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SYNTHESIS, CHARACTERIZATION, AND APPLICATION OF WATER-SOLUBLE CHIRAL CALIX[4]ARENE DERIVATIVES IN SPECTROSCOPY AND CAPILLARY ELECTROKINETIC CHROMATOGRAPHY

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Chemistry

by
Kim Hamilton
B.S., Southern University and A & M College, 1995
August 2003
DEDICATION

To my grandmother

Mrs. Laverne Ridley Monroe

Though you did not have the privilege of completing your formal education, you have shown the Ph.D. spirit all along…Patience…Humility…Determination. Most importantly, God has enriched you with gifts of the Spirit whose lessons transcend far beyond the curriculum of any earthly institution of higher learning. Through your thoughts, words, and deeds, you taught your children and grandchildren to dream big. In particular, you have taught me that all things are possible when I dare to dream and be guided by Spirit through even the worst of situations. Thank you for giving me the gift of your unconditional love. I love you more than words could ever say.

and

To my mother and father

Delia and Macura Hamilton

Both of you have exemplified the synergism between art, philosophy, and science. Thank you for your example, your love and your support.

“Never underestimate the impact of your lightest touch on someone’s life”
ACKNOWLEDGMENTS

How can I say thanks
For the things you have done for me?
Things so undeserved,
That you did to prove your love for me.
The voices of a million angels
Cannot express my gratitude
All that I am or ever hope to be
I owe it all to thee.

-by Andrae Crouch

from My Tribute, Keep on Singin’ ©1971

The author gratefully acknowledges the following individuals for their contributions to the completion of this work:

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TABLE OF CONTENTS

DEDICATION .......................................................................................................................... ii

ACKNOWLEDGMENTS........................................................................................................ iii

LIST OF TABLES ................................................................................................................ vii

LIST OF FIGURES ............................................................................................................... viii

ABSTRACT ......................................................................................................................... xi

CHAPTER 1. INTRODUCTION .............................................................................................. 1
  Part I. Calixarenes ........................................................................................................ 1
  Part II. Introduction to Molecular Spectroscopy ....................................................... 20
  Part III. Introduction to Capillary Electrophoresis ..................................................... 47
  References .................................................................................................................... 62

CHAPTER 2. SYNTHESIS AND CHARACTERIZATION OF CALIXARENES ....... 70
  Part I. Synthesis and NMR Assignments of Water-Soluble Chiral
  N-Acylcalix[4]arene L-Amino Acid Derivatives ...................................................... 70
  Part II. Synthesis and Characterization of Calixarene-Bonded
  Silica Stationary Phases ......................................................................................... 85
  References .................................................................................................................. 90

  CHIRAL SELECTORS IN ELECTROKINETIC CHROMATOGRAPHY ................... 92
  Part I. Introduction ..................................................................................................... 92
  Part II. Experimental Section .................................................................................. 93
  Part III. Results and Discussion ............................................................................. 95
  Part IV. Conclusions ............................................................................................... 105
  References ................................................................................................................ 106

CHAPTER 4. SPECTROSCOPIC STUDIES OF CALIXARENE
  COMPLEXATION ......................................................................................................... 108
  Inner-Filter Effects in Fluorescence ........................................................................ 108
  Part II. Spectroscopic Investigation of Sulfonated Calixarenes
  in the Presence of Metals ...................................................................................... 129
  References ................................................................................................................. 133

CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS .............................................. 136

APPENDIX: ADDITIONAL FIGURES DISCUSSED IN
PREVIOUS CHAPTERS .................................................................................................. 140
LIST OF TABLES

Table 1.1 pKa Values of Water-soluble Calix[4]arenes .......................................................... 9
Table 1.2 Properties of Calixarenes and Cyclodextrins .......................................................... 18
Table 1.3 Spin Quantum Numbers of Various Nuclei .......................................................... 36
Table 1.4 Significant Aspects of CEC, HPLC, and CE .................................................. 58
Table 2.1 ¹H and ¹³C Chemical Shifts (δ) of Calix[4]arene Derivatives ......................... 78
Table 2.2 Elemental analysis of three batches of CVAPS stationary phase ............... 87
Table 2.3 Surface coverage of calixarene-modified silica beads .................................. 88
Table 4.1 Mean concentration of metals in soft and hard plaque (mg/g)................. 130
LIST OF FIGURES

Figure 1.1 Naming scheme of calixarenes ................................................................. 2
Figure 1.2 Conformations of substituted calix[4]arenes .......................................... 4
Figure 1.3 Examples of water-soluble calixarenes ................................................... 8
Figure 1.4 Unselective (A) and selective (B) inclusion of TMA by \( p \)-sulfonato-calix[4]arenes (H\(_2\)O fixes cone conformation) ................................................. 14
Figure 1.5 The electromagnetic spectrum ............................................................... 22
Figure 1.6 Jablonski diagram ................................................................................... 23
Figure 1.7 Mirror Image Rule and Franck-Condon Principle .................................... 28
Figure 1.8 Schematic diagram of a typical fluorometer .............................................. 34
Figure 1.9 Generation of \( \alpha \)- and \( \beta \)-spin states upon application of magnetic field ...... 36
Figure 1.10 Precession of nucleus in external magnetic field \( B_0 \) ............................... 39
Figure 1.11 Spin coupling of neighboring nuclei in different environments .............. 42
Figure 1.12 Carbon-13 NMR spectra obtained by different methods ....................... 45
Figure 1.13 Schematic of NMR spectrometer ......................................................... 46
Figure 1.14 Diagram of CE Instrumentation ............................................................. 48
Figure 1.15 Generation of EOF ............................................................................... 50
Figure 1.16 Solute migration in CE ......................................................................... 53
Figure 1.17 Migration time window of neutral solutes in EKC ................................. 55
Figure 1.18 Examples of pseudostationary phases .................................................. 56
Figure 1.19 Interior of packed capillary in CEC ....................................................... 57
Figure 1.20 Procedure for the formation of slurry-packed capillaries ....................... 59
Figure 2.1 Synthetic scheme of \( N \)-L-aminoacylcalix[4]arenes ................................. 71
Figure 2.2 Structure and number assignments of calixarene esters ......................... 76
Figure 2.3 Proton NMR spectrum of tetraacetic acid tetraethyl ester of 4-tert-butylcalix[4]arene (Compound 2, Figure 2.1) .......................................................... 77
Figure 2.4 COSY Spectrum of CX4-VAL t-butyl ester .................................................. 81
Figure 2.5 HMBC Spectrum of CX4-VAL t-butyl ester ................................................ 82
Figure 2.6 TOCSY Spectrum of (N-L-leucinoacyl)calix[4]arene t-butyl ester .............. 84
Figure 2.7 Synthetic scheme of CVAPS stationary phase ........................................... 86
Figure 2.8 Solid State $^{13}$C NMR spectra .................................................................. 89
Figure 3.1 Structure of $N$-Acylcalix[4]arene L-amino acid derivatives .................... 94
Figure 3.2 Structures of binaphthyl derivatives and benzodiazepines ....................... 94
Figure 3.3 Separation of BNP (1), BOH (2), and BNA (3) using CX4-L-VAL .......... 96
Figure 3.4 Separation of BNP, BOH, and BNA using 10 mM CX4-L-LEU ................. 97
Figure 3.5 Effect of pH on the resolution of binaphthyl derivatives ......................... 98
Figure 3.6 Effect of urea concentration on resolution of binaphthyl derivatives ........ 99
Figure 3.7 Separation of oxazepam enantiomers ....................................................... 101
Figure 3.8 Effect of pH on the chiral interaction ($\alpha$) ................................................ 102
Figure 3.9 Effect of CX4-ILE concentration on the resolution of benzodiazepines .... 103
Figure 3.10 Separation of lorazepam enantiomers ..................................................... 104
Figure 3.11 Separation of oxazepam enantiomers ..................................................... 104
Figure 4.1 Absorption and Fluorescence Spectra of a Two Component Host-Guest System ........................................................................................................ 111
Figure 4.2 Structures of host calixarenes and guest ($R$)-BNA ................................. 112
Figure 4.3 Calibration plot of CX4-LEU absorbance ................................................ 115
Figure 4.4 Absorbance spectra of $1 \times 10^{-5}$ M $R$-BNA, $1 \times 10^{-5}$ M CX4-LEU, and $1 \times 10^{-4}$ M CX4-LEU .................................................. 115
Figure 4.5 Emission spectrum of $10^{-5}$ M $R$-BNA in $3.5 \times 10^{-4}$ M CX4-LEU .......... 116
Figure 4.6  Emission spectra of R-BNA in increasing concentrations.......................... 117

Figure 4.7  Emission intensity of R-BNA with and without absorbance correction..... 119

Figure 4.8  Double reciprocal plot of intensity of R-BNA as a function of CX4-LEU concentration........................................................................................................... 120

Figure 4.9  Double reciprocal plot of fluorescence intensity of R-BNA as a function of CX4-LEU concentration............................................................................. 120

Figure 4.10  Orientations of tandem cuvette in conventional fluorimeter .................... 122

Figure 4.11  Effect of CX4-LEU concentration and cuvette position on fluorescence intensity................................................................................................................... 123

Figure 4.12  Fluorescence emission spectra of R-BNA ............................................. 125

Figure 4.13  Relationship between R-BNA intensity and CX4-LEU concentration.... 126

Figure 4.14  Double reciprocal plots assuming A) 1:1; and B) 2:1 stoichiometry between CX4-LEU and R-BNA............................................................................................ 127

Figure 4.15  A) Ultraviolet absorbance spectra of 1.47x10^{-5} M SCX4......................... 133
ABSTRACT

This dissertation is an account of the synthesis, characterization, and application of novel water-soluble chiral calixarenes in spectroscopy and capillary electrophoresis. It is divided into four sections. The first part describes the synthesis and nuclear magnetic resonance (NMR) characterization of four \( p-t \)-butylcalix[4]arenes bearing L-amino acid moieties on their lower rims (CX4-AA). The structure and conformation of the derivatives have been determined using one- and two-dimensional NMR techniques. Proton and carbon-13 spectra show that the derivatives are tetra-substituted and adopt a cone conformation. The preparation and characterization of silica-bonded calixarene stationary phases for capillary electrochromatography is also reported in Chapter 2. A novel synthesis has been attempted, in which one monomer unit of the calixarene is covalently attached to aminopropyl silica (APS) via the formation of a peptide bond between the carboxylate terminus of the calixarene-amino acid moiety and the primary amine of APS. Elemental analyses of the calixarene stationary phases show increases in the percentages of carbon, hydrogen, and nitrogen compared to unmodified APS. Carbon-13 cross polarization-magic angle spinning NMR (CP-MAS NMR) is used to confirm successful attachment of the calixarenes to APS.

The third part focuses on the use of these derivatives as pseudostationary phases in electrokinetic capillary chromatography (EKC). Comparisons have been made pointing out the structural influence of each derivative on its selectivity toward enantiomeric and atropisomeric pairs. Electrokinetic parameters such as buffer pH, chiral selector concentration, and organic modifier concentration are varied to yield the best compromise between analyte resolution and elution times.
Spectroscopic studies of calixarene inclusion complexes generally focus on the properties of molecules whose absorption and emission bands do not overlap those of calixarenes. In order to investigate the complexation behavior of CX4-AA derivatives with binaphthyl atropisomers, special considerations had to be taken because CX4-AA emission signals overlap those of binaphthyls. In Chapter 4, a steady-state fluorescence method is proposed which investigates and corrects for inner-filter effects of calixarenes during complexation studies of guests with overlapping absorption and emission. Association constants and complex stoichiometries are then determined from steady state fluorescence measurements and general correlations between EKC and fluorescence data are established.
CHAPTER 1 INTRODUCTION

Part I. Calixarenes

1.1 Evolution of Calixarene Chemistry

Calixarenes are macrocyclic compounds composed of phenolic units connected by methylene bridges to form a hydrophobic cavity that is capable of forming inclusion complexes with a variety of molecules. These molecules were first synthesized by Adolph von Baeyer as products of the reaction of phenols with aldehydes in the presence of strong acids.\(^1\) Because he could not isolate pure materials from these reactions, Baeyer did not have elemental analysis data from which to propose structures of his products. Realizing the marketable qualities of products of phenol-formaldehyde reactions, Leo Baekeland patented a process which used a small amount of base to yield resinous materials he called Bakelite.\(^2\) This patent, issued in 1908, constituted the first large-scale production of a synthetic plastic, but the structural details of the resins were still nebulous. It was not until 1944 that Alois Zinke, a professor of chemistry at the University of Graz in Austria, and his coworker Erich Zeigler proposed a cyclic tetrameric structure of the product of the base-induced condensation of \(p\text{-}{\textit{tert}}\text{-}\)butylphenol and formaldehyde.\(^3\) He later provided unequivocal proof of structure of this and other phenol-formaldehyde products in 1952.\(^4\) Shortly thereafter, scientists at the Petrolite Corporation in Missouri commercialized oxyalkylated alkylphenol-formaldehyde resins as oil demulsifiers. They patented the “Petrolite Procedure” for making cyclic oligomers in the 1970s.\(^5\)

The pioneering work of C. David Gutsche in the late 1970s led to a renewed interest in the chemistry of phenol-formaldehyde products, and his naming such products
calixarenes.\textsuperscript{6} Derived from the Greek word *calix* meaning vase, and *arene* indicating the presence of aromatic rings, calixarenes have been synthesized in a number of sizes. A bracketed number positioned between *calix* and *arene* indicates the number of phenolic units linked to each other by methylene bridges to form the cavity of the molecule. Substitution on the aromatic rings is specified by appropriate prefixes. The naming scheme is illustrated in Figure 1.1.

![Figure 1.1 Naming scheme of calixarenes](image)

If \( n = 3 \), and \( R = t\)-butyl, the name of this compound is \textit{p-t}-butylcalix[6]arene

The motivation behind Gutsche’s experiments was the possibility that the torus shape of calixarenes could be appropriate for enzyme mimic building. Enzymes are highly specific both in the reactions they catalyze and in their choice of substrates. Calixarenes possess a well-defined cavity with simultaneous polar (lower-rim) and nonpolar (upper-rim) properties. Also, they can be derivatized in terms of cavity size and functional group to yield analyte-selective compounds capable of forming inclusion
complexes. Therefore, their applicability to bioorganic chemistry was an important springboard for other researchers interested in calixarene chemistry.

### 1.2 Structural Characteristics of Calixarenes

The basic structure of a calixarene consists of repeating phenolic units linked by methylene groups to form a distinct cylindrical-shaped cavity. The wider side of the cavity is defined as the upper rim, and the narrower hydroxyl side is the lower rim. Because they are easily derivatized, numerous reaction schemes have been reported that produce these compounds with a myriad of functionalities and chemical properties. Calixarenes with as few as three and as many as twenty repeating units are known. The majority of studies deal with derivatives of calix[4]arenes, calix[6]arenes, and calix[8]arenes. A common factor in all the reports is the retention of the calixarene cavity. Cavity sizes of calix[4]arene, calix[6]arene, and calix[8]arene are 3.0, 7.6, and 11.7 Å, respectively.

The spatial orientation of each phenolic unit lends to a conformation which is a function of reaction conditions, the number of phenols linked together, their degree of substitution, and sometimes the length of the linkage between phenols. The unsubstituted parent compound of calix[4]arene, possesses a chair-like conformation with two aromatic rings in one plane and the other two at right angles. The rotations of the methylene groups between phenols bring about variable conformations in substituted calixarenes. For example, \textit{p-}\textit{tert}-butylcalix[4]arenes more often assume the cone, partial cone, 1,2-alternate, or 1,3-alternate conformation. Figure 1.2 shows a simplified representation of each conformation. By convention, when all phenolic rings are pointed up, this signifies the cone. When one or two are pointed down, this signifies one of the
partial cone arrangements. Proton nuclear magnetic resonance (NMR) measurements of several calixarenes in solution show that they mainly exist in the cone conformation, but they are conformationally mobile at room temperature.\textsuperscript{12}

![Conformations of substituted calix[4]arenes](image)

**Figure 1.2** Conformations of substituted calix[4]arenes

The flexibility of calixarenes can be controlled by crystallization, which allows a desired conformation to be fixed in the solid state. Further control of conformation can be achieved by derivatization of the upper and lower rim functionalities with bulkier groups which inhibit rotation. For instance, intramolecular bridging of phenols through a spacer can form bridged\textsuperscript{13} and capped\textsuperscript{14} compounds, depending upon the size of the spacer. Intermolecular bridging results in double calixarenes possessing single\textsuperscript{15} and double bridges.\textsuperscript{16} In one case, two calix[6]arenes linked by six bridges have been reported.\textsuperscript{17}

**1.3 Other Physical Properties**

Measurement of a calixarene’s melting point and range allows one to determine its purity quickly. Most calixarenes have melting points above 250 °C. Even subtle isomeric differences in substituent groups can yield dramatic differences in the melting points of calixarene derivatives.\textsuperscript{18} These same differences can affect solubility.

The most common forms of calixarenes are insoluble in water, due to their aromatic components. They also possess limited solubility in organic solvents, thereby
making their purification and characterization a formidable task. Most forms are sufficiently soluble to allow spectral analyses in a limited number of common organic solvents however. Again, derivatization can be employed to induce different solubilities. It is interesting to note that the organic solvent solubility is increased by those substituents that tend to lower the melting point.\textsuperscript{19} The water-solubility of calixarenes will be discussed separately.

In spite of their low solubilities in organic solvents, the spectral properties of calixarenes have been investigated using infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR), X-ray crystallography, mass spectrometry (MS), and fluorescence spectroscopy. Each technique provides valuable insight about the calixarenes’ structure and other properties using different types of radiation. Some will be discussed in more detail in a separate section, but the key information they give about calixarenes will be touched upon briefly at this time.

A distinctive spectral component of the IR spectra of calixarenes is the low frequency of the OH stretching vibration, ranging from approximately 3150 cm\textsuperscript{-1} to 3300 cm\textsuperscript{-1}, depending upon the size of the molecule. This is attributed to very strong intramolecular hydrogen bonding between the lower rim hydroxyl groups. This circular hydrogen bonding\textsuperscript{20} is strongest in the tetramer and weakest in the pentamer.\textsuperscript{21}

A pair of absorption maxima near 280 and 288 nm occur in the UV spectra of calixarenes. The ratio of the intensity at these two wavelengths is a function of calixarene size, at 1.30, 0.90, and 0.75 for the tert-butyl derivatives of calix[4]arene, calix[6]arene, and calix[8]arene, respectively. The molar absorptivities (\(\varepsilon_{max}\), L mol\textsuperscript{-1} cm\textsuperscript{-1}...
range from 9,800 to 23,100 at 280 nm and 7,700 to 32,000 at 288 nm, increasing as the size of the calixarene increases.

The NMR spectra of calixarenes are relatively simple, because the molecules are symmetrical. The 3.5-5.0 ppm region in the $^1$H NMR spectra provides a very distinct pattern for conformational analysis, because the methylene bridge protons are affected most by conformational changes. In the cone conformation, neither proton is positioned inside the cavity. This results in a splitting pattern in the $^1$H spectrum of calix[4]arene, a pair of doublets, one occurring around 3.2 ppm and the other at 4.9 ppm. The patterns for the other conformers are as follows: partial cone (two pairs of doublets (ratio 1:1) or one pair of doublets and one singlet (ratio 1:1)); 1,2-alternate (one singlet and two doublets (ratio 1:1)); and 1,3-alternate (one singlet). A singlet arising from the OH groups varies in position (8-10 ppm) with the size of the calixarene, but does not correlate with the strength of circular hydrogen bonding.

X-ray crystallography provides conclusive proof of the calixarene structure. The first example appeared in 1979 when Andreetti and coworkers at the University of Parma, Italy, published the single crystal data for calix[4]arene. Mass spectrometry has been successfully used to determine the molecular weights of calixarenes. Though not as revealing of structural detail as X-ray crystallography or NMR spectroscopy, the host-guest features of calixarenes can be investigated. Fluorescence spectroscopy has also been a useful tool for gaining insight into the complexation behavior of calixarenes and into the microenvironments of calixarene complexes.
1.4 Synthesis of Calixarenes

The publication of Gutsche’s one-pot synthetic procedures for tert-butylcalixarenes with four, six, and eight repeating units led to a proliferation of new calixarene derivatives in the literature. One of the most significant results of his procedures was the excellent yield of the major products. It was shown that by choosing the reaction conditions properly, the starting material could be transformed to the cyclic tetramer, cyclic hexamer, or cyclic octamer, each having the same substituent in all the para-positions. Coupled with the fact that the starting materials are readily available, significant strides have been made in the synthesis of calixarenes since the 1980s. A survey of the literature reveals that over 200 articles have been published which report the synthesis of novel calixarene derivatives since 1995. Because this dissertation focuses on water-soluble calixarene derivatives, the subsequent discussion will be limited to that topic.

Calixarenes are inherently insoluble in water. This makes them unsuitable for substrate-binding studies in aqueous solution. Consequently, synthesis of water-soluble derivatives is necessary to extend their utility. The tetracarboxylic acid of \( p\text{-}\text{tert}-\text{butylcalix}[4]\text{arene} \), introduced by Ungaro and coworkers in 1984 was the first example of a water-soluble calixarene. Functionalized with four carboxylic groups on the lower rim, its inclusion properties could be studied in aqueous solution. In the same year, Shinkai reported the preparation of \( p\text{-}\text{sulfonato calix}[6]\text{arene} \). Following the preparation of the sulfonated tetramer and octamer, other anionic water-soluble derivatives containing nitro, phosphonic acid, and carboxyl moieties emerged. Shinkai is also noted for reporting the first example of a cationic water-soluble calixarene
which both strongly bound anionic guest molecules and exhibited enzyme-mimetic activity. Other cationic calixarenes contain tetraalkylammonium groups and primary amines. In order to avoid unspecific binding or repulsion of ionic groups in complexation experiments, neutral water-soluble calixarenes have been synthesized with sulfonamides, hydroxyl group-containing amides, sugars, polyoxyethylene and polyalcohol residues. Examples of ionic and neutral water-soluble calixarenes are shown in Figure 1.3.

![Diagram of calixarenes](image)

**Figure 1.3** Examples of Water-soluble Calixarenes
Acid dissociation constants (p$K_a$) for the OH groups of some water-soluble calixarenes have been determined by potentiometric and photometric titrations. Because the p$K_a$ of p-nitrophenol in water is about 3 units less than that of alkylphenols, Böhmer and associates were able to determine the first dissociation constant in a molecule composed of one p-nitrophenol unit among several alkylphenol units.$^{42}$ The spectroscopic properties of the p-nitrophenol made it possible to monitor the UV-Visible absorbance as a function of the solution pH. By use of this approach, p$K_a$ values have been determined for tetra-substituted p-nitrocalix[4]arenes.$^{43,44}$ The p$K_a$ values obtained for sulfonated (1) and nitro (2) and neutral sulfonamide (3) calix[4]arenes depicted in Figure 1.3 are summarized in Table 1.1.

