WATER-SOLUBLE FLUORESCENT PH SENSORS BASED ON A DIARYLACETYLENE SCAFFOLD

by

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Intracellular pH plays an important role in many biological processes: receptor-mediated signal transduction, enzymatic activity, cell growth and death, ion transport and homeostasis, calcium regulation, endocytosis, chemotaxis, and cell adhesion. Normal cell pH is around 7.40 and can range from 7.35-7.45 without any adverse effects. If it ranges more than 0.1-0.2 in either direction cardiopulmonary and neurologic problems can arise and in the case of extreme variations, death can result. Determination of pH in cells is of great importance and many methods exist for sensing pH; however, fluorescence is the most useful because of its nondestructive nature, high sensitivity, and specificity. We chose to use an intrinsic fluorescent probe in which the protonation site is integrated into the main chromophore. The systems were prepared by Sonogashira coupling of halopyridines with acetylenes. Protonation of the first class of pyridyl receptors synthesized occurred at a pH of 4 and was accompanied by a decrease in fluorescence. Protonation of the second class of pyridyl acceptor synthesized occurs near the pH of 5.5 and is accompanied by an increase in fluorescence intensity. Though water-soluble, model membrane transport studies show that the probes have a high affinity for non polar environments.

WATER-SOLUBLE FLUORESCENT PH SENSORS BASED ON A DIARYLACETYLENE SCAFFOLD

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by

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June 2010

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CHAPTER 1: FLUORESCENT PH SENSORS

1.1 Intracellular pH

Regulation of hydrogen ion concentrations is vital to many biological processes, including receptor-mediated signal transduction, enzymatic activity, cell growth and death, ion transport and homeostasis. Normal extracellular pH is typically 7.40, and can range from 7.35-7.45 without any adverse effects. If it deviates more than 0.1-0.2 in either direction, cardiopulmonary and neurologic problems can arise and in the case of extreme variations, death can result. Even though pH is so important to many biological functions, the cellular effects of changes in $[H^+]$ have not been studied as much as for other ions like Ca^+ or K^+.[1]

Different organelles in the cell have different hydrogen ion concentrations. Endosomes, which sort material before they move on to the lysomes, usually range between pH of 5 to 6.[2] Lysosomes digest the cell's waste and are the most acidic organelle with a pH between 4.5 and 5.5. Their pH gradient is generated by a vacuolar type H^+-ATPase which transports protons in the presence of Mg^{2+}-ATP.[3] Phagosomes are specialized compartments the cell uses to ingest materials that cannot readily pass though the cell membrane and typically have a pH similar to lysosomes. Phagocytosis acts as form of protection against assault from microorganisms. The mitochondria are the power house of the cell and typically have a pH of about 8.0.[4] The mitochondria produce ATP by utilizing the proton gradient. The subunit that produces the ATP is called the F1F0-ATPase.[5] The Golgi apparatus processes and packages macromolecules and has a pH of about 6.17. Most of the enzymes found within the Golgi apparatus work best under slightly acidic conditions.[6]

There are two main ways cells maintain their pH. Short term homeostasis is maintained by cellular buffering. The buffering power of a cell near its normal pH can range from 25-100mM/pH unit.[7] Long term regulation is normally the result of various active transport processes. The typical ion transporters for pH regulation are Na^+/H^+ exchangers, H^+-pumping ATPases, HCO_3^- transporters, and monocarboxylate-H^+ cotransport.[8]

1.2 Measuring pH in Biological Systems

Due to the importance of pH maintenance in cells, biomedical researchers are interested in monitoring cellular pH and must have the ability to measure pH accurately, reliably and in a reasonable amount of time. Many methods exist for sensing pH in a biological system, and a few of the more common methods utilize pH-sensitive electrodes, nuclear magnetic resonance (NMR), and fluorescence. With these techniques one can measure the pH of whole organs, tissue fragments, cell populations, single cells, and even intracellular organelles.[8]

There are two types of microelectrodes that are commonly used: glass-sensitive microelectrodes and liquid-ion exchange microelectrodes. Both types come in a variety of sizes, but differ in their selectivity, response time, and resistance. Measurements performed with microelectrodes allow for an immediate and precise measurement of the intracellular pH; however the cell must be impaled at least once to perform the measurement.[9] Many biologists use microelectrodes to measure the extracellular pH. When one uses a microelectrode to measure the intracellular pH, the cell membrane potential has to be taken into account since the electrochemical potential across the tip of the membrane is in series with the membrane potential of the cell. Thus, the membrane potential of the cell must be measured independently with a second microelectrode (potentially piercing the cell twice) or by using a microelectrode with a

double barreled tip, in which one barrel measures the intracellular pH and one measures the cell membrane potential.[10]

NMR is occasionally utilized for the determination of intracellular pH in intact animals as the technology is becoming more widely available. ^{31}P-NMR is typically used as phosphate is a relatively abundant species and can be titrated from $H_2PO_4^-$ to HPO_4^{2-} within the biological pH range. The shifts of the phosphate are typically compared to the creatine signal, 3.0 ppm, which does not change with pH. NMR determination of pH is hampered, however, by the lack of accurate calibration, long measurement times, and expensive equipment.[11]

1.3 Fluorescent Dyes

Fluorescence is widely considered most useful for studying intracellular pH because of its nondestructive nature, high sensitivity, and specificity. Many of the fluorescent dyes and indicators are cell permeant and do not have to be injected into a cell. Fluorescence is normally measured in the μM range. Additionally, only a small amount of the endogenous molecules that absorb light fluoresce, and two molecules that absorb at the same wavelength will probably not emit at the same wavelength and vice versa. Measurements via fluorescence can be made in any lab with a fluorometer; however, special optical configurations are needed to take advantage of the spectral properties of some dyes and high resolution microscopy still necessitates expensive equipment.[8]

A fluorescent pH probe usually binds protons directly with the binding event resulting in a change in the fluorescence of the molecule. Fluorescent probes belong to two classes. In the first class, binding of the analyte either turns the fluorescence of the molecule "on" or "off" resulting in an "on-off" sensor. This is the most common type of sensor. Members of this class

usually contain a derivative of fluorescein, anthracene, or pyrene as the chromophore. In the second class of sensors, binding of the analyte results in a shift in the absorption or the emission spectra. These sensors are capable of ratiometric measurement where the ratio of the two wavelengths is independent of the overall dye concentration, photobleaching, and changes in instrumental conditions such as optical path length, excitation, intensity, or detector sensitivity.[12]

The three best understood mechanisms of fluorescence response involve charge transfer, photoinduced electron transfer, and excimer/exciplex formation probes. For any of these responses to take place, probe design must include a fluorophore attached to an analyte-responsive receptor. In charge transfer probes, the fluorophore and the receptor are in direct electronic conjugation with the spacer being a π-system. The two subunits of the probes are chosen such that one subunit can act as a donor and the other subunit can act as an acceptor. Once the analyte of interest is recognized by the probe at the acceptor or donor subunit, charge flows from the donor to the acceptor. For photoinduced electron transfer systems, the receptor and the fluorophore are not in direct electronic communication as the spacer is typically a short alkyl chain. In the unbound state, when the molecule is excited, a fast electron transfer from the donor to the acceptor module quenches the fluorescence of the molecule. When the analyte is bound, it changes the redox potential of the molecule and thus electron transfer can no longer occur. This revives the fluorescence of the molecule. The opposite situation can also occur with the unbound state fluorescing and the bound state not capable of fluorescence. In contrast, excimer or exciplex sensors are made up of multiple flexible dyes usually connected by a short alkyl chain. These probes fold over upon recognizing the species of interest which results in strong intramolecular orbital overlap. The fluorescence emission wavelength undergoes pronounced shifts from the unbound to the bound state.[13]

The techniques used to incorporate an electrically charged sensor into a cell there are microinjection, scrape loading, hypertonic lysis, and carrier mediated endocytosis. If the molecule is small and uncharged another approach exists, namely simple diffusion across the cell membrane. Lipinski's "rule of five" states that for a molecule to be membrane permeable it needs to have no more than five hydrogen bond donors, no more than five hydrogen bond acceptors, a molecular weight less than 500, and a partition coefficient greater than five. If the molecule of interest disobeys only one of the rules, there is a good chance it will be able to cross the cell membranes. These rules were compiled to be used for drug design; however, they are broadly applicable to any membrane-permeable molecule one wishes to prepare. There are a few classes of drugs that don't necessarily follow these rules that can still permeate the cell membrane. These compounds belong to four different classes: antifungals, antibiotics, vitamins, and cardiac glycosides. Structural components of these compounds are recognized by naturally occurring transporters.[14]

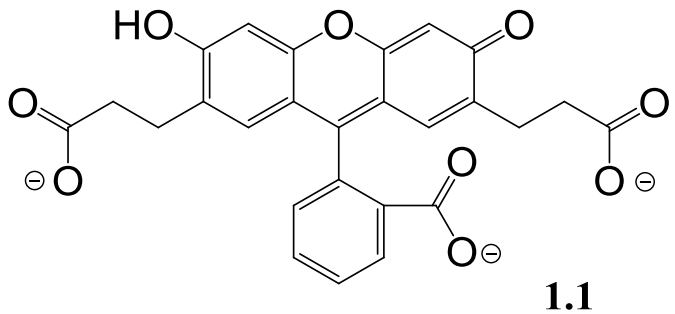
Many modern fluorescent sensors have electronic characteristics that change upon protonation, so the protonated (or deprotonated) sensor absorbs or emits at a different wavelength. Examples of such molecules include fluorescent pH sensors which are based upon carboxyfluorescein and carboxy-seminapthorodafluor derivatives. Both of these probes derive their pH dependence from a titratable side group attached to a fluorophore. The useful range of the sensors is largely determined by the pKa of the attached side group. These molecules enter the cell in one of two ways: either by microinjection or more commonly by passive diffusion while in a membrane-permeant, non-fluorescent form. Later cleavage in the cell by non-specific esterases yields the fluorescent form. Though both of these classes of sensors are very useful, they are relatively expensive.[8]

1.4 Common Types of Sensors

1.4.1 Fluorescein Derivatives

The most widely used are the carboxyfluorescein derivatives. BCECF (**1.1**) has a pKa of 7.0 and has an absorption maximum very close to the 488-nm argon-ion laser line. This absorption profile makes it ideal for flow cytometry and confocal microscopy applications. BCECF also has 4-5 negative charges at pH 7-8 which aids in cellular retention. Additionally, the acetoxymethyl ester derivative is membrane-permeant which allows for non-invasive bulk loading. The ester derivative is non-fluorescent and is efficiently converted to the fluorescent BCECF by intracellular esterases; this action can be used as an indicator of cellular viability. This probe also does not partition into any particular compartments in the cell and the ionic strengths of the surrounding solution does not have a large affect on spectral properties of the probe.[15]

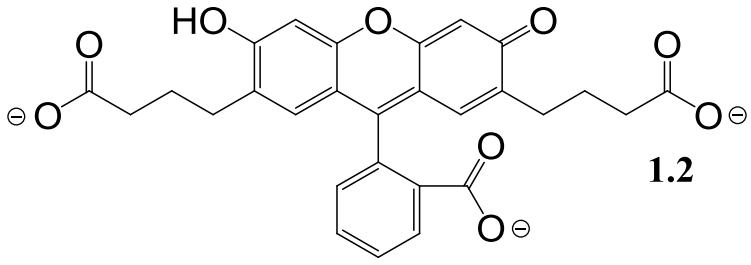
One of the drawbacks of this indicator is that is emission isosbestic point is quite far from the excitation wavelength, and consequently it displays poor signal to noise ratio. Additionally the pH-dependent changes in the spectra are relatively small so dual emission ratios are rarely performed. Also, the conversion from the ester derivative to the active derivative results in the formation of methanol and acetic acid which can be toxic to the cell and may produce abnormal cell events. Another downfall is that BCECF photobleaches fairly quickly. BCECF can also leak out of the cell by as much as 10% over 10-20 minutes at 25°C. This issue can be



1.1

avoided if a dextran conjugate is utilized as this conjugate has a much higher cell retention. However, the BCECF-dextran conjugate is not membrane permeable so it must be introduced using a more invasive method, like microinjection.[15] BCECF has been used in cell viability and cytotoxicity, apoptosis, adhesion, multidrug resistance, and chemotaxis studies.[16]

