing the percentage of each sample that was excited. Based on this factor, the 100 μM sample shows only 50% of the sample is excited by incoming light while the sample at 10 μM has 93% of the sample excited, minimizing inner filter effects while also maintaining a stepwise equilibrium constant comparable to literature values. An additional factor regarding the inner filter effects is the common cut-off of 0.1 absorbance units for samples used in calculating quantum yields. As an additional measure, the LoD of 2.14 was calculated for each concentration shown in Table 3.2. This shows the unfortunate effect of the rise in concentration causing a decrease in sensitivity of detection. The LoD is given in nanomolar concentration for comparison to the goal LoD of 0.02-0.54 nM.

Table 3.2

Attenuated fluorescence calculated for various concentrations of 2.14 in methanol showing absorbance at λ\text{max} = 310 nm.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>log $K_1$</th>
<th>log $K_2$</th>
<th>Absorbance</th>
<th>($F_{att} \cdot 100)$%</th>
<th>LoD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μM</td>
<td>9.39</td>
<td>32.18</td>
<td>0.6</td>
<td>50</td>
<td>8106</td>
</tr>
<tr>
<td>10 μM</td>
<td>6.81</td>
<td>27.96</td>
<td>0.06</td>
<td>93</td>
<td>553</td>
</tr>
<tr>
<td>1 μM</td>
<td>5.43</td>
<td>23.58</td>
<td>0.006</td>
<td>99</td>
<td>87</td>
</tr>
</tbody>
</table>

Based on the equilibrium constant near the literature values of log $K_2 = 30.5$, the primary speciation being the 2:3 complex, and the attenuated fluorescence of 93%, 10 μM has been found to be the working concentration without compromising either thermodynamic factors or inner filter effect factors. The limit of detection did not reach the initial goal in the lowest concentration studied, with lower concentrations not viable, and thus is neglected in the choice of concentration. While it is useful to define these factors, all studies at this point were performed in methanol. Consideration must be given to aqueous solutions and the effect of pH on the binding of Fe$^{3+}$ ions to the probes.
Preliminary Solvent Studies

The molecular probes reported here need to function in simulated sea water. The effects of solvent system and pH have been mentioned previously and must be tested. In Figure 3.7 a 10 µM solution of compound **2.14** is shown titrated with FeCl$_3$ in various solvent systems starting with 100% methanol to 100% water (unbuffered). These titrations show drastic differences in the binding isotherm upon the introduction of water to the system with the 50:50 system showing a shallower isotherm. For the systems with 100% methanol, 99% methanol, and 50% methanol the data was modeled in HypSpec, Table 3.3. Unfortunately the data for 100% water was not able to be modeled; this is expected due to use of only water in the system which gives no control over pH while a Lewis acid in the form of Fe$^{3+}$ ions is being added to make the overall solution acidic. The isotherm for 100% water is included only for completeness. The effect of water on the system gives a clear trend, causing a decrease in the apparent equilibrium constants for the system. This comes from the ability of water to compete more strongly than methanol against the binding moieties of the DFB ligand for the Fe$^{3+}$ ions in the solution. Based on the modeled data, the log $K_1$ of the complex decreases upon increasing amounts of water in the solution, as would be expected as a pH effect (**vide infra**).
Figure 3.8. \(\textbf{2.14}\) titrated with \(\text{FeCl}_3\) in various ratios of methanol and water. \(\lambda_{\text{em}} = 330\) nm, \(\lambda_{\text{ex}} = 400\) nm, Slits = 1.25 mm.

Table 3.3

*Thermodynamic data for \(\textbf{2.14}\) with various amounts of water added, conditions given in Figure 3.6. *Not modeled.*

<table>
<thead>
<tr>
<th>Compound</th>
<th>% H(_2)O</th>
<th>log (K_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\textbf{2.14})</td>
<td>0%</td>
<td>6.81</td>
</tr>
<tr>
<td>(\textbf{2.14})</td>
<td>1%</td>
<td>6.42</td>
</tr>
<tr>
<td>(\textbf{2.14})</td>
<td>50%</td>
<td>5.99</td>
</tr>
<tr>
<td>(\textbf{2.14})</td>
<td>100%</td>
<td>--*</td>
</tr>
</tbody>
</table>

As mentioned the impact of water on the binding of \(\text{Fe}^{3+}\) ions to the system comes from competition between water molecules and the binding sites of \(\textbf{2.14}\) as well as the solvation of the metal ions. Specifically, water as a more polar molecule is able to solvate the \(\text{Fe}^{3+}\) ion better than the relatively less polar methanol.
With the effect of solvent established on the system, the function of the probe in a buffered system must be explored. As previously discussed, the speciation of DFB complexes such as 2.14 is predominantly a 2:3 complex above pH 6.4, and predominantly a 1:1 complex below. To confirm this, a 10 µM solution of 2.14 was titrated with FeCl$_3$ in acetate buffer at pH 5.8 (Figure 3.8). At this pH there should be a mixture of species with the 1:1 complex being predominant. The isotherm given by this titration is strong, with 95% of the initial emission quenched by one equivalence of Fe$^{3+}$ ions. Inset in Figure 3.8 is the HypSpec model of the isotherm, showing the speciation of the free ligand (red), the 1:1 complex (maroon) and the 2:3 complex (blue). Thermodynamic data from the model (Table 3.4) indicates a higher log $K_f$ constant compared to other titrations performed at this concentration.
To further confirm the effect of pH on the speciation of the complex between 2.14 and Fe$^{3+}$ ions, higher pH tests are needed. This additionally serves the purpose of testing the 2.14 probe in an aqueous solution at oceanic pH, which is one of the objectives of the probe (Chapter I).

A 10 μM solution of 2.14 titrated in phosphate buffer at pH 8.2 (PBS-0) is shown in Figure 3.9 along with a second experiment using PBS-0 with the addition of saline (3.2%) to simulate the salinity of sea water (PBS-32). In these solutions the PBS-0 solution shows a reasonably steep isotherm, with 70% of fluorescence quenched upon addition of 10 equivalences of Fe$^{3+}$ ions, comparative in strength to the methanol.

**Figure 3.9.** 2.14 at 10 μM in acetate buffer at pH 5.8. $\lambda_{ex} = 330$ nm, $\lambda_{em} = 400$ nm, Slits 1.25 mm. Inset: HypSpec model of isotherm. Speciation shown: free ligand (red), 1:1 complex (maroon), 2:3 complex (blue). Data being modeled shown by blue diamonds; red crosses show HypSpec model.
isotherms shown previously. The saline solution influences the shape of the isotherm, with only 25% of the initial emission quenched by 10 equivalences of Fe\(^{3+}\) ions. Both isotherms were modeled in HypSpec, Table 3.4. The log \(K_i\) for 2.14 varies by a magnitude when moving from buffer at pH 5.8 to pH 8.2, then by an additional magnitude with the presence of saline solution. This shows a clear effect of decreasing the ability of 2.14 to bind Fe\(^{3+}\) ions at higher pH and in the presence of saline.

Limit of detection for 2.14 in these buffered systems was calculated (Table 3.4) for comparison with the LoD calculated for the probe in methanol at 10 \(\mu\)M (Table 3.1). The move from methanol to water, which is a more competitive solvent, has caused an increase in LoD as expected. Additionally the presence of buffer has been shown to alter the thermodynamics of the system, which will further increase the LoD. The LoD in AB-58 is shown to be 513 nM, comparable to that in methanol at the same concentration (553 nM), while in PBS-0 the LoD is nearly a magnitude larger at 4273 nM. The difference in LoD between these two buffers is likely due to the change in pH from the pH 5.8 of AB-58 to pH 8.2 with PBS-0. Additionally the LoD of 2.14 in PBS-32 was found to be 7694 nM, another increase this time attributable to the saline in the buffer.

Table 3.4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>(\log K_i)</th>
<th>LoD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.14</td>
<td>AB-58</td>
<td>7.60</td>
<td>513</td>
</tr>
<tr>
<td>2.14</td>
<td>PBS-0</td>
<td>6.29</td>
<td>4273</td>
</tr>
<tr>
<td>2.14</td>
<td>PBS-32</td>
<td>5.72</td>
<td>7694</td>
</tr>
</tbody>
</table>

*Thermodynamic data of 2.14 in various buffers at 10 \(\mu\)M along with limit of detection. Ex = 330 nm, Em = 400 nm, Slits = 1.25 mm for AB-58 and 1.50 mm for PBS-0 and PBS-32.*
With the effect of solvent, pH, and concentration on 2.14 explored, we now turn our attention to the lanthanides with the molecular probes.

**Binding of Eu\(^{3+}\) Ions to 2.12, and 2.14**

The use of Ln\(^{3+}\) ions as a reporter group in our molecular probes first needs to be investigated. A general discussion of spectroscopic studies with lanthanides is detailed in Chapter I. The use of the probe 1.41 with the Tb\(^{3+}\) ion in an indicator displacement assay has been discussed previously and will be discussed further (*vide infra*).\(^{126}\) To achieve this we investigated the spectroscopy of the of the antenna fluorophore rather than through the emission of the lanthanide. The reason for this is the quenching of the antenna fluorescence when the lanthanide is bound. This quenching effect comes from

*Figure 3.10. 2.14 at 10 µM in phosphate buffer (PBS-0) at pH 8.2 and phosphate-buffered saline (PBS-32) at pH 8.2 with 0.5 mM NaCl and 0.5 mM KCl. \(\lambda_{\text{ex}} = 330\) nm, \(\lambda_{\text{em}} = 400\) nm, Slits = 1.50 mm.*
the lanthanide-resonance energy transfer (cf. Figure 1.14) and is useful in determining whether a lanthanide is being sensitized then quenched by a different process, or if the lanthanide is not being sensitized in the first place. A typical titration of 2.12 and 2.14 (10 μM) with EuCl₃ is shown by Figure 3.10.

In Figure 3.10 the titration of 2.14 with Eu³⁺ ions shows a steep isotherm that quenches 50% of the initial emission of the 400 nm band of the coumarin fluorophore by 10 equivalences of metal. Modeling of the isotherm in HypSpec shows an excellent fit, Table 3.5. The iron data taken under the same conditions has been included for comparison. The log $K_2$ for the Eu³⁺ ion is notably two magnitudes lower than that for the Fe³⁺ ion as would be expected based on literature values for Eu³⁺.¹²⁷ Notably the log $K_1$ value for the Eu³⁺ ion is slightly higher than the equivalent value for the Fe³⁺ ion; due to this the speciation for Eu³⁺ is shown to be close to 50:50 between the 1:1 and 2:3 complexes.

Table 3.5

<table>
<thead>
<tr>
<th>Compound</th>
<th>Metal Ion</th>
<th>log $K_1$</th>
<th>log $K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.14</td>
<td>Fe³⁺</td>
<td>6.81</td>
<td>27.96</td>
</tr>
<tr>
<td>2.14</td>
<td>Eu³⁺</td>
<td>7.62</td>
<td>26.3</td>
</tr>
<tr>
<td>2.12</td>
<td>Eu³⁺</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
The titration between \textbf{2.12} and \textit{EuCl}_3 showed no visible binding and the fluorescence signal is quenched by less than 6\% when ten equivalences of \textit{Fe}^{3+} ions are added. Attempts to model the isotherm failed, suggesting an inability of the \textit{7-diethylamino-coumarin} fluorophore in \textbf{2.12} to efficiently transfer energy to the \textit{Eu}^{3+} ion. This relates to the energy levels of an antenna necessary to sensitize a lanthanide as discussed in Chapter I (\textit{cf.} Figure 1.14). The emission band at 460 nm of \textbf{2.12} is shown by this experiment to be too low in energy to allow the fluorophore to act as an antenna for \textit{Eu}^{3+} which requires an antenna emission of <425 nm for an efficient energy transfer. Similarly for \textit{Tb}^{3+} an energy <417 nm is necessary.\textsuperscript{103} \textbf{Probe 2.14}, however, shows that the coumarin fluorophore utilized in its design is able to sensitize the \textit{Eu}^{3+} ion from its 400 nm emission band, which thus is transferring energy to the lanthanide to effectively quenches the fluorophore emission.
The data shown in Figure 3.10 demonstrates 2.14 to be the best probe for the indicator displacement assay system with lanthanides. Therefore the spectroscopic studies discussed from this point forward will be of 2.14 unless otherwise stated. The proof of principle that 2.14 can transfer energy to the Eu$^{3+}$ ion leads us to an interest in the details of how DFB itself alters the photophysics of the lanthanides (Chapter I).

