Synthesis of a Regenerative Thiol Chemosensing Fluorescent Probe

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Abstract:

The purpose of this research project was to replicate the synthesis and test for the effectiveness of a biological chemosensor. The ringed structure of the molecule, 7-chloro-2,3-dihydro-1H-cyclopenta[b]chromene-1 -one, had been previously shown to be opened by a thiol and closed by mercury ions.[1] The exact opening and closing of the ring structure is important due to its control of the fluorescent light emission of the molecule. The chemosensor was synthesized and then tested for its viability as a marker for both thiols and mercury. Findings were consistent with the proposed lock mechanism of the probe molecule in that quantitative differences were seen upon the addition of a thiol and a subsequent regeneration of the probe through the addition of mercury ions. All work was conducted at the Middlesex County College labs with the synthesis and results from the testing subjected to various analytical tools such as IR spectroscopy, column chromatography, UV-Vis and Fluorescent spectroscopy.

Introduction:

Sensor chemistry has been an intensely researched area of chemistry in the last 10 years because of the multitude of potential benefits it may offer to areas such as healthcare, environmental monitoring, manufacturing quality control, biological research, and food safety. The precise measurement of a chemical quantity allows for better monitoring, control, and synthesis of a desired product.

Specifically, the creation of an effective thiol chemosensor that can measure the minute concentrations present in the human body is representative of the next generational methods and devices that may be used in health care. Sensing implants may one day allow for constant

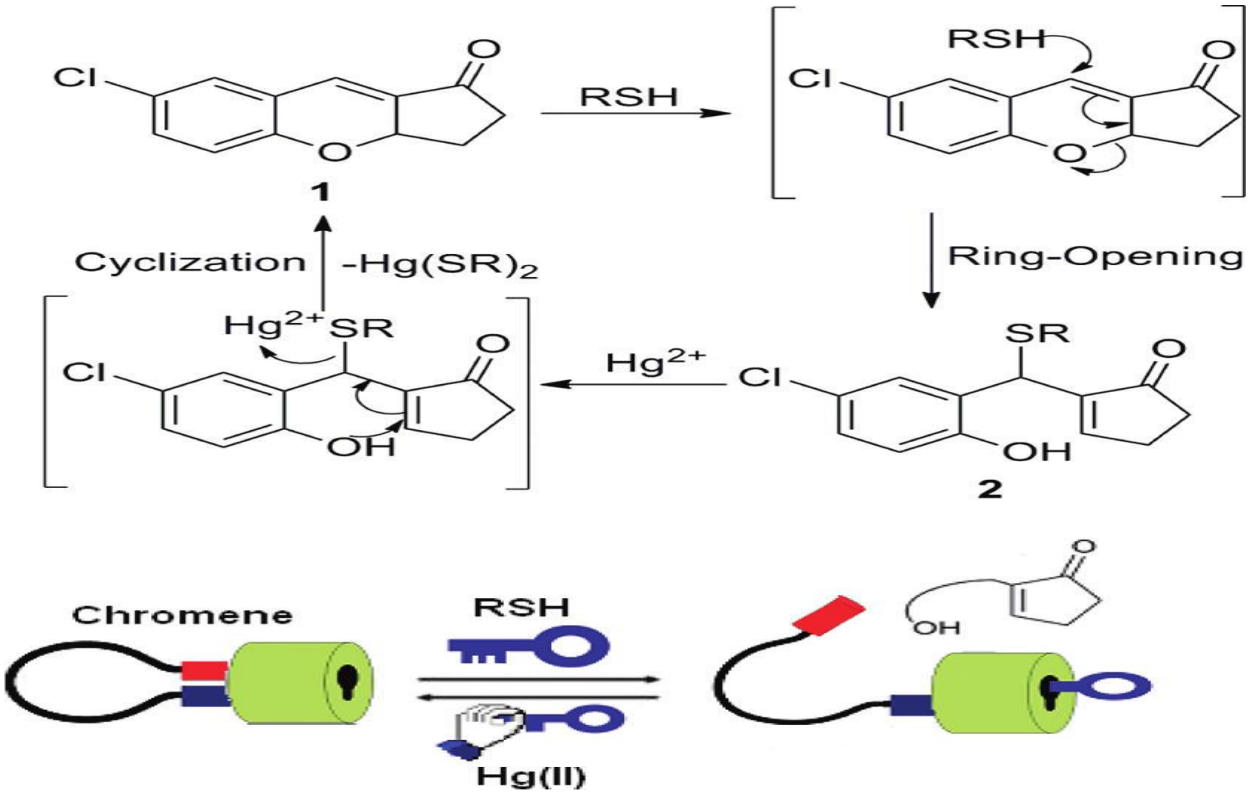
biological monitoring of the human body, providing both early detection and the opportunity to counteract conditions that can lead to various pathologies. Thiols play a particularly important role in the human body because they are found in the amino acid cysteine. The sulfhydryl group attached to cysteine makes it extremely reactive and a basic building block of proteins through the natural formation of disulfide bridges. Featuring in the complex shaping of protein structure, cysteine and its residues are essential components of enzymes and thereby potentially important biological markers of enzymatic activity.[2]

Two residues of cysteine, homocysteine and glutathione, are particularly important as biological markers because of the implication of their measurable levels. Homocysteine features as a component in many enzymes and an elevated level of concentration suggests that enzymatic functionality may be compromised. High concentrations of homocysteine may be indicated in cardiovascular disease, thrombosis, or renal failure.[3] Glutathione is an important reducing agent and antioxidant that protects cells from oxidative stress. Low concentrations suggest a risk factor to cell health and can be an indicator for a wide range of pathologies from cancer to Alzheimer's.[4]

Thiol chemosensors are being synthesized in a multitude of strategies in order to find the most effective methods. Fluorescence is a popular strategy because it is generally uncommon human biological characteristic and is relatively harmless. Quantitative analysis of the chemical to be measured is possible through the altered intensity of fluorescence of the probe in the presence of that chemical. Fluorescence is possible through the absorption of electromagnetic radiation at one excitation wavelength and the emission of that energy at a longer wavelength. In the case of the probe, 7-chloro-2,3-dihydro-1H-cyclopenta[b]chromene-1-one, fluorescence is a characteristic of the molecule because absorption peaks occur in the UV range at 380nm and are

emitted as radiant energy in the visible range at 550nm. When the ringed structure of the probe (1) is opened by the nucleophilic addition of a thiol, a new molecule is formed (2) that no longer shows absorption peaks in the UV range. Subsequently, there is a change in the emission at a longer wavelength and the fluorescence of the molecule has been quenched. The level of change for absorbance and intensity of electromagnetic energy depend on the reaction kinetics between the probe and the thiol.

