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**Studies on The Process of Electrospray
and Applications of Electrospray Mass Spectrometry**

by

Fu Ke, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

Ottawa-Carleton Chemistry Institute
Department of Chemistry
Carleton University
Ottawa, Ontario

September 1, 1998

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acceptance of the thesis

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Thesis Supervisor

Chair, Department of Chemistry

Carleton University
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Abstract

This thesis work was designed to investigate the mechanism of electrospray and applications of electrospray mass spectrometry.

As one of a continuous series of studies on the mechanism of electrospray, a long-chain *n*-alkanol, such as stearyl or cetyl alcohol, was used to retard the droplet's evaporation since it can form a layer on the droplet's surface. If solvent evaporation from electrospray-generated droplets played a crucial role in the electrospray mechanism, the presence of a long-chain *n*-alkanol would interfere with the gas-phase ion formation and cause a drastic decrease in analyte response. As it turned out, the electrospray responses of proteins tested with and without the presence of *n*-alkanols were comparable. In these experiments, the *n*-alkanols were added either as solutes in water/methanol solutions of proteins or in hexane solutions delivered coaxially to aqueous protein solutions during their electrospray. These results lend further support to the hypothesis that solvent evaporation is not a necessary prerequisite to ion formation in electrospray.

In the studies on applications of electrospray mass spectrometry, first, collision-induced dissociation of alkali metal-containing anionic tripeptides was investigated. Both *N*-terminal and *C*-terminal product ions were observed from the precursor ions $[M-2H+X]^-$ and $[M-3H+2X]^-$ where X stands for an alkali metal. The results are consistent with precursor ion structures in which an alkali metal ion is centrally located for bonding with the amino

terminus, the amide nitrogen atoms, and an oxygen atom of the carboxylate terminus; the second alkali metal ion in $[M-3H+2X]^-$ is likely attached to the other oxygen atom of the carboxylate terminus. Second, windows of 30 m/z units wide of ions generated in electrospray and sampled under lens conditions of nominally zero potential gradient conditions were mass selected with Q1 in a triple-quadrupole mass spectrometer, fragmented in Q2, and their product ions were mass analyzed with Q3. A variety of analytes (equine myoglobin, bovine insulin, leucine enkephalin and PPG 1000) were examined. The windows selected sometimes contained desolvated (bare) analyte ions; more often they did not and contained only 'background' ions. For protein samples, multiply charged product ions having m/z values both lower and higher than those of the precursor ions were observed even when the precursor ion window could not have contained naked protein dimer ions. To observe significant product ions, the precursor ion window typically contained ions residing within background 'humps' that trailed protein peaks. These ions were speculated to be diverse, solvated multimeric clusters of the analyte which fragmented in Q2 to yield the characteristic production spectra. Finally, low-flow-rate electrospray mass spectrometry was investigated. So-called microelectrospray (microES) and nanoelectrospray (nanoES) were used to analyze peptides, proteins and oligonucleotides. Due to much smaller inside diameters of microES and nanoES probes, the flow rates of sample solutions in microES and nanoES were 50-500 times lower than that in conventional electrospray, typically in the range 10-500 nL min⁻¹. Thus, the sample consumption was only 50-2000 femtomole per scan, and microES and nanoES provided more stable electrospray and higher sensitivity as well. Since only 0.5-5 μ L of sample solution is needed to produce a mass spectrum, nanoES is particularly useful

for the analysis of some precious and volume-limited biological samples.

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Chapter 1

Introduction

Over the last two decades, mass spectrometry (MS) has become one of the most widely applied techniques in analytical chemistry due to its ability to yield definitive qualitative and quantitative information on molecules. Tremendous advances have been made in all aspects of mass spectrometer hardware, computer interfacing, ionization techniques, interfacing with separation techniques, tandem mass spectrometry, and the performance/cost ratio. Thus MS is now an ideal tool for solving analytical problems and has been widely used in a huge variety of fields, such as chemistry, biochemistry, space research, health, archaeology, forensic science, pharmaceutical chemistry, and environmental science.

A mass spectrometer is typically designed to perform the following three basic functions: (1) produce gas-phase ions from sample compounds; (2) separate gas-phase ions according to their mass-to-charge (m/z) ratio; and (3) detect and record the separated ions. The ionization, by which gas phase ions are generated from sample compounds, is the starting point for mass spectrometric analysis, as well as one of the most important aspects in mass spectrometry. Therefore, a lot of research work focuses on improving ionization techniques and developing new ionization methods.

Electron ionization (EI) was the first ionization method employed and is still a widely used method in mass spectrometry. It can provide much more structural information because more fragment ions can be obtained by using EI. Chemical ionization (CI) is another widely used ionization technique in MS. However, because of the requirement for sample vaporization before the ionization, EI and CI are limited in use to thermally labile and

involatile compounds, such as peptides and proteins, which are often encountered in biological research. Some other ionization methods, including fast atom bombardment (FAB) [1,2], thermospray ionization (TS) [3], field desorption (FD) [4], plasma desorption (PD) [5, 6], secondary ion mass spectrometry (SIMS) [7], and laser desorption (LD) [8, 9], are based on ion desorption, which means that gas phase ions are directly produced from solid or liquid samples. These methods do not require sample volatilization prior to ionization, and minimize the thermal degradation of samples. Compared with EI, all other ionization methods are usually called 'soft' or 'mild' ionization techniques because the molecular ions are produced with less excess of internal energy, so that fewer fragments and greater abundances of molecular ions can be obtained using these soft ionization techniques.

In the last decade, a new ionization technique, so-called electrospray (ES) [8-12], along with matrix-assisted laser desorption ionization (MALDI) [13-15], has dominated developments in mass spectrometry and provided new possibilities for the mass spectrometric analysis of high molecular weight compounds including proteins, nucleotides and synthetic polymers. Electrospray is the dispersion of a liquid into small charged droplets by applying a high voltage; subsequently gas-phase ions can be produced from the small charged droplets and transferred to a mass analyzer. Due to the low internal energy of molecular ions generated by ES, electrospray is considered to be the softest of all ionization techniques. In fact, the structures of the gas phase ions produced by ES for the mass spectrometric analysis often reflect those in solution. Another distinguishing feature of ES is the ability to produce multiply charged ions; it enables mass spectrometers to measure

molecular weights for biomolecules (or relative molecular masses, M_r) smaller than 200 kDa with a precision of better than 0.05%. By means of tandem mass spectrometry, collision induced dissociation (CID) of multiply charged ions produced by ES is an effective method to generate fragments, so that much information on the molecular structure of analytes can be obtained from ES-CID mass spectra. Because samples under analysis must be introduced in solution for ES, electrospray is one of the most suitable methods to couple MS with many separation techniques, such as liquid chromatography (LC) and capillary electrophoresis (CE). This thesis work will focus on studies of the process of electrospray and applications of electrospray mass spectrometry.

1.1 Developments of Electrospray

Electrospray is a phenomenon in which a flow of a liquid through a capillary tube is dispersed into small charged droplets by a high electrostatic field at atmospheric pressure. The first experiments to study this phenomenon, by using an electrostatic sprayer that was similar to those used in today's ES-MS experiments, were carried out by Zeleny early in this century [16]. In the late 1960s and early 1970s, Dole and co-workers [17-20] first used electrospray as an ionization technique to produce gas phase ions from macromolecules in a liquid for mass spectrometric analysis. In the experiments, they built a nozzle-skimmer system (see Figure 1.1). A dilute solution of polystyrene was delivered to a hypodermic needle, onto which high voltages were applied, and sprayed out to form the gaseous mixture of macroions, solvent, and nitrogen molecules in an evaporation chamber. The ion beam

Figure 1.1 Schematic diagram of the early ES-MS apparatus for the production and detection of gas phase macroions [3].

went through the nozzle and skimmer, and was detected by a Faraday cage. By this experiment, Dole *et. al.* found that polystyrene macroions ($M_r=51$ kDa) tended to form singly charged dimers and trimers in the ion beam, while larger polystyrene macroions ($M_r=410$ kDa) were mostly multiply charged monomers.

Stimulated by Dole's work, in the late 1970s, Iribarne and Thompson [21-23] first successfully demonstrated that macroions ($X^\pm$) could be desorbed from the surface of highly charged droplets produced by electrospray from aqueous solution at atmospheric pressure, and detected by a quadrupole mass spectrometer. Both positive and negative ions were observed, and the majority of ions were $X^\pm(H_2O)_n$, in which the number of waters would be dependent on the nature of the ion ($X^\pm$). But, unfortunately no multiply charged ions were observed in their experiments. Based on the results of their experiments, Iribarne and Thompson proposed a mechanism, the so called "ion evaporation model" (IEM), for the production of gas phase ions from small charged droplets (more details will be discussed in Section 1.3).

It is common knowledge that the pioneering work for modern electrospray mass spectrometry was done by Yamashita and Fenn [24, 25] in the early 1980s. They successfully coupled an electrospray ion source to a mass spectrometer for the analysis of both positive and negative ions. Another research group, Aleksandrov and co-workers [26-28], simultaneously and independently reported the combination of ES and MS, and demonstrated the accomplishment of on-line coupling an electrospray magnetic sector mass

spectrometer with liquid chromatography. Over the following few years, both groups applied ES-MS to the analysis of peptides, polymers and proteins. They reported that the dominant contributions from polypeptides, such as bradykinin ($M_r=1060$) and gramicidins ($M_r=1141$), were double charged ions [29, 30]. Most significant was the report by Fenn and co-workers that up to 23 charges were observed in the analysis of polyethylene glycols with an average molecular weight from 200 to 17,500 [31], which proved that multiple charging, a distinct and useful feature, could be achieved by electrospray ionization. In 1988, Fenn and co-workers [32] first reported ES-MS analysis of proteins with masses of 40,000 Da, having up to 45 charges. From then on, the potential of applying electrospray ionization to mass spectrometry for the analysis of high molecular weight compounds, including proteins and polymers, began to be widely appreciated.

Within a few years of Fenn's and Aleksandrov's reports, the groups of Henion [33-36] and Smith [37-39] applied ES-MS for the analysis of all types of nonvolatile, polar, or thermally labile compounds. Henion and co-workers developed a new method of pneumatically assisted electrospray, so called "ionspray". In this technique, a co-axial nebulizing gas of nitrogen was applied to assist the dispersion of a sample solution, so that the ion current was more stable. Another major advantage of ionspray is that a much wider range of the flow rate of sample solutions, from 0.1 to 200 $\mu\text{L}/\text{min}$, could be used compared to the flow rate of 1 to 20 $\mu\text{L}/\text{min}$ for electrospray. Therefore, ionspray became a more suitable interfacing technique for coupling high performance liquid chromatography (HPLC) to MS. At the same time, Smith and co-workers [37] demonstrated the combination of ES-

MS and capillary electrophoresis. More recently, the benefits of low flow rates of electrospray have been recognized. So called "microelectrospray" and "nanoelectrospray" ionization techniques have been developed [40-43]. The names come from the use of flow rates in the microliter or nanoliter per minute range. Microelectrospray and nanoelectrospray provide advantages of low sample consumption, stable electrospray and high sensitivity, so that they are particularly useful for volume-limited biological samples. Today, the applications of ES-MS have undergone a tremendous growth, from the mass analysis and structure determination for biomolecules to coupling with separation techniques, such as LC and CE, for the analysis of mixtures.

1.2 Electrospray Process

Electrospray is an ionization technique by which analyte ions can be transferred from sample solution to the gas phase, and then these ions can be subjected to mass spectrometric analysis. Figure 1.2 shows the process of electrospray. A solution of the analyte is delivered to the capillary tube (either metal or fused silica) by means of a syringe pump with flow rates typically from 1 to 20 $\mu\text{L}/\text{min}$. The capillary is located 1-3 cm from the counter electrode (ie. interface plate in MS), and a potential difference of typically 2-6 kV is applied between the capillary and the plate. Because the tip of the capillary is very thin, the electric field E around the capillary tip is very high. It can be evaluated approximately by the following equation [44, 45]:

Figure 1.2 Typical electrospray set-up.

$$E = \frac{2V}{r \ln(4d/r)} \quad (1)$$

where V is the applied potential, r is the outside radius of the capillary, and d is the distance between the capillary and the plate. Typically, when $V=3000$ V, $r=100\mu\text{m}$, and $d=1$ cm, E is about 1×10^7 V/m.

When a potential is applied to the capillary, the high electric field at the capillary tip results in charge separation in the solution and charge accumulation on the liquid surface at the capillary tip (the type of charges depends on the applied potential, ie. positive potential causes positive charge accumulation and negative potential leads to negative charge accumulation). As the charge is accumulated on the surface, the mutual electrostatic repulsion, called Coloumbic repulsion, between the ions at the surface increases. When the Coloumbic repulsion is large enough to overcome the surface tension of the liquid, the liquid will be drawn out of the capillary in a downfield direction to form a so-called "Taylor cone". Taylor [46] found that, when the half angle of the cone tip was 49.3° , the cone would be stable. If the applied potential is sufficiently high, a fine jet starts at the Taylor cone tip to form small charged droplets, and then gas phase ions are produced from these small charged droplets (the detailed mechanism of formation of gas phase ions from small charged droplets will be discussed in the next section).

The flow rate, resistivity, and surface tension of the liquid play an important role in

the production of charged droplets. A proper flow rate ensures that the liquid spray generates fine droplets; high flow rates result in the formation of large droplets. At room temperature, the solution conductivity is required to be less than $10^{-5} \Omega^{-1}$ for a stable electrospray at an appropriate flow rate [47]. A liquid with a higher surface tension needs a higher threshold potential to produce a stable electrospray. Typically, the potential required for a stable electrospray is *ca.* 3.3 kV for water and *ca.* 2.8 kV for methanol at a distance of 1 cm between the capillary tip and plate.

1.3 Mechanism of Electrospray

The production of gas phase ions by electrospray ionization can be divided into three major steps: (1) generation of charged droplets from an ES capillary tip by applying a high potential; (2) shrinkage and rupture of the charged droplets caused by solvent evaporation, and followed by the formation of very small and highly charged droplets; (3) production of gas phase ions from the very small and highly charged droplets.

The understanding of the mechanism is clear for the first two steps of the production of gas phase ions. But some debate still remains regarding the last step of the formation of gas phase ions. There are two proposed mechanisms for the formation of gas phase ions from small charged droplets. The first mechanism of note is the so-called "Charge Residue Model" (CRM) put forward by Dole and co-workers [17] and advanced by Röllgen and co-workers [48, 49]. Figure 1.3(a) shows the schematic diagram of the CRM process of electrospray

Figure 1.3 Schematic diagrams: (a) the CRM process of electrospray [50]; and (b) the ion evaporation process [8].

