ANALYSIS OF SELF-ASSEMBLING COMPLEXES VIA SUPRAMOLECULAR MASS SPECTROMETRY

By

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To Oliver, Ella, and Jake
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Self-assembling supramolecular complexes are stable configurations of two or more molecules stabilized by noncovalent intermolecular interactions, which aggregate independently according to molecular recognition principles. Noncovalent, e.g., cation-\(\pi\) and hydrogen-bonding, interactions make important contributions to the structure and function of many biomolecules. They are vital to protein folding and stabilization and so to substrate specificity and enzyme action. Host-guest inclusion complexes stabilized by these interactions are used in areas such as enzyme mimicry, catalysis, and therapeutic drug development and delivery. Modeling and analysis of these noncovalent interactions are essential to the development of selective synthetic hosts. Thorough analytical characterization of diverse supramolecular systems is necessary to contribute to the wealth of data required to gain a better understanding of the inherent chemical behavior involved in molecular recognition and noncovalent complex formation. Mass spectrometry, as a gas-phase analytical technique, has the ability to provide vital information concerning supramolecular chemistry in the absence of interference from a solvent shell. The advancement of supramolecular mass spectrometry was the major goal of this research.
A pair of designed synthetic receptors for alkali-metal cations based on the natural product 1,3,5-trihydroxybenzene (phloroglucinol) was thoroughly characterized in the gas phase using advanced supramolecular mass spectrometric techniques. Following additional solution-phase characterization using absorbance spectroscopy in the ultraviolet region, it was determined that the mass spectrometric approach and the optical spectroscopic approach combined to yield useful complementary characterization information.

Following the configuration of a home-built source with a time-of-flight mass analyzer, desorption electrospray ionization (DESI) was developed and validated as a useful novel analytical tool for this area of application, through the design and implementation of a rapid screening experiment for potential guest compounds for supramolecular encapsulation by a β-cyclodextrin host. Comparison experiments, using nuclear magnetic resonance spectroscopy as the standard solution-phase validation technique, revealed that DESI is a superior ionization technique to the commonly-employed electrospray ionization (ESI) for this type of work. DESI is not as prone to the detection of false-positive nonspecific complexes resulting from the formation of artifacts during the electrospray process. Thus a useful addition to the supramolecular mass spectrometry toolkit has been contributed.
CHAPTER 1
INTRODUCTION

The term supramolecular chemistry was first used by Jean-Marie Lehn in 1978 to encompass all of the previously divided areas breached by his groundbreaking work exploring the noncovalent chemistry of macropolycycles.\(^1\) He has defined it as ‘the chemistry of the noncovalent bond’,\(^2\) briefly comprising intermolecular binding, catalysis, molecular recognition, self-assembly, directed molecular design, and self-replication phenomena.\(^3\) Over the preceding decade, various alkali and alkaline-earth cations had been observed to form stable complexes with macrocyclic ligands, the most famous example being the crown ether family discovered by Charles Pedersen,\(^4\) and Lehn had been working on synthesizing macropolycycles with designed molecular recognition properties.\(^1\) As more supramolecular species were synthesized and their complexation characteristics explored, an entire discipline evolved. Lehn, Pedersen, and Donald Cram, a synthetic chemist specializing in cyclophane chemistry, were subsequently awarded the 1987 Nobel Prize in Chemistry for work resulting in creation of the field.\(^5\) Today, supramolecular chemistry is an accepted interdisciplinary area with far-reaching biological significance.

Mass spectrometry, measurement of the mass-to-charge ratio of ions, is an analytical technique applicable not only to the structural elucidation and quantitative analysis of molecules but also to kinetic and conformational studies. Inherently a gas-phase technique, it is well-suited to the study of supramolecular complexes because it enables the study of intrinsic properties of ions without the interference of molecules of solvation.\(^3,6\) Recently developed soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have addressed the challenge of providing charge to the complexes without
disrupting the weak stabilizing noncovalent interactions,\textsuperscript{6} making mass spectrometry accessible to a plethora of supramolecular applications.

The research described in this thesis contributes to the development of advanced supramolecular mass spectrometry beyond general stoichiometric complex detection, and demonstrates how combining useful characteristics of different types of instrumentation and innovative experimental design can maximize acquisition of information about supramolecular complexes via mass spectral analysis. Electrospray ionization and tandem mass spectrometry were used to probe the noncovalent cation-bound homodimer formation behavior of a novel pair of designed alkali-metal cation receptor synthons. Electrospray ionization mass spectral characteristics were used to analyze the effects of structural differences between monomers on the noncovalent interactions responsible for stabilization of the corresponding heterodimer. Solution-phase optical spectroscopy provided insight into the supramolecular structure of the homodimers, but highlighted the necessity of gas-phase analytical techniques for the determination of supramolecular complexes without the complication of competition for binding sites between the complex components and molecules of solvation. Fragmentation pathways for alkali-metal cation adducts of some phloroglucinol derivatives were elucidated using electrospray ionization tandem-in-time mass spectrometry.

A home-built desorption electrospray ionization (DESI) source was configured to two high-resolution mass analyzers individually, and some varying configurations and DESI parameters were compared. Subsequently, the recently-developed soft desorption/ionization technique, DESI, was applied to rapid screening of supramolecular host-guest inclusion complexes for the first time. Data obtained were compared to the results of an analogous ESI screening experiment and demonstrated significant inconsistencies. Following solution-phase
validation using nuclear magnetic resonance (NMR) spectroscopy, reactive DESI was
determined to be the superior ionization technique for the application. Computational chemistry
confirmed that the tendency of ESI to yield nonspecific artifacts, which were detected as false-
positives in the cyclodextrin guest screening analysis, is a disadvantage of the technique which is
not shared by DESI. The reactive DESI screen resulted in positive confirmation of only those
complexes determined by NMR spectroscopy. The ESI screen did not yield detection of the
weakest binding inclusion guest, and consequently did not demonstrate comparable sensitivity to
the reactive DESI analysis. No evidence to suggest preferential detection of kinetic rather than
thermodynamic products using DESI was observed, despite the short interaction time between
host and guest molecules during the DESI event.

**Supramolecular Chemistry**

**Concepts**

Supramolecular chemistry is the structural and functional behavior of organized entities
formed through association of two or more chemical species in the absence of a traditional
covalent bond. The species involved in the association, or complexation, can be referred to as
the host and guest or the molecular receptor and substrate, and they associate uniquely via
electrostatic forces differing from full covalent bonds to form complexes. The host is formally
defined as the possessor of convergent binding sites such as Lewis base donor atoms while the
guest possesses divergent binding sites such as Lewis acid donor atoms or hydrogen-bond-
accepting halides.

The complexes form through molecular recognition interactions stemming from
information pre-programmed into the associated molecules which is manifested in their
geometric and chemical characteristics and defines the nature of the governing interactions. This
concept of encoded molecular information is responsible for selective binding of individual
substrates by specific molecular receptors and is the principle underlying the phenomenon of
self-assembly.

The process by which molecules spontaneously form ordered aggregates with no external
interference is molecular self-assembly, but self-assembly is not limited to molecules. Self-
assembly has been reported for aggregation of varying species up to macroscopic dimensions on
the order of centimeters; the major pre-existing condition for the event on any scale is
complementarity in terms of geometric shape, or topology.\textsuperscript{10} Species form self-assembling
complexes if their binding sites are of complementary electronic character and are spaced
appropriately for interaction. A preorganized host will undergo minimal conformational changes
during the binding event,\textsuperscript{5} which is important in terms of the thermodynamic favorability of
binding. In supramolecular complexation, alternatively referred to as noncovalent synthesis, the
products are equilibrating structures. Therefore, a complex must correspond to a thermodynamic
minimum in order to self-assemble.\textsuperscript{11} Kinetic driving forces are also relevant, and an important
consideration here is that a conformationally rigid preorganized molecule may be slow to bind a
guest because of the difficulty it experiences passing through a transition state necessary for
complexation. Thus a balance must be achieved to promote self-assembling complex formation.\textsuperscript{5}

Broadly, supramolecular complexes may be divided into two categories: cavitates and
clathrates. This distinction is based on the position of the bound guest. Hosts possessing
intramolecular cavities are cavitands and guest binding, or inclusion, results in formation of a
cavitate, a complex in which the guest or guests are encapsulated by a pocket in the host
molecule. Conversely, clathrates are formed when guests are bound in extramolecular cavities
formed by aggregation of two or more molecules.\textsuperscript{12} For clarity, see Figure 1-1. The designed
synthetic alkali-metal cation receptor phloroglucinol derivative dimers characterized in chapters
two and three are clathrates binding the cation between two monomers. The cyclodextrin 
complexes investigated in chapter five are cavitands with the cyclodextrin host encapsulating 
guests inside a hydrophobic intramolecular cavity.

Types of Noncovalent Bonds

Non-covalent interactions are labile and confer on supramolecular complexes an important 
intrinsic property, dynamic character. Practically, this attribute enables supramolecules to 
reversibly associate and dissociate, yielding selective self-organization as individual molecules 
are able to undergo conformational changes to promote favorable molecular recognition 
interactions.9 Supramolecular complexes are stabilized by several types of noncovalent 
interactions but another important consideration is the nature of their surroundings.6 The relative 
influences of the presence of molecules of solvation and crystal close-packing effects can greatly 
impact the thermodynamic stability of these weakly-stabilized entities.5

The major types of noncovalent bonds are all based on electrostatic interactions arising 
from the presence of small directional and nondirectional electrical charges on the interacting 
molecules. Two partial electrical charges interact to produce either attractive or repulsive forces; 
charges with opposite polarities will yield an attractive force while like-charges repel.13

Ion-ion interactions

Ions will interact with electrostatic dipoles resulting from the electron density on the 
potential surface of a molecule. Ion-ion interactions are the strongest purely electrostatic 
interactions with bond energies of ~100-350 kJ mol\(^{-1}\), and bond strength is dependent on 
interionic distance and the extent of charge delocalization.13 This is the major reason that crystal 
close-packing forces can have such a significant effect on supramolecular complex stability and, 
therefore, must be taken into consideration in the design of analytical methodologies involving 
supramolecular co-crystallization.
Ion-dipole interactions

Ion-dipole interaction strength is dependent on the orientation of the dipole with respect to the ionic charge and is of the order of 50-200 kJ mol\(^{-1}\). Co-ordinate or dative bonds fit into this category but they also incorporate some covalent contribution.\(^5\)

Dipole-dipole interactions

Interactions between two appropriately-aligned dipoles can yield attractive forces with bond energies in the region of 5-50 kJ mol\(^{-1}\). Possible orientations include matching of a pair of opposite poles on adjacent molecules or opposing alignment of two adjacent dipoles (Figure 1-2).\(^5\)

Hydrogen bonds

A specific instance of a dipole-dipole interaction corresponding to the attractive force between a hydrogen atom attached to an electronegative atom and a neighboring dipole results in a hydrogen bond. Bond energies between 4-120 kJ mol\(^{-1}\) are known and various bond lengths from 1.2 to 3.2 Angstroms have been reported.\(^5\) One important consideration that must be addressed in the analysis of hydrogen-bonded supramolecular assemblies is the solvation strategy, because protic solvents can weaken or even destroy hydrogen-bonds.\(^3\) This is another reason that environment is a major factor in supramolecular chemistry.

Cation-\(\pi\) interactions

Aromatic rings consist of a partially positive \(\sigma\)-scaffold and a partially negative \(\pi\)-cloud above and below the plane of the ring. This confers a quadrupole moment on species such as benzene which can result in an attractive force of the order of 5-80 kJ mol\(^{-1}\) between the \(\pi\)-cloud and an appropriately located cation (Figure 1-3).\(^{14}\)
\textbf{π-Stacking interactions}

Attractive forces of 0-50 kJ mol\(^{-1}\) can be attributed to π-stacking of aromatic rings. The presence of the previously described quadrupole moment enables attractive electrostatic interactions between oppositely charged regions of the rings. Edge-to-face and face-to-face orientations exist as well as various intermediate geometries, but face-to-face configuration never corresponds to direct overlap which would yield a repulsive force.\(^{15}\)

\textbf{Biological and Physical Significance}

Noncovalent intermolecular interactions make myriad contributions to the natural world. Structure and function of many important biomolecules are delineated by noncovalent interactions. The immediately recognizable double-helix structure of deoxyribonucleic acid, DNA, is partially stabilized through hydrogen bonds\(^{16}\) and π-stacking, or arene-arene, interactions, which also govern the higher-order structure and thermal stabilization behavior of some proteins.\(^{17}\) Active site binding between an enzyme and ligand often involves cation-π interactions,\(^{13}\) hence biological catalysis is inherently dependent on supramolecular forces. Active transport across lipid bilayers, which form structures such as cell membranes, is also facilitated by noncovalent interactions, cation-π in the case of transport of ions by ionophores, and the bilayers themselves are stabilized by hydrogen-bonds.\(^{5}\)

Molecular devices are organized, functionally-integrated chemical systems built into supramolecular architectures. They are capable of performing a specific function upon activation by some external stimulus.\(^{7}\) These molecular devices based upon supramolecular chemistry principles are used as sensors and switches for multiple applications.\(^{18}\) Crystal engineering is inherently a self-assembly field, and crystal growth is based on noncovalent interactions.\(^{5}\)
Consequently, supramolecular chemistry has considerable significance beyond the frequently-discussed application to biologically-relevant molecules and processes.

Synthetic chemists seek to exploit supramolecular chemistry for various reasons. Production of synthetic species capable of mimicking the catalytic and selective binding phenomena observed in biomolecules like enzymes and proteins has significant implications to drug design and delivery research. Host-guest inclusion complexes stabilized by noncovalent interactions are currently used in areas such as catalysis and the encapsulation of drugs. Design of hosts capable of specific molecular recognition behavior is a rapidly-expanding area of research. Capitalization on self-assembly can aid synthetic strategy development in areas like supramolecular catalysis and if appropriately employed can increase synthetic yields and enhance synthetic selectivity. A current aim of supramolecular chemists is to achieve realistic theoretical modeling of noncovalent interactions, and that will require an abundance of physical data so appropriate analytical techniques are in high demand.

**Mass Spectrometric Approaches**

Mass spectrometry, the measurement of the mass-to-charge ratio (m/z) of ions, is an analytical technique known for the three S characteristics: speed, sensitivity, and selectivity. The m/z is the ratio of the mass of a particle to the number of electrostatic charge units carried by the particle, and it can be used to aid identification of the elemental composition of said particle. Mass spectrometric analysis requires several major stages. The first stage, following sample introduction, is ionization, for which a strategic explanation is outside the scope of this section. The ionization stage combines, in most cases, nebulization and incorporation of a charge onto the analyte or analytes of interest. Nebulization is necessary because mass spectrometry is inherently a gas-phase technique as particles must be separated for detection. Ionization is essential to charge the particles for separation according to m/z. Arguably, the second stage is
fragmentation of the ionized particles. In instances where ‘hard’ ionization is used, this is combined with the ionization stage, and in some cases, for example when only the m/z ratio of the whole particle is required, this stage is omitted. Fragmentation will be discussed in greater detail in the tandem mass spectrometry section. Differentiation of the ions introduced on the basis of their m/z follows and may be referred to as the mass analysis stage, and ion detection completes the experiment. Data are presented as a spectrum of absolute or relative signal intensity versus m/z, and the signal detected at each m/z is represented as a peak.

Ionization may be achieved in a variety of ways. The most appropriate method for a specific analysis depends on the sample, the analyte, and the desired information. Techniques used for ionization are generally classified as either hard or soft, based on the energy required and the resulting ion types. Hard ionization methods such as electron ionization (EI) or chemical ionization (CI) break apart the ionized particle and produce only low abundances of molecular ions or protonated molecules but yield several characteristic fragment ions. Soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) result in minimal fragmentation but yield significantly more intense signals corresponding to the intact ion. Although a vast array of fragmentation strategies exist, all the mass spectral data presented here were produced using ESI or desorption electrospray ionization (DESI), a related technique. Consequently, the extended discussion here will be limited to a brief description of ESI.

The ESI technique, developed by John Fenn, enables the transfer of solution-phase ions into the gas phase, and so has been widely adopted as an interface for liquid chromatography (LC) and capillary electrophoresis (CE). Electrospray ionization (see Figure 1-4) involves electrochemical charging of a flowing liquid sample via the application of a high voltage across a
fine-tipped spray needle and an endplate; charged droplets of solution form. The droplets exit the spray needle in a Taylor cone, the apex of which narrows to form a liquid jet and ultimately an aerosol.\(^{23}\) The mechanism by which this aerosol yields ions is the subject of debate. Two convincing possibilities are commonly cited: droplet fission at the Rayleigh limit and direct field evaporation of ions. Both can be referred to as the desolvation mechanism or step, and desolvation efficiency is significantly enhanced at elevated temperatures.\(^{24}\) For this reason, charged droplets are introduced into a heated capillary prior to mass analysis via a potential gradient. Generally, the opposing ends of the heated capillary are charged to aid focusing of the ion beam into the mass spectrometer. This feature confers a useful capability on the ESI technique, source-skimmer collisionally induced dissociation (SCID)\(^{27}\) which will be discussed further in the tandem mass spectrometry section. As noted, the ESI process results in very limited ion fragmentation and, therefore, has the benefit of promoting determination of molecular weight. Another important advantage of ESI is its ability to produce multiply charged intact ions which makes it amenable to the analysis of large molecules. Increasing the charge decreases the m/z, bringing the signal into more efficient ranges for mass analysis.\(^{24}\)

Different instrumental approaches define the type of information obtained and the way that mass spectrometry can be applied to a specific analytical issue. Maximizing the information obtained in a mass spectrometric analysis is generally achieved in one, or sometimes both, of two ways: high-resolution mass spectrometry and tandem mass spectrometry. Although a combination of both these approaches can be used, this is not always desirable due to constraints of expense and time, as well as control of the amount of data acquired, which is a very real and problematic issue in terms of storage and processing capacities in mass spectrometry labs today.
High-Resolution Mass Spectrometry

Concepts

Resolution is a measurement of the degree of separation between adjacent peaks corresponding to ions of different m/z. It is defined below, and should not be confused with resolving power which is an indication of the theoretical resolution capability of an instrument.

\[ R = \frac{m}{\Delta m} \]  

(1.1)

In equation 1.1, \( m \) is the m/z value of the peak for which resolution is being defined and \( \Delta m \) is either the difference in m/z between two adjacent peaks or the width of the peak, but some indication of the shape and separation of the spectral peaks must be included in the definition to give it a tangible meaning. In some cases, a measure of the height of the valley between the peaks relative to the height of the peaks themselves, commonly 10 or 50%, is used to account for this. Alternatively, a measure of the width of the peaks at some specified fraction of their height is substituted; often the peak width at half the peak height or full-width half maximum, FWHM, is employed (see Figure 1-5). Resolution between 100-1,000, is usually termed low resolution, medium resolution corresponds to \( R=2,000-10,000 \), and \( R>10,000 \) is classified as high-resolution.28

Calculated exact mass, a term often incorrectly substituted for high-resolution, is used to describe the sum of the exact mass of the individual isotopes that compose a single ion,29 and is often reported to a certain point of precision, for example four decimal places. Each element has a specific monoisotopic mass, e.g., carbon by definition has a monoisotopic mass of 12.00000 atomic mass units, the exact value of which corresponds to the mass of the lowest number of subatomic particles one of its atoms can comprise. In mass spectrometry, the term measured accurate mass is used to mean the measured m/z reported with four decimal places and less than
15 ppm error. Since each isotope of an element has a specific and precise mass, as the mass accuracy is improved, a particle corresponding to a certain m/z can be more accurately identified in terms of elemental composition. Fewer permutations of elemental combinations can yield a specific m/z than a nominal m/z. High-resolution mass spectrometry yields accurate mass values, so it can be one important approach to increase the useful information obtained via mass spectrometric analysis. Combined with soft ionization techniques which produce protonated, deprotonated, or adducted, e.g., sodiated, molecules or molecular ions, the elemental composition of an ion can often be narrowed down to a manageable number of possibilities. Peak distributions corresponding to the isotopic distributions and mass defect values are also valuable contributors to the determination of elemental composition. The major disadvantage, however, is the lack of structural information inherent in resultant spectra.

