

METHODOLOGY

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Ionization Methods in Protein Mass Spectrometry

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Mass spectrometry (MS) has become one of the most powerful and popular modern physical-chemical methods to study the complexities of elemental and molecular processes in nature. The advent of new methods of ion generation, novel mass analyzers, and new tools for data processing has made it possible to analyze almost all chemical entities by MS, ranging from small organic compounds, large biological molecules, to whole living cells/tissues. As proteins fulfill a plethora of biochemical functions within every living organism, equally spectacular efforts and advances have been seen for protein ionization methods. In particular, the invention of matrix-assisted laser desorption ionization (MALDI) [1] and electrospray ionization (ESI) technologies [2,3] allow one to measure protein molecular weights, to determine sequences, and to probe conformations and post-translational modifications of proteins. In addition the mass range of species amenable for MS analysis has been increased immensely, enabling the transfer into the gas phase of ionized noncovalent species with masses well over one million (e.g., a 100 MDa single DNA ion [4]). These advances move MS into the range of intact protein oligomers and functional machineries.

This chapter is an introduction to various ionization methods for proteins. As this is a broad topic with an immense literature coverage including many excellent books [5,6] and reviews [7–17], we will emphasize some types of spray or laser-based protein ionization techniques, including atmospheric pressure MALDI, surface-enhanced laser desorption/ionization (SELDI), nanostructure-initiator MS (NIMS), sonic spray ionization (SSI), electrosonic spray ionization (ESSI), desorption electrospray ionization (DESI), fused-droplet electrospray ionization (FD-ESI), electrospray-assisted laser desorption ionization (ELDI), and matrix-assisted laser desorption electrospray ionization (MALDESI). We begin with the introduction of some historic facts for the

development of protein ionization methods, followed with the description of each method including the ionization principles, strengths, and analytical applications.

1.1 HISTORY OF THE DEVELOPMENT OF PROTEIN MASS SPECTROMETRY

MS originates from nineteen-century physics. The first known mass spectrometer was built by J. J. Thomson in the early 1900s to study and measure the mass (m)-to-charge (z) (m/z) values of the “corpuscles” that make up “positive rays” [18], a type of radiation initially observed by German physicist Eugen Goldstein. Following the seminal work of Thomson, MS underwent countless improvements in instrumentation, ionization methods, and applications. The classical ionization method, electron ionization (EI), was devised by Dempster and improved later by Bleakney [19] and Nier [20], and became a widely used standard for ionization of volatile organic compounds. This ionization technique requires extensive derivatization and evaporation of a nonvolatile analyte into the ion source, and it involves numerous fragmentation and rearrangement reactions.

Applications of MS to peptides (derivatized via acylation) begun in the late 1950s by Biemann [21] and McLafferty [22]. The first methods that allowed analysis of nonderivatized peptides were field desorption (FD) and chemical ionization (CI) developed in the 1960s [23,24]. Ionization by CI is achieved by interaction of its volatile molecules with reagent ions. CI allows ionization without significant degree of ion fragmentation but still requires gas-phase samples. Field desorption was reported by Beeky in 1969 [25], in which electron tunneling triggered by a very high electric field results in ionization of gaseous analyte molecules.

It was plasma desorption (PD) [26] and fast atom bombardment (FAB) [27] that opened the way to protein analysis. PD ionization, invented by R. D. Macfarlane in 1976 [28], a breakthrough in the analysis of solid samples, involves ionization of materials in the solid state by bombardment with ions or neutral atoms formed as a result of the nuclear fission of the Californium isotope ^{252}Cf . In 1982 Sundqvist and coworkers obtained the first spectrum of a protein, insulin (Figure 1.1), using bombardment with a beam of 90 MeV $^{127}\text{I}^{20+}$ ions from a tandem accelerator [26]. Later, FAB involving focusing the sample in liquid matrix with a beam of neutral atoms or molecules, was implemented for the ionization of proteins up to 24 kDa [29]. In 1983 Blakely and Vestal [30] introduced electrospray ionization (ESI) to produce ions from an aqueous solution sprayed directly into a mass spectrometer. Electrospray is a form of atmospheric pressure ionization in MS, transferring ions from the liquid phase to the gas phase for analysis. It was particularly useful in coupling liquid chromatography with mass spectrometry [31].

