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GENERAL STUDIES IN MASS SPECTROMETRY. PART A: MATRIX ASSISTED LASER DESORPTION IONIZATION (MALDI) ANALYSIS OF OLIGONUCLEOTIDES PART B: ELECTROSPRAY IONIZATION (ESI) STUDIES OF METALLOPORPHYRINS

A Dissertation

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In partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by

Victor Vandell
B.S., Chicago State University, 1991
M.S., Rochester Institute of Technology, 1994
May, 2000
For Genita, Alec and Greg
".....and now I rise!"
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ABSTRACT

The application of mass spectrometry for the analysis of biomolecules and metalloporphyrins is the aim of the work presented within this document. Part A of this document addresses the use of Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) for the analysis of oligonucleotides. The goal of Part A is to develop a better understanding of the matrix/analyte relationship in the condensed phase required for MALDI analysis of oligonucleotides. Part A also focuses on the employment of novel co-matrices to help improve oligonucleotide mass resolution and ion abundance.

The objectives for Part B was to study the use of Electrospray Ionization (ESI) for the analysis of metalloporphyrins. The first half of Part B focuses on the characteristic behavior observed for each of the model porphyrin systems used in this study. The second half of Part B presents the data collected from the positive ion ESI-MS analysis of homogeneous and heterogeneous porphyrin solutions. The ion abundance of each of the porphyrins present in these solutions was monitored as a function of the ESI flow rate, porphyrin concentration, ESI needle voltage, and solvent polarity. These systematic investigations revealed the influence of these parameters on the ion abundance of one porphyrin relative to another porphyrin in a heterogeneous solution. Furthermore, certain experimental conditions have been observed to separately optimize the detection of each of the components of such mixtures.
PART A:
MATRIX ASSISTED LASER DESORPTION IONIZATION (MALDI)
ANALYSIS OF OLIGONUCLEOTIDES
CHAPTER 1. MASS SPECTROMETRY OF BIOMOLECULES: A GENERAL OVERVIEW

1.1 Introduction

Mass determination for large biomolecules is generally conducted using gel electrophoresis. This technique is time consuming and is often found to yield a mass accuracy variance of as much as 10%. The quick analysis time of mass spectrometry offers several attractive features for biomolecule analysis. By the early 1980's, the analysis of non-volatile, thermally labile bio-organic molecules greater than 10,000 daltons could be conducted using mass spectrometric techniques that utilize one of several newly developed ionization processes. Such ionization process included liquid secondary ion mass spectrometry (LSIMS) (Perreault 1994), thermospray (TS) (Blakley 1983), fast atom bombardment (FAB) (Caldwell 1989; Domon 1994; Kovacik 1994) or plasma desorption (PD) (Hunt 1981; Chait 1984). The biggest limitation with these ionization methods was their inability to ionize compounds of higher masses (>50,000 Da). Upper mass limitations for biomolecule mass analysis using FAB and PD were reported by the late 1980s to be 25,000 Da and 45,000 Da, respectively (Barber 1987; Jonsson 1989). The development of new ionization techniques in the late 1980s, like matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), finally enabled researchers to surpass the 50,000 Dalton mass limitation. Biomolecules upwards of 150 kDa have been analyzed using both the MALDI and ESI techniques (Karas 1988; Fenn 1989; Karas 1989). Some of the advantages of MALDI and ESI include: 1) the small amount of sample necessary to obtain a good signal; 2) the expedience of the procedure; and 3) the possibility of reaching mass ranges well above those that had been achieved by other desorption techniques. Such advantages have made MALDI and ESI the premier ionization methods for the mass spectrometric analysis of biomolecules.
The goal of this chapter is to discuss the general application of these techniques for the mass spectral analysis of biomolecules (e.g., proteins, peptides, and oligonucleotides). Table 1.1 summarizes the types of mass spectral applications used in the analysis of the different types of biomolecules mentioned above. For more detailed discussions on the various mass spectral ionization techniques please review the references provided herein (Senko 1994; Loo 1995).

1.2 Ionization Sources

1.2.1 Fast Atom Bombardment (FAB)

FAB was one of the first “productive” techniques employed in biomolecule analysis, specifically peptides and proteins. The FAB source is designed to bombard the sample, which has been mixed in a glycerol matrix, with high energy atoms (i.e. Ar). As a result, a continuous beam of ions is generated and either a quadrupole instrument or a double focusing sector instrument can be used for mass analysis (Rinehart 1982; Burlingame 1984). The FAB ionization process generally yields protonated, deprotonated and odd electron type ions, with little to no fragmentation of the analyte. Hence, FAB is a “soft” ionization process. The decreased fragmentation observed with FAB is extremely beneficial for spectral interpretation. The biggest limitation for FAB is it’s inability to generate ions routinely greater than 10,000 Da. The ionization efficiency of the FAB source decreases with increasing analyte mass. To date, FAB has proven to be quite successful in the analysis and structural determination of small biomolecules (< 5000 Da) (Cerny 1987).

1.2.2 Plasma Desorption (PD)

The application of PD to the analysis of large biomolecules was actually reported before FAB and was believed to be extremely promising (Senko 1994). This claim was based on the ionization of biomolecules in the mass range of 20 kDa to 40 kDa using PD. Ultimately of importance was the analysis of biomolecules over the 200 kDa range, which was well out of the range of FAB, but seemed hopeful for PD.
Table 1.1 Summary of mass spectrometry ionization sources and mass analyzers used to analyze various biomolecules.

<table>
<thead>
<tr>
<th>BIOMOLECULE</th>
<th>IONIZATION SOURCES</th>
<th>MASS ANALYZERS (commonly used)</th>
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<tr>
<td>Proteins/Peptides</td>
<td>PD</td>
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<td></td>
<td>FAB</td>
<td>Quadrupole, Sector</td>
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<td>MALDI</td>
<td>TOF, FT-MS</td>
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<td>Oligonucleotides</td>
<td>PD</td>
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<td>ESI</td>
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The ionization process in the PD source involves the bombardment of the sample, mixed in a nitrocellulose matrix, with fission products from Californium 252 which generates a plasma. Within this plasma the sample can be ionized via complicated collisional and charge transfer processes (McNeal 1982). A low energy pulsed ion beam is generated from this source making it difficult to couple to any mass analyzer other than a time-of-flight (TOF) mass analyzer (Senko 1994). The TOF mass analyzer is limited in mass range only by the detector being used. It was found that the TOF mass analyzer could be used to successfully analyze the high mass ions generated in the PD source. A major disadvantage with the TOF mass analyzer is its inherently poor resolving power. This inefficient resolution made exact mass determination extremely difficult. Compounding the poor resolution problems inherent to the TOF mass analyzer was the broad initial kinetic energy distribution for the ions as they are generated in the PD source (McNeal 1982).

Relative to the FAB source, the PD ionization source was found to work adequately at producing a large enough abundance of high mass molecular ions for...
sample analysis. The downside, however, was the extensive fragmentation that also results from the process. This event made spectral interpretation very difficult and the overall process not as practical as was anticipated. The nature of the source (i.e. use of high energy radioactive particles for ionization) also lowered the marketability of these instruments, and the use of PD was not widespread as a means of biomolecule analysis.

1.2.3 Matrix Assisted Laser Desorption Ionization (MALDI)

The introduction of MALDI for biomolecule analysis became the turning point in the analysis of large biomolecules. Initial reports of MALDI analysis of biomolecules over 100 kDa offered high expectations for this new ionization technique (Karas 1988; Karas 1989). The MALDI source generates ions from a sample, mixed with a “suitable” matrix, via laser energy deposition onto the sample. The desorption/ionization process governing the transition of large biopolymers from the condensed phase to the gas phase has not been fully elucidated. Many research groups have investigated the desorption/ionization process to formulate a better understanding of these mechanisms, which will help to increase the mass range of biomolecules that can be successfully ionized using this technique (Parr, 1992; Schneider 1993; Tang, 1993; Siegert 1996; Limbach 1998). A more detailed discussion of the overall process for MALDI will be addressed in Chapters 2-5.

The MALDI source typically generates both negative and positive ions and neutral molecules. The neutral molecules are in far abundance (ca. 1000 fold) relative to the negative and positive ions generated within the source (Johnson 1994). The MALDI source is a pulsed ion beam source typically coupled to the TOF mass analyzer. Recent developments in instrument design now allow for the MALDI source to be coupled to ion traps (Alexander 1993). The sensitivity of MALDI was found to be extremely high for biomolecule analysis and has been reported in the subferatomolar level (Tseng 1997). A higher efficiency for molecular ion production
in a MALDI source relative to FAB and PD has also been observed. MALDI typically yields spectra with predominately the molecular ion and little to no fragmentation, hence it too is labeled as a "soft" ionization process. The production of high molecular weight ions and subsequent analysis of these ions makes the MALDI-TOF combination a powerful tool for biomolecule analysis. While fragmentation still occurs in the MALDI source, the extent relative to FAB and PD is considerably lower.

MALDI sources are generally coupled to TOF instruments. Unfortunately, resolution for a TOF instrument is relatively poor as stated earlier which results in broad peaks. One of the contributors to peak broadening is cationization that occurs quite frequently in biomolecule analysis. The adduction of sodium and potassium can broaden a molecular peak out as much as 100 mass units. The poor resolving power of the TOF mass analyzer results in decreased mass accuracy. Some of the techniques currently employed to improve resolution in a MALDI experiment will be addressed in Chapters 4 and 5.

MALDI sources were later coupled to a Fourier transform ion cyclotron resonance mass spectrometer (FTICRMS) and successfully used to analyze moderately large (ca. 15 kDa) biomolecules (Loo 1995; Tseng 1997). These instruments are limited in mass range relative to the TOF instrument, but have excellent resolving power. The upper mass capability for FTICRMS instruments is about 20,000 Da, which limits the use of MALDI-FTICRMS for analysis of high mass biomolecules.

1.2.4 Electrospray Ionization (ESI)

The use of an ESI source in the analysis of biomolecules has offered even more advantages than MALDI. Early reports of ESI analysis of biomolecules demonstrated the successful ionization of large biomolecules up to 130 kDa (Fenn 1989). The ESI source ionizes "preformed ions" via an applied electrical potential to a needle from which the ions are subsequently sprayed. Both negative and positive
ions are formed in solution, but only one type of ion is sprayed from the needle tip depending on the polarity of the applied electrical potential. The ES ionization of biomolecules yields molecular ions with little to no fragmentation and is also labeled as a soft ionization technique. One of the advantages of the ESI source is that the analyte is sampled atmospherically. This feature allows the source to be coupled to liquid chromatographic or electrophoretic systems and sets the stage for LC-MS experiments (Senko 1994; Loo 1995). Due to the high sensitivity of the ESI source (ca. sub-micromolar levels) analysis of an effluent directly from a chromatographic column can be performed. The mechanism for ESI is still not fully understood. Ion production within an ESI source not only yields positive and negative ions (as is the case with FAB, PD, and MALDI) but also multiply charged ions.

Multiple charging results from the loss or addition of hydrogen atoms or metal atoms (e.g., potassium or sodium) resulting in a decreased mass-to-charge ratio \( (m/z) \) for the molecular ion of a large biomolecule. The multiple charging effect is advantageous because it allows for the analysis of large biomolecules using mass analyzers with low upper mass limits. The molecular weight of the biomolecule, if highly multiply charged, can be mass analyzed at a substantially lower mass than the singly charged molecular ion (e.g., actual molecular ion 100,000 Da and 50 charges yields an \( m/z \) value of 100,050/50 = 2000 Da). The disadvantage of multiple charging is that a spectrum has to be deconvoluted and thus spectral interpretation is complicated. Biomolecules have numerous acidic and basic sites, and multiple charging is quite often observed for ESI generated spectra. The extent of multiple charging of the biomolecule can be a direct function of the pH of the solution containing the analyte.

ESI sources are generally coupled with quadrupole instruments, FTICRMS instruments and sector instruments. Mass resolution for the latter two configurations is very good because these instruments have relatively high mass resolving power.
This feature results in better mass accuracy during an experiment. As a result of improved mass accuracy, structural elucidation and protein modification studies can be conducted with higher sensitivity, thus allowing for the successful analysis of extremely high molecular weight samples.

1.3 Mass Spectral Analysis of Biomolecules

1.3.1 Proteins and Peptides

The development of new ionization techniques in mass spectrometry allowed scientists, for the first time, to analyze large biomolecules as intact gas-phase ions. The analysis of large proteins and peptides were among the first mass spectral experiments conducted by researchers (Karas 1988; Caldwell 1989).

Protein and peptide samples are typically analyzed in positive-ion mode, but they can also be analyzed in the negative-ion mode as well. The abundance of negative and/or positive ions generated during the ionization of proteins and peptides is a function of the type of sample being analyzed in the experiment and the type of ionization source being utilized.

The structural elucidation of peptides and proteins can be performed using FAB, MALDI, or ESI. FAB analysis of peptides and proteins has been demonstrated with limited success due to the inherent mass limitations of this technique (Senko 1994). Both ESI and MALDI have proven to be the most effective methods for analysis of these types of compounds. There are some limitations, however, even for ESI and MALDI ionization sources. One important factor in the ESI ionization process is that the analyte has to be ionizable in solution. These ions will exist as stable singly or multiply charged gas-phase ions. Peptides and proteins are very stable as gas-phase ions with single or multiple charges, as evidenced by the abundant parent ions generated for proteins and peptides during ESI analysis (Fenn 1989; Loo 1995). MALDI generated ions for peptides and proteins also demonstrate this high ion stability. Multiple charging in MALDI experiments occurs to a much lesser extent.
than is observed for ESI analysis. The overall stability of peptides and proteins as charged ions in the gas phase enhances analysis of these molecules.

Tandem mass spectrometry (MS-MS) experiments are often performed to determine the primary amino acid sequence of peptides and proteins (Senko 1994). This goal can be accomplished using collisionally induced dissociation (CID) experiments to generate fragment ions that can then be isolated and mass analyzed to determine the parent ion from which they originated. The use of enzymes to selectively digest peptides and proteins are also employed in experiments designed to sequence these molecules (Loo 1995). The fragments resulting from an enzymatic digest can be analyzed in the mass spectrometer to determine the sequence of amino acids of the target molecule. Protein sequencing and identification of post-translational modifications of peptides can be determined using MALDI and ESI very effectively.

1.3.2 Oligonucleotides

Polymers of nucleotides, whether the monomer units are all the same nucleobase (homopolymer) or mixed nucleobases (heteropolymers), are called oligonucleotides. Naturally occurring oligonucleotides are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Mass spectral analysis of oligonucleotides was initially performed with FAB, with a satisfactory level of success (Cerny 1987). The application of MALDI and ESI for the analysis of oligonucleotides again proved to be a far more effective means of analysis for these compounds (Karas 1988; Chou 1999a). The ionization of oligonucleotides, and subsequent analysis in a mass spectrometer, began with dimers of nucleotides and has progressed to the ionization of oligomers up to 150 base units in length (Tang 1994; Tang 1997). Oligonucleotides are generally analyzed in the negative-ion mode.

Advances in the analysis of high molecular mass oligonucleotides have been much slower than those in the protein and peptide field. Mass spectral analysis of
these biomolecules is inherently more difficult because of the susceptibility of oligonucleotides towards fragmentation in the gas-phase (Zhu 1995; Tang 1997; Tang, 1997). The instability of gas-phase ions has made higher molecular mass analysis relatively limited to about 90,000 Da thus far due to the extensive fragmentation of oligonucleotides in the MALDI ionization source. On the other hand, the analysis of high molecular weight oligonucleotides using ESI has been much more successful with oligomers as large as 2 MDa being analysed by Smith et al. (Smith, 1990; Smith, 1991). Fragmentation of oligonucleotides in the MALDI source has been shown to be base dependent because certain nucleotide bases are more resistant to fragmentation than others (Parr, 1992; Tang, 1993; Wu, 1993). The base-dependent propensity towards fragmentation is discussed in greater detail in Chapter 3. Of all the nucleobases, thymine is the most resistant towards fragmentation. Homopolymers of thymidine are often used as model compounds when designing MS experiments for this class of compounds. Compounding the difficulties for oligonucleotide analysis is the adduction of alkali metal ions (e.g., sodium and potassium) to the phosphodiester backbone of the oligonucleotide (Cheng 1996). Oligonucleotides have a higher affinity for cationization than peptides and proteins because of their highly charged phosphodiester backbone, thus lowering the overall mass accuracy during a typical MALDI or ESI analysis. The effects of oligonucleotide cationization during the MALDI analysis of this class of biomolecules is discussed in more detail in Chapter 3 and 4.

Techniques designed, in general, to help decrease fragmentation of oligonucleotides during ESI and MALDI analysis include chemical modification of the oligomer (Kirpekar, 1995; Chait 1995) and the use of matrix additives (co-matrix) (Currie 1993; Pieles 1993). The latter topic will be addressed in Chapter 5.

Structural determination of large oligomers using MALDI and ESI can be accomplished readily by enzymatic cleavage of the oligomer and subsequent analysis
of the fragmentation products. Chemical modifications to the nucleobase and the sugar moiety of a nucleotide have also been shown to aid in structural elucidation studies of oligomers by increasing the stability of the gas-phase ions of the oligomer (Parr, 1992). CID studies of chemically modified oligonucleotides in conjunction with tandem MS-MS experiments are also performed to yield nucleobase sequencing information (Senko 1994; Loo 1995).

1.4 Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry: Background Review and Current Applications

1.4.1 The Parameters Governing Matrix-Assisted Laser Desorption/Ionization

Several important parameters which govern the mechanism of matrix-assisted laser desorption/ionization (MALDI) have been identified. Parameters like laser power, matrix-analyte crystal morphology, sample preparation and type of matrix are key factors governing the outcome of the MALDI experiment (i.e., high signal to noise, ion abundance and resolution). Recent studies from various laboratories have been directed at understanding how these parameters affect the desorption/ionization process, and what factors can be introduced to optimize these parameters. While the overall process is still not fully understood, MALDI has become one of the most commonly used techniques in the analysis of proteins and oligonucleotides.

1.4.2 Instrument Configuration

As stated earlier in this dissertation, the MALDI process involves the use of radiant energy by means of a laser onto the surface of a crystalline matrix-analyte complex spotted on a probe tip (Zhang 1997). The angle of incidence for lasers used in MALDI experiments is typically 60°. Samples are generally spotted on the probe surface as a small droplet (1-5 μm in diameter) and allowed to dry at atmospheric pressure. The probe is generally loaded into an instrument where the internal pressure is typically pumped down to $10^{-6}$ to $10^{-8}$ torr.
Ions are generated from the probe surface by photons, which employ mechanisms still not fully understood. Models proposed by others probing these mechanisms are discussed in upcoming sections. Ions generated by the laser pulse are extracted by high voltage lenses which direct the ion packets to a field-free drift region in the TOF instrument. This field free region, or time-of-flight tube, is typically 1.2-m in length, and allows the ions to fly towards a detector with mass-dependent velocities. Detection of ions is generally accomplished using an electron multiplier detector.

1.4.3 Time-of-Flight (TOF) Mass Spectrometry

Time-of-flight mass spectrometry is based on the measurement of the time necessary for ions to travel from the ionization source to the detector. More specifically, most instruments measure this flight time as a function of the time at which the laser fires and the time at which the detector registers ion current. In the TOF instrument, ions are generated by an energy transfer process from a pulsing laser. The ions generated from this process differ in mass and initial kinetic energies. These ions travel in ion packets normal to the plate surface from which they were generated with varying kinetic energies. All of the ions are extracted and accelerated to a constant kinetic energy via an applied electrical potential normal to the plate surface. At this point the velocity of the ions is now inversely proportional to the square root of the mass to-charge-ratio for individual ions as expressed in equation 1.1 where \( m \) is

\[
K = qV_s = \frac{mv^2}{2}
\]

mass in Daltons, \( V_s \) is the acceleration potential, \( q \) is the total charge, and \( v \) is the velocity of the ions. The ions are then allowed to drift through a field-free region toward the detector. The ions separate in the field-free region as a function of their
velocity before striking the detector. The relationship between flight-time \( t \) and mass-to-charge ratio is expressed in equation 1.2 where \( D \) is the drift tube length in meters and \( e \) is the charge in Coulombs. The mass resolution is defined in equation 1.3

\[
\text{Resolution} = \frac{m}{\Delta m} = \frac{t}{2\Delta t}
\]

where \( \Delta m \) is the difference in mass between the \( (A) \) and \( (A+1) \) peaks and \( \Delta t \) is the width of an ion packet of constant mass.

1.4.4 Instrument Design Improvements

Mass resolution is typically < 1000 for simple linear TOF mass spectrometers. Factors which contribute to poor resolution in TOF instruments are 1) ion production time, 2) initial ion velocity distributions and 3) the extraction time for ion packets leaving the plate surface (Hillenkamp 1991). Figure 1.1 is a layout of a standard MALDI-TOF instrument, with the ion flight path outlined, used in the studies presented in Chapters 4 and 5.

Improvements to instrument design have vastly increased the resolution for TOF spectrometers to ca. > 5000. This improved mass resolution also increases the mass accuracy for the instrument as well. These design improvements address the issue of initial ion velocity distributions, post source ion focusing, and ion injection/formation by employing techniques like time-delayed extraction (Bahr 1997), ion mirrors or reflectron technology (Hillenkamp 1991) and orthogonal injection (Chernushevich 1999), respectively. A review of these techniques can be found in a recent text by Cotter. (Cotter 1999).

1.4.5 Laser Contributions to the MALDI Process

Laser wavelength has been shown by several researchers to be a very instrumental factor for MALDI to be effective. Various wavelengths have been used
Figure 1.1 Diagram of basic MALDI Linear Time-of-Flight Spectrometer
in MALDI experiments. The most common wavelength utilized in MALDI is the 337 nm line from a nitrogen laser. The relationship between the laser and the sample/matrix is still not fully understood. It is understood, however, that one of the main criteria for the matrix being used in an experiment is that it absorb at the wavelength of the laser being used in the experiment. Other matrix criteria are that the matrix must be able to host the analyte within it's crystal lattice, and the matrix-analyte complex should crystallize upon evaporation of the solvent.

The importance of laser power to the MALDI process has been investigated by many research groups. The type of laser irradiation used in a MALDI experiment is extremely critical to the outcome of the experiment. The energy transfer process between the matrix and analyte during ion/molecule reactions stems from the energy input into the system by the laser. This energy can be input into the system by electronic excitation from a UV laser source or vibrational excitation from an IR laser source (Bencsura 1995; Bencsura 1997; Berkenkamp 1998). Demirev et al. compared the ion intensities of insulin collected from the desorption processes promoted by an excimer laser and a nitrogen laser (Demirev 1992). They reported that the amount of energy transferred to the sample as a function of the laser power was the important parameter in the desorption process as opposed to the laser power being emitted. Laser fluence threshold values were studied by Beavis in an effort to determine the minimal amount of laser power necessary to generate protein ions (Beavis 1992). Beavis also reported an observed dependence on the protein ion intensities as a function of the laser fluence. Further support of the importance of laser fluence in the MALDI process was offered by Riahl et al. who reported the observation of a laser fluence threshold plateau for the emission of ions (Riahl 1994). Beyond this plateau, ion emissions level off. Riahl also reported that below the fluence threshold, ions are not generated.
Quist et al. took a closer look at the laser ablation of a sample and found that a thermal component exists in the laser desorption process (Quist 1994). This component was found to be a function of laser fluence. This thermal component was also reported by Maechling (Maechling 1996). Quist also observed that some of the ions generated post laser depart from the surface of the probe at various angles relative to the probe surface. These finding suggest that the signal intensity for mass spectral analysis can be lost due to the way an ion leaves the surface of the probe. A radial component for ion ejection was observed by Zhang and Chait, but was found to be substantially less than the axial ion ejection component (Zhang 1997).

The importance of the focal point of the laser was also studied by Riahl (Riahl 1994) and Hillenkamp (Dreisewerd 1995). It was determined that ion emissions were not increased by increasing the illuminated area. A detailed account of laser contribution to the overall desorption process can be reviewed in the report submitted by Johnson (Johnson 1994). Laser influence on the MALDI process is a critical parameter to account for, and is one that is difficult to constantly vary when trying to achieve signal optimization. Thus, it is commonly a parameter that is set and held constant throughout an experiment. Typical settings for laser power and focal point are 70 w/cm² and 1 μm respectively. Optimization of this parameter can also be addressed sufficiently by empirically determining the fluence threshold necessary to obtain signal for the sample, and holding this value constant throughout the experiment.

1.4.6 Matrix/Analyte Chemical Interactions

Analyte/matrix crystal morphology and the chemical interactions between an analyte and the host matrix are also important parameters in the MALDI process. Heise and Yeung studied the qualities of several commonly used ultraviolet matrices and the degree of surface ablation caused by laser beam deposition on the crystals of
the matrices (Heise 1995). Differences were observed for the degree of crystal surface ablation relative to the type of matrix.

The ion/molecule reactions which occur during the desorption/ionization process between the matrix and analyte have been the main target of many research groups looking to probe this system. Optimizing the desorption process by understanding and exploiting the chemical interactions between the matrix and analyte has been by far the most feasible approach in achieving signal optimization during a MALDI experiment.

Researchers have been trying to understand the chemical aspect of the MALDI process for years. The chemical interactions of the sample and the matrix at the molecular level are believed to be the key factor in the desorption process. It is believed by some researchers that the analyte is ionized in the gas phase shortly after it is desorbed from the probe tip (Lienes 1992; Dean 1993). Others believe that an analyte can already be a "pre-formed" ion species in the solid state, e.g., after recrystallization with the matrix (Lehmann 1997). If the latter of these two scenarios is true, then the importance of the chemical interaction between the matrix and the sample at the molecular level is indeed worth exploring. Factors to be considered when probing the desorption process via chemical interaction studies in the condensed phase are mixture procedures for the sample and matrix, types of matrix, co-matrix assistance, sample/matrix recrystallization techniques, and sample deposition within the matrix crystals.

Some studies have been already conducted targeting some of these factors. Tomer et al. studied sample preparation procedures and found that a decrease in analyte signal can be attributed to sample preparation methods (Amado 1997). In this study, the dried drop preparation method was compared to the homogeneous film method for sample preparation directly on the probe tip. A dependence on a samples hydrophobicity and basicity was found for crystal formation and sample deposition.
from within the matrix crystals. Gusev et al. support these observation as well, showing that analyte distribution within the matrix crystal was increasingly irregular as a function of analyte size (Gusev 1995). Typically during sample spotting and evaporation, distribution of analyte molecules throughout the crystals formed on the probe tip surface are far from homogeneous. Examination of matrix/analyte crystal shows no regular or generally favored distribution of analyte molecules within the matrix crystal lattice. Upon evaporation of the solvent, analyte molecules have been reported to be generally distributed around the outer parameter of the evaporated droplet. This process, known as the Marangoni effect, is a mass transport phenomenon caused by changing surface tensions throughout the evaporating droplet (Amado 1997). Figure 1.2 is a diagram illustrating this process.

Many techniques for sample preparation aimed at addressing this problem have been reported as well. They include the formation of big crystals, formation of microcrystal, pellet pressing, and crystal crushing (Amado 1997). Of all these techniques, crystal formation was shown to be an important factor, particularly microcrystal formation. Microcrystals yielded better ion signal intensities, and less discrimination for the type of analyte being studied (Amado 1997).

It is generally agreed upon that crystal formation is a critical factor in the ion formation process. The effects of matrix/analyte interactions on this crystallization process has been the focus of many studies. Amado et al. were able to demonstrate that co-crystallization of the matrix and analyte together, however, is not necessary (Amado 1997).

Further support of microcrystalline sample preparation comes from Sadeghi and Vertes who point out the degree of inhomogeneity of sample concentration in larger crystals (Sadeghi 1998). It was shown by Seheghi and Vertes that the amount of sample in a matrix crystal ideally should be the same from crystal to crystal. This
Figure 1.2 Diagram of sample droplet spotted on MALDI plate. Matrix and analyte molecules migrate in opposite directions during the evaporation of solvent.
pattern, of course, was not the case for the more commonly used sample preparation method (e.g., dried drop). They observed for this preparation method a varying distribution of analyte deposition in the matrix crystals and a large variation of crystal size and structure, thus making signal reproducibility difficult.

This varying distribution of analyte deposition during the evaporation process can also be attributed to the difference in surface tension between the metal probe and the liquid droplet (Figure 1.3). The surface tension for the liquid droplet \( \gamma_l \) and the surface tension for the metal surface \( \gamma_s \) are labeled in Figure 1.3. The interfacial tension can be referred to as \( \gamma_w \). A constant change in the liquid volume (e.g., by evaporation) causes instability at the interfacial region, hence energy is required to stabilize the interfacial tension \( \gamma_w \). The system tries to counter the interfacial tension by the shifting of analyte and matrix molecules to the interfacial region. The greatest instability for the evaporating system occurs at the contact angle \( \theta \) shown in Figure 1.3. As a result of the stabilization process, most of, if not all of, the analyte is deposited at the contact angle or on the outer parameter of the solution droplet once all of the solvent has been lost to evaporation.

