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# STUDY OF CYANINE DYE BINDING TO AMINO ACIDS AND ITS ANALYTICAL UTILITY

by

YONATHAN MERID

Under the Direction of Gabor Patonay

## ABSTRACT

Investigation of the NIR cyanine dye MHI-36 shows binding affinity to charged amino acids. This cyanine dye showed aggregation and dimerformation at higher dye concentration ( $2.0 \times 10^{-3} \text{M}$ ) induced by lysine. When dye concentration decreased to  $1.0 \times 10^{-4} \text{M}$  no strong aggregate formation was viewed. Dye shows strong binding and selectivity properties towards charged amino acids lysine and arginine, compared to neutral leucine. It's believed the positively charged presence was able to break and disrupt the conjugated  $\pi$ - $\pi$  bonds at lower dye concentration. Computational work showed intramolecular aggregation of the phenyl groups on the dye. These aggregates are believed to create electron rich environment suitable for lysine interaction.

INDEX WORDS: Aggregation, Near infrared, Protein labeling, Quenching

STUDY OF CYANINE DYE BINDING TO AMINO ACIDS AND ITS ANALYTICAL UTILITY

by

YONATHAN MERID

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Masters of Science

In the College of Arts and Sciences

Georgia State University

2010

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2010

# STUDY OF CYANINE DYE BINDING TO AMINO ACIDS AND ITS ANALYTICAL UTILITY

by

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May 2010

## DEDICATION

I would like to dedicate this work too my father, mother and sister. Thank you for your support.

## ACKNOWLEDGEMENTS

Even though this seems like a personal achievement I believe anyone that has been in contact with me the past two years has played some part in this accomplishment. First and foremost I would like to thank God because he provided the strength I needed to reach my mountain top. My supportive and loving parents who believed in me. My out of town sister (lol), who has helped me understand the importance of education and to the rest of my family, all my Uncles, Aunts, cousins, and friends who have encouraged me more than they know.

Dr. Patonay, thank you for providing more questions than answers. The freedom and flexibility you provided me helped me understand how to be a true scientist.

Garfield, all the numerous conversation we had, majority non-chemistry related. Sang Hooh Kim, Kristen Ashbey and other members of Patonay group. The rest of chemistry students I have met along the way.

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## 1. INTRODUCTION

### 1.1 NIR energy

The near-infrared region (NIR) of the emission spectrum has found numerous applications in different division, ranging from use in semi-conductor laser technology to bio medical fluorescence spectroscopy. NIR has become very effective and continues to become more popular. Absorbance in the NIR region transpires when energy gap between the excited and ground state energy level is minuscule. Difference between dyes that fluoresce in the visible and near infrared-region is due to the higher energy gap between the ground and excited states, thatvisibleregion fluorescing dyes have<sup>1</sup>. This smallenergy gap exhibited that NIR dyes have isa result oflong  $\pi$ -conjugated bonds or insertion of heavy substituent on polyene chain.

In NIR energy N-H, C-H and O-H are some more common bonds of interest due their stretching and bending capabilities<sup>2</sup>. These active molecules have vibrating dipoles with specific frequency and amplitude. Frequency is defined as vibrations per unit of time, usually in units of sec<sup>-1</sup> or Hetz<sup>2</sup>. Amplitude is reference to the extreme ends traveled by vibrating dipole, which is dependenton energy the infrared active bond takes in<sup>2</sup>. When a spectrophotometer emits photons from a lamp and it strikes molecules in a sample, two outcomes arise: the energy does not equally match the natural vibrational frequency of the molecule or the entering frequency does match the vibrational frequency of the molecule<sup>2</sup>. When the incoming frequency from the light matches the molecules vibrational frequency, the molecule turns the absorbed energy into increased vibrational amplitude of the absorbing dipole. It's important to note this frequency from absorbed energy of the vibration remains constant. The availability of commercial semiconductor laser diodes used for excitation is an advantage of NIR region. The powerful monochromatic excitation that is provided by the laser improves low limit detection<sup>3</sup>.

## 1.2 Dye Aggregation

Dye aggregation in aqueous solution has been an intriguing researched topic. Research has shown that pH change leads to aggregation in aqueous solution of which hydrophobicity of solvent plays a significant role in dye aggregation<sup>4</sup>. Formation of aggregates can occur as hydrophobicity of solvent decreases. NIR dyes form dimers in aqueous solutions at low concentrations, but no dimerization have been known to form below  $10^{-5}$ M concentration<sup>5</sup>. Dye aggregation occurs through intramolecular and or intermolecular assembly, orientation is based on angle of slippage  $\alpha$  of the stacked molecules. The angle of slippage is defined as angle between molecular transition moment and the long axis of aggregate. The angle slippage is derived from the geometric shape of the aggregate that is formed from repulsion from of dye charge and attraction from hydrophobic interaction. J-aggregate posse's relatively small angle of slippage, less than  $32^\circ$ , which form intermolecular aggregation that causes shift in red wavelength direction along with narrower band absorption. Reports have also shown J-aggregate forming side-to-side stacking aggregate pattern similar to "brickwork" grouping. Due to this alignment J-aggregate are believed to exist mainly in lower monomer energy level. While H-aggregates embodies geometric framework referred to as "card-pack" and larger angle slippage greater than  $32^\circ$ . Researching possible cause of aggregation can lead to insightful information used for improving applications like bio-imaging and protein labeling.

Since only a few molecules exhibit intrinsic fluorescence in the NIR region, the application of NIR cyanine dyes has become very useful for fluorescent labeling and other bio-analytical techniques<sup>6</sup>. Majority of proteins or other biomarkers fluoresce in UV and visible region, therefore NIR assays have minimum background fluorescence due to low autofluorescence in the NIR region<sup>6</sup>. Autofluorescence occurs when tissue, proteins or other bio-molecules fluoresce naturally. This explains why higher background signals are seen if the dye in use is in the UV and visible region<sup>7</sup>. This knowledge is a momentous reason that makes NIR region very relevant in

medical science. By using NIR dyes for bio-imaging, most tissues and cells will not interfere with the signal since they don't fluoresce in the region. Therefore this application becomes more advantageous since it decreases unwanted fluorescence signal. Minimal absorption takes place in ground state in the NIR wavelength range from biomolecules that fluoresce in UV/Vis region, which also leads to reduced light scattering<sup>8</sup>. This theory makes NIR dyes more advantageous towards vivo imaging due to the fact that no absorption takes place of photons in tissue and blood samples<sup>3</sup>. This makes NIR dyes very useful for disease detection such as cancer imaging as well as drug development and treatment evaluation<sup>1</sup>. Unlike infrared dyes, NIR dyes have also showed great purpose as chemical sensors for metal ions detection as well<sup>3</sup>.

NIR dye metal binding has also shown remarkable growth and potential. Through electron transfer from fluorophore to metal ions, while forming a charge-transfer complex, the ion is able to quench the fluorophore<sup>3</sup>. It's believed that the charge-transfer complex formed disrupts the conjugated structure of the dye caused by quenching. Metal-dye complex can quench in dynamic or static method<sup>3</sup>. Both of these methods are temperature dependant. For dynamic quenching, increasing the temperature leads to larger diffusion coefficient, this increases the bimolecular quenching constant, which leads to decreased fluorescence intensity. While static quenching forms when dissociation of ground state complex leads to increase of fluorescence intensity, when temperature rises<sup>3</sup>. Fluorescence resonance-energy transfer has been used to investigate protease kinetics<sup>9</sup> and nucleic acid hybridization<sup>10</sup>. Covalently attached fluorescent donor and non-fluorogenic chromophore are attached to enzyme substrates which in resonance-energy transfer results in quenching<sup>11</sup>. Quenching is important in NIR dyes, the understanding of quenching at different concentrations can possibly lead to longer fluorescence capabilities.

### 1.3 Protein Labeling

To continue to understand the interaction of NIR dyes with biomolecules, one must first study the interaction between NIR cyanine dyes and the amino acids found in the proteins that

make up biomolecule. Labeling of biomolecule occurs either through covalent or noncovalent attachment. Covalent linkages are more stable but do not occur in relative fast time or in physiological pH range<sup>12</sup>. Functional groups located on the dye form the covalent bond with a reactive group on the particular biomolecule. Noncovalent bonds form a more stable complex with biomolecule, at a faster time either thru ionic, electrostatic, hydrophobic or hydrogen bonding interactions<sup>12</sup>. Straightforward technique like mixing protein and labeling fluorophore produces noncovalent attachment. Cyanine and squarylium dyes are two main classes of dyes used for labeling proteins<sup>13</sup>. The study of using fluorescent molecules and their interaction with proteins has increased knowledge in understanding the intrinsic workings of biological systems. One of the main proteins in study is HSA protein.

#### 1.4 Human Serum Albumin

Human serum albumin (HSA) is the most abundant plasma carrier protein (35-59 g/L human serum), which has molecular weight of 66.5 kDa<sup>14</sup>. Synthesized in the liver and produces roughly 10-15 g daily, HSA has an average half-life of 19 days. HSA has multiple functions that range from bilirubin binding, metabolism of lipids through solubilization of long chain fatty acids and binding of therapeutic agents<sup>15</sup>. It is the ability of using HSA as a transport molecule for delivering agents like penicillin, sulfonamids, indole compounds and others that makes it attractive for drug delivery<sup>16</sup>. HSA bound to fluorophore has been used for cancer tumor detection<sup>17</sup>. Along with quantity and reversibly binding ability, it is also one of the smallest proteins in the blood plasma, which in part explains its effectiveness as transporters of metabolic and therapeutic compounds<sup>15</sup>. Research shows that HSA has two primary hydrophobic pockets, and sites labeled I and II, allocated as the binding sites.

HSA is made of 534 amino acid residues with three similar homologous, majority helical domains (marked I, II, III), and within each domain, contains two subdomains labeled A and B<sup>16</sup>. In



subdomain IIA, and IIIA are two main binding sites. Subdomain IIA, and IIIA have deep hydrophobic pockets with positively charged compounds located at the entrances, while subdomain IA hydrophobic core has no known pocket<sup>18</sup>. Within subdomain IIA is site I, also referred to as warfarin site, which is considered to be the most popular drug binding site. Drug or molecules that bind to this site tend to be bulky heterocyclic anions with a charge located in the center of the molecule<sup>(19,20)</sup>. Site I is understood to bind multiple ligands or molecules which give it a large flexible area or region with multiple overlapping sites. Early studies suggest that lysine is involved in warfarin binding to HSA<sup>(21,22)</sup>.

The other binding location, site II, located in subdomain IIIA, known to prefer aromatic carboxylic acids with negative charge on the end of molecule<sup>23</sup>. HSA interaction with negatively charged bilirubin has shown electrostatic interaction between positively charged amino acid of HSA molecule. The negative charge from oxygen atom of bilirubin is believed to interact with positive charged lysine and arginine amino acids<sup>23</sup>. Lysine is thought to have high affinity for bilirubin located at the binding site. Research was conducted to show through acetylation of lysine, bilirubin affinity was greatly reduced<sup>24</sup>. The presence of lysine in binding site II is located in loop 4 of subdomain IIA<sup>25</sup>. HSA, bilirubin binding is assumed to form salt bridges that occur between carboxyl group of bilirubin with the  $\epsilon$ -NH<sub>2</sub> group of lysine<sup>26</sup>. These interactions have led to believe that lysine 195, and 199 are charged while lys 190 is neutral<sup>27</sup>.

The ability of fluorescent dyes to bind HSA can occur through hydrophobic interaction for labeling purposes. This has attracted considerable attention especially in HSA complexation interaction with small hydrophobic molecules. When covalent binding takes place, there isn't much conformational change that occurs to the entire HSA molecule but it is believed that when HSA non-covalently binds through hydrophobic interaction, conformational change takes place<sup>28</sup>. By studying the interactions of positively charged amino acids found in HSA molecule, can lead to

understanding the complex that's formed. If a dye has affinity for specific amino acid, we can possible predict conformational changes that occur due to positioning of amino acids in HSA molecule. Or identification of molecules found in active site.

