Fluorescence-Based Studies of the Host-Guest Inclusion of Vitamin Molecules in Various Supramolecular Hosts

By

Katie Dunn

Thesis submitted to the Department of Chemistry
In partial fulfillment of the requirements for the degree of Bachelor of Science (Honours)

This thesis has been accepted by:

[redacted]
Dr. Brian Wagner
Chair, Department of Chemistry

[redacted]
Dr. Nola Etkin
Dean of Science

Supervisor: Dr. Brian Wagner
Chemistry 4900
Department of Chemistry, Faculty of Science
University of Prince Edward Island, Charlottetown, Prince Edward Island

This work is dedicated to my family, friends, and colleagues, who have given their continued support to me throughout every new experience of my life.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

β-CD	beta-Cyclodextrin
CB7	Cucurbit[7]uril
CDEQWIN	Cyclodextrin Equilibrium Constant for Windows
HP-β-CD	2-Hydroxypropyl-beta-cyclodextrin
HP-γ-CD	2-Hydroxypropyl-gamma-cyclodextrin
K	Kelvin
mM	Millimolar
nm	Nanometers
NMR	Nuclear Magnetic Resonance
SCX4	para-sulfonatocalix[4]arene
SCX6	para-sulfonatocalix[6]arene
UV	Ultraviolet
Vis	Visible

Acknowledgements

I would like to extend my deepest thanks to my supervisor, Dr. Brian D. Wagner. You have provided me with continuous support as a professor, a supervisor, and as a mentor throughout my degree; and for that I am forever grateful. You have constantly believed in me and have always reminded me to believe in myself. You have played the largest role in shaping me into the chemist I have become. Your enthusiasm and wisdom have shown me how to problem solve, think on my feet, and have fun while doing it. It has been an honour being a part of the Wagner Lab.

Next, I would like to thank my closest friend that I have made during my undergrad. Jocelynne, you have been the greatest bonus to this experience. You have always been my person to turn to when I was in need of advice, encouragement, or a good light-hearted laugh. I am so proud of all of your accomplishments and I know you will succeed in all of your future endeavours.

To all of my professors, lab instructors, and other Department of Chemistry staff, thank you for all of your guidance, knowledge, and contributions to my love for science. Without your time and assistance, this experience would not have been the same. To my family, to my boyfriend Logan, to my friends outside of the field of chemistry, thank you for listening to me and pretending to understand what I was talking about during some of the most challenging moments of my degree; sometimes I just had to get it out.

Last but certainly not least, to my fellow chemistry graduates, members of the chemistry society, lab partners, classmates, and everyone else along the way, you have all contributed significantly to my undergraduate career at the University of Prince Edward

Island. I hope I have left an impression on you like you have on me. This has been the greatest journey with you all.

Abstract

The formation of host-guest inclusion complexes between vitamin molecules as guests and various supramolecular hosts and their properties was investigated through the use of fluorescence spectroscopy. Calix[n]arenes and cucurbit[n]urils are two classes of supramolecular hosts that are underrepresented in the field of supramolecular chemistry involving vitamins as guests. Preliminary fluorescence experiments revealed that the combination of vitamin B2 with para-sulfonatocalix[4]arene, SCX4, para-sulfonatocalix[6]arene, SCX6, and cucurbit[7]uril, CB7, resulted in fluorescence suppression. The binding properties of these three hosts with vitamin B2 were found to vary based on the size, shape, and polarity of the guest molecule relative to the host and the accommodation of this by the internal cavity of the hosts.

Fluorescence titrations were performed with varying concentrations of the two classes of hosts to determine the binding constant, K, for the vitamin with the various hosts. For example, the binding constant of vitamin B2 in SCX6 was determined to be 399 ± 30 M^{-1}, in SCX4 it was 638 ± 24 M^{-1}, and in CB7 it was 7400 ± 3800 M^{-1}. This indicated that inclusion of vitamin B2 in SCX4 was favourable over SCX6 however, it was strongest in CB7. A thermodynamic study of the inclusion of vitamin B2 in SCX4 at varying temperatures was performed to determine the enthalpy and entropy of inclusion. A curved van't Hoff plot was obtained and discussed. This work shows that calix[n]arenes and cucurbit[n]urils are excellent choices for hosts in encapsulating vitamins with potentially useful applications in drug delivery and enhancement of bioavailability, solubility, and

other physicochemical properties of vitamins in biological environments, such as the human body.

Chapter 1: Introduction

Vitamins are an important class of organic molecules that are essential in the maintenance of proper metabolic function in many living organisms. Essential vitamins are those that are either not synthesized at all or not synthesized in adequate quantities by the body and must be obtained through other sources. Whether vitamins are accounted for in the diet through food intake or other supplements, one of the main concerns that remains without a well-defined solution is increasing the bioavailability of vitamins in the human body. It is well-known that the bulk of the vitamins that are introduced to the body are never absorbed and are later excreted as waste. The field of supramolecular chemistry holds a large opportunity for potential improvements in this area.

A number of research studies have been done involving the inclusion of a variety of vitamins with a common class of supramolecular hosts; cyclodextrins, to enhance the solubility and stability and therefore the bioavailability of these vitamins. Cyclodextrins are valued as supramolecular hosts for their water-solubility, extremely low cost, and stamp of approval for safe human consumption. They have shown success in the encapsulation of some vitamins, however, lack the ability to encapsulate others. This area requires further investigation of vitamins that are incapable of binding in cyclodextrins with other supramolecular hosts such as calixarenes and cucurbiturils. The objective of this project was to study the inclusion of vitamins with not only cyclodextrins but calixarenes and cucurbiturils as well through the use of fluorescence spectroscopy. Binding constants for inclusion complexes of different vitamins in varying hosts can be determined using fluorescence titrations. The results of the binding studies from the fluorescence titrations

will provide a better understanding of the inclusion of vitamins in supramolecular hosts, and the potential benefit this may have in enhancing vitamin delivery to the site of absorption for greater bioavailability.

1.1 Supramolecular Chemistry

Supramolecular chemistry is described as the interaction of two or more chemical species resulting in the self-assembly of a complex through reversible non-covalent intermolecular interactions. Such interactions include but are not limited to van der Waals forces which encompasses dipole-dipole interactions and London dispersion forces, hydrogen bonding, and the hydrophobic effect.[1,2] The formation of a supramolecular host-guest inclusion complex involves a smaller "guest" molecule being held within a larger "host" molecule through the interactions described above in the well-defined internal cavity of the host.[3] A schematic representation of the formation of a host-guest inclusion complex is shown in Figure 1.1 below. These interactions between the guest and the host are of particular interest because their complexation has the potential to enhance or suppress some of the physicochemical properties of both the guest and host such as stability, solubility, reactivity, colour and fluorescence in comparison to when they are dissociated into their individual components.[4] In enhancing the solubility or stability of a drug, this could enhance the absorption of the drug and therefore the bioavailability; decreasing the amount that is excreted as waste. This has important applications in the area of pharmaceuticals and drug delivery as the inclusion of a drug in a supramolecular host

and its reversible nature has the ability to enhance the overall bioavailability of the drug in the body, helping to ensure the patient receives the maximum benefit in taking the drug.[5]

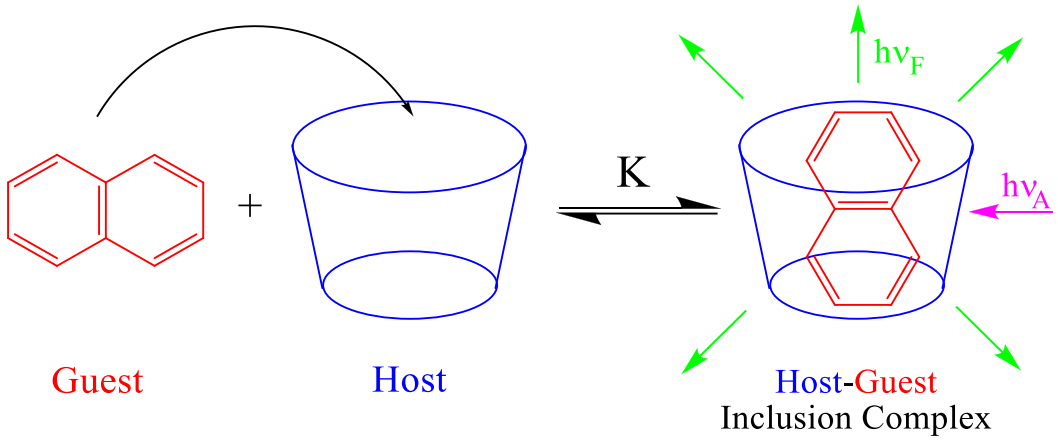


Figure 1.1: Schematic representation of the assembly of a host-guest inclusion complex.

The non-covalent intermolecular interactions of the guest and host provide the stabilization of the inclusion complex. Their stability can be described through the use of the Gibbs free energy equation shown in Equation 1.1 where ΔH is change in enthalpy, ΔS is change in entropy, and T is temperature in Kelvin.

$$\Delta G = \Delta H - T\Delta S \qquad \textbf{Equation 1.1}$$

A negative Gibbs free energy indicates the self-assembly of an inclusion complex will occur spontaneously. The spontaneity and stabilization are driven by a negative change in enthalpy upon complexation. On the other hand, the formation of a complex from two free components results in a negative entropy change because the number of free components; one guest and one host in the case of 1:1 complexation, is decreased upon complexation.

The formation of the complex with a negative enthalpy and entropy change will never be guaranteed, as the resulting Gibbs free energy may be positive depending on the temperature at which the reaction is performed. If the reaction is performed at a high enough temperature, the entropy change would overrule the enthalpy change, resulting in a positive Gibbs free energy indicating a non-spontaneous process. Under these conditions the complex would not form. However, a strong enthalpy driven stabilization effect can overcome this unfavourable negative entropy change and result in the spontaneous formation of the host-guest inclusion complex. A positive change in entropy is more favourable than negative because when accompanied by a negative change in enthalpy, the change in Gibbs free energy is guaranteed to be negative; the inclusion complex will be formed. A positive entropy change can occur as the result of solvent molecules being expelled from the internal cavity of the host upon inclusion of the guest.

Shown above in Figure 1.1 was one of the infinite stoichiometries in which an inclusion complex can form. The schematic representation shows the use of a naphthalene guest being encapsulated by the host. The stoichiometry of an inclusion complex is best described using a ratio x:y where x is the number of host molecules and y is the number of guest molecules. From this, the stoichiometry in Figure 1.1 can be seen to be 1:1 where one molecule of host encapsulates one molecule of naphthalene guest. Aside from this, other host:guest stoichiometries are also possible. For example, 1:2 complexes involve one host molecule encapsulating two guest molecules, 2:1 complexes involve two host molecules encapsulating one guest molecule and, 2:2 complexes involve two host molecules encapsulating two guest molecules are often observed. These four common inclusion complex stoichiometries are depicted in Figure 1.2 below. Variation in

stoichiometries is largely attributed to the difference in size of the guest and host as well as the accommodation of electronic and steric factors.[6] The resulting stoichiometry will favour the most optimal interaction of the guest with the internal cavity of the host, resulting in the largely negative ΔG.

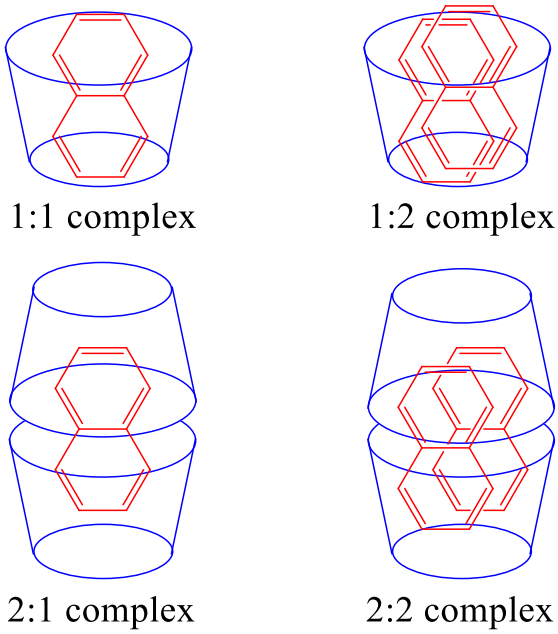


Figure 1.2: Stoichiometries of host-guest inclusion complexes.

Supramolecular hosts are characterized by their large macrocyclic structure and well-defined internal cavity. Their structure is typically made up of repeating monomeric units that differ depending on the family of supramolecular hosts from which they originate. Some of these families are cyclodextrins, having conjoined glucose units through $\alpha - (1 \rightarrow 4)$ glycosidic bonds as seen in Figure 1.3; calixarenes, having linked phenolic units through meta-substituted methylene bridges shown in Figure 1.4, and cucurbiturils,

having glycoluril units linked by two methylene bridges as seen in Figure 1.5.[7-9] The cavity of these macrocyclic hosts tend to be non-polar; allowing them to interact favourably with the guest through hydrophobic non-covalent interactions. The internal cavity of the host molecule can vary in size depending on how rigid or flexible it is. Cucurbiturils are known to have more rigid cavities while cyclodextrins are known to be more flexible.[10,11] The compatibility of the size of the guest molecule with the size of the internal cavity of the host allows for the encapsulation of a guest molecule that is neither too small nor too large but, just the right fit. While there are more limitations in structures that can serve as supramolecular hosts, the same cannot be said for guests that can be encapsulated in the hosts. The most prominent feature that must be true is that it must have a compatible size with the host cavity. Other than size, the only other important feature is its ability to participate in non-covalent interactions with the guest, which can vary depending on structure. There have been countless different types of molecules that have been studied as guests in host-guest inclusion complexes such as drugs and metal ions to name a couple.

As mentioned, the formation of an inclusion complex depends on having a guest that matches the geometry and size of the host cavity. The spontaneity of the formation of the inclusion complex can be determined through Equation 1.1. However, this equation does not provide a picture of the stability of the complex if and when it is formed. The stability of the complex is determined by the magnitude of the equilibrium or binding constant, K, which measures the bonding affinity of the host and guest at equilibrium. The binding constant is equal to the concentration of the ratio of the host-guest complex over the concentration of the host and guest as shown in Equation 1.2 below. Strong binding of the host and guest in complexation with each other is characterized by a binding constant

with a large magnitude. This is an indication that the host and guest are well matched, compatible in size and geometry, and interact favorably.

$$K = [H:G]/[H][G] \qquad \textbf{Equation 1.2}$$

The value of K then provides the ability to quantify the interaction that is occurring between a guest, such as a drug, and a host. If an inclusion complex is formed with a strong binding constant, this can have important implications in enhancing the important characteristics of effective drug delivery and drug absorption in the body. As mentioned, in the case of vitamins, there is often difficulty in maximizing the amount absorbed by the time the vitamin reaches the site of absorption. Supramolecular chemistry has played an important role in attempting to find a solution to this problem and to this point there has been interesting preliminary work done involving the use of cyclodextrin hosts. However, the expansion of this field to include calixarenes and cucurbiturils could be very beneficial in enhancing drug delivery and bioavailability moving forward as cyclodextrins are not capable of successfully encapsulating all vitamin molecules.

1.2 Application of Host-Guest Inclusion to Pharmaceutics

Host-guest inclusion complexes have become a tool that is increasingly more valuable in the world of pharmaceuticals and drug delivery. There are many obstacles that are faced in formulating new drugs, one being the challenge of getting the drug to the site of absorption without disintegrating or without it being excreted from the body as waste

prior to absorption. This is a challenge that is seen across all classes of drugs, from antibiotics to antineoplastics. Drug developers want the most effective, cost-efficient, and safe method to overcome this obstacle. Up to this point, there has been prevalent work done in the use of cyclodextrins in the enhancement of solubility of water-insoluble drugs, stability, and bioavailability across many different methods of drug delivery; oral, transdermal, and rectal to name a few, and across a broad spectrum of drug classes.[12] For the purposes of this research, the use of host-guest inclusion complexes to enhance the bioavailability of select vitamins is of particular interest.

1.2.1 Vitamins and Current Research

Unlike the body's ability to synthesize non-essential amino acids, the body is incapable of synthesizing some of the vitamins that are required in maintaining proper metabolic function on a day-to-day basis. The human body relies heavily on the diet for the intake of adequate sources of vitamins. There are very few vitamins that are synthesized by the body without full reliance on dietary sources. Vitamin D of course is the vitamin that is obtained when the skin is exposed to sunlight, vitamin B3 is obtainable through the amino acid tryptophan and vitamins B7 and K are produced by the bacteria in the gastrointestinal tract.[13] Aside from these, it is up to the individual to provide their bodies with reliable sources of all other vitamins.

A topic with a large area for debate is determining appropriate disintegration times for vitamin tablets to ensure they are being effectively absorbed. A study performed by Bhagavan *et al.* focused on the disintegration times of vitamin C tablets and how they

played a role on the bioavailability of vitamin C to the body.[14] Four different formulations of 500 mg vitamin C tablets with varying mean disintegration times ranging from 9 to 120 minutes were compared with a standard vitamin C solution and administered to 15 male subjects in a double-blind clinical trial. The bioavailability of the vitamin C to the body was measured through urine ascorbate analysis. Through this study it was determined that the standard vitamin C solution and a fast-disintegrating tablet had lower bioavailabilities than vitamin C tablets with longer disintegration times.[14] The expansion of this work to include the complexation of vitamin C with a supramolecular host to increase disintegration times for a water-soluble vitamin could have important contributions to this field.

Further debate involving appropriate disintegration times of vitamins encompasses the more ethical concern of the amount of information that should be provided on commercially available over-the-counter vitamin labels about the release mechanism of the vitamin. Often these labels are flooded with scientific information and "buzz words" to attract the consumer without allowing them to make informed decisions about their health. They become under the impression that a guaranteed dose of a vitamin is promised in one tablet. The reality, however, is that many commercially available vitamin tablets and capsules will not properly disintegrate, as indicated through the use of a simple dissolution test. Different vitamins are absorbed at different locations along the digestive tract. Disintegration is the first step in the absorption process.

A study performed by Loboenberg *et al.* involved a series of disintegration tests involving vitamin and mineral supplements on the Canadian market.[15] One series of tests involved the use of a pH 6.8 simulated intestinal fluid. There were 46 different supplements tested with varying formulations including tablets, capsules, and timed-release products.

Out of the 46 products, 25 of them failed to disintegrate. The second series of tests was performed under United States Pharmacopeia, USP, disintegration standards on the products that failed the first test. Seven of the products did not fully disintegrate however, and four of the products failed the test completely.[15] This creates concern that there are products on the Canadian market that are not holding up to the standards with which they are marketed. Patients may be misled and become under the impression they are receiving a guaranteed dose of vitamin or mineral when really the disintegration quality of the product is low-quality. This is evidence that there is a place in the Canadian pharmaceutical market for improvement in disintegration quality of OTC vitamin and mineral products that could be contributed to with the help of supramolecular chemistry. This need has received some attention already in several projects involving the use cyclodextrins as inclusion hosts.

