Observation Of Spectral Changes To Trp-214 Residue In Human Serum Albumin Upon Binding With Mangiferin And Near Infrared Dyes

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OBSERVATION OF SPECTRAL CHANGES TO TRP-214 RESIDUE IN HUMAN SERUM ALBUMIN UPON.Binding with MANGIFERIN AND NEAR INFRARED DYES

by

JENNIFER DEE NOVAK

Under the Direction of Gabor Patonay, PhD

ABSTRACT

A novel approach of using near infrared region (NIR) dyes is applied to elucidate the binding interaction between human serum albumin (HSA) and mangiferin (MGF). HSA is a blood carrier protein used for drug delivery, while mangiferin is a natural polyphenol found in mangoes that possesses numerous beneficial health properties. The NIR dyes are used as a probe to investigate MGF binding interaction with HSA via monitoring fluorescence of Trp-214 residue. Molecular modeling is used for docking and semi-empirical analysis. The investigation of the binding interaction between Trp-214 and MGF is significant, for it may offer broader pharmacological insight and applications for the polyphenol. Mangiferin in proposed to bind with a 2:1 stoichiometric ratio with HSA to the Trp-214 residue in subdomain IIA and another possible binding site to be determined in future studies. Spectral changes suggest a stabilized protein conformation upon mangiferin binding with the addition of NIR dye E-06 and MHI-06.

INDEX WORDS: NIR dyes, human serum albumin, mangiferin, anticancer, antibacterial, antiviral, competitive binding, absorbance, fluorescence spectroscopy, molecular modeling
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JENNIFER DEE NOVAK

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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2015
DEDICATION

I would like to dedicate this accumulation of work to my family.

Suzanne and Shandor Novak (Mommy and Daddy), thank you for your continuous financial support and unwavering moral support. You have been here for me through my greatest triumphs and darkest hours. I am proud to be your daughter and I love you.

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS............................................................................................................vi

LIST OF TABLES.........................................................................................................................x

LIST OF FIGURES.......................................................................................................................xi

1 INTRODUCTION.....................................................................................................................1
  1.1 Mangiferin Indica....................................................................................................................1
  1.2 Human Serum Albumin ..........................................................................................................2
    1.2.1 Sudlow’s Binding Sites ....................................................................................................3
  1.3 Near Infrared Region Dyes.....................................................................................................4
    1.3.1 Electromagnetic Spectrum ..............................................................................................4
    1.3.2 Chromophores..................................................................................................................4
    1.3.3 Woodword-Fieser Rules and Conjugation.......................................................................6
  1.4 Jablonski Diagram..................................................................................................................7
    1.4.1 Absorbance......................................................................................................................7
    1.4.2 Fluorescence...................................................................................................................8
  1.5 Molecular Modeling..............................................................................................................10
  1.6 Aim of Investigation............................................................................................................11

2 EXPERIMENTAL ...................................................................................................................11
  2.1 Reagents.............................................................................................................................11
  2.2 Sample Preparation..............................................................................................................12
  2.3 Instrumentation...................................................................................................................13
  2.4 Molecular Modeling............................................................................................................13
LIST OF TABLES

Table 1 Detection and Quantification Analysis ..............................................................19
Table 2 Statistical Summary of Conformational Changes in Trp-214 Fluorescence............34
Table 3 Association Constants for Probing Agents with HSA........................................35
LIST OF FIGURES

Figure 1 Mangifera Indica L ................................................................. 1
Figure 2 Human Serum Albumin Crystal Structure .................................... 2
Figure 3 Electromagnetic Spectrum ......................................................... 4
Figure 4 Chromophore Visual ............................................................... 5
Figure 5 Jablonski Diagram .................................................................. 7
Figure 6 Inner Filter Effect .................................................................... 9
Figure 7 Human Serum Albumin Absorbance Investigation ....................... 14
Figure 8 Trp-214 Mangiferin Absorbance .............................................. 15
Figure 9 Mangiferin Calibration Curve ................................................... 15
Figure 10 Trp-214 Mangiferin Binding Fluorescence Spectra ..................... 17
Figure 11 Calibration Curve for Trp-214 Fluorescence ............................. 18
Figure 12 Deconvolution of Trp-214 Spectral Shoulder Peaks .................... 18
Figure 13 Stern Volmer Plot .................................................................. 20
Figure 14 Scatchard Plot ...................................................................... 21
Figure 15 Kapparent Binding Constant Determination ............................. 22
Figure 16 Method of Continuous Variation ............................................. 23
Figure 17 Molecular Docking of Mangiferin with Human Serum Albumin .... 24
Figure 18 Probing Agent Solutions ......................................................... 26
Figure 19 E-06 and MHI-06 Absorbance ................................................. 27
Figure 20 Effect of E-06 Serial Dilution on Fluorescence Intensity of Trp-214 .... 28
Figure 21 Graphical Representation of Changes in Trp-214 Fluorescence Intensity Upon Serial Dilution of E-06 .......................... 29
Figure 22 Effect of E-06 and Ibuprofen Competitive Binding on Trp-214 Fluorescence .... 30
Figure 23 Effect of Competitive Binding on Fluorescence Intensity of Trp-214………………..31
Figure 24 Effect of E-06 Concentration on Mangiferin Bound Trp-214…………………………..32
Figure 25 Effect of E-06 Addition to Mangiferin Bound Trp-214………………………………33
Figure 26 Effect of MHI-06 Concentration on Mangiferin Bound Trp-214 Residue…………33
Figure 27 Effect of MHI-06 Addition to Mangiferin Bound Trp-214…………………………...34
1 INTRODUCTION

1.1 *Mangifera indica* L.

*Mangifera indica* L., more commonly known as the fruiting mango tree, is rich in various therapeutic compounds. The most abundant compound making up 10% of the stem bark and roughly 42% of dry mango peel weight is mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthen-9-one).\(^2\) Mangiferin (MGF) is a polyphenol glucosylxanthone and may also be obtained from the honey bush and other sources.

**Figure 1. Mangifera indica** L.

Fresh mangoes rich in mangiferin purchased from the local Dekalb Farmers Market.

The clinical applications of Mangiferin include treatment of allergies, dysentery, scabies, infection, inflammation, cancer, viral infections and as a vermifuge\(^3\). Mangiferin has also been found to chelate iron\(^2,3b,4\).