**Instrumentation**

There are three main types of high-resolution mass spectrometers and they are each suited to different situations. All three are capable of high resolving power either because they rely on detection by measurement of time, for which extremely accurate measurement capabilities are available, or because they employ combinations of mass analyzers for superior ion beam focusing. They all require frequent calibration with known mass standards to maintain performance. Double-focusing sector instruments incorporate two mass analysis stages using both magnetic and electric fields to separate and focus ions on the basis of kinetic energy and velocity, and are excellent for configuration with high-energy ionization and dissociation techniques. Resolving power high enough to yield resolution of the order of 50,000 has been reported. Sector instrumentation, however, was not used in this body of work and further discussion is therefore outside the scope of this dissertation.
Time-of-flight, TOF, mass analyzers pulse packets of ions along a field-free flight tube of known path length. The ions entering the drift tube are pulse accelerated so they all have the same initial kinetic energy. The velocity of their flight, therefore, is dependent on their mass according to the equation below, where \( E \) is kinetic energy, \( m \) is mass, and \( v \) is velocity.

\[
E = \frac{1}{2}mv^2
\]  

(1.2)

Since the path length is known, measurement of the flight-time of each ion enables determination of the mass, or in this case the \( m/z \), using the following.\(^{22}\)

\[
\frac{m}{z} = 2eV \left( \frac{t}{L} \right)^2
\]  

(1.3)

In equation 1.3, \( m/z \) has been defined, \( e \) is the charge on an electron, \( V \) is the potential used to accelerate the ions, \( t \) is the flight time, and \( L \) is the path length. Resolving powers of the order of 10,000 have been reported for orthogonal-acceleration reflectron TOF mass analyzers\(^{33}\) such as the one used in this body of work. Manufacturer claims of 15,000 have been made.

Fourier transform ion cyclotron resonance (FTICR) mass analyzers incorporate a superconducting magnet. Ions are introduced into the magnetic field where they are trapped electrostatically and forced into ion cyclotron motion, a circular orbit with a frequency inversely proportional to the \( m/z \) ratio of each ion.\(^{34}\) Briefly, orthogonal RF excitation of the ions yields a coherent packet of ions traversing one circular path. The combined time domain signal of the ion orbits is measured via the induced current on a pair of parallel plates and the signal is subjected to Fourier transformation for conversion to the frequency domain.\(^{35}\) The relationship between the frequency and the \( m/z \) is shown below, where \( f \) is frequency and \( B \) is magnetic field strength.\(^{35}\)

\[
\frac{m}{z} = \frac{B}{2\pi f}
\]  

(1.4)
High mass-accuracy, typically below 5 ppm relative mass error, and resolving power of 150,000 can be achieved using an intermediate magnetic field of 4.7 Tesla,\textsuperscript{31} and the Marshall group continues to observe increased resolution as they increase the strength of the incorporated magnetic fields.\textsuperscript{32} The trapping capabilities of this type of mass analyzer also make it suitable for tandem mass spectrometric analysis, but the expense of acquiring and maintaining a superconducting magnet, slow spectral acquisition, and large size of data files limit the use of FTICR-MS.

**Tandem Mass Spectrometry**

Tandem mass spectrometry is the measurement of the m/z of ions prior to and following a reaction within the mass spectrometer. The term encompasses a plethora of instrumental and experimental approaches,\textsuperscript{36} but only a very limited discussion of the strategies relevant to this body of work will be presented here. Major reasons for the popularity of tandem mass spectrometry, sometimes referred to as MS/MS or MS\textsuperscript{n}, are its usefulness in structural elucidation of molecules and analysis of complex mixtures.\textsuperscript{36-38} As mentioned, soft-ionization high-resolution work often only yields information about elemental composition\textsuperscript{39} so structural geometry and the configuration of functional groups remain unaddressed. In tandem work, the term precursor, or parent, ion refers to the detected ion prior to the reaction event, and the ion or ions generated via the reaction, for example a dissociation event, are the product, or daughter, ions. Precursor ions are not necessarily molecular ions.\textsuperscript{36}

Appropriate experimental design is an important caveat to the application of tandem mass spectrometry. The type of problem under investigation and the information required define the design. Traditionally, mass spectrometry may be considered tandem-in-time or tandem-in-space. Experiments involving ion selection, reaction, and detection in different regions of the mass spectrometer are considered tandem-in-space. Sector instruments and triple quadrupole
instruments are commonly employed for tandem-in-space experiments,\textsuperscript{40,41} although the aforementioned SCID technique was incorporated in a tandem-in-space configuration with a TOF mass analyzer as part of this research. Tandem-in-time approaches involve selection and isolation of an ion, reaction, and detection in a single region of the mass spectrometer and events occur sequentially. Ion trapping instruments such as the FTICR and various geometry ion trap (IT) instruments are employed for this type of experiment.\textsuperscript{40,41} Data presented in Chapter 3 were acquired using a linear quadrupole ion trap.

Briefly, trapping instruments for tandem work employ electrostatic trapping of ions following ionization. A precursor ion is selected by mass or energy and the unwanted ions are ejected from the trapping region electrostatically. Introduction of the other components necessary for the reaction, for example an inert gas for collision and fragmentation, into the region occurs, and the resultant product or daughter ions are detected.\textsuperscript{42} Theoretically this process can repeat indefinitely, yielding data following multiple events, but experimentally some limiting factor, for example initial signal intensity or low mass cutoff, will govern the number of successive reactions successfully monitored.\textsuperscript{36}

When an ESI source comprises the front end of a mass spectrometer, tandem mass spectral data can be acquired even in instruments not traditionally designed for it. As discussed, the exit end of the desolvation capillary is held at a high voltage, as is the skimmer succeeding it, to force the ions to travel into the mass analyzer region of the instrument. Increasing the voltage difference between the exit end of the desolvation capillary and the skimmer, or skimmer-cone, increases the corresponding energy imparted to the desolvated ions which can result in dissociation. This strategy is called source-skimmer or nozzle-skimmer collisionally-induced...
dissociation because the millibar pressure in the fragmentation region of the source promotes collision between ambient molecules and the sprayed ions.  

Tandem mass spectra acquired using trapping or sequential quadrupole or sector instruments yield spectra where parent ion selection and isolation enables determination of the true relationships between sequentially detected ions. The SCID technique suffers from an inability to select the precursor or parent ion, so ions detected following the reaction have uncertain parentage. Therefore SCID is not the technique of choice for elucidating dissociation pathways or uncovering structural information, although it is often used for these applications because of its simplicity, low cost, and accessibility. It has found its niche, however, in experiments designed for comparison of relative ion stabilities, and it is also useful for isomer differentiation and improved analyte detection. In-cell collisionally-induced dissociation, CID, can be problematic for ion stability determination of noncovalent complexes because the high-energy collisions generally employed break covalent bonds of the parent ion causing rapid and extensive fragmentation over a very narrow energy-range, making construction of reliable dissociation curves difficult.

**Supramolecular Mass Spectrometry**

**Gas-Phase Supramolecular Chemistry**

Elucidation of the chemistry of gas-phase supramolecules is of vital importance to the development of supramolecular chemistry as a field. Particles in the gas-phase are essentially isolated entities; they experience negligible interference from surrounding gas-phase particles and are not surrounded by molecules of solvation. Consequently gas-phase analyses are capable of characterizing the intrinsic properties of the system under investigation. In order to further the understanding, modeling, and ultimately synthetic utilization of the physical and chemical bases of noncovalent bonds, analytical strategies to characterize intrinsic behavior of
supramolecular complexes are essential. In a field where stabilizing forces are relatively weak, this is a very relevant consideration, because molecular chemistry can no longer be studied and understood without some appreciation of environmental effects. This concept of approaching molecules and their surroundings as complete individual chemical entities is one of the fundamental principles underpinning supramolecular chemistry.

Two crucial benefits are derived from the absence of a solvation shell surrounding gas-phase molecules. One is that, using traditional analytical techniques to determine solution-phase chemistry, direct comparisons between solution-phase and gas-phase chemistry can be made, elucidating the direct effects of solvation on the supramolecular system. The second is more specific to supramolecular chemistry. Species stabilized by noncovalent bonds such as hydrogen bonds, which are known to compete with some solvents for binding sites, can be destabilized upon dissolution. Hydrogen bonds may be weakened or even destroyed in the presence of protic solvents. Hence, secondary and higher-order structures of complex molecules like proteins can be significantly altered leading to the acquisition of entirely misleading data.

Gas-phase chemistry, therefore, has an important place in the field of supramolecular chemistry as a whole, but characterization of gaseous supramolecules specifically by using the unique analytical capabilities of mass spectrometry can be justified by several factors. As previously discussed, mass spectral data have traditionally been used to determine elemental and structural composition, charge states, and stoichiometric relationships pertaining to analytes of interest. In this vein, mass spectrometry is extremely useful for topological studies to define secondary structures and conformations of supramolecular complexes and to investigate the stoichiometry of binding between molecular recognition elements. However, the benefits of
Mass spectral analysis for supramolecular chemistry extend far beyond these traditional approaches.

Mass spectrometry enables determination of the reactivity of noncovalent complexes, not only in terms of fragmentation behavior, but also in comparative preferential substrate binding and hydrogen/deuterium exchange experiments for mechanistic studies. Additionally, exciting developments in mass spectrometry for the determination of thermodynamic data have demonstrated the capabilities of the technique as a detection method for representation of solution-phase equilibria and as a strategy for the calculation of both relative and absolute gas-phase thermodynamic quantities. Supramolecular chiral analyses have been successfully performed.\textsuperscript{3,6}

**Survey of Recent Literature**

Some recent reviews and developments in supramolecular mass spectrometry accessible in the literature are highlighted here for the purpose of clarifying the context of the work presented in subsequent chapters. Two excellent reviews examining the role of gas-phase analysis within the field of supramolecular chemistry have been presented by the Schalley group.\textsuperscript{3,6} An in-depth overview of molecular recognition by mass spectrometry was published by De Angelis and colleagues.\textsuperscript{26} Independent reviews concerned with mass spectral analysis of noncovalent complexes for the determination of quantitative thermodynamic quantities were authored by Armentrout \textit{et al.}\textsuperscript{47} and the Zenobi group.\textsuperscript{21} Specific to biomolecules, Kaltashov and Eyles reviewed mass spectrometry applied to the study of conformations and conformational dynamics,\textsuperscript{48} Kriwacki \textit{et al.} presented an overview of the mass spectral characterization of protein complexes,\textsuperscript{49} and Liesener and Karst authored a critical review on monitoring enzymatic conversions by mass spectrometry.\textsuperscript{50}
Supramolecular mass spectrometry for determination of the elemental composition of synthetic products and structural elucidation continues to be prolific. Lately, novel bis-crown ethers\textsuperscript{51} and rotaxanes,\textsuperscript{52} cyclic dipyridyl-glycolurils,\textsuperscript{53} large phosphorus macrocycles,\textsuperscript{54} and mixed-metal, mixed-pyrimidine self-assembling metallacalix\textsubscript{[n]}-arenes\textsuperscript{55} have been synthesized and characterized using soft-ionization mass spectrometry. The modification of a tolylpyridine-bridged cavitand with water-solubilizing groups for the promotion of aqueous-phase self-assembly was determined using mass spectral analysis,\textsuperscript{56} and a detailed structure of the amyloidogenic protein wild-type transthyretin was obtained via HPLC-nanospray MS/MS and MALDI-MS.\textsuperscript{57} The first report of sonic spray ionization mass spectrometry for the characterization of metal-assembled cages was published in 2006.\textsuperscript{58}

Topological experiments incorporate mass spectrometry for conformational analysis and higher-order structural elucidation. Electrospray ionization has been applied to examination of the supramolecular architectures of complementary self-assembling host-guest complexes of the softball type,\textsuperscript{59} of cucurbit\textsubscript{n}urils,\textsuperscript{60} and the chelating bidentate catechol ligand with various polyatomic cation guests.\textsuperscript{61} An interesting study on the dynamic topological control over subcomponent self-assembly of synthetic helicates, macrocycles, and catenates employed ESI-FTICR mass spectrometry for the elucidation of secondary structures.\textsuperscript{62} Additionally, ESI-MS monitoring of the folding and assembly of hemoglobin was achieved using a novel on-line dialysis system.\textsuperscript{63}

Several groups have recently published topology and reactivity studies on biological macromolecules. Limited proteolysis MALDI-TOF-MS was used to probe the kinetics of cyclin-dependent kinase inhibition.\textsuperscript{64} Noncovalent complex formation between the protein Link module from human tumor necrosis factor stimulated gene-6 and hyaluronan oligosaccharides was
studied over time using hydrogen/deuterium (H/D) amide exchange ESI-MS. Collisionally-activated dissociation ESI-MS/MS experiments elucidated fragmentation and preferential binding behaviors of differentially-substituted perylene diimide ligands complexed with DNA, revealing correlation between gas and solution-phase behavior. A similar project focusing on DNA complexed with several drug candidates incorporated KMnO₄ oxidation with ESI-MS/MS and compared topological and reactivity data using infrared multiple photon dissociation (IRMPD) and CAD dissociation strategies. Dissociation pathways for complexes of a single-stranded DNA with a polybasic guest were obtained using nanoelectrospray ionization and tandem mass spectrometry.

Mass spectral analysis of supramolecular synthon reactivities is also experiencing rapid growth. The Dearden group, who have made myriad contributions to quantitative gas-phase thermodynamics, have investigated the dissociation and reactivity properties of the cucurbituril derivatives as hosts for inclusion of small-molecule guests using FTICR-MS and have exploited the inherent trapping capabilities of the technique to study ion-molecule chemistry in the ICR cell. Solution-phase kinetic data for the noncovalent binding between chiral resorcinarenes and ammonium guests were obtained using FTICR-MS, and a series of elegant experiments also used FTICR-MS but employed H/D exchange and ion-molecule reactions to investigate inclusion of alkyl ammonium ions by substituted resorcarenes. Additionally, fragmentation and rearrangement of substituted oxadiazoles and their noncovalent complexes with cobalt cation and cyclodextrin were studied using a plethora of mass spectral approaches combined with isotope-labeling. Elucidation of a ring-closure mechanism subsequently incorporated into the design of a method for synthesis of interlocked π-conjugated macrocycles was achieved using FTICR-MS and tandem mass spectrometry. The utility of FTICR-MS in this area was also
demonstrated in the study of noncovalent complexes of the protein *Mycobacterium tuberculosis* adenosine-5’-phosphosulfate reductase; reactivity and thermodynamics were investigated for the delineation of a mechanistic model.\(^{74}\)

Quantitative gas-phase thermodynamic data are desirable for the reasons outlined earlier. Major developments in the representation of solution-phase thermodynamic behavior by ESI-MS have been made just in the last few years. The Brodbelt group in particular has contributed to this progress with the early design and application of a method for determination of binding constants by ESI-MS using reference complexes with known thermodynamic data.\(^{75}\) Subsequent work developed mathematical models based on equilibrium partitioning theory to predict the ESI-MS response to host-guest complexation\(^{76}\) and to relate ESI-MS ion abundances with solution concentrations of host-guest complexes.\(^{77}\) Complexes of crown ethers with alkali metal cations were used for validation. A novel quinoxaline-containing crown ether ligand-metal-ligand sandwich complex stabilized by \(\pi\)-stacking interactions was characterized via ESI-MS resulting in quantitative determination of free-energy gains achieved by modification of the crown ether with electron-rich functional groups.\(^{78}\) Binding stoichiometry and relative binding affinities of nucleic acid aptamer/small molecule complexes were obtained using ESI-MS and compared to values deduced using traditional solution-phase techniques.\(^{79}\) Evaluation of protein-DNA binding affinities, using the DNA-binding domain of a transcription factor, c-Myb, and several double-stranded DNA substrates, was demonstrated using both ESI\(^{80}\) and laser spray mass spectrometry.\(^{81,82}\)

Mass-spectrometric determination of gas-phase thermodynamic quantities for elucidation of intrinsic supramolecular thermochemistry is an additional area of activity. Progress continues toward the goal of determining absolute quantitative thermodynamic data using mass
spectrometry. Valuable contributions made by Dearden and colleagues include accurate pressure
determination strategies to enable the measurement of exchange equilibrium binding constants
for crown ethers and alkali-metal cations and entropies and enthalpies associated with
discrimination between enantiomers of chiral amines by dimethyldiketopyridino-18-crown-6
during the molecular recognition event.83 Experimental gas-phase alkali-metal binding energies
of dibenzo-18-crown-6 obtained using FTICR-MS have been compared to theoretical data
derived computationally.84 The effects of size of noncovalent complexes on their stability during
collision-induced dissociation were studied using complexes of metallated porphyrins with
histidine-containing peptides and model compounds.45 More recently, energy-resolved SCID for
the evaluation of relative stabilities of noncovalent complexes of crown ethers, nucleic acid
bases, and amino acids with alkali metal cations has been investigated for determination of
solvent influences.43 Complexes formed between alkali metals and polyether ionophore
antibiotics were analyzed in a study comparing data analysis methods for gas-phase stability
determination by CID.85

The background, significance, and recent developments in supramolecular chemistry and
mass spectrometry have been introduced. The remainder of this dissertation will describe in
detail the specific contributions to supramolecular mass spectrometry made though this research.
Chapters 2 and 3 describe the characterization of a novel alkali-metal cation synthetic receptor
platform via ESI and tandem mass spectrometry. A technical chapter presenting the
configuration of a home-built DESI source to two high-resolution mass analyzers follows.
Chapter 5 describes the development of a novel DESI technology for supramolecular
applications using a cyclodextrin model system. The work culminates in a general conclusion
and future directions section.
Figure 1-1. Representation of a) a cavitand including a guest to form a cavitate and b) clathrands forming a clathrate with a guest molecule.

Figure 1-2. Two possible orientations of dipole-dipole interactions.

Figure 1-3. Representation of cation-π interaction showing the quadrupole moment on an aromatic ring and the interaction between the partially negative π cloud and a cation.
Figure 1-4. Generalized configuration of an electrospray source showing a) solution flowing through a spray needle, with co-axial nebulizing gas, across a potential difference, b) a desolvation capillary, c) a skimmer cone, d) some ion optics (no specific type), and e) the direction of flow into the mass analyzer.

Figure 1-5. Quantities used to calculate resolution where a) represents the 15% valley definition and b) shows the full-width half maximum, FWHM, approach.
CHAPTER 2
CHARACTERIZING NONCOVALENT DIMERIZATION BEHAVIOR OF DESIGNED PHLOROGLUCINOL DERIVATIVES USING ELECTROSPRAY IONIZATION HIGH RESOLUTION MASS SPECTROMETRY

Introduction

A major facet of supramolecular chemistry is the design of synthetic compounds capable of supramolecular interactions with their surroundings. Synthetic supramolecular systems are under exploration because they offer simplification of synthetic strategies and a collection of novel, manipulable properties.86 The principle of chemical information encoded into molecules and supramolecules is the foundation for the design of synthetic species capable of specific molecular recognition behavior. Information can be stored at the molecular level by topological design9,87 in a manner similar to the way that biological information is stored in DNA.

