Sequencing and Characterization of Larger Oligonucleotides by Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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ABSTRACT

The rapidly emerging technique of electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry is reviewed with respect to DNA-related analyses. The focus of this review is mainly the analysis of intact molecular ions that are generated using enzymatic methods (*e.g.*, Sanger cycle sequencing chain termination products) with some emphasis placed on multistage MS. Although this review is dedicated to ESI-FTICR mass spectrometry, several other important issues of direct relevance, including sample preparation techniques and mathematical methods for mass spectrometry, are presented.

I. INTRODUCTION

The ability to promote high molecular weight thermally labile or nonvolatile compounds into the gas-phase without extensive fragmentation has only become a reality over the past two decades. These ionization techniques have been dubbed "soft" for their ability to produce intact molecular ions and include fast-atom bombardment (FAB) (1), static-secondary ion (SIMS) (2), and ^{252}Cf plasma

desorption (PDMS) (3, 4). These ionization methods allowed the production of intact molecular ions, for species in the low kDa range, but were found to lack general applicability to most species of masses greater than 5 kDa.

The mass spectrometric analysis of intact higher molecular weight biopolymers can be largely attributed to the advent of electrospray ionization (ESI) (5) and matrix-assisted laser desorption ionization (MALDI) (6). The multiple-charging phenomenon inherent in ESI allows fast, accurate, and precise molecular weight measurement, identification of modifications and more detailed structural studies for very high mass biopolymers (> 20,000 Da) using conventionally limited *m/z* mass analyzers. In general, the number of charges depends on the chemical nature and structure of the biopolymer (*e.g.*, protein, ss-DNA) as well as the solution and gas-phase environments; however, high molecular weight compounds typically exhibit broad charge-state distributions centered at relatively low mass-to-charge ratios ($m/z < 2000$).

The introduction of ESI and MALDI are two significant milestones from which the biological mass spectrometry era has not only emerged, but rapidly matured to a level allowing biochemical problems to be solved. Although both of these ionization techniques have allowed significant progress in the analysis of DNA, this review will solely focus on the progress and promise of ESI in nucleic acid research. In addition, although ESI has been coupled with a wide variety of mass analyzers, including double sector (7), linear quadrupoles (8), time-of-flight (9-11), quadrupole ion traps (12), and Fourier transform ion cyclotron resonance (FTICR) (13-15) (which in large part is chosen for the specific needs demanded by the application and even possibly by the available spectrometer), this review focuses on the progress and potential of ESI-FTICR-MS for DNA characterization.

ESI-FTICR-MS has the potential for making a significant impact in DNA analysis. Clearly, a rapid and accurate method for the characterization of DNA (synthetic or natural) would be invaluable for characterization of primers (which are utilized in DNA sequencing, polymerase chain reactions, and site-directed mutagensis),

characterizing modified oligonucleotides for use as therapeutic agents, detection of DNA damage products, large scale genomic DNA sequencing, polymerase chain reaction (PCR) product characterization, etc. Throughout this review, the terms nucleic acids, DNA, and oligonucleotide are used interchangeably; however, it should be noted that nucleic acids, of which DNA is a subset, are naturally derived biopolymers of cellular origin and oligonucleotides are of synthetic origin (*e.g.*, a DNA synthesizer) or degradation products from a nucleic acid. For example, a PCR amplification of a region of genomic DNA yields an x-mer oligodeoxynucleotide; however, what if long-PCR (*e.g.*, 25 kbp) is conducted? This PCR product would be correctly referred to as a 25,000-mer oligodeoxynucleotide. Thus, confusion arrives; but, it is important to at least recognize the difference (*e.g.*, a 25,000-mer produced by the PCR, which is *in vitro*, is not the same product as when the replication is conducted *in vivo*). Thus, specifically stating oligonucleotide clearly delineates the "source" of the sample under investigation. Throughout this review, we use the nomenclature that appeared in the original reference.

This review covers related practical issues (*e.g.*, desalting oligonucleotides) and several applications in which ESI-FTICR-MS has demonstrated significant potential. We do not, however, cover the ion chemistry associated with dissociation processes or the unimolecular reactions of multiply charged oligonucleotides as well as related ion/molecule reactions. Although the scope of this review pertains to ESI-FTICR-MS of DNA, several other reports that have significant relevance (*e.g.*, an algorithm for *de novo* sequencing (16) and solution modifications for spectral enhancement (17-19)) will also be covered where appropriate. The principles and characteristics of ESI (20-22) and FTICR (23-27) have been reviewed elsewhere, and those readers who are unfamiliar with these topics are referred to that literature (20-27). However, an important attribute of FTICR needs to be mentioned; **isotopic resolution allows direct charge-state measurement** (therefore, mass) (28). Throughout this review, many of the applications, such as multistage MS, would not be possible without knowing the charge-state of the multiply charged oligonucleotide anions. For example, fragmentation of a 50-mer into

several product ions does NOT produce a charge-state distribution which precludes charge-state assignment. Because the *m/z* spacing of the $^{12}C/^{13}C$ isotopic envelope for a *singly* charged ion is 1 Da, the addition of successive charges decreases the 1 Da isotopic spacing by a predictable and measurable quantity (28). It should be noted that other MS platforms (*e.g.*, ESI-TOF and quadrupole ion traps) are capable of direct charge-state measurement, albeit over a more limited MW range.

This review is written at a very exciting time and is intended to clearly indicate how much progress has been made in the field of biological mass spectrometry pertaining to DNA-related research since the first report nearly a decade ago by Covey *et al.* that demonstrated oligonucleotides ions (14-mer) could be analyzed by ESI-MS (29). Admittedly, due to continuous progress, several aspects of this review will become rapidly dated. This review covers a very broad spectrum of research, and therefore each section will be preceded by a short introduction relating to the particular topic(s) to be discussed. For the readers convenience, Table 1 lists the monoisotopic and average masses of the four constituent bases of DNA. Readers are also referred to several other recent reviews that pertain to the use of mass spectrometry in nucleic acid research (30-33).

II. Practical Issues Related to Electrospray Ionization of DNA

It was evident early on that analysis of DNA based upon ESI presented a greater challenge than proteins and peptides; that fact is partly reflected by the size of that literature. The difficulty with obtaining quality ESI mass spectra of DNA has largely been attributed to the highly anionic phosphate backbone, which results in substantial adduction of non-volatile cations (*e.g.*, Na^+, K^+). However, it is well known that "volatile salts" such as ammonium acetate are readily removed in the ionization/desolvation process, and are therefore widely used as buffers. Most research to date has focused on methods that remove buffers and salts prior to ESI-MS analysis because the adduction of salts and buffers reduces sensitivity and places more stringent demands on mass resolution (it has been shown that in the presence of even a minuscule amount of Na, mass measurements can

Table 1: Monoisotopic and Average Masses of the Four Deoxyribonucleic Acids of DNA

Deoxyribonucleic Acid	Monoisotopic Mass (Da)	Average Mass (Da)
A	313.054	313.210
G	329.054	329.209
C	289.045	289.185
T	304.049	304.197

be precluded for larger oligonucleotides). In addition, we will also address the important issue of the stability of single-stranded (ss) and double-stranded DNA (ds-DNA) because promotion of intact molecular ions in the gas phase is of primary importance for most applications, including tandem MS studies.

Sample Preparation Techniques for Oligonucleotides

The efficient removal of buffers and salts is crucial for the analysis of oligonucleotides by ESI-MS to avoid adduction, which degrades the mass spectrum. There are numerous steps at which salt can be introduced, such as the storage of samples in glass (or even plastic) vials or from more obvious sources such as the buffers and salts that are necessary for the wide range of DNA manipulations in molecular biology (*e.g.*, DNA sequencing and PCR). Therefore, in order for ESI-MS to be a useful technique, simple and rapid methods are needed to remove ubiquitous as well as large amounts of salts and buffers from relatively small samples (picomoles). There have been numerous methods reported for the removal of these types of contaminants from biological materials prior to mass spectrometric analysis, including multiple buffer exchange (typically accomplished using a Centricon filter) (34), precipitation (17, 35), C_{18} columns (36), high-performance liquid chromatography (HPLC) (35), additives such as organic bases (19, 34, 35, 37, 38) and solvents (18), microdialysis methods (39-42), collisional activation in the nozzle-skimmer region (17, 43), infrared dissociation in the FTICR cell (44), and cation-exchange resins (45, 46).

Several researchers have investigated a variety of methods for the removal of ubiquitous cations (*e.g.*, Na^+ and K^+) to facilitate the precise mass measurement of nucleic acids. Stults and Marsters observed as many 20 Na adducts to a 30-mer, which clearly delineates the significance of the problem (17). They reported that, by precipitating an oligonucleotide out of ammonium acetate with cold ethanol, a substantial decrease in the amount of sodium adduction was observed; this process proved effective for oligonucleotides that ranged from a 30-mer to a 77-mer. It was noted that this step could be repeated several times to improve desalting efficiency. Using this

procedure, the analysis of a mixed base ss-DNA 77-mer with only one sodium adduct remaining was observed, allowing a mass measurement accuracy of 0.03%. Stults and Marsters also reported that collisional activation of the precipitated oligonucleotides with residual gas molecules in the source region produced relatively sharper peaks, which was attributed to the loss of the adduct ions. However, fragmentation of the biomolecule was also observed that proved difficult to control (17).

Potier, *et al.* also utilized a precipitation method to allow the analysis of large synthetic oligonucleotides (35). Briefly, each oligonucleotide was HPLC-purified and precipitated from an ammonium acetate (10 M) solution overnight, at -20°C in ethanol, and this procedure was repeated **three times** to effectively remove cations. Using this desalting method with subsequent addition of high concentations of triethylamine (1%), mass spectra were obtained for synthetic oligonucleotides of a 28-mer, 40-mer, 72-mer, and a 132-mer size with a mass measurement accuracy of better than 0.01% for all but the 132-mer (35).

Limbach *et al.* also utilized ammonium acetate precipitations and the addition of chemical additives, which compete for adduct ions in solution, to reduce mono- and divalent cation adduction to nucleic acids (47). Specifically, they showed that using either ammonium acetate precipitations or organic base addition (or both) was not sufficient to remove all of the cations from the sample (47). Because tRNAs are known to bind Mg^{2+} strongly, therefore, they used chelating agents such as trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) to remove divalent metal ions and triethylamine to displace monovalent cations from the analyte, which allowed accurate mass measurements to be obtained (~0.01%) (47). Thus, some knowledge of the nucleic acid being investigated can help in determining the best salt "removal" method. However, precipitation methods in general are time-consuming, require relatively large sample amounts, the effectiveness increases with oligonucleotide length, and significant samples losses can be incurred.

Bleicher and Bayer reported on the use of organic solvents to improve the ESI mass spectra of oligonucleotides (18). They first investigated the effect of organic phase concentration on signal intensity for a 6-mer. They concluded that the higher the percentage of organic phase, the higher the ion abundance; a six-fold increase in ion intensity was observed when the infused solution contained 80% acetonitrile. It was also shown that a sample infused from 0.1 M ammonium acetate under their interface conditions resulted in an essentially useless ESI spectrum; however, after a 2-fold dilution of the sample in acetonitrile and the addition of 10 μL of triethylamine, 0.01% mass measurement accuracy was readily obtained in addition to a 50-fold increase in signal intensity (18). Clearly, the manipulation of solution composition has a dramatic effect on the quality of the resulting ESI mass spectrum. However, it is well known that a variation of the organic content effects the desolvation process, and that different interface designs may lead to different results with other instruments. Such caveats apply to all changes that involve ESI solution compositions, and are especially notable under the gentlest interface conditions that are used to preserve non-covalent complexes.

Deroussent *et al.* have reported that a solid-phase extraction device can be used to bind the DNA on the column, with subsequent washes to remove non-volatile cations prior to elution of the DNA (48). This method allowed the analysis of several oligonucleotides up to a 25-mer with 0.01% mass accuracy using a quadrupole mass spectrometer; however, it should be noted that Na adducts still remained (48).