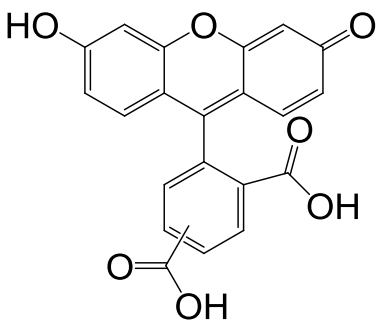
One of the processes used to calibrate BCECF in cells is the high potassium/nigericin clamp protocol of Thomas et al. During this process the internal pH of the cells is set to known values by exposing the cell to nigericin, a K^+/H^+ transporter, in the presence of potassium solutions with known pHs. With the transporter present, the potassium concentrations and the H^+ concentrations equilibrate such that the concentration on the inside of the cell is the same as the concentration on the outside. By clamping the cells between 6.4 and 7.8, the pH values where BCECF changes its fluorescence intensity, a linear relationship is produced. With this graph, one can then convert the experimentally determined ratios to pH values.[17]



1.2

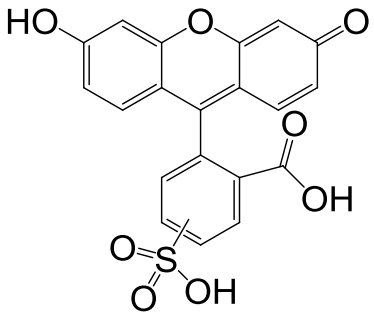
There are many other probes that use the same fluorescein unit as BCECF. A well-known example is BCPCF (2',7'-bis-(2-carboxypropyl)-5-(and-6-)-carboxyfluorescein), a homolog of BCECF with 2-carboxypropyl substituent's at the 2'- and 7'-xanthene positions instead of 2-carboxy groups. The two probes have similar pKa's, absorption and emission profiles, and quantum yields. BCPCF (**1.2**) is a better ratiometric dual excitation probe than BCPCF. BCPCF has the same major flaw as BCECF, however. The fluorescence emission of both the probes is concentration dependent so if the probes congregate

in a certain area of the cell they will indicate internal pH that are based on the concentration of the dye and not on proton concentration.[15]



5(6)-carboxyfluorescence (**1.3**)

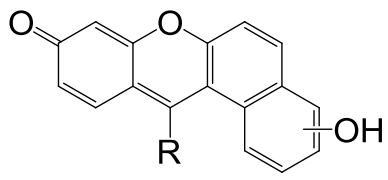
Even thought they are decades old, fluorescein (**1.8**), and especially 5(6)-carboxyfluorescein (**1.3**) and 5(6)-sulfofluorescein (**1.4**) are still used for pH measurements as these probes are cheap and easy to prepare. They are not as widely used as BCPCF or BCECF, however. Fluoresceinsulfonic acid is highly water-soluble and well retained within the cell, but it is membrane impermeant. This feature makes it useful for determinations of barrier permeability. 5(6)-Carboxyfluorescein and 5(6)-sulfofluorescein both have a pKa of ~6.5 and can be used as esters making them membrane permeant. These probes tend to leak out of the cell, however, and 30-40% of the concentration can diminish in the first 10 minutes of washing.[15]



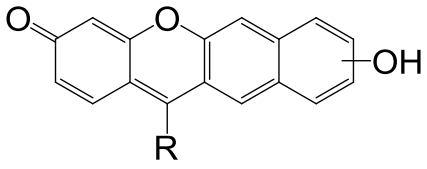
5(6)-sulfofluorescein (**1.4**)

1.4.2 Benzoxanthene Derivatives

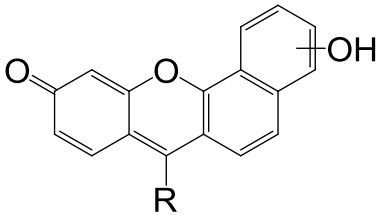
Another widely used class of fluorophores for pH measurements are the benzoxanthene dyes. Of this class there are three main subunits: the SNAFLs (**1.11**), SNAFRs (**1.12**), and the SNARFs (**1.13**). The SNA portion of the acronyms stands for "seminaptho". The SNAFRs do not possess the carboxy substituent at the 3' position (making them fluororones), the SNALFs are derived from fluorescein derivatives and the SNARFs are derived rhodafluoresceins. SNARF is a long-wavelength fluorescent probe that undergoes a pH-based fluorescent shift. This shift allows researchers to measure the ratio of the two wavelengths in order to obtain a more accurate pH reading. Both calcium ion concentrations and pH have been measured simultaneously using this



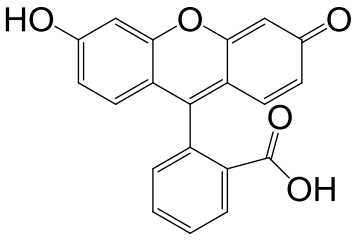
Benzo[a]xanthene (**1.5**)



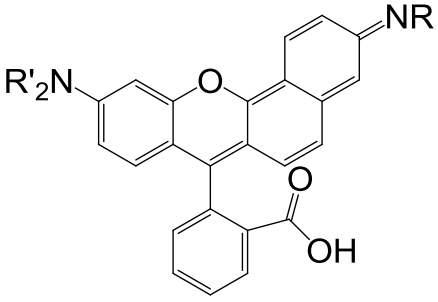
Benzo[b]xanthene (**1.6**)



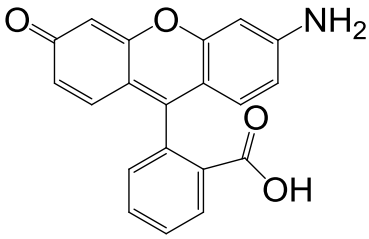
Benzo[c]xanthene (**1.7**)



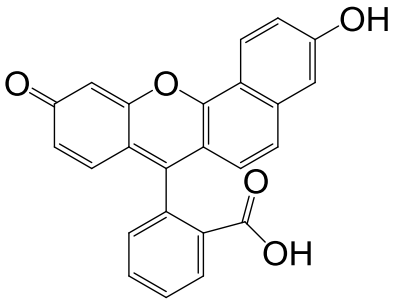
Fluorescein (**1.8**)



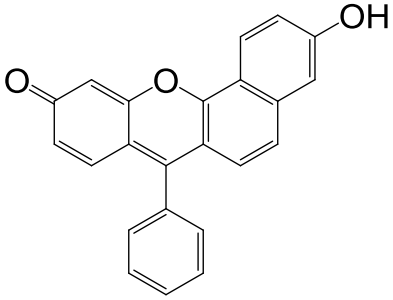
Rhodamine (**1.9**)



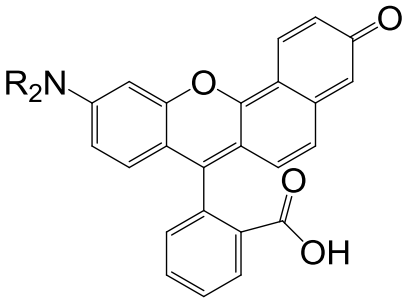
Rhodol (**1.10**)



SNAFL Core (**1.11**)



SNAFR Core (**1.12**)



SNARF Core (**1.13**)

indicator.[15] Synthetic modification of the aryl rings of SNARF is possible, and the fluorinated versions of the probe provide sensitivity at a lower pH. A tetrafluorinated version has a pKa of 6.4 and while the F_5 derivative has a pKa of 7.2. Both of these receptors maintain dual emission.[12]

Carboxy-SNARF(C.SNARF-1) is the second most widely used pH indicator after the BCECF probe. C.SNARF-1 has been used to determine absolute cytosolic, mitochondrial, and nuclear pH values in living cells using flow cytometry, mircoplate readers, confocal imaging, and microspectralfluorometry. While the fluorescent, charged form of the probe cannot enter the cell, it may be masked as an ester, like BCECF, and permeate the cell membrane to later be cleaved by cellular esterases yielding the desired form of the molecule. The ratiometric properties of C.SNARF-1 are not dependent on its concentration or the ionic strength of the surrounding medium and are not susceptible to photobleaching, which are desirable features for a multi-use pH indicator.[15] This probe also has a longer excitation wavelength than many other probes. This longer wavelength aids in the reduction of cell damage due to radiation and also avoids some of the problems associated with intracellular autofluorescence.

Unfortunately C.SNARF-1 has a low quantum yield and its high pKa makes the measurement of pH values under 7 problematic. Furthermore, the spectral properties of the probe are altered by the internal temperatures and environments of the cells. A temperature increase from 25 to 37^{o}C results in a quantum yield decrease of 25%. Interactions with proteins within the cell can cause further dimunition of its fluorescence.[15]

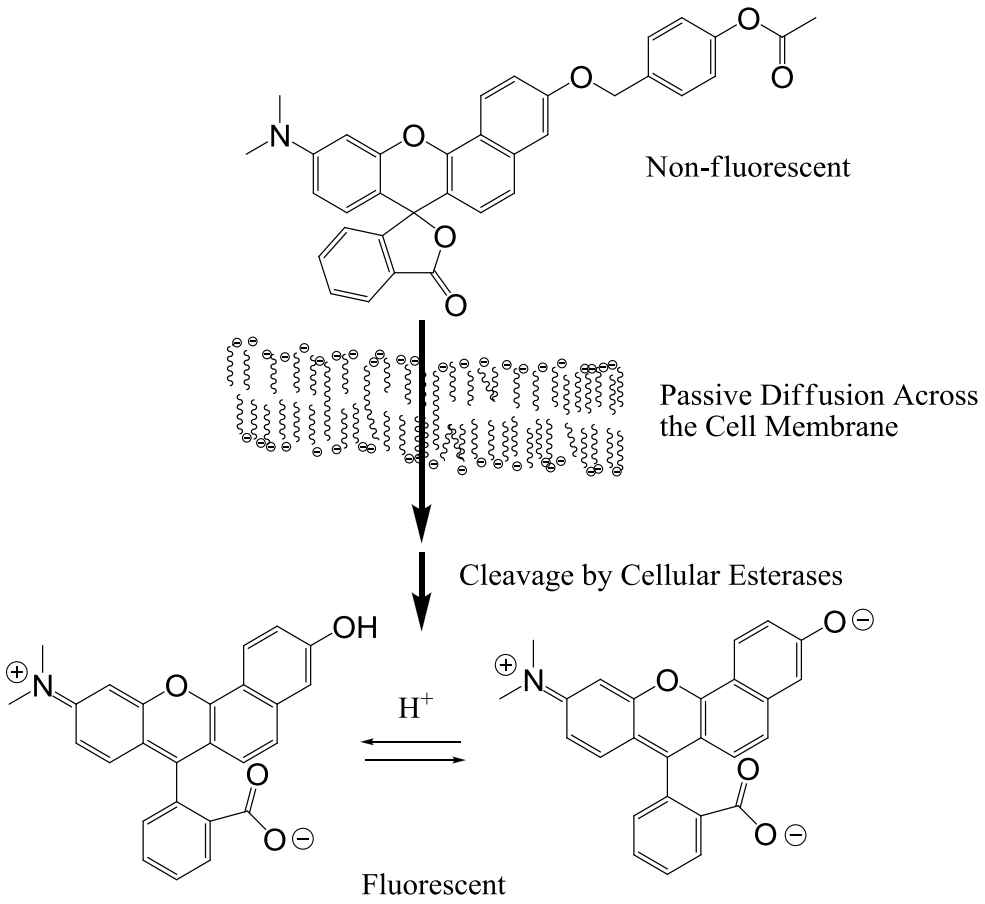


Figure 1.1: Depiction of UTX-40 permeating a cell membrane to be cleaved by cellular esterases[18]

Recently, Nakatao and coworkers produced a cell-permeable SNARF derivative. This new derivative has decreased background fluorescence as it forms nonemissive aggregates in aqueous media. After hydrolysis, the aggregates become diffused and the now monomeric SNARF displays its fluorescent properties.[18] Figure 1.1 shows this SNARF derivative, UTX-40, crossing the cell membrane and then cleavage by cellular esterases to yield the fluorescent form.

1.4.3 Pyrenes, Napthalenes, and "BODIPYs"

Polysulfonation of simple pyrenes can give rise to compounds like 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS). This compound is highly water-soluble and has low toxicity.