1.2.2 Cyclodextrin Inclusion of Vitamins

The use of cyclodextrins as supramolecular hosts has been well explored in the pharmaceutical industry and has proven effective in enhancing stability, safety, solubility and bioavailability of the guest molecules. The use of cyclodextrins as encapsulation hosts of vitamins is of particular interest for this project. One of the most common cyclodextrins, β-cyclodextrin (Figure 1.3), has been shown to be successful in encapsulating two water-soluble vitamins, vitamin B3 and vitamin C, in a study by Saha *et al.*[16] These two vitamins are important in treating hypercholesterolemia and the growth and repair of tissues respectively and the goal of this work was to protect these vitamins from oxidation or

structural modification before reaching the site of absorption, similar to the crisis of disintegration as described above. The confirmation of the complexation of vitamin C in β-CD was completed with a 2D NMR spectral analysis with a Nuclear Overhauser Effect, NOE, cross-correlation, that confirmed that the protons of the internal cavity of the β-CD were in proximity of the protons of the vitamin C. This same process was used to confirm complexation of vitamin B3 in β-CD.[16] Following NMR analysis, UV-Vis spectroscopy was used to determine binding constants and thermodynamic parameters of enthalpy and entropy. It was determined that there was a negative entropy change with an overcoming negative enthalpy stabilization effect, resulting in the thermodynamically favourable inclusion complex.[16] This study demonstrated potential of overcoming stability and bioavailability challenges of the absorption of vitamin B3 and C in the body with the use of a cyclodextrin host.

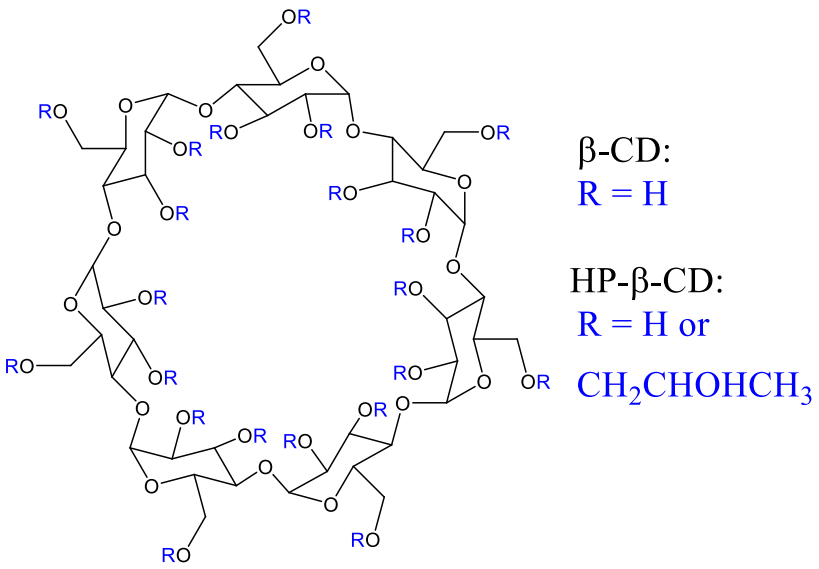


Figure 1.3: Chemical structure of β-CD and HP-β-CD.

The investigation of β-CD as a host was also performed with vitamin D as a guest in a study by Bakirova *et al.*[17] Vitamin D plays a large role in the metabolism of calcium and phosphorus in the body as well as the prevention of many diseases such as cancer, cardiovascular, and diabetes to name a few. Vitamin D is one of the fat-soluble vitamins which presents bioavailability challenges. The use of a cyclodextrin host with a hydrophobic cavity to encapsulate a fat-soluble vitamin can aid in overcoming this challenge. The morphology of the formed inclusion complex of vitamin D in β-CD was confirmed through the use of scanning electron microscopy and a 2:1 host to guest stoichiometric ratio was determined. The formation of the inclusion complex was also confirmed through ^{1}H and ^{13}C NMR analyses.[17] With the strong hydrophobic interaction between the guest and host when complexed, it is expected that the water-solubility would be enhanced, improving the bioavailability of vitamin D in the body.

A similar study was conducted using another fat-soluble vitamin, vitamin A. This study was performed by Xu *et al.* and investigated the complexation of vitamin A and its esters, vitamin A acetate and vitamin A palmitate, in β-CD.[18] As a result of vitamin A being a fat-soluble vitamin, similar solubility challenges are present when being absorbed in the body. A water-soluble vitamin improves upon bioavailability challenges. Solutions of vitamin A and vitamin A esters with β-CD were prepared with ethanol and distilled water and precipitated to be analyzed using FT-IR and NMR techniques. From the spectroscopic methods, the formation of inclusion complexes was confirmed. Phase solubility studies indicated a 2:1 host to guest ratio and that the vitamin A and β-CD had the lowest Gibbs free energy change, indicating this complex to be the most

thermodynamically favourable of the three complexes.[18] Solubility tests were also performed and it was determined that water-solubility was greatly enhanced by the complexation which could have value in increasing the bioavailability of vitamin A in the body.

From these three studies, there is a clear preference for the use of cyclodextrins in forming host-guest inclusion complexes with vitamins; particularly β-CD. While they have proven to be successful in forming thermodynamically stable complexes and have been approved by the FDA for safe ingestion, there is a lack of investigation into the use of alternative supramolecular hosts with the same goal. The introduction of alternative hosts such as calixarenes or cucurbiturils to the pharmaceutical industry particularly for the use of the encapsulation of vitamins could bring many advantages and is an opportunity that should not be missed. Both calixarenes and cucurbiturils have the ability to interact with guest molecules through electrostatic interactions and calixarenes have the ability to interact with aromatic molecules through π - π stacking interactions. These are two types of intermolecular interactions that are major driving forces of inclusion that cyclodextrins are incapable of participating in. With further exploration of other families of supramolecular hosts with vitamin molecules, additional reversible binding relationships at temperatures close to body temperature could be discovered that have major applications to the pharmaceutical industry in the enhancement of the bioavailability of these molecules.

1.3 Alternative Hosts for Host-Guest Inclusion

As mentioned, there are families of supramolecular hosts not including cyclodextrins, that tend to receive less attention from the pharmaceutical industry in being used to enhance the solubility, stability, and bioavailability of vitamins. Considering alternative supramolecular hosts such as calixarenes or cucurbiturils could be an avenue that could greatly expand this field and provide many more formulary options for drug developers in synthesizing a product that is best designed for effective absorption by the body. While the use of these supramolecular hosts aims to reach the same goal, their structural and geometrical differences make them unique, and may offer advantages over cyclodextrins.

1.3.1 Calix[n]arenes

Calix[n]arenes are a class of macrocyclic host molecules with unique structural characteristics that were first discovered in 1944 by Zinke *et al* through a two-step synthesis involving p-tert-butylphenol and formaldehyde that resulted in the formation of a cyclic tetramer.[19] Calix[n]arenes are made up of repeating phenolic units which are joined by meta-substituted methylene bridges that form a molecular "basket" with a small rim on the bottom where the hydroxyl groups are located and a larger upper rim as seen in Figure 1.4 below.[20] The nomenclature of calix[n]arenes is straightforward as the number of phenolic units, n, is what differentiates the name. For example, calix[4]arene is a calix[n]arene made up of four repeating phenolic units. The hydroxyl groups that are oriented ortho to the

methylene bridges of the lower rim interact with each other through hydrogen bonding to stabilize the basket-like structure. The upper rim of the calix[n]arene can be functionalized with varying substituents such as sulfur, amines, imines, and alkyl groups among others to tailor the macrocycle in having varying degrees of solubility, molecular recognition, and hydrophobicity.[20]

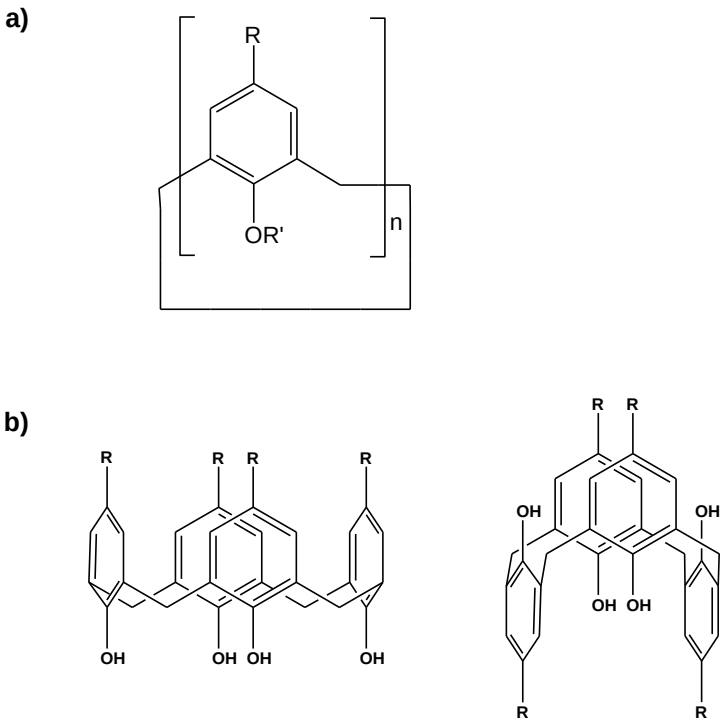


Figure 1.4: a) General structure of calix[n]arenes. b) Cone and 1,3-alternate calix[4]arene conformations.

The ability to functionalize the upper rim of the calix[n]arene is of particular importance to the pharmaceutical industry. With the ability to functionalize the upper rim with a hydrophobic group and having a hydrophilic lower rim, this opens up the potential

of having an amphiphilic macrocycle capable of self-assembling into a liposome, micelle, or vesicle structure capable of effectively transporting drugs throughout the body at a low cost and cytotoxicity. The introduction of a sulfonic group in the para position has shown to demonstrate success in the encapsulation of drugs to increase solubility, bioavailability, oral absorption and stability under varying heat, light, and acidic conditions.[20] This type of functionalization and the use of para-sulfonated calix[n]arenes played an important role in this project in the analysis of their formation of inclusion complexes with vitamins. The only limitation of their use is that to date calix[n]arenes have not been approved for use in human medicine; however, they have not demonstrated human toxicity or heightened immune responses and further investigation of this type of supramolecular host remains important.

Calix[n]arenes have a prominent difference from the popular cyclodextrins hosts in their water-solubility. Typically, calix[n]arenes are largely water insoluble, however, they can be made to be water soluble via functionalization with a charged group. The hydroxyl groups of the phenolic units can also be deprotonated in aqueous solution depending on the pH.[21] With the deprotonation of the hydroxyl groups and the functionalization with charged groups, the electrostatic forces between the calix[n]arene and guest are increasingly important in driving the encapsulation. The importance of these electrostatic forces is highlighted throughout this work with the use of water-soluble para-sulfonated calix[n]arenes.

Another unique characteristic of the calix[n]arenes is their ability to participate in conformational changes. The calix[4]arenes can adopt four different isomeric forms; the cone, the partial cone, the 1,2-alternate, and the 1,3-alternate. The cone conformation is the

standard conformation demonstrated in Figure 1.4 above. The partial cone involves the inversion of one of the phenolic units. The 1,2-alternate involves the inversion of the phenolic units in the first and second positions and the 1,3-alternate inverts the phenolic units in the first and third positions.[20] The ability to adopt these different conformations is important in molecular recognition and expanding the variety of guests the calix[n]arenes can encapsulate.

There are a variety of calix[n]arenes that are commercially available with the most popular ones being calix[4]arene, calix[5]arene, calix[6]arene, calix[7]arene, and calix[8]arene as they have the optimal cavity sizes ranging from 3.0 to 11.7Å to accommodate small molecules.[22] A recent study by Guerineau *et al.* involved the synthesis and characterization of "giant" calixarenes containing up to 90 phenolic units.[23] Similar to other supramolecular hosts such as cyclodextrins, calix[n]arenes have been successfully used in encapsulating guests through non-covalent interactions such as van der Waals forces and hydrogen bonding. However, as a result of calix[n]arenes having repeated aromatic rings in their macrocycle, they are capable of contributing to $\pi - \pi$ stacking interactions with aromatic guests to act as an additional driving force of the inclusion process and further stabilize the complex. This is an advantage over other hosts such as cyclodextrins that do not contain aromatic rings, however, could have disadvantages when considering the safety of the use of aromatics in pharmaceutical products.

1.3.2 Cucurbit[n]urils

Cucurbit[n]urils are a class of macrocyclic host molecules that were first discovered in 1905 by Robert Behrend through the condensation of glycoluril with formaldehyde, however, it was not until 1981 that their structure was first elucidated.[24] Their general structure involves the repetition of glycoluril units joined by two methylene bridges. The oxygens of the glycoluril units point inwards, partially closing the cavity at the top and bottom giving them a pumpkin-like appearance. Because of this, they are referred to as the "jack-o-lanterns" of supramolecular hosts. Their nomenclature is simple, involving the number of glycoluril units, n, to determine their name. For example, a cucurbit[n]uril formed by seven glycoluril units is named as cucurbit[7]uril. For shorthand they are denoted CBn. The general cucurbit[n]uril structure is shown in Figure 1.5 below.

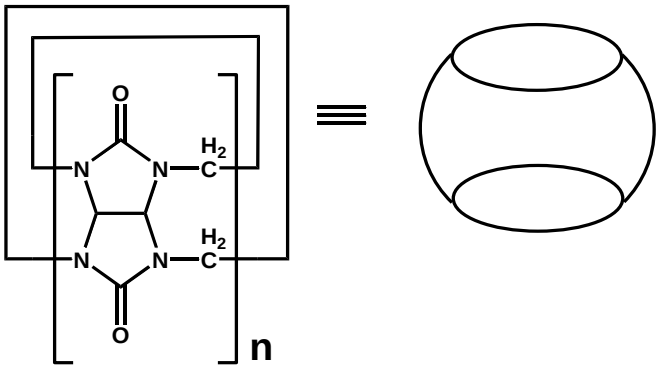


Figure 1.5: General structure of cucurbit[n]urils.

The use of cucurbit[n]urils in the encapsulation of drugs to enhance bioavailability in the body has been a highlight of their capabilities as supramolecular hosts. Cucurbit[n]urils have been shown to fully encapsulate a variety of drug classes such as vitamins, hormones, and antineoplastics among others.[9] The inclusion of drugs in supramolecular hosts is often monitored through spectroscopic methods, one main technique being fluorescence spectroscopy. A study by Miskolczy *et al.* investigated the inclusion of berberine, an anti-microbial agent, in cucurbit[7]uril through the use of a stopped flow method coupled to the fluorimeter, fluorescence titrations, and isothermal calorimetry.[25] Their investigations resulted in the observation that CB7 did include the berberine in its cavity with a 1:1 host to guest ratio and resulted in a 500-fold enhancement in fluorescence intensity. A similar study from the same group investigated the encapsulation of berberine in CB8 using the same fluorometric techniques.[26] Their findings demonstrated the formation of 1:1 and 2:1 complexes. A positive entropy change was observed due to the expulsion of water molecules from the inner cavity of the CB8 to accommodate the guest molecule. This indicated a highly thermodynamically favourable inclusion complex. This implies that the formation of berberine-CB8 inclusion complexes could have large applications in its delivery mechanism in the body or potential in creating a controlled release product.

These are two important examples of the use of cucurbit[n]urils for the encapsulation of drugs. Cucurbit[n]urils are also known to be reliable supramolecular hosts for medicinal purposes because of their low cytotoxicity and high biocompatibility in the human body. They have also demonstrated higher binding affinities for drug guest molecules in comparison to the popular cyclodextrin supramolecular hosts that are

commonly studied because of their low cost and high water-solubility.[9] Cucurbit[n]urils also have electronegative carbonyl groups that are capable of participating in electrostatic interactions with charged guest molecules which is unique from cyclodextrins that are incapable of participating in these same interactions. There are a number of commercially available cucurbit[n]urils such as CB5, CB6, CB7, CB8, and CB10. Unfortunately to this point, a successful synthesis for CB9 has not been determined. Cucurbit[n]urils are known to be less water-soluble in comparison to cyclodextrins, however, for the purpose of this work, all experiments were performed using water as the solvent and this did not present any challenges.

Applicative research for the use of cucurbit[n]urils in the encapsulation of vitamins has also been presented in the literature. A study by Li *et al.* demonstrated the use of CB7 in the encapsulation of vitamin B6.[27] Experimental methods included the use of UV-Vis titrations and ^{1}H NMR for structural confirmation. It was determined that a 1:1 inclusion complex was formed with a binding constant of $(4.0 \pm 0.5) \times 10^{3}$ M^{-1}. This indicated that the vitamin B6 had high binding affinity in the cavity of CB7 and holds future applications in the potential for developing new formulations of vitamin B6 with enhanced stability and bioavailability in the body. This study was of particular importance to this project as it demonstrates the ability of cucurbit[n]urils to be used in enhancing the biocompatibility of vitamins and provided motivation in expanding this area to vitamins outside of vitamin B6.

1.4 Fluorescence Study of Host-Guest Inclusion

Spectroscopic techniques are often applied to measure and confirm the inclusion of a guest in a host and determine their stoichiometric binding ratio. A spectroscopic method that is commonly used is fluorescence spectroscopy. Luminescence is the emission of a photon from an electronically excited state and encompasses the phenomena of fluorescence and phosphorescence. Fluorescence and phosphorescence vary in the multiplicity of the electronic states participating in the transition. Multiplicity refers to the spin configuration of the electron occupying the ground and excited electronic states involved in the transition of the luminescence process and can be determined using Equation 1.3 where S is the total spin angular momentum.

$$M = 2S + 1 \qquad \textbf{Equation 1.3}$$

An excitation of one of the two opposite spin electrons from the ground singlet state (S_0) to a higher energy level singlet excited state denoted S_1, S_2, etc. results in a total spin angular momentum of 0 and by Equation 1.3 the multiplicity would be 1. On the other hand, the inversion of the spin of the electron upon excitation to the higher energy level would result in a total spin angular momentum of 1 and by Equation 1.3, a multiplicity of 3. These two types of electronic states are shown in Figure 1.6 below.

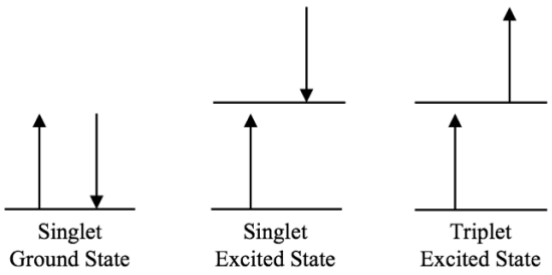


Figure 1.6: Schematic diagram of singlet and triplet state electronic excitations.