The utilization of organic bases moderate Na^+ and K^+ adduction to DNA has been reported by several researchers (18, 19, 35, 37, 38, 41, 47, 49). Greig and Griffey reported that a number of organic bases can effectively reduce alkali adduction to ss-DNA (37). They showed that the use of piperidine resulted in a substantial decrease in Na adduction; however, it was accompanied by a signal suppression of the analyte material. The addition of a weak base, imidazole, slightly reduced cation adduction but improved the signal intensity. The co-addition of triethylamine and imidazole or piperidine and imidazole resulted in the enhancement of signal

intensity, an almost complete suppression of alkali adduct formation, and a slight shifting of the charge-state distribution to higher *m/z* (37).

Muddiman *et al.* investigated a number of solution compositions, including organic acids, bases, and solvents (19), for reducing charge states concurrent with reduction in alkali metal adduction to facilitate complex mixture analyses. These studies confirmed previous findings (18, 37) and showed that a cocktail containing acetonitrile, piperidine, and imidazole enhanced spectral intensity and significantly reduced cation adduction (19). However, it was shown that non-covalent adduction of piperidine (9 adducts of piperidine could be detected on an 18-mer) to nucleic acids could be readily detected at lower desolvation capillary temperatures (19). It was unclear whether piperidine adduction to larger oligonucleotides would be problematic at modest capillary temperatures because all oligonucleotides that were investigated up to this point utilizing organic bases were smaller than 25-mers. Preliminary results have shown the removal of piperidine adducts from DNA in the FTICR cell using a low energy sustained off-resonance irradiation (SORI) event (19); over 10 adducts of piperidine to a 18-mer could be completely removed in a single soft SORI step. This process is analogous to the efficient removal of at least some non-covalent adducts by infrared irradiation (IR). Little, *et al.* (50) showed that the application of a 200 ms IR pulse facilitated the analysis of a 100-mer DNA strand that otherwise yielded an essentially useless spectrum. It was noted that an accurate mass measurement was obtained due to the high resolution capabilities of FTICR and that significant adduction was still present. To further test their approach, they added 1,4-diaminobutane (DAB) to a synthetic 50-mer and observed adduction of up to 14 DAB molecules prior to IR dissociation (50). After a 17 ms IR irradiation pulse, the adduction of DAB to the DNA appeared negligible (50). It should be noted that, in many cases (*e.g.*, IR dissociation), it is not clear what adducts are being removed.

Recent evidence suggests that piperidine adduction is not problematic even when larger oligonucleotides are investigated, as reported by Wunschel *et al.* (49) and Muddiman *et al.* (41). Arguably, the most striking evidence that the co-addition of piperidine and

imidazole to the sample containing the nucleic acid was reported by Wunschel *et al.* (49); these data also serve to highlight the effectiveness of this approach. Figure 1A shows the ESI-FTICR mass spectrum of a synthetic mixed base 49-mer that was HPLC-purified three times in attempts to remove the salt. This sample was infused in 80 vol % acetonitrile (as previously shown to enhance spectral intensity) (18) and resulted in an uninformative mass spectrum. A range of 5 to 15 Na adducts was detected, which precluded effective mass measurements (it should be noted that this same sample 2 weeks previously yielded a satisfactory ESI mass spectrum with no Na adduction; thus, the amount of Na contamination is expected to be

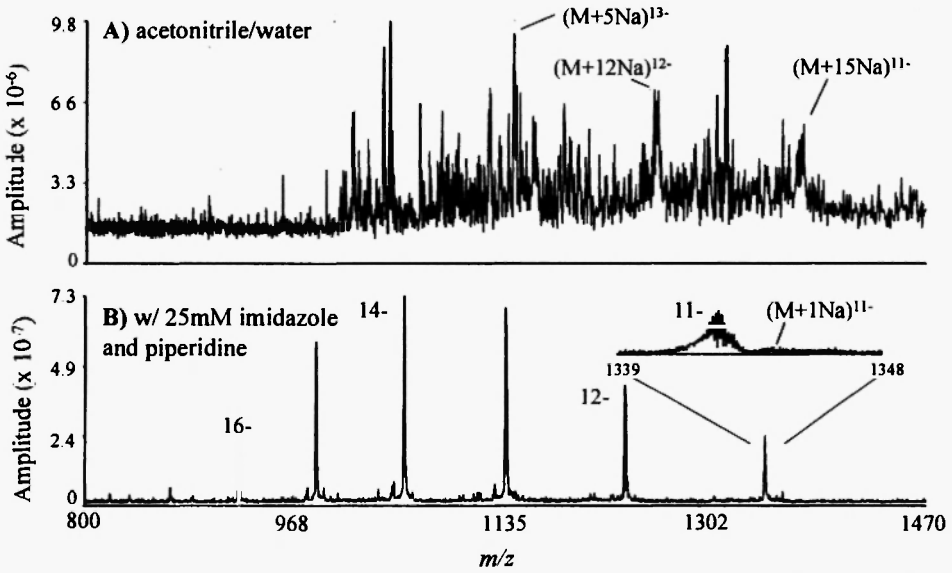


Figure 1: a) The ESI-FTICR spectra for a mixed-base 49-mer infused from a solution containing 80 vol % acetonitrile acquired with a 10 second selective-accumulation with quadrupolar noise excitation corresponding to the *m/z range* 1000-2000. (b) ESI-FTICR spectrum produced with a 3 second accumulation and a similar solution as in 2a except for the addition of 25 mM piperidine and 25 mM imidazole. The inset in the spectrum shows an expansion of the 14- charge state. (Figure adapted from *Rapid Commun. Mass Spectrom.* **1996,** *10*, 29-35)

minimal and to result from the short-term storage conditions), a striking demonstration of the high affinity of Na for DNA. Addition of piperidine and imidazole resulted in the ESI-FTICR mass spectrum shown in Figure 1B. Clearly, this simple step results in a tremendous gain in mass spectral quality; Na adduction is essentially non-existent and isotopic resolution was obtained.

Doktycz *et al.* investigated ESI-MS for the analysis of tRNA's and DNA, utilizing a high percentage of organic phase (methanol) to reduce surface tension and thus facilitating efficient desolvation (51), but did not rid the tRNA of adduction with metal ions (resulting in a mass spectrum with an error of over 2%!). These workers also investigated the analysis of a mixture of a 72-mer and 75-mer (51). In this case, the alkali ions were exchanged with ammonium ions prior to ESI-MS analysis, using either anion-exchange columns or precipitation methods; both processes proved ineffective and accurate mass measurements were precluded due to residual sodium adduction (51). This report clearly illustrates that sample preparation is crucial for the ESI mass spectrometry analysis of larger oligonucleotides. One possible explanation for this poor mass accuracy could be that the organic solvents are contaminated with nonvolatile cations that are inadvertently added to the tRNA sample, as indicated in a recent report by Simmons *et al.* (38). It should be mentioned that another method using cation-exchange resin to convert the oligonucleotide to the ammonium form has proven successful for MALDI (45) and FAB (46), but that there are as yet no reports concerning its use with ESI-MS.

Recently, Smith and coworkers have developed a microdialysis method for the rapid and effective removal of buffers and salts from proteins and nucleic acids (39-42). This work has clearly shown that microdialysis allows effective handling of small sample volumes (μL) and sample amounts (picograms), more efficient and rapid desalting, and flexibility (*e.g.*, buffer composition, ability to couple on-line with mass spectrometry). Recently, the use of this microdialysis clean-up approach has permitted the analysis of PCR products by ESI-FTICR-MS (41). Prior to the utilization of microdialysis, the analysis of PCR products was problematic (49).

The microdialysis procedure for desalting nucleic acids is illustrated here for an 89-base pair PCR product generated using a bacterial genome as the template DNA (41). PCR products initially consist of a complex mixture of buffers, salts, enzymes, and nucleotidetriphosphates (dNTP's). The mixture was poured over a silica resin column, which preferentially binds large DNA (over 65 base pairs) in the presence of a chaotropic salt (*e.g.*, guanidinium hydrochloride). While the PCR product was bound to the column, the DNA was washed extensively with ammonium acetate, water, and ethanol prior to elution from the column; most of the salt was removed. Figure 2A shows the ESI-FTICR mass spectrum after addition of acetonitrile, piperidine, and imidazole. The poor signal-to-noise ratio is attributed to cation adduction, which precluded peak assignments. Figure 2B shows the ESI-FTICR mass spectrum of the same PCR product after a 5 minute microdialysis clean-up step. The spectrum clearly shows the coding and non-coding strands of the PCR product (*i.e.*, the single-stranded products), allowing accurate mass determination of each strand. Thus, the 5 minute microdialysis step (which can also be implemented on-line) significantly improved the overall quality of the ESI mass spectrum of the PCR products. Clearly, the combination of microdialysis with organic base addition appears to be the current "state-of-art" methodology for desalting nucleic acid samples.

Stability of Large Single-stranded and Double-stranded DNA

Due to early difficulties, it was often speculated that large DNA ions were inherently unstable in the gas phase. For most mass spectrometric applications, it is extremely important that there is not an inherent instability in single-stranded (ss) or double-stranded (ds) DNA (*i.e.*, the transfer of intact DNA from solution to the gas-phase is of the most fundamental importance). The largest ss-DNA reported to date is a synthetic 132-mer by Potier, *et al.* In that study, they utilized a triple quadrupole mass spectrometer equipped with ESI; they observed three peaks in the mass transformation spectrum and assigned them to the intact molecular ion and to the loss of one and two internal bases, presumably due to failures in the synthesis. Due to

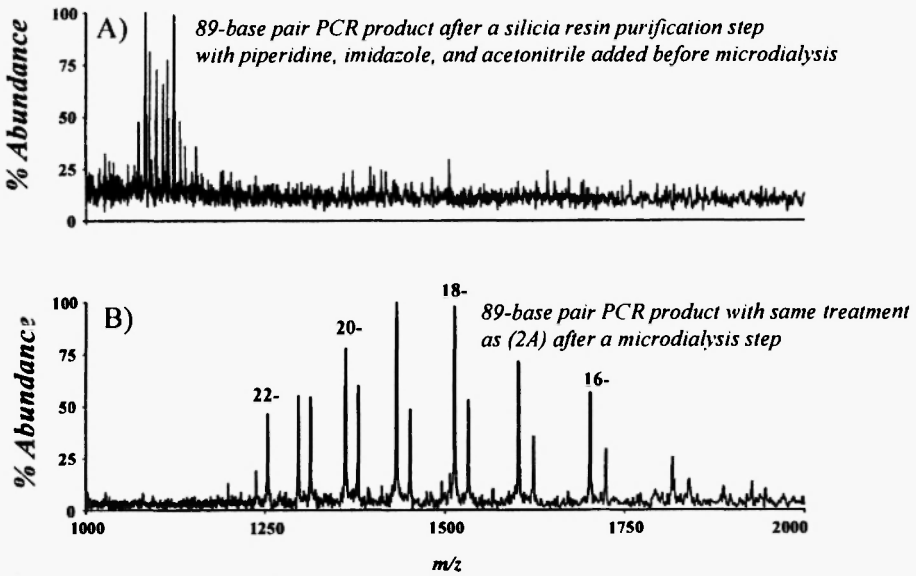


Figure 2: ESI-FTICR mass spectra of the 89 bp PCR product amplified from *B. anthracis*. Figure 2A represents the spectra obtained after removal of reaction components and addition of piperidine and imidazole, but without microdialysis. Figure 2B shows the spectrum of the 89 bp product, which was dialyzed prior to the addition of piperidine and imidazole. Note the shift in intensities to lower charge-states (higher *m/z*) for the charge states of the individual strands of the product, as well as an improved S/N. (Figure from *Anal. Chem.* **1996,** *68*, 3705-3712)

the limited resolution and possible alkali metal adduction, it was difficult to ascertain whether any fragmentation of the intact species occurred. However, this result clearly indicated that large ss-DNA molecules are amenable to ESI-MS analysis.

There are a number of other examples in the literature of large ss-DNA (> 45-mers) that indicate that ss-DNA is (or can be) stable during the electrospray process (41, 44, 52-55). It is certainly possible to collisionally dissociate oligonucleotides in the ESI interface; the essential issues are whether there are any fundamental limitations of the gas-phase stability of DNA and whether useful ESI conditions can be readily determined. Little *et al.* has reported partial sequence

verification for a 108-mer (52), indicating in another report that dissociation is problematic (53). The transfer and effective detection of intact deprotonated DNA ions after ESI might have been limited in these studies due to the extensive cation adduction to the highly charged polyanionic backbone of DNA. Severe adduction would have required harsher interface regions (collisional activation) for adduct removal during ESI to produce spectra with sufficient quality to allow a detectable DNA ion current (See Figure 1A as an example of how Na adduction results in a degraded mass spectrum).