The importance of studying the interaction between protein and fluorescent dyes has led to the investigation of individual amino acids by NIR spectroscopy. The significance of studying charged amino acid is due to their presence in the entrance and in actual binding sites of HSA hydrophobic pocket<sup>29</sup>. The importance of this study is conducted to investigate key amino acids found at active site in HSA used for drug binding. The participation of lysine residues of HSA with bilirubin occurs through ion pair, which has been researched, but the interaction of lysine and other positively charged amino acids individually with fluorescent dyes have yet to be investigated.

### 1.5 NIR Fluorescent Probe

Research for improving already existing NIR fluorescent techniques for in-vivo imaging is always in demand. Increasing efficiency and detect ability for deep penetrating live tissue imaging can lead to enhanced understanding to target and combat diseases. These techniques can range from cancer imaging to cardiovascular research<sup>29</sup>. NIR fluorescent dyes are used as probes for detection due to their optical abilities. A good biological NIR probe must be stable over a range of pH values (due to observed pH changes in diseased tissue), show no phototoxic effects, and have strong excitation and fluorescence emission in NIR range<sup>30</sup>. Due to those characteristics as well as having low auto-fluorescence, NIR dyes are attractive agents for biological probes<sup>31</sup>. The use of probes can be classified based on their method of use, non-specific, targeted and activatable probes<sup>32</sup>.

There are several difficulties that could prevent probes from arriving at their desired target tissue. Some of the well-researched barriers like epithelial, endothelial and blood brain barriers<sup>33</sup> can provide problems to the probes based on the location of the target. Damage of barriers can lead to non-specific probes reaching the tissue compartment due to leakage<sup>32</sup>. Even though this gives an direct application for non-specific probes, it can imply that targeted and activatable probes would not be completely site specific and error arises from leakage<sup>(32,34)</sup>. Therefore increasing selectivity for tissue specific probes is an area that's continues to be researched for improvement. Developing better affinity for probes-receptor binding is measured through target-to-background noise ratio. Increasing sensitivity and specificity of the probe can increase the target-to background ratio, which can be done through adding different substituent's on the dye molecule<sup>34</sup>.

Non-specific fluorescent probes are fluorescent dyes that have ability to show differences between healthy and diseased tissue<sup>32</sup>. They are able to find alterations in vascular permeability and perfusion, while having low target-to-background ratio, due to their non specific functionality<sup>34</sup>. While target probes have higher target-to-background ratio, and give a better insight on biological and patho-physiological properties of tissues<sup>35</sup>. That's because they have targeting biopolymerlike antibodies, and peptides attached to them to increases their specificity. The more affinity the biopolymer has for the receptor the higher the signal to noise ratio<sup>32</sup>. Cyanine dyes are good NIR fluorescent dyes when used with biopolymer able to form targeted fluorescent probes. Activatable probes are closely attached NIR fluorescent dyes that are initially quenched, but upon enyme-mediated cleavage dye molecules are freed and fluorescence emission is gained<sup>32</sup>. The advantage of these types of probes is that the quenched state has minimal to none noise to background ratio, thus the fluorescence signal cannot be detected which increases its accuracy.

## 1.6 Cyanine Dyes

Cyanine dyes are fluorescent derivatives that have the ability to be used for a wide range of applications from spectral sensitization of photographic emulsion<sup>36</sup> to wideband semiconductor<sup>37</sup> to nonlinear optical materials<sup>38</sup>. Due to their capacity of absorbing light in the visible/NIR region (380-1100nm) cyanine dyes also have the capability of color imaging in biological systems. Cyanine dyes mainly consist of two heterocyclic unit connected by a polyene chain<sup>39</sup>. Within one of the heterocyclic ring there contains at least one cationic molecule. By every vinyl ( $\text{CH}=\text{CH}$ ) that is increased on the polyene chain, this can cause a bathochromic shift by 100nm. Cyanine dyes have tendency to self-aggregate in aqueous solution. Due to distinctive characteristic and wide range of application J-aggregation receives added interest. J-aggregation (bathochromic shift) occurs in assorted conditions by adding salts, surfactants, polymers, polyelectrolytes and addition of a meso-substituent<sup>40</sup>. The meso position is located at the central position of the polyene chain, which can result in an increased bathochromic shift. The size and shape of the group at meso position has a direct effect in aggregation of the dye<sup>39</sup>. It's because of these special characteristics that cyanine dyes are preferably used for bio-imaging.

Cyanine dyes make good fluorescent probes due to their large molar extinction coefficients, high quantum yield and adjustable wavelength. One of main disadvantages using cyanine dyes is their poor solubility in water. Increasing solubility of these cyanine dyes has led to many synthesized derivatives. Cyanine dyes like cy5.5 and cy7 are well known and used target probes for *in vivo* imaging<sup>41</sup>. Cyanine dyes are able to attach to a lysine residue of the biopolymer. These classes of MHI dyes are positively charged that absorb in NIR region. MHI-36, a cyanine dye with good fluorescent and good solubility in hydrophobic solvent, was used in this research to show possible selectivity for positively charged residues.

Amino acids are the building blocks of biological systems. They join together to make up proteins, enzymes and other elements and molecules. We wanted to look at amino acids either commonly found in active site of HSA or ones used in applications, to view their interactions with NIR dyes individually. There might be some relation between dye-protein and dye-amino acid complex formation. All reactions were preformed in physiological pH environment; therefore we can propose that all negatively charged amino acids will have a protonated side chain and treat them as similar uncharged amino acid.

The aim of was to better understand dye-protein complex by examining hydrophobic interaction that takes place with amino acids. The overall goal is to assess results from positively charged amino acid and cyanine dye.

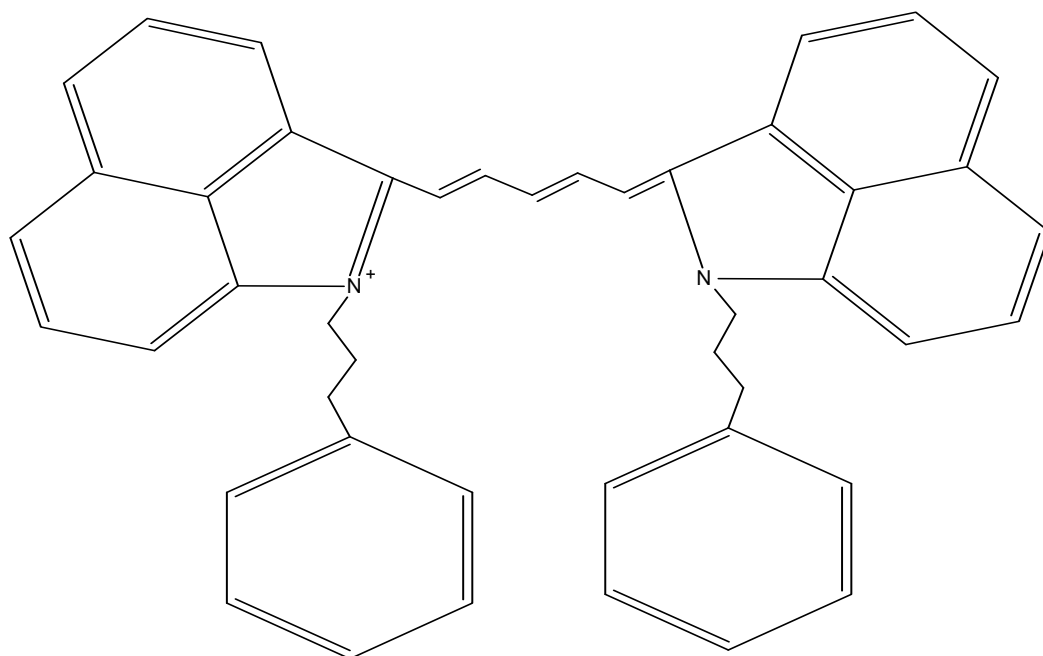


Figure 1: MHI-36 Dye. Two phenol groups attached to the propyl group.

## 2. INSTRUMENTATION

Absorption measurement was taken using Perkin-Elmer Lambda UV/VIS/NIR (Lambda 50) Spectrophotometer (Norwalk, CT). Measurements were taking using 1 cm quartz cuvette (VWR, Suwanee, GA, USA). The pH was calibrated using Orion Model 210.

## 3. EXPERIMENTAL

A stock solution of MHI-36 at 2.0mM concentration made by dissolving appropriate calculated mass in methanol HPLC grade from Aldrich Chemical Company (Milwaukee, WI). All stock solution was kept in refrigerator in the dark. Solution of L-Lysine (Sigma Chemical Company, St.Louis, MO 63178, lot #52H5616), D-Arginine (Aldrich Chemical Company, Milwaukee, WI 53201), L-Leucine (Sigma Chemical Company St Louis, MO 63178, lot# 62H0243) and Sodium Acetate (Fisher Scientific ACS Certified lot # 930631A Fair Lawn Neew Jersey 07410) were made by dissolving appropriate calculated amount in Phosphate buffer. Phosphate buffer was prepared mixing sodium phosphate monobasic (Fisher Scientific ACS Certified lot# 792465) and sodium phosphate dibasic (EM Science 480 S. Democrat Rd. Gibbstown, NJ 088027 CAS 7558-79-4) and adjust the pH to 7.2. Nanopure water was used (Bernard Model D4751 ultrapure water system).

## 4. RESULT

### 4.1 Concentration difference of MHI-36 with Amino Acid

The spectral characteristics of MHI-36 dye in the presence of lysine shows spectral changes different compared to the other compounds reacted with the dye at given concentration. Figure 4.1 illustrates the excitation spectrums obtained from dye without lysine then at 50mM, 150mM and 300mM concentrations. As the concentration of lysine increases, not only does the shoulder peak at 750nm region increase, but the twin peaks around the 640nm and 660nm region also increase.

Initially at no lysine present the peak at 640nm region shows lower absorption than at the 660nm region. As the concentration of lysine increases, absorption of the overall spectrum increases. In the 640nm and 660nm region both show equal peak intensity at 300mM concentration of lysine. The peak at 640nm is believed to be an h-aggregate since it increases as lysine in solution increases, and the peak at 660nm is the monomer peak.

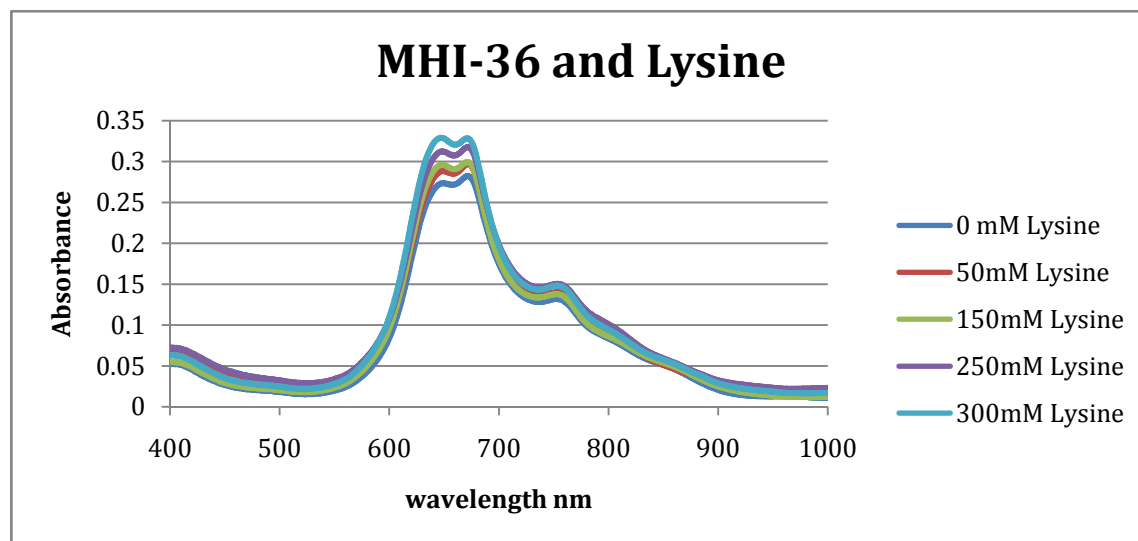


Figure 4.1: Absorption Spectrum  $2.0 \times 10^{-3}$  M Concentration MHI-36 dye, with Lysine in pH 7.2 Phosphate Buffer. Lysine concentration is at  $1 \times 10^{-4}$  M.