[50]. Droplets that are emerging from the ES capillary tip are "charged" by the high electric field. They are ejected into the surrounding gas once Coloumbic repulsion among the charges overcomes the surface tension of the liquid. Evaporation of the solvent causes the droplets to shrink, but the charge of the droplets remains constant. Due to the decrease of droplet radius (R) with constant charge (q), the electrostatic repulsion of the charges at the droplet surface increases until the Rayleigh stability limit is reached [51]:

$$q = 8\pi(\epsilon_0\gamma R^3)^{1/2} \quad (2)$$

where ϵ_0 is the permittivity of vacuum and γ is the surface tension of the solvent. Once its radius R and charge q satisfy the above equation, ie. the electrostatic repulsion is equal to the force caused by the surface tension of solvent to hold the droplet together, the charged droplet becomes unstable and is going to divide. Depending on the relative sizes of the new generation of droplets, the fission is described as even or uneven [50, 52]. For an even fission, the droplet fragments into two or a few particles with equal mass and charge; in an uneven fission, the smaller of the offspring droplets carries a higher charge-to-mass ratio relative to that carried by the larger droplet, typically with 15% of the parent's charge and only 2% of the mass [53]. Kobarle *et al.* [50, 52, 54] has speculated that the dominant fission in ES is uneven. The cycle of evaporation and Rayleigh fission continues until each droplet contains only one analyte ion. Complete evaporation of the solvent in these droplets results in individual gas-phase analyte ions that may be mass analysed.

The second mechanism proposed by Iribarne and Thomson [21-23] is called "Ion

Evaporation Model" (IEM). It shares the CRM's droplet evaporation and Rayleigh fission components, but differs from it in the gas phase ion formation step. In the IEM, a charged droplet undergoes a series of evaporation and Rayleigh division cycles to produce a droplet that is so small and so high in charge density that the electrostatic field on the droplet surface is large enough to overcome ion solvation energy and results in ion desorption from the droplet (the so-called "ion evaporation", see Figure 1.3(b) [8]). By comparison with CRM, IEM would need fewer sequential Rayleigh divisions to reach the limit of the droplet size for the ion evaporation than those required to produce eventual droplets containing only one ion. That means, gas phase ions would be produced much earlier in IEM than in CRM.

The exact process of the formation of gas phase ions from very small and highly charged droplets is still not known. When the charged droplets become very small ($R < 10$ nm), there are no experimental methods that could be used to observe such droplets to prove what would happen, ie. whether the evaporation and Rayleigh fission continue and eventually result in the droplets containing only a single ion, or ions directly desorb into the ambient gas from the small charged droplets. The relative importance of the two competitive mechanisms of CRM and IEM still remains a subject of discussion and research.

1.4 Electrospray Mass Spectrometry

Although the phenomenon of electrospray was first studied by Zeleny early in this century and suggested to be used for the mass analysis of large molecules by Dole and co-

workers in the late 1960's, electrospray ionization did not attract the attention of mass spectrometrists until Fenn and co-workers' pioneering work on coupling ES with mass spectrometers to determine the molecule masses of proteins in the late 1980s. Electrospray as an ionization technique for mass spectrometric analysis has undergone very quick development and great progress, and has been successfully used in all types of mass spectrometers, such as quadrupole mass spectrometers, magnetic-sector mass spectrometers [26, 55, 56], time-of-flight (TOF) mass spectrometers [57-59], quadrupole ion-trap mass spectrometers [60-62] and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers [63-65]. Today electrospray mass spectrometry has become one of the most powerful tools in the bioanalytical laboratory.

Electrospray ionization has some unique features which make it different from other mass spectrometric ionization techniques, so that electrospray mass spectrometry possesses some distinct advantages:

(1) Electrospray is the softest ionization technique compared to other ionization methods for mass spectrometric analysis, and the structures of the gas phase ions produced by ES often reflect those in solution. Therefore, by means of studies on the gas phase ions through ES mass spectra, it is possible to obtain a lot of information regarding the noncovalent interactions between molecules and the three-dimensional molecular conformations that originally exist in solution [66].

(2) The ability to produce extensively multiply charged ions is the unique feature of electrospray ionization (more details will be discussed in section 1.5). It is possible for all types of mass spectrometers to determine very large molecular weight compounds by means of interfacing with electrospray ionization to produce highly charged ions. So far, the largest protein determined successfully by ES-MS is human complement C4 with the molecular weight of more than 190 kDa, having up to 150 charges [67].

(3) Samples, which are introduced to the electrospray capillary to produce gas phase ions for mass spectrometric analysis, must be in solution. This distinct feature of electrospray ionization makes it one of the most suitable methods to interface MS with many types of solution-based separation techniques, such as liquid chromatography and capillary electrophoresis, which are powerful separation methods for mixtures of larger bio-molecules.

Due to the softness of electrospray ionization, ES mass spectra of biomolecules are mostly multiply charged molecular ions and there are very few fragments if any. It is most helpful for accurate determination of molecular weights, but provides little information about molecular structures. Therefore, tandem mass spectrometry has been widely applied to interface with electrospray ionization, and many more fragments of multiply charged ions can then be obtained by collision induced dissociation in an ES tandem mass spectrometer (see Section 1.6).

During the last few years, quick development and further improvement on ES-MS

have made it a powerful analytical technique in biochemistry and pharmaceutical chemistry, and it is widely used for the analysis of peptides, proteins, nucleic acids, carbohydrates and lipids, drugs and drug metabolites, and inorganic and organometallic complexes. It is not only one of the best methods to determine molecular weights with very high accuracy, but also can provide a lot of information regarding noncovalent interaction, three-dimensional molecular conformations and the sequence of peptides and proteins.

1.5 Mass Spectra of Multiply Charged Ions

Producing multiply charged ions is a unique feature of electrospray ionization. An ES mass spectrum typically consist of a distribution of molecular ion charge states, ie. a series of molecular ions with only the differences in the number of charges. Figure 1.4 is a typical ES mass spectrum of the protein, cytochrome c. The signal intensity of molecular ions varies with the charge states.

Fenn and co-workers [31, 68] and Covey and co-workers [69] reported a method to determine the charge state of each molecular ion and the protein molecular mass based on the following assumptions: (1) the charge difference between adjacent peaks is only one; (2) charging is due to proton attachment to the protein (for almost all protein studies, alkali metal attachment is usually not significant); (3) the molecular integrity is preserved in ionization, ie. all peaks are only the contribution of the intact molecules with the same mass (not fragmentation and multimeric species). Given these conditions, the mass-to-charge ratio

Figure 1.4 ES mass spectrum of 10 μ M cytochrome c in 50/50 water/methanol with 0.2% acetic acid.

(m/z) of the i th peak can be described by the following equation:

$$(m/z)_i = \frac{(M_r + M_a z_i)}{z_i} \quad (3)$$

where M_r is the molecular weight of the protein, and M_a is the mass of the charge carrying adduct (M_a is typically a proton). For the j th peak, where $(m/z)_j > (m/z)_i$ and $z_j = z_i - n$ ($n=1$ for the adjacent peak), the mass-to-charge ratio is given by:

$$(m/z)_j = \frac{(M_r + M_a z_j)}{z_j} \quad (4)$$

ie.

$$(m/z)_j = \frac{(M_r + M_a (z_i - n))}{(z_i - n)} \quad (5)$$

From equation (3) and (5), the charge of the i th peak can be solved:

$$z_i = n \frac{((m/z)_j - M_a)}{((m/z)_j - (m/z)_i)} \quad (6)$$

By the equation (6), the charge carried by each peak can be easily obtained from an ES mass spectrum. For example, the charge of the peak at m/z of 773.8 in Figure 1.4 is +16. After solving the charge state for every peak in a spectrum, the molecular weight of the protein can

be obtained by:

$$M_r = ((m/z)_i - M_a) z_i \quad (7)$$

Therefore, the number of possible measurements, to determine the molecular weight in a single spectrum, is the total number of peaks minus one. Table 1.1 gives the calculations of the molecular weight for the spectrum in Figure 1.4. The mass accuracy is 0.025%, obtained by comparison with the expected value from the sequence data. Generally the accuracy of M_r measurements by using ES-MS is 2 to 3 orders of magnitude better than that by using denaturing gel electrophoresis [10], and about one order of magnitude better than that by using a TOF mass spectrometer interfaced with laser desorption [70] or plasma desorption methods [71]. Typically, the mass accuracy of ES-MS is in the range of 0.1% to 0.005%. With more careful operation, the mass accuracy of ES quadrupole mass spectrometers may reach 0.0006% [72], and a mass accuracy of 0.0001% may be obtained by using ES magnetic-sector mass spectrometers or ES FT-ICR mass spectrometers [9].

1.6 Tandem Quadrupole Mass Spectrometry

As indicated above, a distinct feature of ES-MS is to produce spectra for biomolecules with abundant multiply charged molecular ions and fewer fragments. It gives very high accuracies for molecular weight measurements, but very little information to characterize or confirm molecular structures. Therefore, producing fragments of ES multiply charged ions is very important for studies on the conformation and sequence of peptides and

$(m/z)_i$	$(m/z)_i$	z_i (rounded)	M_r (Da)
589.8	619.2	21	12364.7
619.2	651.6	20	12363.9
651.6	687.8	19	12361.3
687.8	728.2	18	12362.3
728.2	773.8	17	12362.3
773.8	825.2	16	12364.7
825.2	884.2	15	12362.9
884.2	952.0	14	12364.7
952.0	1031.2	13	12362.9
1031.2	1124.6	12	12362.3
Average Molecular Weight		12363.2	
Standard Deviation (Rel. Std. Dev.)		1.16 (0.0094)	
Expected Molecular Weight		12360	
Mass Accuracy (%)		0.025	

Table 1.1. Calculation of the molecular weight of cytochrome c from the spectrum in Figure 1.4.

proteins. Tandem mass spectrometry (MS/MS) is a very effective method and has been widely used for the fragmentation of multiply charged ions, in which multiply charged ions are dissociated by the gas phase collision with a neutral target gas, such as argon or nitrogen.

Due to its unique capabilities, MS/MS with quadrupole mass filters has received widespread interest [56, 73-76]. Tandem quadrupole mass spectrometers (also called triple quadrupole mass spectrometers) have advantages of high sensitivity, high selectivity, tolerance of high pressures and low cost. Figure 1.5 illustrates the fundamental principle of the collision induced dissociation (CID) analysis for a mixture containing A, B and C molecules by a tandem quadrupole mass spectrometer [77]. The first quadrupole (Q1) performs the function of the precursor ion selector from multiply charged ions generated by an ES ion source (for example, B is selected by Q1). The second quadrupole (Q2) is not a mass filter, but a collision cell. A neutral target gas (argon or nitrogen) is supplied to this region. When the precursor ions selected by Q1 pass through Q2, collision induced dissociation occurs in this region to produce fragment ions (or so-called product ions) from the precursor ions (e.g. fragments of D, E, and F are produced from precursor ion B). Then the product ions are mass analyzed by the third quadrupole (Q3), and a product ion spectrum is recorded by a detector.

Because CID of multiply charged ions is created by the gas phase collision between precursor ions with certain kinetic energy and a neutral gas in Q2, the changes of kinetic energy of precursor ions, target gas pressure and target gas species result in changes of the

Figure 1.5 Schematic diagram of the isolation of product ions from a mixture of A, B and C in a tandem quadrupole mass spectrometer [77].

CID mass spectrum. When precursor ions collide with a target gas, a part of the kinetic energy of the precursor ions may be converted to internal vibrational excitation energy to cause the fragmentation of precursor ions. Therefore, the kinetic energy of precursor ions determines the degree of fragmentation of precursor ions. Commonly the kinetic energy of precursor ions is measured at the laboratory frame (E_{lab}). Since only a part of E_{lab} is available for vibrational excitation, this part of E_{lab} is known as the collision energy (E_{cm}) and can be described by the following equation [78]:

$$E_{cm} = E_{lab} \frac{m_g}{m_p + m_g} \quad (8)$$

where m_g is the mass of the target gas, and m_p is the mass of the precursor ion. For a heavy precursor ion incident on a light target gas, E_{cm} is only a very small fraction of E_{lab} . Typically E_{lab} used in tandem quadrupole mass spectrometers is from 10 to 100 eV.

Another important factor for CID of precursor ions is the thickness (or pressure) of the neutral target gas in the Q2 region. It determines the frequency of the collision between precursor ions and the target gas; in other words, the relative intensity of a product ion produced by CID depends on the thickness of the target gas in the Q2 region (see Figure 1.6 [78]).

MS/MS is an effective technique for the fragmentation of multiply charged ions produced by ES. It can yield product ions with the same charge state or a lower charge state

Figure 1.6 The relationship between the intensity of fragment ions of $(\text{H}_2\text{O})_4\text{H}^+$ and the thickness of target gas argon at $E_{lab}=24$ eV; the lines were calculated from the kinetic scheme [78].

than that of precursor ions. Figure 1.7 shows (a) the negative ion ES mass spectrum of a tripeptide, LGG, and (b) the CID mass spectrum of the precursor ion $[M-H]^-$ of LGG. All fragment ions can be easily deduced as indicated in the CID mass spectrum. However, if charge states of multiply charged ions are unknown, the interpretation of CID mass spectra presents special difficulties. The CID spectra of doubly charged ions are typically easy to interpret because dissociation of the 2+ precursor ion produces mostly singly charged fragments. The residues at the C-terminus and the N-terminus generated by tryptic digestion of peptides and proteins are usually expected to yield doubly charged precursor ions. Therefore, the combination of tryptic digestion with ES-MS/MS has been widely used to obtain sequence information of peptides and proteins [79, 80]. Further interfacing with LC or CE separation techniques provides a very powerful and rapid analytical tool for peptide and protein sequencing [81].

Figure 1.7 1 mM LGG in 50/50 water/methanol with 1 mM NaOH: (a) ES mass spectrum; and (b) CID spectrum of $[M-H]^-$ ions.

1.7 Objectives

The objectives of the following research work include three aspects: (1) further studies on the mechanism of electrospray; to determine if solvent evaporation is necessary for the generation of gas phase analyte ions from charged droplets in electrospray. Long-chained n-alkanols were used to retard the droplet's evaporation in ES-MS of proteins in order to obtain the answer. (2) applications of ES-MS/MS; fragments of alkali metal-containing anionic complexes of peptides are different from those of protonated peptides, and can provide additional information for peptide sequencing. These complexes of tripeptides were studied in negative ion detection mode by means of ES-MS/MS; CID of the electrospray-generated background was also performed in mild lens conditions in order to investigate the abundance of multimeric species. (3) studies on low-flow-rate electrospray MS; microelectrospray and nanoelectrospray MS of peptides, proteins and oligonucleotides were investigated by comparison with conventional electrospray MS to give the advantages of low-flow-rate electrospray MS.

Chapter 2

Experimental

All experiments were carried out on two SCIEX (Thornhill, ON, Canada) atmospheric pressure ionization triple-quadrupole mass spectrometers: TAGA 6000E and API 300.