HPTS is capable of being used for ratio imaging as it has two absorbance maxima which decrease or increase as the pH varies from 5-8. This probe also has a high cellular retention as it can have 3 or 4 negative charges in the physiological pH range. A very significant limitation to the use of HPTS is that it is not cell permeable and cannot be readily masked with protecting groups that might aid its passive diffusion across the cell membrane. This leaves only more invasive methods of inserting HPTS into the cell like microinjection, electroporation, and scrape loading.[15]

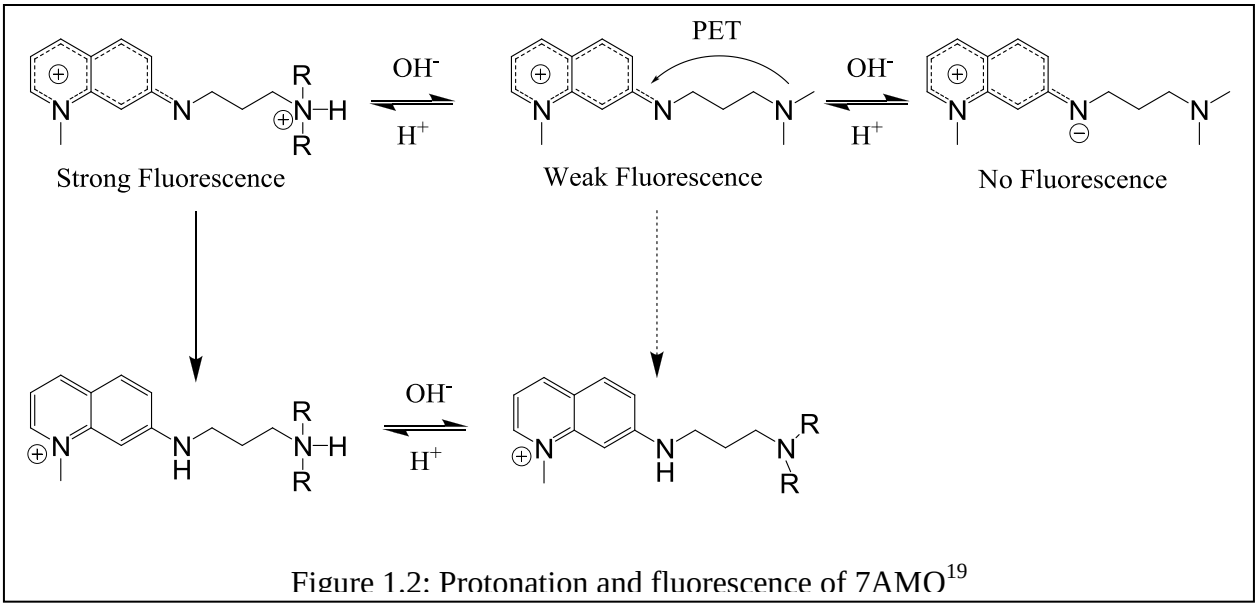
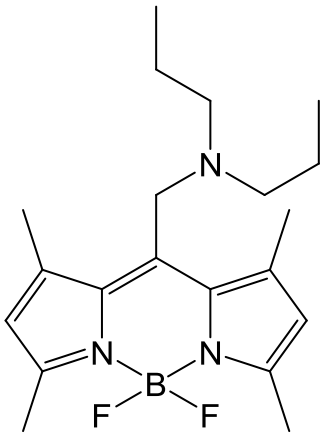


Figure 1.2: Protonation and fluorescence of 7AMQ[19]

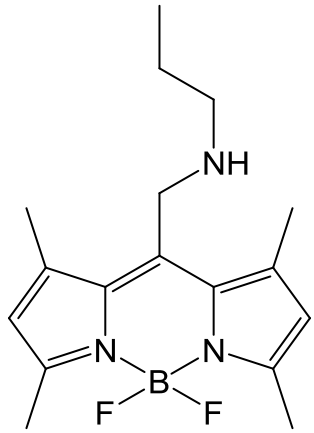
Recently, a group of highly sensitive water-soluble fluorescent pH sensors based on 7-amino-1-methylquinolinium (7AMQ) was reported by Jager et. al.[19] These sensors feature a peripheral alkyl amino moiety that makes photoinduced electron transfer (PET) possible. Behavior of the molecules was found to lie between those of a ratiometric sensor and a "light-up" sensor. 7AMQ has a high fluorescent yield when fully protonated ($\phi_F = 0.85$), and a fluorescence lifetime of up to 13 ns. At low pH the absorption shifts from 414 nm to 401 nm and

the emission shifts from 517 nm to 490 nm. The enhancement in emissive quantum yield (ϕ_F = 0.05 to ϕ_F = 0.85) is caused by a PET from the receptor nitrogen to the chromophore. The various substituents on the nitrogen also have a direct effect on the fluorescence, either enhancing it or detracting from it. At high pH values the fluorescence was also quenched by OH^- ions.[19]



1.14

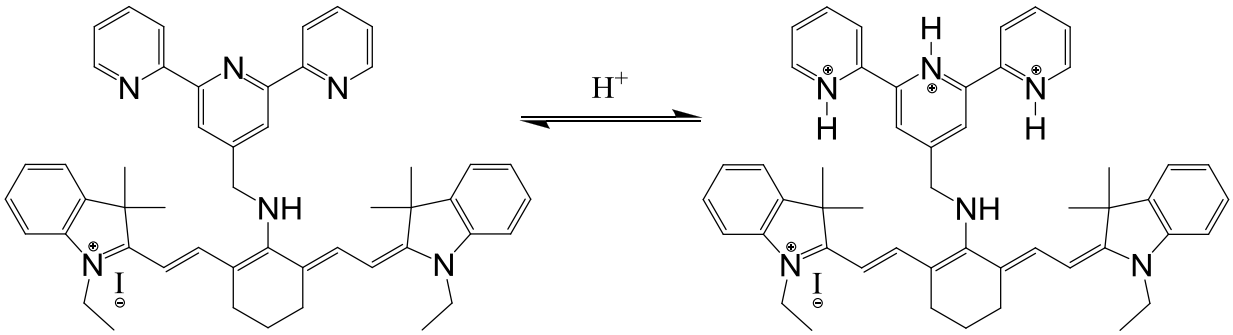
Organic and inorganic chemistry come together in the 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) fluorescent dyes (**1.14** and **1.15**). These dyes have high absorption coefficients, high fluorescent quantum yields, and long wavelength emission. They are also photochemically stable and insensitive to oxygen content. The quenching mechanism of this probe involves PET from either propylamine or dipropylamine. Protonation of these sites stops the electron transfer from occurring and turns on the fluorescence on the probe. Two of these dyes were synthesized by Tian et al. Their excitation maxima are 493-503 nm and their emission maxima was 506-515 nm depending on the solvent. The pKa's of the probes were found to be 7.75 and 7.38 for probe **1.14** and probe **1.15** respectively.[20]



1.15

A new sensor based using tricarbocyanine (Cy) as a fluorophore and 2,2':6'2"-terpyridine (Tpy) as a protonation site was reported by Tang et al. The probe, Tpy-Cy, has a pKa of about 7.1 and is able to respond to pH fluctuations within the pH range of 6.70-7.90. Tpy-Cy's excitation wavelength is 648 nm and its emission wavelength is 750 nm. The fluorescence of

Scheme 1.1: Protonation of Tpy-Cy[1]



Tpy-Cy is quenched by PET between the receptor and the fluorophore. When one or more of the pyridyl N atoms become protonated this transfer can no longer occur, so the fluorescence is switched on. The probe has high sensitivity, good photostability, excellent cell membrane permeability, and avoids the influence of autofluorescence in biological systems. Real-time imaging of cellular pH changes was performed using this probe in living HepG2 and HL-7702 cells, which are cancerous human liver cells and normal human liver cells, respectively.[1]

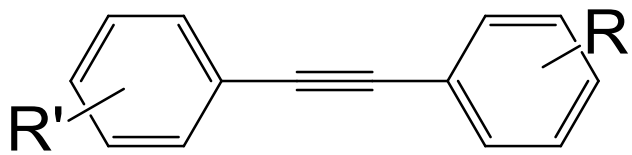
To develop a low-molecular weight, electrically-neutral (cell-permeant) pH sensor, we have chosen to use a diarylacetylene scaffold in which the protonation site is a component of the main chromophore. The subunits of the probe are such that one unit can act as an electron acceptor and the other as the donor. Thus, the modules are capable of undergoing intramolecular charge transfer.[3] Previous work in our group has established that diarylacetylene systems display ratiometric behavior in organic solution. In these cases the probes posses two different emission wavelengths, with emission at one wavelength increasing and the other wavelength in decreasing upon the addition of the analyte (H^+). In principle, once a standard curve is generated, one can calculate the pH by measuring the ratio of the intensities. Ratiometric fluorescent probes have a distinct advantage over probes that display only increased (or decreased) intensity: namely, the

response of ratiometric probes are not concentration-dependent. This feature is very useful for cell studies as it can be difficult to get probes to cross the cell membrane in high concentration. Errors resulting from leakage, photobleaching, and nonuniform distributions can also easily be corrected when using a ratiometric probe.[21] The following chapter with describe the photophysical properties of diarylacetylenes in general and our efforts to incorporate them into amphiphilic H^+ sensors.

CHAPTER 2: DESIGNED SENSORS

2.1 Introduction

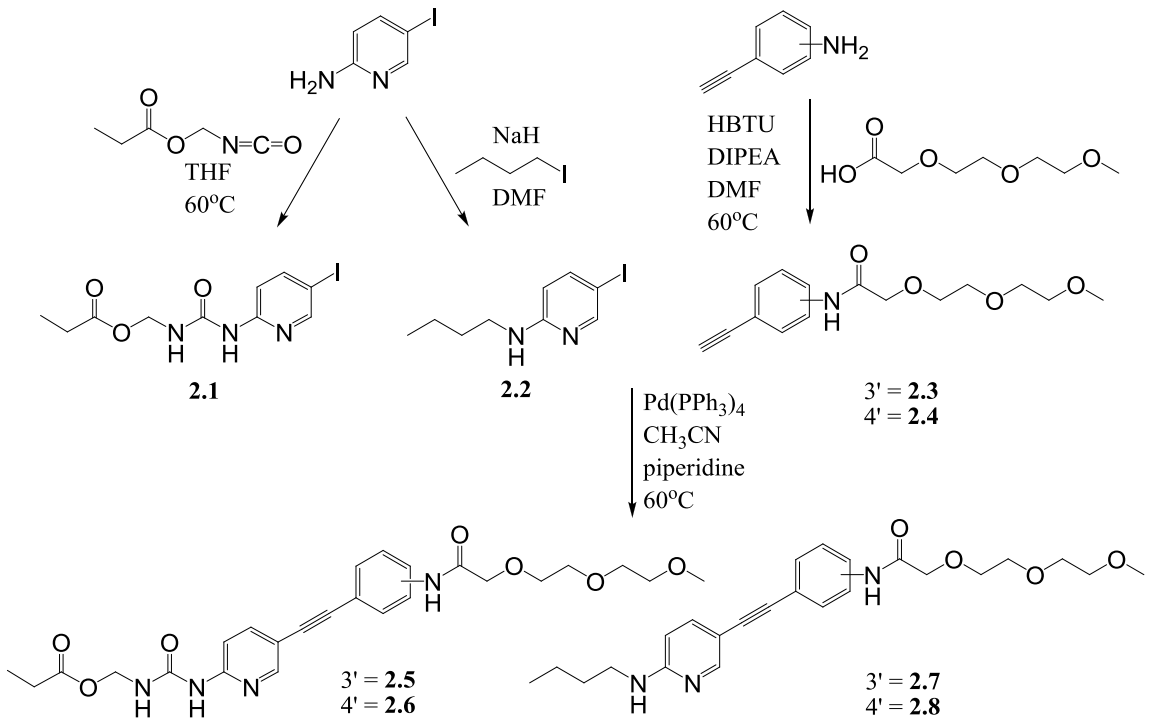
To produce a library of sensors with different electronics, we decided to use a diarylacetylene scaffold (see below). Diarylacetylenes are small and easily decorated with electron acceptor and the donor moieties. Conformational restriction about the central alkyne can lead to moderately intense luminescence (Φ>0.1).



