Spring 2015

Investigation Of The Binding And Thermodynamic Properties Of Polycyclic Aromatic Hydrocarbons On Biological Specimen And Cytotoxic Effect On Triple Negative Breast Cancer Cells

Jr. Derick Jones
North Carolina Agricultural and Technical State University

Investigation of the Binding and Thermodynamic Properties of Polycyclic Aromatic Hydrocarbons on Biological Specimen and Cytotoxic Effect on Triple Negative Breast Cancer Cells

Derick D. Jones, Jr.

North Carolina A&T State University

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Chemistry Department

Major: Chemistry

Major Professor: Dr. Sayo O. Fakayode

Greensboro, North Carolina

2015

The Graduate School
North Carolina Agricultural and Technical State University

This is to certify that the Master's Thesis of

Derick D. Jones, Jr.

has met the thesis requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2015

Approved by:

Sayo O. Fakayode, Ph.D.
Major Professor

Margaret I. Kanipes-Spinks, Ph.D.
Committee Member

Julius Harp, Ph.D.
Committee Member

Checo J. Rorie, Ph.D.
Committee Member

Margaret I. Kanipes-Spinks, Ph.D.
Department Chair

Sanjiv Sarin, Ph.D.
Dean, The Graduate School

Biographical Sketch

Derick D. Jones, Jr. was born in Greenville, North Carolina. Upon graduation from high-school in 2009 he decided to pursue his baccalaureate degree in human biology from North Carolina State University. After graduating in May 2013, Derick advanced to a Master of Science degree in Chemistry North Carolina Agricultural and Technical State University. Derick has a unique interest in research and education. Through research he hopes to find solutions to some of the major health disparities that has plagued this nation for decades. Through education he hopes to encourage and motivate the generation to come. His ultimate passion is to be a part of a team that implements a program to graduate more minorities in PhD disciplines.

Dedication

This is dedicated to my late grandparents, Ernest L. Jones and Verna D. Jones. I never thought this would ever be possible. I am thankful for the time that I had and will cherish them forever. I would also like to dedicate this to those who thought life was impossible. To those who are in an unfavorable situation, I hope my life will serve as a reminder to keep moving forward even when the odds are against you.

Acknowledgments

All glory to God, for His guidance during this process. I am very grateful to the Chemistry Department at North Carolina Agricultural and Technical State University. I am thankful for their belief in me to be successful in such a rigorous program. I am also grateful to Dean Goldie Byrd for initiative and believing in me well enough to offer me a RISE (Research Initiative for Scientific Enhancement) fellowship.

Thank you to my Major Professor, Dr. Sayo O. Fakayode for your introduction to analytical chemistry. All of the encouragement and support of my project is greatly appreciated. Thank you for seeing the "Derick the scientist" when I could not see it myself. All of the knowledge and wisdom you have shared was not in vain. The people of the Fakayode Research Group are beyond phenomenal. Without all of the help and support provided from them, this would have been impossible.

To my Thesis committee, Dr. Margaret I. Kanipes-Spinks and Dr. Julius Harp, thank you for all of your help during this process. Your ideas, thoughts and suggestions have challenged me to think outside of the box. To Dr. Checo R. Rorie, I am very grateful for the time and training. Thank you for considering all of my thoughts and ideas.

Table of Contents

List of Figures

List of Tables

Nomenclature

HSA	Human Serum Albumin
ΔG	Gibbs Free Energy
ΔH	Enthalpy
ΔS	Entropy
K	Binding Constant
BSA	Bovine Serum Albumin
PAH	Polycyclic Aromatic Hydrocarbon
POCs	Persistent Organic Compounds
FTIR	Fourier Transform Infrared Spectroscopy
TNBC	Triple Negative Breast Cancer Cells
ER	Estrogen Receptor
PR	Progesterone Receptor
HER2	Human Epidermal Growth Factor 2
mg	Milligram
mL	Milliliter
g	Gram
nm	Nanometer
cm	Centimeter
M	Molar
a.u.	Arbitrary Units
min	Minutes

Abstract

Pesticides, weed control chemicals, and polycyclic aromatic hydrocarbons (PAHs) are cytotoxic, carcinogenic, and toxic chemicals of concern. The toxic effects and health related issues of PAHs in humans necessitate the urgent need to investigate the binding mechanism of PAHs with biological specimen for biomedical diagnosis. This study investigated the binding of three PAHs (anthracene, pyrene, and naphthalene) with human serum albumin (HSA) and bovine serum albumin (BSA), proteins responsible for the delivery of drug molecules and other important metabolites to various targets. Specifically, the influence of the temperature, PAH type, and PAH concentrations on HSA/BSA emission property was investigated. The binding constant (K) and stoichiometry of PAH-HSA/BSA complexes were also determined from the emission data using a modified Stern-Volmer equation. The thermodynamic properties (Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS)) of PAH-BSA/HSA complexation were also determined from emission data using Van't Hoff equation. The emission spectra, the calculated *K* and thermodynamic parameters of PAH-HSA/BSA complexation were found to be PAH and temperature dependent. The large values of the calculated binding constant and ΔG indicated high affinity and strong bindings of PAH with HSA/BSA. The influence of PAHs on triple negative breast cancer (TNBC) cells viability was further investigated. The result of cytotoxicity suggested that the viability of TNBC cells is PAH and concentration dependent.

CHAPTER 1

Introduction

Many infer the root behind many illnesses are family history and/or genetics oriented. While it is indeed important to take the family history aspect into consideration, it is justifiably important to consider diet and environmental exposure. There are environmental and food scientists who research and take into account the exposure of toxic compounds through air and food and drink consumption. Many questions in the biomedical community are being researched for answers. Understanding the interaction of polycyclic aromatic hydrocarbons (PAHs) with human serum albumin (HSA) and bovine serum albumin (BSA) along with its cytotoxic effects on triple negative breast cancer (TNBC) cells was the primary focus of this thesis. It is evident that PAHs are found pervasively in the environment. Preventing human exposure to compounds of this sort is nearly impossible because of its ubiquities in nature. These hydrocarbons in particular are evident to have interaction with human specimen as it will be seen throughout this thesis.

1.1 Research Objectives

While it is impossible to eliminate human exposure, this research was conducted in hopes of investigating interactions of the PAHs with humans on a molecular level. Not only for purposes of this thesis is this research relevant, but the understanding of the cytotoxic chemicals of interest is imperative because it enables scientists to understand the interactions of the PAHs with the human and the animal specimen. The objectives of the study are:

1.1.1. To investigate the influences of PAHs on the binding and emission spectra of human and bovine serum albumin. The emission spectra of serum albumin will allow accurate determination of how the PAHs are interacting with the serum proteins. This involves determining the binding constant and stoichiometry of PAH-serum albumin complexes.

1.1.2. To examine the thermodynamic properties of PAH-BSA/HSA complexes. The calculated thermodynamic variables will aid in showing the feasibility of the reactions. Thermodynamic variables to be calculated are enthalpy *(*ΔH*)*, entropy *(*ΔS*)*, and Gibbs free energy *(*ΔG*)*. This objective will also show if the effects of these properties are analyte and temperature dependent.

1.1.3. To investigate the effect of PAHs on cell-viability and on TNBC cells. This objective will provide the interdisciplinary focus by understanding the biological effects on the cellular level. This objective was investigated in collaboration with Dr. Checo J. Rorie Assistant Professor in the Biology Department at North Carolina Agricultural and Technical State University (NCAT).

CHAPTER 2

Literature Review

2.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs or polyaromatic hydrocarbons) are known to be very ubiquitous in nature. These persistent organic compounds (POCs) in their purest form can be anywhere from colorless to a white or pale yellow-green color [1, 2]. These preponderant constituents of air are formed vastly by the incomplete combustion of products (i.e. garbage, oil, coal, gas and vegetation) [3]. They are known to exist in more than 100 different combinations. Because of their potential cytotoxic, mutagenic and genotoxic effects to the human population they are of increasing interest in the biomedical community [1-13]. These hydrocarbons have the feasibility of staying in the environment for long periods of time because of their inability to burn easy [2,9]. Although PAHs exist in the environment as a whole, their concentrations vary significantly throughout. There are substantiated differences in the concentrations of PAHs in rural areas versus that of urban areas [10]. This is due to more anthropogenic emissions of PAHs in the urban areas. It was suggested that the influence of vehicle and domestic emissions had major influences on the elevated PAH emissions in urban areas [1-8, 10].

Polyaromatic hyrdrocarbons are often described as simple or complex carbon and hydrogen containing compounds containing at least 2 or 3 fused benzene rings (other rings that are not 6-membered may be fused to the benzene rings as well) [1-2, 4-7, 8-15]. There still remains some controversy whether to count naphthalene as a PAH because in actuality it is a bicyclic compound (see Figure 2.2.1). However, naphthalene exhibits many of the same characteristics as all other PAHs. Polycyclic aromatic hydrocarbons are distributed throughout the atmosphere dependent upon location. These compounds of interest possess very distinct ultraviolet (UV) absorbance

spectra. The spectra is dependent upon each aromatic ring and its location to the rings it may be fused with. As a result, no single PAH having the same spectra [8]. As it will be shown in the results section, most PAH are also fluorescent as well, emitting distinct wavelengths of light upon excitement. They are known to have low solubility in water (especially those with three or more fused rings) along with a low vapor pressure [3]. As a result, at ambient temperature most PAHs are present in the air [2-3]. The PAHs with a higher molecular weight are mostly present in the gas phase. On the contrary, PAHs with a lower molecular weight are found adsorbed on particles dispensed in the air [3]. Thus, resulting in these compounds being one of the first to be identified as a potential carcinogen [3]. It was suggested that there is increased suspicion that increase in molecular weight increases the carcinogenicity while decreasing the adverse effects of exposure within 24 hours (better known as acute toxicity) [3]. These lipophilic hydrocarbons and derivatives are formed through the incomplete combustion of organic material. Some of this combustion is what some may classify as naturally occurring or natural combustion such as volcanic eruptions and forest fire. However, most of this emission is caused by humans; from vehicular emissions to industrial emissions [3]. Because of the exposure being able to be carcinogenic, many countries have put non-mandatory limits on anthropogenic emissions. PAHs can be synthesized through a host of mechanistic approaches. Many scientists have geared their research toward unraveling how these PAHs are found through nature. Polyaromatic hydrocarbons are the most stable form of hydrocarbons. They usually appear as complex mixtures rather than single compounds [3]. Because PAHs do not burn very easily (extreme temperatures are need to cause decomposition) they can remain in the environment for long periods of time [2].

Most PAHs are used to conduct research, however, some are still used to make dyes, plastic, pesticides, and even some are used in synthesizing some medications [2-3, 6, 7]. Whilst it is

known that many of these compounds in the human body can have adverse effects, it is also imperative to understand the human body is variant in response. One of the most common ways these contaminants are presented to the body is through the inhalation of contaminated air [2, 7, 9-10]. If a family lives near a hazardous waste site, they are easily exposed to these PAHs by the air they breathe. Because of their high volatility it makes it easier for the PAHs to convert into the vapor phase thus causing them to be one of the constituents in the air. Also, if a family has an open wood fireplace in their home, they could be exposed to these hazardous compounds. Another method of contact is through eating and drinking food and water that are contaminated with the PAHs. A lot of times when grilling food with coal, the meats are then exposed to these PAHs formed and those partaking in eating the grilled food are exposed to them. This could explain the reasoning for it being strongly advised to limit the intake of grilled food and to avoid charring food. They can also enter the body through skin contact with contaminated products (heavy oil, coal tar, etc.) [1-16]. Once PAHs enter the body they are readily stored in fats and tissues. The primary target organs for these compounds include the liver and kidneys. It is known that the body rids (~90%) most of the PAH contamination in a matter of days [2].

There is no clinical or scientific proof that PAHs are the primary cause of cancer or other health defects in humans. However, a number of selected PAHs have caused tumors in laboratory animals that were exposed to them in high concentrations. Mice were exposed to PAHs through their food and by skin contact [2]. As a result of the exposure, these laboratory animals developed stomach, skin and lung cancers. The affects PAHs will have on human health depends mainly on the length of time exposed, the amount of PAH exposure and the toxicity with regards to the types of exposure [3]. However, some non-cancer health effects of long term PAH exposure may include cataracts, kidney damage, liver damage and jaundice. The ability of most PAH mixtures to cause

or lead to human cancer has been thought of since the late 1700s. This was discovered when Sir Percival Pott was able to successfully demonstrate the correlation between exposure of chimney soot and the incident of scrotum cancer [13].

Polyaromatic hydrocarbons are mostly formed during the incomplete combustion and pyrolysis (followed by pyrosynthesis) of fossil fuels or wood, and from the release of petroleum products (such as oil spills). They are also found in coal combustion which is the key primary fossil fuel that is responsible for about 27% of the world's energy consumption [1,4,6,7,8,10]. It is responsible for about 70% of energy consumption in China (about 45% greater than the world average). Whilst being a global provider of energy, coal combustion, gasification, carbonization and liquefication are all major sources of the formation of PAHs. In the conversion process, coal pyrolysis and pyrosynthesis are important factors in the formation of the PAHs [3]. Pyrolysis shall be defined as the thermochemical decomposition of organic material at elevated temperatures. At a specific temperature, a larger organic compound is in its molecular vibrational state. Textbook knowledge suggests that the frequency at which a molecule vibrates is directly proportional to the amount of heat (energy) applied [3]. Therefore, at very high temperatures, the molecule will have elevated vibrations. At this point in time, the molecules are stretched and begin breaking down into smaller molecules.

After pyrolysis of the bigger molecules takes place pyrosynthesis, the thermochemical synthesis (fusion) of free radicals (as a result of pyrolysis) to form more complex compounds (PAHs), takes place. Pyrolysis is subsequently followed by pyrosynthesis. Thus yielding some of the most dangerous PAHs known to date. Figure 2.1 below gives a general schematic of pyrolysis followed by pyrosynthesis thus resulting in the formation of the PAHs. It was noted the following in investigation of PAH emission from pyrolysis of Chinese coal; PAH production reached a peak

at around 800°C. They were also able to conclude that the lower temperature PAH emission were already present in the coal [4].

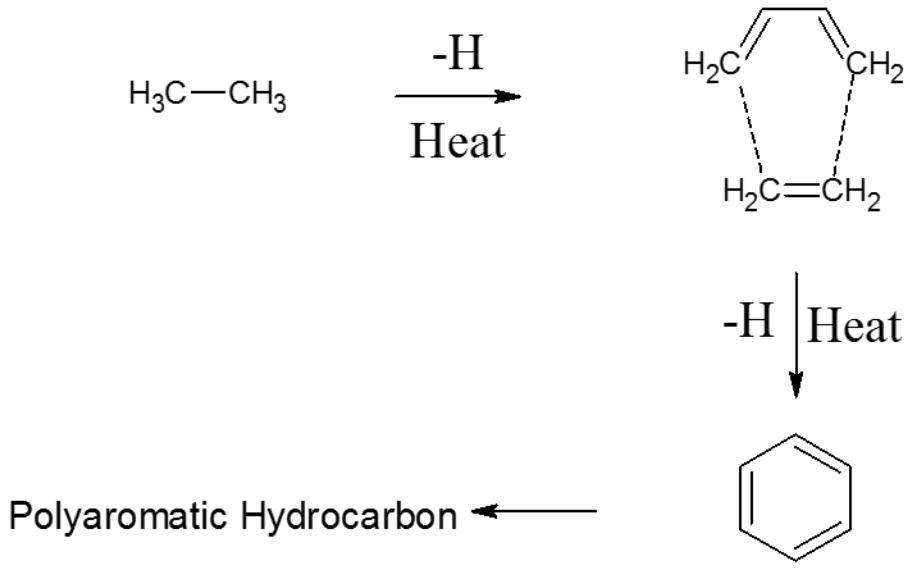

Figure 2.1. Pyrosynthesis of PAHs starting with ethane.

Ravindra et al. (2008) suggested through review that low hydrocarbons such as ethane are able to form PAHs by pyrosynthesis [3]. They noted that when temperatures get as high as 500°C the carbon-hydrogen and the carbon-carbon bonds are broken to form free radicals. These radicals can then bind with other compounds during the process are then able to combine to form aromatic rings which are now resistant to further thermal degradation.

Ravindra et al. (2008) further concluded in their review for three mechanistic approaches for PAH formation during combustion [3]. PAH formation could happen through slow Diels-Alder condensation reactions, rapid radical reactions, and ionic reaction mechanisms. It was later suggested that the radical formation mechanism was the favored mechanism because of the combustion process happens rapidly [3].

The United States Agency for Toxic Substances and Disease Registry (ATSDR/US) has considered 17 PAHs to be priority PAHs, seen in Table 2.1 [3,8].

Table 2.1

ATSDR/US Priority PAHs, and Phase Distribution [3,8]

PAHS	**Particle/gas phase distribution**
Acenaphthene	Gas phase
Acenaphthlene	Gas phase
Anthracene	Particle gas phase
Pheanthrene	Particle gas phase
Pyrene	Particle gas phase
Benz[a]anthacene	Particle phase
Chrysene	Particle phase
Benzo[b]fluoranthene	Particle phase
Benzo[j]fluoranthene	Particle phase
Benzo[k]fluoranthene	Particle phase
Benzo[a]pyrene	Particle phase
Benzo[e]pyrene	Particle phase
Fluoranthene	Particle gas phase
Flourene	Gas phase
Dbenz[a,h]anthracene	Particle phase
Benzo[g,h,i]perylene	Particle phase
Indeno[1,2,3-c,d]pyrene	Particle phase

The pre-dominate sources of the PAHs are motor vehicles and wood smoke. Although no single PAH is exactly like another PAH, it is suspected that these selected priority PAHs are more harmful and are exemplified through other PAHs. Another reason why these are chosen as priority PAHS is because there is a greater chance for human exposure than the exposure of other PAHs [3, 8]. A few methods have been developed to assess the amount of PAHs absorbed by the body and possibly distributed to different organs and tissues. Of the methods, the most widely used is a urine

test. The urine test looks for low to high levels of 1-hydorxy pyrene. This test is not recommended to the public, but for those with high risk exposure occupations [7].

2.1.1 PAHs of interest in this study. Figure 2.2 shows the structure of the PAHs of interests. These PAHs were chosen from lowest to increasing aromaticity. Naphthalene is the PAH many people smell when using moth balls (it is the main ingredient found in mothballs). It is composed of two fused benzene rings, making it the smallest PAH.

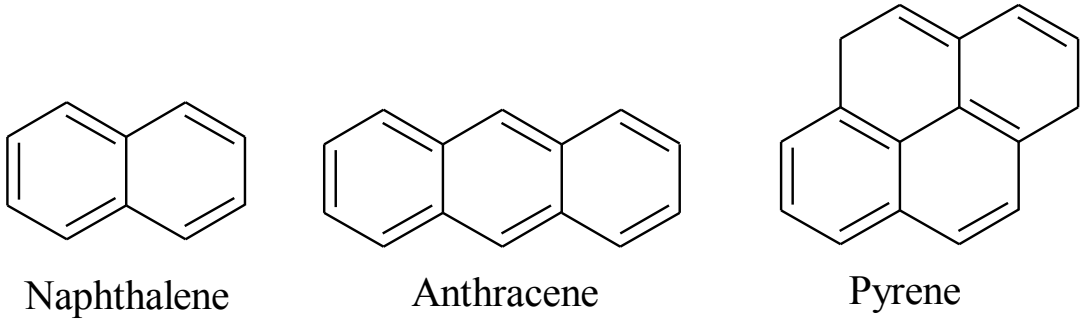

Figure 2.2. Three PAHs of Interest.