The regeneration of probe (1) can be accomplished through the addition of mercury ions to the open ring structure of probe (2). Mercury and thiols have long been known to have an especially strong affinity for one another. The mercury ions will cleave the carbon-sulfur bond of probe (2) and a subsequent intramolecular Michael addition will reclose the ring to reform probe (1). Absorption at the UV range of 380nm will be restored, leading to a reigniting of the fluorescence of the molecule. The entire reaction scheme of ring opening and reclosing is diagrammed below:



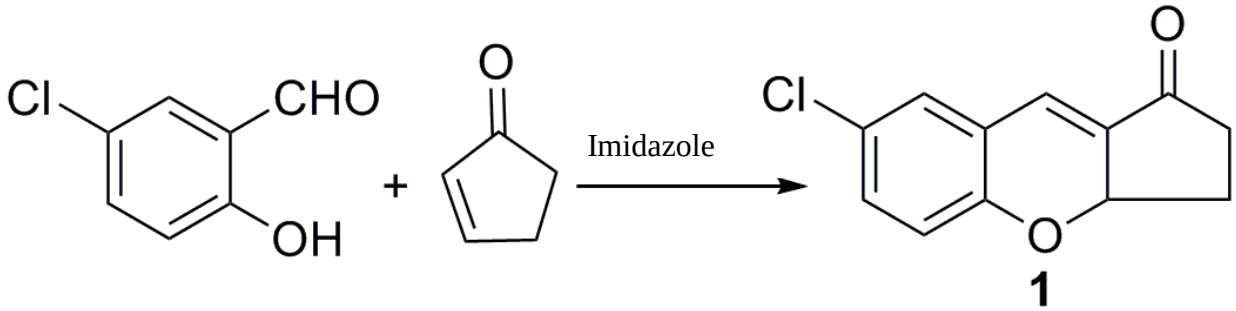
The research project was conducted in a number of stages. First, the chemosensing probe was synthesized from a base catalyzed reaction of 5-chlorosalicylaldehyde and cyclopent-2-enone. A pure product was obtained through flash chromatography and tested by solid state IR and UV-Vis spectroscopy. Second, the probe (1) was tested in the presence of varying concentrations of L-cysteine and measured with UV-Vis and Fluorescent spectroscopy. Third, the open ringed molecule, probe (2), was tested for reactivity with mercury chloride and measured again for absorbance and emission intensity. Finally, a different sample of probe (1) was reacted with an additional thiol, mercaptopropionic acid (MPA), in order to gauge reaction affinity and provide a point of comparison. All attempts were made to create as accurate measurements as possible given the limitation of time and equipment available.

Experimental Section:

All chemicals were obtained from Sigma-Aldrich Corp. Lab equipment and instrumentation were all provided by Middlesex County College.

Lab and Safety Procedures: Waste solutions must be places in proper waste containers. Special precautions should be taken with mercury chloride and mercaptopropionic acid which are toxic.

Part 1 – Synthesis of chemosensing molecule, probe (1)



5-chlorosalicylaldehyde 2-cyclopentenone 7-chloro-2,3-dihydro-1*H*-*cyclopenta[b]chromen-1-one*

Synthesis of the probe is to take place at room temperature. Imidazole is to act as a base catalyst in a Baylis-Hillman reaction between the 2-cyclopentenone and the 5-chlorosalicyaldehyde. A subsequent intramolecular Michael addition reaction closes the ring structure to form probe (1).

1. In a clean and oven dried 50mL round bottom flask, add 313mg of 5-chlorosalicyaldehyde (2mmol).

2. Add 3mL of tetrahydrofuran (THF) to the reaction flask.

3. Add 250uL of chilled 2-cyclopentenone (3mmol) in the reaction flask with micropipette

4. Add 3mL of deionized water to reaction flask.

5. Add 204mg of imidazole (3mmol) and a magnetic stirring rod to flask. Contents of flask will immediately turn yellow to signify reaction is taking place.

6. Stir flask with magnetic rod for a period of 48hrs to complete reaction.

7. Transfer to separatory funnel and dilute mixture with 20mL of 1M HCL.

8. Extract product by adding 30mL of ethyl acetate. Remove ethyl acetate layer containing extracted crude product. Repeat 2 more times to in order to obtain more product.

9. Add drying agent (sodium sulfate) in order to remove moisture. Decant into clean container.

10. Concentrate under pressure with rotovap if available. If rotovap not available, set up a water bath and a simple distillation at 80˚C. Proceed until product has been separated from ethyl acetate.

11. Set up TLC chromatography plate. Prepare a solvent of ethyl acetate to petroleum ether in a 1:4 ratio. Record a definite separation of product in order to proceed to flash chromatography.

12. Set up Flash Chromotography with eluant of ethyl acetate to petroleum ether in a 1:4 ratio. Saturate silica gel of column with eluant and remove any air pockets in column.

13. Add crude product to top of column and use air pump to push air through the column.

14. Collect eluted product in test tubes for further analysis. Make sure to continually add eluant every few minutes in order to maintain the integrity of the column.

15. Take TLC plates all test tubes in order to isolate the tubes that carry the pure product as matched by earlier test TLC plate.

16. Allow pure product to evaporate slowly over one week, giving yellow crystal as probe (1).

Part 2 – Testing reactivity of probe (1) with L-cysteine.

The probe (1) was tested under biological condition by using Tris-HCL 10mM as a buffer solution. Different concentration of L-cysteine were added to solutions of probe (1) and tested for quantitative differences in absorbance and emission.

1. Add 363mg of Tris to 300mL of deionized water. Calibrate a pH meter to accurately identify pH of solution. Add single drops of HCL and test until a pH of 8.0 is reached. Cover as Tris-buffer is easily contaminated.

2. Measure 1.32mg of probe (1) and dissolve in 1mL of ethanol. Mix until fully dissolved yielding a yellow solution.

3. Add solution of probe (1) to 199mL of Tris-buffer yielding a concentration of 30uM.

4. Divide 30uM solution of probe (1) into 6-25mL volumetric flasks and mark 1-6.

5. Weigh 36mg of L-cysteine and add to 25mL volumetric flask. Dissolve with 25mL of Tris-buffer to make concentration of 12mM.

6. In flask of probe (1) #1, add 15uM concentration of L-cysteine by adding 31.25uL from 12mM solution with a micropipette. Shake for three minutes and then take UV-Vis spectrum of solution.

7. Repeat for probe (1) #2-5, by adding an increasing concentration of L-cysteine in each flask for the values of 30uM, 45uM, 60uM, and 75uM. After each addition, shake for three minutes and take UV-Vis.

8. Take probe (1) #6, and add 180uM concentration of L-cysteine by 375uL of 12mM solution. Shake for 3 minutes and take UV-Vis.

9. This process was repeated for a fluorescence reader but with different concentrations in order to account for the length of the well in the 96-well plate and the limitations of the instruments. Emission intensity could only be measured at 590nm, necessitating a scaling up of concentrations in order to see predictable changes.

10. Fluorescent readings were done with a probe (1) concentration of 75uM. The addition of L-cysteine to the probe was done from a 1:1 to a 6:1 stepped increment with total additions of 75, 150, 225, 300, 375, and 450uM concentrations. Fluorescent emissions were recorded after 5 minutes on one 96-well plate with an excitation wavelength set at 360nm.

Part 3 – Regenerating probe through the addition of mercury ion.