Some synthetic chemists use noncovalent interactions and self-assembly phenomena to promote selective synthesis or for catalysis of the synthesis process.9 Alternatively, designed synthetic receptors capable of mimicking the noncovalent interactions responsible for a plethora of biological processes like enzyme-substrate action and active transport across cell membranes may be sought. This type of work is of major importance in the improvement of drug design and lead optimization, for example.17 Novel properties of supramolecular entities such as unique photo- and electro-activity give impetus to synthesis of designed noncovalent receptor molecules.7

Despite the wealth of macrocyclic ion receptors such as the crown ethers, few designed \( \pi \)-receptors for alkali-metal cations are known.88 Effective design of a synthetic receptor capable of molecular recognition with a specific substrate requires that functional groups on a molecular platform be aligned to form complementary binding interactions with the substrate. One way to achieve this is by fixing the conformation of the receptor by using a rigid platform such as a
benzene ring as a foundation for the functional regions of the synthon. The desired conformation can be permanent or may only be achieved during the molecular recognition event. Following this design approach, two novel synthetic compounds, derived from naturally-occurring phloroglucinol (Figure 2-1) have been investigated for their ability to form dimers capable of selectively binding a size-appropriate alkali-metal cation. The 1,3,5-trisubstituted 2,4,6-triethylbenzenes have demonstrated host-guest inclusion complex capabilities for both cation and anion guests, so there is a good precedent for this work.

The synthetic phloroglucinol derivatives 2,4,6-tribenzoylphloroglucinol and 2,4,6-(3,5-dimethyl)-tribenzoylphloroglucinol, hereafter referred to as phloro 1 and phloro 2 respectively, are shown in Figure 2-2. It was hypothesized by the Castellano group at the University of Florida that phloro 1 could potentially form a cage-type dimer capable of housing an alkali-metal cation in the cavity of the resultant hollow, spherical complex. Preferential complexation of appropriately-sized cations would support this hypothesis. Conversely, the two methyl groups on each benzoyl substituent of the central aromatic ring of phloro 2 should prevent formation of a cage-type dimer via steric hindrance, which would be supported by complex formation exhibiting no dependence on cation radius. These postulates were explored using mass spectrometric strategies for determination of the order of binding preference for several metal cations with each phloroglucinol derivative.

In the study of noncovalent preferential binding by mass spectrometry, two major approaches are commonly employed. Traditional competitive binding experiments consist of dissolving the host in a suitable solvent and adding equimolar amounts of the two or more competing guests. Assuming similar ionization efficiencies and minimal signal suppression for the resulting complex ions, subsequent comparison of mass spectral peak intensities
corresponding to the respective complexes is used to assign binding preferences. The more intense the peak corresponding to a given complex, the higher the standing of the related guest in the preferential binding order. The aforementioned assumptions can mean that this strategy is inappropriate under some circumstances, but its speed and simplistic experimental design result in its frequent utilization. A more rigorous approach capitalizes on the amenability of mass spectrometry to tandem experimental design. Detection of a peak corresponding to a complex of interest is followed by some dissociation event involving controlled introduction of energy to the chemical system. The extent of dissociation following the addition of the energy is monitored by comparing the absolute peak intensity after energy addition relative to the initial absolute peak intensity. The energy applied is varied and several data points acquired. Construction of a dissociation or stability curve which is a graphical representation of the data plotting the previously-described normalized peak intensity against the amount of energy incorporated follows. Some point on the curve, for example the point at which 50% of the original peak intensity is lost, is used to compare the energetic stabilities of the respective complexes. Common strategies for providing the energy for dissociation include increasing the voltage gap applied during SCID or CID, or introducing electromagnetic radiation in the form of laser light. Although this is the more rigorous approach, it is slower and slightly more complex in nature than performing traditional competitive binding experiments and therefore is not always the method of choice.

**Experimental**

**Sample Preparation**

The phloroglucinol derivatives 2,4,6-tribenzoylphloroglucinol and 2,4,6-(3,5-dimethyl)-tribenzoylphloroglucinol were synthesized and purified by members of the Castellano group at the University of Florida. All samples were dissolved in HPLC grade methanol (Honeywell...
Burdick & Jackson, Muskegon, MI), and the cations Na\(^+\), Li\(^+\), K\(^+\), and NH\(_4\)\(^+\) were introduced as chloride salts (Sigma-Aldrich, St. Louis, MO). The samples were prepared to concentrations of 3x10\(^{-4}\) M host and 3x10\(^{-5}\) M guest for the ESI-MS competitive binding and dissociation curve experiments. For the heterodimer analyses, equimolar amounts of phloro 1 and 2 yielding a total host concentration of 3x10\(^{-4}\) M were used. Each sample prepared for mass spectrometric analysis contained 1% formic acid (ThermoFisher Scientific, Waltham, MA) to improve current stability and ionization efficiency throughout the analysis.

The samples prepared for UV/Vis spectroscopic analysis did not contain additional acid. Concentrations of 0.05-0.5 mM host in increments of 0.05 mM were used for titration-type experiments and 3x10\(^{-5}\) M guest was incorporated where appropriate. Successive addition of \(\mu\)L amounts of a concentrated host stock solution to a 3 mL sample volume followed by spectral acquisition comprised the titration approach for the chromic shift observation. The analytical measurement of the chromic shift used solutions with the aforementioned guest concentrations and 3x10\(^{-5}\) M and 3x10\(^{-4}\) M concentrations of the phloroglucinol derivatives.

**Mass Spectrometry**

All the mass spectrometry incorporated electrospray ionization. Mass spectra were acquired using a 4.7 T Bruker Bioapex II Fourier Transform Ion Cyclotron Resonance mass spectrometer equipped with an Apollo API 100 Source (Bruker Daltonics, Billerica, MA) and an Agilent 6210 Time-of-Flight mass spectrometer configured for ESI (Agilent Technologies, Inc., Santa Clara, CA).

Samples were introduced at flow rates of 2 \(\mu\)L/min for the ESI-FTICR-MS and 5 \(\mu\)L/min for the ESI-TOF-MS using direct infusion via a Harvard Apparatus PHD 2000 syringe pump, with a nebulizing N\(_2\)(g) pressure of 20 psi. The potential difference between the ESI needle and
the desolvation capillary inlet was always 3.5 kV. The capillary exit voltage was held at 90 V for the initial FTICR-MS analyses, while the skimmer voltage was 20 V. During the dissociation curve SCID-FTICR-MS experiments, the potential difference between the desolvation capillary exit voltage and the succeeding skimmer was varied from 50-250 V in 10 V increments by increasing the exit voltage while maintaining a constant skimmer voltage. Hexapole accumulation time was 2 s and 50 scans were acquired and averaged for each data point plotted. Each run was repeated three times using individually-prepared samples and the averaged peak intensities were plotted versus the potential difference. For the SCID-TOF-MS dissociation curve experiments, the potential difference was manipulated by varying the fragmentor voltage from 75-300 V in 15 V increments. Signal for each data point was acquired for 20 s and the average integrated intensity used for data analysis. Again, the average of three runs using three different samples was plotted for determination of VC₅₀ values.

To determine VC₅₀ values following construction of the stability curves, linear regression was performed on the data points corresponding to the region of decline in signal intensity to calculate the equation of the line associated with the data points. Subsequently, the equation was solved to determine the potential difference value (the x-variable) for which the percentage dissociation (the y-variable) was 50 % in order to obtain VC₅₀. Standard error propagation techniques were applied to the calculation of corresponding 95% confidence intervals.

**Ultraviolet/Visible Absorbance Spectroscopy**

Optical spectra were acquired using a Hewlett-Packard HP8450 Diode Array UV/visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA) set to absorbance or derivative absorbance mode. Cuvettes with a path length of 1 cm were used to hold samples for absorbance measurement. A reference solution of methanol was used for blank and background correction. Derivative absorption spectra were used for determination of the wavelengths corresponding to
maximum absorbance. Three measurements of each wavelength of maximum absorbance were made using three different solutions and the average was plotted for comparison.

Results and Discussion

Electrospray Ionization Mass Spectrometry for Dimer Detection

Analysis of each host compound in the presence of one of each of the cations revealed that phloro 1 forms cation-bound dimers with Na\(^+\), Li\(^+\), K\(^+\), and NH\(_4\)^+ (Figure 2-3). Initial observation of peak intensities corresponding to the complexes suggested a preferential binding order of Na\(^+\)>Li\(^+\)>K\(^+\)>NH\(_4\)^+, but the variation in peak intensity could also have been due to differing ionization efficiencies for the respective complexes. Extensive carryover was noted as different salts were incorporated and after injection of three samples, signal suppression was significant. Consequently, after analysis of each individual cation-bound dimer, the electrospray line and needle were cleaned with heated HPLC water and the source region was vented for removal and thorough cleaning of the desolvation capillary. Generally the successive skimmer and accessible parts of the hexapole assembly also required cleaning at this point. This was an issue for both the FTICR-MS and the TOF-MS experiments.

Traditional Competitive Binding Approach

The initial approach toward elucidating the preferential binding order of Na\(^+\), Li\(^+\), K\(^+\), and NH\(_4\)^+ used the traditional competitive binding experimental design. Since the ionization technique chosen for the work was ESI, only two cations were added to each sample because signal suppression due to high salt concentrations was a concern. Following mass spectral analysis of samples containing all possible combinations of two of the four cations under investigation, peak intensities corresponding to the complexes would have been compared to determine an overall binding order. Analysis of the host compounds phloro 1 and 2, however, revealed the presence of significant amounts of adventitious sodium (Figure 2-4). The sodium
needed to be removed to ensure controlled addition of guest cations in equimolar amounts, a fundamental requirement for the acquisition of valid data using the traditional competitive binding approach.

Modification of the initial experiment to incorporate a chromatographic separation step followed by post-column addition of the competing guests was subsequently attempted, but it proved impossible to develop a chromatographic method to remove all of the sodium. The traditional approach was therefore abandoned in favor of the more rigorous dissociation curve approach which would not be adversely affected by the presence of adventitious sodium.

**Dissociation Curve Approach**

Following the strategy of Rogniaux and colleagues, solutions of one phloroglucinol in the presence of each of the alkali metal chloride salts were electrosprayed into the FTICR mass analyzer. The intensity of the mass spectral peak corresponding to the cation-bound dimer of interest was recorded at a range of desolvation capillary exit voltages while the adjacent skimmer voltage was held constant as described in the experimental section. The peak intensity relative to its observed maximum was plotted as a percentage against the potential difference in volts, and the equation of the linear sloping portion of the curve was used to determine the potential difference at which 50% of the maximum signal intensity corresponding to the complex was lost. This value, \( VC_{50}\), was calculated for each cation-bound dimer and the respective quantities were used to rank relative stability of the complexes. The ESI-FTICR-MS dissociation curves for phloro 1 are shown in Figure 2-5 and those corresponding to phloro 2 are presented as Figure 2-6. The calculated \( VC_{50}\) values are presented in Table 2-1. Preferential binding order for the cations was determined using this method to be \( \text{Li}^+ \geq \text{Na}^+ > \text{K}^+ > \text{NH}_4^+ \) for phloro 1 and \( \text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{NH}_4^+ \) for phloro 2. The \( \text{Na}^+ \) and \( \text{K}^+ \)-bound dimer \( VC_{50} \) values for phloro 2 had to be extrapolated from the stability curves because 50% dissociation of the phloro 2 complexes bound
by these cations was never observed even at the maximum potential difference capabilities of the instrument. The ammonium-bound dimer of phloro 2 was not stable enough for construction of its dissociation curve.

The alkali-metal cationic radii are given in Table 2-2, and show that cation size increases in the order Li$^+$>Na$^+$>K$^+$>NH$_4^+$. Apparent size-dependent preferential binding of the cations for phloro 1 but not for phloro 2 supported the original hypothesis that phloro 1 would form a sandwich-type dimer while steric hindrance should prevent phloro 2 from doing the same. A major problem with the obtained stability curves, however, was poor reproducibility, evidenced by large error bars on the plots, which show the average of three trials, and large 95% confidence intervals in the VC$_{50}$ value calculations. Configured to ESI, FTICR-MS can show significant current, and consequently peak intensity, fluctuation because only one packet of ions is detected at a time, following hexapole accumulation. Signal averaging can be achieved using a multiplexing strategy, but this is extremely time-consuming. The current and peak intensity stability for an oaTOF-MS can be superior because the signal from multiple packets of ions is detected and averaged in a very short time. For this reason, and because 50% dissociation of two of the phloro 2 cation-bound dimers was not achieved using the FTICR-MS, the dissociation curve experiment was repeated using the same sample preparation protocol but introducing the electrosprayed complexes into an oaTOF mass analyzer.

The dissociation curves obtained using the TOF-MS instrument (Figure 2-7) addressed both of the major problems associated with the FTICR-analysis. The reproducibility of the curve data obtained via TOF mass analysis showed distinct improvement over that obtained using the FTICR-MS, particularly for phloro 2. This was as expected for the reason outlined above. A more perplexing result was that more than 80% dissociation of the Na$^+$ and Li$^+$ bound phloro 2
dimers was observed in the TOF analysis. This enabled the interpolation of VC₅₀ values for both complexes. Reasons for this remain unclear.

The calculated VC₅₀ values for each observed complex determined using the TOF mass analyzer are presented in Table 2-3. The smaller confidence intervals associated with the values and the better reproducibility observed for the dissociation curves are the reasons that the TOF values are assumed to be more representative of the system chemistry. The quantities calculated using the different mass analyzers exhibit similar trends. Dimers bound with Li⁺ and Na⁺ cations are more stable than those bound with K⁺ and NH₄⁺ for phloro 1 supporting some size-dependence in binding preference. The dimers observed for phloro 2 are significantly more stable than those observed for phloro 1, with the exception of the ammonium ion bound dimers which were not observed for phloro 2. This seems indicative of differences in binding position of the cations by each host molecule. According to the stability curves constructed using data acquired by the TOF mass analyzer, the binding preference order of the cations under investigation is Li⁺>Na⁺>NH₄⁺>K⁺ for phloro 1. The order of binding preference for phloro 2 is Li⁺>Na⁺>K⁺>NH₄⁺. Therefore, size-dependent binding is again exhibited for phloro 1 but not phloro 2.

**Heterodimer Studies**

The size-dependent preferential cation binding observed for phloro 1 but not phloro 2 supports the original conformation hypothesis, but the technique implemented does not address the effect on cation-π binding of the structural differences between the host molecules, so an ESI-TOF-MS approach was used to analyze cation-bound heterodimers of phloro 1 and phloro 2. The intention was to determine, using relative ion peak intensities, whether or not the phloro 1 and phloro 2 are a good geometric fit despite the methyl substituents on the benzoyl groups of
phloro 2. Heterodimer formation was observed with Li\(^+\), Na\(^+\), and K\(^+\) cations but not with NH\(_4\)^+.

Statistically, if phloro 1 and phloro 2 were a good geometric fit, the peak intensity ratio of the cation-bound dimers should correspond to 1:2:1 where the intensity of the signal observed for the heterodimer should be twice that of the signal intensities corresponding to the homodimers. This is because two distinct species can combine separately in four permutations. If we represent phloro 1 as P1 and phloro 2 as P2, then they can combine to form P1P1, P1P2, P2P1, or P2P2, but in this analysis P1P2 is identical to P2P1 so the ratio given above would be expected unless the phloro 1 and phloro 2 are poor complementary binders.

A representative ESI-TOF mass spectrum for a sample containing equimolar amounts of phloro 1 and phloro 2 with the addition of LiCl salt is shown as Figure 2-8. Note the presence of adventitious sodium. It clearly shows that the cation-bound phloro 2 dimers are the most stable of the three dimer types. This correlates with the VC\(_{50}\) data obtained from the dissociation curves for the homodimers. The spectrum also shows that phloro 1 and 2 do not bind well together and so are a poor geometric fit as the heterodimer peak intensities are smaller than those corresponding to the phloro 2 homodimers. This supports the theory that the addition of methyl groups to the benzoyl substituents on the phloroglucinol core has a profound effect on the cation-\(\pi\) binding interactions associated with phloroglucinol derivative cation-bound dimerization, and hence on dimer conformation. Dissociation curves constructed using SCID-TOF-MS are shown in Figure 2-9. They show that the heterodimers exhibit the same preferential cation binding behavior as the phloro 2 homodimer, but the large error bars suggest poor reproducibility so specific VC\(_{50}\) values were not determined.

The mass spectral analyses were able to elucidate the cation-binding preferences for dimers of phloro 1 and phloro 2 as well as the related heterodimers. Size-dependent preferential binding
for phloro 1 but not phloro 2 or the heterodimers supported the postulated sandwich-type dimer conformation for phloro 1 and end-to-end dimer conformation for phloro 2. A mass spectrometric study into heterodimer stability revealed that the addition of methyl groups to the benzoyl substituents of the phloroglucinol core had a significant effect on the noncovalent cation-π interactions responsible for stabilization of the cation-bound dimers.

### Ultraviolet/Visible Absorbance Spectroscopy

The mass spectral analyses described so far yielded data elucidating the cation-binding preferences of the phloroglucinol derivative dimers and provided some insight into the effect of the structural differences between phloro 1 and 2 on the cation-π interactions responsible for dimer stabilization. So far, however, no direct information about the conformation of the dimers under investigation had been obtained. A spectrophotometric analysis based on absorbance of ultraviolet (UV) and short wavelength visible (vis) light was consequently employed to study solution-phase dimer conformation. An absorption spectrum for phloro 1 in methanol is presented as Figure 2-10, and a Beer’s Law plot constructed using a titration-type experiment is shown in Figure 2-11. Beer’s Law is stated in equation 2.1 where $A$ is absorbance, $\varepsilon$ is molar absorptivity, $b$ is path length, and $c$ is analyte concentration.

$$A = \varepsilon bc$$  \hspace{1cm} (2.1)

Using a Beer’s Law plot, the molar absorptivity value at 310 nm was determined from the slope of the line to be $2330 \pm 130$ L mol$^{-1}$ cm$^{-1}$ for phloro 1 and $2200 \pm 27$ L mol$^{-1}$ cm$^{-1}$ for phloro 2 which yields a very similar UV/vis absorption spectrum. These molar absorptivity values are consistent with $n\rightarrow\pi^*$ electronic transitions.\textsuperscript{92}

A titration-type experiment where UV/vis absorption spectra were obtained for phloro 1 concentrations ranging from 0.05-0.5 mM varied in 0.05 mM increments was performed to
search for observable changes in spectral characteristics which could correspond to dimerization. The same experiment was performed for phloro 2. Representative data are presented as Figure 2-12.

Comparison of the absorption spectra acquired for low and high concentrations of phloro 1 revealed a shift of the longer wavelength peak to slightly lower wavelengths, a hypsochromic or blue shift, and a shift of the shorter wavelength peak to slightly higher wavelengths, a bathochromic or red shift. The spectra acquired for phloro 2, however, only evidenced bathochromic shift of the shorter wavelength peak and no shift of the longer wavelength peak was observed. An observed anomaly in the spacing between the spectral curves for the third highest concentration phloro 1 solution may be due to instrumental error resulting from accidental disturbance of the plotter, or could be the result of erroneous solution preparation.

Long-established molecular exciton theory has described two ideal homodimer conformations: sandwich dimers and end-to-end dimers. When two identical molecules interact electronically, as in dimerization, molecular orbitals which were not degenerate in the individual molecule exist in close proximity, rendering them degenerate molecular energy levels in the dimer form. Consequent splitting of the newly degenerate energy levels results in changes in the magnitude of resultant electronic transitions. This is observed in optical spectroscopy as chromic peak shifts corresponding to molecular aggregation. Hypsochromic shift ideally corresponds to sandwich-type dimers while bathochromic shift corresponds to end-to-end dimers.

The observed chromic shift began to occur at 0.05 mM phloro 1, so an experiment was designed to measure the wavelength of maximum absorption, \( \lambda_{\text{max}} \), of each peak before and after the shift for both phloroglucinol derivatives in the absence and presence of each of the alkali
metal cations. Derivative spectroscopy was used to determine the \( \lambda_{\text{max}} \) values for improved precision. Specific conditions are delineated in the experimental section. The resultant data are presented as Figure 2-14.