**Photophysics Methods for Lanthanide Ions**

The basic concepts of lanthanide photophysics has been covered previously (Chapter I). To expand on this, we now discuss more specific aspects of lanthanide photophysics related to their interaction with ligands. The lanthanide ions each have a unique spectroscopic set of emissions they produce from their transitions and hence their

**Figure 3.11.** Solutions of 2.12 and 2.14 at 10 µM run with EuCl$_3$ in 100% methanol. For 2.14 $\lambda_{ex} = 330$ nm, $\lambda_{em} = 400$ nm, Slits = 1.25 mm. For 2.12 $\lambda_{ex} = 420$ nm, $\lambda_{em} = 460$ nm, Slits = 0.60mm.
own unique fingerprint. Many of these transitions correspond to orbitals that align with the electrical dipole of the lanthanide ion. The alignment of the dipoles are therefore hypersensitive, whereby any change to the metal ion’s environment, namely by the coordination of ligands, causes substantial changes in the efficiency of the transition which alters the emission. An example of this effect utilized in a sensor system comes from our own work, where the sensor 1.41 was shown capable of inducing an emission in the Tb$^{3+}$ ion (Figure 3.11). The Tb$^{3+}$ ion shows its strongest emission band at 550 nm, which is its hypersensitive transition corresponding to $J = 5$. The Eu$^{3+}$ ion has an intense emission band at 620 nm which is hypersensitive and corresponds to $J = 2$. In both cases these are the most efficient transitions and thus give the strongest emissions for these ions (Chapter I).
A luminescence technique that is useful for determining changes in the solvent shell of the lanthanide is a phosphorescence excitation experiment. In a phosphorescence excitation experiment the function of the two monochromators are inverted; the emission monochromator is held constant at $I_{\text{max}}$ for the lanthanide while the excitation monochromator sweeps a window of wavelengths giving the respective spectra of the particular lanthanide ion. This technique works exceptionally well with lanthanide ions due to their sharp emissions given by their $f-f$ transitions (Chapter I). In a Ph Ex scan, a spectrum is taken that is effectively the absorption of the lanthanide due to the movement of the excitation monochromator showing what wavelengths lead to the strongest emissions. In a solution the Eu$^{3+}$ and Tb$^{3+}$ ions both do absorb a small amount of light in
the UV range giving them a very low molar absorptivity (less than 10 L·mol⁻¹·cm⁻¹). This absorption comes from disturbances in the degeneracy of the f-f orbitals of the lanthanide due to ligands interacting with the electric dipole-aligned orbital. This means this technique is very useful for confirming that the lanthanide ion has coordinated to the ligand based on changes in the absorption of the lanthanide. Additionally it is useful for determining where to best excite the antenna sensitizing a lanthanide. Changes occurring in this absorption are important for confirming a lanthanide has been coordinated (blueshift or redshift) and sensitized (hyperchromic increase). To show that this phenomena exists in our systems, the Ph Ex spectrum was recorded for the salt EuCl₃ and then for the Eu³⁺ ion coordinated by DFB, Figure 3.12. The spectra shows a single transition for the EuCl₃ salt at 280 nm, while the coordination of the hydroxamic acid moieties of DFB to displace methanol solvent molecules, chloride counter ions, and water from the metal shows a new band at 310 nm. Both bands increase substantially in the complex of Eu³⁺ with DFB, but are scaled in Figure 3.12 to show the shifts of the signals more clearly (the coordinated Eu³⁺ is 8-fold stronger in emission). The new band at 310 nm is potentially the ⁵D₀ emitting state of the Eu³⁺ ion, which is commonly found as the emitting state for Eu³⁺ ligand complexes. This would suggest that the 280 nm band seen is either the ⁵D₁ or ⁵D₂ emitting state of the Eu³⁺ ion—both of which the lanthanide has access to and are higher in energy (cf. Figure 1.14).¹²⁹ This shift in emitting states is due to the effect of the ligand while the increase in emission is both due to the effect of the ligand as well as the protection of the lanthanide from water and methanol solvent. Additionally, the noise in the spectra shown in Figure 3.12 demonstrates the need for an antenna to sensitize the lanthanide; the slit widths for this study are 5.00 mm and 2.50
mm for the EuCl$_3$ alone and the DFB complex of the Eu$^{3+}$ ion. Compare to previously shown slit widths for fluorescence spectra in the range of 0.60 mm to 0.85 mm.

Figure 3.13. Comparison of EuCl$_3$·6H$_2$O and Eu$^{3+}$ bound to DFB (0.1 mmol in dried methanol. Slits for EuCl$_3$ at 5.00mm, slits for Eu$^{3+}$ bound to DFB at 2.50mm).

The same experiment was then run with TbCl$_3$ and the Tb$^{3+}$ ion coordinated to DFB, Figure 3.13. The results for the Tb$^{3+}$ ion are similar to that of the Eu$^{3+}$ ion, but due to differences in the spectroscopic properties of the individual lanthanides the Tb$^{3+}$ ion undergoes a blue shift rather than a red shift, with the ligand interaction with the Tb$^{3+}$ ion shifting absorption from 315 nm ($^5$D$_4$) to 270 nm ($^5$D$_3$). Notably the absorption of the Tb+DFB complex is relatively sharper than that shown by the Eu+DFB, indicating an almost total shift from the $^5$D$_4$ emitting state to the $^5$D$_3$ emitting state of Tb$^{3+}$ (cf. Figure 1.14). Once again the spectra are normalized to highlight the blue shift of the Tb$^{3+}$ ion.
The actual comparison of intensities would show the Tb\(^{3+}\) ion with DFB to have an intensity roughly 8-fold stronger than that of TbCl\(_3\).

![Graph showing fluorescence intensity vs. wavelength]

*Figure 3.14.* Comparison of TbCl\(_3\) · 6H\(_2\)O to Tb\(^{3+}\) coordinated by DFB, each 0.1 mmol in dried methanol. Slits for TbCl\(_3\) at 5.00mm, slits for Tb\(^{3+}\) coordinated to DFB at 2.50mm.

The spectra in Figure 3.14 show a clear change has occurred between a solvated (methanol) lanthanide ion and a lanthanide ion chelated by a siderophore as well as increasing the emission due to protection from the binding sites of the DFB. When sensitized by an antenna, the phosphorescence excitation experiment should reveal a novel spectrum that shares overlap with the absorption of the sensitizing antenna, which is confirmed by Figure 3.14. This is a good indication of not only coordination by the ligand, but also sensitization by the antenna fluorophore.
Figure 3.15. Comparison of Ph Ex scan of EuCl$_3$·6H$_2$O to Eu$^{3+}$ coordinated by DFB and the fluorescence of 2.14, each at 0.1 mmol in dried methanol. Slits for EuCl$_3$ at 5.00mm, slits for Eu$^{3+}$ coordinated to DFB at 4.00mm, slits for 2.14 were at 0.65mm. Scaled for comparison.

The same experiments are then performed using Tb$^{3+}$ ions in place of the Eu$^{3+}$ ions, as shown in Figure 3.15. Results are similar to those shown with the Eu$^{3+}$ ion, with the Ph Ex spectrum of the Tb$^{3+}$ coordination complex with 2.14 being of most note. In this case, rather than a broad spectra such as the Eu$^{3+}$ ion showed, there is a much narrower and intense absorption band, so much so that the slits were set to 0.85 mm which is comparable to settings used for fluorescence samples. This compares to the slit widths of 4.00 mm used for the Eu$^{3+}$ ion equivalent, indicating that the Tb$^{3+}$ ion is not only sensitized by the antenna of 2.14, but sensitized much more so than the Eu$^{3+}$ ion under the same conditions. The sensitization appears to specifically come from the higher
energy of the two $\pi - \pi^*$ transitions of 2.14 ($I_{max} = 298$ nm) hence the complex $I_{max}$ at 305 nm.

![Graph showing comparison of Ph Ex scan](image)

**Figure 3.16.** Comparison of Ph Ex scan of TbCl$_3 \cdot$ 6H$_2$O to Tb$^{3+}$ coordinated by DFB and the fluorescence of 2.14, each at 0.1 mmol in dried methanol. Slits for TbCl$_3$ at 5.00mm, slits for Tb$^{3+}$ coordinated to DFB at 0.85mm, slits for 2.14 were at 0.65mm. Scaled for comparison.

The phosphorescence excitation experiments demonstrate the differences in efficiency when it comes to sensitizing Eu$^{3+}$ and Tb$^{3+}$ ions. While the Tb$^{3+}$ ion shows more promise from these tests, both lanthanides were used in the indicator displacement assay. While the Tb$^{3+}$ ion shows greater intensity, the Eu$^{3+}$ ion shows an emission of interest for applications in *in vivo* studies (Chapter II).
External Blocking Group for the Eu$^{3+}$ Ion

The purpose of the blocking group was discussed in Chapter I. When sensitized by 2.14 the Eu$^{3+}$ ion does show a detectable signal, but it is of interest to maximize the signal-to-noise ratio and thus improve the detection limit. The β-diketone acetylacetone (acac) was chosen as a blocker for this purpose, being a well-studied ligand used with various lanthanides.\textsuperscript{131,132} To utilize acac as a blocker, titrations were performed with acac added to a solution of 2.14 coordinating the Eu$^{3+}$ ion. This test determines the concentration of blocker necessary to most efficiently block the Eu$^{3+}$ ion in its position coordinated by 2.14. In Figure 3.16, the titrations are shown which demonstrated that the presence acac in a 5 mM concentration caused a 16-fold increase in the emission of the Eu$^{3+}$ ion.

![Graph](Figure 3.17. Titrations of Eu$^{3+}$ ion complex of 2.14 with acac. $\lambda_{ex} = 310$ nm, $\lambda_{em} = 620$ nm, Slits = 2.50 mm.)
The impact of the acac is shown in part by the slit widths used in its presence (2.50 mm) compared to the slit width required for the detection of the Eu$^{3+}$ ion signal without acac (4.00 mm). Based on this, each solution used for the titration of Eu$^{3+}$ ion complex of 2.14 is brought to 5mM of acac using a stock solution before the titration is performed.

Solvent Tests for Eu$^{3+}$ & Tb$^{3+}$ Complexes of 2.14

An early study performed with our modified DFB systems was the use of 1.11 and the Tb$^{3+}$ ion in an indicator displacement assay.$^{126}$ This study was performed in methanol:water (50:50) with 2.14 bound to the Tb$^{3+}$ ion (10 µM) titrated with Fe$^{3+}$ ions, the isotherm of which is shown as Figure 3.17, which uses data from Figure 3.11. The isotherm shown here is relatively steep, with 80% of the emission of Tb$^{3+}$ quenched by the addition of 10 equivalences of Fe$^{3+}$ ions. While a good isotherm is produced by this study, of note is the high slit widths required to detect the signal from Tb$^{3+}$, which is exceedingly high at 5.00 mm. This study acts as a baseline that tests what the 2.14 probe can be tested against.
Figure 3.18. Titration of a 10 µM solution of the Tb$^{3+}$ complex of 1.11 with FeCl$_3$ in 50:50 methanol:water. $\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 550$ nm, Slits = 5.00 mm.

For the 2.14 probe and what, methanol was used for the initial studies with the goal of moving into aqueous systems. In these studies the Eu$^{3+}$ or Tb$^{3+}$ ion complex of 2.14 was titrated with Fe$^{3+}$ ions to determine what solvent systems the IDA of each lanthanide would function in. Of particular interest is the result of titrating Fe$^{3+}$ ions into the complexes of Eu$^{3+}$ and Tb$^{3+}$ ions with 2.14 in dried methanol, as shown by Figure 3.18. In both IDA an increase in emission is seen. The Eu$^{3+}$ ion emission reaches a maximum around nine equivalences of Fe$^{3+}$ ions before beginning to quench. The Tb$^{3+}$ ion shows a sharp increase of emission to over four times the starting intensity ($I_0$) by one equivalence of Fe$^{3+}$ ions.
There are two things that seem to be significant to this increase in emission:

- Water is coordinated to the lanthanides within the IDA, even in dried methanol. As the Fe$^{3+}$ ion enters, an equilibrium is established between the water coordinated to the lanthanide ion and water coordinated to the Fe$^{3+}$ ion.