<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>p$K_a$ Values of Water-soluble Calix[4]arenes</th>
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<tr>
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<td>Calixarene</td>
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The tetra-substituted calix[4]arenes in Table 1 have very low p$K_{a1}$ values. In the p-nitrocalix[4]arenes (2) for example, the p$K_{a1}$ value is lower by seven p$K_a$ units than in its non-cyclic analog. This indicates that strong intramolecular hydrogen bonds bring about a very acidic OH group, i.e., the hydrogen bonds through the three remaining protons stabilize the mono-anionic species. The p$K_{a4}$ values are very high, because the last proton is trapped in a ring of four oxygen atoms. Shinkai and coworkers believe that the
unusually strong hydrogen-bonding in the calixarene cavity is responsible for the large differences in $pK_a$ values and that this hydrogen-bonding stabilizes the cone conformation.\textsuperscript{45}

1.5 Complexation Properties of Water-soluble Calixarenes

Interest in calixarenes is largely due to their ability to include other entities within themselves. This inclusion of metal cations (main group, transition metal, actinides and lanthanides), organic cations and anions, and neutral molecules has driven the understanding of factors which determine selective molecular recognition, and consequently the design of molecular systems which perform highly efficient and selective chemical, physical, and biomimetic duties. The capability of calixarenes for forming 1:1 inclusion complexes with aromatic guest molecules,\textsuperscript{46} was first demonstrated in 1979. However, this was not in water. Since most biological processes take place in water, researchers have taken various approaches to solubilize calixarenes in aqueous systems over the years. A full literature review is beyond the scope of this dissertation. Therefore, only selected results on complexation, solvent extraction and membrane transport will be discussed.

1.5.1 Complexation of Cationic Guests

The first water-soluble calixarenes, carboxylic acid derivatives of $p$-tert-butylcalix[4]arene, were very efficient in the extraction of uranyl ($\text{UO}_2^{2+}$), lead, and alkaline earth cations from water to dichloromethane, but not very selective.\textsuperscript{47} A hexacarboxylated derivative of $p$-sulfonated calix[6]arene was synthesized and deemed a “super-uranophile” by Shinkai,\textsuperscript{48} because of its ability to selectively extract uranium from sea water\textsuperscript{49,50} over other metal ions present in a greater excess. They later used a chromatographic column containing the same calixarenes immobilized in a polymer resin to acquire $\text{UO}_2^{2+}$ directly from the
They spectroscopically evaluated the selectivity of the super-uranophile by monitoring changes in the absorption band of the \( \text{UO}_2^{2+}\text{-calixarene complex} \) upon addition of competing metal cations (\( M^{n+} \)). They obtained the following selectivity factors (\( K_{\text{UO}_2^{2+}/M^{2+}} \)): \( >10^{+17} \) for \( \text{Mg}^{2+} \), \( 10^{+15.3} \) for \( \text{Ni}^{2+} \), \( 10^{+13.1} \) for \( \text{Zn}^{2+} \), and \( 10^{+12} \) for \( \text{Cu}^{2+} \). This extremely high selectivity was explained by a coordination-geometry concept, wherein the calixarene provides ligand groups arranged in the appropriate manner for pseudoplanar hexacoordination of \( \text{UO}_2^{2+} \). The other cations usually require either square planar or tetrahedral coordination geometry for favorable binding. Another possible explanation suggesting hole-size selectivity related \( K_{\text{UO}_2^{2+}} \) to the rigidity of the calixarene and the size of the guest ions. Less emphasis was placed on this explanation because the cavity sizes of calixarenes are variable due to their conformational flexibility, and the radii of the competing metal cations are similar to that of \( \text{UO}_2^{2+} \). In any event, authors alluded to complexation through the carboxylate groups.

Complexation in solution generally occurs through the hydroxyl or the carboxylate groups, but sulfonated calix[4]arenes have also been reported to form a complex of two \( \text{Pb}^{2+} \) ions coordinated both by sulfonate oxygens and water molecules. Carboxylated derivatives of \( p\)-sulfonatocalix[4]arene were shown to form \( \text{Cu}^{2+} \) complexes as well. However, a 1:1 metal•calixarene stoichiometry could not be assumed solely, because monoprotonated, diprotonated, and hexaprotonated species were also detected. Sulfonated and carboxylated calix[4]arenes bearing crown ether moieties have also been reported to form 1:1 complexes with cesium.

An environmentally relevant application of calixarenes is the extraction of radioactive metals from acidic or high salinity media generated from the reprocessing of
spent nuclear fuels. Uranium and plutonium are effectively extracted from the fuels by tri-\textit{n}-butylphosphate, then purified and reused. Other actinides and fission products with long half-lives remain in the acidic (1 M HNO\textsubscript{3}) and high salinity (4 M NaNO\textsubscript{3}) solutions arising from this process. Some of the products ($^{93}$Zr, $^{107}$Pd, $^{129}$I, $^{135}$Cs, and $^{237}$Np) have half-lives on the order of $10^6$ years. Others like $^{90}$Sr and $^{137}$Cs have half-lives around 30 years, but they account for about 90\% of the heat released by high activity wastes. To this end, researchers are focusing efforts on calixarenes which exhibit high selectivity for strontium, cesium, and actinides over alkali metals using membrane transport\textsuperscript{55,56} and basic extraction and complexation studies\textsuperscript{57,58}.

Calix[4]arene-lanthanide complexes have been synthesized and may find utility as luminescent probes for imaging and bioassays, thus replacing radioimmunoassays.\textsuperscript{59} Typical emission of Eu$^{3+}$ and Tb$^{3+}$ luminescence occurs at 618 nm and 545 nm, respectively, which is spectrally distinct from the fluorescence of most biological systems. The attachment of a sensitizer (preferably with an excitation wavelength above 350 nm) allows the use of standard optics to monitor the emission, shields the ions from the surrounding water, and maximizes the intensity of emitted light.\textsuperscript{60} Reports of calixarene-lanthanide complexation in aqueous systems are geared toward the optimization of complex solubility and enhancement of long-wavelength absorptivity of sensitizers to produce complexes suitable for practical applications.\textsuperscript{61}

Complexation of Eu$^{3+}$ and Tb$^{3+}$ ions in calix[4]arenes are reported to yield highly luminescent species with millisecond lifetimes, three orders of magnitude higher than the background fluorescence. Complexation in a tetraamide derivative of $p$-$t$-butylcalix[4]arene gave a neutral water-soluble system.\textsuperscript{62} The tricarboxylic acid
calix[4]arene derivatives yielded neutral species as well, the lanthanide ions being effectively shielded from the solvent.\(^{63}\) Calixarene derivatives tetra-substituted with carboxylic esters and one aromatic sensitizer group formed charged Eu\(^{3+}\) and Tb\(^{3+}\) complexes and enabled excitation of their luminescence up to 350 nm. Other lanthanide complexes were prepared via complexation of the phenolic oxygen atoms of \(p\)-sulfonated calixarenes, but these complexes were also positively charged.\(^{64}\) To circumvent aspecific binding, neutral complexes are recommended. Other examples of complexes of calix[4]arenes with cations in biological systems show clinical promise. Kinetically stable \(^{81}\)Rb complexes of bridged calix[4]arenes have been synthesized with the ultimate goal of rubidium immobilization for organ imaging.\(^{65,66}\)

After the ability of tetracarboxylic acid calix[4]arenes to extract \(^{225}\)Ac\(^{3+}\) from an aqueous to an organic phase was demonstrated,\(^{67}\) the development of potential calix[4]arene-based radiotherapeutic agents in mice was reported.\(^{68}\) This premier study of bioconjugation of \(^{225}\)Ac\(^{3+}\)-chelating calix[4]arenes revealed that conjugation does not impair immunoreactivity when calixarenes are bound to a mouse monoclonal antibody. Also, immune response was strongly influenced by the carrier type, the dosage, and the injection method used.

The crucial role that weak cation-π interactions play in molecular recognition is exemplified by the complexation of quaternary ammonium cations.\(^{69,70}\) Selective guest inclusion is determined by host preorganization to an extent. For example, conformationally mobile \(p\)-sulfonatocalix[4]arene includes trimethylanilinium (TMA) unselectively. In contrast, one molecule of water bridges the two opposite phenolate oxygens in the lower rim of \(p\)-sulfonatocalix[4]arene-1,3-dicarboxylic acid, fixing it in
the cone conformation. Consequently, the aromatic portion of TMA is selectively included in the calixarene cavity (Figure 1.4). This induced selectivity is also possible in the complexation of the neurotransmitters choline and acetylcholine with the tetrameric and hexameric forms of \( p \)-sulfonatocalixarene through electrostatic interactions and hydrophobic effects.

![Figure 1.4](image-url)

**Figure 1.4** Unselective (A) and selective (B) inclusion of TMA by \( p \)-sulfonatocalix[4]arenes. (\( \text{H}_2\text{O} \) fixes cone conformation)
1.5.2 Complexation of Anionic Guests

Anion recognition has not been as widely explored as cation recognition, because anions possess unique properties that must be considered when synthesizing appropriate receptors. The negative charge is generally delocalized over a number of atoms, and the shape can be spherical, linear, planar, tetrahedral, or octahedral. Complexation in water is a difficult undertaking because anions are strongly hydrated and must be desolvated before binding can occur. Still, the need exists for systems which could sequester anionic species such as phosphates, which are environmental contaminants and important components of biologically significant compounds like ATP. However, few examples of anion complexation by water-soluble calixarenes exist.

To gain an understanding of the roles of peptide-carbohydrate and carbohydrate-carbohydrate interactions in biological processes, a family of calixsugars which are capable of carbohydrate recognition have been synthesized. Attachment of carbohydrate moieties to calix[4]arenes induced water-solubility while retaining the hydrophobic cavity, and was presumed to enhance binding properties of polar chiral substrates. The lone water-soluble derivative formed complexes with charged guests D-glucosamine hydrochloride and anionic dihydrogen phosphate, as evidenced by shifts in the $^1$H NMR spectra. Neutral carbohydrate and $N$-protected amino acids were not complexed. In another report, researchers used a water-soluble azacalix[4]arene to bind anionic carbohydrates. Inositol triphosphate and fructose 1,6-phosphate were included in the cavity, which is larger than a normal calix[4]arene cavity.

Complexation of naphthalenesulfonate derivatives by charged calixarenes was investigated spectroscopically by monitoring their fluorescence. Upon comparison of the
results with those of their hydrophobic analogs, they were reported to complex by inclusion of their hydrophobic portions in the calixarene cavity.\textsuperscript{77,78} Also, a series of $\pi$–metallated calixarenes were synthesized, incorporating positively charged redox active transition metal centers directly onto the calixarene aromatic rings.\textsuperscript{79} X-ray crystal structures confirmed the complexation of tetrafluoroborate, sulfate, and iodide ions.\textsuperscript{80}

\subsection*{1.5.3 Complexation of Neutral Guests}

Most investigations of the complexation of neutral molecules by water-soluble calixarenes were performed during the 1980s.\textsuperscript{9,46} A correlation between binding constants and host-guest complementarity was established by studies of the complexation of aromatic hydrocarbons by water-soluble $p$-carboxyethyl calix[4]arenes.\textsuperscript{33,81} As expected, larger calixarenes were able to include larger aromatic hydrocarbons. Tetrameric calixarenes were not selective and did not form inclusion complexes with compounds as small as naphthalene. In contrast, their hexameric and octameric analogs were found to be selective for and capable of including larger polyaromatic hydrocarbons such as perylene.

The complexation of $p$-sulfonated calix[6]arenes with neutral analytes has also been reported. When the complexation with the neutral species ferrocenylmethanol was compared to those of cationic ferrocenyl derivatives, the binding constant value measured with the neutral guest was slightly lower. This indicates that nonelectrostatic interactions bring about most of the free energy change in the complexation process.\textsuperscript{82}

Comparative studies have been conducted which determined the selectivity for nitriles, ketones, and alcohols by various water-soluble calixarenes (specifically, upper rim $p$-sulfonated, $p$-sulfonated calix[4]arenes with lower rim carboxylic acids, and $p$-$t$-
butylcalix[4]arene tetracarboxylic acids). The \( p \)-sulfonated derivatives complexed with all guests, but the tetracarboxylated \( p \)-\( t \)-butylcalix[4]arene only complexed acetonitrile. In fact, it complexed acetonitrile more efficiently than the other two hosts molecules. The authors surmised that negative interactions between the nitrile dipole and the sulfonate groups caused inefficient complexation with these hosts.\(^\text{83}\) Also, ketone selectivity was exhibited by the \( p \)-sulfonated derivative, whereas its carboxylated form was selective for alcohols, indicating that the charges on the sulfonated hosts assisted in the hydrophobic binding of the guests. More recently, the association constants of \( n \)-alcohols with \( p \)-sulfonated calixarenes were determined by headspace gas chromatography in order to investigate differences between complex formation in calixarenes and cyclodextrins.\(^\text{84}\)

The coupling of calix[4]arenes to cyclodextrins has brought about the construction of new receptor molecules for neutral analyte binding and optical sensing.\(^\text{85}\) Typically, a fluorophore is attached to a cyclodextrin. Sensing is evidenced by the optical change of the fluorophore when it is in competition with an analyte for accommodation in the cyclodextrin cavity. In the case of covalently coupled calix[4]arene-\( \beta \)-cyclodextrin molecules, the fluorophore is attached to the calixarene instead. The calixarene brings about a favorable orientation for intramolecular inclusion of the fluorophore within the \( \beta \)-cyclodextrin and an enlargement of the \( \beta \)-cyclodextrin cavity, thus increasing the hydrophobic surface for analyte binding. Amphiphilic molecules composed of calix[4]arenes coupled to \( \beta \)-cyclodextrins have been reported to complex steroids, terpenes and other neutral natural products, with the sensitivity for steroids being approximately 10 times higher than that for terpenes.\(^\text{86}\) These covalently
coupled molecules possess complexation properties which are different from those of native β-cyclodextrin.

1.6 Comparison to Cyclodextrins

The structures of the simplest calixarenes bear a likeness to those of cyclodextrins, which are composed of oligosaccharide units covalent bound together to form a hydrophobic cavity. Both have been reported to selectively form inclusion complexes with smaller compounds. Cyclodextrins are natural products whereas calixarenes are largely synthetic. With an inner diameter of 5.7 Å and possessing six monomer units, α-cyclodextrin is the smallest. Beta-cyclodextrin is composed of seven monomer units and has an inner diameter of 7.8 Å. The largest one, γ-cyclodextrin, has a diameter of 9.5 Å and is composed of eight monomer units. Similarly, the diameters of calixarenes bearing four, six, and eight units are 3.0, 7.6, and 11.7 Å, respectively. Table 1.2 summarizes some similarities and differences between calixarenes and cyclodextrins.

<table>
<thead>
<tr>
<th></th>
<th>Calixarenes</th>
<th>Cyclodextrins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer units</td>
<td>Phenolic units</td>
<td>oligosaccharides</td>
</tr>
<tr>
<td>Cavity inner diameter (Å);</td>
<td>3.0 ; four</td>
<td>5.7 ; six</td>
</tr>
<tr>
<td>No. of monomer units</td>
<td>7.6 ; six</td>
<td>7.8 ; seven</td>
</tr>
<tr>
<td></td>
<td>11.7; eight</td>
<td>9.5 ; eight</td>
</tr>
<tr>
<td>Cavity properties</td>
<td>Flexible heterogeneous</td>
<td>Rigid homogeneous</td>
</tr>
<tr>
<td>Solubility</td>
<td>Not water-soluble</td>
<td>Water-soluble</td>
</tr>
<tr>
<td>Spectroscopic properties</td>
<td>Strong UV absorption</td>
<td>UV transparent</td>
</tr>
<tr>
<td>Derivatization</td>
<td>Easy</td>
<td>Difficult</td>
</tr>
<tr>
<td>Inherent Chirality</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.7 Chiral Calixarenes

Chiral recognition plays an important role in biological systems. For example, only L-amino acids are constituents of proteins. Hydrogen bonding of the amino acids within a protein contributes to its three-dimensional structure and subsequent functionality. Macrocyclic platforms anchoring amino acids and peptides have been designed to model the organizational, assembly and molecular recognition processes in biological systems.\textsuperscript{87,88} Even though they possess a cavity suitable for molecular recognition, the highly symmetrical structures of simple calixarenes render them achiral. Therefore, in order for calixarenes to be more useful in biomimetic chemistry, they must be chiral.

Chirality can be induced in calixarenes by the introduction of chiral substituents on the rims, by inducing asymmetry by attaching different functional groups on the rims or phenols, and by conformational isomerism. This was first accomplished by Muthukrishnan and Gutsche, who reported the synthesis and circular dichroic characteristics of mono- and di-camphorsulfonyl esters of \textit{p-\textit{t}}-butylcalix[8]arene.\textsuperscript{89} The complexation properties of water-soluble \textit{p}-sulfonated calixarenes bearing (\textit{S})-2-methylbutoxy groups as chiral substituents were also studied.\textsuperscript{90,91} Circular dichroism investigations revealed that conformational fluctuations of hexamers and octamers were minimized upon inclusion of guest molecules, whereas tetramers remained fairly rigid irrespective of guests.\textsuperscript{92} Further study of the hexamers and Auramine O dye revealed that the fluorescence of the dye is enhanced as a result of its association with both the chiral and achiral calixarenes.\textsuperscript{93} However, the achiral calix[6]arene did not restrict the rotation of the diphenylanilnine groups of Auramine O as completely as the chiral calix[6]arene.
did. The chiral hexamer was also shown to discriminate between enantiomers of propranolol.\textsuperscript{94}

Amino acids have also been attached to calixarenes to induce chirality and in some cases water-solubility. First, L-cysteine residues were attached to the upper rims of calix[4]arene and calix[6]arene to yield products which are water-soluble at acidic and basic pH. Fluorescence studies were then conducted which determined that the pH of the medium affected not only the hydrophobicities of the cavities but also their selectivities toward guests.\textsuperscript{95} Calixarenes bearing amino acid functionalities have also shown promise in biological applications. For instance, calix[4]arenes bridged with D- and L-alanine units are reported to be biologically active vancomycin antibiotic mimics.\textsuperscript{96} Other amino acid-derivatized calixarenes are reported to discriminate between binaphthyl atropisomers,\textsuperscript{97} chiral ammonium ions,\textsuperscript{98} and phenylglycinol enantiomers.\textsuperscript{99}

**Part II. Introduction to Molecular Spectroscopy**

Electromagnetic radiation (ER) is a form of energy that moves through space at very high speed. It manifests itself in the forms of gamma-ray, X-ray, ultraviolet, visible, radiant heat, microwave, and radio-frequency radiation, each form varying in wavelength and frequency. It possesses both wave properties and those of discrete particles called photons (packets of energy). The wave model describes ER as electric and magnetic fields which travel from the source at in-phase, sinusoidal oscillations much like waves of water across the surface of a body of water. The fields are at right angles to each other and to the direction of propagation. The electric field is responsible for the majority of physical processes of interest to spectroscopists (including transmission, reflection, refraction, and absorption), whereas the magnetic field is specifically responsible for
absorption of radio-frequency radiation. In this model, multiplication of the frequency of ER by the wavelength will give the velocity of propagation. The frequency of incident ER is independent of the medium through which it passes, but the velocity of ER is not. Consequently, the wavelength of radiation is also dependent upon its velocity, except when it is in a vacuum or air. For those conditions its velocity is equivalent to the speed of light \((3.00 \times 10^8 \text{ m/s})\) and is represented by Equation 1.1

\[
c = \lambda \nu ,
\]

where \(c\) is the velocity of light in a vacuum or air, \(\lambda\) is the wavelength of ER in m, and \(\nu\) is the frequency of ER in hertz \((\text{seconds}^{-1})\).

The particle model describes the permanent energy transfer of ER to the absorbing medium or from the emitting object. A consequence of the photoelectric effect, it depicts ER in a form capable of transferring enough kinetic energy to some electrons on a surface to the extent that they are almost instantaneously released from the surface. The number of electrons released is proportional to the intensity of the incident ER. The relationship of the dual characteristics of ER is expressed by the Einstein-Planck equation (1.2), which proportions the frequency of ER with the net kinetic energy of the ejected photoelectron

\[
E = h \nu ,
\]

where \(E\) is the energy of a single photon of ER in Joules, and \(h\) is Planck’s constant \((6.626 \times 10^{-34} \text{ Joule\cdotseconds})\).

The electromagnetic spectrum qualitatively depicts the position of each major type of radiation based upon its energy, i.e., wavelength, frequency, and interaction with
molecules (Figure 1.5). Although adjacent regions overlap each other, they can be categorized by the methods of generating and detecting the various kinds of radiation.

![Figure 1.5 The electromagnetic spectrum](image)

Spectroscopy is a branch of science which identifies and measures interactions of ER with matter, whether absorbed, emitted, or scattered. The transition of matter from a lower energy state to a higher energy state is the basis of absorption spectroscopy. The matter which is promoted depends upon the frequency of the radiation absorbed. For instance, X-ray radiation oscillates at a high frequency. Therefore, it is energetic enough to excite core electrons. Conversely, lower frequency ultraviolet and visible radiation only excites bonding (valence) electrons. Lowest energy transitions occur in the vibrational and rotational levels of molecules and nuclei and are induced by infrared and radio waves. Emission spectroscopy, on the other hand, deals with the descent of an atom or molecule from a higher energy state to a lower one. This transition is usually
accompanied by the emission of radiant energy, which can be measured by a variety of spectroscopic techniques specific to the frequency of the radiation. Common emission spectroscopies are X-ray fluorescence, inductively coupled plasma, and phosphorescence.

The various processes associated with the absorption and emission of radiation by organic molecules containing an even number of electrons can be graphically represented by the Jablonski diagram (Figure 1.6). The orbital angular momentum of an organic molecule is either of singlet or triplet multiplicity.

![Jablonski diagram](image)

**Figure 1.6  Jablonski diagram**

Multiplicity is determined by the overall spin quantum number $S$ in Equation 1.3,

$$M = 2S + 1,$$

where $S$ is the sum of the electron spins (+1/2 and -1/2). When all the electrons are paired, $S$ equals zero (the sum of +1/2 and -1/2). Therefore, the multiplicity is singlet. When an electron from an occupied orbital is excited to a higher, previously unoccupied
orbital, it may flip its spin. Consequently, the absolute value of S is unity and the multiplicity is triplet. The ground, first, and second singlet electronic states of the molecule are given by $S_0$, $S_1$, and $S_2$ and the first excited triplet electronic state is given by $T_1$ in the diagram. Superimposed on each electronic energy level are vibrational energy levels, denoted by $v_0$, $v_1$, $v_2$, etc.

### 1.8 Absorption

When a solution absorbs electromagnetic radiation emitted from a given light source, the quantity of radiation absorbed follows definite physical laws.  The amount of radiation absorbed by the solution ($A$) is the logarithm of the ratio of the intensity of the incident radiation ($I_0$) and the intensity of radiation which is transmitted through the solution ($I$), described mathematically as

$$A = \log \frac{I_0}{I}.$$  \hspace{1cm} 1.4

The absorbance is linearly related to the concentration of the solution by the Beer-Lambert law, which is expressed as

$$A = \varepsilon bc,$$  \hspace{1cm} 1.5

where $\varepsilon$ is the molar absorptivity, $b$ the light path length, and $c$ the concentration of the solution. Absorptivity is the inherent ability of a chemical species to absorb light and is constant at a given wavelength. The path length is the distance the light travels through the measured solution.