The bar graphs a and b show the bathochromic shift of the lower wavelength peak which evidences end-to-end aggregation of both phloro 1 and 2, although this shift is minimal for phloro 2 and is not observed for the samples in the presence of sodium and lithium cations. A shift is considered to be represented by adjacent bars with non-overlapping 95% confidence intervals which are shown as error bars. Overlap of the confidence intervals for the bars representing the pre- and post-shift peaks is taken to mean that there is no conclusive evidence of chromic shift.

The graphs labeled c and d show data obtained concerning the \( \lambda_{\text{max}} \) of the higher wavelength peak at concentrations preceding and succeeding that corresponding to the expected hypsochromic shift consistent with sandwich-type dimerization. This shift is only observed in the phloro 1 data, so sandwich-type aggregation is only evidenced for phloro 1. The optical spectra clearly show evidence of solution-phase end-to-end aggregation of both phloroglucinol derivatives but only show evidence of sandwich-type aggregation of phloro 1. These findings are consistent with the original hypothesis and the gas-phase mass spectral data. The addition of each individual alkali metal cation under investigation, in the form of a chloride salt, to the phloroglucinol derivatives in solution appeared to have no effect on the spectroscopic data. This is as expected because each cation should be surrounded by a solvent shell preventing cation-\( \pi \) interaction between the cation and the phloroglucinol derivative hosts.
Conclusions

Electrospray ionization mass spectrometry enabled determination of the preferential binding order of alkali metal cations complexed with dimers of two phloroglucinol derivatives differing by the presence or absence of methyl groups on the benzoyle substituents of the phloroglucinol core. The binding order for phloro 1 was elucidated as Li⁺>Na⁺>NH₄⁺>K⁺ but for phloro 2 as Li⁺>Na⁺>K⁺>NH₄. Size-dependent preferential binding was therefore observed for phloro 1 but not phloro 2. Traditional competitive binding experiments were found to yield inconclusive results due to the presence of adventitious sodium, but the dissociation, or stability, curve approach using SCID offered superior results. It was noted that the rapid averaging capabilities of an oaTOF mass analyzer resulted in faster acquisition of more reproducible data than that acquired using an FTICR instrument. Heterodimer studies via ESI-TOF showed that the presence of additional methyl groups on phloro 2 had a profound effect on the cation-π interactions responsible for stabilization of the cation-bound dimers. Mass spectral analysis, therefore, was able to characterize the intrinsic noncovalent binding chemistry between the two phloroglucinol derivatives and the cations, but no direct information about dimeric conformation could be obtained via this technique. Ion mobility spectrometry could potentially address this issue in the gas-phase.

Solution-phase optical spectroscopy provided evidence of both sandwich and end-to-end molecular aggregation for phloro 1, but only end-to-end aggregation of phloro 2. This conformational information corroborated the gas-phase data. It also supported the original hypothesis stating that phloro 1 should be capable of forming a sandwich or cage-type dimer potentially housing a small cation in an intermolecular cavity while the addition of methyl groups to the extremities of phloro 2 should sterically hinder formation of a sandwich complex.
No chemical information about cation binding was attainable via solution-phase analysis, however, due to solvent interference.

For this analysis, it was determined that, while lacking in conformation-probing capabilities, mass spectrometry was able to provide more useful information concerning the chemical behavior of the supramolecular phloroglucinol derivative-cation complexes than solution-phase spectrophotometry. It should be noted, however, that solution-phase optical spectroscopy did yield useful complementary conformational data, despite its inability to probe the noncovalent interactions between the organic hosts and the ionic guests.
### Table 2-1. Phloro 1 and 2 dissociation curve VC$_{50}$ values and their associated 95% confidence intervals for cation-bound dimers determined using SCID-FTICR-MS

<table>
<thead>
<tr>
<th>Complex Ion</th>
<th>Phloro 1 VC$_{50}$, V</th>
<th>Phloro 2 VC$_{50}$, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2M+Li]$^+$</td>
<td>129 ± 22</td>
<td>151 ± 26</td>
</tr>
<tr>
<td>[2M+Na]$^+$</td>
<td>128 ± 19</td>
<td>177 ± 213</td>
</tr>
<tr>
<td>[2M+K]$^+$</td>
<td>89 ± 14</td>
<td>176 ± 110</td>
</tr>
<tr>
<td>[2M+NH$_4$]$^+$</td>
<td>71 ± 217</td>
<td>—</td>
</tr>
</tbody>
</table>

### Table 2-2. Alkali-metal cationic radii in Angstroms

<table>
<thead>
<tr>
<th>Cation</th>
<th>Radius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li$^+$</td>
<td>0.76$^a$</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>1.02$^a$</td>
</tr>
<tr>
<td>K$^+$</td>
<td>1.38$^a$</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>1.43$^b$</td>
</tr>
</tbody>
</table>

$^a$ Reference 95  
$^b$ Reference 96

### Table 2-3. Phloro 1 and 2 dissociation curve VC$_{50}$ values and their associated 95% confidence intervals for cation-bound dimers determined using SCID-TOF-MS

<table>
<thead>
<tr>
<th>Complex Ion</th>
<th>Phloro 1 VC$_{50}$, V</th>
<th>Phloro 2 VC$_{50}$, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2M+Li]$^+$</td>
<td>168 ± 21</td>
<td>218 ± 22</td>
</tr>
<tr>
<td>[2M+Na]$^+$</td>
<td>153 ± 25</td>
<td>190 ± 21</td>
</tr>
<tr>
<td>[2M+K]$^+$</td>
<td>128 ± 21</td>
<td>162 ± 23</td>
</tr>
<tr>
<td>[2M+NH$_4$]$^+$</td>
<td>131 ± 70</td>
<td>—</td>
</tr>
</tbody>
</table>
Figure 2-1. Phloroglucinol structure

Figure 2-2. Structures of the phloroglucinol derivatives a) 2,4,6-tribenzoylphloroglucinol (phloro 1) and b) 2,4,6-(3,5-dimethyl)-tribenzoylphloroglucinol (phloro 2).

Figure 2-3. Electrospray ionization mass spectra showing phloro 1 forming cation-bound dimers in the presence of a) Na\(^{+}\), b) Li\(^{+}\), c) K\(^{+}\), and d) NH\(_4\)\(^{+}\) cations
Figure 2-4. ESI Spectra of a) phloro 1 and b) phloro 2 in the absence of added salt showing the presence of adventitious sodium.
Figure 2-5. Phloro 1 dimer dissociation curves for dimers bound with a) Na\(^+\), b) Li\(^+\), c) K\(^+\), and d) NH\(_4\)\(^+\) obtained using SCID-FTICR-MS. Note the poor reproducibility, although VC\(_{50}\) values were calculated from these curves.
Figure 2-6. Phloro 2 dimer dissociation curves for dimers of bound with a) Na\(^+\), b) Li\(^+\), and c) K\(^+\) obtained using SCID-FTICR-MS. The NH\(_4\)\(^+\) bound dimer was not stable enough for construction of a stability curve. Note the poor reproducibility. Again VC\(_{50}\) values were calculated from these curves but had to be extrapolated for a) and b).
Figure 2-7. Dissociation curves for all four cation-bound dimers of a) phloro 1 and b) phloro 2 obtained using SCID-TOF-MS. Note the improved reproducibility which translated to smaller confidence intervals for the calculated VC_{50} values.
Figure 2-8. Spectrum obtained using ESI-TOF-MS to analyze cation-bound homo and heterodimers of phloro 1 (P1) and phloro 2 (P2). Both homo and heterodimers were observed to form with Li$^+$ and Na$^+$ although only LiCl was added to the sample.

Figure 2-9. Dissociation curves for the cation-bound heterodimers with Li$^+$, Na$^+$, and K$^+$. No heterodimer formation with NH$_4^+$ was observed.
Figure 2-10. Absorption spectrum for 0.05 mM phloro 1 in methanol.

Figure 2-11. Beer’s Law plot showing absorbance intensity varying with concentration of phloro 1. Adherence to Beer’s Law is observable for several UV wavelengths.
Figure 2-12. Ultraviolet/visible absorption spectra for concentrations of 0.05-0.5 mM host in increments of 0.5 mM of a) phloro 1 and b) phloro 2 in 50:50 methanol:water. The higher wavelength peak exhibits hypsochromic shift with increasing concentration of phloro 1 but not phloro 2. The lower wavelength peak exhibits bathochromic shift for both phloro 1 and 2.
Figure 2-13. Ultraviolet/visible absorption spectra for 0.03 mM and 0.3 mM solutions of a) phloro 1 and c) phloro 2. The corresponding derivative spectra are shown as b) and d), respectively.

Figure 2-14. Bar charts showing measured $\lambda_{\text{max}}$ chromic shift with corresponding 95% confidence intervals of the lower wavelength peak for a) phloro 1 and b) phloro 2 and the higher wavelength peak for c) phloro 1 and d) phloro 2.
CHAPTER 3
ELUCIDATING THE DISSOCIATION MECHANISM OF NOVEL PHLOROGLUCINOL DERIVATIVES

Introduction

Phloroglucinol, 1,3,5-trihydroxybenzene (Figure 2-1), exists in various natural products. Various phloroglucinol derivatives have been isolated as naturally-occurring bioactive compounds, and synthetic phloroglucinol derivatives have been widely reported.97 Interest in designed synthetic species based on phloroglucinol is growing.98-100 Members of this important class of compounds have demonstrated therapeutic and pharmacological properties such as antiviral, antibacterial, and vasodilator activities.101 In particular, hyperforin and adhyperforin, constituents of St. John’s Wort (Hypericum perforatum), have been well-characterized for their antidepressant action.102-109 Phloroglucinol derivatives comprise important secondary metabolites in several dicotyledonous plants,97 and have been reported as useful environmentally-responsible dye agents110 and feeding inhibitors.111 Additionally, acylphloroglucinols have demonstrated potential as lead structures for degenerative disease drug development.112 Isolation, quantitation, and characterization of diverse types of phloroglucinol derivatives are therefore relevant to many applications, and are of increasing importance in pharmacokinetic and toxicological studies.

Pharmaceutical development relies on rapid, sensitive, and quantitative analytical techniques for the determination of drugs and metabolites in complex biological matrices.113 High-performance liquid chromatography, HPLC, combined with mass spectrometry, MS, and tandem mass spectrometry, MS/MS or MSn, is a widely-employed tool in this area because it benefits from several important advantages. HPLC provides separation capabilities to improve analysis of complex or dirty samples, and MS is useful because it enables rapid and selective detection of compounds simultaneously.114 MS/MS is essential for structure elucidation which
can be especially important in metabolite identification. Another important aspect of tandem work, however, is selected reaction monitoring, SRM, the capability of detecting a compound by monitoring fragmentation of one ion to another following a characteristic and specific pathway for a particular analyte. Multiple reactions may be monitored during a single chromatographic separation, and SRM confers the ability to distinguish between structurally similar compounds with different fragmentation behaviors increasing both selectivity and sensitivity of an assay designed for a specific application. Confident assignment of a fragment ion to its parent, however, is an important part of method development for this type of work and consequently an understanding of dissociation mechanisms for systems under investigation is necessary.

Elucidation of the fragmentation behavior of the phloroglucinol derivatives as a class of compounds is therefore of growing importance due to the wealth of applications evolving for these species. Several reports of qualitative and quantitative LC-MS for determination of hyperforin and adhyperforin in extracts of St. John’s Wort have recently been published.

Two synthetic phloroglucinol derivatives substituted at the 2,4 and 6 positions with benzoyl groups (Figure 2-2) have been characterized using tandem and accurate mass spectrometry. One compound has pure benzoyl substituents (phloro 1), but the benzoyl groups of the second are methylated at both meta positions of the benzene ring (phloro 2). Alkali-metal-bound dimers of both phloro 1 and 2 have been observed to form with Li⁺, Na⁺, and K⁺, and proton and NH₄⁺-bound dimers of phloro 1 have also been detected, as described in Chapter 2. Both phloro 1 and phloro 2 were observed as protonated molecules and as molecular adducts of each alkali-metal cation. Following fragmentation pathway elucidation for a simple compound, 2-hydroxybenzophenone (Figure 3-1), which contains similar functional groups to phloro 1 but has no C₃ character, a dissociation mechanism for the initial fragmentation of the cationized
molecules of phloro 1 and 2 was deduced. Dissociation pathways for the alkali-metal cation and proton-bound dimers, as well as the protonated and ammonium adducted molecules of phloro 2, were determined in this research. A recent publication elucidated the fragmentation behavior of protonated and deprotonated hyperforin and adhyperforin,\textsuperscript{109} but to date no dissociation mechanism information has been made available for alkali metal adducts of phloroglucinol derivatives. This information is very valuable because electrospray ionization is an extremely important interface for HPLC with MS. Alkali metal adducts, particularly sodiated and potassiated species, are known to be prevalent in mass spectra obtained using ESI. Additionally, in analytes lacking a significantly acidic or basic site, formation of an alkali metal cation adduct may be the only available route to ionization and consequently detection where ESI is the sole accessible ionization technique.

**Experimental**

**Sample Preparation**

The phloroglucinol derivatives 2,4,6-tribenzoylphloroglucinol and 2,4,6-(3,5-dimethyl)-tribenzoylphloroglucinol were synthesized and purified by members of the Castellano group at the University of Florida. Structures were confirmed by ESI-FTICR-MS and \textsuperscript{1}H and \textsuperscript{13}C nuclear magnetic resonance spectroscopy. All samples were dissolved in HPLC grade methanol (Honeywell Burdick & Jackson, Muskegon, MI), and the cations Na\textsuperscript{+}, Li\textsuperscript{+}, K\textsuperscript{+}, and NH\textsubscript{4}\textsuperscript{+} were introduced as chloride salts (Sigma-Aldrich, St. Louis, MO). The samples were prepared at concentrations of 3x10\textsuperscript{-4} M host and 3x10\textsuperscript{-5} M guest, and contained 1% formic acid (ThermoFisher Scientific, Waltham, MA) to improve current stability and ionization efficiency throughout the analysis. The test compound 2-hydroxybenzophenone (Sigma-Aldrich, St. Louis, MO) was prepared in the same solvent at a concentration of 3x10\textsuperscript{-4} M.
**Mass Spectrometry**

All mass spectra were acquired using direct infusion electrospray ionization with an appropriate mass analyzer. The tandem mass spectral data for phloroglucinol derivative fragmentation pathway elucidation were obtained using an LTQ linear ion trap mass spectrometer, monitoring the fragmentation through MS$^5$ (ThermoFisher Scientific, Waltham, MA). The 2-hydroxybenzophenone fragmentation was observed via an LCQ Deca quadrupole ion trap (ThermoFisher Scientific, Waltham, MA). The precursor mass selection window was set to 1 amu and optimal CID cone voltages between 25 and 30 V were applied. High-resolution exact mass measurements of precursor and product ions were made using SCID-ESI-MS with either a 4.7 T Bruker Bioapex II Fourier Transform Ion Cyclotron Resonance mass spectrometer equipped with an Apollo API 100 Source (Bruker Daltonics, Billerica, MA) or an Agilent 6210 Time-of-Flight mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA). Samples were introduced at flow rates of 2 μL/min for the FTICR-MS and 10 μL/min for all other mass spectrometers using direct infusion. The potential difference between the ESI needle and the desolvation capillary inlet was 3.5 kV. Each run was repeated three times using individually-prepared samples for mapping of dissociation pathways. The ESI-SCID-TOF data presented are data obtained via the experiments described in Chapter 2; consequently, experimental configurations and conditions may be found in the Chapter 2 experimental section.

**Results and Discussion**

**Electrospray Ionization Source-Skimmer Collisionally Induced Dissociation Justification**

Chapter 2 describes ESI-SCID-TOF experiments yielding dissociation curves for the alkali-metal cation-bound dimers of the phloroglucinol derivatives phloro 1 and 2. The data were reanalyzed by plotting the ratio of cation-bound dimeric peak intensity to adducted monomeric peak intensity against the SCID fragmentation voltage difference. The curves for phloro 1 are
presented as Figure 3-2. Comparison of the shapes of these curves highlighted a difference between the sigmoidal decline of the curves corresponding to the Na\(^+\) and Li\(^+\) adducts and the plateaus corresponding to the K\(^+\) and NH\(_4^+\) adducted species. Analogous curves for phloro 2 are shown in Figure 3-3; all three curves corresponding to the Na\(^+\), Li\(^+\), and K\(^+\) adducts are of a similar shape and differ predominantly in magnitude. This type of plot represents the dissociation of an alkali-metal cation-bound phloroglucinol derivative dimer to a monomeric adduct of the same cation, so differences in shape could be indicative of different cation-dependent dissociation pathways for the phloro 1 host. Initial attempts at dissociation pathway elucidation based on the ESI-SCID-TOF data were hindered by ambiguity concerning the origin of apparent fragment ions, which is a limitation of SCID. These observations illustrated the need for a tandem mass spectrometric investigation incorporating specific precursor ion selection capabilities, as opposed to SCID which does not enable direct determination of precursor-product ion relationships. Consequently, fragmentation of the mass selected cation-bound dimers of phloro 1 and phloro 2 was investigated using CID in an ion trap mass analyzer, but to aid in dissociation pathway elucidation, a similar experiment with 2-hydroxybenzophenone was first performed. The SCID-TOF data were used to obtain exact mass measurements for the fragment ions which were observed via both tandem mass spectrometric approaches.

**2-Hydroxybenzophenone Experiments**

The compound 2-hydroxybenzophenone (Figure 3-1) was selected to aid in the elucidation of the dissociation behavior of the phloroglucinol derivatives. It has similar structural characteristics to the phloro 1 molecule as it is comprised of a benzene ring modified by a benzoyl substituent, and so has the important functional groups involved in the phloro compound fragmentation. The two phloroglucinol derivatives, however, have a repeating pattern of central benzene ring substituents which confers C\(_3\) symmetry on their molecular structures, and which
complicates determination of dissociation mechanisms and pathways because structural rearrangement during the high-energy CID event becomes a significant issue. The smaller and less complex 2-hydroxybenzophenone has minimal structural reconfiguration capabilities and so proved extremely useful as a tool for the derivation of the initial fragmentation mechanism of both phloro 1 and 2.

Following MS$^3$ of electrosprayed 2-hydroxybenzophenone, a fragmentation pathway for the protonated molecule was determined and is presented as Figure 3-4. Primary cleavages were observed between the carbonyl carbon and each of its neighboring carbon atoms, as was expected. Interestingly, the major fragment ion observed following the initial CID event corresponded to the loss of a benzene ring and indicated the involvement of keto-enol tautomerization in the dissociation process. This was evidenced by the loss of a complete benzene ring which would only be possible if cleavage of the bond between the carbonyl carbon and carbon 1 on the benzene ring were accompanied by the abstraction of a hydrogen atom from a different region of the precursor ion. As stated previously, the rearrangement capabilities of 2-hydroxybenzophenone are limited so the most accessible hydrogen atom available for abstraction would be that originating in the alcohol group, following keto-enol tautomerization. This deduction coupled with the similar fragmentation behavior of hyperforin and adhyperforin reported by Sleno et al.$^{109}$ enabled elucidation of the primary mechanism of covalent dissociation which is shown in Figure 3-5 for 2-hydroxybenzophenone.

**Protonated Molecule Dissociation Pathways**

Dissociation pathways for the protonated molecules of phloro 1 and phloro 2 are presented as Figures 3-6 and 3-7, respectively. Both phloroglucinol derivatives exhibit identical fragmentation behavior and adhere to the mechanism deduced for the smaller 2-hydroxybenzophenone. Following keto-enol tautomerization, the complete neutral benzene
ring is lost from one of the phloro 1 benzoyl groups and the complete neutral dimethylated benzene ring is lost from the corresponding substituent of phloro 2. This neutral loss occurs for all three benzoyl groups until only the protonated core of the molecule C₉O₆ remains, suggesting that the proton is noncovalently bound to one or more of the core oxygen atoms and is not solely stabilized by arene interaction, as the central carbon cycle is no longer conjugated at this point. No evidence of structural rearrangement beyond the initial keto-enol tautomeration was observed.