- Water is necessary for the efficient quenching of the lanthanide by the mechanism previously discussed (Chapter I). Each lanthanide varies in sensitivity to water—the Eu$^{3+}$ ion is particularly sensitive while the Tb$^{3+}$ is less sensitive. An insufficient amount of water means the lanthanide ion can be displaced from the 2.14 ligand but continue to emit due to the through-space nature of the LRET mechanism.$^{100}$

The first can be attributed to water coordinated to the lanthanides within the IDA.

Figure 3.19. Lanthanide complexes of 2.14 titrated with FeCl$_3$ in methanol. Slits at 2.50 mm for Eu$^{3+}$ and 0.85 mm for Tb$^{3+}$. 
Based on the aforementioned two reasons, water was added to the solution and each titration was carried out in 99:1 methanol:water, Figure 3.19. In this case the Tb$^{3+}$ ion shows a three-fold increase in emission. The Eu$^{3+}$ ion, on the other hand, no longer shows an increase and instead quenches as expected. The differences in these titrations can be attributed to the differences in sensitivity to water between the two lanthanides. This also shows that water plays a role in the operation of a lanthanide-based IDA by quenching the indicator signal. Increasing the amount of water in the system further shows its impact on the system; the Tb$^{3+}$ ion signal no longer rises before being quenched, as shown in Figure 3.20. The Eu$^{3+}$ ion, more sensitive to the presence of water, does not show a detectable emission in 50:50 methanol:water, much less in pure water. The Tb$^{3+}$ ion complex, on the other hand, shows an improvement in detection of the Fe$^{3+}$ ions in pure water compared to 50:50 methanol:water. This improvement is likely due to there no longer being a competition between methanol and water to interact with the Tb$^{3+}$ ions, and thus a more efficient quenching once the lanthanide is displaced from 2.14 by the Fe$^{3+}$ ion. The limit of detection for the isotherms shown in Figure 3.20 were calculated, showing the IDA in 50% water to have a high limit of 30,732 nM, while in 100% water the IDA showed a LoD of 3895 nM. The value for the IDA in 100% water can be compared to those of the 2.14 probe alone in buffer (cf. Table 3.4), which shows a comparable LoD to the PBS-0 buffer study of 2.14 (4273 nM). Additionally the LoD of 2.14 in 100% water can be calculated from the isotherm shown in Figure 3.7, indicating a LoD of 6140 nM. This shows the IDA in the same solvent does improve upon the detection of the Fe$^{3+}$ ion compared to the 2.14 probe reporting by fluorescence.
Figure 3.20. Lanthanide complexes of 2.14 titrated with FeCl₃ in 99:1 methanol:water. Slits at 2.50 mm for Eu⁢³⁺ and 0.85 mm for Tb⁢³⁺.
Attempts were made to titrate the Tb$^{3+}$ complex with 2.14 in phosphate buffer and phosphate-buffered saline, but the presence of buffer was found to fully quench the Tb$^{3+}$ ion emission, likely due to the presence of the phosphate anion coordinating with the Tb$^{3+}$ ion. In the end the Tb$^{3+}$ ion complex of 2.14 has been shown to effectively operate as an indicator displacement assay for detection of Fe$^{3+}$ ions in water. Attempts at operating the IDA in buffer were unsuccessful.

Selectivity Studies

An important part of this work is the selectivity of the molecular probes. A probe that does not show a large difference in response between a target analyte and other analytes is not selective for the target analyte. If a probe is not selective for the target analyte over other metals found in the same environment it will potentially give false
positives (Chapter I). While siderophores are generally considered to be highly selective for Fe$^{3+}$ ions, recent literature suggests that under biological conditions the siderophores show affinity for divalent and trivalent metals other than iron ions. In addition to being iron transport agents, siderophores play a role in the solubilization, binding, and biological uptake of both divalent and trivalent transition metals. This makes metals found in the ocean in similar or higher concentration than the Fe$^{3+}$ ion (0.07-0.2 nmol·kg$^{-1}$) that are also divalent or trivalent with similar ionic radii to the Fe$^{3+}$ ion of particular concern (Al$^{3+}$, Cr$^{3+}$, Cu$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, Ni$^{2+}$, Zn$^{2+}$). The Al$^{3+}$ and Cu$^{2+}$ ions are the most likely to compete with molecular probes or interfere with the selectivity of 2.14. In the case of the Al$^{3+}$ ion this is due to its similar ionic radius (67.5 pm) compared to the Fe$^{3+}$ ion (69 pm), giving it a stepwise stability constant with DFB of log $K_2 = 24.1$, relatively close to that of the Fe$^{3+}$ ion, log $K_2 = 30.5$. While this means that the iron complex is more thermodynamically favorable, it also means that the Al$^{3+}$ ion is competitive compared to other metals such as the Cu$^{2+}$ ions with log $K_2 = 13.5$. The primary effect of Cu$^{2+}$ ions is not the direct competition per se, but it is effective at quenching fluorophores by a ligand-to-metal charge transfer. To study the selectivity of 2.14, a standardized solution is used with a concentration of 10 µM (vide supra), to which five equivalences of a metal ion are added. The decrease in fluorescence intensity of the coumarin fluorophore is then measured and compared to the initial emission (Figure 3.21). The experiment shows a clear divide in quenching effects of the different metals studied which show a maximum quenching of 45% for the Cu$^{2+}$ ion compared to the 80% of the initial emission quenched by the Fe$^{3+}$ ion. The reason for quenching from the Cu$^{2+}$ ion was discussed above, while quenching for the Fe$^{2+}$ ion suggests it is in its high-spin
state due to its coordination to weak field ligands, and is able to quench through a ligand-to-metal charge transfer. As a borderline Lewis acid, the Fe$^{2+}$ ion is not expected to associate very strongly with the oxygen atoms of 2.14, yet still has an equilibrium constant of log $K_2 = 7.2$ with DFB.\textsuperscript{38} These studies show that while the Fe$^{3+}$ ion demonstrates the largest quenching of emission from the fluorophore of probe 2.14, the threshold for emission (as marked on Figure 3.21) is at 45% with anything less being questionable as to whether it is induced by the Fe$^{3+}$ ion or not. The impact of these metals on the emission of the coumarin fluorophore of probe 2.14 is not unprecedented, with similar effects from various divalent metals on the emission of coumarin being shown previously by Yao et al.\textsuperscript{63}

![Figure 3.22. Selectivity of 2.14. Standard solution of 2.14 (10 μM) in methanol titrated with 50 μM of metal chloride salts. Dotted line is the threshold for quenching at 45%. $\lambda_{ex} = 330$ nm, $\lambda_{em} = 400$ nm, Slits = 1.25 mm.](image-url)
While the ability of the above metals to quench 2.14 has been demonstrated, this does not fully consider their impact on the effectiveness of 2.14 as a sensor for ferric ions. The equilibrium constants for Al^{3+} ions with DFB and their comparison to that of Fe^{3+} ions has been previously discussed. In spite of this strong binding to DFB, the Al^{3+} ion quenches less than 15% of the emission of the fluorophore. To better show the impact of the Al^{3+} ion on the function of probe 2.14, as well as the other metals previously shown, a competition study was performed.

Solutions of 2.14 at 10 µM concentration were prepared in 100% methanol. To each solution 50 µM of a metal chloride salt was added, followed by 50 µM of FeCl_3, as shown in Figure 3.22. To show the impact of the metals on the quenching of 2.14 a dashed line is drawn at the level of the Fe (III) blank where 50 µM of FeCl_3 is added, but no other metal is present. This sets the baseline whereby any emission over the dashed line is remaining emission due to interference from the metal ions present. The metals Mg^{2+}, Ni^{2+}, Zn^{2+}, and Cr^{3+} show only a 10% or less increase in emission compared to the sample without them, indicating little interference from these metals. As would be expected, the metals with the largest effect on quenching and the largest equilibrium constant with DFB compared to the Fe^{3+} ion show the greatest interference. As previously mentioned, the selectivity experiment did not show the Al^{3+} ion as quenching 2.14 effectively in spite of having a strong affinity for the DFB binding moieties. In Figure 3.22 it is clear that Al^{3+} is binding strongly to 2.14 and competing with the Fe^{3+} ion, thus decreasing the ability of the Fe^{3+} ion to coordinate and quench the fluorophore of 2.14. The metals Fe^{2+} and Cu^{2+} have substantially lower equilibrium constants than the Fe^{3+} ion, but also have been shown to have a stronger quenching effect on the
fluorophore of 2.14. In the case of the Fe$^{2+}$ and Cu$^{2+}$ ion the interference comes from not only their small amount of competition with the Fe$^{3+}$ ion, but also from their quenching of the 2.14 fluorophore before the Fe$^{3+}$ ion is added to the solution. The results of this competition study show that for the 2.14 probe alone the ions Fe$^{2+}$, Al$^{3+}$, and Cu$^{2+}$ show a great deal of interference. While a probe that detects both ferric and ferrous ions is acceptable, the effects of the Al$^{3+}$ and Cu$^{2+}$ ions giving false positive signals are problematic to the function of the probe due to the presence of both in the ocean.\textsuperscript{140}

![Figure 3.23. Competition of 2.14. Standard solution of 2.14 (10 μM) in methanol titrated with 50 μM of metal chloride salts followed by the addition of 50 μM of FeCl$_3$·6H$_2$O. $\lambda_{ex} = 330$ nm, $\lambda_{em} = 400$ nm, Slits = 1.25 mm.](image)

With the responses of 2.14 to metal ions in mind, the next step of determining selectivity and competition was to study the indicator displacement assay function in regards to the metal ions. The previous parameters were repeated for this experiment with
a 10 µM solution of the Eu$^{3+}$ complex of 2.14 used as a blank. Samples of this solution were then used with 50 µM of the previously shown metal chlorides added each time, the data shown in Figure 3.23. Data was captured via the 620 nm phosphorescent emission of the Eu$^{3+}$ ion rather than by the fluorophore emission of the 2.14. While this is described as a selectivity experiment, it is technically a competition due to the presence of Eu$^{3+}$ in the system when the metal chloride salts are added. For this reason, the results were found to be drastically different. As each of these metals is known to have an affinity with the DFB ligand, each one shows a significant impact by displacing the Eu$^{3+}$ ion from the ligand complex. The least effect is seen by the Mg$^{2+}$ ion which shows a 10% decrease in the emission of the Eu$^{3+}$ ion. The metals Zn$^{2+}$, Fe$^{2+}$, and Al$^{3+}$ show less ability to displace the Eu$^{3+}$ than the Fe$^{3+}$ ion, which is to be expected from their lower equilibrium constants (cf. Table 3.5). The Fe$^{3+}$ ion itself shows a quenching of 40%. Notably the Cu$^{2+}$, Ni$^{2+}$, and Cr$^{3+}$ ions show a stronger ability to quench the fluorescence of the Eu$^{3+}$ ion than the Fe$^{3+}$ ion. The quenching for the Cr$^{3+}$, Cu$^{2+}$ and Ni$^{2+}$ ions is due to an energy transfer from the Eu$^{3+}$ ion to the low-energy spin-forbidden d-d orbitals of Cr$^{3+}$, Cu$^{2+}$ or Ni$^{2+}$. The energy transfer is made possible due to a dipole-dipole overlap between the $^5D_0$ emitting state of the Eu$^{3+}$ ion and the absorption bands of the Cr$^{3+}$, Cu$^{2+}$, and Ni$^{2+}$ ions which lead to an energy transfer to the transition metal ion followed by a non-radiative relaxation.$^{129,141,142}$ This causes an obvious interference in the function of the IDA for detection of Fe$^{3+}$ ions. To further explore the impact of metals on the IDA, a competition study was carried out.
Figure 3.24. Selectivity of Eu\textsuperscript{3+} coordinated to 2.14 (10 μM) with 50 μM of various metal chloride salts added in 100% methanol. λ\textsubscript{ex} = 310 nm, λ\textsubscript{em} = 620 nm, Slits = 4.00 mm, Time Captured 60 s, Slits = 2.50 mm.