The absorptive process involves a transfer of energy from a photon to a molecule. Electrons are then promoted from the lowest energy level $S_0$ to higher energy levels ($S_1$ and above) called excited states. The energy difference between the two states is equal to the energy of the incident photon. The electronic transition takes place in approximately
10^{15} seconds, which is much faster than nuclear reorganization (>10^{-14} sec). Therefore, the higher energy state is reached without rearrangement of the nuclei. This hypothesis, the Franck-Condon principle, also states that a specific transition with the highest probability in the absorption phenomenon will have the highest probability of the reciprocal transition in the emission phenomenon. After absorption, a molecule can return to the ground state by radiative and non-radiative processes. The radiative processes are fluorescence and phosphorescence, and the non-radiative pathways are vibrational relaxation, internal conversion, intersystem crossing, fluorescence quenching, and other deactivation processes.

1.9 Vibrational Relaxation

When a molecule has achieved an electronically excited state, it undergoes vibrational relaxation in order to return to the lowest vibrational level of the electronic excited state. Vibrating at the frequency characteristic of the excited state, it relinquishes its excess vibrational energy in the form of heat through collisions with other molecules in the excited state and through collisions with solvent molecules. Occurring in the time frame of 10^{-14} to 10^{-12} seconds, it is among the more rapid deactivation processes, allowing thermal equilibration in the excited state prior to radiative processes. It is represented by gray wavy arrows in Figure 1.6.

1.10 Internal Conversion

After vibrational relaxation to the lowest energy level in the highest excited state, energy can only be lost by moving to a lower electronic energy level. When an overlap exists between the lower vibrational levels of the higher electronic level and the higher vibrational levels of a lower electronic level, a crossover from the higher to the lower
excited singlet state is favorable. Such a transition happens in approximately $10^{12}$ seconds, and is represented in Figure 1.6 by a pink wavy arrow between $S_2$ with $S_1$. A slower internal conversion ($10^8$ seconds) can also occur between $S_1$ and vibrational levels in $S_0$, but this is favored when the molecule has a large number of vibrational levels in the lower state, thereby lessening the energy difference between the upper and lower states.

1.11 Intersystem Crossing

Non-radiative transition from the lowest singlet excited state to the triplet state in a manner similar to internal conversion is called intersystem crossing. Represented in Figure 1.6 by a red wavy arrow between $S_1$ and the vibrational levels of $T_1$, it involves the change in spin of the excited electron. Consequently, there is a $10^6$-fold lower probability of this spin-forbidden electronic transition occurring compared to internal conversion. Nevertheless, excited electrons in molecules containing transition-metal ions or heavy atoms such as iodine, almost exclusively deactivate through intersystem crossing. With a lifetime of $10^8$ seconds however, it can compete with fluorescence for deactivation of $S_1$.

1.12 Phosphorescence

Another radiative process, phosphorescence, occurs from the lowest vibrational level of $T_1$ to vibrational levels in $S_0$. It is longer-lived than fluorescence, typically lasting from $10^6$ to 10 seconds because it arises from the spin-forbidden process of intersystem crossing. It is denoted in Figure 1.6 by red downward arrows originating from $T_1$, and it occurs at wavelengths longer than those resulting from fluorescence. Because triplet states are so long-lived, solution phosphorescence is often precluded by
triplet-to-singlet intersystem crossing, solvent collisional deactivation, quenching, photochemical reactions, and energy transfer processes.

1.13 Fluorescence

Also possessing a lifetime on the order of $10^{-8}$ seconds, fluorescence is a radiative process which can compete with internal conversion and intersystem crossing. Demotion of excited electrons from the lowest vibrational level of $S_1$ to the vibrational levels of $S_0$ may come about by the emission of visible or ultraviolet light, i.e., fluorescence. Very rarely does this process take place between two electronic states other than $S_0$ and $S_1$. Shown in Figure 1.6 by green downward arrows, the wavelength of the emitted light is longer than that which is initially absorbed, because of energy dissipation by vibrational relaxation and other competing processes. The energy of the emitted photon is equivalent to the difference in energy between $S_1$ and the vibrational level of $S_0$ to which the transition occurs. Characteristics such as spectral position and shape, and environmental factors affecting fluorescence appear in subsequent sections of this chapter.

1.13.1 Characteristics of Fluorescence Emission

Emission of a photon after relaxation of an electronically excited molecule from the lowest singlet excited state to the ground state is called fluorescence. Typically identical to the absorption spectrum, the fluorescence excitation spectrum is a plot of the relative efficiency of exciting radiation at various wavelengths to produce emission at a given wavelength. It can be obtained by fixing the emission wavelength even though its shape is independent of the monitored emission. The fluorescence emission spectrum is a plot of the relative intensity of emitted radiation as a function of emission wavelength (nm). It is dependent upon the chemical structure and the environment of the molecule,
but it is typically independent of the excitation (absorption) wavelength. Vibrational relaxation, solvent effects and excited state reactions cause a loss of energy when the molecule is in the excited state, thus causing fluorescence to occur at longer wavelengths than absorption. This is the basis of the Stokes shift phenomenon which is illustrated in Figure 1.7 and can be estimated by:

\[
\text{Stokes Shift} = 10^7 \times \left( \frac{1}{\lambda_{\text{ex}}} - \frac{1}{\lambda_{\text{em}}} \right),
\]

where \( \lambda_{\text{ex}} \) and \( \lambda_{\text{em}} \) are the wavelengths (in nm) of maximum excitation and emission, respectively.\(^{101}\)

**Figure 1.7**  **Mirror Image Rule and Franck-Condon Principle**

Excitation \( (S_0 \rightarrow S_1) \) and emission \( (S_1 \rightarrow S_0) \) spectra are generally mirror images of each other because they represent reciprocal transitions. Recall that the Franck-Condon principle states that the position of the nuclei does not change during an excitation or emission event. Furthermore, if the vibrational energy levels are equally separated in
both the ground and lowest excited singlet states of the molecule, the same transitions are involved for both excitation and emission events. For example, the \((S_0)_0 - (S_1)_2\) absorption will most probably result in the \((S_1)_0 - (S_0)_2\) emission. Examples of such transitions are shown by vertical arrows in the potential energy distance diagram in Figure 1.7. Polycyclic aromatic hydrocarbons generally obey the mirror image rule, but deviations occur when nuclear displacements or chemical reactions occur in the excited state.

### 1.13.2 Fluorescence Kinetics

Because two other deactivation processes (internal conversion and intersystem crossing) occur along the same time frame as fluorescence, not all molecules possessing fluorescent potential will actually do so to return to the ground state. Therefore, two parameters can be employed to measure fluorescence with respect to the other processes. The fluorescence quantum yield, \(\Phi_F\), is the fraction of excited molecules that fluoresce, and the lifetime, \(\tau\), is the average time that an excited molecule will spend in the excited state. Both parameters are dependent upon changes in the chemical environment around the molecule, such as solvent, temperature, viscosity, or quencher concentration.

Fluorescence efficiency or quantum yield, \(\Phi_F\), is the ratio of the number of photons emitted to the total number of excited molecules. It is determined by the relative rate constants of the competing processes by the following equation:

\[
\Phi_F = \frac{k_F}{k_F + k_{IC} + k_{ISC} + k_{VR} + k_Q[Q]}, \quad 1.7
\]

where \(k_F\), \(k_{IC}\), \(k_{ISC}\), \(k_{VR}\), and \(k_Q\) are rate constants for fluorescence, internal conversion, intersystem crossing, vibrational relaxation, and quenching, respectively. The term \([Q]\) is
the concentration of quencher, which is a species which reduces the intensity of fluorescence in a solution. When $\Phi_F$ approaches zero, nonradiative processes are more prevalent. Conversely, when $\Phi_F$ approaches unity, fluorescence is the dominate process.

The mean fluorescence lifetime ($\tau$) is the reciprocal of the sum of the rate constants of all deactivation processes listed in Equation 1.7. The reciprocal of $k_F$ is called the intrinsic lifetime ($\tau_0$), which describes the lifetime of fluorescence in the absence of the other deactivation processes:

$$\tau = \frac{1}{k_F + k_{IC} + k_{ISC} + k_{VR} + k_Q[Q]}, \quad 1.8$$

$$\tau_0 = \frac{1}{k_F}. \quad 1.9$$

Combining Equations 1.7 and 1.8 produces

$$\Phi_F = k_F \times \tau. \quad 1.10$$

The relationship between quantum efficiency and lifetime is thus established by combining Equations 1.9 and 1.10

$$\Phi_F = \frac{\tau}{\tau_0}, \quad 1.11$$

which suggests that a decrease in the lifetime results in a decrease in quantum efficiency. In general, when more processes are competing with fluorescence, the shorter the actual lifetime.

### 1.13.3 Fluorescence Intensity and Concentration (Revisiting Beer-Lambert Law)

The combination of Equations 1.4 and 1.5 give a representation of the Beer-Lambert law, which establishes the relationship between fluorescence intensity and solution concentration.
\[ \frac{I}{I_0} = 10^{-\epsilon_{ebc}}. \]  \hspace{1cm} 1.12

Since Equation 1.12 represents the fraction of light intensity transmitted after excitation, then the fraction of light absorbed is given by

\[ 1 - \frac{I}{I_0} = 1 - 10^{-\epsilon_{ebc}}. \]  \hspace{1cm} 1.13

Multiplying Equation 1.13 by \( I_0 \) yields

\[ I_0 - I = I_0 (1 - 10^{-\epsilon_{ebc}}). \]  \hspace{1cm} 1.14

Since the fluorescence has a quantum efficiency, the fraction of absorbed light that appears as fluorescence (F) is

\[ F = \Phi_p (I_0 - I) = \Phi_p I_0 (1 - 10^{-\epsilon_{ebc}}). \]  \hspace{1cm} 1.15

The exponential term \( 1 - 10^{-\epsilon_{ebc}} \) can be expanded through the Taylor’s series to give

\[ 1 - 10^{-\epsilon_{ebc}} = 2.3\epsilon_{ebc} - \frac{(2.3\epsilon_{ebc})^2}{2!} + \frac{(2.3\epsilon_{ebc})^3}{3!} - \frac{(2.3\epsilon_{ebc})^4}{4!} + \ldots \]  \hspace{1cm} 1.16

If \( \epsilon_{ebc} \) is less than 0.05, then the higher order terms are negligible. Therefore, fluorescence intensity is related to concentration by

\[ F = 2.3\Phi_p I_0 \epsilon_{ebc}. \]  \hspace{1cm} 1.17

1.13.4 Environmental Influences on Fluorescence

The degree of fluorescence a molecule exhibits is largely influenced by solvent characteristics such as composition, polarity, temperature, and pH.\footnote{103} The structure of the molecule and its concentration are also important factors in the fluorescence phenomenon. These chemical and physical properties not only make it a powerful technique for the elucidation of molecular structure, but also for the detection of impurities within a given sample.
The choice of an appropriate solvent is extremely pertinent to the impact which competing deactivation processes have on fluorescence. The electrostatic properties of solvents, namely dipole-dipole and hydrogen bonding, determine their mode of solvation of potentially fluorescent species, thereby influencing their electronic spectra. When the solute absorbs light, its electronic dipole moment becomes different from that of its ground state. However, the solvent molecules have yet to reorient themselves to accommodate the dipolar change, according to the Franck-Condon principle. Upon solvent reorientation, the energy of the excited electronic state is lowered, creating a metastable state. When fluorescence occurs, the solute achieves a metastable ground state. Upon solvent reorientation, the energy of the state falls to its original ground state. The energy losses due to solvent reorientation are reflected in the magnitude of the Stokes shift. With increasing solvent polarity and hydrogen-bonding capacity, such solvent relaxation processes shift fluorescence to longer wavelengths.

The temperature and viscosity of the solvent also influence fluorescence. As the temperature increases, viscosity decreases. This causes an increase in the number of molecular collisions which results in more nonradiative deactivation versus fluorescence. Conversely, decreases in temperature will increase the viscosity of the solvent, thereby decreasing the number of molecular collisions in the excited state, allowing more molecules to fluoresce.

The pH of the solution influences the rate of protonation and deprotonation of a fluorophore with acid/base properties. When the acidity or basicity of a molecule is changed in the excited state, its electron distribution will be different from that in the ground state. Consequently, these chemical reactions compete with fluorescence and
often result in multiple fluorescence bands originating from a single excited species. The spectra may also vary with the concentration of proton donors or acceptors, resulting in quantum yields and lifetimes which vary with pH. A shift in fluorescence to longer wavelengths (red shift) results from protonation of electron-withdrawing groups of the fluorophore, indicating an increase in basicity upon excitation. In contrast, a blue shift results from protonation of electron-donating groups, indicating a decrease in basicity.

The structure of a molecule is most important in determining its fluorescent character. Chromophores and multiple conjugated arrays endow a molecule with increased π electron mobility and consequential resonance stability. Such structures exhibit fluorescence, the degree of which increases with the extent of conjugation and resonance stability. Whereas aliphatic molecules do not normally fluoresce, rigid aromatic molecules with planar structures exhibit high fluorescence. Aromatic molecules that contain heteroatoms produce weak fluorescence, if any, because there is a greater probability that intersystem crossing will occur. This can be attributed to overlap of vibrational bands in $S_1$ and $T_1$, attributed to spin-forbidden transitions such as n-π*.

Typically phosphorescence is favored in this case. Electron-donating groups tend to enhance fluorescence, whereas electron-withdrawing substituents decrease fluorescence. Heavy atoms such as bromine or iodine tend to cause a mixing of singlet and triplet states, thereby increasing the rate of intersystem crossing.

Inner-filter effects produce a reduction in the observed fluorescence intensity in two ways. First, the amount of radiant power available to excite the fluorophore may be decreased by the presence of an artifact which absorbs in the same wavelength as the analyte. Secondly, the artifact may absorb at the wavelength at which the analyte
fluoresces, thus causing a decrease in emission. It is also interesting to note that even too high a concentration of the analyte of interest can produce self-absorption. This leads to a nonlinear negative deviation in the concentration-response plot.

1.14 Fluorescence Instrumentation

The main components of a fluorometer are an excitation source, monochromators or wavelength selectors, a sample holder, a detector, and a recorder.

![Schematic diagram of a typical fluorometer](image)

**Figure 1.8  Schematic diagram of a typical fluorometer**

Light from the excitation source passes through an excitation monochromator, which transmits radiation at a wavelength that will induce fluorescence in the sample. The photons signifying the fluorescence event are emitted in all directions, but are observed at right angles to the excitation beam. This right angle configuration eliminates any interference from the transmitted light from the source. Increased photon scattering from
the solution and the walls of the sample holder could result in errors in the measurement of intensity if fluorescence would be observed at other angles. After passing through the sample, the emitted light passes through the emission monochromator which selects the fluorescence peak for measurement. The fluorescence is then detected by a suitable detector (e.g., photomultiplier tube) and the data recorded on a computer.

1.15 Nuclear Magnetic Resonance Spectroscopy

As previously discussed, radiation of different wavelengths and frequencies can induce a variety of transitions in molecules. The nuclear magnetic resonance (NMR) event involves nuclear spin energy transitions occurring in a magnetic field. These transitions are caused by the absorption of radio-frequency ER, and are governed by the characteristics of certain nuclei in the sample. These nuclei, along with the influence of the molecular environment on their absorption, can be correlated with molecular structure. A brief discussion follows.

Just as the spinning electrons of a given atom have two allowed spin states, $+\frac{1}{2}$ and $-\frac{1}{2}$, some nuclei have spin with allowed spin states of $+\frac{1}{2}$ and $-\frac{1}{2}$. As a consequence, the nuclei have angular momentum which can be described in terms of the spin quantum number ($I$). This quantum number determines the number of orientations a nucleus can have in an applied magnetic field by the formula $2I + 1$. The quantum number can be determined by the atomic mass and the atomic number of a particular atom. Generally speaking, nuclei with an odd atomic mass number have half-integral spin. Those with an even mass number and an odd atomic number have integral spin. Those with an even mass number and atomic number have zero spin. Values of $I$ for several nuclei are displayed in Table 1.3.
Table 1.3 Spin Quantum Numbers of Various Nuclei

<table>
<thead>
<tr>
<th>$I$</th>
<th>Atomic Number</th>
<th>Atomic Mass</th>
<th>Isotopes ($I$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>Even</td>
<td>Even</td>
<td>$^4$He (0), $^{12}$C (0), $^{16}$O (0), $^{32}$S (0)</td>
</tr>
<tr>
<td>Half-integer</td>
<td>Even or Odd</td>
<td>Odd</td>
<td>$^1$H (1/2), $^{13}$C (1/2), $^{17}$O (5/2)</td>
</tr>
<tr>
<td>Integer</td>
<td>Odd</td>
<td>Even</td>
<td>$^2$H (1), $^{14}$N (1)</td>
</tr>
</tbody>
</table>

The circulation of nuclear charge in nuclei possessing spin generates a magnetic dipole along the nuclear axis. The magnitude of the dipole is expressed in terms of magnetic moment, $\mu$. In the absence of an external magnetic field, the orientations of magnetic moments are random. When a magnetic field is applied, however, the magnetic moments of the nuclei orient themselves either in the same direction as the field or opposite that of the field. Recalling $2I + 1$, there are two possible orientations (or quantum states) that a nucleus with $I$ equal to $1/2$ can have in a magnetic field. They are either $+1/2$ which is aligned with the field (called the $\alpha$-spin state) or $-1/2$ which is aligned against the field (β-spin state) (Figure 1.9).

![Figure 1.9](image.png)

**Figure 1.9** Generation of α- and β-spin states upon application of magnetic field

The energy of the $\alpha$-spin state is lower than that of the $\beta$-spin state. When a nucleus in the $\alpha$-spin state absorbs enough radiation, it will flip its spin and enter the $\beta$-spin state.
This energy difference between the two states is a function of the magnitude of the magnetic field \((B_0)\). The greater the strength of the applied magnetic field, the greater the difference in energy is between the states. This energy difference \((\Delta E)\) is given by

\[
\Delta E = \frac{h\gamma}{2\pi} B_0 ,
\]

1.18

where \(B_0\) is the magnitude of the applied magnetic field measured in tesla (T), \(\gamma\) is the magnetogyric ratio (a proportionality constant between \(\mu\) and \(I\); units of radian T\(^{-1}\) s\(^{-1}\)), and \(h\) is Planck’s constant. The size of \(\gamma\) depends on the magnetic properties of the nucleus. Recall that \(\Delta E = hv\). Therefore,

\[
v = \frac{\gamma}{2\pi} B_0 ,
\]

1.19

which is called the Larmor frequency, establishing the relationship between \(\Delta E\) and \(B_0\). So, not only can one obtain the frequency of radiation required to bring about the absorption, but also detect the absorption event in which nuclei flip their spins back and forth in response to the applied radiation. For example, if a magnet in an NMR instrument provides a field strength of 4.69 T, the hydrogen nuclei \((\gamma = 2.68 \times 10^8\) rad T\(^{-1}\) s\(^{-1}\)) would require an energy source of 200 MHz to flip their spins. Likewise, for carbon nuclei \((\gamma = 6.73 \times 10^7\) rad T\(^{-1}\) s\(^{-1}\)) to flip their spins, a 50 MHz energy source would be needed. At these given values, the systems are said to be in resonance, i.e., nuclei absorb energy and are raised to a higher energy state. This flipping manifests itself in the form of a signal in the NMR spectrum.

As previously stated, the energies of the \(\alpha\)- and \(\beta\)-spin states are not identical. Furthermore, there is a slight excess of nuclei in the \(\alpha\)-spin state. To determine the extent to which those in the \(\alpha\)-spin state predominate, the Boltzmann equation can be used
\[
\frac{N_j}{N_o} = \exp\left(\frac{-\gamma h B_0}{2\pi k T}\right),
\]

where \(N_j\) and \(N_o\) are the number of protons in the \(\beta\)-spin state and \(\alpha\)-spin state, respectively, \(k\) is the Boltzmann constant (1.38x10\(^{-23}\) J K\(^{-1}\)), and \(T\) is the absolute temperature. For instance, if 10\(^6\) protons were in the \(\beta\)-spin state of a sample placed in a 4.69 T field at 293 K, there would be a 33 part per million excess of protons in the \(\alpha\)-spin state. Although this is a small difference, it is the basis of NMR spectroscopy. Without this excess, there would be no net absorption of radiation to observe.

From Equation 1.19 it is established that a proton absorbs energy when \(\Delta E\) between the \(\alpha\)- and \(\beta\)-spin states equals the energy defined by the frequency of the spectrometer. If all nuclei were in the same environment, the same \(B_0\) would be required to cause \(\Delta E\) to match the operating frequency of the NMR spectrometer. This would result in NMR spectra with only one absorption signal, which would not be sufficient for structure determination. Instead, nuclei experience only a portion of \(B_0\) because their electron cloud and their position in a molecule in relation to other nuclei defines their environments. These environments shield them from \(B_0\) in varying degrees, thus allowing the analyst to determine where the nuclei are within a compound. Nuclei that are less shielded from \(B_0\) sense more of it to achieve \(\Delta E\). Those nuclei which are more shielded require a higher \(B_0\) to achieve \(\Delta E\).

### 1.15.1 Absorption and Relaxation in NMR

Consider an external stationary magnetic field \(B_0\) whose axis is oriented in the positive \(z\)-direction. When this field is applied to a nucleus spinning on its own magnetic
axis, the magnetic axis of the nucleus precesses around the $z$ axis of the external field (Figure 1.10).

![Precessional Orbit]

**Figure 1.10 Precession of nucleus in external magnetic field $B_0$**

The frequency of this precession is equal to the magnetic field strength $B_0$ times the magnetogyric ratio. The potential energy of the precessing nucleus is given by

$$E = -\mu B_o \cos \theta,$$

where $\mu$ is the magnetic dipole of the nucleus and $\theta$ is the angle at which $\mu$ precesses from the magnetic dipole of the external magnetic field. So when the nucleus absorbs radio-frequency radiation, $\theta$ changes. For a nucleus possessing spin of $\frac{1}{2}$ or $-\frac{1}{2}$, the absorption causes a flipping of the magnetic moment to the opposite direction. The nucleus is then allowed to relax back to its original position.

Relaxation of a precessed nucleus is accomplished by two processes. The first process is called the spin-lattice or longitudinal relaxation process. It is characterized by a time, $T_1$, which is the average lifetime of the nuclei in the excited state. In this process,
energy is transferred from the excited nuclei to surrounding nuclei which are in violent vibrational and rotational motion, thereby increasing the amplitude of their thermal vibrations or rotations. This time depends not only upon the magnetogyric ratio, but also the mobility of the atoms in the sample. The second process is called spin-spin or transverse relaxation and is designated as $T_2$. It involves a transfer of energy between neighboring nuclei having identical precession rates but different magnetic quantum states. Although there is no net change in the spin state population, the average lifetime of the excited nucleus is shortened. This usually results in line broadening.