2.1 Probes and Spraying Conditions

TAGA 6000E. The electrospray probe used in TAGA 6000E was laboratory-built (see Figure 2.1(a)). It was fabricated from a 3-cm long, 33-gauge stainless steel tube (Hamilton, *ca.* 100 μ m ID) and a length of 1/16 in. OD stainless steel tube. They were first electropolished to yield a smooth and polished surface. It is particularly important for the tip of the 33-gauge tube to ensure proper electrospraying. The 33-gauge tube was then partially inserted into the 1/16 in. tube and fixed with epoxy glue.

The optimum probe position was established on a daily basis for the analyte of interest, but was typically *ca* 1-2 cm from the interface plate with the spray off-axis from the orifice. The probe was held by a plastic clamp for electrical isolation. Biasing of the probe tip was achieved via a 50 M Ω current-limiting resistor in series with a high-voltage power supply (Tennelec, Model TC950). The potential for maintaining a stable electrospray depended on the composition of sample solution, typically between 2.5 and 3.5 kV for positive ion detection and -2.5 to -3.5 kV for negative ion detection. The electrospray current was monitored via a laboratory-built microammeter that could be floated above ground. For a stable electrospray, the current was usually between 0.1 and 0.2 μ A.

Figure 2.1 Schematic diagrams of (a) a laboratory-built electrospray probe used in TAGA 6000E; and (b) an ionspray probe used in API 300.

Sample solutions were continuously infused into the 33-gauge tube of the probe by means of a syringe pump (Harvard Apparatus, Model 22, South Natick, MA, USA) at a typical flow rate of 1-20 $\mu\text{L min}^{-1}$ via a poly (tetrafluoroethylene) (Teflon) connection tubing.

API 300. Figure 2.1 (b) shows an ionspray probe used in API 300[82]. A 26-gauge stainless steel tubing (*ca.* 250 μm ID, 450 μm OD) encased a 50-100 μm ID, 150 μm OD fused silica tube (Polymicro Technologies, Phoenix, AZ, USA), and was inserted coaxially through a 21-gauge stainless steel sprayer needle (*ca.* 800 μm ID). The tips of the fused silica tube and the steel guide tubing were flush with each other, and they protruded 1 to 2 mm beyond the sprayer needle. Nitrogen was used as the nebulizer gas, and was delivered at the annular space between the steel tubing and the sprayer needle. The flow rate of the nebulizer gas was between 0 and 1.84 L/min, typically *ca.* 1.2 L/min.

High voltage from the ion source power supply (a part of the API 300) was biased to the sprayer needle in the range of 5 to 6 kV for positive ion detection. The optimum probe position was established from time to time, but was typically with the tip about 2 cm from the interface plate and with the spray off-axis from the orifice. Sample solutions were continuously infused into the ionspray probe by using a Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) via the fused silica tube at flow rates from 1 to 10 $\mu\text{m/min}$.

2.2 ES-MS Interfaces

Since analyte ions are produced at atmospheric pressure in ES-MS, and mass analysis and detection are performed in the vacuum chamber at approximately 1×10^{-5} torr, the drastic pressure change between the ion source and the vacuum chamber requires a well-design ES-MS interface and an efficient pumping system. The interface should have the following two functions: (1) allow analyte ions transfer from the ion source into the mass analyzer; and (2) restrict solvent and ambient air from entering the vacuum chamber. The "gas curtain" and orifice are important components of the interface to perform these functions.

TAGA 6000E. Figure 2.2 shows the interface of TAGA 6000E [77]. The interface is comprised of the interface plate and the orifice plate. The vacuum chamber is isolated by the orifice plate which contains a $110 \mu\text{m}$ orifice. Dry nitrogen is delivered as the curtain gas to the space between the interface plate and the orifice plate, typically at a flow rate of 1-2 L/min. The orifice, through which analyte ions enter the vacuum chamber, is covered by the curtain gas to minimize neutral sample and solvent entering the vacuum chamber. Approximately 60 mL min^{-1} of curtain gas flows through the orifice into the vacuum chamber, while the remainder flows out to the ion source region through the aperture (*ca.* 3 mm in diameter) in the interface plate. The single-stage pumping system of TAGA 6000E consists of a cryogenic pump backed by a rotary pump. The cryogenic pump is highly efficient, so that it is able to pump down the vacuum chamber and maintains an operating pressure around 5×10^{-6} torr.

Figure 2.2 TAGA 6000E interface [77].

Due to the large pressure difference between the two sides of the orifice, the sample ions, nitrogen gas and residual solvent undergo a supersonic jet expansion when they are drawn by the pressure difference through the orifice into the vacuum chamber. It causes cooling of the ions, gas and solvent in this region. When this happens, residual solvent tends to combine with the sample ions to form solvated clusters. These clusters result in more complicated spectra which are difficult to identify. To facilitate mass spectral interpretation, these clusters must be broken down to ions and solvent molecules prior to mass analysis. This task can be accomplished via collision induced dissociation in the RF-only quadrupole (Q0) region between the clusters and the fraction of curtain gas which enters into the vacuum chamber. The CID energy is determined by the potential difference between the orifice (OR) and Q0.

API 300. Most features of the interface of API 300 are similar to those of TAGA 6000E. There are, however, some important differences (see Figure 2.3 [82]):

(1) The orifice of API 300, approximately 254 μm in diameter, is much larger than that of TAGA 6000E. Therefore, the amount of the curtain gas which flows into the vacuum chamber through the orifice is much larger than that in TAGA 6000E. The flow rate of the curtain gas in API 300 is typically between 1 and 1.8 L/min, and the fraction of the curtain gas that flows into the vacuum chamber is about 600 mL min⁻¹.

(2) Differential pumping system is employed in the API 300. Since a skimmer is

Figure 2.3 API 300 interface [82].

mounted behind the orifice plate, the interface of API 300 is comprised of two distinct pressure chambers: the gas curtain interface and the differentially pumped interface. These two interface regions are isolated by the curtain plate, the orifice plate and the skimmer. The differentially pumped interface is the first low pressure stage between the atmospheric pressure ion source and the vacuum chamber. The D16 rotary vane interface pump is used to pump down this region and maintain the pressure at *ca.* 2.2 torr. The vacuum chamber is divided into two distinct regions and two turbo pumps are employed to evacuate them: a 50 L/sec turbo pump is used for the Q0 region whose pressure is at 8×10^{-3} torr and a 360 L/sec turbo pump is used for the rest of the chamber which is maintained at *ca.* 1×10^{-5} torr.

2.3 Mass Analyzers and Operating Parameters

TAGA 6000E. The m/z range of TAGA 6000E triple quadrupole mass spectrometer is from 15 to 1400. Figure 2.4 [77] shows the quadrupole rod sets and ion optics of TAGA 6000E inside the vacuum chamber. The quadrupole rod sets include mass filters, the first quadrupole (Q1) and the third quadrupole (Q3), and RF-only quadrupoles (Q0 and Q2). The ion optics are comprised of the interface (IN), orifice (OR), shield lens (L7), exit lens (L9) and Faraday plate (FP).

Sample ions produced from the electrospray probe are attracted towards the interface plate, because the potential of IN is lower than that on the probe. The orifice plate separates atmosphere and vacuum and is at a lower voltage than IN, so that the electric field is

Figure 2.4 Schematic diagram of the quadrupoles and ion optics of TAGA 6000E triple quadrupole mass spectrometer [77].

Description	Parameter	Q1 MS	MS/MS	Operating Range
Interface Plate	IN	+650	+650	+200 to +700
Orifice Plate	OR	+40	+40	0 to +250
Quadrupole 0	Q0	+32	+32	0 to +250
Quadrupole 1	Q1	+30	+30	0 to +130
Shield Lens	L7	-100	-100	0 to -250
Quadrupole 2	Q2	0	as optimized	0 to +250
Quadrupole 3	Q3	0	as optimized	0 to +130
Exit Lens	L9	0	as optimized	0 to +250
Faraday Plate	FP	+100	+50	0 to +250
Ion Detector	MU	as optimized	as optimized	0 to -5000

Table 2.1. Typical voltage settings of the quadrupoles and ion optics for the positive ion detection mode of Q1 MS and MS/MS in TAGA 6000E triple quadrupole mass spectrometer.

attractive between IN and OR. Q0 is a RF-only quadrupole and only plays the role of transferring all ions into Q1. Quadrupole Q1 is a DC and RF mass filter, which selectively filters ions based on their m/z ratios. L7 is the shield lens to assist ion extraction from Q1 and transmission into Q2. RF-only quadrupole Q2 is the collision "cell", and transports the ions into Q3. Quadrupole Q3 is another DC and RF mass filter same as Q1. Exit lens L9 helps to extract ions from Q3. FP deflects the transmitted ions into the detector. Finally the ions are detected by the channel electron multiplier (CEM). Typical voltage settings of the quadrupoles and ion optics of TAGA 6000E are listed in Table 2.1 for the positive ion detection mode of Q1 MS and MS/MS. For the negative ion mode, the voltages are the same but the polarity is reversed.

The collision energy for the declustering in Q0 region depends on the potential difference between OR and Q0, which is typically from 5 to 15 V. The voltage difference between OR and Q2 determines the CID energy (E_{lab}) for MS/MS analysis in Q2 region, typically about 40 V for efficient fragmentation of singly charged ions. Mass spectra were recorded with a measurement time of 10-50 ms per m/z unit, and five to ten scans were averaged to generate a mass spectrum.

API 300. Compared to that of TAGA 6000E, the m/z range of API 300 is approximately double, from 30 to 3000. The ion optics designs are similar although API 300 has a few more parts. Figure 2.5 shows the API 300 ion path [82]. Due to the introduction of the skimmer, the focus ring is employed to focus the ions through the skimmer into the

Figure 2.5 Schematic representation of the quadrupoles and ion optics of API 300 triple quadrupole mass spectrometer [82].

Description	Parameter	Q1 MS	MS/MS	Operating Range
Ion Source	IS	+5000	+5000	0 to +8000
Curtain Plate	-	+1000	+1000	+1000
Orifice Plate	OR	+30	+30	0 to +200
Focus Ring	RNG	+300	+300	0 to +400
Quadrupole 0	Q0	-10	-10	0 to -17
Interquad Lens 1	IQ1	-12	-12	0 to -17
Stubby Rod	ST	-15	-15	0 to -40
Quadrupole 1	Q1	-12	-12	0 to -20
Interquad Lens 2	IQ2	-50	-30	0 to -200
Quadrupole 2	Q2	-25	-25	0 to -200
Interquad Lens 3	IQ3	-100	-55	0 to -200
Quadrupole 3	Q3	-100	-30	0 to -200
Exit Lens	EX	0	0	0
CEM Deflector	DF	0	0	0 to -400
Ion Detector	CEM	as optimized	as optimized	0 to -3200

Table 2.2. Typical voltage settings of the quadrupoles and ion optics for the positive ion detection mode of Q1 MS and MS/MS in API 300 triple quadrupole mass spectrometer.

vacuum chamber. The interquad lenses, IQ1, IQ2 and IQ3, assist the transmission of ions into the respective quadrupoles. The stubby focussing rod helps to transfer the ions from the Q0 region into the high vacuum region. The remaining parts play the same role as in TAGA 6000E. Table 2.2 lists the typical voltage settings of the quadrupoles and the ion lenses of API 300 for the positive ion detection mode of Q1 MS and MS/MS. The voltage of the curtain plate is fixed at 1000 V by the manufacturer. As in TAGA 6000E, the typical voltages for the negative ion detection mode are identical to those for the positive ion detection mode but the polarity is reversed.

2.4 Peptides, Proteins and Chemicals

All peptides, proteins, oligonucleotides and poly(propylene glycol) (PPG) were purchased commercially from Sigma Company and were used as received. Table 2.3 lists all samples used and their molecular weights calculated from sequence data [83]. All tripeptides are described using standard one-letter abbreviations for the α -amino acids; oligonucleotides by one-letter abbreviations for the bases.

The concentrations of the protein solutions used in this work were typically 10 μ M; 1 mM for the tripeptide solutions. Solvents were usually water or water/methanol (50/50). Acetic acid, ammonia, ammonium acetate and alkali hydroxides were used for pH adjustment.

All solvents and chemicals were reagent grade or better, and were used without further purification. Table 2.4 gives a list of all chemicals used and their suppliers.

Peptide and Protein	Source	Molecular Weight (Da)
AGG	Tripeptide	203.2
LGG	Tripeptide	245.3
VGG	Tripeptide	231.3
GGA	Tripeptide	203.2
GGL	Tripeptide	245.3
GGV	Tripeptide	231.3
PGG	Tripeptide	229.2
APG	Tripeptide	243.3
GPA	Tripeptide	243.3
Leucine enkephalin	Peptide	555.6
Bradykinin	Peptide	1060
Gramicidin s	Bacillus brevis	1142
d(pT) ₁₈	Oligonucleotide	5493.5
d(GGAATTCC)	Oligonucleotide	2409.6
Cytochrome c	Equine skeletal	12360
Bovin Insulin	Bovine pancreas	5733
Equine myoglobin	Equine muscle	16954
PPG 1000	poly(propylene glycol)	1000

Table 2.3. List of all samples used in this work and their molecular weights calculated from sequence data.

Chemical	Supplier
Acetic acid	BDH Chemicals
Ammonia	Aldrich
Ammonium acetate	Anachemia
Cesium hydroxide	Anachemia
Cetyl alcohol	Aldrich
Hexane	Anachemia
Hydrogen peroxide	Anachemia
Lithium hydroxide	Anachemia
(3-mercaptopropyl)trimethoxisilane	Anachemia
Methanol	BDH Chemicals
Potassium hydroxide	Anachemia
2-propanol	BDH Chemicals
Sodium hydroxide	Anachemia
Stearyl alcohol	Aldrich
sulfuric acid	Anachemia

Table 2.4. List of chemicals used in this work and their suppliers.

Chapter 3

The Effects of Stearyl and Cetyl Alcohol on the Electrospray Mass Spectrometry of Proteins

3.1 Introduction

Electrospray mass spectrometry has been quickly transformed from a scientific curiosity to one of the most powerful tools in the bioanalytical laboratory since Fenn and co-workers' pioneering work on applying this technique to determine the molecular masses of proteins [31, 68]. As in the case of many analytical techniques, the pace of our understanding of the Electrospray process and its associated mechanism lags far behind the pace at which the technique is being used in new applications. Two competing mechanisms, "Charge Residue Model" and "Ion Evaporation Model", have been proposed for electrospray mass spectrometry (see Section 1.3); these have stimulated a considerable number of interesting and cleverly designed experiments aiming to resolve specific areas of contention. However, these two proposed models are not completely independent and share many common features. The effectiveness of ion desorption in competition with evaporation and Rayleigh fission in smaller droplets has been questioned by Röhlgen and co-workers [84, 85]. They argued that the actual field strength required for Rayleigh fission is almost always lower than that for ion evaporation from even the smallest charged droplets; as a result, the cycle of evaporation and Rayleigh fission would continue until each droplet contains only one sample ion. However, a subsequent flourish of experimental evidence amassed by Fenn and co-workers [31, 86, 87] and others [9, 88, 89] convinced many that the IEM is a more accurate description of what goes on in electrospray.