Recently, utilizing effective desalting procedures (*i.e.*, microdialysis and addition of organic bases to "bind" cations), the coding and non-coding strands (*i.e.*, single-strands) of a 114 base pair PCR product were both detected intact with no evidence of fragmentation (41). Figure 3 shows a spectrum for a 114 base pair PCR product. Thus, with improved sample manipulation techniques, including desalting, as well as a better understanding of the electrospray ionization technique, the transfer of significantly larger intact large ss-DNA to the gas-phase should be possible.

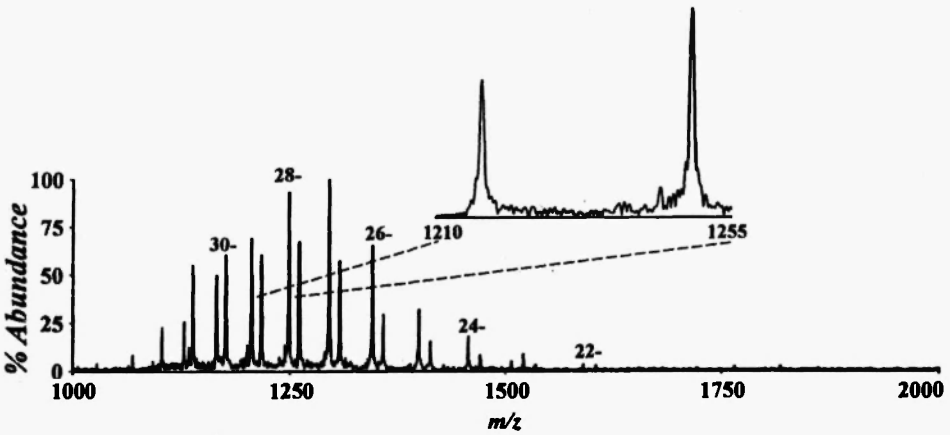


Figure 3: ESI-FTICR mass spectrum of a 114-bp PCR product amplified from *B. subtilis* with the coding and non-coding strands easily resolved. The inset shows an expansion of the *m/z* range 1210 - 1255, indicating that no fragmentation of the the 114-bp ss-DNA was observed. (Figure adapted from *Anal. Chem.* **1996,** *68*, 3705-3712)

Studies with ds-DNA macroions have clearly indicated that there are no significant molecular weight limitations related to the ESI process (56-58). This laboratory has shown that individual ions of T4 DNA with a nominal molecular weight of 1.1 x 10^8 Da from ESI can be trapped and detected using FTICR-MS, allowing accurate mass measurements to be obtained (56). These experiments relied on a direct charge measurement, using the image current amplitude on the detection plates and calculated cyclotron radius; those experiments resulted in a mass measurement accuracy of 5-10%. Another later study by Fuerstenau and Benner utilized a related charge detection scheme, in which the image current from a single ion is measured as it passes through a tube while simultaneously defining the ion's velocity; that instrument allows the mass to be determined (57). Analysis of several DNA samples with molecular weights exceeding 1 MDa were obtained for ions in the *m/z* 2000 to 12,000 range, based upon measurements of about 3000 individual ions (57). A third report appeared by Cheng *et al.*; they measured the precise charge on a series of individual highly charged ions of plasmid DNA using a charge-state shifting approach (58). That study involved the ESI of 2 MDa plasmid DNA with subsequent trapping of the macroions in a FTICR cell. The ions were allowed to slowly undergo gas-phase reaction with acetic acid to shift the charge-states in a stepwise fashion. That method of making mass measurements of large ds-DNA ions allowed a very high mass accuracy for individual ions. Interestingly, although individual masses could be accurately measured, those measurements only showed an agreement to within 0.2% of the expected mass for the center of the distribution of measured ions; the measured mass of 1.95 $\pm$ 0.07 MDa (expected 1.946 MDa) (58).

Combined, these reports indicate that ds-DNA can be transferred intact into the gas phase via ESI without any significant dissociation. Available results indicate that ds-DNA is more stable in the gas phase than ss-DNA; that observation can be partly attributed to its higher *m/z* (which reduces dissociation in the interface region) and the high stability of the double-stranded structure and the internal location of the more labile bases. If the latter point is correct, and the double helix structure is preserved in the gas phase, then adducted bases may be more readily lost for ds-DNA because it is known that at

least some structures involve a "swing out" of the modified DNA and its exposure on the "outside" of the double helix. However, a likely alternative to these experimental observations is the limitations imposed on obtaining large ss-DNA species of high purity, which are typically obtained by synthetic means; ds-DNA can be obtained from biologically derived sources; therefore, more and larger species are readily available for analysis.

III. The Characterization of DNA Sequencing Mixtures by Mass Spectrometry

One of the most challenging and potentially significant applications using ESI to analyze complex mixtures is in the area of DNA analyses; specifically, the ability to rapidly determine the primary structure (*i.e.*, base sequence) of DNA (31). Mass spectrometry offers the potential to sequence DNA utilizing a wide variety of collisional dissociation techniques as well as rapidly "sizing" Sanger sequencing products or sequencing ladders many orders of magnitude faster than electrophoretic techniques. Also, as the Human Genome Project goals are realized, it is evident that the need for comparing and rapidly identifying sequence differences for specific regions of DNA will increase.

The ultra high mass resolution that is inherent in FTICR potentially provides an improved basis for the characterization of dissociation products for large multiply charged oligonucleotide ions (see next section) as well as complex sequencing mixtures as a result of the enzymatic synthesis or degradation process (*e.g.*, ladder creation or digestion). In addition, point mutations (*e.g.*, base substitutions, additions, or deletions) can be derived by comparison of the mass corresponding to the expected sequence with the measured mass; that process has recently been demonstrated by observing a "shift" in molecular weight that could be correlated to a specific base substitution (41, 59). In this section, we describe the use of ESI mass spectrometry for analyzing chain termination products as well as to the analysis of DNA sequencing ladders > 20-mers, and briefly describe the current status of tandem MS approaches to obtain structural information about large DNA fragments. Readers are referred to an

excellent review by Limbach, which provides a comparison of the different enzymatic methods as well as the gas-phase sequencing methods (30).

Sanger Cycle Sequencing

Although traditional DNA sequencing is based almost entirely upon the electrophoretic analysis of nested sets of oligonucleotides of various lengths, faster, more sensitive, and more accurate methods are desired to support the goals of the various Genome programs. Improved sequencing methods generally must be capable of addressing mixtures with hundreds of unique components that can span up to two orders of magnitude difference in molecular weight. Currently, DNA sequencing is conducted primarily using slab gel electrophoresis methodology. Traditional slab gel electrophoretic analysis of dideoxy sequencing (*i.e.*, Sanger method) (60) mixtures typically requires a full day for preparation, separation, and analysis. Classically, sequencing requires four separate reactions and four lanes of gel; however, automated methods that are based upon fluorescent labels now permit sequencing from a single reaction, have decreased the separation and data acquisition time to 4 to 6 hours. Although separations upon conventional gels appear to be approaching a practical limit (*i.e.*, limitations imposed by Joule heating using higher electric fields) for dideoxy-sequencing mixtures, capillary electrophoresis and ultra thin slab gels (that dissipate heat more effectively at higher electric fields) have recently shown promise for generating several hundreds of bases of sequence information in less than an hour and for running many parallel separations. Despite these advances, such methods are still developmental, and even faster alternative technologies remain attractive.

Recently, Wunschel *et al.* have utilized the ability of ESI to transfer a complete dideoxy sequencing reaction mixture into the gas phase with subsequent analysis using the high resolution capabilities of FTICR (61). The initial work used cycle sequencing to generate a sequence ladder from a synthetic double-stranded PCR-amplified template (coding strand sequence: **5'-CCT ACA ACA CCA GGA AGT ACG AGT GCT GAG CCG AGA TCT ACC CGG ACA TCA CC-3'**) to

evaluate the potential of ESI-FTICR-MS for DNA sequencing. The 53-mer PCR amplification template (~200 picomoles) was denatured and annealed to a 20-mer primer complementary to the template strand (offset by 5 bases inward from the 3' end). The cycle sequencing was carried out in the presence of **all four** dideoxy analogs and normal dNTP's. Thus, the template strand is terminated for a fraction of the products at each base after the primer to the end of the template to yield the desired set of oligonucleotides. In addition to the 28 different oligonucleotides obtained from these dideoxynucleotide polymerization termination events, which occur at every possible base position from primer to the end of the template, there are also two strands for the PCR amplification sequencing template (coding and non-coding), the primer, and a polymerization product without incorporation of dideoxynucleotides. Thus, over 30 components of varying size and concentration are present in this mixture (61).

The ESI-FTICR mass spectrum shown in Figure 4 was obtained from the direct infusion of the sequencing reaction mixture after a HPLC step and addition of piperidine and imidazole (61). Clearly, there are numerous peaks spread over a wide range of *m/z* values (750 - 2000) that have different relative intensities. Expansion plots for two narrow *m/z* ranges are also shown in Figure 4, with two additional insets to illustrate the resolved isotopic distributions for two of the peaks that directly provide the charge state for the corresponding molecular species, (*i.e.*, charge state (z) = 1 / *m/z* spacing) (28). Table 2 lists the assignments of the peaks depicted in the two expanded *m/z* regions in Figure 4. The adenylation of the template DNA is a result of the non-templated nucleotide addition when using DNA polymerases that lack proofreading activity, such as *Thermus Aquaticus* that was used in the PCR phase of these experiments (62). Table 3 gives the "deconvoluted" masses derived from these measurements, the expected molecular weights (based on the known sequence), and the number of charge states detected for each species. Because sometimes only a single charge state is detected, isotopic resolution was required to determine charge state (and therefore mass) for these species. The sequence is readily obtained from the mass difference between sequential termination products (Δ), and these results and base assignment are also shown in

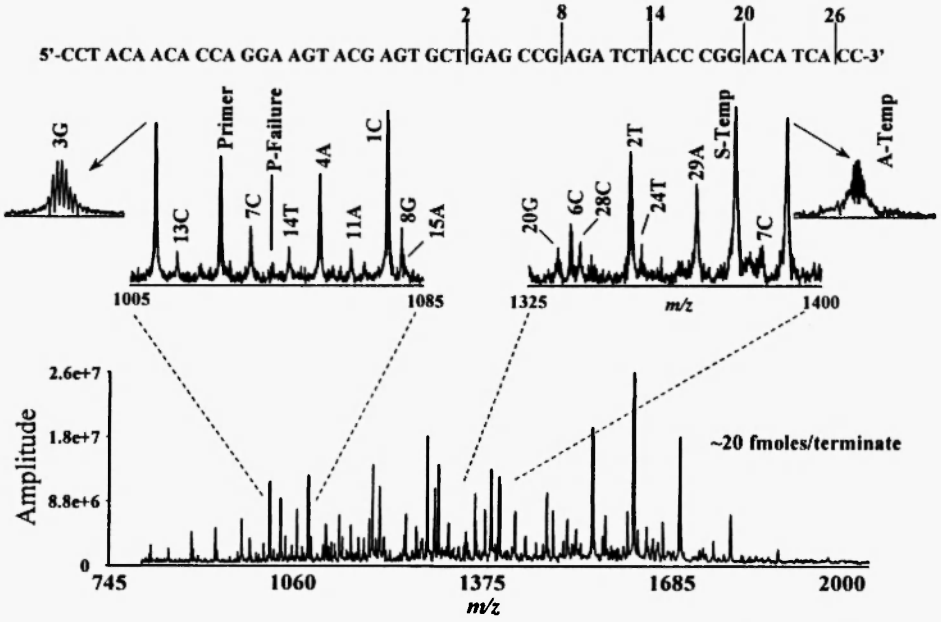


Figure 4: Electrospray ionization Fourier transform ion cyclotron resonance mass spectrum of the DNA sequencing reaction containing all four ddNTP's. The sample was electrosprayed from a solution containing 30% acetonitrile, 33 mM piperidine, and 33 mM imidazole. Broadband quadrupolar excitation was applied over the *m/z* range from 600 - 2500 at 10 V_{pp}.