The side chain of arginine and lysine are similar in structure and charge, a quick glance at Figure 4.2 shows that the peak intensity and overall structure is similar to Figure 4.1 above. But as the concentration of arginine increases, the intensity decreases drastically, (at 50mM of arginine, it is believed that it has already quenched) this explains why as the concentration increases the peak intensity decreases, increasing the concentration of arginine will have no more further visible or obvious effect on the dye. In fact it shows to have an inverse and repulses the charged amino acid. The peak of arginine at a concentration of 50mM in Figure 4.2 is identical to 300mM of lysine in Figure 4.1. Can this be caused by the side chain charge difference between lysine and arginine? The side chain of arginine has a pKa of 12.48, which is higher than that of lysine at 10.79.

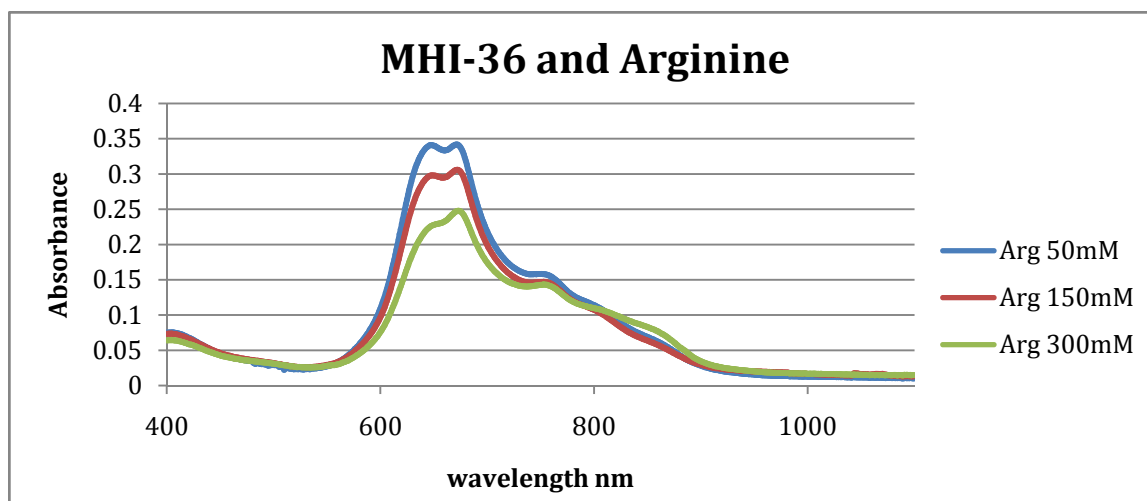


Figure 4.2: Absorption Spectrum  $2.0 \times 10^{-3}$  M Concentration MHI-36 Dye with Arginine in pH 7.2 Phosphate Buffer. Arginine concentration is at  $1 \times 10^{-4}$  mM.

The absorbance spectrum of arginine, leucine and lysine are overlaid at 50mM and 300mM in Figures 4.3 and 4.4 respectively. The interaction of leucine with the dye shown below is similar to arginine at both concentrations. Therefore the theory that the side chain pKa has a binding affect on the dye can be disregarded. At 300mM, lysine shows a slightly stronger selectivity compared to both arginine and leucine.

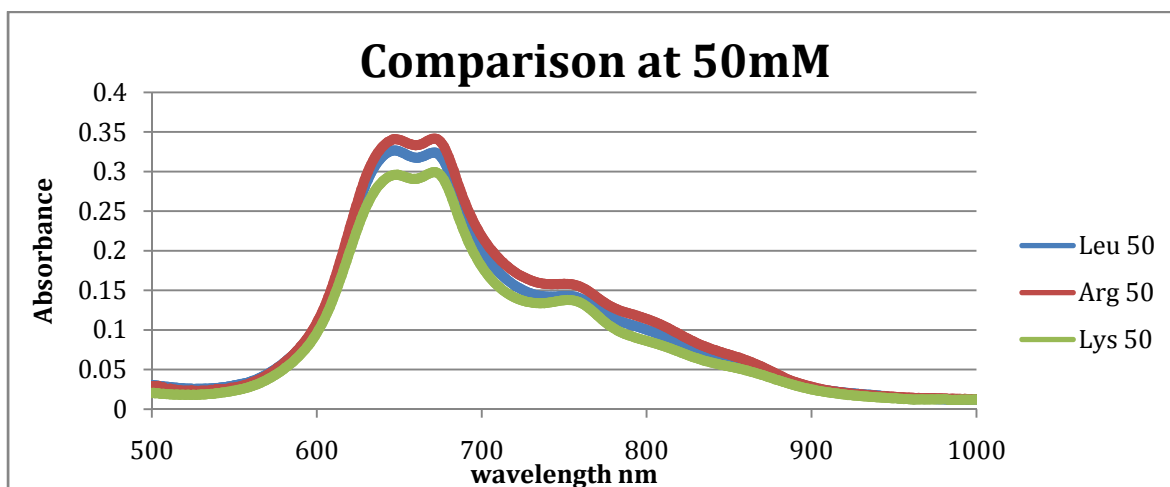


Figure 4.3: Absorption Spectrum comparison  $2.0 \times 10^{-3}$  M Concentration MHI-36 Dye with Arginine, Leucine and Lysine at 50mM Concentration in pH 7.2 Phosphate Buffer. Amino Acid concentration is at  $1 \times 10^{-4}$  mM.



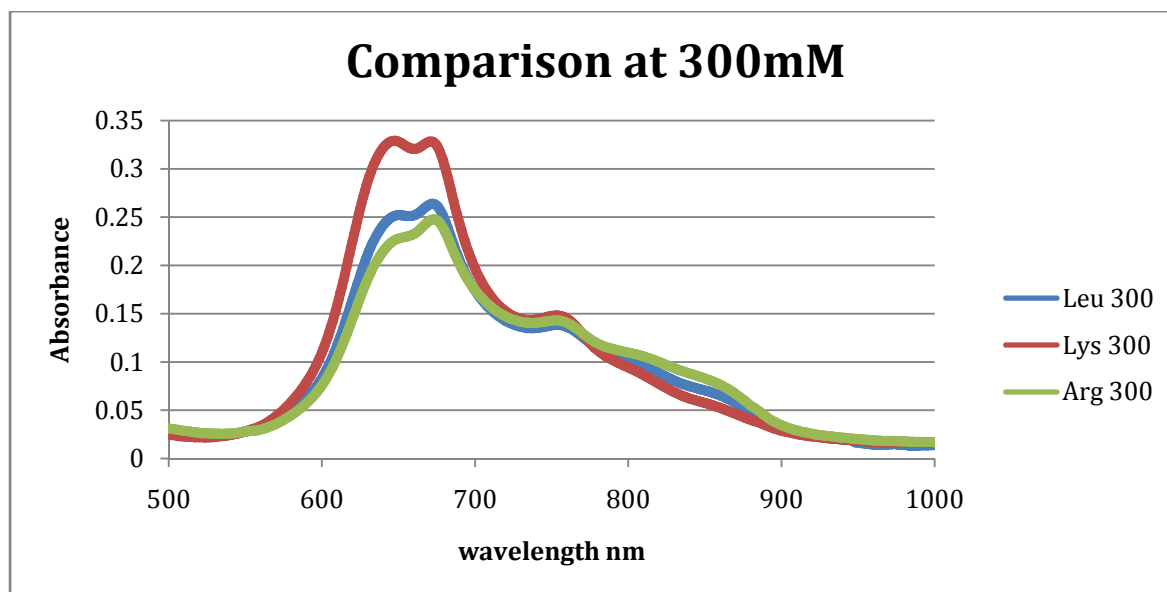


Figure 4.4: Absorption Spectrum comparison  $2.0 \times 10^{-3}$  M Concentration MHI-36 Dye with Arginine, Leucine and Lysine at 300mM Concentration in pH 7.2 Phosphate Buffer. Amino Acid concentration is at  $1 \times 10^{-4}$  mM.

After different amino acids interact with the dye, Figures 4.5 and 4.6 shows the dye when reacted with sodium acetate and valeric acid. If interaction with lysine and the dye forms a salt bridge, then the data will show sodium acetate reacting with the dye, in similar manner to lysine. Valeric acid does not have an amine group, but has the same length identical carbon chain group as lysine's side chain. The interaction of the dye with valeric acid is seen at a lower intensity, which could be due to the lack of nitrogen group.

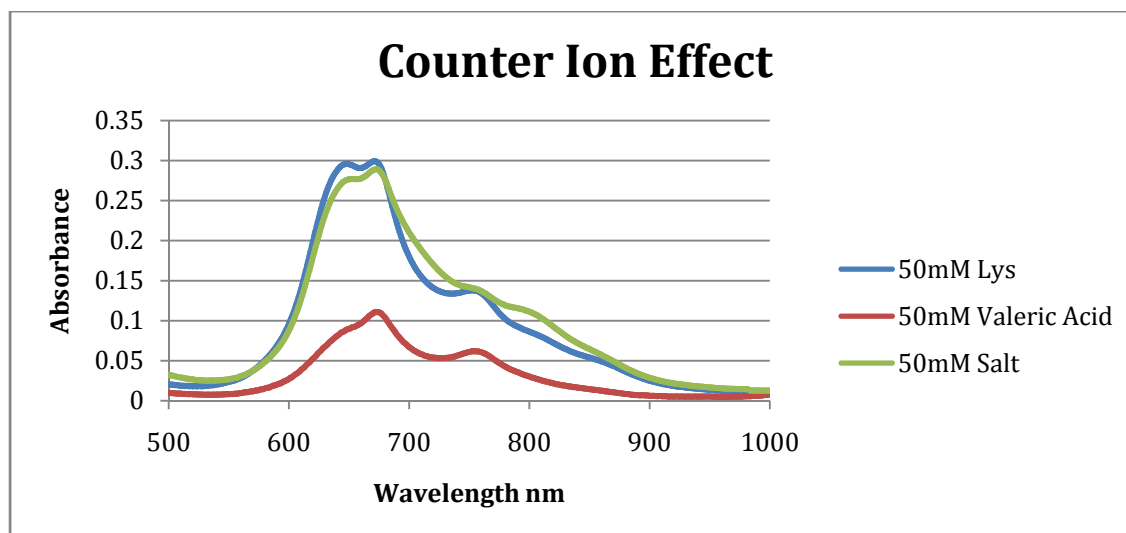


Figure 4.5: Absorption Spectrum  $2.0 \times 10^{-3}$  M Concentration MHI-36 Dye with Salt(Sodium Acetate), Lysine and Valeric Acid at 50mM Concentration in pH 7.2 Phosphate Buffer. Lysine, Valeric Acid and Salt concentration  $1 \times 10^{-4}$ M.