**1.15.2 Pulsed Fourier Transform NMR**

When short pulses of intense radio-frequency radiation are applied to nuclei in a strong magnetic field, a time-domain, radio-frequency signal is emitted as nuclei return to their original state. This signal is called the free induction decay (FID). The length of the pulses is on the order of $\mu$s, and the interval between pulses is on the order of seconds. The FID signal is representative of the difference between the frequency of the pulse and the frequencies of all the nuclei as they relax. The signal approaches zero by decreasing exponentially as relaxation progresses. A radio-receiver coil orthogonal to the stationary magnetic field detects the FID signal, which is then converted to a frequency-domain signal by Fourier transformation. The resulting NMR spectrum is therefore a plot of the absorption as a function of frequency.

**1.15.3 Chemical Shift**

Nuclei experience the effects of small magnetic fields generated by electron clouds which either oppose or reinforce the external magnetic field. When the electron cloud opposes the external magnetic field, the nucleus is said to be shielded from the
influence of the field. Conversely, the nucleus is deshielded from the magnetic field when the electron cloud reinforces the external magnetic field. The magnitude of the electron cloud defines the chemical environment of the nucleus, thereby affecting the frequency of its absorption. Each type of nucleus may have a slightly different frequency depending upon what it is bonded to. The difference between the absorptive frequency of a nucleus in a particular chemical environment and that of a reference standard divided by the frequency of the spectrometer is the chemical shift. It is usually expressed in parts per million (ppm) because of the large difference between the frequency of the spectrometer (MHz) and that of the chemical shift range (Hz). In proton NMR measurements, tetramethylsilane (TMS) is normally used as the reference standard. Chemical shifts can be expressed in hertz as long as the applied frequency is specified. Alternatively, they can be expressed in dimensionless units (δ), by multiplying the quotient of the resonance frequency (Hz) and the applied frequency (Hz) by 10^6. The identification of functional groups and the determination of structural arrangements based upon chemical shifts has been tabulated previously. The nature of the solvent and the concentration of the nuclei of interest may cause slight shifts in δ, but these tables give a general idea of their assignments.

1.15.4 Spin-Spin Coupling

When the spin of a neighboring nucleus influences the resonance behavior of another, a splitting pattern is observed in the NMR spectrum. Basically, nuclear spins are indirectly coupled to each other by the intervention of bonding electrons. For two nuclei with spins I_a and I_b, the resonance of spin I_a is split into 2I_b+1 peaks of equal intensity. Likewise, the resonance of spin I_b is split into 2I_a+1 peaks of equal intensity. The
spacing between the split peaks is measured in Hz and is called the coupling constant \((J)\). In simple NMR experiments, coupling is not observed past three bonds. Coupling through two bonds is called geminal coupling \((^2J)\), and coupling through three bonds is called vicinal coupling \((^3J)\). Figure 1.11 illustrates \(^3J\) coupling in the simulated spectrum of a compound with hydrogens in significantly different environments. One hydrogen (HA) is bonded to a carbon which is attached to electronegative substituents. Therefore, it will have resonance at a higher frequency than the other hydrogen (HB). Because they are neighboring hydrogens, however, each one’s resonance will be affected by the other, thus creating the doublet pairs whose peaks are separated by the value of the coupling constant, \(J\). Other nuclear couplings occur through \(\pi\) electrons, long-range (>3 bonds), and through space and are determined with multidimensional NMR techniques.105

Figure 1.11  Spin coupling of neighboring nuclei in different environments

1.15.5 Two-dimensional NMR Techniques

Two-dimensional NMR can be utilized to enhance the interpretation of the information in more complex NMR spectra by determining the coupling relationships among the atoms in a molecule. A second time domain is created by the introduction of a
second pulse of radio-frequency radiation. The time delay between pulses is called the evolution time. Spectra are arranged orthogonally to one another, and correlations are made in a manner similar to the assignment of Cartesian coordinates. Correlation spectroscopy (COSY) establishes proton-proton connectivity through up to three bonds. Long range COSY establishes connectivity through four bonds, and total correlation spectroscopy (TOCSY) through even longer ranges. Nuclear Overhauser enhancement spectroscopy (NOESY) establishes through-space interactions between protons within a distance of $\leq 5$ Å from each other. Heteronuclear multiple quantum coherence (HMQC) identifies $1^J$-coupling relationships between carbons (or other nuclei) bearing hydrogens. Heteronuclear multiple bond coherence (HMBC) identifies proton-carbon long range couplings and is useful in assigning quaternary carbons.

1.15.6 Solid-State NMR

In liquid-state NMR, fast molecular tumblings average out the anisotropies in the local fields. The result is well-resolved NMR spectra. In contrast, the anisotropies in the local fields are not averaged out in solid-state NMR. In fact, the spectral lines in early solid-state studies were broadened to the extent that no spectral resolution was observed except for isolated spin pairs or methyl groups. Consequently, the measurement of nuclear relaxation times as a function of the external field or the temperature was the focus of early solid-state NMR studies. Information such as motions of a part of the molecule such as the rotations of methyl groups or motions of parts of the molecular chains in polymers were acquired from these broad, featureless spectra.

The main approaches to achieve line narrowing in solid-state NMR impose artificial motions on a solid. The technique of magic angle spinning (MAS) was
introduced by Andrew et al.\textsuperscript{106} and Lowe\textsuperscript{107} as a means to suppress anisotropic interactions which cause line broadening. In this technique, the sample is rotated about an axis which is 54.7° with respect to the external magnetic field. Motion at this “magic angle” reduces dipolar interactions to zero while retaining isotropic chemical shift information. Artificial motion has also been induced via special radiofrequency pulse sequences which eliminate the decay due to dipolar interaction and partially eliminate the decay due to the chemical shift.\textsuperscript{108,109} Therefore, after Fourier transformation, a spectrum which contains only chemical shift information is generated. For abundant spin systems such as protons, the spectra still lack resolution due to the remaining dipolar interactions. Multiple-pulse programs have thus been combined with MAS to address this issue, thereby affording spectra with narrow lines determined by the isotropic chemical shift information only. The CRAMPS (combined rotation and multiple-pulse spectroscopy) method has even been reported to yield proton spectra which have resolution comparable to that of liquids.\textsuperscript{110}

All proton signals are not sufficiently resolved by the above techniques due to the relatively small chemical shift range for many compounds. More detailed information can be obtained for rare-spin nuclides such as $^{13}$C and $^{29}$Si because of their large chemical shift ranges. However, their NMR signals are very weak because they have small magnetogyric ratios and low natural abundances. Signal averaging is time-consuming because the relaxation times of these nuclides are very long. Pines et al.\textsuperscript{111} used cross-polarization (CP) to obtain $^{13}$C spectra in solids in a considerably shorter time. With this method, the signals are increased and the measuring time necessary to obtain a reasonable spectrum is reduced considerably, because the waiting time between successive
experiments can be decreased. Combining the CP method with MAS removes chemical shift anisotropies and results in high-resolution spectra only when combined with dipolar decoupling.\textsuperscript{112} An example of resolution differences between NMR techniques is shown in Figure 1.12. No signal would be detected from a solid sample subjected to solution conditions such as a single-pulse Fourier transform experiment. A signal would be acquired with a CP experiment, but chemical shift anisotropy patterns may overlap, resulting in line broadening. The isotropic chemical shifts can be retained and line broadening could be removed by employing CP-MAS. The solution-state $^{13}$C spectrum of the compound dissolved in an NMR solvent is included for comparison of resolution.

\textbf{A.} \hspace{2cm} \textit{Solid sample; solution conditions}

\textbf{B.} \hspace{2cm} \textit{CP solid state spectrum}

\textbf{C.} \hspace{2cm} \textit{CP-MAS solid state spectrum}

\textbf{D.} \hspace{2cm} \textit{Solution state spectrum}

\textbf{Figure 1.12} Carbon-13 NMR spectra obtained by different methods: A) solid sample under solution-state NMR single pulse experiment; B) cross polarization solid-state NMR; C) cross polarization magic angle spinning solid-state NMR; D) solution state NMR spectrum of compound dissolved in suitable solvent.
1.15.7 NMR Instrumentation

The main components of an NMR spectrometer are a magnet, radiofrequency transmitters, radio-frequency receivers/detectors, and a computer for Fourier transformation. A schematic diagram of a simple NMR spectrometer is presented in Figure 1.13.

![Schematic of NMR spectrometer](image)

**Figure 1.13 Schematic of NMR spectrometer**

The north and south poles of the magnet face each other. Precise scanning of the magnetic field is facilitated by a pair of coils positioned parallel to the magnet poles. The radiofrequency transmitter emits a precise radio frequency which can be amplified if necessary. Two designs exist for the radiofrequency receiver/detector: 1) a coil wrapped around the sample tube as the transmitter, and a second coil in an orthogonal relationship to the transmitter coil as the detector; and 2) a single coil wrapped around the sample tube which acts as the transmitter and the receiver. Once the signal is received, it is Fourier transformed from a time domain into a frequency domain by the computer.
Capillary electrophoresis (CE) is a collective term for the family of separation techniques which have arisen from the merger of electrophoresis and chromatography principles. Chromatography is a general term for techniques which separate components in a mixture on the basis of their differential partitioning between a stationary phase and a mobile phase. In electrophoresis, the separation of charged molecules is accomplished on the basis of their mobilities in an electric field. The separation principle of CE is electrophoretic, based upon the differential mobilities of analytes in an electric field. Its chromatographic component stems from the use of a miniature column (termed “capillary”) as the separation platform, and the use of a mechanical pump to introduce substances onto the column. When a packed column is used, the mechanical pump facilitates the movement of substances within the column. The uniqueness of CE stems from its miniature size. The inner diameter of the capillary is 25 to 100 µm, which allows for efficient heat dissipation and the use of high voltage to drive the separation. Consequently, only nanoliter and milliliter quantities of reagents are used. Furthermore, detection is accomplished in the same capillary in real time.

1.16 CE Instrumentation

The instrumentation of CE is relatively simple (Figure 1.14), consisting of a separation capillary placed between two electrode reservoirs filled with buffer solution. Platinum electrodes connect the high voltage power supply. The sample is introduced in the capillary, and upon application of a voltage, buffer and analyte ions migrate toward the opposite end of the capillary. Analyte zones are analyzed by a detector at the
opposite end of the capillary, where a section of the polyimide coating on the capillary has been removed to make a detector window.

Figure 1.14  Diagram of CE Instrumentation

The high voltage supply has a range of 0 to 30 kV. Samples can be introduced on the capillary either by hydrodynamic injection (application of external pressure) or by electrokinetic injection (application of electric field). Detection systems which have been used in CE are absorption, fluorescence, conductivity, electrochemical reaction, refractive index, NMR and mass spectrometry.

In CE, the mobility of analytes in solution is based upon their charge-to-size ratio. In addition, the velocity of these analytes depends upon the strength of the applied electric field, properties of the solution (viscosity, temperature, pH, and ionic strength), and properties of the analytes themselves (mass, three-dimensional structure, and charge). Fundamental parameters in CE are the migration time ($t_m$), electrophoretic mobility ($\mu_{ep}$, cm$^2$/V$\cdot$s), electrophoretic velocity ($v_{ep}$, cm/s), and the field strength ($E$, V/cm). The
migration time is defined as the time an analyte takes to move from the beginning of the capillary to the point of detection.\textsuperscript{113} The determination of $E$, $v_{ep}$, and $\mu_{ep}$ is accomplished by the following equations:

$$E = \frac{V}{L_t}, \quad 1.22$$

$$v_{ep} = \frac{L_d}{L_m}, \quad 1.23$$

$$\mu_{ep} = \frac{v_{ep}}{E}, \quad 1.24$$

where $V$ is the applied voltage, $L_t$ is the total capillary length, and $L_d$ is the effective capillary length (capillary length from injection end to detection window). Equation 1.24 defines the observed (or apparent) mobility of an analyte in the capillary. The actual mobility is independent of voltage and capillary length and can be determined by subtracting the mobility of the electroosmotic flow (EOF) from the apparent mobility.

The EOF is the flow of liquid in the capillary resulting from the effect of the applied electric field on the interior of the capillary wall. The capillary is made of fused silica whose surface silanol groups are deprotonated at pH values above two. Cations from the bulk liquid (buffer) build up in two layers (stern and diffuse) near the surface of the negatively charged capillary in to maintain a charge balance. The stern layer is comprised of those cations tightly adsorbed to the wall (Figure 1.15), whereas the diffuse layer contains loosely bound cations. The potential difference created by this double layer is called the zeta potential ($\zeta$). Upon the application of voltage, the cations in this layer are attracted to the cathode.
The movement of these solvated cations drags the bulk solution toward the cathode also, thus creating EOF. The magnitude of the EOF can be expressed mathematically as follows

\[ v_{\text{EOF}} = \mu_{\text{EOF}} E = \frac{\varepsilon \zeta}{\eta} E, \tag{1.25} \]

where \( v_{\text{EOF}} \) is the velocity of the EOF, \( \mu_{\text{EOF}} \) is the mobility of the EOF, \( \varepsilon \) is the solution dielectric constant, \( \zeta \) is the zeta potential, \( \eta \) is the solution viscosity, and \( E \) is the applied electric field strength. Because \( \zeta \) is pH dependent, the magnitude of the EOF is also pH dependent.

In contrast to the parabolic flow resulting from pressure driven systems, the flow profile of the EOF is flat because the driving force of the flow is along the capillary
walls. This is beneficial because it allows all of the analyte molecules to experience the same velocity component. Therefore, analytes elute in narrow zones, giving high efficiency peaks in the electropherogram.

1.17 Efficiency in CE

The flat flow profile of CE makes the achievement of high peak efficiency relatively simple. The peak efficiency of capillary electrophoresis can be expressed as the number of theoretical plates, \( N \), given by

\[
N = \left( \frac{L_d}{\sigma} \right)^2,
\]

where \( L_d \) is the effective capillary length and \( \sigma \) is the standard deviation of the peak. Dispersion, spreading of the solute zone, is a common variable in chromatography and CE. Because of the small inner diameter of the capillary in CE, dispersive forces such as molecular diffusion, Joule heating, injection plug length, and solute-wall interactions can significantly impact the separation if not properly controlled. Assuming molecular diffusion to be the only contributor of dispersion in CE, the efficiency can be related to molecular diffusion by

\[
\sigma^2 = 2Dt = 2D \frac{L_d L_{e}}{\mu_{ep} V},
\]

where \( D \) is the diffusion coefficient of the solute, and \( V \) is the applied voltage. Substitution of Equation 1.27 into Equation 1.26 yields

\[
N = \frac{\mu_{ep} VL_d}{2 DL_{e}} = \frac{\mu_{ep} EL_d}{2 D},
\]

The number of theoretical plates can also be determined directly from the electropherogram using
\[ N = 5.54 \left( \frac{t}{w_{0.5}} \right)^2, \]  

where \( t \) is the analyte migration time and \( w_{0.5} \) is the peak width at half height. However, this equation only applies to Gaussian peaks.

1.18 Resolution

How well adjacent peaks are separated from each other is termed resolution (R). Resolution is mathematically expressed as

\[ R = \frac{2(t_2 - t_1)}{w_1 + w_2}, \]  

where \( t_1 \) and \( t_2 \) are the migration times of the former and latter eluting solutes, respectively, and \( w_1 \) and \( w_2 \) are the peak widths of the former and latter eluting solutes, respectively. Resolution as it relates to efficiency is given by

\[ R = \frac{N^{0.5}}{4} \frac{\Delta \mu}{\mu_{avg}}, \]  

where \( \Delta \mu \) is the difference in mobility between two species, and \( \mu_{avg} \) is the average mobility of the two. Substituting Equation 1.28 into Equation 1.31 yields a resolution equation that does not require the calculation of \( N \). It also describes the influence of the EOF on resolution:

\[ R = \left( \frac{\Delta \mu}{4\sqrt{2}} \right) \left( \frac{V}{D(\mu_{avg} + \mu_{EOF})} \right)^{0.5}. \]  

1.19 Migration in CE

The EOF velocity affects the movement of all species in the capillary. Regardless of charge, all species are moved in the same direction as the EOF. If the flow is from anode to cathode, anions will be moved towards the cathode because their electrophoretic
mobilities are less than that of the EOF. As previously mentioned, analytes are separated in CE on the basis of their charge-to-size ratios. This means that those analytes with higher charge-to-size ratios have comparatively higher mobilities and velocities. Therefore, the migration of cations will be fastest, neutrals will migrate at the velocity of the EOF, and anions will migrate slowest. This process is illustrated in Figure 1.16.

![Figure 1.16  Solute migration in CE](image)

Because neutral analytes do not have electrophoretic mobility, they are swept to the detector by the EOF. Therefore, they cannot be separated from each other using capillary zone electrophoresis. Either the capillary surface must be modified or a substance must be added to the buffer to serve as the separation platform for neutral molecules. Reminiscent of the biphasic chromatographic system employing a mobile phase and a stationary phase, this substance is called a pseudostationary phase because it moves at a slower rate than the mobile phase. It possesses either low or no electrophoretic mobility, depending upon its charge state. Neutral molecules are separated based upon their differential partitioning between the pseudostationary phase and the surrounding buffer. This additional partitioning component forms the basis of electrokinetic capillary chromatography (EKC).
1.20 Electrokinetic Capillary Chromatography

Electrokinetic capillary chromatography (EKC) is a hybrid of chromatography and electrophoresis, employing the separation mechanisms of both families into one widely used technique. It is the one free-solution CE technique in which both neutral and ionic molecules can be separated in the electric field.\textsuperscript{114, 115} For neutral molecules, the separation in EKC is based upon differential binding of the analyte with the pseudostationary phase. The capacity factor, $k'$, describes this relationship. In its conventional form,

$$k' = \frac{t_r - t_0}{t_0},$$ \hspace{1cm} 1.33

where $t$ is the retention time of the solute ($r$) and the EOF marker ($0$). In CE, the migration time ($t_m$) replaces retention time. However, this equation does not take into consideration the mobility of the pseudostationary phase in EKC. It can be modified to include

$$k' = \frac{t_m - t_0}{t_0 (1 - \frac{t_m}{t_{PSP}})},$$ \hspace{1cm} 1.34

where $t_{PSP}$ is the migration time of the pseudostationary phase. The resolution in EKC is related to the capacity factors of the solutes as follows

$$R = \left(\frac{N0.5}{4} \right) \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k'_2}{k'_{2}+1} \right) \left( 1 - \left( \frac{t_0}{t_{PSP}} \right) \right),$$ \hspace{1cm} 1.35

where $\alpha$ is the selectivity factor given by $k'_{2}/k'_{1}$. The migration time of the pseudostationary phase is measured by using a very hydrophobic compound such as
Sudan III or IV which will be strongly retained by the pseudostationary phase. The elution window for neutral solutes is illustrated in Figure 1.17.

![Figure 1.17 Migration time window of neutral solutes in EKC](image)

The pseudostationary phases incorporated into this technique have brought about the separation of a wide range of hydrophilic and hydrophobic substances. Examples of these substances are antifungals, peptides, vitamins, aromatic hydrocarbons, prescription pharmaceuticals, drugs of abuse, enzymes, fatty acids, warfare explosives, industrial chemicals, and environmental pollutants. A full review of the applications of pseudostationary phases in EKC is outside the scope of this dissertation. However, they can be categorized into two general groups, dynamic aggregates and covalently bonded organized assemblies. Examples of dynamic aggregates are vesicles, micelles and bile salts. Covalently bonded organized assemblies are cyclodextrins, crown ethers, polymeric surfactants,
calixarenes\textsuperscript{136} and dendrimers\textsuperscript{137}. Examples of pseudostationary phases are shown in Figure 1.18.

![Figure 1.18 Examples of pseudostationary phases](image)

1.21 Capillary Electrochromatography

Capillary electrochromatography (CEC) is a separation technique which combines the high efficiency of CE with the high selectivity of high performance liquid chromatography (HPLC). In CEC, a stationary phase is created by packing silica particles in a capillary and keeping them in place by frits on both sides of the packed bed. Uncharged molecules are separated on the basis of their differential partitioning into the stationary phase, whereas the separation of charged molecules is both chromatographic and electrophoretic. The mobile phase is electrically driven as opposed to pressure driven. The experimental configuration of CEC is similar to that of CE with the exception of the packed capillary and an external pressure source which minimizes
bubble formation in the capillary. Figure 1.19 is a schematic of the interior of a packed capillary.

![Figure 1.19 Interior of packed capillary in CEC](image)

Just as in CE, the capillary walls and silica beads are negatively charged when in contact with the run buffer. The accumulation of buffer cations at these surfaces results in the formation of an electrical double layer. Upon application of a voltage, cations migrate toward the cathode dragging the bulk liquid with them. Consequently, the run buffer moves around each bead. If the bead diameter is too small, the electrical double layers around the beads will overlap and the EOF will stop. This explains why mobile phase does not flow within the bead. Depending upon how the capillary is packed, the bead diameter must be between 20 and 40 times the thickness of the electrical double layer around it in order for an effective EOF to be generated. The advantages of this EOF
are its low flow rates and flat flow profile which result in high peak efficiencies. Other significant aspects of CEC are compared with HPLC and CE in Table 1.4.

<table>
<thead>
<tr>
<th>Feature</th>
<th>CEC</th>
<th>HPLC</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates (N)</td>
<td>100,000-700,000</td>
<td>&lt; 50,000</td>
<td>~200,000</td>
</tr>
<tr>
<td>Peak capacity</td>
<td>100+</td>
<td>~50</td>
<td>~100</td>
</tr>
<tr>
<td>Solvent consumption</td>
<td>nL-µL range</td>
<td>mL-L range</td>
<td>nL-µL range</td>
</tr>
<tr>
<td>pH operating range</td>
<td>3-11</td>
<td>2-9</td>
<td>2-11</td>
</tr>
<tr>
<td>Frits</td>
<td>Experimental problem</td>
<td>Not used</td>
<td>Not used</td>
</tr>
</tbody>
</table>

Packed capillaries are produced by three methods: draw packing, electrokinetic packing, and slurry packing. Slurry-packed capillaries are most common and will be discussed in a separate paragraph. Drawn packed capillaries are so named because a glass-drawing machine draws a large-bore capillary filled with dry stationary phase to the desired inner diameter.138 Electrokinetically packed capillaries are formed by the migration of charged silica particles into the capillary upon application of a voltage to a silica slurry.139,140

The formation of slurry-packed columns is generally accomplished in six steps. First, a slurry of the stationary phase and appropriate packing mobile phase is added to the capillary. Second, a frit is formed by heating the capillary momentarily. Third, pressure is applied in order to push excess stationary phase out of the region beyond the frit and to compress the packed region. In step four, a second frit is formed by heating the capillary momentarily, thereby establishing the final boundary for the stationary phase. In step five, pressure is applied to remove stationary phase outside the packed region. Lastly, a detection window is formed by burning off the polyimide coating of the
capillary immediately adjacent to one of the frits. The slurry-packing process is illustrated in Figure 1.20.