2.2 Synthesis

Scheme 2.1 shows the overarching synthesis of this project**.** Intermediate **2.1** was produced by coupling a commercially available isocyanate with a commercially available iodopyridine. The exocyclic amine of the pyridine was deprotonated with NaH in order to enhance nucleophilic attack upon the 1-iodobutane to form **2.2**. Two isomeric ethynylanilines

Scheme 2.1: Synthesis of the Sensors



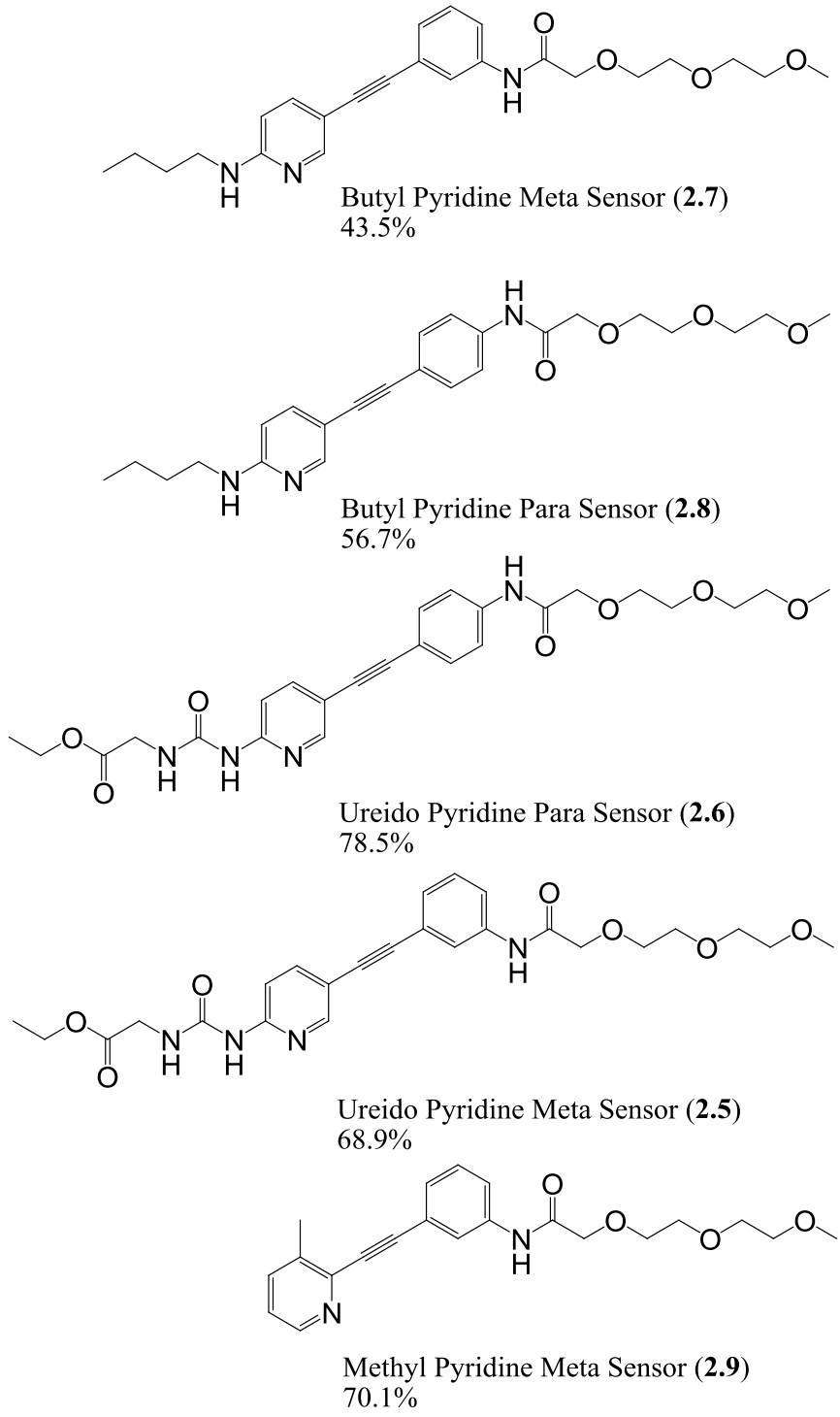


Figure 2.1: Prepared Sensors

were coupled with a PEG-carboxylic acid under typical peptide synthesis conditions to produce **2.3** and **2.4**. The PEG (polyethylene glycol) was added to aid in water-solublizing the probes. Next, the decorated pyridines were appended to the water-solublizing side of the sensors using Cu-free Sonogashira conditions to produce **2.5**, **2.6**, **2.7** and **2.8**.

The completed receptors and their synthesis yields are shown in Figure 2.1. They will be referred to by both their shortened names and their respective numbers throughout the rest of this thesis.

2.3 Fluorescence

The meta-substituted probes were significantly more fluorescent that the para isomers. Table 2.1 shows the electronic absorption data and relative emission intensities of **2.5-2.9**.

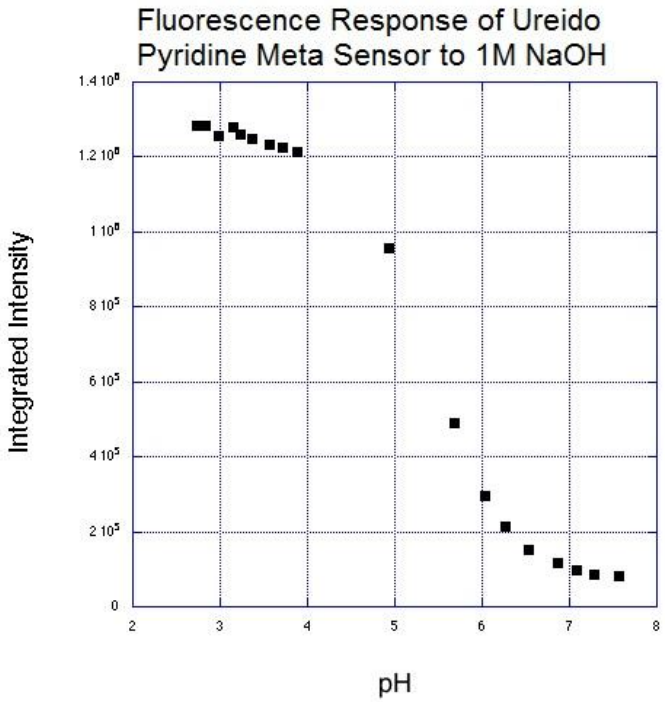
Table 2.1: Electronic absorption and emission data for the prepared sensors

Sensor	λ abs	Abs.	Conc.	ε	λ emiss	relative intensity
2.7	328	0.0520	2.06 µM	29000	406	1
2.5	324	0.0327	3.812 µM	8600	360	0.458
2.8	329	0.0639	1.976 µM	32000	420	0.228
2.6	330	0.0809	2.006 µM	40000	360	0.303
2.9	295	0.0309	2.172 µM	14000	n/a	n/a

Fluorescence titrations were performed on all the sensors. The titrations were carried out in aqueous 40 mM HEPES buffer with 0.1 M NaCl to keep a constant ionic strength throughout the experiments. The titrations were performed first from low pH to high pH and then from high pH to low pH to check the reversibility of the reaction. Each titration was carried out with a fresh solution. For the titrations starting out at low pH, the solutions were treated with 1M NaOH. After each aliquot was added the pH was checked using a pH meter. A fluorescence spectrum was then acquired and more base was titrated in. These steps were repeated until a pH higher than 8.0 was reached. The titrations from high pH to low pH were performed in a similar fashion using 1M HCl.

For uriedo systems **2.5** and **2.6** when the species of interest, H^+, is in high concentration, the fluorescence is quenched. While the ureido sensors were clearly luminescent in the biological pH range, their fluorescence did not *change* in this range. The emission changes for the ureido sensors only occurred in the pH range of 2-5. The titrations from low pH to high pH

and from high to low were very similar and this similarity in the data shows that the process is reversible. The fluorescence response for **2.5** is shown in Figures 2.2 and 2.3.



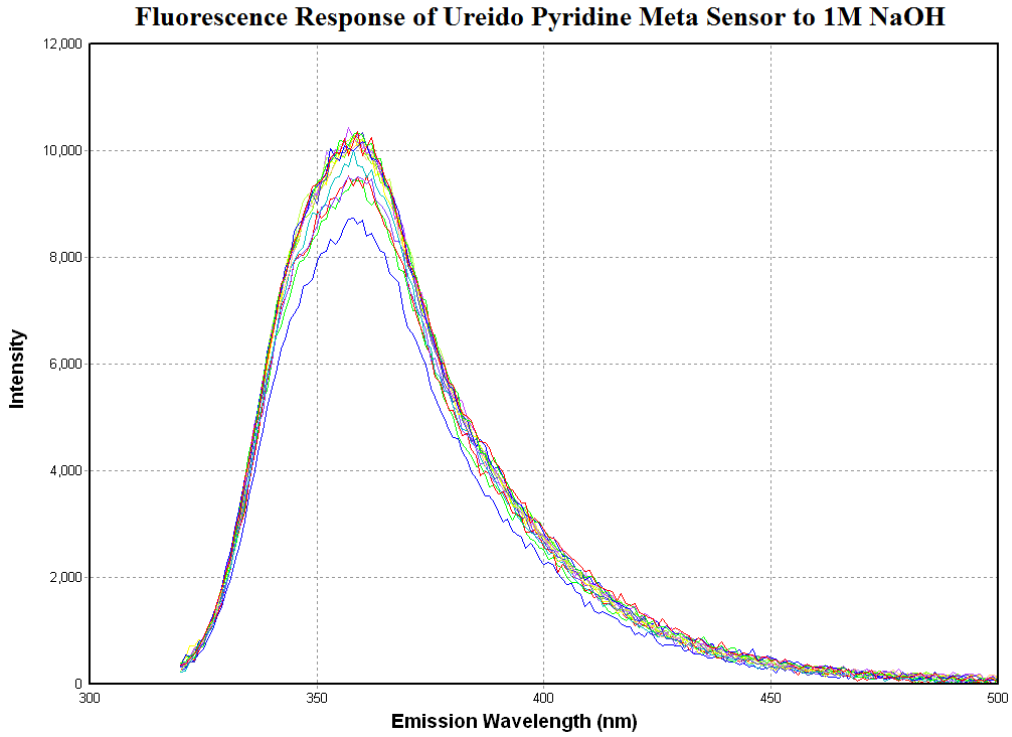
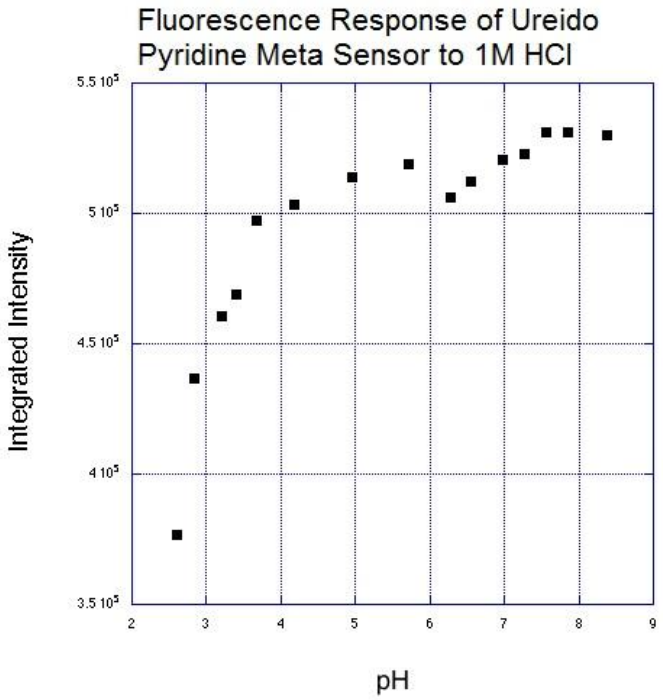