Fluorescence is the emission of a photon by a molecule undergoing relaxation between electronic states of the same spin multiplicity. Typically, this occurs between the first excited singlet state, S_1, to the ground singlet state, S_0. In contrast to this, phosphorescence is the photophysical process of the emission of a photon by a molecule during the relaxation between electronic states of different spin multiplicities. Typically, this occurs between the first excited triplet state, T_1, to the ground singlet state, S_0.[21] Fluorescence and phosphorescence are known as radiative processes as the excess energy of the excited state exits the system via the removal of a photon. Aside from radiative processes, there are non-radiative processes that allow excited electronic states to relax to a lower energy state without the emission of a photon.[21] These radiative processes are demonstrated in Figure 1.7 of a Jablonski diagram.

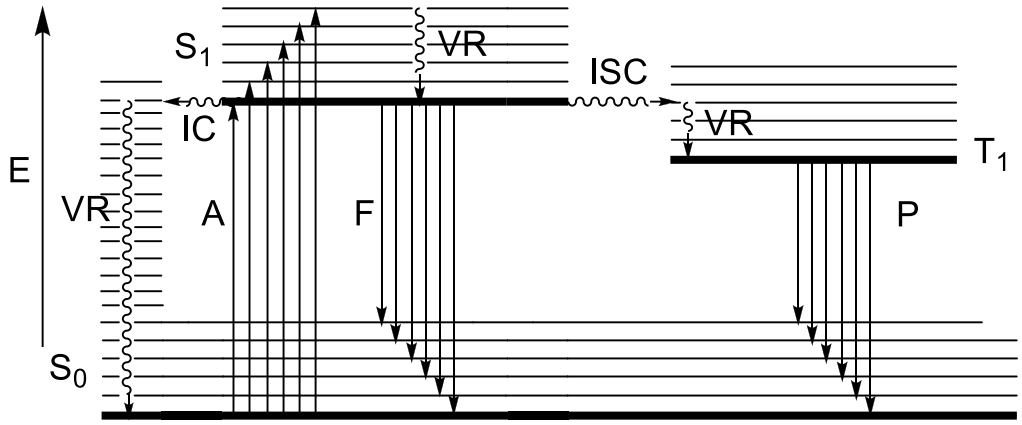


Figure 1.7: Jablonski diagram.

The Jablonski diagram provides a clear visualization of both radiative and non-radiative processes. Radiative processes such as absorbance (A), fluorescence (F), and phosphorescence (P) are denoted with straight arrows. Non-radiative processes such as internal conversion (IC), intersystem crossing (ISC), and vibrational relaxation (VR) are denoted with the use of waved arrows.[21] Vibrational relaxation involves relaxation in energy of an excited vibrational level within an electronic state to a ground vibrational level within an electronic state. This process occurs very rapidly and is the reason for which electronic transitions typically occur from ground vibrational levels. For example, fluorescence typically originates from the ground vibrational level of S_1. Internal conversion is another non-radiative transition that occurs between electronic states with the same spin multiplicity. Intersystem crossing is a non-radiative transition that occurs between electronic states of different spin multiplicities.[21]

For the purposes of this work, fluorescence played an important role in confirming the inclusion of guest molecules in supramolecular hosts through the use of fluorescence

spectroscopy. This technique is most commonly used when a fluorescent guest and non-fluorescent host are being investigated.[21] The most common method to perform fluorescence spectroscopy is through steady-state fluorescence spectroscopy. This method uses a constant source of illumination to excite the fluorophore molecules to the S_1 state at a constant rate of excitation. Fluorescence spectra are produced by measuring fluorescence intensity as a function of wavelength of emission. From total integrated areas of the emission spectrum, F/F_0 values can be determined through the use of Equation 1.4 where F_∞ is the total fluorescence of the fully complexed guest and $[host]_0$ is the concentration of added host, to draw conclusions on the fluorescent properties of the guest molecule assuming the inclusion complex has a 1:1 host to guest ratio. The fluorescence of the guest included in a host is denoted F and the fluorescence of the guest in the absence of host is denoted F_0. A F/F_0 value greater than 1 indicates fluorescence enhancement of the guest upon inclusion, while a F/F_0 value less than 1 indicates fluorescence suppression of the guest upon inclusion. From this, the most important parameter can be determined; the binding constant, K. Through the performance of fluorescence titrations and measuring the F/F_0 value as a function of added host concentration, the data can be plotted and used to determine K through the use of a non-linear least-squares fitting curve and represent the binding affinity the guest molecule has for the internal cavity of the host molecule.[21]

$$\frac{F}{F_0} = 1 + \left(\frac{F_\infty}{F_0} - 1\right) \frac{[host]_0 K}{1 + [host]_0 K} \qquad \textbf{Equation 1.4}$$

1.5 Thermodynamic Study of Host-Guest Inclusion

Using Equation 1.4 from above and the non-linear least-squares fitting curve to determine K at varying temperatures, the thermodynamics of the host-guest inclusion process can be evaluated. As discussed earlier in Section 1.1, the formation of an inclusion complex can be explained using Equation 1.1 which uses enthalpy, entropy, and temperature to determine the Gibbs free energy change of the inclusion process; whether the complex is thermodynamically favourable over the host and guest components existing separately. The binding constant, K, is related to the change in Gibbs free energy through Equation 1.5 shown below with R being the ideal gas constant and T being the temperature in Kelvin at which the experiment was performed.

$$K = e^{-\Delta G/RT} \qquad \textbf{Equation 1.5}$$

Once the binding constants from each of the different temperatures are known, a van't Hoff plot of the natural logarithm of the binding constant versus the reciprocal of the temperature in Kelvin can be used to determine the thermodynamic parameters for the inclusion process using the van't Hoff equation shown in Equation 1.6. If the enthalpy of inclusion, ΔH, and entropy of inclusion, ΔS, remain constant over the temperature range, the van't Hoff plot will be linear and the enthalpy of inclusion can be determined from the slope and the entropy of inclusion is obtained from the y-intercept.[21] This is indicative of the binding constant of inclusion being dependent on the temperature. Under these

conditions, the heat capacity, C, is constant for the inclusion process ($\Delta C = 0$).[28] A van't Hoff plot with a positive slope is indicative of an exothermic reaction; implying a thermodynamically favourable negative change in enthalpy.

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad \textbf{Equation 1.6}$$

Aside from linear van't Hoff plots, it is also possible to have curved van't Hoff plots. A curvature in the plot is indicative that the enthalpy or entropy of inclusion changes with temperature and/or the heat capacity is not constant over the temperature range. Enthalpies of inclusion can change with a change in conformation of the inclusion complex or changes in inter- or intramolecular interactions that are temperature dependent.[28] As mentioned, inclusion complexes with calixarenes as hosts are known to be capable of undergoing significant host conformational changes.[20,21] With the changes in enthalpies of inclusion, the slope of the van't Hoff plot will inevitably change. Because of this, it is common to see inflection points in the van't Hoff plot resulting in an S-shaped distribution of the data.[28] Depending on the extent of curvature of the van't Hoff plot, a series of data points that demonstrate linearity can be plotted separately to approximate the enthalpy and entropy of inclusion or these values can be determined individually for each data point if the change in heat capacity is known.

1.6 Project Objectives

The main objective of this project is to determine the ability of different types of supramolecular hosts, namely calix[n]arenes, cucurbit[n]urils, and cyclodextrins, to form inclusion complexes with various vitamins which have not been previously reported in the literature. Fluorescence experiments can be performed to determine whether enhancement, suppression, or no fluorescence effect is observed upon inclusion of the guest in the host in comparison to the free guest. If either enhancement or suppression is observed, fluorescence titrations will be conducted to acquire binding constants which indicate the affinity and strength the host and guest share in forming an inclusion complex. This project was chosen out of personal interest in pharmaceuticals and wanting to contribute to finding a solution for the disintegration crisis of vitamins and enhancing their bioavailability in the human body.

A second part of this project involves the thermodynamic study of the formation of inclusion complexes with vitamins and the effect of temperature on the binding constant, to determine its contribution to the thermodynamic favourability of complexation over the free host and guest components. Alternative hosts such as para-sulfonatocalix[4]arene, para-sulfonatocalix[6]arene and cucurbit[7]uril will be used to investigate their abilities as supramolecular hosts in the encapsulation of vitamins in aqueous solution. The contribution of electrostatic and $\pi - \pi$ stacking interactions from these hosts in the formation of inclusion complexes with vitamins will be highlighted to demonstrate the importance of further investigation of supramolecular hosts other than cyclodextrins and the advantages their introduction into the pharmaceutical industry could have.

Chapter 2: Experimental

2.1 Materials

Table 2.1: Vitamins used as guests in fluorescence enhancement, suppression, and titration experiments.

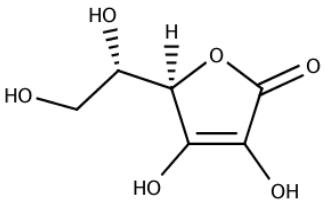
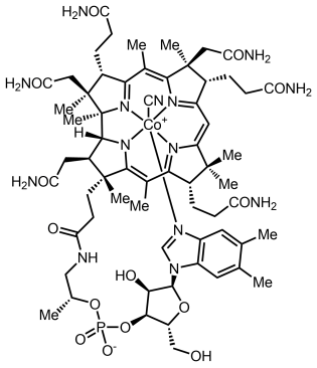
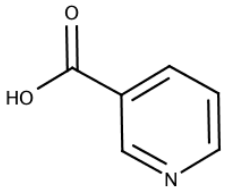
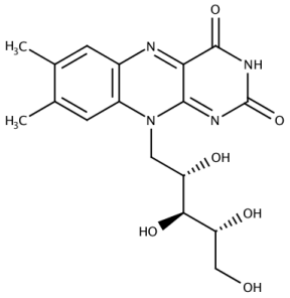
Chemical	Structure	Manufacturer
Ascorbic Acid (Vitamin C)		Sigma-Aldrich
Cyanocobalamin (Vitamin B12)		Sigma-Aldrich
Niacin (Vitamin B3)		Sigma-Aldrich
Riboflavin (Vitamin B2)		Sigma-Aldrich

Table 2.2: Hosts used for fluorescence enhancement, suppression, and titration experiments.

Chemical	Manufacturer
β-cyclodextrin	Sigma
Hydroxypropyl-β-cyclodextrin	Aldrich
Hydroxypropyl-γ-cyclodextrin	Aldrich
Cucurbit[7]uril	Aldrich
4-Sulfocalix[4]arene	ACROS Organics
4-Sulfocalix[6]arene	ACROS Organics

2.2 Guest and Host-Guest Solution Preparation

All guest solutions used for fluorescence spectroscopy experiments were prepared in volumetric flasks. The concentration of guest was not important for these experiments, therefore the mass of guest used was not measured. Instead, guest solutions were prepared to an appropriate absorbance that was measured using UV-Vis spectroscopy. Once a guest solution was obtained with an absorbance in the range of 0.28-0.33 at the excitation wavelength used for fluorescence experiments, the guest solution was deemed to be ready for analysis.

After preparing a guest solution with an appropriate absorbance measurement, host-guest solutions were prepared by first weighing out a sample of host into a glass vial. This

was performed accurately by weighing the host directly into the vial using an analytical balance. Then, 3.00 mL of guest solution was pipetted into the vial containing the host using a 3.00 mL volumetric pipette in order to obtain the desired concentration. The host-guest solutions were then sonicated using the Branson 200 Ultrasonic Cleaner prior to fluorescence experiments to ensure thorough mixing and solubilization.

2.3 UV-Vis Absorption Spectroscopy Measurements

All absorption measurements were conducted using a Cary Bio UV-Vis spectrophotometer. To obtain an absorption spectrum, 3.0 mL of a prepared solution was pipetted into a 1 cm^2 quartz cuvette using a glass pipette. Using a dampened Kimwipe with deionized water, fingerprints and other residues were removed from the cuvette. The cuvette was then dried and polished using a dry Kimwipe to remove moisture from the outside. The cuvette was placed in the sample holder of the spectrophotometer and the chamber was closed to stop any room lighting from affecting the measurement. A baseline correction with a solvent blank was then performed prior to each measurement. All absorption measurements were performed between 500-250 nm at a medium scan rate to obtain the absorption spectra and determine the excitation wavelength of maximum absorbance.

2.4 Fluorescence Spectroscopy Measurements

All fluorescence measurements were performed using the Photon Technology International RF-M2004 Luminescence Spectrometer. The Felix software was used to run all experiments and collect data. The slit widths of the fluorimeter were set to 0.38 mm which corresponded to a 1 nm band width. For each experiment, an appropriate excitation wavelength was chosen based on the absorption spectrum of where the guest demonstrated absorbance, but the host did not. The temperature was controlled for all samples at 25°C except for experiments that are discussed in Chapter 5 involving a thermodynamic study in which temperature was manipulated.

Fluorescence spectra were collected by first pipetting 3.0 mL of a prepared solution into a 1 cm^2 quartz cuvette using a glass pipette. The cuvette was then cleaned of fingerprints and residues using a dampened Kimwipe with deionized water, followed by a dry Kimwipe to get rid of excess moisture on the outside of the cuvette. The cuvette was then loaded into the sample holder of the fluorimeter and the sample chamber was closed, blocking out any room light that would affect the experimental measurements. The scan was then performed through a range of wavelengths reaching no more than twice the excitation wavelength. The fluorescence spectrum of fluorescence intensity versus wavelength of emission was then collected. This procedure was followed for all fluorescence enhancement, suppression, and titration experiments. Fluorescence titrations were performed on the condition that enhancement or suppression was seen as measured by the resulting F/F_0 value obtained using Equation 1.4. Enhancement is characterized as

having a F/F_0 value greater than 1, while suppression is characterized as having a F/F_0 value less than 1.

2.5 Fluorescence Titrations using SCX4 and SCX6

Fluorescence titrations were conducted using riboflavin (vitamin B2) as the guest. From initial fluorescence experiments using riboflavin, para-sulfonatocalix[4]arene (SCX4) and para-sulfonatocalix[6]arene (SCX6) separately, it was determined that the resulting effect of their host-guest combination was suppression. The maximum concentration of SCX4 and SCX6 used was 15.0 mM. This maximum concentration was decided upon by evaluating the fluorescence spectrum of 15.0 mM SCX4 and 15.0 mM SCX6 with riboflavin and determining that this concentration resulted in a plateau of the fluorescence in comparison to the guest solution in absence of calixarene. Host-guest solutions of riboflavin and the appropriate calixarene were prepared at concentrations of 1.0 mM, 3.0 mM, 5.0 mM, 7.5 mM, 10.0 mM, 12.5 mM, and 15.0 mM. For each titration, a water blank was measured, as well as a guest solution blank in absence of host.

From the resulting emission spectra, the integration function of the Felix software was used to determine total integrated areas. The total integrated areas were used to determine F/F_0 values that are used to characterize the enhancement or suppression of the fluorescence once the guest molecule is encapsulated in the host. In order to get true F and F_0 values, the total integrated area of the fluorescence of the solvent; which in this case is water, was subtracted from the F and F_0 values to account for any fluorescence that is emitted by the solvent. The binding constant was then calculated by inputting the F/F_0

values for each host concentration used into the CDEQWIN (Cyclodextrin Equilibrium Constant for Windows) computer program. This program is an in-house program that uses the non-linear least squares fitting method to fit 1:1 or 2:1 host-guest inclusion complexes. Although denoted in its name as a cyclodextrin equilibrium constant calculator, it may be used for other hosts such as calixarenes and cucurbiturils that are prevalent in this work.

2.6 Fluorescence Titrations using CB7

Fluorescence titrations using cucurbit[[7]uril were carried out using riboflavin as the guest. An initial fluorescence experiment using riboflavin and CB7 confirmed that the effect of CB7 on riboflavin was a suppression of its fluorescence. The titrations were carried out by starting at a maximum concentration of 1.0 mM CB7 and diluting with appropriate aliquots of guest solution to obtain CB7 concentrations of 0.8 mM, 0.6 mM, 0.4 mM, 0.2 mM, 0.1 mM, and 0.05 mM as the lowest concentration. This method was chosen as CB7 is one of the more expensive supramolecular hosts and this procedure would conserve as much host material as possible and use it efficiently. Along with the host-guest solutions, the emission spectrum for a water blank and a guest solution blank were obtained for each titration. Similar to the titrations performed with SCX4 and SCX6 as outlined above, the total integrated areas for each solution of desired CB7 concentration were used to determine the F/F_0 values which were then inputted into CDEQWIN to determine the binding constant for each of the trials conducted.

2.7 Thermodynamic Study using Fluorescence Titrations

A thermodynamic study of the host-guest inclusion of riboflavin in SCX4 was carried out through the use of fluorescence titrations. The same procedure as described in Section 2.5 was carried out to perform this series of titrations with the only difference being the manipulation of the temperature. For this thermodynamic study, fluorescence titrations were performed at 7, 15, 25, 35, 45, and 55°C in triplicate at each temperature. Upon loading the cuvette with the sample into the sample holder of the fluorimeter, the sample was left to equilibrate to temperature for 10 minutes prior to beginning the scan for each sample. The sample's temperature was measured using a digital thermometer and the scan was started once the temperature was read to be within 0.1°C of the temperature that had been set for that experiment. After the three titration trials were performed at each temperature, a van't Hoff plot was created. From this, the enthalpy and entropy of the inclusion of riboflavin in SCX4 was analyzed.

Chapter 3: Preliminary Studies with Various Vitamins in Various Hosts

Preliminary fluorescence studies were performed with Vitamins B2, B3, B12, and C (see Table 2.1 for chemical structures) in varying hosts to determine whether fluorescence enhancement, suppression, or any effect would be seen to indicate the formation of a host-guest inclusion complex. These guests were chosen due to a personal interest in pharmaceuticals and the lack of literature surrounding the inclusion of vitamins in supramolecular hosts. The solvent chosen for these studies was water, as each of the chosen vitamins are water-soluble and the effect of solvent on the formation of an inclusion complex was not one of the main priorities of this research

3.1 Preliminary Studies of Vitamin B3, B12, and C in HP-β-CD

The first guest of interest in these fluorescence studies was vitamin B3. The chosen host was HP-β-CD as its properties as a supramolecular host are well reported in the literature and solubility issues were not a concern. The emission spectrum for vitamin B3 with 10.0 mM HP-β-CD in water is shown in Figure 3.1.