It is becoming more and more agreed upon that the homogeneity of the analyte within matrix crystals is an important factor to address. The formation of microcrystals is one approach to address this issue. Another approach is to load highly uniform sample films onto the probe tip (e.g., electrospray of sample onto probe tip) (Allwood 1996). Both of these option, however, do require special procedural methods which can vary as a result of the type of matrix/analyte system being used, thus making reproducibility difficult to achieve.
Figure 1.3 Diagram of solution droplet and metal probe surface tension interactions.
1.4.7 Matrix-Analyte Surface Ejection: The Desorption Event

The desorption event can be viewed as a non-equilibrium, hydrodynamic process. Intrinsic to this process is the ejection of matrix and analyte molecules. The ejection process involves the transfer of energy or momentum from the matrix molecules to the analyte molecules during the input of laser energy into the matrix-analyte crystal.

The assumptions for this process, when developing models for ion yield determination, are that the ejection process for both matrix and analyte is collective and the laser power and matrix molecules may exhibit some non-uniformity (Johnson 1994). Models developed to define ion yields are discussed by Johnson in great detail. These models are subjected to the assumptions discussed above. Total ion yields for matrix molecules ejected from the surface per laser pulse can be defined in equation 1.4 where Y is the yield or number of matrix molecules removed from the surface per laser pulse, $V_s$ is the volume of matrix molecules ablated at the site of laser energy deposition and $n_m$ is the number of matrix molecules in the area excited. The collective desorption event described for MALDI makes the process different than other types of laser desorption events where individual ion ejection occurs as a direct function of photon energy absorbed from the laser. In MALDI the desorption process can be described as the area for the molecules excited where laser deposition occurs times the amount of material ablated from the laser deposition site which will be referred to as $\Delta z$ (Johnson 1994). Thus equation 1.5 reads:

$$Y = (V_s)(n_m) = n_m(A_p)(\Delta z)/\cos(\theta)$$

where $A_p$ is the area of the laser pulse used, and $\theta$ is the angle of incidence for the laser pulse. Based on the equation above, it is assumed that the ion yield is directly dependent on the laser fluence (photons/area) delivered to the ablation site. Equations
1.4 and 1.5 are the most basic mathematical account of matrix ablation and subsequent ion generation. Further definitions of this event accounting for other variables like the angle of incidence for the laser pulse, ion ejection velocities, and time scales for the excitation process to occur are reviewed in full detail in Hillenkamp (1991) (Hillenkamp 1991).

1.4.8 Desorption Models for the MALDI Process

Several models can be proposed for the desorption event based on ion yield calculations. While all of these models hold true at some point or another during the desorption event, no one model clearly defines the process. All of these models are laser fluence dependent and ion yields generated as explained by these models only occur at or above the experimental determined laser threshold.

Model A defines the desorption event as a spotted crystalline surface expansion of the matrix-analyte molecules. This expansion is generated by the localized energy of a laser pulse. The process occurs as a function of the layers generated at the site of laser ablation on the MALDI crystal. Sample depletion subsequently occurs rapidly and signal inconsistency will occur. The overall process is described as a non-thermal transport mechanism for the matrix-analyte collective desorption process.

Model B describes a quasi-thermal process which increases in magnitude as a function of the surface vapor pressure of the crystalline spot. As laser power is delivered to the crystalline surface, an instantaneous build up of temperature at the laser deposition site occurs. A thermal transport mechanism is proposed for the actual desorption event and subsequent ejection of matrix-analyte ions occurs. This particular process is heavily dependent on the type of matrix and analyte being used.

Model C describes a laser pulse generated expansion of the matrix-analyte volume, via a rapid, impulsive expansion process which occurs, not as a function of...
the laser fluence, but rather as a function the molecular net momentum generated at the site of laser pulse deposition.

Regardless of which model explains the ion ejection event, it can be agreed upon that the matrix molecules transfer energy or momentum over to the analyte molecules during the non-uniform desorption process. This event would imply that the threshold fluence necessary to eject matrix molecules may be different from that needed to eject analyte molecules. Relative surface concentrations of analyte and matrix molecules will obviously differ by a vast margin. Thus concentration differences of analyte molecules relative to matrix molecules could offer some explanation to the differences in laser fluence dependency.

1.4.9 The Ionization Process

The analyte ionization process for MALDI is an even more complex process to model due to the fact that it is still unclear whether this process occurs in the condensed phase or the gas phase. Quist et al. observed that laser fluence for the ionization event of analyte molecules is substantially lower than that necessary for ablation. This observation was based on relatively low laser fluences necessary for electron ejection. The probability of forming analyte ions is much lower than the formation of analyte neutrals. The ratio of neutral to analyte ions has been reported to be around $1.0 \times 10^5$: or the entire area being irradiated during a single laser pulse (Quist 1994). Analyte ion yields tend to be roughly on the order of $10^{-4}$ to $10^{-5}$ which is considerably lower than neutral yields (Johnson 1994). A probability factor, $P_{Mi}$, can be added to equation 1.5 to estimate analyte ion yield. The new expression can be written as equation 1.6 where $M$ denotes the values calculated for the analyte molecules (use $m$ when referring to matrix molecules).

$$Y_M = n_M (P\Delta z)_M (A_p / \cos \theta)$$  

1.6
Models designed to explain the ionization event have been developed based on the theoretical linear relationship between ion flight time and ion mass. This linear relationship is supported under the ideal conditions of instantaneous ion formation at or near the probe surface (Kinsel 1997). The most fundamental relationship to define the kinetic or translational ion energy, E, for a theoretically ideal ionization model is equation 1.7 where m is the mass of the ion and v is the ion velocity.

\[ E = \frac{1}{2}mv^2 \quad 1.7 \]

The mechanism mostly agreed upon for the desorption/ionization event is the absorbance of laser photons by the matrix molecules which subsequently causes excitation on the electronic energy levels. This energy is transferred to the vibrational and transitional levels of the molecule. Axial ion velocity distribution studies by Beavis and Chait suggested that ions can be generated normal to the sample surface with velocities that were mass independent (Zhang 1997). This same mass independent velocity was observed by Verentchikov et al. for polypeptides with broad mass ranges (Zhang 1997). Thus, it may be assumed that ion kinetic energy distributions are mass independent.

An extension of the energy definition (equation 1.7) to further define the MALDI ionization event would be equation 1.8 where t is the ion flight time,

\[ t = \left[ \frac{1}{2E^{1/2}} \right] m^{1/2} + b \quad 1.8 \]

l is the length of the flight tube, and b is an offset constant which accounts for the delay between the actual ionization event and acquisition of the ion signal. A more generalized term can be developed from equation 1.8 if a couple of assumptions are made: the flight path for all ions, regardless of mass, is the same, and the translational energy for all ions eventually will be of the same order of magnitude.
(with the influence of electrical extraction fields). Hence equation 1.9 can be derived. Kinsel et al.'s experimental measurements of this linear relationship between ion flight time and ion mass suggested that two ionization components exist for the ionization phenomenon in MALDI. By measuring the flight times for a mass range of bovine insulin ions, they were able to offer supporting data for what is believed to be a probable explanation for the broad ion cluster distribution observed in many MALDI experiments.

It was proposed that the two components of MALDI ion formation are independent of each other and occur as a function of source and instrumental conditions. Component 1 involves an instantaneous ionization event at or near the probe surface. The ions formed from this event have initial kinetic energies that are mass-independent. These ions are then accelerated to translational energies that are mass dependent. Ions formed from component 1 conditions can also conceivably be pre-formed ions in the condensed phase.

Component 2 ion formation suggests that ions are formed under a delayed mechanism in the gas phase. Based on the dynamics of this component, analyte ionization occurs via a plume of neutral molecules ejected from the surface at a constant velocity. Molecules in this plume can absorb a second photon of energy in the gas phase leading to ionization. Ion translational energy derived from the component 2 mechanism are mass dependent. Factors that contribute to the magnitude of ion energy are the plume velocity, instrumental electrical fields and time at which ionization occurs.

Another analyte ionization mechanisms was proposed by Karas and Hillenkamp in which the formation of matrix ions was found to occur more readily than the formation of analyte ions (Karas 1989). As a result it was proposed that
during the collective desorption process, the matrix possibly transfers a proton to the analyte. This theory has gathered a lot of support based on results reported by those who study biological molecules, in particular. Incorporation of the Karas and Hillenkamp analyte ionization theory into the component 2 ionization model proposed by Kinsel et al. helps to further define the overall ionization event and the data reported in the literature to date.

1.5 Conclusion

The mass spectral analysis of biomolecules of is great importance to the biomedical research fields. As mentioned earlier, the most effective mass determination technique for biomolecules was gel electrophoresis. The attractiveness of mass spectrometry as a means to not only analyze the mass of biomolecules, but also aid in the structural elucidation of these compounds, has made it the primary focus of many research groups. To date, several ionization techniques have been employed in the analysis of high molecular weight biomolecules. Peptides and proteins have proven to be the most easiest to analyze while oligonucleotides have proven to be the most difficult. A chronological account of the introduction and development of MALDI-MS has been presented up to this point. The applications of this process for the mass spectral analysis of various compounds, as well as the criteria necessary for optimal performance of this technique, have also been addressed in this chapter.

The first part of this dissertation will focus on the mass spectral analysis of oligonucleotides using MALDI-TOFMS. Part A of this dissertation will focus on the problems associated with the analysis of oligonucleotides using MALDI-TOFMS. Some of the primary areas of focus will be on: 1) the solution-phase chemistry between the analyte and the matrix and how these interactions affect the desorption/ionization event; 2) sample preparation techniques; and 3) the use of novel co-matrices to help improve the mass spectral quality. Subsequent chapters will
address the specific applications of MALDI towards a class of compounds targeted in this study, namely deoxyribonucleotide polymers. The objectives and results of this study shall be discussed in the following chapters.
CHAPTER 2. PAST TO PRESENT ACCOUNTS OF OLIGONUCLEOTIDE ANALYSIS USING MALDI-MS

2.1 Developments in MALDI-MS Analysis of Oligonucleotides

The analysis of proteins has been extremely successful using MALDI-MS. Matrices like 2,5-dihydroxybenzoic acid (2,5-DHB) (Strupat 1991) and various cinnamic acid derivatives like sinapinic acid (Beavis 1989) have enabled researchers to analyze proteins upwards of 500,000 Da. The analysis of other classes of biomolecules using MALDI-MS soon followed. High expectations for the analysis of oligonucleotides (Figure 2.1) using MALDI-MS excited researchers in the biological sciences. Oligonucleotides were prepared and analyzed using the same MALDI experimental protocol as used in protein analysis. Experiments directed towards the analysis of oligonucleotides did not prove as successful as those experimental results gathered from protein analysis. Masses were analyzed up to only 30,000 Da. This limited success is primarily attributed to the high fragmentation tendencies of oligonucleotides. The fragmentation seemingly occurs during the desorption process and results in low sensitivity and complicated spectra. The high rate of fragmentation has been found to be a direct function of the type of nucleotide base and matrix that is used in the experiment (Zhu 1995).

2.2 Oligonucleotide Fragmentation

The mass limitation on the analysis of oligonucleotides has been of great interest to many researchers and is the topic of many papers. Experimental analysis of homopolymers of nucleobases adenine (Ade), guanine (Gua), cytosine (Cyt), and thymine (Thy) (Figure 2.2) ranging from 4-10 base units long revealed that certain nucleotide bases were more susceptible to fragmentation than others (Zhu 1995; Tang 1997; Tang, 1997). Specifically, it was found that cytosine, quanine and adenine are more likely to fragment than thymine.

The ionization of oligodeoxyribonucleotides occurs quite readily in solution. The mechanisms for the gas-phase ionization of these oligomers is still under debate.
Figure 2.1 Structures for DNA 5'- and 3'- monophosphate nucleotides.
Figure 2.2 Major nucleobases which compose DNA.
The ideal scenario for MALDI generation of oligonucleotide ions would be the neutralization of all of the phosphate groups on the oligomer backbone except one as shown in Figure 2.3. In this scenario, the analyte would be characterized in the negative-ion mode.

The susceptibility of certain nucleotide bases to protonation can be explained by comparing the proton affinities of the matrix and the analytes. The proton affinities for guanine, adenine, cytosine, and thymine are 227.4, 224.2, 225.9 and 209.0 kcal/mol, respectively (Tang, 1997). The lower proton affinity of thymine may reduce it’s propensity to protonation, relative to the other bases. The data obtained in these and other experiments suggest that the thymidine nucleotides are protonated at the phosphodiester backbone and not at the nucleotide base as in the case of adenine, cytosine, and guanine (Tang, 1997). This theory, however, has not been conclusively proven. As a result, an increased amount of fragmentation is commonly observed during the MALDI analysis of this particular class of compounds. Hence high molecular weight analysis of DNA oligomers is a difficult and still very limited process.

Positive-ion mode and negative-ion mode analysis of small oligonucleotides (< 10 bases) reveal that fragmentation occurs extensively at Ade, Gua, and Cyt bases in heteropolymers. Homopolymers of oligonucleotides show extensive fragmentation for dAₙ, dCₙ, and dGₙ (where n=4, 6, 8, and 10), and dTₙ showed little to no fragmentation (Zhu 1995; Tang, 1997). The degree of fragmentation was found to be related to the bases present, the length of the polymer, and the matrix. In all cases, however, fragmentation was found to occur in both the positive-ion and negative-ion mode, but at a varying degree.

A closer look at the factors involved in the fragmentation event for oligonucleotides reveals that, while there are several types of fragmentation which
Figure 2.3 Neutralization of oxygen on phosphorous backbone would be the desired mechanism for ionization of oligomer.
occur during the MALDI process, there is one particular type of fragmentation which seems to be the most prevalent. This fragmentation pathway involves the loss of a nucleobase from the oligomer via internal cleavage of the N-glycosidic bond. Studies into the mechanisms surrounding this particular fragmentation pathway suggest that the fragmentation occurs due to the instability of the N-glycosidic bond. This instability is believed to be caused by the protonation of the nucleobase (Zhu 1995). Evidence from collisional activation studies by McLuckey et al. reinforces this hypothesis (McLuckey 1993). It is believed that protonation of the nucleobase followed by cleavage of the N-glycosidic bond is the primary fragmentation pathway. Figures 2.4a and 2.4b illustrate a proposed mechanism for this fragmentation event (Nordhoff, 1993). It is believed that the protonation of the nucleobase can occur during the desorption/ionization event.

While most researchers would support the conclusion that this protonation process occurs in the gas phase, there is still some debate that protonation of the nucleobase can occur in the condensed phase resulting in "preformed ions" prior to the desorption event. Protonation of the nucleobase can create a zwitterion which is energetically unfavorable for the molecule. Hence once the protonation occurs at the nucleobase it seems to be energetically favorable for the cleavage of the N-glycosidic bond to occur. This cleavage process has been proposed to occur as a direct result of the abstraction of the 2' hydrogen on the sugar moiety of the protonated nucleobase by a neighboring phosphate group. It is conceivable that the 2' hydrogen could also be abstracted by the matrix anion generated from the protonation process. On the other hand, if protonation of the nucleobase does not occur, then abstraction of the 2' hydrogen by the neighboring phosphate group may not occur as readily and thus the fragmentation event does not occur. A subsequent secondary fragmentation pathway has also been observed with the cleavage of the phosphodiester bond 3' C-O bond or the 5' C-O bond occurring after base loss. Experiments aimed at elucidating the
Figure 2.4a First step in fragmentation process involves the abstraction of a proton from matrix molecule.
Pi bond rearrangement leads to cleavage of N-glycosidic bond

Loss of nucleobase results

Figure 2.4b Second step in oligonucleotide fragmentation mechanism which results in the loss of a nucleobase.
protonation process suggest that electronically excited matrix molecules generated by the emission of the laser into the matrix/analyte sample are responsible for the protonation process (Gimon 1992).

2.3 Matrix Contribution to MALDI-MS of Oligonucleotides

The selection of a suitable matrix is a critical part of the MALDI experiment. This decision is contingent on the type of analyte being analyzed. Criteria for a suitable matrix are: 1) The matrix has to be able to incorporate the oligonucleotide in its crystal lattice (crystal morphology); 2) The matrix should be able to assist in ionizing the sample, (i.e. proton abstraction or protonation); and 3) Structural homology for matrices suggest that acidic proton sites (e.g., carboxylic acid functionality), along with the presence of a chromophore that absorbs within the wavelength of the laser being used in the experiment, are an important criteria for oligonucleotide analysis. Figure 2.5 gives the structures of several common matrices used in the MALDI analysis of oligonucleotides.

The analysis of low molecular weight oligonucleotides (<10 bases) can be accomplished satisfactorily with the matrices commonly used in protein analysis. A commonly used matrix for low molecular weight analysis (< 5000 Da) is 2,5-dihydroxybenzoic acid. Fragmentation problems are still prevalent, however, with 2,5-dihydroxybenzoic acid in positive-ion and negative-ion mode analysis of oligonucleotides over 10 base units long. It was later observed, however, that a dramatic decrease in fragmentation can be obtained when analyzing mixed nucleotides bases (< 5 bases) using 2,5-dihydroxybenzoic acid in negative mode (Tang, 1997). This data suggests that the fragmentation process may be slightly dependent on the mode of analysis but the limited range of effectiveness (oligonucleotide length) makes this factor insignificant as a possible means of approach to the problem at hand, which is the need to find better ways to increase the desorption/ ionization efficiencies for
oligonucleotides which will ultimately allow researchers to analyze much larger oligonucleotides.

6-aza-2-thiothymine

3-hydroxypicolinic acid

2,4,6-trihydroxyacetophenone

2,5-dihydroxybenzoic acid

Figure 2.5 MALDI matrices commonly used for the analysis of oligonucleotides.

The limited success of mixed base oligonucleotide analysis (> 10 bases) using the same matrices used in the MALDI analysis of proteins prompted the search for alternative matrices. 3-hydroxypicolinic acid (3-HPA) was found to be much more effective for the analysis of higher molecular weight oligonucleotides than the other matrices used in protein analysis (Tang 1997). One possible explanation for the effectiveness of 3-HPA relative to 2,5-DHBA could be the estimated proton affinities for both of these matrices. The proton affinities for 3-HPA and 2,5-DHB have been determined from their heats of formation values to be 230 and 213 kcal/mol (Zhu 1995). The higher proton affinity value for 3-HPA would suggest that nucleotide base protonation does not occur as readily with 3-HPA. It is speculated that the proton affinity of the matrix coupled with the type of nucleotide base being analyzed
has a direct effect on the degradation of the oligonucleotide during the desorption process. This theory, however, has not been proven yet.

Results from Zhu et al. show that small oligonucleotide mixed base analytes analyzed with 3-HPA compared to the same analytes mixed with 2,5-DHB produced substantially less fragmentation with 3-HPA (Zhu 1995). Tang et al. have reported the use of 3-HPA in the analysis of a double stranded DNA, but the ion abundance and signal resolution was extremely low (Tang 1994). More recent results of MALDI experiments using 3-HPA demonstrated successful analysis of a single stranded heteropolymer oligonucleotide 89 nucleobases in length with better sensitivity and increased resolution than had been reported before for larger mass oligonucleotide analytes (Wu 1994). At higher molecular weights, a dramatic decrease in signal intensity is observed (Wu 1994). While the analysis of an 89-mer mixed base oligonucleotide is a substantial increase in mass analysis range, it is still very limited compared to the mass ranges achieved for proteins. To date, 3-HPA is the most popular choice for single-stranded oligonucleotide analysis.

2.4 Use of Co-matrices

One approach used by researchers to address the problem of excessive fragmentation in nucleotide analysis has been the use of co-matrices. Incorporation of a select component into the matrix crystal lattice along with the analyte has been shown to help decrease fragmentation and increase signal intensity and resolution. The explanation behind the effectiveness of co-matrices stems from the chemical interactions occurring between the matrix, the analyte, and the co-matrix. Whether these interactions occur in the condensed phase or the gas phase has yet to be determined.

Pieles et al. were able to increase signal resolution and ion intensity by adding diammonium hydrogen citrate to a trihydroxyacetophenone matrix (Pieles 1993). Similar results were observed by Currie and Yates when they analyzed oligomers.
from 9 base units in length up to 60 base units in length using ammonium salts (Currie 1993). Currie and Yates tested various combinations of matrices like 2,5 DHB and 3-hydroxy-4-methoxy-benzaldehyde (which were found to yield the best results out of all the matrices they tried) with different ammonium salts. Ammonium acetate proved to be the most effective co-matrix. Ion intensity and signal resolution increased in the presence of ammonium acetate along with a considerable decrease in salt adduction. In some cases no signal was observed without ammonium acetate. The chemical effect of the ammonium acetate on the desorption process could not be adequately explained. It was hypothesized that possible disruption of ionic interactions within the matrix crystal lattice in the condensed phase could increase analyte incorporation into the crystal lattice of the matrix. Another explanation offered suggested that the proton transfer mechanism between the co-matrix and the matrix in the excited state is enhanced creating a competitive proton transfer between the co-matrix and the oligonucleotide which aids in limiting the protonation of the nucleotide bases and subsequent oligomer fragmentation.

Simmons and Limbach demonstrated the correlation of proton affinities of a co-matrix to the increased ion signal of an analyte (Simmons 1998). In that study, organic bases with proton affinities above and below the proton affinities of the four nucleotide bases were used as co-matrices. The organic bases used in these experiments were triethylamine, piperidine, and imidazole whose proton affinities are 234.7, 228.0, and 225.2 kcal/mol respectively. Two homopolymers of thymidine d[(Tp)nT] and adenine d[(Ap)nA] were used for those experiments. It was found that the degree of fragmentation for the adenine homopolymer decreased as a function of the increasing proton affinity of the organic bases used as a co-matrix. The three different matrices used in these experiments, 3-HPA, THAP, and ATT, all exhibited similar behavior. The homopolymer of thymidine did not display any fragmentation with or without the co-matrix in any of the above matrices. This resistance towards
fragmentation was attributed to thymidine’s relatively high resistance to base protonation, and thus served as a reference point. It was also observed that the molar ratio of the co-matrix to matrix was a critical factor for improving signal intensities. The chemical explanation for these observations was presented as a direct effect of the organic base’s ability to act as a "proton sink" for excess protons from the matrix. The ability of the co-matrix to compete for excess protons decreases the protonation event of the oligonucleotide base. While more studies have to be done, it does seem apparent that the relative proton affinities of the analyte, the matrix, and the co-matrix are essential factors in improving mass spectral signal. The effects of novel co-matrices is one of the areas of research reported in this dissertation. Thus a more detailed discussion of co-matrix effects will be covered in Chapter 4.

2.5 Conclusion

The success of oligonucleotide analysis using MALDI has been extremely limited compared to the success reported for MALDI analysis of proteins. Part of this lack of progress in the analysis of high molecular weight oligonucleotides can be attributed to the limited understanding of the desorption/ionization event during MALDI. Experimental protocols thus far used to optimize MALDI performance are employed consistently without a full understanding of how the protocol affects the experimental outcome. Until a better understanding of how these current protocols affect the MALDI process, improvements in the upper mass limits for oligonucleotide analysis will be more difficult to achieve. One aim of this doctoral study is to explore the current experimental protocols used in the MALDI analysis of oligonucleotides. Data collected from this study will be employed to aid in overcoming the mass limitation of oligonucleotide analysis.
CHAPTER 3. PRELIMINARY STUDIES INTO STANDARD MALDI-TOF MASS SPECTROMETRY ANALYSIS OF OLIGONUCLEOTIDES

3.1 Introduction

The initial studies into the MALDI analysis of oligonucleotides were designed to formulate a better understanding of the desorption/ionization process. A series of experiments were designed to help define the solution-phase matrix/analyte behavior of the oligonucleotides and matrices selected as model systems for this study. The effects of this behavior on the overall dynamics of the MALDI experiment were observed to be quite critical to the experimental outcome. Several model systems were initially studied to establish 1) the quality of data characteristic for our instrument, 2) the chemistry of the matrix/analyte system, 3) various sample preparation protocols, and 4) the development of research projects designed to help further the development of MALDI strategies and techniques for sample analysis.

The exact mechanisms for the desorption process and the subsequent production of ions during the MALDI process is still the subject of much debate. The desorption process is believed to involve the transfer of laser energy, absorbed by the matrix molecules, to analyte molecules imbedded in the matrix’s crystal lattice. As a result of this laser energy transfer process, the subsequent sublimation of both the matrix and analyte molecules occurs via a rapid thermalization process at initial crystal surface temperature calculated at ca. 1000K - 3000K (Bencsura 1995; Maechling 1996; Bencsura 1997).

The production of MALDI ions is argued to occur either in the gas-phase (Beavis 1992; Demirev 1992) or as pre-formed ions in the solution-phase (Lehmann 1997). One model for the analyte gas-phase ionization process suggests that ionization occurs as a result of the photoionization of matrix molecules after the desorption event (Mowry 1993). During the photoionization process, a proton transfer mechanism is proposed to occur between the matrix and analyte. The mechanism for this proton transfer process is believed to involve excited-state matrix
protons generated during the absorption of laser energy by the matrix molecules. The proton transfer and abstraction process which generates positive and negative ions, respectively, occurs simultaneously along with the production of a large ratio of neutral molecules (Mowry 1993). Two gas-phase parameters which have been studied as factors which may govern the gas-phase ionization process are the relative matrix/analyte proton affinities (Nordhoff, 1993, Nelson, 1996) and ionization efficiencies (Chou 1999).

One of the difficulties of analyzing the MALDI desorption/ionization process stems from the fact that the gas-phase components are inherently difficult to probe. Gas-phase ion/molecule studies are difficult to perform and usually require extremely sophisticated instrumentation. Instead indirect methodologies aimed at probing the desorption/ionization event have been sought by many researchers. The most obvious method for this indirect analysis is through solution-phase chemistry.

Solution-phase dynamics have been shown by many researchers to affect the efficiencies of the desorption/ionization process and subsequently the quality of mass spectral data collected for a sample (Tang, 1993; Cohen 1996; Amado 1997). Solution-phase studies have demonstrated that mass spectral signal improvements can be achieved based on the solution-phase interactions between the matrix and analyte molecules. Researchers have focused on how solution-phase chemical interactions between the matrix and analyte molecules govern the quality of the ion signal observed in a MALDI experiment. The effects of solution-phase interactions between the matrix and analyte molecules have prompted many experiments aimed at elucidating any correlations between the solution-phase dynamics and gas-phase dynamics for a standard MALDI experiment. One attractive feature for this approach is that solution-phase studies are easier to implement with results that can improve the outcome of a MALDI experiment. Solution-phase studies can also be utilized to indirectly study the gas-phase dynamics of a MALDI experiment. As a result,
solution-phase studies have emerged as the primary method for increasing the ionization/desorption efficiencies for MALDI experiments.

The conditions of the solution and the method by which the matrix and analyte molecules are prepared has been one of the concentrated areas of research. Factors like type of matrix, type of analyte, sample solution pH, sample crystal morphology and sample preparation protocols limit the general application of various solution-phase developments to specialized circumstances. Often, these circumstances usually have to be empirically determined. Empirically derived results reported as successful for any and all solution-phase methodologies are typically tried by other researchers to see if they too can capitalize on the success. Other factors which can be taken into account are analyte size (Karbach 1998), hydrophobicity (Amado 1997), and relative pKₐ values (Chiarelli 1993).

Various sample preparation methods have been developed and adapted as general protocols during a MALDI experiment. One such method is the dried-drop method in which the analyte and matrix are prepared as individual solutions (Karas 1988). These solutions are mixed together on a small volumetric level (ca. several μL of each solution) and subsequently spotted (ca. 1-2 μL) on a MALDI plate and allowed to dry. Many different preparation methods have been developed since the dried-drop method (Amado 1997).

One preparation method found particularly effective for the studies presented in this dissertation involves the mixing of both analyte and matrix as individual solutions, as described above, and subsequent spotting of the analyte component (ca. 1-2 μL) on the MALDI plate first. The analyte solution is allowed to dry, and then the matrix solution would be spotted directly on top of the analyte. Both of the techniques discussed thus far are commonly used in the preparation of samples for MALDI analysis and were empirically determined as the best methods to employ for these experiments.
The distribution of analyte molecules into the matrix crystal lattice is an area also addressed by researchers. The crystal morphology of a matrix-analyte sample after the spotting process is very critical to the outcome of the experiment having a direct effect on the signal quality generated for the sample (Amado 1997; Karbach 1998, Cohen, 1996). From these studies, it was demonstrated that the solution-phase dynamics for the matrix/analyte system directly affect the distribution of analyte molecules in the matrix's crystal lattice. No one report has definitively explained this phenomenon, but, rather, many have put forth experimental data that support the hypothesis that crystal morphology does effect the outcome of a MALDI experiment. One point that is generally agreed upon is the relative ratio of matrix to analyte (ca. > 10,000 to 1) necessary to achieve MALDI ion signal (Mowry 1993).

A series of experiments were conducted to test various solution-phase experimental parameters for the MALDI-TOF analysis of the model oligonucleotides and matrices. These experiments set the stage for understanding the behavior of the matrix/analyte systems discussed in this dissertation. Experimental parameters studied were 1) the chemical interactions of various matrices and oligonucleotides during sample preparation, 2) improved preparation protocols, 3) MALDI plate surface effects on sample crystal morphology, 4) the effects of pH on matrix performance and 5) the introduction of novel co-matrices. This chapter will address the chemical interactions observed between the matrix and analyte in the condensed phase during sample preparation.