Alkali-Metal Cation-bound Dimer and Adducted Monomer Dissociation

The dissociation pathways for the lithium cation-bound dimers of phloro 1 and phloro 2 were determined using the same strategy and are shown in Figures 3-8 and 3-9. The lithium cation-bound dimer of phloro 1 dissociates to a monomeric lithium cation adduct via the neutral loss of a phloro 1 molecule. The monomeric lithium adduct is subsequently fragmented in one of three different ways. The neutral loss of one complete benzene ring, previously observed for the protonated molecules, is the primary pathway observed, but neutral loss of either a water molecule or carbon dioxide also occurs. Analogous to the fragmentation of the protonated molecules, the benzene ring loss is followed by successive benzene losses until only the oxygen and carbon monoxide-substituted cyclohexane core remains. The lithium cation, however, remains a component of the detected ion through the entire process, suggesting that it is interacting with the lone electron pair(s) of one, or more probably, several of the core oxygen atoms. Evidence that the lithium cation remains is based not only on the fragment nominal and measured accurate m/z data, but also on the isotopic distribution observed in the detected ions. Lithium cation is the only one of the cations employed which has a nonstandard isotopic distribution. This characteristic combined with the fact that the less abundant monoisotope has a high enough abundance for detection above the noise threshold aid in tracking of the cation.
throughout fragmentation. The loss of carbon dioxide from the monomeric lithium cation adduct is of particular interest because it provides evidence for structural rearrangement of the phloroglucinol derivative during the high-energy CID process. Carbon dioxide can only be cleaved from phloro 1 following relocation of the benzene ring portion of one of the benzoyl groups, but the position of the benzene ring after the rearrangement is currently indeterminate.

The phloro 2 lithium cation adducts exhibit analogous behavior. The lithium cation-bound dimer dissociates to the lithium cation adducted monomer, but the monomeric adduct only fragments via neutral loss of either a dimethylated benzene substituent or a carbon dioxide molecule; no water loss occurs. Successive cleavages of the bonds between the carbonyl carbons and the dimethylated benzene ring portion of the benzoyl groups follow until only the core remains, similar to the dissociation behavior of the other phloroglucinol derivative compound.

The sodium cation-bound dimers of both phloro 1 and phloro 2 adhere to the same dissociation pathway as those bound by lithium cation; the specific pathways are presented as Figures 3-10 and 3-11, respectively. Smaller initial signal intensity, however, meant that the dissociation process could not be observed through MS$^5$ so only two of the benzoyl substituents were observed to fragment. Both monomeric sodium cation adducts exhibit water and carbon dioxide loss, further evidencing the structural rearrangement during CID observed for the lithium adducts.

Fragmentation behavior for the less stable phloroglucinol derivative dimers bound by the potassium or ammonium cations proved somewhat different to the previously characterized dissociation process. For both phloro 1 and phloro 2 dimers bound by potassium cation, the initial step yields monomeric potassium adducts and the successive step, for which the mechanism has been proposed, involves the neutral loss of either the conventional or
dimethylated benzene ring. The respective pathways are delineated in Figures 3-12 and 3-13. No alternative dissociation pathways are evidenced; neither water nor carbon dioxide losses from the monomeric adducts are observed, hence there is no evidence to suggest that the potassium cation phloroglucinol derivative adducts undergo structural rearrangement during CID. Low initial signal intensity only enabled detection through MS$^3$, so only one fragmentation step for the monomeric adducts could be followed.

Adducts of the phloroglucinol derivatives with ammonium cation showed the most deviation from the dissociation pattern observed for the cationized host compounds. The corresponding pathways are presented as Figures 3-14 and 3-15. Although the initial fragmentation of the phloro 1 ammonium cation-bound dimer involves the neutral loss of a phloro 1 monomer yielding the monomeric ammonium cation adduct, consistent with the dissociation behavior already described, no stable ammonium cation-bound phloro 2 dimer could be observed. Consequently, the dissociation pathway for phloro 2 in the presence of ammonium chloride is shown for the monomeric precursor ion. The successive step for phloro 1, however, results in detection of the protonated molecule following the neutral loss of ammonia. No loss of a neutral benzene ring occurs during the dissociation process, but subsequent to the ammonia loss, cleavage of the bond between the carbonyl carbon of one benzoyl substituents and a carbon atom on the core benzene ring is observed, yielding a carbonium cation. This can be further fragmented to yield a carbonium cation of one of the benzoyl substituents carrying the charge on the carbonyl carbon.

The fragmentation behavior of the ammonium cation adducted phloro 2 monomer shows two alternative dissociation pathways. The traditional phloro 2 neutral loss of a dimethylated benzene ring occurs, consistent with data obtained for each of the other phloro 2 monomeric
adducts. An alternative competing dissociation pathway involves the loss of neutral ammonia yielding the protonated molecule, consistent with data obtained for the phloro 1 ammonium cation adducted molecule.

**Conclusions**

Dissociation behavior for two phloroglucinol derivative compounds has been elucidated using ESI-MS and a combination of tandem mass spectrometric techniques. Coupling of SCID followed by high resolution mass analysis and mass selective ion trapping followed by CID led to the acquisition of complementary data for thorough delineation of the dissociation pathways associated with the C₃ symmetric molecules.

Initial justification for the dissociation pathway study was based on the increasing pharmaceutical significance of phloroglucinol derivative compounds, but data obtained during the relative stability experiments conducted on the alkali metal cation-bound dimers of phloro 1 and phloro 2 also highlighted a need for this information. Graphical representations of the dissociation of the dimers to monomeric adducts suggested the possibility that different cations could result in different fragmentation behavior.

Since no information concerning dissociation characteristics of alkali metal adducts of phloroglucinol derivatives is currently available, the goal was initially pursued through fragmentation of the smaller and less complex, but structurally similar, 2-hydroxybenzophenone. Elucidation of the dissociation pathway observed via CID of the protonated molecule was achieved and yielded useful data which led to the determination of the mechanism for the initial dissociation step. The proposed mechanism involves keto-enol tautomerization followed by the neutral loss of an alkyl group.

Adherence to the proposed mechanism was ultimately observed for CID of the protonated molecules of both phloroglucinol derivatives. Successive occurrences of the fragmentation step
corresponding to the determined mechanism were observed until no benzoyl substituents remained. The carbon and oxygen core of the molecule retained the charge-supplying proton throughout all the cleavages associated with this behavior suggesting that the proton noncovalently interacts with the core region of the host molecule.

Dissociation pathways for both phloroglucinol derivative dimers bound by Na⁺, Li⁺, and K⁺ were delineated, and showed that the dimers fragment to the corresponding cation-adducted monomers. The primary route to fragmentation of the monomeric adducts adhered to the dissociation mechanism observed for the 2-hydroxybenzophenone. Again, retention of the cation throughout dissociation showed that the major stabilizing noncovalent interactions probably involve the lone electron pairs on the oxygen atoms and not only the arene system, which is predominantly destroyed by the loss of the final benzoyl substituent. Competing pathways involving water loss and carbon dioxide loss were also evidenced, but only for the lithium and sodium cation adducts. The loss of neutral carbon dioxide from the complete cation-adducted monomers provided evidence for structural rearrangement of the host molecules during the CID process.

The ammonium adducts behaved differently to the other adducts under investigation. Although the dimeric adduct of phloro 1 initially dissociated to the analogous monomer, no stable phloro 2 ammonium cation-bound dimer could be isolated for fragmentation. The monomeric adduct of phloro 1 was only observed to fragment via the loss of neutral ammonia, and successive steps showed no evidence of adherence to the dissociation mechanism determined for the other adducts. The monomeric ammonium cation adduct did evidence fragmentation by the proposed mechanism, but a competing pathway involving the loss of neutral ammonia was also observed.
Electrospray ionization mass spectrometry was successfully employed to characterize the
dissociation behavior of the noncovalent adducts of the two phloroglucinol derivatives for future
pharmaceutically-relevant LC-MS and LC-MS\textsuperscript{n} analyses. Differences in fragmentation behavior
of protonated and differentially cation-adducted species have been highlighted for this class of
compounds. Evidence for stabilizing noncovalent interactions between the oxygen-rich core of
the phloroglucinol derivatives and the cations rather than between the arene system and the
cations has been obtained.
Figure 3-1. Structure of the test compound 2-hydroxybenzophenone.

Figure 3-2. Dissociation of dimers of phloro 1 bound by a) Na$^+$, b) Li$^+$, c) K$^+$, and d) NH$_4^+$ to their respective monomeric alkali-metal cation adducts as potential difference voltage is varied in an ESI-SCID-TOF experiment. These curves are based on data presented in Chapter 2.
Figure 3-3. Dissociation of dimers of phloro 2 bound by a) Na$^+$, b) Li$^+$ and c) K$^+$ to their respective monomeric alkali-metal cation adducts as potential difference voltage is varied in an ESI-SCID-TOF experiment. The NH$_4^+$-bound dimer was not stable enough for curve construction. These curves are based on data presented in Chapter 2.
Figure 3-4. Proposed fragmentation pathway for protonated 2-hydroxybenzophenone.
Figure 3-5. Proposed initial dissociation mechanism for protonated 2-hydroxybenzophenone. The cationized molecules of the phloroglucinol derivatives phloro 1 and 2 also adhere to this mechanism.
Figure 3-6. Proposed fragmentation pathway for the protonated molecule of phloro 1 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-7. Proposed fragmentation pathway for the protonated molecule of phloro 2 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-8. Proposed fragmentation pathway for the lithium cation-bound dimer of phloro 1 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-9. Proposed fragmentation pathway for the lithium cation-bound dimer of phloro 2 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-10. Proposed fragmentation pathway for the sodium cation-bound dimer of phloro 1 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-11. Proposed fragmentation pathway for the sodium cation-bound dimer of phloro 2 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-12. Proposed fragmentation pathway for the potassium cation-bound dimer of phloro 1 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-13. Proposed fragmentation pathway for the potassium cation-bound dimer of phloro 2 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-14. Proposed fragmentation pathway for the ammonium cation-bound dimer of phloro 1 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF.
Figure 3-15. Proposed fragmentation pathway for the ammonium cation adducted monomer of phloro 2 showing observed fragments with exact masses and mass error values where appropriate. MS/MS stages were obtained using the LTQ instrument and exact mass data were determined via SCID-TOF. The ammonium cation-bound dimer was too unstable to be mass selected for dissociation.
CHAPTER 4
CONFIGURATION OF A HOME-BUILT DESORPTION ELECTROSPRAY IONIZATION SOURCE WITH A COMMERCIAL TIME-OF-FLIGHT MASS ANALYZER

Introduction

In 2004, the Cooks group reported the development of desorption electrospray ionization, DESI, which, combined with direct analysis in real time (DART) formed the basis of an innovative platform for ambient ionization. The term ambient is used here to imply that it is an atmospheric pressure technique maintaining sample accessibility throughout analysis. Consisting of a pneumatically-assisted electrospray solution used to desorb and ionize analytes from an ambient surface, the DESI technique has demonstrated ESI-like characteristics in the ionization of analytes amenable to electrospray ionization such as peptides. DESI also benefits from an ability to ionize species traditionally ill-suited to ESI, however, via an apparent additional ionization mechanism. Inherent sample accessibility throughout analysis confers on the technique the advantages of real-time manipulation of ionization conditions such as temperature and solvent composition and also the configuration referred to as reactive DESI, a variant of the technique which is described in greater detail in Chapter 5. The DESI technique is related to both ESI and desorption ionization techniques such as MALDI and desorption/ionization on porous silicon, DIOS. Currently, DESI involves a nebulized solvent, usually water or a mixture of water and methanol, under high voltage conditions sprayed in an optimal geometry at an analyte adsorbed onto a surface or an in situ analyte. Desorbed ions are focused into a mass spectrometer via an atmospheric pressure interface.

Two mechanisms of ionization have been postulated, but they are each relevant to different applications. One involves the formation of charged solvent droplets which ionize analyte molecules on the desorption surface by charge transfer; the resultant buildup of static
charge forces desorption of the analyte ions from the surface, a phenomenon known as chemical sputtering. The second mechanism explains the strong resemblance in spectral characteristics of DESI data to that obtained via ESI. Electrosprayed droplets impact the surface resulting in the dissolution of analyte within the droplet; subsequent evaporation of the solvent yields analyte ions. Both ESI and DESI generally yield significant amounts of the protonated molecular ion, multiply charged ions, and alkali metal adducts in positive mode. An interesting study investigating droplet dynamics and ionization mechanisms of DESI using phase Doppler particle analysis was recently published by Venter et al.

Primarily, DESI is advantageous because it is an ambient method, which requires little or no sample pretreatment. It is sensitive, fast, and versatile, being suitable for solid, liquid, and adsorbed gaseous samples. Traditional DESI has been applied to an enormous number of analytes, including pharmaceuticals, chemical warfare agents, plant alkaloids, and lipids. Novel sampling surfaces like porous silicon, thin-layer chromatography plates, and solid-phase microextraction fibers have been analyzed. A major application area of the DESI technique is the trace level detection of explosives on ambient surfaces. Native explosives and their plastic compositions have been analyzed on metal, plastic, paper, polymer, and living human tissue surfaces; promising results were obtained using complex matrices such as lubricants and detergents. Pharmaceutical applications include high throughput analysis of various commercial dosage forms, drug and metabolite identification in blood and urine, and coupling of DESI to thin layer chromatography for over-the-counter preparations analysis. Food chemistry applications and determination of alkaloids in plant tissues have been demonstrated. Mass spectrometric profiling of intact, untreated bacteria employing DESI has been described. A study describing the complementary application of nuclear magnetic
resonance spectroscopy and DESI-MS, and incorporating principal component analysis for data interpretation, for urine metabolite determination in patients with inborn errors of metabolism was recently contributed by the Cooks group. Imaging mass spectrometry under ambient conditions has been achieved by the application of DESI, and tissue imaging demonstrated.

The preceding applications employed standard bench top ion trap or triple quadrupole mass analyzers. An interesting addition to the types of trapping instruments with which the DESI technology has been interfaced is a custom-built portable mass spectrometer fitted with a miniature cylindrical ion trap mass analyzer. The Cooks group has also demonstrated the capabilities of DESI coupled to an Orbitrap mass spectrometer for exact mass measurements on therapeutic drugs and peptides, and several other mass analyzers have been configured for high resolution and exact mass DESI applications. DESI has been interfaced with TOF-MS for the direct determination of counterfeit, commercial, and illicit drug tablets and for the determination of CID fragmentation pathways of over-the-counter drugs. An atmospheric pressure interface for the coupling of ESI, electro-sonic spray ionization, ESSI, an extremely soft form of ESI utilizing a supersonic gas jet, and DESI to FTICR-MS was developed by the Bruce group, and successful analysis of proteins and peptides was recently achieved using DESI-FTICR-MS.

A home-built DESI source originally designed for configuration with a Bruker Bioapex FTICR-MS has been successfully interfaced with first the FTICR-MS instrument and subsequently an Agilent TOF-MS. The DESI technique has compatibility issues with FTICR-MS, hence the DESI-TOF-MS configuration was undertaken. The unique geometry of the source housing on the Agilent TOF mass spectrometers poses particular challenges for DESI source
integration, as do some of the instrument-control-specific features of the accompanying Analyst software. Strategies for addressing those challenges are described herein.

Experimental

Desorption Electrospray Ionization Source Design

The DESI source was constructed by the University of Florida Chemistry Department Machine Shop. It was fabricated using anodized aluminum (6061-T6), and consists of one xyz-stage for the manipulation of an electrospray head and a second xyz-stage for the manipulation of a sample holder platform. A schematic showing an overhead view is presented as Figure 4-1, and photographs of the source configured with the FTICR-MS are included as Figures 4-2 and 4-3. The dimensions shown in the schematic are those used for configuration with the FTICR-MS; alterations made for configuration with the TOF-MS are described in the results and discussion section. The electrospray head is mounted on a cylindrical block which can be rotated for manipulation of the angle made between the spray needle and the deposition surface. A commercial ESI needle is housed inside the coaxial gas needle which is visible in the schematic. A Teflon® spacer between the block and the solution/gas introduction port insulates the remainder of the source from the high-voltage applied to the solution. Solution introduction is via direct infusion using a Harvard Apparatus PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA), and high-voltage is applied to the spray solution via an external 210-10R high voltage power supply (Bertan Associates, Hicksville, NY). The sample surface is chassis ground. The sample holder is a Teflon® platform mounted on an aluminum steel block with an indentation for housing of a deposition surface the size and shape of a standard microscope slide.

Mass Spectrometry

Mass spectra were acquired using a Bruker Bioapex II 4.7 T Fourier Transform Ion Cyclotron Resonance mass spectrometer (Bruker Daltonics, Billerica, MA), or an Agilent 6210
Sample Preparation

Rhodamine 6G (Figure 4-4) samples were prepared by drawing a layer of red Sharpie (Sandford Corporation, Oak Brook, IL) ink onto the sampling surface. Cytochrome C (horse heart, Sigma-Aldrich, St. Louis, MO) samples were prepared by dissolving 1 mg/mL of the solid in 50:50 methanol:water. Ten microliter spots were deposited onto the sampling surface using a micropipettor and were dried under ambient conditions for thirty minutes. HPLC grade solvents were employed (Honeywell Burdick & Jackson, Muskegon, MI).

Results and Discussion

Configuration of DESI Source with FTICR-MS

Initially the DESI source was interfaced with the FTICR using the original dimensions and attachment mechanism specifically designed for this purpose. The FTICR-MS instrument used is ordinarily equipped with an ESI source, and so has an atmospheric pressure inlet orifice accessible for DESI. The hollow center of the desolvation capillary is the inlet and must be immediately adjacent to the deposition surface for DESI compatibility. The ESI spray head housing attaches to the desolvation capillary housing via a hinged door and latch, so the whole spray head can be removed easily. Removal of the ESI spray head housing makes the inlet orifice accessible. The original design of the DESI source included a hinged door, of the same dimensions as that of the ESI spray head housing, placed adjacent to the sample holder platform (see Figure 4-2). A circular hole cut in the door enabled access to the desolvation capillary for ion entrance.

The applied high-voltage necessary to charge the spray solution is ordinarily achieved in the standard ESI source for this instrument by maintaining the spray needle at ground potential
and applying a voltage of 3-4 kV to the desolvation capillary entrance. The applied voltage is negative for detection of positive ions and positive for detection of negative ions. A safety feature of this instrument prevents the high-voltage from being applied unless a certain switch in the capillary housing is closed; the switch is depressed when the hinged door is closed and tightly latched. If the high-voltage does not turn on, the software will not allow the instrument to acquire data. Therefore, the switch must also be depressed during DESI spectral acquisition. To overcome this issue, a small screw was placed through the DESI source door and screwed into position to depress the switch upon latching the replica door. This can be seen in Figure 4-3. The screw is above and to the right of the desolvation capillary. Interestingly, to obtain DESI signal, the high-voltage had to be applied to the electrospray solution while the desolvation capillary was held at 100 V. Reducing the desolvation capillary entrance to ground potential resulted in unstable current and loss of signal.