Naphthalene is the most soluble compound in water (addition of more benzene rings decreases aqueous solubility of other PAHs) [7]. Naphthalene is classified as a possible human carcinogen and its presence in both internal (homes) and external (outdoors) environment captivated the interest of this thesis. Naphthalene is ubiquitous in the environment mainly due to the burning of biomass, gasoline and oil combustion, use of mothballs and also tobacco smoking. In addition, naphthalene is also used as an intermediate during the production of phthalic anhydride, surfactants and in pesticides which the US collectively produces about 107,000 tons a year of naphthalene [9]. Because of its vapor pressure of about 0.087 mmHg at room temperature (25^0C) it is the most volatile PAH [9]. Exposure to this toxic chemical of concern has already been linked to a number of adverse health effects [9]. Some of the health concerns associate with naphthalene include but are not limited to hyperplasia (respiratory epithelium) and metaplasia

(olfactory epithelium) and nasal tumors and cancer [9]. Naphthalene can also cause the breakdown of red blood cells if inhaled or ingested in large amounts [7]. Since as early as 1980, it has been reported that naphthalene has been a subject of exposure and risk assessment because of the negligible health effects. In most recent years, naphthalene was classified as a possible human carcinogen due to the laboratory assessment of cancer in rats. Its formation, as well as the formation of other PAHs, has been studied with much rigor. It is determined that combustion is its largest emission supply. When formed through combustion, 90% of the naphthalene was in the vapor phase. Interestingly, food cooking can involve the pyrolysis and pyrosynthesis of naphthalene, researchers have reported that 0.25 micrograms per cubic meter of excess naphthalene concentrations of fried fish and pork chops while boiling contributed to lower concentrations being distributed. Another constant supply is through pesticides [9]. The major source of human exposure is through inhalation. Most of the outdoor inhalation is a result of the combustion of coal, wood and vegetation [10, 11]. Second hand smoke is another major source of inhalation. Indoor inhalation is via open fireplaces, moth repellent and burning of incense sticks [9]. More generally, naphthalene is used a moth fumigant to repels animals and insects in closets and other spaces. Napthalene is also used as a deodorizer and toilet refresher. As a result it is to inhale and come into contact with indoors [9, 10]. Naphthalene can attach to clothing and as a result can be a direct irritant to through skin irritant. It can also be ingested through both contaminated, grilled and fried foods [9]. Extensive research has been done on naphthalene, but little to no research has focused on human serum attachment and transport. Neither has much research been conducted on the cellular uptake and toxicity of naphthalene on triple negative breast cancer cells.

Anthracene is a polycyclic aromatic hydrocarbon consisting of three linear fused benzene rings as shown in Figure 2.2. Like many other PAHs, anthracene is used to make dyes, plastics

and pesticides. It has also been used to make smoke screens [13]. Anthracene and its derivatives are emitted from some of the same sources as all other PAHs. This includes but not limited to combustion of coal, coal tar, emission of fuels and oil seepage. Like naphthalene, anthracene is also found iin cigarettes and tobacco, therefore, one can be exposed through the inhalation of secondhand smoke. Eating grilled food is another source of anthracene exposure (combustion of charcoal). Anthracene is classified as one of the seventeen priority PAHs by the United States Agency for Toxic Substances and Disease Registry. This due is to the abundance of anthracene and its derivatives found throughout the environment [1-7,16]. It has been suggested that once anthracene enters the body it tends to target the skin, lymph system, stomach and intestines [16].

Pyrene is a polyaromatic hydrocarbon consisting of two pairs of linear fused benzene rings stacked on each other as shown in Figure 2.2. Amongst being present in combustion processes, pyrene is used in to manufacture pigments. While much research on the purest form of pyrene have not been investigated in detail, much research on pyrene containing PAHs has been investigated. These compounds along with pyrene itself have proven to be very toxic and carcinogenic to the human population. Because of its ubiquities in nature, pyre is also listed amongst the 17 US EPA priority PAHs [1-7]. Minimum research has been done to focus on the practical modes of transport via biological specimen. Also, no research to date has been shown in the toxicity of the compounds to triple negative breast cancer.

2.2 Human Serum Albumin and Bovine Serum Albumin

Human serum albumin and bovine serum albumin are major biological proteins. Human serum albumin (HSA) found in the plasma. The typical concentration found in the plasma is about 3.5-5g/100ml [17-18]. Human serum albumin is an anionic, hydrophilic protein with three major binding regions which will be discussed throughout this section. The protein's molecular weight

is about 66,500 Daltons. Like many other important proteins found in the human body, HSA is synthesized in the liver [18]. Human serum albumin (HSA) has a wide affinity for a variety of things including metals such as copper and zinc, fatty acids, amino acids, metabolites and many drug compounds. These are many of the reasons why HSA is of great pharmacological and biomedical interest. One of the most important physiological roles of the albumin is to deliver these solutes to the bloodstream and to their suggested target organs. Serum albumin is also important for pH and osmotic pressure maintenance of the plasma. Since as early as the 1970s, many scientists and other investigators have attempted to use the protein as a carrier to deliver specified drugs [17, 19-23].

Human serum albumin is known to be a single polypeptide amino acid sequence consisting of 585 amino acid residues. HSA has 17 pairs of disulfide bonds and one free cysteine. HSA in its monomeric form contains three domains (they are known as domain I, II and III) composed of alpha-helices. In other words, the amino acids of the protein are arranged in a helical format where there is little to no free space on the inside and the side chains are pointed towards the outside. This conformation is usually supported/stabilized through hydrogen bonding [20]. Each of the three domains contain ten helices. These helices are broken in to two groups, A and B. Group A contains six helices and group B contain four helices. Groups A and B are anti-parallel to one another in each of the three major domains [20].Over the last 40 years, many biomedical researchers have gravitated towards understanding the nature of the binding sites on the protein [20]. Several crystal structure analyses have revealed that many binding sites, especially those of drugs are found in subdomains IIA and IIIA [21]. Domain IIA presents a large hydrophobic pocket. While it is known that subdomain IIA and IIIA are similar topologically similar, in that they are composed of the same six-helices and hydrophobic pocket/cavity which has distinguishable polar attributes. Several crystal forms of HSA were studied and reported in the early 1970s [17 19]. During this time there was

not much structural information provided about the protein because of the poor crystal reproducibility. In the late 1980s through the late 1990s, carter and coworkers along with another group were able to produce crystal structures of the protein make it better for the structural features of the protein to be understood. Also, it shed light to the three–dimensional structure which clarified how the protein-molecule interactions of many drugs, metabolites and potential environmental contaminants [17]. It is necessary in the biomedical and pharmacological community to understand the binding and transport functions of HSA to provide necessary therapies and treatments to biological systems. Figure 2.3 gives a more detailed representation of HSA [19]. Because of the three fluorescent amino acids, tryptophan, tyrosine and phenylalanine (these compounds are fluorescent due to the conjugation), we were able to observe the fluorescent and identify the binding affinity of PAHs to this protein.

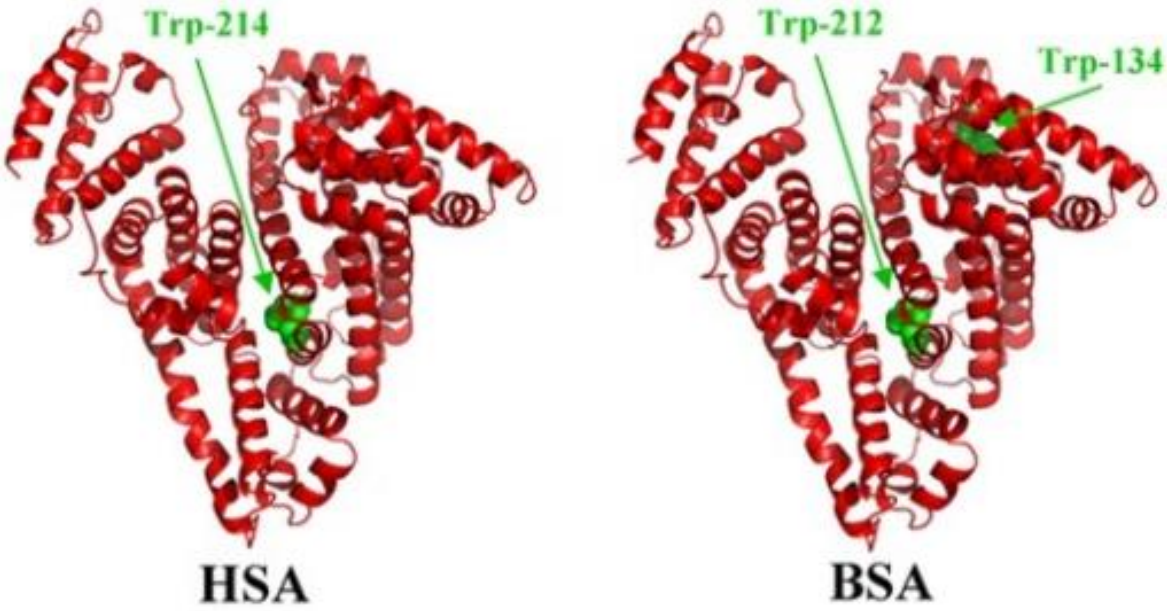

Figure 2.3. Three-dimensional structure of HSA and BSA [24].

Bovine serum albumin (BSA) (Figure 2.2.1), like HSA is a biological protein. This protein is one of the most abundant found in the cow serum. It differs from HSA in many ways. One of the main differences is that BSA has two tryptophan amino acids [21, 26-29]. One is located in domain

IA (amino acid 135) and the other is located in domain IIA (amino acid 212). For this cause both domains IA and IIA are the two major binding sites of this protein [18, 21, 26]. Bovine serum albumin has a molecular weight of about 66,500 Daltons. It used and studied for many of the same reason as discussed with HSA.

2.3 Cell Cycle, Cancer, Triple Negative Breast Cancer

While it is extremely pharmacologically important to understand how PAHs bind to both human and bovine serum albumin, we must take into account with how these particular compounds interacts with human cells. In collaboration with Dr. Checo Rorie (Biology Department, NCAT) the interaction of PAHs with triple negative breast cancer cells (TNBC) was investigated. Before advancing further into detail on the focus of the PAH-cell interaction, it is imperative to have an understanding of the cell cycle and cancer (specifically triple negative breast cancer).

A cell is classified as the basic unit of life and can only arise from pre-existing, living cells. In other words, cells do not just magically appear. To be more specific, this study focuses on human breast cells. Like all human cells, these cells must go through the cell cycle. Many scientists agree that there are two phases of the cell cycle, interphase and mitosis as shown in Figure 2.4.

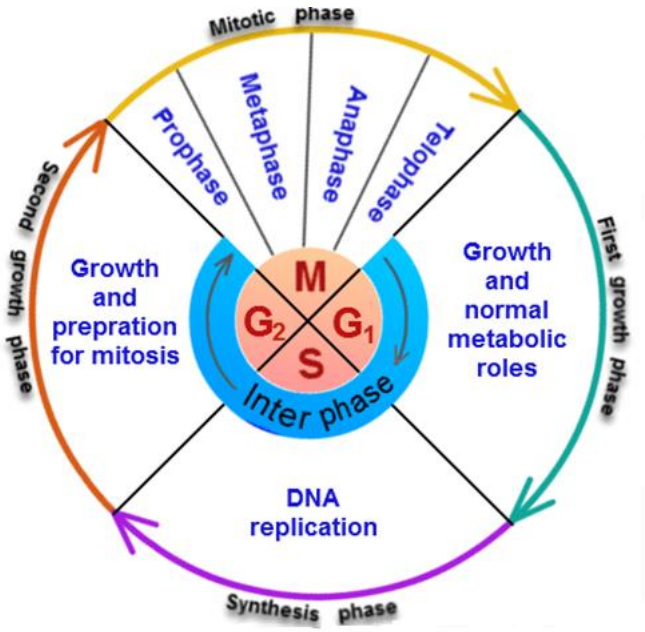

Figure 2.4. A brief overview of the cell cycle. Interphase and mitosis are the major components thereof [30].

In the first phase of the cell cycle, interphase, it is further broken down into three distinct periods (some may consider it as four periods if they consider growth period zero). During the first growth period, the cell maintains growth and metabolic roles. This is usually the longest of them all. This period is the period where the cell is living its normal life [30]. During this period of maintaining its metabolic roles, the cell is said to be fulfilling its purpose in life. For example, if it is a white blood cell, it is fighting off infections. However, at some point during the cells lifetime, it has to make the decision to divide [30]. Once this decision is made by the cell it enters its first check point (from now on these check points will be referred to as stop lights). It is very important that the cell stops at the stop light. While at the stop light the cell is given time to check itself for any malfunctions and mutations [30]. If the cell recognize that something is not right the cell will fix it with its repair enzymes. Sometimes the cell cannot fix the problem, so in a healthy individual, the cell will kill itself via apoptosis and will not move on to the next period. Once the cell has

repaired itself and have the go ahead, it then advances to the next period. This period (S-period) is referred to the synthesis period. During this period, the cells goes through deoxyribonucleic acid (DNA) replication [30]. After DNA replication the cell goes sees another stop light. If it cannot repair anything that is it sees wrong it goes through apoptosis. However, if it repairs the inconsistencies, it to the last period of interphase. This period is known as the second growth period [30]. During this period the cell is awaiting to advance to mitosis. During the second growth phase, the cells are making the last organelles necessary to advance to cell division. Once it is has succeeded at the stop light the cell is now ready to undergo mitosis (also known as cell division). There are four steps that happens before a cell successfully undergoes mitosis as shown in Figure 2.5.

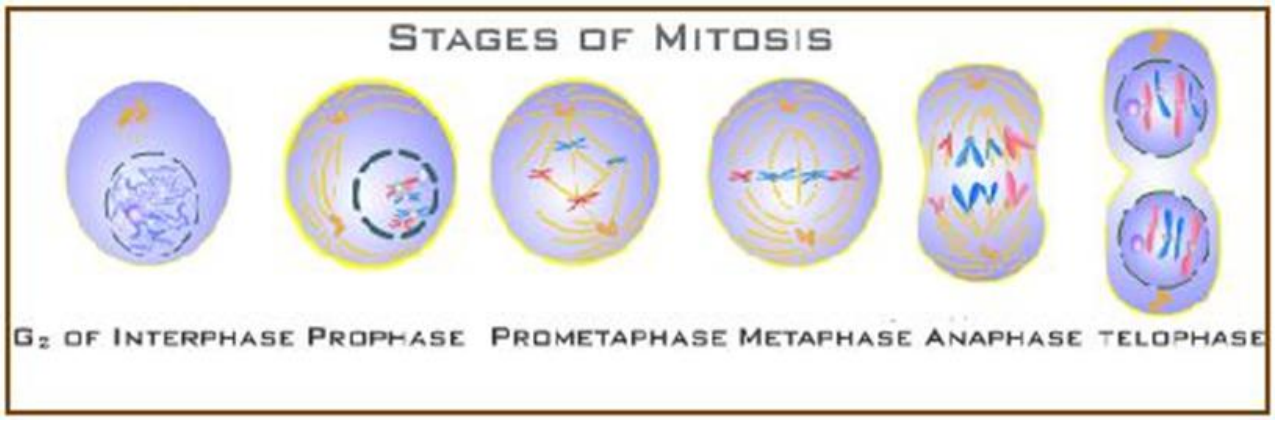

Figure 2.5. The four primary stages of mitosis [31].

The very first step is prophase. During this step the cell has now full committed to undergoing division. At this point, the nucleolus has now disappeared. The genetic material has now become loosely coiled (known as chromatin). Mitotic spindles begin formation as centrioles are moving to opposites sides of the cell. During the second step, metaphase the chromosomes and the centromeres begin to line along the equatorial plane of the cell (many biologist refer to this as the metaphase plate. This aid the cell in ensuring that when these chromosomes are separated, each nucleus will get a copy of each. During anaphase, which is the third step in mitosis, each pair of chromosomes are now separated and are pulled to opposite ends of the cell via the microtubules

(microtubules degrade after this). During the last step in mitosis, telophase, new membranes begin to form around the prospective organelles. It is also important to note at this point the chromosome spread is not noticeable. Toward the end of telophase one will notice that there are two copies of all organelles and also two cells that have not yet been detached. At this point the cell goes through cytokinesis where the cell membranes are separated. Cytokinesis is a separate process from mitosis, though many will include it with telophase. This is a result of the process beginning the same time as telophase and ends upon the completion of telophase.

The description above is how a normal, healthy cell were to behave under normal conditions. When cells do not operate according to how they should, many diseases including cancer may develop. Cancer is known to many as one to the most terrifying ailments to deal with. Cancer is a result of the cells passing the stop light during the cell cycle. Instead, these cells metaphorically put their feet on the gas pedal and go through rapid and uncontrolled cell proliferation. There are seven hallmarks of cancer which is shown in Figure 2.6.

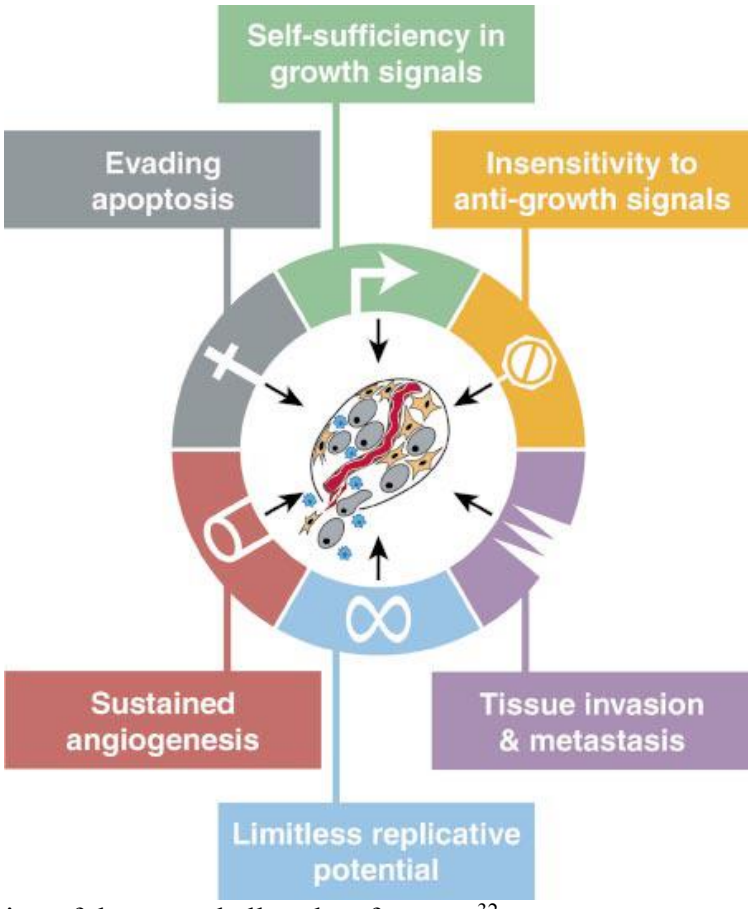

Figure 2.6. Illustration of the seven hallmarks of cancer [32].

In no particular order, one of the hallmarks of cancer is self-sufficiency in growth signals. In normal cells, growth occurs upon expression of growth factors. In most cancer cells there is an overexpression of these growth factors. Another hallmark of cancer is the insensitivity to anti-growth signals. The cancer cells no longer have receptors for the anti-growth factors it is presented with so they are unable to recognize and respond to it. Another hallmark of cancer is the tissue evasion and also metastasis. This could be broken into different hallmarks. One of the most common hallmarks of cancer is limitless replicative potential. Once cancer is more advanced in its stages (stages of cancer will be discussed later in this section) it begins angiogenesis. In laymen terms, the cancer now has generated/created its own blood supply. The last hallmark of cancer is evading apoptosis. That is some cancer cells has the ability to ignore signals to kill itself [33]. Many

oncological professionals warn that if one of the hallmarks of cancer are present, then one could have cancer. A person does not have to exhibit all of the hallmarks of cancer because every biological system responds differently.

Modern day medicine and therapies have advanced significantly to ensure that that those living with cancer are able to manage it. Some of the treatment options for cancer is dependent on the overall health and preference of the patient. Treatment options include, chemotherapy, immunotherapy, and targeted therapy to name a few. These options are also dependent upon type and progression of the cancer. There are five stages of cancer. Stage 0 is known as in-situ cancer. At this stage, the cancer is in place and have not invaded any of its nearby tissues. During this stage the cancer is said to be curable. This is because the cancer could easily be removed by simply removing the tumor. Stage I is known as localized cancer, also It is most notably referred to as early-stage cancer. The cancer has not yet invaded deep into the tissues or reached the lymph nodes. During stages II and II the tumor have now grown more vastly into the nearby tissues. At this point angiogenesis has now begun and the cancer has spread to the lymph nodes. Lastly, stage IV is the most malignant form of cancer. At this point, the cancer have now spread throughout the rest of the body and now begun attacking other tissues and organs (this is known as metastasis) [33]. This is the most difficult type of cancer to treat.

People are diagnosed with various types of cancers in the United States alone. Figure 2.7 illustrates many of the different types of cancer found in both men and women. Table 2.1 shows a more statistical view of cancer and its statistics as it relates to the most prevalent cancers and deaths in the USA.