Considering mangiferin’s versatility and broad range of therapeutic potential, the mechanism behind its pharmacological behavior is of great interest. Elucidation of the interactions between MGF and macromolecules may provide insight as to the how the numerous therapeutic benefits from MGF may be maximized, allowing for advances in pharmacy and health care.

The macromolecule selected for binding analysis is human serum albumin. This is the most abundant protein in the human body and also a carrier protein, which participates in drug distribution and delivery. Thus, the mangiferin must come into contact with the albumin during transport. Previous literature has proposed that MGF binds to the hydrophobic binding cavity within subdomain IIA to the Trp-214 residue\(^5\). This Trp-214 residue is the only tryptophan in...
the entire macromolecule and is responsible for protein fluorescence. Thus, absorbance and fluorescence spectroscopy will be applied in order to investigate the binding interaction between human serum albumin and MGF. Spectroscopic analysis was accompanied by molecular modeling to examine the mangiferin ligand binding to the Trp-214 receptor. Similar investigations have been previously performed with a spectrophotometric focus on the binding ligand rather than the fluorescent tryptophan residue itself. This study will specifically focus on the binding interactions from the perspective of the Trp-214 residue.

1.2 Human Serum Albumin

Human serum albumin (HSA) is a carrier protein in *homo sapiens*. HSA is the most abundant protein found in blood plasma and has several significant physiological functions. These functions include the transport of fatty acids, hormones, amino acids and toxic metabolites as well as the regulation of oncotic pressure, which is the pressure proteins exert in a blood vessel pulling water into capillaries. HSA is also responsible for the distribution of pharmaceutical drugs and other key analytes to the necessary organelles and tissues. Through this distribution process, upon ligand binding to HSA the pharmokinetic and pharmodynamic properties of the drugs may become altered. Thus, it is important to study interactions between this protein and ligands possessing therapeutic properties to gain insight on how binding to the different subdomains of HSA may influence this phenomenon.

**Figure 2. Human Serum Albumin Crystal Structure**

Image taken with permission from Dr. Andrew Hamilton on June 20, 2015. Original crystal structure obtained from PDB (1ha2).
1.2.1 Sudlow’s Binding Sites

Human Serum Albumin contains two prominent binding sites known as Sudlow’s Sites. These may be seen in Figure 2 labeled as Site I and Site II\(^1\). Site I is found in subdomain IIA of HSA. This site binds bulky heterocyclic anions with centrally located charge\(^7\). Pharmaceutical compounds such as Warfain and Azapropazone are known to bind to this site\(^6\-^7\). Site II is located in subdomain IIIA and tends to bind aromatic carboxylates with extended conformation. Site II is the most active and accommodating site of the two\(^7\). Ligands such as Ibuprofen bind to this site. Some therapeutic agents such as the thyroid hormone Thyroxine may bind to both of the Sudlow Sites due to allosteric effects of concurrent binding with other drugs or fatty acids\(^7\).

HSA may undergo reversible conformational isomerization. Through pH- and allosteric-effectors, HSA conformation may change into various forms including fast (pH 2.7-4.3), neutral (pH 4.3-8.0) and basic (pH 8.0+). The fast form is characterized by decrease in alpha-helix content, the neutral maintains a heart-shape structure, while the basic form also has a decrease in alpha-helix with an increased ligand infinity. The conformational changes taking place in HSA during drug binding may change the pharmacological behavior of drugs bound to the protein\(^7\).

Due to the large binding capacity of human serum albumin, the effect of binding multiple ligands simultaneously is difficult to study. Multiple compounds may be bound to the protein at one time, although the binding of one may affect the binding of another due to allosteric effects or conformational changes.

Through fluorescence spectroscopy, the HSA residue Trp-214 was used to observe conformational changes due to its fluorescence emission wavelength of 350 nm. By binding compounds to the protein, fluorescence analysis is an invaluable tool for the elucidation of protein behavior and mechanism of multidrug binding action.
1.3 Near Infrared Dyes

1.3.1 Electromagnetic Spectrum

The electromagnetic spectrum is the foundation of spectrophotometry and the study of complex biomolecules. Molecules become excited by energy in the form of light, absorbing some of that energy at a specific wavelength, exciting electrons to a higher energy state, then falling back down at another wavelength. The wavelength and energy at which the electrons excite and emit are summarized by the electromagnetic spectrum. For the purpose of this investigation, the UV-visible-near infrared region (260-700 nm) will be discussed. As seen in Figure 3, the ultra-violet (UV) region spans roughly 250-400 nm, the visible region spans 400-700 nm and the near infrared region (NIR) spans 600-1200 nm. HSA absorbs in the UV region, MGF absorbs in the visible region and the dyes used as a probe for HSA binding absorb in the near infrared region.

1.3.2 Chromophores

Fluorescence spectroscopy is one method used to investigate the spectral properties of near infrared dyes in binding investigations, specifically for biological applications. NIR dyes fluoresce approximately between 600-1200 nm\[^8\]. Although biological molecules including proteins, DNA and hemoglobin are virtually non-fluorescent; their emission wavelength does not interfere with that of the dyes allowing the distinction between dye and macromolecule\[^8-9\]. The NIR dyes are referred to as chromophores, due to their vibrant colors visible with the naked eye.
An example of this may be seen in Figure 4. The biological applications of the chromophores come specifically from their characteristic colors. These dyes are used as contrast agents during Computerized Tomography (CT), nuclear Magnetic Resonance Imaging (MRI) scans and also with newer healthcare instrumentation that utilize Raman Scattering during surgery. These dyes are used as an aide in diagnosis and a real time surgical guide.

It is believed that the dyes bind to HSA and are transported in the central circulatory system to target tissues and organelles, filtered out via the kidneys and thus excreted through urine. Dyes are currently being designed for the primary purpose of disease prevention and diagnosis. In our studies two dyes (E-06 and MHI-06) are currently investigated for how they influence the conformation of HSA when bound to the therapeutic agent mangiferin.

Previous studies have revealed that both of the dyes and mangiferin bind to HSA, mangiferin in subdomain IIA and the dyes in subdomain IIIA\(^{[9-10]}\). The near infrared dyes will be used as a probe to study any conformational changes to HSA as a result of their addition to mangiferin bound protein. The Trp-214 residue will be examined because it is the binding site of mangiferin. Upon dye addition, any resulting spectral changes at this binding site may give insight as to the effect of the dye on protein conformation and the effect of mangiferin on the stability of protein conformation. Through competitive binding of the dyes with mangiferin, the interaction between HSA, therapeutic agent and dye may be assessed.
1.3.3 Woodword-Fieser Rules and Conjugation

The most frequently used NIR dyes have absorption in the range between roughly 600-1200 nm. The closer a wavelength maximum is to 400 nm or below is known as a hypsochromic shift or a blue shift. The closer a wavelength maximum is to 700 nm or beyond is known as a hypochromic shift or a red shift\textsuperscript{[11]}. Each compound has an inherent absorbance and fluorescence maxima, though this may change due to solvent polarity or aggregation. Another way a compound may change its wavelength maxima is through extended conjugation, which in turn makes a new compound.