Table 3. Failures in the primer synthesis, both the 17 and 19-mer, were also detected. These results clearly indicated that ESI-FTICR-MS has the ability to sequence DNA; however, high mass resolution detection is required for the analysis of Sanger sequencing reactions.

The complexity of the mixture is obviously a major issue for the extension of ESI mass spectrometric approaches to the sequencing of larger DNA segments. Although the full sequence could be read in the present case, the extension to larger DNA fragments will certainly increase the demands related to dynamic range. The dynamic range is defined (or limited) by the number of charges that is required to obtain a signal-to-noise ratio of 3 and the maximum number of charges that

Table 2: The peak assignments shown in Figure 4 (*i.e.* a through t) of the components that were detected in the two different expanded *m/z* ranges. The *m/z* value, the charge state (N), and the corresponding assignment are listed.

Peak Label	*m/z* value	N	Assignment
a	1011.96	7	23-mer
b	1017.88	10	33-mer
c	1029.70	6	Primer (20-mer)
d	1037.95	8	27-mer
e	1043.35	5	17-mer
f	1048.40	10	34-mer
g	1056.71	7	24-mer
h	1065.26	9	31-mer
i	1075.22	6	21-mer
j	1079.12	8	28-mer
k	1079.74	10	35-mer
l	1332.82	9	40-mer
m	1336.09	6	26-mer
n	1338.44	11	48-mer
o	1351.30	5	22-mer
p	1354.25	10	44-mer
q	1368.36	11	Primer + 28 bp + dA
r	1378.01	12	PCR (C) Template + dA
s	1384.40	6	27-mer
t	1391.26	12	PCR (N) Template + dA

Table 3: From the data shown in Figure 4, the assignment, the expected molecular weight, the number of charge states detected (N), the calculated molecular weight, and the associated standard deviation. The mass error for the majority of the assignments was less than 10 parts-per-million.

MW (calculated)[a]	N[b]	MW (observed) ± 2s	Assignment	Δm (Da)
16707.38	4	$16707.30 \pm 0.16_4$	N Template + dA	-
16548.29	4	$16548.40 \pm 0.14_4$	C Template + dA	-
15063.27	3	$15063.10 \pm 0.20_0$	Primer + 28 bp + dA	-
14734.05	1	14733.92	Primer + 28 bp	-
14444.86	2	$14444.80 \pm 0.01_4$	-C	289.12
14155.67	1	14155.81	-C	288.99
13842.45	2	$13842.7 \pm 0.12_7$	-A	313.11
13553.26	2	$13552.60 \pm 0.06_4$	-C	290.10
13249.05	2	$13249.40 \pm 0.14_8$	-T	303.20
12935.83	1	12935.41	-A	313.99
12646.64	2	$12646.40 \pm 0.03_5$	-C	289.01
12333.42	1	12333.51	-A	312.89
12004.20	3	$12004.40 \pm 0.07_2$	-G	329.11
11674.98	2	$11675.00 \pm 0.16_9$	-G	329.40
11385.79	1	11385.44	-C	289.56
11096.60	1	11096.69	-C	288.75
10807.41	1	10807.49	-C	289.20

Table 3:

10494.19	1	10494.11	-A	313.38
10189.98	2	$10189.50 \pm 0.81_9$	-T	304.61
9900.79	2	$9900.87 \pm 0.13_7$	-C	288.63
9596.58	1	9596.39	-T	304.48
9283.36	1	9283.37	-A	313.02
8954.14	3	$8954.01 \pm 0.24_5$	-G	329.36
8640.92	3	$8640.84 \pm 0.16_6$	-A	313.17
8311.70	4	$8311.87 \pm 0.40_2$	-G	328.97
8022.51	4	$8022.58 \pm 0.30_0$	-C	289.29
7733.32	5	$7733.27 \pm 0.15_1$	-C	289.31
7404.10	5	$7404.06 \pm 0.04_8$	-G	329.21
7090.88	5	$7090.81 \pm 0.04_6$	-A	313.25
6761.66	5	$6761.57 \pm 0.02_7$	-G	329.24
6457.45	4	$6457.38 \pm 0.01_9$	-T	304.19
6184.26	4	$6184.25 \pm 0.01_1$	-C (Primer)	273.13[c]
5855,04	2	$5855.08 \pm 0.22_6$	19-mer	
5221.61	1	5221.79	17-mer	

[a]based upon known sequence
[b]N is the number of charge states used to calculate the molecular weight
[c]Note: Primer is not dideoxy terminated

can be trapped in the ICR cell; the detection of multiply charged ions produced by ESI significantly adds to the latter problem, which is commonly referred to as the space-charge limit. Several reports describe the dynamic range and detection limit of FTICR (63-67).

One obvious approach to that limit would be to divide the Sanger method into the traditional "four pot" reaction, thereby decreasing the complexity of the reaction by *ca.* a factor of four. Recently, using the same DNA sequencing reaction, the dynamic range was enhanced by using broadband quadrupolar excitation accumulation waveforms (64, 65, 68-72) and "stepping" through the entire *m/z* domain in 225 *m/z* increments (73) to selectively accumulate a specific ion population. This method and related approaches (64) essentially circumvents the space-charge limit (*i.e.*, the finite number of charges that the FTICR cell can hold), and effectively increases the dynamic range (73).

Mass Analysis of Exonuclease Digestions of DNA

The generation of DNA sequencing ladders based upon an exonuclease (phosphodiesterase), which sequentially cleaves an oligonucleotide at the phosphate backbone from the 3' or the 5' end, can be used to produce sequencing mixtures for MS analysis. Under the appropriate reaction conditions, an oligonucleotide can be rapidly degraded into a ladder that ranges from the intact oligonucleotide down to a single nucleoside (74, 75). For short oligonucleotides (<10-mers), one can use a single reaction time and "read back" the sequence based on the mass differences. However, for larger oligonucleotides, a time-sampling approach to the digestion process is necessary to create a "sequence window"; appropriate control of the reaction time to ensure overlap allows the entire sequence to be read. There are many more reports in using MALDI as a method to analyze exonuclease digestion products ((76), and references therein), in part, due to its spectral simplicity; however, the discussion here will be limited to ESI because neither technique has been demonstrated superior to the other for such analyses.

McCloskey and coworkers used this approach and were able to almost completely sequence a 10-mer using a continuous injection

approach coupled with ESI quadrupole mass spectrometry (74). Glover *et al.* digested a 10-mer, with subsequent separation of the products using off-line liquid chromatography followed by ESI mass spectrometry (75).

Recently, Smith and co-workers have demonstrated that the partial sequence of a mixed-base 22-mer could be determined by digestion of the template, using an exonuclease and sampling the reaction mixture in time with subsequent analysis by ESI-FTICR mass spectrometry (77). Figure 5 shows the ESI-FTICR spectrum that was obtained upon direct infusion of the partial 3'-5' exonuclease digest mixture which provided over 45% of the sequence in a single spectrum. Although multiple charge-states were not observed for all species (problematic for low resolution measurements), the charge-states were readily determined from the isotopic spacings and thus the mass ladder (*i.e.*, sequence) was easily obtained. Analysis of five different reaction times allowed over 65% of the sequence to be defined and, due to the size (22-mer), no significant demands were placed on the mass accuracy or resolution afforded by FTICR; therefore, much longer read lengths could be obtained. The results presented here indicate that this approach may provide an alternative DNA sequencing strategy by using ESI mass spectrometric detection for shorter oligonucleotides.

Collisional Activation Approaches for DNA Sequencing

The use of multistage mass spectrometry has been the focus of several reviews (16, 33, 78-80), with others specifically dedicated to discussions pertaining to oligonucleotide sequencing (30, 31, 33). In two recent reviews (Nordhoff *et al.* (33) and Limbach (30)), the apparent differences in fragmentation pathways of oligonucleotides were summarized; and readers are referred to those reviews and references therein.

There have been numerous reports in the literature that describe tandem mass spectrometry of normal and modified oligonucleotides of known sequence that range from 2-mers up to 108-mers by utilizing a wide variety of dissociation techniques and mass

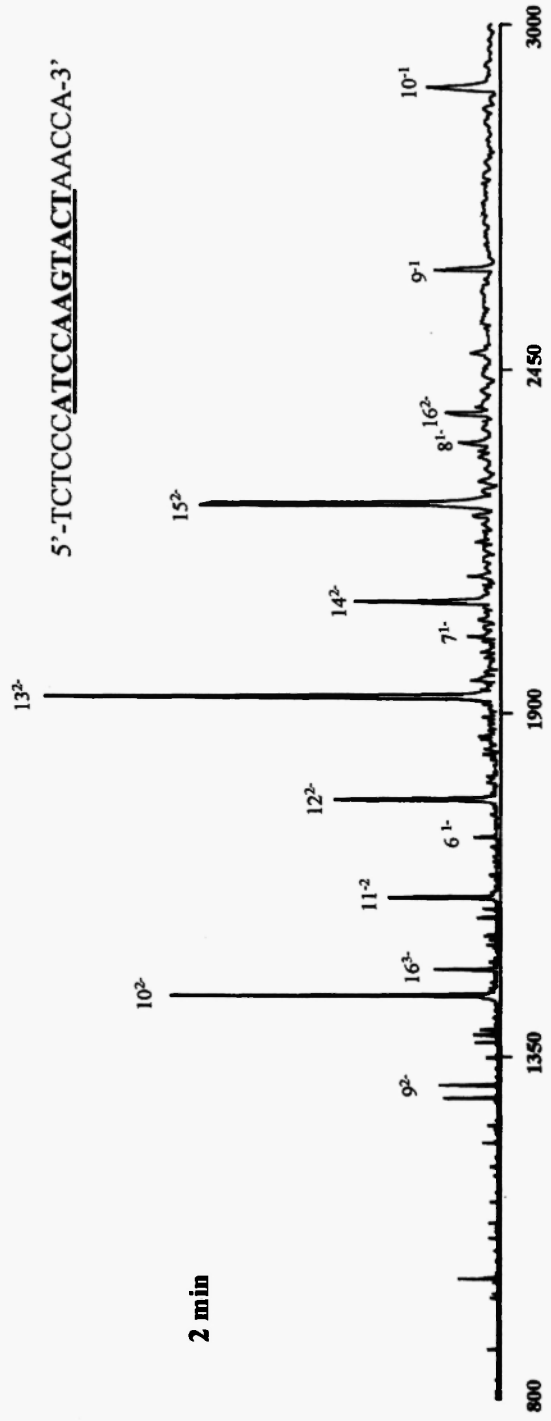


Figure 5: ESI-FTICR mass spectrum of a partial exonuclease digestion of a mixed base 22-mer. Splicing results from five different reaction times allowed ~70% of the 22-mer to be rapidly sequenced.

analyzers (16, 31, 44, 52-54, 80-89). Although each report (as well as others) demonstrates specific capabilities of tandem MS of electrosprayed oligonucleotide ions, we focus here on the gas-phase fragmentation of large oligonucleotide ions for the purposes of DNA sequencing or characterization (16, 52-54, 82).

Building upon the early work of McLuckey *et al.* (80, 83), ESI coupled with FTICR mass spectrometry has proven the ability to rapidly sequence small oligonucleotides (< 15-mers) by using collisional dissociation in the intermediate pressure region of the ESI source (nozzle skimmer - NS) (82). Using NS, collisional activation methods and infrared multiphoton-dissociation (IRMPD), have demonstrated the potential for providing sequence information for several oligonucleotides up to a 108-mer (52).