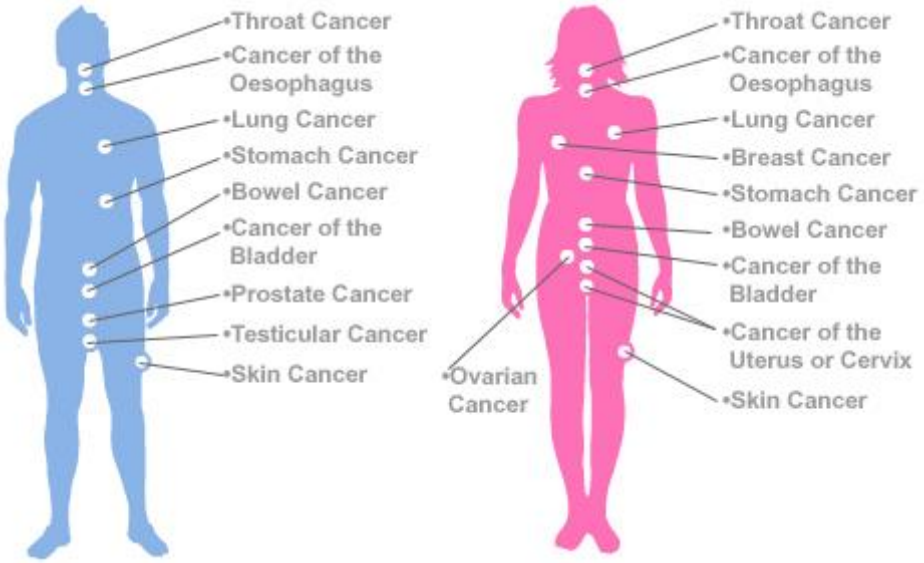

Figure 2.7. Types of cancers found in both men and women [34]

Table 2.2

Estimated New Cases of Cancer and Cancer Deaths in 2014 [35]

Estimated New Cases and Deaths		
Male		
Type	**Number of Cases (thousand)**	**Deaths (thousand)**
Prostate	233	29.48
Lung and bronchus	116	86.93
Colon and rectum	71.83	26.27
Female		
Breast	232.67	40
Lung and bronchus	108.21	72.33
Colon and rectum	65	24.04

Age, race, diet, genetic, and gender are among the major risk factors of a person getting cancer in his or her lifetime. The focus of this thesis is the investigation on TNBC cells. Breast cancer is the most common type of cancer a female is diagnosed with in the United States. As Table 2.2 suggests, an estimated 232,670 women were diagnosed with cancer in the year 2014. Of the women diagnosed with cancer an approximate 40,000 died of breast cancer. There are many forms of breast is more than one form of breast cancer, however this study focused on TNBC cells.

Triple-negative breast cancer is a more recently coined term in the medical and scientific community. It was first mentioned in 2005 but ever since then it has been mention in nearly 1,000 publications. This is a result of increasing interest by health and other professionals because of the 12% to 17% of women with breast cancer who have triple negative breast cancer [36]. Treatments are not as readily available for women with this type of breast cancer. Triple-negative breast cancer is the only type of breast cancer that lack expressions of the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor type 2 (HER2) [36-39]. Triple negative breast cancer occur more frequently in young black and Hispanic women than those of other racial or ethnic groups [36]. Approximately 20-40% of African American women diagnosed with breast cancer have triple-negative breast cancer [38]. Doctors use the same types of tests, treatments and surgeries for possible treatment of triple negative breast cancers as they do for other types of breast cancers. The treatment for those diagnosed with this form of breast cancer is chemotherapy. This is a type a medicine that kills cancer cells everywhere in the body [38]. However, it is very important to note that this type of treatment is called systemic, meaning that it is a whole-body therapy. Therefore, this therapy is killing off a majority of the cancer cells, it also kills some of the healthy cells as well which may cause one to feel pain, sickness, and fatigue. Medical and other oncologic professionals are making every effort to encourage women to have thorough breast examinations and also check for mutations in one of the two genes (BRCA1 and BRCA2) as it may very well increase the risk of a her developing breast cancer in her lifetime [38].

The p53 tumor suppressor gene was first recognized in 1979 as the transformation-related protein that accumulates in the nuclei of cancer cells [40]. It belongs to a three member protein family: p53, p63, and p73 [40]. They are all structurally and functionally related, however, it was noticed that in the higher organisms, the p53 has evolved to prevent (suppress) tumor development.

The p63 and p73 had major roles in functioning normal cellular development. The p53 protein is found on chromosome 17 and is made up of a 393 amino acid sequence. It has several structural and functional domains. A few years after this identification it was recognized that this wild type p53 protein had no oncogenic properties of the cell. It was not until the early 1990s that a mutation of p53 was discovered and classified as a gain of oncogenic function [40]. It was then noticed that the wild type p53 protein was the major guardian of the genome. That is the p53 protein contributed to many vital functions of the human system. It is responsible for cell cycle regulation, the induction of apoptosis, and genomic integrity.

As a tumor suppressor gene, one of the major physiological functions of p53 is to prevent unwarranted cell proliferation. Activation of p53 occurs after an intracellular or extracellular stimuli has caused damaged. This damage includes an array of factors such as UV radiation and cytotoxic chemicals and drugs. Once activated, p53 can induce cell cycle arrest. Cell cycle arrest could happen in any of the phases associated with interphase. By induced cellular arrest the p53 gene is given the cell additional time to repair any genomic damage. For this reason p53 is known as a cellular gatekeeper. As a gatekeeper, it monitors cellular stress and can also induce apoptosis if necessary. Figure 2.8 gives a structure of the three proteins with major emphasis on the p53 protein.

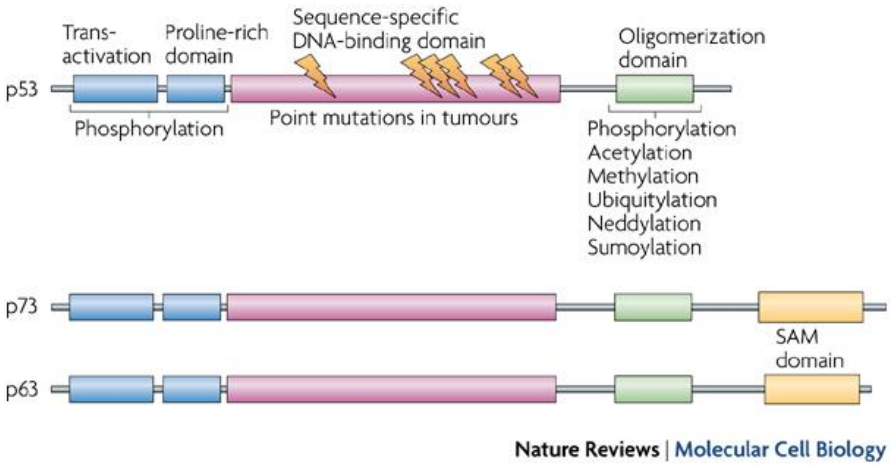

Figure 2.8. The functional domains of the p53 protein. [41]

The central region of the p53 protein is extremely hydrophobic and is a sequence-specific binding domain for damaged DNA. The p53 protein can be activated by in a variety of ways. One mode of activation is through phosphorylation from protein kinases [40]. Lack of the p53 gene activation is known to play a role in all types of cancers and is associated with more than 50% of all cancer cases worldwide [40].

As previously mentioned, once activated p53 can cause cell arrest or apoptosis. Apoptosis is a term coined to describe programmed cell death. Apoptosis is a distinctive form of cell death [42]. Apoptosis occurs in normal cell development as mechanism to maintain cell growth. It is also used as a defense response when cells are damaged by disease and other toxic concoctions. Whilst there are both pathological and physiological stimuli that may induce apoptosis, not all cells will die via the same stimulus. DNA-damaged cells die via a p53 dependent pathway of apoptosis, while other cells are unaffected [42]. The apoptotic pathways of cell death is very sophisticated and complex. Two of the major apoptotic pathways are the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathways [42]. Figure 2.9 gives a general diagram of how the p53 protein engage in the apoptotic cycle.

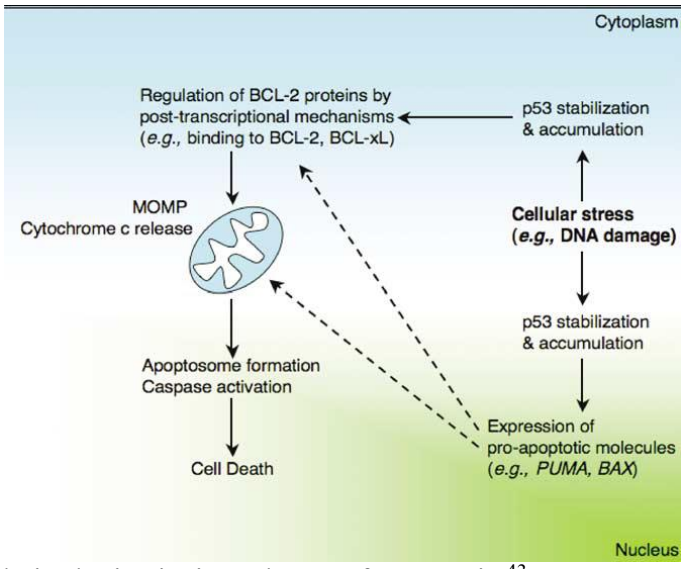

Figure 2.9. p53 role in the intrinsic pathway of apoptosis [43]

The p53 dependent apoptotic pathway is a form of the intrinsic pathway. Cellular stressors, such as DNA damage, stabilize and activate the p53 protein by activation [43]. The stabilized forms of p53 accumulates in the nucleus and cytoplasm of the stressed cells and regulates the expression of the pro-apoptotic genes that are directly associated with inducing mitochondrial outer-membrane permiabilization. Once this happens an apoptosome formation is initiated and a cell death cascade is activated [43].

2.4 Fluorescence

Luminescence comes from the Latin root lumen which means light [44]. In 1888, Eilhard Wiedemann came up with this term to describe the phenomena of light that is not incandescence (conditioned by the rise of temperature). The present day definition of luminescence is "a spontaneous of emission of radiation from an electronically excited species (or from a vibrationally excited species) not in thermal equilibrium with its environment" [44]. In more simple terms, luminescence can be defined as the "transformation of diverse modes of energy into a form of visible light" [44]. There are nine different types of luminescence. They are classified according to

their mode of excitation. The type of luminescence that was focused on for purposes of this thesis paper was photoluminescence.

Photoluminescence can be defined as the production of light "from the direct photoexcitation of the emitting species" [45]. Photoluminescence is divided into three sub-groups: (1) fluorescence, (2) phosphorescence and (3) delayed fluorescence. The term fluorescence was first introduced by G.G. Stokes in the mid-nineteenth century. At this point in time the distinction between fluorescence and phosphorescence was that fluorescence was the emission light that disappears simultaneously at the end of excitation. Whereas with phosphorescence, the light emitted will persist for some time even after excitation [45]. After the discovery of delayed fluorescence, those distinctions became inadequate. Molecular photochemists now say that spin multiplicity is retained for fluorescence, however, phosphorescence would involve a change in multiplicity usually from singlet to triplet or vice versa (shown in Figure 2.10) [45].

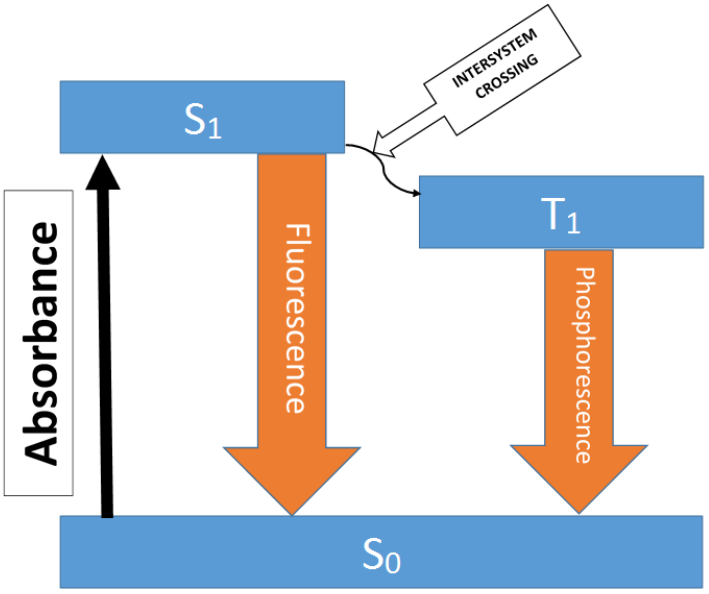

Figure 2.10. The simplified Perrin-Jablonski Diagram. Non-radiative decay is not shown in figure.

In the simplified Perrin-Jablonski diagram (see Figure 2.10), the distinction between fluorescence and phosphoresce. In the diagram the electron are excited (absorb the energy,

absorption spectrum), after excitation the electron loses its energy either through radiative relaxation (emits light, emission spectrum) or non-radiative relaxation (does not emit light, not pictured). Since the purpose of this thesis did not focus on phosphorescence, the rest of this section will focus primarily on steady state fluorescence. The period where the fluorophore is absorbing energy (or when the electron is excited) is when the electron in the lowest unoccupied orbital (better known as the LUMO) is being shifted to the highest unoccupied molecular orbital (HOMO). Fluorescence typically occurs from aromatic molecules. The first known fluorophore was quinine. Quinine played a key role in the development of the spectrofluorometers in the 1950s [46].

As seen in Figure 2.11, after an electron is excited (period of absorption) it is rapidly rotates downward to the next electronic sub-stable level (kasha's rule). This is known as internal conversion. It should be noted that excitation happens very rapidly ($\sim 10^{-15}$ seconds). Internal conversions happens in as little as $\sim 10^{-13}$ seconds. After internal conversion, during fluorescence the electron experiences both non-radiative relaxation (no emission as it travels to the ground state (this is not pictured). and also radiative fluorescence (emission of light). This is said to happen as quickly as ~10-9secs. It should be noted that these times are were taken from one source [47]. The author did make a note that there were minor variations of time throughout the scientific community. The time for the electron to reach its stable state (S_0) is relatively faster in fluorescence than that of phosphorescence (said to happen anywhere from 10^{-3}-10 seconds). This has to do with the intersystem crossing (shown in Figure 2.11). During intersystem crossing the electron is going from a singlet stat to triplet state, to get back to the stable singlet state (S_0) [47].

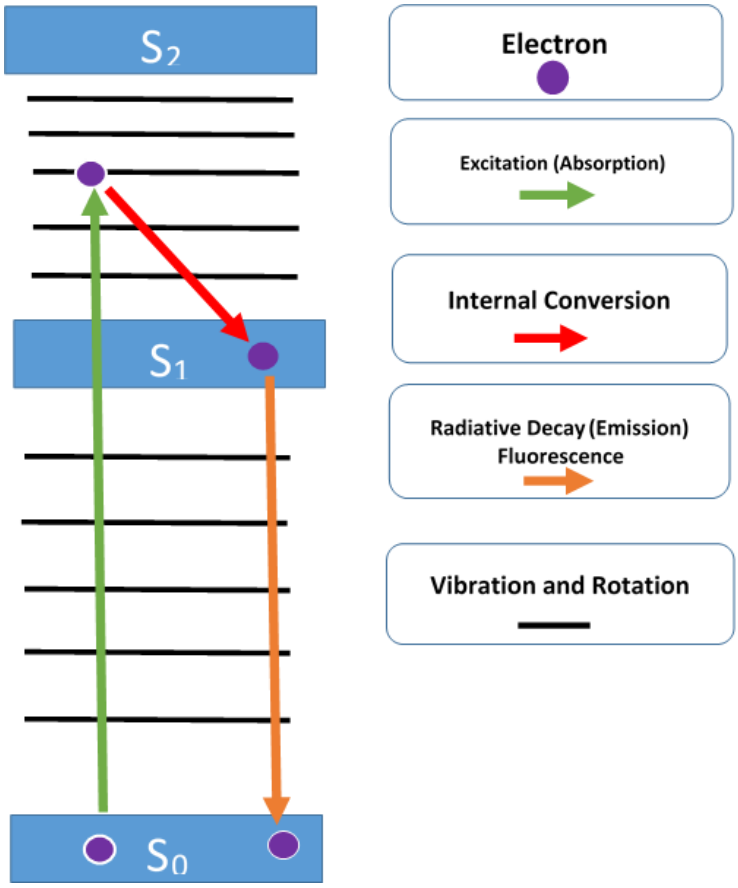

Figure 2.11. Jablonski's diagram of fluorescence. Legend to the right gives brief description. Non-radiative decay not shown in figure.

The Jablonski diagram is named after Professor Alexandar Jablonski and he is regarded as the father of fluorescence because of his many contributions to the field [46]. While Jablonski's diagram of fluorescence gives us a great idea of adsorption and emission of light, it also gives some generalization of what to expect when observing different fluorophores. Upon adsorption of light, flourophores, tend to emit light at longer wavelengths. This is known as a red shift (Stokes shift) [44-45]. This could be easily explained through use of the Figure 2.11. Once excited, the electron then experiences internal conversion. A red shift is described in general chemistry as the point when energy shifts from shorter wavelength toward longer wavelengths (toward the red region of the visible spectrum). Having longer wavelengths would result in the decrease in frequencies thus

having lower energy. This is what happens during internal conversion. If the electron is excited to the vibrational and rotation region, it will rotate and vibrate down to the next sub-stable state via Kasha's rule [44]. Therefore, the electron will be emitting light at a lower energy than it had absorb, resulting in the red (Stoke's or Bata chromic) shift.

It is also important to understand the principles of measuring fluorescence. Figure 2.12 gives an illustrated overview of fluorescence measurement.

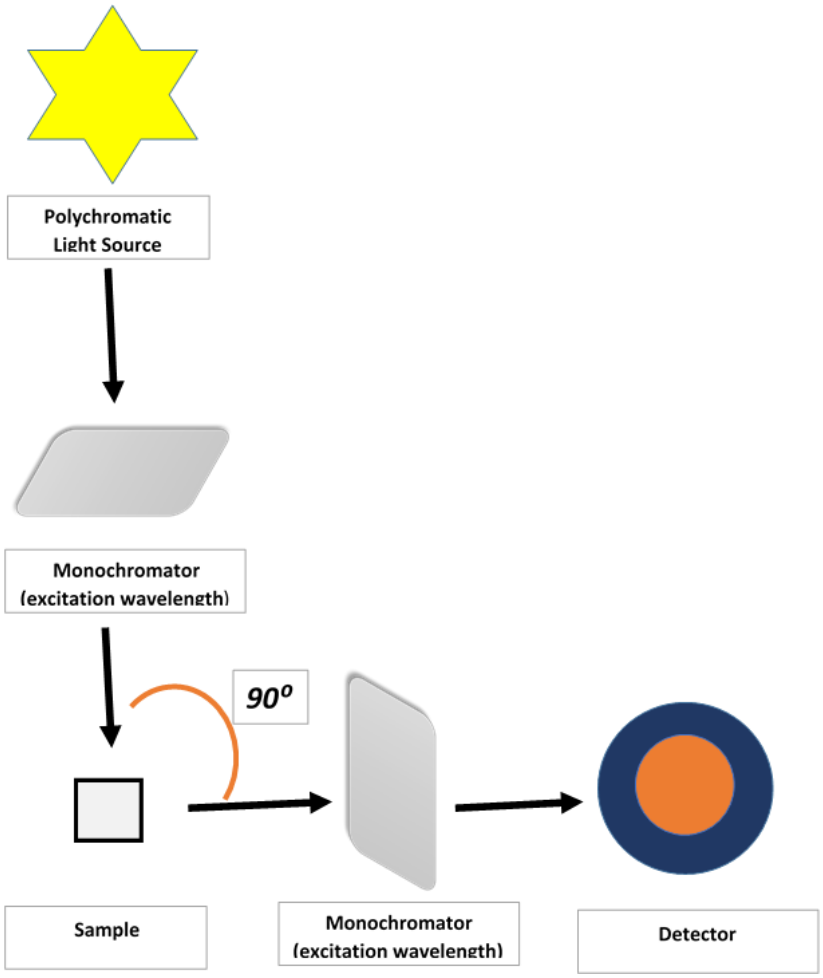

Figure 2.12. Instrumentation, measuring fluorescence

First the polychromatic light (our light source) goes through a monochromator (prism). The first monochromator is responsible for the excitation wavelength. This monochromator will only allow the wavelength chosen to shine through to the sample. Once the wavelength hits the samples

there is a 90^0 turn which make the fluorescence more sensitive than UV absorption. Once the monochromatic light shines through the sample the fluorescence given off through the sample goes through a monochromator responsible emission wavelength. This monochromator will allow the emission wavelength chosen to shine through to the detector. The detector will supply the reading [47]. Fluorescence is now one of the most used methodologies in an array of biomedical and biotechnology studies [46].

CHAPTER 3

Methodology

3.1 Sample Preparation

Acid 3-morpholino-propanesulfonique (MOPS) buffer, HSA, BSA, naphthalene, anthracene, pyrene, dimethyl sulfoxide (DMSO) and acetonitrile were purchased from SIGMA-ALDRICH Company. Sodium hydroxide was purchased from Fisher-Scientific Company. Throughout the whole project 30M MOPS buffer solution was used. The pH of the buffer solution was adjusted to 7.4 (physiological pH) sodium hydroxide solution. The buffer solution was stored in the refrigerator for 24 hours before use.