The original flasks from Part 2 were unusable due to the degradation of cysteine and other unknowns related to time. Fresh flasks of probe (1) which were unlocked by L-cysteine to form probe (2) were used for the regeneration of the probe through the addition of mercury ion.

1. 2-25mL volumetric flasks of probe (1) were prepared with a concentration of 30uM.

2. Weigh 81mg of mercuric chloride and add to a 25mL volumetric flask. Add 25mL of Tris-buffer to make 12mM concentration.

3. A concentration of 12mM L-cysteine is prepared as in Part 2. 90uM L-cysteine is added to one flask and 180uM to the other. The flasks are shaken and a UV-Vis spectrum is taken 5 minutes later.

4. A concentration in slight excess of 90uM and 180uM of mercuric chloride is added to the respective flask of probe (2). The flasks are shaken and allowed to sit for 10 minutes before they have a UV-Vis spectrum taken.

5. Different concentrations were used again for the fluorescent reading of the regeneration of probe (1) through the addition of mercury ions. The 96-well plate from part 2 was used immediately after the fluorescent reading of the unlocking of the ring structure by cysteine. Mercuric chloride was added in equal concentration as the addition of L-cysteine. A reading was taken after 5 minutes under the same conditions of excitation at 360nm and emission at 590nm.

Part 4 – Testing with an alternative thiol (mercaptopropionic acid)

Repeat tests were conducted with mercaptopropionic acid (MPA) in order to observe the kinetics of the reaction and test for the affinity of the reagents for each other.

Tests were conducted with a probe (1) concentration of 100uM. This was near the upper limit of the concentration of the probe as higher concentrations were observed to precipitate out of solution.

1. Pour probe (1) in a concentration of 100uM into a 10mL volumetric flask.

2. Add 109uL of mercaptopropionic acid into a 25mL volumetric flask and fill with Tris-buffer to create a 50mM concentration.

3. Add 20uL of 50mM mercaptopropionic solution in order to add 100uM concentration into probe (1) flask. Shake for 3 minutes and take UV-Vis spectrum

4. Repeat procedure every 5 minutes for 2 additional increments of 100uM concentrations to a final total added mercaptopropionic concentration of 300uM. Flask of probe (1) should turn color from yellow to clear.

5. Weigh 339mg of mercuric chloride and pour into 25mL volumetric flask. Fill with Tris-buffer to create concentration of 50mM.

6. Add 20uL of 50mM mercuric chloride solution in order to add 100uM concentration of mercuric chloride to flask containing probe (2). Shake and wait 10 min. Take UV-Vis spectrum.

7. Repeat procedure every 15 minutes for 3 additional increments adding a final total concentration of 400uM mercuric chloride. Flask of probe should turn color from clear to yellow.

Results and Discussion:

Part 1:

1. The synthesis of the probe was a straightforward process as the reagents only needed to be added to reaction flask and stirred for 48 hrs. The yield, however, was lower than expected with only 24mg of pure product.

The percent yield is calculated:

.313g of 5-chlorosalicylaldehyde x (1mol/156g) x (219g/1mol Probe(1) = .439g expected yield

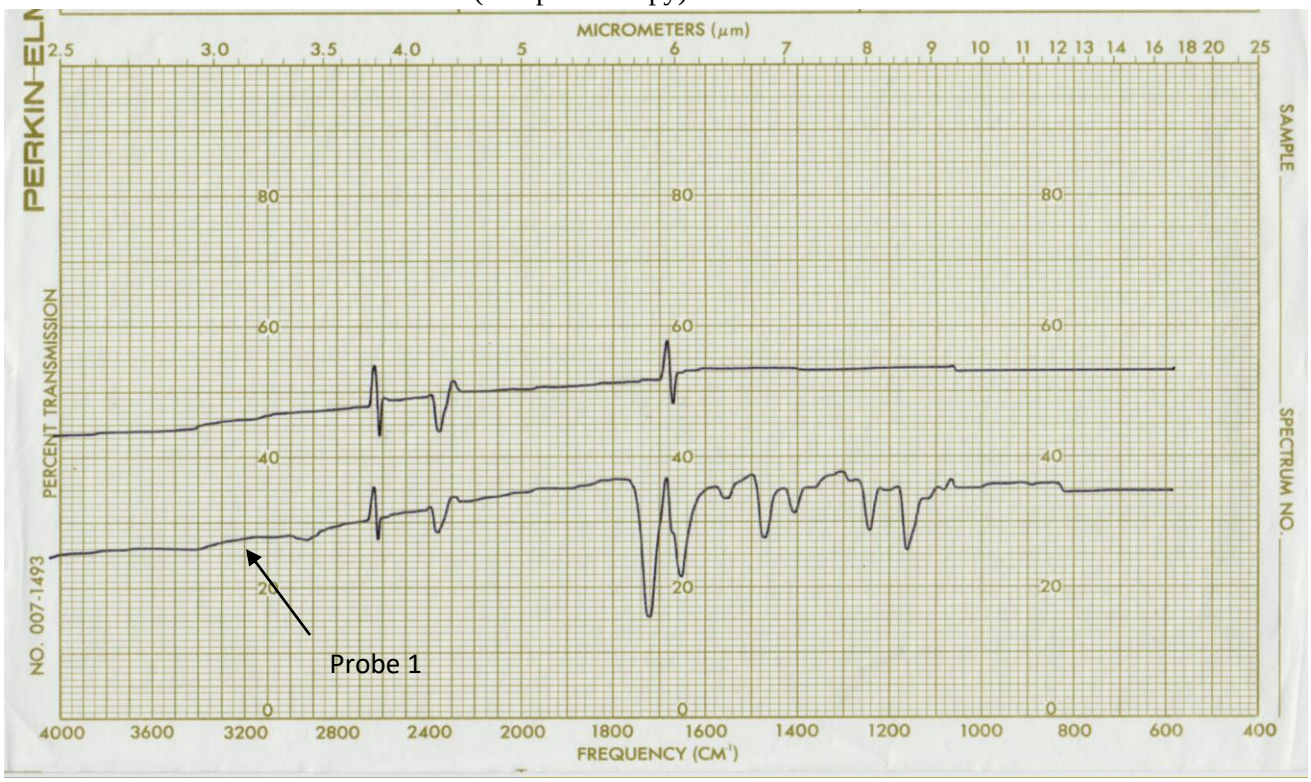
.024g / .439g = 5.47% percent yield

The low percent yield may be due to a number of reasons. The lack of a rotovap may have caused some of the product to have been lost with the ethyl acetate. Also, flash chromatography may have played a role as a long column was used to separate the crude product. Some of the pure product may have become stuck in the column and could not be eluted.