![Procedure for the formation of slurry-packed capillaries](image)

**Figure 1.20  Procedure for the formation of slurry-packed capillaries**

As previously mentioned, frits keep the stationary phase in place. Reported methods of frit preparation involve filling the capillary with either wet or dry stationary phase. Boughtflower *et al.* heated wet silica stationary phases within the capillary until the silica melted slightly, but this yielded frits with low porosity. Heating for shorter periods of time at a moderate temperature yielded more uniform and porous frits. Benhke *et al.* prepared frits by three methods. In the first method, they heated a solution of formamide and potassium silicate to polymerize it. The second and third methods involved filling the capillary with silica gel wetted with potassium silicate solution and bare silica gel, respectively. Amorphous frits with irregular channels were produced in the first and second methods. A more uniform frit was produced in the third method, but it was unstable at the pressures necessary to pack the capillary with more stationary phase.
Factors affecting the separation of molecules in CEC include the temperature of the buffer, mobile phase composition and ionic strength, applied voltage, capillary inner diameter, and most importantly, the type of stationary phase employed. About two-thirds of typical stationary phases are silica-based. The most common stationary phase used in CEC is octadecylsilane (ODS). Use of other stationary phases has resulted in formation of sol-gel, continuous-bed, ion-exchange, and open-tubular columns.

1.22 Scope of this Dissertation

The research presented in this dissertation focuses on the synthesis, characterization, and application of novel amino acid-derivatized calix[4]arenes in spectroscopy and EKC. Calixarenes in their basic forms are very hydrophobic molecules which are capable of forming inclusion complexes. The resurgence of interest in calixarenes in the latter part of the 20th century was due to their potential as enzyme mimics. By virtue of their symmetry, they are achiral and thus incapable of chiral recognition. Therefore, their utility as enzyme mimics cannot be fully realized until they possess chirality. They can be functionalized to induce chirality and water-solubility. Such properties would extend their utility in biological applications and other aqueous environments.

Chapter 2 is divided into two parts. The synthesis of N-L-aminoacylcalix[4]arene derivatives from \( p-t \)-butylcalix[4]arene is described in Part I. Because amino acids have an essential role in biological systems, L-alanine, L-valine, L-leucine, and L-isoleucine have been chosen as lower rim functionalities on \( p-t \)-butylcalix[4]arene. The goal is to attach the amino acids to the calix[4]arene via peptide coupling to yield compounds which are chiral and water-soluble, while maintaining the integrity of the calixarene
cavity. One- and two-dimensional NMR techniques are employed to show that tetrasubstituted calix[4]arenes bearing amino acid moieties have been successfully synthesized. In Part II, the synthesis and characterization (elemental, IR, and solid-state $^{13}$C NMR) of calixarene-bonded silica stationary phases is reported.

In Chapter 3, the application of the four amino acid-derivatized calix[4]arenes in EKC is described. Drawing from previous separations using calixarene-modified EKC, I separated atropisomeric binaphthyl derivatives in a single run using urea as an organic modifier in the run buffer. Even though the hydrophobic nature of aliphatic amino acids would suggest that their use in aqueous EKC systems would be very limited, these amino acid-derivatized calixarenes were water-soluble at pH $\geq$ 7. In addition to the binaphthyl derivatives, the calixarenes were used as chiral selectors for a group of neutral benzodiazepines. The effects of pH, organic modifier concentration and chiral selector concentration on capacity factors, resolution, and chiral selectivity are discussed.

Slight differences in the elution orders of the binaphthyl derivatives in the L-leucine- (CX4-LEU) and L-isoleucine-derivatized (CX4-ILE) calixarene buffer systems suggest differential interaction, even though the mobilities of the calixarenes are equivalent. Therefore, in Chapter 4, the complexation of a binaphthyl atropisomer with CX4-LEU and CX4-ILE using steady-state fluorescence spectroscopy is investigated. It was reported previously that sulfonated calix[6]arene would not be suitable as a host molecule because it produces inner-filter effects. However, a proposed method by which to obtain spectra of the complexed guests while minimizing inner-filter effects of the hosts is discussed. The second part of Chapter 4 reports the investigation of
spectroscopic properties of sulfonated calixarenes in the presence of metals. Chapter 5 summarizes the previous chapters and gives accounts of future directions to consider.

References


<table>
<thead>
<tr>
<th>Reference</th>
<th>Authors</th>
<th>Journal</th>
<th>Year</th>
<th>Pages</th>
</tr>
</thead>
</table>


139. Inagaki, M., Kitagawa, S., Tsuda, T. *Chromatography* 1993, 55R.


CHAPTER 2. SYNTHESIS AND CHARACTERIZATION OF CALIXARENES


2.1 Introduction

As noted in Chapter 1, calixarenes are cyclic oligomers originating from the base-catalyzed condensation of p-substituted phenol with formaldehyde. Their characteristic architecture, phenolic units linked by methylene bridges to form a hydrophobic cavity, make them capable of host-guest interactions with molecules.1 The earliest examples of calixarene complex formation appeared in the mid 1980s involving nonaqueous systems with calix[4]arenes and amines,2 and aqueous systems with water-soluble p-sulfonatocalixarenes.3,4 Interest in our laboratory is primarily centered on water-soluble calixarenes and their effectiveness as buffer additives for capillary electrophoresis (CE). Since most calixarenes are not water-soluble and do not have chiral recognition ability, functionalization of the upper and lower rims is necessary to fully exploit their utilities.5,6 Recently, the syntheses and utility of water-soluble p-t-butylcalix[4]arenes with L-alanine and L-valine amino acid groups as CE buffer additives has been demonstrated.7,8 This chapter reports the synthesis of (N-L-leucinoacyl)calix[4]arene (CX4-L-Leu) and (N-L-isoleucinoacyl)calix[4]arene (CX4-L-Ile) and the characterization of all four amino acid-functionalized calix[4]arenes by nuclear magnetic resonance spectroscopy (NMR).

NMR spectroscopy is an important analytical chemistry tool which aids in the proof of structure of our calixarenes.1 Two-dimensional techniques such as total correlation spectroscopy (TOCSY), homonuclear (H,H)-correlation (H,H-COSY), nuclear Overhauser enhancement (NOESY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple-bond connectivity (HMBC) spectroscopies were
employed to determine proton-proton connectivity overall and through bonds ($^1J_{\text{H-H}} - ^3J_{\text{H-H}}$), proton interactions through space (distance < 5 Å), short-range proton-carbon connectivities ($^1J_{\text{H-C}}$), and long-range proton-carbon connectivities ($^2J_{\text{H-C}} - ^4J_{\text{H-C}}$), respectively.

2.2 Experimental Methods

2.2.1 Synthesis

The synthetic scheme of chiral $N$-acylcalix[4]arene L-amino acid derivatives was performed according to the literature (Figure 1). Some modifications were made in the reactions and are summarized in subsequent sections. Numbering of compounds corresponds with those in Figure 1.

![Figure 2.1 Synthetic scheme of N-L-aminoacylcalix[4]arenes](image)

2.2.1.1 Distillation of N,N-Dimethylformamide and Ethyl Bromoacetate

Before use, both $N,N$-dimethylformamide (DMF) and ethyl bromoacetate ($\text{Br}_2\text{CH}_2\text{COOEt}$) were distilled. DMF (150 ml) was added to a 250 ml round-bottom flask, equipped with a condenser with thermometer joint. Five CaH$_2$ pellets were added to the flask to absorb water and the mixture was stirred for ten minutes. The flask was then heated at approximately 90 °C and covered with aluminum foil. The distilled DMF
was collected in a 250 ml Schlenk flask and stoppered with a rubber septum. The distillation of \( \text{Br}_2\text{CH}_2\text{COOEt} \) was carried out in a similar fashion, except for the amount of solvent and the temperature. It is a strong lachrymator and therefore must be carried out at a lower temperature. Because only a small amount is necessary for the synthesis, only 25 ml is distilled.

2.2.1.2 Synthesis of \( \text{p-t-Butylcalix[4]arene-O,O',O",O‴-Tetraacetic Acid Tetraethyl Ester} \)

All reactions were performed in a fume hood. Four mmoles of Compound 1 were added to a 2-necked 250 ml Schlenk flask. In an argon glove box, 24 mmoles of NaH were added to a curved ground glass joint Schlenk tube. This tube was secured to a neck of the Schlenk flask and the second neck was closed with a septum. All valves were closed and care was taken to prevent premature introduction of NaH to Compound 1 when the apparatus was removed from the glove box. The reaction flask was placed on a heating mantle in the fume hood. The valve on the DMF flask was flushed with argon for 30 seconds before the argon hose was connected to it and the valve was opened. After one minute, the septum of the DMF flask was pierced with the needle of a glass syringe of which the plunger had been removed. The plunger was then replaced and 100 ml of DMF was drawn. The DMF was added to the reaction flask by piercing the septum. After Compound 1 was completely dissolved in the DMF, the argon hose was attached to the reaction flask, and a distillation column equipped with a mineral oil bubbler was attached to it. The NaH was slowly added by gently twisting the curved Schlenk tube until all NaH had been added to the reaction flask. The mixture was stirred for ten minutes. The curved tube was then removed and replaced with a glass stopper. The
argon valve was closed and the mixture was allowed to react at room temperature for 24 hours.

Before attaching the argon hose to the valve of the Br₂CH₂COOEt flask, the stem of the valve was flushed with argon before the valve was opened and the hose attached. The septum was then removed. A borosilicate graduated pipet was held in the flask above the level of the liquid so that it too could be flushed with argon. As a precaution, argon was taken up and expelled through the pipet and bulb before the tip was immersed in the Br₂CH₂COOEt to draw approximately 32 mmoles. The glass stopper of the reaction flask was removed and the Br₂CH₂COOEt released into the reaction mixture. The glass stopper was immediately replaced after the reaction flask was flushed with argon. The transparent yellow reaction mixture was stirred for 24 hours.

To remove DMF, the reaction flask was placed on a heating mantle and the condenser was replaced with a fused glass distillation apparatus equipped with a vacuum pump and a super-cooled collection flask. Under reduced pressure, the heat was slowly adjusted until a gentle reflux occurred. DMF was collected until only ~ 5 ml and a precipitate remained in the reaction flask. The reaction mixture was transferred to an Erlenmeyer flask. Distilled water was added to the reaction flask and to the Erlenmeyer flask, and both were stirred until the precipitate became gummy. The water was poured off and warm 100% ethanol (EtOH) was added to dissolve impurities. At this point, the contents of both flasks were consolidated because the precipitate had a powdery consistency, indicative of the partial dissolution of the impurities. The flask was cooled in the refrigerator overnight before the precipitate was filtered, washed with 100% EtOH, and dried in a heated desiccator. The filtrate, which contained pure Compound 2, was
returned to the Erlenmeyer flask and cooled in the refrigerator for 3 hours. Water was then added to the flask and a milky white precipitate formed. This precipitate (Compound 2) was collected on a filter and dried in a heated vacuum desiccator. The yield was 70%.

2.2.1.3 Formation of \( p-t \)-Butylcalix[4]arene-O,O’,O”,O’”-Tetraacetic Acid

Compound 2 was dissolved in 50 ml of dry THF. Aqueous 10% tetramethylammonium hydroxide was added and the mixture stirred for 24 hours. The mixture was then acidified with hydrochloric acid and stirred for 16 hours. The crude product was isolated from the solvent by rotary evaporation, was thoroughly washed with distilled water and dried in a vacuum desiccator. The yield is 97%. It is interesting to note that this compound is water-soluble.

2.2.1.4 Formation of \((N-L\text{-Aminoacyl})\text{calix}[4]\text{arene } t\text{-Butyl Ester}\)

In this highly efficient reaction, 0.5 mmoles of the hydrolysis product, 2 mmoles of the \( t \)-butyl ester\( \text{HCl}\) of either L-alanine, L-valine, L-leucine, or L-isoleucine, and 4.4 mmoles of diisopropylethylamine were added to 25 ml of DMF and stirred until completely dissolved. Next, 2.2 mmoles of the peptide coupling reagent HATU were added to the reaction mixture and stirred at 0 °C for one hour, and at room temperature for four hours. The reaction was stopped and water was added. The precipitate (Compound 4) was filtered, washed thoroughly with water, and dried in a heated vacuum desiccator. The yield was 86%.

2.2.1.5 Hydrolysis of \((N-L\text{-Aminoacyl})\text{calix}[4]\text{arene } t\text{-Butyl Ester}\)

Compound 4 was dissolved in 5 ml of methylene chloride. The mixture was cooled in a dry ice/acetone bath while 10 ml of trifluoroacetic acid was added dropwise.
The reaction was rapid after removal of the flask from the cooling bath. After 30 minutes, the solvent was removed by reduced pressure distillation, leaving crude product which was washed with distilled water and dried in a heated vacuum desiccator. The overall yield was 97%.

2.2.2 NMR experiments

NMR experiments were executed in 5-mm NMR tubes on a Bruker AMX-500 spectrometer without sample spinning. Deuterated chloroform (CDCl$_3$; $\delta_H$ 7.27 ppm, $\delta_C$ 77.0 ppm) was used as the solvent. Chemical shifts are expressed in $\delta$ (ppm) scale downfield from tetramethylsilane (TMS), the internal reference standard. TOCSY experiments were carried out using the following acquisition parameters: recycling delay (D1), 2.0 s; dummy scans (DS) = 4; number of scans (ns) = 72; D0 increment, 3 $\mu$s; temperature 303 K. Literature pulse sequences were used for other 1D and 2D experiments. The $^1$H-$^1$H NOESY connectivity maps were obtained with the following parameters: 512x512 data matrix size; time domain (td) = 512 in F1 and 1024 in F2; D1 = 2 s; D0 = 3 ms; NS = 96; mixing time = 800 ms.$^{12}$ The $^1$H-$^1$H COSY parameters were D1 = 1.5 s; DS = 4; NS = 64; D0 increment, 3 $\mu$s; spectral width in F2, 2400 Hz, and in F1, 1200 Hz; temperature 298 K.$^{13}$ HMQC parameters are 512x512 data matrix size; td 512 in F1 and 1024 in F2; D1 = 2 sec; ns = 48; ds = 4.$^{14}$ HMBC parameters are 512x512 data matrix size; td 512 in F1 and 1024 in F2; rd = 2 sec; ds = 16; ns = 84; delay for evolution of long range couplings = 0.06 s.$^{15}$

2.3 Results and Discussion

Number assignments for Compound 2 and esters of each (N-L-aminoacyl)calix[4]arene derivative are shown in Figure 2.2.
A number of interesting points can be raised about the NMR data. First, it is evident in Figure 2.3 that the derivatives adopt a cone conformation as do most of the esters for...
which X-ray structures have been obtained. In this conformation, the methylene bridge protons (11a, b) are positioned outside the cavity, one axial and the other equatorial with respect to each benzene ring. This results in a splitting pattern, a pair of doublets in the methylene region (3.0-5.0 ppm), with each doublet $J$-coupled at a frequency of approximately 13 Hz. It is important to note that different conformers of calix[4]arenes exhibit distinctive patterns in the methylene region of the $^1$H NMR spectra: (1) cone: one pair of doublets; (2) partial cone: two pairs of doublets (ratio 1:1) or one pair of doublets and one singlet (ratio 1:1); (3) 1,2-alternate: one singlet and two doublets (ratio 1:1); and (4) 1,3-alternate: one singlet. Calixarenes most often assume the cone or partial cone conformation. Proton and $^{13}$C NMR spectral data are summarized in Table 2.1.

The assignment of each peak was made on the basis of one and two-dimensional $^1$H and $^{13}$C spectra using tetramethylsilane (TMS) as a reference. The TOCSY experiment displayed overall coupling of protons throughout the molecules, whereas the COSY experiment identified short range coupling as a result of through-bond interaction of neighboring protons. The observed geminal coupling of the methylene protons (11a,
b) was an important factor in establishing the nonequivalence of each, and in determining their positions (equatorial or axial) with respect to the benzene ring. A cross peak indicating $^4J$ coupling was also observed between the aromatic protons (3) and one of the methylene bridge protons (11a). This supports the theory of nonequivalence of the bridge protons (11a, b), and led to the assignment of 11a as the equatorial proton, because it is in the shielding region of the benzene ring. This shielding causes its resonance signal to occur at a more upfield region than that of 11b.

Table 2.1 $^1$H and $^{13}$C Chemical Shifts ($\delta$) of Calix[4]arene Derivatives

<table>
<thead>
<tr>
<th>Compound*</th>
<th>$\delta$ (in ppm) ($^1$H label)</th>
<th>$\delta$ (in ppm) ($^{13}$C label)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calixaryl tetraethyl ester</td>
<td>1.07 (8,9,10); 1.28 (15); 3.19 (11a); 4.20 (14); 4.80 (12); 4.85 (11b); 6.77 (1,3)</td>
<td>17.79 (15); 31.36 (8,9,10); 31.89 (11); 33.81 (7); 60.28 (14); 71.32 (12); 125.34 (1,3); 133.44 (4,6); 145.14 (2); 152.98 (5); 170.52 (13)</td>
</tr>
<tr>
<td>(1)</td>
<td>1.07 (8,9,10); 1.39 (20); 1.44 (17,18,19); 3.15 (11a); 4.61 (14); 4.62 (12); 4.63 (11b); 6.75 (1,3); 7.76 (N-H)</td>
<td>17.82 (20); 27.96 (17,18,19); 31.34 (8,9,10); 31.84 (11); 33.82 (7); 48.55 (14); 74.47 (12); 81.41 (16); 125.60 (1,3); 132.82 (4,6); 145.31 (2); 153.35 (5); 169.69 (13); 172.16 (15)</td>
</tr>
<tr>
<td>(2)</td>
<td>0.88 (21,22); 1.06 (8,9,10); 1.44 (17,18,19); 1.67 (20); 3.15 (11a); 4.48 (14); 4.60 (11b); 4.77 (12); 6.78 (1,3); 7.33 (N-H)</td>
<td>17.85 (20); 19.82 (21); 20.50 (22); 27.06 (17,18,19); 31.75 (8,9,10); 31.89 (11); 33.93 (7); 53.51 (14); 73.57 (12); 78.14 (16); 125.56 (1,3); 132.78 (4,6); 145.22 (2); 153.84 (5); 169.66 (13); 172.41 (15)</td>
</tr>
<tr>
<td>(3)</td>
<td>0.90 (22,23); 1.06 (8,9,10); 1.44 (17,18,19); 1.61 (21); 1.66 (20); 3.16 (11a); 4.54 (12); 4.67 (14); 4.77 (11b); 6.72 (1,3); 7.81 (N-H)</td>
<td>22.23 (22); 23.03 (23); 25.09 (21); 28.23 (17,18,19); 31.58 (8,9,10); 32.39 (11); 34.00 (7); 41.39 (20); 51.36 (14); 74.70 (12); 81.57 (16); 125.53 (1,3); 132.66 (4,6); 145.16 (2); 153.97 (5); 170.38 (13); 173.04 (15)</td>
</tr>
<tr>
<td>(4)</td>
<td>0.82 (21); 0.88 (23); 1.06 (8,9,10); 1.24 (22); 1.43 (17,18,19); 1.84 (20); 3.15 (11a); 4.53 (12, 14); 4.84 (11b); 6.72 (1,3); 7.37 (N-H)</td>
<td>11.89 (23); 15.42 (21); 25.85 (22); 28.28 (17,18,19); 31.57 (8,9,10); 32.22 (11); 34.02 (7); 38.10 (20); 56.74 (14); 74.54 (12); 81.56 (16); 125.46 (1,3); 133.09 (4,6); 145.23 (2); 152.86 (5); 169.76 (13); 171.08 (15)</td>
</tr>
</tbody>
</table>

*Numbered compounds correspond to compound names in Figure 2.2.

A 2D NOESY experiment was performed in order to identify the three-dimensional conformation of the esters. Intramolecular interactions were observed
between \(t\)-butyl (8,9,10) and aromatic protons (1, 3), and between bridging methylene protons (11a, b) as expected. Interestingly, unequal interaction of 11a and 11b with nearby protons was observed. Only one bridging methylene proton (11b) showed spatial interaction with acyl methylene (12) protons, whereas the other (11a) interacted with the aromatic protons (1, 3). This dramatic difference between the bridging methylene protons’ interactions is a strong indication that neither proton is positioned inside the cavity of the calix[4]arene. Also, it further supports the theory that 11a and 11b are not equivalent.

In order to assign the carbons bearing hydrogens, an HMQC (\(^1J_{\text{H-C}}\)) inverse experiment was performed. It was observed that carbon 11, though directly attached to the benzene ring is located upfield, indicating that it is strongly shielded from the applied magnetic field. The arene carbons (1, 3) are located downfield in the aromatic region as expected. Carbons 12 and 14 were unambiguously assigned, with carbon 12 being located more downfield due to its proximity to electronegative substituents.

Structure determination required an extensive long-range \(^1\text{H-}^{13}\text{C}\) correlation experiment, HMBC, because of the high number of quaternary carbons in the calixarene derivatives. The sequence employed was able to connect quaternary carbon atoms to any second, third, or in some cases fourth neighbor. Carbon 13, located most downfield was found to correlate with protons 12 and 14, exhibiting \(^2J\) and \(^3J\) coupling respectively. Carbons 4 and 6 correlated with the aromatic (1, 3) and bridging methylene (11a, 11b) protons through two bonds, and with acyl protons (12) through four bonds. Their location in the \(^{13}\text{C}\) NMR spectrum (more downfield than carbons 1 and 3) is based upon their closer proximity to the electronegative oxygen. The disappearance of cross peaks of
carbons 14 and 15 between their corresponding protons unequivocally showed that the hydrolysis of the calixaryl tetraethyl ester (Compound 2, Figure 2.1) had been successful.

The NMR spectral data for t-butyl esters of CX4-L-ALA, CX4-L-VAL, CX4-L-LEU, and CX4-L-ILE are summarized in Table 2.1 also. Hydrolysis of each ester to its acid was shown by the disappearance of $^1$H and $^{13}$C peaks associated with the t-butyl group of the amino acid.

A TOCSY experiment was performed for two main reasons. First, it gave confirmation that the protected amino acid had been successfully attached to the calix[4]arene tetracarboxylic acid by formation of an amide bond. Secondly, it enabled a more definitive assignment of the protons along the amino acid portion of the calix[4]arene, starting from the amide proton (N-H) ($\delta \sim 7.7$ ppm), whose doublet is $J$-coupled at a frequency of approximately 6 Hz. Because the side chain of alanine is a single methyl group, only two cross peaks are noteworthy in its COSY spectrum: one occurring between the amide proton (N-H) and the $\alpha$ proton (14); and the other between 14 and the $\beta$ protons (20), the methyl protons of the side chain.

An HMQC correlation between carbon 20 and its corresponding protons confirmed that it is located most upfield in the $^{13}$C NMR spectrum of CX4-L-ALA t-butyl ester. Another correlation was observed between the stereogenic carbon 14 and its corresponding proton. As expected, a correlation between the $t$-butyl carbons of the protected amino acid (17, 18, 19) and their corresponding protons was observed. All other correlations occurred between protons and carbons of the calix[4]arene skeleton.