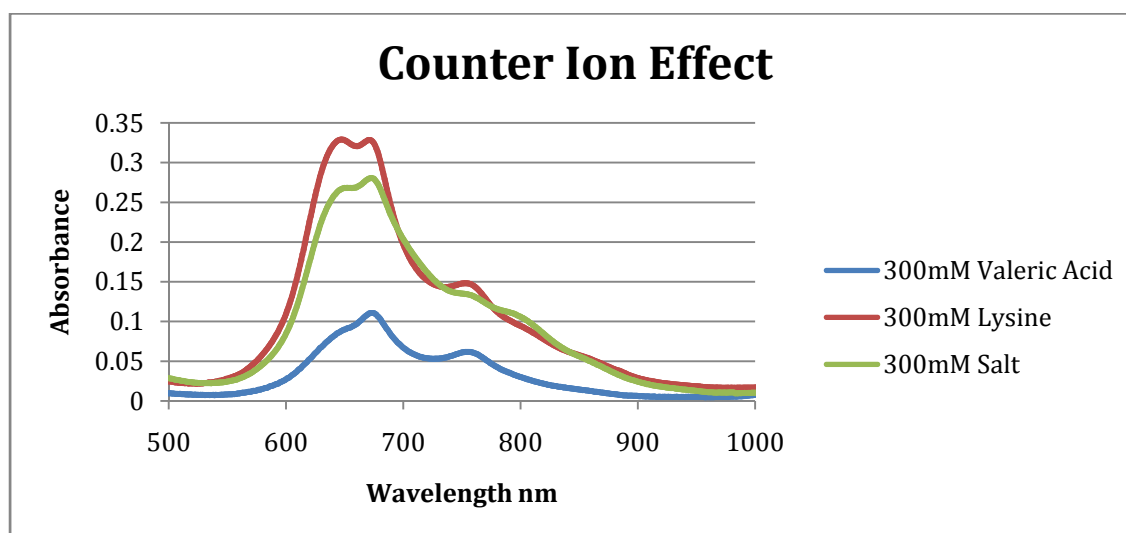


Figure 4.6: Absorption Spectrum  $2.0 \times 10^{-3}$  M Concentration MHI-36 Dye with Salt(Sodium Acetate), Lysine and Valeric Acid at 300mM Concentration in pH 7.2 Phosphate Buffer. Lysine, Valeric Acid and Salt concentration  $1 \times 10^{-4}$ M

At both concentrations the salt does not show the shoulder peak in the 740nm region. At 50nm the peak intensity of the salt is similar to lysine. Even though it does have two similar peaks similar to lysine in the 640 and 660nm region, the peak intensity of the 640nm does not increase as

the concentration increases. Therefore came to the conclusion that lysine interaction is not likely to be a 'salting out' effect. Although actual binding specifics are not known at this time, I presume the amino acid interacts with phenyl side chain attached to the charged nitrogen. Since the salt peaks are almost similar to lysine in 640 and 660nm range, my belief is one of the nitrogen atoms of lysine interact with the dye side chain. There showed no shifts in spectrum from amino acid interaction with the dye. The absence of the shoulder peak in 740nm area with the salt is present in valeric acid spectrum, which implies possible involvement of c-terminal carbon. Therefore one can assume the side chain involvement in binding process. Since analyzing leucine and arginine absorption spectrums and possibly eliminating the theory of side chain pK participation, we can speculate the backbone nitrogen not the side chain nitrogen is bound to the dye.

The following data collected shows the interaction of amino acids with MHI-36 dye at a lower concentration of the dye. When dye concentration decreases aggregation pattern changes as expected. The same characteristic of increasing shoulder peak at 740nm and monomer at 660nm are present, while aggregate peak at 640nm increases proportionally with concentration. Even at lower dye concentration, lysine spectrum still shows distinguished peaks as concentration increases, while arginine spectrum shows the dye to be quenched at 50mM. At lower dye concentration it's believed that one of the nitrogen on arginine side chain could be involved in binding process. At  $1 \times 10^{-4}$  M dye concentration less dye is in solution, therefore the guanidinium group of arginine could act as scavenger looking to align near the phenyl side chain bound to the dye. Therefore increasing arginine concentration leads to no visible change in absorption spectrum, which could explain arginine interaction at lower dye concentration.

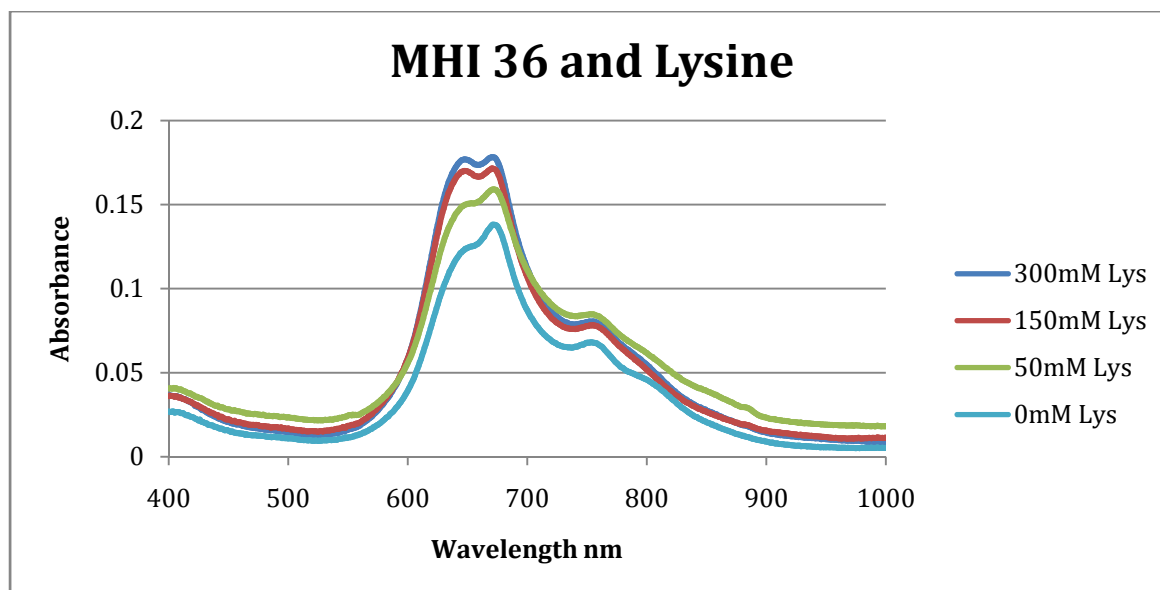


Figure 4.7: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with Lysine, at increasing Concentration in pH 7.2 Phosphate Buffer. Lysine concentration  $1 \times 10^{-4}$  M.

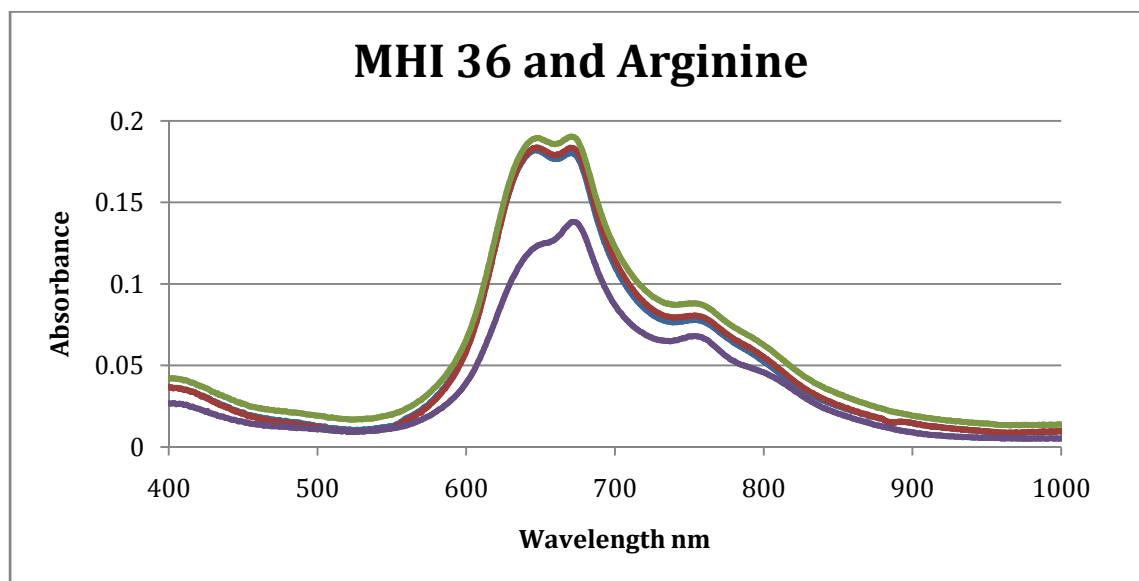


Figure 4.8: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with Arginine, at increasing Concentration in pH 7.2 Phosphate Buffer. Arginine concentration  $1 \times 10^{-4}$  M.

#### 4.2 Selectivity of Amino Acid in Solution

This experiment, analyzing selectivity of amino acid, while using MHI-06 dye was conducted by mixing positive and neutral charged amino acid. The Figure 4.9 and 4.10 below shows the spectrum of lysine and leucine in mixture together as concentration of both varies. Figure 4.9 displays concentration of lysine increasing while leucine is held constant. The spectrum shows directly as ratio of lysine increase peak height increase. It is distinguishably visible in Figure 4.9, that as the ratio of lysine is increased in solution the peak height increases therefore it's believed more unbound dye can bind to lysine. Now in Figure 4.10 the concentration of leucine increases while holding lysine constant is shown. When ratio of 1:3 and 1:12 of lysine to leucine are compared their peaks overlap, which confirms that as more leucine is present in solution, there is no visible difference. There was no observed shift in overall spectrums but an increase of peak height according to increase of lysine concentration could indicate unbound MHI-06 binds stronger and more selective towards the charged lysine over leucine. Increasing the presence of positive ion can promote monomer complex formation. Aggregate formation was also noticeably lower when figure 4.10 was compared to figure 4.9, which showed increasing concentration of non-charged amino acid did not have same effect as increasing positively charged amino acid.

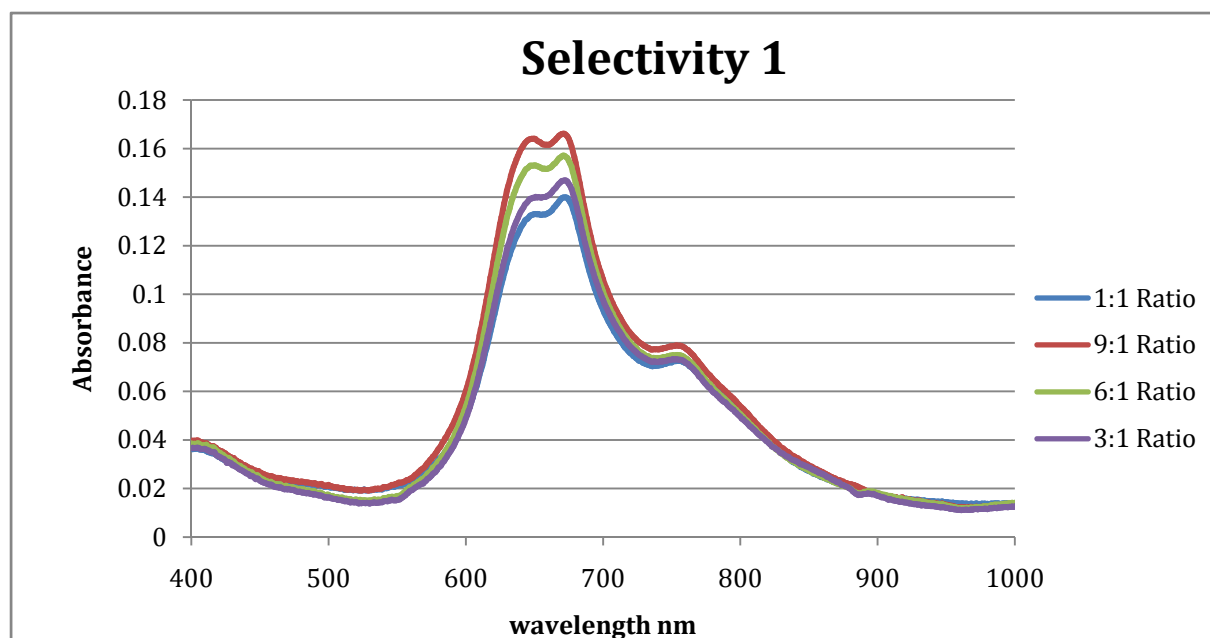


Figure 4.9: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with Lysine and Leucine in solution (mixture 1) as concentration of lysine is increased while leucine is held constant at pH 7.2 Phosphate Buffer. Amino Acid concentration  $1 \times 10^{-4}$  M