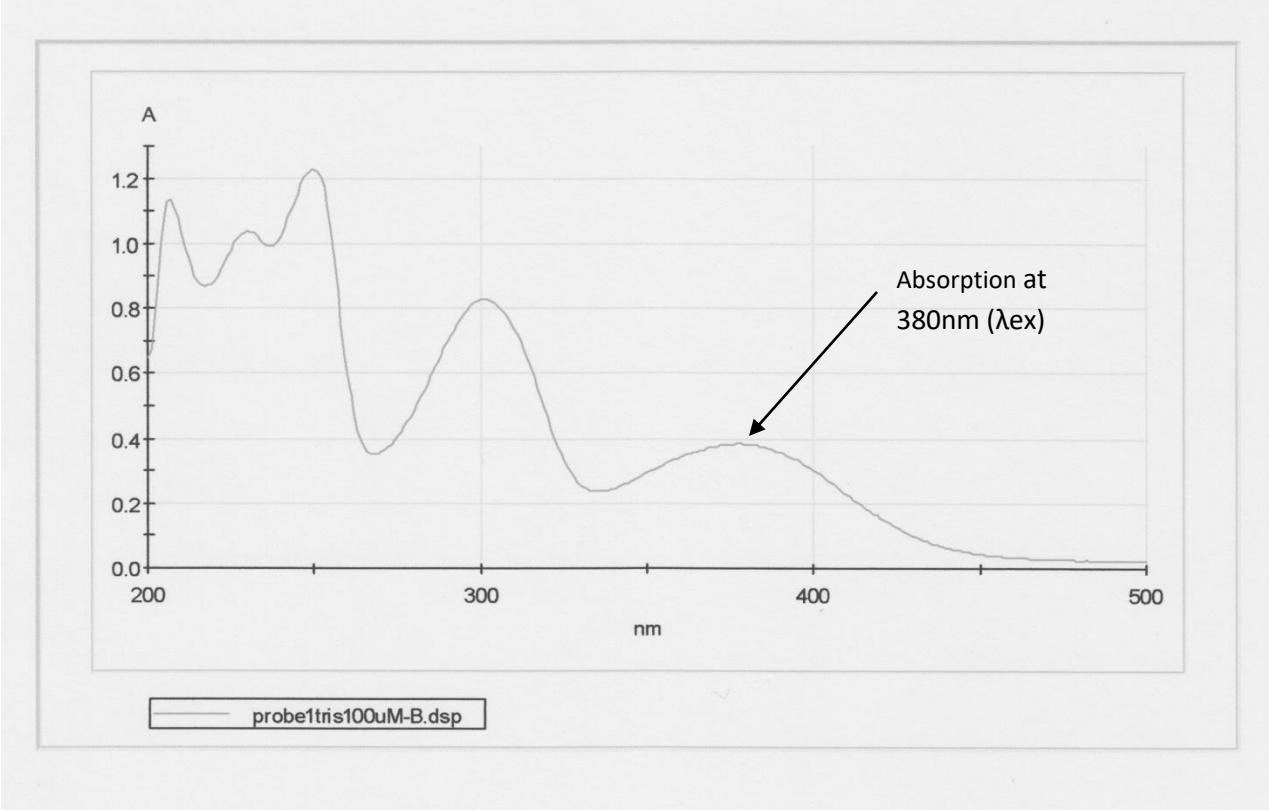
2. The solvent of ethyl acetate to petroleum ether in 1:4 ratio was previously reported in the original literature and proved to be a dependable solvent for compound separation. Further, the dots on the TLC plate were early evidence of fluorescence as the spots glowed purple under the UV lamp.

3. Spectrum analysis was done on the pure product to confirm the synthesis of the desired compound. Both IR spectroscopy and UV-Vis spectroscopy were consistent with expectations. Solid state IR spectroscopy was done with a KBR baseline and displayed a functional group at expected readings for a carbonyl group and benzene ring. The UV-Vis spectrum of a 100uM concentration of probe 1 displayed an absorption peak at 380nm, the expected UV wavelength of excitation.

(IR Spectroscopy): below



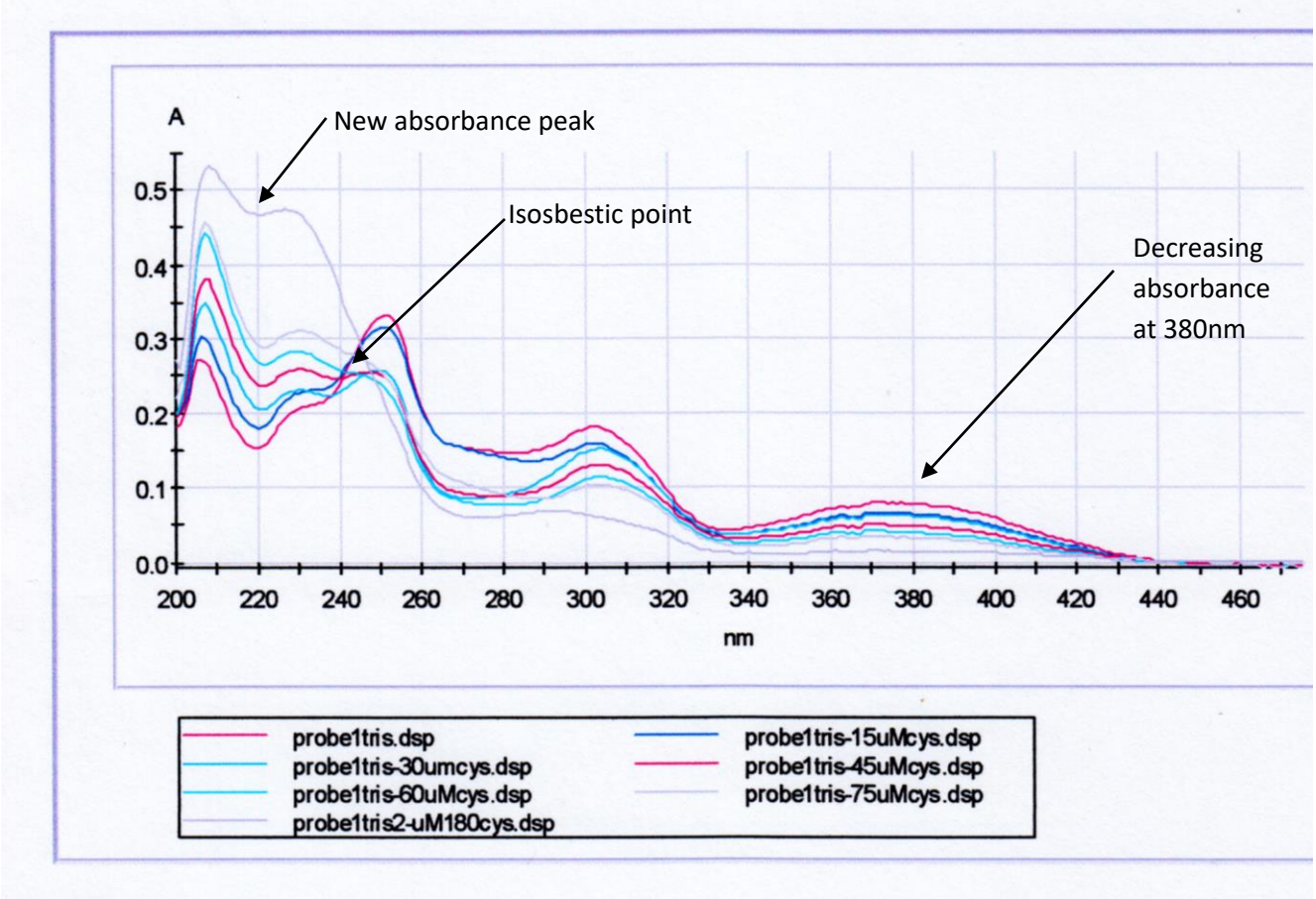
(UV-Vis Spectrum of Probe (1) at 100uM): below



Part 2:

1. The reactivity of the probe (1) with L-cysteine was consistent with the expected results. There was an incremental decrease in the wavelength of excitation at 380nm until a flattening of the curve at 6 times the concentration of thiol to probe.