A key component of both the CRM and the IEM is solvent evaporation; through it

a larger droplet decreases in size, increases in charge density and reaches the Rayleigh limit. Based on the results of three studies [90-92], Siu and co-workers have recently questioned the importance of this role in relation to the production of gas-phase ions. The debate was not on whether droplets evaporate and/or undergo fission, but rather whether solvent evaporation is necessary for the generation of gas-phase analyte ions in electrospray. In one study [90], the electrospray mass spectra of cytochrome c and myoglobin in aqueous solutions of 0.2% acetic acid and propionic acid were compared. From equilibrium data, the pHs of these two acid solutions change in opposite directions on evaporation; the pH of the acetic acid solution increases whereas that of the propionic acid solution decreases with increasing evaporation. The pHs of aqueous solutions of proteins determine the charge state of the protein ion [93], which means that the distribution profile of multiply charged protein ions in solution shifts with solution pH. Since the pKa values of acetic and propionic acid are comparable, the distribution profiles of protein dissolved in these two acid solutions should be shifted from each other if solvent evaporation were a prerequisite to ion formation in electrospray mass spectrometry. As it turned out, the spectral profiles of the same protein dissolving in the same concentration of acetic and propionic acid were almost identical, suggesting that the ions observed must have been formed (desorbed) from acetic and propionic acid solutions of identical pH. This could only mean that the ions must have been desorbed from droplets that had undergone minimal evaporation.

This conclusion is also supported by an earlier observation on electrospraying 0.2% acetic acid solutions of proteins at temperatures ranging from approximately 25 to 91°C [92].

In this study, heating was supplied by means of a hot-air gun; therefore, both the electrospray probe and the adjoining gas were heated. The only significant effect of changing temperature observed was one due to thermal-induced denaturation of some proteins, e.g. cytochrome c and lysozyme, in the acetic acid solutions. For proteins that do not undergo significant conformational changes within that temperature range, no significant changes in the electrospray mass spectra were discernible. This lack of a universal thermal effect, in the authors' opinion, is one of the key arguments against the requirement of solvent evaporation in any electrospray mechanism. If solvent evaporation were a necessary step prior to ion formation, one would expect that raising the source temperature, which expedites evaporation, should have resulted in an increased electrospray response, especially of solutions containing relatively high boiling-point solvents, such as water. This expected increase was not observed.

In the third study [91], the electrospray mass spectra of gramicidin s, myoglobin and tetrabutylammonium bromide in ethylene glycol, a non-volatile solvent, were compared to those of the same analytes in water. Computer modelling of the evaporation of droplets of these two solvents revealed that under conditions in which a water droplet's radius would shrink by ~30%, an ethylene glycol droplet shrinks only negligibly; furthermore, a droplet, be it ethylene glycol or water, that is initially hot (such as one ejected from a heated electrospray needle) cools very rapidly owing to evaporation and heat loss to ambient air, and subsequently evaporates much like a droplet that is initially at room temperature. For gramicidin s, the ion abundances in ethylene glycol as the solvent were ~200 times lower

than those in water/methanol under room temperature operating conditions; in experiments where the spray probe was heated to $\sim 100^{\circ}\text{C}$ to reduce the viscosity of ethylene glycol, the gramicidin ion abundances were only ~ 40 times lower than those in water/methanol. Similar trends were obtained for the other analytes. The authors argued that the observed ion abundances in ethylene glycol, relative to those in water, are orders of magnitude too large to be accounted for using models that require solvent evaporation. Furthermore, the observed increase in electrospray response from solutions containing ethylene glycol as the solvent, when the electrospray probe was heated, was due to a decrease in ethylene glycol's viscosity, hence, an increase in the analyte ions' drift velocity in these solutions, and consequently, an increase in the total number of ions desorbing from the air/solution interface of the Taylor cone during electrospray.

The work described in this chapter may be considered as the fourth study of the series. The approach continues to be designing experiments to test the physics/chemistry of electrospray such that one can deduce, or at least have an educated guess, if solvent evaporation is indeed necessary for the observation of electrospray response. It has been shown that promoting evaporation via heating has no universal effect in increasing the electrospray response of analyte ions in aqueous solutions [92]. It has also been shown that minimizing evaporation via the use of ethylene glycol as the solvent does not preclude the observation of analyte ions [91]. In this study, a long chain *n*-alkanol, such as stearyl (C18) or cetyl (C16) alcohol, was added to aqueous-based analyte solutions to deter solvent (water) evaporation. These non-ionic surfactants have found an application as evaporation retardants

in lakes and reservoirs [94], and can also be used to increase the life time of water droplets [95].

3.2 Experimental

Mass spectra were recorded on the TAGA 6000E using the laboratory-built electrospray probe [see Chapter 2]. For experiments that required addition of a hexane solution of stearyl or cetyl alcohol to the analyte solution during electrospray, a laboratory-built coaxial sprayer was used (see Figure 3.1): It had a 26-gauge stainless steel tube encasing the 33-gauge tube, and delivering the surfactant solution in the annular space between the two coaxial tubes. At the probe tip, the 33-gauge tube protruded ~2 mm beyond the outer tube. With the aid of a microscope, the hexane solution was seen to flow over the protruding inner tube and over the Taylor cone during electrospray. As previously described, all stainless steel tubes were electropolished prior to use.

Ten micromolar protein solutions in water and water/methanol with or without <1% *n*-alkanol were used in this work. When needed, a second syringe pump was used to deliver a solution of <1% *n*-alkanol (stearyl or cetyl alcohol) in hexane into the annular space between the outer and the inner probe tube at a typical rate of 0-20 $\mu\text{L min}^{-1}$.

3.3 Effect of a monolayer of *n*-alkanol on water evaporation

Figure 3.1 Schematic diagram of a laboratory-built coaxial electrospray probe.

It is well known that the existence of a surface monolayer of a long-chained *n*-alkanol, such as cetyl or stearyl alcohol, on a pool of water greatly retards its evaporation. A monolayer of cetyl alcohol can reduce the rate of evaporation by as much as 40% [94]. To achieve maximum retardation, a close-packed monolayer is required; other desirable properties are self-healing and fast spreading. Cetyl alcohol and stearyl alcohol possess properties that make them the appropriate choices [94]. The effect of a monolayer of *n*-alkanols on a water droplet is more pronounced than that of a monolayer on a flat water surface. Eisner and co-workers [95] reported that the presence of 0.05-1.2% of a mixture of C₁₄ to C₁₈ *n*-alkanols in water droplets, in the range 2-20 μm , stabilizes these droplets by reducing their evaporation rate, and droplet life time has been increased by factors up to several hundreds.

In this work, we have devised methods to create a surface monolayer of *n*-alkanol on the droplet produced in electrospray. This was carried out in two ways. In the first approach, the analyte (cytochrome c or myoglobin) was dissolved in a 50/50 water/methanol mixture at pH~3 containing typically 0.1% or 0.5% of cetyl or stearyl alcohol (methanol was needed to increase the solubility of these surfactants to 0.1% and 0.5%). We shall provide evidence that an *n*-alkanol monolayer is formed on the surface of such a solution. Furthermore, we shall argue that this surface film can also be formed on a droplet within the time scale relevant to electrospray. In the second approach, the analyte, cytochrome c or myoglobin, was dissolved in water at pH~3; the *n*-alkanol was added in the form of a dilute solution in *n*-hexane and delivered to the Taylor cone via a coaxial stream. We envisaged

that a droplet sprayed out under such a condition would have an external layer of *n*-alkanol in hexane encasing the inner aqueous droplet. Similar to the spreading of *n*-alkanol on bulk liquid [94], evaporation of the hexane would leave behind a surface film of *n*-alkanol on the water droplet.

3.4 Electrospraying protein solutions containing *n*-alkanols

Figure 3.2 shows the electrospray mass spectra of 10 μ M myoglobin in 50/50 water/methanol, acidified with 0.2% acetic acid, and containing 0, 0.1 and 0.5% stearyl alcohol. Apparently, the presence of up to 0.5% stearyl alcohol had a minimal effect on the spectra of myoglobin; the variation of abundances among the spectra is within typical variation of repetitive runs of the same solution. Similar results were obtained for 10 μ M cytochrome c solutions containing stearyl alcohol (Figure 3.3), and for similar solutions containing cetyl alcohol. That is to say, the presence of up to 0.5% of an *n*-alkanol had no significant effect on the electrospray response of proteins. The question then becomes: Could and did a surface film of *n*-alkanol form on droplets produced in these electrospray experiments?

A comparison of the evaporation rates of bulk liquids of 50/50 water/methanol and 50/50 water/methanol containing stearyl and cetyl alcohols at various concentrations revealed that the 0.1 and 0.5% *n*-alkanol containing solutions evaporated at a rate about half of that of water/methanol; this relative difference between the solutions was similar to the

Figure 3.2 Electrospray mass spectra of 10 μ M myoglobin in 50/50 water/methanol, acidified with 0.2% acetic acid, and contained, from top to bottom panel, 0, 0.1 and 0.5% stearyl alcohol.

Figure 3.3 Electrospray mass spectra of 10 μ M cytochrome c in 50/50 water/methanol, acidified with 0.2% acetic acid, and contained, from top to bottom panel, 0, 0.1 and 0.5% stearyl alcohol.

relative difference reported for aqueous solutions with and without *n*-alkanol [94]. Furthermore, spreading tests consisting of dropping hexane on *n*-alkanol solutions in water/methanol, and dropping hexane solutions of *n*-alkanols on water/methanol [94], indicated strongly the presence of a surface active compound on water/methanol. Thus there appears little doubt that a surface film forms on an *n*-alkanol-containing water/methanol solution.

The question remains whether a surface film would form fast enough to exert an effect on evaporation. The flight time of an ion from the electrospray needle to the orifice is generally believed to be in the order of a millisecond or so. The flight time (therefore lifetime) of a non-evaporating charged droplet of a few micrometers in radius would probably be in the order of ten milliseconds (see Appendix 1, Page 127). For a droplet of 1.5 μm in radius, the averaged time, τ , that it takes for a surfactant molecule released at the centre of the droplet to diffuse to the surface is given by [96]

$$\tau = \frac{(r/\pi)^2}{D_1} \quad (9)$$

where D_1 is the diffusion coefficient of the surfactant in the droplet. Assuming a D_1 of $1 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ and $r=1.5 \text{ um}$, $\tau=2.3 \times 10^{-4} \text{ s}$. Therefore, there is ample time for the *n*-alkanol molecules to diffuse to the droplet surface during its life time. However, they may not form a surface film instantaneously; dynamic surface tension measurements of aqueous solutions

of *n*-alkanols carried out in the 1940s indicated that an activation energy barrier which hinders the establishment of an adsorbed film may exist, and overcoming this barrier becomes the rate determining step for these solutions [97, 98]. Results for solutions of $\leq 1\%$ isoamyl alcohol showed that surface equilibrium was reached in the order of a few milliseconds [98]. Therefore, although a surface film of stearyl or cetyl alcohol would form on a micrometer size droplet during its life time in electrospray, and that a surface film does not have to be complete before it would be effective, we cannot rule out that such a droplet might have a chance to evaporate as rapidly as a droplet without the surfactant during its first fraction of a millisecond of its life time.

However, since protein solutions with and without stearyl or cetyl alcohol appeared not to differ in their electrospray responses, one would have to state that solvent evaporation is unlikely to be an important step leading to the production of gas-phase ions. This conclusion is not surprising to us, and is in accord with the earlier interpretations of the Siu's group. Owing to the fact that we cannot eliminate the above uncertainty concerning the surface film status at the initial stage of a droplet's life time, we decided to add the *n*-alcohol using a different method, which eliminates the need for diffusion and adsorption, and in many respects is more similar to what is practised on bulk liquids.

3.5 Addition of *n*-alcohol coaxially during electrospray

A surfactant, such as an *n*-alcohol, is typically added to an aqueous surface in a

solution of a volatile solvent, e.g. hexane. Evaporation of the volatile solvent leaves behind the surfactant on the aqueous surface [94]. This approach also avoids the problem of the limited solubilities of *n*-alkanols in water. Figure 3.4 shows the mass spectra of electrospraying a 10 μ M solution of myoglobin in water with a sheath of 0.1% stearyl alcohol in hexane. The inner aqueous protein solution flow rate was 10 μ L min⁻¹. The mass spectra were practically identical. Similar results were obtained in electrospraying a cytochrome c solution in water with a cetyl alcohol sheath (Figure 3.5).

If one were to accept that water could evaporate substantially from an electrospray-generated droplet during its lifetime, then one had to assume that hexane, which is considerably more volatile than water, would evaporate and leave behind a layer of surfactant on a droplet produced in these experiments. This being the case, the evaporation of water from such a droplet would be severely retarded; as before, no apparent effect on the production of electrospray ions was observable. It may be appropriate to reiterate that the so-called parent droplets in electrospray, from which the bulk of the sprayed liquid is evaporated, are believed to have initial radii centre about 1.5 μ m [49], and that the lifetime of droplets having initial radii of 2-20 μ m was demonstrated to increase by factors up to several hundreds via the introduction of 0.05-0.2% of a mixture of fatty alcohols, whose major components were cetyl and stearyl alcohol [95].

3.6 Evidence against the need for solvent evaporation in producing electrospray ions

Figure 3.4 Electrospray mass spectra of 10 μM myoglobin in water continuously infused at 10 $\mu\text{L min}^{-1}$ via the inner coaxial probe; a solution of 0.1% stearyl alcohol in hexane was continuously infused via the annular space at a flow rate of, from top to bottom panel, 0, 2 and 4 $\mu\text{L min}^{-1}$.

Figure 3.5 Electrospray mass spectra of 10 μM cytochrome c in water continuously infused at 10 $\mu\text{L min}^{-1}$ via the inner coaxial probe at a source temperature of $\sim 50^\circ\text{C}$ (to denature thermally a significant portion of the cytochrome c); a solution of 0.5% cetyl alcohol in hexane was continuously infused via the annular space at a flow rate of, from top to bottom panel, 0, 3 and 5 $\mu\text{L min}^{-1}$.

As stated earlier, the current study may be considered the fourth part of a series of studies on the origin of electrospray ions. The first study considered the effect of heat on the ion abundances of electrosprayed ions; when thermal denaturation was absent, heating has no effect on the mass spectra [92]. The second study compared the electrospraying of analyte solutions whose initial pH values were identical but are expected to diverge on evaporation; the electrospray mass spectra of the same analyte were virtually identical [90]. The third study compared the electrospraying of analyte solutions in water and in ethylene glycol; the electrospray mass spectral intensities of the same analyte were not sufficiently different [91]. This fourth study compares the electrospraying of analyte solutions in water with and without surfactants that are proven evaporation retardants; the electrospray mass spectra of the same analyte are virtually identical. In all cases, the experimental results tell us that solvent evaporation, occur as it may, is not a necessary step leading to gas-phase ion formation in electrospray. One may argue that, as some of our colleagues have [50, 99], taken individually each of the studies has its associated experimental and conceptual points of contention. However, taken collectively, the four studies, because they each probe the evaporation phenomenon from a different angle, paints, in our mind, a convincing picture that electrospray-generated ions, at least the majority of them, do not originate from droplets that have undergone significant solvent evaporation.