The breakthrough for large molecule laser desorption ionization came in 1987 when Tanaka combined 30-nm cobalt particles in glycerol with a 337-nm nitrogen laser for ionization and showed that singly charged protein molecular ions up to about 35 kDa can be introduced to a mass spectrometer [32]. During that time, MALDI [15,33], first reported in 1985 by Hillenkamp, Karas, and their colleagues,

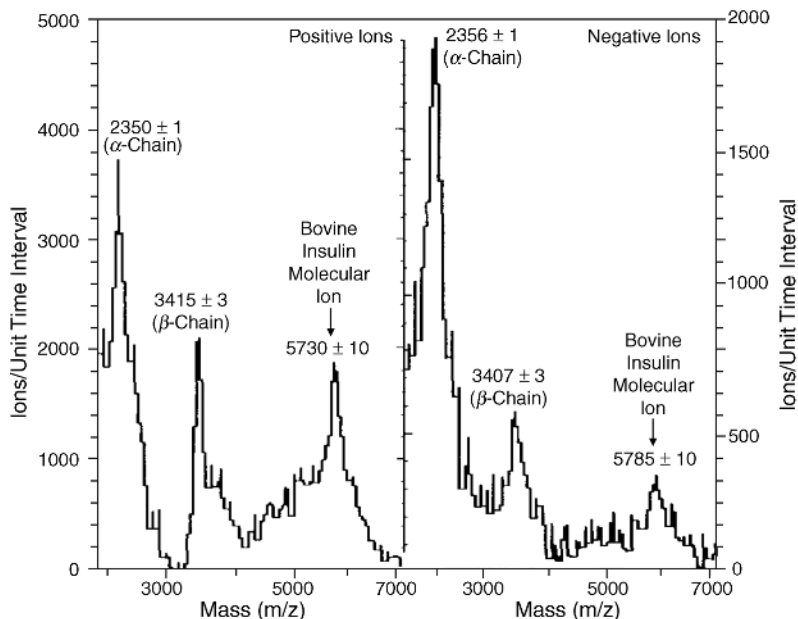


FIGURE 1.1 ^{127}I -PDMS spectra of bovine insulin recorded over a 1.5-h period with a 90-MeV ^{127}I (+20) beam current of 2000 s^{-1} . From [26]. Copyright permission was obtained from ACS.

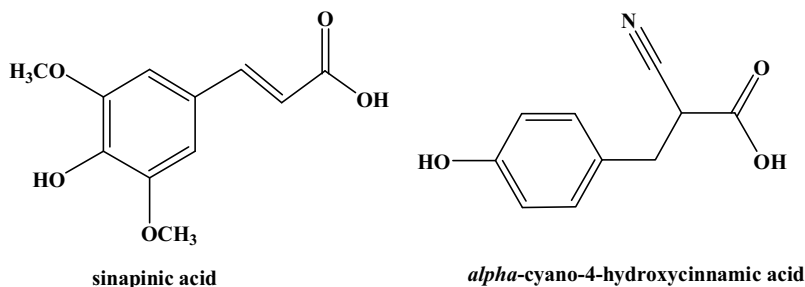
emerged as the culmination of a long series of experiments using desorption ionization (DI). MALDI is a soft ionization technique for the analysis of biomolecules and large organic molecules and has gained wide success in protein analysis, particularly when coupled with time-of-flight (TOF) instruments [34,35].

Another breakthrough occurred in 1984 when Fenn and coworkers used electrospray to ionize biomolecules [2]; the first ESI analyses of biopolymers including proteins were published in 1989 [3]. MALDI and ESI have revolutionized protein mass spectrometry since their invention in 1980s, and they have triggered the explosion in application of mass spectrometry for protein studies [36].