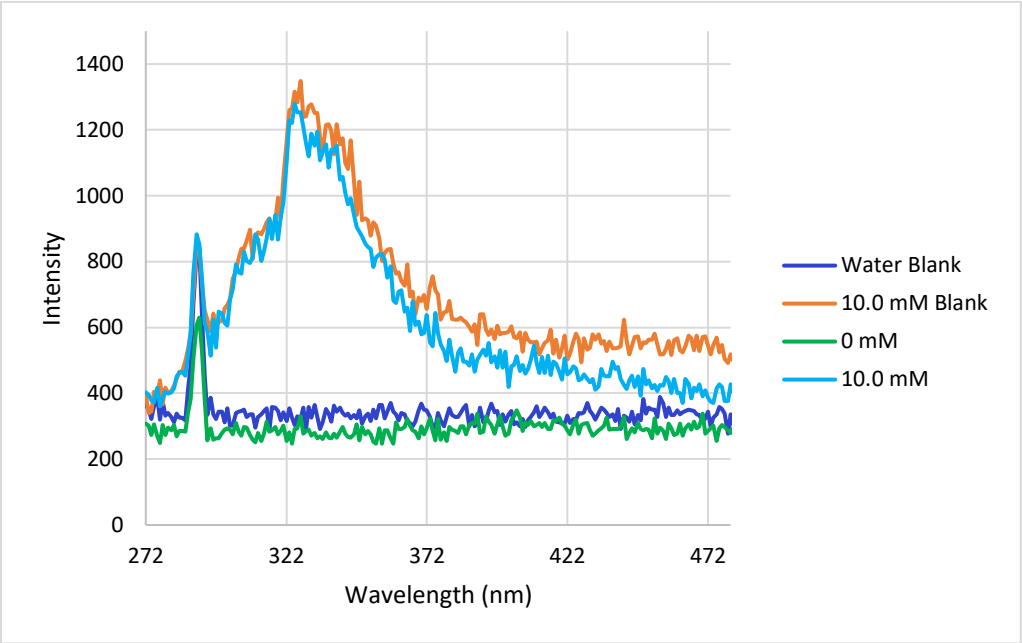


Figure 3.1: Emission spectrum of vitamin B3 with and without 10.0 mM HP-β-CD.

Fluorescence of vitamin B3 was not observed in this preliminary fluorescence experiment. As seen above, the vitamin B3 scan overlaps closely with that of the water blank. Water is known to be a non-fluorescent solvent. Since no fluorescence effect was observed, vitamin B3 was abandoned as a guest for further experimentation as all binding studies were to be carried out through fluorescence measurements. Similar results were observed from preliminary studies using vitamin B12 and C as guests with 10.0 mM HP-β-CD. These two vitamins did not demonstrate fluorescence and the addition of the cyclodextrin host did not affect the fluorescence of these molecules therefore, vitamin B12 and C were abandoned for further fluorescence experimentation. Vitamin B2 was then chosen as the next guest of interest and showed fluorescence both with and without the

presence of host. Vitamin B2 showed varying effects with different hosts and these effects are further discussed in Sections 3.2 and 3.3.

3.2 Preliminary Studies of Vitamin B2 with Cyclodextrins

Vitamin B2 showed strong fluorescence in comparison to the previous three vitamins that were discussed. Because of this, vitamin B2 was first combined with HP-β-CD, β-CD, and HP-γ-CD to determine if there was fluorescence enhancement or suppression of vitamin B2 as a result of potential complexation with these different cyclodextrins. These hosts were chosen primarily because of their favourable water solubility. The emission spectrum of vitamin B2 with 10.0 mM HP-β-CD is shown in Figure 3.2.

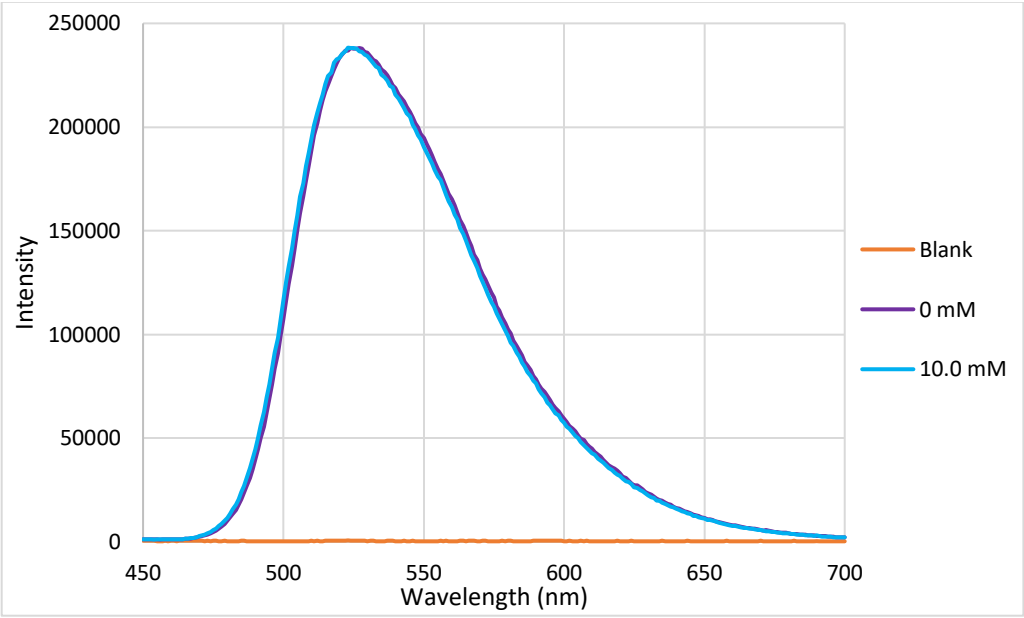


Figure 3.2: Emission spectrum of vitamin B2 with and without 10.0 mM HP-β-CD.

As shown above, the 10.0 mM HP-β-CD did not have an effect on the fluorescence of vitamin B2. The F/F_0 value was determined to be 1.00 because the fluorescence spectrum of the guest in the presence and absence of the cyclodextrin host was identical. As a result, a fluorescence titration of vitamin B2 with HP-β-CD was not conducted because there was no effect on the fluorescence of the guest in the presence of the host. Similar results were gathered for preliminary fluorescence measurements involving the use of 8.0 mM β-CD in an attempt to reproduce results obtained by Mukherjee *et al.* that reported vitamin B2 and β-CD inclusion complexes in 1:1 and 1:2 stoichiometries using fluorescence titrations.[29] The F/F_0 value from initial fluorescence measurements with vitamin B2 and β-CD was 1.09. This was indicative of only a 9% increase in fluorescence of vitamin B2 in the presence of β-CD which was not a great enough effect to proceed with fluorescence titrations and the complexation reported by Mukherjee *et al.* could not be reproduced. Unfortunately, it was unknown why these reported results were not reproducible and further investigation is required to understand this outcome. A preliminary experiment with vitamin B2 and 10.0mM HP-γ-CD was also performed which resulted in an F/F_0 value of 1.08 which again was not a great enough effect to proceed with fluorescence titrations and binding studies. These three cyclodextrin hosts all showed either no effect on fluorescence or enhancement of less than a 10% change in the fluorescence of vitamin B2 upon addition therefore, fluorescence titrations were not performed and the use of cyclodextrins were eliminated from future experimentation.

3.3 Preliminary Studies of Vitamin B2 with Calixarenes and Cucurbiturils

Aside from cyclodextrins, two other types of supramolecular hosts were explored during preliminary fluorescence measurements involving vitamin B2; calixarenes and cucurbiturils. Two water soluble calixarenes were chosen; SCX4 and SCX6, as well as one cucurbituril; CB7. These hosts were chosen based on their water solubility and approximation that their cavity size could be potentially compatible in encapsulating a molecule of riboflavin. The emission spectra of vitamin B2 with 5.0 mM SCX6, 5.0 mM SCX4, and 1.0 mM CB7 can be found in Figures 3.3-3.5. CB7 is one of the more expensive supramolecular hosts and for that reason a lower starting concentration was chosen in comparison to the calixarenes to ensure that as little material was used as possible while still being able to observe and quantify its effect on the fluorescence of vitamin B2.

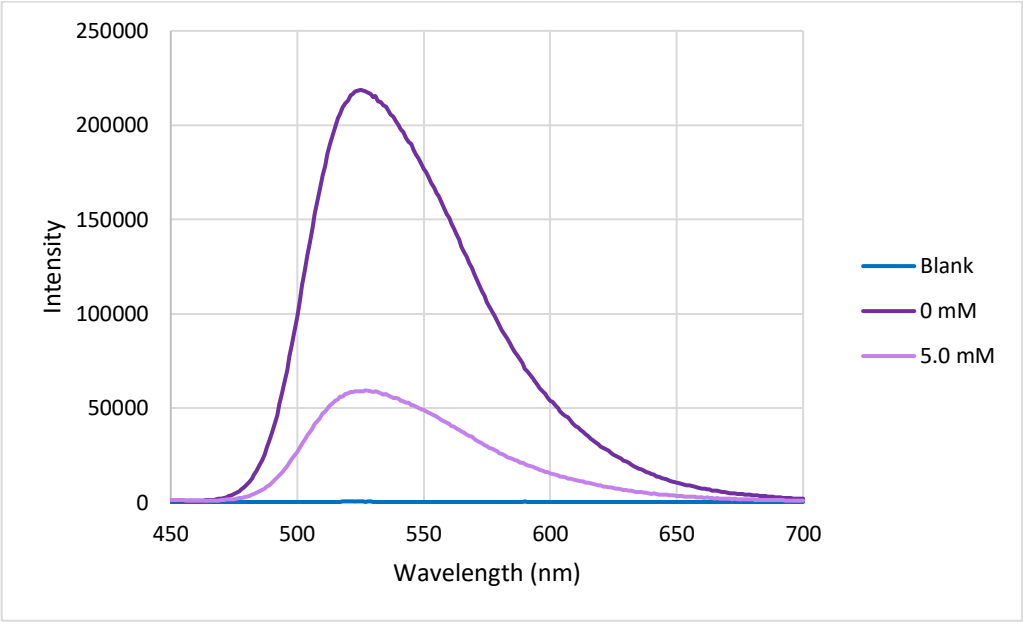


Figure 3.3: Emission spectrum of vitamin B2 with and without 5.0 mM SCX6.

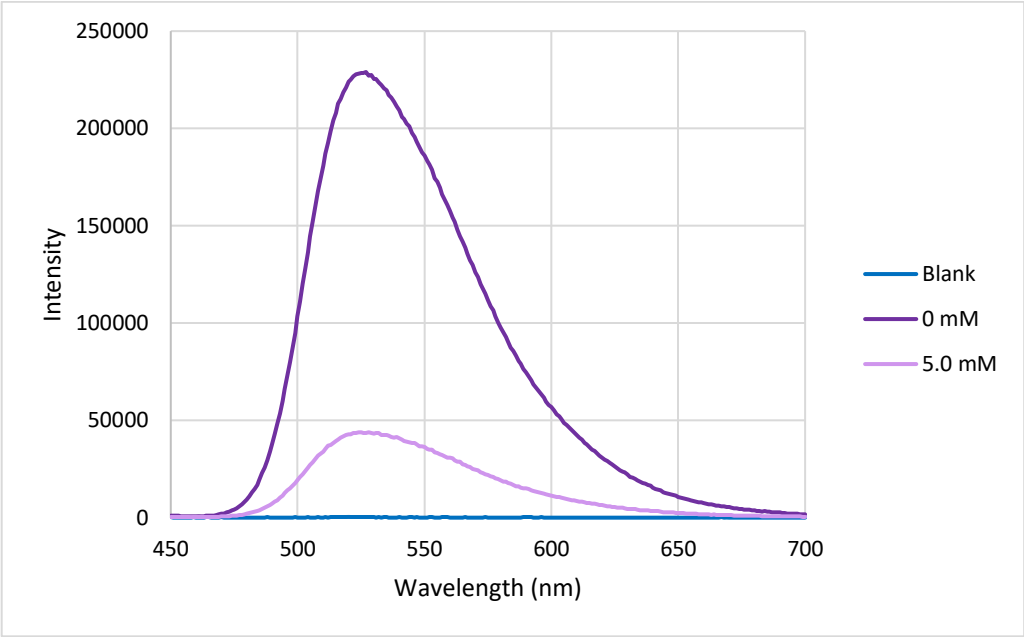


Figure 3.4: Emission spectrum of vitamin B2 with and without 5.0 mM SCX4.

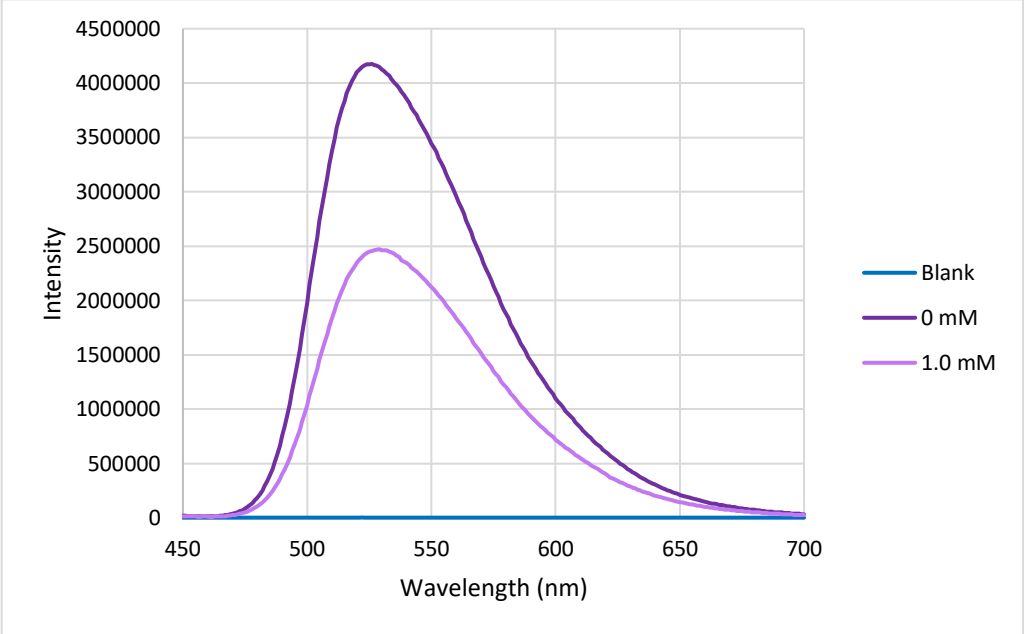


Figure 3.5: Emission spectrum of vitamin B2 with and without 1.0 mM CB7.

Upon addition of SCX6, SCX4, and CB7 to the vitamin B2 guest solution, significant fluorescence suppression was observed. F/F_0 values of these initial suppression experiments were 0.28, 0.19, and 0.60 respectively. These values were an indication that inclusion of vitamin B2 in these three hosts was occurring. This was a significant result compared to the negligible effect observed with the cyclodextrin hosts that were chosen for preliminary fluorescence experiments, motivating the inclusion in this work of the exploration of hosts belonging to families other than cyclodextrins. The two water-soluble calixarenes demonstrated a 70 to 80% decrease in the fluorescence of vitamin B2 while CB7 demonstrated a 40% decrease. This was a major motivation to continue with fluorescence titrations and binding studies using these three different hosts and justified the abandonment of cyclodextrins which demonstrated no effect. The calixarene hosts have π systems that are capable of interacting with the aromatic vitamin B2 guest molecule through $\pi - \pi$ interactions resulting in the favourable intermolecular interaction for inclusion over the cyclodextrin hosts that are incapable of this non-covalent interaction due to the lack of a π system in their structure. Cucurbituril hosts have carbonyl rims and a highly positive internal cavity which also may provide favourable interaction conditions with the vitamin B2 molecule. These additional intermolecular interactions experienced by calixarenes and cucurbiturils with vitamin B2 over cyclodextrins are a potential explanation for the fluorescence effect seen with these alternative hosts and not with cyclodextrins. It is not clear at this point why these hosts cause suppression of the vitamin B2 fluorescence; this will require further examination in future work. These intermolecular

interactions as well as the fluorescence titrations and binding studies of vitamin B2 with SCX6 and SCX4 are further discussed in Chapter 4 and CB7 in Chapter 6.

Chapter 4: Binding Studies of B2 in Calix[n]arenes

As described in Chapter 3, upon initial exploration in pairing vitamin B2 with two para-sulfonated calixarenes, strong fluorescence suppression was observed. This was an indication of the occurrence of complexation between vitamin B2 and the two para-sulfonated calixarenes. Following these initial experiments, fluorescence titrations were performed in order to determine the binding constants of this complexation.

4.1 Binding Studies of Vitamin B2 in SCX6

The first para-sulfonated calixarene that was chosen to conduct a fluorescence titration of vitamin B2 was SCX6 as a result of the initial fluorescence suppression discussed in Section 3.4. Fluorescence titrations were carried out following the method that was outlined in Section 2.5 and were performed in triplicate. For these fluorescence titrations the concentration of host ranged from 0 mM to 15.0 mM. Based on the absorption spectrum, the excitation wavelength was set to 440 nm. The emission spectra of a fluorescence titration of vitamin B2 with SCX6 in a representative trial are shown in Figure 4.1.

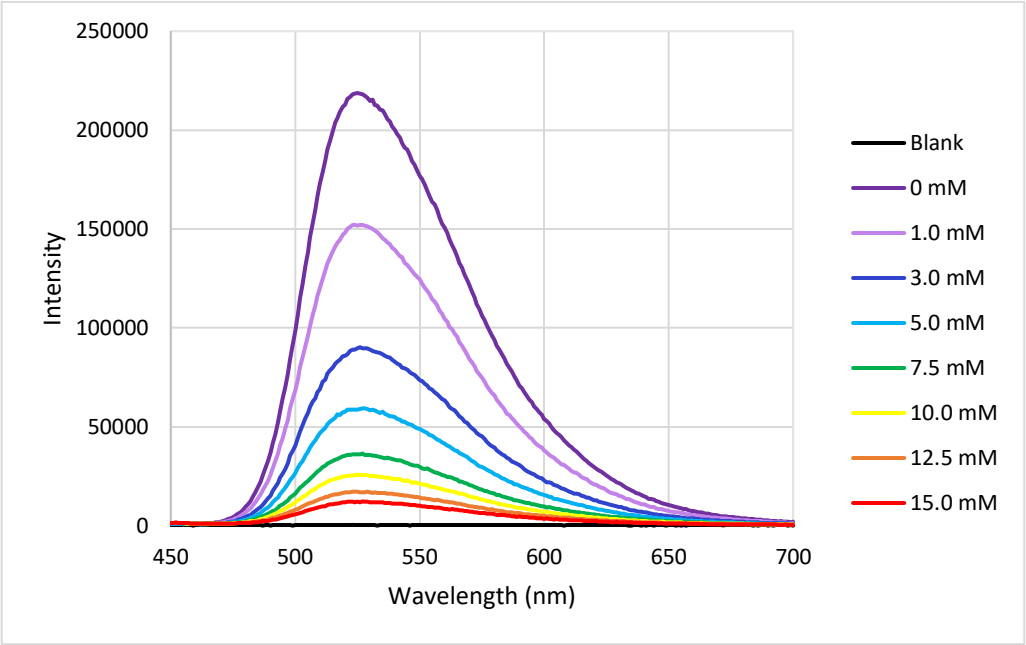


Figure 4.1: Fluorescence titration of vitamin B2 with SCX6.