Figure 2.2: Fluorescence Response for **2.5**



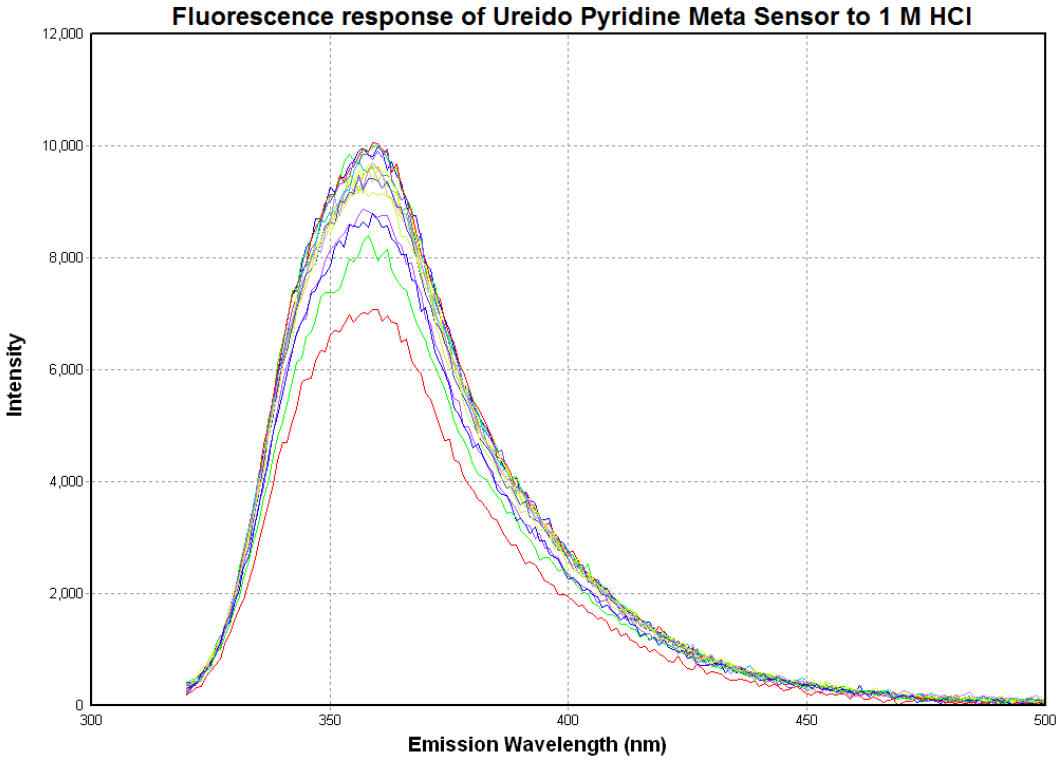
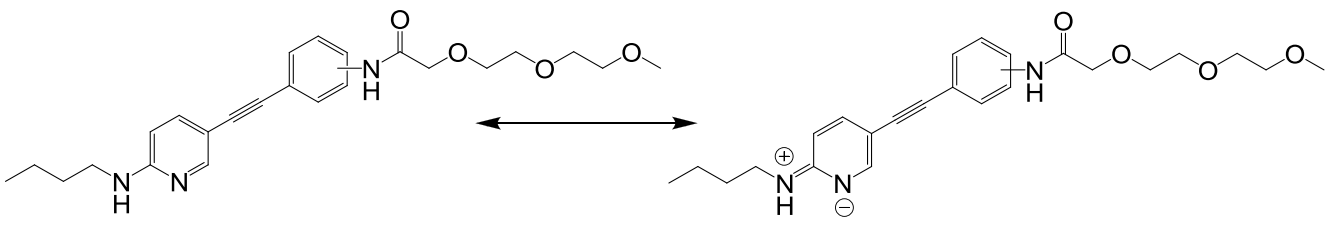


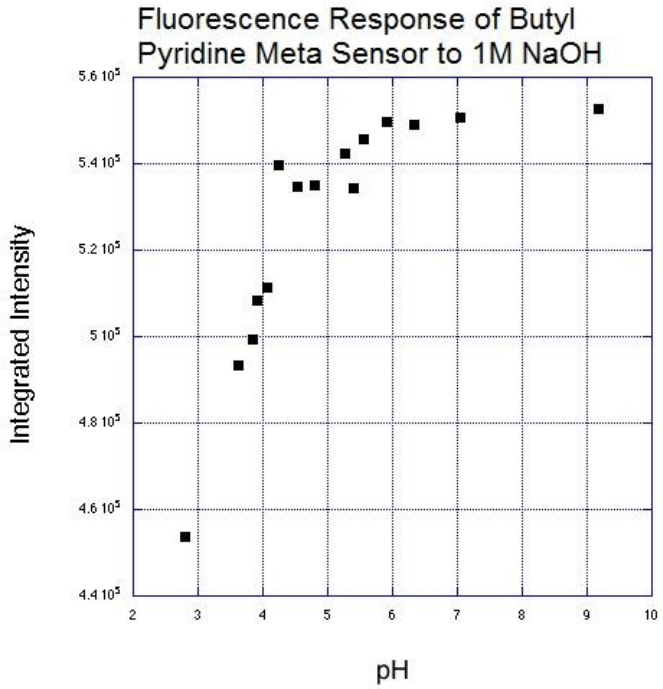
Figure 2.3: Fluorescence response of 2.5 to 1M HCl

In an effort to shift the pKa of the sensors toward higher (biologically relevant) values, the exocyclic amine was alkylated. The low nucleophilicity of the amine rendered mild alkylation conditions (e.g., K_2CO_3/acetone) ineffective. This low nucleophilicity is presumably caused by the exocyclic amine being in direct electronic conjugation with the nitrogen of the pyridine ring.

In the butyl sensors, when $[H^+]$ increased, the fluorescence emission was intensified. This finding is consistent with an internal charge-transfer process in which the pyridine unit acts as an electron acceptor. Protonation at this site would serve to stabilize a forming negative charge. Furthermore, the pKa of the sensor was shifted from 3.5 to 5.5 which is closer to the biological pH range. The change in the pKa may be explained by the electronic analysis in Scheme 2.2. While the charge-separated resonance contributor is significant when the exocyclic amine is alkylated, it is not favored when the amine is part of a urea. The fluorescence experiments produced the same "s-shaped" response curves characteristic of acid/base titrations regardless of whether the titration was begun from high pH or low pH. The fluorescence data for **2.7** is shown in Figures 2.4 and 2.5. The fluorescence spectra for **2.8**, the Butyl Pyridine Para Sensor, is shown in Figure 2.6.



Scheme 2.2: Resonance contributor for the butyl sensors



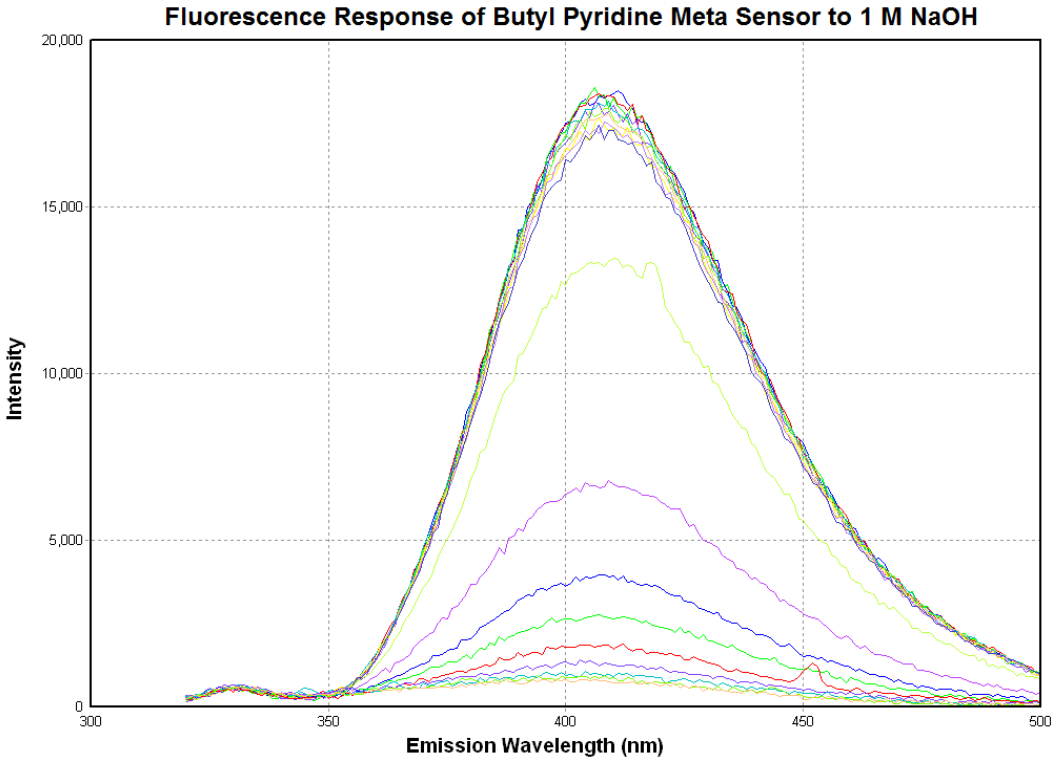
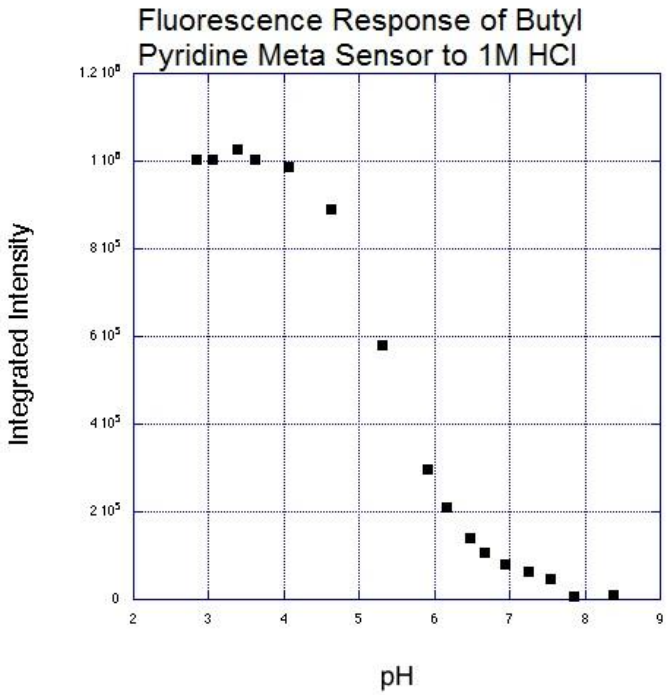


Figure 2.4: Fluorescence response for **2.7**



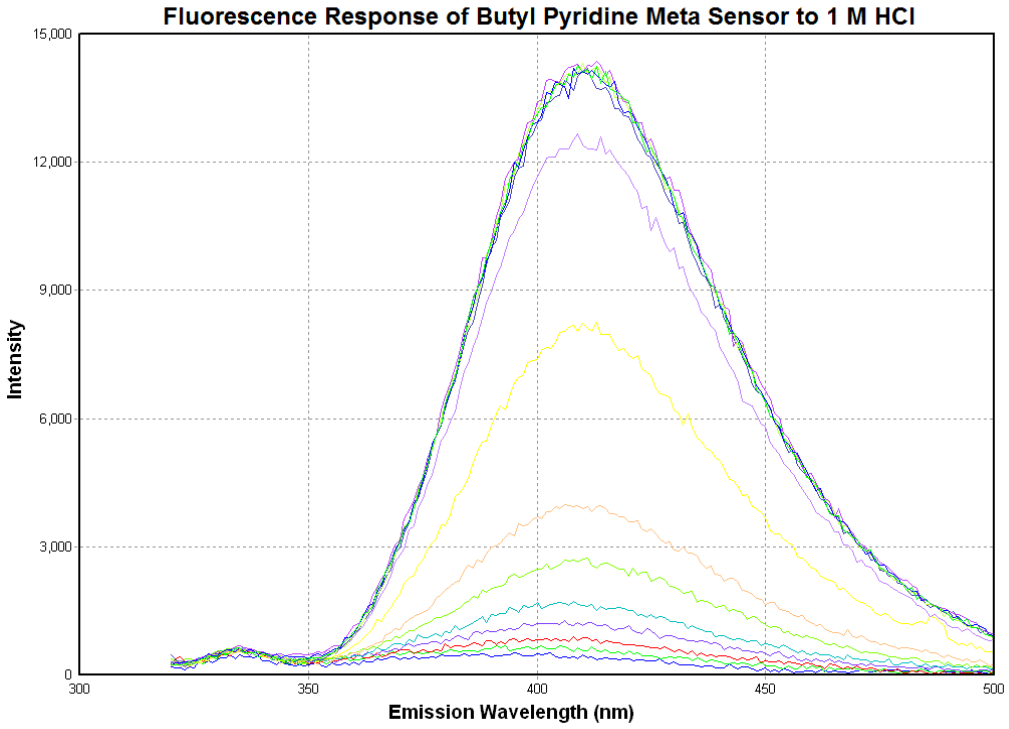
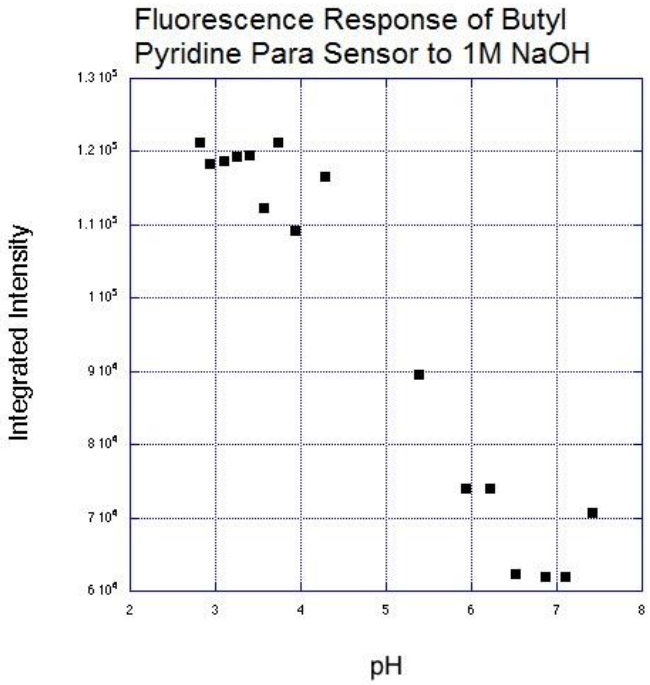