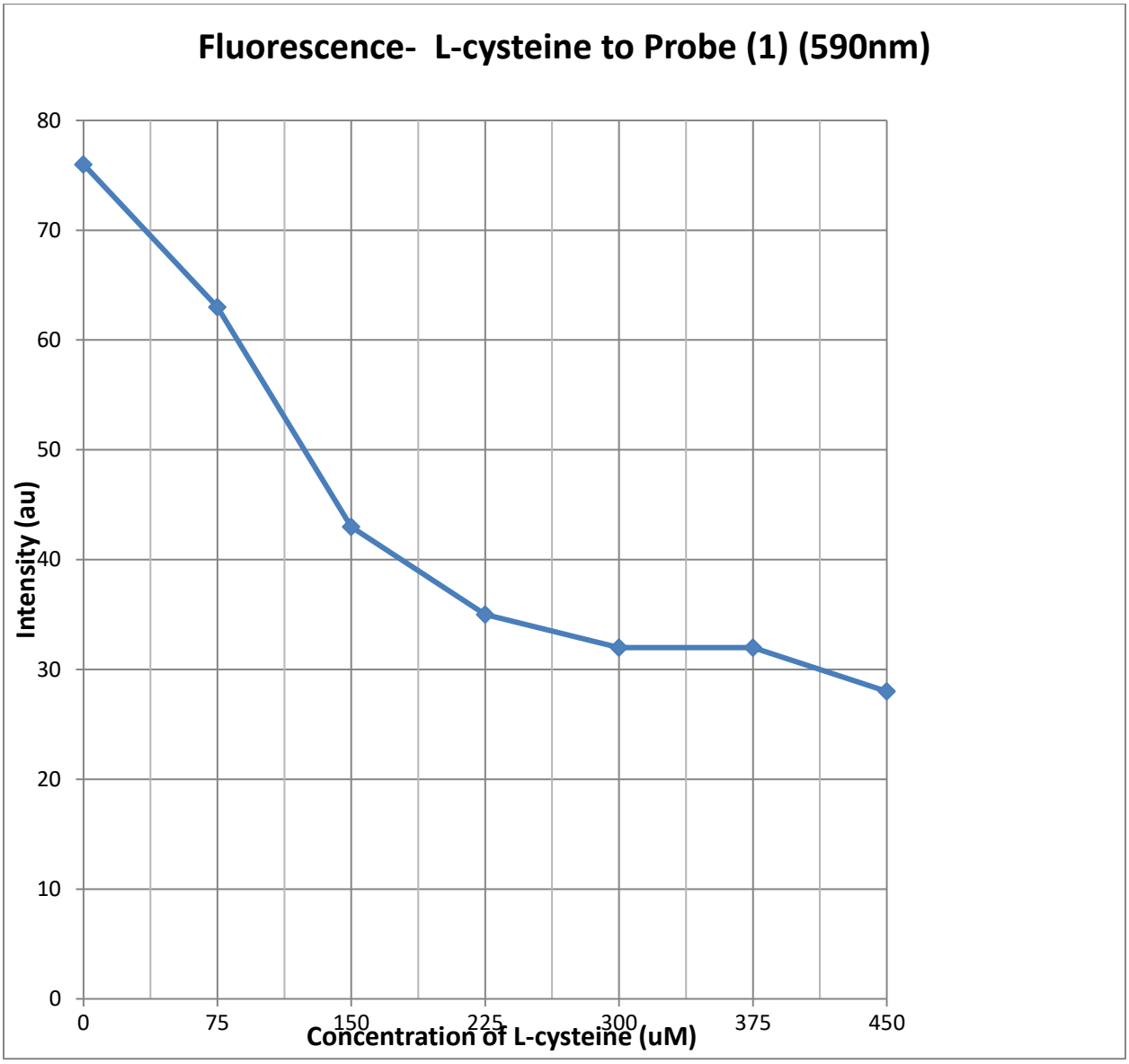
(UV-Vis Spectrum of Probe (1) at 30uM with L-cysteine Concentrations)



2. Also evident in the UV-Vis spectrum is the formation of a new absorbance peak at 215nm. This is indicative of a new compound being formed. The isosbestic point at 240-250nm shows a clean transition of one compound into another with no intermediates.

3. The fluorescence reader was limited to only a single wavelength for excitation and emission. The provided a wavelength of excitation (360nm) and emission (590nm) that were not exact matches for the maximum peaks of the probe (1) at 380nm and 550nm respectively. Results showed a decrease in fluorescence emission but without a defined rate of decline. The rate of decline slowed at higher concentrations of L-cysteine and even showed a leveling between 300 and 375uM.