As seen in the fluorescence titration above, significant suppression of the fluorescence of vitamin B2 was observed which was dependent on the concentration of the host. The maximum concentration, 15.0 mM, was chosen as this concentration was where a significant plateau in the fluorescence of vitamin B2 was seen; indicating the approach to the maximum suppression in the fluorescence of the guest as a result of all free guest molecules being encapsulated by a host molecule. Using the total integrated areas from the emission spectrum, F/F_0 values were calculated using the method described in Section 2.5. The plot of F/F_0 versus the concentration of SCX6 is shown in Figure 4.2.

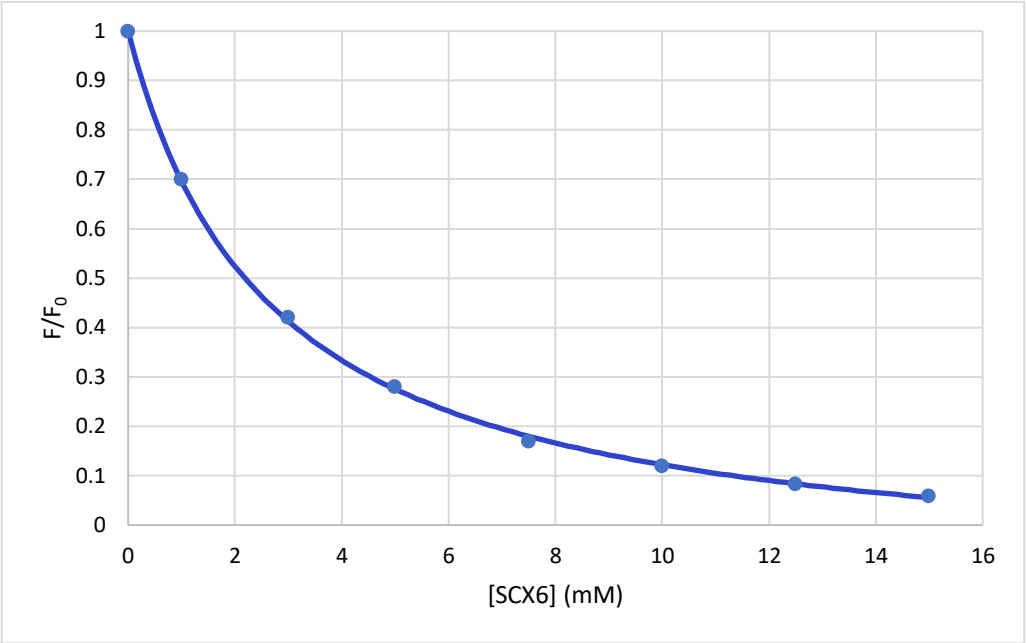


Figure 4.2: F/F_0 versus the concentration of SCX6 with vitamin B2.

In the event that fluorescence enhancement was seen upon addition of SCX6 to vitamin B2, a double reciprocal plot of F/F_0-1 versus concentration of SCX6 would have been used to determine the ratio of host:guest complexation occurring. The double reciprocal plot however cannot be used for this purpose in the case of fluorescence suppression. In the case of a double reciprocal plot, a linear trend would prove that the type of binding occurring would be classified as 1:1.[21] However, as seen in Figure 4.2, the data was well fit to the curve allowing for the assumption that the SCX6:vitamin B2 complex has 1:1 stoichiometry. If the data did not show a good fit, it would have been assumed that the stoichiometry was different from 1:1 requiring further investigation. Binding studies were performed using CDEQWIN to determine the binding constant, K, for the three titration trials. The binding constant, K, obtained for the three trials are listed below in

Table 4.1. The average binding constant for the inclusion of vitamin B2 in SCX6 was determined to be 399 ± 30 M^{-1}.

Table 4.1: Binding constants obtained for trials 1-3 of vitamin B2 in SCX6.

Trial	K (M^{-1})
1	374
2	383
3	442

As discussed briefly as the end of Chapter 3, the encapsulation of vitamin B2 in SCX6 was believed to be successful over encapsulation in cyclodextrins due to the π - π stacking interactions between the aromatic vitamin B2 molecule and the aromatic phenolic units of SCX6. This is an additional driving force for inclusion that is not experienced with cyclodextrins. The inclusion of guest molecules in the cavity of cyclodextrins is largely driven by the hydrophobic effect and hydrogen bonding. The ability for other supramolecular hosts, such as water-soluble calixarenes, to participate in the same, as well as additional, non-covalent interactions that cyclodextrins cannot, increases their likelihood for successful encapsulation of the guest. In the case of calixarenes, their structures are made up of repeating aromatic phenolic units with π-electrons capable of participating in $\pi - \pi$ stacking interactions with other aromatic guests, such as vitamin B2. Cyclodextrins do not have this ability because their structures are not aromatic. This was an advantage of calixarene hosts over cyclodextrins; not only having hydrophobic cavities but also their

ability to participate in another form of intermolecular interaction to further drive the encapsulation of the vitamin B2.

Vitamin B2 is known to be excreted from the human body in urine at high concentrations.[29] Around 60% of a single dose of vitamin B2 is not absorbed by the body and for this reason the confirmation of the complexation of vitamin B2 with SCX6 was a significant finding. Although it could not be encapsulated in cyclodextrins, if approved by the FDA or another health agency for human ingestion, SCX6 could have a great importance for drug developers who are looking to formulate vitamin B2 tablets or capsules that would have a greater bioavailability than 40%. This was an important finding that highlighted the benefits of calixarenes as hosts over cyclodextrins and why other options for supramolecular hosts should be approved for use in pharmaceutics. Although cyclodextrins have great qualities that have been previously outlined such as high water-solubility, low cost and low toxicity, they are not capable of successfully encapsulating all drug molecules.

4.2 Binding Studies of Vitamin B2 in SCX4

Following the binding studies of vitamin B2 and SCX6, the next host of interest was SCX4. As mentioned, following preliminary fluorescence measurements involving vitamin B2 and SCX4, fluorescence suppression was observed as a result of the complexation of vitamin B2 in SCX4. Fluorescence titrations were then carried out following the method outlined in Section 2.5. The range of concentrations of SCX4 that was examined was 0 mM to 15.0 mM, similar to the titrations involving SCX6. This range

of concentrations was chosen as these two para-sulfonated calixarene hosts exhibit similar solubility properties and were expected to have similar fluorescence suppression effects on vitamin B2. The excitation wavelength was maintained at 440 nm based on the absorption spectrum of vitamin B2. The emission spectra of a fluorescence titration of vitamin B2 with SCX4 in a representative trial is shown in Figure 4.3.

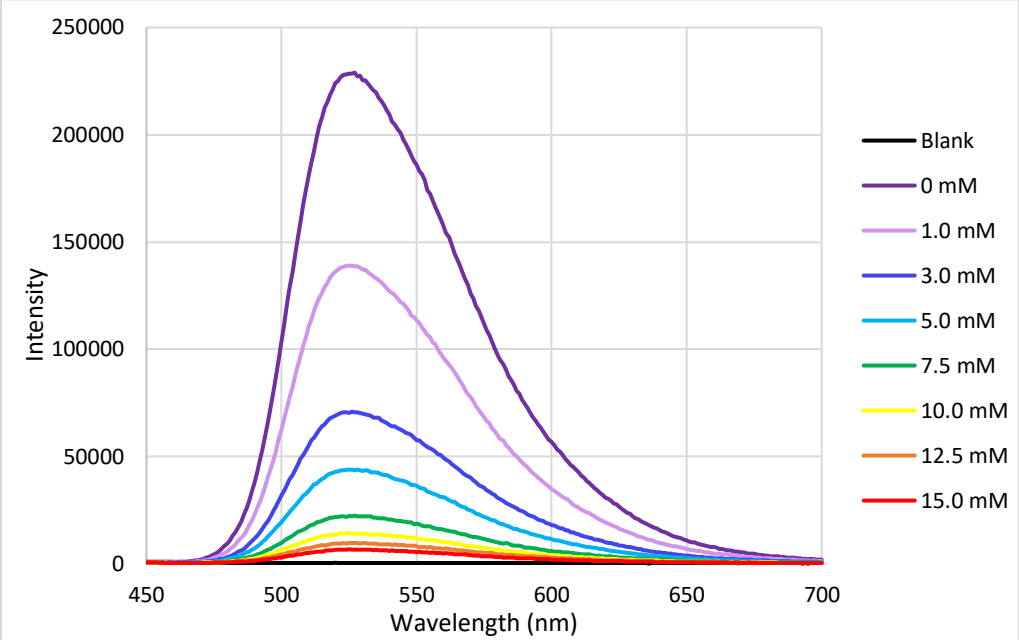


Figure 4.3: Fluorescence titration of vitamin B2 with SCX4.

As seen above, significant suppression in the fluorescence of vitamin B2 occurred upon increase in the concentration of SCX4 added. Similar to the fluorescence titrations involving the use of SCX6 as the host, the maximum concentration of SCX4 was held at 15.0 mM as this was the concentration where a plateau in the fluorescence of vitamin B2 was observed; indicating maximum fluorescence suppression and encapsulation of all free

guest molecules by a host molecule. Using the total integrated areas extracted from the emission spectrum, F/F_0 values were calculated. The plot of the F/F_0 values versus the concentration of SCX4 is shown in Figure 4.4.

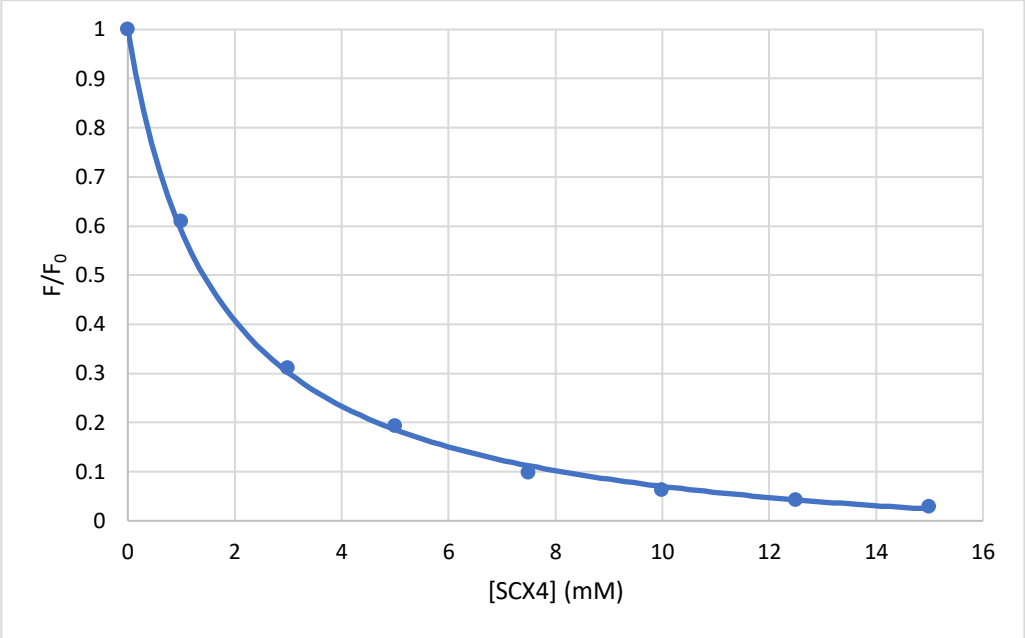


Figure 4.4: F/F_0 versus the concentration of SCX4 with vitamin B2.

In the comparison of Figure 4.2 and 4.4, it can be seen that similar plots were obtained for SCX6 and SCX4 however, Figure 4.4 demonstrates a steeper decrease in the F/F_0 value as the concentration of SCX4 is increased. This is indicative that stronger binding is occurring between vitamin B2 and SCX4 than with SCX6. To confirm this, binding studies were performed using the CDEQWIN software to obtain binding constants for the three trials. Similar to the case of SCX6, the data seen in Figure 4.4 is well fit to the curve, indicating 1:1 binding occurring between the host and guest. The resulting binding

constants of the three trials are displayed in Table 4.2 below. Similar $\pi - \pi$ stacking interactions were believed to be a driving force in the inclusion of vitamin B2 in SCX4 as seen with SCX6. The average binding constant for the inclusion of vitamin B2 in SCX4 was determined to be 638 ± 24 M^{-1}. From this it was determined that vitamin B2 was experiencing stronger binding with SCX4 than SCX6.

Upon first glance of the chemical structure of vitamin B2 (shown in Table 2.1), it was noted that its aromatic structure consists of three rings and a five-carbon side chain with four hydroxyl groups. There was no definite prediction that could be made as to whether the four-membered or six-membered para-sulfonated calixarene would be more accommodating of this structure. Upon completion of the binding studies, it was clear from the greater magnitude of the SCX4 binding constant that the cavity size of SCX4 was more accommodating for the aromatic riboflavin structure and stronger interactions occurred between vitamin B2 and SCX4 over SCX6. In this case, the smaller cavity, and different cavity shape of the four-membered para-sulfonated calixarene must provide a better size and shape match to that of the riboflavin guest molecule. For example, $\pi - \pi$ interactions require close contact between the host and guest, and a more snug fit in a tighter cavity would provide this environment. Also, six-membered calixarenes may not provide as well a defined cavity due to higher "floppiness".

Table 4.2: Binding constants obtained for trials 1-3 of vitamin B2 with SCX4.

Trial	K (M^{-1})
1	604.3
2	647.0
3	661.5

As mentioned in Section 2.5, fluorescence titrations with SCX4 were carried out at a constant temperature of 25℃. After completion of the triplicate binding studies outlined above, further fluorescence titrations and binding studies were performed at five different temperatures to assess the effect of temperature on the binding constant of vitamin B2 with SCX4. SCX4 was chosen as the host for this thermodynamic study because its binding constant with vitamin B2 had a greater magnitude over that of SCX6 therefore it only made sense to choose SCX4 to conduct the thermodynamic study. Information about the enthalpy and entropy were extrapolated from the fluorescence titrations and binding studies through the use of a van't Hoff plot. The results of this thermodynamic study are discussed thoroughly in Chapter 5.

Chapter 5: Thermodynamic Study of Vitamin B2 in SCX4

Upon completion of binding studies of vitamin B2 with SCX4 at room temperature, the effect of temperature on the binding constant was further explored. A series of temperatures both above and below 25°C were chosen to obtain a picture of the potential effect of temperature on the binding constant. The temperature range was from 7 to 55°C and was chosen based on the solvent properties. Water of course having a boiling point of 100°C and a melting point of 0°C, it was important to choose temperatures within this range to eliminate the chance of evaporation of solvent, manipulating the concentration of the sample, or formation of condensation on the sides of the cuvette. Triplicate series of fluorescence titrations were performed at temperatures of 7, 15, 35, 45, and 55°C. All fluorescence titrations were performed following the methods outlined in Section 2.7. The excitation wavelength remained at 440 nm for each of the titrations as determined through the absorption spectrum of vitamin B2 and the concentrations of SCX4 remained the same as those used in Section 4.2. The effect of varying the temperature on the binding behavior of vitamin B2 and SCX4 are fully discussed in future subsections.

5.1 Binding Studies of Vitamin B2 in SCX4 at 7°C

The first temperature of interest for the thermodynamic study was 7°C. This was the lowest temperature chosen to conduct titrations to avoid the possibility of condensation forming on the outside of the cuvette, which would increase the room for error in the

experiments. The emission spectrum of a fluorescence titration of vitamin B2 with SCX4 from a representative trial at 7°C is shown in Figure 5.1.

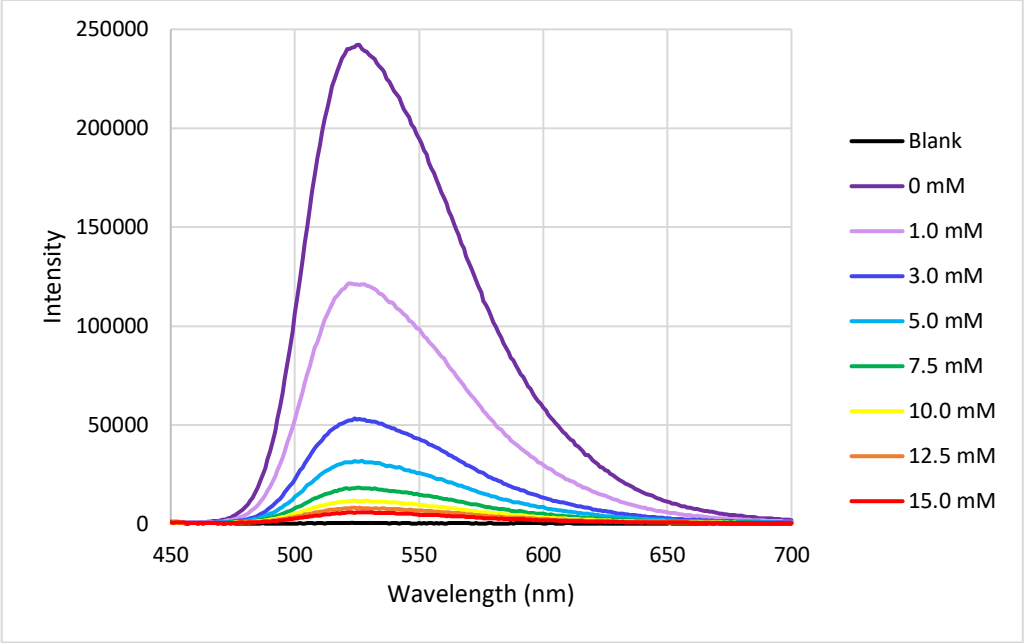


Figure 5.1: Fluorescence titration of vitamin B2 with SCX4 at 7°C.

In comparison to the emission spectra of fluorescence titrations performed at room temperature in Section 4.2, there are slight differences in some of the intensities of the samples at this lower temperature. The sample of the guest in the absence of host (0 mM) has a higher fluorescence intensity at 7°C than at 25°C partially due to the fact that different host-guest solutions were prepared for titrations performed at 7°C than from 25°C and the absorbances of the vitamins would have been slightly varied but within the 0.28-0.33 threshold. This may also be an indication that the fluorescence of riboflavin is temperature dependent. There are also significant changes in the samples containing 1.0 and 3.0 mM

SCX4. Their fluorescence intensities are lower at this lower temperature, indicating a greater suppression of the fluorescence at increasing concentrations of host. From this, it was predicted that as the temperature is decreased, the binding constant will increase. This hypothesis was confirmed by using the total integrated areas of the emission spectrum to determine the F/F_0 values. These values were plotted against the concentration of SCX4 and shown in Figure 5.2.