3.2 Experimental

3.2.1 Oligonucleotide Synthesis and Purification

The reagents for oligonucleotide synthesis were purchased from Perkin Elmer/Applied Biosystems (Foster City, CA). All of the oligonucleotides were synthesized using standard phosphoramidite chemistry on 1-μmol columns using a Perkin Elmer/Applied Biosystems Model 394 DNA/RNA synthesizer. After
synthesis, the deprotected oligonucleotides were purified using oligonucleotide purification cartridges (OPC) purchased from Perkin Elmer to separate out any failure sequences. Purification of the oligonucleotide was performed by retaining the terminal dimethoxytrityl (DMT) protected oligonucleotide on the OPC column in 10% aqueous ammonium hydroxide. The OPC was washed with ammonium hydroxide to remove any impurities. The DMT group is then removed using 3% trifluoroacetic acid and the deprotected oligonucleotide was eluted with 20% aqueous acetonitrile. The oligonucleotide collected from the OPC column was evaporated to dryness using a LabConco centrivap (Kansas City, MO). The lyophilized sample was redissolved in 100 μL of nanopure water.

3.2.2 Sample Preparation

The matrices used for these experiments were 3-hydroxypicolinic acid (3-HPA), 6-hydroxypicolinic acid (6-HPA), 2,4,6-trihydroxyacetophenone (THAP), and 6-aza-2-thiothymine (ATT). 3-HPA and ATT were mixed in nanopure water and heated as necessary to fully dissolve the matrix into solution. THAP was dissolved in a 50/50 mixture of water/ethanol. All of these reagents were purchased from Aldrich (Milwaukee, WI, USA). All chemicals were used as received without further purification.

The matrices were prepared at three different concentrations for the sample preparation and surface effect experiments. These concentrations were .030 M, 0.36 M and a saturated solution (0.72 M). The sample solutions were spotted on a stainless steel MALDI sample plate.

3.2.3 Mass spectrometry

The MALDI-MS experiments were carried out using a PerSeptive Biosystems Inc. Voyager linear MALDI-TOF instrument (Farmingham, MA, USA) equipped with a nitrogen laser (λ=337 nm). All data reported here were collected at a laser setting of 690-750 which was empirically determined to be the threshold range for ionization of
the various samples. Between 60 and 128 scans were averaged for each mass spectrum acquired. Data was collected in both negative- and positive-ion mode.

### 3.2.4 Potentiometric Titrations

The potentiometric titration protocol followed was that of Albert and Serjeant (Albert 1971). The pH values for all of the oligonucleotides and matrices were measured using a Orion-Ross glass body semi-micro combination pH electrode. (Beverly, MA., USA). All of the matrices were prepared at a concentration of 0.01 M in nanopure water. The matrices were titrated with a standardized 1.0 N potassium hydroxide (KOH) solution. Three runs were performed for each titration experiment and an average pKₐ value was determined. The pKₐ values for each of the matrices were calculated statistically following the protocol of Albert and Serjeant.

### 3.2.5 Oligonucleotide Concentration Determination

The concentration of the oligonucleotides was determined by ultraviolet (UV) absorption measurements. A series of oligonucleotide solution dilutions are usually prepared and their absorbances at 260 is measured. Several UV absorbance readings are taken for each dilution and averaged to determine the optical density unit (ODU) for the oligomer using equation 3.1:

\[
\text{ODU/mL} = \frac{(A_{\text{meas.}})(1 \text{ mL})}{\text{volume of solution}}
\]  

3.1

The OD value is then converted to mass μg/mL using the standard DNA OD conversion factor (equation 3.2):

\[
1 \text{ ODU of DNA} \equiv 50 \text{ mg of DNA}
\]  

3.2

The UV-determined concentration for all of the oligomers was on the order of 1.0 x 10⁻⁶ M.
3.3 Results and Discussion

3.3.1 Standard Sample Preparation Protocols for Model Oligonucleotide/Matrix Systems

The most commonly used matrix-analyte protocols require the mixing of excess matrix with the analyte. Typically, the matrix is prepared as an individual solution whose concentration is at least 5-fold greater than that of the analyte solution concentration. Equal volumes of the two solutions are mixed together. A 1-2 μL aliquot of the mixture is then spotted on the MALDI plate and allowed to dry (dried-drop method). The plate is then loaded into the mass spectrometer for MALDI analysis.

The oligonucleotides used in the preliminary studies were mixed base oligomers referred to as “12mer”, “16mer” and “24mer” with the base sequences of d(GATC)3, d(GATCT)4, and d(GATC)6 respectively. Other oligomers used as standards and instrument calibrants were homopolymers of thymidilic acid with base sequences of dT14, dT20, and dT30.

The matrix reported in the literature as the most effective matrix for the analysis of mixed base oligomers is 3-HPA (Tang, 1997). The 3-HPA matrix was generally used for the analysis of the mixed-base oligomers because it was observed to yield the best molecular ion signal of all the matrices tested. The 3-HPA matrix was often prepared at a concentration of 0.36 M. This concentration was empirically derived to be the most effective for these systems and hence, 0.36 M 3-HPA matrix solutions were used for all further experiments. The homopolymers of thymidilic acid were analyzed using THAP as the matrix at concentrations ranging from 30 mM to a saturated solutions.

The mixed base oligomers were observed to characteristically yield a strong molecular ion signal, [M-H]− or [M+H]+, plus some fragment ions corresponding to the loss of predominately guanine and adenosine bases. All of the oligomers were
studied in negative-ion mode to reduce the occurrence of fragmentation. Base loss is not normally expected to occur as readily in negative-ion mode for mixed-base oligomers, but was commonly observed for our samples. The doubly charged molecular ion species, \([\text{M-H}]^{-2}\), and the dimer ion species, \([2\text{M-H}]^{+}\), were also commonly observed for these oligomers. Figure 3.1 is a typical MALDI negative-ion mode mass spectrum of several mixed-base oligonucleotides analyzed using the dried-drop method.

3.3.2 Matrix Dilution Effects and Development of Novel Preparation Protocols

The importance of the relative matrix/analyte concentration for sample preparation is demonstrated in Figure 3.2. Here we see the same 12mer mentioned above, now prepared with a 10-fold dilution of 3-HPA matrix solutions. The loss of the molecular ion signal is directly proportional to the decrease in matrix solution concentration. This observation agrees with similar observations reported in the literature (Fitzgerald 1993; Cohen 1996). The effects of matrix concentration shown here, as well as those reported in the literature, support the claim that excess matrix is a necessity in MALDI sample preparation techniques. One of the reasons for this fundamental criteria has been attributed to the inability of "one" matrix molecule to facilitate the desorption of a much larger oligonucleotide molecule via the energy transfer process (Karbach 1998). From this observation it was hypothesized that it takes more than one matrix molecule to interact with the embedded analyte and facilitate the desorption process. It is believed that when the "optimum" number of matrix molecules have surrounded and can associate with the embedded analyte in the condensed phase, an increase in desorption efficiency may result.

A different and more effective protocol for mixing the matrix with the oligomer was developed. A small volume (ca. 2 μL) of the oligomer solution was added to a
Figure 3.1 Typical MALDI spectra for model oligonucleotides a) d(GACT)$_3$, b) d(GACT)$_4$, and c) d(GACT)$_6$. All of the mixed base oligomers were prepared with 0.36 M 3-HPA.
Figure 3.2 MALDI spectra of d(GACT)$_3$ with 3-HPA at a) 0.036 M, b) 0.0036 M, and c) 0.00036 M concentrations. Loss of ion signal is directly proportional to decrease in matrix concentration.
microcentrifuge tube. A single crystal of 3-HPA was added directly to the oligomer solution. At room temperature (25 °C) the crystal of 3-HPA did not fully dissolve into the oligomer solution. The solution was placed in a hot water bath for 30 seconds to completely dissolve the solution. An aliquot of the hot matrix/analyte solution was spotted on the MALDI plate and allowed to dry. As a result of this method, crystal formation was much more dense than had been observed previously. This method will be referred to as the direct crystal addition method.

A benefit of the direct crystal addition method was that a more homogenous distribution of analyte molecules throughout the matrix crystal lattice was observed. As mentioned in Chapter 2, the Marangoni effect occurs when the bulk of the analyte molecules are distributed around the outer parameter of the dried sample spot, despite the formation of matrix crystals on the inner parameter of the sample spot. This phenomenon occurs quite readily when using the dried-drop method (Amado 1997). The Marangoni effect was decreased substantially when the direct matrix crystal addition method was used as the sample preparation method. The increased homogeneous distribution of analyte molecules was found to create more “sweet spots” for analyte analysis. The only downside to the direct addition protocol was the formation of excessive crystals. This was found to occur if too many crystals of matrix are added to the oligomer solution. As a result, a decrease in spectral quality was subsequently observed.

3.3.3 Effects of Sample Substrate

The MALDI substrate is typically stainless steel. Several research groups have explored the effect the MALDI substrate has on ion signal. Various substrates have been shown to yield improved ion signal with an increased sensitivity (Bai 1994; Liu 1995; Zhan 1997).

A plastic film was tested in our lab as a MALDI substrate and found to yield increased ion abundance and mass resolution as well as an increase in sensitivity. A
standard stainless steel plate used for MALDI analysis was modified by mounting a 1-mm thick plastic film. Various oligonucleotides were spotted on the film using the mixed solution method and direct matrix crystal addition method.

Figure 3.3 compares the effects of MALDI substrates on signal sensitivity and mass resolution for d(GACT)$_3$. Figure 3.3a shows the oligomer spotted on the stainless steel plate and analyzed at the arbitrary laser power of 745. A comparison of the same oligomer now spotted on the plastic surface at the same laser power shows substantial increase in ion abundance (Figure 3.3b). A decrease in laser power to 690 now gives ion signal comparable to that acquired on the metal surface at the much higher laser power (Figure 3.3c). All of the samples shown in Figure 3.3 were prepared using the direct addition preparation method. Similar results were observed using the mixed solution preparation method as well.

These results demonstrate an increase in sensitivity as a function of plate surface composition. The surface effects were tested on a larger oligomer to establish if size of oligomer limits the effectiveness of changing the surface composition. Figure 3.4 shows the 24-mer tested on both metal and plastic surfaces at the same laser power. An increased in ion abundance is observed for the larger oligomer as a result of the plastic surface. Again, both samples were prepared using the direct addition method. Similar results were observed for the various oligomers that were prepared using the standard solution mixing method for 3-HPA, ATT and THAP matrices as well. The best overall results however were achieved using the direct addition sample preparation protocol.

The effects of the plastic surface on the desorption/ionization process are not fully understood. It may be speculated that a change in surface tension effects (Chapter 2) between the plate surface and droplet angle are favorably different from the droplet surface tension on stainless steel. This effect could enhance the distribution properties for the matrix/analyte system during the solvent evaporation process yielding
Figure 3.3 The MALDI spectra of d(GACT)₃ demonstrating the effects of plate surface on signal quality. The oligomer is analyzed a) on the standard stainless steel plate. The same oligomer is then analyzed on plastic surface b) at the same laser power used for analysis of oligomer on stainless steel plate, and c) at a much lower laser power than used with stainless steel plate.
Figure 3.4 The effects of plate surface on larger oligomer d(GACT)$_6$. The oligomer solution was analyzed by a) using a stainless steel surface and b) using a plastic surface.
more desorbed analyte molecules per laser deposition area and leading to an increase in analyte ions per laser shot.

3.3.4 Testing of Novel Matrix Compound

The discovery of effective new compounds as MALDI matrices has been seemingly serendipitous in nature. The criteria for oligonucleotide matrices were once believed to be very specific for the molecule to be active as a MALDI matrix. The criteria were 1) the molecule had to be acidic or have an acidic site, 2) facile desorption and 3) strong UV absorption. These criteria have been shown to not always hold true for compounds. Compounds which meet all of the criteria have been shown to be poor matrix compounds (Tang, 1993), while on the other hand, compounds which meet only a few of the criteria have been shown to act as good matrices (Fitzgerald 1993). There is often no general application rule for potential MALDI matrix compounds. The best methodology for discovering potential matrix candidates is by simple trial and error.

One factor believed to govern the effectiveness of matrix is it’s solution-phase acidity. Researchers, however, have shown that solution-phase acidity is not necessarily the main factor in oligonucleotide matrix effectiveness. Compounds which are very basic have been also employed as MALDI matrices (Fitzgerald 1993). These compounds, however, are extremely limited in their applications and thus are regarded as not very useful. A more recent model for matrix effectiveness now suggests that it is the difference in gas-phase acidities of the matrix and analyte that governs the matrix molecule’s ability to facilitate the desorption/ionization process (Lehmann 1997; Green-Church 1999). Other researchers have supported the claim that an increase in a matrix molecule’s gas-phase acidity via absorption of the laser power, and a subsequent excited state proton transfer process, defines the molecules ability to act a MALDI matrix (Cornett 1992; Gimon 1992). These reports however
do not explain how solution-phase parameters like pH changes or matrix concentration changes dramatically affect the outcome of a MALDI experiment.

Experiments were conducted to test a structural isomer of 3-HPA for matrix activity and for possible use in the analysis of the mixed-base oligonucleotides. The isomer chosen for this study was 6-HPA. 6-HPA is structurally different from 3-HPA only by the hydroxyl group on the pyridine ring which is moved from the 3 position to the 6 position. This particular molecule has a strong UV absorption around 310 nm and contains very labile acidic hydrogens which undergo intramolecular shifting similar to 3-HPA (Wu 1994). These attributes seem to make 6-HPA an attractive candidate as a possible MALDI matrix. 6-HPA was actually studied by Wu et al. earlier. They found that 6-HPA did not work as a matrix (Wu 1994). They attributed the lack of effectiveness for 6-HPA, and several other structural isomers looked at in their study, to a tautomerism effect in which the hydroxyl hydrogen shifts to the neighboring pyridine nitrogen, subsequently forming a pyridinone molecule (Figure 3.5).

![Figure 3.5](image_url) The tautomeric equilibrium of 6-HPA to the pyridinone species in solution.

The pyridinone molecule was reported to be more stable in solution while the hydroxypyridine tautomer is more stable in the gas-phase (Wu 1994). The ineffectiveness of the 6-HPA was attributed to the lower gas-phase acidity for the hydroxypyridine species which dominates in the gas-phase.
Our investigation of 6-HPA yielded quite different results which proved to be influenced by the sample preparation protocol. 6-HPA was first prepared as a 0.30 M solution in 50:50 water:ethanol. As observed by Butler et al., no substantial signal was obtained for several mixed-base oligonucleotides tested with 6-HPA (Butler 1996). However, when the direct addition sample preparation protocol was tested using 6-HPA, analyte signal was obtained. Figure 3.6a shows the signal obtained for a 30-mer of thymidilic acid when a crystal of 6-HPA was added directly to the oligonucleotide solution. Similar results were observed for the mixed base oligomers as well (Figure 3.6b). The use of a different MALDI substrate also helped improve the performance of the matrix and the overall quality of ion signal as well. Figure 3.6c shows the same mixed base oligomer prepared by the direct addition of 6-HPA, and subsequent spotting onto the plastic surface mentioned earlier. An increase in ion abundance and mass resolution is observed for the oligomer on the plastic surface using 6-HPA as a matrix. The overall performance of 6-HPA as a matrix was improved dramatically based on the sample preparation protocol. The matrix role of 6-HPA was still limited relative to 3-HPA and ATT. The increased concentration of 6-HPA seemed to benefit its role as a matrix. The reasons for this improved performance are not clear at this point.

These observations support the importance of solution-phase dynamics and sample preparative methods on MALDI generated ion signal. The effects of gas-phase dynamics and possible ion/molecular reactions can apparently be affected by solution-phase conditions. This observation raises questions about the proposed gas-phase mechanisms for the desorption/ionization process.

3.3.5 pH Effects on Matrix Performance

The solution-phase dynamics of a matrix/analyte system have been shown to directly affect the outcome of a MALDI experiment. The matrix/analyte chemical
Figure 3.6 MALDI spectra of a) a homogeneous oligonucleotide d(T<sub>30</sub>) on a stainless steel surface and a mixed base oligonucleotide d(GACT)<sub>4</sub> on b) a stainless steel surface and c) a plastic surface using 6-HPA as a matrix.
interactions which occur in the condensed phase prior to the sublimation process have been shown to be a critical factor in the desorption, and, more than likely, the ionization events. The energy necessary to promote an analyte to the gas phase via this sublimation process is supplied by a single laser pulse. The pathway by which this energy is channeled from the excited state matrix molecules to the analyte molecules embedded in the matrix crystal lattice has not been elucidated. One could speculate, though, that analyte orientation within the matrix crystal lattice and the relative number of matrix molecules associating with individual analyte molecules are factors that contribute to this energy transfer process. The most likely type of chemical interaction that the matrix and analyte molecules undergo in the solution-phase with one another is ionic and/or hydrogen bonding. The functional groups on both the matrix and oligonucleotide molecules sets the stage for ionic or hydrogen bonding interactions. The extent or preference of one type of bond over another is difficult to determine or verify after the desorption process because the amount of laser energy absorbed by the matrix/analyte system is more than enough to dissociate any matrix/analyte molecules adducting by either ionic or hydrogen bonds. This dissociation is not instantaneous, however, and models predicting the desorption process do suggest that matrix/analyte complexes sublime together before finally falling apart in the gas phase (Bencsura 1995). Experiments like the ones conducted thus far help to emphasize the contribution of solution-phase dynamics to the desorption/ ionization process and thus offer viable options for enhancing this process.

To gain a better understanding of the nature of the matrix and analyte bonding interactions in the solution-phase, a series of experiments were conducted which focus on changes in the matrix solution pH and how these changes affect the matrix’s performance. It has been hypothesized that the acidic hydrogens on the matrix molecules are the active site for matrix activity. To test the importance of the acidic
functionality of the matrix molecule, a set of experiments were designed using standard potentiometric techniques to monitor matrix activity as a function of pH.

Several matrices were titrated with 1.0 N KOH to determine their relative pKₐ values. The matrices titrated were 3-HPA, 6-HPA and ATT. All of the matrices were prepared at 0.01 M concentrations and titrated with the 1.0 N KOH to a point just past the matrix's equivalence point. Figure 3.7 shows the titration curves for ATT and 3-HPA titrated with KOH.

The pKₐ values for all of the matrices were calculated using a method by Albert and Serjeant so that statistical error for the data could be evaluated. This method is more precise for determining the pKₐ rather than the standard graphical method. The KOH was standardized with potassium hydrogen phthalate (KHP). The concentration of the standardized base was determined to be 0.82704 M. 50 mL solutions of 1.0 N 3-HPA and ATT were prepared. Both of the matrices were titrated with 1.0 N KOH. The titration was conducted at a constant temperature of 25 °C. The data collected for the pKₐ determinations were tabulized in a spreadsheet for computational purposes. The tabulized data generated for the 3-HPA pKₐ determination is shown in Table 3.1. The same procedure was followed for all of the matrices and their pKₐ values are reported in Table 3.2.

The trends for matrix behavior, based on observations in our lab, suggest that matrices with pKₐ values around 5 or 6 were more effective than matrices with pKₐ values more acidic (e.g., 6-HPA). This correlation contradicts, to some degree, the importance of the matrix acidic functionality.

Further experiments to probe the solution-phase interactions between the matrix and analyte molecules were performed using the standard potentiometric techniques discussed above. The importance of the matrix acidic hydrogens was tested based on their availability for interaction with the analyte molecules. This point
Figure 3.7 Titration curve for a) ATT and b) 3-HPA titrated with 1.0 N KOH using standard potentiometric protocol.
Table 3.1 Titration data generated for 3-HPA using protocol by Albert and Serjeant.

<table>
<thead>
<tr>
<th>Volume (mL)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.83N KOH</td>
<td>[HA]</td>
<td>[A⁻]</td>
<td>[HA]/[A⁻]</td>
<td>LOG([HA]/[A⁻])</td>
<td>pH</td>
<td>pKa (#5+#6)</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
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<td>0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.56</td>
<td>0.09</td>
<td>0.01</td>
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<td>4.37</td>
<td>5.3242</td>
<td></td>
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<td>0.08</td>
<td>0.02</td>
<td>4</td>
<td>4.71</td>
<td>5.3121</td>
<td></td>
</tr>
<tr>
<td>1.68</td>
<td>0.07</td>
<td>0.03</td>
<td>2.333333333</td>
<td>0.367976785</td>
<td>4.95</td>
<td>5.318</td>
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<td>2.24</td>
<td>0.06</td>
<td>0.04</td>
<td>1.5</td>
<td>0.176091259</td>
<td>5.15</td>
<td>5.3261</td>
</tr>
<tr>
<td>2.8</td>
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<td>1</td>
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<td>5.33</td>
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<td>3.36</td>
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<td>0.66666667</td>
<td>-0.176091259</td>
<td>5.52</td>
<td>5.3439</td>
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<td>0.07</td>
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<td>5.372</td>
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<tr>
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<td>0.08</td>
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<td>-0.602059991</td>
<td>6.02</td>
<td>5.4179</td>
</tr>
<tr>
<td>5.04</td>
<td>0.01</td>
<td>0.09</td>
<td>0.11111111</td>
<td>-0.954242509</td>
<td>6.54</td>
<td>5.5858</td>
</tr>
<tr>
<td>5.6</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>10.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average 5.37
Std Dev. 0.08748

was tested by monitoring matrix behavior at the matrix equivalence point, where no acidic hydrogens should be available, to see if the matrix would remain effective.

Since the MALDI sample is typically prepared on a very small scale, titrations of the matrix were performed on a small scale as well. A protocol was developed for these experiments which were referred to as micro-titrations.

Table 3.2 The pKa values calculated for the model matrices.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Calculated pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HPA</td>
<td>5.37 ± 0.09</td>
</tr>
<tr>
<td>6-HPA</td>
<td>2.92 ± 0.04</td>
</tr>
<tr>
<td>ATT</td>
<td>6.56 ± 0.04</td>
</tr>
</tbody>
</table>
The micro-titrations were conducted in a J-shaped glass container designed specifically to allow for the addition and extractions of solutions while constantly monitoring the pH of the overall solution. The matrix solutions were prepared at concentrations of 0.291 M and 0.087 M for 3-HPA and ATT, respectively. These concentrations are in the range for optimum matrix performance established from the earlier studies. It took about 150 μL and 70 μL of the 0.82704 N KOH to completely titrate a 1 mL aliquot of the 3-HPA and ATT matrix solutions, respectively. Each of the 1 mL matrix solutions were titrated to a point just past the equivalence point. Along the course of the titration, 1 μL aliquots of the titrated matrix were drawn out and added to a 1 μL aliquot of an oligonucleotide sample. Once the titrated matrix and oligomer were mixed, they were spotted on a standard MALDI plate. Several homogeneous and mixed-based oligomers were subjected to MALDI analysis following this titration protocol.

MALDI analysis was performed on each of the titrated matrix/analyte spotted samples to determine at what point along the matrix's titration curve the matrix becomes inactive. Figure 3.8 shows the titration curves generated for ATT and 3-HPA. Labeled on the ATT titration curve are the points at which the matrix becomes inactive for dT_{14} and d(GATC)_3. Surprisingly, the point at which the matrix becomes inactive are well before the equivalence point. 3-HPA was observed to become inactive even further from it's equivalence point (Figure 3.8b). The concentrations for each of the matrices at the point of inactivity were calculated to be about 0.20 M and 0.04 M for 3-HPA and ATT, respectively. The concentrations of the oligonucleotides were on the order of 1.0 x 10^{-7}. From the dilution studies conducted earlier (section 3.3.2), the minimum concentration necessary for matrix activity was established. The calculated matrix concentrations at the titrated point of inactivity is still well within the range for the matrix to be active based on the dilutions study data.
Figure 3.8 Micro-titration curves of 1 mL matrix solutions of a) ATT and b) 3-HPA with 1.0 N KOH. The matrix became inactive well before it’s equivalence point was reached.
The fact that the matrix becomes inactive well before it's equivalence point, despite the 10,000 fold excess of matrix still present in the titrated solution suggest that the matrix concentration is not the primary factor responsible for matrix activity.

A better correlation factor to focus on for the matrix performance may be the role of the matrix acidic hydrogens. The absence of the matrix acidic hydrogens seems to directly affect the performance of the matrix. The acidic hydrogen concentration dependence for matrix performance may be a more specific area of correlation than that of overall matrix concentration. Figure 3.9 shows the reaction associated with the titration of 3-HPA. The acid species [HA] dissociates into it's conjugate acid [A\(^-\)] in the presence of KOH. The dissociation also occurs for each of the matrices without base present. The pK\(_a\) of all of the matrices gives an indication of the extent of this dissociation in solution. Based on their pK\(_a\) values, 3-HPA and ATT are weaker acids than 6-HPA. Given that all of the matrices are weak acids, it is hard to establish a criteria based on the relative acidities of the matrices. It may be inferred, however, that the degree of dissociation which occurs for one matrix relative to another may be a key factor for matrix effectiveness.

\[
\text{[HA]} + \text{KOH} \rightarrow \text{[A\(^-\)]} + \text{K}^+ + \text{H}_2\text{O}
\]

**Figure 3.9** Deprotonation of 3-HPA in the presence of KOH.

The titration experiments suggest that it is the [HA] species that is responsible for the matrix activity. The concentration of this species is directly affected by titration. The deprotonation leading to an increased concentration of the [A\(^-\)] species ultimately suppresses the matrix activity. This observation explains the low activity
observed for 6-HPA which has a much higher concentration of the $[A^-]$ species present in solution in the absence of base.

The percent of ionized anion species $[A^-]$ in solution as a function of pH can be calculated using equation 3.3:

$$%\text{Ionized} = \frac{100}{1 + 10^{(pK_a-pH)}}$$

From equation 3.3 we can get an idea of how the concentration of $[A^-]$ changes with the overall matrix concentration. Increased $[A^-]$ concentration suppresses matrix activity, thus a decrease in the anion concentration is desired. Table 3.3 list the calculated % of the $[A^-]$ species present in solution for 3-HPA, ATT and 6-HPA at various concentrations.

Table 3.3 Calculated concentration of $[A^-]$ present in matrix solutions as a function of pH and matrix concentration.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Conc.(M)</th>
<th>pH</th>
<th>$pK_a - pH$</th>
<th>Anion % ionized</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HPA</td>
<td>0.01</td>
<td>3.62</td>
<td>1.75</td>
<td>1.747</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>3.05</td>
<td>2.32</td>
<td>0.498</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>2.75</td>
<td>2.62</td>
<td>0.2505</td>
</tr>
<tr>
<td>ATT</td>
<td>0.01</td>
<td>5.36</td>
<td>1.20</td>
<td>5.93</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>4.27</td>
<td>2.29</td>
<td>0.4987</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>3.93</td>
<td>2.63</td>
<td>0.2505</td>
</tr>
<tr>
<td>6-HPA</td>
<td>0.01</td>
<td>2.36</td>
<td>0.56</td>
<td>21.59</td>
</tr>
<tr>
<td></td>
<td>0.144</td>
<td>2.92</td>
<td>0.0</td>
<td>50</td>
</tr>
</tbody>
</table>

The percentage of ionized anions for 3-HPA and ATT decreases with increasing concentration, which agrees with the improved performance observed for these matrices at the higher matrix concentrations. The 6-HPA, however, has an increase in
anion concentration as the concentration for the matrix increases. This would explain why the standard sample preparation protocol does not work well for 6-HPA.

The direct addition protocol, however, did work well for 6-HPA and improved the overall performance of all of the matrices. The addition of matrix crystals directly into oligonucleotide should create an environment similar to a saturated solution. While this should be ideal for 3-HPA, and ATT, it is still not fully understood why it worked for the 6-HPA as well. One possible explanation could be that the improvement in 6-HPA matrix performance was a direct result of the increased concentration of the [HA] species, irrespective of the overall [A⁻] concentration, now available to interact with analyte molecules and facilitate the desorption/ionization process.

The titration results also imply that ionic bonding dictates the chemical interaction between the matrix and analyte rather than hydrogen bonding. The most titratable hydrogens on 3-HPA are the carboxylic hydrogens and the thiol hydrogens for ATT. Hydrogen bonding between the matrix and analyte can still occur at the hydroxyl sites on both 3-HPA and ATT. If hydrogen bonding effects are necessary to facilitate the desorption/ionization process, then matrix activity should not have been suppressed as a result of the titrations. Based on these observations, the importance of the hydroxyl groups may be relevant only towards influencing the absorption spectra for the matrix molecule. These observations also suggest that the energy transfer process by which the analyte molecule is promoted to the gas-phase occurs through the acidic proton site on the matrix molecule (e.g., carboxylic group in 3-HPA and the thiol group in ATT). This point is quite interesting because it demonstrates the importance of a specific physical association between the matrix and analyte necessary for the matrix to facilitate the desorption/ionization process.
3.4 Conclusion

The experiments reported in this chapter have laid the fundamental groundwork to establish a better understanding of the matrix and oligonucleotide interactions for MALDI-TOF analysis. The studies into the sample preparation protocols demonstrate their effectiveness and limitations. It was important to work with the oligonucleotides under varied types of experimental circumstances to identify any limitations for their analysis, and, from this information, develop experiments to address these limitations. Further experimentation has helped to identify the active component of the matrix molecule, thus increasing our understanding of the matrix/analyte interactions in the condensed phase. Collective observations have helped to explain the ineffectiveness of 6-HPA based on its substantially lower pKₐ value. It seems that the higher degree of dissociation for 6-HPA in solution decreases the degree of ionic bonding between the matrix and analyte at the carboxylic acid site of 6-HPA which has now been shown to be important in matrix activity. The 6-HPA performances as a matrix was improved by using the direct addition protocol for sample preparation seemingly because the direct addition protocol creates a saturated matrix/analyte system.