Competition experiments for the metal ions were run using the methods previously described. Beginning with a 10 μM solution of the Eu\textsuperscript{3+} ion complex of 2.14, 50 μM of FeCl\textsubscript{3} was added to act as a blank. For each subsequent sample, 50 μM of a metal chloride salt was added followed by the addition of 50 μM of FeCl\textsubscript{3} with data shown in Figure 3.24. As before, a dashed line is drawn to show deviations from the blank, and thus interference in the detection of the Fe\textsuperscript{3+} ion. The Fe\textsuperscript{3+} ion shows a 45% quenching of the emission of the Eu\textsuperscript{3+} ion in the blank. The Cr\textsuperscript{3+} ion shows an inhibition of further quenching by the Fe\textsuperscript{3+} ion due to the large amount of the Eu\textsuperscript{3+} emission already quenched by the Cr\textsuperscript{3+} ion, similar to the effect of Fe\textsuperscript{2+} and Cu\textsuperscript{2+} shown above, Figure 3.22. Other ions studied show a further decrease in the emission of the Eu\textsuperscript{3+} ion compared
to their absence. This is due to the indicator displacement assay function, where the addition of more competitive metals will displace more of the Eu$^{3+}$ from the system. The most efficient effects are shown by Al$^{3+}$ due to its comparable equilibrium constant to the Fe$^{3+}$, and by the Cu$^{2+}$ ion which is potentially quenching the fluorophore antenna through an energy transfer mechanism as previously discussed.

![Figure 3.25. Competition of Eu$^{3+}$ coordinated to 2.14 (10 μM) titrated with 50 μM of various metal chloride salts then 50 μM of FeCl$_3$ in methanol. $\lambda_{ex} = 313$ nm, $\lambda_{em} = 620$ nm, Time Captured 60 s, Slits 2.50 mm.](image)

The complex of Tb$^{3+}$ bound to 2.14 was studied for its selectivity using the ions of most concern from the previous studies both for selectivity and competition issues, as shown in Figure 3.25. For the Tb$^{3+}$ ion a threshold is established at 70% for the metals studied, with Cr$^{3+}$ producing the most quenching. The Al$^{3+}$ ion is once again likely highly competitive with the Tb$^{3+}$ ion leading to the 50% quenching shown by it. Both Cu$^{2+}$ and
Ni$^{2+}$ ions quench by less than 50%, in contrast to the quenching caused by these ions with Eu$^{3+}$. The lower sensitivity of Tb$^{3+}$ to the Cu$^{2+}$ and Ni$^{2+}$ ions—and to the Cr$^{3+}$ ion as well—is not without precedent. While both Eu$^{3+}$ and Tb$^{3+}$ ions are known to be quenched via energy transfers to these transition metals, the Tb$^{3+}$ ion is less sensitive to these effects due to its $^5D_4$ emitting state being higher in energy than the $^5D_0$ emitting state of the Eu$^{3+}$ ion (cf. Figure 1.14). Unlike with the previously shown 2.14 bound to Eu$^{3+}$, the displacement of the Tb$^{3+}$ ion in methanol has shown to be most selective for the Fe$^{3+}$ ion by a margin of 15%. In competition between the Fe$^{3+}$ ion, the interfering metals studied, the Cr$^{3+}$ and Al$^{3+}$ ions showed a 15% interference in the quenching of the indicator displacement assay (Figure 3.26). The Ni$^{2+}$ and Cu$^{2+}$ showed small effects on the competition, quenching by an additional <5%, which is expected for metal ions that do not interfere with the coordination of the Fe$^{3+}$ ion to 2.14 but instead directly quenching the emission of the Tb$^{3+}$ indicator.
Figure 3.26. Selectivity of Tb$^{3+}$ coordinated to 2.14 (10µM) in methanol with various metal chloride salts (50 µM) to demonstrate the selectivity of the sensor. $\lambda_{\text{ex}} = 310$ nm, $\lambda_{\text{em}} = 550$ nm, Time Captured 60 s, Slits = 0.85 mm.
Figure 3.27, Competition of $\text{Tb}^{3+}$ coordinated to 2.14 (10 $\mu$M) with various metal chloride salts (50 $\mu$M) followed by $\text{FeCl}_3$ (50 $\mu$M) in methanol. $\lambda_{\text{ex}} = 310$ nm, $\lambda_{\text{em}} = 550$ nm, Time Captured 60 s, Slits = 0.85 mm.

Spectroscopy Summary

We have demonstrated the function of three molecular probes, 1.11, 2.12, and 2.14 for the detection of the $\text{Fe}^{3+}$ ion both as on-OFF probes and IDA systems utilizing the $\text{Tb}^{3+}$ or $\text{Eu}^{3+}$ ion as the indicator. Solvent studies have indicated a concentration of 10 $\mu$M is ideal based on the inner filter effect and the thermodynamics of the probe with the $\text{Fe}^{3+}$ ion. The limit of detection for these probes falls short of the stated goal (Chapter I) of a sensor able to detect the 0.02-0.6 nM concentration of iron in the ocean, particularly in simulated sea water (PBS-32). The $\text{Eu}^{3+}$ ion was found not to be functional as an indicator in aqueous systems even with the presence of the external blocking group acac. The $\text{Tb}^{3+}$ ion was found to give an excellent emission response in aqueous systems.
comparable in intensity to those of fluorophores. The probe 2.14 was shown to be reasonably selective for Fe$^{3+}$ ions with a clearly stronger response to this metal than others tested, while in competition the Al$^{3+}$, Fe$^{2+}$, and Cu$^{2+}$ ions showed an impact on detection. The IDA of 2.14 with Eu$^{3+}$ was shown to not be selective for Fe$^{3+}$ ions due to the direct quenching effect of Cu$^{2+}$, Cr$^{3+}$, and Ni$^{2+}$ on the Eu$^{3+}$. The Tb$^{3+}$ IDA of 2.14 improved on the selectivity, but still showed only a narrow margin of difference between the effect of the Fe$^{3+}$ ion and the effect of the Cr$^{3+}$ ion, with both Cr$^{3+}$ and Al$^{3+}$ ions interfering with the detection of Fe$^{3+}$ ions. While the Tb$^{3+}$ IDA of 2.14 functions in pure water, it does not show a functional signal in buffered systems.
CHAPTER IV

SOL-GEL ENCAPSULATION OF MOLECULAR PROBES

Sol-gels, named for their synthesis via the networking of particles (gels) from a colloidal solution (sol), are of interest as a method of encapsulating molecular probes in a biologically inert matrix. The modern interest in silica gels began over 50 years ago with the work of Stöber et al. in the synthesis of micrometer-scale spheres capable of suspension in solvent.\textsuperscript{144,145} Silica gels can take many forms based on their treatment, from glass monoliths,\textsuperscript{146} to sol-gel fibers,\textsuperscript{147} to aerogels,\textsuperscript{148} depending on the exact synthetic procedure and purification process used. This leads to the importance of the Stöber Process in controlling the size and integrity of the sol-gel microspheres.\textsuperscript{149}

While there are a wide variety of starting materials and methods of synthesis for sol-gels, of interest were silicon alkoxide reagents which react by hydrolysis followed by polymer condensation.\textsuperscript{150} The focus on these reagents and this method is due to the well-studied nature of the reagents and the mild conditions of the synthesis.\textsuperscript{145}

Silicon alkoxides such as tetramethylorthosilicate (TMOS) or tetraethylorthosilicate (TEOS) can be reacted with either an acid or a base catalyst. The difference in these catalysts is the pathway for the formation of sol-gels. In acid-catalyzed systems, the hydrolysis step is slower which leads to the formation of linear chains which then cross-link during polycondensation to form the sol-gel particle (Figure 4.1a). In base-catalyzed systems, the hydrolysis step is fast, making the formation of highly-branched gels more likely, which then aggregate into the sol-gel (Figure 4.1b).\textsuperscript{149} In both cases, a porous silsesquioxane cage is formed which is capable of encapsulating a molecular probe or other molecule of interest.
Between these two polymerization pathways, the base-catalyzed one is of more interest in this project as it is more stable due to the higher amount of cross-linking during its polymerization. While the synthetic method is important to the end product, the removal of solvent from the system is also very important to the pore size and consistency of the resultant sol-gels. Once the polymerization is complete, the sol-gels can be aged, which will increase their end size, or the reaction can be immediately quenched by the removal of the catalyst followed by the removal of the solvent.

The production of sol-gels and the encapsulation of our molecular probes offer three interesting points:

- Sol-gel encapsulation will effectively act as a blocker for the lanthanide while allowing for the entry of analyte ions (Chapter I).
- Immobilization of the probe in a sol-gel provides a gateway to thin-film techniques for maturing our molecular probes as discrete sensing systems.
- Improve solubility of molecular probes in aqueous buffer.

With these design goals in mind, we now look at previous work on encapsulating molecular probes in sol-gel networks.
Figure 4.1. Mechanisms for hydrolysis and condensation steps of the sol-gel reaction, showing both the (a) acid-catalyzed and (b) base-catalyzed mechanisms.

Background for Encapsulated Molecular Probes

A key part of the encapsulation of molecular probes by sol-gels is their use in biological systems. In this case, the encapsulated probe is referred to as a PEBBLE (Probe Encapsulated by Biologically Localized Embedding), which is intended to avoid the cytotoxicity brought by a probe, while allowing it to function in vivo. A relatively popular type of probe to encapsulate in a sol-gel is one involving Eu$^{3+}$, which is sensitive to water (cf. Figure 1.16). A Eu$^{3+}$ complex of 4-methyl-7-hydroxycoumarin (Figure 4.2)
encapsulated in a sol-gel was synthesized by Tang et al. to take advantage of this.\textsuperscript{152} This probe is intended for \textit{in vitro} imaging, which the inert sol-gel capsule is important to. The probe itself reports by the fluorescence of the coumarin moiety, rather than the emission of the Eu\textsuperscript{3+} ion. In this probe the purpose of the lanthanide is not as an indicator, but instead to increase the emission of the coumarin fluorophore \textit{via} a back transfer of energy into the fluorophore’s triplet state due to the similarity of energy for the 4-methyl-7-hydroxycoumarin to the Eu\textsuperscript{3+} ion (\textit{cf}. Figure 1.16). Encapsulation of the probe in an organically modified silane (ormosil) sol-gel using 3-aminopropylmethyldiethoxysilane gave 45 nm sol-gel particles that greatly improved the solubility of the fluorescent probe. The emission of the probe while encapsulated showed a roughly 35\% increase in emission compared to the probe free in solution.

\textit{Figure 4.2}. The coordination of the Eu\textsuperscript{3+} ion by the sodium salt of 4-methyl-7-hydroxycoumarin.

While it is useful to demonstrate the blocking effect of the sol-gel cage for lanthanides, a true sensing method requires ions of interest to enter the pores of the sol-gel to modulate the emission of the probe. Tan et al. demonstrated the sensitization of the Tb\textsuperscript{3+} ion coordinated to carboxylic acid moieties with an imidazole antenna, Figure 4.3. Encapsulation of the probe into a TEOS-based sol-gel showed sufficient protection of the Tb\textsuperscript{3+} ion from water to allow for the probe to function in aqueous solution. The porous
nature of the sol-gel allows for ions to enter, demonstrated by this probe being quenched by the presence of Cu\(^{2+}\) and H\(_2\)PO\(_4^-\) ions (Figure 4.4). This gives a dual-purpose probe with both a cationic and anionic detection function.