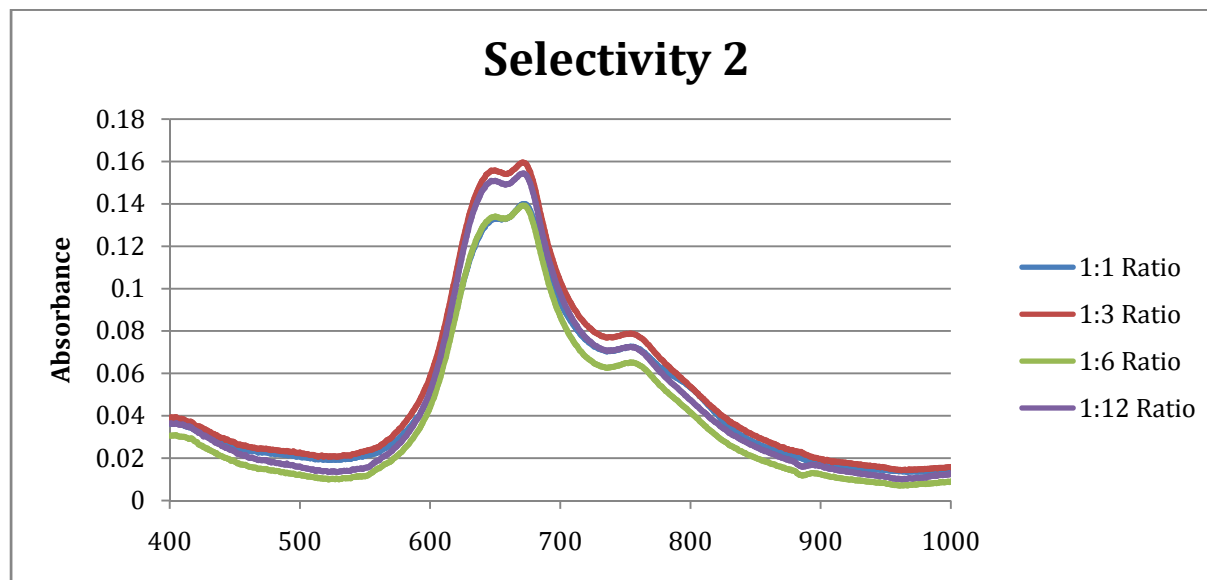


Figure 4.10: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with Lysine and Leucine in solution (mixture 2) as concentration of leucine is increased while lysine is held constant, at pH 7.2 Phosphate Buffer. Lysine Leucine concentration  $1 \times 10^{-4}$  M

The same procedure was used for studying lysine and arginine in solution at different concentration ratios. Figure 4.11 shows when the concentration of arginine is held constant while lysine is increased, the overall spectrum increases. It is interesting to note that the ratio of 1:1 and 3:1 (lysine to arginine) overlap while the ratio of 6:1 and 12:1 slightly overlap. This could imply that the dye shows no preference to lysine as concentration is increased. But looking at selectivity 4, as the concentration of arginine increases, the peaks become more distinguishable. This could imply that since arginine has a stronger positive charge due to its side chain, the dye favors a stronger charged amino acid at a lower dye concentration. It was shown earlier that at higher dye concentration lysine showed to have stronger affinity towards the MHI dye. But it's believed that at lower dye concentration there is preference for arginine over lysine, which could be due to charge difference. At a higher dye concentration, only minimal amount of arginine was needed to reach

absorption maximum, therefore the stronger positively charged arginine would be limited at 150 and 300mM because it showed a decrease in peak intensity. Figure 4.11 and 4.12 shows higher absorbance at aggregate peak around 640nm range and monomer peak at 660nm. This could suggest that the presence of positively charged residue could increase aggregate and dye formation. The next set of experiments will examine the charged and non-charged amino acids at equal molar absorptivity.

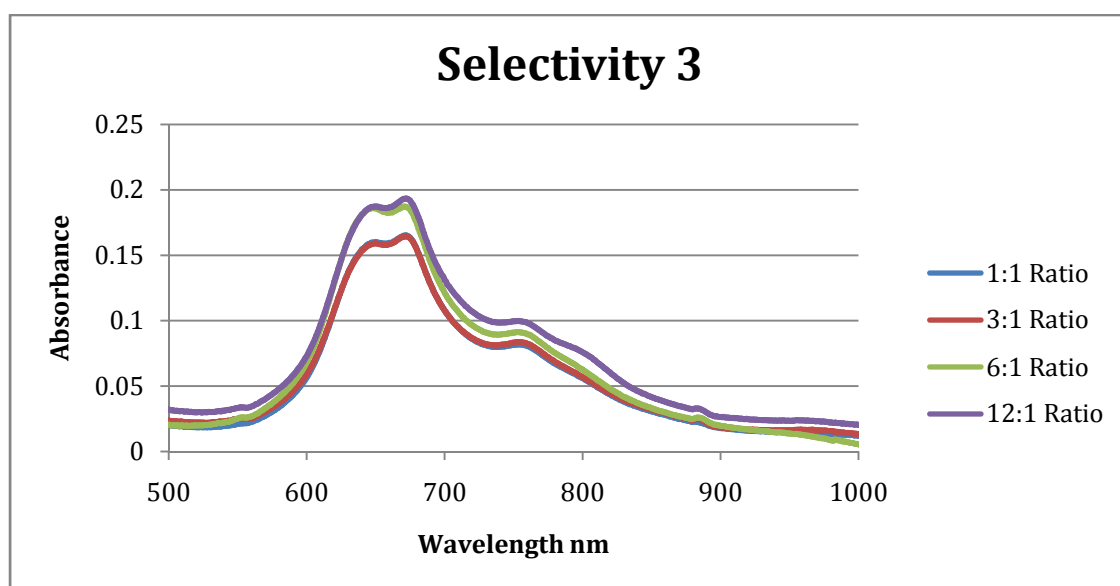


Figure 4.11: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with Lysine and Arginine in solution (mixture 3) as concentration of Lysine is increased while Arginine is held constant, at pH 7.2 Phosphate Buffer. Lysine Arginine concentration  $1 \times 10^{-4}$  M

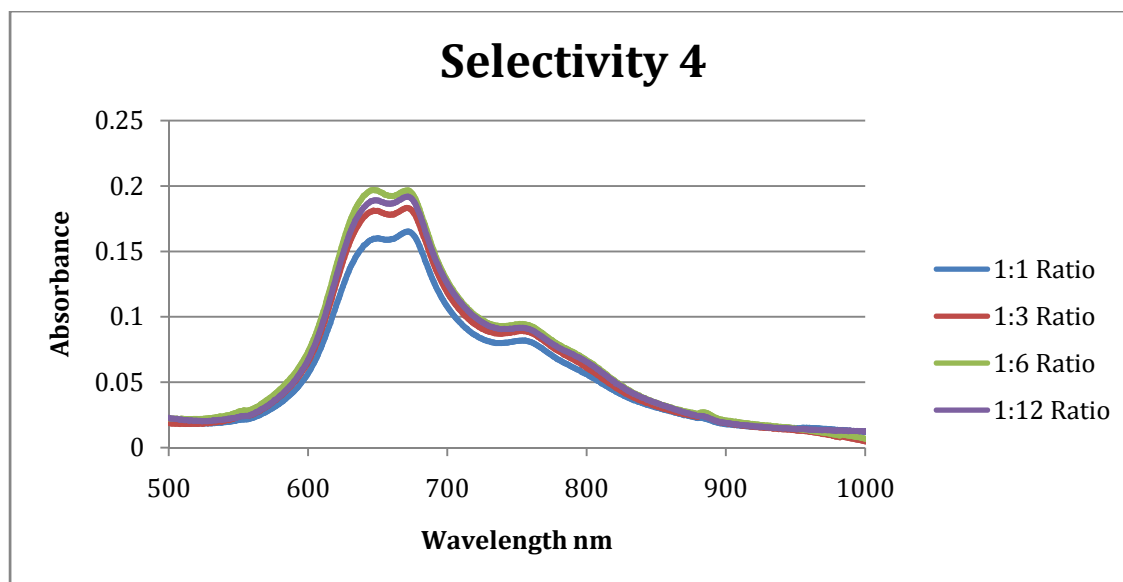


Figure 4.12: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with Lysine and Arginine in solution (mixture 4) as concentration of Arginine is increased while lysine is held constant, at pH 7.2 Phosphate Buffer. Lysine Arginine concentration  $1 \times 10^{-4}$  M

#### 4.3 Effect of Positive Charge and Job Plot

Next experiment focused on setting the total amount of amino acid at 300mM, while varying the individual amount of amino acid to view changes based on charge or structure. Maintaining the total volume constant while increasing a charged amino acid, which is in solution with a non-charged amino acid, will show if charge does play a part in dye binding. The spectrum below, Figure 4.13; shows that as leucine decreases and lysine increase, while keeping total volume of lysine and leucine equal at 300mM, peak height increases. Distinguishable peaks are seen in this spectrum, at all three points: 740nm, 660nm and 640nm, all increase as the more positively charged amino acid is present in solution. The same increase in peak is observed when lysine is substituted with arginine, figure 4.13. Comparing figures 4.13 and 4.14 shows that substituting arginine for lysine has possibly led to decrease in aggregate formation. Possible diminishing of aggregate formation can be accounted for the difference in side chain rigidity between arginine and



lysine. The more flexible side chain of lysine could be more advantageous when in presence of another molecule to motivate aggregate development.

Figure 4.15 shows arginine in solution with histidine. Even in presence of another charged amino acid, and due to the bulky aromatic rings of histidine, arginine was able to show better binding towards the dye. Aggregate and monomer formation is significantly decreased when histidine replaces leucine. It can be seen on figure 4.15 that absorbance intensity is reduced in the presence of histidine. This gathered information insinuates that size of amino acid is possibly involved, but not to the extent of positive charge, for aggregate and monomer complex formation. Jobs plot was preformed to show stoichiometry relationships between dye and amino acid, show in figure 4.16 and 4.17 respectively. Both Jobs plot show 1-1 stoichiometry with the dye.