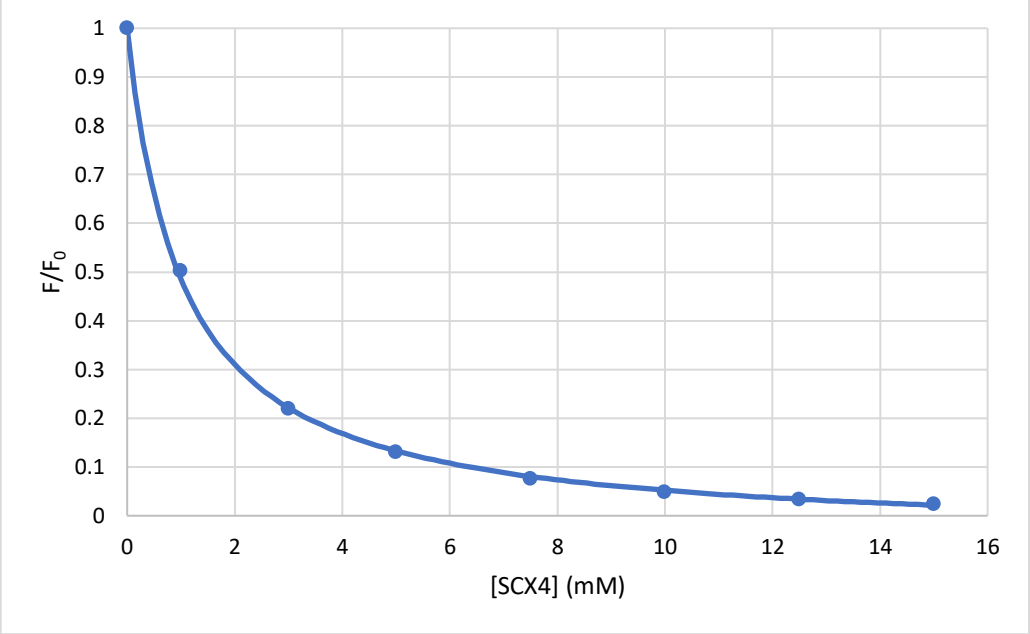


Figure 5.2: F/F_0 versus the concentration of SCX4 at 7°C.

Upon comparing Figure 5.2 with Figure 4.4, a difference in the steepness of the decrease of the curve was noted. The curve in Figure 5.2 is significantly steeper than the one pertaining to the fluorescence titrations performed at 25°C. This was an indication that the binding constant between vitamin B2 and SCX4 for titrations at this lower temperature

would be higher; agreeing with the prediction that was made. To confirm this, the binding constants of the three trials were calculated using the CDEQWIN program. The host to guest ratio was also confirmed to be 1:1 and unchanged as a result of the lower temperature. The relevant binding constants of the three titration trials are shown in Table 5.1. The average binding constant for the inclusion of vitamin B2 in SCX4 at 7°C was determined to be 904 $\pm$ 50 M^{-1}. This binding constant of the inclusion of vitamin B2 in SCX4 at 7°C was higher than that of the average binding constant of the fluorescence titrations performed at 25°C. A higher binding constant at this lower temperature was indication that the strength of the non-covalent interactions between the host and guest were dependent on temperature.

Table 5.1: Binding constants obtained from trials 1-3 of vitamin B2 with SCX4 at 7°C.

Trial	K (M^{-1})
1	854
2	885
3	971

5.2 Binding Studies of Vitamin B2 in SCX4 at 15°C

The next temperature of interest to be analyzed was 15°C. The emission spectrum of a fluorescence titration of vitamin B2 with SCX4 from a representative trial at 15°C is shown in Figure 5.3.

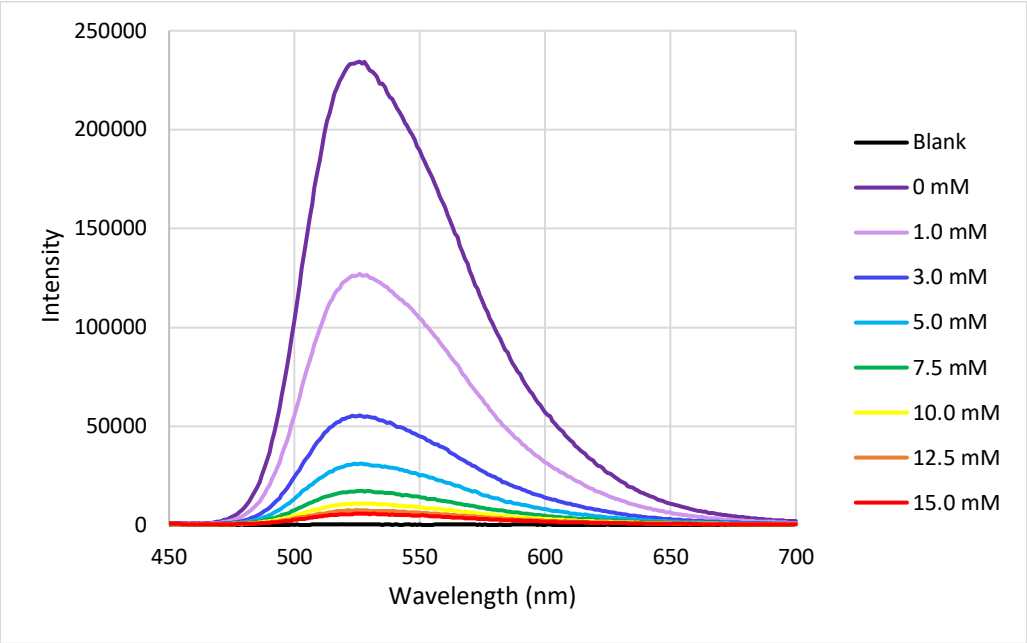


Figure 5.3: Fluorescence titration of vitamin B2 with SCX4 at 15°C.

The emission spectrum of the fluorescence titration at 15°C closely resembles that shown in Figure 5.1 of the 7°C fluorescence titration. With the slight increase in the temperature from 7°C to 15°C, it was expected that the binding constant would decrease to remain consistent with the prediction that was made; when the temperature is increased, the binding constant is decreased. The total integrated areas of the emission spectrum from Figure 5.3 were used to calculate F/F_0 values to conduct further binding studies to determine the binding constant for these titrations at 15°C. The plot of F/F_0 versus the concentration of SCX4 is shown in Figure 5.4.

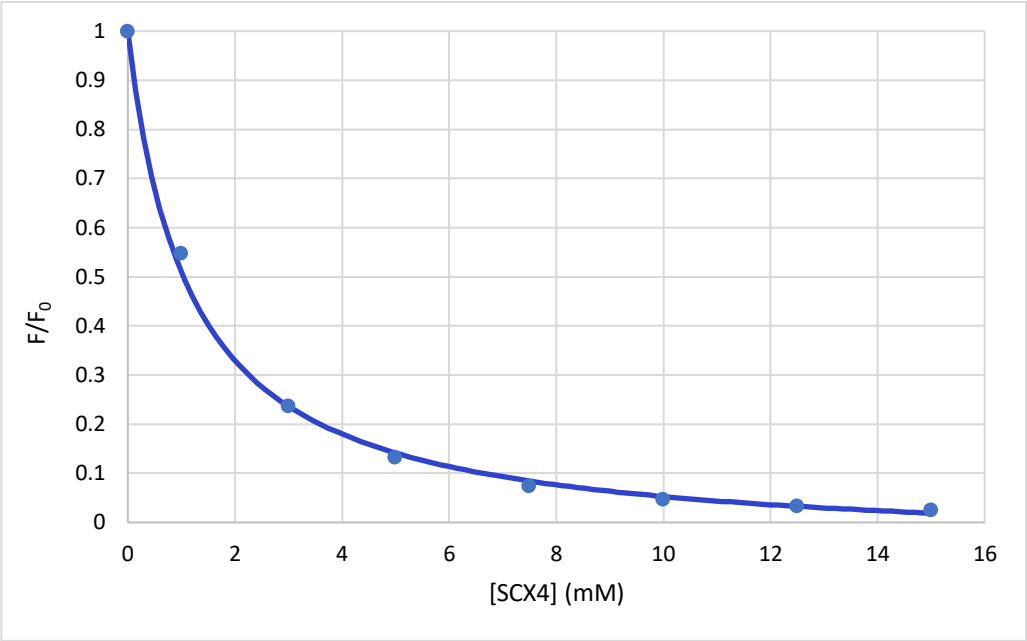


Figure 5.4: F/F_0 versus the concentration of SCX4 at 15°C.

In comparing Figures 5.2 and 5.4, there are sharper decreases in the F/F_0 values at the lower concentrations of SCX4 than seen with the titrations at higher temperatures. This indicates that the binding of vitamin B2 with SCX4 is stronger at lower temperatures than higher temperatures. The binding constants for the three trials at 15℃ were calculated using CDEQWIN and are displayed in Table 5.2. The average binding constant was determined to be $861 \pm 8\ M^{-1}$. The binding constants of these three trials were in high agreement with each other and demonstrated promising reproducibility at this temperature. This was favourable in considering the future for the development of vitamin B2 formulations containing SCX4 as the complex demonstrated strong binding with exceptional reproducibility which would be two factors of great importance to drug developers.

Table 5.2: Binding constants obtained from trials 1-3 of vitamin B2 with SCX4 at 15°C.

Trial	K (M^{-1})
1	870
2	851
3	860

5.3 Binding Studies of Vitamin B2 in SCX4 at 35°C

The next temperature at which fluorescence titrations were performed for this thermodynamic study was 35°C. The emission spectrum of a fluorescence titration of vitamin B2 with SCX4 performed at 35°C from a representative trial is shown below in Figure 5.5.

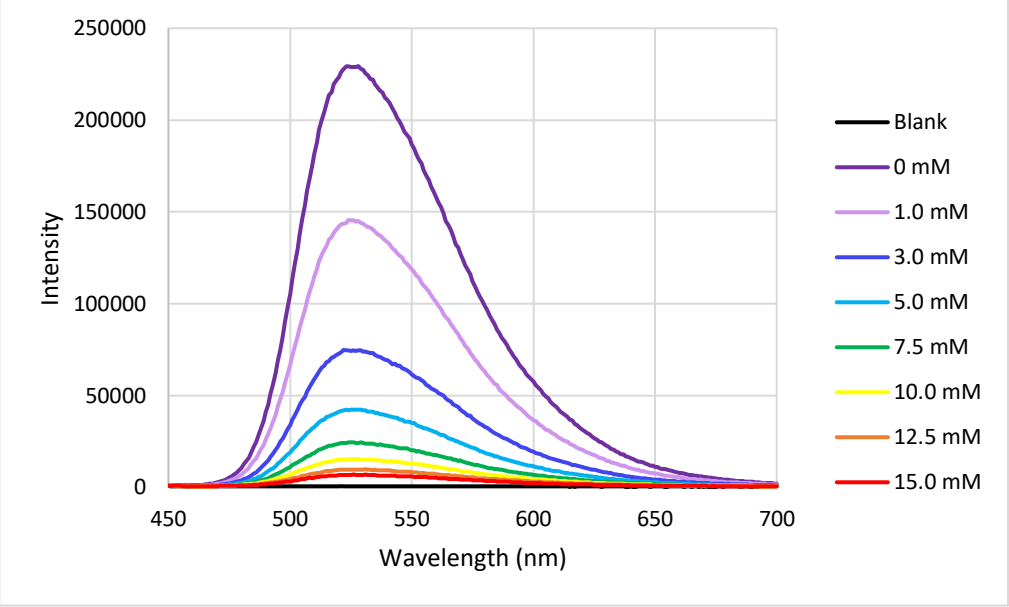


Figure 5.5: Fluorescence titration of vitamin B2 with SCX4 at 35°C.

As seen above, a similar plateau in the fluorescence at the maximum concentration of SCX4 occurred for the fluorescence titration performed at 35°C as well as those performed at 7 and 15°C in comparison to the preliminary experiment described in Section 4.2 that was performed at room temperature. This was an indication that whether the temperature is decreased or increased, the overall observation of fluorescence suppression of vitamin B2 is maintained however varies slightly as shown in the resulting binding constant. The total integrated areas of the emission spectra were used to calculate the F/F_0 values of each concentration of SCX4 used. The plot of F/F_0 versus the concentration of SCX4 is shown in Figure 5.6 below.

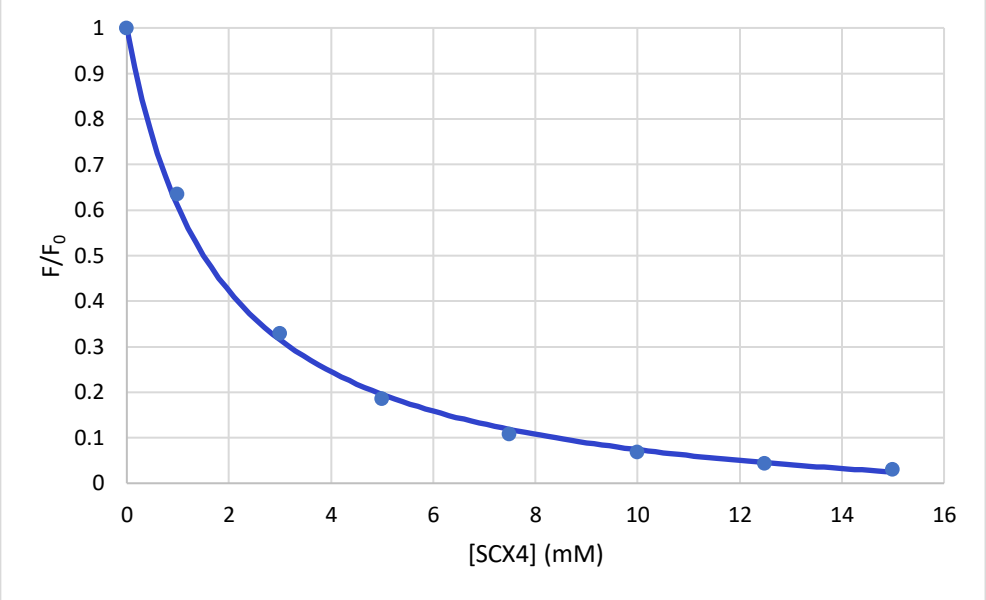


Figure 5.6: F/F_0 versus the concentration of SCX4 with vitamin B2 at 35°C.

From the figure above, the type of binding between the host and guest was confirmed to be 1:1 because the data was well fit to the curve. Conclusions about the

strength of the inclusion of vitamin B2 in SCX4 at 35°C were also made through the use of this figure. The curve shown in Figure 5.6 is not as steep as the curve shown in Figure 4.4, such that it was determined that the binding between the vitamin B2 and SCX4 at this elevated temperature was weaker. This was confirmed using the CDEQWIN program to determine the binding constants from the three trials at this temperature. The resulting binding constants from the three trials are listed in Table 5.3. The average binding constant was determined to be 553 ± 22 M^{-1}. As predicted from Figure 5.6, this binding constant was indeed lower than the fluorescence titrations conducted at all temperatures previously discussed. Again, the binding constants of the three trials were in agreement with each other and the increase in temperature did not appear to cause difficulty with reproducibility. This concluded that whether the temperature is increased or decreased, the strength of the binding can vary but the overall ability to reproduce the complexation is not an obstacle.

Table 5.3: Binding constants obtained from trials 1-3 of vitamin B2 with SCX4 at 35°C.

Trial	**K (M^{-1})**
1	564
2	573
3	522

5.4 Binding Studies of Vitamin B2 in SCX4 at 45°C

The next temperature of interest was 45℃. The emission spectrum of a fluorescence titration of vitamin B2 with SCX4 of a representative trial performed at 45°C is shown in Figure 5.7.

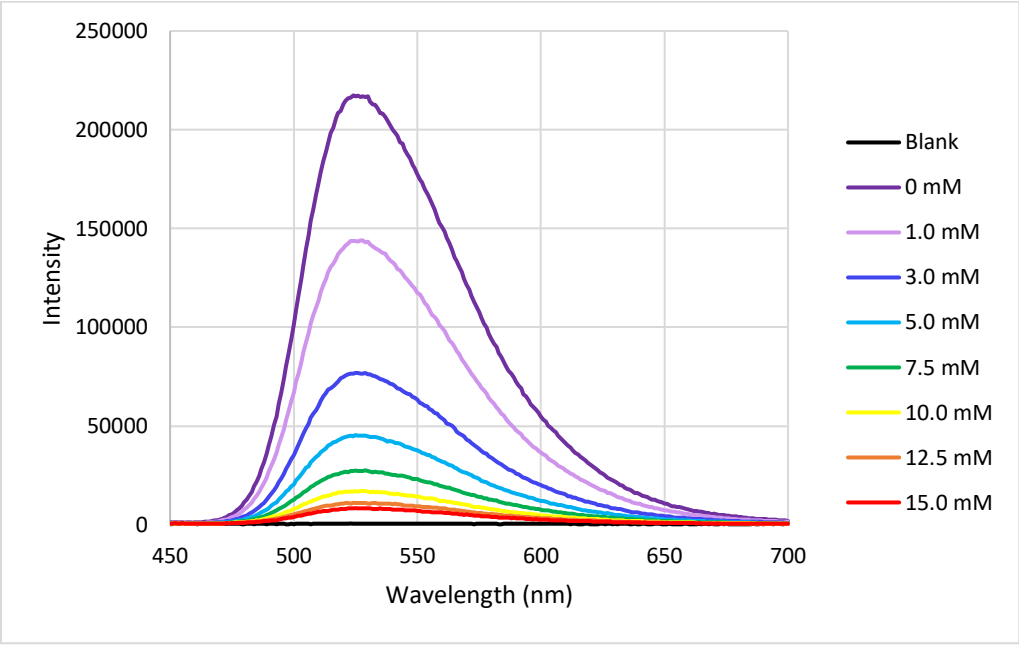


Figure 5.7: Fluorescence titration of vitamin B2 with SCX4 at 45°C.

Upon completion of the triplicate set of fluorescence titrations at 45°C, it was noted that their emission spectra and the intensities of each sample looked almost identical to those from the titrations performed at 35°C. In order to determine if the binding constant did decrease at the increased temperature as predicted, the total integrated areas from the

emission spectrum were used to calculate the F/F_0 values which were then plotted versus concentration of SCX4 as seen in Figure 5.8 below.