Conc. Of L-cysteine	Intensity (au)
0	76
75	63
150	43
225	35
300	32
375	32
450	28

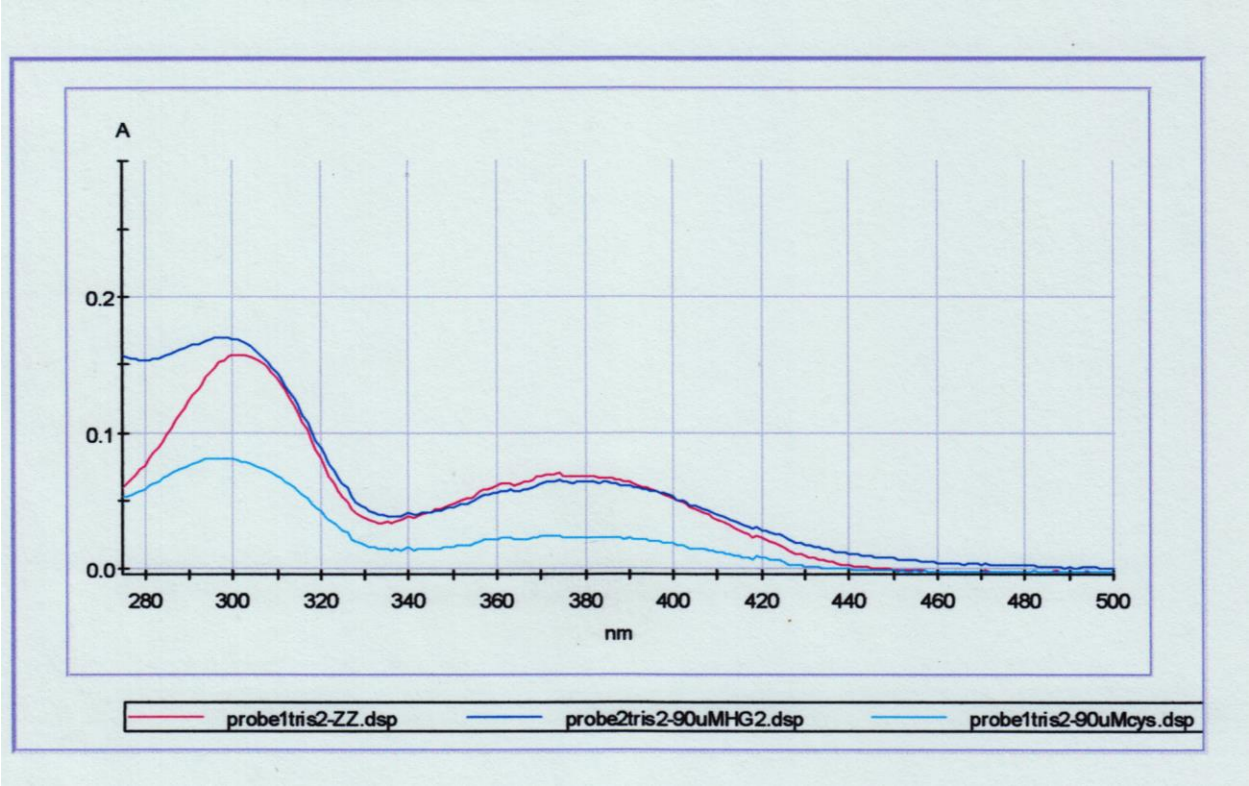


4. The leveling off of the rate of decrease may be due to a number of factors. The less than optimal instrument locked wavelengths of excitation and emission may have yielded poorer results than normal. Further, the reagents were added together in a 96-well plate that may have not provided opportunity for adequate mixing. Finally, there is the possibility of experimental error such as the a mistake with the delivery of the L-cysteine with the micropipette. When dealing with microliters of volume, an incorrect delivery of the solution will likely compromise the data. Additional attempts to replicate the data were not possible due to time constraints and the availability of the fluorescent reader.

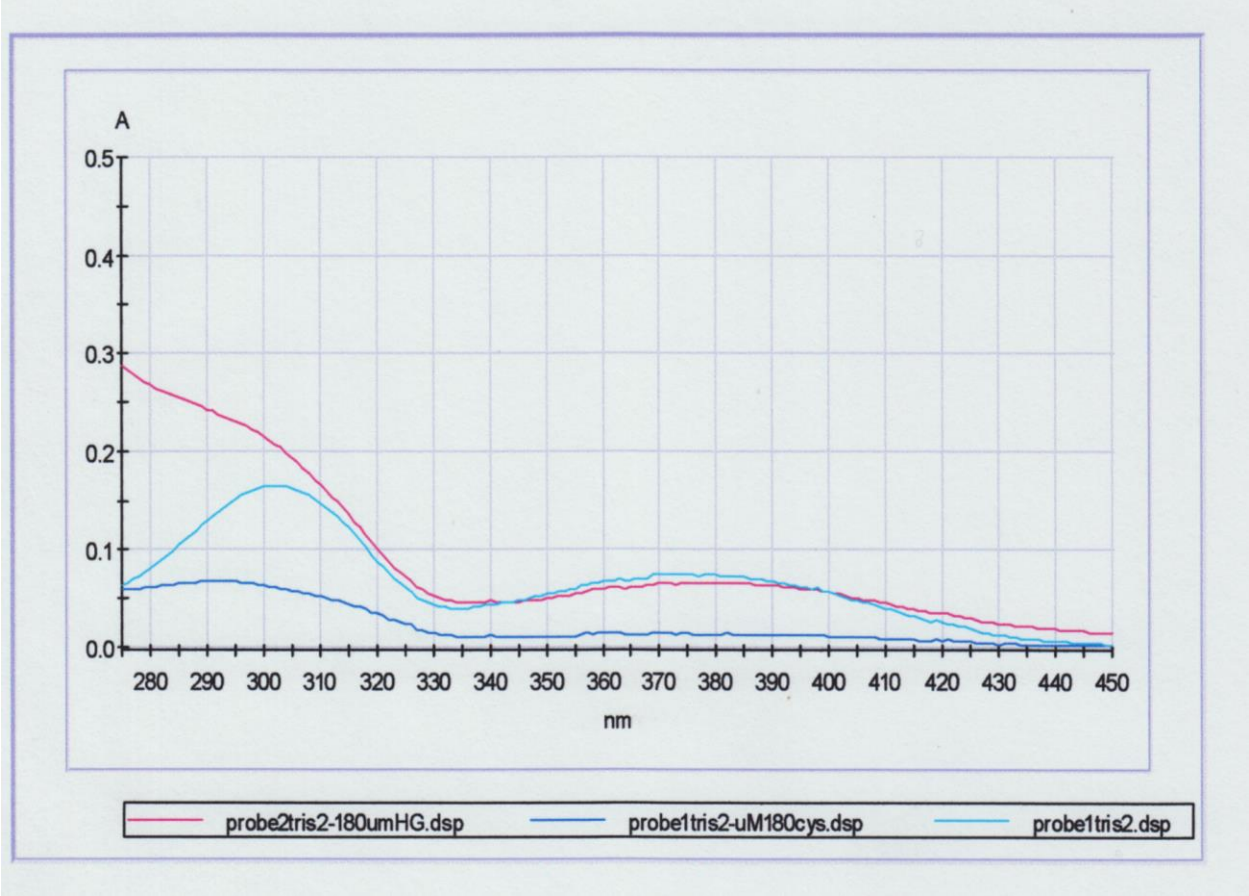
Part 3:

1. Results for the regeneration of the probe (1) with the addition of mercury ions were consistent with expectations. Both the 90uM and 180uM flasks of L-cysteine added probe (2) showed a low absorbance at the excitation wavelength of 380nm, with the 180uM addition showing a flattened curve. An addition of mercury ions in equal concentrations of 90uM and 180uM to the respective flask was able to reset the absorbance at 380nm to the level of the original probe (1). The relocking of the ring structure through desulfurization allowed for absorbance at the UV range and a likely reigniting of fluorescence.