The next two chapters detail the explorations conducted into these limitations and the solutions offered to address them. Areas of interest for oligonucleotide improved analysis have been focused on the introduction of novel matrix additives and their effects on increasing the desorption/ionization efficiencies for the oligonucleotides. As will be demonstrated in the upcoming chapters, matrix additives can be used to enhance the MALDI-TOF experiment. A comprehensive study into how these additives interact with the oligonucleotides and enhance the matrix performance will be presented.
4.1 Introduction

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is now recognized as a powerful technique for the analysis of biomolecules, including peptides, proteins and nucleic acids (Gusev 1995; Limbach 1998). The analysis of proteins has been extremely successful using MALDI-MS. Unfortunately, the analysis of oligonucleotides has not been as successful. It is now well established that the primary limitation for MALDI-MS analysis of high molecular samples of oligonucleotides is molecular ion instability arising from nucleobase protonation (Wu 1993; Gut 1995; Kirpekar 1995; Nordhoff 1995; Zhu 1995; Gut 1997; Tang 1997; Tang 1997).

The current explanation for the limited molecular ion abundances of higher molecular weight oligonucleotides is that protonation of the nucleobases initiates base loss which leads to strand scission along the phosphodiester backbone (Nordhoff 1993; Zhu 1995). A plausible mechanism for nucleobase loss via protonation of the nucleobase and a more detailed discussion of the process was covered in Chapter 3. The propensity for base protonation correlates with the known proton affinities (PAs) of the four nucleobases, with guanine and adenine bases being the most likely sites of protonation and thymine being the least likely site of protonation. Indeed, ribonucleic acids (RNA) and deoxyribonucleic acid (DNA) with selected modifications form more stable ions upon MALDI-MS than does unmodified DNA. For example, no prompt fragment ions have been observed from poly-(T)$_n$ (polythymidylic acid) analyzed with a variety of matrices (Parr 1992; Schneider 1993; Tang, 1993; Tang 1993; Wu 1993; Tang 1997). 7-deaza analogs of guanosine and adenosine have been shown to result in reduced fragmentation in MALDI (Kirpekar 1995; Schneider 1995).
Based on the observations reported in Chapter 2 (sec. 2.4) from previous research on MALDI co-matrices, it appears that the success of organic base co-matrices can be attributed to their amino functionality. Thus, it would be logical to propose the introduction of co-matrices with multi-amino functional sites. Here, we have extended the prior studies on organic base co-matrices to include the polyamino organic bases spermidine and spermine (Figure 4.1). Mechref and Novotny previously reported the use of spermine as a co-matrix for the MALDI-MS analysis of acidic glyco-conjugates (Mechref 1998). They reported that spermine was highly effective at increasing ion abundance and signal resolution (via reduction of analyte cation adduction) with DHB as the matrix. Improved crystal formation as a result of mixing spermine with DHB was also observed. Recently, Asara and Allison reported similar results with spermidine during the analysis of oligonucleotides and metal-DNA complexes (Asara 1999).

The goal of this research was to test the effects of polyamines as possible co-matrices in the analysis of oligonucleotides. Spermine and spermidine were both used as co-matrices in various oligonucleotide:matrix mixtures. Results from the analysis of homopolymers and mixed-base oligonucleotides are reported. The influence of co-matrix:matrix mole ratio on the effectiveness of the polyamine as co-matrices was investigated. In addition, the solution-phase dissociation constants and gas-phase proton affinities of spermine and spermidine were determined to denote correlations between these properties and the effectiveness of each co-matrix at improving oligonucleotide analysis.

4.2 Experimental

4.2.1 Oligonucleotide Synthesis and Purification

The reagents for oligonucleotide synthesis were purchased from Perkin Elmer/Applied Biosystems (Foster City, CA). The oligonucleotides \(d\text{T}_{12}, d\text{T}_{14}, \text{dT}_{20}, \)
Figure 4.1 Structures of spermidine and spermine as the free amines. Both of these polyamines were used as MALDI co-matrices in this study.
dT$_{30}$ and d(TGAC)$_{3}$ were synthesized using standard phosphoramidite chemistry on 1-μmol columns using a Perkin Elmer/Applied Biosystems Model 394 DNA/RNA synthesizer. After synthesis, the deprotected oligonucleotides were purified using oligonucleotide purification cartridges (OPC) purchased from Perkin Elmer to separate out any failure sequences. Purification of the oligonucleotide was performed by retaining the terminal dimethoxytrityl (DMT) protected oligonucleotide on the OPC column in 10% aqueous ammonium hydroxide. The OPC was washed with ammonium hydroxide to remove any impurities. The DMT group is then removed using 3% trifluoroacetic acid and the deprotected oligonucleotide was eluted with 20% aqueous acetonitrile. The oligonucleotide collected from the OPC column was evaporated to dryness using a LabConco centrivap (Kansas City, MO). The lyophilized sample was re-dissolved in 100 μL of nanopure water. The concentrations of the stock solution of dT$_{12}$, dT$_{20}$, dT$_{30}$ and d(GACT)$_{3}$ were determined by UV-Vis spectrophotometry to be 190 μM, 298 μM, 340 μM and 78 μM, respectively. Sample solutions for MALDI were prepared by diluting the stock solution 100-fold.

**4.2.2 Sample Preparation**

2,4,6-trihydroxyacetophenone (THAP), 6-aza-2-thiothymine (ATT), 3-hydroxy-picolinic acid (3-HPA), spermidine, spermidine trihydrochloride, spermine, and spermine tetrahydrochloride were all purchased from Aldrich (Milwaukee, WI, USA). All of the chemicals were used as received without further purification.

THAP, ATT and 3-HPA were prepared at a concentration of 30 mM in nanopure water and as a saturated solution (.1g/mL w:v) in ethanol. The two co-matrices, spermine and spermidine, were prepared at a concentration of 30 mM in a 3:1 ethanol:water mixture and as saturated solutions (.1g/mL w:v) in nanopure water. The matrix, analyte and co-matrix were combined at various ratios as listed in the text.
After mixing, a 1 µL aliquot was spotted on the MALDI sample plate and allowed to air dry.

4.2.3 Titration of MALDI Co-Matrices

Spermidine and spermine were titrated following standard potentiometric methods. A 0.003 M solution of spermidine in nanopure water was titrated with a 0.09 N solution of aqueous hydrochloric acid. A 0.0025 M solution of spermine in nanopure water was titrated with a 0.01 N solution of aqueous hydrochloric acid. The titrant was added in 2.5 mL increments and the temperature during titration was held at 20 °C. The pKb values for spermidine and spermine were determined graphically.

4.2.4 Mass Spectrometry

The kinetic method experiments were performed using a Finnigan MAT 900 (Bremen, Germany) double focusing sector mass spectrometer. The ionization source utilized was a FAB source designed to deliver a 15 keV Cs+ ion beam at a neutral current of 15 µA. Spermidine and spermine were prepared with glycerol as the matrix. The reference bases used in these experiments were cytidine, adenosine and arginine. Typically 1 µL of polyamine and 1 µL of reference base were combined and spotted in glycerol on the FAB probe tip. The product ions analyzed from the proton-bound dimer were generated via metastable decay in the second field-free region of the instrument.

The MALDI-MS experiments were carried out using a PerSeptive Biosystems Inc. Voyager linear MALDI-TOF instrument (Farmingham, MA, USA) equipped with a nitrogen laser (λ=337 nm). All data reported here were collected at a laser setting of 460 which was empirically determined to be the threshold for ionization. Between 14 and 174 scans were averaged for each mass spectrum acquired. All of the data was collected in the negative ion mode. A two-point calibration was performed using dT12 and dT30.
4.3 Results and Discussion

4.3.1 Base Dissociation Constants for Spermine and Spermidine

To our knowledge, the pKb’s of spermine and spermidine have not been reported previously. Here, the pKb values are estimated using standard potentiometric techniques. As would be expected, several equivalence points for spermidine were observed. The spermine sample was not as straightforward, as only one equivalence point was found for this compound. The pKb’s for spermidine were determined graphically to be 2.74, 3.33, and 4.34. The pKb for spermine was determined to be 4.02. Figure 4.2 shows the titration curves and the 1st derivative inflection point curves generated from the data collected for both spermidine and spermine. The first derivative plot for equivalence point determination is overlaid on each titration curve. It was anticipated that several equivalence points would be observed for both compounds. The fact that only one equivalence point was determined for spermine suggests that either the potentiometric titration technique was not sensitive enough to resolve the closely overlapping neutralization events, or all of the amino sites have very similar dissociation constants.

The measurement of overall matrix-analyte solution pH as a result of spermine or spermidine addition revealed that the relatively high basicity of both spermidine and spermine can dramatically affect the overall pH of the matrix-analyte solution. For example, the pH value of 1000 µl samples of 0.3 µM d(GACT)_3 and THAP were individually measured. The pH of the d(GACT)_3 and THAP samples, prior to any mixing, were measured to be 5.87 and 4.04, respectively. The pH of the solution resulting from the addition of 300 µl of the THAP solution to the 1000 µl of d(GACT)_3 was measured as 4.38.
Figure 4.2 Titration curves with 1st derivative curve overlay for a) spermidine and b) spermine. All of the inflection points for spermine were not determinable from our measurement.
Subsequent addition of 300 µl of a dilute sample of spermidine (5 µl of a saturated stock solution was diluted in 1000 µl of nanopure water) caused the pH of the overall solution to increase to 6.93.

4.3.2 Apparent Proton Affinity of Spermidine

To confirm that the basic character of the polyamines seen in solution is also a characteristic of the gas-phase properties of these co-matrices, the apparent proton affinity of spermidine was determined using the kinetic method. Briefly, the kinetic method involves the formation of a proton-bound dimer between the analyte of interest and a reference base of known proton affinity (Cooks 1994). The proton-bound dimer is allowed to dissociate and the relative abundances of the protonated analyte and protonated reference base are used to calculate the relative proton affinity of the analyte see Cooks (1994) (Cooks 1994) for a more detailed discussion. Here, the reference compounds of known proton affinity utilized were cytosine (PA 234.8 kcal/mol), (Hunter 1998) adenosine (PA 236.4 kcal/mol), (Hunter 1998) and arginine (PA 242.0 kcal/mol) (Hunter 1998).

Figure 4.3 is a plot of the data collected from the kinetic method experiments for spermidine. The apparent proton affinity value is the x-intercept of this plot. As calculated from the linear regression fit, the apparent proton affinity of spermidine was found to be 240.5 kcal/mol. As initially suspected, the proton affinity for this polyamine is significantly higher than the proton affinities of other mono-functional bases, including imidazole (PA 225.2 kcal/mol), (Hunter 1998) piperidine (PA 228.0 kcal/mol) (Hunter 1998) and triethylamine (PA 234.7 kcal/mol), (Hunter 1998) which have been used as co-matrices for MALDI-TOFMS analysis of oligonucleotides previously (Simmons 1997; Simmons 1998). Although three distinct base
dissociation constants were found for spermidine during the solution-phase experiments, only a single apparent proton affinity value was determined using the kinetic method. As the kinetic method can be sensitive to multiple sites of protonation, (Cooks 1994) there is a possibility that the value obtained here represents the average of the three distinct sites of protonation available on spermidine. However, as the quality of the data obtained were high, and the error associated with this measurement is low (< 2 kcal/mol), it may be that the three distinct sites of protonation available in the gas phase have nearly equivalent enthalpy's of protonation.
Unfortunately, attempts to determine the apparent proton affinity of spermine using the kinetic method were unsuccessful for unknown reasons. Based on the similar chemical structure of spermine to spermidine and the resulting gas-phase behavior of spermine during MALDI-MS analysis of oligonucleotides (vide infra), it seems reasonable to assume that the apparent proton affinity of spermine is close to that value obtained for spermidine.

4.3.3 Effectiveness of Spermidine and Spermine as Co-Matrices

Figure 4.4 contains representative mass spectra obtained from the analysis of dT20 in the absence and presence of spermidine and spermine co-matrices. In Figure 4.4a, dT20 was analyzed using a 30 mM solution of THAP as the matrix with no co-matrix present. A broad molecular ion is detected in negative-ion mode. The presence of the [M–Thy]– peak is from a failure sequence obtained during the solid-phase synthesis of this sample which could not be removed during the OPC purification step.

As previously seen when monofunctional amine co-matrices were added, (Simmons 1997) the addition of spermidine (Figure 4.4b) or spermine (Figure 4.4c) results in a general improvement in the quality of the mass spectral data. In Figure 4.4b, a 1:1 (v:v) ratio of 30 mM solutions of THAP and spermidine were used for the characterization of the polythymidylic acid sample. A slight improvement in resolution of the molecular ion is found in this case.

The addition of spermine has a much more dramatic effect on the mass spectral data. As seen in Figure 4.4c, when a 1:1 (v:v) ratio of saturated solutions of THAP and spermine are used for the analysis of dT20, a significant improvement in the mass spectral resolution of the molecular ion is obtained. Unfortunately, under these experimental conditions, significant adduction of spermine to the polythymidylic acid sample is also detected. As will be discussed further below, spermine adducts are a common problem with this co-matrix. However, the overall trend seen with the
Figure 4.4 Negative-ion mode MALDI-TOF mass spectra of dT₂₀ analyzed with THAP as the matrix: a) no co-matrix added; b) spermidine trihydrochloride added as the co-matrix; c) spermine tetrachloride added as the co-matrix. Both co-matrices improved the mass spectral quality as assessed by improvements in molecular ion resolution and ion abundance. Spermine is more effective than spermidine, but typically yields adducts which interfere with the mass spectrometric characterization of the analyte. The low abundance [M-Thy]⁻ peak is due to a failure sequence which was not removed after solid-phase synthesis.
addition of the polyamine co-matrices matches that expected. Improved mass spectral data, as assessed by the resolution of the molecular ion and the signal-to-noise ratio, are obtained in the presence of these co-matrices. Similar results are seen during the analysis of dT30. It is well-known that polythymidylic acid is more resistant to protonation-induced fragmentation than other homopolymers or mixed-base oligonucleotides (Parr 1992; Schneider 1993; Tang 1993; Tang 1993; Wu 1993; Tang 1997). Thus, a more rigorous test of the effectiveness of the polyamine co-matrices requires analyzing mixed-base oligonucleotides in the absence and presence of these co-matrices. Figure 4.5a is a mixed-base oligodeoxynucleotide, d(GACT)3, analyzed using ATT as the matrix without the addition of a co-matrix (similar results are obtained using THAP and 3-HPA as the matrix). Similar to the results seen during the analysis of dT20 (Fig. 4.4), a broad molecular ion is detected in the absence of the co-matrix.

Figures 4.5b and 4.5c show the improved spectral resolution and increased ion abundance obtained for d(GACT)3 using saturated solutions of ATT with spermidine trihydrochloride and spermine tetrahydrochloride as co-matrices, respectively. With both co-matrices, a dramatic improvement in the resolution of the molecular ion is found, with spermine yielding the best resolution (m/Δm = 209). In both Figures 4.5b and 4.5c, a small ion signal due to loss of the cytosine base is seen. The amount, however, is minimal and cannot be compared to the case where no co-matrix was utilized, (Figure 4.5a) due to the lack of resolving power for the instrument.

Following the hypothesis put forth by Simmons and Limbach, (Simmons 1998) the polyamine co-matrices probably serve to improve the molecular ion stability of the oligonucleotide samples due to the higher proton affinity of the co-matrix as compared to the nucleotide residues (Green-Church 1999). In addition, as mentioned by Simmons and Limbach, the improved resolution could be due to improved desorption conditions which serve to cool the analytes during the MALDI event.
Figure 4.5 Negative-ion mode MALDI-TOF mass spectra of d(TGAC)₃ analyzed with ATT as the matrix: a) no co-matrix added; b) spermidine trihydrochloride added as the co-matrix; c) spermine tetrachloride added as the co-matrix. A dramatic improvement in the mass spectral data is found upon addition of the polyamine co-matrices.
There are two potential advantages to the use of these co-matrices compared to the monofunctional amine co-matrices previously investigated. The first is the higher proton affinity value of these polyamine co-matrices. From those investigated previously, triethylamine has the highest proton affinity (PA 234.7 kcal/mol). As noted above, we have determined that spermidine has a proton affinity which is significantly higher (PA 240.5 kcal/mol) and spermine is estimated to be of similar proton affinity. Thus, these co-matrices may be more thermodynamically favorable proton sinks during the proton-transfer reactions occurring in the laser desorbed plume in the MALDI event.

The second difference is the presence of multiple sites of protonation which are present on the polyamine co-matrices. These multiple sites of protonation may result in more favorable kinetics for the proton-transfer reaction. Alternatively, spermine and spermidine are known to have high affinities for the phosphodiester backbone in solution (Tabor 1984). This high solution-phase affinity may improve the desorption conditions, as the polyamine serves to carry away excess energy upon dissociation from the oligonucleotide after the desorption/ionization step.

4.3.4 Concentration Effects on Co-Matrix Performance

Once we confirmed that the polyamine co-matrices were effective at improving the mass spectral data during MALDI-MS analysis of oligonucleotides, we were interested in determining how the matrix:co-matrix mole ratio influenced the resulting data. Figure 4.6 shows the effect of varying the relative ratios of saturated solutions of co-matrix and THAP. In Figure 4.6a, the mole ratio of spermidine:THAP:dT_{20} is 10^{5}:10^{6}:1, while in Figure 4.6b the mole ratio of spermidine:THAP:dT_{20} is 10^{6}:10^{5}:1. Although a slight improvement in the molecular ion resolution is seen in Figure 4.6b, as compared to Figure 4.6a, the most noticeable effect upon addition of excess spermidine is the presence of spermidine adducts.
Figure 4.6 Negative-ion mode MALDI-TOF mass spectra of dT20 analyzed with THAP as the matrix: a) spermidine:THAP:analyte 10^5:10^6:1 mole ratio; b) spermidine:THAP:analyte 10^6:10^7:1 mole ratio.
Similar results were found for THAP:spermine hydrochloride mixtures. Figure 4.7a is the mass spectrum of dT20 analyzed at a spermine:THAP:dT20 mole ratio of 10^5:10^6:1. Although an abundant molecular ion for dT20 is detected, spermine adduct peaks are prevalent. Increasing the co-matrix:matrix mole ratio further exacerbates the adduction problem. In Figure 4.7b, the same mixture as in Figure 4.7a was analyzed at a spermine:THAP:dT20 mole ratio of 10^6:10^5:1. Not only is there a significant reduction in the relative abundance of the dT20 molecular ion, but the relative abundances of the spermine adduct peaks increase relative to the analyte molecular ion.

Thus, it appears that, unlike the monofunctional amine co-matrices, the polyamine co-matrices readily form adduct peaks with the oligonucleotide analyte when the amount of co-matrix becomes too high. Typically, minimal adduction for spermidine is seen when the mole ratio of co-matrix to matrix is similar, and the co-matrix is not in great excess to the analyte. Spermine is more problematic, and care must be taken to minimize adduction in this case by minimizing the amount of spermine added relative to the analyte. Whether these trends are specific to a particular combination of co-matrices and matrices, or are universal for other co-matrix and matrix combinations is beyond the scope of this study.

4.3.5 Effectiveness of Free Amine Form of Spermidine and Spermine as Co-matrices

The improvements found during MALDI-MS analysis of oligonucleotides by addition of polyamine co-matrices is not limited to the hydrochloride salts of spermidine and spermine. It was observed that an overall improvement in the mass spectral analysis of oligonucleotides is also found when the free-amine forms of spermidine and spermine were used. No significant differences were found in the sample handling steps for the spermidine or spermine salts or free-amines. The free amines did have a tendency to interfere with crystal formation in some cases.
Figure 4.7 Negative-ion mode MALDI-TOF mass spectra of dT₃₀, analyzed with THAP as the matrix: a) spermine:THAP:analyte 10⁴:10⁵:1 mole ratio; b) spermine:THAP:analyte 10⁶:10⁴:1 mole ratio. When the polyamine co-matrices are present in too high of excess relative to the analyte, significant adduction occurs.
Figure 4.8 Negative-ion mode analysis of dT_{14} with saturated THAP as the matrix: a) no co-matrix added; b) 1 μM of free amine form of spermidine added.
Figure 4.9 Negative-ion mode analysis of d(GACT)₃ with saturated THAP as the matrix: a) no co-matrix added; b) 1 μM of free amine form of spermidine added.
However, it was found that a 1000-fold dilution of the free-amine stock solutions prior to mixing with the analyte:matrix mixture helped improve the crystallization process. Figure 4.8 shows the results of dT_{14} with and without the presence of the free amine form of spermidine. As observed with the salt forms of these compounds, a dramatic improvement in ion abundance and ion resolution is observed.

Similar results are observed for the mixed base oligomer of d(GACT)_3 when the free amine form of spermidine is added to it as well (Figure 4.9). A saturated solution of THAP was used as the matrix for this run as well. Again there was some crystallization problems observed if too much of the free amine solution was used during the sample preparation.

4.4 Conclusion

The polyamine co-matrices, spermine and spermidine, are found to be effective at stabilizing oligonucleotide molecular ions during MALDI-MS. Addition of either co-matrix to the standard MALDI matrix:analyte solution improves signal resolution and molecular ion abundance. Spermine was found to be more effective than spermidine, but yielded adducts to the oligonucleotide molecular ion in many instances. The apparent proton affinity of spermidine was also determined via the kinetic method to be 240.5 kcal/mol. Thus, as was found with monofunctional amine organic base co-matrices, the addition of a co-matrix with a proton affinity higher than the proton affinities of the nucleotide residues reduces nucleobase protonation and improves molecular ion stability. Crystal formation is not inhibited by the presence of spermine or spermidine. The polyamine organic base co-matrices are easily incorporated into standard MALDI sample preparation protocols and should prove to be an effective means of improving MALDI-MS characterization of oligonucleotides.
CHAPTER 5. PEPTIDE NUCLEIC ACIDS AS NOVEL COMATRICES IN MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY: NUCLEOBASE INTERACTIONS VS. BACKBONE INTERACTIONS FOR OLIGOMER SIGNAL IMPROVEMENT

5.1 Introduction

Peptide nucleic acids were first synthesized by Berg et al. as peptide/DNA hybrids which were designed specifically to bind to complementary nucleic acid strands (Egholm 1992). PNAs were structurally designed with an uncharged backbone consisting of (2-aminoethyl) glycine repeat units. Attached to each of these glycine repeat units is an acetyl linker containing a nucleobase. PNAs are well documented in the literature and are being studied as possible antisense therapeutic agents (Kim 1992; Will 1995; Nielson 1997). The structures of two of the PNAs used in this study are shown in Figure 5.1 and Figure 5.2. The PNA in Fig. 5.1 is a typical PNA with four nucleotide base units (denoted by A, C, G, and T) attached. This PNA is referred to as just “PNA” in this dissertation. The second PNA (Fig. 5.2) is a relatively more complicated structure. Here a rhodamine pendent group has been attached to the 5’ end of the PNA backbone. This particular PNA is referred to as “PNA-Rho” in this chapter.

The uncharged backbone of PNAs and their high affinity for duplexing with complimentary nucleic acid strands via Watson-Crick, and to a lesser extent Hoogsten, binding makes them ideal candidates for matrix additives which can base-base interact with an oligonucleotide analyte. Here we show for the first time, co-matrices which can specifically interact with the nucleobase of the oligomer strand as opposed to the backbone interaction generally observed for co-matrices with the amine functionality.

It will be shown herein that base-base interaction does indeed result in increased ion abundances and resolution for a group of mixed base oligonucleotides.
Figure 5.1 PNA mixed-base tetramer structure.

Conditions optimal for PNA effectiveness as co-matrices along with some of their limitations are reported. The results for the PNA co-matrices were found to be comparable to that of spermidine during the MALDI analysis of the oligonucleotide analytes. A comparison between the two classes of co-matrices, backbone vs. nucleobase interactions, is conducted to determine which type of interaction yields the most improved signal quality.
Figure 5.2 PNA mixed-based tetramer with rhodamine pendant group.
5.2 Experimental

5.2.1 Oligonucleotide Synthesis and Purification

The reagents for oligonucleotide synthesis were purchased from Perkin Elmer/Applied Biosystems (Foster City, CA). All of the oligonucleotides were synthesized using standard phosphoramidite chemistry on 1-μmol columns using a Perkin Elmer/Applied Biosystems Model 394 DNA/RNA synthesizer. After synthesis, the deprotected oligonucleotides were purified using oligonucleotide purification cartridges (OPC) purchased from Perkin Elmer to separate out any failure sequences. Purification of the oligonucleotide was performed by retaining the terminal dimethoxytrityl (DMT) protected oligonucleotide on the OPC column in 10% aqueous ammonium hydroxide. The OPC was washed with ammonium hydroxide to remove any impurities. The DMT group is then removed using 3% trifluoroacetic acid and the deprotected oligonucleotide was eluted with 20% aqueous acetonitrile. The oligonucleotide collected from the OPC column was evaporated to dryness using a LabConco centrivap (Kansas City, MO). The lyophilized sample was redissolved in 100 μl of nanopure water. The concentration range of the stock solution of oligonucleotides was determined by UV-Vis spectrophotometry to be 2.94 x 10⁻⁴ M to 6.6 x 10⁻⁴ M.

5.2.2 Sample Preparation

The matrix used for these experiments was 3-hydroxypicolinic acid (3-HPA). Spermidine trihydrochloride was used as the oligomer backbone interaction co-matrix. Both of these reagents were purchased from Aldrich (Milwaukee, WI, USA). Peptide nucleic acids were used as oligomer nucleobase interaction co-matrices. All of the peptide nucleic acids were purchased from Perkin Elmer Biosystems (Framingham, MA.). All chemicals were used as received without further purification.

3-HPA was prepared at a concentration of .36 M in nanopure water. The solution was heated mildly to fully dissolve the matrix into solution and cooled to room
temperature. The spermidine trihydrochloride was prepared at 0.05 M, 0.005 M, and 0.0005 M concentrations in nanopure water. All of the PNAs were received as dehydrated samples at concentrations ranging from 1 μmol to 200 nmol. Each of these samples was dissolved into 1000 μL of nanopure water. The stock PNA solution was then divided into 100 μL fractions to be used as sample solutions.

Typically, the matrix, co-matrix, and analyte sample were all mixed at volumetric ratios of 1:1:1, where 1 μL of each component was mixed together in a 0.5 ml micro-centrifuge tube. In some cases, the analyte: matrix:co-matrix was mixed volumetrically at a 5:1:1 ratio.

The sample solution was spotted on the MALDI sample plate by two different methods. The first method involved the pre-mixing of all components together and then subsequent spotting of 1 μL of sample solution which was allowed to air-dry. The second method involved the mixing of the analyte and co-matrix together and subsequent spotting of 1 μL of the sample solution. Upon drying of the sample, 0.5 μL of the matrix was added directly on top of sample film and all air-dried. The first method will be referred to as "pre-addition" and the second method will be referred to as "post-addition". In both cases, crystal growth was generally adequate and similar for all samples.

5.2.3 Temperature Melt Curve Determination

Melting curves were generated for several of the PNA-DNA systems to demonstrate the occurrence of base-base pairing in the model systems. The sample melt temperatures ($T_m$) were measured using a Gilford spectrometer (Norwood, MA.). The samples were prepared at concentrations of $10^{-6}$ M, $10^{-5}$ M, and $10^{-4}$ M in 1-cm, 0.1-cm, and 0.01-cm cells, respectively. The absorbance measurements were collected at a temperature gradient of 5°C to 95°C at 0.5°C increments. A buffer solution of 10 mM sodium phosphate and 0.1 mM EDTA at pH 7.0 was used for the $T_m$ curve determination.
5.2.4 Mass spectrometry

The MALDI-MS experiments were carried out using a PerSeptive Biosystems Inc. Voyager linear MALDI-TOF instrument (Farmingham, MA, USA) equipped with a nitrogen laser ($\lambda=337$ nm). All data reported here were collected at a laser setting of 690-750 which was empirically determined to be the threshold range for ionization of the various samples. Between 60 and 128 scans were averaged for each mass spectrum acquired. Data was collected in both negative ion and positive ion mode. A two-point calibration was performed using $dT_{12}$ and $dT_{30}$.