Approximately 0.025% solutions of HSA and BSA were prepared using MOPS, 7.4 buffer solution. Upon preparation, these protein solution were store in the refrigerator for a minimum of 48 hours to ensure activation. It is also important to note that none of the protein solutions were used pass twenty days to ensure protein activity.

Stock solutions of analytes (naphthalene, anthracene, and pyrene) were made using solvent acetonitrile. This solvent was used for spectroscopic results. All of the solutions were made so that the PAH concentrations were 1.00×10^{-03}M. All stock solutions were stored in the refrigerator for at least 24 hours before use.

The PAHs were titrated, using eppendorf pipets, into HSA/BSA solutions and buffered to the 3 ml mark. While naphthalene concentrations ranged between 2.00×10^{-06}M and 1.40×10^{-05}M, while anthracene concentrations ranged between the values of 2.00×10^{-06}M and 6.60×10^{-05}M. The pyrene concentration ranged between 8.00×10^{-07}M and 1.28×10^{-05}M. All samples including control (HSA/BSA and buffer mixed only) and blank (buffer only) were equilibrated at room temperature for at least 1 hour to ensure protein-analyte interaction. The samples were subjected

to fluorescent and ultraviolet-visible measurement using Spectrofluorometer (Jasco-8500) and Shimadzu UV-Vis NIR Spectrophotometer respectively.

3.2 Ultraviolet-Visible (UV-Vis) Spectroscopy

Different molecules can absorb different wavelengths of light. Since the proteins of interest (BSA and HSA) absorb in the UV-Vis region, UV-Vis spectroscopic techniques was used for the study. The visible of the electromagnetic region ranges from 400 nm to 780 nm. The ultraviolet region of the electromagnetic region ranges from 200 nm to 315 nm. For ultraviolet-visible (UV-Vis) spectroscopy, a Shimadzu UV-3600 UV-Vis NIR Spectrophotometer was used collect all data. Data was collected for all of the protein-analyte complexes at five different temperatures; 25°C, 37°C, 45°C, 55°C, and 65°C. UV-Vis main purpose was to obtain the absorbance of HSA and BSA. The lambda-maximum of the tryptophan residue(s) in the proteins were used for excitation wavelength when doing fluorescence. Questions were raised because some thought tyrosine was in this region, but this question was justified through internal fluorescent quenching. Also, by using this spectroscopy, the energy transitions (transitions will be discussed further in results and discussion) of the samples were determined. The blank used for protein-analyte complexes was MOPS buffer. To get a general scheme of where the analytes absorption took place, acetonitrile was as the blank. The results of these studies were used to accurately determine the concentrations of HSA and BSA, using equation 4. The concentration of BSA for this study was 1.3×10^{-06} M, and 2.5×10^{-06} M for HSA.

3.3 Fourier-Transform Infrared (FTIR) Spectroscopy

For FTIR data collection, a Shimadzu IR Affinity-1 FTIR Sectrophotometer was used. Solid samples of all proteins and analytes of interests were used to get results. Enough of each

solid sample were placed on top of the red crystal (the crystal was completely covered). All backgrounds were ran at resolution four, from 300 cm^{-1} to 4000 cm^{-1}.

3.4 Fluorescent Spectroscopy and Calculations

All fluorescent data were collected using a Jasco Spectrofluorometer JP-8500. The samples were excited at 280 nm and the emission of the samples were collected between 285 nm and 550 nm. A 1 cm cuvette was used in all experiments. Scan speed was typically at 200 nm/minute at high sensitivity with emission and excitation bandwidths of 2.5 nm.

3.4.1 Determination of binding association constant and stoichiometry of PAH-HSA/BSA complexes. The binding constants and stoichiometry were determined by using the modified Stern Volmer equation shown in Equation 1.

$$\log\left(\frac{F - F_0}{F_0}\right) = \log K + n\log[PAH]$$

Equation 1. Modified Stern Volmer equation

F is the final fluorescent intensity which is the intensity after the protein (HSA or BSA) has been exposed to naphthalene, anthracene and pyrene. F_o is the initial fluorescent intensity of the protein only. K is the binding constant and n is the stoichiometry Also, [PAH] is representative of the concentration of the PAHs.

3.4.2 Determination of thermodynamic property of PAH-HAS/BSA complexes. Thermodynamic parameters were also calculated to determine the types and feasibility of bonding. These parameters were calculated using the Van't Hoff equation shown in Equation 2.

$$\ln K = \frac{\Delta S}{R} + \frac{-\Delta H}{R}\frac{1}{T}$$

Equation 2. Van't Hoff equation

Where K the binding constant, ΔS is the entropy of the protein-analyte complex, and ΔH is the enthalpy. T is the temperature in Kelvin. R is the ideal gas constant. The Gibbs free energy (Δ*G*) was calculated using Equation 3.

$$\Delta G = \Delta H - T\Delta S - T\Delta S$$

*Equation 3.*Gibbs free energy equation

3.5 Investigation of PAHs on Triple Negative Breast Cancer: Protocol and Sample Preparation

The human breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). HCC1806 is a basal cell line that expresses the triple negative phenotype which is a result of the lack of expression from progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor 2 (HER2) receptor. This cell line was derived from a 60 year old African-American woman on July 31, 1995. She suffered from a primary squamous cell carcinoma. The tumor was considered to be a grade 2 tumor. MEGM bullet kit growth media, soybean trypsin inhibitor, 5 ml pipets and 10 ml pipets, and tissue/cell culture plates were all purchase from Fisher-Scientific Company. Upon 80% confluency of TNBC they were ready to be exposed to the PAHs of interests (naphthalene, anthracene and pyrene).

The first thing done to prepare was the placement of growth medium in 37°C water bath. After this, pre-treatment pictures of the cells were taken with a microscope at magnification 10X and 20X. With a total of thirteen cell cultured plates. Concentrations used for all three PAHs were the same (1.8×10^{-5}M, 2.5×10^{-5}M, 3.6×10^{-5}M). Standard cell plates of each concentration using dimethyl sulfoxide (DMSO) were made to compare results with results of those cells treated with PAHs. Stock solutions of PAHs were made using DMSO instead of the acetonitrile. Preliminary studies showed that acetonitrile is very toxic to TNBC cells. Old media and non-adhesive cells (apoptotic cells) were extracted from each of the thirteen cell plates. Known concentrations of

PAHs were titrated into each of the plates and buffered with medium to obtain a total volume of 2 mL. One of the thirteen plates were just treated with the medium (no other chemicals were added) this was done so that we could compare DMSO damage to cells. Treated cells were placed back in the incubator for 24 hours. The incubator was at appropriate temperature and gas mixture (37°C and 5% CO_2).

After 24 hours incubation period pictures were taken of each plate (shown in results) at the same magnifications previously described. After completion of picture taking, a cell viability stud of all plates containing concentration of 2.5×10^{-5}M of the PAH, the untreated cells, and the control (DMSO). The very first step in cell viability preparation is the collection 2 eppendorf tubes per cell plate and 1 conical tube per sample plate. Tubes were labeled according to cell plate label. A major aspect of the process is to keep the volume low. Since bigger sized cell plates were used, it was very important that conical tubes did not exceed 5 mL final volume. Secondly, media and apoptotic cells were removed and placed into the corresponding conical tube (this is about 2 mL). Next, 1 mL of phosphate buffered saline solution (purchased from Fisher-Scientific Company) and gently swirled. Solution-along with any of the residual media-was to conical tubes (conical tubes contain 3mL total volume). Addition of trypsin-ethylenediaminetetraacetic (EDTA) to the plates were allowed to 5-15 minutes. The trypsin-EDTA is responsible for chelation and is used to detach the living cells from the plate. During the process of incubation, 10 microliters of trypan blue were added to each labeled eppendorf tubes. The trypan blue is responsible for coloration of the dead cells. After all of the cells detached from the plates (a slight finger tap to ensure cells have lifted), splashing technique was use to prevent reattachment to the cell plate. After this, all contents were transferred to conical tubes (all have 5 mL total volume). Conical tubes were inverted several times and immediately transferred to the two of the appropriately labeled eppendorf tubes (10

microliters). Eppendorf tubes were vortexed for approximately 20 seconds and 10 microliters were transferred to one of two sides of the cell counting slides. A total of 2 eppendorf tubes were used per counting side (1 eppendorf tube per side). Each side of the cell counting slide was placed into the counter and data was reported.

CHAPTER 4

Spectroscopic Results

4.1 FTIR Study

FTIR study was used to identify the major functional groups of the HSA and BSA.

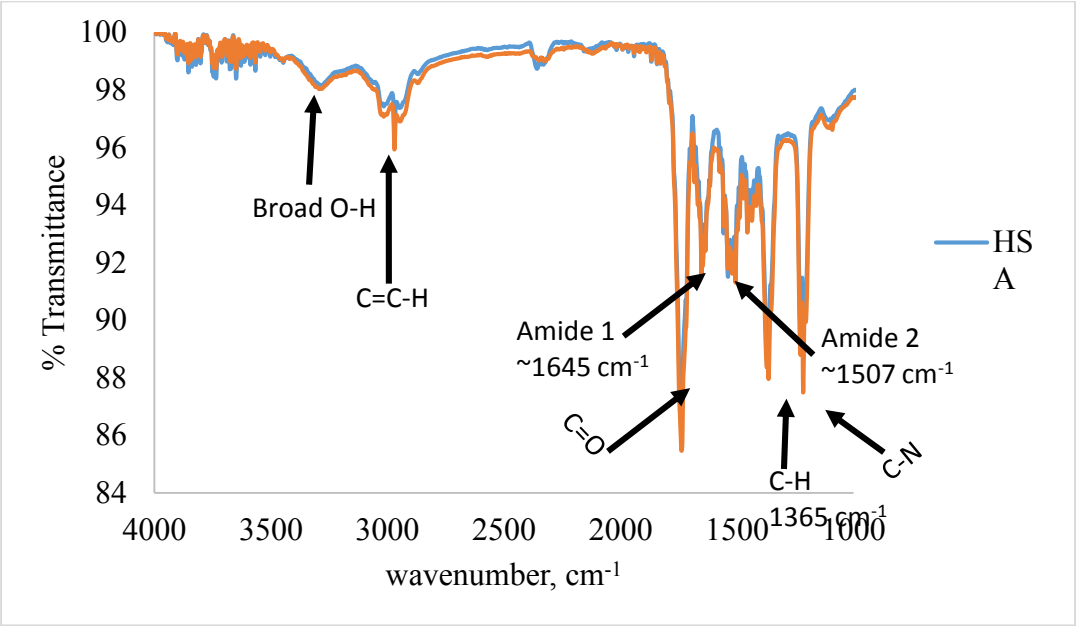

Figure 4.13. FTIR Sprctra of HSA and BSA

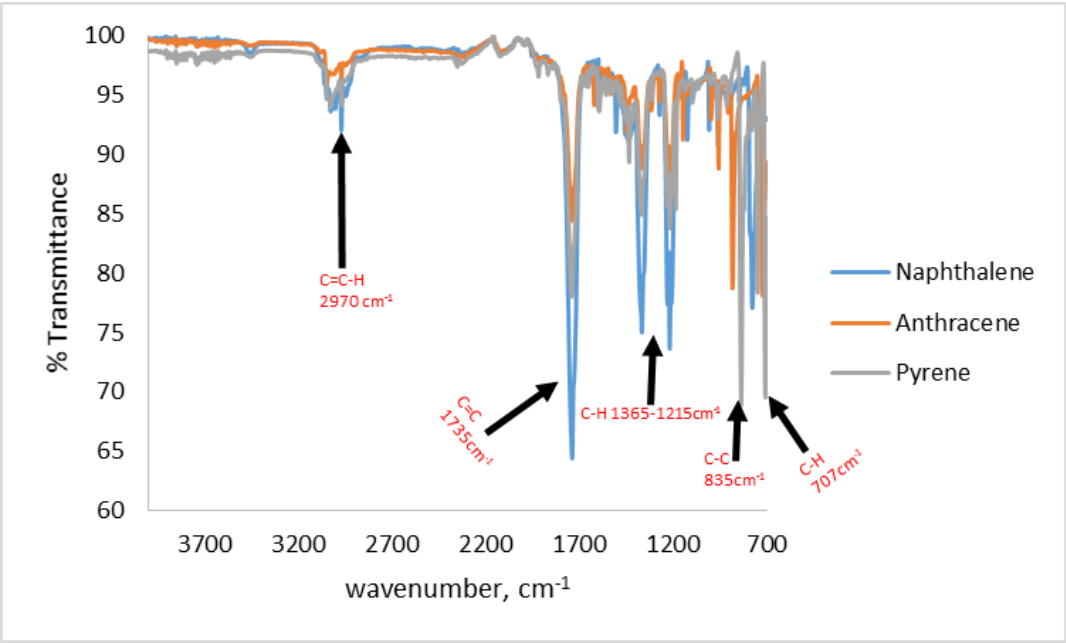

Figure 4.14. FTIR Spectra of Naphthalene, Anthracene, and Pyrene

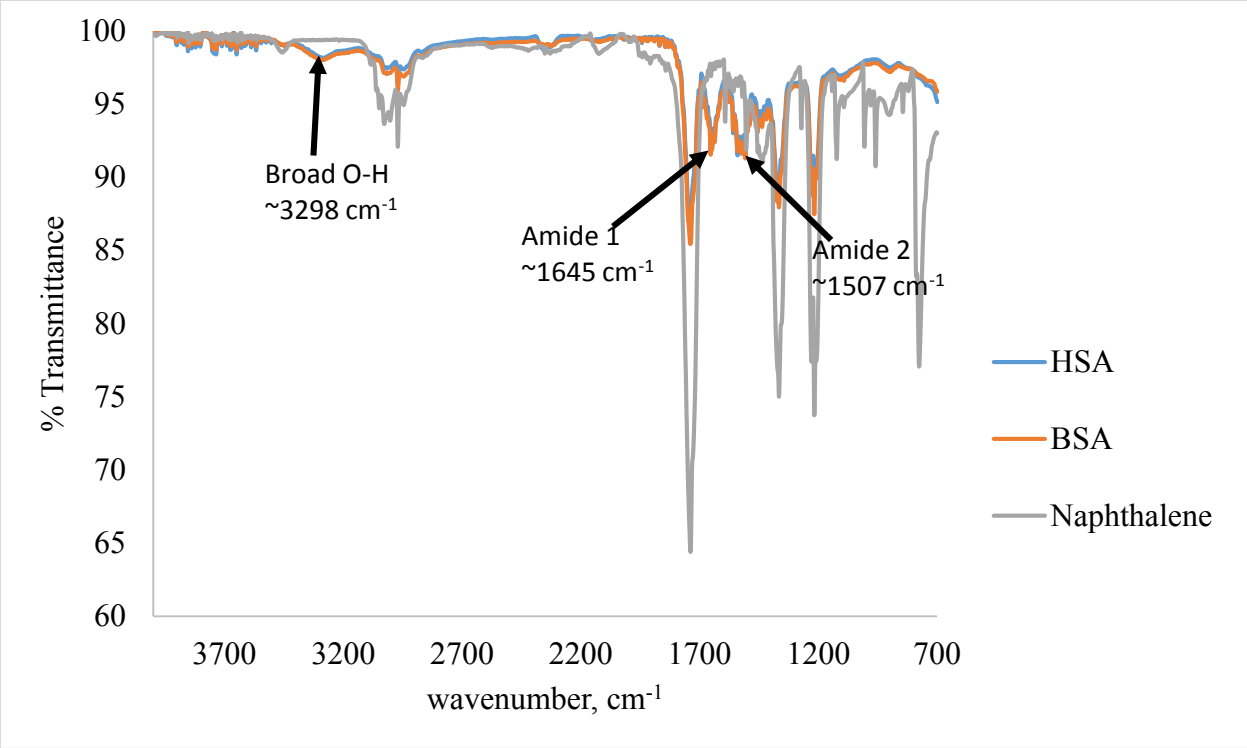

Figure 4.15. FTIR Sprectra HSA, BSA, and Naphthalene

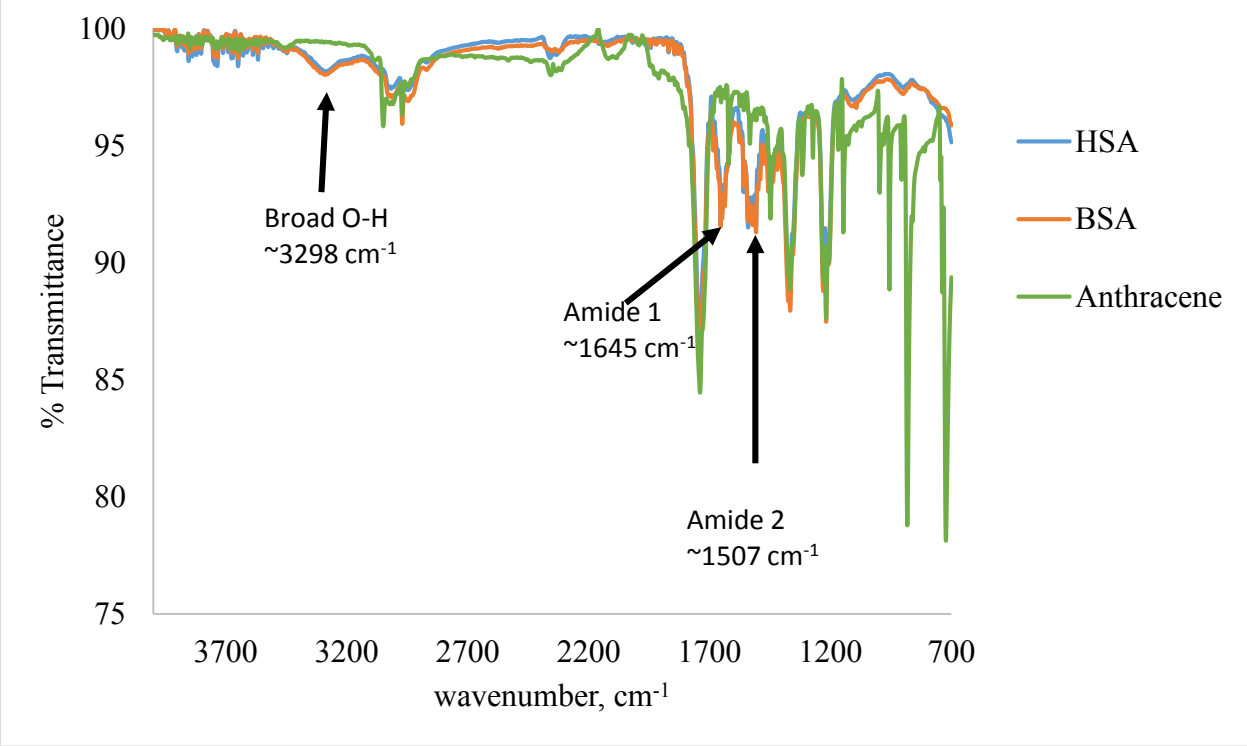

Figure 4.16. FTIR Spectra HSA, BSA, and Anthracene

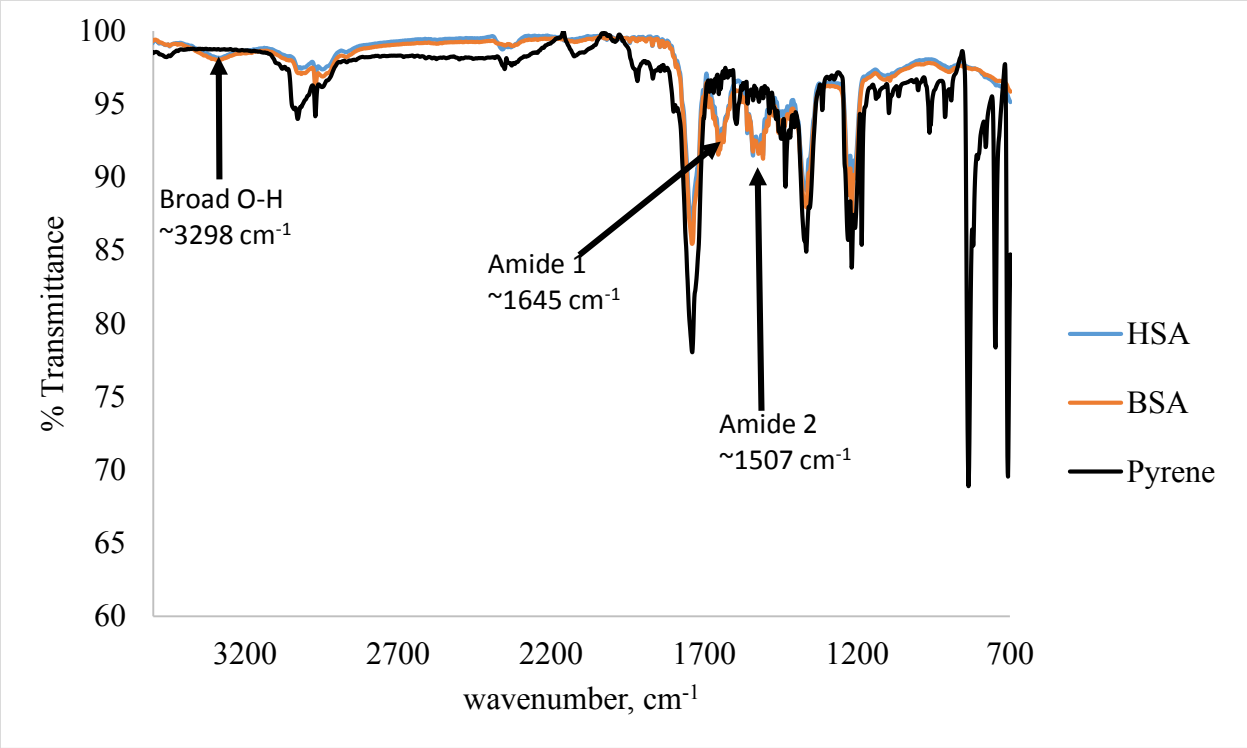

Figure 4.17. FTIR Spectra HSA, BSA, and Pyrene

Figures 4.1-5 is the FTIR spectra of the overlaid HSA and BSA proteins, overlaid PAHs, and overlaid PAH, HSA, and BSA. The broad alcohol stretch is at ~3298 cm^{-1}, C=C—H stretch at ~2900-3000 cm^{-1}, C=C stretch ~1735 cm^{-1}, and C=O stretch ~1737 cm^{-1}. Two amide regions are located at 1650 cm^{-1} and 1500 cm^{-1}. This is due to the C=O (amide 1) and the N—H (amide 2) stretch. There is also C-H bending ~1365-1215 cm^{-1}, C-C stretch ~835 cm^{-1} and C-H rock ~707 cm^{-1}.