Conjugation from the presence of alternating double bonds is what gives a fluorophore (fluorescence compound) its fluorescence. The greater the conjugation the greater the red shift hence wavelength maxima. Theoretically, the conjugation of a compound may be calculated such that a compound with a specific absorbance wavelength may be synthesized. This may be done through the application of Woodword-Fieser rules. These rules give a rough estimation of where a compound will yield an absorption maximum based on its conjugation and substituents. These rules require the calculation of wavelength maximum for the base, which is the longest conjugated chain present and then additional nanometers are added based on attached substituents. These rules allow for the absorbance wavelength maximum estimate for conjugated diene correlations, $\alpha$, $\beta$ unsaturated carbonyl compounds or ketones and aromatic compounds. The rough estimation of the absorbance wavelength maximum based on conjugation is key to ensure there is no autofluorescence from biological molecules.
1.4 Jablonski Diagram

The Jablonski Diagram depicts the basic electronic transitions in Spectrophotometry. The energetically induced transitions described within include Absorption, Fluorescence, Intersystem crossing and Phosphorescence.

1.4.1 Absorbance

Absorbance is the process of electron excitation from the ground singlet state to the Sn (n=2,3…) excited state. Electrons become excited by photons from a light source. Upon absorption, electrons undergo vibrational relaxation, where the electrons lose energy within own self-molecule, down to the lowest vibrational level of their highest excited state. Once at the lowest energy of the excited state, internal conversion takes place. This is due to the excited singlet states being so close in energy and once internal conversion occurs, vibrational relaxation occurs down to the lowest energy in the first singlet excited state. From this lowest level back down to the ground state is known as fluorescence.

Absorbance of a sample is measured via the transmittance of a beam of light through the sample in a cuvette where the transmitted light is measured by a detector. From the transmittance, a spectrum of wavelength scanned vs. absorbance measured is produced. As seen in Equation 1, absorbance equals negative log base ten of transmittance. This is how the UV-VIS instrument converts the percent transmittance signal into the absorbance. During spectral analysis a calibration curve is produced to assess linearity of data obtained. This linearity range
is due to the Beer-Lambert range circa 0.1-1.0 absorbance units. The Beer-Lambert Law is expressed in Equation 2.

\[ A = -\log_{10}T \]  \hspace{1cm} (1)

\[ A = \varepsilon \iota c \]  \hspace{1cm} (2)

In this equation, the absorbance \( A \) is equal to molar attenuation coefficient \( \varepsilon \), cell path length \( \iota \) and concentration \( c \). Each compound has a specific wavelength where maximum absorbance occurs and this is known as absorbance maximum \( \lambda_{\text{max}} \). The absorbance is not necessarily compound specific, as multiple compounds can have absorbance in similar or even the same region. Though, the molar attenuation coefficient is inherent to each individual compound making it compound specific. The \( \varepsilon \) expresses how strongly a compound attenuates light at a given wavelength. This value may be obtained from the slope of a calibration curve, as a calibration curve plots concentration against absorbance. Though, the cell path length is typically 1 cm, this may change based on what diameter cuvette is used and should be taken into consideration for calculation. As the absorbance examines the excitation of a sample, the fluorescence examines the emission of the sample.

1.4.2 Fluorescence

Fluorescence occurs from the lowest vibrational level in the excited singlet state down to the ground singlet state. During this transition, several routes may lead to dispersion or loss of energy from the target molecule. A molecule can have a decrease in fluorescence known as quenching due to interactions with itself (dynamic/collisional quenching), interactions with other molecules (static quenching) or via photon loss. This utility of analyzing this transition is the focus of this research.
As previously mentioned, the linearity range for absorbance analysis should fall between 0.1-1.0 absorbance units. Although, for samples, which require fluorescence measurements, the linearity range falls between 0.05-0.1 absorbance units, as the fluorescence instrumentation is highly sensitive. This required sensitivity is due to the Inner Filter Effect (IFE). As seen in Figure 6, excitation occurs at a 90° angle from emission detection in a spectrophotometer. A light source emits a beam that penetrates the sample cuvette causing the excitation of the sample, leading to its fluorescence and measurement by the detector.

The IFE occurs when a sample is too concentrated and the excitation beam causes sample attenuation at the wall of the cuvette, in turn resulting in an uneven distribution of fluorescence and therefore distorted spectra is observed. By diluting a sample to a lower concentration with absorbance below 0.1-0.5 absorbance units, also depending on the sensitivity of the instrument, the sample fluorescence will occur evenly distributed throughout fluorescence cuvette resulting in an undistorted spectra.

Several other sources may be the cause of spectral distortion. In order to rule out instrumental issues prior to fluorescence measurements, a Raman peak validation should be run. During this validation, DI water is placed into a cuvette and validation is run with excitation parameters set by the user\cite{12}. The utility of using DI water for validation includes the lack of toxic chemicals, waste, accessibility and reliability. The input excitations may vary using the same or different instruments as the energy between the excitation and Raman peak will be equivalent, universally producing a reliable validation peak\cite{12}. 

![Figure 6. Inner Filter Effect](image)
This process examines the width, height and relative location of the Raman peak. In doing so, the signal to noise ratio is calculated and reported. If the report indicates a failure of this validation, the most likely errors are the instrument did not have sufficient time to warm up or the light source requires replacement. A passed validation is vital to obtaining fluorescence spectra.

Intersystem crossing and phosphorescence will be outlined briefly as they are not examined in this research. Intersystem crossing occurs from the lowest vibrational level of the singlet excited state to an equivalent energy level in the triplet excited state. The single excited state refers to an anti-parallel electron that has become excited into a higher energy state. When the electron remains anti-parallel it satisfies the Pauli Exclusion Principle which states that electrons fill their orbitals beginning with the lowest energy and the electrons are paired in anti-parallel fashion. During intersystem crossing, an electron transitions from the singlet-excited state to the triplet-excited state undergoing a spin forbidden transition and becomes parallel. The procession of this electron back down to its anti-parallel orientation and back down to the ground state takes time and fluoresces at a slower rate. This is known as phosphorescence.