Most of the progress in FTICR-MS has been made by Little *et al.* in a series of papers (52-54, 82) that have described the use of high resolution FTICR tandem mass spectrometry using NS (43), dissociation in the FTICR cell using multiple collisional activation steps (MECA) (90), SORI (91), and/or IRMPD (50, 52). They have investigated a number of oligonucleotides of varying sizes with a variety of fragmentation methods and have found that, even under apparently similar experimental conditions, different results are observed (52). They found that NS, IRMPD, and MECA provide complementary information with the later providing significant information on the internal ions (or sequence) whereas the lower energy of SORI was found to result in primarily base loss, which is consistent with other findings (89). Aaserud *et al.* has also utilized an approach that also allows the controlled deposition of energy into DNA to produce structurally relevant fragments (92). In that study, they utilized the Blackbody Infrared Radiative Dissociation technique introduced by Williams and co-workers (93) and found that, even at low cell temperatures (~150 °C), similar dissociation processes were observed; however, additional sequence information was obtained (92). Although dissociation in the ESI interface (*i.e.*, NS) region is also effective, all components in a mixture undergo dissociative processes to produce a complex mass spectrum. A fragment ion can, however, be pre-selected, accumulated, and gas-phase purified with

subsequent multi-stage dissociation being conducted in the trapped ion cell (65, 69, 94). McLafferty and co-workers also reported the development of mathematical approaches based on the IRMPD of oligonucleotide anions to assist in the interpretation of these highly complex product-ion spectra (52). Although FTICR-tandem-MS can identify the site and base substitution of a point mutation in a synthetic oligonucleotide, many applications of this type do not require sequence information. For example, flanking a point mutation in a genomic region with two 20-mer primers would result in a 41-bp PCR product (*vide infra*). Because the primer sequences are known *a priori*, and because they become part of the PCR product, the only unknown is the base-pair that is being flanked. The presence of a mutation can thus be determined by a simple, accurate mass measurement. However, when screening for unknown mutations, multi-stage MS (*i.e.*, MS^n) should prove to be a valuable technique to determine the polymorphic locus.

An interesting observation was reported by Bartlett *et al.*; they investigated the effect that backbone charge has on the preferred dissociation pathway of oligonucleotides (95). Briefly, they modified the phosphodiester linkages with methylphosphonate to effectively concentrate the charge on the 5' or 3' ends of the molecule, with an even distribution along the backbone. Collisional activation of these three different cases, as well as the unmodified control, produced "w" and "a-B" ions for every residue. These data indicate a formal charge on the phosphodiester is not required using multiple collisional conditions.

Recently, a series of papers that describe the analysis of **polyprotonated** oligonucleotides have appeared (96-98). A systematic investigation of the tandem MS of **positively charged** oligonucleotides was reported by Ni *et al.* (98) and by Martin *et al.* (97). In the initial studies, they found that, for polyprotonated oligonucleotides up to the 19-mer level infused from a water/propon-2-ol solution in an ammonium acetate buffer (2.5 mM to 30 mM), polyprotonated spectra could be readily obtained (98). CID of the positively charged oligonucleotide ions showed three primary product series: 1) loss of a protonated base, whose abundance correlated with

the base proton affinity; 2) phosphodiester chain cleavage at C3'-O3', which describes the sequence in the 3'-5' direction; and 3) furan-type ions from chain cleavage concominant with base loss which describe the sequence in the 5'-3' direction (98). Martin *et al.* reported that sequence information could be readily obtained, primarily "w" and "a-B" ions, as typically observed in polydeprotonated CID spectra (97). However, for the analysis of the homopolymers of A, G, C, and T, the latter produced strikingly different results. The difference was that poly T produced exclusively x-2H and z-2H ions, which were thought to be a result of the phosphate backbone being the site of protonation rather than the base (97); that conclusion is consistent with the results reported by McCloskey and coworkers (98).

At this time, it is difficult to ascertain which approach will ultimately be best for the determination of the primary structure of oligonucleotides. Whether using a multistage MS or an enzymatic approach, a complex mixture is produced; thus the application to larger DNA segments clearly benefits from the high resolution FTICR affords. Enzymatic methods are attractive because they do not require precise experimental conditions to produce useful fragments, and also the experiment itself, including data interpretation, is more straightforward. However, enzymatic methods require reproducible reaction conditions, and often require a significant investment in time because the molecular techniques are commonly carried out in tandem to generate sufficient material for analysis (*e.g.*, PCR followed by a Sanger cycle sequencing step). In addition, complex mixture analysis requires a large dynamic range, which is limited in FTICR (and which could potentially be circumvented by using on-line capillary electrophoresis (99)). There are a multitude of choices for ion activation when employing multistage MS for determining a DNA sequence by ESI-FTICR, including NS, IRMPD, SORI, BIRD and MECA and combinations such as a top-down approach (NS dissociation followed by multistage MS in the ICR cell). To date, the dissociation of large oligonucleotides have been compared and contrasted only using FTICR (52), which is partly attributed to the complexity of the experiment and the availability of the instrumentation (all of those experiments were conducted at 6.2 T). From these extensive studies, it is difficult to "choose" the method

because reproducibility is lacking; this could be attributed to both sample and instrumental conditions. Thus, at this stage in DNA sequencing using ESI-FTICR mass spectrometry, no overwhelming evidence exists to indicate which approach will be most suitable for large-scale genomic sequencing, particularly because much larger oligonucleotides are sequenced in Sanger-type approaches (> 300-mers). As a result, no "hard and fast rules" can be suggested at the writing of this review to those readers who are currently considering either approach.

IV. Polymerase Chain Reaction Mass Spectrometry

The PCR (100) has proven to be an extremely powerful and versatile technique for the amplification of regions of genomic DNA. The PCR reaction can be used to characterize microorganisms, to screen for new mutations or to diagnose known mutations, to fingerprint DNA, and to produce PCR products for direct sequencing, among many other applications.

The steps in the PCR and subsequent analysis generally include isolating the template DNA from the sample matrix, amplifying a targeted DNA region, and determining the size or molecular weight of the products. Currently, the relative molecular weights are determined by relative electrophoretic mobilities of the PCR products using conventional agarose gel electrophoresis (101), which generally takes several hours (depending upon the resolution desired, the size of the PCR product, and the nature of the modifications). The quality of the "molecular weight" determination and limited resolution provided by agarose gel electrophoresis can restrict the utility of PCR in situations where single-base deletions, additions, or substitutions or where an investigation of several mutations simultaneously (*i.e.*, with mixtures) are of interest. Clearly, a method for the detection of PCR products that is accurate and that provides a significant reduction in analysis time compared to electrophoretic methods would be of significant practical utility. Mass spectrometry fulfills these requirements and offers a potentially rapid, sensitive, and accurate method for "sizing" PCR products.

The first reports of using ESI mass spectrometry for the detection of PCR products appeared independently and simultaneously by two research groups (49, 102). In both initial studies, the preparation of the crude PCR reaction prior to ESI-MS analysis was time-consuming. In one initial report, ESI was used with a high resolution FTICR-MS to investigate 105 and 106 base pair PCR products of the spacer region between the 16S and 23S rRNA genes of two members of the *B. cereus* group of bacteria. The results yielded a mass precision and accuracy that was only comparable with gel electrophoresis (49); that poor result was attributed to the spectral complexity that arises from the non-templated 3' addition of deoxyadenosine phosphate (dA) and to residual cation adduction (49). Naito *et al.* recognized the former problem and eliminated the non-templated dA by incorporating restriction sites in the primers with subsequent endonuclease digestion prior to ESI-MS analysis (102). Using a magnetic sector instrument fitted with an array detector, their results indicated that ESI-MS could provide high mass accuracy (~0.005%) and precision for the individual strands of the resulting shorter 57 bp PCR product. However, this approach requires additional enzymatic manipulations and incurs a considerable lengthening of sample preparation time (as much as a day or more) (102). Two recent reports by Naito *et al.* (103) and Tsuneyoshi *et al.* (104) demonstrated the detection of a 5-base pair deletion of the adenomatous polyposis coli gene of a 57 base pair PCR product and a single-base pair substitution (C to G) in a 52 base pair product, respectively (103, 104). Both of these reports utilized the incorporation of the *Eco*R1 restriction sites in the primers; that approach has the disadvantage of requiring a significant amount of time (103, 104).

From a mass spectrometry perspective, the analysis of PCR products presents a similar analytical challenge regardless of the nature of the PCR product (*e.g.*, bacterial vs. human genome, arbitrarily primed vs. normal PCR). Because PCR reaction mixtures essentially contain identical components (*i.e.*, nucleotides, oligonucleotide primers, and buffers), the PCR product constitutes only one component of this complex mixture. Thus, some cleanup of the PCR reaction is highly beneficial prior to ESI mass spectrometric analysis. We will describe here several recent results that relate to both bacterial and human genomes and provide the reader with the potential that ESI-FTICR-MS has for the characterization of PCR products.

Taxonomic Classifications using a PCR-MS Approach

The ability to rapidly classify bacteria using nucleic acid methodology has practical implications in areas such as bioremediation and monitoring drinking water supplies for bacterial contamination. The generation of nucleic acid biomarkers (*i.e.*, PCR of a targeted genomic region common to several bacteria) that provide rapid identification of bacteria in specific environments allows one to monitor microbial populations. Relying on the micro-heterogeneity of the biomarker produced from each bacteria (*i.e.*, small sequence additions, deletions, or substitutions), the entire microbial community could potentially be monitored in "real time".

Recently, Muddiman *et al.* investigated the use of mass spectrometry to classify four different bacteria using the same highly conserved genomic region common to all four species (41). An 89-base pair nucleotide portion of the spacer region between the 16S and 23S ribosomal rRNA genes was amplified from the genome of three members of *B. cereus* group (*i.e.*, *B. anthracis, B. cereus, and B. thuringiensis*) and a 114-base pair region from one member of *B. subtilis* group. Due to the large mass difference between the *B. cereus* and *B. subtilis* group (*i.e.*, 25-base pairs), ultra high mass resolution was not required for this model investigation. However, the expected mass of each strand (*i.e.*, coding and non-coding) based on reported sequence information did not match the experimental values. Two members of the *B. cereus* group (*B. thuringiensis* and *B. anthracis*) indicated a ~15 Da mass deficit for the coding strand and an additional ~16 Da in the non-coding strand (single-stranded data were not obtained for the *B. cereus* member - *vide infra*). The measured mass for the coding strand of *B. subtilis* was determined to be ~39 Da higher than the predicted mass and the non-coding strand ~41 Da lower than the predicted mass. These mass discrepancies for the two strands possibly indicated a C to G switch between the two strands, which results in a 40.03 Da addition to one strand and a corresponding 40.03 Da subtraction in the other. The results indicated simple single or double base-substitutions for all the four strains investigated (41).

Another interesting observation that was noted in this paper pertained to the detection of three **double-stranded** products for the *B.*

cereus member (41). Since that time, we also analyzed the single-stranded PCR products, which have further defined the origin of these observations (105). Figure 6A shows the ESI FTICR mass spectrum of the PCR product obtained from *B. cereus*, which yielded three **double-stranded** PCR product with the largest being the expected 89 bp species (also refer to Figures 2 and 3 for the ESI-FTICR mass spectra of two other PCR products). The other two double-stranded products in the spectrum correspond to a single (heterodimer) and double base deletion (88 bp product), respectively. Thus, the unexpected products are lower in mass by 308.5 (for the heterodimer; *i.e.*, the average of the two 88-89 bp species) and 617.1 for the 88 bp species. However, because the sum of the mass of an adenine (A) and a thymine (T) is 617.4 and the sum of the mass of a cytosine (C) and guanine (G) is 618.4, it was difficult to