4.2 UV-Visible Spectroscopic Study

A UV-Visible spectra presented in this section were used to accurately determine the concentration of HSA and BSA. Through successful application of beer-lambert's law (Equation 4) the concentrations of the proteins were obtained.

$$A = e \times b \times c$$

Equation 4. Beer-Lambert law

Where A is the absorbance, e is the molar absorptivity or extinction coefficient in L mol^{-1} cm^{-1}, b is the path length of the sample in cm, and c is the concentration of the compound (protein in this case) in solution reported in M.

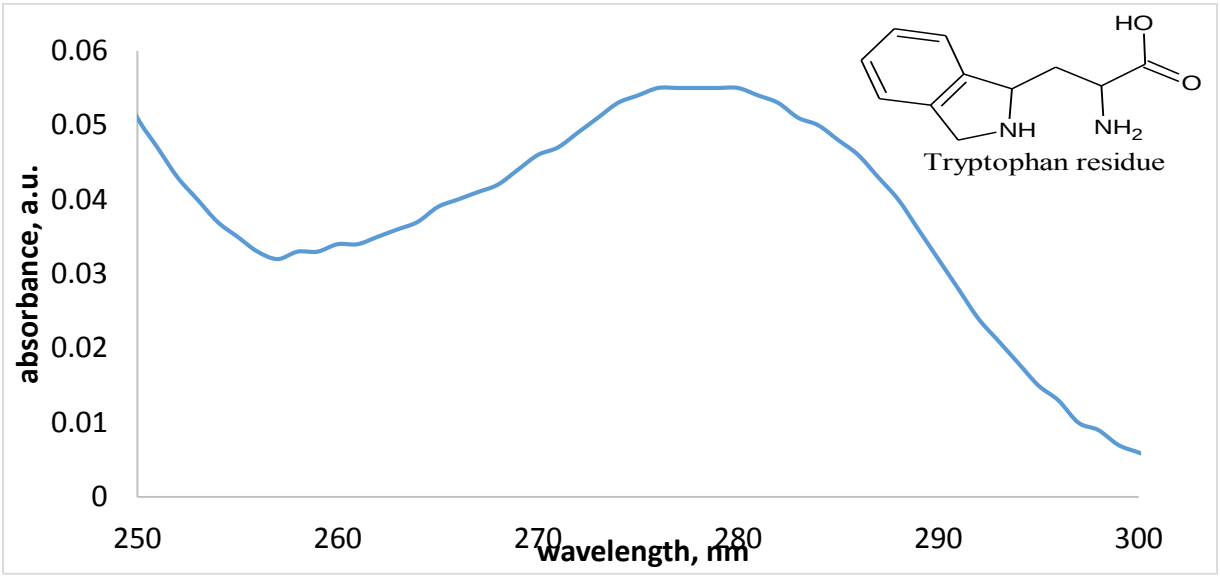

Figure 4.18. BSA UV-Vis Absorbance

Bovine serum albumin absorbance is shown in Figure 4.6. The absorbance at λmax 280 nm is 0.055 units (au). The molar extinction coefficient of BSA is 43,824 L mol^{-1} cm^{-1}. The concentration of BSA for this study was 1.3×10^{-06} M.

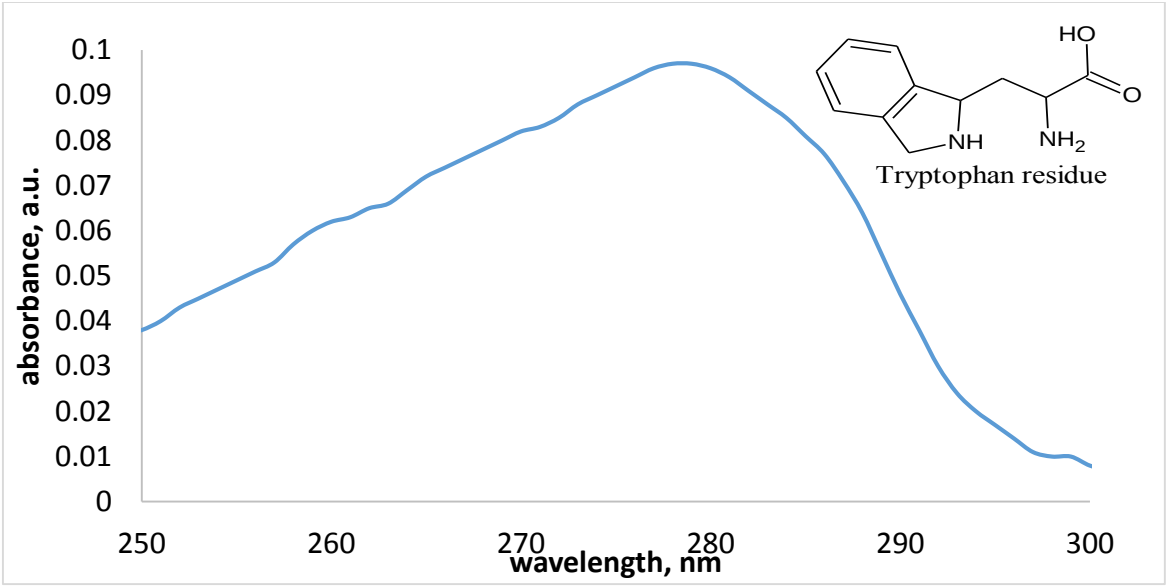

Figure 4.19. HSA UV-Vis Absorbance

Human serum albumin absorbance is shown in Figure 4.7. The absorbance at λmax 280 nm is 0.097 units (au). The molar extinction coefficient of HSA is 38,553 L mol^{-1} cm^{-1}. The concentration of BSA for this study was 2.5×10^{-6}M.

Of the three fluorescent amino acids, major focus was on tryptophan. Due to more conjugation, tryptophan is the lowest in energy so it would be seen at a longer wavelength. According to research literature, it is understood to have a maximum absorbance at 280nm. At this absorbance, tryptophan is also internally quenching the fluorescent tyrosine residue.

From previous literature review naphthalene has a broad band in between 250 nm and 270 nm. There was a hypsochromic shift due to the interference of the naphthalene with the tryptophan and in the presence of the acetonitrile solvent. In reference to tryptophan absorbance in the presence of naphthalene; as a reminder naphthalene, has a broad band in between 250 nm and 270 nm. Anthracene has a broad band around 260 nm and a secondary broad band at 340 nm. Referring back to figures mentioned already in this paragraph, there is a hypsochromic shift due to the interference of the anthracene with the tryptophan. It is evident temperature is in part causing the hyperchromic effect of this the molecule. Also it is noticeable that as the concentration of naphthalene is increased, so is the absorbance of my tryptophan region. This is a result of the anthracene with the tryptophan. UV-Vis did not make it evident. Due to more conjugation, pyrene absorb at lower energy than anthracene and naphthalene. Pyrene was affected in the same way.

4.3 Fluorescence Spectroscopy (Emission spectrum) for Both Protein-Naphthalene Complexes

In this section, results of fluorescent spectroscopic measurements are shown between HSA-naphthalene complex and also BSA-naphthalene complex. The proteins go through structural changes as they are complexed with PAHs. These structures are dependent upon concentration of

PAH and temperature. Data collected in this section were used to calculate the binding constants and thermodynamic variables.

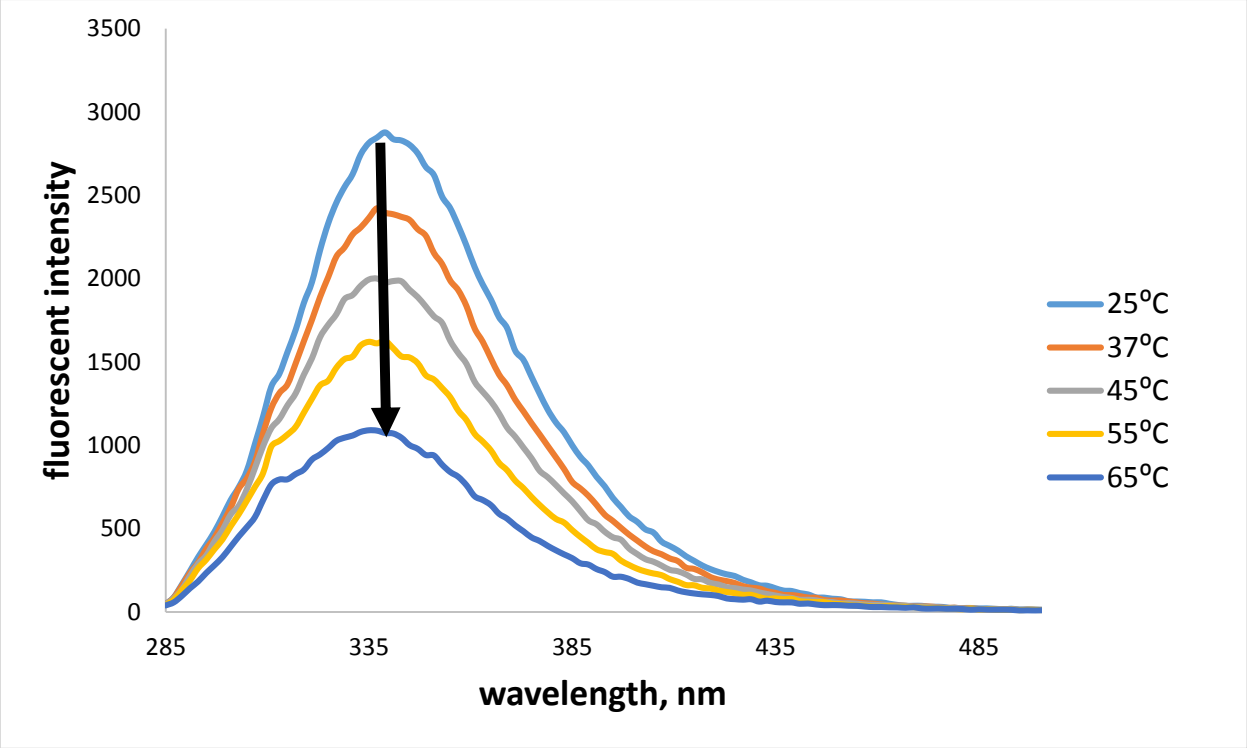

Figure 4.20. General BSA Study at Different Temperatures

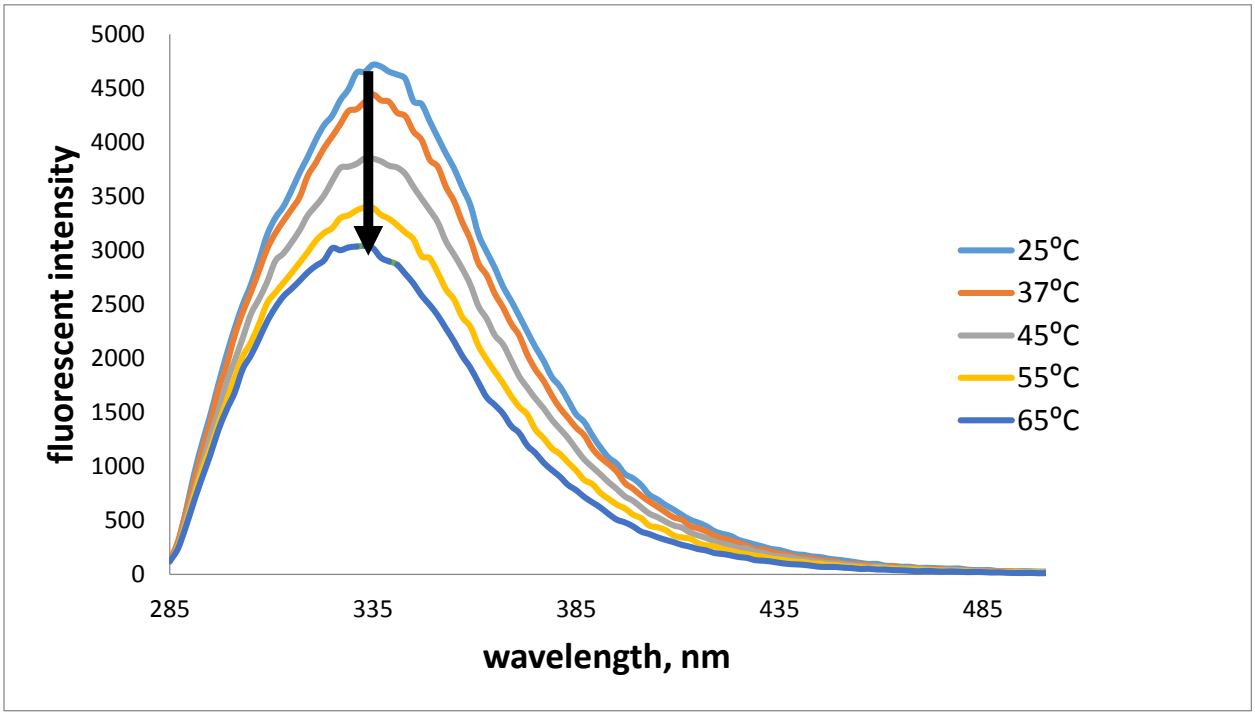

Figure 4.21. General HSA Study at Different Temperatures

Figures 4.8 and 4.9 are emission spectrum of both proteins (BSA and HSA respectively) as they are introduced to a variety of temperatures. Increased temperature resulted in a decrease in fluorescent intensity on BSA and HSA, thus causing the fluorescent intensity to decrease. The fluorescence of the protein is decreased because of denaturation of the protein. The wavelength of highest fluorescence was obtained for BSA and HSA. BSA had highest fluorescence at 341nm and HSA at 335 nm. These wavelengths were used to calculate binding constants, stoichiometry and thermodynamic variables.