5.3 Results and Discussion

5.3.1 Oligonucleotide/PNA Duplexing Determination

One of the benefits of using PNAs that specifically undergo base-base interactions with the target oligonucleotides as co-matrices is their reportedly high affinity for DNA strand duplexing (Nielsen 1991; Egholm 1992; Kim 1992; Betts 1995). Table 5.1 lists the series of synthesized oligonucleotides and the complimentary and non-complimentary PNA oligomers used in this study. The verification of any base-base interactions or duplexing between complimentary and non-complimentary strands of DNA and PNA was conducted by temperature melt experiments to support the notion that condensed phase DNA/PNA complexes were in fact forming.

If duplexing is occurring for the DNA/PNA systems, the $T_m$ curves should show an increase is hypochromicity (ca. >10%) due to base stacking as a function of decreasing temperature (Breslauer 1994). A typical $T_m$ curve generated for our complimentary strands of DNA and PNA is shown in Figure 5.3. The DNA/PNA sample is that of DNA-8mer/PNA-8mer(a). These two oligomers are complimentary strands and should demonstrate a high propensity for duplex formation. Indeed, the $T_m$ curve in Figure 5.3a shows the occurrence of duplexing for this system. Both of the first two cells (1.0 cm, 0.1 cm) show somewhat staple duplexing. The highest concentration sample (0.01 cm) gave a very unusual curve where over 35%
hypochromicity is observed, indicating a unusually high concentration of base stacking. The increased hypochromicity at the highest concentration cell was at first believed to be due to possible base pairing effects characteristic for this particular system. A closer look however at the pre-scan and post-scan absorbance data at 25 °C for the 0.01 cm cell indicates a 35% difference in absorbance values before and after the experiment. This difference indicates that a significant amount of sample evaporation had occurred during the experiment run. Hence, the concentration in the cell is changing and subsequently an increased absorbance is recorded. This event overshadows any duplexing which may be occurring in this cell. The other two cells however showed an evaporation of less than 10% which is the ideal scenario.

Table 5.1 List of names, nucleobase sequences and molecular weight for the series of oligonucleotides and PNAs.

<table>
<thead>
<tr>
<th>Oligomer Name</th>
<th>Oligomer Sequence (3' =&gt; 5')</th>
<th>Calc. mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-12mer</td>
<td>TGACTGACTGAC</td>
<td>3645.44</td>
</tr>
<tr>
<td>DNA-24mer</td>
<td>TGACTGACTGACTGACTGACTGAC</td>
<td>7352.84</td>
</tr>
<tr>
<td>DNA-8mer</td>
<td>TTGTCATT</td>
<td>2390.62</td>
</tr>
<tr>
<td>DNA-16mer</td>
<td>CGATCGATCGATCGAT</td>
<td>4881.24</td>
</tr>
<tr>
<td>DNA-16mer(b)</td>
<td>CGATCGATTTTTTTTTT</td>
<td>4843.2</td>
</tr>
<tr>
<td>DNA-16mer(c)</td>
<td>TCCCTCCCGATCGAT</td>
<td>4768.15</td>
</tr>
<tr>
<td>DNA-17mer</td>
<td>TCGATCGATCGATCGAT</td>
<td>5185.43</td>
</tr>
<tr>
<td>DNA-32mer</td>
<td>CGATCGATCGATCGATCGATCGATCGATCGATCGAT</td>
<td>9824.44</td>
</tr>
<tr>
<td>DNA-32mer(b)</td>
<td>ACTGACTGACTGACTGACTGACTGACTG</td>
<td>9824.44</td>
</tr>
<tr>
<td>PNA</td>
<td>ATCG</td>
<td>1102.07</td>
</tr>
<tr>
<td>PNA-Rho</td>
<td>ATCG-Rhodamine</td>
<td>1805.84</td>
</tr>
<tr>
<td>PNA-Lys</td>
<td>CAGT-Lysine</td>
<td>1375.41</td>
</tr>
<tr>
<td>PNA-8mer(a)</td>
<td>AATGACAA</td>
<td>2203.15</td>
</tr>
<tr>
<td>PNA-8mer(b)</td>
<td>ATCGATCG</td>
<td>2186.11</td>
</tr>
</tbody>
</table>
Figure 5.3  a) $T_m$ plot for complimentary DNA/PNA oligomers of DNA-8mer/PNA-8mer(a). Curve for 0.01 cm cell shows effects of sample evaporation during melting experiment. b) $T_m$ plot repeated for same system, without evaporation in 0.01 cm cell.
The 0.01-cm cell was repeated as a result of the increased evaporation and a more acceptable curve was generated for this cell. The new plot now indicates a consistent duplexing over a 100-fold concentration difference for this DNA/PNA system (Figure 5.3b).

The data collected for the complimentary strands of DNA-16mer(c) and PNA-8mer(b) was also supportive of strand duplexing for the DNA/PNA oligomers. Again some instability at the lowest concentration cell (1.0 cm) was observed and a lower percent of hypochromicity (ca. 8%) was observed for this system. The 0.1-cm and 0.01-cm cells, nevertheless, had very distinctive sigmoidal transitions which indicated possible duplexing.

The complimentary strands of DNA-32mer(b) and PNA-Lys gave slightly different Tm curves (Figure 5.4). The melting curve data collected for this system suggests that some base stacking may be occurring, but the paired bases are relatively unstable. This effect may be due to either the inability of the PNA-Lys to base pair with the DNA-32mer(b) due to possible oligonucleotide intramolecular base stacking (i.e., hairpin formation) or possible oligonucleotide backbone interactions with the lysine pendent group on the PNA. A look at a system similar to that in Figure 5.4a was conducted with DNA-32mer and PNA-Lys. Here we have a non-complimentary relationship between the two oligomers. The Tm curves generated for this system (Figure 5.4b) suggest that there is some degree of hypochromicity occurring which indicates base stacking. The fact that the two oligomers are non-complimentary suggest that the 32mer oligonucleotide may be base pairing with itself (intramolecular base pairing). It should be noted that the increased stability of the base pairing for this system is occurring and may be a direct result of the decreased base-base interactions between the DNA-32mer and the PNA-Lys. There is also the probability of DNA backbone interactions from the lysine pendent group of the PNA which may also help
Figure 5.4 a) T_m plot of complimentary strands DNA-32mer(b) and PNA-Lys. b) T_m plot of non-complimentary strands DNA-32mer and PNA-Lys.
stabilize any intramolecular duplexing which may be occurring within the PNA-32mer oligomer.

The $T_m$ data collected for the DNA/PNA systems studied supports the occurrence of duplexing for complimentary systems in the condensed phase. The correlation for this data to non-specific DNA/PNA complexing observed in the gas phase is difficult, if not impossible, to establish. There was, as will be demonstrated, some correlation to the effectiveness of the PNA as a co-matrix and its condensed phase relationship to the DNA oligomer. These correlations will be outlined in the upcoming sections.

5.3.2 Mass Spectral Analysis of Peptide Nucleic Acids (PNAs)

Mass spectral data for the PNAs was collected to establish an idea of just how amenable these molecules would be to MALDI analysis. The MALDI-TOF characterization of PNAs was first reported by Butler et al. using a variety of peptide and oligonucleotide matrices (Butler 1996). Figure 5.5 is the MALDI spectra of several of the PNA compounds used in this study. MALDI analysis of these compounds reveals that the PNAs have extremely high desorption/ionization efficiencies because of the high ion abundances commonly observed for these compounds. While all three of the PNAs shown in Figure 5.5 were acquired in negative-ion mode, comparable spectra were obtain for each of the PNAs in positive-ion mode as well. The strong negative-ion mode signal for the PNAs is unusual in the sense that upon inspection of the compound's structure, there appears to be a limited number of sites that can support the negative charge needed for negative-ion mode analysis. The source of the negative charge may come from the carbonyl groups in the PNA backbone. There appears to be little to no alkali cation adduction observed for the negative-ion mode MALDI analysis of these compounds, as would be expected due to the PNA's uncharged backbone. Commonly observed in the MALDI spectra generated for this study was the formation of PNA-PNA complexes ranging from dimers (Fig. 5.5b) to tetramers (Fig. 5.5c). Also observed, as will be
Figure 5.5 Negative-ion mode MALDI spectra of a) PNA-Rho, b) PNA-8mer(a) and c) PNA-Lys.
seen later, was the formation of DNA-PNA complexes. The PNA-Rho (Fig. 5.5a) was found not to form PNA-PNA and DNA-PNA complexes as readily as some of the other PNAs. This effect may be attributed to steric interference from the rhodamine pendent group. The two pendent groups used in this study introduced some interesting properties which may have played a part in the molecule’s overall performance as a co-matrix. These properties, along with the overall effectiveness of the PNAs as co-matrices, will be discussed in the upcoming sections. Significant correlations between the condensed-phase interactions and the gas-phase interactions of the DNA and PNA oligomers were also observed and will be addressed as well in the upcoming sections.

5.3.3 Use of Matrix Additives in MALDI Analysis of Oligonucleotides

The use of co-matrices to aid in the reduction of cation adducts and the increase in molecular ion abundances has been reported throughout the literature (Currie 1993; Pieles 1993; Leo 1998). Based on the type of compounds generally employed as co-matrices (i.e., compounds with an amine functionality), it could be proposed that the resultant interaction between the analyte oligomer and the co-matrix is through oligomer backbone interactions (Simmons 1998; Asara 1999; Vandell 1999). The co-matrix, subsequently aids the matrix which generally facilitates desorption and ionization of the analyte via energy and excited state proton transfer reactions post laser deposition onto the spotted sample (Cornett 1992; Gimon 1992; Chiarelli 1993; Amado 1997; Bencsura 1997; Karbach 1998). These events lead to the desorption/ionization event which yields the gas-phase ions generated during the experiment. The exact matrix structural affiliation with the oligonucleotide in solution is not known. Evidence presented in the case of several types of structurally different matrices would suggest that the structural affiliation between the oligomer and the matrix yields optimal MALDI signal when the matrix molecules align or interact with the oligonucleotide’s backbone (Tang 1993; Bencsura 1995; Tang 1997). The
instability of large oligonucleotide gas-phase ions generated during the MALDI event is partially attributed to the overall charge destabilization on the oligomer's backbone (Zhu 1995; Nelson 1996; Gut 1997). To offset this charge destabilization, counter ions (preferably hydrogen ions from the matrix) are picked up by the analyte. This process unfortunately, is also the driving force for the increased cation adduction characteristic to this particular class of analytes. Thus, it seems only logical to introduce matrix additives which can help in backbone charge stabilization by competing with alkali cations for counter-ion sites along the phosphodiester backbone of the oligonucleotide. As a result, the overall signal improvement observed with backbone interaction co-matrices has been consistent and turned out to be a logical approach to help stabilize the oligomer analyte during the complex desorption/ionization event.

Nucleobase interactions between the matrix and analyte are less well understood. A better understanding of the importance, if any, for nucleobase interactions should be established to help further elucidate the mechanisms for the desorption/ionization events. Hence it would seem an appropriate extension in co-matrix investigations to identify and test compounds that interact with the nucleobase component of the oligonucleotide.

To the best of our knowledge, there has been no report in the literature of co-matrices which specifically interact with the nucleobase of an oligonucleotide. The use of PNAs for this task stems from the exceptionally high base pairing affinity these compounds have for complimentary DNA oligomers (Nielsen 1991; Egholm 1992). The various oligonucleotides were tested with PNAs that were complimentary and non-complimentary to themselves to see if any improvements were observed for the oligonucleotide signal. A definite pattern of signal improvement was observed for DNA/PNA complimentary strand relationships.
The first examples of this effect can be seen in Figure 5.6. Here we show a comparison of DNA-8mer without any co-matrix present (Fig. 5.6a) and then with the complimentary PNA strand of PNA-8mer(a) added (Fig. 5.6b) at the same laser power for each sample. A dramatic decrease in salt adduction and an increase in mass resolution is observed. Also observed is the PNA molecular ion along with the PNA-DNA duplex molecular ion and the DNA dimer. Figure 5.6b demonstrates the effectiveness of the PNA as a co-matrix in a situation where every nucleobase of the oligonucleotide can be base paired with its complimentary base from the PNA strand.

Ideally, the PNA duplexing effect would be an even bigger asset if the PNA complimentary strand did not have to base pair with every base on the oligonucleotide strand to be effective. To test the importance of relative strand sizes for DNA/PNA systems, the oligomer of DNA-32mer was mixed with its strand compliment PNA-Rho. It should be noted that the number of the base units for the oligonucleotide is 8 times that of the PNA-Rho. Figure 5.7b and Figure 5.7a show the oligonucleotide with and without, respectively, the presence of the PNA-Rho as a co-matrix. An increase in ion abundance and overall signal enhancement is, again, observed with the co-matrix present. Similar results were observed for the systems of DNA-17mer/PNA-Rho, DNA-16mer/PNA-8mer(b) and DNA-32mer/PNA-8mer(b).

Even more interesting is the effect of the pendent groups on base-pairing interactions. MALDI studies conducted on PNA-Lys with its complimentary DNA oligomer of DNA-32mer(b) yielded unexpected results. The PNA co-matrix demonstrated little to no effect on the oligomer ion stability. The lack of effectiveness may be due to a competitive effect between the base pairing and backbone interactions occurring as a result of PNA-Lys structure. In some cases signal suppression for the DNA-32mer(b) was also observed. The melting curves generated for DNA-32mer(b)/PNA-Lys system (Fig. 5.5a) suggest that some duplexing seems to be
Figure 5.6 MALDI mass spectra of a) DNA-8mer and b) DNA-8mer after the addition of the complimentary strand of PNA-8mer(a) as a co-matrix.
Figure 5.7 MALDI mass spectra of a) DNA-32mer and b) DNA-32mer after the addition of complimentary strand of PNA-Rho.

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occurring between these two complimentary strands, but as stated earlier the base stacking appears to be very unstable. The duplexing behavior observed for this system may predominately be due to intramolecular base pairing within the DNA-32mer(b), which would hinder the PNA-Lys from base pairing and subsequently limit its effect as a co-matrix.

In a similar situation, the complimentary DNA/PNA system of DNA-16mer/PNA-Rho was tested. While this particular system seems to be capable of undergoing base-base interaction, the fact that the rhodamine pendent group present also introduces the possibility for backbone interaction which could ultimately interfere with base pairing interactions can not be discounted. MALDI analysis for this system showed signal enhancement for the oligonucleotide in the presence of the PNA suggesting that the competitive forces of intermolecular backbone vs. nucleobase interactions may not be a debilitating factor for some PNAs. Whether or not the effectiveness of PNAs which can undergo nucleobase interactions and/or backbone interactions is increased or decreased as a function of the type of pendent group present has not been determined yet.

5.3.4 Sample Preparation Effects

A closer look at the method of preparing the samples was also performed. Ideally, a sample preparation method that would increase the occurrence of base pairing for complimentary DNA/PNA systems would help to support the results collected up to this point. To increase the occurrence of base pairing interactions and compare to data collected thus far, the 3-HPA matrix was added in a "pre" and "post" addition fashion to the DNA/PNA samples under analysis. It is suggested here that due to possible ionic repulsion effects of the excess matrix molecules around the oligonucleotide during the mixing, spotting and drying process, the probability of the PNA/DNA complexing may increase substantially if the DNA and PNA are allowed to dry first and then the 3-HPA is added later (post-addition).
Further tests for base-base interaction effects between a PNA co-matrix and its complimentary DNA oligomer after post addition of the matrix were run. Here the effects of the sample preparation method are compared for optimal signal. The oligonucleotide DNA-17mer was tested with its complimentary strand PNA-Rho. It should also be pointed out that the base pairing effect for these two oligomers is not complete because the DNA-17mer has four times as many nucleobase units as the PNA-Rho. Figures 5.8b and 5.8a are the spectra of the DNA-17mer with and without the PNA co-matrix present, respectively. Both of these samples were prepared via the pre-addition of 3-HPA to the samples. Signal improvement is observed with the PNA present as a co-matrix. Also observed is the PNA-DNA duplex molecular ion (Fig. 5.8b). The post addition of 3-HPA to the same DNA/PNA system with and without the addition of PNA-Rho can be observed in Figure 5.9b and 5.9a, respectively. While some base loss is observed for the oligonucleotide, a dramatic increase in ion stability is observed. An increase in signal resolution and a reduction in cation adduction is also observed. Similar results were observed for the complimentary strand systems of DNA-32mer/PNA-8mer (b), DNA-32mer/PNA-Rho and DNA-16mer/PNA-8mer (b). Hence, post addition of the 3-HPA seemed to benefit the analyte/co-matrix relationship. This condition was a consistent factor throughout the entire study. As stated earlier in this text, PNAs have been reported to undergo a secondary binding function for DNA-PNA complexes known as Hoogsteen bonding (Kim 1992; Betts 1995; Nielson 1997). This particular bonding is not as likely to occur as that of Watson-Crick. While it can not be ruled out that Hoogsten interactions may account for some of the PNAs effectiveness as a co-matrix, it is not the aim of this report to determine whether or not either Hoogsten or Watson-Crick interactions are occurring. It will be assumed that the interactions for the complimentary strand systems is of a Watson-Crick nature and any occurrence of Hoogsten interactions will be to a much lesser extent.
Figure 5.8 MALDI mass spectra of a) DNA-17mer and b) DNA-17mer plus the addition of complimentary PNA-Rho. Both of these samples were prepared via the pre-addition of the matrix 3-HPA.
Figure 5.9 MALDI mass spectra of a) DNA-17mer and b) DNA-17mer with PNA-Rho. These samples were prepared via the post-addition of the matrix 3-HPA.
5.3.5 MALDI analysis of Non-complimentary DNA/PNA Systems

The effects demonstrated thus far from the introduction of complimentary strand PNA co-matrices to the oligonucleotide analyte systems suggest that base-base interactions can be important in the desorption/ionization event during the MALDI process.

A more direct approach to studying backbone interactions between the DNA and PNA strand is the introduction of pendent groups onto the PNA oligomer. Specifically, pendent groups which can ironically interact with the DNA backbone were used in this study. Pendent groups like rhodamine and lysine are attached to the 5' end of two of the PNA molecules via F-moc linker groups. Both of these pendent groups can carry a positive charge which can ultimately adduct with the oligonucleotide backbone. This effect now introduces new oligonucleotide interaction properties for the PNA molecule which can lead to the PNA working as a backbone interaction type co-matrix.

To test for PNA backbone interaction effects on signal enhancement, a DNA/PNA system which was of a non-complimentary nature was tested. The analysis of DNA-8mer and PNA-Rho was conducted. The negative-ion mode analysis of DNA-8mer with and without the addition of PNA-Rho, Figures 5.10b and 5.10a respectively, demonstrates a dramatic decrease in alkali cation adduction and increased ion abundances. Exactly one month later, the same sample was run in positive-ion mode. An increase in alkali cation concentration had resulted over the course of time (Figure 5.10c). The addition of PNA-Rho to this sample results again in the reduction of salt adduction and an increase in ion abundances (Figure 5.10d). All of the samples were prepared by post-addition of the matrix. Similar results were observed for PNA-Rho in the presence of DNA-12mer and DNA-24mer. Further support of the PNA-Rho undergoing oligonucleotide backbone interactions was gathered when both the DNA-
Figure 5.10 MALDI mass spectra of a) DNA-8mer b) DNA-8mer and PNA-Rho, c) DNA-8mer and d) DNA-8mer and PNA-Rho. Spectrum a and spectrum b were collected in the negative mode, while spectrum c and spectrum d were collected in the positive mode. All of the samples were prepared via post addition of the matrix 3-HPA.
12mer and DNA-24mer were tested with the non-complimentary co-matrix PNA. PNA is structurally the same as PNA-Rho minus the rhodamine pendent group. The results of these runs showed no obvious signal enhancement for the oligonucleotide in the presence of PNA as a co-matrix. Because PNA did not interact with either the nucleobase or the backbone of the oligonucleotide and subsequently no signal improvement resulted, it appears that both of these interactions are extremely important factors for co-matrix effectiveness.

Another case which demonstrates the effects of pendent group backbone interaction was that of the non-complimentary DNA/PNA relationship of DNA-8mer and PNA-Lys. Again it was observed that the presence of PNA-Lys helped increase ion abundances and reduce cation adduction. The non-complimentary relationships for the two oligomers would suggest that backbones interactions may be the influencing factor. Melting curve analysis of DNA-8mer/PNA-Lys yielded T_m curves that indicated a non-duplexing system thus suggesting that PNA-Lys is not base-base interacting with the oligonucleotide, but instead is probably interacting with the DNA backbone. The non-complimentary system of DNA-32mer(b) and PNA-Rho also yielded MALDI results similar to the ones reported above, where an increase in ion abundance is observed for the oligonucleotide in the presence of the PNA-Rho co-matrix.

5.3.6 Temperature Dependence for PNA Effectiveness as a Co-Matrix

In an effort to further probe the base-base interactions and subsequent increased ion stability effects observed for the PNA co-matrices, temperature variations were introduced to see if PNA effectiveness is enhanced or decreased as a function of temperature. Duplexing should increased for complimentary systems as a function of lowered temperatures. All of the samples were prepared via the "pre" and "post" addition of matrix for this set of experiments. Various DNA/PNA solutions were prepared at room temperature. An aliquot of the sample was withdrawn and
spotted on the MALDI plate. The remainder of the sample was placed in an ice bath, held at a temperature of 5 °C, for several minutes and were also spotted on the MALDI plate. A comparison of data collected for each sample at 25 °C and 5 °C showed a remarkable increase in ion abundance and signal resolution as the temperature is lowered for the complimentary DNA/PNA system. This improvement in co-matrix performance at low temperatures was consistent for all of the DNA/PNA complimentary strand systems. Figure 5.11a shows the oligonucleotide of DNA-8mer at 25 °C. The addition of the PNA complimentary strand of PNA-8mer(a) again shows a dramatic improvement on oligonucleotide signal at 25 °C (Fig. 5.11b). At the lowered temperature of 5 °C an even better signal enhancement effect is observed for the oligonucleotide with the addition of PNA-8mer(a) (Fig. 5.11c). All of the samples shown here were prepared via the post-addition of the matrix. Similar results were observed for the complimentary systems of DNA-32mer/PNA-8mer(b), DNA-32mer/PNA-Rho, DNA-16mer(c)/PNA-Rho and DNA-16mer/PNA-Rho. This data further suggest that base-base interactions for DNA-PNA complexes is important for co-matrix effectiveness and can aid in overall analyte ion stability. Also observed during this set of experiments was an increase in DNA-PNA ion abundances at the lower temperature, which suggest that not only was the occurrence of duplexing in the condensed phase increased at the lowered temperatures, but the condensed phase conditions are apparently carried over into the gas phase.

The effects of lowered temperatures on non-complimentary systems was tested as well. The non-complimentary systems of DNA-32mer(b)/PNA 8mer(b) and DNA-32mer(b)/PNA-Rho were both tested at 25 °C and 5 °C. The MALDI data showed no substantial improvements on oligonucleotide signal in the presence of the PNA co-matrix at the lowered temperature. Analysis of DNA-32mer(b)/PNA-Rho where the co-matrix can undergo both oligonucleotide backbone and nucleobase interactions, showed no dramatic increase in analyte ion stability at either temperature as well.
Figure 5.11 MALDI mass spectra of a) DNA-8mer, b) DNA-8mer with the addition of PNA-8mer(a) at 25 °C and c) DNA-8mer with the addition of PNA-8mer(a) at 5 °C. All of the samples were prepared via post addition of the matrix 3-HPA.
Figure 5.12 MALDI mass spectra of a) DNA-8mer, b) DNA-8mer with the addition of PNA-Rho and c) DNA-8mer with the addition of 0.005 M spermidine.
Several of the complimentary and non-complimentary DNA/PNA systems were tested at temperatures above 25 °C. Temperature studies were conducted at 25 °C, 37 °C, 58 °C, and 83 °C. No signal improvement compared to data collected at 25 °C was observed for any of the oligonucleotides in the presence of the PNA co-matrices at any of these higher temperatures. In fact, the co-matrices were observed to become more ineffective at the higher temperatures.

5.3.7 Comparison of Spermidine and PNA as Co-Matrices

A look at whether a co-matrix is more effective based on it's interaction with the backbone or the nucleobase of the oligonucleotide is an interesting comparison which may offer some insight into the desorption/ionization process. The most obvious approach for this investigation is to look at the effects of spermidine relative to several of the PNAs and note relative signal enhancement of one class of co-matrix relative to another.

Figure 5.12a shows the oligonucleotide analyte of DNA-8mer without any co-matrix additives. The addition of PNA-8mer(a) (complimentary strand) and spermidine (Figs. 5.12b and 5.12c respectively) demonstrated different levels of matrix effectiveness. It is apparent that the spermidine has the greatest effect on the system. This observation would suggest that backbone interaction may be the most important factor needed for a co-matrix to be optimally effective. The data presented here for base-base interactions can not be discounted however, and may offer some alternatives as a co-matrix. Results similar to the one discussed above were observed for the DNA/PNA systems of DNA-16mer(c)/PNA-Rho, and DNA-17mer/PNA-Rho. It should be noted that all of these samples were tested with "pre" and "post" addition of the matrix and the latter sample preparation method offered the best results for this set of experiments in the case of both PNAs and spermidine co-matrix addition. There was also no temperature dependence observed for the effectiveness of spermidine.
5.4 Conclusion

The effects of a new class of co-matrices demonstrated here introduces more possibilities for MALDI sample preparation protocols. The increased gas-phase ion stability of an analyte due to co-matrix interactions with either the analyte's backbone or nucleobases has been shown here to be an important factor in co-matrix effectiveness in MALDI experiments. It seems that both types of interactions are important and can benefit the desorption/ionization event given the right conditions. Optimal performance for a co-matrix can be accomplished as a function of many parameters like temperature and sample preparation methods. Backbone interactions seem to be the most crucial in obtaining substantially increased gas-phase ion stability. However, the base-base interactions can also play an important role in increase gas phase ion stability with the extent of this increase relevant to the type of oligonucleotides being analyzed. By far the use of PNAs as co-matrices has yet to be fully investigated. Future studies into base-base interacting co-matrix compounds may lead to a whole new class of very effective co-matrices for MALDI experiments.
CHAPTER 6. CONCLUSIONS

6.1 Summary

The analysis of oligonucleotides using MALDI-TOF mass spectrometry is not as straightforward as the analysis of peptides and proteins. Problematic areas like gas phase ion instability which leads to oligonucleotide fragmentation, low desorption/ionization efficiencies, and high mass analysis limitations makes this area an active center of much research. Many research groups have targeted these problem areas with efforts to overcome the limitations of oligonucleotide analysis using MALDI. Some of their approaches involve improving sample preparation, identifying more effective matrices, use of matrix additives and instrumentation modifications.

In our lab many experiments were designed to probe the MALDI oligonucleotide analysis process. The solution-phase matrix/analyte interactions were studied to gain a better understanding of the chemistry between the matrix and analyte prior to the desorption/ionization event. As a result of my initial studies into the chemistry of the matrix/analyte system, concepts and research plans were formulated to address the issues of low desorption/ionization efficiencies for oligonucleotide analysis. The sets of experiments designed and discussed herein were developed with the intent to offer solutions that help increase the desorption/ionization efficiencies for a set of model oligonucleotides. Strategies that were employed to address low desorption/ionization efficiencies were 1) development of improved sample protocols, 2) the identification of matrix active site which facilitates the desorption/ionization event and 3) the introduction of novel matrix additives.

The way a MALDI sample is prepared can dramatically affects the quality of the MALDI signal acquired during an experiment. Sample protocols which increased solution-phase interactions between the matrix and analyte were shown to improve the spectral quality tremendously. These improved preparation protocols were used to
enhance the matrix activity of compound which is typically ineffective using the standard sample preparation protocols.

The chemical interactions between the matrix and analyte in the condensed phase are believed to be a key component for the matrix to facilitate the desorption of the analyte when laser energy is absorbed by the matrix. The nature of these interactions are still not fully understood. The titration experiments discussed herein have offered a reasonable model to define the bonding nature between matrix/analyte complexes believed to be formed in the condensed phase. The data collected from the titration experiments demonstrates that the active matrix species is the acid form of the matrix [HA] and not it’s conjugate base [A']. Increased concentrations of the conjugate base form leads to matrix activity suppression. A better understanding of why matrices that are weak acids work better for oligonucleotide analysis has been established from the data put forth here.

The addition of novel matrix additives which can help increase the efficiencies of the desorption/ionization event was also discussed herein. The solution-phase chemical interactions between the matrix and analyte can be both desired and detrimental to the MALDI process. It is believed that certain undesirable interactions can be disrupted by the introduction of co-matrices. Two strategies employed by our lab to optimize the effects of co-matrices were the introduction of polyamine type co-matrices (spermidine and spermine) and the use of compounds that interact with the oligonucleotide bases (PNAs). Here it was demonstrated that both of these methodologies offer great potential, with further development, as new emerging classes of MALDI co-matrices.

6.2 Future work

The titration experimental data which suggested that the acidic species of the matrix molecule is the active form of the matrix opens the door for the exploration of other molecules that meet the specific criteria of having low acid dissociation rate.
Perhaps compound can be tested that contain functional group which prevent the
dissociation of this acidic proton. Of course, the other matrix criteria have to be
followed as well, thus any modified matrix compounds should still be able to absorb
in the UV and crystallize out of an evaporating solution.