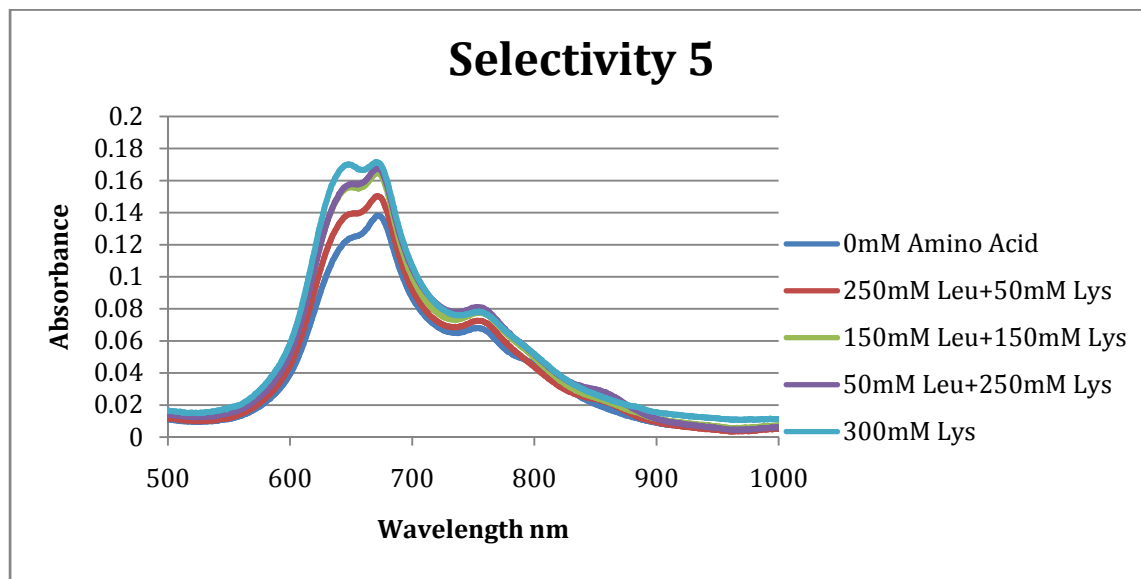


Figure 4.13: Absorption Spectrum  $1 \times 10^{-4}$  M Concentration MHI-36 Dye with total volume of 300 mM Lysine and Leucine in solution as their concentration vary, at pH 7.2 Phosphate Buffer. Lysine and Leucine concentration  $1 \times 10^{-4}$  M

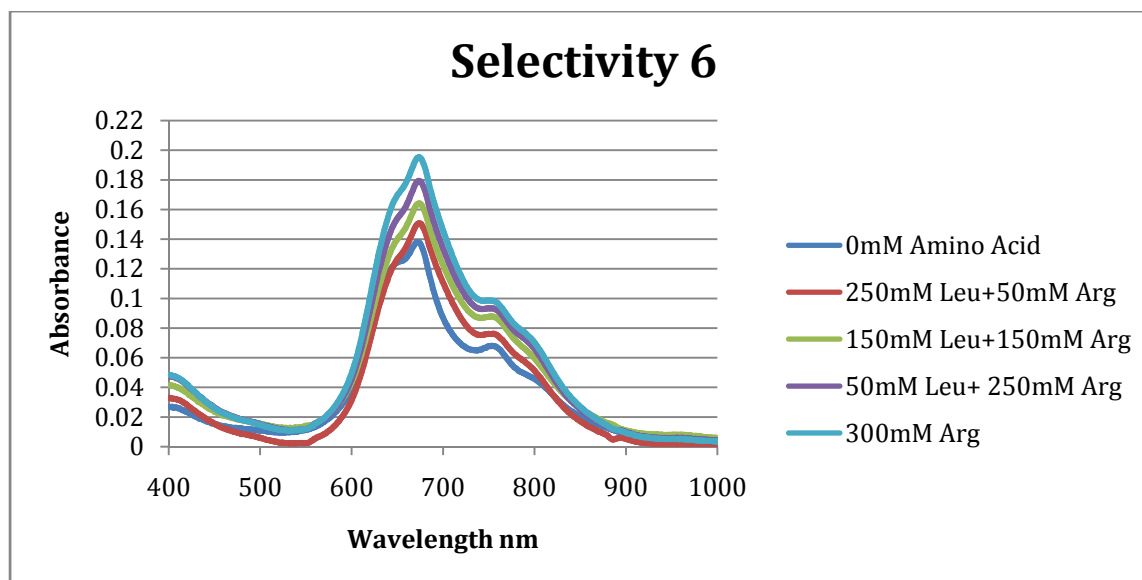


Figure 4.14: Absorption Spectrum 1x10<sup>-4</sup> M Concentration MHI-36 Dye with total volume of 300 mM Arginine and Leucine in solution as their concentration vary, at pH 7.2 Phosphate Buffer. Arginine and Leucine concentration 1x10<sup>-4</sup>M

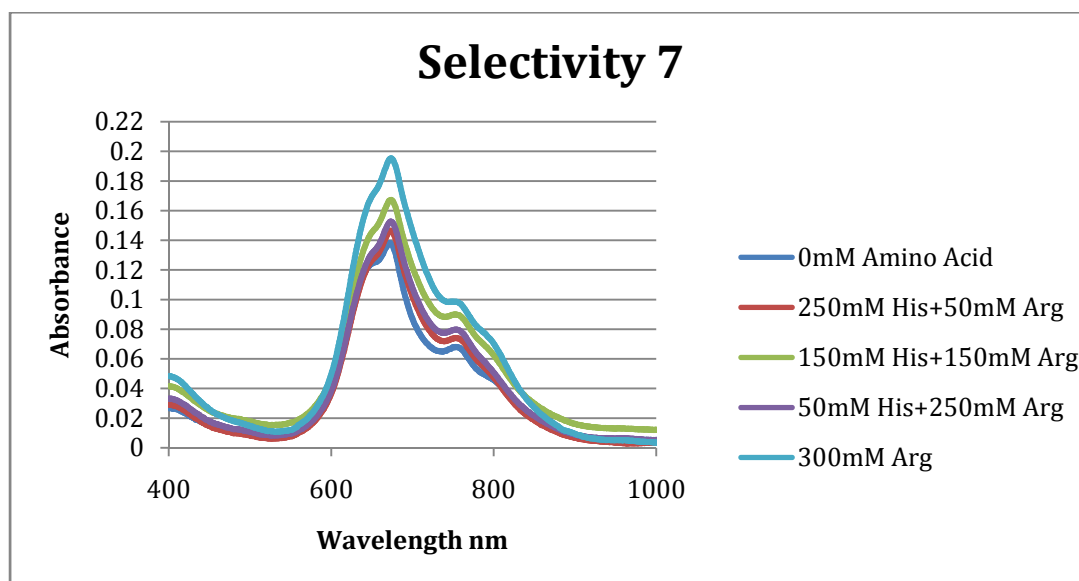


Figure 4.15: Absorption Spectrum 1x10<sup>-4</sup> M Concentration MHI-36 Dye with total volume of 300 mM Histidine and Arginine in solution as their concentration vary, at pH 7.2 Phosphate Buffer. Arginine and Histidine concentration 1x10<sup>-4</sup>M



Figure 4.16: Job Plot of Arginine and Cyanine Dye MHI-36



Figure 4.17: Job Plot of Arginine and Cyanine Dye MHI-36

#### 4.4 Peak Ratio and Aggregation Formation

Data was interpreted to show amino acid increase in solution resulted indye-aggregation breaks up while increasing complex dimerization. Peak ratio of the believed aggregate and monomer formation are taken and plotted versus their concentration values. Peak ratio is

measured by dividing aggregated peak (640nm) over dimer peak (660nm). Figure 4.18 below is taken at higher dye concentration. As the ratio value moves closer to 1, indicates equal amounts of aggregate and dimerization are present. From understanding of protein-dye relations, increasing protein concentration could be cause of aggregation break-up. The same belief could be applied here, as concentration of amino acid increases, the lower the ratio value should become, since more monomer should be present and aggregate is broken up. This is observed for arginine and leucine, but not for lysine. At low lysine concentration (0-100mM) the ratio slightly decreases after its starting position, which might indicate that aggregation formation decreases (I will return to this point later). But as concentration increases so does ratio value as it approaches 1. This trend for lysine gives an unexpected result for aggregation and monomer formation increasing collectively. What is seen for leucine and arginine are ratio value decreasing closer to 0 when their concentration increases. This could be interpreted as less aggregate and more monomer present when concentration of either amino acid is increased. The direction of the peak for arginine and leucine was expected, along with premise that HSA and other protein are able to break up dye aggregation and promote complex formation as their concentration increases.

This same flow is seen when dye concentration changed from  $2.0 \times 10^{-3}$  mM to  $1.0 \times 10^{-4}$  mM as seen in figure 4.19. All three amino acids show similar direction, but their initial slope indicates selectivity the dye has for lysine over arginine and leucine at  $1.0 \times 10^{-4}$  mM concentration. From the 0-100mM concentration range leucine and arginine display similar characteristics and are possibly indistinguishable. But lysine which has a lower slope it easier to differentiate. Lysine displays this rare feature because it's able to form less aggregate at those low concentrations. This assimilation can also be spotted in figure 4.18; lysine is shown to have the lowest slope from 0-100mM concentration. These believed to be unique features of lysine's ability for untimely aggregate/monomer formation different from arginine and leucine, regardless of dye concentration. When dye concentration is at  $1.0 \times 10^{-4}$  mM and lysine concentration is kept below

100mM, it's believed to make less aggregate compared to arginine and leucine. But when dye concentration is at  $2.0 \times 10^{-3}$ M it's presumed that aggregate formation increases when lysine concentration is between 200-300mM. With pH environment at 7.2 it can be assumed that C-terminal carboxylate is protonated, for lysine, leucine and arginine. For lysine the N-terminal nitrogen and side chain are understood to be deprotonated because of their higher pKa's of 9.0 and 10.5 respectively. The same is understood for arginine with similar N-terminal pKa of 9.0 and slightly higher side chain pKa of 12.5. One possible explanation could be the slack sidechain of lysine. The floppy side chain could promote multiple binding with itself, or dye-aggregation.

Since actual binding mechanism is not fully understood, the data collected has led me to believe lysine is able to demonstrate different features at varying concentrations. Even though jobs plot was able to show 1-1 stoichiometry with the dye, it could be deduced that lysine is able to stimulate aggregate formation through multiple-dye lysine multiplex. It is also likely that lysine has greater potential over arginine and leucine to affix itself to dye aggregate structure and demote dye breakup. It's within the realms that at high lysine and dye concentration the  $\pi$ - $\pi$  stacking that occurs with dye aggregation is not affected and possibly encouraged. Perhaps the four carbon lysine side chain adopts multiple conformational positions, which lead to various binding possibilities<sup>42</sup>. It can be imagined that if the conformation takes on position to expose the interior carbons of the side chain, they could attach or influence either for dye aggregate, lysine-dye complex or other lysine residues. This could explain the phenomena of increasing aggregate and monomer formation simultaneously. At low lysine and dye concentrations it could be realistic that more lysine is needed to imitate the aggressive character that is seen in arginine and leucine. It's likely the side chain of lysine discourages aggregate formation due to lack of presence of other lysine molecules in solution. Arginine and leucine both exemplify qualities for monomer and aggregate formation at low concentrations, while lysine does not show this trait, which could be attributed to its floppy side chain. Since less dye molecules are in solution, possible conformational

structure remodeling become useless and discourages aggregate formation. At these low lysine concentrations, it becomes a good candidate for aggregation break up. It's probable that side chain interactions occur which explains why as more lysine is in solution more aggregation is present so therefore unbound lysine could interact with each other. Therefore it can be inferred that lysine is not activated for promoting dye aggregation at low concentrations. It can be concluded that at low dye concentration lysine does not manifest characteristic results similar to what is seen with lysine at higher concentration in fig 4.18.

Possible explanation of the interaction viewed could arise from hydrogen binding seen from amino acid to dye in similar fashion to dye-aggregation. The distinct properties that lysine presents could be due to the side chain equivalent to dye-dye aggregation. The extra nitrogen atoms found in side chain of arginine could change the polarity due to its stronger charge, which lead to the repulsive behavior seen. The nitrogen atom on the side chain of lysine, could be the main difference (none in leucine, extra in arginine) and gives us insight at the sensitivity for aggregation pattern in cyanine dyes.