1.5 Molecular Modeling

Molecular modeling is a technique used to aid in investigations of chemical species by observing molecular dynamics in specified environments. The environment surrounding a molecule of interest can be manipulated in a variety of ways to suggest basic behavior that may be applicable to dynamic systems. For instance, temperature, solvent, molecules interacting with one another and adding specific constrains are a few of the ways a molecule can be observed to gather insight on basic molecular dynamics. Simulations and calculations may be obtained for insight on the basic interactions of a molecule. This technique is a preliminary technique used to
suggest simplified models of real dynamic systems. Molecular modeling programs use a semi-empirical approach to data gathering. The semi-empirical approach uses data that has been previously tested hands on and combines that with algorithms to calculate probable thermodynamic constants. This approach can only give preliminary suggestions as to chemical dynamics and is not representative of dynamic in vivo systems.

1.6 Aim of Investigation

Mangiferin is a natural compound currently being studied for its various health benefits. This polyphenol is distributed throughout the blood via the carrier protein human serum albumin. In order to gain insight on any possible changes in the pharmokinetic and pharmodynamic properties of the mangiferin-human serum albumin binding interaction, the fluorescence Trp-214 residue in HSA will be studied using fluorescence spectrophotometry. The effect of the mangiferin on the Trp-214 fluorescence will initially be studied, followed by the addition of NIR dyes to examine if there is any change in Trp-214 spectra upon competitive binding. By competitively binding the NIR dyes to the HSA, the protein may change conformation, potentially causing a change in affinity for either the dye or mangiferin therefore potentially changing distribution and efficacy of the polyphenol.

The human serum albumin and mangiferin binding interaction will be examined using absorbance, fluorescence, molecular modeling and competitive binding techniques.

2 EXPERIMENTAL

2.1 Reagents

Sodium phosphate dibasic anhydrous, free-flowing, Redi-Dri™, ACS reagent, ≥99%
(CAS 7558-79-4) and sodium phosphate monobasic monohydrate, ACS reagent, ≥98% purchased from Sigma-Aldrich, Saint Louis, MO 63103, USA (CAS 10049-21-5). Mangiferin from *Mangifera indica* bark (CAS 4773-96-0), Albumin, from human serum, lyophilized powder ≥97% (agarose gel electrophoresis) obtained from Sigma-Aldrich, Saint Louis, MO 63103, USA (CAS 70024-90-7). Near Infrared region dyes MHI-06 and E-06 synthesized in house by Eric Owens under the advisement of Dr. Maged Henary. HPLC grade methanol ≥99.9% obtained from Sigma-Aldrich, Saint Louis, MO 63103, USA (CAS 200-659-6).

2.2 Sample Preparation

Phosphate saline buffer solution was prepared by following FDA laboratory method guidelines. One liter of 0.2 M monobasic phosphate solution is prepared by dissolving sodium phosphate monobasic monohydrate into triple distilled water. One liter of 0.2M dibasic phosphate solutions is prepared by sodium phosphate dibasic anhydrous into triple distilled water. A final 0.02 M PBS is prepared by mixing a dilute monobasic and dibasic solution to 1:10 with DI water. The buffer is adjusted to pH 7.31–7.41. All further dilutions use this pre-made buffer.

Human serum albumin (HSA) solution is prepared in PBS 0.02 M to a stock concentration of 1x 10⁻³ M. Mangiferin stock solution is prepared in PBS to a concentration of 1x 10⁻⁴ M and sonicated for 10 minutes until all particulates have dissolved in full. Dyes MHI-06 and E-06 were previously prepared in HPLC grade methanol to a concentration of 1x 10⁻³ M and sonicated for 10 minutes following further sample preparation. During sample preparation, samples in which dyes are present contain methanol content remaining constant at 1.4%. HSA is initial constituent added to solution where as methanol is added last in order to prevent HSA
denaturing.

2.3 Instrumentation

Buffer is adjusted using Schott pH electrode BlueLine and Thermo Orion VWR sb20 Symphony pH meter. Triple distilled water obtained from Georgia State University Core Facilities.

Absorbance spectra obtained from Cary WinUV (Santa Clara, CA, 95051), using Cary WinUV software. Absorbance also obtained from Perkin Elmer Lambda 25, (Waltham, Massachusetts 02451) using UV Winlab software. Fluorescence spectra obtained from Perkin Elmer Fluorescence Spectrophotometer LS-55 (Waltham, Massachusetts 02451) using FL Winlab software.

2.4 Molecular Modeling

Molecular modeling performed using WaveFunction Spartan 14 and HyperChem Professional 8.0 software. Docking investigation completed using AutoDock 4.2 and Visualization Molecular Docking v1.9.1 (VMD).

3 RESULTS AND DISCUSSION

3.1 Absorbance

Human serum albumin (HSA) solutions are prepared in 0.02M PBS. Initially, HSA behavior was analyzed via UV-Vis at 10%, 50% and 90% HSA in PBS. Figure 7 shows representative spectra. Since, 10% HSA in PBS falls into the Beer-Lambert linearity range of absorbance, this is the maximum percentage of HSA used for the absorbance investigation of
HSA-mangiferin binding. HSA-mangiferin samples were prepared with a constant 50 μL volume of HSA (10% HSA) and incrementally increasing volume of mangiferin from 0-110 μL increasing by 10 μL. Buffer added to adjust total final volume up to 5 mL.

Figure 8 shows the resulting absorbance spectra for human serum albumin binding to mangiferin. The absorbance maximum of Trp-214 is 282 nm and 383 nm for mangiferin. Mangiferin absorbance shows an increasing linear trend upon increase of concentration. The Trp-214 experiences an increase in absorbance and a slight blue shift from 282 nm to 263 nm upon binding. The inset in Figure 8 indicates that the mangiferin absorbance overlaps with the Trp-214 causing an increase in absorbance. The phenomena of a blue shift is suggested to occur due to the peptide strand extension occurring in HSA as reported by Yuanyuan Yue, et.al [14]. The linearity of the trend is observed through a calibration curve in Figure 9 with a R² value of 0.99877.