Figure 2.5: Fluorescence response of **2.7** to 1M HCl



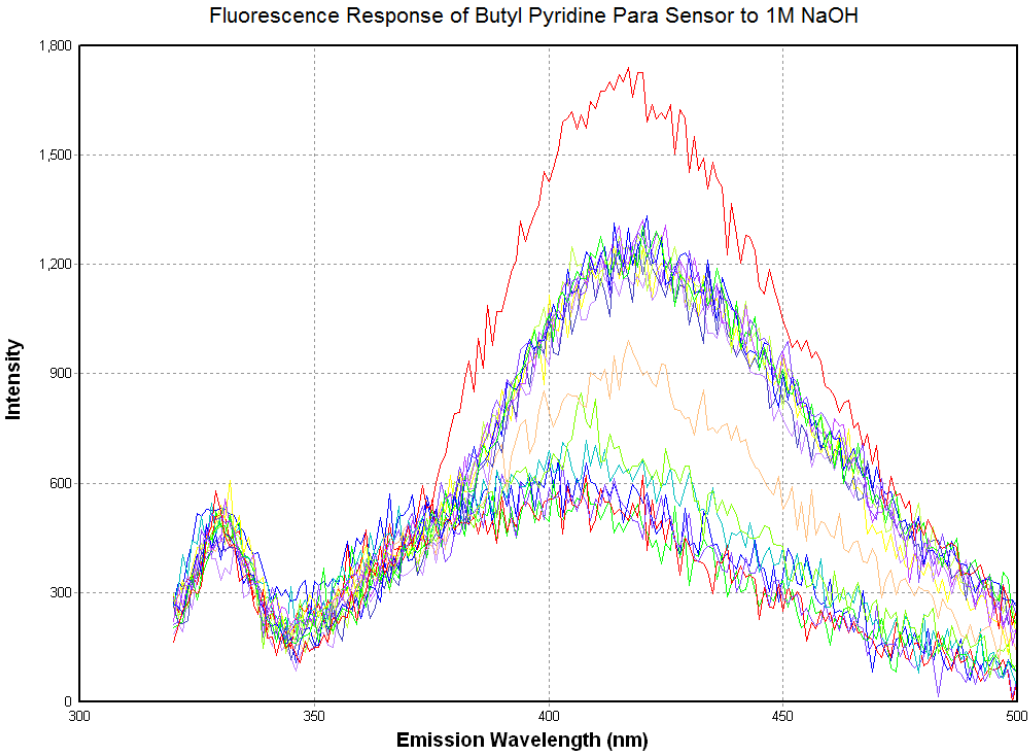
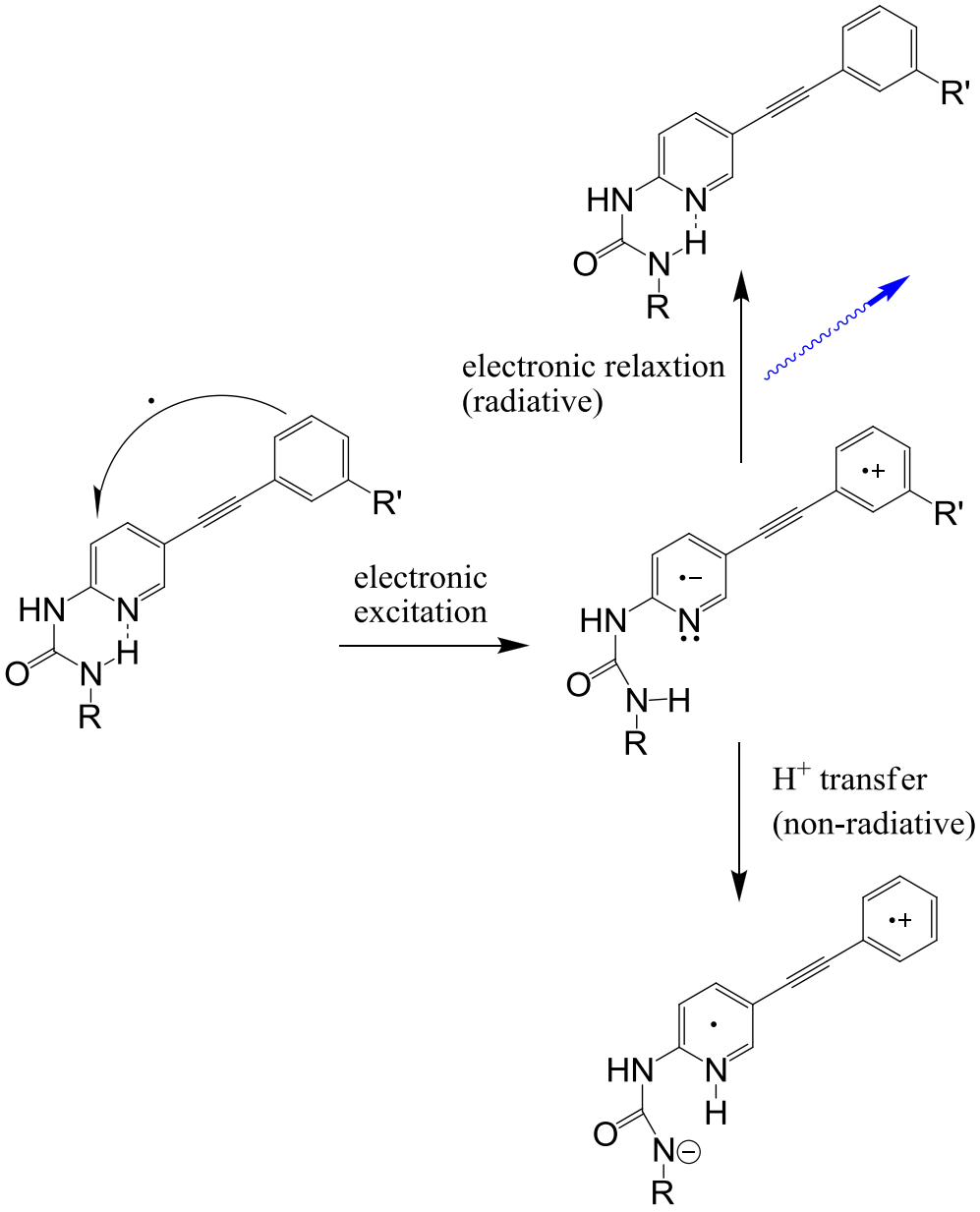


Figure 2.6: Fluorescence response of **2.8** to 1M NaOH



Scheme 2.3: Excited-state proton transfer of the ureido sensors

Additionally, the ureido sensors were not as fluorescent as the butyl sensors proved to be. 2-Pyridyl ureas are known to adopt an intramolecularly H-bonded conformation as shown in Scheme 2.3. Charge transfer to the pyridine would serve to enhance its basicity, making migration of H^+ from the urea moiety more likely. Thus, ureido sensors have an additional non-radiative pathway accessible to them that is not available to the butylated systems.

The next step to produce a sensor that would provide useful data over a biological pH range was to incorporate imidazole in place of pyridine. Imidazole has a pKa of 6.99 while pyridine has a pKa of 5.21. Unfortunately, after exhaustive synthetic attempts, a probe with imidazole for the site of protonation was not forthcoming.

2.4 NMR Titrations

^{1}H NMR titrations were performed with all the sensors, by treating millimolar solutions with methanesulfonic acid or with tetrabutylammonium hydroxide. These experiments were conducted in 1:3 CD_3CN:D_2O. In the base titrations, there was not an appreciable shift of the hydrogens. The acid titrations, however, demonstrated that for both the ureido and the butyl sensors the site of protonation was the pyridyl nitrogen. This process is characterized by downfield shifts in most of the pyridine hydrogens indicating that the protons have become more deshielded.

A sensor with a weakly electron donating group on the pyridine (-CH_3) was also prepared (**2.9**). Unfortunately, it was not fluorescent. The NMR titrations gave similar results as the other pyridine probes, however, with the protonation occurring at the pyridyl nitrogen. The NMR data for **2.8**, **2.9**, and **2.6** is given in Figures 2.9, 2.10, 2.11, and 2.12.