An HMBC experiment was performed to assign the two quaternary carbons correctly. Correlations were observed between carbon 15 and protons 14 and 20, indicating $^2J$ and
$^3J$ coupling, respectively. In addition, a correlation indicating $^2J$ coupling between carbon 16 and t-butyl protons (17, 18, 19) was observed. The occurrence of cross peaks signifying $^2J$ coupling between carbon 13 and proton 12 and the amide proton (N-H) was a valuable indicator of the successful synthesis of (N-L-alaninoacyl)calix[4]arene t-butyl ester. All other correlations were between carbons and protons of the calix[4]arene cavity.

A double doublet in the aliphatic region of the $^1$H NMR spectrum of CX4-L-VAL t-butyl ester led to its assignment to the geminal methyl protons (21, 22) of the isopropyl side chain. The COSY spectrum of CX4-L-VAL t-butyl ester (Figure 2.4) revealed one noteworthy cross peak, occurring between the methine proton (20) and the terminal methyl protons (21, 22) of the isopropyl side chain.

![Figure 2.4 COSY spectrum of CX4-VAL t-butyl ester](image-url)
In the HMQC spectrum of CX4-VAL t-butyl ester, strong cross peaks were observed between carbons 14, 20, 21, and 22 and their attached protons. The most useful information was obtained from the HMBC spectrum (Figure 2.5). This spectrum not only showed the correlations between carbons and protons of the calixarene cavity but also showed residual HMQC correlations. For example, the correlation of carbon 21 with proton 14, and carbon 22 with protons 21 and 22 is evident in both the HMQC and HMBC spectra. Other correlations were carbons 13 and 15 with proton 14, and carbon 16 with t-butyl protons (17, 18, 19), which led to the unambiguous assignment of all carbons in the molecule.

Figure 2.5   HMBC Spectrum of CX4-VAL t-butyl ester
The TOCSY NMR spectral data for CX4-L-LEU t-butyl ester are shown in Figure 2.6. A pair of doublets indicating the geminal methyl protons (22, 23) of the isobutyl side chain, suggests that the protons are nonequivalent. The COSY spectrum of the acid form of this derivative shows indistinguishable differences in the interactions of these protons with nearby protons. Therefore, both of these protons are presumed to be equivalent. As was the case for CX4-L-VAL, cross peaks were observed between the amide proton (N-H) and protons 12 and 14. Strong interactions were observed between proton 14 and protons 20 and 21. This may be attributed to the linearity of the isobutyl chain, by which proton 14 feels the influence of its neighboring protons more, with less interaction between it and a bulky methyl branch, as is the case with the sec-butyl side chain of isoleucine. As expected, there were cross peaks between 20 and 21, between 20 and protons 22 and 23, and between 21 and protons 22 and 23, indicating strong coupling between these protons. Extraneous cross peaks observed in the 1H NMR spectra may be due to the existence of other conformers of calixarene derivatives. Studies are underway to determine their origin and identity.

Peaks representing the terminal methyl protons (21, 23) of the sec-butyl side chain of isoleucine were resolved as a doublet (21) and a triplet (23) in the 1H NMR spectrum of CX4-L-ILE t-butyl ester. A cross peak between the amide proton (N-H) and the α proton (14) was observed. Also, cross peaks indicating 3J coupling of the α proton (14) to the β proton (20) were observed. Couplings between the β proton (20) and protons 21, 22, and 23 were indicated by strong cross peaks observed in the aliphatic region of the spectrum (0.18 - 2.0 ppm).
2.4 Conclusions

characteristic splitting patterns of methylene bridge protons in the spectra confirmed that the derivatized calixarenes maintained their cone conformation.

**Part II. Synthesis and Characterization of Calixarene-Bonded Silica Stationary Phases**

2.5 Introduction

As discussed in Chapter 1, calixarenes are a versatile class of compounds capable of forming inclusion complexes with a variety of guest molecules. Their simple structure and ease of derivatization make the exploitation and modification of their chemical and physical properties an attractive undertaking. It has been demonstrated that calixarenes can be employed as stationary phases in capillary gas chromatography, liquid chromatography, and more recently capillary electrophoresis. The utility of calixarenes in aqueous capillary electrophoresis is limited by three key factors. First, complete dissolution of the calixarenes in a suitable run buffer may be problematic. Buffer solutions may become turbid upon standing, thus discouraging long experimental sequences. Secondly, their high UV absorption may impair selectivity and efficiency if UV detection is used. Thirdly, the inability to reuse the derivatives after dissolution in the run buffer amplifies the overall man-hours devoted to buffer replenishment. Immobilization of the derivatives onto the walls of the capillary or onto silica beads within the capillary would offer a reusable capillary which would be stable in a variety of environments and selective for a variety of molecules (charged, neutral, and chiral) in a single run.

In this report, I have attempted to tether CX4-L-VAL to aminopropyl silica beads (APS) for use as stationary phases in capillary electrochromatography. As their name suggests, APS beads are silica beads which have been chemically modified to possess
aminopropyl moieties. The primary amine is very reactive with carboxylic acids, therefore I aim to deprotonate one of the carboxylic groups of CX4-L-VAL in order to ultimately form an amide bond.

2.6 Experimental Section

The peptide coupling agent O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) is used to facilitate the bond formation in the presence of the base N,N-diisopropylethylamine (DIPEA). The synthetic scheme of the stationary phase (CVAPS) is displayed in Figure 2.7.

![Synthetic scheme of CVAPS stationary phase](image_url)
Approximately 0.15 g of CX4-L-VAL was dissolved in DMF in a round bottom flask. To this solution, DIPEA was added to deprotonate the carboxylic acid. The peptide coupling agent HATU was added next, and finally APS beads were slowly added over a 20 minute period. The reaction was stirred for 24 hours. The mixture was filtered and washed with several aliquots of DMF. The product was dried under vacuum for 24 hours before being analyzed by infrared spectrometry and elemental analysis. Infrared spectra of CX4-L-VAL, APS, and CVAPS were obtained, but the presence of a broad peak indicative of strong silicon-oxygen stretching in CVAPS rendered IR ineffective for characterization (see appendix). Although IR results were inconclusive, elemental analysis indicated carbon, hydrogen and nitrogen percentages increased. Results are summarized in Table 2.2. Most importantly, the %C in the three trials compared to the %C of APS represent a 9.44, 6.64, and a 7.08% increase, respectively.

Table 2.2 Elemental analysis of three batches of CVAPS stationary phase

<table>
<thead>
<tr>
<th>Sample</th>
<th>%C</th>
<th>%H</th>
<th>%N</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>3.14</td>
<td>0.30</td>
<td>0.66</td>
</tr>
<tr>
<td>Trial 1</td>
<td>12.58</td>
<td>0.69</td>
<td>0.89</td>
</tr>
<tr>
<td>Trial 2</td>
<td>9.78</td>
<td>0.88</td>
<td>1.25</td>
</tr>
<tr>
<td>Trial 3</td>
<td>10.22</td>
<td>0.96</td>
<td>1.37</td>
</tr>
</tbody>
</table>

Surface coverage is a parameter which is normally used to characterize bonded silica beads in HPLC. After elemental analysis is used to determine the percentage of carbon on the beads, Equation 2.1 is used to calculate the surface coverage:
\[ \text{surface coverage (\(\mu\text{mol m}^{-2}\))} = \frac{\%C \times 10^6}{1200nA}, \]  

where \(\%C\) is the carbon percentage (w/w) acquired from elemental analysis, \(n\) is the number of carbon atoms in the molecule, and \(A\) is the surface area of the silica gel (in m\(^2\) g\(^{-1}\)), respectively. Results of the samples in Table 2.2 are summarized in Table 2.3.

**Table 2.3** Surface coverage of calixarene-modified silica beads

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface coverage ((\mu\text{mol m}^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>0.21</td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.82</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.64</td>
</tr>
<tr>
<td>Trial 3</td>
<td>0.67</td>
</tr>
</tbody>
</table>

It has been reported that the percentage of carbon revealed by elemental analysis is the average of the entire bulk of the silica gel. Furthermore, it does not allow for correction of carbon present from organic contaminants or materials which are normally present on the surface of samples\(^{23}\). Consequently, elemental analysis introduces significant error in the calculation of surface coverage by the aforementioned equation. However, elemental analysis results have still been included in this chapter for comparative purposes.

Solid-state \(^{13}\text{C}\) NMR spectra of APS, CX4-L-VAL, and CVAPS were acquired with the aim of monitoring carbonyl spectral shifts which would infer the establishment of a covalent bond between CX4-L-VAL and APS. As expected, three carbon signals were obtained with APS. The spectra of APS, CX4-L-VAL, and CVAPS are shown in Figure 2.8.
A shift in the carbonyl resonance around 170 ppm would indicate the formation of an amide bond between CX4-L-VAL and APS. However, if only one monomer unit of CX4-L-VAL bonded with APS through an amide bond, the overall change in internal
field strength may be too small to notice. As evidenced by Figure 2.8c, there is no noticeable shift in the carbonyl resonance, but the downfield shifts of the APS carbons suggest that they are being subjected to a stronger internal field strength than before. Such a shift could indicate bond formation as well.

References


Part I. Introduction

Electrokinetic capillary chromatography (EKC) is an attractive analytical technique because of its wide application range, cost, ease of operation and method development, speed, extreme sensitivity, and minimal use of solvents and analytes, compared to traditional separation techniques such as high-performance liquid chromatography (HPLC). It is an electrophoretic technique capable of separating charged and neutral analytes in the presence of an electric field. The separation of charged analytes comes about by their differential mobilities in the electric field, whereas neutral compounds are separated on the basis of their variable interactions with a pseudostationary phase. Typical pseudostationary phases are cyclodextrins, crown ethers, dendrimers, surfactants, polymerized micelles, and calixarenes, all of which can possess chiral recognition ability inherently or via derivatization.

Interest in calixarenes in analytical chemistry has spawned from the seemingly endless possibilities of their application. They are composed of repeating phenolic units linked by methylene bridges to form a torus-shaped macrocycle consisting of a hydrophobic cavity, similar to that of cyclodextrins. Unlike cyclodextrins, the calixarene cavity structure is not rigid, and may undergo conformational changes and at times collapse altogether depending upon experimental conditions. The majority of calixarene research focuses upon the exploitation of their complexation ability. In the solid state, simple substituted benzene-calix[4]arene complexes have been reported. Many reports of solution-state calixarene complexation focus upon complexes formed in nonaqueous solvents because the most common calixarenes are not water-soluble. Only after the results of Shinkai and co-workers were reported did the study of calixarene complexation in aqueous media gain momentum. Without derivatization
however, their simple symmetrical structures render them incapable of discriminating between chiral enantiomers.

In this investigation, we synthesized calix[4]arene derivatives bearing amino acid moieties (namely L-alanine, L-valine, L-leucine, and L-isoleucine) on their lower rims in order to produce water-soluble compounds which are capable of chiral recognition in EKC. Experimental parameters (pH, calix[4]arene concentration, and organic modifier concentration) were varied in order to determine their effect on analyte selectivities, capacity factors, and resolution of binaphthyl and benzodiazepine derivatives.

**Part II. Experimental Section**

3.1 Chemicals and Reagents

The synthesis of each N-L-aminoacylcalix[4]arene derivative was performed according to the procedure reported by Sánchez-Peña and co-workers.31 The structure is shown in Figure 3.1 with \( R \) representing the components of each amino acid side chain as indicated. The analytes (±)-1,1′-bi-2-naphthol (BOH), \((R)-(+)\)1,1′-bi-2-naphthol \((R\text{-}BOH)\), \((S)-(\_)-1,1′\text{-bi-}2\text{-naphthol (S-BOH)}\), \((R)-(\_)-1,1′\text{-binaphthyl-2,2′-diamine (R-BNA)}\), \((S)-(\_)-1,1′\text{-binaphthyl-2,2′-diamine (S-BNA)}\), \((\_)-1,1′\text{-binaphthyl-2,2′-diyl hydrogen phosphate (BNP)}\), \((R)-(\_)-1,1′\text{-binaphthyl-2,2′-diyl hydrogen phosphate (R-BNP)}\), and \((S)-(\_)-1,1′\text{-binaphthyl-2,2′-diyl hydrogen phosphate (S-BNP)}\) were dissolved in either 50:50 methanol/water 80:20 methanol/water, or 100% methanol to yield an approximate concentration of 0.33 mg/ml. The benzodiazepine derivatives lorazepam, oxazepam and temazepam were obtained in 1.0-mg/ml methanol solutions and diluted to a concentration range of 0.3–0.5 mg/ml with 100% methanol before use. The structures of the analytes are shown in Figure 3.2. A PureLab UV/UF water system was used to deionize distilled water to 18 MΩ.
Figure 3.1  Structure of $N$-Acylcalix[4]arene L-amino acid derivatives

Figure 3.2  Structures of binaphthyl derivatives and benzodiazepines
3.2 Capillary Electrophoresis Procedure

Binaphthyl derivative separations were performed using a BioFocus 3000 automated capillary electrophoresis system employing a multiwavelength UV absorbance detector at 254 nm, 280 nm, and 300 nm. A 50 cm (effective length) bare fused-silica capillary with 50 µm inner diameter was thermostated at 25 ºC by a 20% methanol solution and used as the separation platform. Benzodiazepine separations were performed using a Hewlett-Packard 3D capillary electrophoresis system equipped with a UV-Visible absorbance detector which monitored the separation at a wavelength of 254 nm. A 56.5 cm (effective length) bare fused-silica capillary with 50 µm inner diameter was used as the separation column and was air-cooled to 12 ºC to minimize analyte racemization. In both systems, a constant voltage of +25 kV was used to induce analyte and electrolyte mobility within the capillary.

3.3 Background Electrolyte Preparation

Dry quantities of each calix[4]arene derivative were dissolved in 100 mM Tris(hydroxymethyl)-aminomethane (Tris) to produce buffered solutions possessing calix[4]arene concentrations ranging from 0.1 mM to 15 mM. The pH of each buffer was adjusted with 1.0 M NaOH or 1.0 M HCl to produce solutions over a pH range of 6.5 to 11. All solutions were sonicated and filtered through a 0.45 µm nylon filter prior to use.

Part III. Results and Discussion

3.4 Binaphthyl Derivative Separations

Some binaphthyl derivatives are known to be effective chemical shift reagents in nuclear magnetic resonance spectroscopy. Their chirality is a result of an asymmetrical plane as opposed to a stereogenic carbon; hence these non-superimposable mirror-imaged compounds are called atropisomers as opposed to enantiomers. Sánchez-Peña and co-workers effectively
separated binaphthyl atropisomers using \( N\text{-}L\text{-}\text{alaninoacylcalix[4]arene (CX4-L-ALA)} \) and \( N\text{-}L\text{-valinoacylcalix[4]arene (CX4-L-VAL)} \) as chiral selectors in EKC at pH 11.\(^{33}\) The efficacy of CX4-L-ALA as a chiral discriminator was enhanced by the addition of sodium dodecyl sulfate (SDS) to the running buffer at concentrations above its critical micelle concentration (~8 mM). In contrast, CX4-L-VAL was an effective chiral selector for the analytes without the addition of a surfactant (Figure 3.3), affording baseline resolution of them all.

![Graph](image)

**Figure 3.3** Separation of BNP (1), BOH (2), and BNA (3) using CX4-L-VAL. Conditions: 40 mM phosphate buffer, pH 11; applied voltage, 25 kV; detection at 300 nm; analyte concentration, 0.33 mg/mL.

Two more calix[4]arene derivatives (\( N\text{-}L\text{-leucinoacylcalix[4]arene (CX4-L-LEU)} \) and \( N\text{-}L\text{-isoleucinoacylcalix[4]arene (CX4-L-ILE)} \)) were synthesized to study the effect of the amino
acid side chain length on the separation of the binaphthyls. Because the amino acid side chains of leucine and isoleucine are more hydrophobic than those of alanine and valine, water-solubility of the calixarene derivatives of these compounds was enhanced by the use of Tris as the running buffer. It was inferred that the larger size of these calixarenes would cause longer migration times of the analytes upon complexation. As indicated in Figure 3.4, the new calix[4]arene derivatives are effective chiral selectors for binaphthyl separations at a much lower pH, therefore preserving capillary integrity over a longer period of time. As predicted, the new calixarenes moved slowest in the capillary, forming strong complexes with analytes and causing them to elute at longer migration times as a consequence.

Figure 3.4. Separation of BNP, BOH, and BNA using 10 mM CX4-L-LEU (A) and 10 mM CX4-L-ILE (B) buffer systems. Conditions: 100 mM Tris buffer, 8 M urea, pH 8.7; applied voltage, 25 kV, detection at 300 nm; analyte concentration 0.33 mg/mL.
A study of the effect of pH on the separation of a mixture of the binaphthyl derivatives was investigated using 100 mM Tris containing 10 mM CX4-L-LEU or CX4-L-ILE. The effect of pH on atropisomeric resolution indicated that the workable pH range of these calixarenes is from 7 to 11. At pH values below 7, the calixarenes precipitated out of solution due to a decrease in the ionization of the carboxylate functionalities. When CX4-L-LEU was used as the chiral selector, BNP atropisomers eluted first, followed by those of BOH and BNA, respectively, at each pH. In every case, the \( R-(+)- \) atropisomer eluted before its corresponding \( S-(-) \) antipode. A decrease in resolution of BNP was observed at pH values above 7, while increases in resolution were observed for BOH and BNA. The effect of pH on the observed resolution of the binaphthyl derivatives is shown in Figure 3.5.

![Figure 3.5 Effect of pH on the resolution of binaphthyl derivatives; Conditions: 10 mM CX4-LEU/6 M Urea/100 mM Tris; others same as Figure 3.4.](image)

The addition of urea was necessary to achieve separation of the binaphthyl derivatives. It has been shown that achiral modifiers can enhance chiral recognition of chiral pseudostationary phases.\(^{34}\) In previous studies, the addition of the surfactant sodium dodecyl sulfate (SDS)
enhanced resolution of the binaphthyl derivatives. In contrast, SDS did not enhance resolution and urea was employed as an achiral modifier in this study. A high concentration of urea increases the solubility of hydrophobic compounds into water as well as hinders hydrogen bond formation. Therefore, a urea concentration range of 0 to 10 M was employed to investigate its effect on chiral recognition of the binaphthyl derivatives. As the concentration of urea increased, the viscosity of the buffer, the migration times of the analytes, and baseline noise increased. Despite these observations, baseline resolution was achieved for each binaphthyl derivative at some point. At urea concentrations below 6 M, BNP was not resolved with either calixarene. However, BNP atropisomers were baseline resolved without a significant change in resolution ($Rs \sim 1$) between 5.5 and 8.5 M urea. A complete loss of resolution was observed at concentrations above 8.5 M. In contrast, resolution values higher than one ($Rs = 1$, baseline resolution) were achieved at each urea concentration employed (Figure 3.6). In all cases, the $R$-atropisomer eluted before the $S$-atropisomer, suggesting that a stronger complex forms between the $S$-atropisomer and each calix[4]arene derivative.

![Figure 3.6](image)

**Figure 3.6** Effect of urea concentration on resolution of binaphthyl derivatives
It is interesting to note the difference in analyte selectivity of CX4-L-LEU compared to that of CX4-L-ILE. The analyte elution order was BNP>BOH>BNA when CX4-L-LEU was used as the chiral selector, in agreement with previous studies using CX4-L-ALA and CX4-L-VAL. When CX4-L-ILE was employed in the running buffer, the overall elution order was BNP>BOH>BNA, but the R-atropisomer of BNA eluted before the S-atropisomer of BOH. Because leucine and isoleucine are constitutional isomers, their mobilities within the capillary are identical. A significant difference between the structures of the isomers is the presence of an additional stereogenic carbon in isoleucine. We believe that this additional chiral center contributes to the difference in observed analyte selectivity.

3.5 Benzodiazepine Separations

The benzodiazepines employed (lorazepam, oxazepam, and temazepam) are a group of pharmaceutical compounds presently sold to consumers in racemic mixtures to treat anxiety and insomnia. Under the conditions studied, all three analytes were electrically neutral, while the calixarenes were anionic. As a result of their negative charge, the calixarenes moved in a direction opposite that of the analytes, but the bulk electroosmotic flow ultimately caused all species in the capillary to be moved away from the point of injection towards the detector.

3.5.1 Effect of Amino Acid Chain Length

Because of the difference in aliphatic chain length of the amino acid residues, the hydrophobicities of the calixarenes vary. Valine, leucine, and isoleucine have hydrocarbon side chains that are three and four carbons long, whereas alanine possesses a methyl side chain. The larger side chains are hydrophobic and are most likely to cluster in order to avoid contact with water. We believe that conformational changes in the side chain influence the complexation ability of each calixarene with each analyte, therefore producing changes in the migration times
of the analytes under investigation. Separations were carried out using a 10 mM concentration of each calixarene derivative in 100 mM Tris buffer. Electropherograms of the separation of oxazepam enantiomers using each calixarene derivative as a buffer additive in EKC are shown in Figure 3.7. When CX4-L-ALA was used as the buffer additive, the enantiomers were only partially resolved, whereas baseline resolution was achieved when the longer chain amino acid-derivatized calixarenes were used. Baseline resolution in the shortest time with a minimum amount of band broadening was achieved with the CX4-L-LEU buffer system. This suggests that a weaker binding interaction between each enantiomer and CX4-L-LEU took place. Such were the same observations with oxazepam and temazepam enantiomers in the CX4-L-LEU buffer system.

![Electropherograms](image)

**Figure 3.7** Separation of oxazepam enantiomers using 5 mM CX4-L-ALA (A), CX4-L-VAL (B), CX4-L-LEU (C), and CX4-L-ILE (D). Conditions: 100 mM Tris (pH 9); applied voltage, 25 kV; temperature, 12 °C; analyte concentration 1 mg/mL.
3.5.2 Effect of pH

The effect of pH on the resolution of the benzodiazepines was investigated using a 100 mM Tris buffer and a 10 mM concentration of each calixarene derivative. Because calixarene water-solubility could be induced at pH values of 8 and higher with this buffer system, the pH range studied was between 8 and 11. Buffer systems containing CX4-L-ALA were found to be unsuitable to achieve resolution values of at least 1.0 throughout the pH range investigated, but lorazepam and oxazepam enantiomers were separated with a resolution of at least 0.85 at pH 10. The highest resolution values were realized at pH values of 10 and 11 for buffer systems containing CX4-L-VAL and CX4-L-LEU (Figure 3.8).

Figure 3.8 Effect of pH on the chiral interaction ($\alpha$) of lorazepam (-♦-), oxazepam (-■-), and temazepam (-▲-) with CX4-L-ALA (A), CX4-L-VAL (B), CX4-L-LEU (C), CX4-L-ILE (D). Conditions same as in Figure 3.7.
3.5.3 Effect of Chiral Selector Concentration

A concentration range of 0.25 to 15 mM calixarene was used to investigate the effect of calixarene concentration on the resolution of the benzodiazepines. The electrolyte system employed was a 100 mM Tris buffer at pH 10. The relationship between CX4-L-ILE concentration and the resolution of the benzodiazepines is illustrated in Figure 3.9. Among all calixarenes employed, it was discovered that a 5 mM concentration yielded the best resolution values. Band broadening, longer migration times, and a decrease in resolution were observed at concentrations higher than 5 mM.