Figure 7. HSA Absorbance Investigation

HSA prepared in 0.02M PBS pH 7.4 at 10%, 50% and 90%. Spectra run on Cary WinUV using Cary Win software. Spectra obtained using a quartz cuvette. Scan range 250-350 nm. SBW 5 nm. Scan speed 150 nm/min. HSA λ_max=280 nm.
Figure 8. Trp-214 Mangiferin Absorbance

Solution prepared in 0.02M PBS pH 7.4 with 10% HSA. Magiferin volume is increasing from 0-110 µL (a-l). Graphical inset shows the absorbance of 110 uL of Mangiferin in PBS with no HSA. Spectra run on Cary WinUV using Cary Win software. Spectra obtained using a quartz cuvette. Scan range 250-450 nm. SBW 5 nm. Scan speed 150 nm/min. Trp-214 $\lambda_{\text{max}}$=282-263 nm. Mangiferin $\lambda_{\text{max}}$=383 nm.

Figure 9. Mangiferin Calibration Curve

Calibration curve is plotting mangiferin concentration (M) vs. mangiferin absorbance from spectra found in Figure 12. Linearity of $R^2$=0.99877.
3.2 Fluorescence

For fluorescence analysis, the Perkin Elmer LS-55 instrument is utilized. HSA-mangiferin samples were prepared with a constant 3500 uL volume of HSA and incrementally increasing volume of mangiferin from 0-110 uL, increasing in 10 uL increments. Buffer added to adjust total final volume up to 5 mL. Figure 10 shows the fluorescence spectra for Trp-214-mangiferin binding. The spectral behavior of Trp-214 indicates a binding event between itself mangiferin. The calibration curve shown Figure 11 suggests a linear fluorescence response from the Trp-214 upon increasing the concentration of mangiferin. Previous literature has suggested mangiferin’s propensity for binding with HSA in the hydrophobic binding pocket of subdomain IIA, where the sole tryptophan residue resides (Trp-214). This residue is majorly responsible for the fluorescence of HSA and is also a dominant residue in drug binding. The Trp-214 residue fluoresces at 349 nm in PBS. Upon the addition of the mangiferin to the HSA, the fluorescence decreased. This observed effect is proposed to be quenching of the Trp-214 residue by the MGF. In the Trp-214 MGF spectra, an isosbestic point appears to be forming around 430 nm, although the increase in fluorescence is due to the second order peak at 520 nm, as a result of the Rayleigh Peak at the excitation wavelength of 260 nm. Extending the spectra farther, the third order peak is also visible at 680 nm.
**Figure 10.** Trp-214-Mangiferin Binding Fluorescence Spectra

(a) Constant concentration of human serum albumin (7.5 µM) is titrated with an increasing concentration of mangiferin a-l (0-110 µM). Trp-214 emission maximum is 349 nm. Shoulder peaks are labeled as 1, 2 and 3. (b) Mangiferin fluorescence at concentrations of 10 µM, 50 µM and 110 µM, without the presence of albumin. Mangiferin emission maximum is at 309 nm. Solvent is PBS with a pH of 7.4. Spectra obtained via Perkin Elmer LS-55, excitation wavelength 260 nm, 5 SBW, 150 nm/s.

Figure 10 is the representative spectra for Trp-214 binding to mangiferin. Three shoulder peaks are present in addition to the maximum emission wavelength, which is due to Trp-214. Peak 1 seen on the far left is due to overlapping mangiferin fluorescence. The inset in Figure 10 shows the fluorescence of mangiferin at 10 µM, 50 µM and 110 µM. Mangiferin appears to be self-quenching as shown by the inset and also visible by the gradual disappearance of the peak on the Trp-214 spectra. Though, two other peaks are present, peak 2 and peak 3. In order to further investigate the nature of these peaks, basic manual peak deconvolution was performed.
**Figure 11.** Calibration Curve for Trp-214 Fluorescence

The calibration curve for the Trp-214 mangiferin binding shows a linear decrease in fluorescence with a $R^2 = 0.99323$. Concentration is on the x-axis and fluorescence intensity is on the y-axis.

**Figure 12.** Deconvolution of Trp-214 Spectral Shoulder Peaks

Calibration curve plots the ratio of shoulder to Trp-214 fluorescence intensity vs concentration of Mangiferin (0-110 uM). Right shoulder, left shoulder and far left shoulder emission maximums are as follows 373 nm, 331 nm and 309 nm.

As seen in Figure 12, peak deconvolution isolates the three peaks of interest by plotting the concentration of mangiferin to the ratio of the shoulder peaks fluorescence to Trp-214 fluorescence. This figure shows strong linearity between each of the peaks, suggesting that the shoulder peaks are consistently part of the binding event and not randomly occurring events. The
shoulder peaks were initially believed to possibly be due to the phenylalanine and tyrosine residues in the human serum albumin protein, although were discounted due to their weak fluorescence and that their fluorescence wavelengths do not coincide with that of the shoulder peaks. The occurrence of shoulder peaks is believed to be due to conformational change within the protein itself. Obaidur Rahaman et al. investigated the unfolding pathways of human serum albumin and found that under varying pH, solvent or even binding conditions, slight shoulder peaks may be found in fluorescence spectra which are indicative of protein unfolding\cite{15}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Ka & 0.15 E 5 M$^{-1}$ \\
LOD (6-12 uM) & 0.424 \\
LOQ (6-12 uM) & 1.29 \\
%RSD (6-12 uM) & 1.49 \\
% Error & 0.61 \\
\hline
\end{tabular}
\caption{Detection and Quantification Analysis}
\end{table}

Table 1 presents the associative binding constant, limit of detection, limit of quantification, percent relative deviation and percent error of fluorescence data for binding between HSA and mangiferin between the concentrations of 6-12 µM of mangiferin.
**Figure 13. Stern Volmer Plot**

Stern Volmer Plot of the binding between HSA and mangiferin shows an upward curvature, which is indicative of cooperative binding between the two species.

Table 1 presents the calculated binding constant, limit of detection (LOD), limit of quantification (LOQ), percent relative standard deviation (% RSD) and percent error. The LOD and LOQ are both below 2.0 suggesting that quantification of HSA binding to 6-12 µM of mangiferin is accurate and precise.