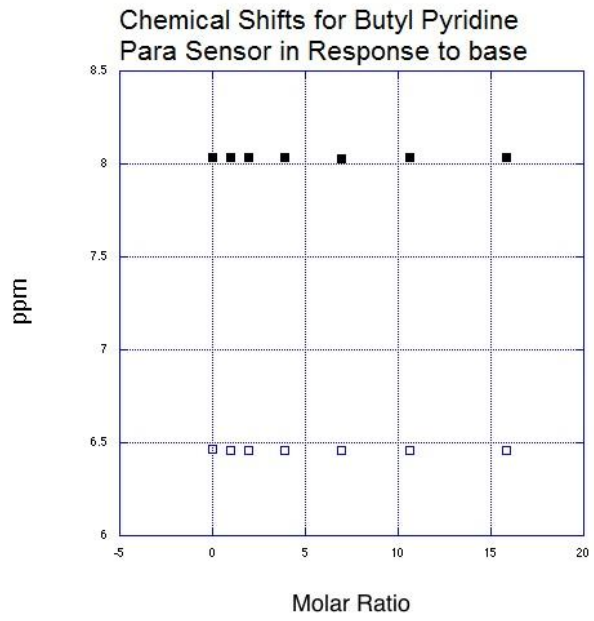


Figure 2.7: Chemical shifts of **2.8** in response to base

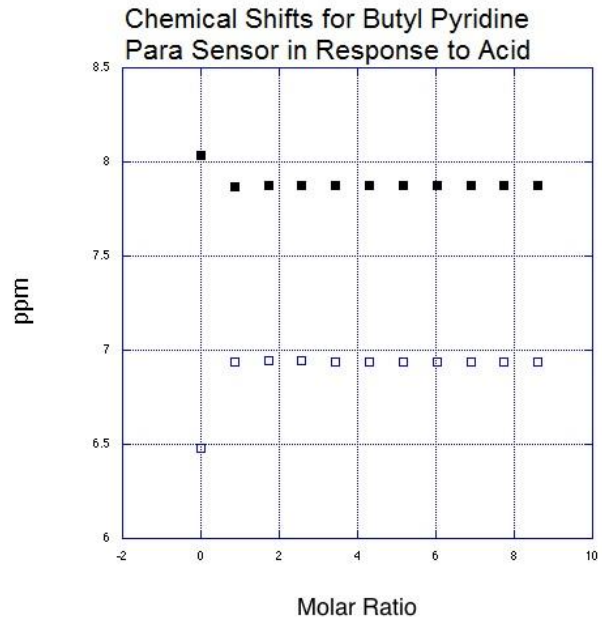


Figure 2.8: Chemical shifts for **2.8** in response to acid

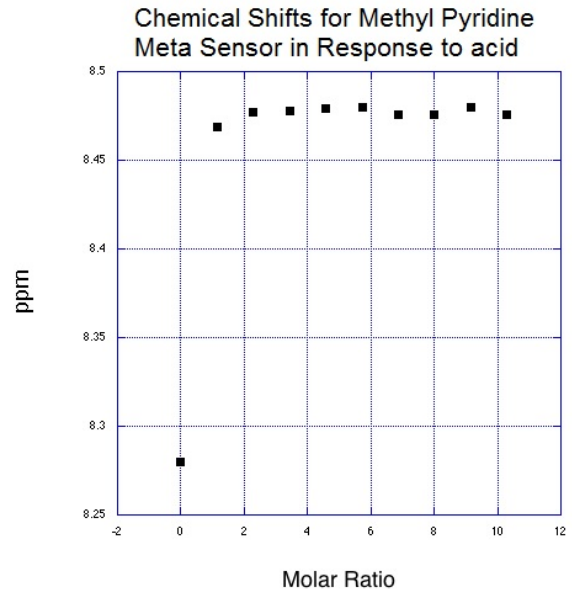


Figure 2.9: Chemical shifts of **2.9** in response to acid

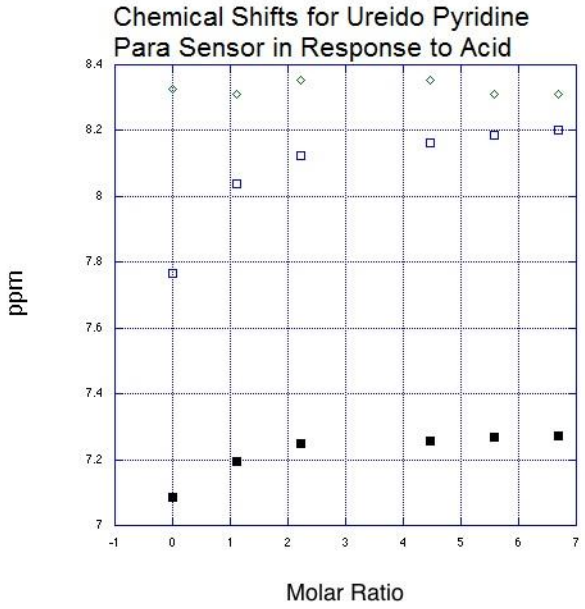


Figure 2.10: Chemical shifts for **2.6** in response to acid

2.5 Model Membrane Partitioning

Simulated cell membrane permeability studies were also performed, using a simple "U-tube" apparatus (Figure 2.11). An organic solvent (10 mL) was placed in the bottom of the tube

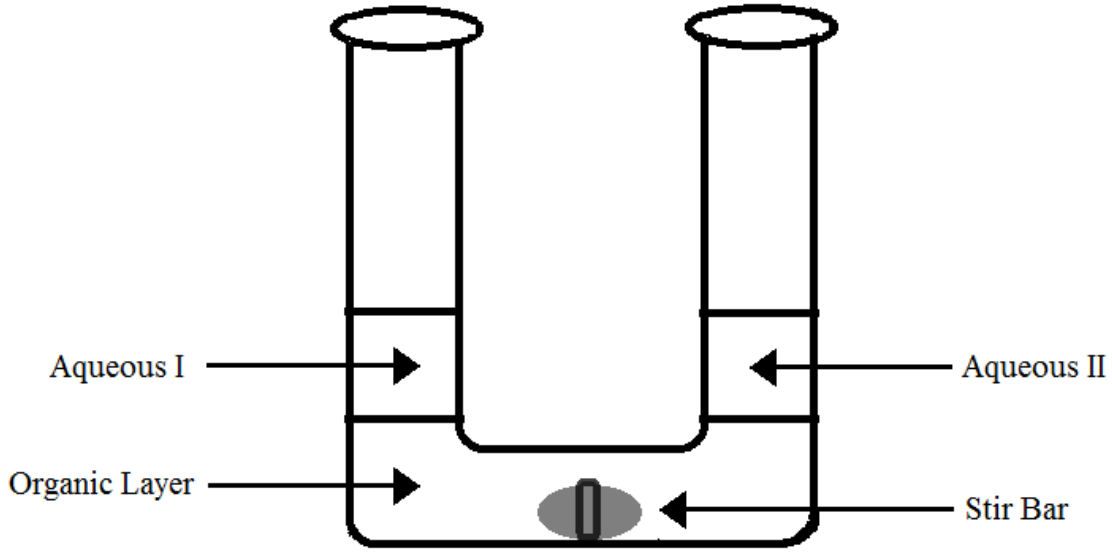


Figure 2.11: Diagram of the U-shaped tube used in the simulated cell membrane studies

with 5 mL of buffered solution floated on either side. The organic layer was gently stirred throughout, and samples were taken from each aqueous side and monitored via UV/vis. In the first attempt, both aqueous layers had a pH of 8.4 and dichloromethane was used as the organic layer. In the second attempt aqueous I had a pH of 8.4, and aqueous II had a pH of 5.9, and dichloromethane was again used as the organic layer. In the third attempt the pH of aqueous I was again 8.4, and aqueous II was 5.9, and the organic layer was dichloromethane with 30% octanol present. Both the ureido and the butyl sensors did partition into the organic layer, however, no evidence for the appearance of the dye into aqueous II was obtained. In addition,

the modifications to the solvents did not dramatically change the rate the sensor partitioned into the organic layer. The fact that the sensors never transferred into aqueous II suggests that modifications should be made to the sensors to make them more water-soluble or less organic

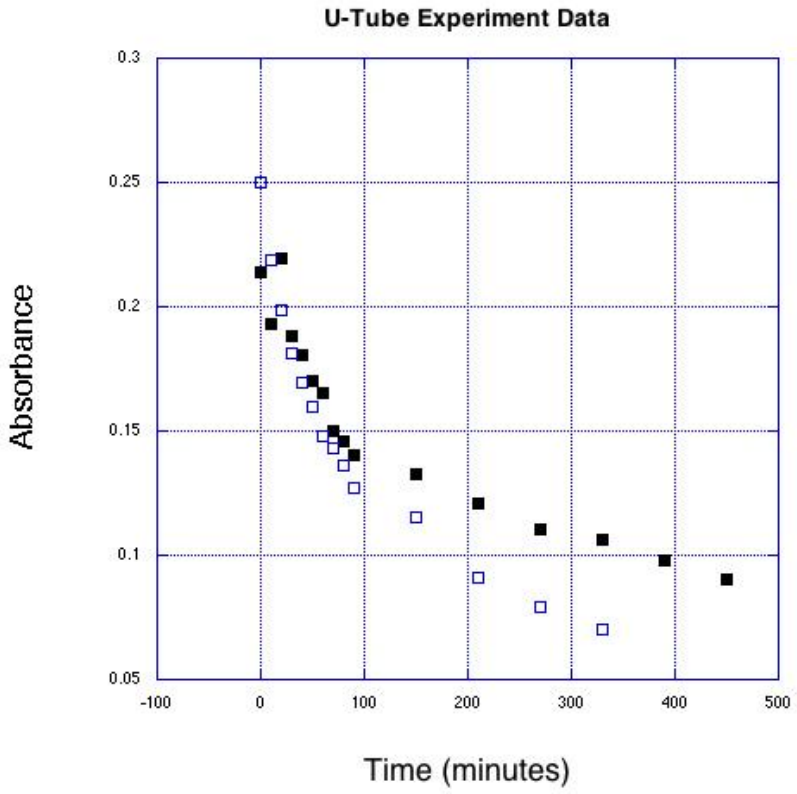


Figure 2.12: This graph represents the partitioning of **2.7** into the organic layer from aqueous I. The closed black squares represent the experiment done in which 30% octanol was present in the organic layer and the open blue squares represent the experiment in which the organic layer was pure dichloromethane. In both experiments, aqueous I had a pH of 8.4 and aqueous II had a pH of 5.9.

soluble. Figure 2.12 shows the rates of diffusion from aqueous I into the organic layer for the studies done with two different pHs.

2.6 Conclusions

In conclusion, fluorescence titrations on the ureido-based receptors showed that they do not respond in the biological pH range. In order to develop sensors that would be more useful, alkylation of the exocyclic amine of the pyridine ring was performed. The pKa of the butylamino products is estimated to be 5.5, which is still outside of the useful range for the cytosol. Interestingly, the emission of the ureido receptor class is quenched when the species of interest was in high concentration, while the butylamino receptor displays an increase in fluorescence under similar conditions. Also, the location of the amido-PEG solubilizing group on the benzene ring (i.e.,para vs meta) had a pronounced effect on the fluorescence intensity. Para-amides display weak fluorescence, whereas meta-amides are highly fluorescent.

The butylamino probes are a new class of small, photostable, water-soluble fluorescent pH probes. While the pKa of the probes is outside of the pH range of the cytosol, it is in the pH range of some of the acidic organelles (i.e. lysosomes and endosomes). These new sensors could prove useful for examining pH fluctuations in these organelles. Future work on this class of sensors will focus on further raising the operational pKa of the probes and enhancing their membrane transport properties.

2.7 Experimental

Ethyl 2-(3-(5-iodopyridin-2-yl)ureido)acetate (2.1). 2-Amino-5-iodopyridine (0.3404 g, 1.547 mmol) and ethyl isocyanatoacetate (0.2460 g, 1.857 mmol) were combined in a 50 mL round bottom flask containing 10 mL of anhydrous THF. The solution was allowed to reflux under nitrogen for 48 hours. The solution was cooled, diluted with 200 mL of ethyl acetate, washed 4x with 20 mL of 10% HCl, washed 2x with 20 mL of saturated sodium bicarbonate, and finally

once with 20 mL of deionized water. The organic layer was dried and the solvent was removed in vacuo. ^{1}H NMR (CD_3CN) δ 1.20 (t, 3H), 1.94 (q, 2H), 2.17 (s, 2H), 4.21 (s, 2H), 5.50 (s, 1H), 7.14 (d, 1H), 7.25 (d, 1H), 7.55 (t, 1H), 7.94 (s, 1H).

***N*-(4-Ethynylphenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (2.4)**. 2-[2-(2-Methoxyethoxy)ethoxy]acetic acid (1.8610 g, 9.551 mmol), 4-ethynylaniline (1.0172 g, 8.683 mmol), diisopropylethylamine (2.2638 g, 17.37 mmol), and HBTU (4.2857 g, 11.28 mmol) were added to a 100 mL RB flask that was charged with 30 mL of DMF. The reaction was allowed to stir at room temperature for three hours. The solvent was removed in vacuo and the residue was dissolved in 300 mL of ethyl acetate. After washing 4x with 20 mL of water, the organic layer was dried and the solvent removed in vacuo. The product was further purified by flash chromotagraphy using ethyl acetate as the eleunt. ^{1}H NMR (CD_3CN) δ 2.76-3.76 (m, 10H), 4.09 (s, 3H), 7.26-7.96 (m, 5H), 8.97 (br, 1H).

***N*-(3-Ethynylphenyl)-2-(methoxyethoxy)ethoxy)acetamide (2.3)**. 2-[2-(2-Methoxyethoxy)ethoxy]acetic acid (2.0352 g, 10.507 mmol), 3-ethynylaniline (1.190 g, 9.552 mmol), diisopropylethylamine (2.5072 g, 19.104 mmol), and HBTU (4.8267 g, 12.417 mmol) were added to a 100 mL RB flask that was charged with 30 mL of DMF. The reaction was allowed to stir at room temperature for three hours. The solvent was removed in vacuo and the residue was dissolved in 300 mL of ethyl acetate. After washing 4x with 20 mL of water, the organic layer was dried and the solvent removed in vacuo. The product was further purified by flash chromatography using ethyl acetate as the eluent. ^{1}H NMR (CD_3CN) δ 3.32-3.73 (m, 10H), 4.09 (s, 3H), 7.47-7.27 (m, 4H), 7.93 (d, 1H), 8.97 (s, 1H).

***N*-Butyl-5-iodopyridin-2-amine (2.2)**. 2-Amino-5-iodo-pyridine (0.1967 g, 0.894 mmol) and sodium hydride (0.2145 g, 8.91 mmol) were combined in a 50 mL RB flask with 10 mL of DMF. The solution was allowed to stir for 10 minutes, and then iodobutane (0.2248 g, 0.984 mmol) was added to the flask. The reaction was allowed to stir at room temperature under nitrogen for 24 hours. The sodium hydride was quenched by dropwise addition of deionized water and the volatiles were removed in vacuo. The residue was taken up in ethyl acetate and then filtered. The solvent was then again removed in vacuo with no further purification necessary. ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3H), 1.41 (q, 2H), 1.58 (q, 2H), 3.24 (d, 2H), 4.53 (br, 1H), 6.23 (d, 1H), 7.60 (dd, 1H), 8.213 (s, 1H).