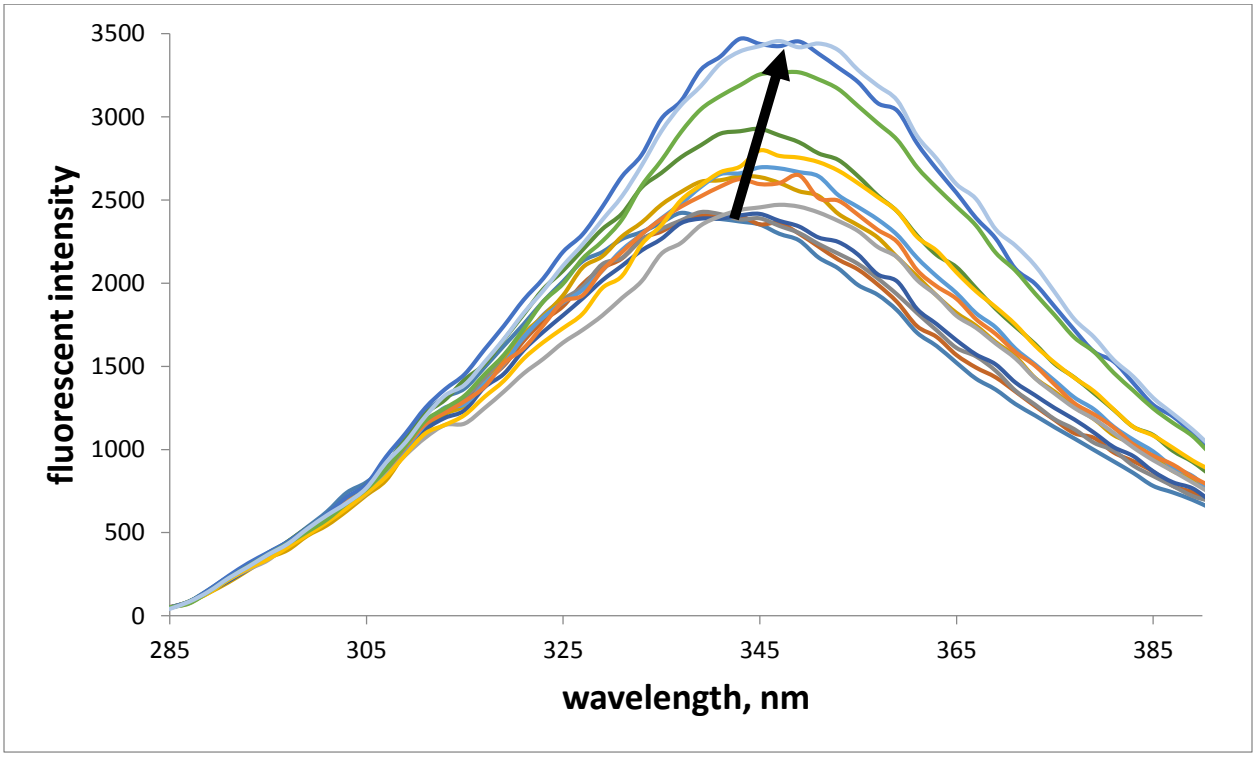

Figure 4.22. BSA-Naphthalene at 37°C

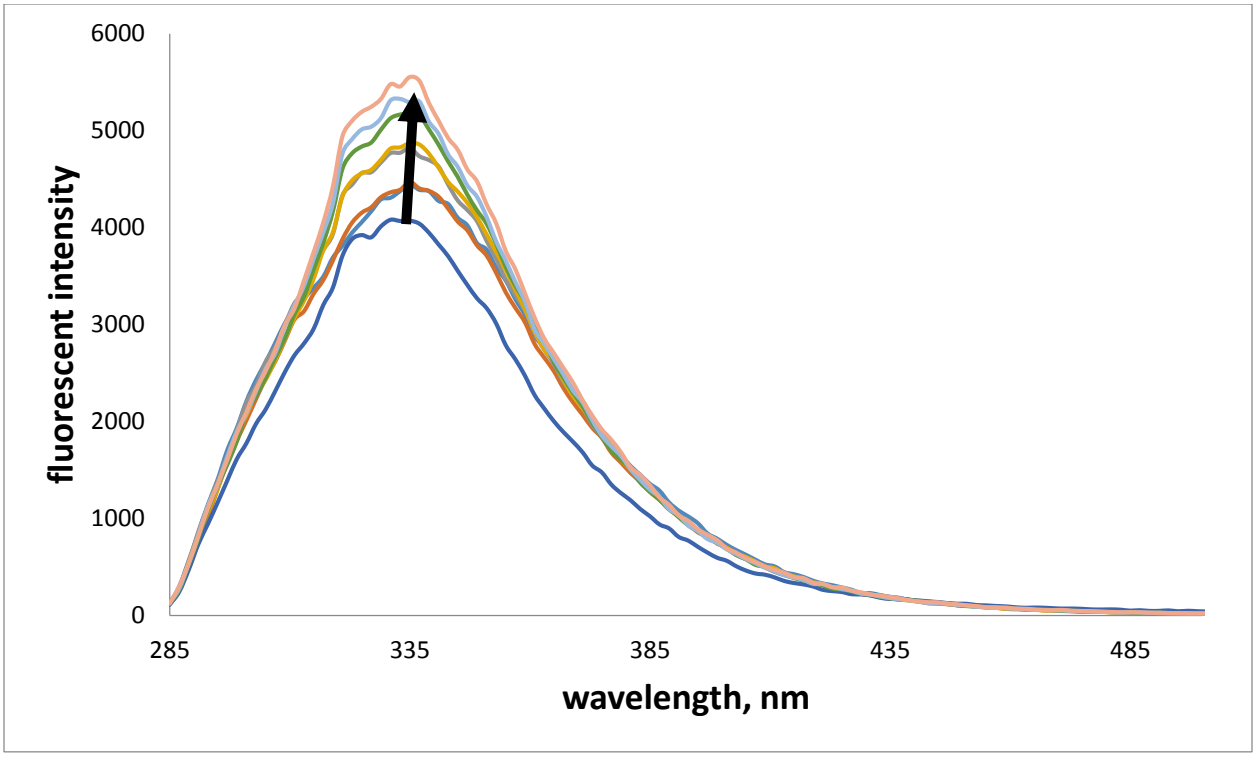

Figure 4.23. HSA-Naphthalene at 37°C

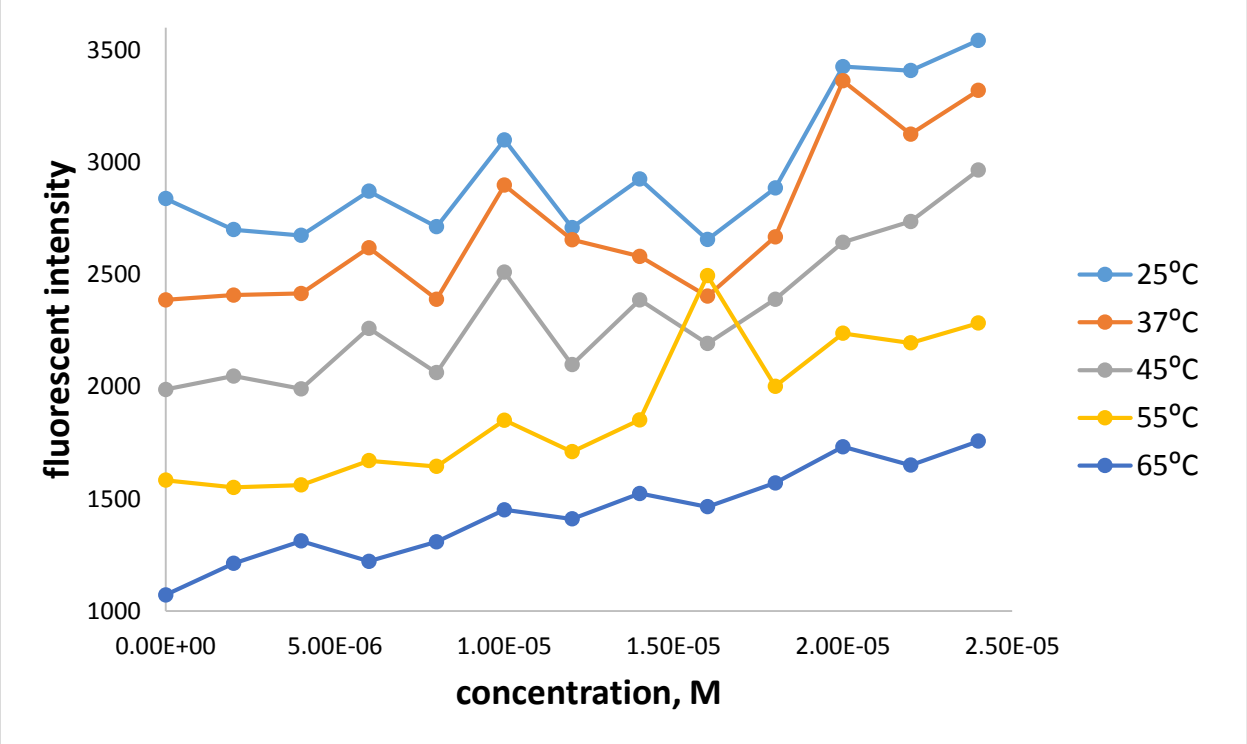

Figure 4.24. BSA-Naphthalene Temperature Comparison at λmax= 341 nm

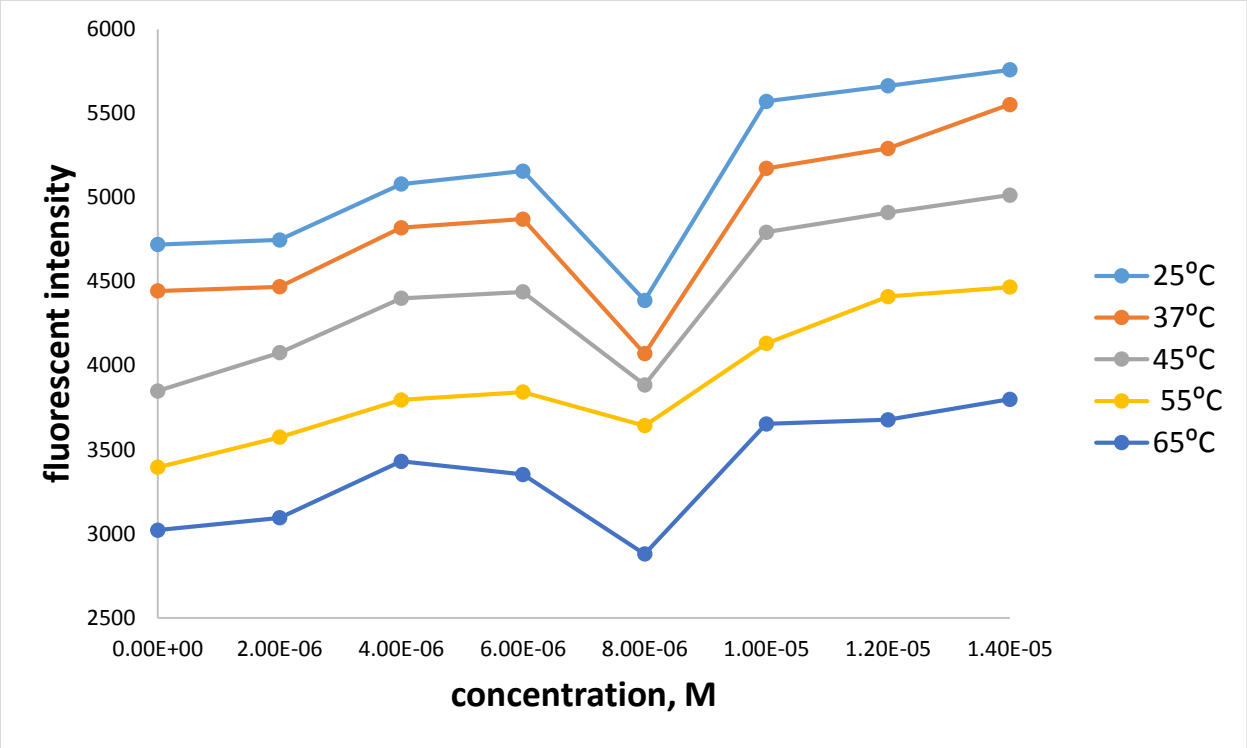

Figure 4.25. HSA-Naphthalene Temperature Comparison at λmax= 341 nm

Figures 4.10-13 shows the emission of samples containing fixed BSA and HSA at varying naphthalene concentrations. The increased naphthalene concentrations resulted in an increase of BSA and HSA intensity. This is best known as a hyperchromic effect. It also show that temperature's major role in fluorescence for this particular cause is through by quenching. A complexation of naphthalene with BSA and HSA also resulted in a bathochromic shift. Due to naphthalene bicyclic aromatic characteristics its fluorescence is seemingly in the same region as that of tryptophan.

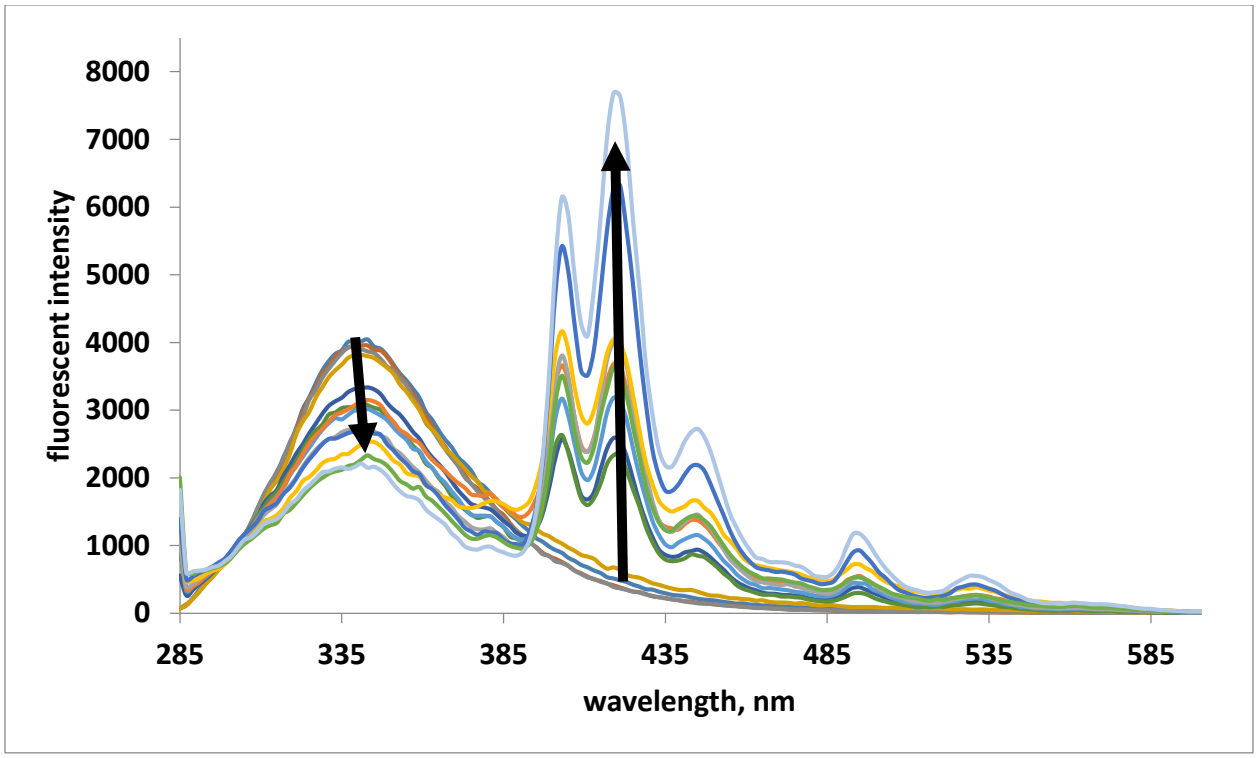

Figure 4.26. BSA.Anthracene at 37°C

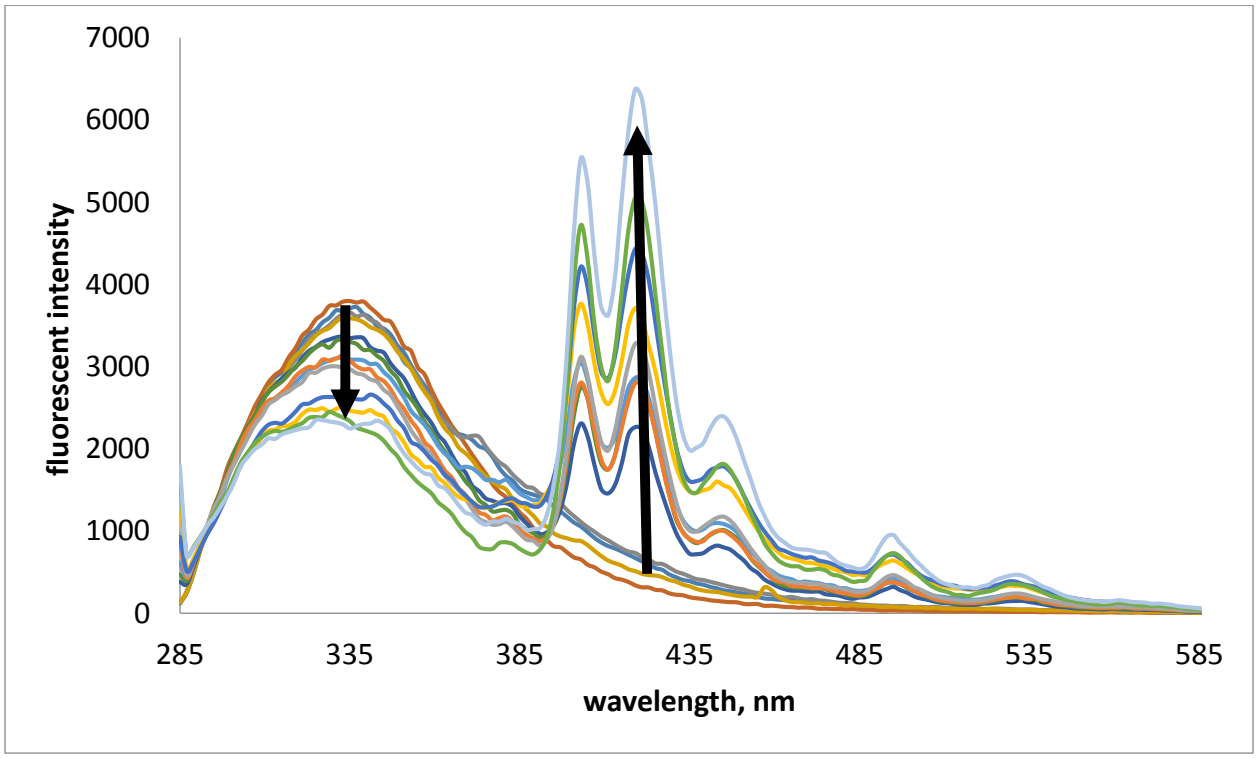

Figure 4.27. HSA-Anthracene at 37°C

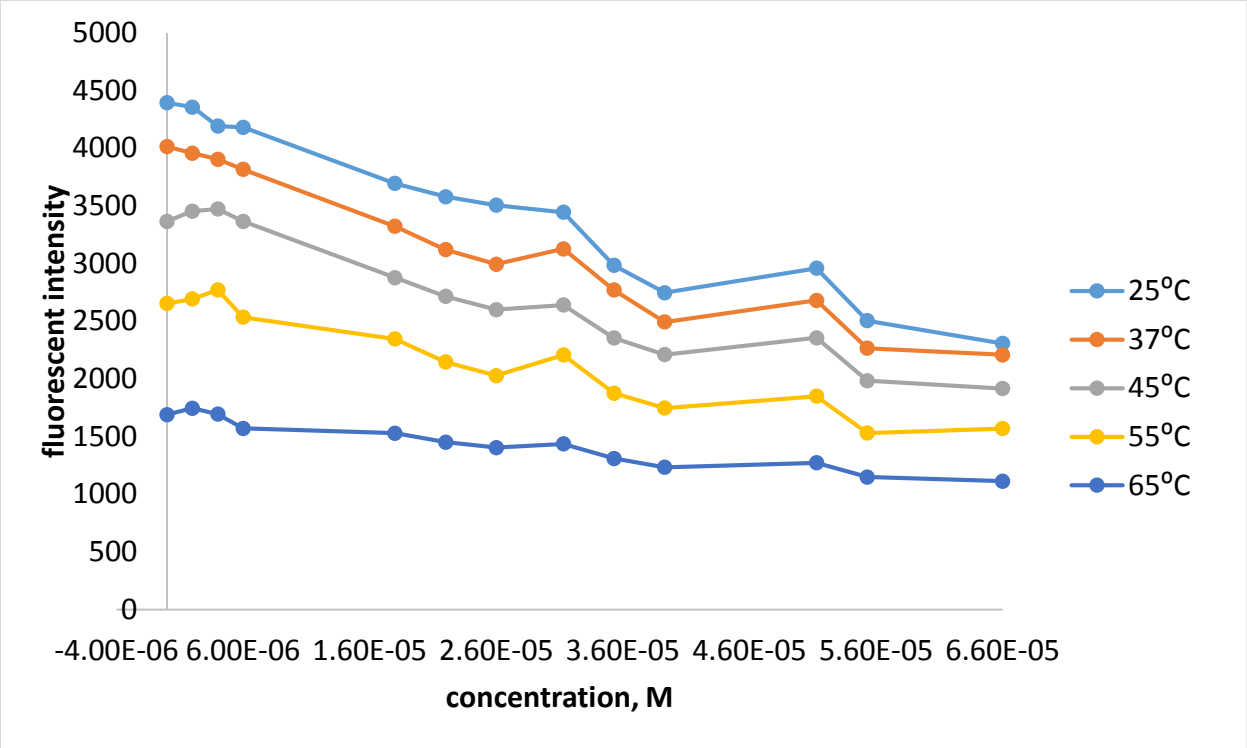

Figure 4.28. BSA-Anthracene Temperature Comparison at λmax= 341 nm

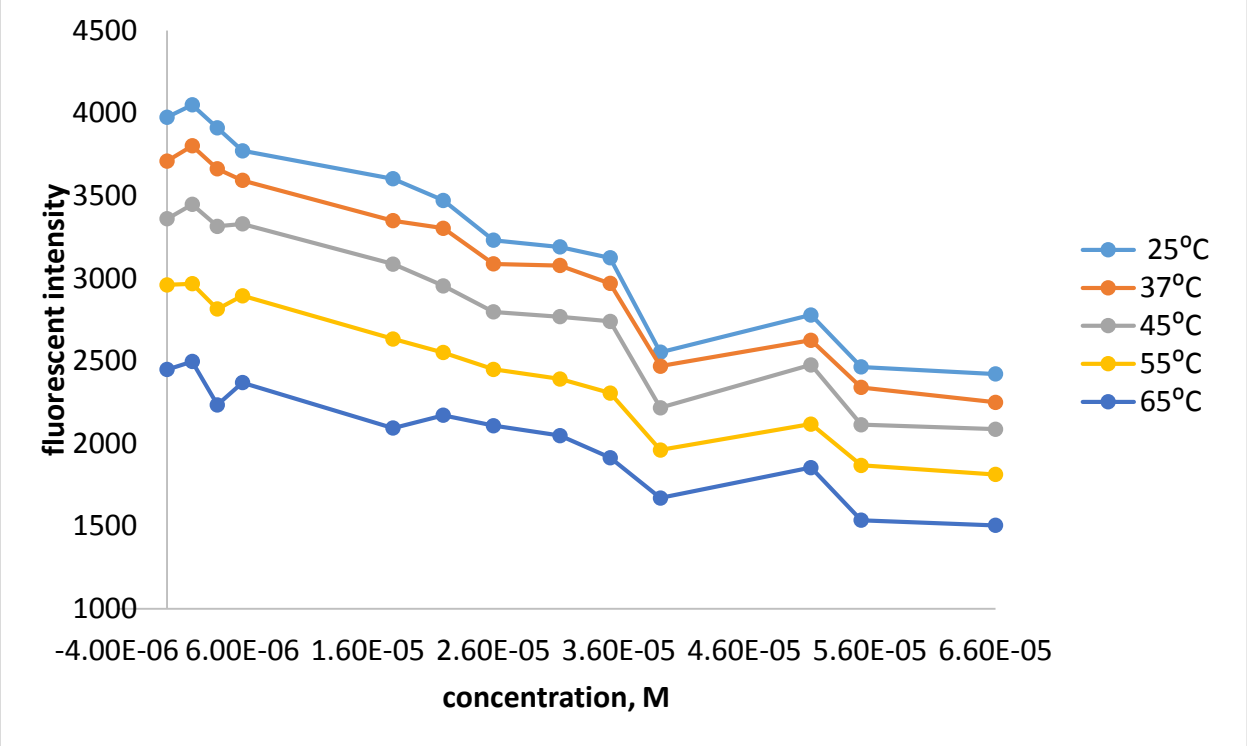

Figure 4.29. HSA-Anthracene Temperature Comparison at λmax= 335 nm

Figures 4.14-17 indicates anthracene's hypochromic and batachoromic effect and shift respectively. As the concentration of anthracene is increased the fluorescence of BSA and HSA decreases. Because anthracene is a conjugated system, it is expected to see it longer wavelengths (lower energy) than less conjugated systems (naphthalene in this case). Interaction between anthracene is observed by the hypsochromic shift in the anthracene region. Increased temperature had a hypochromic effect in both regions of fluorescence. In Figures 4.16-17 the effect of anthracene along with the BSA and HSA were further investigated. Due to the complexation, both proteins go through structural changes.