![Figure 4.3. The coordination of the Tb\(^{3+}\) ion by the imidazole 4.1.](image)

*Figure 4.3. The coordination of the Tb\(^{3+}\) ion by the imidazole 4.1.*

![Figure 4.4. Sol-gel encapsulated complex from Figure 4.2 with TBAH\(_2\)PO\(_4\) and CuCl\(_2\).](image)


The probes described here have demonstrated the features of the sol-gels we wish to utilize in our own work, both the protection of the lanthanide within the sol-gel and the ability of metal cations to enter the sol-gel pores to quench the molecular probe within.
Synthesis of Sol-Gel Microparticles

To approach the synthesis of sol-gel microparticles, TMOS was first tested as a reagent, but found the reaction to be too fast to be controlled, with a monolithic sol-gel glass forming immediately. To gain control of the reaction we shifted our focus to TEOS, which slows the hydrolysis and condensation steps of its reaction down due to the bulkier ethyl groups.

As the synthesis of sol-gels is a polymerization reaction, care must be taken to control the reaction parameters to ensure a consistent product is acquired. A strict protocol was established and used for all reactions discussed in this work. An additional step taken to improve the consistency of size in the sol-gel microparticles was the addition of 5,000 M.W. polyethylene glycol monoethyl ether (PEG). The role of PEG in the reaction is to become embedded in the walls of the microparticles as they aggregate, leading to the effective functionalization of the sol-gel particle surfaces with PEG chains. These chains are useful as bulky groups that, by steric hindrance, limit the aggregation of the sol-gel particles during the polycondensation phase of the reaction, thus controlling the size of the particles.\(^{153,154}\)

To synthesize the sol-gels, PEG is first dissolved in ethanol (200 proof) before TEOS is added. Once these reagents have fully mixed, NH\(_4\)OH is added as a catalyst, triggering the reaction which was stirred for two hours. Of note is the function of water as a reagent in the hydrolysis step—or more specifically the hydroxide ion as a reagent. Due to this, the amount of water in the reaction also controls the end product of the reaction. A convenient source of water for the reaction is from the NH\(_4\)OH solution, which, while a catalyst, is controlled to keep consistent the amount of water added to the reaction. The
ratio of reagents is 1:4:250 for PEG:TEOS:H$_2$O. While the reaction parameters are important, the work-up of the reaction is equally important. The sol-gel reaction was quenched by the addition of an excess of water followed by concentration in an ultrafiltration cell with a 100 kDa membrane, water being added during this process until all monomer, ethanol, and free PEG are removed. To determine the size of the sol-gels produced dynamic light scattering (DLS) is utilized.\textsuperscript{155} For DLS, samples are allowed to settle for 24 hours to remove any particles not suspended in the solution, the data from which is shown by Figure 4.4. This reaction was very inefficient, producing few sol-gel particles that did not settle out of solution overnight. The small amount of sol-gel detected showed a median size of 1 nm—that is the lower limit of the DLS instrument, with the rest visible as having precipitated out of the solution overnight.

Figure 4.5. DLS of sol-gel after 24 hours, median diameter 1 nm.

To decrease the amount of sol-gel material lost due to aggregation into macroscopic particles, we added a step to our procedure. Once the sol-gel was quenched
by water, the reaction mixture was then subjected to 30 minutes of sonication before being concentrated as before. Allowed to settle overnight after this time period, the sol-gel material was visible at the bottom of the sample vial, while the DLS showed an increase in average particle size to 300 nm. This increase in average size shows sonication breaks up the post-reaction aggregation of the sol-gel particles, thus giving a more consistent size.

![Graph](image.png)

*Figure 4.6. Sol-gel after 30 minutes of sonication and 24 hours of settling, median diameter 300 nm.*

While DLS showed a consistent size of our sol-gels when used straight away in solution, we also dried other sol-gels by filtration through a 0.8 µm membrane using vacuum filtration to remove the larger particles and then passed the filtrate through a 0.1 µm membrane to capture the correctly sized particles. The particles on this membrane were dried under vacuum for seven days before being suspended in water, at which point DLS was performed once more on the sample, Figure 4.6. This showed a consistency in size after drying, indicating the sol-gels were not destroyed in the process.
Our method at this point provided consistently sized sol-gel particles, which we then were interested in encapsulating our probes into. For encapsulation, our synthetic method was altered with the addition of a molecular probe to be encapsulated to the solution containing PEG before the addition of TEOS or NH$_4$OH. The efficiency of a molecular probe being encapsulated by a sol-gel matrix can vary wildly. Due to this, we began with our probe, Tb$^{3+}$ bound to 1.11, at a tenth of an equivalence to the TEOS monomer (more would have been cost prohibitive). Once the sol-gels were produced and dried, we attempted to calculate the amount of Tb$^{3+}$ complex of 1.11.

Attempts to detect the 1.11 within the sol-gel were unsuccessful. Fluorometric studies did not show a signal at $\lambda = 420$ nm for the 1.11 fluorophore. Similarly UV-Vis studies did not show a band at $\lambda = 340$ nm. IR likewise did not show any signals indicating the presence of 1.11, including the normally strong and broad signal of the hydroxamic and amide carbonyl stretches at 1620 cm$^{-1}$. The previously shown probes

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**Figure 4.7.** Sol-gel after 30 minutes of sonication and drying for seven days under vacuum. Re-dissolved in ddH$_2$O for DLS measurement. Median diameter 300 nm.
encapsulated in sol-gels (*vide supra*) each contain a hydroxyl group that allow them to be potentially incorporated into the sol-gel cage itself. The probe 1.11 does not have this, thus decreasing the efficiency with which it is encapsulated, with only van der Waals forces and hydrogen bonding with the amide groups of the DFB chain to retain it in the sol-gel. Attempts to recover the Tb$^{3+}$ complex of 1.11 from the waste of the reaction were not effective. After the concentration step a solution is produced that is a mixture of un-encapsulated Tb$^{3+}$ complex of 1.11 along with sol-gels below 50 nm in size, unreacted monomer, and the NH$_4$OH catalyst. The DFB derivatives do not purify well from silica columns (Chapter II) and recrystallization from methanol and ether did not work to separate out the DFB complex.

Sol-gels were successfully produced from TEOS in the size range of 400 nm, which allows for the particles to be suspended in solution for fluorometric studies. Attempts to encapsulate a modified DFB-based probe into the sol-gels showed that the process is highly inefficient for this particular class of probes. A relatively large amount (gram scale) of the modified DFB would be necessary before a detectable amount is encapsulated in the sol-gel, which becomes cost prohibitive. For these reasons the studies of the encapsulation of our modified-DFB probes into sol-gels were not continued.

**Synthetic Procedures**

General. Concentration of solution utilized an Amicon Stirred Cell 8200 system (Millipore Corp.) with a 100 kDa membrane, pressurized to 10 psi using argon. Sol-gels were recovered from 0.1 µm and 0.8 µm filter membranes (Millipore Corp.). UV-Vis samples were run using a Beckman-Coulter DU-800 spectrophotometer. Fluorescence
experiments utilized a PTI QuantaMaster 40 with a 75 W xenon lamp. Infrared
spectroscopy studies were performed using a Nicolet Nexus 470 FT-IR.

Sol-Gel from Tetraethylorthosilicate. PEG monomethyl ether (3.0 g, 6 x 10^{-4} mol) placed into solution in EtOH (200 proof, 6 mL). To this solution TEOS is added (500 µL, 2.0 x 10^{-3} mol) with the solution remaining clear until the addition of NH_{4}OH (30%, 3.9 mL, 0.15 mol H_{2}O) at which point the solution becomes cloudy. The solution was stirred for two hours then quenched with EtOH (190 proof, 200 mL) then sonicated for 30 minutes. After sonication the solution was washed in the ultrafiltration cell using EtOH (190 proof, 2 x 200 mL) with a 100 kDa membrane and then concentrated to 20 mL. The concentrated solution was then passed through a 0.8 µm filter using vacuum filtration, the filtrate then passed through a 0.1 µm to give the end product sol-gel which was dried in a vacuum desiccator for seven days before use. DLS (ddH_{2}O): 250-500 nm.

Sol-Gel Encapsulating Tb^{3+} Complex of 1.41. PEG monomethyl ether (3.0 g, 6 x 10^{-4} mol) and Tb^{3+} bound to 1.41 (169 mg, 2.0 x 10^{-4} mol) placed into solution in EtOH (200 proof, 6 mL). TEOS (500 µL, 2.0 x 10^{-3} mol) added to solution followed by NH_{4}OH (30%, 3.9 mL, 0.15 mol H_{2}O). Solution was allowed to stir for two hours then quenched with EtOH (190 proof, 200 mL) then sonicated for 30 minutes. After sonication the solution was washed in the ultrafiltration cell with EtOH (190 proof, 2 x 200 mL) with a 100 kDa membrane, followed by concentration to 20 mL. The product sol-gels were collected using vacuum filtration with a 0.8 µm then 0.1 µm membrane before drying for seven days in a vacuum desiccator. No embedded Tb^{3+} complex of 1.41 was detectable via UV-Vis, fluorometry, or FT-IR.
Synthesis and Spectroscopy Conclusions

Early attempts at imine coupling of fluorophores to DFB were unsuccessful, but we have shown a successful peptide coupling that is efficient (>80% yield) in the production of the coumarin-DFB probes 2.12 and 2.14. We have successfully shown 1.11, 2.12, and 2.14 to act as fluorescent sensors for Fe$^{3+}$ ions in methanol and form thermodynamically stable complexes in both methanol and aqueous buffer. From this, the use of probes 1.11 and 2.14 was expanded to an IDA for Fe$^{3+}$ ions with the Tb$^{3+}$ ion as an indicator in methanol and water respectively. The IDA of 2.14 with Tb$^{3+}$ ions partially meets the project goal of a system functional in phosphate-buffered saline. The detection limits of these probes does not meet our target for detection of Fe$^{3+}$ ions in the ocean; under oceanic conditions the probes are several magnitudes less sensitive to the Fe$^{3+}$ ion than required. For selectivity, the results of the probe 2.14 showed a stronger response to Fe$^{3+}$ ions than to any other metal, but metals such as Cu$^{2+}$ did show a quenching effect on the fluorophore. The use of Eu$^{3+}$ as an indicator was found to be questionable due to direct quenching effects on the Eu$^{3+}$ ion by the Cu$^{2+}$, Cr$^{3+}$, and Ni$^{2+}$ ion, all of which are present in some quantity in the ocean. The IDA of 2.14 with the Tb$^{3+}$ ion showed more promise by retaining its ability to discriminate the Fe$^{3+}$ ion over other metal ions, meeting the selectivity goal. Attempts to encapsulate the probe 1.11 within a sol-gel network were shown to be cost prohibitive due to the inefficient nature of the sol-gel encapsulation process and the difficulty in recovering starting material that went unreacted or underwent side reactions.
In solution, solvent molecules play a significant role in the interactions between the host and guest species via a variety of interactions. These include hydrogen bonding, dipole-dipole, dipole-ion, and ion-ion interactions as well as van der Waals interactions. Collectively, these solvent interactions can sometimes be stronger than the individual host–guest interactions. To overcome solvent effects, the thermodynamics of the host–guest system has to be favored. This can be achieved by incorporating multiple interactions by taking advantage of the chelate effect, macrocyclic effect, or macrobicyclic effect (these influence both the enthalpy and entropy of the system), Figure 5.1. For example, the chelate effect can help stabilize the host–guest complex, such as the interaction of siderophores with the Fe$^{3+}$ ion, which are generally able to form six dative covalent bonds with the metal ion (Chapter I). While siderophores are able to overcome solvent competition due to a positive effect on entropy of the system in their coordination, the competition is still present. The determination of binding affinities in the gas phase can be argued to be a true interaction, as the host and guest are no longer competing with solvent molecules. Additionally, for other ligand systems that interact with guests through hydrogen bonding or van der Waals forces, the bonds tend to strengthen in the gas-phase due to the absence of competing protic solvents.
Figure 5.1. Examples of the chelate effect (Fe$^{3+}$ bound to DFB), the macrocyclic effect (K$^+$ bound to [18]-crown-6 ether), and the macrobicyclic effect (K$^+$ bound to [2.2.2]-cryptand).