The co-matrix work has opened the door for the development of new classes
of MALDI co-matrices. Chemical modifications to both spermine and spermidine
(e.g., polymerizations) may increase their effectiveness. Hybrid molecules which
incorporate the a UV absorption center into the polyamine chain may create a
compound which has the best of both matrix and co-matrix worlds for MALDI
analysis.

The interactions of the PNAs with the oligonucleotide is interesting and may
offer the potential for selective ion signal enhancement during mixture analysis. The
PNA work is still very new and further studies identifying their effectiveness and their
limitations needs to be conducted. Other avenues to pursue is the synthesis of
specialized PNAs with pendant groups that interact with the oligomer nucleobase and
or backbone in a more highly specific manner than the groups discussed in this
dissertation.
PART B:
ELECTROSPRAY IONIZATION (ESI) STUDIES
OF METALLOPORPHYRINS
CHAPTER 7. ELECTROSPRAY IONIZATION (ESI): BACKGROUND AND OVERVIEW.

7.1 The Phenomenon of Macroion Molecular Beams

The phenomenon of producing a spray of charged droplets by flowing a solution through an applied electric field (typically 3-8 kV) was first reported two centuries ago by Bose around 1784 (Hayati 1987). Further development of this phenomenon was conducted by Dole et al. in the 1960s (Dole 1968; Mack 1970). Dole’s group was interested in finding alternative ways to volatilize macromolecules so as to study their gas-phase ions. Unfortunately, most macromolecules would degrade before they could be volatilized into the gas phase. Recalling the possibility of producing gas-phase ions by generating an electrosprayed solution from a steel needle via an applied electrical potential led Dole and his group towards the development of the process for macroion molecular beam production. The initial investigations reported by Dole et al. involved a series of experiments which were geared towards producing an in vacuo beam of ions by electrospraying a dilute solution of polystyrene molecules into a heated gas bath of nitrogen at atmospheric pressure (Dole 1968). The beam of ions produced from the electrospray process were concentrated and focused using a nozzle-skimmer system. The beam of ions were then sampled by a faraday cage to measure the faradaic current produced of the beam. The faraday cage was tuned to repel smaller ions (i.e., solvent ions) so that the measured current would be attributed solely to that of the gas-phase macroions (i.e., polystyrene).

7.2 Initial Observations of Essential Experimental Parameters for the Electrospray Process

Dole et al.’s initial observations of the electrospray process allowed them to establish certain criteria critical to the electrospray process. The formation of the charged droplet and subsequent evaporation of this droplet to produce successively smaller charged droplets was one of the primary focuses for their study. They noted that as the droplet shrinks (i.e., solvent evaporation) it becomes more and more...
unstable. The point at which the surface charge on the droplet exceeds the droplet's surface tension causing the droplet to break apart into smaller droplets is known as the Rayleigh instability limit (Rayleigh 1882). This limit was calculated to occur at alpha (α) equals 4 in equation 7.1, where q equals the charge in Coloumbs

\[ \alpha = \frac{q^3}{(3Vye)} \]  

7.1

on the droplet, V is the volume of droplet in m³, γ is the surface tension on droplet in N m⁻³, and ε represents the dielectric constant of the solvent. Further evaluation of the validity of α was conducted by Ryce and Patriarche who found that α at a value of 4 gives the most stable state for droplet disintegration into 4 individual droplets (Ryce 1965). An important factor is that the charge needs to remain on the droplet throughout the process.

Dole et al. also recognized that parameters like solution flow rate, analyte concentration, and needle potential all play an important role in the overall electrospray process. These parameters were adjusted accordingly to get the maximum gain in faradaic current generated for the macroion beam. Hayati et al. who later investigated the mechanism of the electrospray process reported that certain experimental parameters do indeed affect the electrostatic atomization process. These parameters were: liquid flow rate, applied potential, field geometry, electrical conductivity, viscosity, surface tension and dielectric constant of the solvent system used (Hayati 1987).

7.3 Formation of Gas Phase Ions from Charged Droplets

Further studies into the electrospray process were conducted by Dole et al. into the overall process and possible application for the process (Mack 1970). Dole sought to understand the transition of the condensed-phase ions to the gas phase through further
experimentation. Dole proposed a model for the production of gas phase ions which explained the phenomenon as a product of Coloumbic explosions at the Rayleigh limit for an initial droplet. By this proposed mechanism, the initial droplet with x amount of analyte molecules reaches a critical radius, via evaporation, at which the surface charge of the droplet exceeds the surface tension of the solvent. The repulsive forces of the excess surface charges cause the droplet to break apart into smaller droplets. This process continually occurs until a charged droplet with one analyte molecule is produced. The last bit of solvent is evaporated from this droplet and the single analyte molecule is charged and promoted to the gas phase.

This model was generally accepted until further investigations by Iribarne and Thomson were reported in 1975 (Iribarne 1976). The additional studies into the electrospray process conducted by Iribarne and Thomson produced important data which helped to increase the understanding of the process and the critical parameters associated with it's success. Iribarne and Thomson conducted experiments aimed at understanding the mechanism of the charged droplet formation and the subsequent formation of gas-phase ions from these droplets (Iribarne 1976). Their method of ion formation was different from Dole et al. in that a solution containing an ionized analyte was atomized by the cross flow of a high velocity gas. The total charge of the droplets was enhanced by the applied voltage to an induction electrode which polarizes the charge distribution in the droplet. The result is the formation of a mixture of droplets with opposite polarities. The increased population of one type of ion over the other was determined by the polarity of the induction electrode. This particular method for ion generation was later adopted by Bruins et al. and was termed ion spray (Bruins 1987).

From the Iribarne and Thomson studies a model for ion formation was proposed which suggested that analyte ions are desired from the droplet surface as the applied electric field is increased and the surface charge on the droplet increases. A critical
radius, at which the droplet surface charge is great enough and the droplet radius is small enough to permit ion evaporation, has to be reached before the Rayleigh instability limit is reached and the droplet is disrupted. The ion desorption model is now the generally accepted model over that proposed by Dole et al.

7.4 The Development of Electrospray Ionization for Mass Spectrometry

The coupling of ESI with mass spectrometry is attributed to Fenn et al. who conducted a series of investigations into the parameters associated with the charged droplet formation and the sampling of these ions by mass spectrometry (Yamashita 1984; Yamashita 1984; Whitehouse 1985; Fenn 1989; Fenn 1993). Experimentation with the same set-up used by Dole et al. lead Fenn's group to investigating a way to definitively identify the macroions generated via electrospray by their mass. They started out analyzing small molecules using a quadrupole mass analyzer with a maximum mass range of about 450. Their primary objective was to study the ion beam generated and the mechanism associated with this process. Fenn et al. observed that the droplet in the absence of any applied potential would form at the tip of the needle and then drop off at certain point due to gravitational forces. When an increasing potential is applied to the needle the droplet gains a horizontal component and is drawn toward the end plate (usually held close to ground). At higher applied potentials, the liquid flowing through the needle takes on an elongated shape toward the end plate. This elongation is known as the Taylor cone which results from the destabilization of the flowing liquid which is drawn outward by the influence of a decreasing potential field (Taylor 1964). Fenn reported that the type of solvent, the shape and finish of the needle or capillary, and the pressure of the ambient desolvation gas affects the formation of the Taylor cone at the tip of the capillary.

The solvent systems used by Fenn were predominately volatile because it was observed that the vapor pressure of water was too high to be fully evaporated away in the time necessary to desolvate the ions and feed them through the nozzle-skimmer.
into the high vacuum region of the mass spectrometer. Solutions consisting of 50:50 (v:v) methanol:water were used in most of their experiments. Fenn also noted that the current generated from the electrospray could be increased with the addition of electrolytes such as LiCl, (NH₄)₂CO₃, and (CH₃)₄NI.

Fenn and Yamashita offered supporting evidence to Iribarne and Thomson’s model for ion formation by measuring the faradaic current from the ion beam, for an analyte of known concentration, at the end plate. From this they were able to calculate the number of elementary charges corresponding to this current. The mass flux of the analyte was found to exceed that of the charge flux calculated for the ion current. From this calculation measurement they discounted Dole’s theory of successive charged droplet formation down to a single analyte molecule per droplet.

Probably the most important development by Fenn and Yamashita for the electrospray process was the application of the high voltage to the source of the liquid flow. This resulted in the generation of all the ions of the same charge based on the polarity of the applied electrical potential. This method of atomization of the analyte molecules was also shown to be beneficial because it allowed for the control of the droplet charging process by adjustment of the applied voltage.

Several small non-volatile, thermally labile molecules were mass identified in the Fenn instrument. These analyte species were the protonated (M+H)⁺, cation adduct (M+Cation)⁺, and dimers (2M+H)⁺. This particular study was geared at identifying positive ion species.

Other studies reported by Fenn and Yamishita address the mass spectral analysis of negative ions via electrospray (Yamashita 1984). One addition to the instrumental set-up was the introduction of a nebulization gas (i.e., oxygen). The production of negative ions resulted in an increase in corona discharge at much lower ion currents than was observed for positive-ion mode. Oxygen acts as an electron scavenger and suppresses the tendency for corona discharge. Fenn also demonstrated the benefits of
interfacing the electrospray ionization source to a liquid chromatographic system (Whitehouse 1985).

7.5 Further Elucidations of the Mechanisms Governing the Ion Formation Process

Additional studies into the mechanism of charged droplet formation and subsequent ion desorption were later reported by other research groups (Hayati 1987; Ikonomou 1990; Blades 1991; Ikonomou 1991). These groups all further defined the phenomenon of charge droplet formation as an electrophoretic process via charge balancing of ions opposite in polarity to that of the applied field. This hypothesis is well accepted in conjunction with that put forth by Iribarne and Thomson.

Electrophoretic charging occurs when the positive and negative ions in the solution are caused to partially separate due to the applied electric field. Ions of the same polarity as the applied potential migrate towards the liquid surface junction within the capillary. This phenomenon causes an excess ion of the same charge to build up on the surface of the liquid and subsequently be pulled out towards the tip of the capillary into a Taylor cone. Ions of the opposite charge migrate back into the solution away from the needle tip. The ions which migrate away from the needle tip can eventually generate an imposed field which will lower the overall net charge of the solution. The imposed field, if strong enough, can suppress the ion current and hinder the electrospray process. The fact that the electrospray process can continue under the proper conditions suggest that this imposed field is somehow disrupted by charge balancing (Ikonomou 1991). Figure 7.1 illustrates the electrophoretic ion production process observed for electrospray.

The charge balancing process for the ions which migrate away from the needle tip is believed to result from either a chemical or electrochemical process. The ions generated from either of these processes are the molecular cation ($M^+$) or molecular anion ($M^-$) for oxidation or reduction, respectively. The electrochemical process is thought to be the case based on studies by Blades et al. and Ikonomou et al. in which
redox generated ions (e.g., iron and zinc) from the capillaries used in their studies were observed in the mass spectra (Ikonomou 1990; Blades 1991; Ikonomou 1991). The electrochemical oxidation (for positive-ion mode analysis) of ions migrating away from the tip of the capillary enables the pumping of electrons back to the metal capillary to maintain the ion current for the system.

![Diagram](image)

**Figure 7.1** Electrophoretic ion generation from the electrospray capillary. A positive applied potential is inferred due to the migration of positive ions towards the capillary tip. Oxidation of the negative ions occurs at the liquid-metal interface.

Kebarle refers to the electrospray device as an electrolytic cell of a “somewhat special kind”. The cell is considered special because ion transport occurs in the gas phase as well as the solution phase. From the work of Blades and Ikonomou it was shown now that not only could the electrospray process transport preformed ions to the gas phase, but also actually generate ions via a redox mechanism. Their work allows for the labeling of the electrospray source as a “true ionization source”.

### 7.6 The Analysis of Metalloporphyrins Using ESI in the Redox Mode

The ionization of large biological macromolecules is very amenable to the ESI process due to the ionizability of these molecules in solution prior to introduction to
the applied electrical field. The Lowry-Brønsted acid/base chemistry of biological molecules works to the advantage of ESI and is initially the grounds on which ESI was developed. The performance of the ESI source under these conditions, where preformed ions in the condensed phase are promoted to the gas phase, does not qualify it to be a "true" ionization source. Nevertheless the technique is extremely successful for the mass spectral analysis of preformed in the solution phase.

The fact that the ESI source can be operated in the redox mode allows for the mass spectral analysis of a new class of compounds which are non-polar, thermally labile and non-volatile. Certain criteria, however, for the redox mode operation of the ESI source have to be met to actually analyze this class of molecules. Of particular interest to this report is the redox mode ESI analysis of metalloporphyrins. Chapter 8 will focus on the ESI mass spectral analysis of metalloporphyrins and conditions necessary to achieve the optimum ion signal for this class of compounds. Several model porphyrin systems will be studied using ESI-MS and their behavior will be reported in Chapters 8 and 9.
8.1 Introduction

8.1.1 Mass Spectral analysis of Metalloporphyrins: An Overview

Metalloporphyrins are of great importance to many industrial processes in the areas of biology, catalysis and geology, among others. Metalloporphyrins have been analyzed using a variety of mass spectrometric methods including electron ionization (EI), (Jackson 1965; Shaw 1978) chemical ionization (CI), (Shaw 1981; Wolff 1984; Evershed 1985; Tolf 1986; Van Berkel 1989; Van Berkel 1990) fast atom bombardment, (Kurlansik 1983; Naylor 1990; Naylor 1990; Naylor 1992) plasma desorption, (Hunt 1981; Chait 1984) thermospray, (Blakley 1983) laser desorption, [Brown, 1986 #34; Forest, 1989 #105; Dale, 1996 #106; Irikura, 1991 #31] field desorption, (Schronk 1982) atmospheric pressure chemical ionization,(Mele 1996) and electrospray ionization (ESI) (Van Berkel 1991; Van Berkel 1992; Van Berkel 1993). Traditionally, EI and CI are useful for structural elucidation studies but complicate the analysis of mixtures as they produce mass spectra containing a large number of fragment ions. The remaining ionization methods generally produce a high abundance of molecular ions with little fragmentation, and, thus, are useful for identification purposes. Of all the ionization methods mentioned above, electrospray ionization has been shown to offer the greatest potential as a selective ionization process which can be utilized in the mixture analysis of metalloporphyrins.

Van Berkel et al. were the first to report the electrospray ionization analysis of porphyrins (Van Berkel 1991). In that work, the ionization of various porphyrins with and without metal centers was accomplished using a variety of protic and aprotic solvents. A general ionization pattern was observed for the porphyrins in the ESI source. It was observed that the free base porphyrins yielded a protonated species (M+H)+ while the metalloporphyrins yielded 1) the protonated species, 2) the
molecular cation species ($M^+$), and 3) the alkali metal adduct species ($M+Na)^+$
depending on the type of solvent used. Based on these observations other
compounds which were non-polar and thermally labile were tested by Van Berkel et
al. to determine the circumstances surrounding the ionization event (Van Berkel 1994;
Van Berkel 1995). Compounds like metallocenes and polycyclic aromatic
hydrocarbons (PAHs) were found to also be amenable to ES ionization under the
proper source conditions.

The method of ionization for the compounds studied by Van Berkel was a key
focus of his research. The transfer of preformed ions from the solution phase to the
gas phase had been well established as viable route for the ESI analysis of compounds
which could be ionized in solution. This method of ionization will be referred to as
chemical ionization to distinguish this process from the other alternative ionization
process which occurs in the ESI source known as electrochemical ionization.

The ability of the ESI source to ionize compounds which are not ionizable in
solution was not as fully understood or utilized as a means to analyze non-polar
compounds. Ikonomou et al. had already proposed that electrochemical reactions
were more than likely occurring in the ESI source during charge balancing (Ikonomou
1991). This method of ESI source ionization was investigated as a means to actually
ionize analytes in the ESI source. The operation of the ESI source for electrochemical
ionization is sometimes referred to as redox mode. Van Berkel observed that chemical
and electrochemical ionization of compounds was indeed possible under the proper
source conditions (Van Berkel 1991; Van Berkel 1992; Van Berkel 1994). It was
demonstrated that a charge-transfer reagent (e.g., trifluoroacetic acid (TFA), 2,3-
dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and antimony pentafluoride) assisted
if facilitating the chemical and electrochemical oxidation process in the ESI source
(Van Berkel 1994). Other researchers have also reported similar observations and

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subsequently have analyzed many compounds using ESI (Hiraoka 1992; Bond 1995; Caldas 1995; Xu 1996).

8.1.2 Research Objectives for Porphyrin Analysis

The mass spectral analysis of a series of model porphyrins was conducted by our lab to establish their behavior in our electrospray source. Of interest to our lab is the ability of our ESI source to electrochemically ionize our model porphyrins. The ESI source used for these experiments was designed by Analytica and is of a “non-conventional” arrangement in that the electrical configuration is reversed from that of conventional ESI sources. Our ESI has the applied potential on the end plate and the needle is held at ground.

This unique ESI source arrangement has drawbacks for redox mode ionization because of the decreased time for analyte exposure to the high electric field. A direct correlation for the time the analyte experiences the applied electric field can be expressed by the solution flow rate. Equation 8.1 gives a general relationship between ion current (I) and flow rate (FR): where m is a constant.

\[ I \propto (FR)^m \]  

Thus, it would be assumed that the source used for our analysis may yield lower ion currents due to the decreased time of analyte exposure to the applied electric field. To offset this drawback, it may be necessary to operate our source at higher flow rates than those used for the conventional sources.

The performance of our non-conventional source was evaluated for analysis of the model porphyrin systems. It was hypothesized that the non-conventional source would facilitate both chemical and electrochemical ionization of the model porphyrins. The conditions for one type of ionization versus the other were determined. It was believed that under the right source conditions, one type of ionization process could be promoted over another in the non-conventional source. Porphyrin ionization is the single most important aspect of the study being conducted, for without it no mass
spectrum would be attainable. It was observed that the porphyrins were, in fact, ionizable in the ESI source chemically via protonation of the porphyrin ring or electrochemically. It was also observed that the metalloporphyrins were more readily ionizable when the electrospray source was operated in redox mode than the free base porphyrin systems. Once it was demonstrated that ionization of model porphyrins can be accomplished, mixture analysis for the model systems was conducted.

In this chapter of the dissertation, the half wave oxidation potentials (\(E_{1/2\alpha}\)) for several porphyrin systems will be reported. These \(E_{1/2\alpha}\) potentials were determined using cyclic voltammetry (CV). It is hypothesized that the half wave potentials for each of the porphyrins in simple binary mixtures determine the selectivity of ionization for one type of porphyrin over another in the non-conventional electrospray source. It is hoped that a qualitative correlation can be established for porphyrin identification based the \(E_{1/2\alpha}\).

### 8.2 Experimental

#### 8.2.1 Chemicals

The following porphyrins were obtained from Midcentury Chemicals (Posen, IL) and were used without any further purification: nickel (II) octaethylporphyrin (NiOEP), magnesium (II) octaethylporphyrin (MgOEP), zinc (II) octaethylporphyrin (ZnOEP), vanadyl (IV) tetraphenylporphyrin (VOTPP), nickel (II) tetraphenylporphyrin (NiTPP), mesoporphyrin (XI) (Meso), cobalt protoporphyrin (CoPP) and copper (II) tetraphenylporphyrin (CuTPP). The following porphyrins were obtained from Strem Chemical (Newburyport, MA) and were used without any further purification: meso-tetraphenylporphyrin (TPP), magnesium meso-tetraphenylporphyrin (MgTPP), zinc meso-tetraphenylporphyrin (ZnTPP), and vanadyl octaethylporphyrin (VOOEP). HPLC-grade dichloromethane (Curtis Matheson Scientific, Houston, TX) and acetonitrile (Burdick and Jackson, Muskegon, MI) were used without further purification.
8.2.2 Mass Spectrometry

All electrospray ionization mass spectra were acquired using a Finnigan-MAT 900 double focusing mass spectrometer (San Jose, CA) equipped with an Analytica electrospray ionization source utilizing a platinum tipped glass capillary. Data acquisition was performed on a personal DEC station 5000 (Digital Equipment Corp., Maynard, MA). Samples were infused by using a Harvard Apparatus (South Natick, MA) Model 22 syringe pump at flow rates of 0.5 - 3.0 µL/min for all experiments.

Stock solutions for all homogeneous solution porphyrins were prepared in dichloromethane. The porphyrin sample solutions were electrosprayed at a concentration in the range of 25 pmol/µL- 1000 pmol/µL in acetonitrile (ACN), anhydrous dimethyl sulfoxide (DMSO) and methanol/water (MeOH). Twenty-five consecutive scans were collected for each sample at a scan rate of 10 s/decade with a scan range of 100-1300 amu. The mass spectrometer was calibrated with gramicidin-S (571.3 amu) at a concentration of 35 pmol/µL.

8.2.3 Cyclic Voltammetry

Cyclic voltammograms were recorded with a Model 273 Bi-Potentiostat (Princeton Applied Research, Princeton, N.J., USA) in potentiostat mode with a Yokogawa 3025 x-y recorder (Yokogawa Corp., Newnan, GA, USA). Porphyrin solutions were prepared at a 1.0 x 10^-3 M concentration in dichloromethane. The supporting electrolyte used was tetrabutylammonium fluoroborate (Sachem Inc., Austin, TX, USA) at a concentration of 0.1 M. The experiments were run in a three-neck glass electrochemical cell with a platinum disk working electrode, a platinum auxiliary electrode and an saturated calomel electrode (SCE) reference electrode.
8.3 Results and Discussion

8.3.1 ESI Analysis of Model Porphyrin Systems in the Non-Conventional Source

The mechanisms behind the ES ionization of porphyrins, or of any compound for that matter, are complex. Variables like solvent polarity, needle voltage, flow rate, temperature, solvent dielectric constant, volatility of solvent and ion stability are just a few of the parameters governing the achievement of a detectable ion signal. Based on the observations from data collected, a better understanding for the behavior of porphyrin systems is being sought.

Electrochemical ionization produces a radical cation of the analyte. Radical cations are formed via the removal of an electron from the highest occupied orbital. Radical cations can be formed from chemical oxidants, photoionization or anodic oxidation. The lower the ionization potential and/or oxidation potential for a compound, the easier it is to oxidize. Metalloporphyrins are reported to have $E_{1/2}^{\text{ox}}$ potentials in the range of 0.6-0.9 volts, and free base porphyrins are reported to have $E_{1/2}^{\text{ox}}$ potentials of greater than 1 volt. Van Berkel has demonstrated the electrochemical oxidation of octaethylporphyrins in aprotic solvent systems. He states that the oxidation event is solvent dependent and that protic solvents can consume the radical cation formed. Van Berkel also used strong charge-transfer reagents (e.g., trifluoroacetic acid) to help stabilize the radical cation generated. Trifluoroacetic acid has been documented to act as a chemical oxidant in the stabilization of radical cations (Bard 1976). It was found in our lab, however, that the radical cations for several porphyrin systems were formed consistently in protic solvents and the addition of additives was not necessary.

The porphyrins studied were grouped into three different classes, the octaethylporphyrins (OEP), tetraphenylporphyrins (TPP), and “natural” porphyrins. Figure 8.1 shows the structures of the different porphyrin families. The various types of porphyrins were chosen to demonstrate the electrospray ionizability of different
Figure 8.1 Structures of model porphyrin systems analyzed by ESI-MS.
types of porphyrins in the source. The analyte diversity also aids in formulating ionization patterns for different types of porphyrin classes which ultimately may help in the structural elucidation of the metalloporphyrin compounds found in sample mixtures.

Figures 8.2 and 8.3 are typical mass spectra generated for a metalloporphyrin and free-base porphyrin, respectively. The isotopic resolution of the sector instrument yields a characteristic isotope peak pattern for all of the porphyrins, especially the metalloporphyrins. Subsequently, these unique peak patterns served as “fingerprints” for porphyrin identification.

8.3.2 Chemical Ionization of Porphyrin Systems

It is well documented that metalloporphyrins and free base porphyrins can be ionized in solution to yield the protonated species (McEwen 1936; Grigg 1972; Van Berkel 1991; Van Berkel 1992). The basicity of free base metalloporphyrins is greater than metalloporphyrins and thus protonation is highly favored for these systems. It should be noted that ring protonation specifically occurs at pyrrole nitrogens which are not coordinating to a metal or hydrogen (in the case of a non-metalloporphyrin). The pyrrole nitrogen has a $pK_a$ value of 17.0 (for the free base porphine). It is also known that the pyrrole nitrogens of a alkyl substituted porphyrin are more acidic than the free base porphine (Van Berkel 1992).

The $pK_a$ values for N-methyl-etioporphyrin and etioporphyrin I were reported as 14.0 and 16.0 respectively (McEwen 1936). Thus, ring protonation in solvents like methanol should be more favored to occur for the free base unsubstituted porphine than the alkyl substituted porphyrin. This relative assumption can be extended one more step when comparing free base porphyrins, which are alkyl substituted, to alkyl substituted metalloporphyrins.

The protonation of pyrrole nitrogens can also occur in the gas phase. This phenomenon is a function of the proton affinity of the pyrrole and the donating
Figure 8.2 ESI mass spectrum of VOOEP in MeOH at 222 pmol/μL. The applied potential equals -2466 V and the solution flow rate was set at 1.7 μL/min.
Figure 8.3 ESI mass spectrum of OEP in MeOH at 55 pmol/μL. The applied potential equals -2651 V and the solution flow rate was set at 2.2 μL/min.
proton. The gas-phase basicity or proton affinity of a compound is determined by the measurement of the heat of formations for the newly formed protonated ion species. The proton affinities of methanol, acetonitrile and pyrrole (representing the porphyrin) were compared to support the possibility of gas-phase protonation of the porphyrin ring even in the case of acetonitrile as the solvent system. The proton affinities were reported to be 213 kcal/mol, 188 kcal/mol, and 182 kcal/mol for pyrrole, acetonitrile, and methanol, respectively (Franklin 1979). A compound with a higher proton affinity will not transfer a proton to a compound with a lower proton affinity, but the converse is possible. Thus, proton exchange from methanol and acetonitrile to the porphyrin ring is favored based on the proton affinity values.

Porphyrin systems with ionizable side chains (e.g., carboxylic groups, amine groups etc.) are even more amenable to chemical ionization. The contribution of side chain groups to the ionization process was briefly investigated by our lab. We observed, in the case of the free base porphyrins, that ring protonated ions were the only species observed for the mass spectral analysis of these compounds, as compared to the metalloporphyrins in which the molecular ion was exclusively formed (except in the case of the vanadyl octaethylporphyrin). The fact that ring protonation was also observed in aprotic solvents like acetonitrile suggests that gas-phase kinetics may also be responsible for promoting protonation. The protonation of the porphyrin system can also occur at a side ligand depending on the basicity of the ligand. In the case of the systems being studies here, the ligands were secondary factors contributing to the protonation step. The ligands increased or decreased the basicity of the pyrrole nitrogen but were not themselves protonated. Figure 8.4 is a mass spectrum for mesoporphyrin XI (Meso) which is a porphyrin with ionizable side chains. The Meso yields an intense (M+H) signal at 567.5 u. Two more ion signals at 589.5 u and 611.5 u, which correspond to one and two sodium adducts, respectively, are also observed. It is feasible to propose that the sodium...
Figure 8.4 ESI mass spectrum of Meso in MeOH at 55 pmol/μL. The applied potential equals -2678 V and the solution flow rate was set at 2.2 μL/min.
molecules are coordinating to the carboxylic acid groups (of which there are two in the case of the mesoporphyrin). The cation adduction at the CO$_2$' groups may be hindered due to competition between solvent protons and metal adducts. This competition may be a function of the adduct ion concentration and is something to be explored in the future. The electrochemical ionization of this porphyrin, however, is difficult. No radical cation (M$^+$) was ever observed for this porphyrin. Side chain participation in the electrochemical oxidative process is believed to play a major role in the ionization process.

8.3.3 Electrochemical Oxidation of Porphyrins Compounds

The influence of analyte oxidation potential on the ionization efficiency of the ESI source is of utmost importance to our mixture analysis. To help understand the limitations of the oxidation potentials, cyclic voltammetry (CV) studies were conducted on several of the porphyrin systems to obtain their oxidation potentials. The measured $E_{1/2\text{ox}}$ potentials for the porphyrins offered some insight as to what porphyrins could be reasonably oxidized in the conventional electrospray source. Porphyrins with low $E_{1/2\text{ox}}$ potentials were found to be to be more easily ionized in the ESI source than porphyrins with higher $E_{1/2\text{ox}}$ potentials. In mixture analysis, this observation may be explained based on the hypothesis proposed by Van Berkel that the electrospray source is actually a controlled-current electrolytic cell (Van Berkel 1995). In this model, the ESI source generates current that depends on the relative redox potentials of all the components in the cell (ESI source), including the solvent and metals from the needle.