It should be emphasized that there is no *experimental evidence* that offspring droplets would undergo uneven (and not even) fission [50]; their importance in the electrospray mechanism is conjectural. Furthermore, it is our opinion that it is unreasonable to assume

offspring droplets as small as 3 nm in radii should play such important roles in electrospray mass spectrometry [50] in view of the fact that the radii of many globular proteins studied by means of this technique have values of a few nm [100]. It may be of interest to note that uneven Rayleigh fission may actually be viewed as a mechanism bridging the first generation mechanism which requires extensive solvent evaporation plus even Rayleigh fission [50], and the speculation of direct ion desorption near the Taylor cone [90, 91]. The *extreme* form of uneven Rayleigh fission is the ejection of a solvated ion, in other words, ion desorption.

3.7 Conclusion

Based on the experimental results of these studies, which show the lack of correlation between the extent of solvent evaporation and the extent of the electrospray response, one has to conclude that solvent evaporation is not a necessary prerequisite to ion formation in electrospray, and that the gas-phase ions measured in electrospray mass spectrometry must have been formed early rather than late during the electrospray process. The earliest that this can take place is at the Taylor cone [90, 91].

Chapter 4

Electrospray Tandem Mass Spectrometry of Alkali Metal-containing Anionic Complexes of Tripeptides

4.1 Introduction

The need to develop efficient analytical techniques for sequencing biologically important peptides has led to considerable advances in the application of tandem mass spectrometry in bioanalytical chemistry. Alkali metal ion adducts of peptides are readily observable under fast atom bombardment (FAB) conditions [102-112]. Since the fragmentation pathways of these adducts are rather different from those of protonated peptides, some effort has been spent on evaluating them for peptide sequencing [111]. Aside from this practical aspect, the application of MS/MS in this area has principally been spurred on by the desire to better comprehend the interaction between alkali metal ions and peptides.

Alkali metal ions, especially sodium and potassium, interact with a number of important peptides and proteins and play an important biological role [113-115]. The gas phase chemistry of alkali metal ion-peptide adducts, as imparted in MS/MS, can reveal the intrinsic properties of the metal ion-peptide bonds unencumbered with solvation. Adams *et al.* [102-105], Gross *et al.* [105-110], and Leary *et al.* [111, 112] reported extensively on the gas phase decomposition of peptide adducts containing a single metal ion. Both positively [102-104, 106, 107] and negatively [105, 108-110] charged adduct ions were observed. These adduct ions were typically produced by means of FAB using a matrix of glycerol and thioglycerol saturated with the appropriate alkali metal hydroxide or halide.

Owing to its compatibility with aqueous samples, electrospray introduction is

preferred over FAB in many bio-applications. Adducts between alkali metal ions and peptides have been observed using electrospray mass spectrometry under a variety of conditions [116-118]. Siu *et al.* [118] reported that from a sample of a tripeptide dissolved in a mixture of water and methanol adjusted to pH 12 with an alkali metal hydroxide, several cationic alkali metal-containing peptide adducts were evident in the gas phase. These were, in decreasing relative abundance, $[M-H+2X]^+$, $[M-2H+3X]^+$ and $[M-3H+4X]^+$ where M denotes the tripeptide and $X=Li, Na$ and K . The abundance of $[M-H+2X]^+$ ions is typically low in FAB while it was high in this study using electrospray; the existence of $[M-2H+3X]^+$ and $[M-3H+4X]^+$ ions had not been previously reported. Tandem mass spectrometry revealed that, in these multiple alkali metal-containing peptide adducts, the metal ions are both attached to carbonyl oxygen atoms and centrally coordinated to the deprotonated carboxylate terminus as well as the peptide nitrogen atoms. Low energy fragmentation of $[M-H+2X]^+$ and $[M-2H+3X]^+$ ions yielded both N-terminal and C-terminal product ions. They were $[c_1+2X]^+$ and $[y_1+2X]^+$ from the precursor ion $[M-H+2X]^+$, and $[a_2+2X]^+$ and $[y_2-H+3X]^+$ from $[M-2H+3X]^+$. Note that since the precursor ions and product ions contained variable numbers of metal ions, to avoid ambiguity, the assignments, c_1 , y_1 , etc., adopted refer to the fragments of non-zwitterionic, non-metal ion containing peptides. For instance, y_1 of alanylglycylglycine (AGG) is $NH-CH_2-COOH$ and, therefore, $[y_1+2X]^+$ is $[NH-CH_2-COOH+2X]^+$; c_1 of the same tripeptide is $NH_2-C(CH_3)H-CO-NH$ and, similarly, $[c_1+2X]^+$ is $[NH_2-C(CH_3)H-CO-NH+2X]^+$. We shall continue using the same nomenclature in this thesis work.

Although not explicitly stated, gas phase anionic multiply alkali metal-containing peptide adducts were also observable from the same samples that yielded the $[M-H+2X]^+$ and $[M-2H+3X]^+$ ions when the mass spectrometric detection mode was switched from positive to negative [118]. In this study, we investigate the structure of these anionic alkali metal-containing adducts and their collision induced dissociations.

4.2 Experimental

Mass spectra were recorded on TAGA 6000E in the negative ion detection mode. The electrospray probe and spraying conditions are described as in Section 2.1. Most of the experiments were carried out with a potential difference between OR and R0 of 8V. Argon was used as the collision gas for the low energy CID in Q2 region, and at a thickness of approximately 1.2×10^{14} atoms cm^{-2} . At this value, the precursor ion underwent, on average, one collision event within the collision chamber [119, 120]. E_{lab} was typically about 40 eV.

All tripeptides, AGG, LGG, VGG, GGA, GGL, GGV, PGG, APG and GPA, were dissolved in 50/50 methanol/water in concentrations of 1 mM. These were adjusted to pH 12 with alkali hydroxide before electrospraying. Deuterium-labelling of exchangeable hydrogen atoms was accomplished by dissolving the tripeptides in 50/50 $\text{CH}_3\text{OD}/\text{D}_2\text{O}$ adjusted to pH 12 with alkali deuterioxides; the equilibrium was typically reached within a few minutes with the fully deuterated products being one of the major species. Acylation of the *N*-terminal amino group was performed by dissolving the tripeptide of interest in 1%

acetic anhydride in 50/50 methanol/water, allowing to react for one hour, and adjusting to pH 12 with the appropriate alkali hydroxide.

4.3 Results

As in earlier studies [102-105, 108-110, 118], to ascertain examination of the "fundamental" interactions between the alkali metal ion and the peptide, all the tripeptides selected for this study contained only neutral sidechain groups. Figure 4.1 shows negative ion electrospray mass spectra (Q1 scans) of two peptides, LGG and GGV, in solutions at pH 12 with potassium hydroxide. Three peptide-containing ions are discernible: $[M-H]^-$, $[M-2H+K]^-$ and $[M-3H+2K]^-$, in decreasing abundance (the rest of the peaks were background ions). Spectra of other tripeptides were very similar except for those of APG and GPA in which the $[M-3H+2K]^-$ ion intensity was frequently below the limit of detection. In this study, we focus our attention on the two alkali metal ion-containing ions, $[M-2H+X]^-$ and $[M-3H+2X]^-$.

4.3.1 Fragmentation of the $[M-2H+X]^-$ Ions

Figure 4.2 shows the CID mass spectra of $[M-2H+Na]^-$ ions of six tripeptides: AGG, GGA, LGG, GGL, VGG and GGV. The abundances of the product ions are relatively low since single-collision conditions were employed to eliminate the possibility of forming second generation product ions. Four major product ions are apparent: $[x_1-2H]^-$, $[b_2-2H]^-$,

Figure 4.1 Electrospray mass spectra of 1 mM LGG and GGV in 50/50 water/methanol at pH 12 with potassium hydroxide.

Figure 4.2 Collision induced dissociation mass spectra of the $[M-2H+Na]^+$ ions of AGG, GGA, LGG, GGL, VGG and GGV.

$[y_2-H+Na]^-$ and $[y_2-H+Na-CO_2]^-$; the first three are present in the CID spectra of all six tripeptides while the last one is present only in those tripeptides whose last two amino acid residues are glycine. These same product ions were also present in the product ion spectra of the $[M-2H+X]^-$ ions of GPA, APG and PGG. Equivalent product ions were also observed for the fragmentation of $[M-2H+X]^-$ ions of other alkali metal ions. Figure 4.3 shows a comparison of the CID spectra of the $[M-2H+X]^-$ ion from GGL with $X = Li, Na, K$ and Cs . In general, fragmentation chemistry becomes increasingly richer moving from left to right of the series, $Cs < K \sim Na < Li$ although the same major product ions are present for all four alkali metal ions.

Acylation of the *N*-terminus altered the fragmentation of the $[M-2H+X]^-$ ions considerably. Figure 4.4 shows the results for the acylated $[M-2H+Na]^-$ ions of VGG and GGV. The major product ions are now: $[x_1-2H]^-$, $[b_1-2H]^-$, $[x_2-3H+Na]^-$, $[x_2-HCOOH]^-$ and $[c_1-H+Na]^-$, and for VGG $[y_2-H+Na]^-$ as well.

4.3.2 Fragmentation of the $[M-3H+2X]^-$ Ions

The CID mass spectra of the $[M-3H+2Na]^-$ ions of the six non proline-containing tripeptides are shown in Figure 4.5. Only one major product ion is discernible: $[y_2-2H+2Na]^-$. This product ion was also prominent when *M* was PGG. For GPA and APG, the $[y_2-2H+2Na]^-$ product ion became relatively less intense.

Figure 4.3 Product ion spectra of the $[M-2H+X]^-$ ions of GGL for $X = Li, Na, K$ and Cs .

Figure 4.4 Collision induced dissociation mass spectra of the $[M-2H+Na]^+$ ions of N-acetylated VGG and N-acetylated GGV.

Figure 4.5 Collision induced dissociation mass spectra of the $[M-3H+2Na]^+$ ions of AGG, GGA, LGG, GGL, VGG and GGV.

4.4 Discussion

4.4.1 Structures of the Gas Phase Precursor Ions, $[M-2H+X]^-$ and $[M-3H+2X]^-$

The fragmentation patterns of the precursor ions are consistent with structures **1** and **2** (Figure 4.6) for $[M-2H+X]^-$ and $[M-3H+2X]^-$, respectively. Note that, as before (Ref. 118), **1** is one of several possible interconverting gas-phase structures of $[M-2H+X]^-$ (**3** and **4** are other possible structures), and likewise **2** is one of the several possible interconverting gas-phase structures of $[M-3H+2X]^-$; structures of the same family may interconvert with low energy barriers prior to fragmentation. The core structure of **1** and **2**, where an alkali metal ion is centrally located for bonding with the amino terminus, the amide nitrogen atoms, and an oxygen of the carboxylate terminus, is very similar to structures proposed by Gross *et al.* [108-110] and Adams [105]. Both **1** and **2** have an alkali metal ion bonded to the deprotonated carboxylate anion; this is required by our MS/MS results and is also in accord with recent *ab initio* calculations [121, 122] which showed that alkali ion attachment to the zwitterionic carboxylate terminus is energetically feasible.

4.4.2 Fragmentation of the $[M-2H+X]^-$ Ions

The fragmentation of the $[M-2H+X]^-$ ions to form the information rich $[y_2-H+X]^-$ and $[y_2-H+X-CO_2]^-$, $[x_1-2H]^-$, and $[b_2-2H]^-$ are shown in Figures 4.7, 4.8 and 4.9, respectively, for X=Na.

Figure 4.6 Scheme of gas-phase precursor ion structures: 1 for $[M-2H+Na]^-$; and 2 for $[M-3H+2Na]^-$.

The formation of y fragment ions requires transfer of a hydrogen from the neutral to the ionic product. Both the β - [103, 118] and the γ -hydrogen [123] (i.e. the $-\text{CH}-$ and the $-\text{NH}-$ hydrogen) of the preceding residue have been identified as the hydrogen transferred. Siu *et al.* showed with d_4 - $[\text{M}-2\text{H}+\text{X}]^+$ ions that the $[\text{y}_1+2\text{X}]^+$ product ion is d_1 , meaning that it receives a $-\text{CH}-$ and not an $-\text{NH}-$ hydrogen from the previous amino acid residue. The opposite is true here for the fragmentation of $[\text{M}-2\text{H}+\text{X}]^-$ ions; CID spectra of d_3 - $[\text{M}-2\text{H}+\text{X}]^-$ ions show that both the $[\text{y}_2-\text{H}+\text{X}]^-$ and $[\text{y}_2-\text{H}+\text{X}-\text{CO}_2]^-$ ions are d_2 . This means that the y_2 anion must have received a hydrogen from the N-terminus as shown in Figure 4.7. Note that the mechanism depicted for the formation of $[\text{y}_2-\text{H}+\text{X}]^-$ is almost identical to that of Gross *et al.* [109]. As far as we know, the observation of $[\text{y}_2-\text{H}+\text{X}-\text{CO}_2]^-$ ions has never been previously reported. Proline-containing tripeptides contain only four replaceable hydrogen atoms. Therefore, their fully deuterated $[\text{M}-2\text{H}+\text{X}]^-$ ions were d_2 . Fragmentation of d_2 - $[\text{M}-2\text{H}+\text{X}]^-$ ions of GPA and APG yielded d_1 - $[\text{y}_2-\text{H}+\text{X}]^-$ ions but those of PGG yielded d_2 - $[\text{y}_2-\text{H}+\text{X}]^-$ ions, in accordance with Figure 4.7.

Figure 4.8 depicts the formation of the product ion, $[\text{x}_1-2\text{H}]^-$, from the precursor ions 1, 3 and 4. The $[\text{x}_1-2\text{H}]^-$ ion has been reported by Gross *et al.* [109] under a different notation. The mechanisms shown in Figure 4.8 are supported by deuterium-labelling experiments in that the fragmentation of d_3 - $[\text{M}-2\text{H}+\text{X}]^-$ ions from non-proline containing and d_2 - $[\text{M}-2\text{H}+\text{X}]^-$ ions from proline containing tripeptides yielded d_0 - $[\text{x}_1-2\text{H}]^-$ ions. Figure 4.8 shows that, for precursor ions 3 and 4, the rearrangement involves the loss of the $-\text{NH}-$ hydrogen on the third residue to the α -carbon of the second residue thus making $[\text{x}_1-2\text{H}]^-$

Figure 4.7 Scheme of the formation of $[y_2-H+X]^+$ and $[y_2-H+X-CO_2]^+$ from the precursor ion $[M-2H+Na]^+$.

Figure 4.8 Scheme of the formation of $[xI-2H]^-$ from the precursor ion $[M-2H+Na]^-$.