When the DESI source was first interfaced with the FTICR-MS, it was observed that the dimensions of the source did not allow the full range of motion that should have been available to the spray head on its mounting block, and the spray head was too far away from the inlet for DESI signal to be attained. This issue was unforeseen during the design process because the additional length of the spray needle and the length of the desolvation capillary were difficult to measure accurately in position in the enclosed ESI spray head housing. A useful feature of the DESI source design was exploited to address the problem. Between the spray head mounting block and the x-y-z stage is an entirely removable piece, labeled “a” in Figure 4-1. An analogous piece is used to connect the sample holder platform and its x-y-z stage. Piece “a”, which was originally 9 cm long, was replaced with a 12.75 cm piece. This enabled the movement of the spray head towards the inlet orifice, and subsequently DESI signal of Rhodamine 6G was
observed. The first DESI-FTICR-MS data acquired using the DESI source are spectrally represented in Figure 4-5, and a close-up view of the DESI needle coaxial gas tubing adjacent to the orifice inlet is shown as Figure 4-6, to provide some idea of the small distance between the needle tip and the inlet of the mass spectrometer necessary for spectral acquisition. Also visible is a portion of the sample holder stage and the deposition surface from which the detected ions were desorbed.

Optimization of DESI parameters is an involved process which requires signal stability. Unfortunately, sample ablation during DESI can be quite fast, of the order of tens of seconds. The small colorless spots on the slide shown in Figure 4-6 are areas where sample ablation via DESI has occurred. An important step in the acquisition of mass spectral data via FTICR-MS is the hexapole accumulation of ions prior to their transfer to the ICR cell for detection. This usually takes between 1 and 2 seconds. Additionally, several complete scan events involving accumulation time, ion transmission time, and time for cyclotron resonance detection and multiplexing are necessary to obtain usable signal intensity and signal-to-noise ratio, S/N, values. This makes optimization of DESI parameters for FTICR-MS extremely challenging, as, during the time it takes to determine the effect of slight variation of one parameter using the FTICR-MS, sample ablation has resulted in loss of signal. For this reason, it was determined that it would be useful to configure the home-built DESI source with an Agilent TOF-MS, which has the advantage of a very high scan rate, conferring the ability to maintain high-resolution while acquiring around 1 spectrum per second, depending on the selected mass range.\textsuperscript{139}

**Configuration of DESI Source with TOF-MS**

To capitalize on the fast spectral acquisition capabilities of the TOF mass analyzer, the DESI source was moved onto the TOF-MS described in the experimental section. The original
design was not compatible with the unusual geometry of the Agilent source (see Figure 4-7), so several modifications had to be made.

The first major obstacle to the DESI-TOF configuration was the height of the desolvation capillary housing on the TOF. To address this, a specially built platform for the DESI source had to be made out of stainless steel. The 13” stand is visible in Figure 4-8. However, although the stand brought the source to an appropriate height, the slanting front of the desolvation capillary housing made positioning of the sample holder platform adjacent to the inlet orifice difficult. The front of the base of the DESI source was held at a certain distance from the capillary by the protruding lower edge of the desolvation capillary housing, and the original dimensions of the arm connecting the sample holder platform and its x-y-z stage limited movement of the platform toward the mass spectrometer, so that 7 cm between the sample surface and the inlet orifice was the minimum distance achievable. This issue was overcome by exchanging the removable 7.5 cm long piece labeled b in Figure 4-1 with a new piece of length 14 cm. Since the spray head was now incapable of reaching either the sample surface or the inlet orifice of the mass spectrometer, piece “a” was replaced again, this time with a 19 cm long piece.

At this point, the spray head was able to reach the inlet orifice, but the slanting geometry of the desolvation capillary housing was still preventing movement of the sample holder platform to within the necessary distance of a few mm from the inlet orifice. At its nearest point, the edge of the sample platform or the arm of its mounting block jarred against the edge of the capillary housing, 1.25 cm away from the orifice. As stated briefly in the introduction section of this chapter, in 2006 the Bruce group at Washington State University reported the use of a flared capillary adapter to improve collection efficiency of ions resulting from ESI, ESSI, and DESI ionization techniques.\textsuperscript{137} This strategy was modified to address the issue of the distance
remaining between the needle of the home-built DESI source and the inlet orifice of the TOF mass analyzer. A capillary extender designed to fit snugly onto the end of the desolvation capillary and extend to the sample surface was used to bring the orifice inlet to the sample (see Figure 4-9). A gold canted coil spring was placed into a groove milled on the inside of the piece which fits over the capillary to hold the extender firmly in place while promoting conduction for the electrospray. This was essential, because in the absence of the spring, the capillary extender will be pulled off the end of the desolvation capillary and stick to the edge of the sample holder stage due to charge build-up upon application of the potential difference necessary to achieve an electrospray. An additional useful feature of the capillary extender is that it enlarges the area of the sampling orifice, which has been shown to improve ion transmission and spectral characteristics.137 The diameter of the desolvation capillary inlet orifice is 0.6 mm, but the diameter of the capillary extender inlet orifice is 1.5 mm.

The Agilent TOF mass spectrometer can only acquire spectra when it is configured to a source. Since several commercial atmospheric ionization sources have been designed for it, a source recognition mechanism incorporating strategically positioned magnets is employed to allow control of the sources via one software suite. Magnets on the inside of the source attachments, which interface by latching onto the desolvation capillary housing, are in locations specific to each source type. Consequently, when a nonstandard source is interfaced with the desolvation capillary, a configuration of appropriately-sized magnets is necessary to enable source recognition by the software. The standard ESI source has the simplest magnetic configuration, so the mass spectrometer was configured for ESI whenever the DESI source was attached. One small bar magnet on the inside of the ESI spray head housing is used for ESI source recognition. The addition of a small bar magnet correctly positioned external to the
desolvation capillary source housing and held in place with Scotch tape enabled the instrument to “recognize” the ESI source and allowed spectral acquisition in the absence of a commercial source. A second issue in terms of nebulizer gas settings also had to be addressed prior to data acquisition. The ESI nebulizing gas is not used for the DESI source, and the outlet has to be covered to minimize unnecessary loss of N₂(g). However, for the instrument to come out of standby mode, the internal flow meter has to register the flow rate set by the user. This is achieved by setting the nebulizing gas flow rate to the number that is registered prior to spectral acquisition. A setting of zero may or may not be appropriate, despite the fact that nebulizing gas is not flowing.

Following the described modifications and conditions, DESI-TOF-MS data were obtained for the Rhodamine 6G sample. A representative mass spectrum is shown as Figure 4-10.

Following the Rhodamine 6G tests, another standard, the protein cytochrome c, was used for confirmation of the DESI-TOF ionization capabilities. Cytochrome C was successfully analyzed using the DESI-TOF-MS configuration and provided evidence of the success of the interface for compounds other than Rhodamine 6G which is known to exist as a preformed ion. The first spectral data obtained for cytochrome C are presented as Figure 4-11.

**Important Optimization Considerations**

Optimization strategies, and corresponding effects on spectral characteristics, for the geometric and physical parameter conditions important in DESI mass spectral analyses have been extensively documented elsewhere. Consequently, the discussion here is limited to important considerations in the optimization process which are frequently omitted from the published literature. DESI conditions must be optimized specifically for each type of analyte and surface, although similar analytes often have similar optimal conditions. Following the determination of the specific surface used for sample deposition, parameters requiring
optimization may be broadly grouped into two categories: electrospray conditions and geometric parameters.

Examples of the electrospray conditions requiring optimization include solution-phase composition, solution flow rate, applied high voltage difference, and nebulizing gas flow rate. These can be optimized to some extent using real-time visual cues, such as the extent of sample ablation or surface wetting. Monitoring the current measured on the desolvation capillary for stability is a significant aid in optimization of solution composition and applied voltage values. Solution and nebulizing gas flow rate optimization can be performed speedily by monitoring wetting of the sample surface. An additional aid in significantly increasing the efficiency of optimization of the electrospray conditions is monitoring the total ion chromatogram (TIC) instead of the signal corresponding to a particular analyte or analytes. This is useful because it renders the ESI optimization process largely independent of the geometric DESI parameters, upon which analyte-specific signal intensity and S/N are dependent.

Important geometric DESI parameters have been defined by the Cooks group,118 and are delineated in Figure 4-12. Optimization of these parameters is a very interactive and iterative process. Signal intensity and S/N are particularly sensitive to variation of the angle \( \alpha \), which is additionally the most difficult parameter to manipulate using the home-built DESI source, so this is the most challenging parameter to optimize. A good starting point for analytes traditionally amenable to ESI is \( \alpha = 60^\circ \). Again, monitoring the TIC instead of signal intensity or S/N is an efficient way of making an initial assessment of the appropriate range of geometric parameter values for subsequent, more rigorous optimization.

For each type of DESI analysis, it is important to optimize parameters for maximum signal intensity and maximum S/N specific to the analysis. This is because the variable values yielding
maximal signal intensity sometimes do not correspond directly to those yielding maximal S/N, although overlap in terms of a value ranges for some parameters may be observed. Figure 4-13 presents some representative parameter optimization data and shows the effects on signal intensity and S/N resulting from variation of the electrospray flow rate. The optimal flow rate range for maximizing both signal intensity and S/N is 4-6 μL/min, but the peak maxima do not necessarily correspond. Consequently, in some situations 5 μL/min could be most desirable flow rate, but for analysis of a limited amount of sample, where SCID would be employed for structural elucidation or determination of relative stabilizing forces, it is conceivable that the lower flow rate would be selected for the advantage of increased spectral acquisition time it would confer.

**Conclusions**

A home-built DESI source engineered by machinists at the University of Florida department of chemistry machine shop specifically for configuration with a Bruker Bioapex FTICR-MS instrument has been successfully interfaced with an Agilent 6210 API Time-of-Flight mass spectrometer. Initial configuration of the DESI source with the FTICR was successful in that DESI mass spectral acquisition was achieved; mass spectra of the dye Rhodamine 6G were acquired. A significant challenge, the fact that sample ablation during DESI often occurred prior to adequate data acquisition to determine the effects of parameter variation during optimization, highlighted the poor compatibility of DESI with FTICR-MS in terms of slow scan rate of the FTICR instrument.

Several modifications to the original design were necessary to accommodate the significant height difference between the inlet orifice of the FTICR instrument and that of the TOF mass spectrometer, and the unorthodox geometry of the desolvation capillary housing. The fabrication
of a 13” custom built stand overcame the first issue. Extension of the range of motion in the
direction of ion transmission of both the DESI spray head and the sample holder platform
coupled with the fabrication and incorporation of a specifically-designed capillary extender
addressed the latter. Following some additional steps to compensate for the source recognition
and condition-monitoring processes integral to the function of the commercial instrument,
successful acquisition of DESI-TOF mass spectra of deposited Rhodamine 6G and cytochrome C
was attained.

DESI optimization is an iterative and interactive process which has been documented
elsewhere, but some important considerations and strategies for increasing the efficiency of the
process have been outlined to aid the novice user.
Figure 4-1. Schematic of the overhead view of the original home-built DESI source showing major parts and dimensions, in centimeters, for configuration with the FTICR-MS. The pieces marked a and b were altered for configuration with the TOF-MS and the door and hinge were removed.
Figure 4-2. Desorption electrospray ionization source interfaced with the FTICR-MS showing door and hinge for attachment to the desolvation capillary housing.

Figure 4-3. Close-up view of DESI source configured with FTICR-MS showing the spray head, the sample holder platform, and the front end of the desolvation capillary.
Figure 4-4. Structure of the Rhodamine 6G preformed ion.

Figure 4-5. First spectrum obtained using the home-built DESI source configured to the FTICR-MS, showing the molecular ion of Rhodamine 6G.
Figure 4-6. Close-up view of the DESI-FTICR-MS setup used to acquire the spectrum presented in Figure 4-5. The relative positions of the DESI spray needle and the desolvation capillary orifice are shown. The deposition surface, a sandblasted glass slide, and its layer of Rhodamine 6G are visible and small colorless spots on the sample surface show areas of sample ablation.
Figure 4-7. Close-up view of the DESI source interfaced with the TOF-MS showing the unusual slanting geometry of the desolvation capillary housing. Note that the capillary itself is not visible, as only the capillary extender protrudes from the housing. The DESI spray head solution/gas introduction port and fluid connections can be seen. The bar magnet taped to the upper right side of the desolvation capillary housing is responsible for ESI source recognition by the software.
Figure 4-8. Side view of the DESI-TOF-MS configuration. The 13” stand constructed to bring the DESI source to the height of the desolvation capillary is shown and the connection for the application of the high voltage to the electrospray solution is visible.

Figure 4-9. Representation of the capillary extender, with dimensions given in mm, for the DESI-TOF interface showing a) the side view and b) the view from the back. A canted coil spring is fitted into a circular groove on the inside of the extender to hold the extender firmly in place on the desolvation capillary.
Figure 4-10. Mass spectrum of Rhodamine 6G obtained using the DESI-TOF-MS configuration.

Figure 4-11. Positive-mode DESI-TOF mass spectrum of cytochrome C desorbed from a glass slide. A multiprotonated envelope containing charge states ranging from $+9$ to $+19$ was observed.
Figure 4-12. Schematic representation of the DESI spray head and inlet orifice showing the major geometric DESI parameters which require optimization.

Figure 4-13. Representative optimization data for the DESI-TOF-MS configuration using Rhodamine 6G. The effects of variation of the solution flow rate on a) signal intensity and b) S/N for the molecular ion are shown.
CHAPTER 5
REACTIVE DESORPTION ELECTROSPRAY IONIZATION FOR RAPID SCREENING OF GUESTS FOR SUPRAMOLECULAR INCLUSION COMPLEXES

Introduction

The need for a better understanding of noncovalent molecular recognition interactions is fueling the development of analytical techniques appropriate for the characterization of both natural and synthetic supramolecular systems.\textsuperscript{26} Methods like optical and nuclear magnetic resonance spectroscopies and circular dichroism have proven extremely useful for solution-phase analyses, and X-ray crystallography is indispensable for solid phase experimentation.\textsuperscript{17,21} Gas-phase techniques, on the other hand, enable characterization of supramolecular species without the added complications of interference from solvating molecules and crystal close packing effects.\textsuperscript{3,6,26} Mass spectrometry is therefore an appropriate tool for this application and has the benefits of sensitivity, selectivity, and the ability to provide complex stoichiometric information.\textsuperscript{19,26}

ESI is inherently applicable to complexation based on solution-phase self-assembly, and ESI mass spectrometry has been reported for the analysis of a plethora of noncovalent systems from enzyme-substrate complexes\textsuperscript{26,79} to macrocycles, such as calixarenes\textsuperscript{140} and cucurbiturils,\textsuperscript{60,68,69} which are capable of forming inclusion complexes with both neutral and charged guest molecules. ESI is known for a tendency to form nonspecific artifacts which can be confused with true supramolecular complexes in this type of work;\textsuperscript{143,144} this can be particularly problematic in the study of alkali-metal complexation. Additionally, while ionization occurs at atmospheric pressure, the technique does not satisfy a recent definition of the term ambient, which includes the requirement of sample accessibility for manipulation during analysis.\textsuperscript{115} In this application area, sample accessibility is useful because it enables real-time manipulation of
parameters like solvent character and availability of different guest molecules for faster analysis in terms of guest screening.

A major impetus for the development of high-throughput noncovalent receptor screening techniques is the wide applicability of molecular recognition interactions to fields like drug discovery and lead optimization.\textsuperscript{17,26} High-throughput enzyme-substrate assays are well-documented, but predominantly reliant on solution-phase analytical techniques such as immunoassays using spectrophotometric or radiochemical detection, and consequently often subject to interferences from solvent and assay reagent molecules.\textsuperscript{50,145} Structural modifications of ligands are sometimes required to yield required chromophoric properties for detection of the binding event which can alter natural kinetic behavior, and the use of radiochemicals for scintillation-based assays involves the generation of radioactive waste.\textsuperscript{50} Mass spectrometry has the selectivity to minimize some of these effects and consequently reduce the instance of false positive results, particularly when high-resolution or tandem mass analyzers are employed. Mass spectrometry has been applied to receptor screening as a technique for the analysis of combinatorial libraries,\textsuperscript{146-148} but in this context complexation is often driven by solution-phase equilibria in complex matrices, complicating spectra and increasing the likelihood of signal suppression from competing analytes. All of the above suffer from an inability to refine experimental design on a real-time basis for faster method development. A fast mass spectrometric screening method for supramolecular receptors with an appropriately soft ionization technique, manipulable chemical conditions, and minimizing interference from competing species would address some of these issues.

The DESI technique appeared promising for supramolecular applications initially because of its reported similarity to ESI characteristics in the ionization of some analytes well-known to
be amenable to electrospray ionization. Sample accessibility throughout analysis confers on the technique a major advantage in the form of reactive DESI, a variant of the technique in which reagents are incorporated into the spray solution for reaction with the deposited analyte at the sampling surface. Reactive DESI using hydrochloric acid and trifluoroacetic acid has been shown to improve selectivity and detection limits.\textsuperscript{120,121} Traditional DESI is not well suited to the study of supramolecular complexation because deposition and drying of pre-mixed host and guest can add the complication of close-crystal packing forces to the analysis, potentially resulting in the detection of complexes which would not form under useful and realistic conditions. Reactive DESI, however, does not incorporate this disadvantage and so is the more promising configuration of the technique for this application.

Conventional DESI has been applied to an enormous number of analytes, including pharmaceuticals, chemical warfare agents, plant alkaloids, and lipids.\textsuperscript{116} Large biomolecules such as cytochrome C have been shown to maintain nativelike folded conformations during DESI with appropriate conditions,\textsuperscript{149} providing evidence for the applicability of DESI to supramolecular systems. Reactive DESI, predominantly incorporating alkali metal and ammonium cations as spray reagents but also using chloride anions, has been reported for the analysis of pharmaceuticals and metabolites,\textsuperscript{140} and for cation adduction ionization of explosives and chemical warfare agents.\textsuperscript{150} Trace detection of the explosive triacetone triperoxide further demonstrated the utility of reactive DESI for analysis of alkali metal complexes,\textsuperscript{151} and heterogeneous reactions between ions formed by the DESI spray head and solids deposited on a surface have been shown to yield covalently-bound products identified by mass analysis.\textsuperscript{152,153} A natural progression, therefore, is the application of reactive DESI for receptor screening in small-molecule host:guest inclusion complex studies. Very recently, a reactive DESI supramolecular
screen for the determination of counterfeit antimalarial tablets was described by Nyadong et al.,\textsuperscript{154} but to date DESI has not been applied to the analysis of cyclodextrin host:guest inclusion complexes. Additionally, no direct comparison between DESI and ESI for supramolecular applications or for characterization of solution-phase representative data has been made.

Cyclodextrins, cyclic polymers of monosaccharides, are extremely well-characterized toroidal molecules with a hydrophilic exterior and a hydrophobic intramolecular cavity in which a small neutral guest molecule can be encircled to form a true inclusion complex which self-assembles under suitable solution-phase conditions.\textsuperscript{26,155} The cyclodextrins have been shown to complex a multitude of guest species and several ESI-MS studies of this behavior have been reported,\textsuperscript{19,143,144,156-158} making them a useful model system for reactive DESI method development. β-cyclodextrin is composed of seven α−1,4-linked glucopyranose units and consequently has a versatile cavity size for small molecule encapsulation. It has been shown to house various pharmaceuticals and nitro-compounds among other candidates. Its versatility led to its selection as the test host compound. The initial test guest nortestosterone was selected for the documented ability of similar steroids to complex with β-cyclodextrin\textsuperscript{144,159} and the guests used in the screening analysis were chosen on the basis of size and appropriate functional groups.

**Experimental**

**Sample Preparation**

All solutions were prepared using HPLC grade solvents (Honeywell Burdick & Jackson, Muskegon, MI). β-cyclodextrin (Trappsol, CTD, Inc., High Springs, FL) was diluted to 0.05 mM with 50:50 methanol:water. Nortestosterone was used for proof-of-concept and the twelve screened potential guest compounds were acenaphthalene (G1), acetalanilide (G2), caffeine (G3), 4-chloro-3-nitrobenzonitrile (G4), cyclam (G5), 1,3-dinitronaphthalene (G6),
diphenylglyoxime (G7), formanilide (G8), guanosine (G9), L-arginine (G10),
2-methylnaphthalene (G11), and 4’-nitroacetanilide (G12) shown in Figure 5-1 (G5,G10: Acros
Organics, ThermoFisher Scientific, Waltham, MA; all others: Sigma-Aldrich, St. Louis, MO).
They were selected as potential guests on the basis of their sizes and appropriate functional
groups.