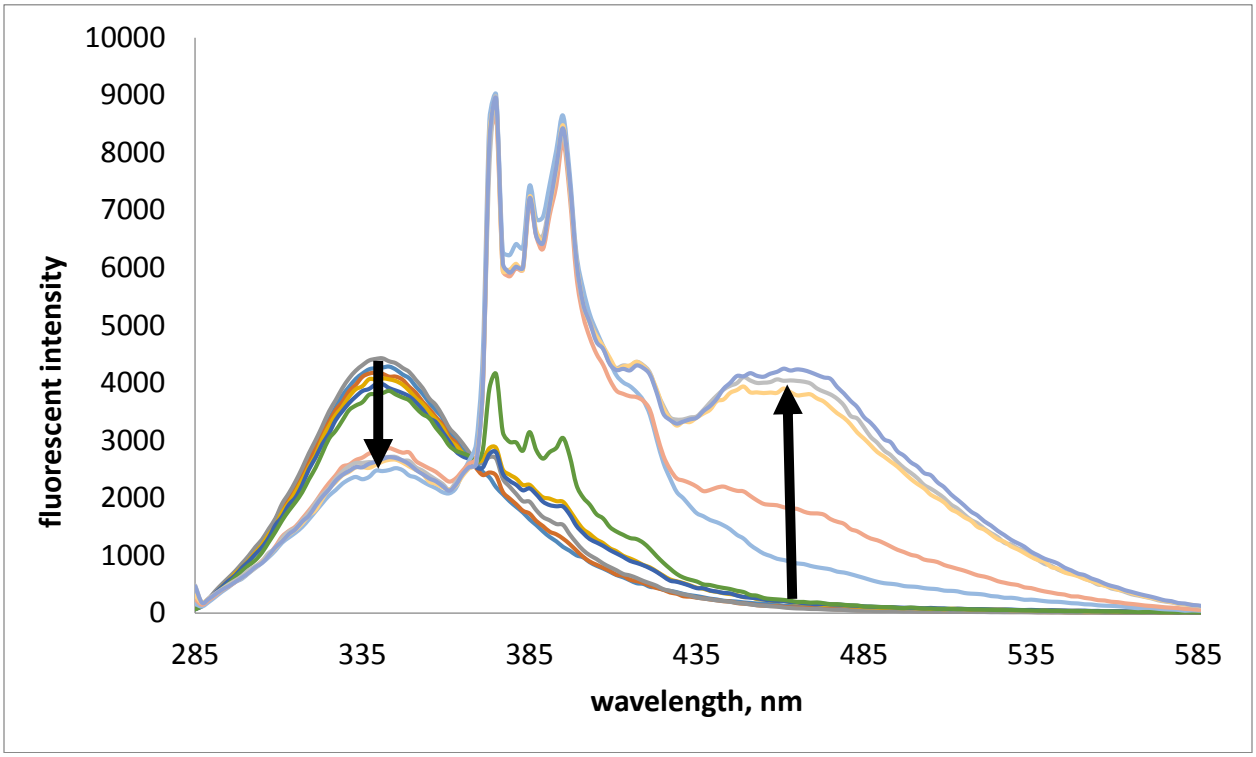

Figure 4.30. BSA-Pyrene at 37°C

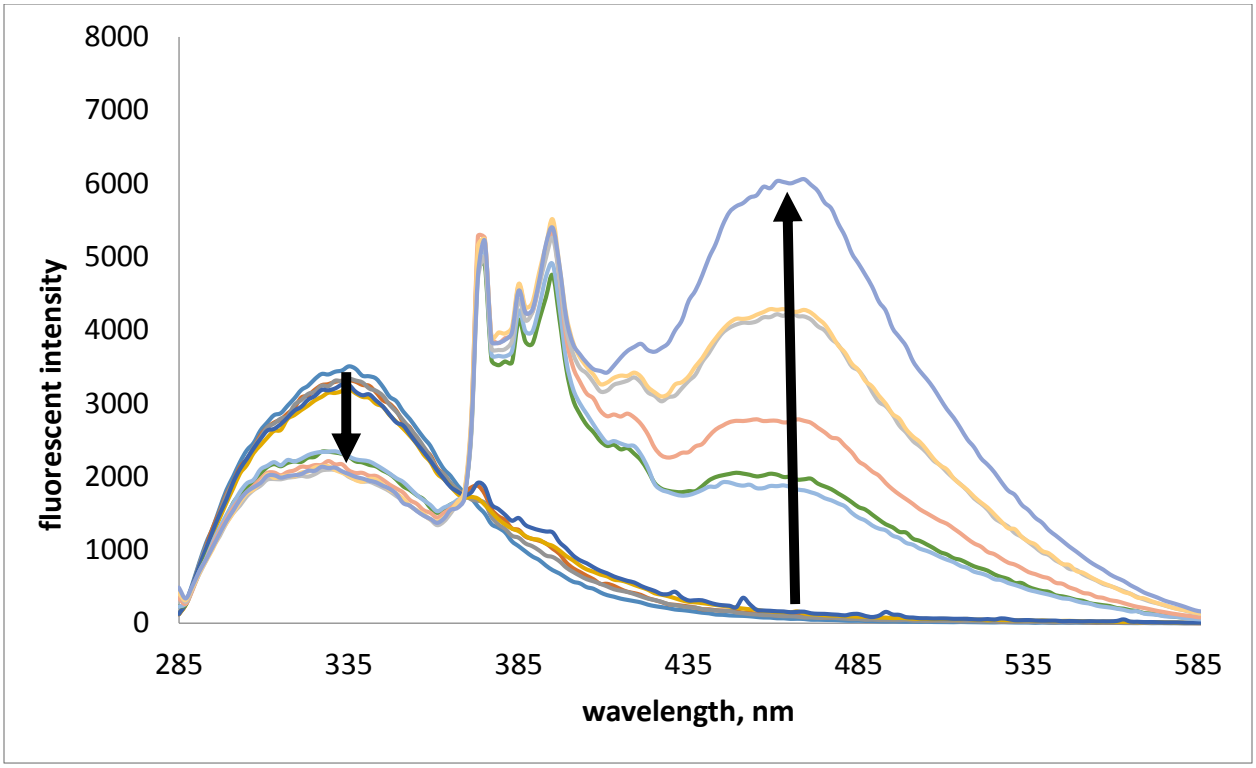

Figure 4.31. HSA.Pyrene at 37°C

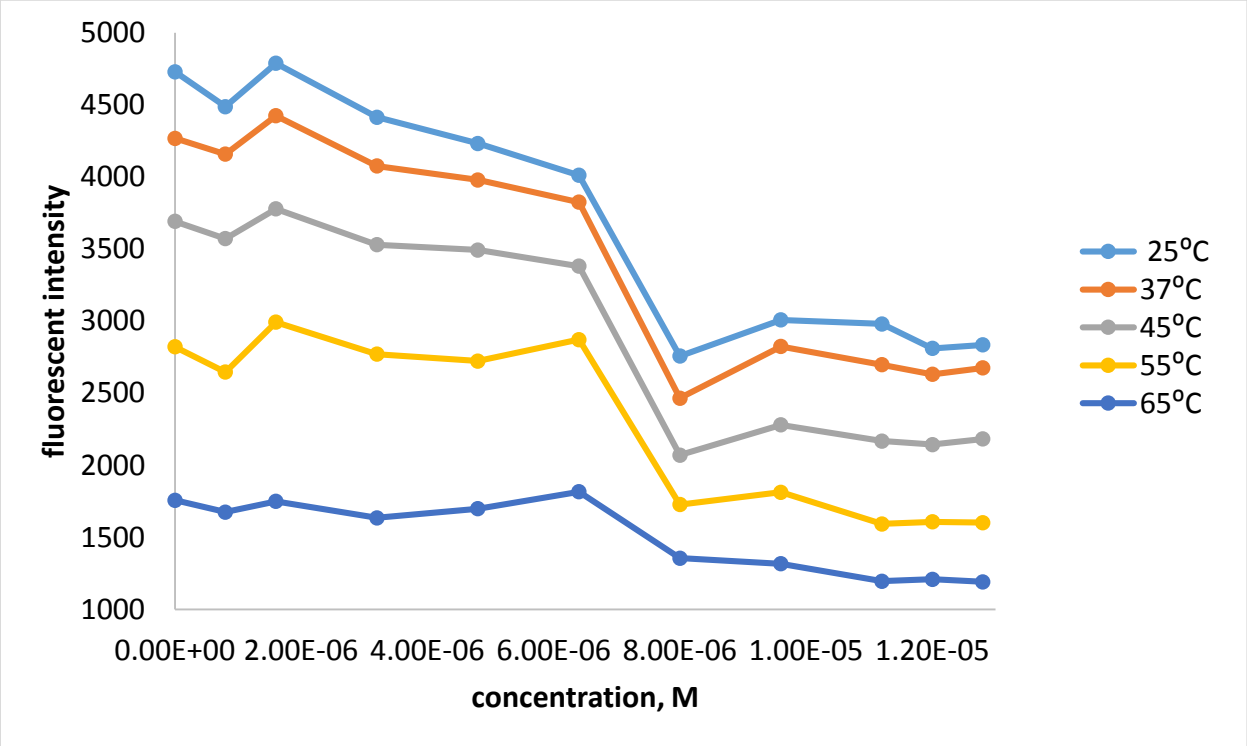

Figure 4.32. BSA-Pyrene Temperature Comparison at λmax= 341 nm

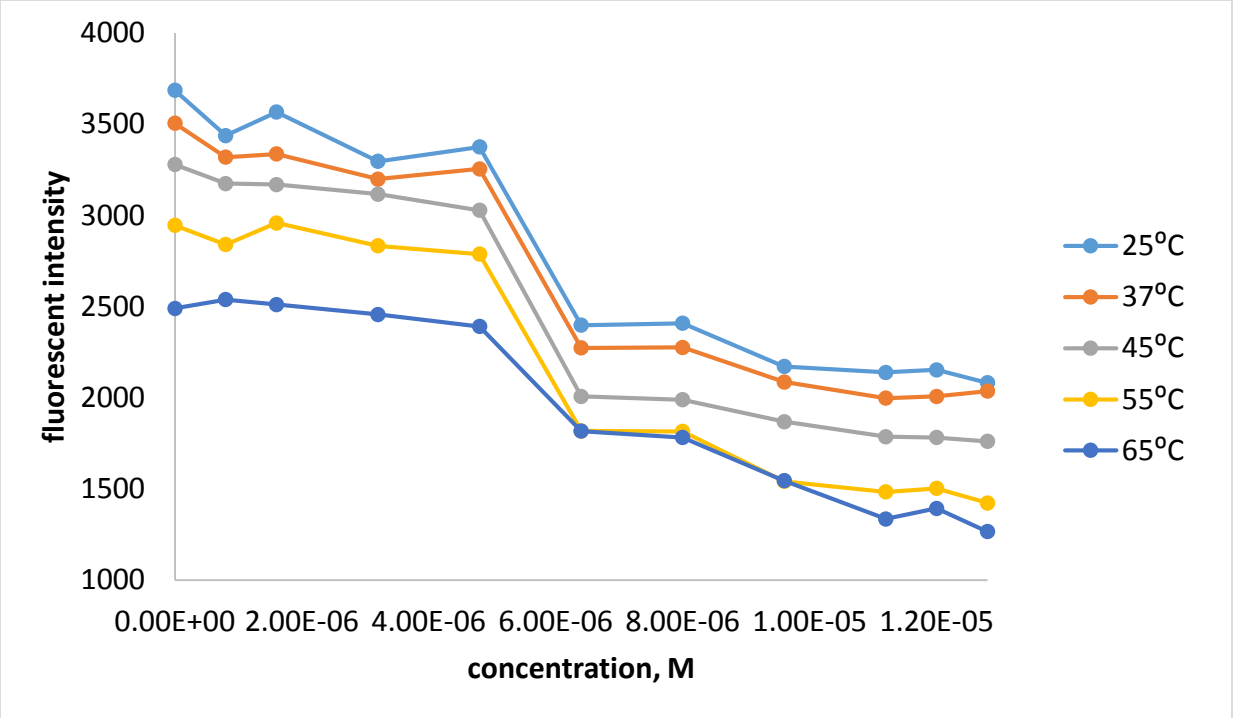

Figure 4.33. HSA-Pyrene Temperature Comparison at λmax= 341 nm

Figures 4.18-21 shows pyrene's hypochromic effect on BSA and HSA. Figures 4.20-21 shows that as temperature and concentration of pyrene is increased, the intensity of the HSA and BSA are decreased. Pyrene is known as a quenching agent in the protein region. During equilibrium period (discussed in methods) pyrene complexes with the protein.

4.4 Determination of Binding Constant and Stoichiometry of PAHs-BSA and PAHs-HSA Complexes

Binding constants and stoichiometry were accurately determined using the modified Stern Volmer equation (Equation 1). Wavelengths of maximum fluorescence were 335nm and 341nm for HSA and BSA respectively. Tables are shown as a comparison of the effects the PAHs have on HSA and BSA.

Table 4.1

Comparison of Binding and Stoichiometry on Naphthalene-BSA and Naphthalene-HSA Complexes

Temperature, Kelvin	Binding Constant (M^{-1})		Stoichiometry	
	BSA	**HSA**	**BSA**	**HSA**
298	2.65E+01	4.65E+07	0.53	1.694
310	9.07E+05	5.20E+07	0.6	1.7001
318	4.50E+06	1.81E+02	1.54	0.6202
328	5.16E+06	3.31E+04	1.53	0.8444
338	5.80E+02	1.91E+04	0.652	1.0133

In Table 4.1, temperature has a major effect to how the naphthalene binds the protein. As temperature increases, the binding increases in the naphthalene-BSA complex, with exception to 338 K. At this temperature binding could be affected majorly because of structural and conformational changes due to temperature. The binding for the naphthalene-HSA complex decreases as temperature is increased. Because of the tryptophan regions in both HSA and BSA, it was expected that there be a 1:1 and 2:1 molar ratios of naphthalene-HSA and naphthalene-BSA complexes, respectively. The conformational changes in the protein could be a factor. Current studies are being done to understand this further.

Table 4.2

Comparison of Binding and Stoichiometry on Anthracene-BSA and Anthracene-HSA Complexes

Temperature, Kelvin	Binding Constant (M^{-1})		Stoichiometry	
	BSA	**HSA**	**BSA**	**HSA**
298	1.92E+03	4.94E+03	0.86	1.09
310	4.25E+02	4.85E+03	0.87	1.14
318	8.50E+02	1.08E+04	0.79	1.14
328	2.34E+03	1.03E+05	0.89	0.98
338	2.58E+03	5.78E+02	0.92	0.94

In Table 4.2 temperature did not play a role in the binding association of anthracene to BSA and BSA because the binding constants are relatively the same. The binding affinity of anthracene to the proteins is relatively high. The stoichiometric ratios of anthracene-BSA and anthracene-BSA are around 1:1 as temperature is increased.

Table 4.3

Comparison of Binding and Stoichiometry on Pyrene-BSA and Pyrene-HSA Complexes

Temperature, Kelvin	Binding Constant (M^{-1})		Stoichiometry	
	BSA	HSA	BSA	HSA
298	1.20E+05	1.45E+04	1.12	0.92
310	1.12E+05	1.65E+04	1.12	0.94
318	2.08E+05	3.14E+05	1.17	1.19
328	6.42E+03	1.49E+07	0.9	1.52
338	8.47E+04	2.28E+07	1.13	1.58

In the Table 4.3, as temperature increases the binding increases in the pyrene-BSA complex, with exception to 328 K. At this temperature binding could be affected majorly because of structural and conformational changes due to temperature. The binding association constant for the pyrene-HSA complex increases as temperature as temperature. The stoichiometry for pyrene-BSA complex is 1:1, temperature increase do not have any major impact. On the contrary, when pyrene is bound to HSA at 328 K and 338 K, a 2:1 pyrene-HSA stoichiometric ratio was observed.

4.5 Investigation of Thermodynamic Property on PAH-BSA and PAH-HAS Complexes

Table 4.4

Comparison of Thermodynamic Property on Naphthalene-BSA and Naphthalene-HSA Complexes

Temperature, Kelvin	ΔH (J)		ΔS (J/K)		ΔG (J)	
	BSA	HSA	BSA	HSA	BSA	HSA
298	2.18E+03	1.33E+04	1.07E+02	1.16E+02	-2.98E+04	-2.13E+04
310					-3.11E+04	-2.27E+04
318					-3.19E+04	-2.36E+04
328					-3.30E+04	-2.48E+04
338					-3.41E+04	-2.59E+04

In the naphthalene-BSA and naphthalene-HSA complex in Table 4.4, the positive enthalpy and entropy values indicate that the binding is due to hydrophobicity. The high negative Gibbs free energy at any moment indicates that the binding of naphthalene to the two protein complexes is spontaneous.

Table 4.5

Comparison of Thermodynamic Property on Anthracene-BSA and Anthracene-HSA Complexes

Temperature, Kelvin	**ΔH (J)**		**ΔS (J/K)**		**ΔG (J)**	
	BSA	**HSA**	**BSA**	**HSA**	**BSA**	**HSA**
298	1.88E+05	6.43E+04	6.71E+01	8.13E+01	-1.25E+04	3.88E+04
310					-1.33E+04	3.77E+04
318					-1.38E+04	3.70E+04
328					-1.45E+04	3.62E+04
338					-1.52E+04	3.53E+04

In the anthracene-BSA and anthracene-HSA complex in Table 4.5, the positive enthalpy and entropy values indicates hydrophobic binding. The high negative gibbs free energy at any moment indicates that the binding of anthracene to BSA is spontaneous. However the to the two protein complexes is spontaneous. This is not the same for HSA. Because of the high positive free energy values, anthracene-HSA complexation is not favored. In other words, energy is required for complexation.

Table 4.6

Comparison of Thermodynamic Property on Pyrene-BSA and Pyrene-HSA Complexes

Temperature, Kelvin	**ΔH (J)**		**ΔS (J/K)**		**ΔG (J)**	
	BSA	**HSA**	**BSA**	**HSA**	**BSA**	**HSA**
298	1.68E+05	7.56E+05	9.90E+01	1.29E+02	1.39E+05	7.18E+05
310					1.38E+05	7.16E+05
318					1.37E+05	7.15E+05
328					1.36E+05	7.14E+05
338					1.35E+05	7.13E+05

In the pyrene-BSA and pyrene-HSA complex in Table 4.6, like naphthalene and anthracene, the positive enthalpy and entropy values indicates hydrophobic binding. The positive free energy indicates this is not spontaneous. This is an endothermic complexation, because of the energy required.

CHAPTER 5

PAH Cytotoxic Effects on Triple Negative Breast Cancer Cells

The protein-analyte complex considerations above were used as a preliminary basis of cell study. While it is vital to understand how these analytes are interacting with serum and other transport proteins, it is of great value to understand what is also happening on the cellular level. The results shown below are the preliminary results.

This section is to focus mainly on cell appearance. All pictures in this section are reported at 10X magnification. Magnification pictures at 20X were also taken but are not shown. Because of acetonitrile's highly toxic effect interactions with cells, dimethyl sulfoxide (DMSO) was used as the solvent at three different concentrations (the same concentrations of analyte was used).