8.3.4 Cyclic Voltammetry Determination of Porphyrin Oxidation Potentials

Several groups have demonstrated a correlation between the electrochemical behavior observed in the electrospray source and the relative oxidation potentials of the analytes under investigation (Ikonomou 1991; Van Berkel 1992; Xiaoming 1994; Van Berkel 1995; Van Berkel 1995). Because the electrospray ionization source is a
controlled-current electrolytic cell, the relative mass spectral intensities of oxidized species in a mixture will be determined by the electrospray current and the relative oxidation potentials of the analytes. Thus, in this study, the measured halfwave oxidation potentials of the metalloporphyrins used in the experiments should serve as a reference point of comparison for the predicted and measured signal intensities of the same metalloporphyrins in solution mixtures. Table 8.1 lists the oxidation potentials measured for each of the porphyrins studied using cyclic voltammetry. As our electrospray ionization source is equipped with a platinum tipped glass capillary, the oxidation potentials in Table 8.1 were determined using a platinum working electrode.

It was initially thought that the metal contained within the porphyrin was the major contributor towards the oxidation potential for the porphyrin in the ESI source. To test this hypothesis, a mixture of a metalloporphyrin and free-base porphyrin (both with ionizable side chains) was conducted. In Figure 8.5, the cobalt protoporphyrin (CoPP) and Meso are mixed in equimolar amounts (55 pmol/μL). The ion abundances for the metalloporphyrin are much greater than those for the free base. Based on these observations, it may be inferred that the metal coordinated in the ring somehow helps facilitate the ionization process in the ESI source, perhaps through electrochemical oxidation of the metal in the porphyrin ring. Non metal-containing porphyrins, on the other hand, must facilitate ionization via ring oxidation, which can occur electrochemically but at much higher oxidation potentials, or protonation of the ring and/or it's substituents.

To determine which one of these pathways dictates the method of ionization for a porphyrin analyte, mixtures of metalloporphyrins with the same metal center
Table 8.1 Measured oxidation potentials of metalloporphyrins that are the focus of this study.

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>Halfwave Ox. Potential (E_{1/2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium Etioporphyrin</td>
<td>0.515</td>
</tr>
<tr>
<td>Vanadyl Etioporphyrin</td>
<td>1.01</td>
</tr>
<tr>
<td>Nickel Etioporphyrin</td>
<td>0.845</td>
</tr>
<tr>
<td>Copper Etioporphyrin</td>
<td>0.830</td>
</tr>
<tr>
<td>Zinc Etioporphyrin</td>
<td>0.690</td>
</tr>
<tr>
<td>Magnesium Octaethylporphyrin</td>
<td>0.545</td>
</tr>
<tr>
<td>Vanadyl Octaethylporphyrin</td>
<td>1.030</td>
</tr>
<tr>
<td>Nickel Octaethylporphyrin</td>
<td>0.860</td>
</tr>
<tr>
<td>Copper Octaethylporphyrin</td>
<td>0.840</td>
</tr>
<tr>
<td>Zinc Octaethylporphyrin</td>
<td>0.745</td>
</tr>
<tr>
<td>Magnesium Tetraphenylporphyrin</td>
<td>1.05</td>
</tr>
<tr>
<td>Nickel Tetraphenylporphyrin</td>
<td>1.09</td>
</tr>
<tr>
<td>Vanadyl Tetraphenylporphyrin</td>
<td>1.22</td>
</tr>
<tr>
<td>Copper Tetraphenylporphyrin</td>
<td>1.07</td>
</tr>
<tr>
<td>Zinc Tetraphenylporphyrin</td>
<td>0.870</td>
</tr>
</tbody>
</table>

but different ring substituents were analyzed using the non-conventional source.

Figure 8.6 demonstrates the ESI-MS analysis of a mixture of a two octaethylporphyrins (VOOEP and NiOEP) and two tetraphenylporphyrins (VOTPP and NiTPP). All of the porphyrins were prepared as equimolar solutions (55 pmol/μL). The ion signal for both of the octaethylporphyrins was more intense than the tetraphenylporphyrin signals, which were barely detectable above the background noise. All of the porphyrins were ionized electrochemically in the ESI source. The
Figure 8.5 ESI mass spectrum of a 1:1 mixture of CoPP and Meso in acetonitrile. Both of the porphyrins were prepared at 55 pmol/μL. The applied potential equals -2877 V and the solution flow rate was set at 1.5 μL/min.
Figure 8.6 ESI mass spectrum of an equimolar mixture of VOOEP, NiOEP, VOTPP and NiTPP in MeOH at a concentration of 55 pmol/μL. The applied potential equals -2031 V and the flow rate was set at 1.0 μL/min. The ion signal for VOOEP and NiOEP (599.4 u, and 590.3 u, respectively) dominate the spectrum. The NiTPP ion signal (670.3 u) is barely detectable above the background noise.
fact that the OEPs were almost preferentially ionized over the TPPs suggests that the type of porphyrin ring has a greater influence on the electrochemical ionization process than the metal. It is apparent, however, that the metal somehow facilitates the electron transfer process and is still an important part in the overall scheme.

Free base porphyrins were reported by Van Berkel to be ionized by chemical ionization preferentially to electrochemical ionization in the ESI source while the metalloporphyrins were more readily ionized electrochemically (Van Berkel 1991; Van Berkel 1992). These observations are supported by reports in the literature of the increased stability of the radical cation of metalloporphyrins in solution over those of the free base porphyrins. The basicity of the pyrrole nitrogens of the free base porphyrin is much greater than those of the metalloporphyrins, and thus the protonated species is expected to be the ion of choice for the free base system. This behavior is, indeed, observed in both our data and that of Van Berkel.

The oxidative process, in general, for metalloporphyrins has not been fully established. The role of the metal versus that of the ring ligands is still under debate. The oxidation phenomenon, while not directly stated, has been attributed mostly to the metal center of the porphyrin which would result in the generation of the d-orbital radical cation. Oxidation of the metal in metalloporphyrins is well documented in the literature and is reported to occur at the transition metal site in general.

Fuhrhop and Mauzerall reported that metals with electrons in the d-orbital play an important role in the oxidation process for metalloporphyrins (Fuhrhop 1969). Electron spin resonance experiments conducted by this group offered evidence that the electron removal process involves the orbitals on the metal in the case of nickel, copper, and palladium octaethylporphyrins. They also found evidence that an electron can be taken from the ring of a metalloporphyrin resulting in the pi-radical cation. Conversely it was also reported that the ring oxidized species for the nickel and palladium metalloporphyrin systems were generated in the presence of Fe(ClO₄)₃.
suggesting that the type of radical cation formed is dependent on the conditions of the overall system. It was concluded that the more negative charge there is on the porphyrin ring, the easier it will be to remove an electron. Thus ring electron density would be a function of the ligands attached to it and the electronegativity of the metal incorporated in the ring.

8.3.5 The Unusual ESI Source Behavior for Vanadyl Octaethylporphyrin (VOOEP)

The VOOEP system was a peculiar system to study because of its ability to be electrochemically and chemically ionized. This phenomenon is still not understood but is constantly observed even when experimental parameters like flow rate, needle voltage and solvent are changed. Whether or not a particular combination of these variables can promote one type of ionization event over another is still being investigated. This particular phenomenon is not observed in any of the other metalloporphyrin systems studied. It is suspected that the chemistry of the vanadium-oxygen group plays a part in this phenomenon.

The VOOEP was found to be soluble in all of the solvents employed in this study. The ideal solvents for the ES process are MeOH and acetonitrile, both of which have very close dielectric constants. Dimethyl sulphoxide (DMSO) was found by our lab to also work well as an electrospray solvent. Rapid solvent evaporation was an issue of concern for using this solvent, but was found to not as critical a factor as was originally thought. The results observed in DMSO were quite interesting and are discussed in more detail below. All of the experimental samples (10 pmol/μL to 200 pmol/μL) were prepared in both methanol and acetonitrile from the stock solution (1000 pmol/μL) made up in DCM. Figure 8.2 shows a typical mass spectrum acquired for VOOEP where the M⁺ ion (599.2 u) is observed.
A review of all the data collected on the VOOEP system suggests that the protonation is occurring at the oxygen atom of the vanadyl moiety. Because the pyrrole nitrogens are tied up with the vanadium, it is highly unlikely that protonation is occurring at the nitrogen sites. This fact is well documented for all metalloporphyrins, thus leaving only the ethyl ligands and the oxygen atom as possible candidates for sites of protonation. Reduction of one of the ethyl groups would have to result for protonation to occur at this site. This event is also highly unlikely. Protonation of a heteroatom such as oxygen, however, is a more reasonable pathway for the protonation mechanism. This hypothesis is further supported by Scott and Drew who reported on the ability of the oxygen in the vanadyl moiety to indeed hydrogen bond with both water molecules and other vanadyl molecules (Drew 1984).

Further evidence is presented in Figure 8.7. It was observed that when the VOOEP was analyzed in MeOH at various concentrations, both electrochemical and chemical ionization occurred. Note the appearance of the isotopic distribution pattern for the parent ion compared to the isotopic pattern for VOOEP in Figure 8.2. A slightly more intense peak is observed for the protonated species (600.4 u) than the electrochemically ionized species (599.4 u) indicating the competition of the ionization processes. A common fragment peak for VOOEP is seen at 583.49 u. The 583.49 u fragment corresponds to the loss of the oxygen atom on the vanadyl moiety. The isotope pattern for this fragment correlates with the molecular ion species (M+) for the oxygen free vanadium porphyrin (VaOEP). A protonated species was never observed for this fragment ion but the sodium adduct was, thus suggesting that the oxygen is the site of protonation in the vanadyl porphyrin. In acetonitrile at various concentrations, both types of ionization processes were also represented. It was later observed that a nearly 1:1 relationship of the \([\text{M}^+]/[\text{M}+\text{H}]^+\) could be established in methanol at a lower flow rate and higher needle
Figure 8.7 ESI mass spectrum of VOOEP in MeOH at 25 pmol/μL. The applied potential equals -2754 V and the flow rate was set at 0.8 μL/min. The competition between electrochemical ionization and chemical ionization in the ESI source is demonstrated by the intense (M+H) ion at 600.4 u compared to the radical cation at 599.4 u.
voltage. This unique characteristic may later aid in the identification of the vanadyl porphyrins systems during mixture analysis.

The VOOEP was also analyzed in anhydrous DMSO to limit the source of free protons. The use of DMSO as an electrospray solvent has never been reported before. The behavior of the VOOEP in DMSO yielded very unexpected results. Figure 8.8 shows the ion signal obtained for the VOOEP system in DMSO at a concentration of 100 pmol/μL. The applied potential used was relatively high, but can be justified by the fact that the oxidation potential for VOOEP was measured and found to be much higher in DMSO (1.18 V) than in DCM (0.95 V). The radical cation was formed in DMSO. The conditions of this particular solvent system promote the formation of the radical cation because the polarity is great enough to help stabilize the cation, and the absence of abstractable protons helps suppress the formation of the protonated species. The data presented here is the first report of exclusively generating the molecular ion for VOOEP without the presence of the protonated species in an aprotic solvent. The mass spectrum (Figure 8.8) also contains the sodium adduct ion signal. At a higher flow rate it was found that the abundance of the molecular ion peak could be increased over that of the sodium adduct signal. Characteristic fragmentation for VOOEP was often observed as the loss of the oxygen from the Vanadyl moiety resulting in an ion observed at 583.49 u which will be referred to as the VaOEP species.

8.4 Conclusion

The initial studies for the ES analysis of porphyrins using our non-conventional source demonstrated the ability of this source to effectively ionize the model porphyrin systems via chemical and electrochemical ionization. The preliminary mixture analyses suggested the possibility of selectively ionizing mixtures of the model porphyrins in our source as well. The most important variables established as critical to controlling the selective ionization process were applied
Figure 8.8 ESI mass spectrum of VOOEP in anhydrous DMSO at 100 pmol/μL. The applied potential equals -3191 V and the flow rate was set at 1.0 μL/min.
Figure 8.9 ESI mass spectrum of NiOEP in MeOH at 22 pmol/μL. The applied potential equals -2873 V and the flow rate was set at 2.0 μL/min.
potential and analyte oxidation potentials. A more detailed study focusing on the influence of analyte oxidation potentials is presented in Chapter 9. It is believed that the oxidation potentials for various porphyrin models will dictate the ES source preference for analyte ionization.
CHAPTER 9. MIXTURE ANALYSIS OF BINARY METALLOPORPHYRINS SYSTEMS USING ELECTROSPRAY IONIZATION MASS SPECTROMETRY

9.1 Focus of Study

The focus of this set of experiments was to closely examine the electrochemical ionization process responsible for the ionization of metalloporphyrins in the electrospray ionization (ESI) source of a double focusing sector mass spectrometer. Chapter 8 has established the ability of the ESI source to ionize various types of porphyrins individually and in mixtures. The mixture analysis study demonstrated the inherent preference for the ionization of one porphyrin over another. The reasons for this “selectivity” were not completely understood. It has now been proven, however, that this source selectiveness depends on the $E_{1/2}$ potentials of the analytes being sprayed from conventional sources. Hence, further studies were conducted to establish a better understanding of the capabilities for the non-conventional ESI source for ionization selectiveness for one type of porphyrin over another.

A variety of experimental parameters associated with this particular ionization process are varied and the signal response monitored to identify the optimal settings necessary to achieve the maximum ion abundances for each metalloporphyrin studied. This information is then used to conduct mixture analysis on binary metalloporphyrin solutions with the intent to determine the factors governing the selective ionization of one metalloporphyrin over another in the ESI source. Cyclic voltammetry data was collected on each of the metalloporphyrins to establish the relative $E_{1/2}$ potentials for all of the porphyrins used in this study. The $E_{1/2}$ potentials were used to predict the expected results and correlate the observed results.

9.2 The Electrospray Analysis of Metalloporphyrins

Van Berkel and coworkers were the first to demonstrate the use of ESI as a means to ionize porphyrins (Van Berkel 1991). In their original study, they showed that the porphyrin systems with protonatable side chains (e.g., carboxylic acids, alcohols) and
free base porphyrins (no metal in the center cavity) were chemically ionized in the ESI source via protonation of the porphyrin (Van Berkel 1991). The additional use of protic solvents was found to promote the chemical ionization of the various porphyrin systems used in their study. It was also pointed out that typically the strongest ESI signal was achieved for samples that were already ionized in solution (Van Berkel 1993).

Van Berkel was the first to report the observation of radical cations \( (M^+) \) of metalloporphyrins in the ESI source (Van Berkel 1991). The radical cation species was believed to occur via the electrochemical oxidation of the metalloporphyrins being analyzed in the ES source. A closer look at how the ESI source was operating in the electrochemical or redox mode found that variables such as solvent polarity, needle voltage, flow rate, solvent dielectric constant and analyte oxidation potentials govern the capabilities of the electrospray source to electrochemically ionize various analytes (Van Berkel 1991; Van Berkel 1992; Van Berkel 1993). Formation of the porphyrin radical cation in the ESI source was hypothesized to occur as a result of either chemical oxidation in solution via redox coupling reactions of the analyte with the solvent or electrochemical oxidation at the liquid metal interface of the liquid flow system between the analyte and the ES capillary (Van Berkel 1991; Van Berkel 1992). Both of these explanations seemed acceptable since it is documented that oxidation of metalloporphyrins can occur quite readily and metalloporphyrin radical cations were found to be very stable in solution (Van Berkel 1992). Numerous studies have been performed on the electrochemical oxidation and reduction of porphyrins (Davis 1965; Fuhrhop 1969; Heiling-Peychal G. 1971). These studies show that redox behavior is quite common to metalloporphyrins. Thus, it seems feasible to propose that metalloporphyrins can undergo electrochemical ionization in the electrospray source.

Other experiments involving the spraying of metalloporphyrin solutions from different ESI configurations suggested that the radical cation formation occurs at the
solution-metal interface (Van Berkel 1992). This observation also supports the electrophoretic charge separation mechanism discussed earlier in this text. It was proposed that the ESI source, when operating in the redox mode, acts as a controlled-current electrolytic cell because of the electrolytic process which must occur in the ES capillary to charge balance the loss of each ion polarity in the charged droplets (Van Berkel 1995; Van Berkel 1995).

The analysis of individual porphyrin systems by Van Berkel allowed him to determine some of the factors governing the ES source redox process. The critical variables governing this process were believed to be the applied voltage, the $E_{1/2ox}$ potentials of the analytes and concentration of the analytes in solution. The $E_{1/2ox}$ potentials of the analytes were found to be a direct function of the ES current (Van Berkel 1992; Van Berkel 1995; Van Berkel 1995). In continuing studies of the electrochemical mechanism occurring in the ESI source, it was also found that the addition of charge-transfer complexes and the use of certain solvents helped to promote the electrochemical ionization of various analytes as evidenced by increased ion currents (Ikonomou 1991; Van Berkel 1992; Van Berkel 1994; Van Berkel 1994; Xiaoming 1994).

The studies conducted by Van Berkel and other research groups into the electrochemical ionization process have all focused on the behavior of individual analytes. All of the previous studies and the preliminary studies conducted by our group (Chapter 8) suggest that, under controlled conditions, the electrochemical ionization process may be used to promote the selective ionization of one porphyrin over another in a heterogeneous solution containing equimolar concentration of two different metalloporphyrins. This process ultimately may prove useful for metalloporphyrin mixture analysis in complex mixtures like crude oil.
9.3 Research Objective

Of particular interest to this author is the electrochemical ionization pathway and the possibilities of exploiting the variables which govern its mechanism for selective ionization of metalloporphyrins in binary mixtures as a function of the half-wave oxidation potentials of the metalloporphyrins. The generation of metalloporphyrin ions in the positive-ion mode is a direct function of the porphyrin's half-wave oxidation potentials (Van Berkel 1995; Van Berkel 1995). This evidence is supported by the fact that the molecular ion (M+) is observed exclusively for metalloporphyrins (Van Berkel 1991; Van Berkel 1992). There are some limitations, however, to the electrochemical ionization process for mixtures that will be discussed in the upcoming sections.

It has been shown in earlier reports, as well as in this report, that the oxidation potential of the analyte and the actual ionization process can be correlated to data obtained from cyclic voltammetry experiments on the analytes prior to running the porphyrin analysis in the mass spectrometer (Van Berkel 1992; Van Berkel 1994). This correlation can be used as a prediction tool for the behavior of the metalloporphyrins in the ESI source once an understanding of how the half-wave oxidation potentials influence the ionization process for porphyrin mixtures.

Three different porphyrin families were used as standards for this study. Figure 9.1 gives the chemical structure of these porphyrin families along with the metals housed in the center cavity of the porphyrins studied. Two of the families, etioporphyrin I and octaethylporphyrin are structurally similar to porphyrins found in crude oil samples. These three families were chosen because they vary in oxidation potential range. The objective of this research project was to examine the applicability
Figure 9.1 Selected metalloporphyrins studied by electrospray ionization mass spectrometry.
of ESI-MS operating in the redox mode for characterizing mixtures of metalloporphyrins and understanding the relative limitations of the ionization process for these mixtures.

The ion abundances for several porphyrins in homogeneous and heterogeneous solutions were measured while varying experimental parameters. It was found that under certain conditions, the signal intensity of one porphyrin relative to another could be enhanced as a function of certain experimental parameters (i.e., analyte concentration and analyte \( E_{1/2} \) potential). In this study, we demonstrate that selective ionization of metalloporphyrins in a mixture is feasible using ESI mass spectrometry. Further, limits on the selectivity of ESI-MS for metalloporphyrin mixture analysis are established.

9.4 Experimental

9.4.1 Chemicals

The following porphyrins were obtained from Midcentury Chemicals (Posen, IL, USA) and were used without further purification: copper etioporphyrin I, magnesium etioporphyrin I, vanadyl etioporphyrin I, zinc etioporphyrin I, nickel etioporphyrin I, copper (II) octaethylporphyrin, nickel (II) octaethylporphyrin, magnesium (II) octaethylporphyrin, zinc (II) octaethylporphyrin, nickel (II) tetraphenylporphyrin, vanadyl (IV) tetraphenylporphyrin, nickel (II) tetraphenylporphyrin, and copper (II) tetraphenylporphyrin. The following porphyrins were obtained from Strem Chemical (Newburyport, MA, USA) and were used without any further purification: \( meso- \) tetraphenylporphyrin, magnesium \( meso- \) tetraphenylporphyrin, zinc \( meso- \) tetraphenylporphyrin, and vanadyl octaethylporphyrin. HPLC-grade dichloromethane was obtained from Curtis Matheson Scientific, (Houston, TX, USA) and acetonitrile from Burdick and Jackson (Muskegon, MI, USA). All chemicals were used without further purification.
9.4.2 Mass Spectrometry

All electrospray ionization mass spectra were acquired using a Finnigan-MAT 900 double focusing mass spectrometer (San Jose, CA, USA) equipped with a first generation Analytica electrospray ionization source utilizing a platinum tipped glass capillary (0.246” o.d., 0.020” i.d.) and a 33 gauge stainless steel needle. Data acquisition was performed on a personal DECstation 5000 (Digital Equipment Corp., Maynard, MA, USA). Samples were infused by using a Harvard Apparatus (South Natick, MA, USA) Model 22 syringe pump at flow rates of 2.0 μL/min - 3.0 μL/min for all experiments except where noted otherwise.

Stock solutions for all homogeneous solution porphyrins were prepared in dichloromethane. The porphyrin homogeneous sample solutions were electrosprayed at a concentration of 25 pmol/μL in acetonitrile. The heterogeneous porphyrin stock solutions (binary mixtures) were electrosprayed at a final concentration of 2.0 nmol/μL - 5 pmol/μL in acetonitrile. Twenty-five consecutive scans were collected for each sample at a scan rate of 10 s/decade with a scan range of 400–800 u.

9.5 Results and Discussion

9.5.1 ESI of Homogeneous Solutions of Metalloporphyrins

Initially, a series of different homogeneous metalloporphyrin solutions were characterized by ESI-MS (Chapter 8). As the purpose of our investigations was to characterize the use of an ESI source alone as an electrolytic cell, no supporting electrolytes (Van Berkel 1995) or charge-transfer reagents (Van Berkel 1991; Van Berkel 1994; Van Berkel 1994; McCarley 1997) were used in any of these experiments to enhance the ion current. Supporting electrolytes may result in ion signal suppression and the use of charge-transfer reagents would complicate interpretation of the results obtained from the analysis of heterogeneous solutions of metalloporphyrins (Kebarle 1993). Further, no trifluoroacetic acid was added to stabilize the radical cation in the binary mixture experiments as aprotic solvents were
used throughout for experiments designed to promote electrochemical ionization of the porphyrin in the ESI source (redox mode) (Van Berkel 1991). The major ions observed for each of the metalloporphyrins analyzed while the source was operated in redox mode are shown in Figure 9.1. Except for vanadyl octaethylporphyrin (VOOEP), which was detected as both the radical cation and the protonated molecular ion (Chapter 8), the metalloporphyrins were detected exclusively as radical cations. Van Berkel has found also that VOOEP can be detected as either the radical cation or protonated molecular ion (Van Berkel 1991).

These results confirmed that our electrospray ionization source was operating in the electrochemical mode. In addition, the ESI source parameters were adjusted to reduce nozzle-skimmer fragmentation of the metalloporphyrins resulting in almost exclusive production of the molecular ion for all metalloporphyrins studied.

9.5.2 Preliminary Mixture Analysis of Porphyrin Systems in the Non-Conventional ESI Source

A study of various porphyrin mixtures was conducted to get a better understanding of the ionizability of one porphyrin system over another in various solvent systems and at various applied voltages. It was found that both of the above variables played a role in the selective ionization of one porphyrin system over another in simple mixtures. It was also observed that metalloporphyrins were ionized more readily than the free base porphyrin systems when the ESI source was operated in redox mode. This event is partially attributed to the fact that experimental conditions were set to promote electrochemical ionization rather than chemical ionization. Electrochemical ionization is of particular interest because it is hypothesized in this dissertation that this process can promote the selective ionization of certain porphyrins in the ESI source.

During oxidation, the metalloporphyrins can undergo the formation of either a π or d-orbital radical cation from the porphyrin ring or transition metal, respectively. The former of these two oxidation pathways is the hardest to promote. The chemical
ionization mechanism can be somewhat suppressed in the ESI source by the use of aprotic solvents. The use of aprotic solvents has been found in our lab to indeed suppress the formation of the protonated species for the free base porphyrin systems. More experimentation was later conducted to find out what conditions favor one type of ionization over another for the porphyrin systems. It was found that the signal intensity for both types of porphyrins (free base and metalloporphyrin) was a function of the needle intensity and the flow rate. These were the most obvious variables to test for possible ionization selectivity in the ESI source.

A series of equimolar porphyrin mixture solutions were studied to establish the solvent dependence on the ionization process. The solvents used in the preliminary studies were MeOH and acetonitrile to compare the effects of protic vs. aprotic solvents. The data acquired from these preliminary studies support the hypothesis that selective ionization of the porphyrins in mixtures may be attainable in the ESI source. The data is reviewed in the upcoming sections.

9.5.2.1 VOOEP: NiOEP: VOTPP: NiTPP (25-55 pmol/µL)

The electrochemical ionization of mixtures of the mOEP and mTPP, as was stated earlier, favors the former of the two types of porphyrin. Analysis of porphyrin mixtures containing these two types of porphyrins was performed. Mixtures of VOOEP, NiOEP, VOTPP, and NiTPP in MeOH and acetonitrile were analyzed to see if the ESI source would preferentially ionize one type of porphyrin over another via electrochemical ionization. Experimental parameters like applied potential and porphyrin solution concentrations were varied to observe their effects on the ionization process as well. It was found that the ESI source generated molecular ion signals exclusively for both of the mOEP, while ion signal for mTPP was dramatically suppressed in both acetonitrile and MeOH (Figure 9.2).

Figure 9.2 is a mixture of all four porphyrins in MeOH with 0.01% of TFA added to help stabilize cation formation. The TFA was used initially to compare its
Figure 9.2 ESI mass spectrum of VOOEP, NiOEP, VOTPP, and NiTPP prepared at equimolar concentrations, 55 pmol/ul, in MeOH with 0.1% TFA. The applied voltage equals -2899 V and the flow rate is set at 1.7 mL/min. Both of the mOEPs dominate the spectrum. NiTPP (670.3 u) is just detectable above the background noise.
effects on the ion signal. It was later determined that TFA was not necessary for the mixture experiments to be discussed in the upcoming sections. Signal for NiTPP (670.3 u) can be seen in the spectra but the signal intensity is just above the background noise. At various needle voltages and solution concentrations, the mOEP ion signals were always observed with changes in intensity relative to the experimental parameters. The mTPP ion signal, on the other hand, was barely detectable, if not totally suppressed, at the different experimental parameters. Similar results were observed for the mixture analysis conducted in acetonitrile with and without TFA.

To verify that the mTPPs could be ionized in the source from a mixture, both of the mTPPs were mixed together without mOEP to see if the ion signal for each porphyrin would be generated. Figure 9.3 is the mass spectrum collected for this mixture. The mTPPs, when mixed, were both detectable with very strong ion signals suggesting that the oxidation potential for both the VOTPP and NiTPP systems are close to each other relative to the oxidation potentials for the mOEPs. This can be verified by the measured $E_{1/2}$ reported in Table 8.1. Oxidation potentials for the mOEPs and mTPPs were measured, and found to be much lower for the mOEPs. A fragment peak (597.1 u) was observed for the mTPP mixture. This fragment is attributed to the NiTPP system. The fragment peak is the same type of fragment observed during the analysis of NiTPP. The fragment is the loss of a phenyl group.

The mTPP mixture sample was accidentally contaminated with a trace amount of the VOOEP. Figure 9.4 shows the mass spectrum for this sample. Surprisingly, not only was ion signal for the VOOEP detectable, but the VOOEP had a much stronger intensity than both of the mTPPs. The fact that both of the mTPPs are detectable in the presence of a trace amount of a mOEP suggest that the ionization efficiencies for the analytes are concentration dependent.
Figure 9.3 ESI mass spectrum of NiTPP and VOTPP at 55 pmol/μl in MeOH with TFA. The applied potential equals -2199 V and the flow rate is set at 1.3 μL/min. The fragment ion peak at 597.1 u is believed to be a loss of a phenyl group from the NiTPP.
Figure 9.4  ESI mass spectrum of NiTPP and VOTPP in MeOH/TFA at equimolar concentrations of 55 pmol/μL.
The applied potential equals -2199 V and the flow rate was set at 1.3 μL/min. The peak at 599.3 u is a trace contaminant of VOOEP. The ion abundance for the VOOEP is still substantially larger than both of the mTPPs.
The ESI source has demonstrated a preference for the mOEPs in the presence of mTPPs. The basis for this preference is directly related to the oxidation potentials of the porphyrins. Based on these observations it is believed that the π radical cations are being formed for all of the porphyrins in the ESI source. This theory is formulated from the fact that while both sets of porphyrins have the same metal centers, there is still a large difference in the ionizability of the porphyrins. The difference structurally for each set of porphyrins is the ring.