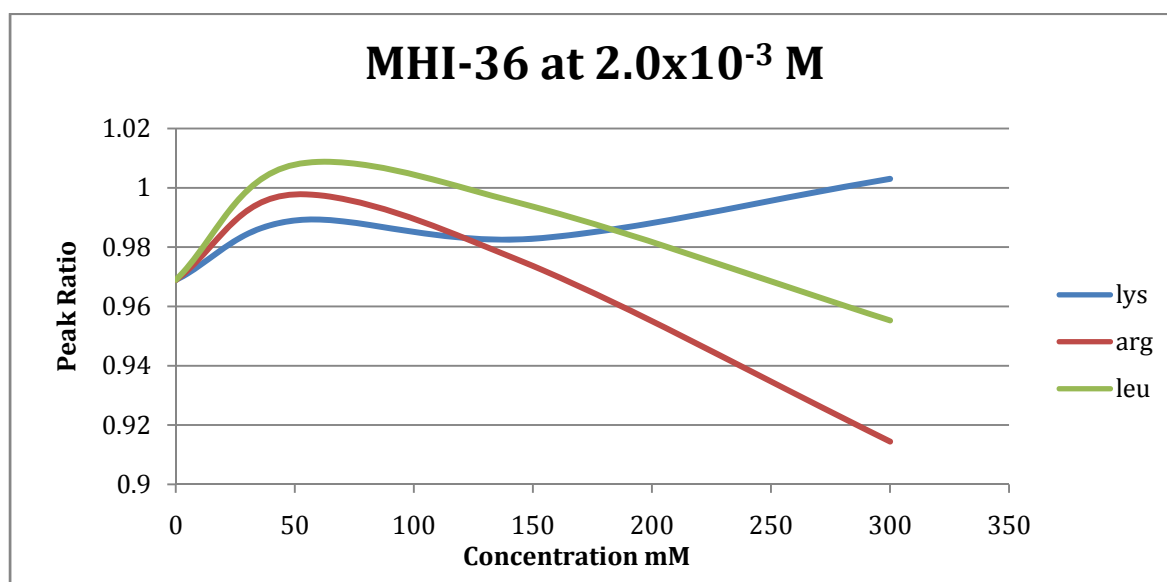


Figure 4.18: Plot of peak ratio versus concentration at  $2.0 \times 10^{-3}$  mM dye concentration and  $1.0 \times 10^{-4}$  M amino acid concentration.

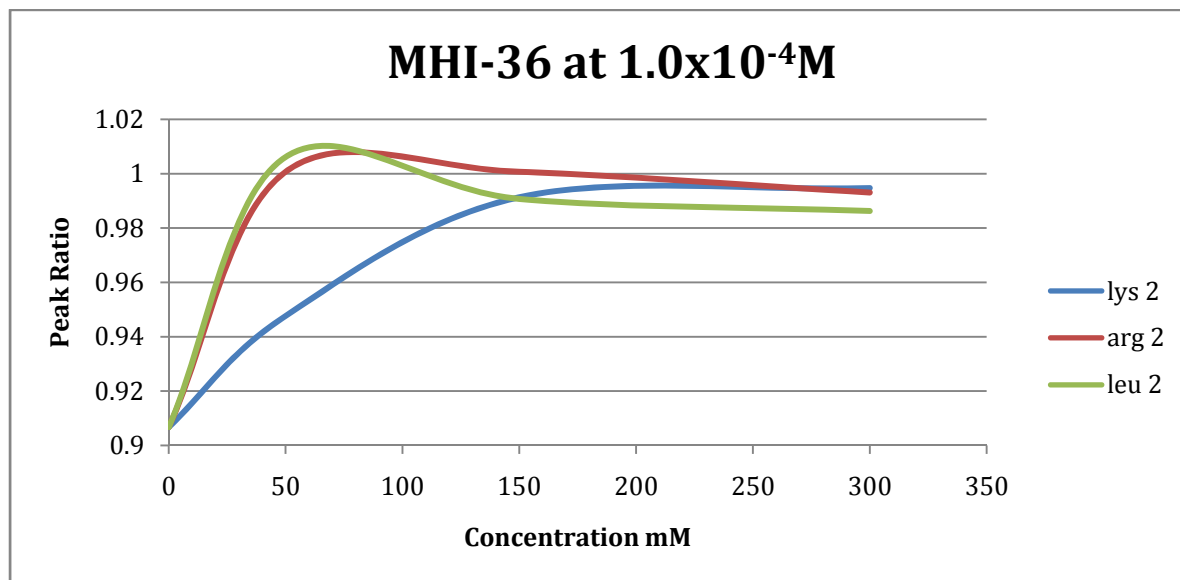


Figure 4.19: Plot of peak ratio versus concentration at  $1.0 \times 10^{-4}$  mM dye concentration and  $1.0 \times 10^{-4}$  M amino acid concentration

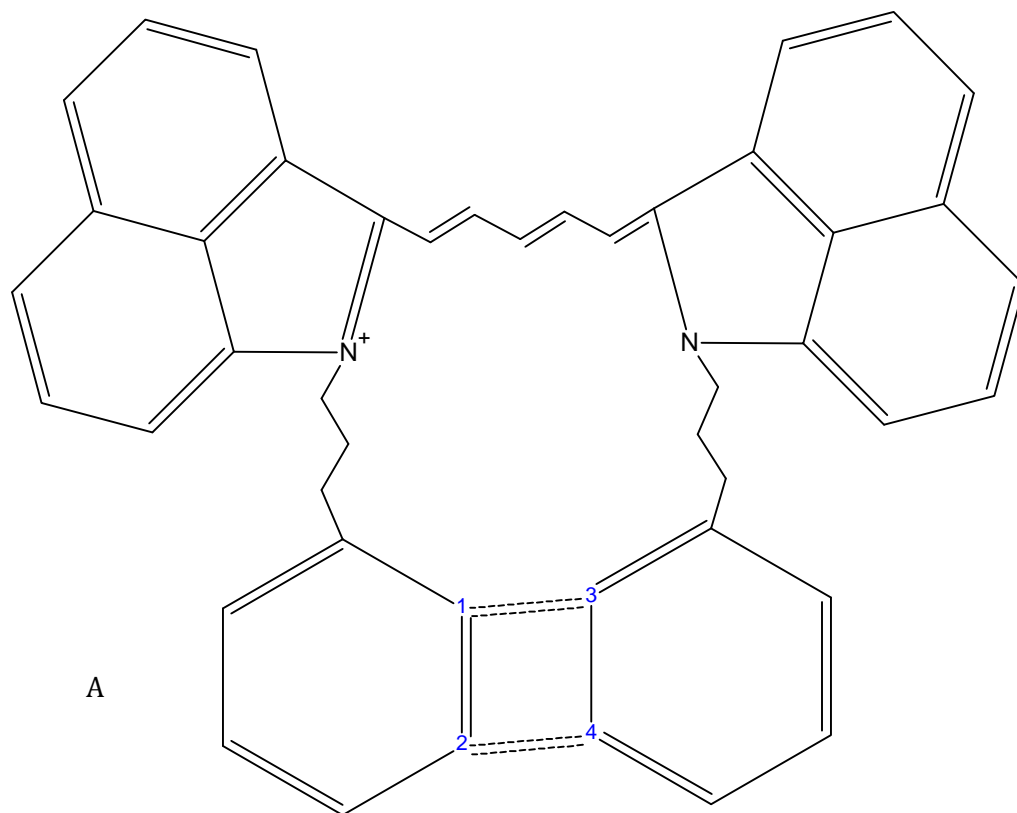
#### 4.5 Computational Derived Intramolecular Aggregation

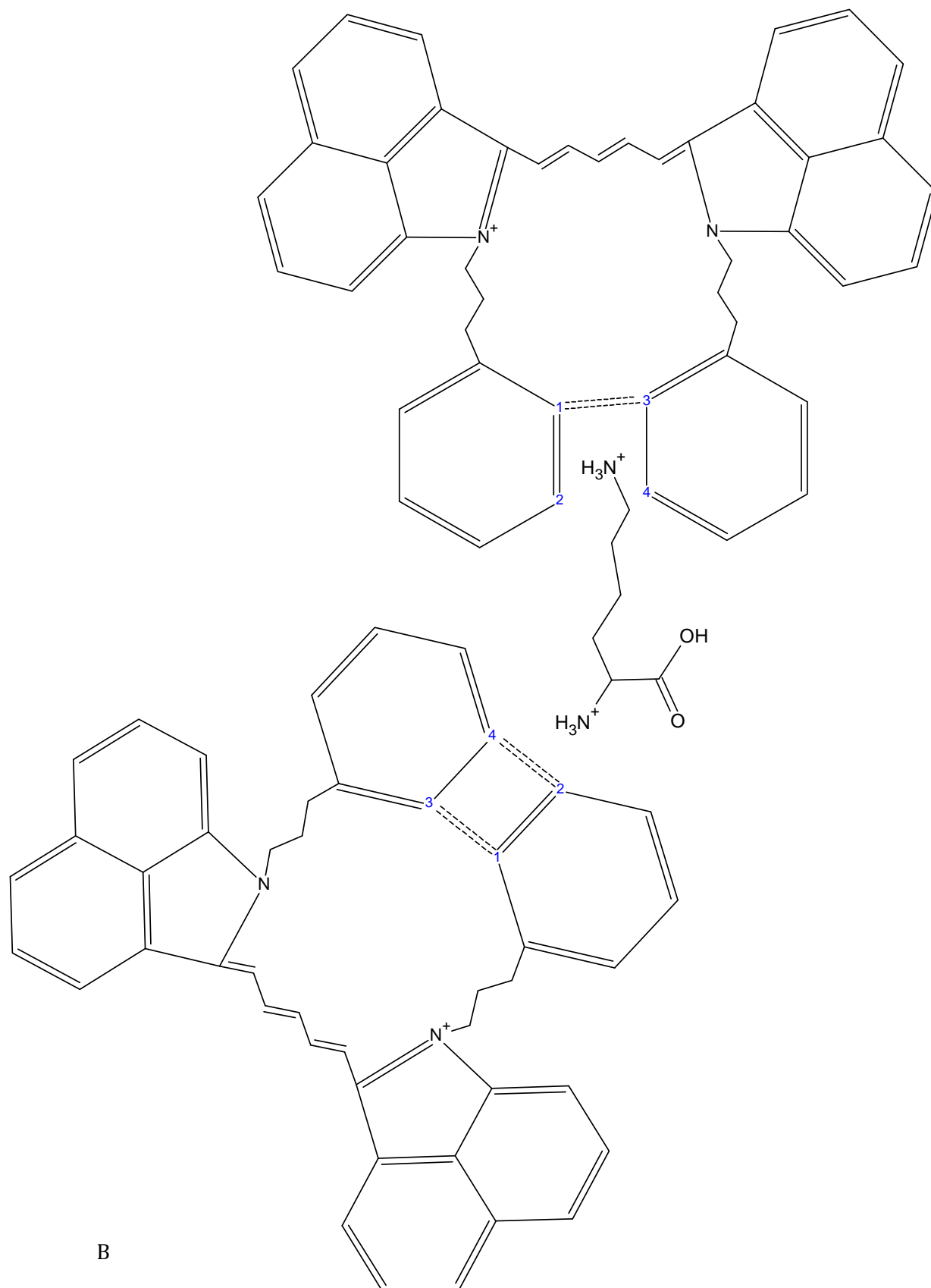
Computational chemistry is a good tool for understanding theoretical orientation of dye molecule. At lowest energy of dye, the alignment the dye presents can be penetrating view of dye in phosphate buffer solution. Furthermore this gives supplementary grasp of viable positioning of amino acid to positively charged cyanine dye. Gaussian view program revealed possible orientation of dye molecule at lowest energy level, which was transferred to chem draw and presented on figure below. Chem-draw was not able to fully display the cis/trans orientation but it captured a main feature, which showed close proximity between the two phenyl groups. Originally the N-ethyl benz[cd] indolieniu groups were believed to place themselves closer due to strong intramolecular forces. But the polymethine chain that connects these big rings retains a planar conformation because of  $sp^2$  hybridized carbons, therefore limiting the molecule movement. The selected carbons (labeled 1, 2, 3, and 4 Figure 4.20 A) of the phenyl groups can position themselves closer