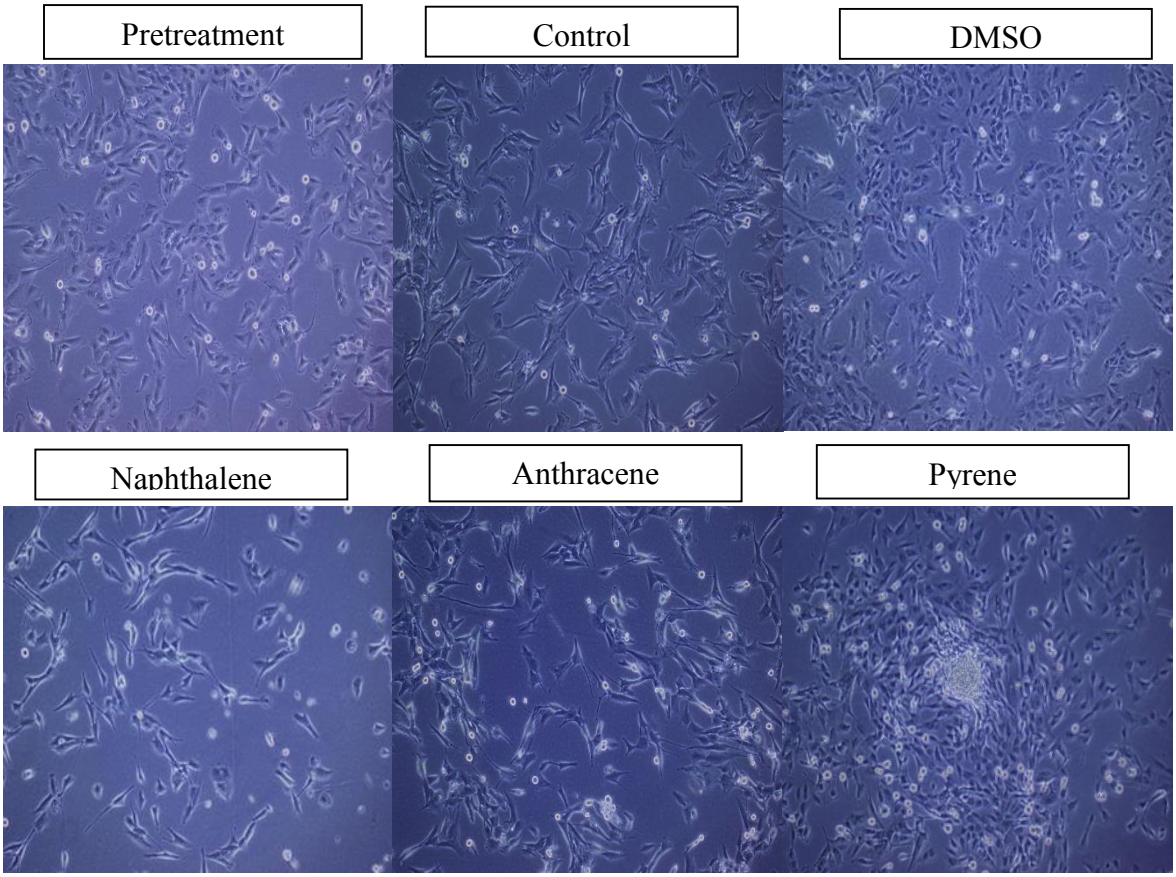

Figure 5.34. Pre-treatment of TNBC cells compared to the Non-treated cells. These results are them compared to solvent and analyte introduction at 1.8×10^{-05} M concentration.

Figure 5.1 shows cellular activity after exposure to the lowest concentration of PAHs. The solvent had a slight toxic effect on the cells. As the cells were treated with naphthalene, apoptosis of the cells were induced. Anthracene and pyrene effects were beginning to induce apoptosis. The cells were aggregating more with anthracene and pyrene, as opposed to naphthalene. However, differences in the rate of apoptosis were not as noticeable at this concentration.

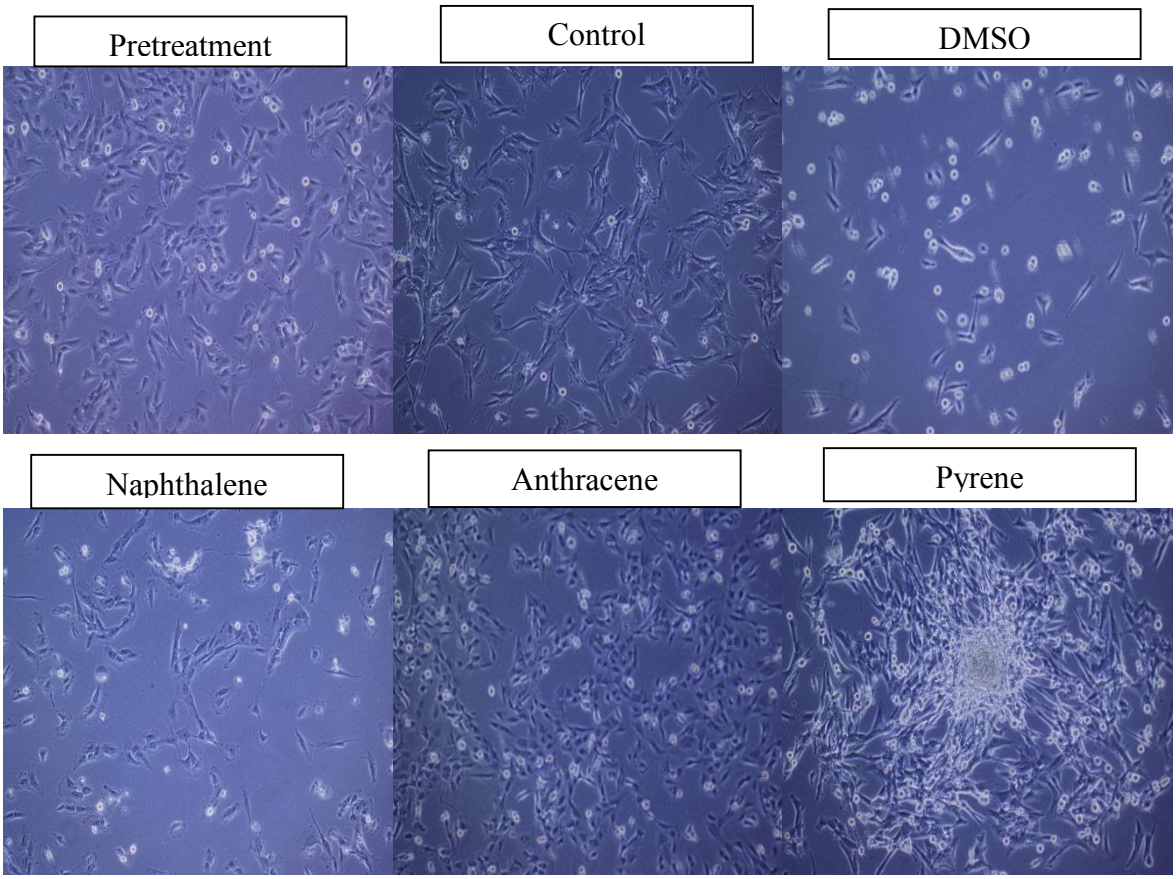

Figure 5.35. Pre-treatment of TNBC cells compared to the Non-treated cells. These results are them compared to solvent and analyte introduction at 2.5×10^{-05} M concentration.

Figure 5.2 shows cellular activity after exposure to slightly higher concentration of PAHs than those in Figure 5.1. The solvent had a slight toxic effect on the cells. Apoptosis is more prevalent in naphthalene. Congregation and cell structural changes are noted in anthracene exposure. The cells were aggregating more with pyrene. These structural changes are suggested to

be the beginning of apoptosis according to the observances in Figure 5.3. Differences in the rate of apoptosis was observed and further assessed by a cell viability assay (not shown).

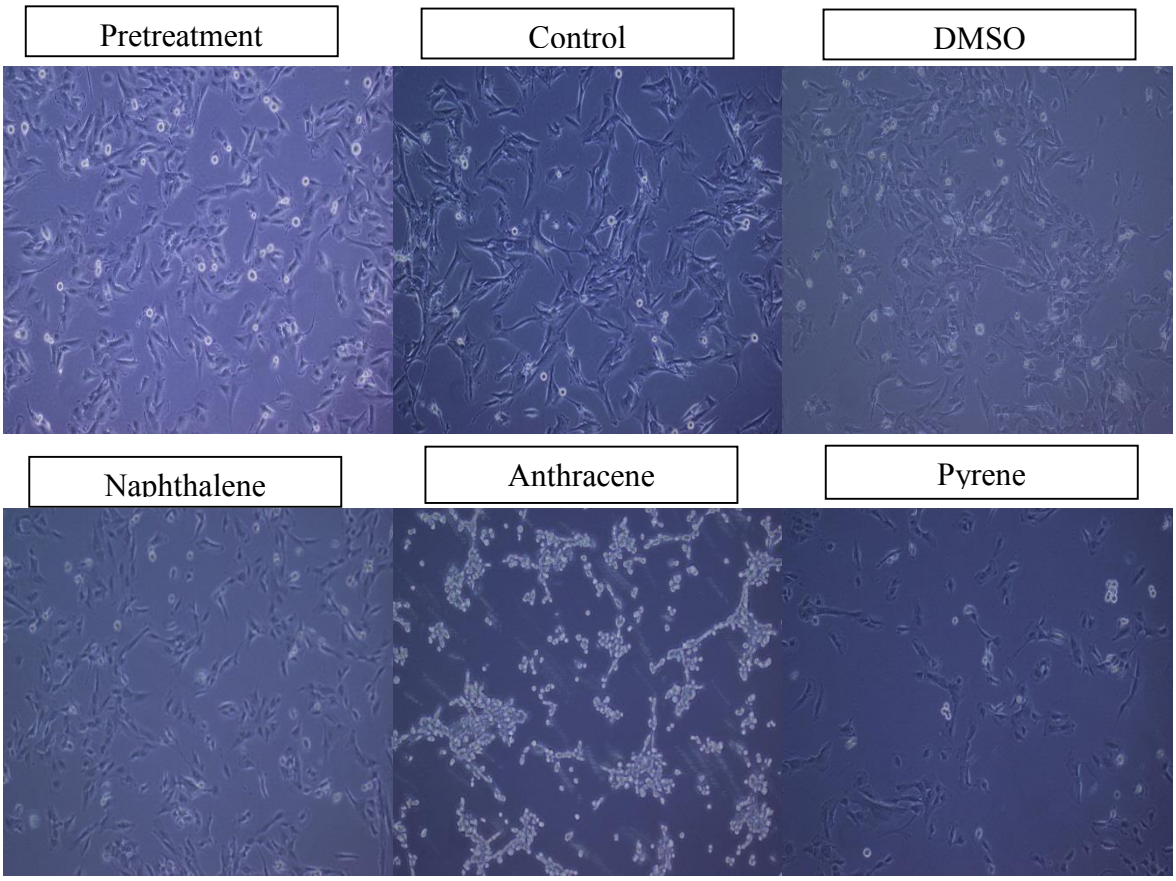

Figure 5.36. Pre-treatment of TNBC cells compared to the Non-treated cells. These results are them compared to solvent and analyte introduction at 3.6×10^{-05} M concentration.

Figure 5.3 shows that pyrene has the strongest toxic affect to the cells. Pyrenes increased conjugation by the addition and stacking of aromatic benzene rings could be a result of the apoptotic cells.

CHAPTER 6

Conclusion and Moving Forward

6.1 Conclusion and Limitations

All of the results and discussion of this thesis suggest that the activity of the analytes with BSA and HSA are dependent on concentration and temperature. Since one of the main characteristics of the analytes are high-hyrdrophobicity, the major contributors of PAH-BSA/HSA interactions is by the hydrophobic pockets found in the major binding sites of both proteins. The experimental studies evaluated throughout the duration of this thesis is vital not only for the scientific community, but the world as a whole. Cell studies, though in the beginning stages are showing promising results that will be studied further. It seems that the analytes are having apoptotic effect on the triple negative breast cancer cells. One of the major limitations were time and detachment of cells. When trying to perform cell viability, some difficulty and challenges in detaching the cells from the cell plate were encountered. Cell viability testing was done but is not reported because of this. There is an ongoing effort toward the development of alternative methods of cell exposure and cleavage.

6.2 Moving Forward

Although a lot of analytical and spectroscopic were done on these particular compounds there are still more aspects to be investigated. Next, anisotropic studies will be conducted in order to understand more the molecular structure and the influence of PAHs on the mobility of BSA and HSA in solution. Normalized fluorescent studies will be conducted to confirm and suggest more definitive motion and mode of action. Studies of the interaction of more conjugated PAHs on BSA and HSA to evaluate the role that they play in binding will be conducted. In everyday living, we are exposed to a mixture of analytes, so in hopes understand the binding, consequently, further

studies involving how a mixture of PAHs would bind to the proteins will be conducted. Hopefully this will bring clarity to the competition of binding.

References

1. Yan, L.; Bai, Y.; Zhao, R.; Li, F.; Xie, K., Correlation between coal structure and release of the two organic compounds during pyrolysis. *Fuel* **2015,** *145*, 12-17.
2. Agency, U. S. E. P., Polycylic Aromatic Hydrocarbons (PAHs). Management, O. o. S. W., Ed. United States Environmental Protection Agency: Internet, **2008**.
3. Ravindra, K.; Sokhi, R.; Vangrieken, R., Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation. *Atmospheric Environment* **2008,** *42* (13), 2895-2921.
4. Dong, J.; Cheng, Z.; Li, F., PAHs emission from the pyrolysis of Western Chinese coal. *Journal of Analytical and Applied Pyrolysis* **2013,** *104*, 502-507.
5. Hu, Y.; Li, G.; Yan, M.; Ping, C.; Ren, J., Investigation into the distribution of polycyclic aromatic hydrocarbons (PAHs) in wastewater sewage sludge and its resulting pyrolysis bio-oils. *The Science of the total environment* **2014,** *473-474*, 459-64.
6. Moreira dos Santos, C. Y.; de Almeida Azevedo, D.; de Aquino Neto, F. R., Atmospheric distribution of organic compounds from urban areas near a coal-fired power station. *Atmospheric Environment* **2004,** *38* (9), 1247-1257.
7. Health, D. o., Polycyclic Aromatic Hydrocarbons (PAHs): Health effects. Health, D. o., Ed. Department of Health, Government of South Australia: Internet, **2009**; p 2.
8. Lima, A. L. C.; Farrington, J. W.; Reddy, C. M., Combustion-Derived Polycyclic Aromatic Hydrocarbons in the Environment—A Review. *Environmental Forensics* **2005,** *6* (2), 109-131.

9. Jia, C.; Batterman, S., A critical review of naphthalene sources and exposures relevant to indoor and outdoor air. *International journal of environmental research and public health* **2010,** *7* (7), 2903-39.
10. Sudakin, D. L.; Stone, D. L.; Power, L., Naphthalene Mothballs: Emerging and Recurring Issues and their relevance to Environmental Health. *Curr Top Toxicol* **2011,** (7), 13-19.
11. Batterman, S.; Chin, J. Y.; Jia, C.; Godwin, C.; Parker, E.; Robins, T.; Max, P.; Lewis, T., Sources, concentrations, and risks of naphthalene in indoor and outdoor air. *Indoor air* **2012,** *22* (4), 266-78.
12. Guerrero, P. A.; Corsi, R. L., Emissions of p-dichlorobenzene and naphthalene from consumer products. *Journal of the Air & Waste Management Association* **2012,** *62* (9), 1075-1084.
13. Baird, W. M.; Hooven, L. A.; Mahadevan, B., Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environmental and molecular mutagenesis* **2005,** *45* (2-3), 106-14.
14. Daisy, B. H.; Strobel, G. A.; Castillo, U.; Ezra, D.; Sears, J.; Weaver, D. K.; Runyon, J. B., Naphthalene, an insect repellent, is produced by Muscodor vitigenus, a novel endophytic. *Microbiolgy* **2002,** (148), 3737-3741.
15. Khalili, N. R.; Schieff, P. A.; Holsen, T. M., PAH Source Fingerprints for Coke Ovens, Diesel and Gasoline Engines, Highway Tunnels, and Wood Combustion Emission. *Atmospheric Environment* **1994,** *29* (4), 533-542.
16. Registry, A. f. T. S. a. D., Anthracene. Agency for Toxic Substances and Disease Registry: Internet, **1991**.

17. Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K., Crystal structure of human serum albumin at 2.5 A resolution. *Protein Engineering* **1999,** *12* (6), 439-446.

18. Moghaddassi, S.; Eyestone, W.; Bishop, C. E., The Large-Scale Production of Recombinant Human Serum Albumin in the Milk of Transgenic Cattle: Strategy and Implication. *Jounal of Science and Applications* **2014,** *2* (2), 6-19.

19. Meloun, B.; Moravek, L.; Kostka, V., Complete Amino Acid Sequence of Human Serum Albumin. *FEBS Letters* **1975,** *58* (1), 134-137.

20. Petitpas, I.; Grüne, T.; Bhattacharya, A. A.; Curry, S., Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids1. *Journal of molecular biology* **2001,** *314* (5), 955-960.

21. Sułkowska, A., Interaction of drugs with bovine and human serum albumin. *Journal of Molecular Structure* **2002,** *614* (1–3), 227-232.

22. Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S., Structural basis of the drug-binding specificity of human serum albumin. *Journal of molecular biology* **2005,** *353* (1), 38-52.

23. Swaney, J. B.; Klotz, I. M., Amino Acid Sequence Adjoining the Lone Tryptophan of Human Serum Albumin. A Binding Site of the Protein. *Biochemistry* **1970,** *9* (13), 2570-2574.

24. Belatik, A.; Hotchandani, S.; Carpentier, R.; Tajmir-Riahi, H.-A., Locating the Binding Sites of Pb(II) Ion with Human and Bovine Serum Albumins. *PLoS ONE* **2012,** *7* (5), e36723.

25. Sułkowska, A.; Równicka, J.; Bojko, B.; Sułkowski, W., Interaction of anticancer drugs with human and bovine serum albumin. *Journal of Molecular Structure* **2003,** *651–653* (0), 133-140.

26. Kandagal, P. B.; Ashoka, S.; Seetharamappa, J.; Shaikh, S. M.; Jadegoud, Y.; Ijare, O. B., Study of the interaction of an anticancer drug with human and bovine serum albumin: spectroscopic approach. *Journal of pharmaceutical and biomedical analysis* **2006,** *41* (2), 393-9.

27. Bi, S.; Sun, Y.; Qiao, C.; Zhang, H.; Liu, C., Binding of several anti-tumor drugs to bovine serum albumin: Fluorescence study. *Journal of Luminescence* **2009,** *129* (5), 541-547.

28. Bojko, B.; Sułkowska, A.; Maciążek-Jurczyk, M.; Równicka, J.; Sułkowski, W. W., Investigations of acetaminophen binding to bovine serum albumin in the presence of fatty acid: Fluorescence and 1H NMR studies. *Journal of Molecular Structure* **2009,** *924-926*, 332-337.

29. Huang, B. X.; Kim, H. Y.; Dass, C., Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry. *Journal of the American Society for Mass Spectrometry* **2004,** *15* (8), 1237-47.

30. Cell Cycle Control System. http://biology.tutorvista.com/cell/cell-cycle.html (accessed March 16).

31. Phases of Mitosis. http://biology.tutorvista.com/cell/mitosis.html (accessed January 15).

32. Hanahan, D.; Weinberg, R. A., The Hallmarks of Cancer. *Cell* **2000,** *100* (1), 57-70.

33. Stages of Cancer. http://www.cancer.net/navigating-cancer-care/diagnosing-cancer/stages-cancer (accessed March 1).

34. Kolte, S. Cancer Treatment. http://www.doctorshangout.com/profiles/blogs/cancer-treatment (accessed March 16).

35. Cancer Facts and Figures 2014. http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2014/index (accessed March 3).

36. Foulkes, W. D.; Smith, I. E.; Reis-Filho, J. S., Triple-Negative Breast Cancer. *New England Journal of Medicine* **2010,** *363* (20), 1938-1948.

37. Your Guide to the Breast Cancer Pathology Report. http://www.breastcancer.org/symptoms/diagnosis/trip_neg (accessed December 15).

38. Sachs, J.; Dinerman, H. Guide to Understanding Triple-Negative Breast Cancer. http://www.lbbc.org/Understanding-Breast-Cancer/Guides-to-Understanding-Breast-Cancer/Guide-to-Understanding-Triple-Negative-Breast-Cancer# (accessed December 14).

39. Dawood, S., Triple-negative breast cancer: epidemiology and management options. *Drugs* **2010,** *70* (17), 2247-58.

40. Bai, L.; Zhu, W.-G., p53: Structure, Function and Therapeutic Applications. *Journal of Cancer Molecules* **2006**.

41. Vousden, K. H.; Lane, D. P., p53 in health and disease. *Nat Rev Mol Cell Biol* **2007,** *8* (4), 275-283.

42. Elmore, S., Apoptosis: a review of programmed cell death. *Toxicologic pathology* **2007,** *35* (4), 495-516.

43. Chipuk, J. E.; Green, D. R., Dissecting p53-dependent apoptosis. *Cell death and differentiation* **2006,** *13* (6), 994-1002.

44. Valeur, B.; Berberan-Santos, M. r. N., A Brief History of Fluorescence and Phosphorescence before the Emergence of Quantum Theory. *Journal of Chemical Education* **2011,** *88* (6), 731-738.

45. Melhuish, W. H.; Zander, M., Nomenclature, symbols, units and their usage in spectrochemical analysis—VI molecular luminescence spectroscopy. *Spectrochimica Acta Part B: Atomic Spectroscopy* **1982,** *37* (3), 259-272.

46. Introduction to Fluorescence. In *Principles of Fluorescence Spectroscopy*, Lakowicz, J., Ed. Springer US: 2006; pp 1-26.

47. Quenching of Fluorescence. In *Principles of Fluorescence Spectroscopy*, Lakowicz, J., Ed. Springer US: 2006; pp 277-330.