![Figure 3.9](image_url)

**Figure 3.9** Effect of CX4-ILE concentration on the resolution of benzodiazepines

3.5.4 Effect of the Number of Chiral Centers

It was observed that the CX4-L-ILE buffer system most often produced the most pronounced and unpredictable results in terms of resolution, selectivity, and migration times. Because L-leucine and L-isoleucine are constitutional isomers, their corresponding calixarene derivatives are expected to share a similar mobility in the capillary. Also, we expect their chiral interaction (represented by $\alpha$) with the analytes to be different, because leucine possesses one chiral center while isoleucine possesses two. However, it is evident in Figure 3.10 that while lorazepam enantiomers are experiencing more binding interaction (e.g., higher $R_s$ values) with
CX4-L-ILE when compared to CX4-L-LEU, they are experiencing a chiral interaction ($\alpha=1.09$) similar to that of CX4-L-LEU ($\alpha=1.04$).

Figure 3.10 Separation of lorazepam enantiomers using CX4-L-LEU (A) and CX4-L-ILE (B). Conditions same as in Figure 3.7, pH 10.

This means that neither enantiomer is favored over the other by the calixarene in terms of chirality. At the optimum calixarene concentration (5 mM), oxazepam enantiomers were more resolved in the CX4-L-ILE buffer system and at shorter migration times (Figure 3.11).

Figure 3.11 Separation of oxazepam enantiomers using CX4-L-LEU (A) and CX4-L-ILE (B). Conditions same as in Figure 3.7, pH 10.
Also, the second chiral center of the isoleucine side chain in CX4-L-ILE played a greater role in the separation as evidenced by a corresponding $\alpha$ value of 1.26 in comparison to an $\alpha$ value of 1.09 for CX4-L-LEU. Like lorazepam, the temazepam enantiomers were more resolved in the CX4-L-ILE buffer system at longer migration times than those achieved in the CX4-L-LEU system. However, there was more chiral interaction in the CX4-L-ILE system. More detailed studies are underway to investigate the role of the additional chiral center on the separations we observed.

**Part IV. Conclusions**

The study presented in this chapter compared the effectiveness of four L-amino acid-derivatized calix[4]arenes as chiral selectors in EKC for the separation of binaphthyl and benzodiazepine derivatives. Parameters optimized for these separations were buffer pH and chiral selector concentration. The optimum buffer pH for the separations was 10 and the chiral selector concentration was 5 mM in 100 mM Tris. Under neutral and basic conditions, it was demonstrated that calix[4]arenes bearing amino acids with longer side chains (CX4-L-VAL, CX4-L-LEU, and CX4-L-ILE) are strong chiral selectors when compared to CX4-L-ALA. Because we observed a different elution order of the binaphthyl derivatives when CX4-L-ILE was employed in the buffer system, it was deduced that CX4-L-ILE exhibited a different analyte selectivity than CX4-L-ALA, CX4-L-VAL, and CX4-L-LEU. This observation led us to investigate the role of its second chiral center in the separation of benzodiazepines. In terms of chiral selectivity, it was shown that CX4-L-ILE provided the most chiral interaction between itself and the analytes. Based upon resolution and migration times however, it is concluded that CX4-L-LEU is the best chiral selector to use as a buffer additive for the separation of benzodiazepines in EKC.
References


CHAPTER 4. SPECTROSCOPIC STUDIES OF CALIXARENE COMPLEXATION


4.1. Introduction

Calixarenes are a versatile class of materials, composed of substituted phenolic units which are linked by methylene bridges to form a hydrophobic cavity. They are known to form host-guest type inclusion complexes with a variety of molecules and are employed in areas such as bioorganic chemistry,\(^1\) metal-ion complexation,\(^2\) and catalysis.\(^3\) Hydrophobic effects are reported to be responsible for the inclusion phenomenon, and are important in molecular recognition in water. Water-soluble calixarenes facilitate the study of hydrophobic interactions (cation-\(\pi\), \(\pi-\pi\), and CH-\(\pi\)) by enhancing the complexation of apolar guests in their cavity. In fact, it was shown that a water molecule was hydrogen-bonded to the \(\pi\) cloud of the \(p\)-sulfonatocalixarenes. Most biological processes take place in water. A water molecule was even shown to be included in the cavity of \(p\)-sulfonatocalixarenes via hydrogen bonding to the \(\pi\) cloud of the ring systems.\(^4\) This arrangement bears a resemblance to that of the hydrophobic pocket of proteins. Therefore, water-soluble calixarenes show promise in the mimicry of natural processes like molecular recognition.

The complexation of water-soluble calixarenes with different guests has been discussed in Chapter 1. Guest size and hydrophobicity are key determinants in the selectivity of the calixarene. Complexation properties of calixarenes have been evaluated by microcalorimetry,\(^5\) induced circular dichroism,\(^6\) NMR,\(^7\) X-ray crystallography,\(^8\)
electronic absorption and fluorescence spectroscopy. In this chapter, steady-state fluorescence spectroscopy is employed to investigate the complexation of calixarenes.

The impetus behind this study stems from results obtained in early EKC experiments using CX4-LEU and CX4-ILE as pseudostationary phases. The observed difference in the elution orders of the atropisomers of BNA is suggestive that CX4-LEU may bind more strongly to R-BNA than does CX4-ILE. A comparison of the association constants of CX4-LEU:R-BNA and CX4-ILE:R-BNA complexes would be sufficient to confirm this inference. In addition, determination of the stoichiometry of the complexes could lead to a better understanding of their formation. In order to adequately correlate EKC results with spectroscopic results, solution conditions in spectroscopy must closely reflect those used in EKC.

Solution-state NMR spectroscopy would be best suited for this type of study. From Chapter 2 of this dissertation, it was reported that the chemical shifts of the geminal protons of the methylene bridges of the calixarene cavity occur in the region between 4.5 and 5.5 ppm with reference to TMS. These protons are most sensitive to inclusion complexation. In an aqueous system, the broad resonance peak from water (~4.8 ppm) masks the doublets of the calixarene, thereby obviating accurate determination of shifts upon inclusion complexation. X-ray crystallography would also be quite definitive in the determination of association constants and complex stoichiometry. However, crystallization of amino acid-derivatized calix[4]arenes has been unsuccessful thus far. Consequently, steady-state fluorescence will be employed to determine association constants of CX4-LEU:R-BNA and CX4-ILE:R-BNA.
Fluorescence spectroscopy is a powerful analytical technique for the investigation of complex formation. In a two species host-guest system, the changes in the fluorescence signal are monitored as a function of host or guest concentration. If only one of the species fluoresces, its fluorescence is monitored as a function of the other species’ concentration. The absorption and fluorescence spectra of such a system are illustrated in Figure 4.1a. If both species fluoresce, the wavelengths of excitation and emission must be optimized in order to minimize signal overlap (Figure 4.1b). In both cases, differences between the fluorescence signals of the free fluorophore and the complexed fluorophore lead to the determination of complex stoichiometry and association constants. In the most complicated two species host-guest system, both species fluoresce in the same wavelength range (Figure 4.1c), thereby increasing the difficulty of complex stoichiometry and association constant determination.

Spectra like those represented in Figure 4.1a are obtained in cyclodextrin complexation studies because cyclodextrins are UV-transparent. Also, similar spectra can be obtained in calixarene complexation studies provided the calixarene is the sole fluorophore. Other fluorescence studies of calixarene complexation focus on guest molecules that do not absorb and fluoresce in the same wavelength region as the calixarene. Though powerful in this regard, it is limited to species whose excitation and emission wavelength regions do not severely overlap. Otherwise, calixarene inner-filter effects will adulterate the observed fluorescence signals. This brings up the most relevant question in the current investigation. Can the true fluorescence spectrum of a complexed guest be extracted from the overlapping spectrum of its host? In other words, what information can be acquired from spectra similar to those represented in Figure 4.1c?
When the fluorescence signal of an analyte is altered due to interferences caused by other sample constituents, this is called the inner-filter effect. In one form of the inner-filter effect, the interferent absorbs in the same wavelength range as the analyte.

Figure 4.1. Absorption and Fluorescence Spectra of a Two Component Host-Guest System
This decreases the amount of radiation available to excite the analyte, thereby producing a lower fluorescence quantum efficiency. Also, the quantum efficiency is decreased if the interferent absorbs at the wavelength at which the analyte fluoresces. In this instance, the number of emitted photons that reach the detector is decreased. High concentrations of the fluorophore make self-absorption more likely as well. In this phenomenon, some molecules of the fluorophore absorb the fluorescence. Another cause of inner-filter effects is sample turbidity. In this case, molecules at the face of the sample holder nearest to the incident radiation source absorb more radiation than those which are further away.

Recall that the naphthyl moieties of \( R \)-BNA and the phenolic units of calixarenes are strongly absorbing species in the UV region of the spectrum. The structures are shown in Figure 4.2.

\[
\begin{align*}
\text{N-L-leucinoacylcalix[4]arene} & \quad \text{(CX4-LEU)} \\
\text{N-L-isoleucinoacylcalix[4]arene} & \quad \text{(CX4-ILE)} \\
\text{(R)-(+)\text{-1,1'-binaphthyl-2,2'-diamine}} & \quad \text{(R)-BNA}
\end{align*}
\]

\text{Figure 4.2 Structures of host calixarenes and guest (R)-BNA}
Zhang and coworkers reported that the inner-filter effects caused by sulfonated calixarenes are seriously disadvantageous for the study of their complexation with certain fluorescent probes. Therefore, the photophysics of calixarenes must be considered in these luminescence probe studies, as inner-filter effects could lead to errors in spectroscopic measurements. In this chapter, I propose a simple model by which the inner-filter effects of CX4-LEU and CX4-ILE may be studied and minimized in the determination of association constants and complex stoichiometry.

4.2 Experimental Section

4.2.1 Chemicals

The synthesis of N-L-leucinoacylcalix[4]arene (CX4-LEU) and N-L-isoleucinoacylcalix[4]arene (CX4-ILE) was performed as reported in Chapter 2. Stock solutions of each were prepared at 1×10⁻³ M and 5×10⁻³ M in 100 mM trishydroxyaminomethane (Tris) which was purchased from Aldrich Chemical Company (Milwaukee, WI). The sample pH of 10 was determined by pH paper and left unadjusted. Serial dilutions were made to produce duplicate solutions in borosilicate tubes of the following concentrations (×10⁻⁴ M): 1, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10, 15, 25, 35, 45, and 50. The guest compound (R)-(+) -1,1'-binaphthyl-2,2'-diamine (R-BNA) was purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. A dry quantity of R-BNA was dissolved in methanol to yield a stock solution with a concentration of 1×10⁻³ M. Equal aliquots of the R-BNA stock solution were added to one set of borosilicate tubes such that the final concentration of R-BNA was 1×10⁻⁵ M in each calixarene solution. The second set of calixarene solutions did not contain R-BNA and were used as blanks.
4.2.2 Apparatus

Steady-state fluorescence measurements were taken with a Jorbin-Yoba Fluorolog (τ-3) spectrofluorometer equipped with a cell compartment and circulation inlets and outlets for temperature control. The excitation and emission slit bandwidths were set at 5 nm. Fluorescence emission spectra were obtained at room temperature at an excitation wavelength of 340 nm. Absorption spectra were acquired with a Shimadzu UV-3101PC double beam scanning spectrophotometer equipped with a thermostat held at 20 °C. Because the calixarene solutions contained 100 mM Tris, all absorbance measurements were obtained using 100 mM Tris as a reference.

4.3 Results and Discussion

4.3.1 Absorption Spectra

The structural isomers CX4-LEU and CX4-ILE differ by an additional chiral center on the monomer units of CX4-ILE. From the previous chapter, it was shown that they both can be effective chiral selectors in EKC. However, a slightly different elution order was obtained when the CX4-ILE buffer was employed. Also, their absorption spectra differ slightly. Both contain two broad absorption bands at ~227 nm and ~280 nm, which correspond to the transitions from the ground state to the second and the first excited electronic states, respectively. However, the second band has more fine structure in CX4-LEU than in CX4-ILE. The molar absorptivities were determined from a calibration plot in the linear range of absorbance versus calixarene concentration (Figure 4.3). The molar absorptivity at 340 nm is 214 L mol\(^{-1}\) cm\(^{-1}\) according to the plot. The molar absorptivity of a CX4-ILE solution of equal concentration at 340 nm is 237 L mol\(^{-1}\) cm\(^{-1}\). Additional data are shown in the appendix.
$y = 222.13x + 0.0076$

$R^2 = 0.9988$

**Figure 4.3  Calibration plot of CX4-LEU absorbance**

Overlaid absorbance spectra of $1 \times 10^{-5}$ M *R*-BNA, $1 \times 10^{-5}$ M and $1 \times 10^{-4}$ M CX4-LEU are shown in Figure 4.4. The spectra severely overlap each other in a manner which is similar to that in Figure 4.1c. Only in the region >330 nm is the intensity of the *R*-BNA trace greater than that of CX4-LEU (at concentrations $\leq 6.5 \times 10^{-4}$ M).

**Figure 4.4  Absorbance spectra of $1 \times 10^{-5}$ M *R*-BNA, $1 \times 10^{-5}$ M CX4-LEU, and $1 \times 10^{-4}$ M CX4-LEU**
4.3.2 Fluorescence Studies

4.3.2.1 Single Cuvette Experiments

The mole ratio method was applied in the determination of association constants. The concentration of \( R\)-BNA was held constant (1×10\(^{-5} \) M), while the concentration of the calixarene was increased from 1×10\(^{-4} \) M to 5×10\(^{-3} \) M. In order to obtain association constants, corrections must be made for the emission and absorption of the calixarene, the contributor of inner-filter effects. Because calixarene emits photons in the same wavelength region as \( R\)-BNA, the calixarene emission must be subtracted from the \( R\)-BNA in calixarene (\textit{labeled instrument in plots}) emission as illustrated in Figure 4.5.

![Emission spectrum of 10\(^{-5} \) M R-BNA in 3.5×10\(^{-4} \) M CX4-LEU; correction for CX4-LEU emission achieved by subtraction of blank emission from instrument emission](image)

**Figure 4.5** Emission spectrum of 10\(^{-5} \) M R-BNA in 3.5×10\(^{-4} \) M CX4-LEU; correction for CX4-LEU emission achieved by subtraction of blank emission from instrument emission

All the fluorescence emission spectra produced when corresponding blanks were subtracted from each spectrum to eliminate the influence of the CX4-LEU emission are shown in Figure 4.6. By subtracting the calixarene (blank) spectra from the instrument
The resulting R-BNA spectra represent fluorescence of both free and complexed R-BNA. However, association constants cannot be calculated at this point because a correction for the amount of photons which the calixarene absorbed during the excitation and emission events of R-BNA has not been made. For this reason, the intensity of R-BNA must be multiplied by a suitable correction factor. Recall the following Beer-Lambert relationships from Chapter 1:

$$A = \log \frac{I_0}{I} = \varepsilon C_x$$

$$\frac{I_0}{I} = 10^{\varepsilon C_x}$$

$$I = I_0 \ast 10^{-\varepsilon C_x} \quad (4.1)$$

where $A$ is absorbance of CX4-LEU at 340 nm; $I_0$ and $I$ are the intensities of R-BNA in the absence and presence of CX4-LEU, respectively; $\varepsilon$ is the molar absorptivity of the calixarene at 340 nm; and $C_x$ is the concentration of CX4-LEU. The intensity ($I$) of R-BNA is constant and is proportional to $I_0$. Therefore, its intensity as a function of CX4-LEU concentration is constant theoretically, assuming there is no interaction between R-BNA and CX4-LEU.

**Figure 4.6** Emission spectra of R-BNA in increasing concentrations ($x10^{-4}$ M) of CX4-LEU; spectra corrected for background emission of CX4-LEU
However, the observed emission intensity sharply increased to a point and gradually decreased afterwards, indicating either complexation of $R$-BNA with CX4-LEU or concomitant absorption and emission of photons by CX4-LEU. In the second case, the CX4-LEU is sharing the photons with $R$-BNA, thereby attenuating the incident radiation. Therefore, a decrease in the emission intensity of $R$-BNA is observed as the concentration of CX4-LEU increases. To correct for this absorption, and assuming linear dependence, the intensity of $R$-BNA was multiplied by the intensity of the calixarene at 340 nm as indicated:

$$I'_{BNA} = I_{BNA} \times 10^{ECx}.$$  \hspace{1cm} (4.2)

My interest is to keep the model as simple as possible. Therefore, the corrections are limited to linear approximation. Fluorescence intensities in the linear concentration range are shown in Figure 4.7. The impact of the absorption of CX4-LEU during the $R$-BNA emission event is indicated by the consistently higher intensities of species with absorbance correction compared to those without. At concentrations above $1 \times 10^{-3}$ M CX4-LEU, the $R$-BNA fluorescence intensities appear to increase exponentially when corrected for calixarene absorbance. This is in agreement with raw data, which indicate that the presence of increasing concentrations of CX4-LEU causes an increase in the fluorescence measured. Logically, most of this fluorescence is attributed to the greater excess of CX4-LEU compared to $R$-BNA. Conversely, without absorbance correction, $R$-BNA fluorescence intensities decreased at CX4-LEU concentrations above $3.5 \times 10^{-3}$ M. This indicates that CX4-LEU is concentrated to the extent that self-absorption is attenuating not only the radiation available to $R$-BNA, but also the $R$-BNA fluorescence that is detected. It is interesting to note that $R$-BNA fluorescence increased linearly over
the entire concentration range studied when corrected for CX4-LEU absorption. Without this absorption correction, \( R \)-BNA fluorescence gradually decreased above a CX4-LEU concentration of \( 1.5 \times 10^{-3} \) M. Again this indicates that above this concentration, CX4-LEU not only absorbs radiation from the source, but also re-absorbs photons emitted as fluorescence by \( R \)-BNA. Consequently, the number of photons reaching the detector as fluorescence decreases.

![Graph showing emission intensity of \( R \)-BNA with and without absorbance correction](image)

**Figure 4.7  Emission intensity of \( R \)-BNA with and without absorbance correction**

At this point an association constant can be calculated using Scott’s modification\(^{12}\) of the Benesi-Hildebrand equation for 1:1 stoichiometry:\(^{13}\)

\[
[I'_{\text{BNA}} - I_0]^{-1} = [I'_{m\text{BNA}} - I_0]^{-1} + [K_a (I'_{m\text{BNA}} - I_0)]^{-1} \times [C_x]^{-1} \quad (4.3)
\]

where \( K_a \) is the association constant and \( I'_{m\text{BNA}} \) is the fluorescence intensity when all \( R \)-BNA is complexed with CX4-LEU. Because the above equation is a linear relationship, \( K_a \) can be calculated by dividing the y-intercept \( ([I'_{m\text{BNA}} - I_0]^{-1}) \) by the slope.
\([K_a(I_m^{\text{BNA}} - I_0)]^{-1}\). Also, the fluorescence intensity of completely complexed \(R\)-BNA can be calculated by adding the inverse of the y-intercept to \(I_0\). By doing so, \(K_a\) approaches zero. In a simple system, \(K_a \leq 0\) indicates that there is either extremely weak or no complexation occurring (Figure 4.8).

![Graph](image)

**Figure 4.8** Double reciprocal plot of intensity of \(R\)-BNA as a function of CX4-LEU concentration, assuming 1:1 stoichiometry; association constant is negative.

In order to assume a 2:1 calixarene:BNA stoichiometry, \([Cx]^{-1}\) was squared plotted against \([I'BNA-I_0]^{-1}\) (Figure 4.9). The resulting value of \(K_a\) was \(8.0 \times 10^6 \text{ M}^{-1}\).

![Graph](image)

**Figure 4.9** Double reciprocal plot of fluorescence intensity of \(R\)-BNA as a function of CX4-LEU concentration assuming 2:1 stoichiometry.
The curvature of the plots in Figures 4.8 and 4.9 indicate that the stoichiometry of any CX4-LEU:R-BNA association is mainly 1:1 even though the intercept of the plot in Figure 4.8 is negative.

The value of $K_a$ when CX4-ILE was the host compound was determined using the aforementioned procedures. Similarly, values determined from a double reciprocal plot assuming 1:1 CX4-ILE:R-BNA stoichiometry over a linear concentration range were negative. When a 2:1 CX4-ILE:R-BNA stoichiometry was assumed, a line fit was achieved throughout the entire concentration range. The resulting $K_a$ value was $1.5 \times 10^6$ M$^{-1}$ which is indicative of strong association. Supplemental data are shown in the appendix.

**4.3.2.2 Tandem Cuvette Experiments**

Because the absorption regions of CX4-LEU and CX4-ILE overlap that of R-BNA, inner-filter effects contributed by the calixarenes must be taken into consideration. The wavelength of maximum absorbance of a $1 \times 10^{-5}$ M R-BNA solution is 240 nm, and its corresponding molar absorptivity is 121430 L mol$^{-1}$ cm$^{-1}$. However, the molar absorptivities of $1 \times 10^{-4}$ M solutions of CX4-LEU and CX4-ILE at this wavelength are 12697 and 12121 L mol$^{-1}$ cm$^{-1}$, respectively, at pH 10. At 340 nm the highest molar absorptivities of solutions of CX4-LEU and CX4-ILE are 257 and 289 L mol$^{-1}$ cm$^{-1}$, respectively, compared to a molar absorptivity of 7140 L mol$^{-1}$ cm$^{-1}$ for R-BNA.

Inner-filter effects can be produced when fluorophores closer to the front face of the cuvette (and the excitation source) decrease the number of photons which are available to excite fluorophores located further away from the source. In some cases, changing the orientation or shape of the sample holder minimizes inner-filter effects.
Because the current study employs two fluorescing species, a simple theoretical model has been proposed which will establish the extent of absorption and emission of the individual species.

Measuring fluorescence using a tandem cuvette in a conventional fluorimeter allows one to monitor the emission of two solutions without mixing them (Figure 4.10). Information about emitting species as a function of their proximity to the excitation source could lead to the identification of the sources of photon absorption (and emission) in a mixture of fluorophores. As indicated below, when two solutions containing individual fluorophores with overlapping emission spectra are excited, the fluorophore in the solution nearer to the excitation source will absorb and emit more photons. Therefore, its relative fluorescence intensity will be higher in the spectrum. This could help to further explain increases in intensity accompanied by shifts in wavelength as functions of individual fluorophore concentrations. Ultimately, these measurements could be used to confirm the legitimacy of single cuvette data.