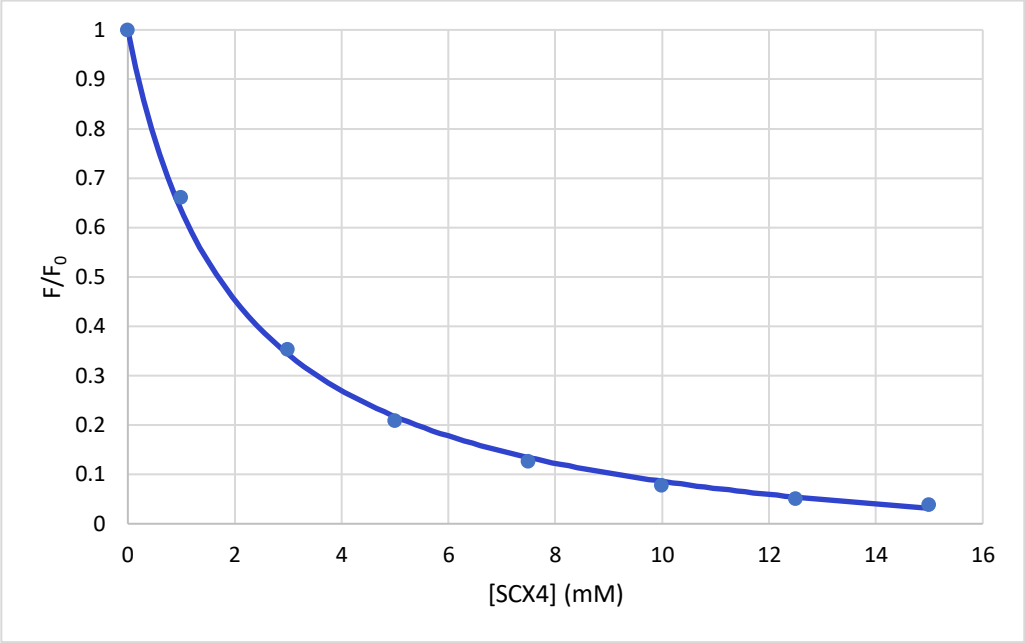


Figure 5.8: F/F_0 versus the concentration of SCX4 at 45°C.

In comparison to the similar plot seen in Figure 5.6, the F/F_0 values from the titration performed at 45°C decreased less intensely as the concentration of SCX4 was increased. This was an indication that binding between vitamin B2 and SCX4 at this temperature was weaker as predicted. From this plot, the 1:1 host to guest binding relationship was also confirmed as a result of the data being fit perfectly to the curve. Further binding studies were performed using the CDEQWIN program to calculate the binding constant from each of the three titration trials. These binding constants are displayed in Table 5.4. The average binding constant of vitamin B2 with SCX4 was

determined to be 520 $\pm$ 17 M^{-1}. This binding constant was lower than that of the 35°C titration trials as expected.

Table 5.4: Binding constants obtained from trials 1-3 of vitamin B2 with SCX4 at 45°C.

Trial	K (M^{-1})
1	496
2	536
3	528

5.5 Binding Studies of Vitamin B2 in SCX4 at 55°C

The next, and highest temperature of interest was 55°C. This was chosen to be the highest temperature to eliminate any possibility of evaporation of the solvent which would result in a change in the concentration of the sample. This temperature was also around 30°C higher than room temperature and gave a good picture of how the binding constant changes in response to an increase in temperature. It was important to confirm if the binding constant would further decrease as a result of the higher temperature. The emission spectrum of a fluorescence titration of vitamin B2 with SCX4 at 55°C is shown in Figure 5.9.

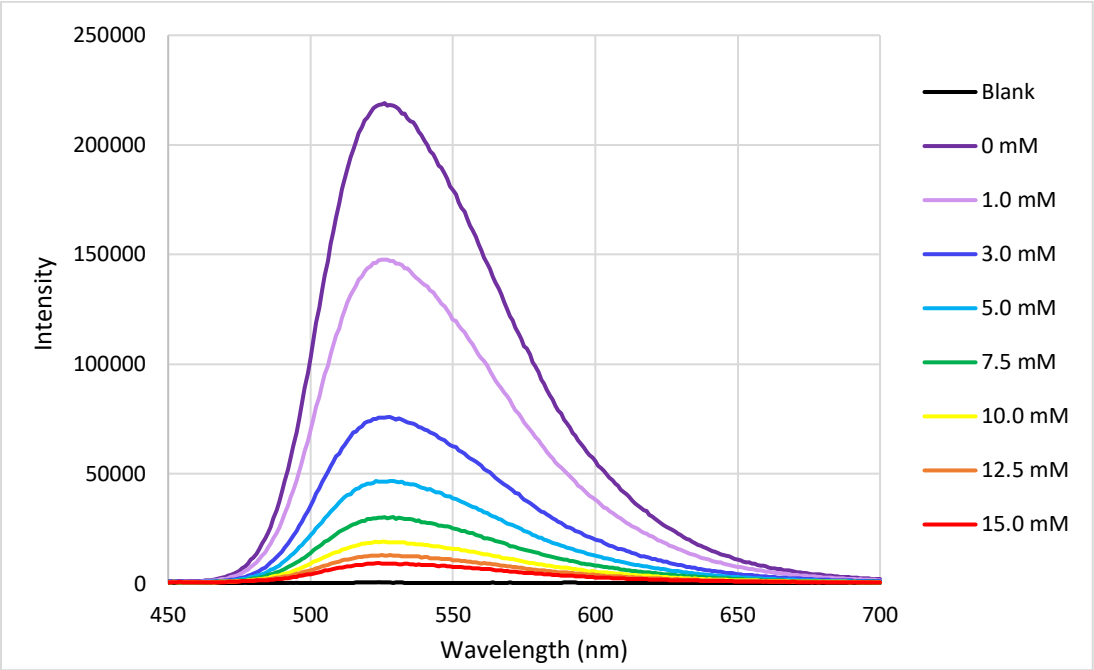


Figure 5.9: Fluorescence titration of vitamin B2 with SCX4 at 55°C.

The emission spectrum shown above very closely resembles the emission spectra of the titrations performed at 35 and 45°C. It was expected that increasing the temperature would not inhibit the ability of SCX4 to suppress the fluorescence of vitamin B2 however, as mentioned, as the temperature was increased, the binding constant was decreasing. In order to confirm this prediction at the 55°C temperature, the total integrated areas from the emission spectrum were again used to calculate the F/F_0 values and plotted against the concentration of SCX4 as shown in Figure 5.10 below.

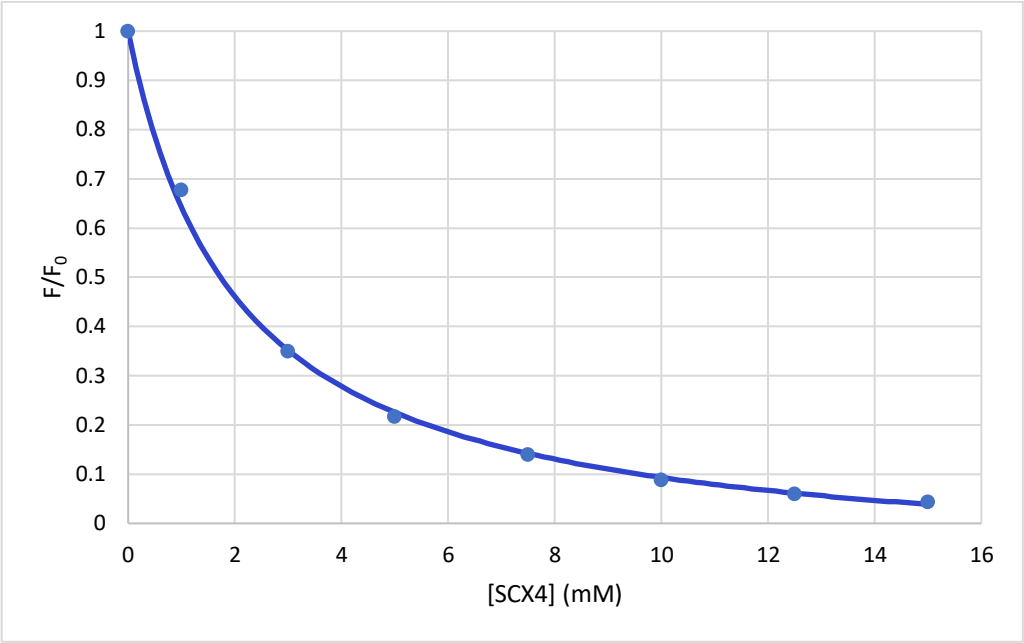


Figure 5.10: F/F_0 versus concentration of SCX4 at 55°C.

In observing the plot above, it became more difficult to make a conclusion on whether the binding of vitamin B2 with SCX4 was weaker, stronger, or the same at 55°C as it was at 45°C. In order to calculate the binding constant and determine if the binding was weaker as predicted due to the increased temperature, the data from Figure 5.10 was inputted into the CDEQWIN program. The binding constants of the three trials are listed below in Table 5.5. The average binding constant for the three trials was determined to be $541 \pm 48\ M^{-1}$.

Table 5.5: Binding constants obtained from trials 1-3 of vitamin B2 with SCX4 at 55°C.

Trial	K (M^{-1})
1	485
2	602
3	536

It was predicted that with the increase in temperature to 55°C, that the binding constant would decrease, following the trend that had been occurring with the two previous increases in temperature from 25°C. However, the average binding constant of the three titrations at 55°C was higher than that of the average binding constant from the three titrations at 45°C as seen in Table 5.6. The relationship that was established changed with the increase in temperature. This outcome is further discussed in Section 5.6.

Table 5.6: Average binding constants of vitamin B2 with SCX4 from thermodynamic study.

Temperature (°C)	K (M^{-1})
7	904 ± 50
15	861 ± 8
25	638 ± 24
35	553 ± 22
45	520 ± 17
55	541 ± 48

5.6 Thermodynamic Analysis of Vitamin B2 in SCX4

The change of the binding constant of vitamin B2 and SCX4 in response to the change of temperature was closely monitored at six temperatures ranging from 7 to 55°C. In general, as the temperature was increased, the binding constant was decreased. This was consistent with temperatures from 7 to 45°C. When the final increase in temperature was made to 55°C however, the binding constant had slightly increased. This was inconsistent with the trend that had been previously established. A van't Hoff plot (Figure 5.11) of ln K versus 1/T was plotted to create a visualization of the dependency of the binding constant on temperature.

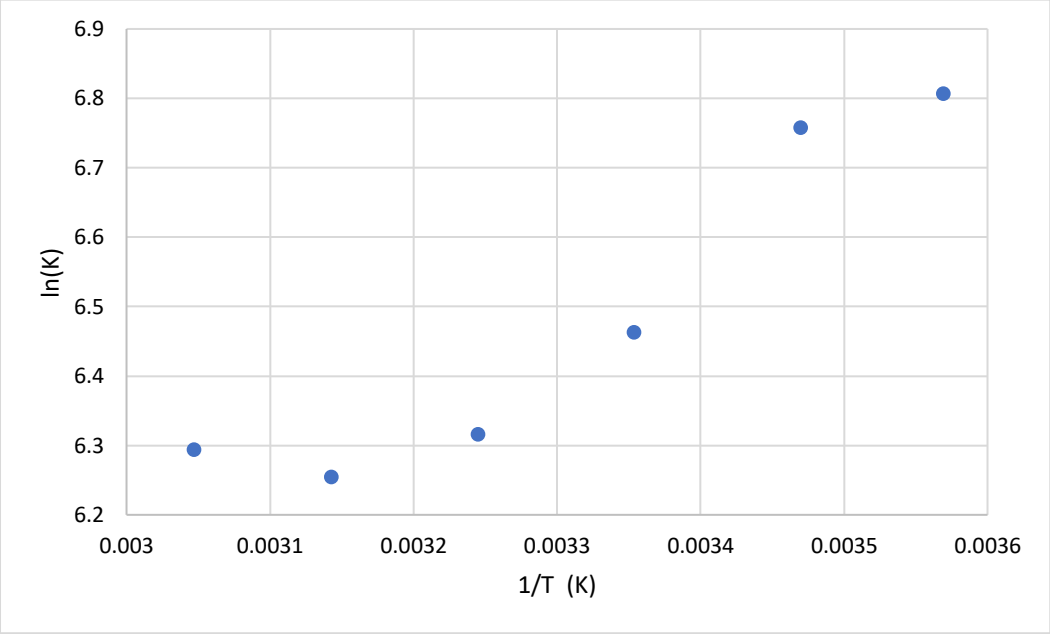


Figure 5.11: van't Hoff plot of the inclusion of vitamin B2 in SCX4.

In examining the van't Hoff plot, it was clear that the data did not demonstrate linearity and instead it demonstrated an S-shaped distribution. This was indicative that the enthalpy of inclusion did not remain constant throughout the temperature range, or that the heat capacities were temperature dependent. From this, it was proposed that as a result of the change in temperature, SCX4 was participating in conformational changes and because of this, the electrostatic forces between the host and guest were being manipulated, resulting in changes of heat capacity and therefore enthalpy at different temperatures. As mentioned, calixarenes are capable of undergoing conformational changes upon encapsulation of the guest. Prior to encapsulation, because of the "floppy" nature of the calixarene, its conformation as a free host can be different than with a guest encapsulated in its internal cavity. This change in conformation can have an effect on the heat capacity depending on the temperature of the system, resulting in a non-linear or in this case an S-shaped curve. In an effort to determine the enthalpy and entropy of inclusion, a subset of the van't Hoff plot from temperatures 15 to 45℃ was plotted. This range of temperatures demonstrated more linearity however, still showed clear curvature, and would be useful in approximating ΔH and ΔS. This subset of the van't Hoff plot is shown in Figure 5.12 below.

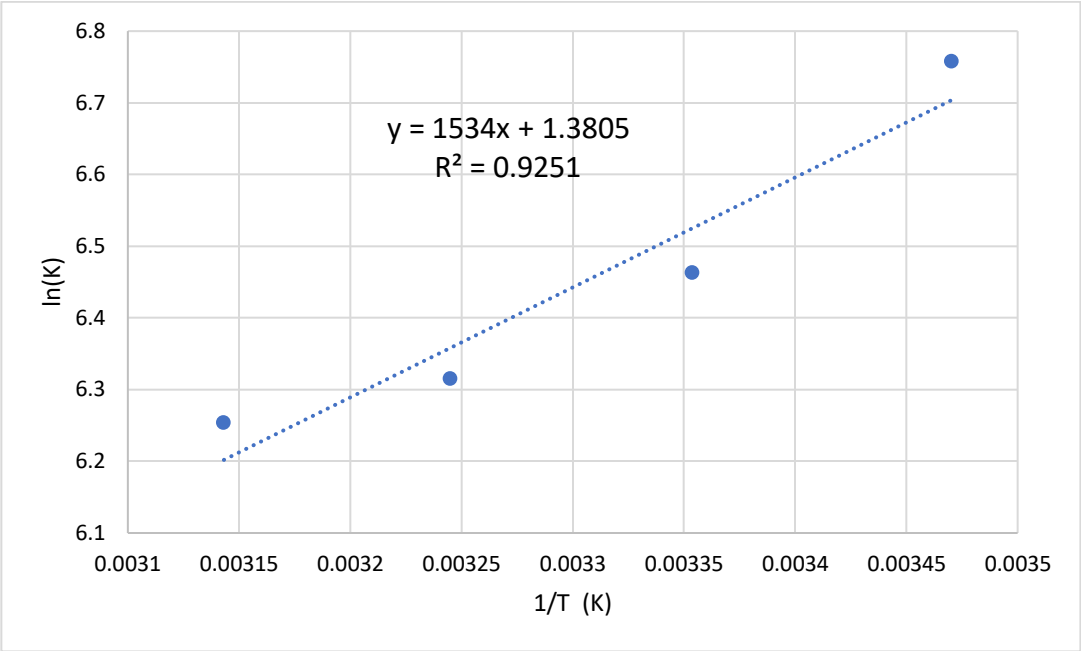


Figure 5.12: van't Hoff plot of vitamin B2 in SCX4 from 15 to 45℃.

Using Equation 1.7, the enthalpy of inclusion of vitamin B2 in SCX4 was estimated to be approximately -13 kJ/mol from the slope in Figure 5.12. It was important to note that this was only an approximation of the enthalpy of inclusion because the subset of data still demonstrated curvature. The enthalpy of inclusion of vitamin B2 in SCX4 was negative; indicating that the inclusion complex is favourable over the host and guest existing separately. The entropy of inclusion was determined to be 12 J/Kmol from the y-intercept of the van't Hoff plot in Figure 5.12. It is important to note that there would be high error in this value due to the extrapolation of data from only the middle portion of the van't Hoff plot. The positive entropy of inclusion was favourable in guaranteeing a negative ΔG indicating the spontaneous formation of the inclusion complex. The formation of the vitamin B2 in SCX4 complex was thus seen to be both enthalpically and entropically

driven, at least based on the approximation of the linear behavior in the middle portion of the van't Hoff plot.

The curved van't Hoff plot was an indication that the enthalpy and/or entropy of inclusion were changing as temperature was changed. The heat capacity was also changing with temperature as a linear van't Hoff plot would indicate constant heat capacity of the free host and guest as well as the complex throughout the temperature range.[28] As mentioned, this was assumed to be the result of conformational changes occurring in the inclusion complex with changes in temperature. This method however, proved to be successful in providing a picture of the thermodynamic properties of the inclusion complexation of vitamin B2 in SCX4 at different temperatures.

As seen in Figure 5.11, the van't Hoff plot was non-linear, and because of this the enthalpies and entropies of inclusion that were determined were only approximations of the true values. Other methods, such as the use of isothermal calorimetric titrations, can be used to take care of the discrepancies in the enthalpy and entropy of inclusion that are approximated using a curved van't Hoff plot. Using isothermal calorimetric titrations that are performed over a small range of temperatures allows for the assumption that the change in heat capacity is temperature independent. A study conducted by Naghibi *et al.* aimed to quantify the discrepancies of van't Hoff and calorimetric enthalpies.[30] In general, it was determined that between the two methods there was a 1-2 kcal/mol difference in the enthalpies. This highlights that curved van't Hoff plots can be used solely for approximation purposes in determining the enthalpy and that the use of high-level calorimetry will guarantee the more accurate experimental value.

Chapter 6: Binding Studies of Vitamin B2 in Cucurbit[7]uril

The final host of interest in performing fluorescence titrations and binding studies with vitamin B2 was CB7. Initial fluorescence measurements described in Section 3.4 revealed the suppression of the fluorescence of vitamin B2 upon addition of CB7. These fluorescence titrations were performed following the dilution method described in Section 2.6. This method was chosen as CB7 is a much more costly supramolecular host in comparison to the para-sulfonated calixarenes and it was more cost effective in using the dilution method to complete the fluorescence titrations. The range of concentrations of CB7 used for the fluorescence titrations was from 1.0 mM to 0.05 mM. This range was chosen as the maximum concentration was successful in showing a significant suppression in the fluorescence of vitamin B2 without using too much material and the lowest was chosen in an effort to approach pure guest without introducing overwhelming error to the results. The emission spectrum of a fluorescence titration of vitamin B2 with CB7 from a representative trial is shown in Figure 6.1.