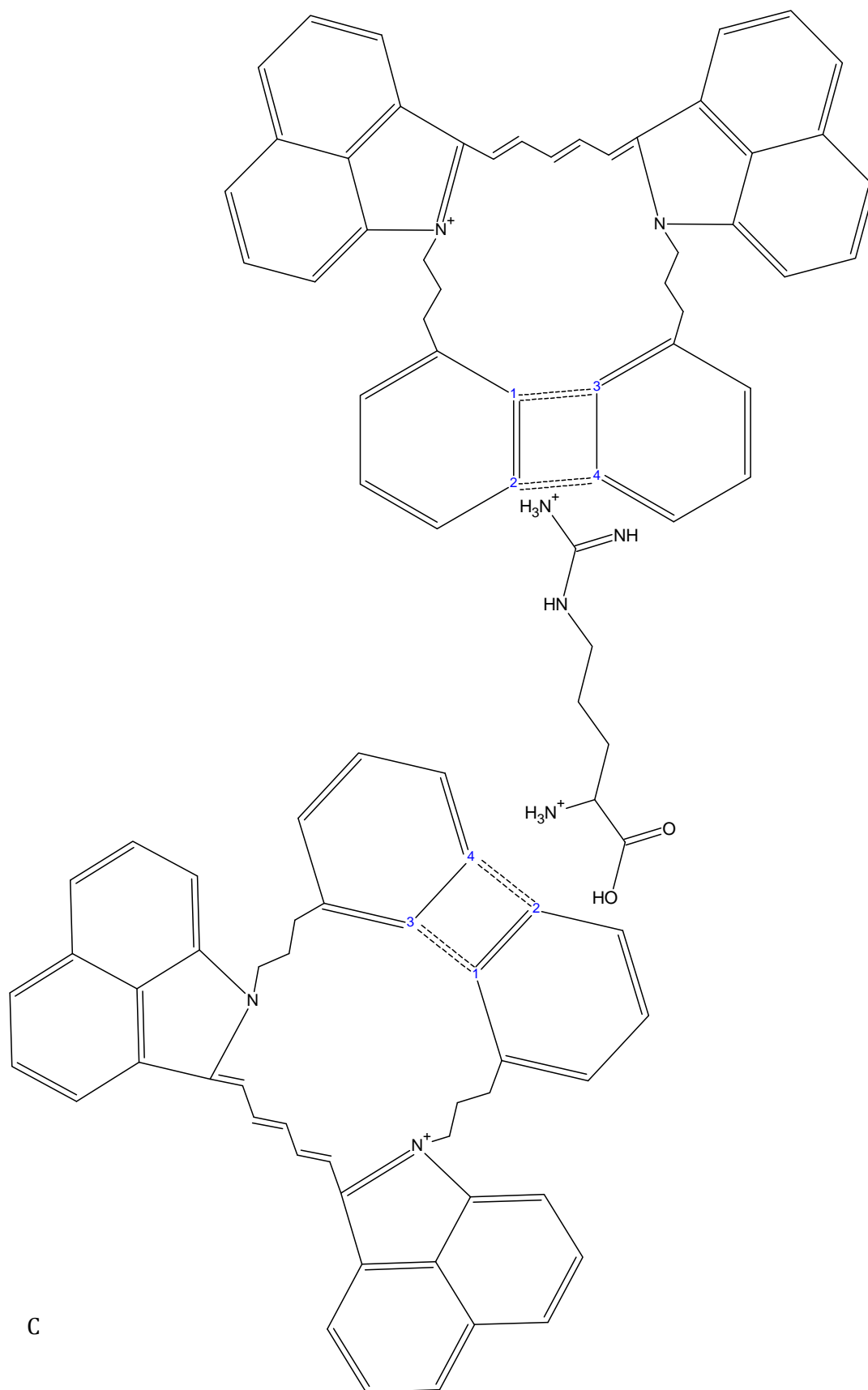
together to form hydrogen bonding. These interactions would change the electron density around those 4 carbons possibly making them more susceptible to variety of interactions. The gathering of electron around that area can increase electron density around those 4 carbons therefore can yield a negative cloud around that environment.

With the incorporation of positive charged residue like lysine, it can position itself around or even in amidst the two phenyl groups, which cause the electron dense region. This premise clarifies the difference distinguished when dye concentration is changed during the experiments. At higher dye concentrations there seemed to be more aggregation present. It's possible the arginine side group bulkiness hinders it to increase aggregation formation and can unsettle the negative environment which leads to disruption of aggregation formation. The ability of the lysine side chain to orientate itself between the two phenyl groups gives it the distinct advantage of not disturbing aggregation formation. It's probable the backbone nitrogen can also align itself close to electron dense environments and be involved in aggregation construction. These illustrations can be seen in Figure 4.20. Intermolecular stacking aggregation is still believed to occur in staircase format.









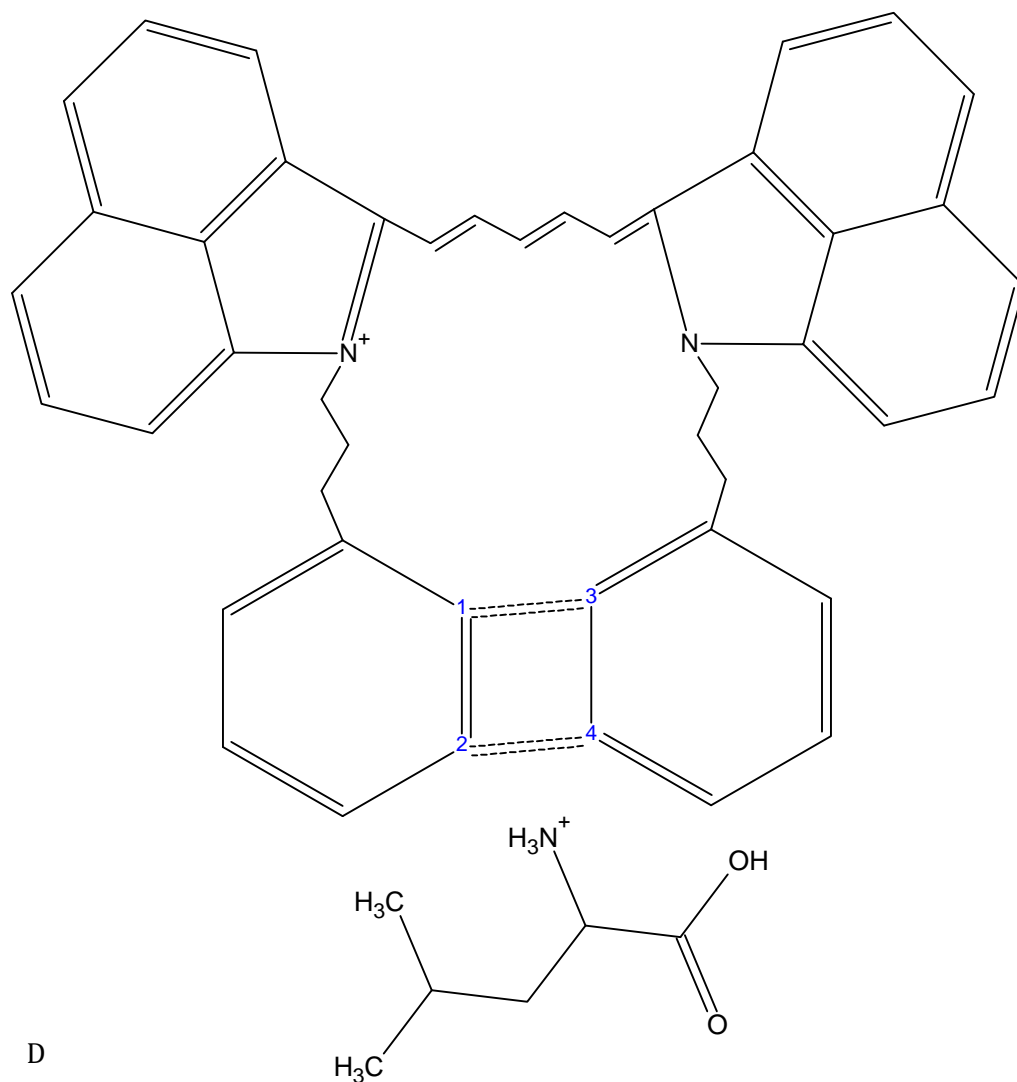


Figure 4.20 Illustration of possible orientation A) intramolecular aggregate B) lysine side chain and backbone nitrogen interaction for possible aggregate formation C) arginine side chain and backbone nitrogen interaction D) leucine backbone nitrogen interaction

## 5. CONCLUSION

With many uses of NIR cyanine dyes provides, new applications and methods are bound to arise. Comparing the reaction the MHI-36 dye had with leucine, lysine and arginine, the dye

showed the selectivity towards positively charged amino acid. The MHI-36 dye shows a high binding affinity for lysine when in solution with the neutral charged amino acid, leucine. Since charged amino acids are found in wide range of proteins, this method of using positively charged NIR dye to increase binding strength through hydrophobic interaction. This information can be applied to further study HSA binding within both known hydrophobic pockets.

The experiments were able to show that aggregation patterns are different at different dye concentrations. At higher dye concentrations the dye showed better reactivity with lysine than arginine. Since lysine's side chain charge is weaker than arginine, at higher dye concentration lysine is believed to be the superior substrate. When the dye concentration is decreased by more than half, arginine is able to react better to the dye. It's believed that as dye concentration decreases, more positively charged compound is needed to reach the absorption maximum. That is the difference viewed between lysine and arginine at a lower dye concentration. A non-charged amino acid leucine, was used in mixture with charged amino acid, lysine or arginine. As charged amino acid increased in solution, peak height increases accordingly. The ratios of charged versus non-charged amino acid were also compared and again peak height increased as charged amino acids increased in ratio. Spectral changes occur not through shift in wavelength but increase in absorption intensities. Jobs plot of arginine and lysine showed one to one stoichiometry with the positively charged MHI dye.

The presence of a charged amino acid is believed to present some of the changes in peak height ratios in the spectra. These changes can be related to dye aggregation, especially at different dye concentration. Increasing charged amino acid has a desired effect believed to cause aggregation break up at high dye concentration. This explains why this cyanine dyes shows selectivity towards lysine and other positive charged amino acid. This study was able to show MHI-36 dye's ability to increase binding and affinity towards positively charged amino acids. This application can be

applied towards NIR probes, protein labeling and other assays where these types of cyanine dyes can react better with positively charged amino acids.

MHI-36 cyanine dye lacks the functionality to effectively form covalent attachment with biomolecules. However, this research establishes a theory that lysine can be a good target for attachment point on biopolymer. The initial belief that at lower dye concentrations, the dye has better selectivity towards arginine was disproved upon comparison of peak ratio versus concentration. Those results found that at low amino acid concentrations, arginine and leucine were nearly identical while lysine showed contrasting outcome. At higher dye concentrations lysine showed uncommon results compared with leucine and arginine, when amino acid concentration was between 200-300mM. Computational chemistry was able to show achievable positions for the amino acid in solution with the NIR dye. Lysine and arginine can align themselves to electron dense areas which are believed to have some negative charge due to gathering of electrons.

Future studies can look at aggregation patterns caused by amino acids with change in different solvents. Solvent involvement has been known to increase and decrease aggregation due to difference in dipole-dipole moment. Since aromatic groups contain  $\pi$ -conjugated carbons, a localized charge can travel through the phenol ring. Therefore future work would be compelling to explore this idea of localized charge having different directions for travel.

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