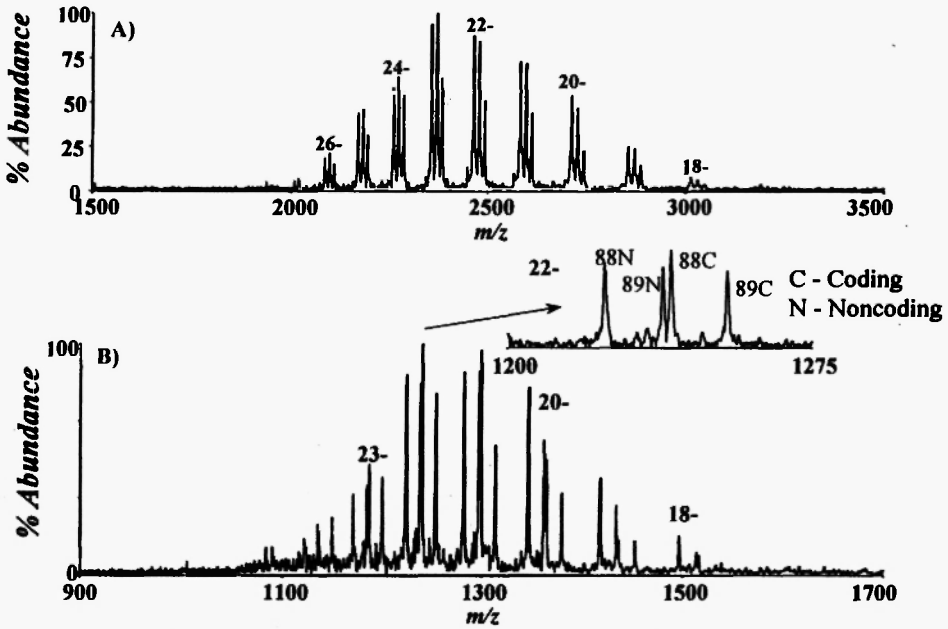


Figure 6: ESI-FTICR mass spectra of the PCR products observed after amplification of the 16S/23S rRNA gene in *B. cereus*. **A**) Detection of 3 double-stranded products and **B**) detection of 4 single-stranded products. Inset shows the 88 and 89 coding and non-coding strands. (Figure adapted from *Anal. Chem.* **1996,** *68*, 3705-3712)

assign with any certainty which Watson-Crick base pairs were missing from the 89-base pair expected product. Figure 6B shows the ESI-FTICR mass spectrum of the same PCR product in the **single-stranded** form (105). This spectrum yielded 4 peaks that correspond to the 88 base-pair coding (C) non-coding (N) and the 89N and 89C with an expansion shown of the 22- charge-state. These results clearly indicated that the difference between the 89C and 88C was a T (measured 304.1; expected 304.2) and the difference between the 89N and 88N was an A (measured 312.7; expected 313.2); these data are consistent with Watson-Crick base pairing. Comparison of these results with the reported sequence information suggested the presence of inter-operon variability in the 16S/23S rRNA spacer region (105). It is also important to note that, for DNA mass measurements, double-stranded masses (*i.e.*, the sum of the two single strands) are only slightly sequence-dependent. This situation arises because the *average* mass nucleotide base residues of an A and a T is 308.703 and that of a G and C is 309.197. Thus, even the extreme variations in sequence for dsDNA lead to mass differences of only 0.15%. This example highlights the importance of making mass measurements on single-stranded DNA for elucidation of polymorphisms.

High Resolution Capabilities of ESI-FTICR for the Detection of Point Mutations

The presence of a polymorphic site in genomic DNA can produce a relatively large mass difference (*i.e.*, base deletion) or a subtle mass difference (*i.e.*, base substitutions). The mass accuracy and precision obtainable by mass spectrometry are far superior to electrophoretic techniques. Electrospray mass spectrometry readily affords mass measurement errors of less than 0.01% (*i.e.*, 1 Da at 10,000), and 0.001% is achievable with similar mass precision. Clearly, the most demanding case is for a single base substitution, and involves an A to T or T to A substitution, which corresponds to only a 9 Da difference. For example, Figure 7 shows the ESI-FTICR mass spectrum of a 9 Da mass difference for the 10-charge state for PCR products of the size presently studied corresponds to a *m/z* difference of only 0.9 *m/z* units for the 10- charge state. FTICR analysis is

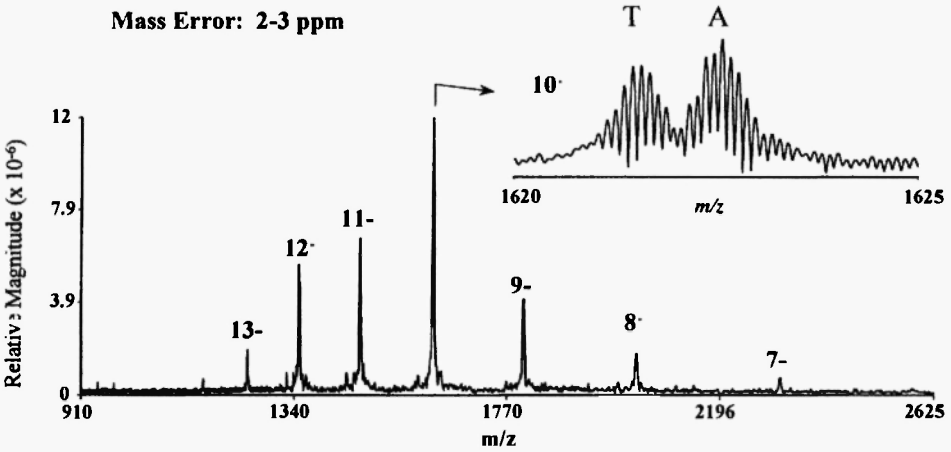


Figure 7: ESI-FTICR mass spectrum of two mixed-base 53-mers modeling an A to T polymorphism. Quadrupolar excitation from *m/z* 900 - 2500 was employed during ion injection (5 seconds)and for 1 second following injection. The high resolution afforded by FTICR-MS allows the resolution of the even the 14- charge state (*i.e.*, *m/z* separation is 0.64). The inset clearly illustrates that isotopic resolution is obtained allowing facile mass measurements (See Table 4).

particularly well-suited for such analysis because resolving power is inversely proportional to *m/z*. To demonstrate the utility of ESI mass spectrometry coupled with FTICR, two synthetic systems were investigated that model single or double base substitution polymorphisms. The first system involved the measurement of two 53-mer mixed based oligonucleotides, which have identical sequences except for a base substitution (A to T) at the 29 position (where W = A or T) from the 5' end:

5'-CCT ACA ACA CCA GGA AGT ACG AGT GCT GWG CCG AGA TCT ACC CGG ACA TCA CC-3'

The calculated molecular weights (most abundant isotope) based on the sequence for A and T in the W position are listed in Table 4 (105).

Table 4: Predicted and Measured Molecular Weights of a Mixed-Base 53-mer, that models a T to A Polymorphism

W	Predicted Mass (Da)[a]	Measured Mass (Da)[a]
T	16225.77	16225.73
A	16234.78	16234.76

a - based on the most abundant isotope

When the locus of the polymorphism is flanked on both sides by using primers in the PCR reaction (*i.e.*, amplification of a single base polymorphic site), the final PCR products in principle will have the same sequence except for the targeted single base site. Figure 7 shows the ESI-FTICR mass spectrum of equilmolar concentrations of the mixture, with the inset showing an expansion of the 10- charge state (105). The mass resolution (>30,000 FWHM) is adequate to distinguish the 9 Da difference at the 53-mer level, even with multiple charging (*i.e.*, the *m/z* difference of the 10- charge state is less than 1). The calculated molecular weight for each of the two strands, based on the most abundant isotope peak, is listed in Table 4. The mass accuracy for the two strands was determined to be significantly better than 5 ppm (which is 0.5 Da in 10,000 Da), even without the use of an internal calibration!

To demonstrate the capabilities of ESI-FTICR to resolve multiple alleles, 32-mer oligonucleotides were analyzed (106). The oligonucleotide had a total of four degeneracies:

5'-CAU CAU CAU CAU AAA CTY AAA KGA ATT GAC GG-3'

where Y = C or T and K = G or T. The calculated molecular weights (mono-isotopic) are listed in Table 5. These primers, which are 32 bases in length, have 2 sites where double degeneracies can occur (resulting in a total of 4 species). The ESI-FTICR mass spectrum of the 9- charge state of these primers is shown in Figure 8 and allows

the accurate mass measurement of all four products. The monoisotopic masses calculated using the 9- charge state are listed in Table 5. Clearly, ESI-FTICR-MS has the potential to rapidly and accurately determine subtle allelic differences that can be problematic for conventional electrophoretic methods.

Table 5: Predicted and Measured Molecular Weight of a 32 Base Pair Oligonucleotide with Four Degeneracies

Y	K	Predicted Mass (Da)[a]	Measured Mass (Da)
C	G	9748.64	9749.28
C	T	9723.63	9724.29
T	T	9738.63	9739.29
T	G	9763.64	9764.28

a - based on monoisotopic mass

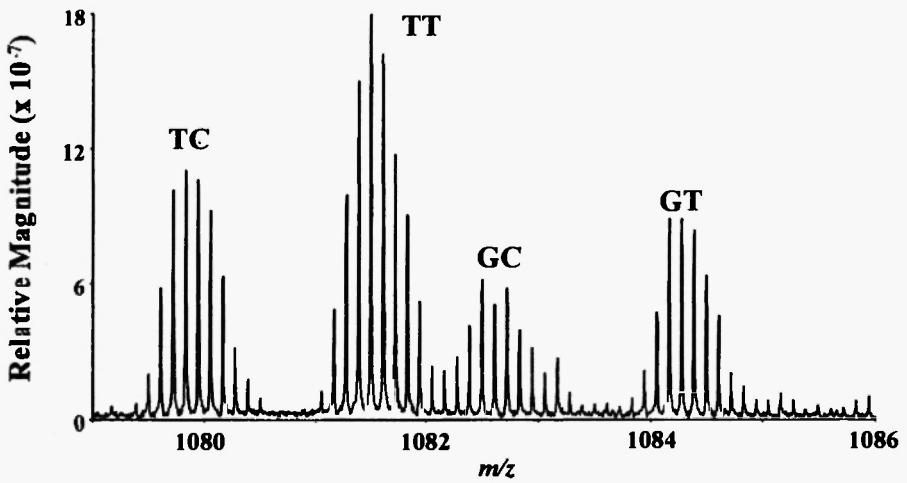


Figure 8: ESI-FTICR mass spectrum of degenerate mixed base 32-mers (see text). Quadrupolar excitation from *m/z* 1100 - 1200 was employed during ion injection and for 1 second following ion injection. The average resolution (> 100,000 (FWHM)) is more than adequate to resolve the ^{13}C isotopic envelope of each species and to unambiguously assign base substitutions (See Table 5).

V. Mathematical Methods for DNA analysis by Mass Spectrometry

DNA Sequencing using Tandem Mass Spectrometry

An algorithm reported by McCloskey and coworkers for the *de novo* sequencing of DNA is of significant importance (16). The algorithm allows the sequence of an **unknown** oligonucleotide to be determined by utilizing the typical dissociation pathways (*e.g.*, w and a-B ions, as well as their intensity) of oligonucleotides (16) by distinguishing the 3' and 5' terminal residues, alignment of the overlapping sequences obtained from each terminus, and utilizing the accurate mass measurements in conjunction with compositional constraints (described in the next section) (107) to reconstruct the original sequence. This methodology allows, for the first time, the sequencing of short oligonucleotides (< 15 mers) **without** any prior knowledge of the sequence; that situation has obvious implications.

Recently, Pomerantz *et al.* have utilized this algorithm (16) to allow the "deconvolution" of a combinatorial oligonucleotide library by ESI-MS (108). Injection of different libraries of limited size (up to 27 components), using tandem MS for the larger libraries, allowed the sequencing of the complex mixtures where the components are similar in mass and sequence (108). The success of their approach was in the use of the predominate fragmentation pathways of oligonucleotides (16). Admittedly, these researchers note that the extension of their approach to much larger libraries would place more demands on resolution (*e.g.*, isobaric species), which is something that FTICR would be directly suitable for because the analysis of complex mixtures would be facilitated by the direct determination of mass and charge from a single peak (28).

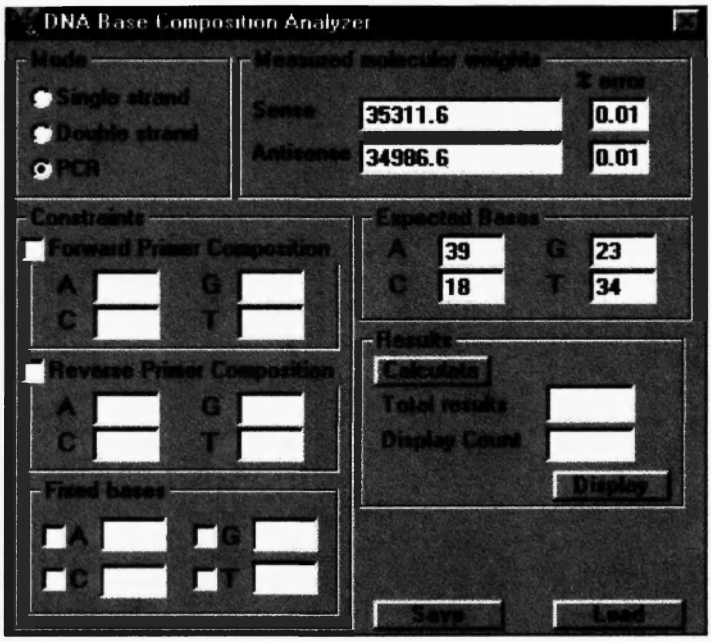
Characterization of PCR Products

McCloskey and co-workers demonstrated that the number of compositional possibilities for RNA becomes extremely large as molecular weight increases if no constraints are imposed upon base composition (107). However, they also showed that the number of possible base compositions was significantly reduced by restricting the

number for a particular base (e.g., by elucidating the compositional value for any one residue via chemical modification or enzymatic methods). It was shown that, for base compositions of RNA in which the abundance of one base is known, compositions up to the 14-mer level could be uniquely defined given a mass measurement precision of 0.01% (100 ppm); similar results were noted for DNA (107). The key contribution of this work is that, by using information about the sample in conjunction with an accurate mass measurement, the algorithm is constrained, thus producing a manageable number of base compositions.