Figure 4.9 Scheme of the formation of $[b_2-2H]^-$ from the precursor ion $[M-2H+Na]^+$.

devoid of exchangeable hydrogen. The same product ion is also formed from precursor 1, whose fragmentation results in the transfer of the alkali metal from the ionic to the neutral product. The lack of an amide hydrogen atom in the proline residue rules out precursor structure 3 and the fragmentation depicted for precursor 1 for GPA and APG. For these two tripeptides, $[x_1-2H]^-$ can be formed from precursor 4.

Formation of the $[b_2-2H]^-$ product ion from $[M-2H+X]^-$ requires the elimination of $NH_2C(R_3)HCOOX$ as depicted in Figure 4.9. The structure of the $[b_2-2H]^-$ ions is different depending on the nature of the second residue. As shown by Gross *et al.* [109], a cyclic $[b_2-2H]^-$ structure is proposed here for tripeptides whose second residue is not proline; in other words, the mechanism for the formation of this ion involves transfer of an N-terminal hydrogen to the neutral product [Figure 4.9(a)]. This hypothesis is supported by the fragmentation of $d_3-[M-2H+X]^-$ precursor ions, which yielded $d_1-[b_2-2H]^-$, and the fragmentation of $d_2-[M-2H+X]^-$ from PGG, which yielded $d_0-[b_2-2H]^-$. The other two proline-containing tripeptides, GPA and APG, lack a critical amide hydrogen and, hence, cannot form the cyclic $[b_2-2H]^-$ ion shown in Figure 4.9(a); instead, an open $[b_2-2H]^-$ structure is proposed, of which the formation proceeds via transfer of a $-CH-$ hydrogen on the proline ring to the neutral product [Figure 4.9(b)]. The $[b_2-2H]^-$ ion depicted in Figure 4.9(b) contains one exchangeable hydrogen atom. This is supported by collision induced dissociation of $d_2-[M-2H+X]^-$ ions of GPA and APG, which yielded $d_1-[b_2-2H]^-$.

4.4.3 N-acylated $[M-2H+X]^-$ Ions

As mentioned previously, the *N*-acylated $[M-2H+X]^-$ ($[AcM-2H+X]^-$) precursor ions displayed a much richer fragmentation chemistry. Major product ions, aside from the aforementioned $[x_1-2H]^-$ and $[y_2-H+X]^-$, were readily observable. For $[b_1-2H]^-$ ion, fragmentation of $d_2-[AcM-2H+X]^-$ showed that the majority of that product ion was d_0 ; a $d_0-[b_1-2H]^-$ ion could be formed in a mechanism similar to that of the $[b_2-2H]^-$ ion [Figure 4.9(c)].

Figures 4.10-4.12 show schemes for the formation of $[x_2-3H+X]^-$, $[x_2-HCOOH]^-$ and $[c_1-H+X]^-$, respectively. The formation of the $[x_2-3H+X]^-$ product ion from *N*-acetylated $[M-2H+X]^-$ requires transfer of a hydrogen from the ionic to the neutral product. Collision induced dissociation of $d_2-[AcM-2H+X]^-$ ions of non-proline containing tripeptides showed that the $[x_2-3H+X]^-$ ion is d_0 . This means that the hydrogen transferred is an $-NH-$ and not a $-CH-$ hydrogen. Figure 4.10 shows the formation of the $[x_2-3H+X]^-$ ion from the *N*-acetylated version of 1 that involves transfer of the $-NH-$ hydrogen of the second residue and elimination of $R_1CH_2NHCOCH_3$. The tripeptides GPA and APG lack the $-NH-$ hydrogen on the second residue and no $[x_2-3H+X]^-$ ions were evident from their *N*-acetylated $[M-2H+X]^-$ ions; for PGG, however, the critical $-NH-$ hydrogen is present and the $[x_2-3H+X]^-$ ions were observed.

Figure 4.11 depicts the formation of the $[x_2-HCOOH]^-$ ion from the $[AcM-2H+X]^-$ precursor ion of non-proline containing tripeptides and PGG. For non-proline containing tripeptides, transfer of the $-NH-$ hydrogen on the first residue to the β -carbon of the same

Figure 4.10 Scheme of the formation of $[x_2-3H+X]^-$ from the precursor ion of *N*-acetylated $[M-2H+X]^-$.

Figure 4.11 Scheme of the formation of $[x_2\text{-HCOOH}]^-$ from the precursor ion of N -acetylated $[M-2H+X]^-$.

Figure 4.12 Scheme of the formation of $[c_1-H+X]^+$ from the precursor ion of N -acetylated $[M-2H+X]^-$.

residue and loss of $R_1CH=NCOCH_3$ leads to the $[x_2-H+X]^-$ intermediate. Elimination of $HCOOX$ from this intermediate yields $[x_2-HCOOH]^-$. This mechanism is supported by the presence of $d_1-[x_2-HCOOH]^-$ when $d_2-[AcM-2H+X]^-$ was fragmented, and the absence of $[x_2-HCOOH]^-$ when $[AcM-2H+X]^-$ from GPA and APG was analyzed. For PGG, the $[x_2-H+X]^-$ intermediate is formed by transferring a $-CH-$ hydrogen on the proline ring to the β -carbon of the first residue and eliminating the proline ring. This mechanism leads to $[x_2-HCOOH]^-$ that is devoid of any original exchangeable hydrogen; an hypothesis supported by the observation of $d_0-[x_2-HCOOH]^-$ from $d_1-[AcM-2H+X]^-$.

Figure 4.12 shows the formation of the $[c_1-H+X]^-$ product ion from the *N*-acetylated derivative of **3**. Formation of this product ion requires cleavage of the *N*-C bond on the second residue plus separation of the ionic from the neutral fragment; this is impossible for the tripeptides APG and GPA, and the $[c_1-H+X]^-$ ion was not observed. Collision induced dissociation of $d_2-[AcM-2H+X]^-$ of non-proline containing tripeptides yielded $d_1-[c_1-H+X]^-$ as expected.

4.4.4 Fragmentation of the $[M-3H+2X]^-$ Ions

Collision induced dissociation of the $[M-3H+2Na]^-$ ions to the $[y_2-2H+2Na]^-$ ions is depicted in Figure 4.13. Two precursor ion structures, **2** and **5**, can give rise to the same product ion; from precursor ion **2**, a hydrogen on the *N*-terminus is transferred to the ionic fragment while from **5**, the centrally coordinated Na is transferred. The mechanisms are in

Figure 4.13 Scheme of the formation of $[y_2-2H+2Na]^-$ from the precursor ion $[M-3H+2Na]^-$.

accord with *d*-labelling experiments, in which fragmentation of $d_2\text{-[M-3H+2Na]}^-$ precursor ions from non-proline containing tripeptides yielded $d_1\text{-[y}_2\text{-2H+2Na]}^-$.

4.4.5 Comparison with FAB results

Based on the results of the previous [118] and this study, it is apparent that the alkali metal ion-peptide adducts produced by means of electrospray have structures that are very similar to those produced by means of FAB. We saw an abundance of adducts containing two and even three and four metal ions [118] using electrospray while studies involving FAB [102-110] typically reported adducts containing a single metal ion. Our assumption is that the relative intensities of multiple metal ion-peptide adducts in FAB are likely low.

4.5 Conclusion

The product ions that have been observed from the collision induced dissociation of alkali metal-containing anionic tripeptides are consistent with a precursor ion structure in which an alkali metal ion is centrally located for bonding with the amino terminus, the amide nitrogen atoms, and an oxygen atom of the carboxylate terminus; the second alkali metal ion in [M-3H+2X]^- is likely attached to the other oxygen atom of the carboxylate terminus.

Chapter 5

Collision Induced Dissociation of Electrospray-generated Background Ions Sampled Under Mild Lens Conditions

5.1 Introduction

Electrospray is arguably one of the most versatile sample introduction methods in mass spectrometry, being amenable to a variety of analyte ions ranging from simple inorganic ions [124] to complex biological macro ions [125]. For most analytes, pre-formed ions in solution are electrosprayed (sometimes with the aid of a nebulizer gas [126]) into air at 1 atm pressure. The mechanism of ion production in electrospray is a subject of much interest and discussion [50, 127]. However, there is a little doubt that a variety of charged species, including ions, clusters and droplets, are produced and can potentially be sampled for analysis through an orifice. In order to minimize sampling of solvent and other polyatomic molecules, it is common practice to employ a ‘curtain gas’ or ‘counter-current bath gas’, which sweeps the region immediately upstream from the orifice and presumably keeps this region relatively free of these molecules. On some instruments, e.g. the SCIEX TAGA 6000E, turning off the curtain gas results in a significant reduction of the electrospray signal, presumably due to a significant increase in clustering in the lens region of the mass spectrometer. Furthermore, it is also common practice to transmit the sampled ions under an appropriate potential gradient in that region to ensure efficient transmission as well as fragmentation of any clusters that may be present.

The importance of this ‘front-end’ collision induced dissociation has been highlighted in studies carried out by Smith and co-workers [66, 125] and Thomson [128]. In studies of fragmentation of multiply charged protein ions, Smith *et al.* [125] observed that the product

ion spectra of $[M+nH]^{n+}$ ions of cytochrome *c* ($n=9\sim 12$) contain low abundances of other multiply charged cytochrome *c* ions, e.g. for $n=10$, low abundances of $[M+9H]^{9+}$, $[M+10H]^{10+}$ and $[M+11H]^{11+}$ were also present. They attributed these low-abundance ions to the presence of a small percentage of dimer ions that were sampled; these dimers fragmented to monomer units of different charges during CID. However, in a subsequent study where mild interface conditions were emphasized and where highly disparate monomer abundances were observed, Smith *et al.* [66], reassigned those monomer ions with m/z values lower than that of the precursor ion to products of CID of the solvated clusters that they mass selected. They proved, by means of a precursor ion scan, that some of the $[M+16H]^{16+}$ product ion of cytochrome *c* could be formed from declustering of adducts of the $[M+16H]^{16+}$ ion with solvent molecules. In Thomson's work [128], model protein ions, e.g. myoglobin, were also electrosprayed and sampled into the mass spectrometer under mild lens conditions. In some experiments, the first quadrupole (Q1) of a triple-quadrupole mass spectrometer was operated as a high-pass filter to transmit multiply charged protein ions larger than a given m/z value; these transmitted ions were mass resolved by means of the third quadrupole (Q3). In the absence of collision gas in the second quadrupole (Q2), only the 'expected' multiply charged protein ions were observed; that is, if Q1 was set to transmit ions beyond an m/z value of 700, only ions with $m/z > 700$ were observed in mass scanning Q3. In the presence of collision gas, however, 'unexpected' ions were encountered; in the same example, more highly charged protein ions having m/z values smaller than 700 would be recorded. In other experiments, Q1 was set to pass a multiply charged protein precursor ion under unusually low resolution, and Q3 was scanned under unit mass resolution to resolve the product ions

after CID in Q2. What was observed was that, aside from the unreacted precursor ion, other multiply charged protein ions having charges both larger and smaller than that of the precursor ion were also present. Agreeing with the earlier interpretation of Smith *et al.* [125], Thomson [128] attributed his observations to the presence of a small fraction of protein dimers that were mass selected along with the monomers.

Stimulated by these reports, we decided to investigate these phenomena, and this chapter describes results of some of these investigations.

5.2 Experimental

Experiments were carried out on API 300. Samples were typically 10 μM in concentration dissolved in either water or mixtures of water and methanol; acetic acid, ammonia and ammonium acetate were used for pH adjustment. Measurements were carried out using the ionspray probe, and air was used as the nebulizer gas.

Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a typical scan speed of 10 ms per 0.3 m/z unit. Ten scans were summed to produce a mass spectrum. Most experiments were carried out with Q3 being the resolving quadrupole, and with the orifice (OR) and the quadrupole lens (Q0) biased to ground potential; that is, ions were sampled under a nominally zero potential gradient from OR to Q0 (the skimmer, immediately downstream from the orifice, is permanently grounded). As

a result, a Q3 scan would produce a mass spectrum collected under nominally zero collision energy conditions in the lens region. To generate a product ion spectrum, Q1 was set to pass a precursor ion window of typically ~ 30 m/z units at 10% peak maximum; nitrogen was used as the collision gas in Q2 at a pressure of 2-4 mTorr (1 Torr=133.3 Pa). E_{lab} was typically 10-50 eV for singly charged ions.

5.3 Results and Discussion

Our first attempts were to reproduce the findings of Smith and co-workers [66, 125] and Thomson [128]. Figure 5.1 shows product ion spectra of an ~ 30 m/z unit wide window centred on the $[M+11H]^{11+}$ ion of equine myoglobin: (a) in the absence of collision gas in Q2 and (b) and (c) with 4 mTorr of nitrogen. It is evident in Fig. 5.1(c) that multiply charged equine myoglobin ions from +8H to +17H were present. In fact, it was not necessary to select a precursor ion window that included any of the $[M+nH]^{n+}$ ions to observe this effect. Figure 5.2 shows product ion spectra of a 30 m/z unit wide precursor window centred on m/z 1842, which is sandwiched between the $[M+9H]^{9+}$ and the $[M+10H]^{10+}$ ion at m/z 1884 and 1696, respectively. Again, it is evident that the product ions include multiply charged myoglobin ions from +8H to +18H. Ions such as these have been ascribed to the presence of a small percentage of protein dimers along with the monomer that was selected [125, 128]. The abundance of the multiply charged product ions seen in fig. 5.2 (b), their charges and the precursor ion window chosen appear to cast doubt on this interpretation. First, the m/z values of the relevant equine myoglobin dimer ions are: 1885 ($[2M+18H]^{18+}$), 1785

Figure 5.1 Product ion spectra of 10 μM equine myoglobin in water-methanol (1:1) containing 0.2% acetic acid; ~ 30 m/z unit precursor ion window centred on m/z 1542; axial acceleration voltage (Q0-Q2), 52 V, i.e. for the $[M+9H]^{9+}$ ion, $E_{lab} \approx 468$ eV: (a) no collision gas in Q2; (b) 4 mTorr of nitrogen; (c) results from (b) shown with an expanded abundance scale.

Figure 5.2 Product ion spectra of 10 μ M equine myoglobin in water-methanol (1:1) containing 0.2% acetic acid; ~ 30 m/z unit precursor ion window centred on m/z 1842; axial acceleration voltage (Q0-Q2), 52 V: (a) no collision gas in Q2; (b) 4 mTorr of nitrogen.