The upward curvature of the Stern Volmer analysis in Figure 13 suggests a possible combination of static and dynamic quenching. Quenching is the term used to describe any process that results in decreased fluorescence intensity of a sample\(^{[11]}\). Static quenching refers to the formation of a non-fluorescent ground state complex between a fluorophore and quencher. Dynamic quenching, also known as collisional quenching, occurs when a fluorophore and a quencher have a collisional encounter during the excited lifetime of the fluorophore. Upon contact, the fluorophore returns to ground state without photon emission\(^{[11]}\). Analysis by the Scatchard Plot (Double Reciprocal Plot), as seen in Figure 14, resulting in the associative
binding constant of $K_a = 0.15 \times 10^5 \text{ M}^{-1}$, which agrees reasonably to previously obtained literature value of $0.33 \times 10^5 \text{ M}^{-1}[14, 16]$. Initially, the goal was to determine whether the quenching of the Trp-214 residue was static or dynamic through $K_{\text{apparent}}$ calculations from linear regression analysis (Figure 15). The suggested combined quenching by the Stern Volmer Plot in Figure 13 is found to be unlikely upon further analysis. Equation 3 found below is the Stern Volmer Equation$^{[17]}$. The variables are filled in with the slope $(K_S*K_D)$ and $y$-intercept $(K_S + K_D)$ from $K_{\text{app}}$ plot and then the apparent binding constant is solved by using the quadratic equation. The values could not be obtained, for calculations resulted in an imaginary number. This result suggests combined quenching is not occurring. Upon further investigation, proof has also been presented through the analysis of HSA and MGF through Method of Continuous Variation, also known as the Jobs Method for Determining Binding Stoichiometry.

**Figure 14. Scatchard Plot**

The binding constant is determined by plotting the inverse of quencher concentration (mangiferin) by the inverse of change in fluorescence. Dividing the $y$-intercept by the slope results in the associative binding constant $K_a = 0.15 \times 10^5 \text{ M}^{-1}$. 

$$y = 4E-08x + 0.001$$

$R^2 = 0.98479$
Figure 15. $K_{\text{app}}$ Binding Constant Determination

$K_{\text{app}}$ binding constant determination is performed by plotting the quencher concentration (mangiferin) vs. the Kapp term that represents $((F_0/F - 1)/[Q])$, initial fluorescence, divided by final fluorescence minus 1 divided by quencher concentration.

$$y = 0.0021x + 0.0388$$
$$R^2 = 0.96756$$

As seen in Figure 16, a peak forms at the top of the graph. A line can be drawn on the data points to the left and right of this apex. An arrow is drawn downward toward the x-axis from the point where these lines intersect. This tip of the arrow points to the stoichiometric ratio of binding. During the Method of Continuous Variation, the concentration of both components remains constant, but the mol ratio of one compound decreases as the other increases. The arrow in Figure 16 points to roughly 0.600 mol fraction, which is indicative of a 2:1 binding ratio of mangiferin to HSA.
The graph plots mol fraction of mangiferin in the solution vs. absorbance. The point of intersection at the apex results in stoichiometric mol fraction of the mangiferin to the HSA. The point of intersection results in roughly 0.600 mol fraction.

Through Jobs analysis, the MGF binds to HSA with a 2:1 stoichiometry. Due to this 2:1 binding there are multiple binding events, although they may not take place with the same residue. Due to this, the $K_{app}$ cannot be calculated using fluorescence spectra alone and takes more advanced methods for determination of the second possible binding residue. These results suggest a cooperative binding event where multiple mangiferin ligands bind to multiple residues within human serum albumin. Determined binding stoichiometry was applied during the competitive binding analysis.
Molecular modeling software was used in order to visualize mangiferin followed by a docking study in order to observe binding interaction of mangiferin with human serum albumin. Initially, mangiferin was modeled using HyperChem software, then input in AutoDock 4.2 as a ligand. Human serum albumin crystal structure was retrieved from protein data bank (1H9Z). Macromolecule (HSA) was set as rigid and ligand glycosidic bond was rotatable. Upon ligand docking with macromolecule, resulting docking (Figure 17) shows the lowest obtained Gibbs free energy upon binding to Trp-214 residue. Further examination via enlargement of grid box also presented a low Gibbs free energy with Tyr-401 residue, suggesting mangiferin may also bind to this residue. Considering the 2:1 binding stoichiometry between mangiferin and HSA, there is a possibility that mangiferin can bind to both the Trp-214 and Tyr-401 residues simultaneously, which would support the cooperative binding proposition.

**Figure 17.** Molecular Docking of Mangiferin with Human Serum Albumin

HSA receptor obtained from protein data bank (1H9Z). VMD used to create model and Autodock used for molecular docking of mangiferin to human serum albumin. Orange residue is that of Trp-214. MGF ligand is 3.91 angstrom from receptor.
5.1 Effect of E-06 on Trp-214 Fluorescence

Near infrared dyes are examined for their utility in biological applications. Several in vivo applications exist in the medical field, such as real time scans including computerized tomography (CT), nuclear Magnetic Resonance Imaging (MRI) and laser guided surgery. Due to the importance of these medical procedures and the utility of using dyes to assist with them, they are currently being investigated as to their influence upon binding to human serum albumin and their affect on mangiferin. Previously reported data have revealed that the dyes studied are believed to bind to subdomain IIIA (Suldow’s Site II). Although the dyes do not bind to the same subdomain as mangiferin (subdomain IIA), they may still play a vital role in the conformational changes of HSA. The binding of the dye may elicit a response from the HSA that may affect its binding interaction with mangiferin.

Due to the beneficial properties of mangiferin and its binding to subdomain IIA, protein interactions are examined at its binding site, the Trp-214 residue. Initially the effect of E-06 on Trp-214 is examined via a serial dilution of the dye, followed by competitive binding between E-06 and ibuprofen, both which bind to subdomain IIIA. This spectral analysis was used to compare the effect of mangiferin on Trp-214 versus the dye alone.

In Figure 18, three sets of samples are present, on the left the magenta (E-06), in the middle the turquoise (MHI-06) and on the right the colorless (Ibuprofen). The Ibuprofen is used as a competitive binding agent against the E-06, for both are suggested to bind to the same subdomain. The spectral differences between binding two agents to the same subdomain and binding two agents to differing subdomains are analyzed. All four of these agents, including
mangiferin, are used as probes to analyze any conformational changes exhibited through the fluorescence of Trp-214.

**Figure 18. Probing Agent Solutions**

The probing agents include the two NIR dyes E-06 (magenta) and MHI-06 (turquoise) as well as Ibuprofen (colorless). Concentrations of $1 \times 10^{-4}$ M, $1 \times 10^{-5}$ M and $1 \times 10^{-6}$ M. Solvent in use is 0.2 M PBS, pH 7.39-7.4 for the Ibuprofen. Methanol is solvent for E-06 and MHI-06.