In section IV, this review article discussed the potential role for mass spectrometry in the characterization of PCR products. Using the accurate molecular weight information obtained in a recent study (41), an algorithm recently introduced by Muddiman, *et al.* determined the base composition of nucleic acids (59). A recent study found that each of the four PCR products that was examined appeared to have a polymorphism (single or double base substitutions) compared to the reported sequence (41). Although the molecular weight determinations unambiguously indicated that a polymorphic site was present, the relatively high mass of these PCR products (ranging from *ca.* 27-35 kDa per strand) precluded the assignment of a unique base composition solely from the molecular weight due to the large number of possible base compositions for the mass accuracy obtained (*ca.* 50 ppm).

A simple mathematical strategy has been invoked to assist the characterization of denatured PCR products, which involves the integration of one or more of the following data: the molecular weight measurement, mass precision, and primer composition constraints (59) as shown in Figure 9. Typically, the base composition of PCR products is substantially constrained, and one benefits substantially if this information is used. In addition, knowledge of the chain length is also of importance when characterizing PCR products because polymorphisms can be base deletions or insertions, and it was demonstrated that this approach unambiguously defines this parameter (59). The only assumption built into the approach is Watson-Crick base-pairing for blunt-ended DNA. In the algorithm (Figure 9 right



Computer Screen Illustrating the Different Attributes of the Program

Algorithm

$$(G)_w(C)_x(A)_y(T)_z = \text{MW(S)} \pm \text{precision}$$

$$(G)_z(C)_w(A)_x(T)_y = \text{MW(A)} \pm \text{precision}$$

Figure 9: Top: Computer Screen; Bottom: Algorithm. The sole assumption built into the algorithm is Watson-Crick base-pairing. Each base composition that can satisfy the sense strand experimental mass within the measured precision is then compared with the antisense strand mass because the # of G's in the sense strand is equal to the the # of C's in the antisense strand, etc. If the base composition for the sense strand matches the experimental mass and if the complementary base composition matches the antisense mass measurement, then the result is retained. In addition, the program allows provisions for forward and reverse primer compositions (because these compositions become part of the final PCR product) as well as constraints for a specific base(s). (Figure adapted from *Anal. Chem.* **1996,** *68*, 3705-3712)

hand side), the MW of the sense strand (S) must first be satisfied with a user-defined mass precision (e.g., 0.01%); the mass precision (in Da) is determined by multiplying the measured molecular weight by the user-defined precision, which automatically scales with molecular weight. Any base composition that is consistent with MW (S), assuming Watson-Crick base-pairing, must also be consistent with the MW of the antisense strand (A) or the composition is discarded (*e.g.*, the number of G's in the coding strand must equal the number of C's in the non-coding strand). Using this approach, the number of possible combinations for a 114-bp PCR product (*ca.* 35 kDa per strand) was reduced from 5994 to 92 and uniquely defined the length (# of base pairs) (59).

A recent report by McLafferty and co-workers demonstrated that, utilizing the complementary nature of DNA in conjunction with very high mass accuracy data (0.5 Da at 39 kDa), a single base composition could be delineated (55). In that approach, it is possible to determine the number of base pairs by using the double-stranded mass measurement. In addition, by utilizing the number of base pairs and the high accuracy, a unique base composition could be determined. Although this approach leads to similar results as those obtained by Muddiman *et al.* (59), this algorithm is more limited in its applicability because the mass accuracy that is needed is not routinely obtainable by conventional mass spectrometers. It is also important to note that it is unnecessary to measure the double-stranded mass to determine the number of base pairs in the PCR product when both individual strands are measured individually.

VI. Characterization of Modified and Damaged DNA using Tandem MS

The analysis of DNA adducts and damage products as indicators of human exposure to environmental levels of carcinogenic compounds represents an extreme challenge to analytical chemists for trace analyses. Damage to DNA is a critical event in mutagenesis, and the necessity to degrade a large DNA segment to the monomer level has invariably complicated the analysis. Thus, a method that could

investigate a wide variety of lesions at the oligonucleotide level would allow sequence-specific information to be obtained. The characterization of modified or damaged DNA using a tandem MS approach presents a similar problem as that described in Section III (*i.e.*, determining the primary sequence of DNA). For example, following environmental exposure levels of benzo[a]pyrene (a known carcinogen), one can expect less than one adduct per 10^7 base pairs, or *ca.* 30 femtomoles of adduct per mg DNA. The characterization of DNA damage is a significant challenge, and great difficulties are posed by structural analysis of damage in larger oligonucleotides. At present, it is extremely difficult to determine the chemical nature and site of oligonucleotide modifications, and little is known regarding the extent of sequence specificity of most types of adduction and damage, and an ultra-sensitive mass spectrometric approach constitutes an attractive means to acquire such insights. The literature contains several reports that use ESI-MS as a method for the analysis or characterization of modified and damaged DNA (35, 52, 88, 109-118). Readers are referred to the current literature on the goals and challenges for the detection of DNA damage (111, 114, 119).

Potier *et al.* analyzed several synthetic oligonucleotides that were modified with a fluorescent marker that allowed them to deduce the DNA was not only modified correctly, but with the high mass accuracy, allowed them to confirm that the label was still in its lactone form (35). A small oligonucleotide that was modified with a tetra-peptide (KDEL) was identified, as well as the detection of impurities (35). Although these studies do not elucidate the site(s) of the modification(s), tandem MS could potentially provide such information (*i.e.*, if one can observe the intact molecular ion, proper collisional-induced dissociation conditions can often provide the desired information).

Barry *et al.* used capillary zone electrophoresis coupled with ESI triple-quadrupole MS for the detection and identification of the adducts that were formed when DNA was reacted with an active metabolite of benzo[a]pyrene (BPDE) (109). Using a tandem-MS approach, they found that BPDE reacts predominantly with the exocyclic amino group of guanine. They further applied these

techniques to the analysis of the adducts that were formed from the *in vitro* reaction of BPDE with DNA, and demonstrated that a CZE-ESI-MS approach allows the detection of as few as four adducts in 10^7 unmodified bases or less (109).

Linschield *et al.* have shown that in-source CID ESI-MS is effective for modified oligonucleotides when combined with CZE (118). Specifically, this group investigated the distributions of modifications that occur in oligomeric DNA, which were induced by styrene oxide (118). Using both endonucleases in combination with insource-CID, it was possible to determine the modifications in short oligonucleotides; however, an extension to larger oligomers would place significant demands on mass resolution (118). Hofstadler *et al.* have investigated DNA damage in a model system by X-irradiating a tetranucleotide, using HPLC to separate the damage products, and capillary isotachophoresis (CITP) to further separate and concentrate the products with subsequent on-line analysis by ESI-FTICR (117). These high resolution results (< 25,000 FWHM) with low ppm mass accuracy coupled with subsequent multistage MS, could potentially provide the location and type of trace levels of damage should allow for the analysis of unidentified damage products of larger size.

Pasa-Tolic *et al.* demonstrated methods for the characterization of modifications/damage present in oligonucleotides (120). This approach is based upon the use of ESI coupled with FTICR and exploits NS and SORI CID methods, as well recently developed selective ion accumulation (SIA) techniques. The experimental sequence is complicated and requires isolation of a precursor ion using SIA (and, if necessary, further isolated by use of stored wave inverse Fourier transform (SWIFT) ejection of Na and/or K adducts (121)) and subjected to low-amplitude on-resonance or SORI excitation in the presence of collisional gas (*i.e.*, N_2). (The IR or SORI dissociation techniques discussed earlier might also prove to be beneficial for enhancing sensitivity by removing non-covalent adducts (44).) Axialized product ions are excited/detected, or SWIFT-isolated for yet another MS^n stage. To allow several stages of MS by enhancing the ion population in each stage (*i.e.*, increasing the S/N), they demonstrated that simultaneous dipolar SORI excitation of the

precursor ion and quadrupolar SIA excitation of pre-selected product ion during ion injection can be used.

Because cyclobutane pyrimidine dimers represent 90% of all the DNA damage upon exposure to UV radiation, a thymine-thymine (TT) crosslink was chosen in a mixed base 12-mer as one model system. This lesion is particularly challenging (from the MS point of view) because the modified strand has the same mass as the unmodified strand of the same sequence, but also because the distortions of DNA structure caused by this lesion are surprisingly small. Therefore, the subtle structural differences between the normal and modified DNA may not be able to be elucidated using mass spectrometric approaches. Comparison of MS^3 spectra of a TT crosslinked 12-mer and its unmodified analog obtained using SORI CID are shown in Figure 10, which reveals the formation of the w_5 ion as a distinguishing feature induced by lesion. The w_5 ion (McLuckey nomenclature (80)), corresponding to cleavage of C-O bond adjacent to TT crosslink, is absent from the CID spectrum (as well as from the NS - data not shown) for the modified oligonucleotide (120).

An N^2-(10R)-trans-opened benzo[a]pyrenediol epoxide adduct of guanine in a mixed base 22-mer was chosen as a representative of oligonucleotides that are modified by an interaction with carcinogenic agents (122). The analysis of the MS^2 spectrum of the modified oligonucleotide confirmed that guanine was the damaged base in the 22-mer; that conclusion was confirmed by loss of 7R,8S,9S-trihydroxy-N2-deoxyguanosyl-3'-phosphate-7,8,9,10-tetrahydrobenzo[a]pyrene (~453 Da) from the molecular ion, in the NS and CID MS^2 spectra. A molecular ion was observed that corresponded to two BPDE adducts; that result is not unexpected because adenines are known to be adducted at low levels. However, the primary dissociation pathways were the loss of neutral bases and loss of BPDE; these losses precluded obtaining site/sequence information from these CID spectra (122).

From the several dissociation techniques available, NS and off- or on-resonance CID both produce a significant number of structurally informative fragments ("w" and "a-B" series, mostly); NS proved to be more effective. However, the complexity of NS spectra (e.g., the TT

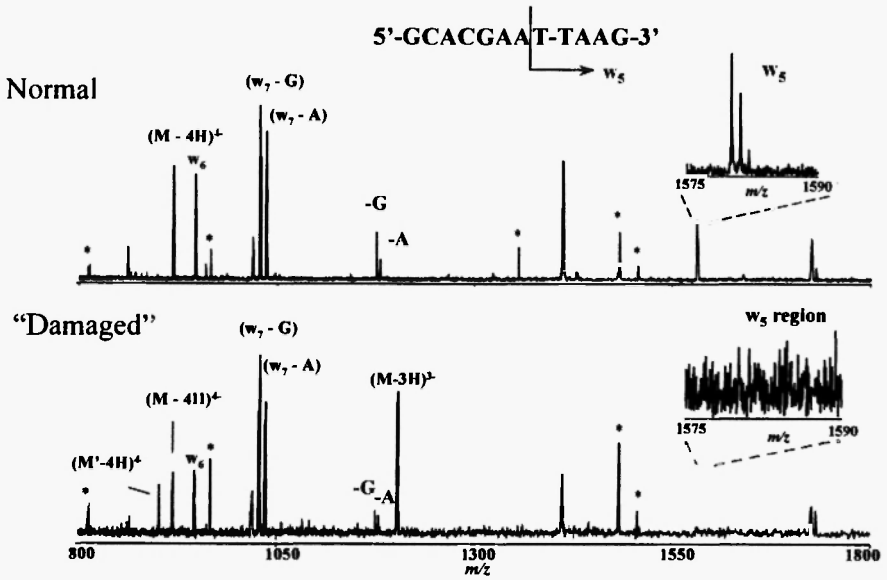


Figure 10: ESI-FTICR MS^3 spectra of a normal and "damaged" 12-mer; the modified oligonucleotide contains a thymine-thymine crosslink. The intact molecular species was dissociated in the ICR cell using a SORI collisional-induced dissociation (CID) step, which was conducted simultaneously with a quadrupolar excitation step (1 Vp-p) to accumulate the w_7 ion. This was followed by an additional SORI CID step to dissociate the w_7 ion which resulted in an essentially identical MS^3 spectrum for the normal and damaged 12-mer. The single distinguishing feature is the presence of the w_5 ion for the normal 12-mer and its absence for the damaged 12-mer. The w_5 ion corresponds to the cleavage of the C-O bond adjacent to the thymine-thymine crosslink.

crosslinked sample described above, where ss-DNA strands as well as the ds-DNA are both present) due to contributions from multiple dissociation steps complicates interpretation. The approach using dissociation in the interface suffers from the inability to select the precursor species; a major liability in many applications where impurities and multiple products are present. However, CID in the

ICR cell allows MS^n (n>2) experiments, and thus provide more extensive structural information provided that the predominate fragmentation pathway is not the loss of the DNA adduct.