Figure 4.10 Orientations of tandem cuvette in conventional fluorimeter
Solutions of $R$-BNA, $R$-BNA in increasing concentrations of CX4-LEU, and CX4-LEU were analyzed in both orientations. Fluorescence spectra of increasing concentrations of CX4-LEU on both sides of the cuvette were obtained and used as blanks. The cuvette held a solution of $R$-BNA in one side and a solution of $R$-BNA in increasing concentrations of CX4-LEU in the other. The concentration of $R$-BNA was held at $1 \times 10^{-5}$ M, while that of CX4-LEU increased from 0 to $5 \times 10^{-3}$ M. When one side was filled with $R$-BNA and the other was filled with $R$-BNA in increasing concentrations of CX4-LEU, the fluorescence intensity not only changed as a function of CX4-LEU concentration, but also as a function of solution position (Figure 4.11). Plot B represents $R$-BNA in CX4-LEU oriented closer to the excitation source, whereas curve A represents $R$-BNA in CX4-LEU further from the excitation source.

![Figure 4.11](image_url)  

**Figure 4.11** Effect of CX4-LEU concentration and cuvette position on fluorescence intensity. Tandem cuvette contains $1 \times 10^{-5}$ M $R$-BNA and $R$-BNA in increasing concentrations of CX4-LEU. Plot A (♦): $1 \times 10^{-5}$ M $R$-BNA closer to the excitation source; and Plot B (■): $R$-BNA in CX4-LEU closer to the excitation source.
The lower intensity of Plot A can be attributed to $R$-BNA in CX4-LEU re-absorption of $R$-BNA fluorescence, because the $R$-BNA concentration remains constant whether it is free or mixed in CX4-LEU solutions. In Plot B, the main absorbing and emitting species ($R$-BNA in CX4-LEU) are closer to the excitation source. Therefore, re-absorption of $R$-BNA in CX4-LEU fluorescence by $R$-BNA is less pronounced in Plot B. The magnitude of the increase in Plot B is mainly due to the increase in CX4-LEU concentration and is reminiscent of the plots obtained in single cuvette experiments. Also, fluorescence from an $R$-BNA:CX4-LEU association may contribute to the increase in intensity. In Plot A the fluorescence intensity is lower because the photons which $R$-BNA emits are re-absorbed by the two components of the second solution. In Plot B, the intensity increases more dramatically because the concentration of absorbing species closer to the excitation source increases by approximately one order of magnitude. The gradual decrease in intensity at high concentrations of CX4-LEU is indicative of the inner-filter effect regardless of complex formation.

When CX4-LEU solutions containing $R$-BNA are oriented closer to the excitation source than free $R$-BNA solutions more intense fluorescence results. This means that the two species closer to the source are the major participants in the absorption and fluorescence events. The fluorophores in the solution of free $R$-BNA are present in a much lower concentration. Therefore, their relative contribution to the overall fluorescence is small. If they are re-absorbing the fluorescence of the species nearer to the excitation source, it is to a smaller extent than if the reverse orientation were used. The spectra obtained in one orientation are illustrated in Figure 4.12, wherein $R$-BNA is closer to the excitation source than the CX4-LEU solutions containing $R$-BNA. The
shifts in the wavelength of maximum emission and changes in spectral shape indicate that the emission of CX4-LEU is increasing as the concentration of CX4-LEU increases. However, the emission of $R$-BNA is evident until the CX4-LEU concentration is at least twice as high as that of $R$-BNA.

![Graph of fluorescence emission spectra](image)

**Figure 4.12** Fluorescence emission spectra of $R$-BNA (closer to excitation source) and $R$-BNA in increasing concentrations of CX4-LEU (x$10^{-4}$ M).

Conversely, when CX4-LEU solutions containing $R$-BNA are closer to the excitation source, the spectra are similar to spectra of CX4-LEU blanks obtained in single cuvette experiments.

When the individual fluorophores were placed on separate sides of the cuvette, and the side containing $R$-BNA was oriented closer to the excitation source, the fluorescence by $R$-BNA is very distinct at lower CX4-LEU concentrations. However, as the CX4-LEU concentration increased, the spectra resembled spectra of the blanks. Interesting to note is the linear increase in fluorescence intensity over the entire CX4-LEU concentration range (Figure 4.13). When CX4-LEU solutions were oriented closer
to the excitation source, the measured intensity leveled off at higher CX4-LEU concentrations. This indicates that inner filter effects caused by the calixarene contribute to the decrease in the fluorescence.

**Figure 4.13** Relationship between R-BNA intensity and CX4-LEU concentration as a function of R-BNA proximity to excitation source.

Corrections for calixarene fluorescence and absorption were made in a fashion similar to those in the single cuvette experiments. Because the blanks contained twice as much CX4-LEU, one-half the intensity of each blank was subtracted from the spectrum of each sample in order to correct for calixarene fluorescence. Fluorescence was then multiplied by the absorption correction factor. Double reciprocal plots based on Equation 4.4 were generated assuming 1:1 and 2:1 CX4-LEU:R-BNA stoichiometry (Figure 4.14). The plot assuming 1:1 stoichiometry is a straight line, whereas that assuming 2:1 stoichiometry is curved. Therefore, it was deduced that CX4-LEU:R-BNA association is 1:1 and the estimated association constant is $1.25 \times 10^3 \text{ M}^{-1}$. Similar methods were performed with CX4-ILE. The linearity of the double reciprocal plot served as evidence
of a 1:1 CX4-ILE:R-BNA association. Also, the association constant was estimated to be 100 M$^{-1}$.

![Double reciprocal plots assuming A) 1:1; and B) 2:1 stoichiometry between CX4-LEU and R-BNA](image)

Figure 4.14  Double reciprocal plots assuming A) 1:1; and B) 2:1 stoichiometry between CX4-LEU and R-BNA

Typically, the concentration of the host should be at least twenty times higher than that of the guest in order for Ka values to be trustworthy. In light of the fact that the system being studied is more complex, Ka values should be validated by a comparable method.
such as NMR or X-ray crystallography. However, the data in this chapter qualitatively support the original hypothesis concerning the interactions of CX4-LEU and CX4-ILE with $R$-BNA.

4.4 Summary

Steady-state fluorescence spectroscopy has been employed to determine the stoichiometries and association constants of the possible complexes of CX4-LEU and CX4-ILE with $R$-BNA. The concomitant absorption and emission of the two species makes direct determination of the aforementioned parameters a formidable task. Therefore, corrections for calixarene absorption and emission must be made to account for the impact of inner-filter effects on the observed fluorescence intensity of $R$-BNA complexed with CX4-LEU and CX4-ILE. Linearity of Benesi-Hildebrand plots suggested that there was 1:1 association between CX4-LEU and $R$-BNA. However, estimated association constants in single cuvette experiments were indicative of extremely weak or no association. The convex curvature of Benesi-Hildebrand plots generated from single cuvette data suggested that there was a 2:1 (or higher) association between CX4-ILE and $R$-BNA. Tandem cuvette experiments were carried out to provide a theoretical model by which to compare results. Linearity of Benesi-Hildebrand plots not only suggested that there was 1:1 association between CX4-LEU and $R$-BNA, but also suggested the estimated association constant to be $1.25 \times 10^3$ M$^{-1}$. In addition, the stoichiometry of the CX4-ILE:$R$-BNA complex is assumed to be 1:1, with an estimated association constant of 100 M$^{-1}$. Although further method validation is needed, the theoretical model is suitable for qualitative support of EKC data.
4.5 Introduction

Atherosclerosis is a major cause of death in heart patients. It is a disease in which the arteries are hardened and narrowed as a result of plaque that has built up along the inside of the artery walls. The mechanism of formation of plaque is not well understood. However, it is believed that plaque develops in stages, from soft to hard. The earliest form of plaque is the fatty streak. This soft plaque mainly consists of cholesterol and other lipids. Calcified plaque (the hard plaque) forms later and mainly consists of calcium and phosphorus. Other minerals are also contained in plaque, but to a much lesser extent than calcium.

Minerals play a vital role in the proper function of cardiovascular systems. For example, sodium, magnesium, potassium, and calcium play an important role in proper muscle function. However, low levels of certain minerals have been associated with some forms of heart disease. Copper and zinc have been implicated in the degeneration of arterial smooth muscle, coronary aneurysm formation, and increased blood cholesterol levels. Likewise, lead and aluminum have been linked to hypertension. Aluminum interferes with calcium and magnesium metabolism and strongly binds to phosphate and oxygenated groups. Aluminum can also bind to phospholipids thereby altering cell membrane integrity. Lead is known to have a high affinity for sulfhydryl groups and can interfere with membrane function. Other minerals may not have a biological function, but may still be toxic to the body.

There is little information on the relative composition of minerals in different types of plaque. It is important to determine the mineral composition of different plaque
formations to afford better understanding of the possible mechanism of formation of these plaques. Warner and coworkers determined the metal concentrations in calcified and soft plaque using inductively coupled plasma spectrometry (ICP) in an effort to understand the factors that influence metal deposition in plaque, and to understand the possible role of metals in plaque formation. Twenty Eleven of the fifteen minerals detected in the plaques were metals. Concentrations of these metals are shown in Table 4.1. Clearly, calcium is a major component of hard plaque. Consequently, it is useful to determine the different forms of calcium present in each sample. To do so would require either an extraction or a separation technique that would isolate calcium from the other metals that are present in a plaque sample.

Table 4.1. Mean concentration of metals in soft and hard plaque (mg/g)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Soft</th>
<th>Hard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>2.72</td>
<td>3.54</td>
</tr>
<tr>
<td>Mg</td>
<td>1.01</td>
<td>1.36</td>
</tr>
<tr>
<td>Al</td>
<td>0.06</td>
<td>0.004</td>
</tr>
<tr>
<td>K</td>
<td>1.50</td>
<td>0.97</td>
</tr>
<tr>
<td>Ca</td>
<td>2.49</td>
<td>94.45</td>
</tr>
<tr>
<td>Cr</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>Fe</td>
<td>0.25</td>
<td>0.04</td>
</tr>
<tr>
<td>Cu</td>
<td>0.004</td>
<td>0.04</td>
</tr>
<tr>
<td>Zn</td>
<td>0.41</td>
<td>0.44</td>
</tr>
<tr>
<td>Ba</td>
<td>0.007</td>
<td>0.003</td>
</tr>
<tr>
<td>Pb</td>
<td>0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>

As indicated in Chapter 1 of this dissertation, calixarenes with cation-complexing groups attached are known to complex metals quite well. In fact, extraction studies have
shown that esters of calix[4]arenes were selective for sodium compared to other Group 1A and 2A metal ions, whereas calix[6]arene derivatives were selective for cesium and other radioactive ions. Some even proved to be excellent ionophores for sodium in liquid membrane and polyvinyl chloride membrane electrodes. Electrodes based upon calix[4]arene esters were reported to be capable of measuring sodium in blood, and are now used in commercial blood electrolyte analyzers. By changing the nature of the cation-complexing groups, the selectivity of the electrode changed from sodium to silver. Other calixarene-based ion-selective electrodes have been fabricated with high selectivities for potassium, lithium, lead, and mercury.

The metal ion of primary relevance to this study is calcium. Calixarene derivatives have also been used in sensors for calcium. More recently, they have been used as selectivity modifiers in capillary electrophoresis (CE) separations using indirect photometric detection. The overall goal of this study was to develop a CE method to selectively separate calcium from the other metals present in aqueous acidic extracts of plaque. To do so, p-sulfonated calix[4]arene and pentoxyl sulfonated calix[6]arene would be employed as metal-complexing agents in CE. However, the effect of metals on the ultraviolet absorbance of calixarenes must be investigated beforehand.

4.6 Experimental

Metal ion solutions (Na⁺, K⁺, Ca²⁺, Mg²⁺, Fe²⁺, Cu²⁺, Zn²⁺, Mn²⁺, and Ni²⁺) were prepared by dissolution of dry metal chlorides or sulfates in deionized water to yield stock solutions of 0.01 M. They were then diluted to yield solutions ranging in concentration from 6.875x10⁻⁷ to 2.8x10⁻³ M. Because of their strong UV absorption, p-sulfonated calix[4]arene (SCX4) and pentoxyl p-sulfonated calix[6]arene (SCX6) were
dissolved in water and diluted to final concentrations of $1.47 \times 10^{-5}$ M and $1.67 \times 10^{-5}$ M. All samples were sonicated before use. Ultraviolet absorbance spectra were obtained using a Shimadzu ultraviolet-visible-near infrared double-beam spectrophotometer controlled by UV3101-PC software. The instrument was equipped with a cooling chamber which held the sample holder region at 20 °C during the acquisition of spectra. Two quartz cuvettes (1 cm path length) were soaked in concentrated nitric acid and rinsed with copious amounts of deionized distilled water before use. The slit widths were set at 2 nm. The acquisition step size was 0.2 nm.

4.7 Results and Discussion

The absorbance spectra of SCX4 in the presence and absence of Ca$^{2+}$ are shown in Figure 4.15. The absorbance of SCX4 increased until the concentration of Ca$^{2+}$ surpassed it, after which a slight decrease was observed. Conversely, when SCX6 was used in place of SCX4, the maximum absorbance observed at equimolar concentrations of SCX6 and Ca$^{2+}$ decreased. In each case, the wavelength of maximum absorbance was 207 nm. Of the Group 1A metals, more significant increases were observed with K$^+$ compared to Na$^+$. There was no significant difference between the absorbance spectra of SCX4 when the Group 2A metals Ca$^{2+}$ and Mg$^{2+}$ were employed. Of the transition metals, the observed absorbance of SCX4 increased most with Cu$^{2+}$, while those observed with Mn$^{2+}$, Fe$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$ increased only slightly. These spectra are included in the appendix at the end of this dissertation. Because significant decreases in the absorbance of SCX4 were not observed at the various concentrations of the metals, it was decided that SCX4 would not be an appropriate complexing agent for use in CE.
Figure 4.15 A) Ultraviolet absorbance spectra of $1.47 \times 10^{-5}$ M SCX4 in the presence and absence of $3.33 \times 10^{-3}$ M Ca$^{2+}$; B) Change in absorbance of lambda max as a function of Ca$^{2+}$ concentration.

References


CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

The synthesis, characterization, and application of novel amino acid-derivatized calix[4]arenes in spectroscopy and EKC has been presented in this dissertation. The overall objective of calixarene chemistry is to explore and exercise the full potential of these molecules in a myriad of applications. Because the common forms of calixarenes are not chiral or water-soluble, study of their utility in biological systems and aqueous media is limited. A standing goal in the Warner research laboratory is to develop more universal chiral selectors for aqueous chromatographic separations. To this end, cyclodextrins, polymeric surfactants, dendrimers, and calixarenes have been functionalized to modify their selectivity for analytes.

The synthesis of \(N\)-L-aminoacylcalix[4]arenes was described in Chapter 2. Calix[4]arenes bearing L-alanine, L-valine, L-leucine, and L-isoleucine moieties as lower rim functionalities have been synthesized via the formation of a peptide bond. One- and two-dimensional NMR techniques were employed to confirm the synthesis of the calix[4]arenes and to determine their conformations. The existence of the tetrasubstituted calix[4]arenes in the cone conformation was evidenced by characteristic splitting patterns in the NMR spectra. Water-solubility in the calix[4]arenes came about by ionization of the carboxylic acids of the amino acids, yielding an anionic calix[4]arene. Because the inherent nature of the aliphatic amino acids is hydrophobic, water-solubility is limited to pH values \(\geq 7\). Future studies could focus on extending the pH range of water-solubility. For instance, replacement of the calixaryl \(t\)-butyl groups with more hydrophilic groups like sulfonates, polyethoxy alcohols, and quaternary amines would extend the pH range below 7, and would allow for calixarene dissolution in a larger variety of inorganic
solvents such as those commonly used in aqueous CE. Another alternative would be to link an NH$_2$-protected amino acid to the calixarene through the carboxyl terminus of the amino acid, resulting in a cationic calixarene which would be water-soluble at pH values less than 7.

The second part of Chapter 2 describes the synthesis and characterization of calixarene-bonded silica stationary phases for use in capillary electrochromatographic applications. Techniques such as IR, solid-state $^{13}$C NMR and elemental analysis were employed to characterize modified and unmodified batches of silica. Although the presence of a strong silicon-oxygen stretch limits the utility of IR for product characterization, it is effective for the characterization of reactants. Downfield shifts of the aminopropyl carbons in the solid-state $^{13}$C NMR spectra of the samples suggest that there have been changes in their local environments. This leads me to believe that I have successfully attached CX4-VAL to APS. However, further characterization is pertinent in order to adequately explain the lack of a shift in the carbonyl signal. The results of elemental analysis reveal an increase in the percentages of carbon, hydrogen, and nitrogen on APS after synthesis. However, bulk averaging by elemental analysis most likely poses severe limitations on the determination of the actual surface coverage of APS.

Future studies could focus on incorporating a surface-sensitive technique such as X-ray photoelectron spectroscopy as a means by which to correct for the presence of organic contaminants on silica beads while allowing for the calculation of the degree of coverage as a function of elemental signal intensity. Also, improvements could be made to the synthetic techniques in order to increase sample loading. In the meantime,
investigations of the effectiveness of CVAPS and other chiral stationary phases in capillary electrochromatography are currently underway. Such ambitious method development will extend the utility of our chromatographic techniques because they will better lend themselves to online coupling with other characterization and fractionation techniques.

In Chapter 3 I described the application of four novel amino acid-derivatized calixarenes in EKC. Drawing from previous separations using calixarene-modified EKC, I have separated atropisomeric binaphthyl derivatives in a single run using urea as an organic modifier in the run buffer. Separation of the binaphthyl derivatives in a single run produced a slightly different elution order when the buffer containing the isoleucine-derivatized calixarene was used. In addition to the binaphthyl derivatives, the calixarenes were effective chiral selectors for a group of neutral benzodiazepines. By varying experimental parameters such as pH and chiral selector concentration, enantiomers of lorazepam, oxazepam, and temazepam were baseline resolved. Additional electropherograms highlighting the separation of Troger’s base and cationic norlaudanosoline are included in the appendix. Because the calixarenes absorb strongly in the UV region, their utility is limited when a UV or laser-induced fluorescence detector is employed. Future EKC work should incorporate another detection method in order to extend the effectiveness of the chiral selectors. Also, EKC investigations of “real” samples would be an ambitious undertaking since calixarenes are currently being used as ionophores in commercial blood analyzers.

In Chapter 4, the complexation of R-BNA with CX4-LEU and CX4-ILE using steady-state fluorescence spectroscopy was discussed. It was reported previously that
sulfonated calix[6]arene would not be suitable as a host molecule because it produces inner-filter effects. However, the proposed method accounts for inner-filter effects and allows for the calculation of association constants, the fluorescence intensity when all the $R$-BNA is complexed, and complex stoichiometry using a simple theoretical model. Results qualitatively prove the hypothesis that CX4-LEU binds $R$-BNA more strongly than CX4-ILE. Future directions could focus on the investigation of the atropisomeric pairs as a means by which to establish more correlations between EKC results and fluorescence results. Also, because it is important to study the mechanism of chiral recognition for each analyte, this model could also be extended to poorly resolved analytes as a qualitative starting point for predicting analyte elution order based upon interaction other than hydrophobic interactions.
APPENDIX: ADDITIONAL FIGURES DISCUSSED IN PREVIOUS CHAPTERS

Figure A1. Infrared spectra of A) CX4-L-VAL; B) APS; C) CVAPS. Conditions: Mixed sample with dry KBr (1:100) for KBr pellet.
Figure A2. Chiral separations using CX4-LEU as chiral selector in EKC. Analytes: A) Troger’s base; B) norlaudanosoline
Figure A3. Ultraviolet absorbance spectra of increasing concentrations (x10^{-4} M) of CX4-LEU in 100 mM Tris.

Figure A4. Ultraviolet absorbance spectra of increasing concentrations (x10^{-4} M) of CX4-ILE in 100 mM Tris.
Figure A5. Corrected fluorescence emission spectra of $10^{-5}$ M R-BNA in increasing concentrations ($x 10^{-4}$ M) of CX4-ILE

Figure A6. Emission intensity of R-BNA as a function of CX4-ILE concentration (with and without CX4-ILE absorbance correction)
Figure A7. Double reciprocal plot of intensity of $R$-BNA as a function of CX4-ILE concentration, assuming 1:1 stoichiometry; association constant and intensity of completely complexed $R$-BNA are negative.

Figure A8. Double reciprocal plot of intensity of $R$-BNA as a function of CX4-ILE concentration, assuming 2:1 stoichiometry; association constant and fluorescence intensity of completely complexed $R$-BNA are $1.5 \times 10^6$ M$^{-1}$ and 338867, respectively.
Figure A9. Double reciprocal plots assuming 1:1 and 2:1 stoichiometry between CX4-ILE and R-BNA. Straight line means there is 1:1 association. The association constant is 100 M$^{-1}$. 

145
Figure A10. Ultraviolet absorbance spectrum of \( p \)-sulfonated calix[6]arene in the presence and in the absence of Ca\(^{2+} \).

Figure A11. Ultraviolet absorbance spectra of \( p \)-sulfonated calix[4]arene in the presence and absence of alkali metal cations.
Figure A12. Ultraviolet absorbance spectra of $p$-sulfonated calix[4]arene in the presence and absence of alkaline earth cations

Figure A13. Ultraviolet absorbance spectra of $p$-sulfonated calix[4]arene in the presence and absence of transition metals
Figure A14. Ultraviolet spectra of \textit{p}-sulfonated calix[4]arene in the presence and absence of copper and zinc.
VITA

Kimberly Hamilton is the second of two children born to Macura and Delia Monroe Hamilton. A native of Caddo Parish, Louisiana, she attended Louisiana private and public schools and graduated as a Louisiana Board of Regents scholar in the top 10% of her graduating high school class in 1990.

She attended Southern University and A & M College in Baton Rouge, Louisiana. While at Southern, Kimberly was chosen to be a Minority Undergraduate Training for Energy-related Careers (MUTEC) scholar. With this honor came the opportunity to participate in environmental research. Under the direction of Ms. Georgia Brown, Kimberly investigated the microbial degradation of ethylene glycol monomethyl ether. She also worked with Moses Omafuaire on the spectroscopic quantitation of heavy metals in human hair and in drinking water. During her undergraduate tenure, she received a Department of Chemistry scholarship and the Outstanding Inorganic Chemistry student award. She also was a member of Delta Sigma Theta Sorority, Incorporated. Kimberly graduated with a Bachelor of Science degree in chemistry in 1995.

Before entering graduate school at Louisiana State University (LSU), Baton Rouge, Louisiana, she worked as an environmental and industrial laboratory technician at Entek Environmental Lab, Ciba Specialty Chemicals, and Allied Signal. While enrolled at LSU, she received a National Institutes of Health predoctoral fellowship. Her current research in the laboratory of Dr. Isiah Warner focuses on the synthesis, characterization, and application of calixarene derivatives in spectroscopy and electrokinetic capillary chromatography. She also served as public relations chairperson and newsletter editor of
the LSU chapter of the National Organization for the Professional Advancement of Black Chemists and Chemical Engineers for the 2001-2002 term. She also was a member of the American Chemical Society. In April 2003, she was presented the Charles E. Harrington Graduate Student Award by the LSU Black Faculty and Staff Caucus. She will receive the Doctor of Philosophy degree in analytical chemistry in August 2003.