Initially, the absorbance of the dyes is observed. Both dyes are $1 \times 10^{-6}$ M in concentration and the molar attenuation coefficient of MHI-06 is 116,000 M$^{-1}$cm$^{-1}$ and E-06 is 106,900 M$^{-1}$cm$^{-1}$.[9] The MHI-06 peak distortion in comparison to that of E-06 is due to PBS solvent. The MHI-06 is susceptible to changes in polar conditions, therefore resulting in the peak found in Figure 19.[9] The absorbance maximum for E-06 and MHI-06 is 544 nm and 759 nm and the emission maximum is 782 nm and 759 nm, respectively. Neither of these emission wavelengths interferes with that of Trp-214 (349 nm), therefore making them suitable for binding analysis.

Figure 20 displays the effect of E-06 serial dilution on the fluorescence intensity of Trp-214. For the serial dilution trial the HSA concentration was held constant at 20 µL, prepared from a $1 \times 10^{-4}$ M stock solution. The E-06 concentration ranged from 10-110 µL. Due to dye
stock solution preparation in methanol, methanol concentration was constant at 1.4 % in all trials. Each trial was performed in triplicate. Samples consisting of HSA and E-06 alone were also obtained for comparison.

As seen in Figure 20, the fluorescence of the dye is negligible in comparison to the HSA, which fluorescence around 550 intensity units. The increase in concentration of E-06 does not result in any linear response and instead results in a broad distribution of Trp-214 fluorescence. There is no clear correlation between Trp-214 fluorescence upon the addition of E-06. The mean and standard deviation of these trials are summarized below in comparison to the other trials in Table 2.

**Figure 19.** E-06 and MHI-06 Absorbance

Spectra obtained via CaryWinUV. Solvent is PBS pH 7.4. Both solutions are $1 \times 10^{-6}$ M. Both stock solutions prepared in 10% methanol. SBW 2 nm, scan speed 240 nm/min.
Figure 20. Effect of E-06 Serial Dilution on Fluorescence Intensity of Trp-214

Serial dilution of E-06 ranges from 10-110uL. HSA is constant volume of 20 uL. Trp-214 λem= 349nm. Solvent used is 0.2M PBS with pH of 7.39-7.4. λex= 260nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm. E-06 structure on left.

Due to the nature of the E-06 Trp-214 fluorescence spectra, Figure 21 is a visual aide to observe the changes in fluorescence intensity of Trp-214. As mentioned, there is no linearity in the fluorescence response. Qualitative analysis suggests that the Trp-214 residue is sensitive to changes in E-06 concentration and does not react in a predictable fashion upon its binding. This suggests the HSA conformation is sensitive to E-06 concentrations upon binding and although the dye is suggested to bind in subdomain IIIA, the binding event influences subdomain IIA is depicted in Trp-214 fluorescence spectra.
Serial dilution of E-06 10-110uL. HSA constant volume of 20 uL. TRP-214 λem= 349nm. Solvent used is 0.2M PBS with pH of 7.39-7.4. λex= 260nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm.

Probing of Trp-214 is currently conducted through a secondary site subdomain IIIA rather than the primary site of interest, subdomain IIA. Examining the Trp-214 residue under various conditions may give insight as to the influence of the one subdomain on the other during a binding event. Considering the apparent random intensity fluctuations in Trp-214 fluorescence spectra upon E-06 binding, competition between E-06 and Ibuprofen was performed to investigate whether this event would produce the same or different spectral results.

Spectra of E-06, Ibuprofen and HSA are individually obtained, followed by E-06 with Ibuprofen and HSA, E-06 and Ibuprofen in a 1:1:1 stoichiometric ratio. Each following sample contains 25-50 µL of Ibuprofen with 20 µL HSA and 20 µL E-06. Resulting spectra in Figure 22 is also non-linear though the distribution is less spread out than previously with E-06 and HSA.
alone. Note that upon the addition of Ibuprofen to the dye, there is an increase in overall fluorescence intensity. The statistical spread of this data may also be found in Table 2. A graphical aide to visualize this non-linear data may be found in Figure 23. The highest fluorescence intensity results from the 1:1:1 HSA, E-06 and Ibuprofen sample. Trp-214 by itself has the highest fluorescence of these samples suggesting that the tryptophan residue is most exposed and upon addition of the dye and Ibuprofen the fluorescence decreased through conformational change in the protein.

**Figure 22. Effect of E-06 and Ibuprofen Competitive Binding on Trp-214 Fluorescence**

HSA and E-06 have constant volume of 20 uL. Ibuprofen volume is 20-50 uL. Trp-214 λem= 349 nm. Solvent used is 0.2M PBS with pH of 7.39-7.4. λex= 260nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm. E-06 structure found on left and Ibuprofen on right.
Figure 23. Effect of Competitive Binding on Fluorescence Intensity of Trp-214

HSA and E-06 have constant volume of 20 uL. Ibuprofen volume is 20-50 uL. Trp-214 λem= 349 nm. Solvent used is 0.2M PBS with pH of 7.39-7.4. λex= 260nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm.

5.2 Effect of E-06 and MHI-06 on Mangiferin-Trp-214 Fluorescence

Investigation of effect of NIR on mangiferin binding to Trp-214 began with E-06. The HSA concentration remained the same, 20 µL prepared from a 1x10^{-4}M stock solution. Throughout the mangiferin-dye investigation, mangiferin concentrations remained constant with a 2:1 stoichiometric ratio to HSA, as determined previously via Jobs Plot. The concentration of E-06 increased from 40-70 µL. Fluorescence of mangiferin, E-06 and mangiferin with E-06 solutions possess insignificant fluorescence with respect to that of HSA. Upon addition of E-06 to the mangiferin bound Trp-214 mangiferin, the fluorescence intensity resulted in minimal fluctuation, as seen in Figure 24. Although, there is no linear response, the response appears within the same region. Statistical analysis results in acceptable percent relative standard deviation of data. This suggests the binding of E-06 has minimal effect on mangiferin binding to Trp-214. Figure 25 is the visual representation of the fluorescence fluctuations.
**Figure 24.** Effect of E-06 Concentration on Mangiferin Bound Trp-214 Residue

HSA volume constant at 20 µL. MGF volume constant at 40 µL. HSA λem= 349 nm. E-06 volume increases from 40-70 µL. Solvent used is 0.2M PBS with pH of 7.39-7.4. λex= 260nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm. E-06 structure found on left and mangiferin structure on right.