($[2M+19H]^{19+}$) and 1696 ($[2M+20H]^{20+}$). The precursor window centred on m/z 1842 would have contained very few if any dimer ions [see Fig. 5.2(a)] — too few to yield a product ion spectrum [Fig. 5.2 (b)] whose intensity is comparable to that of the precursor ion. Second, product ions as highly charged as +18H are apparent in Fig. 5.2(b). Although some charge disproportion when the dimer fragments is evident in some of the spectra shown in Ref. 6, it is unlikely that one monomer would carry 80-90% of the charges and the other the remaining 20-10% [To counter the argument that to minimize Coulombic repulsion, a highly protonated monomer may preferentially interact with a less highly protonated monomer: In general, Coulombic repulsion would make any cation-cation reaction in the gas phase unfavourable. For any dimerization to occur in the gas phase, the monomers must, therefore, be very heavily solvated (or self-solvating) to reduce the effective charge. If that is the case, this preference will be diminished. Furthermore, there is evidence that protons attached to proteins and peptides in the gas phase are relatively mobile [129, 130]. To reduce Coulombic repulsion, mobile protons would tend to redistribute themselves somewhat evenly between the two monomer units, thus making highly uneven distributions unlikely]. However, if fragmentation of multimers had not occurred, it would have been difficult to reconcile the presence of abundant $[M+8H]^{8+}$ and $[M+9H]^{9+}$ product ions.

To confirm these results, we performed similar experiments on bovine insulin, which yields fewer and thus more widely spaced multiply charged peaks. Figure 5.3 shows the results of these experiments: (a) Q3 scan where $OR=Q0=0$ V; and product ion spectra of an approximately 30 m/z unit wide precursor ion window centred on (b) m/z 1800, (c) m/z 1500,

Figure 5.3 10 μM bovine insulin in water-methanol (1:1) containing 0.2% acetic acid: (a) Q3 scan where $\text{OR}=\text{Q0}=0$ V; and product ion spectra of 30 m/z unit wide precursor ion window centred on (b) m/z 1800, (c) m/z 1500, and (d) m/z 1300.

Figure 5.4 Abundances of the $[M+3H]^{3+}$, $[M+4H]^{4+}$ and $[M+5H]^{5+}$ product ions of insulin versus the m/z values of the precursor ion window: data from Fig. 5.3; $\blacktriangle$, $[M+3H]^{3+}$; $\bullet$, $[M+4H]^{4+}$; and $\blacksquare$, $[M+5H]^{5+}$.

and (d) m/z 1300. Typical of full-scan mass spectra collected in this study, the multiply protonated protein peaks, such as $[M+5H]^{5+}$ and $[M+6H]^{6+}$, were often trailed by wide 'humps' (for lack of a better description), which were indicative of extensive and complex clustering. In the CID experiments, both precursor windows centred on m/z 1800 and 1500 would not be expected to contain significant numbers of insulin dimer ions (expected at m/z 1912, 1639 and 1434). However, their product ion spectra contained abundant multiply charged insulin ions with m/z values both lower and higher than those of the precursor ions. A comparison of the product ion spectra in Fig. 5.3 suggests that the abundances of the multiply charged insulin ions were related to the m/z values of the precursor window. This relationship is more convincingly displayed in Fig. 5.4, which shows the abundances of the $[M+3H]^{3+}$, $[M+4H]^{4+}$ and $[M+5H]^{5+}$ ions (m/z 1912, 1434 and 1149, respectively) with different precursor ion windows. The abundances of the $[M+4H]^{4+}$ and $[M+5H]^{5+}$ ions maximized at precursor ion windows centred on m/z 1500 and 1200, respectively, values just exceeding the m/z values of the ions and where clustering was apparently extensive [see Fig. 5.3(a), m/z 1200, at which a hump was evident]; the abundance of the $[M+3H]^{3+}$ ion was relatively low.

Similar results were obtained in a study of leucine enkephalin, YGGFL. The electrospray mass spectrum of this pentapeptide is simple, showing the $[M+H]^+$ ion (m/z 556) and the $[2M+H]^+$ ion (m/z 1111) at an abundances of a few percent of that of the former. Figure 5.5 shows the abundances of the two leucine enkephalin ions with different precursor ion windows selected. As in Fig. 5.4, the abundances of both ions in the product ion

Figure 5.5 10 μ M leucine enkephalin in water-methanol (1:1) containing 0.2% acetic acid; (a) Q3 scan; (b) abundances of $[M+H]^+$ (●) and $[2M+H]^+$ (■) versus m/z of precursor ion window; (c) expansion of (b); axial acceleration voltage (Q0 – Q2), 20 V, i.e. for singly charged ion, $E_{lab} = 20$ eV.

spectrum maximized at precursor ion windows just exceeding the m/z values of the peptide ions. It is, perhaps, noteworthy that the leucine enkephalin dimer behaved similarly to its monomer ion.

Figure 5.6 shows the Q3 scan of a 1mM polypropylene glycol 1000 solution in methanol/water (1:1) containing ammonium acetate. As expected, we observe a distribution of primarily single ammonium adducts of PPG chains varying in the number of propylene units and maximizing at about 1000 Da; at about m/z 1600, the abundance of the PPG chains drops to a few percent of the maximum although there exist PPG chains whose molecular masses exceed 2000 Da (see Fig. 5.7). Product ion spectra of precursor ion windows of ~ 150 m/z units wide are displayed in Fig. 5.7. The extra wide windows were found necessary to yield product ions that were sufficiently abundant; the presence of NH_4^+ -containing PPG product ions in the product ion spectra is evident. It is, perhaps, noteworthy that the difference between the centre of the precursor ion window and the most abundant product ion of each scan appears to increase with increasing m/z values of the precursor window. It should also be pointed out that, when the precursor ion window was reduced to 1 m/z unit wide and included only a higher m/z PPG ion, no PPG product ions were produced, thus proving that these ions were not fragmentation products of longer PPG chains.

The results that we have presented are consistent with the hypothesis that a significant fraction of the ions exiting Q0 and entering Q1 were very heavily clustered and complex. We speculate that these ions included not only solvated monomers but also large

Figure 5.6 Q3 scan mass spectrum of a 1mM polypropylene glycol 1000 in methanol-water (1:1) containing 1 mM ammonium acetate.

Figure 5.7 Product ion spectra of 150 m/z unit wide windows of polypropylene glycol 1000 centring on (a) m/z 2009, (b) m/z 2241, (c) m/z 2416, (d) m/z 2642, and (e) m/z 2819; axial acceleration voltage (Q0-Q2), 50V.

multimeric species of the analyte ions (e.g. multimeric species of myoglobin, leucine enkephalin and PPG). These multimeric species could be dry particles and/or solvated clusters of the analyte. A reoccurring trend in our experiments was that the m/z values of the precursor ion window selected were non-critical, and there were always some characteristic product ions observed, as long as the precursor window was a few tens of m/z units wide and that it resided within the multiply charged envelope of the protein ions. To produce these results, the clusters themselves must, therefore, have been highly variable; that is, they had to comprise a highly variable number (but not the nature) of monomer units and solvent units as well. The humps displayed in Fig. 5.3(a) and 5.8 (Q3 scan of myoglobin) contained ions that could be deconvoluted as adducts between the protein monomer and a small molecule (or a number of small molecules). For example, the series of peaks marked with asterisks in Fig. 5.8(b) were calculated to have a molecular mass of 17114 ± 3 Da, 162 Da above the molecular mass of myoglobin (16952 ± 2 Da). Obviously, the humps contained many species other than the +162 Da adduct; however, most of them were buried in the 'chemical noise'. It is our interpretation that many multimeric species of varying degree of solvation, thus populating virtually all m/z values within a given range, constituted the ions carried by the hump. The existence of an elevated background between analyte peaks was most evidently displayed in Figs 5.3 and 5.8; it should be noted that such a background also existed in the other Q3 scans although they were less prominent.

Desolvation and fragmentation of mass-selected multimeric species in Q2 accounted for the observation of monomer units of varying degrees of protonation in the product ion

Figure 5.8 Q3 scan of 10 μ M equine myoglobin in water-methanol (1:1) containing 0.2% acetic acid; OR=Q0=0 V: (a) full spectrum; (b) expansion of (a); asterisks indicate a +162 Da adduct of myoglobin.

spectra. The relatively high pressure and, consequently the large number of collision events attributable in Q2 of the API 300 mass spectrometer probably resulted in a relatively low threshold for this process. The fact that few dimeric ions (the only exception being those of leucine enkephalin, Fig. 5.5) were observed in the product ion spectra means that the multimeric species were held by weak forces and few naked oligomers could be produced from or survived the CID process. The monomers within the multimeric protein clusters could bear different numbers of attached protons. For this reason, product ions of proteins having m/z values both higher and lower than those of the precursor ions were possible [Figs 5.1(b) and (c), 5.2(b) and 5.3(b)–(d)]. (Product ions with higher m/z values could not have been formed from cleavage of proton adducts from more highly charged, and hence lower m/z values, ions; no significant small ions were observed in the low- m/z segments of any of the above product ion spectra.)

It should, perhaps, be emphasized that the diverse multimeric clusters referred to in this paper are likely to contain more than a few monomer units. For bovine insulin, the multiply charged ions are separated by a few hundred m/z units (Fig. 5.3). Even with heavy solvation, it is difficult to envisage how relatively intense product ion spectra, such as those shown in Fig. 5.3(b)–(d), could be formed from dimers of insulin. Furthermore, it should also be repeated that these large multimeric clusters were observed to exit from Q0; put differently, their existence could only be traced back to that point. At present, it is unknown whether these species exist in the electrospray plume at 1 atm.

5.4 Conclusion

A significant quantity of electrospray-generated ions sampled under very mild lens conditions appeared to exit Q0 as large multimeric species. In fact, the number of monomer units and exact composition of these species varied to such a large extent that individual ions were typically not resolved and they constituted the chemical noise in the electrospray mass spectrum; their existence, however, was inferred from the CID of a window of background ions, which yielded relatively high abundances of monomeric units. It is not known if these large multimeric species exist in the electrospray plume prior to sampling.

Chapter 6

Electrospray, Microelectrospray and Nanoelectrospray Mass Spectrometry of Peptides, Proteins and Oligonucleotides

6.1 Introduction

Electrospray is one of the most versatile ionization techniques which makes mass spectrometry amenable to the analysis of biological molecules, especially peptides and proteins. Pneumatically assisted electrospray (ionspray) allows the use of 20-100 $\mu\text{L}/\text{min}$ continuous sample introduction, thus bringing to reality the coupling of separation techniques, such as high performance liquid chromatography, to mass spectrometry. However, some of the most challenging applications of mass spectrometric analysis in biological research are the analysis of biomacro ions that are at low concentrations (nanomolar) and in small sample volume (microliters). Furthermore, some low-flow separation techniques, such as capillary electrophoresis, require an ion source that could work under low flow rates. The application of ES-MS to small-volume and low-concentration biological samples necessitate an electrospray ion source that utilizes samples more efficiently, provides higher sensitivity, and generates a more stable electrospray.

Early work on small volume and low flow rate electrospray MS was reported by Smith and co-workers [131]. They employed an etched-tip capillary as the electrospray probe and demonstrated the achievement of high sensitivity and stability. Two other groups, Caprioli *et al.* [40] and Wilm and Mann [42], also devoted considerable efforts to the investigation of low flow rate electrospray MS. Based on the flow rate range and the generated droplet size, so called "microelectrospray" (microES) and "nanoelectrospray" (nanoES) were developed by these two groups, respectively.

As described in Section 2.1, the inside diameter of a conventional ES probe is typically *ca.* 100 μm , and the sample solution flow rate is in the range 1-20 $\mu\text{L min}^{-1}$. This flow rate is suitable for most samples; but for some precious and volume-limited biological compounds, this rate of sample consumption is much too high. The flow rates of sample solutions in nanoES and microES are typically in the range 10-500 nL min^{-1} . Consequently, the sample consumption in microES and nanoES are much lower than that in conventional ES. Because much smaller diameter capillaries (1 to 20 $\mu\text{m ID}$) are used in microES and nanoES, the volume of the droplets produced in microES and nanoES is about 100-1000 smaller than those generated by conventional electrospray [42, 43]. This means that microES and nanoES generate droplets that have comparatively much larger surface-to-volume ratio, which results in a much higher percentage of analyte ions desorbing from the droplets. In other words, higher ionization efficiency can be obtained using microES and nanoES. As a result, sample sensitivity can be greatly increased. In addition, because of their lower flow rates and smaller droplets, microES and nanoES probes can be moved closer to the orifice, resulting in a much higher percentage of desorbed ions being sampled into the mass analyzer.

In this study, we applied microES and nanoES MS to the analysis of peptides, proteins and oligonucleotides in both positive and negative ion detection modes.

6.2 Experimental

Mass spectra were recorded on TAGA 6000E in both positive and negative ion

detection modes with a measurement time of 40 ms per m/z unit; ten scans were summed to generate a mass spectrum. Laboratory-built stainless steel probes as described in Section 2.1 were used in conventional electrospray mass spectrometric analysis. The fabrication of microES and nanoES probes is described as follows:

MicroES probes. A microES probe was made from a fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an inside diameter of 4–40 μm and a length of *ca.* 30 cm [see Fig. 6.1(a)]. The tip of the capillary was sharpened to produce a stable electrospray. Two methods, etching in hydrofluoric acid [131] and mechanical grinding [132], were used. We ground the capillary by means of fine sandpaper; the tip was checked under a microscope until its shape was like that illustrated in Fig. 6.1(a). After ultrasonic cleaning in acetone and flushing with methanol, the rounded end of the capillary (*ca.* 3 cm long) was immersed in a mixture of 30% H_2O_2 and concentrated sulfuric acid at 70 °C for 20 min to clean and to remove the polyimide coating on the tip. Then the capillary was rinsed with deionized distilled water (DDW), blown dry with argon, and dried in an oven for 10 min at 105 °C. Finally the cleaned tapered end of the capillary was gold-coated for 3×3 min by means of a vacuum sputter coater (SC502, Fisons Instruments). The gold layer acted as the electrical contact for electrospray.

NanoES Probes Both fused silica capillaries and glass capillaries can be used to make nanoES probes. A 5–20 μm ID fused silica or glass capillary can be pulled with a microcapillary puller to produce a nanoES tip with an inside diameter of 1–5 μm [133, 43].

Figure 6.1 Schematic diagrams of (a) a microES probe; (b) a nanoES probe.

Glass micropipettes with inside diameters of 1-10 μm and 5 cm long are also available commercially from World Precision Instruments. In this study, glass micropipettes were used to fabricate nanoES probes [see Fig. 6.1(b)] using a procedure that was similar to that for microES. Because these tips had much smaller inner diameters, they were more prone to clogging during cleaning and gold-coating. We used nitrogen (10 psi, i.e. ~ 70 kPa) to declog during cleaning, and optimized the gold-coating time, typically 3×3 min. Typical nanoES probes could not be reused; and a fresh probe had to be used for a new sample.

Comparison of MicroES and NanoES probes. There are some distinct differences between operating microES and nanoES probes (see Fig. 6.1). Because nanoES probes have smaller diameters, the electrospray potentials and sample consumption rates are relatively lower. The potential for a stable electrospray in nanoES is typically 1200-1800 V whereas that for microES is 2200-2800 V. In microES, a syringe pump is needed to infuse a sample solution into the probe while in nanoES it is not needed. Instead the 1-5 μL sample deposited into the tip of the probe is pulled out by electrocapillary action during spraying.