ESI-MS and Non-Covalent Complexes

The ESI-MS technique has distinguished itself as an important tool for exploring supramolecular interaction. The earliest examples were reported by Brodbelt and Maleknia in the early 1990’s when they studied the gas-phase interactions of crown ethers with alkali metals.$^{160}$

The actual ionization method of ESI-MS comes from passing a solution through a spray capillary (needle) that is electrically charged. Upon charging, the spray forms a Taylor cone where the individual droplets are driven away from one another due to Coulomb repulsions, Figure 5.2. Desolvation by heating also causes the droplets to undergo Coulomb explosions, decreasing their size. Two models of this process are currently proposed: the single-ion-in-droplet model holds that the Coulomb explosion process eventually desolvates analytes almost completely, while the ion-evaporation
model proposes that single analyte ions are ejected from the droplet surfaces as charge repulsion increases.\textsuperscript{161}

\textbf{Figure 5.2.} Desolvation of ions on ESI-MS. As the solvated analyte leaves the needle tip a Taylor Cone forms that tapers to a jet which forms a plume of charged droplets that desolvate the analytes through one of two possible pathways: ion evaporation, or a Coulomb explosion. Copyright © 2005, John Wiley and Sons.

In either case, the desolvated analytes are focused by an oppositely charged ion lens before entering the MS. This charging method has the additional benefit of allowing large molecules, such as polymers or proteins, to become multiply charged to a high degree, which in turn decreases the \textit{m/z} ratio of high-molecular weight molecules and allows for the detection of fragmentation with a wide variety of molecular masses. While ESI is a soft technique—that is, ESI causes less fragmentation during the ionization process than earlier techniques such as electron ionization or chemical ionization. ESI is easily coupled with techniques such as collision-induced dissociation (CID) and infrared-
multiphoton dissociation (IRMPD) that allow for information to be gained from further fragmentation. Fragmentation patterns of host–guest complexes is of the greatest importance due to the structural and even thermodynamic information it can reveal.\textsuperscript{111,162} Fragmentation patterns that are identified can be used to investigate host–guest complexes. Once identified, mass spectrometry is useful in that it can both scan an entire range or utilize single ion monitoring (SIM) to focus on a single or few signals.

Once the constituents of a solution containing a host–guest complex have been identified, SIM mode can be used to monitor changes in signal intensities between the signal of the host, the host–guest complex, or any other species formed that is relevant to the host–guest system. A common method used in proteins for host–guest interactions to determine dissociation constants ($K_d$), which is the reciprocal of the association constant ($K_a$), compares relative abundances of a host to the host–guest complex. The relationship for a 1:1 binding is shown in Equation 5.1 where $[H]$ is the area under the curve of the free host, $[G]$ is the free guest area, and $[G_i]$ is the initial guest area.\textsuperscript{163,164} While Equation 5.1 can yield a binding constant from a single point of data, it is useful to rearrange this equation to Equation 5.2 which allows for titration points to be plotted as $[HG]/[H]$ versus $([G_i] – [HG])$ which gives a line with a slope of $1/K_d$.

\[
K_d = \frac{[H][G]}{[HG]} = \frac{[H][(G_i) – [HG]]}{[HG]} \quad \text{Eq. 5.1}
\]

\[
\frac{[HG]/[H]}{[G_i] – [HG]} = \frac{1}{K_d} \quad \text{Eq. 5.2}
\]

Now that the function of the ESI-MS instrument and methods for calculating dissociation constants has been discussed, we will look at examples of ESI-MS used in the analysis of non-covalent complexes and the thermodynamics of the complexes.
ESI-MS Methods for Non-Covalent Complexes

The ESI technique can be used for calculating binding constants, but most of the published work has been carried out with traditional host–guest interactions.\textsuperscript{164-166} Dotsikas and Loukas have explored the gas-phase binding constants for β-cyclodextrin, \textbf{5.1}, with various phenol-like guests (\textbf{5.2-5.4}).\textsuperscript{167}

Samples were injected using a constant concentration for the guests \textbf{5.2-5.4} while the concentration of host, \textbf{5.1}, was increased. As discussed previously, differences in ionization efficiency between host molecules and host–guest complexes must be considered for quantitative experiments such as calculating stability constants. For this, Equation 5.5 was utilized for a calibration curve using linear regression where $\Delta I_r$ is the change in relative intensity of the host–guest complex compared to the concentration of the guest, the difference being used as the initial intensity of the complex is near zero.
The variable $k_c$ is a proportionality constant, $K$ is the stability constant, and $H$ and $G_t$ are the host and total guest concentrations respectively.

$$I_r = k_c K[H] \frac{[G]_r}{1 + K[H]} \quad \text{Eq. 5.5}$$

Equation 5.5 can then be re-arranged as a double reciprocal linear equation to produce a Lineweaver-Burk plot which compares changes in the relative host–guest intensity to the initial total centration of the host and guest, given as Equation 5.6.

Additionally, the difference $\Delta I_r = I_r - I_0 \approx I_r$ can be used in place of $I_r$ due to the initial concentration intensity assumed to be zero.

$$\frac{1}{\Delta I_r} = \frac{1}{k_c G_t K[H]} + \frac{1}{k_c [G]_t} \quad \text{Eq. 5.6}$$

Using the above equation, the intercept and slope of a weighted least-squares regression fit of the data can be used to find the stability constants of 5.2 (1438±75), 5.3 (1084±58), and 5.4 (906±43). The stability constants presented here are for the complexes in gas-phase, which Dotsikas and Loukas have shown to be in agreement with stability constants for the complexes calculated in solution using spectrophotometric methods.\textsuperscript{167} In a similar vein of exploring classical supramolecular systems with ESI-MS, Tristani et al. has presented a study of interactions between an iron-siderophore complex and crown ethers.\textsuperscript{168} The siderophore chosen for this study is DFB, as discussed previously. The terminal amine has been found able to coordinate with the cavity of certain ionophores such as crown ethers, as shown by Figure 5.3, whereby the iron complex of DFB (1.8) is able to coordinate 18-crown-6 ether to the terminal primary amine of the DFB chain to form complex 5.5.\textsuperscript{169}
Figure 5.3. The coordination of 18-crown-6 ether to the primary amine of DFB.

By comparison to standards of similar siderophores and ionophores, the affinity of the crown ether for the terminal amine can be calculated. For titrations, the concentration of guest, 1.8, was held in excess (1 mM) in methanolic solution while the amount of host, 18-crown-6 ether, was varied (6 μM to 400 μM). Tetra-n-butyl ammonium acetate (TBA acetate) was used as an internal standard (13 μM, 0.1 or 1 mM) due to its inability to interact with either the host or guest in an appreciable way. From this method it was calculated that the \( \log K = 3.70 \) for complex 5.5. To validate this method, 18-crown-6-ether coordinating K\(^+\) was determined to have a \( \log K \) of 4.29 which is in good agreement with conductivity experiments in methanol (\( \log K = 4.34 \)).\(^{170} \) To calculate the equilibrium constant, \( K_a \), of 5.5 and other host–guest complexes, a nonlinear regression for a single-site binding as given by Eq. 5.7 was used.

\[
I = \frac{I_{\text{max}} [H]}{1 + [H]} \quad \text{Eq. 5.7}
\]

In this equation \( I \) is the signal intensity of the host–guest complex relative to the standard while \( I_{\text{max}} \) is its maximum intensity, [host] is the molar free concentration of the host (1.8), and \( 1/K_a \) is the reciprocal of the association constant of the host–guest
complex. The results calculated by this method appear to be comparable to other methods in their precision.

Why Anions?

The detection of anions is of interest in a variety of fields:

- Environmental pollutants such as anionic surfactants from industrial sources\textsuperscript{171}
- Environmental pollutants from agricultural sources such as the NO\textsubscript{3} anion\textsuperscript{172}
- Narcotics, halides and oxyanions are of interest due to their presence as counter-
anions for the salt form of illicit drugs\textsuperscript{173}

The driving interest in anion recognition is sensitivity in probes that are either selective, or able distinguish different anions—a universal detection technique. Current methods in detection of anions include instrumentation able to directly detect them such as atomic absorption spectroscopy and ion chromatography paired with UV-Vis, as well as indirect methods with chemosensors that work through other techniques such as cyclic voltammetry (CV) or fluorescence spectroscopy. These techniques, while effective, suffer from limitations. Atomic absorption spectroscopy requires larger sample quantities, UV-Vis requires the anion absorb UV or visible light, and indirect methods \textit{via} chemosensors reporting by fluorescence or CV are often unable to distinguish different anions and instead must focus on selectivity.

Both inductively coupled plasma mass spectrometry (ICP-MS) and electrospray ionization mass spectrometry (ESI-MS) are of interest in anion detection for their ability to distinguish anions and sensitivity (detection limits as low as $10^{-9}$ mol). While ICP-MS offers an extremely low detection limit, it is less effective for detection of polyatomic anions and complex molecules due to being a harder ionization technique than ESI-MS.
For ESI-MS, its ionization method is soft enough that anions, macromolecules, and host–guest complexes are not fragmented to the degree they are with ICP-MS techniques. ESI-MS does present two issues with the detection of anions:

- The sensitivity of ESI-MS in negative mode is impeded by instabilities in the Taylor cone (vide infra) which produces higher background-to-noise ratio.
- The lowest mass-to-charge ratio (m/z) limit for ESI-MS is around 100 m/z, making smaller and more highly charged anions impossible to detect directly.

To overcome both of these issues, the softness of electrospray ionization can be taken advantage of by first coordinating the anion guest to a host that is either cationic—an ion-pairing reagent—or neutral and able to be charged positively by a charging agent such as formic or acetic acids. The host–guest complex of a few hundred Daltons is then able to overcome the lack of sensitivity that ESI-MS gives in negative mode as well as having a signal further from the lower limit of the spectrometer’s range of detection (~100 m/z).

To explore the impact of ion-pairing reagents on the detection of anions, Zhang et al. have synthesized 22 tetra and tri-cations to investigate the detection limit of a set of 18 trivalent anions using ESI-MS. To test the detection limit of the anions, the limits were first calculated in negative mode for the anions alone which were then coordinated with each ion-pairing reagent and tested for detection limit once more. Compounds 5.6-5.8 and the cations that gave them the lowest detection limit (5.9-5.11) are shown.
In the study of the ion-pairing agents 5.6-5.8, the anion analytes 5.9-5.11 were first subjected to negative mode ESI-MS by introducing their sodium salts in a 10 \( \mu \)g·mL\(^{-1}\) solution of water/MeOH (v/v 2:1). The limit of detection was calculated for each anion with the pyranine, 5.9, appearing as its singly protonated form (-2 charge where \( m/z = 227.9 \), assigned to the ion \([M + H]^-\)) with a limit of 2.50 ng while vanadate, 5.11, was present in its doubly protonated form (-1 charge where \( m/z = 117.0 \), assigned to the ion \([M + 2H]^+\)) with a limit of 15.0 ng and oxalomalic tricarboxylate, 5.10, appeared in its doubly protonated form (-1 charge, 205.0 \( m/z \)) with a limit of 50 ng. The 50 ng limit was the maximum limit possible based on the solution concentration used. The experiment establishing the previously described limits of detection was then run once more with the addition of one equivalence of the fluoride salt of each ion-pairing reagent to determine whether the detection limit would be improved by the addition of the fluoride salt or not. For compound 5.9, the agent that lowered its detection limit most was 5.7, which brought the limit down to 0.125 ng, showing a 20-fold increase in sensitivity. For the vanadate,
The macrocycle 5.6 showed the greatest affinity, bringing its limit to 0.425 ng giving it a 35-fold increase in sensitivity. Lastly, 5.8 coordinating with 5.10 lowered its limit to 2.50 ng, which lead to a 20-fold increase in sensitivity. The ion-pairing reagents discussed here have, in a simple way, demonstrated the impact that host–guest complexes can have upon detection of anions via ESI-MS. While the experiments to calculate the anion detection limits above clearly indicate an increase in sensitivity compared to the anions alone, it is questionable as to whether the actual detection limits are quantitative or not. The values given are questionable due to a lack of accounting for differences in charging efficiency between the hosts 5.6-5.8 and the complexes they form with anions 5.9-5.11, which is known to change the detector response of an ESI-MS substantially. Nonetheless, this example is an excellent demonstration of one of the early concepts for anion detection using ESI-MS. The same concept of a host–guest complex to detect anions through ESI-MS is utilized by Rodriguez-Cruz and Carson who have presented a use for a pyrrole-based calixarene moiety with ESI-MS in the analysis of the salts of narcotics and counterfeit pharmaceuticals via anion detection. This work has built on previous uses of 5.12 by Sessler et al. in the field of anion detection. For determining the salt identity of a drug, 5.12 is placed into solution where it coordinates the anion of a drug such as cocaine (5.13). The complex between the anion and 5.12 is then detected in negative mode. With the coordination of the anion of the drug, its cationic form is then detectable in positive mode while the anion is detectable in negative mode. This method allows for the identification of drugs such as cocaine and dextromethorphan as well as the identification of their salt form (Cl⁻, Br⁻, I⁻, NO₃⁻, and CH₃COO⁻) which can give clues to their origin and method of preparation. This method
allows for detection limits as low as 0.10 μg·mL⁻¹ using as little as one nanogram of sample, which runs into the aforementioned issue with the sensitivity of negative mode in ESI-MS, but nonetheless allows for an excellent system of detection.