(UV-Vis Spectrum of Regeneration of Probe (30uM) through addition of Hg+ (90uM))



(UV-Vis Spectrum of Regeneration of Probe (30uM) through addition of Hg+ (180uM))

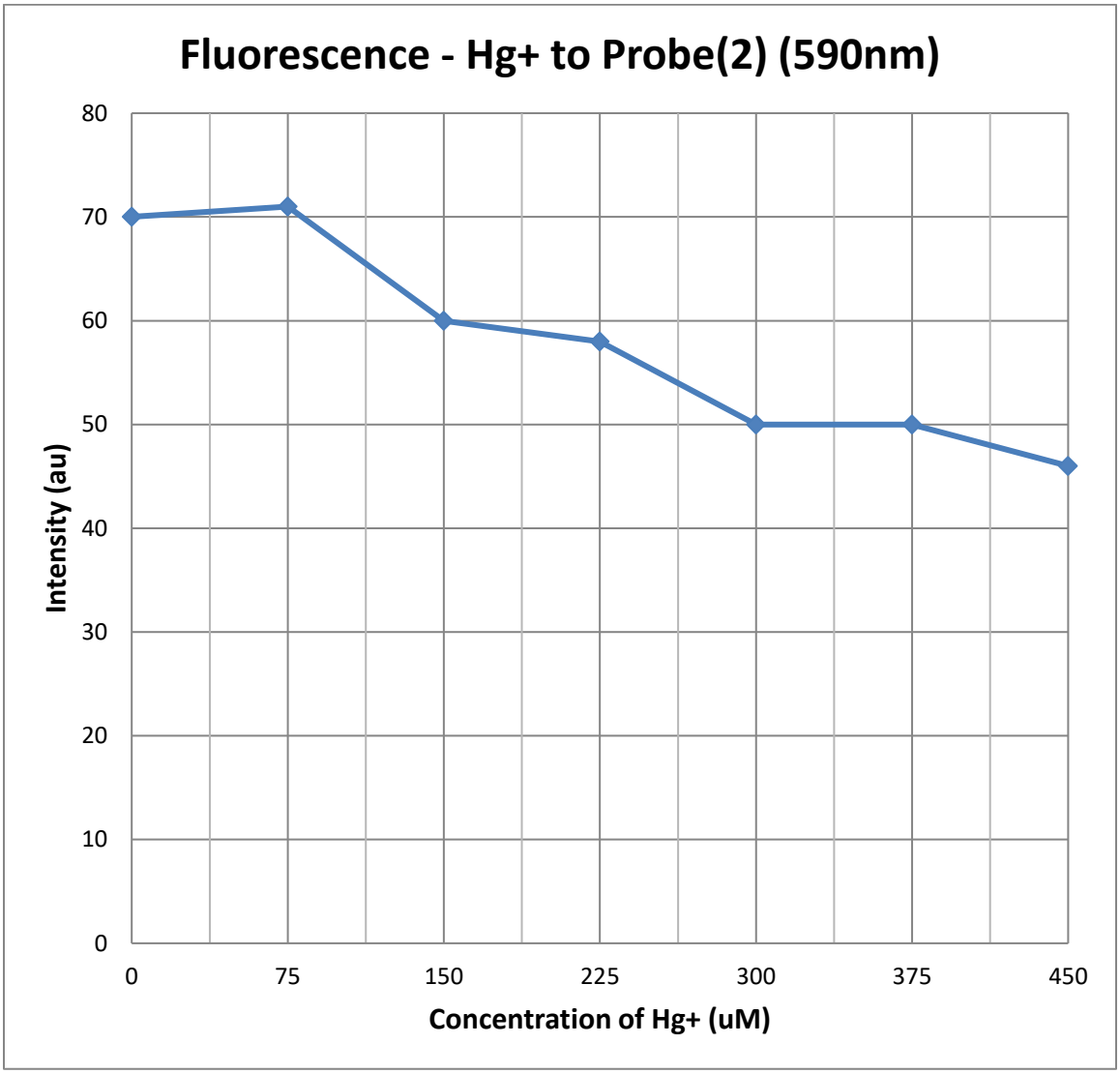


2. A noticeable change in the regenerated probe (1) was a new absorbance reading at 280nm that was previously not recorded. This was likely due to the presence of a new mercury-thiol compound created through desulfurization. Isolation of the new compound from the probe was not attempted but may be performed in future experiments.

3. Fluorescent reading of the original probe (1), ring opened probe (2), or regenerated probe (1) at these concentrations were not done due to time constraints and instrument availability. Excitation wavelength absorbance is a predictable indicator that emission reading would see expected changes.

4. A fluorescent reading of probe regeneration was taken of the samples used in the 96-well plate from Part 2 of the experiment. The 1:1 addition of mercury ions to the cysteine added unlocked probe (2) did not produce the clear results. The measured intensity (au) at 590nm showed a predicted increase in each sample of probe. However, only the 75uM 1:1 addition of cysteine and mercury ions showed a normal level of emission intensity. All the greater concentrations, despite a 1:1 ratio of mercury addition, did not reach a normal level of emission.

Conc. Of HgCl2	Intensity (au)
0	70
75	71
150	60
225	58
300	50
375	50
450	46

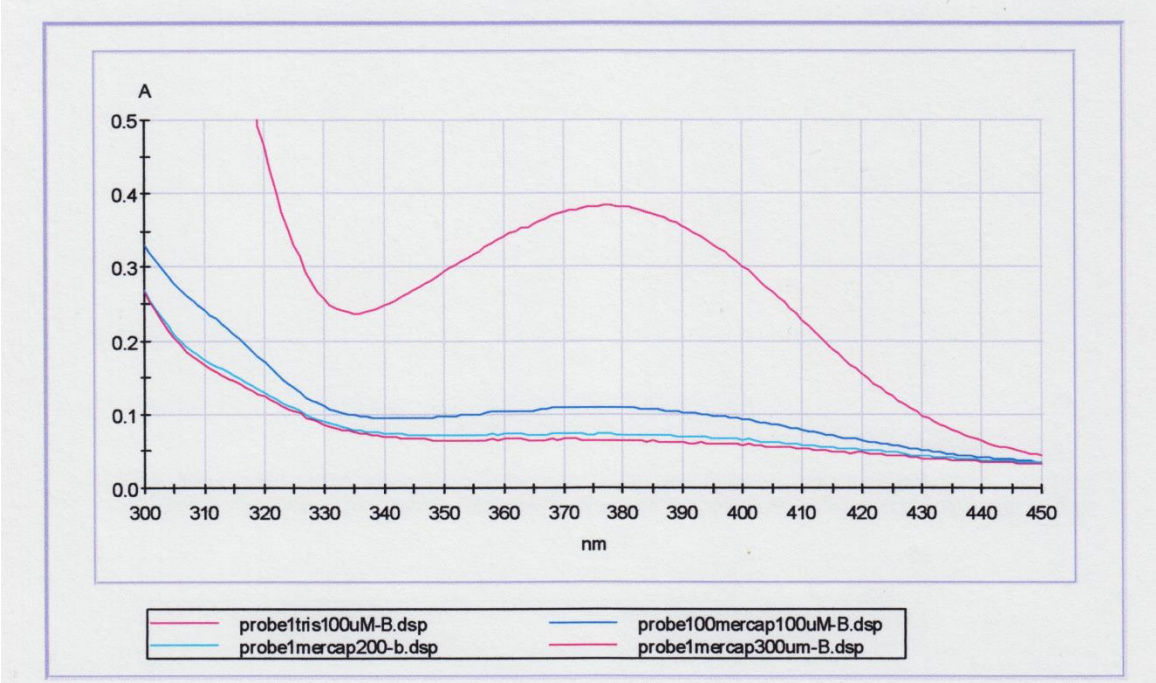


5. A regenerated probe (1) at every concentration after a 1:1 addition of mercury ion would have displayed a flat line at around 70au of intensity. The lowered intensity of the higher concentrations of cysteine/mercury addition may be explained in a number of ways. The reaction done in the flasks were given 10 minutes to react as opposed to 5 minutes in the well plate. A second reading of the well plate may have yielded better results. Instrument limitation may have again caused an issue because the wavelengths defined by the system were sub-optimal. A strong possibility of the lowered rate of regeneration may be due to the reaction kinetics between cysteine opened probe (2) and mercury. All of the wells started with an equal concentration of 75uM of probe (1). The higher concentrations of cysteine and mercury ion additions were as much as 6 times the concentration of the original probe. It is possible the higher concentration additions showed a lower regenerated probe because of a greater affinity of the mercury ion to bind to unattached thiols in the solution. This is possibly due to the necessity of overcoming the activation energy required in the desulfurization of probe (2). In this case, a longer reaction time was most likely necessary in order for the regeneration of the probe (1) to be completed. A normal level of fluorescent emission may have been achievable for each regenerated probe sample if given enough time.