Mass Spectrometry

In preparation for the DESI experiments, the guest compounds were diluted to 1 mg/mL, approximately 5 mM, in 50:50 methanol:water, spotted in 10 μL aliquots onto a sandblasted
glass slide and allowed to dry at ambient temperature and pressure for 30 minutes. The DESI
spray solvent was a 0.05 mM β−cyclodextrin solution in 50:50 or 80:20 methanol:water. ESI
samples comprised 0.05 mM β−cyclodextrin in either methanol or 50:50 methanol:water with
one guest compound at 1 mg/mL. Maltohexaose (Supelco, Bellefonte, PA), 1 mg/mL in 50:50
methanol:water, was used to test for ESI artifacts. For all ESI experiments, 0.5% formic acid was
incorporated into the solvents, and the β−cyclodextrin solution used as the DESI spray solvent
contained 0.5 mg/mL of NaCl; both additives (ThermoFisher Scientific, Waltham, MA) were
used to improve current stability and ionization efficiency.

Mass spectra were acquired using an Agilent 6210 MSD Time-of-Flight mass spectrometer
configured for ESI (Agilent Technologies, Inc., Santa Clara, CA). Acquisition of DESI spectra
required an external syringe-pump connected to a home-built DESI source, for which the
desolvation capillary of the TOF was adapted using a specifically designed capillary extender to
enable sampling close to the desorption surface. The capillary extender was not flared as in the
Bruce design,137 but its orifice was three times the diameter of that of the desolvation capillary.
DESI experiments were performed using a spray flow rate of 3 μL/min and 100 psi head
pressure of nebulizing N\textsubscript{2}(g). A voltage of 4 kV was applied to the spray solution via an external 210-10R high voltage power supply (Bertan Associates, Hicksville, NY), while the sample surface was held at ground and the desolvation capillary at 100 V. The spray needle was positioned 1.5 mm above the deposition surface at a 55° angle from the horizontal. A schematic of the experimental configuration is included as Figure 5-2.

ESI mass spectra were acquired using a 5 μL sample loop injection for sample introduction, with a flow rate of 0.5 mL/min and a desolvation capillary voltage of 3.5 kV. The drying gas temperature was 325°C and the fragmentor voltage was held at 175 V for all experiments. Reference mass correction was enabled throughout the ESI data acquisition to maximize mass accuracy.

**Nuclear Magnetic Resonance Spectroscopy**

An Inova NMR spectrometer operating at 500 MHz was used for acquisition of all \textsuperscript{1}H spectra (Varian, Inc., Palo Alto, CA). Deuterium oxide (ThermoFisher Scientific, Waltham, MA) was selected as the solvent and proton chemical shifts were referenced to the HDO signal at 4.780 ppm. Samples consisting of 1 and 2 mg/mL of a single guest were analyzed alone and in the presence of β−cyclodextrin (ThermoFisher Scientific, Waltham, MA). A titration approach covering the concentration range of 0.5-20 mM β−cyclodextrin was employed for each guest to maximize chances of complex detection. The 2D correlation spectroscopy (COSY) NMR technique was used for unambiguous proton assignment, and 2D nuclear Overhauser effect spectroscopy (NOESY) was employed to elucidate the conformation of the β-cyclodextrin:acetanilide complex for validation of the 1D approach to inclusion complex confirmation. For a basic overview of basic two dimensional NMR techniques the reader is directed to a review article by Reynolds and colleagues\textsuperscript{160}.
Theoretical Calculations

Optimized structures for the guest compounds were obtained using Gaussian 03\textsuperscript{161} with the HF/6-31G* method and basis set. Corresponding dimensions were measured using Molekel 5.0, the latest version of a free molecular visualization software package developed by the Swiss National Supercomputing Center (Manno, Switzerland).

Results and Discussion

Reactive Desorption Electrospray Ionization Receptor Screening

To confirm the applicability of reactive DESI for the analysis of β−cyclodextrin inclusion compounds, a steroid guest, nortestosterone, was used as a model. Steroid and cyclodextrin host:guest inclusion complexes have been well-characterized and the nortestosterone was readily available. The host compound, β−cyclodextrin, was sprayed in methanol:water at the deposited nortestosterone guest under the conditions previously described. The methanol:water solvent system was used here because a major application of cyclodextrin inclusion is the aqueous solvation of small molecules. Therefore, it seemed appropriate to demonstrate the utility of this screening technique using likely real-world conditions. The β−cyclodextrin:nortestosterone 1:1 complex was observed and confirmed using exact mass, showing that complexation on a timescale suitable for the reactive DESI host:guest interaction can occur. A representative spectrum is shown in Figure 5-3.

Following this proof-of-principle experiment, the receptor screening analysis was performed for all twelve potential guest compounds illustrated in Figure 5-1 using the reactive DESI setup described in the experimental section. The sample surface which was mounted on an x-y-z-stage was manually moved in the x-direction (see Figure 5-2) for spatial guest selection, and blank areas between deposited guests were used for blanks. No carryover problems were
experienced. The guests G2, G3, G8, and G12 were detected at \( m/z \) ratios corresponding to theoretical 1:1 complexes with the sodiated \( \beta \)-cyclodextrin host. None of the other eight guests exhibited any evidence of complexation via reactive DESI-TOF-MS. Representative spectra are shown in Figure 5-4 and summarized results are presented in Table 5-1. Sample preparation took about ten minutes, but an additional thirty minutes of drying time were required. Instrumental acquisition time was only twelve minutes including sampling of the blanks.

A concern with this experimental design was that the solvent system could be responsible for false negative results as several of the guests are only sparingly soluble in water, the primary component of the electrosprayed droplets of the \( \beta \)-cyclodextrin solution by the time they reach the deposition surface. One of the major benefits of DESI, its ambient nature, was exploited to rapidly explore this issue. The only alteration necessary to explore the effect of the solvent system was replacing the original spray solution with one containing a higher percentage of methanol. This was done at the end of the screen adding fourteen minutes to the acquisition time, but again only G2, G3, G8, and G12 were detected as complexes.

**Electrospray Ionization Receptor Screening**

For validation of the DESI screen, the twelve guests were screened using ESI, which is an accepted ionization technique for this type of work, in methanol:water solvent. Under these conditions, compounds G2, G5, G7, G8, G9 and G10 were observed to form detectable 1:1 complexes, confirmed via accurate mass calculations, while the other five guests showed no evidence of complexation. Figure 5-5 shows representative mass spectra for analysis of G2 which complexed and G4 which did not for comparison, and results of the ESI screen are summarized in Table 5-2. Similar relative intensities between the sodiated cyclodextrin and the sodiated complex using both DESI and ESI were noted. Again, solvent system effects were a
concern, so the screen was repeated using only methanol for dissolution. Similar results evidencing only marginal changes in signal intensity were obtained.

The entire twelve compound screen took fifty-one minutes of acquisition time, including blank injections, and approximately ten minutes of sample preparation time resulting in an analysis time of sixty-one minutes excluding data analysis. Changing the solvent system required preparation of new samples and repetition of the entire screen, doubling the total analysis time. Although the total analysis time for the DESI screen was significantly longer than for the ESI analysis, this is solely due to the drying time for the deposited guests which does not contribute to either labor or instrument time. Appropriate automation and efficient planning in terms of running screening analyses during drying time for subsequent screens would yield a considerable improvement in analysis time over the analogous ESI experiment.

Comparison of the results obtained using DESI and ESI highlighted significant disparities, challenging the validity of the reactive DESI screen. Both techniques resulted in complex detection for two of the guests, G2 and G8, and four guests, G1, G4, G6, and G11, were not detected in complex form using either technique. Conflicting results were obtained for the six remaining guests. Compounds G3 and G12 were detected as complexes by DESI only, and the other four guests were only observed to complex using ESI. Electrospray ionization is known to be prone to artifacts, nonspecific aggregates formed during the electrospray process and consequently detected as adducts. This is a significant issue in supramolecular receptor screening where artifact formation can potentially lead to identification of false positive receptor candidates, and it may provide a potential explanation for the conflicting results. Initially, maltohexaose, a linear sugar composed of six glucopyranose units, was substituted for the β-cyclodextrin and another ESI screen performed to identify nonspecific complex formation
following the strategy of Bakhtiar and Hop\textsuperscript{144}. This strategy, however, yielded inconclusive information and highlighted the need for a more rigorous validation approach.

**Proton Nuclear Magnetic Resonance Spectroscopic Screening**

ESI has been shown to be representative of solution-phase equilibria in some circumstances,\textsuperscript{76,77} but the relationship between ESI spectra and solution-phase chemistry remains under scrutiny. A recent study investigating the influence of shrinking electrospray droplets on chemical equilibria reported observation of a significant effect on the equilibrium for one fluorescent dye pair, yet no apparent effect on the monomer-dimer equilibrium for a different fluorescent species.\textsuperscript{162} Consequently, the host and guests previously described were analyzed using solution-phase \textsuperscript{1}H NMR spectroscopy to determine which of the ionization techniques yielded the results most consistent with solution-phase behavior. Conditions used for the NMR screen are delineated in the experimental section. The experiments were conducted in deuterium oxide, consistent with the composition of the DESI droplets upon interaction with the surface, as deuterated methanol gives rise to an interferent peak within the spectral range of interest. Acquisition of each spectrum took about five minutes, so one hour was required for the entire screen, omitting sample preparation and data analysis which took approximately one more hour.

Previous studies have shown that the \textsuperscript{1}H NMR signals corresponding to the \(\beta\)-cyclodextrin cavity protons exhibit significant upfield shift upon inclusion of a guest molecule into the cavity.\textsuperscript{163,164} Proton NMR spectra for \(\beta\)-cyclodextrin and each of the twelve guests were obtained at the concentration ranges previously described. Proton assignments were made with the aid of COSY data, and the labeling convention is detailed in Figure 5-6. Representative spectra are presented as Figure 5-7. NMR spectroscopy revealed evidence, in the form of a spectral shift of
the signals corresponding to the H3’, H5’, and to some extent H6’ protons of β-cyclodextrin, of true inclusion complex formation for the guests G2, G3, G8, and G12. The H3’, H5’, and H6’ protons are the protons which line the surface of the hydrophobic cavity of the toroidal host, and therefore would be the protons experiencing environmental change in the event of true inclusion of a guest. No evidence of inclusion of the other eight potential guests was observed. As a secondary validation technique for the evidence of inclusion complexation between β-cyclodextrin and the guests under investigation, 2D NOESY was used to determine complex conformation. Proton assignments for the acetanilide guest are shown in Figure 5-8. An expanded view of the NOESY data obtained for the β-cyclodextrin:acetanilide complex is presented as Figure 5-9. NOESY data indicated interaction between the host cavity protons and the ortho and meta protons on the acetanilide benzene ring, which revealed the complex conformation illustrated in Figure 5-10. It was expected that the aromatic portion of the acetanilide would be encapsulated upon complexation as it is the most hydrophobic region of the guest, so the 2D NOESY derived complex conformation supported the predicted complex, and validated the simpler 1D approach to inclusion complex determination which was used to screen the twelve potential guests.

Solution-phase NMR spectroscopic screening yielded identical results to those obtained via DESI. The four guests detected as 1:1 complexes in the DESI screen were validated as included guests in solution and the eight remaining guests which were not detected as complexes via DESI showed no evidence of inclusion in the NMR experiments. Conversely, the ESI screen proved erroneous for 50% of the guests, resulting in the detection of two false negatives and four false positives. A summary of results obtained for the three screening methods is presented as Table 5-3.
The false positives were thought to be attributable to the aforementioned nonspecific adduct formation commonly associated with ESI. Computational chemistry was used to investigate this hypothesis and is discussed in detail in the following section. Determination of the two false negatives was more difficult to explain. Interestingly, the two guests detected using NMR and DESI but not ESI exhibited the smallest upfield shifts in the NMR experiments. Assuming the magnitude of the signal shift is related to the extent of complexation, an assumption for which there is some precedent, this would mean that these two guests are the least thermodynamically favorable. The organic methanol solvent is in competition with the guests for inclusion in the hydrophobic host cavity, and in ESI is present in higher concentration at the time of host:guest interaction than in DESI. Consequently, for weakly binding guests, the methanol could preferentially move into the cavity, preventing inclusion of the weaker guests during the DESI screen. As previously discussed, this problem would be minimized using reactive DESI. The droplets interacting with the guest compounds would be predominantly aqueous, a substantial fraction of the more volatile organic phase having evaporated during the electrospray process.

The NMR screen showed that reactive DESI is less prone to adduct formation resulting in the detection of false positives in the screening of supramolecular complexes than ESI. Additionally, since all four complexes determined via NMR were identified in the DESI screen, there is no evidence to suggest that complexation during the DESI event is purely kinetically-driven. In fact, if the extent of NMR signal shift is taken as representative of the extent of binding, it appears that the DESI screen spectral intensities may even be consistent with relative binding constants.
Computational Chemistry

To investigate the theory that the four false positive inclusion complexes detected solely using the ESI screen were nonspecific adducts of guest exterior to the host cavity, structures of the twelve guests were optimized using the theoretical calculation method previously outlined. The host compound, β-cyclodextrin, has known dimensions, and the cavity diameter at its widest point is only 6.5 Angstroms (see Fig. 5-11). Since the potential guests were selected only on the basis of potentially appropriate molecular weight and functional groups for β-cyclodextrin inclusion, optimized structures and specific molecular dimensions were desirable to determine whether the screened compounds were actually viable inclusion guests. The calculations revealed that eleven of the guests could theoretically adopt conformations suitable for at least partial encapsulation by the host. Cyclam (G5), however, which has a relatively rigid cyclic structure, cannot fit into the β-cyclodextrin cavity; its optimized structure, with the corresponding dimensions, is presented as Figure 5-12. Repulsion between the hydrogen atoms on the inside of the ring hinders the molecule from adopting a narrower conformation for inclusion. The cyclam guest exceeds the internal cavity diameter of β-cyclodextrin in two dimensions; therefore, it is only capable of forming a nonspecific complex exterior to the cavity and complex detection must be due to nonspecific artifact formation. The β-cyclodextrin:cyclam complex was only detected in the ESI screen experiment and not during either the DESI or the NMR screens, providing evidence for the formation of nonspecific adducts resulting in false positives using ESI, but no evidence to suggest that DESI suffers from a similar drawback in this type of application.

Conclusions

Initially, evidence of complexation between sprayed β-cyclodextrin and deposited nortestosterone steroid, a member of a known family of steroid guests for the cyclodextrin host,
confirmed that inclusion complexation on a suitable timescale for the DESI heterogeneous ion-molecule reaction is possible. A subsequent screening experiment for inclusion of a group of twelve deposited potential guest compounds by the same sprayed host yielded four positive and eight negative results. The appearance of an ion peak at the exact m/z corresponding to the protonated or sodiated complex following the interaction was necessary for a positive result, while absence of a peak at the expected m/z yielded a negative result. The guests observed to complex during the reactive DESI event were three anilide compounds and caffeine.

An analogous screen using ESI, which has been widely reported for supramolecular applications, was conducted. Solutions of pre-mixed host and an individual guest were electrosprayed and again a previously absent peak at the exact m/z of a complex was taken as a positive result. The ESI screen identified six complexes, only two of which had previously been observed using DESI-MS. Disparities between the ESI and DESI screens highlighted the need for further validation, as ESI is known for a tendency to form nonspecific adducts which can result in false positives in supramolecular applications.

Solution-phase validation via NMR was used to assess which of the two ionization techniques yielded more representative data. Upfield shift of the chemical shift values of NMR signals corresponding to the β-cyclodextrin protons known to comprise the surface of the hydrophobic cavity upon guest addition was used to confirm true inclusion complexation of four of the potential guest molecules. A lack of shift of the host signals in the presence of guest indicated that inclusion of the guest into the hydrophobic host cavity was not detected and was recorded as a negative result. Two dimensional NMR techniques were used to confirm guest inclusion and conformation of detected complexes. The NMR screen yielded identical positive
and negative results to the DESI screen, confirming the technique as superior to ESI for supramolecular applications.

The two weaker binders of the four guests observed as inclusion complexes by DESI and NMR were not detected in complex form using ESI. This suggests that ESI may not be as sensitive as reactive DESI for supramolecular complex screening. Further investigation is necessary to corroborate this claim. Computational chemistry helped confirm that ESI is more prone to the formation of nonspecific artifacts detected as false positives in this type of application than reactive DESI.

The utility of reactive DESI-MS for rapid supramolecular complex receptor screening has been demonstrated and the technique has been compared to ESI-MS, an accepted tool for supramolecular mass spectrometry. Comparison between results obtained via the DESI and ESI screens and solution-phase NMR data revealed the superiority of DESI for supramolecular complex guest screening. Reactive DESI has been confirmed as a promising useful tool for supramolecular mass spectrometry.
Table 5-1. Absolute intensities ($x10^4$ counts) and ion type observed with the DESI-TOF-MS screen in methanol:water solvent

<table>
<thead>
<tr>
<th>Guest</th>
<th>[M+H]$^+$</th>
<th>[M+Na]$^+$</th>
<th>[CD+M+H]$^+$</th>
<th>[CD+M+Na]$^+$</th>
<th>[CD+G+2H]$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G2</td>
<td>0.35</td>
<td>6.6</td>
<td>—</td>
<td>0.77</td>
<td>—</td>
</tr>
<tr>
<td>G3</td>
<td>71</td>
<td>6.0</td>
<td>—</td>
<td>0.12</td>
<td>—</td>
</tr>
<tr>
<td>G4</td>
<td>—</td>
<td>—</td>
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<tr>
<td>G5</td>
<td>5.0</td>
<td>2.0</td>
<td>—</td>
<td>—</td>
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<tr>
<td>G6</td>
<td>—</td>
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<tr>
<td>G7</td>
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<tr>
<td>G8</td>
<td>1.7</td>
<td>50</td>
<td>—</td>
<td>0.20</td>
<td>—</td>
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<tr>
<td>G9</td>
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<td>—</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>G10</td>
<td>1.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>G11</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>G12</td>
<td>8.0</td>
<td>4.0</td>
<td>—</td>
<td>0.28</td>
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</tbody>
</table>
Table 5-2. Absolute intensities ($\times 10^4$ counts) and ion type observed with the ESI-TOF screen in methanol:water solvent.

<table>
<thead>
<tr>
<th>Guest</th>
<th>[G+H]$^+$</th>
<th>[G+Na]$^+$</th>
<th>[CD+G+H]$^+$</th>
<th>[CD+G+Na]$^+$</th>
<th>[CD+G+2H]$^{2+}$</th>
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</thead>
<tbody>
<tr>
<td>G1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>G2</td>
<td>120</td>
<td>16</td>
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<tr>
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<td>50</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>G4</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>G5</td>
<td>180</td>
<td>12</td>
<td>—</td>
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<td>2.3</td>
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<td>0.10</td>
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<tr>
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<td>53</td>
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<tr>
<td>G12</td>
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<td>0.79</td>
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</table>
Table 5-3. Potential guests detected as complexes using DESI-MS, ESI-MS, and 1H NMR spectroscopy.