Examination of Trp-214-mangiferin bound to MHI-06 using same procedure resulted in similar results. Figure 26 also depicts a non-linear fluorescence response, though minimally changing as to suggest lack of MHI-06 influence on Trp-214 residue when bound to mangiferin. Figure 27 graphically displays MHI-06 effect on Trp-214-mangiferin binding fluorescence.
Figure 25. Effect of E-06 Addition to Mangiferin Bound Trp-214

HSA volume is constant at 20 µL. MGF volume is constant at 40 µL. HSA \( \lambda_{em} = 349 \) nm. E-06 volume increases from 40-70 µL. Solvent used is 0.2M PBS with pH of 7.39-7.4. \( \lambda_{ex} = 260 \)nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm.

Figure 26. Effect of MHI-06 Concentration on Mangiferin Bound Trp-214 Residue

HSA volume constant at 20 µL. MGF volume constant at 40 µL. HSA \( \lambda_{em} = 349 \) nm. MHI-06 volume increases from 40-70 µL. Solvent used is 0.2M PBS with pH of 7.39-7.4. \( \lambda_{ex} = 260 \)nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm. MHI-06 structure found on right and mangiferin structure on left.
**Figure 27.** Effect of MHI-06 Addition to Mangiferin Bound Trp-214

HSA volume constant at 20 µL. MGF volume constant at 40 µL. HSA λem= 349 nm. MHI-06 volume increases from 40-70 µL. Solvent used is 0.2M PBS with pH of 7.39-7.4. λex= 260nm SBW 5nm. Scan speed 150 nm/min. Perkin Elmer LS-55. Scan range 280-460nm.

5.3 SUMMARY OF NIR DYE AFFECT ON TRP-214 FLUORESCENCE SPECTRA

**Table 2.** Statistical Summary of Conformational Changes in Trp-214 Fluorescence

<table>
<thead>
<tr>
<th>Trial</th>
<th>Mean</th>
<th>Variance</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serial Dilution E-06</td>
<td>563.9</td>
<td>11153.7</td>
<td>10521</td>
<td>18.7</td>
</tr>
<tr>
<td>Competitive Binding of E-06 with Ibuprofen</td>
<td>308.0</td>
<td>1046.0</td>
<td>31.6</td>
<td>10.3</td>
</tr>
<tr>
<td>Mangiferin Binding with E-06</td>
<td>429.1</td>
<td>40.9</td>
<td>6.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Mangiferin Binding with MHI-06</td>
<td>473.5</td>
<td>165.1</td>
<td>12.8</td>
<td>2.7</td>
</tr>
</tbody>
</table>

All calculations performed on fluorescence spectral values at maximum emission of 349 nm. All experiments performed in triplicate. Values obtained from average of triplicate trials. From left to right are the trials, mean, variance, standard deviation and percent relative standard deviation.
Table 2 summarizes statistical calculations of fluorescence fluctuations at Trp-214 emission maximum 349 nm. All four trials were performed in triplicate and table represents averages of these data. The mean, variance, standard deviation (SD) and percent relative standard deviation (% RSD) are displayed. These data were calculated only using comparable samples. Reference samples such as HSA, mangiferin or dye alone were not used in calculations. The most important and revealing portion of this table highlighted in a black box is the % RSD. The serial dilution of E-06 and the competitive binding of the Ibuprofen with E-06 yield large % RSD values quantitatively suggesting the large spread in fluorescence intensities. The mangiferin binding with the E-06 and the MHI-06 result in low % RSD values, roughly >3% suggesting low spread of fluorescence intensities and also the accuracy and precision of this method for quantification purposes.

Table 3. Association Constants for Probing Agents with HSA

<table>
<thead>
<tr>
<th>Probing Agent</th>
<th>$K_a \times 10^5 \text{ M}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGF</td>
<td>0.15</td>
</tr>
<tr>
<td>E-06*</td>
<td>8.74</td>
</tr>
<tr>
<td>MHI-06*</td>
<td>10.6</td>
</tr>
<tr>
<td>Ibuprofen**</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*[9], **[18] Previously reported in literature.

Association constants are present for mangiferin (MGF), E-06, MHI-06 and Ibuprofen. Those probing agents with a star were previously reported in literature. The mangiferin constant was calculated from experimental data.

The association constants for the human serum albumin probing agents are listed in Table 3. Fluorescence analysis revealed upon the binding of mangiferin to HSA, the addition of probing agents E-06 and MHI-06 resulted in no statistical change in fluorescence intensity. This suggests that although the association constant for mangiferin is lower than the other probing
agents, mangiferin and HSA form a stable complex that is not altered by the addition of E-06 or MHI-06. Though, the binding of mangiferin to the HSA protein appears to have a profound effect on the dye binding. Further investigations must be conducted in order to draw conclusions of behavior in subdomain IIIA.

6 CONCLUSION

6.1 Proposed Binding Domain Behavior and Mechanism

Mangiferin binds to human serum albumin in subdomain IIA. The ligand is proposed to bind to residues Trp-214 and possibly Tyr-401. Binding stoichiometry was found to be 2:1 to HSA. Stern Volmer and $K_{apparent}$ analysis suggest cooperative binding between mangiferin and the albumin. Fluorescence analysis suggests hydrogen bond stabilization of the mangiferin molecule in the hydrophobic binding pocket. Addition of a probing agent that binds to subdomain IIIA causes non-linear changes in fluorescence intensity. Although, addition of mangiferin, which binds to subdomain IIA, followed by addition of E-06 or MHI-06, which bind to subdomain IIIA, results in no statistically different fluorescence intensity. This suggests the binding of mangiferin to HSA at a 2:1 ratio according to binding stoichiometry, results in a stable conformation of protein regardless of dye concentration.

The mangiferin binding interactions may be further elucidated by examination via circular dichroism (CD). Through CD the protein binding interactions may be investigated by thermodynamic analysis. The 2-D nuclear magnetic resonance (NMR) technique of Nuclear Overhouser effect spectroscopy (NOESY) NMR can be used to determine hydrogen location through space in order to determine protein conformation. Crystal structure x-ray diffraction of mangiferin bound to HSA is also recommended. Considering the multiple health benefits of the
polyphenol mangiferin and therapeutic potential, the binding interactions between itself and human serum albumin are important to study. The possible interactions between mangiferin and other near infrared dyes are also important to study in order understand the possible effects one may have on the other. Through this research the pharmokinetic and pharmodynamic properties behind mangiferin-HSA binding may be understood and applied to pharmaceutical design.
REFERENCES