In microES, a 26-gauge stainless steel tube encased the microES probe whose tip protruded 1-2 mm beyond the outer tube. Air was used as the nebulizer gas. The optimum position of the microES probe was *ca.* 5 mm from the interface plate with the spray off-axis from the orifice. In nanoES, the probe was held by a stainless steel union. The nanoES probe could be moved even closer to the interface plate, typically 1-5 mm, and on-line with the orifice. No nebulizer gas was needed in nanoES.

6.3 Results and Discussion

6.3.1 Probe lifetime

The two major factors that determined the lifetime of microES and nanoES probes were: clogging by particles (contamination) or precipitated protein or buffer salts, and the loss of the gold coating at the tip due to electrospraying. Because of the relatively large inside diameter of the microES probe, clogging is less of a problem in microES. Kriger *et al.* [132] reported that silylation of the glass with sulfryl-containing silanes improves the adhesion of the gold coating to the capillary. In an effort to improve the lifetime of our probes, we adopted the following modified procedure: After removing the polyimide, the tip of a microES probe (*ca.* 3 cm long) was immersed in a boiling mixture of 5 g of (3-mercaptopropyl)trimethoxysilane, 200 g of 2-propanol and 5 mL DDW for 10 min; the probe was then rinsed, blown, dried and finally gold-coated as described above. This modification extended the lifetime of the probe from a typically value of 40 hours (without silylation) to more than 100 hours with silylation.

Clogging of the probes is a more serious problem for nanoES probes because of their smaller inner diameter. To minimize clogging, all samples were filtered prior to loading. Erosion of the gold coating appeared to be more problematic, most likely due to the higher electric field present at the probe tip. Silylation of these tips to improve adhesion turned out to be less successful as many of them became plugged during silylation. The lifetime of our

nanoES probes was in the range 10-90 min. This time was usually sufficiently long for us to perform our analysis. As previously stated, nanoES probes are typically used only once to avoid cross-sample contamination.

6.3.2 *Advantages of MicroES and NanoES Analysis for Biomolecules*

The sample consumption rate and signal intensity were used to evaluate the relative performance of microES and nanoES. The sample consumption rate was calculated from the concentration, the flow rate of the sample solution, and the time to complete one mass spectrometric scan. Because no external pumping was employed in nanoES, the sample flow rate was estimated from the sample volume injected into the probe and the time that it took to completely exhaust the sample.

Figure 6.2 shows mass spectra of 10 μM cytochrome c in 50/50 water/methanol with 0.2% acetic acid using (a) conventional ES, (b) microES, and (c) nanoES. The sample flow rate in conventional ES was 5 $\mu\text{L min}^{-1}$, corresponding to a sample consumption rate of *ca.* 35 picomole per scan. In microES, a 10 μm ID probe was used, and the flow rate was 100 nL min^{-1} ; the sample consumption rate was 700 femtomole per scan, which was 50 times lower than that in ES. The flow rate in nanoES using a 2 μm ID probe was even lower than that in microES, approximately 20 nL min^{-1} ; the sample consumption rate was about 140 femtomole per scan. Despite their lower sample consumption rates, the microES and nanoES produced spectra that were more intense than a conventional ES spectrum. Similar results

Figure 6.2 Mass spectra of 10 μM cytochrome c 50/50 water/methanol with 0.2% acetic acid using (a) conventional ES (100 μm ID), (b) microES (10 μm ID), and (c) nanoES (2 μm ID); flow rates: 5 $\mu\text{L min}^{-1}$, 100 nL min^{-1} and 20 nL min^{-1} , respectively.

were obtained in a study of gramicidin s, a decapeptide. Five μM gramicidin s in 50/50 water/methanol with 0.2% acetic acid was analyzed by means of conventional ES, microES and nanoES (see Fig. 6.3). The flow rates in conventional ES, microES and nanoES were $5 \mu\text{L min}^{-1}$, 50 nL min^{-1} and 20 nL min^{-1} , respectively. The sample consumption rate in conventional ES was 15 picomole per scan, which was 100 times higher than that in microES and 250 times higher than that in nanoES. In this case, the intensities of microES and nanoES spectra of gramicidin s were still higher than that of the conventional ES spectrum.

The flow rate in microES could be changed within a certain range. Figure 6.4 shows mass spectra of $10 \mu\text{M}$ cytochrome c in 50/50 water/methanol containing 0.2% acetic acid by using a $10 \mu\text{m}$ ID microES probe at different flow rates. The flow rates were changed from 200 to 20 nL min^{-1} thus reducing the sample consumption rate from 1400 to 140 femtomole per scan. In this case, the spray remained stable within the range, and intensities of the spectra were basically identical.

Compared with that in positive ion detection mode of peptides and proteins in ES, the abundance of oligonucleotides in the negative ion detection mode is typically much lower due to the adduction of cations to the multiply charged ions [134]. This limits the usefulness of ES-MS oligonucleotides. Furthermore, oligonucleotides are almost always more costly to produce and are, therefore, typically in smaller amounts than proteins. We applied microES and nanoES to analyze oligonucleotides in the negative ion detection mode. Figure 6.5 shows microES and nanoES mass spectra of $40 \mu\text{M}$ d(pT)₁₈ in 50/50 water/methanol with

Figure 6.3 Mass spectra of 5 μM gramicidin S in 50/50 water/methanol with 0.2% acetic acid by (a) conventional ES (100 μm ID), (b) microES (10 μm ID), and (c) nanoES (2 μm ID); flow rates: 5 $\mu\text{L min}^{-1}$, 100 nL min^{-1} and 20 nL min^{-1} , respectively.

Figure 6.4 Mass spectra of 10 μM cytochrome c in 50/50 water/methanol containing 0.2% acetic acid using a 10 μm ID microES probe at flow rates: (a) 200 nL min^{-1} ; (b) 100 nL min^{-1} ; (c) 50 nL min^{-1} ; and (d) 20 nL min^{-1} .

Figure 6.5 Negative ion mass spectra of 40 μM d(pT)₁₈ in 50/50 water/methanol with 10 mM ammonium acetate using (a) microES (4 μm ID), flow rate, 100 nL min^{-1} ; and (b) nanoES (1 μm ID), flow rate, 10 nL min^{-1} .

10 mM ammonium acetate. The inside diameters of the microES and nanoES probes were $4\mu\text{m}$ and $1\mu\text{m}$, respectively. The flow rates were 100 nL min^{-1} in microES and 10 nL min^{-1} in nanoES corresponding to a sample consumption rate in microES of 2 picomole per scan, and in nanoES of 200 femtomole per scan. With 10 times lower in sample consumption rate, the intensity of the nanoES mass spectrum of d(pT)_{18} was still higher than that of the microES mass spectrum. The molecular weight of d(pT)_{18} calculated from the spectra of Fig. 6.5 is 5493.9; compared to the theoretical value of 5493.5, the mass accuracy was about 0.007%.

In ES-MS, methanol is typically used to facilitate spraying. The solvent used in all of the above experiments was 50/50 water/methanol. Figure 6.6 shows conventional ES and microES ($10\mu\text{m ID}$) mass spectra of $10\mu\text{M}$ myoglobin dissolving in only water containing 0.2% acetic acid. The spectra are of similar quality as those obtained with 50/50 water/methanol. With a sample consumption rate of 700 picomole per scan, 50 times lower than that in conventional ES, the intensity of the microES spectrum of myoglobin was larger than that of the conventional ES spectrum. The flow rate in microES for the same sample solution could even be reduced from 500 to 10 nL min^{-1} [see Fig. 6.7]; at the low flow rate the spray was still stable, and the spectrum was essentially unchanged.

When water (instead of 50/50 water/methanol) was used as the solvent, the nanoES probe could not produce a stable spray; this was likely due to the higher surface tension of water. Figure 6.8 shows the mass spectra of $40\mu\text{M}$ d(GGAATTCC) , an oligonucleotide, with

Figure 6.6 Mass spectra of $10 \mu\text{M}$ myoglobin in water containing 0.2% acetic acid using (a) conventional ES, flow rate, $5 \mu\text{L min}^{-1}$; and (b) microES ($10 \mu\text{m ID}$), flow rate, 100 nL min^{-1} .

Figure 6.7 Mass spectra of 10 μM myoglobin in water containing 0.2% acetic acid using a 10 μm ID microES probe at flow rates: (a) 500 nL min^{-1} ; (b) 200 nL min^{-1} ; (c) 100 nL min^{-1} ; (d) 50 nL min^{-1} ; (e) 20 nL min^{-1} ; and (f) 10 nL min^{-1} .

Figure 6.8 Negative ion mass spectra of 40 μM d(GGAATTCC) containing 10 mM ammonium acetate using a 1 μm ID nanoES probe in different percentage of water and methanol: (a) 90/10; (b) 75/25; (c) 50/50; and (d) 25/75.

10 mM ammonium acetate in different percentage of water and methanol, using a 1 μ m ID nanoES probe in the negative ion detection mode. When the solution contained 10% methanol, a nanoES mass spectrum could be obtained, but it had high background and poor intensity. As the percentage of methanol was increased to more than 25%, very good spectra were obtained with high signal intensities. In our hands, some methanol addition was necessary for nanoES.

6.4 Conclusion

MicroES and nanoES MS have been shown to offer particular advantages for the analysis of biomacro ions, such as peptides, proteins and oligonucleotides. Compared with conventional ES, microES and nanoES exhibit lower sample consumption, and higher sensitivity. Because a very small volume of sample solution (1-5 μ L) is required to produce a mass spectrum, nanoES is particularly useful for mass spectrometric analysis of precious and volume-limited biological samples.

Chapter 7

Conclusion

Although the precise mechanism of how gas-phase ions are formed during electrospray is still not known, two mechanisms, Charge Residue Model and Ion Evaporation Model, have been proposed for electrospray mass spectrometry. A key component of both models is solvent evaporation from electrospray-generated droplets. The presence of a low percentage of a long-chained *n*-alkanol in a micrometer-size water droplets is known to retard significantly the droplet's evaporation rate due to the formation of a layer of the alcohol on its surface. Therefore, the presence of a *n*-alkanol would interfere with the gas-phase ion formation and reduce drastically the analyte response if solvent evaporation were a key step leading to ion formation. However, in our experiments, the electrospray responses of proteins were not affected by the presence of *n*-alkanols, which were added in two different ways. This led to the conclusion that solvent evaporation is not a necessary prerequisite to the formation of gas-phase ions in electrospray.

Tandem mass spectrometry of multiply charged ions produced by electrospray is a powerful technique for structural determination of proteins and peptides. The collision induced dissociation of alkali-metal-ion-containing peptides was investigated in the negative ion detection mode. Both *N*-terminal and *C*-terminal product ions were observed from the precursor ions $[M-2H+X]^-$ and $[M-3H+2X]^-$ where X stands for an alkali metal. The results are consistent with precursor ion structures in which an alkali metal ion is centrally located for bonding with the amino terminus, the amide nitrogen atoms, and an oxygen atom of the carboxylate terminus; the second alkali metal ion in $[M-3H+2X]^-$ is likely attached to the other oxygen atom of the carboxylate terminus.

Electrospray can produce a variety of charged species, including ions, clusters and droplets. Thirty- m/z -unit wide windows of electrospray-generated ions sampled under very mild lens conditions were studied by means of collision-induced dissociation to gain an insight on the various species produced. The precursor ion windows selected needed not contain desolvated analyte ions to generate intense product ion spectra; more often they contained only background ions. For proteins, monomeric analyte ions with m/z values both lower and higher than those of the precursor ions were present even when the precursor ion window could not have contained protein dimer ions. This led to an eventual conclusion that a significant fraction of electrospray-generated ions are heavily solvated multimeric species of the analyte, and this fraction constitutes the chemical noise in the electrospray mass spectrum.

Microelectrospray and nanoelectrospray probes were fabricated and tested. Relative to conventional electrospray probes, they consume much less sample and produce more intense spectra. In our hands, the typical lifetimes for micro- and nanoelectrospray probes are approximately 100 and 1 hour, respectively.

Appendixes

Appendix 1 Calculation for The Flight Time of Charged Droplets

The flight time of a non-evaporating electrospray-generated droplet may be estimated as follows. From Kebarle and Tang [49] a typical parent droplet has a radius, r , of $1.5 \mu\text{m}$; contains 5×10^4 excess ions; and, therefore, has a charge, q , of $(1.6 \times 10^{-19}) \times (5 \times 10^4) = 8.0 \times 10^{-15} \text{ C}$. Applying Milliken's semi-empirical mobility expression for ions and droplets [101], where the mobility,

$$K = \frac{q}{6\pi\eta r} \left(1 + \frac{\lambda[A + B\exp(-Cr/\lambda)]}{r} \right)$$

η = gas viscosity = $1.76 \times 10^{-5} \text{ N s m}^{-2}$ for air at 298 K and 1 atmosphere; λ = mean free path = $5.3 \times 10^{-8} \text{ m}$ for nitrogen at 298K and 1 atmosphere; A, B and C are constants, and equal 1.234, 0.414 and 0.876, respectively. Substituting the numerical values,

$$K = 1.68 \times 10^{-5} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} = 0.168 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$$

The potential difference between the electrospray probe and the interface plate in our mass spectrometer is typically 2,600 V; the separation between the two being 1 cm. Calculation of the electric field gradient on SIMION showed that the potential drop from the probe occurs very rapidly over the first millimetre and becomes essentially linear over the last 8 millimetres which sees a drop of some 700 V. Since the drift velocity,

$$V = KE$$

where E = electric field = $700/0.8 = 875 \text{ V cm}^{-1}$ for the last 8 millimetres. Therefore,

$$V = 0.168 \times 875 = 147 \text{ cm s}^{-1}$$

or, it takes $0.8/147 = 5.4 \text{ msec}$ to traverse the last 0.8 cm of the gap.

Appendix 2 Publications Resulting from This Thesis Work

1. Fu Ke, R. Guevremont and K.W.M. Siu, Collision-Induced Dissociation of 30-m/z-unit wide Windows of Electrospray-Generated Ions Sampled under Lens Conditions of Nominally Zero-Potential Gradient, *J. Mass Spectrom.* Vol. 32, 992 (1997).
2. J. Wang, Fu Ke, K.W.M. Siu and R. Guevremont, Electrospray Tandem Mass Spectrometry of Alkali Metal-Containing Anionic Complexes of Tripeptides, *J. Mass Spectrom.* Vol. 31, 159 (1996).
3. Fu Ke, J.C.Y. Le Blanc, R. Guevremont and K.W.M. Siu, The effects of Stearyl and Cetyl Alcohol on The Electrospray Mass Spectrometry of Proteins and Their Implications on The Electrospray Mechanism, *Eur. Mass Spectrom.* Vol. 1, 253 (1995).

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IMAGE EVALUATION TEST TARGET (QA-3)

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
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