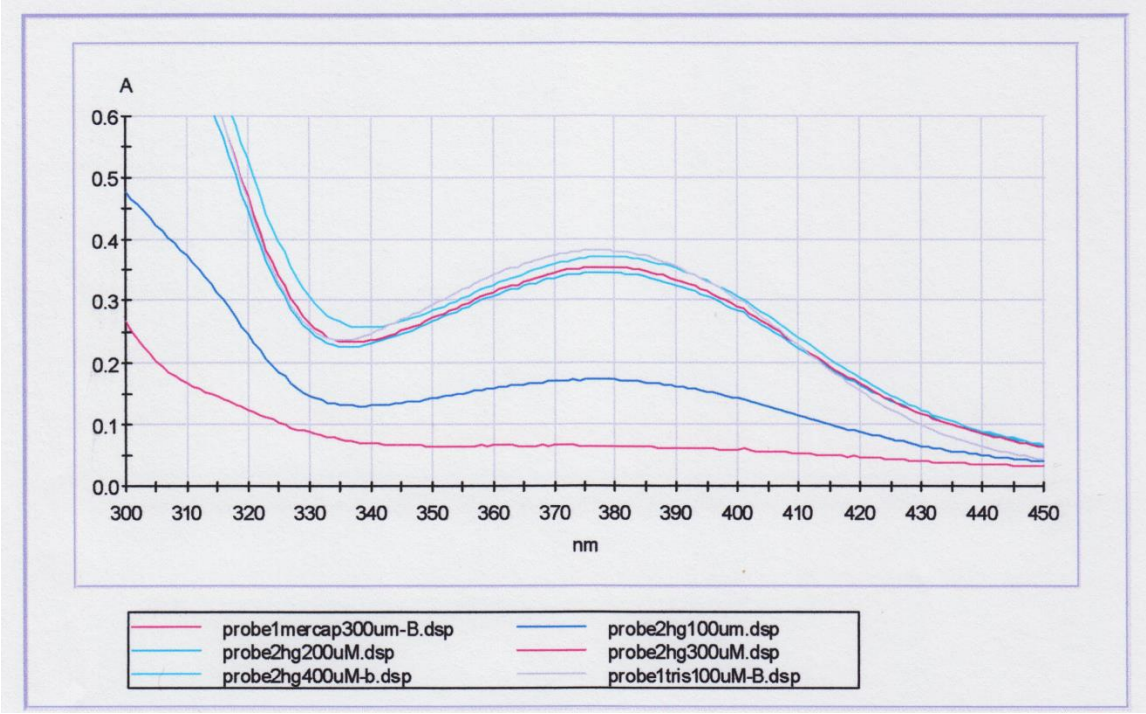
Part 4:

1. A repeat of the thiol unlocking and mercury relocking of the probe was done with mercaptopropionic acid (MPA) to observe difference in reaction kinetics. Results were positive as they showed that mercaptopropionic acid reacted more efficiently in flattening the level of absorbance for probe (1) at the UV wavelength of excitation -380nm. An addition of 1:1 ratio of MPA to probe (1) saw a rapid decrease of absorbance with a flattening out occurring between 2:1 and 3:1 additions of MPA. This was more efficient than L-cysteine which required a 6:1 ratio of addition of the thiol in order to flatten out the absorbance at the excitation wavelength.

(UV-Vis Spectrum of Probe (1) at 100uM with MPA concentrations 100-300uM)



(UV-Vis Spectrum of Regeneration of Probe (100uM) w/MPA through Hg+ (100-400uM)



2. Results of the regeneration of probe (1) were also positive as the mercury ions showed a greater affinity for the sulfur group of probe (2) with MPA than was previously seen with L-cysteine. A 1:3 addition of mercury to MPA showed a half reformation of the absorbance peak at the 380nm wavelength. A 2:3 addition of mercury to MPA showed a near attainment of a regenerated peak of probe (1) at the wavelength of excitation. Increased levels mercury brought the absorbance levels closer to the original by smaller margins.

3. The success of more efficient reaction kinetics with MPA suggests that thiols react with the probe and mercury ions at different rates. The fluorescent readings with the well plate should be conducted with MPA to see if they yield better results.

Conclusion:

The research project met its goal of synthesizing a regenerative chemosensor that displayed an on-off-on mechanism for fluorescence through the opening of a ring structure by a thiol and closing by mercury ions. This chemosensor was novel in that it functioned in the sensing of both thiols and mercury with the promise of a possible future efficiency due to its regenerative characteristics.

Restraints to the lab and equipment lowered expectations in reproducing the exact data as seen in the original literature. More accurate data could be produced by a more efficient fluorescent reader that provides measurement at multiple wavelengths. A rotovap would likely have increased the percent yield while H-NMR could have been used for greater analysis of the product.

The original literature also included tests for other amino acids and common metals as a point of comparison for cysteine and mercury. These tests would have shown the vital selectivity of the probe that makes it potentially useful as a biological sensor. These tests were not possible due to financial and time constraints but are left open for the future. Namely, gold (AU) ions are an

intriguing option as they may be useful as an analog for mercury in this experiment. The position of gold on the periodic table suggests the same reactivity and characteristics. Only further tests will reveal if this chemosensing probe can include gold detection as a possible use.

The development of chemosensors continues to be an exciting area of research in chemistry, with the potential benefits to science and society unknowable at this time. Functionality in mild conditions, efficient yield, reliable data, and thiol differentiation are only some of the characteristics that the thiol sensing implant of the future will need. This chemosensing probe is only a stepping stone upon which more effective thiol probes will eventually be built.

References:

1. Fang-Jun Huo, Yuan-Qiang Sun, et al. *Chromene "Lock", Thiol "Key", and Mercury(II) Ion "Hand": A Single Molecular Machine Recognition System*. 2010. Organic Letters. **12,** *21*. 4756-4759.

2. *Why Cysteine is Special.* http://www.cryst.bbk.ac.uk/pps97/assignments/projects/leluk/project.htm

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4. Hanjing Peng, Weixuan Chen, Yunfeng Cheng, et al. *Thiol Reactive Probes and Chemosensors*