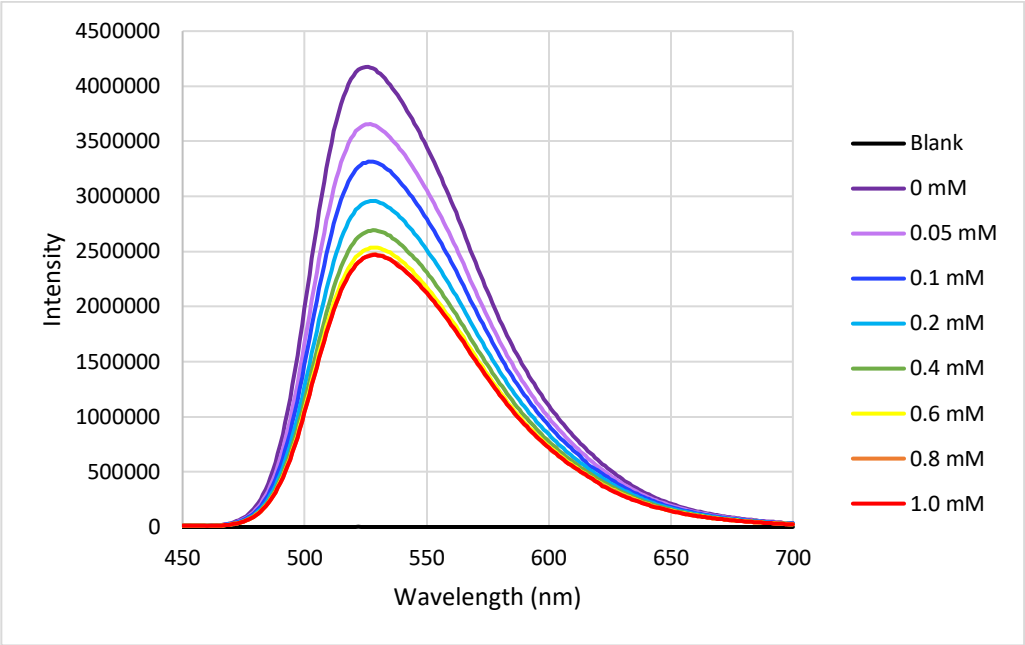


Figure 6.1: Fluorescence titration of vitamin B2 with CB7.

As seen above, as the concentration of CB7 was increased, the suppression of the fluorescence of vitamin B2 increased. Similar to the fluorescence titrations involving SCX6 and SCX4, the complete plateau in the fluorescence of vitamin B2 with CB7 was seen. This was an indication that all free vitamin B2 guest molecules were encapsulated by a CB7 molecule. Using the total integrated areas from the emission spectrum, F/F_0 values were calculated. The plot of F/F_0 values versus the concentration of CB7 is shown in Figure 6.2.

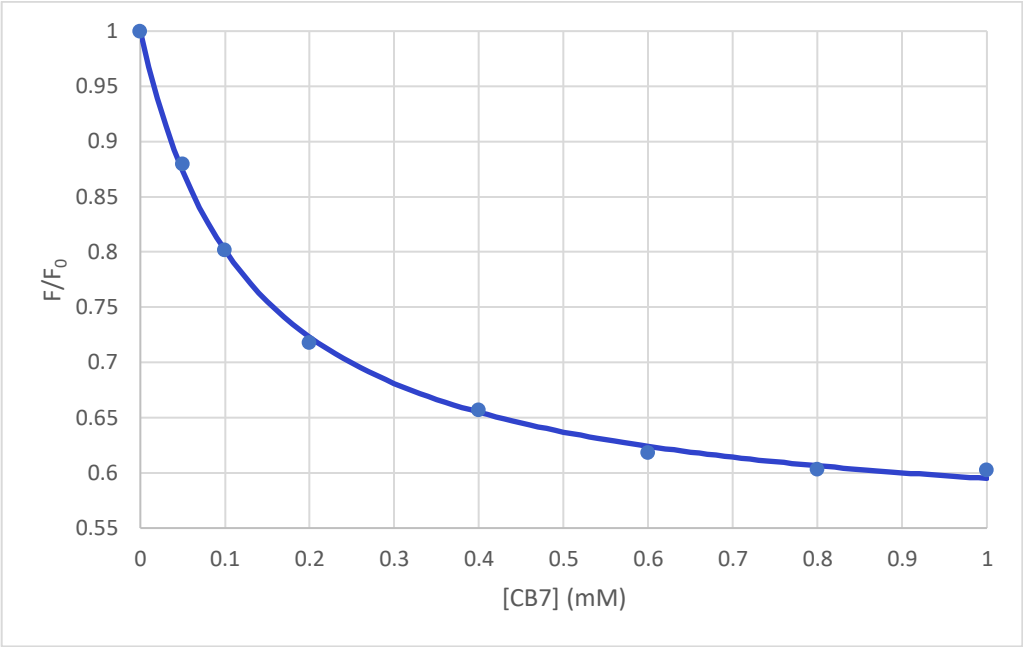


Figure 6.2: F/F_0 versus the concentration of CB7 with vitamin B2.

As seen above, the data in Figure 6.2 is well fit to the titration curve using Equation 1.4, indicating the occurrence of 1:1 host to guest binding between CB7 and vitamin B2. Binding studies were then performed using the CDEQWIN software to calculate binding constants. The binding constants from all trials completed are shown below in Table 6.1. The average binding constant from the five trials for the inclusion of vitamin B2 in CB7 was 7400 $\pm$ 3800 M^{-1}. The average binding constant had a significantly large standard deviation associated with it; greater than 50%. Because of this, a Q-test with a 95% confidence level was completed to determine if the binding constant of the fourth trial could be ruled out as an outlier. The result of the Q-test did not permit this omission of the binding constant of the fourth trial.

Table 6.1: Binding constants obtained for trials 1-5 of vitamin B2 with CB7

Trial	K (M^{-1})
1	3065
2	7659
3	4963
4	14275
5	7131

As seen in the table above, there was a large range in binding constants obtained for the five trials, pointing towards the presence of error in the experiment. This error is expected to be the result of the use of incrementally small concentrations of CB7 to quantify the large magnitude binding with vitamin B2. With increasingly small concentrations of host, the room for error in the experiment increases. This is a potential reason for the large variation in binding constants and consequentially a large standard deviation.

Aside from the error observed with the use of these low concentrations of CB7, CB7 proved to have a stronger binding relationship with vitamin B2 than both SCX6 and SCX4. This was an indication that the non-covalent intermolecular interactions between vitamin B2 and CB7 were stronger than with both SCX6 and SCX4. This was expected to be the result of the difference in cavity sizes between the three hosts with the internal cavity of CB7 being the most optimal for vitamin B2. Although cucurbiturils are not capable of participating in $\pi - \pi$ stacking interactions with vitamin B2 as seen with the para-

sulfonated calixarenes, cucurbiturils have a rigid structure with electronegative carbonyl groups that create an upper and lower rim of the cavity that is smaller in diameter than its internal cavity. Upon encapsulation, many guest molecule are known to interact strongly with the internal cavity through the hydrophobic effect. This was proposed to be a reason for the increase in strength of the inclusion in CB7 over SCX6 and SCX4.

The strong binding relationship that was observed through this work has potential for future implications in drug development. CB7 demonstrated stronger binding for vitamin B2 over the calixarenes and the popular cyclodextrin hosts that demonstrated no binding. Further human trials of the use of CB7 in drug delivery and its safety for ingestion should be further investigated as it demonstrated promise in successfully encapsulating the vitamin B2 molecule and could have the potential for increasing its bioavailability in the body. As mentioned, about 60% of vitamin B2 in a single dose is excreted from the human body in the urine.[28] This percentage could potentially be decreased with the use of a CB7 host to increase absorbance in the body upon approval for medicinal use by an appropriate health agency. This work highlights just how important it was and remains to investigate the use of alternative hosts such as cucurbiturils and calixarenes in addition to cyclodextrins in the pharmaceutical industry.

Chapter 7: Conclusions and Future Work

Host-guest inclusion complexes of vitamins in various supramolecular hosts in aqueous solution were studied. Preliminary fluorescence experiments were conducted with a number of vitamins in varying hosts to determine whether enhancement, suppression, or no effect would be observed. These experiments showed that vitamin B2, also known as riboflavin, showed significant fluorescence suppression in para-sulfonatocalix[6]arene, para-sulfonatocalix[4]arene, and cucurbit[7]uril, but no effect with the more commonly used cyclodextrin hosts. Future work is required to explain why the fluorescence of vitamin B2 is suppressed in the presence of host; this would involve extensive experimental and computational studies of the photophysical properties of riboflavin and their polarity and other dependences.

Fluorescence titrations were performed with all three of these hosts to determine binding constants which described the strength of the complexation between the vitamins and the different hosts. Overall, it was concluded that vitamin B2 demonstrated the strongest binding with cucurbit[7]uril however, a large standard deviation was seen that was concluded to be a result of the use of low concentrations of CB7 in order to conserve as much material possible to maintain a cost efficient procedure. Future work should involve preliminary fluorescence experiments with other cucurbit[n]urils with varying cavity sizes to assess whether an effect on the fluorescence is seen. In the event that an effect is observed, fluorescence titrations and binding studies should be performed to compare the binding affinity of vitamin B2 with cucurbit[7]uril with cucurbit[n]urils of differing sizes.

Host:guest stoichiometries were determined to be 1:1 between vitamin B2 and all three hosts. Between the para-sulfonatocalix[6]arene and para-sulfonatocalix[4]arene hosts, it was determined that the vitamin B2 was more strongly encapsulated in the para-sulfonatocalix[4]arene presumably due to a more accommodating cavity size verified through the comparison of binding constants of which the SCX4 binding constant was larger in magnitude. The vitamin B2 was also believed to interact with these two calix[n]arenes through π - π stacking interactions due to the aromatic nature of both the hosts and guest which increased their likelihood of encapsulation with the calix[n]arenes over the popular cyclodextrin hosts. It was determined however that vitamin B2 experienced the strongest binding relationship with CB7 as the resulting binding constant was the largest in magnitude of the three hosts. This was the result of the stronger non-covalent interactions between vitamin B2 with CB7 than both SCX6 and SCX4. Further study is needed to fully understand the host-guest interaction in these three cases, using NMR spectroscopy and computational studies, for example.

A thermodynamic analysis of the enthalpy and entropy of inclusion of vitamin B2 and SCX4 was performed. This revealed a non-linear van't Hoff plot, indicating that the heat capacity was non-constant and temperature dependent. As a result, the enthalpy of inclusion also varied with temperature. This was proposed to be the result of changes in conformation of SCX4 upon encapsulation of the guest at different temperatures and changes in the electrostatic forces between the host and guest. The enthalpy of inclusion was estimated to be negative, and the entropy of inclusion was estimated to be positive resulting in the spontaneous formation of the inclusion complex (negative ΔG). The formation of the inclusion complex was both enthalpically and entropically driven. Future

work should include a thermodynamic analysis of this same nature of the inclusion of vitamin B2 in SCX6 to assess if a similar trend is observed using the same range of temperatures.

Overall, this work shows that alternative supramolecular hosts, such as calix[n]arenes and cucurbit[n]urils, are capable of successfully encapsulating vitamins. This was an important finding as the area of host-guest chemistry involving vitamins is flooded with research involving cyclodextrins as hosts. Water-soluble para-sulfonated calix[n]arenes and cucurbit[7]uril demonstrated strong binding affinity for vitamin B2 which could have important implications in drug delivery and the enhancement of its bioavailability. The results of this study greatly expanded upon the literature that has been reported for these alternative hosts in encapsulating vitamins.

References

1. Huang, F.; Anslyn, E. Introduction: Supramolecular Chemistry. *Chem. Rev.* **2015**, *115* (15), 6999-7000.
2. Wagner, B.D.; Macdonald, P.J.; Wagner, M.; Betts, T.A. A Visual Demonstration of Supramolecular Chemistry: Observable Fluorescence Enhancement upon Guest-Host Inclusion. *J. Chem. Educ.* **2000**, *77*(2), 178-181.
3. Wagner. B.D. Hydrogen bonding of excited states in supramolecular host-guest inclusion complexes. *Phys. Chem. Chem. Phys.* **2012**, *14*(25), 8825-8835.
4. Szejtli, J. Introduction and General Overview of Cyclodextrin Chemistry. *Chem. Rev.* **1998**, *98*(5), 1743-1754.
5. Roy, A.; Saha, S.; Roy, D.; Bhattacharyya, S.; Roy, M. Formation & specification of host-guest inclusion complexes of an anti-malarial drug inside into cyclic oligosaccharides for enhancing bioavailablity. *J. Incl. Phenom. Macrocycl. Chem.* **2020**, *97*, 65-76.
6. Jankowski, C.; Lamouroux, C.; Jimenez-Estrada, M.; Arseneau, S. Wagner, B.D. Factors Affecting the Formation of 2:1 Host:Guest Inclusion Complexes of 2-[(R-Phenyl)amine]-1,4-naphthalendiones (PAN) in β- and γ-Cyclodextrins. *Molecules* **2016**, *21*(11), 1568-1581.
7. Wimmer, T. Cyclodextrins. *Ullmann's Encyclopedia of Industrial Chemistry.* **2003**, *11*, 23-31.
8. Gutsche, C.D. Chapter 1: From Resinous Tar to Molecular Baskets. In *Calixarenes An Introduction.* 2nd Ed; The Royal Society of Chemistry, 2008, 1-26.
9. Das, D.; Assaf, K.I.; Nau, W.M. Applications of Cucurbiturils in Medicinal Chemistry and Chemical Biology. *Front. Chem.* **2019**, *7*(619), 1-23.
10. Liu, Z.; Nalluri, S.; Stoddart, J.F. Surveying macrocyclic chemistry: from flexible crown ethers to rigid cyclophanes. *Chem. Soc. Rev.* **2017**, *46*(9), 2459-2478.
11. Dodziuk, H. Rigidity versus flexibility. A review of experimental and theoretical studies pertaining to the cyclodextrin non-rigidity. *J. Mol. Struct.* **2002**, *614*, 33-45.

12. Tiwari, G.; Tiwari, R.; Rai, A.K. Cyclodextrins in delivery systems: Applications. *J. Pharm. Bioallied Sci.* **2010**, *2*, 72-79.
13. Weininger, J.; Carpenter, K.; Truswell, A.; Kent-Jones, D. Human Nutrition. *Encyclopedia Britannica.* **2020**. (accessed Mar 23, 2021).
14. Bhagavan, H. N.; Wolkoff, B. I. Correlation Between the Disintegration Time and the Bioavailability of Vitamin C Tablets. *Pharm. Res.* **1993**, *10*(2), 239-242.
15. Lobenberg, R.; Steinke, W. Investigation of vitamin and mineral tablets and capsules on the Canadian market. *J. Pharm. Pharm. Sci.* **2006**, *9*(1), 40-49.
16. Saha, S.; Roy, A.; Roy. K.; Roy, M. Study to explore the mechanism to form inclusion complexes of β-cyclodextrin with vitamin molecules. *Sci. Rep.* **2016**, *6*, 1-12.
17. Bakirova, R.; Nukhuly, A.; Iskineyeva, A.; Burkeyev, M.; Mustafayeva, A.; Minayeva, Y.; Sarsenbekova, A. Obtaining and Investigation of the β-Cyclodextrin Inclusion Complex with Vitamin D_3 Oil Solution. *Scientifica* **2020**, *2020*, 1-8.
18. Xu, X.; Peng, S.; Bao, G.; Zhang, H.; Yin, C. β-cyclodextrin inclusion complexes with vitamin A and its esters: A comparative experimental and molecular modeling study. *J. Mol. Struct.* **2021**, *1223*, 1-11.
19. Kappe, T. The Early History of Calixarene Chemistry. *J. Incl. Phenom. Macrocycl. Chem.* **1994**, *19*, 3-15.
20. Espanol, E. S.; Villamil, M.M. Calixarenes: Generalities and Their Role in Improving the Solubility, Biocompatibility, Stability, Bioavailabilty, Detection, and Transport of Biomolecules. *Biomolecules* **2019**, *9*(3), 90-105.
21. Wagner, B. D. Chapter 1: Fluorescence Studies of Supramolecular Host-Guest Inclusion Complexes. In *Handbook of Photochemistry and Photobiology*. *American Scientific Publishers.* **2003**, 1-57.
22. Hamilton, K. Synthesis, characterization, and application of water-soluble chiral calix[4]arene derivatives in spectroscopy and capillary electrokinetic chromatography. Ph. D. Dissertation, Louisiana State University, **2003**.

23. Guerineau, V.; Rollet, M.; Viel, B. L.; Costa, L.; Saint-Aguet, P.; Laurent, R.; Roger, P.; Gigmes, D.; Martini, C.; Huc, V. The synthesis and characterization of giant calixarenes. *Nat. Commun.* **2019**, *10*(113), 1-14.
24. Freeman, W. A.; Mock, W. L.; Shih, N. Y. Cucurbituril. *J. Am. Chem. Soc.* **1981**, *103*, 7367-7368.
25. Miskolczy, Z.; Biczok, L. Kinetics and Thermodynamics of Berberine Inclusion in Cucurbit[7]uril. *J. Phys. Chem. B.* **2014**, *114*(9), 2499-2505.
26. Miskolczy, Z.; Biczok, L. Sequential inclusion of two berberine cations in cucurbit[8]uril cavity: kinetic and thermodynamic studies. *Phys. Chem. Chem. Phys.* **2014**, *16*, 20147-20156.
27. Li, W.; Li, S.; Wyman, I.; Macartney, D.; Zhang, Q.; Zheng, Y.; Wang. R. Supramolecular Encapsulation of Vitamin B6 by Macrocyclic Nanocontainer Cucurbit[7]uril. *J. Nanomater.* **2015**, *2015*, 1-6.
28. Sharma, G.; First, E. Thermodynamic Analysis Reveals a Temperature-dependent Change in the Catalytic Mechanism of *Bacillus stearothermophilus* Tyrosyl-tRNA Synthetase. *J. Biol. Chem.* **2009**, *284*(7), 4179-4190.
29. Mukherjee, A.; Roy, D.; Deb, N.; Ghosh, B. Inclusion of riboflavin in β-cyclodextrin: A fluorimetric and absorption spectrometric study. *Spectrochim. Acta A.* **2009**, *73*, 201-204.
30. Naghibi, H.; Tamura, A.; Sturtevant, J. Significant discrepancies between van't Hoff and calorimetric enthalpies. *Proc. Natl. Acad. Sci.* **1995**, *92*, 5597-5599.