***N*-(4-((6-(butylamino)pyrin-3-yl)ethynyl)phenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (2.8)**. *N*-butyl-5-iodo-pyridin-2-amine (0.15 g, 0.54 mmol), *N*-(4-ethynylphenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (0.16 g, 0.59 mmol), and piperidine (0.2333 g, 2.72mmol) were added to a pressure tube along with 4 mL of acetonitrile. The solution was sparged for 10 minutes with nitrogen. Tetrakis(triphenylphosphine)palladium(0) (0.0322 g, 27 μmol) was added to the pressure tube and the reaction was heated at 68^{o}C for 5 hours. The reaction was allowed to cool and the solvent removed in vacuo. The residue was taken up in ethyl acetate and washed 4x with 20 mL of deionized water. The organic layer was dried and the solvent removed in vacuo. The residue was purified by flash column chromatography using ethyl acetate as the eluent. ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H) 1.25-1.72 (m, 6H), 3.27-3.77 (m, 10H), 4.12 (s, 2H), 6.35 (d, 1H), 7.27-7.73 (m, 6H), 8.25 (s, 1H), 8.80 (s, 1H); ^{13}C NMR $CDCl_3$ δ 13.39, 14.04, 20.38, 31.77, 42.16, 59.24, 70.40, 70.70, 71.45, 72.03, 82.16, 105.92, 119.96, 122.89, 127.59, 128.66, 128.82, 129.15, 132.19, 132.27, 132.40, 140.34, 151.78; UV/vis (CH_3CN): λ_{max} (ε M^{-1} cm^{-1}) 330 (40000); ESI-MS(m/z) Calcd for $C_{25}H_{30}N_40_7$ + Na [M^+]: 521.52, found: 521.18.

***N*-(3-((6-(Butylamino)pyridine-3-yl)ethynyl)phenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (2.8)**. *N*-Butyl-5-iodo-pyridin-2-amine (0.15 g, 0.543 mmol), *N*-(4-ethynylphenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (0.16 g, 0.598 mmol), and piperidine (0.2333 g, 2.717 mmol) were added to a pressure tube along with 4 mL of acetonitrile and a stir bar. The solution was sparged for 15 minutes with nitrogen, and then tetrakis(triphenylphosphine)palladium(0) (0.322 g, 0.217 µmol) was added. The tube was capped and allowed to heat at 68^{o}C for 5 hours. The reaction was then allowed to cool and the solvent was removed in vacuo. The residue was dissolved in ethyl acetate and the organic solution washed with water. The organic layer was dried and the solvent removed in vacuo. A flash chromatography column was run on the residue using ethyl acetate as the eluent and the slow spot was collected. ^{1}H NMR (CD_3CN) δ 0.94 (t, 3H), 1.35 (m, 5H), 1.52 (d, 2H), 1.94 (s, 2H), 3.29 (s, 5H), 3.52-3.71 (m, 11H), 4.06 (s, 2H), 5.51 (s, 1H), 6.43 (d, 1H), 7.21-7.78 (m, 10H), 8.19 (s, 1H), 8.92 (s, 1H); ^{13}C NMR (CD_3CN) δ 7.47, 7.52, 14.25, 20.88, 30.41, 32.22, 41.96, 49.07, 58.91, 70.76, 71.07, 71.21, 72.03, 72.45, 89.66, 107.77, 107.99, 118.28, 120.69, 123.15, 124.95, 127.62, 129.59, 129.74, 130.12, 132.62, 132.75, 132.99, 139.30, 140.24, 152.35, 159.43, 169.81; UV/vis (CH_3CN): λ_{max} (ε M^{-1} cm^{-1}) 328 (29000); ESI-MS (m/z) Calcd for $C_{24}H_{31}N_3O_4$ + Na [M^{+}]: 448.51, found: 448.18.

Ethyl 3-(5-((3-(2-(2-(2-methoxyethoxy)ethoxy)acetamido)phenyl)ethynyl)pyrindin-2-yl)ureido)acetate (2.6). *N*-(4-Ethylphenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (0.16 g, 0.362 mmol), ethyl 2-(3-(5-iodopyrin-2-yl)ureido)acetate (0.1423 g, 0.401 mmol), and piperidine (0.1311 g, 1.392 mmol) were added to a pressure tube along with 3 mL of acetonitrile and a stir bar. The resulting solution was sparged for 5 minutes with nitrogen. Afterwards, tetrakis(triphenylphosphine)palladium(0) (0.0222 g, 0.139 µmol) was added. The reaction was

then allowed to heat and stir at 75°C for 5 hours. The reaction was then allowed to cool overnight. TLC showed the reaction was complete. ^{1}H NMR (CD_3CN) δ 0.94 (t, 3H), 1.35 (m, 5H), 1.52 (d, 2H), 1.94 (s, 2H), 3.29 (s, 5H), 3.52-3.71 (m, 11H), 4.06 (s, 2H), 5.51 (s, 1H), 6.43 (d, 1H), 7.21-7.78 (m, 10H), 8.19 (s, 1H), 8.92 (s, 1H) ^{13}C NMR (CD_3CN) δ 13.91, 14.06, 39.61, 42.24, 42.30, 46.79, 58.50, 61.44, 62.06, 70.13, 70.45, 70.53, 71.34, 71.84, 88.75, 88.81, 110.99, 111.29, 112.01, 112.42, 120.90, 121.18, 121.36, 121.50, 121.57, 121.98, 122.87, 123.00, 123.95, 127.57, 128.09, 128.58, 129.16, 129.32, 129.80, 132.21, 132.33, 132.67, 132.71, 138.48, 138.83, 139.16, 139.89, 141.35, 153.86, 155.57, 169.58, 170.84, 170.98; UV/vis (CH_3CN): λ_{max} (ε M^{-1} cm^{-1}) 330 (40000); ESI-MS (m/z) Calcd for $C_{25}H_{30}N_4O_7$ + Na [M^+]: 521.52, found: 521.18.

3-(5-((3-(2-(2-(2-Methoxyethoxy)ethoxy)acetamido)phenyl)ethynl)pyridine-2-yl)methyl propionate (2.5). *N*-butyl-5-iodo-pyridin-2-amine (0.15 g, 0.543 mmol), *N*-(4-ethynylphenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (0.16 g, 0.598 mmol), and piperidine (0.2333 g, 2.717 mmol) were placed in a pressure tube along with 4 mL of acetonitrile. The resulting solution was sparged for 5 minutes. At this point, tetrakis(triphenylphosphine)palladium(0) (0.0322 g, 0.27 μmol) was added to the tube. The tube was then capped and the reaction allowed to stir at 68°C for 5 hours. The reaction was allowed to cool and the solvent was removed by rotary evaporation. The residue was taken up in ethyl acetate and washed with water. The organic layer was dried and the solvent removed by rotary evaporation. The product was further purified by flash column chromatography using ethyl acetate as the eluent. ^{1}H NMR (CD_3CN) δ 0.94 (t, 3H), 1.35 (m, 5H), 1.52 (d, 2H), 1.94 (s, 2H), 3.29 (s, 5H), 3.52-3.71 (m, 11H), 4.06 (s, 2H), 5.51 (s, 1H), 6.43 (d, 1H), 7.21-7.78 (m, 10H), 8.19 (s, 1H), 8.92 (s, 1H) ^{13}C NMR (CD_3CN) δ 6.92, 7.00, 7.04, 42.16, 58.52, 61.39, 69.99, 70.37, 71.18, 71.75, 58.94, 91.06, 111.99, 118.55, 120.34,

132.51, 138.92, 141.12, 149.68, 152.84, 155.36, 169.61, 170.98; UV/vis (CH_3CN): λ_{max} (ε M^{-1} cm^{-1}) 324 (8600); ESI-MS (m/z) Calcd for $C_{25}H_{30}N_4O_7$ + Na [M^+]: 521.52, found: 521.18

2-(2-(2-Methoxyethoxy)ethoxy)-N-(3-((3-methylpyridin-2-yl)ethynyl)phenyl)acetamide (2.9). 2-Bromo-3-methyl-pyridine (0.34 g, 2.0 mmol), *N*-(4-ethynylphenyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide, (0.55 g, 2.0 mmol), and piperidine (0.86 g, 1.0 mmol) were combined in a pressured tube along with 8 mL of acetonitrile. The resulting solution was sparged with nitrogen for 5 minutes and then tetrakis(triphenylphosphine)palladium(0) (0.046 g, 0.040 mmol) was added. The reaction mixture was heated at 80^{o}C for 20 hours. The reaction was allowed to cool and the solvent was removed in vacuo. The resulting residue was purified by flash column chromotagraphy using ethyl acetate as the eleunt. ^{1}H NMR (CD_3CN) δ 1.68 (s, 1H), 1.85 (s, 1H), 2.24 (br, 9H), 2.50 (s, 2H), 2.73 (s, 2H), 3.28 (s, 4H), 3.53-3.72 (m, 10H), 4.08 (s, 2H), 5.45 (s, 1H), 7.23-7.37 (m, 4H), 7.90 (s, 1H), 8.41 (s, 1H), 8.99 (s, 1H); ^{13}C NMR (CD_3CN) δ 17.90, 59.31, 69.42, 69.54, 69.58, 86.92, 70.13, 71.67, 90.29, 122.98, 121.40, 121.32, 126.44, 127.91, 128.59, 134.70, 136.23, 138.17, 144.94, 147.49, 169.31; UV/vis (CH_3CN): λ_{max} (ε M^{-1} cm^{-1}) 295 (14000); ESI-MS (m/z) Calcd for $C_{21}H_{24}N_2O_4$ + Na [M^+]: 391.42, found: 391.12.

^{1}H NMR Titrations

For the NMR titrations, samples were made up in a solution of 1:4 CD_3CN:D_2O. The solutions were then titrated with either methanesulfonic acid (made up in deuterium oxide) or tetrabutylammonium hydroxide solution (also in D_2O). The solutions were titrated until an approximately 10 fold excess of H^+ or OH^- was present.

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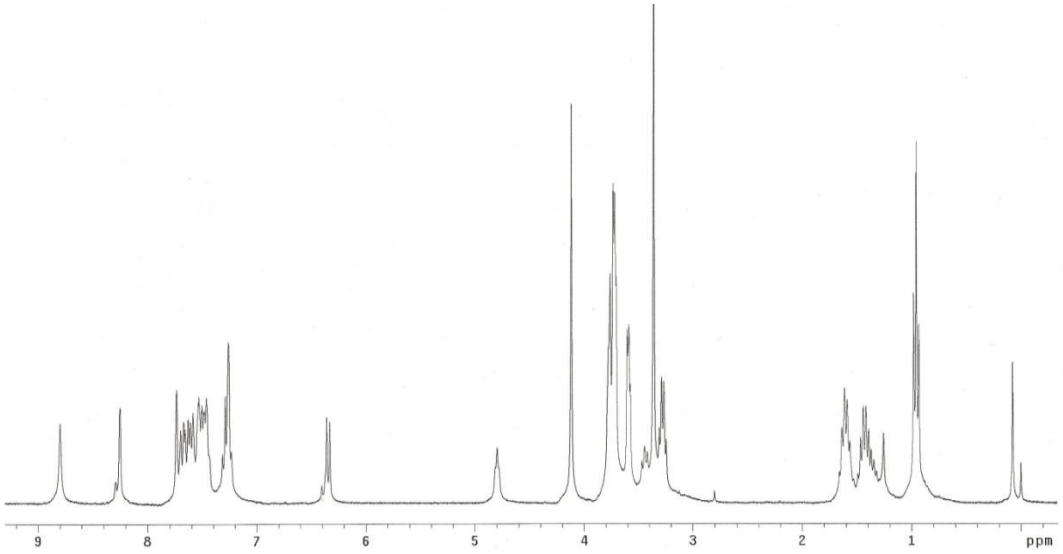
APPENDIX A: SELECTED ^{1}H NMR OF COMPLETED SENSOR



^{1}H NMR of Butyl Pyridine Meta Sensor in $CDCl_3$