VII. Noncovalent complexes of ss- and ds-DNA with proteins and drugs

The ability to transfer ions into the gas-phase intact using ESI has obvious benefits; however, ESI also permits noncovalent complexes present in solution to be transferred into the gas-phase. From an instrumental perspective, there is generally a pragmatic compromise between the preservation of the noncovalent complexes that are formed in solution and obtaining sufficient desolvation to allow ions to be measured; this balance requires the proper selection of interface conditions. Numerous reports now exist in the literature that demonstrate that protein-protein (123-126), receptor-ligand (127-129), DNA duplex (130-132) and multiplex (133), drug-DNA (134-136), protein-DNA (137-139), enzyme-substrate (140, 141), and a host of other specific noncovalent complexes (142, 143) can be successfully transferred into the gas-phase intact by using ESI.

Protein-DNA complexes

Studies have now extensively demonstrated that the complexes that are observed by ESI-mass spectrometry in properly conducted experiments reflect known solution behavior, and that highly specific protein-DNA associations (and non-covalent associations in general) can be detected intact and their constituents analyzed. In particular, protein-DNA complexes can be examined in competitive binding experiments without any adverse effects due to weaker non-specific interactions. Figure 11 shows a series of FTICR mass spectra for complexes of the eukaryotic transcription factor, PU.1, with double-stranded

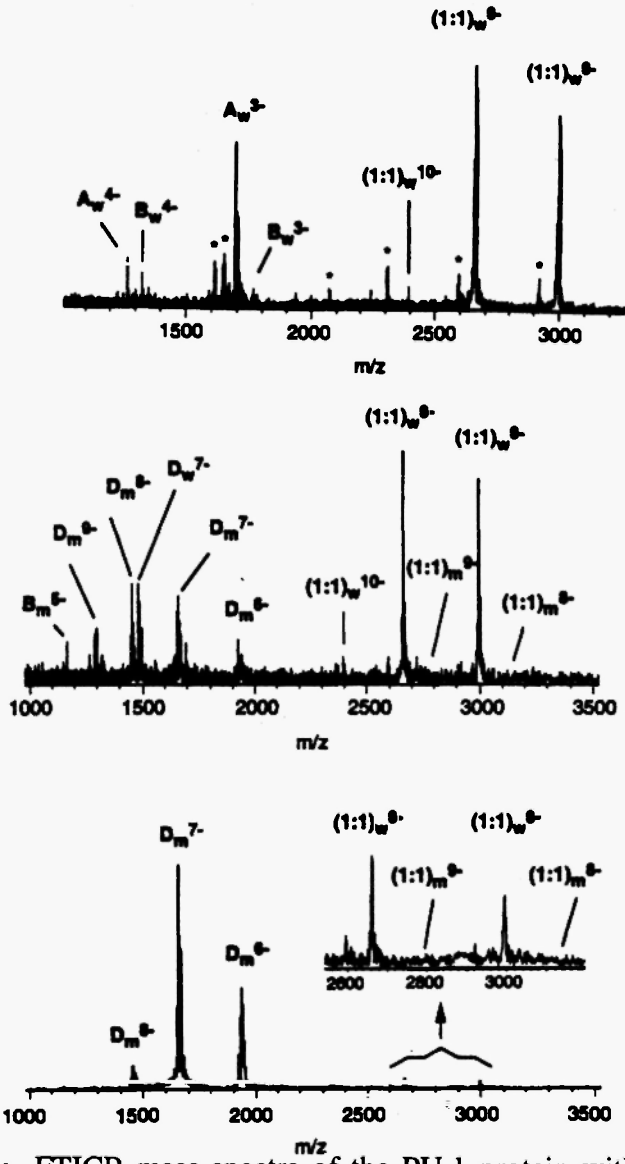


Figure 11: FTICR mass spectra of the PU.1 protein with a double-stranded 17 bp oligonucleotide that has the GGA(A/T) recognition sequence (top), and in competitive binding experiments with (middle) a 1.3-fold and (bottom) a 20-fold excess of a 19 bp oligonucleotide without the recognition sequence. The experiments show the expected 1:1 complexes, and excess DNA duplex is evident at low *m/z*. Gel mobility shift assays confirmed that these results correctly reflected the solution behavior. (Figure from *Anal. Biochem.* **1996,** *239*, 35-40)

oligonucleotides (138). PU.1 is known to bind with high selectivity to the recognition sequence GGA(A/T). The top panel in Figure 11 shows that a solution of the PU.1 protein with an excess of a 17 bp duplex having the recognition sequence results in an FTICR mass spectrum in which the protein-duplex DNA complex dominates (top). The complex is clearly quite stable in the FTICR ion trap, and there is no evidence of protein without DNA or evidence of random 2:1 or 1:2 complexes, and these results clearly establish the stoichiometry for the protein-DNA association. Upon adding PU.1 protein to a mixture of double-stranded oligonucleotides in a competitive binding study, with one having the recognition sequence (wild type) and one not (mutant), showed binding with only the wild type (Figure 11, middle panel) was observed. Repeating the same experiment with a 20-fold excess of the mutant duplex again shows only complex formation with the wild type (bottom panel). Gel shift assays showed that the mass spectrometric method correctly reflected solution behavior (138). Although the exact of scope of these methods remains to be clearly defined, the broad applicability of the mass spectrometric method suggested by studies to date, indicated that complexes having sufficient stability for NMR structural studies will be amenable to study, and also provided the basis for competitive and non-competitive binding studies for mixtures of damaged DNA. In particular, the strong electrostatic contribution to DNA-protein binding suggests that the ESI-FTICR methods will be applicable to even relatively weak complexes.

Cheng *et al.* examined the binding of the Gene V protein (a dimeric protein) to a variety of smaller single-stranded (ss) oligonucleotides by ESI-FTICR (139). Protein-ssDNA complexes having stoichiometries consistent with known behavior were observed; only one dimer was found to bind to a 12-mer oligonucleotide, whereas two dimers were observed as the primary binding motif to the longer 18-mer (*i.e.*, a 4:1 stoichiometry consistent with the known stoichiometry of one Gene V dimer for each 8 bases). The use of competitive binding conditions (*i.e.*, molar excesses of two or more oligonucleotides

relative to Gene V), provided insights into the sequence specificity of complex formation (139). These and other observations reflected solution behavior and indicated that the observed complexes in the gas phase do not arise from non-specific electrostatic interactions (for which little difference would be expected for Gene V interaction with oligonucleotides of different sequence or betweessDNA and dsDNA). A previous study had suggested that the Gene V protein-DNA complex has a stoichiometry in which each protein monomer binds to 3-5 nucleotides, but was ambiguous as to whether each one of these stoichiometries reflects complexes of specific ratio or an average of various stoichiometries. The ESI-FTICR study showed a transition from 2:1 to 4:1 stoichiometry at *ca.* 15 nucleotides. Importantly, it was also demonstrated that collisional dissociation methods could be used to dissociate the complex, and retain the Gene V product in the FTICR cell, after which collisional dissociation of the Gene V protein provided substantial structural information on the protein.

Greig *et al.* determined the relative K_D's of an albumin-oligonucleotide complex by using ESI-MS (144). To accomplish this end, a series of ESI-MS was obtained for albumin as a function of the concentration of the added 20-mer. Using the integrated ion abundances from each ESI-MS spectrum, K_D1 and K_D2 were determined to be 3.1 ± 0.3 and 11.9 ± 0.6 μM, respectively. These measured dissociation constants using ESI-MS were compared with capillary electrophoresis measurements and were in good agreement. This same group also explored the use of microelectrospray (36, 145, 146) for the analysis of duplex DNA (131).

Drug-DNA Noncovalent Interactions

Gale *et al.* reported ESI-MS studies of noncovalent complexes formed between a minor groove binding molecule and a 12 base-pair self-complementary oligonucleotide (134). The association constant for the binding of distamycin (Dm) to a 16 base pair oligonucleotide

duplex is 1.3 x 10^7 M^{-1}. By varying the ratio of distamycin to oligonucleotide, oligonucleotide duplex as well as 1:1 and 2:1 Dm A/oligonucleotide duplex, non-covalent complexes were observed. These observations were consistent with NMR results for Dm to oligonucleotide duplex concentration ratios for the same oligonucleotide sequence (134). An important observation was that Dm was not associated with oligonucleotides without the proper sequence motif. Additionally, these non-covalent complexes were shown to be readily dissociated in the gas phase into their component parts (134). Other results by Gale *et al.* have shown that ESI-FTICR could be effective for the detection of specific drug-oligonucleotide duplex non-covalent complexes (135). In these experiments, 50 μM distamycin and 100 μM oligonucleotide duplex were annealed in 30 μM NH_4OAc from which a charge-state distribution for the 1:1 Dm/oligonucleotide duplex was observed and also showed that specific non-covalent complexes are stable in the ICR cell for extended periods of time (134). Subsequent work extended these studies to other drug molecules (135, 136).

Metal-Oligonucleotide Binding

ESI-FTICR-MS has also been used to study the binding of metal ions to two oligonucleotides, d(pGCTTGCATp) and d(TTGGCCCTCCTT) (89). CID of the metal-oligonucleotide complex revealed that metal ions preferentially bound to the central thymine region of the sequence, and it was suggested that the most probable binding site was the phosphodiester backbone. This binding site was postulated because the sum of the maximum number of metal ions bound to the DNA and the charge state of the whole complex was found to be equal to the number of ionizable protons on the DNA backbone (89). Interestingly, binding was observed for all three of the metal ions studied; however, the binding specificity of UO_2^{2+} ions was significantly greater than for either Mg^{2+} and Na^+ (89).

VIII. FUTURE PROSPECTS

This review has attempted to highlight the accomplishments made using ESI-FTICR-MS as a viable technique in DNA research. It is certain that mass spectrometry will play an ever increasing role in the biological community; however, it is difficult to say with any degree of confidence what role MALDI or ESI mass spectrometry will play in DNA-related research, although MS appears to be almost uniquely suited for the study of modified DNA. It would also be premature (and short-sighted) to state which ionization method and mass analyzer will "rise to the challenge" with respect to a particular DNA analysis. However, ESI coupled with FTICR allows high mass accuracy and superior mass resolution to other existing MS techniques and provides a unique platform to analyze large segments of DNA, thus allowing the characterization of very diverse systems, some of which have been described in this review.

IX. ACKNOWLEDGMENTS

D.C.M. would like to thank Virginia Commonwealth University and the Merck Genome Research Institute for financial support and Mrs. Kimberly E. Muddiman and Professor Patrick A Limbach (Louisiana State University) for their helpful discussions and critical review. The authors would like to thank David S. Wunschel, Ljiljana Pasa-Tolic, Chuanliang Liu, Steven A. Hofstadler, James E. Bruce, and Gordon A. Anderson for helpful discussions. We also thank Professor Alvin Fox and Karen F. Fox of the University of South Carolina for their collaborative efforts pertaining to PCR and Raymond Gesteland and Chris Hopkins (University of Utah) for help with the sequencing effort. This work was supported by the Human Genome Program, Office of Health and Environmental Research, the U.S. Department of Energy. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the U.S. Department of Energy, through Contract No. DE-AC06-76RLO 1830.

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