![Chemical structures](image)

**Project Overview**

This project has two distinct goals:

- Expanding upon the previous work of other groups in the characteristics and attributes of DFB in the gas-phase using ESI-MS
- The application of ESI-MS techniques to a ligand previously unstudied with mass spectrometry

To achieve our first aim, the fragmentation of the DFB-iron complex is studied to compare to literature. The thermodynamics of DFB with the Fe³⁺ iron ion in the gas-phase are then studied to compare to the thermodynamics of modified-DFB in solution-phase (Chapter III). The second goal is targeted by looking at the fragmentation patterns of the ligand 6.1 with CID.
CHAPTER VI

ESI-MS OF CATION AND ANION COMPLEXES

In Chapter V we discussed the background of ESI-MS as a technique for host–guest complexes. We also discussed the use of ESI-MS for structural elucidation and for the calculation of thermodynamic constants of host–guest complexes. We now turn our attention to utilizing ESI-MS with various host–guest systems.

ESI-MS Parameters

For these experiments, HPLC/MS-grade methanol (FisherSci) was utilized. Glassware was cleaned with 1 M HCl and oven-dried before use. Injections for manual titrations were made using a 500 uL SGE gas-tight syringe. Automated titrations were passed to the machine from the Accela autosampler by a direct line. Flow rates of 10 uL min⁻¹ were utilized. Spray voltage for all experiments was maintained at ±5 kV while the sheath gas (N₂) flow rate was set to 20 arbitrary units. Capillary temperature was maintained at 110 °C.

Gas-Phase Thermodynamics of Cation Binding

Previously, Gledhill has performed studies of hydroxamate siderophores including DFB using ESI-MS. Additionally, Tristani et al. has utilized the iron-DFB complex to determine the binding affinity of 18-crown-6 ethers to a primary amine in gas-phase (Chapter V). These studies have been limited to speciation, detection limits, and CID with little discussion of the thermodynamics of the iron-DFB complex itself. However, what was of interest from these previous studies was the fragmentation pattern of the iron-DFB complex, which was shown by Gledhill et al. to fragment at the amide and hydroxamic acid N—C bonds. The charge on these fragments is generally retained
by either the primary amine at the end of the DFB chain, or by the Fe$^{3+}$ ion with the loss of the coordinating hydroxyl group of the hydroxamic acid moiety. To confirm our methods we subjected the [DFB + Fe]$^+$ signal to MS-MS (Figure 6.1). The base peak is found to be 414 $m/z$ corresponding to the [DFB(3) + Fe]$^+$ fragment shown by Figure 6.1, while the next signal is 496 $m/z$ (98% RA) for the [DFB(2) + Fe]$^+$. A third signal corresponding to the loss of the primary amine is shown at 597 $m/z$ (82% RA) corresponding to [DFB(1) + Fe]$^+$. Additionally, an apparent signal for fragmentation across the second amide of DFB, [DFB(4) + Fe]$^+$, appears at 454 $m/z$ (25% RA), in substantially lower abundance. This data agrees well with literature values.$^{162}$ The fragmentation of the DFB-iron complex shows how the bound Fe$^{3+}$ ion templates the fragmentation of DFB by remaining bound to the hydroxamic acid moieties of the fragments.
Figure 6.1. MS-MS of DFB (0.5 µM) with two equivalences of Fe$^{3+}$ ions present, taken at the [DFB+Fe]$^{+}$ (614 m/z) using ESI-MS in positive mode. Capillary temperature at 110 °C, capillary voltage at +5 kV.

As discussed previously (Chapter V) gas-phase host–guest interactions are of interest. For this study both unmodified DFB have been used to study the binding to Fe$^{3+}$ ions in the gas-phase. Samples were made containing variable amounts of Fe$^{3+}$ ions from stock solutions with each sample allowed to stir for five minutes before being run. Each sample was injected into the ESI-MS using the previously described conditions and data was collected in SIM mode recording each identified signal for the complex. The [DFB + Fe]$^{+}$ signal was graphed against the concentration of the Fe$^{3+}$ ion, Figure 6.2.
Figure 6.2. Assay of DFB (10 μM) in methanol with increasing amounts of Fe$^{3+}$ ions. Charged with 5 μL of formic acid, capillary temperature 110° C, capillary voltage 5.00 kV.

The stoichiometry of the coordination complex detected by ESI-MS was of interest. Only the 1:1 complex is detected even with higher concentrations of Fe$^{3+}$ ions, in spite of the 2:3 complex being found as the dominant form in methanol and buffer above pH 6.2 (Chapter III). This suggests a role played by solvent in the different complexes formed between DFB and the Fe$^{3+}$ ion. In the transition from solution to gas-phase, all of the solvent is lost from the solvation shell that could offer competition against the binding sites of the ligand and the guest ion. A result of the loss of solvent, as well, is a strengthening of the chelate effect. For these reasons a 1:1 stoichiometry is exclusively found. The signals in Figure 6.3 are the predominant isotopic envelopes for each ion, which are summed together for binding studies. Additionally, the sodium adduct, [DFB + Na]$^+$, signal is non-negligible and thus summed together with the charged [DFB + H]$^+$ to
give the total host intensity, $I_H$. For the [DFB + Fe + H]$^+$ complex, of note is the change in the isotopic abundances compared to the [DFB + H]$^+$ and [DFB + Na]$^+$ abundances. This difference is due to the abundance of the naturally abundant isotopes of iron; $^{54}$Fe (5.8%), $^{56}$Fe (91.8%), $^{57}$Fe (2.1%), and $^{58}$Fe (0.3%). This gives complexes containing an iron atom a unique isotopic distribution, a theoretical calculation of which is shown in the inset of Figure 6.3.

![Graph showing isotopic abundances](image)

**Figure 6.3.** ESI-MS spectra of the DFB adducts and complex adducts used for calculating the binding constant. Inset: theoretical isotopic distribution of [DFB+Fe+H]$^+$.

The total host intensity, $I_H$, can be used to calculate the relative intensity ($I_r$) as $I_0 - I_H = I_r$. We then are able to use linear regression via a modified Benesi-Hildebrand method to create a calibration curve correlating [DFB + Fe]$^+$ as $1/I_r$ to $1/[Fe^{3+}]$ to
determine $\log K$ for the complex based on Equation 5.6 (Figure 6.4). Our method was validated utilizing a Scatchard Plot. Included for comparison in Table 6.1 is the equilibrium constant of modified DFB, $2.14$, calculated for the 1:1 complex through fluorometric methods (Chapter III). The equilibrium constant in gas-phase is larger than that of the solvent-phase calculation by a magnitude of difference, which is expected from the loss of the solvation shell around the metal ion and the lack of competition between the solvent and the molecular probe in binding the metal (Chapter V).

Table 6.1

*Comparison of thermodynamic data between DFB calculated by ESI-MS and $2.14$ calculated by fluorescence spectroscopy (Chapter III).*

<table>
<thead>
<tr>
<th>Compound</th>
<th>HypSpec (Fluorometric) $\log K$</th>
<th>Benesi-Hildebrand (ESI-MS) $\log K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFB</td>
<td>--</td>
<td>7.11</td>
</tr>
<tr>
<td>2.14 in Methanol</td>
<td>6.27</td>
<td>--</td>
</tr>
</tbody>
</table>
Figure 6.4. Benesi-Hildebrand plot of complex forming between DFB and the Fe$^{3+}$ ion in methanol. The terms $I_0$ and $I_H$ from the [DFB + Fe]$^+$ complex signal at 614 m/z.

Attempts were made to apply these same techniques to the lanthanides Tb$^{3+}$ and Eu$^{3+}$ to DFB. The signals for the complex of DFB with the lanthanides were exceedingly low and insufficient to calculate binding constants or establish clear fragmentation patterns from.

With methods established for the interaction of DFB and the Fe$^{3+}$ ion, we are interested in expanding these techniques to other systems.

Detection and Fragmentation of Compound 6.1

The tripodal receptor 6.1 is a urea-based system bearing $p$-cyanophenyl groups that has been shown to detect sulfate anions.\textsuperscript{179} Interestingly, the same receptor has been shown by Hossain et al. as having an affinity for halide anions, all in solution and solid state.\textsuperscript{180} As we have just discussed in the previous section, we were interested in
investigating the thermodynamics of an anion receptor. Therefore, we employed the same techniques discussed previously, which could lead to useful information about the true non-covalent interactions in the gas-phase.

Samples were prepared in methanol (5 µM) and injected without a charging agent at 110 °C, with the temperature controlled to maximize the signal of the molecule. Compound 6.1 showed a strong response in negative mode with a base signal at 613 m/z and a second signal at 577 m/z (87% RA), corresponding to [M + 2H₂O - H]⁻ and [M - H]⁻ respectively, Figure 6.5a. The presence of water in this molecule is unsurprising as the three urea moieties of 6.1 could each act as both an acceptor and donor for a hydrogen bond. Upon MS-MS on the 613 m/z base peak, the [M - H]⁻ signal at 577 m/z is predominantly seen, indicating the loss of the two water molecules from the 613 m/z signal. Additional signals are 459 m/z (16% RA) and 433 m/z (5% RA) are also observed. When CID is performed on the 577 m/z signal a new set of four distinct signals are found with 459 m/z the new base signal, while 433 m/z (28% RA), 341 m/z (7% RA), and 315 m/z (4% RA) are detectable, Figure 6.5b. The mass difference between these signals is 118 m/z, suggesting the loss of a neutral 4-cyanoaniline molecule.
A known fragmentation product of an urea functional group is an isocyanate derivative. For 6.1 the isocyanate derivative would be a 4-cyanoisocyanate molecule with a mass of 144 Da, which is the apparent loss between the 577 - 433 m/z fragmentation step. The 459 m/z signal, as the most prevalent signal of the CID of [M - H]⁻, does not corresponding to a simple loss of a 4-cyanoaniline or 4-cyanoisocyanate molecule. The 459 m/z fragment appears to be a stable heterocyclic amine intermediate (a pseudo-δ-lactam derivative), [M(H1) - H]⁻, generated due to the nature of charging of the 6.1 receptor which in the negative mode has lost a proton from one of the urea moieties. The anion generated by the proton loss could then reasonably abstract a proton from the α-carbon of the tertiary amine at the center of the molecule. Once the proton is abstracted,
the α-carbon can undergo an intramolecular nucleophilic addition by attacking the carbonyl group of an urea moiety on one of the three arms of the molecule, forming a six-membered heteracyclic amine. The fragment lost in this process is 4-cyanobenzene-1-urea, which, as a neutral molecule, tautomerizes to an imidic acid, then decomposes into 4-cyanoaniline, cyanic acid, and finally ammonia and water. Several of the fragmentation products of 4-cyanobenzene-1-urea (cyanic acid, water, ammonia) are unfortunately below the detection limit of the instrument (~100 m/z), but the signal for [4-cyanoaniline-H]− was detected at 117 m/z from the MS of 577 to 433 to 341 m/z path. Additionally, Isa et al. have confirmed the presence of cyanic acid, water, and ammonia in the fragmentation using thermogravimetric mass spectrometry.181

The CID fragmentation pattern of [M - H]− at 341 m/z and 315 m/z shows a discrepancy in mass, with each signal one Dalton lighter than predicted. Based on the cyclization thought to produce [M(1) - H]− it is possible further cyclization produces the bicyclic heterocyclic amine [M(3) - H]− through the 459 to 341 m/z pathway while the 433 to 315 m/z fragmentation pathway produces the cyclic urea [M(4) - H]−, Scheme 6.1, which would explain the missing proton. Regardless of the pathway, the [4-cyanoaniline - H]− product at 117 m/z is the final assigned molecule after fragmentation steps. These potential cyclic products have not been subjected to high-resolution MS or through deuterium-based studies to confirm the identity of the products, but the proposed products are in excellent agreement with literature sources.181-183 With the fragmentation of compound 6.1 established, we then turned our attention to the fragmentation of the ligand with a bound anion. It was anticipated that the anion would hinder the function of
the cyclic ureas by producing fragments still bound to the anion, in an analogous function to the DFB-Fe discussed in the previous section.