<table>
<thead>
<tr>
<th>Guest</th>
<th>DESI-MS</th>
<th>ESI-MS</th>
<th>$^1$H NMR</th>
</tr>
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<tbody>
<tr>
<td>G1</td>
<td>—</td>
<td>—</td>
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<tr>
<td>G2</td>
<td>Complex detected</td>
<td>Complex detected</td>
<td>Complex detected</td>
</tr>
<tr>
<td>G3</td>
<td>Complex detected</td>
<td>—</td>
<td>Complex detected</td>
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<tr>
<td>G4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G5</td>
<td>—</td>
<td>Complex detected</td>
<td>—</td>
</tr>
<tr>
<td>G6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G7</td>
<td>—</td>
<td>Complex detected</td>
<td>—</td>
</tr>
<tr>
<td>G8</td>
<td>Complex detected</td>
<td>Complex detected</td>
<td>Complex detected</td>
</tr>
<tr>
<td>G9</td>
<td>—</td>
<td>Complex detected</td>
<td>—</td>
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<tr>
<td>G10</td>
<td>—</td>
<td>Complex detected</td>
<td>—</td>
</tr>
<tr>
<td>G11</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G12</td>
<td>Complex detected</td>
<td>—</td>
<td>Complex detected</td>
</tr>
</tbody>
</table>
Figure 5-1. Structures of the twelve screened guest compounds: G1 is acenaphthalene (monoisotopic mass 152.0626 Da), G2 is acetanilide (135.0684 Da), G3 is caffeine (194.0804 Da), G4 is 4-chloro-3-nitrobenzonitrile (181.9883 Da), G5 is cyclam (200.2001 Da), G6 is 1,3-dinitronaphthalene (218.0328 Da), G7 is diphenylglyoxime (240.0899 Da), G8 is formanilide (121.0528 Da), G9 is guanosine (283.0917 Da), G10 is L-arginine (174.1117 Da), G11 is 2-methylnaphthalene (142.0783 Da), and G12 is 4'-nitroacetanilide (180.0535 Da).
Figure 5-2. Receptor screen DESI-MS design showing spray head, sample surface, desolvation capillary, and capillary adapter. The host solution was 0.05 mM β-cyclodextrin solution in 50:50 methanol:water containing 0.5 mg/mL of NaCl and the twelve guests G1-G12 were deposited on a sandblasted glass slide for spectral acquisition.

Figure 5-3. Reactive DESI-TOF spectrum of β-cyclodextrin sprayed onto a deposited sample of nortestosterone. The inset shows a close-up view of the mass range of interest.
Figure 5-4. Reactive DESI-TOF mass spectra showing data for the guests a) G2, b) G7, and c) G8. Insets show close-up views of the complex ion mass ranges of interest.
Figure 5-5. Electrospray ionization TOF mass spectra for the β-cyclodextrin(CD) guest screen in methanol shown for potential guest compounds a) G2 and b) G4 which yielded positive and negative complexation data, respectively.

Figure 5-6. Structure of the glucopyranose monomer of β-cyclodextrin showing the labels used to represent the protons observed via NMR spectroscopy.
Figure 5-7. Proton NMR spectra showing the region 3.5-4 ppm chemical shift for a) lone \( \beta \)-cyclodextrin and b) \( \beta \)-cyclodextrin in the presence of acetanilide (G2). The signals corresponding to H3’, H5’, and to some extent H6’, exhibit an upfield shift consistent with inclusion of a guest upon the addition of acetanilide.
Figure 5-8. Structure of the acetanilide guest, G2, showing the observed chemical shift values for the NMR spectral peaks corresponding to the stable protons.

Figure 5-9. Nuclear Overhauser effect spectrum of \( \beta \)-cyclodextrin in the presence of acetanilide. Signal at the intersection of a line corresponding to the acetanilide with a line corresponding to the host indicates correlation. The signal diameter is indicative of the strength of the through-space interactions.

Figure 5-10. Conformation of the \( \beta \)-cyclodextrin:acetanilide inclusion complex derived using 2D NOESY.
Figure 5-11. Representation of the toroidal β-cyclodextrin host showing cavity diameter.

Figure 5-12. Optimized structure and corresponding molecular dimensions of cyclam, the G5 guest which was observed to complex using ESI supramolecular complex screening only.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Mass spectrometry, currently experiencing an explosion of applications, has become an essential tool in the development of the field of supramolecular chemistry. Its sensitivity, specificity, and ability to obtain information about the inherent supramolecular chemistry of systems in the absence of solvent interferences make it a powerful analytical technique with the potential to fuel rapid advances in this area. The research presented here was conducted to contribute to the evolution of supramolecular mass spectrometry as a technique which can be diversified to address very different needs arising from the growth of the multidisciplinary field of supramolecular chemistry. The major unifying theme is that specific instrumental characteristics and capabilities can be exploited to yield useful information beyond elemental composition or structural elucidation, with a focus on expanding the scope of supramolecular mass spectrometry.

Despite advances in mass spectrometric instrumentation and software for instrumental control and data analysis, the efficient incorporation of mass spectrometry into many labs is limited by the tendency to use mass spectrometers as workhorses where generation of large amounts of data is perceived as more desirable than the acquisition of useful and meaningful information. For some applications, this may be an appropriate use of the techniques comprising mass spectrometry as a whole, but in an economically-driven society, this poses several problems. Primarily, valuable resources of instrument and scientist time can be wasted through poor experimental design. Additional analysis time for superfluous tandem mass spectrometry stages, and time spent wading through unnecessary data acquired due to inefficient planning are a few common examples of laboratory waste. Additional concerns are data handling and storage. Generation of large amounts of data increases the time and resources needed to extract useful
information, but also creates pressure in terms of hardware and software laboratory requirements. Many labs, particularly those in biomedical areas, would benefit significantly in terms of efficiency and productivity by employing a trained and experienced instrumentalist for experimental design and efficient utilization of complex analytical instrumentation.

This research was initially directed toward the characterization of novel designed synthetic receptors for alkali-metal cations based on naturally-occurring phloroglucinol using accepted supramolecular mass spectrometric techniques. Relative binding constants were determined for the homodimers of two phloroglucinol derivatives bound by Li\(^+\), Na\(^+\), K\(^+\), and NH\(_4\)^+ using an SCID-ESI dissociation curve approach. The preferential binding order for 2,4,6-tribenzoylphloroglucinol (phloro 1) was determined to be Li\(^+\)≥Na\(^+\)>K\(^+\)>NH\(_4\)^+ and for 2-4-6-(3,5-dimethyl)tribenzoylphloroglucinol (phloro 2) was revealed as Na\(^+\)>K\(^+\)>Li\(^+\)>NH\(_4\)^+. This size-dependent binding for phloro 1 but not phloro 2 supported the hypothesis that phloro 1 should be capable of forming a cage-type dimer encapsulating an alkali metal cation in the intermolecular cavity, while the additional methyl groups on the benzyol substituents of phloro 2 should sterically hinder that dimeric conformation. No direct conformational information was obtained via mass spectrometry, so UV/vis absorbance spectroscopy was used to probe dimeric conformation in solution. Chromic shifts corresponding to dimerization provided further evidence supporting the hypothesis. In this project, mass spectrometry and solution-phase optical spectroscopy yielded complementary information, but the characterization might have been achievable solely via mass spectrometric techniques, had ion mobility spectrometry (IMS) been available for determination of the dimeric cross-sectional areas.

Characterization of the phloroglucinol derivatives as designed \(\pi\)-receptors benefits supramolecular chemistry in several ways. A significant step towards providing a new synthetic
receptor for encapsulation of alkali metal cations, which has implications in applications like cation scavenging in waste cleanup and active transport mimicry, has been made. Additionally, the effect of the addition of small substituents, like methyl groups, upon the nature of noncovalent $\pi$-system interactions has been shown to be dramatic. This fact highlights the need for more in-depth investigations into the effects on chemical behavior of substituent modification of arene systems, and could ultimately impact synthetic receptor design strategies based on molecular recognition principles. On a more general level, the complementary nature of solution-phase optical spectroscopic techniques and mass spectrometry was demonstrated for supramolecular applications.

A useful addition to the work presented here would be the implementation of an IMS study to explore the dimeric conformations. Following measurement of the cross-sectional area of the homodimers of phloro 1 and phloro 2 in the presence of each of the four cations, comparison between data obtained for each host in the presence of the same guest should enable determination of the dimeric conformations. However, a vital future step for this area of research is expansion of the study to a larger group of phloroglucinol derivative hosts substituted with different heteratoms and functional groups. Comparison of dimeric stability using the SCID dissociation curve approach, for a much greater number of host compounds could significantly benefit the field of synthetic design based on supramolecular recognition principles. The group would have to be large enough to allow the determination of statistically relevant patterns of behavior related to specific types of substitution. If patterns and trends in relative complex stability were observed to correlate with substituent characteristics, this could significantly impact molecular-recognition based designed chemical synthesis, resulting in simplification of the therapeutic drug design process.
Dissociation pathways were proposed for ESI-CID-MS analysis of the cation-bound phloroglucinol derivative dimers. With the aid of the simple 2-hydroxybenzophenone compound, a mechanism for the initial dissociation step was derived, and the more complicated C₃ phloroglucinol derivatives were observed to adhere to this mechanism. The ammonium adducted phloroglucinol derivatives demonstrated slight differences in fragmentation behavior from that observed with the other cations employed. Ion trap mass spectrometry was used for unambiguous determination of the precursor-product ion relationships, and SCID tandem mass spectrometry using one of two high-resolution mass analyzers provided accurate mass measurements for unambiguous determination of elemental composition. The two useful instrumental capabilities of tandem mass spectrometry and high-resolution mass analysis were incorporated into the analysis to maximize the usefulness of the mass spectral data acquired. The growing pharmaceutical relevance of the phloroglucinol derivative family provided a need for this information, as LC-MS and LC-MS/MS analytical methods for determination of this type of compound in various matrices are under rapid development. The most common interface between LC and MS is ESI, and alkali-metal adducts are frequently observed via ESI, so fragmentation information for alkali-metal adducts is vital. The wealth of applications for phloroglucinol derivatives outlined in Chapter 3 renders dissociation pathway information specific to alkali-metal adducts of phloroglucinol derivatives of substantial significance. The contributions of a proposed mechanism for the initial fragmentation step and dissociation pathways for the alkali-metal cation-bound dimers and monomeric adducts have been made, but future application-specific method development could benefit from further insight.

Now that the first mechanism for the initial dissociation of alkali-metal cation-adducted phloroglucinol derivatives has been proposed, the next logical step would involve a detailed
mechanistic study. Exchange of one or all of the $^1$H atoms of the hydroxyl groups with $^2$H followed by an analogous fragmentation study would help to clarify the location of the hydrogen which is abstracted as part of the loss of the benzene or dimethylated benzene ring from the monomeric phloroglucinol derivative adducts. Additionally, in a separate study, strategic replacement of successive $^{12}$C atoms with $^{13}$C atoms could be used to investigate the structural reconfiguration observed via carbon dioxide neutral loss from the monomeric sodium and lithium cation adducts of the two host molecules. Both these experiments would provide detailed information about the dissociation of phloroglucinol derivatives which could be valuable for LC-MS/MS method development for the quantitative and qualitative analysis of this compound class.

An unrelated experiment could help to determine one missing and vital piece of information which could potentially yield insight into the cation-$\pi$ interactions which are involved in ESI adduction by metal cations. The location of the cation, although clearly in the core phloroglucinol region, was not determined by the studies comprising this body of work, but a possible strategy to resolve this issue has been devised. A combination of computational chemistry and gas-phase infrared ion spectroscopy could yield the desired information. Theoretical geometry optimization of the phloroglucinol derivative hosts with an alkali-metal cation in one of a select few positions would enable determination of the adduct conformations corresponding to the lowest energy structures. Theoretical vibrational spectra for each structure could then be obtained. Gas-phase ion spectroscopy using an infrared laser for multiple photon dissociation of the electrosprayed alkali metal cation-adducted phloroglucinol derivatives would provide experimental vibrational spectra. Comparison between the theoretical and experimental
vibrational spectra could confirm the location of the cation. This is a project for which our group has both the resources and the expertise.

Prior to applying the recently-developed ambient ionization technique DESI to the analysis of supramolecular inclusion complexes, a home-built DESI source was interfaced with an Agilent TOF-MS instrument. The source was originally designed for a Bruker FTICR-MS instrument, so several modifications were necessary to accommodate the unusual geometry of the Agilent source housing. Commercial DESI sources have yet to be successfully interfaced with the Agilent TOF instruments, so this work provides a basic approach for possessors of Agilent TOF mass spectrometers aiming to incorporate the DESI technique into their work. The superior scan rate of TOF-MS over FTICR-MS was observed to be useful for DESI parameter optimization, demonstrating a significant advantage of TOF mass analyzers over FTICR mass analyzers for DESI analyses. Successful configuration of the DESI-TOF-MS system was demonstrated using Rhodamine 6G and the protein cytochrome c.

The next step in characterization of the DESI-TOF-MS configuration described in this dissertation is the demonstration of its performance for a diverse range of compound classes. An optimization study resulting in the determination of optimal conditions and surface chemistry for structurally-related compounds such as differentially substituted benzenes or carbonyls could provide useful insight into the effect of different functional groups on the DESI process, and would aid the wider implementation of the DESI technique. Additionally, the high mass range capabilities of the TOF-MS make it very appropriate for the study of large organometallics, which have not been extensively analyzed by DESI, so a future collaborative project with a prolific inorganic synthetic chemist could prove extremely successful. Many large metal
complexes degrade in the presence of acid and DESI is an ionization technique that does not necessarily require acid addition.

The potential of the DESI source interfaced with the FTICR-MS instrument was not thoroughly explored, and recent successful configuration of DESI-FTICR-MS by several groups suggests that the challenges encountered are not insurmountable. The system should be revisited and the optimization procedure exhaustively performed using a standard that is not as rapidly ablated as Rhodamine 6G. During the cyclodextrin work, it was observed that both caffeine and acetanilide yield a relatively long-lived and stable DESI signal; therefore, they could prove to be appropriate analytes for this purpose. Successful integration of the DESI source with the University of Florida FTICR-MS could result in some very exciting work involving infrared dissociation of trapped ions produced using the DESI technique. To date, neither DESI followed by infrared multiple photon dissociation nor DESI followed by gas-phase ion spectroscopy have been reported. Since DESI-FTICR-MS is very desirable for proteomics applications, protein conformation studies using this setup seem a logical and attainable aim.

The reactive DESI technique was subsequently used for the development of a rapid screening technology for supramolecular receptors for inclusion by β-cyclodextrin hosts. Following deposition of several potential guest compounds onto a desorption surface, successful pickup of some of the guests by the host, observed via detection of an ion associated with the specific host:guest complexes, during the DESI event was achieved. An analogous screen using electrospray ionization resulted in the detection of complexes of different guests to those observed to complex via DESI, and illustrated the need for solution-phase validation of the DESI technique for this application. Proton NMR spectroscopic techniques yielded data corresponding to the DESI results and invalidating the ESI results, and computational chemistry showed that
some of the complexes detected via ESI could only be nonspecific adducts. Reactive DESI was confirmed as a valuable analytical technique for supramolecular screening, providing solution-phase-consistent data, and was demonstrated as superior to ESI for the screening of guests for \( \beta \)-cyclodextrin inclusion complexes.

Some additional information would greatly enhance the diversity of potential analytical applications for this technique. Consequently, some suggestions for future experiments are delineated here. The molecular recognition interaction between sprayed \( \beta \)-cyclodextrin and nortestosterone was initially employed to demonstrate that inclusion complexation can occur on the DESI timescale. An attempt at inclusion complexation of several other hormones was made concurrently, but proved inconclusive. The hormones estriol, estrone, dehydrotestosterone, testosterone, and epitestosterone were deposited, but no evidence of desorption was observed. It remains unclear, however, whether the age of the compounds, which had been stored for several years under unknown conditions, was responsible for the lack of signal obtained with these hormones. The fact that no uncomplexed signal was observed for any of these analytes suggests the possibility that unfavorable storage conditions could have led to degradation. It would be extremely useful, however, to determine if this were the case using another ionization technique.

A comprehensive analysis of the inclusion complexation of new samples of these hormones, particularly the testosterones, using a variety of surface chemistries and geometric configurations would be a valuable addition to the data presented here. To increase the chances of successful complexation, a derivatized \( \beta \)-cyclodextrin such as hydroxypropyl-\( \beta \)-cyclodextrin could be used as the spray reagent. Comparison of the complexation behavior of the related guest molecules would be extremely useful both to determination of the scope of this specific technique and to characterization of the capabilities and limitations of reactive DESI in general.
Real-world matrix effects on reactive DESI for supramolecular complexes have yet to be explored. Development of this type of analytical technique in the area of metabolite screening and toxicological applications would require molecular recognition interactions during the DESI process to be robust in biological matrices. A very preliminary experiment to gauge the adaptability of the technology to this type of work would be a simple screen using β-cyclodextrin sprayed at deposited spots of various compositions. Since nortestosterone is known to complex under reactive DESI conditions, solutions of urine, blood plasma, and serum spiked with 2 mg/mL nortestosterone would be appropriate for initial tests. The previously-determined water:methanol testosterone solution would be a necessary control to ensure favorable conditions. Signal corresponding to therapeutic drugs has been obtained using traditional DESI for all three of the suggested matrices, as noted in Chapter 4.

The supramolecular complex screening work presented here has evident potential in the development of high-throughput screening experiments for lead optimization in drug discovery applications. The demonstrated benefits, and the simplicity, rapidity, and potential for automation, confer upon the described technology inherent analytical utility in this area. Prior to expansion into a marketable technique for this type of application, however, several important steps are necessary.

In order to demonstrate the applicability of the technique to real-world receptor substrate chemistry, molecular recognition interactions between a known receptor and substrate must be determined to be specific. An experiment incorporating the receptor region of some well-characterized protein into the spray for interaction with two deposited substrates, one specific substrate for the receptor, and one similar substrate would address this issue. It would also
demonstrate the utility of the technique for systems beyond the small molecule inclusion complex model system, which is essential to its development in biomedical applications.

Following this, the logical approach would be a systematic study of one receptor with a family of known substrates. Subsequently, potential for different types of receptor molecules would have to be investigated.

An additional step involves the determination of the effect of surface chemistry upon the molecular recognition interactions exploited in reactive DESI. To design and implement a screen that would be competitive with a 96-well plate optical spectroscopic assay, for example, the design of a specialized deposition surface would have to be carefully considered. An experiment observing complexation between sprayed β-cyclodextrin and acetanilide on a variety of different surfaces including Teflon, polymethylmethacrylate, and porous silicon is essential to enable the future fabrication of a miniature sample holder along the lines of a MALDI target or desorption ionization on porous silicon (DIOS) chip. These surfaces have the advantage of amenability to the development of a miniature tray with shallow wells drilled or etched onto the surface. The β-cyclodextrin and acetanilide system is suggested as the complex system which resulted in the most intense and stable complex ion signal.

Through careful experimental design and appropriate utilization of different types of mass spectrometric instrumentation, contributions to the development of advanced supramolecular mass spectrometry have been made. Several specific contributions have been detailed and an attempt to define their real-world relevance has been undertaken. The overall goal of this dissertation, however, was promotion of the idea of maximizing the usefulness and efficiency of mass spectrometry as an analytical technique for diverse supramolecular chemistry applications, through the rational implementation of appropriate and complementary instrumental capabilities.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Joanna Barbara was born and grew up in England, but moved to the United States with her husband and then 1-year old daughter to undertake an undergraduate degree in chemistry at the University of Florida in the spring of 2001. Graduating with her B.S. in 2003, and following the birth of her son, she remained at UF to continue her studies in graduate school, pursuing concurrently a non-thesis M.S and a Ph.D. in analytical chemistry. Following coursework completion, she began research in the area of supramolecular mass spectrometry under the joint direction of Dr. John Eyler and Dr. David Powell, and worked as a research assistant in the Mass Spectrometry Service Lab throughout that period. Upon graduation, Joanna will enter the industrial workforce in a metabolism research position with XenoTech in Lenexa, Kansas.