9.5.2.2 CoPP:Meso (1000 pmol/μL)

An equimolar solution of a metalloporphyrin and free base porphyrin was analyzed in the ESI source to observe if any selectiveness could be achieved between a free base and a metalloporphyrin. Acetonitrile was used as the solvent system because it was desired to promote the electrochemical ionization process in the ESI source. Figure 9.5 is the mass spectrum for the CoPP/Meso mixture. It was observed that the CoPP signal intensity was much greater than the Meso system. The applied voltage for this analysis was held at -2827 V. A strong molecular ion signal for the CoPP was observed. An increase in applied potential to -4217 V afforded a 50% increase in the Meso signal (Figure 9.6), demonstrating the correlation between needle voltage and individual porphyrin ion current in the ESI source. While total selectivity was not achieved, it was interesting to see that the ionization efficiency of one porphyrin could be increased in the presence of another porphyrin. This fact offers some support that porphyrin signals may also be selectively ionized by adjusting simple parameters like the applied potential.

9.5.2.3 CoPP:Meso:VOOEP (1000 pmol/μL)

An equimolar solution of CoPP, Meso and VOOEP was now tested to observe the effects of VOOEP on the CoPP:Meso system analyzed above. The VOOEP has a very high ionization efficiency in the ESI source and should be ionized just as readily
Figure 9.5 ESI mass spectrum of CoPP and Meso at equimolar concentrations of 1000 pmol/μL in acetonitrile. The applied potential equals -2827 V and the flow rate was set at 1.5 μL/min.
Figure 9.6 ESI mass spectrum of CoPP and Meso in acetonitrile at 1000 pmol/μL. The applied potential equals -4217 V and the flow rate was set at 1.5 μL/min. The higher applied potential increased the ion abundance of the Meso by about 50%.
despite the presence of the other two porphyrins. The solvent system used for this analysis was acetonitrile with < 1% TFA. Cyclic voltammetry analysis of CoPP, Meso, and VOOEP in acetonitrile gave $E_{1/2}^{ox}$ potentials of 0.990 V, 0.980 V, and 0.863 V respectively. It should be noted that the addition of TFA to the Meso CV run was found to be necessary to stabilize the cation long enough for CV analysis.

Figure 9.7 is the mass spectrum for this particular mixture at -1987 V. All of the porphyrin systems are being ionized in the ESI source. The ionization efficiencies for the CoPP and VOOEP seem to be very close despite the difference in their oxidation potentials. The ionization of Meso was also found to be consistent around this particular applied potential. This mixture proved to be quite interesting because of the effects observed to occur from the adjustment of the applied voltage. The total suppression of the Meso ion signal was found to result at higher applied voltages.

Figure 9.8 is the spectrum generated at the higher applied potential of -2848 V. The CoPP and VOOEP ion signals are equally abundant. Observed at the higher applied voltage is the exclusive formation of the CoPP molecular ion (619.23 u) and the Na$^+$ cation adduct ion for VOOEP (622.38 u). The Meso ion signal suppression caused by the increase in the applied voltage demonstrates the ability to selectively ionize analytes in the ESI source as a function of the applied voltage.

A very abundant Na$^+$ ion is observed (23.15 u) in the spectrum as well. The Na$^+$ ion concentration was unusually high for this sample due to contamination. The increased Na$^+$ ion concentration explains the repeatedly high ion abundances for the VOOEP with an Na$^+$ adduct observed for this set of experiments.

As the CoPP seems to somehow suppress the signal of the Meso, a mixture of just the Meso and VOOEP was run to see if Meso signal would improve. Figure 9.9 is the mass spectrum collected for this mixture at the applied potential of -2870 V. The ion abundances for both the Meso and the VOOEP were very close. It appears that the Meso ionization efficiency has increased in the absence of the CoPP. Also
Figure 9.7 ESI mass spectrum of equimolar concentrations, 1000 pmol/ul, of CoPP, Meso, and VOOEP in acetonitrile/ < 1% TFA

The applied potential equals -1987 V and the flow rate was set at 2.0 µL/min. All of the porphyrins are detected in the mass spectrum. The most abundant ions are the radical cations for CoPP and VOOEP (with a sodium adduct) observed at 619.7 u and 599.8 u, respectively. The Meso (M+H) ion and sodium adduct are observed at 567.9 u and 589.9 u, respectively.
Figure 9.8 ESI mass spectrum of CoPP, Meso, and VOOEP at equimolar concentrations of 1000 pmol/µL in acetonitrile/ < 1% TFA. The applied potential equals -2848 V and the flow rate was set at 2.0 µL/min. The Meso ion signal is totally suppressed at the higher applied potential when the VOOEP is present in solution.
Figure 9.9 The ESI mass spectrum of Meso and VOOEP at equimolar concentrations of 1000 pmol/μL in acetonitrile/1% TFA. The applied potential equals -2870 V and the flow rate was set at 2.0 μL/min. The (M+H), (M+Na) and (M+2Na) ions for Meso are observed at 567.6 u and 589.6 u, 611.6 u, respectively. The only ion detectable for VOOEP is the (M+Na) at 622.7 u.
observed, as seen in previous spectra of the Meso system, is the double sodiated Meso species (611.6 u).

9.5.3 The Effects of Flow Rate on Metalloporphyrin Mixture Analysis

Changing the flow rate showed no significant effect on the mass spectral results for any of the binary mixtures of metalloporphyrins studied. Initially, these results were surprising and were in contradiction to the earlier results of Van Berkel (Van Berkel 1995). As noted by Van Berkel, the electrospray current, \( i_{ES} \), is given by Faraday's law as shown in eq. 9.1, where \( n_j \) is the number of electrons involved in the oxidation of one molecule of species \( j \), \( A_j \) is the concentration of species \( j \) oxidized, \( F \) is the Faraday constant, and \( v \) is the solution flow rate through the electrospray needle. For the binary metalloporphyrin mixtures, once the metalloporphyrin with the lowest oxidation potential is ionized, the remaining ion current will be due to the metalloporphyrin with the higher oxidation potential. Thus, assuming that all of the molecules present in the solution can be oxidized, increasing the flow rate should increase \( i_{ES} \). This increase in the electrospray current should then permit an increase in the generation of radical cations from the metalloporphyrin having the higher oxidation potential, once the metalloporphyrin with the lower oxidation potential is completely ionized. However, we did not observe an increase in the generation of radical cation species from the metalloporphyrin having a higher oxidation potential when the flow rate was increased, irrespective of the concentration of the metalloporphyrins in the solution. Further, reducing the flow rate did not change the relative production of radical cation species, as might be expected if the oxidation process was diffusion limited (Van Berkel 1995).

In addition, the electrospray voltage did not have an appreciable effect on the relative intensities of the metalloporphyrin mixtures analyzed. A minimum
electrospray voltage was required to generate a stable ion current from our source, and this voltage was then optimized for each of the mixtures studied. However, decreasing or increasing this optimal voltage did not result in any change in the relative intensities of the metalloporphyrins in each mixture. Typically, deviation from this optimal voltage resulted in a decrease in the intensities of both metalloporphyrins in the mixture.

The likely explanation for the discrepancy in our results is due to the electrospray source arrangement used in these studies. Data from other laboratories was obtained using a "conventional" electrospray ionization source operating in the electrochemical mode. In the conventional arrangement, the electrospray potential is applied to a metal needle, and the entrance to the mass spectrometer is held at ground. Thus, the residence time for oxidation is determined by the volume of and the flow rate through the metal needle. In our Finnigan MAT900 instrument, the potential is applied to a platinum coated tip of a glass capillary located in the vacuum region of the electrospray source interface and the metal needle on the atmospheric side is held at ground. In this setup, changing the flow rate will have no effect on the residence time of the electrospray produced droplets at the platinum-tipped glass capillary. Further, this arrangement is most likely non-ideal as the total amount of oxidized species is likely controlled by mass transfer of the analyte to the metal-tipped capillary, and will be essentially independent of any operator controlled variables (e.g., flow rate and electrospray voltage).

9.5.4 Influence of Oxidation Potential on the ESI-MS of Heterogeneous Porphyrin Mixtures as a Function of Analyte Concentration

The intent of this set of experiments was to determine the selectivity of the electrospray source relative to the oxidation potentials of the metalloporphyrins in a heterogeneous solution. A series of binary metalloporphyrin mixtures were prepared at different relative concentration values. To determine the effect of concentration on
the ESI-MS results, other operating variables were held constant throughout. The optimum needle voltage was found for each metalloporphyrin mixture at equimolar concentrations, and this voltage was then utilized as the concentration ratios were varied.

9.5.4.1 Metalloporphyrin Mixtures with Nearly Identical Oxidation Potentials

Nickel etioporphyrin (NiEtio) and nickel octaethylporphyrin (NiOEP) was the first mixture characterized. As seen in Table 8.1 (Chapter 8), the oxidation potentials for these two metalloporphyrins are nearly identical (NiEtio 0.845 V, NiOEP 0.860 V). Figure 9.10 is a plot of the ratio of the relative intensities of the molecular ions for these two metalloporphyrins versus the ratio of the molar concentration of these two metalloporphyrins. According to the theory proposed by Van Berkel, when the oxidation potentials are nearly identical, the resulting ion intensities are then governed by the electrospray ion current which is proportional to the analyte concentration (Van Berkel 1995). As seen in Figure 9.10, the molecular ion intensities are indeed determined by the concentration of each metalloporphyrin in solution. When NiEtio and NiOEP are present in the mixture at equimolar concentrations, the ion intensities are nearly identical. As the concentration of NiEtio is increased relative to that of NiOEP, the ion intensity of NiEtio increases accordingly. Further, as one would predict, the response is linear.

We were interested in the effect absolute metalloporphyrin concentration would have on the electrochemical ionization process. Figure 9.10a is the data obtained at a final metalloporphyrin concentration of 5 pmol/µL. Figure 9.10b shows similar behavior when the final metalloporphyrin concentration is 110 pmol/µL. In both cases, the relative ion abundances of the two metalloporphyrins in the mass spectrum are proportional to the relative concentration of each metalloporphyrin present. Below a maximum flow rate (necessary to ensure adequate mass transfer of each analyte to the electrode for ionization) there is no qualitative change in these data as a function of
Figure 9.10  ESI-MS results from the characterization of a mixture of two metalloporphyrins whose ionization potentials are nearly identical. As the molar ratio of nickel etioporphyrin is increased relative to nickel octaethylporphyrin, the relative abundance of the radical cation of nickel etioporphyrin increases accordingly. Each data point is the average of five scans. (a) Final metalloporphyrin concentration is 5 pmol/μL. (b) Final metalloporphyrin concentration is 110 pmol/μL.

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flow rate. The larger error bars in Figure 9.10a are due to the poorer signal reproducibility for the magnesium etioporphyrin and magnesium octaethylporphyrin porphyrins, both of whose oxidation potentials are also quite similar (cf. Table 8.1). These results suggest that ESI mass spectrometry operating in the electrochemical mode may be useful for semi-quantitative analysis of mixtures providing the oxidation potentials of each analyte are nearly equal.

9.5.4.2 Metalloporphyrin Mixtures with Similar Oxidation Potentials

Our next investigations focused on metalloporphyrin mixtures whose difference in oxidation potentials were greater than those studied above. A mixture of NiOEP and zinc octaethylporphyrin (ZnOEP) were studied. The oxidation potentials for these two metalloporphyrins differ by approximately 0.1 V with ZnOEP having the lower oxidation potential (ZnOEP 0.745 V, NiOEP 0.860 V). As seen in Figure 9.11a, at equimolar concentrations (final concentration of mixture: 5 pmol/µl), the molecular ion abundance of ZnOEP (M⁺ 596 u) is nearly five times that of NiOEP (M⁺ 590 u). In this case, the ZnOEP is preferentially ionized because it has the lower oxidation potential of the two metalloporphyrins in the mixture.

Once all of the ZnOEP is oxidized, the remaining electrospray current should be due to the oxidation of the harder to oxidize NiOEP. What happens when one increases the concentration of the harder to oxidize species? One would expect that the ion abundance for the harder to oxidize species would increase as determined by the electrospray current. This event indeed was the case as can be seen in Figure 9.11b where NiOEP is now twice as concentrated as ZnOEP and the relative intensity of the NiOEP molecular ion has increased relative to its intensity in Figure 9.11a. The electrospray ion current for this system is initially due exclusively to the ZnOEP in solution. When all of the ZnOEP has been oxidized, the remaining electrospray ion current is used to oxidized NiOEP. The ionization efficiency is increased for the NiOEP because it is higher in concentration and thus more of it is reaching the metal-
Figure 9.11 ESI-MS results from the characterization of a mixture of two metalloporphyrins (final metalloporphyrin concentration is 5 pmol/µL) whose ionization potentials are within 0.1 V. As the molar ratio of the harder to oxidize metalloporphyrin is increased in the mixture, the relative abundance of its radical cation increases at a non-linear rate. a) 1:1 nickel octaethylporphyrin:zinc octaethylporphyrin mixture in acetonitrile. b) 2:1 nickel octaethylporphyrin:zinc octaethylporphyrin mixture in acetonitrile. c) 9:1 nickel octaethylporphyrin:zinc octaethylporphyrin mixture in acetonitrile.
surface interface to be oxidized as a function of the time it takes the overall process to occur. This same explanation can be used to describe the results shown in Figure 9.11c. In this case, a further increase in concentration of NiOEP to that of ZnOEP results in an increase in the ion abundance of the harder to oxidize species. The mass spectral ion abundances are now slightly in favor of the NiOEP. It appears, however, that the ZnOEP is still preferentially oxidized, due to its lower oxidation potential, in spite of the excess of NiOEP in the mixture. The results shown in Figures 9.11b and 9.11c also suggest that in these studies our electrospray source is operating at its optimal current level. Similar results was observed for the binary system of copper octaethylporphyrin (CuOEP) and copper etioporphyrin whose oxidation potentials also differ by approximately 0.1 V.

As in the case of the mixture of NiEtio and NiOEP, we performed these experiments at various flow rates to ensure that each analyte present had a sufficient residence time in the capillary to undergo oxidation. Qualitatively no change was seen in the relative abundances of the two metalloporphyrins as a function of flow rate. Sensitivity and electrospray current was found to decrease dramatically at flow rates less than 1.5 \( \mu \text{L/min} \), an effect we attribute to the loss of a stable spray from our ESI source.

An increase in the absolute concentration of each component of the mixture does not result in a qualitative change in the mass spectral results for these two metalloporphyrins. In Figure 9.12a, ZnOEP is twice as concentrated as NiOEP and the final metalloporphyrin concentration of this mixture is 2 nmol/\( \mu \text{L} \). ZnOEP is oxidized almost exclusively under these conditions. In Figure 9.12b, NiOEP is now twice as concentrated as ZnOEP (final concentration unchanged), and, as seen in Figure 9.11b, the relative ion abundances are nearly identical. In this case, the ZnOEP is preferentially oxidized, due to its lower oxidation potential, in spite of the
Figure 9.12 ESI-MS results from the characterization of a mixture of two metalloporphyrins (final metalloporphyrin concentration is 2 nmol/μL) whose ionization potentials are within 0.1 V. Even at a higher absolute concentration, nickel octaethylporphyrin can be oxidized once all of the zinc octaethylporphyrin is oxidized. a) 1:2 nickel octaethylporphyrin:zinc octaethylporphyrin mixture in acetonitrile. As zinc octaethylporphyrin has the lower oxidation potential, it is preferentially ionized in this mixture. b) 2:1 nickel octaethylporphyrin:zinc octaethylporphyrin mixture in acetonitrile. Again, the lower oxidation potential of zinc octaethylporphyrin allows for its ionization even in the presence of a higher concentration of nickel octaethylporphyrin.
excess of NiOEP in the mixture. As before, once all of the ZnOEP has been oxidized, the remaining electrospray current obtainable will be due to the oxidation of NiOEP. At such high absolute concentrations, one might expect that the ion current would be due exclusively to the easier to oxidize analyte (ZnOEP). However, as seen in Figure 9.12b, there is sufficient electrospray current to oxidize the ZnOEP and the NiOEP even at these high concentrations. We have found that our electrospray source produces a stable spray when higher concentrations (> 100 pmol/μL) are electrochemically ionized, presumably due to the inherently poor sensitivity of the ESI source and due to the lack of additional supporting electrolyte in these studies. In addition, we noted a slight increase in the electrospray current when the concentration solution (Figure 9.12) are used as compared to those concentrations used to obtain the results shown in Figure 9.11.

To confirm that the results found for NiOEP and ZnOEP at various relative and absolute concentrations are typical of those seen for mixtures whose $E_{1/2}^{ox}$ potentials are close but not identical, we studied also several other mixtures whose $E_{1/2}^{ox}$ potentials differed by 0.1 V or less. As seen in Figures 9.13a and 9.13b, qualitatively similar results for a binary mixture of NiEtio and ZnOEP ($\Delta E_{1/2} 0.1V$) are obtained at 10 pmol/μL and 1 nmol/μL final concentration, respectively. This trend was seen also during the analysis of a mixture of ZnOEP and CuOEP ($\Delta E_{1/2} 0.095 V$)

9.5.4.3 Metalloporphyrin Mixtures with Substantially Different Oxidation Potentials

To determine the selectivity of electrochemical ionization for mixtures whose components have substantially different $E_{1/2}^{ox}$ potentials, a mixture of ZnOEP and VOOEP, at a final concentration of 10 pmol/μL, was studied. The $E_{1/2}^{ox}$ potentials for these two metalloporphyrins differ by 0.285 V (ZnOEP 0.745 V, VOOEP 1.03 V). ZnOEP has the lower oxidation potential, and one would predict that ZnOEP would be ionized preferentially over VOOEP (Figure 9.14). Figure 9.14a is the mass spectrum resulting from the analysis of an equimolar solution of VOOEP and ZnOEP.

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Figure 9.13 ESI-MS results from the characterization of a 9:1 mixture of nickel etioporphyrin and zinc octaethylporphyrin at a) 10 pmol/μL and b) 1 nmol/μL. The difference in oxidation potentials for these two metalloporphyrins is ~0.1 V, and the mass spectral results are similar to those seen for other mixtures with oxidation potential differences in this range (Figures 9.11 and 9.12).
Figure 9.14  a) ESI mass spectrum of an equimolar mixture (10 pmol/µL final metalloporphyrin concentration) of zinc octaethylporphyrin and vanadyl octaethylporphyrin in acetonitrile. Both components are detected in this mass spectrum (see text). b) ESI mass spectrum of a 1:6 mixture of zinc octaethylporphyrin and vanadyl octaethylporphyrin in acetonitrile. As the relative concentration of vanadyl octaethylporphyrin is increased, the abundance of $m/z$ 599 u as compared to $m/z$ 596 u increases accordingly, of the ZnOEP with the A peak for the VOOEP at $m/z$ 599 u complicates
Both of the metalloporphyrins are detected in this mass spectrum. The overlap of the $A+3$ peak interpretation of the relative abundances of these two species detected under these conditions. The $^{67}$Zn isotope is less than 10% as abundant as the $^{64}$Zn isotope (4.11% vs. 48.89% natural abundance, respectively). It is clear from the relative abundances of the ions at $m/z$ 596 u and 599 u that an additional component at $m/z$ 599 u must be present. The abundance of the $m/z$ 599 u peak relative to the $m/z$ 596 u peak increases as the relative concentration of VOOEP increases in the solution (Figure 9.14b). Thus, at these low absolute concentrations, both components of the metalloporphyrin mixture can be detected, with the analyte of lower halfwave oxidation potential being preferentially ionized as before.

However, dramatically different results are obtained when the absolute concentration of the metalloporphyrins in a mixture are increased. Exclusive ionization of one analyte over another is found in the case where the analyte concentration is high and a large difference in the $E_{1/2ox}$ potentials of the analytes exist. For example, when ZnOEP and VOOEP are both present in solution at 1 nmol/μL, only ZnOEP is detected (Figure 9.15a). Further, even when the relative concentration of VOOEP is ten times greater than that of ZnOEP, the radical cation of VOOEP was still not observed (Figure 9.15b). Under these conditions, the analyte with the lower halfwave oxidation potential serves as a "redox buffer" [Van Berkel, 1997 #104] and is the sole source of the electrospray current. Similar results were also observed for binary mixtures of magnesium octaethylporphyrin (MgOEP) and CuOEP, and MgOEP and NiOEP. In all cases, the halfwave oxidation potentials differ by more than 0.2 V for the analytes in each mixture, and the analyte concentrations are relatively high (> 100 pmol/μL).
Figure 9.15 a) ESI mass spectrum of an equimolar mixture (1 nmol/μL final metalloporphyrin concentration) of zinc octaethylporphyrin and vanadyl octaethylporphyrin in acetonitrile. As zinc octaethylporphyrin has a much lower oxidation potential than vanadyl octaethylporphyrin, zinc octaethylporphyrin is exclusively ionized in this solution. b) ESI mass spectrum of a 1:10 mixture of zinc octaethylporphyrin and vanadyl octaethylporphyrin in acetonitrile. Again, the much lower oxidation potential of zinc octaethylporphyrin allows for its exclusive ionization even in the presence of a 10-fold molar excess of vanadyl octaethylporphyrin at these high absolute concentrations.
9.5.4.4 Characterization of Metalloporphyrins Present in Mixtures

As stated in Chapter 8, the metal and porphyrin ring both influence the oxidation potential of the metalloporphyrins. Magnesium containing metalloporphyrins have the lowest oxidation potentials and vanadium containing metalloporphyrins have the highest oxidation potentials for each of the three porphyrin families studied. The etioporphyrins and octaethylporphyrins, due to their similar structure, generally have the same influence on the oxidation potential. The tetraphenylporphyrins have consistently higher oxidation potentials.

The use of ESI-MS for selective ionization of metalloporphyrins can now be addressed. The results of these sets of experiments suggest that limits to the analysis of mixtures whose components have different halfwave oxidation potentials can be established relative to the concentration of the analyte sample. In the case of nearly identical halfwave oxidation potentials ($\Delta E_{1/2} < 0.1$ V), the electrospray response is directly proportional to the concentration ratio of the analytes. Analytes whose halfwave oxidation potentials differ by 0.1 to $\sim0.2$ V can both be detected in ESI mass spectrometry if the relative concentration of the analyte of lower oxidation potential is not significantly greater than the analyte with a higher oxidation potential. If the concentration of the analyte with the lowest oxidation potential dramatically exceeds that of the analyte of higher oxidation potential, preferential ionization and detection of the analyte with the lower oxidation potential will be found. In the case where the halfwave oxidation potentials differ by 0.2 V or more, exclusive ionization of the analyte with the lower oxidation potential results at higher sample concentrations. At lower sample concentration however, both analytes can be ionized to a degree relative to the concentration ratio of the analytes in the mixture.

These results suggest that one can identify and distinguish between etioporphyrins and octaethylporphyrins when only one type of metal, e.g., nickel, is present in both (Figure 9.16a) (Van Berkel 1995). Mixtures of etioporphyrins or octaethylporphyrins
Figure 9.16 ESI mass spectra resulting from the electrochemical ionization of a mixture of a) nickel etioporphyrin and nickel octaethylporphyrin (5 pmol/μL final metalloporphyrin concentration) and b) copper octaethylporphyrin and zinc octaethylporphyrin (5 pmol/μL final metalloporphyrin concentration). ESI-MS offers the potential to characterize mixtures of metalloporphyrins based on metal ligand or porphyrin ring system if the oxidation potentials are within 0.1 – 0.2 V.
with different metals (e.g. Figure 9.16b) should also be amenable to analysis providing that the relative concentrations of each metalloporphyrin are similar. Qualitative analysis of mixtures of metalloporphyrins whose halfwave oxidation potentials are dramatically different (e.g., ZnOEP and VOTPP) is possible only if the concentration of the analyte of lowest halfwave oxidation potential is low in absolute terms ( ~ 1-10 pmol/μL) and relative to the analyte with the higher oxidation potential.

Electrochemical ionization could be used to selectively, but not exclusively, ionize etioporphyrins and/or octaethylporphyrins that are present in a large molar excess in a mixture with tetraphenylporphyrins. Except for zinc tetraphenylporphyrin, all of the other tetraphenylporphyrins have a higher oxidation potential than any of the other etioporphyrins or octaethylporphyrins listed in Table 8.1.

9.6 Conclusion

The behavior of the metalloporphyrin mixtures reported here should aid in gaining a better understanding of the use of ESI-MS for characterizing mixtures of analytes. These experiments have shown that the ability of the electrospray source to ionize one species relative to another is effectively a function of concentration when the oxidation potentials are nearly identical. When the oxidation potentials of two species differ by more than 0.1 V, the ability to ionize one species relative to another in the electrospray source becomes a function of the oxidation potentials of the species, with large differences (> 0.2 V) resulting in the near exclusive production of the analyte of lower oxidation potential. This knowledge will aid future experiments planned at utilizing the selectivity of ESI-MS for characterizing complex mixtures of analytes.

9.7 Future Work

The selective ionization of porphyrin analytes has been demonstrated using the "non-conventional" ESI source described in Chapters 8 and 9. The limitations of the source for single analyte solutions and porphyrin mixtures have been addressed as...
well. The largest limitation to the forward progress of this study is the source configuration utilized in this study. To maximize the electrochemical process, it is believed that an increased residence time for the analyte at the applied voltage will yield higher ion currents and subsequently increased sensitivity for porphyrin detection. The limitations addressed for the selective ionization of the porphyrin using the non-conventional ESI source may be inherent to this particular source configuration. The source configuration does not allow for optimum analyte residence times even with increased flow rates. It was found that the sensitivity for the Analytica source in its present arrangement was much lower than expected. The analysis of porphyrin mixtures seemed to depend heavily on the ESI source parameters. The current source configuration, is believed to have prevented the determination of the true effects of concentration on the porphyrin ionization efficiencies in the mixture studies due to the dependance of the ion current on flow rate (cf. eq. 9.1). For this reason it was believed that a ESI source with the conventional configuration would have aided this study tremendously.

A new source design would be conducive to increasing ion production and subsequently detection limits which would ultimately aid in the analysis of complex mixtures like oil samples. This new source design should allow for an increased analyte residence time in the applied potential field. This effect might allow for analytes with broader $E_{1/2ox}$ potentials to be analyzed and detected simultaneously. The influence of source parameters (e.g., applied potential, flow rates) on ionization efficiencies may be even more effective for analyte mixtures if a longer analyte residence time in the applied potential field could have been used. A source of the conventional configuration allows for greater control on the source parameters which affect the outcome of the experiment.
BIBLIOGRAPHY


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APPENDIX A. LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3-HPA</td>
<td>3-hydroxypicolinic acid</td>
</tr>
<tr>
<td>6-HPA</td>
<td>6-hydroxypicolinic acid</td>
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<tr>
<td>A</td>
<td>adenosine</td>
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<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<tr>
<td>ATT</td>
<td>6-aza-2-thiothymine</td>
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<tr>
<td>C</td>
<td>cystidine</td>
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<tr>
<td>CI</td>
<td>chemical ionization</td>
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<tr>
<td>CID</td>
<td>collision induced dissociation</td>
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<tr>
<td>CoPP</td>
<td>cobalt protoporphyrin</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
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<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<td>DDQ</td>
<td>2,3-dichloro-5,6-dicyano-1,4-benzoquinone</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<td>DMT</td>
<td>dimethoxytrityl</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>Δt</td>
<td>change in time</td>
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<td>EI</td>
<td>electron impact</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
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<td>Etio</td>
<td>etioporphyrin</td>
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<td>eV</td>
<td>electron volts</td>
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<tr>
<td>FAB</td>
<td>fast atom bombardment</td>
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<td>FT-MS</td>
<td>Fourier transform-mass spectrometry</td>
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<tr>
<td>G</td>
<td>guanosine</td>
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<td>liquid secondary ionization mass spectrometry</td>
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<tr>
<td>m/z</td>
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<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
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<td>nm</td>
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<td>OD</td>
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<td>PD</td>
<td>plasma desorption</td>
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<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melt temperature</td>
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<tr>
<td>TOF</td>
<td>time of flight</td>
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<td>thermospray</td>
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<td>u</td>
<td>atomic mass unit</td>
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VITA

Victor E. Vandell was born October 6, 1965 in Chicago, Illinois. He completed his high school education at Scotland High School in Laurinburg, North Carolina. Mr. Vandell earned his Bachelor of Science in Chemistry at Chicago State University in 1991. While at Chicago State University, Mr. Vandell was an active member of Alpha Phi Alpha Fraternity Inc. Mr. Vandell earned his Masters of Science in Chemistry at Rochester Institute of Technology in 1994. Mr. Vandell then attended Louisiana State University where he pursued his doctoral degree under the leadership if Dr. Patrick Limbach. Mr. Vandell was awarded the distinguished Huel Perkins Fellowship in his second year at Louisiana State University. While at Louisiana State University, Mr. Vandell received the Chemistry Department Outstanding Teaching Award, and the Alpha Kappa Alpha Leadership Award. He will receive the degree of Doctor of Philosophy in May 2000.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Victor Vandell

Major Field: Chemistry

Title of Dissertation: General Studies in Mass Spectrometry
Part A: Matrix Assisted Laser Desorption Ionization (MALDI) Analysis of Oligonucleotides
Part B: Electrospray Ionization (ESI) Studies of Metalloporphyrins

Approved:

[Signatures and initials]

EXAMINING COMMITTEE:

[Signatures and initials]

Date of Examination: February 18, 2000