Scheme 6.1. CID scheme of 6.1 (0.5 μM) using ESI-MS in negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.
Fragmentation of Anion Complex of 6.1

The anions of interest are those previously shown to have an affinity with compound 6.1 (F\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), NO\(_3^-\), HSO\(_4^-\), SO\(_4^{2-}\)) and are used in their tetrabutylammonium salt form (the Zn\(^{2+}\) salt of SO\(_4^{2-}\) is used instead). Solutions of 6.1 were made in methanol (5 µM) and to each solution two equivalences of an anion salt were added. Previous studies by Hossain et al. has shown via \(^1\text{H}-\text{NMR}\) that a 1:1 stoichiometry for the complex of 6.1 with spherical halides, which was confirmed by x-ray crystallography. This stoichiometry is thought to be due to the anion residing within the cavity of the host.\(^{180}\) Signals from these samples were shown at 597, 613, 657, and 705 m/z corresponding to the complexes with F\(^-\), Cl\(^-\), Br\(^-\), and I\(^-\) respectively as [M + X]\(^-\) where X\(^-\) is the halide anion. Of the halide anions, free [M - H]\(^-\) is visible for F\(^-\) (93% RA), Cl\(^-\) (<5% RA), Br\(^-\) (15% RA), and I\(^-\) (37% RA). The abundances of the uncoordinated ligand for these samples supports a size selectivity for the Cl\(^-\) anion, which is supported by the previously discussed \(^1\text{H}-\text{NMR}\) experiments by Hossain et al.\(^{180}\) Notably, the F\(^-\) sample shows a base signal for [M + 2H\(_2\text{O}\)]\(^-\) at 613 m/z rather than for [M + F]\(^-\) which appears at 597 m/z (95% RA), Figure 6.6. The appearance of this solvent complex signal is of interest and explored further with the use of oxoanions (\textit{vide infra}).
Figure 6.6. ESI-MS of 6.1 (0.5 µM) with two equivalences of F⁻ ions present, negative mode. (a) [M – H]⁻, (b) [M + F]⁻ (c) [M + 2H₂O]⁻. Capillary temperature at 110 °C, capillary voltage at -5 kV.

When the [M + X]⁻ complexes are subjected to CID, the Br⁻ (Figure 6.7) and I⁻ (Figure 6.8) complexes show a distinct fragmentation pattern with the loss of a neutral 4-cyanophenylisocyanate molecule (144 m/z), with the remaining fragment being [M(2) + X]⁻, Scheme 6.2. This indicates the removal of one of the arms of the tripodal 6.1 and is followed by a second fragmentation step where another neutral 4-cyanophenylisocyanate
molecule is lost. A third fragmentation indicates the loss of the final arm and the removal of the halide from the molecule, confirmed by the detection of [I]⁻ at 127 m/z (the Br⁻ anion is below the detection limit of the instrument). A neutral tris(triethyl)amine would remain after the loss of the last 4-cyanophenylisocyanate molecule. The [M + F]⁻ complex does not show any useful signals in MS-MS, possibly due to the strong hydrogen bonding from the F⁻ ion which leads to the complex requiring high dissociation energies that give no detectable fragments. The Cl⁻ complex presents a different issue; the [M + Cl]⁻ signal appears at 613 m/z, as does the [M + 2H₂O]⁻ signal. This prevents evaluation of the Cl⁻ complex by CID.
Figure 6.7. MS-MS of 6.1 (0.5 µM) with two equivalences of Br⁻ ions present, taken at 657 m/z using ESI-MS in negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.
Figure 6.8. MS-MS of 6.1 (0.5 µM) with two equivalences of I⁻ ions present, taken at 705 m/z using ESI-MS in negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.

Of interest with this fragmentation scheme is the lack of cyclized fragments due to the previously discussed schemes. The template effect of the anion prevents cyclization, which is consistent of 6.1 and its hydrogen bonding to the anion. This prevents the rearrangement necessary to abstract an α-carbon from the tertiary amine. The anion is effectively blocking the proton abstractions and preventing the intramolecular nucleophilic addition to the carbonyl of one of the urea moieties. This also supports the possible mechanism described earlier.
Scheme 6.2. CID scheme of 6.1 (0.5 µM) with two equivalences of Br\(^{-}\) or I\(^{-}\) ions present, taken using ESI-MS in negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.

For the oxoanions a more complex spectrum is found. The base signal for the HSO\(_4^{-}\) anion is found as 675 \(m/z\) corresponding to [M + HSO\(_4\)]\(^{-}\), with another signal at 916 \(m/z\) (21% RA) appearing that corresponds to [M + 3HSO\(_4\) + 2Na]\(^{-}\) with sodium cations scavenged from the glassware used to counter balance the charges of two of the hydrogen sulfate anions to give a total charge of -1, Figure 6.9. From literature, 6.1 forms a 1:1 complex by encapsulating hydrogen sulfate within its cavity, which appears to correspond to the [M + HSO\(_4\)]\(^{-}\) complex.\(^{179}\) The appearance of a 3:1 complex between
hydrogen sulfate and 6.1 indicates it is likely each anion is hydrogen bonding with a
different arm of the molecule in the gas-phase. Other predicted charges of the complex
such as $[M + 3\text{HSO}_4 + 1\text{Na}]^{2-}$ or $[M + 3\text{HSO}_4]^{3-}$ at 462 or 300 m/z were not found.
Additional signals in this spectra are 613 (34% RA), 627 (45% RA), and 640 m/z (15% RA) corresponding to $[M + 2\text{H}_2\text{O}]^-$, $[M + \text{H}_2\text{O} + \text{CH}_3\text{OH}]^-$, and $[M + 2\text{CH}_3\text{OH}]^-$. These solvent complexes with 6.1 are apparent in this spectrum but most are not visible in
spectra with the halide complexes, for example in $[M + \text{Cl}]^-$ the 627 m/z signal for $[M + \text{H}_2\text{O} + \text{CH}_3\text{OH}]^-$ is found at <5% RA. It is possible this is due to an overall lower abundance of the base signal which would effectively raise the relative abundance of the solvent complex signals.
Figure 6.9. ESI-MS of 6.1 (0.5 µM) with two equivalences of HSO$_4^-$ ions present, negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.

An additional explanation for the appearance of these solvent complexes is the removal of a proton from the normally neutral solvent complexes by hydrogen sulfate, giving neutral sulfuric acid in the gas-phase which is not detectable—and would be below the detection limit of the instrument, anyway, at 98 m/z. To lend further evidence to this idea, we look at the scan of 6.1 with SO$_4^{2-}$, which shows a base signal at 627 m/z corresponding to [M + H$_2$O + CH$_3$OH]. The [M - H]$^-$ is strong comparatively (99% RA) while solvent signals are found at 613 (35% RA) and 640 (14% RA) corresponding to [M...
+ 2H₂O]⁻ and [M + 2CH₃OH]⁻. The signals indicating the presence of sulfate are found at 675 (54% RA) and 915 m/z (15% RA) corresponding to [M + HSO₄]⁻ and [3M + SO₄]²⁻, Figure 6.10. The appearance of the signal [M + HSO₄]⁻ appears to support the removal of protons from the neutral complexes by the sulfate anion, based on the substantially higher abundance of both the solvent complexes as well as the hydrogen sulfate complex compared to the divalent sulfate complex.

Figure 6.10. ESI-MS of 6.1 (0.5 µM) with two equivalences of SO₄²⁻ ions present, negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.
The various complexes of both the $\text{HSO}_4^-$ and the $\text{SO}_4^{2-}$ complexes were subjected to CID, with the $[\text{M} + \text{HSO}_4^-]$ signal at 675 $m/z$ showing a similar fragmentation pattern to that shown by the halides previously (Figure 6.11). The CID of the $[3\text{M} + \text{SO}_4^{2-}]^-$ complex at 915 $m/z$ shows the base signal to be $[\text{M} - \text{H}]^-$ at 577 $m/z$ with another signal at 626 $m/z$ (8% RA) that indicates $[2\text{M} + \text{SO}_4^{2-}]^-$. 

![Figure 6.11](image)

*Figure 6.11. CID of 6.1 (0.5 µM) with two equivalences of $\text{HSO}_4^-$ ions present, taken using ESI-MS in negative mode. (a) MS-MS at 675 $m/z$, (b) MS$^3$ at 675 $\rightarrow$ 531 $m/z$. Fragments are labeled to correspond to Scheme 6.3. Capillary temperature at 110 °C, capillary voltage at -5 kV.*
Scheme 6.3. CID scheme of 6.1 (0.5 μM) with two equivalences of HSO$_4^-$ or SO$_4^{2-}$ anions present, taken using ESI-MS in negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.

To add to the studies of 6.1 with oxoanions, as well as to further explore the removal of protons from neutral complexes of 6.1 by oxoanions, samples were run with NO$_3^-$, Figure 6.12. The base signal for this sample shows at 640 m/z for [M + NO$_3^-$]. Solvent signals identified are 613 (59% RA) and 627 (49% RA) m/z corresponding to [M + 2H$_2$O]$^-$ and [M + H$_2$O + CH$_3$OH]$^-$ respectively. When subjected to MS-MS the [M + NO$_3^-$]$^-$ complex does not show any unique fragmentation products, instead showing the same pattern for [M - H]$^-$ (*vide supra*).
Figure 6.12. ESI-MS of 6.1 (0.5 µM) with two equivalences of NO$_3^-$ ions present, negative mode. Capillary temperature at 110 °C, capillary voltage at -5 kV.

The relative abundance of these solvent signals suggests that nitrate can abstract a proton easier than HSO$_4^{2-}$ but is a weaker base than the SO$_4^{2-}$ ion. The ability of these anions appears to follow a series that matches their gas-phase basicity, which is reasonable as this would correspond to the ability of the anion to abstract a proton from the urea groups of 6.1. In this case, the gas-phase basicity of NO$_3^-$ is 1358 kJ·mol$^{-1}$ compared to that of hydrogen sulfate at 1265 kJ·mol$^{-1}$, while sulfate logically is more basic than NO$_3^-$ or HSO$_4^-$ and thus more likely to abstract a proton.\textsuperscript{184}
ESI-MS Conclusions

We have confirmed the effect of bound ions on the fragmentation of ligands using the DFB-iron complex. Our exploration in siderophores using ESI-MS also led to the calculation of the equilibrium constant of DFB with the Fe\(^{3+}\) ion in the gas-phase using a modified Benesi-Hildebrand plot, which is comparable to HypSpec modeling of the 1:1 complex in solution-phase. Attempts to repeat this experiment with lanthanide metals showed insufficient signals to study. We have shown our methods for ESI-MS with DFB can be modified and applied to other systems. We studied the fragmentation of compound 6.1 and demonstrated that the molecule undergoes intramolecular reactions that lead to cyclization of its urea moieties. The anion complexes of 6.1 were shown to be protected from the cyclization reactions by a templating effect much like found in the DFB-iron complex.
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