1.2 LASER-BASED IONIZATION METHODS FOR PROTEINS

1.2.1 Matrix-Assisted Laser Desorption/Ionization (MALDI)

Investigations of the wavelength influence in ultraviolet-laser desorption [33] led to invention of ultraviolet-laser matrix-assisted laser desorption ionization (UV-MALDI) between 1984 and 1986 and summarized in a 1987 paper [37]. In 1988 Karas and Hillenkamp reported ultraviolet-laser desorption (UPLD) of bioorganic compounds in the mass range above 10 kDa [1]. As a soft desorption ionization method, MALDI handles thermolabile, nonvolatile organic compounds, especially those with



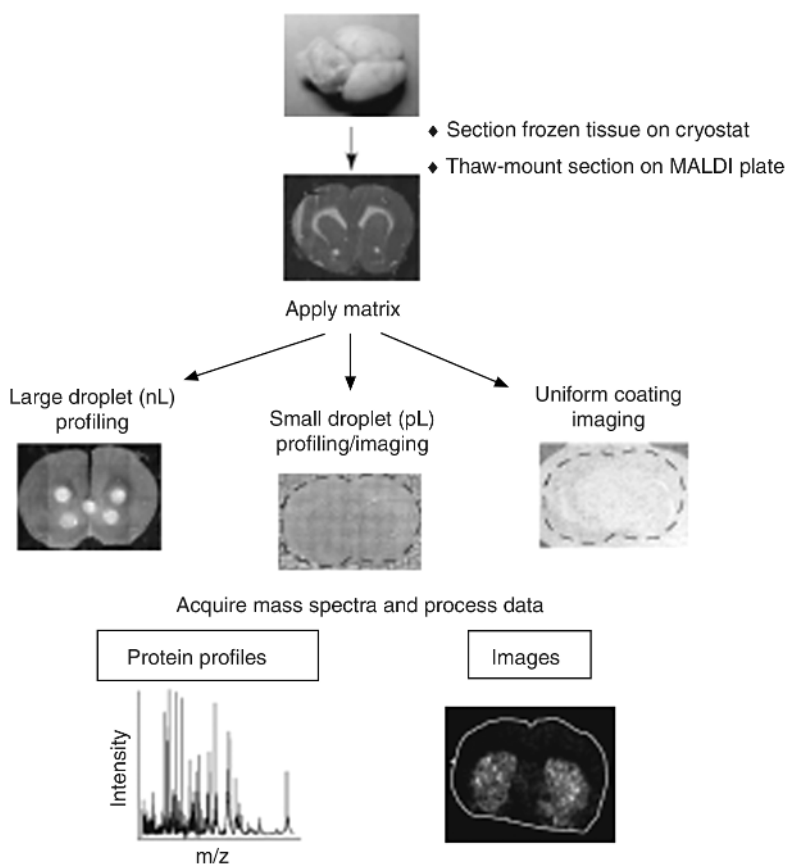
SCHEME 1.1 Structures of two common MALDI matrices.

high molecular weight and can be successfully used for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. Its operation is relatively straightforward, although matrix preparation requires experience and perhaps some artistry.

MALDI is based on the bombardment of sample molecules with laser light, process that allows sample ionization [38]. It requires a specific matrix consisting of small organic compounds (e.g., nicotinic acid) that exhibit a strong resonance absorption at the laser wavelength used. The sample is premixed and diluted with the highly absorbing matrix and allowed to dry on a sample target. A range of compounds is suitable as matrices: sinapinic acid is a common one for protein analysis while α -cyano-4-hydroxycinnamic acid is often used for peptide analysis (the structures of matrices are shown in Scheme 1.1). This kind of acid serves well as a matrix for MALDI owing to the acid's ability to absorb laser radiation and also to donate protons (H^+) to the analyte of interest. Upon laser irradiation, energy is absorbed by the matrix in a localized region of the surface. As a result an explosive break up of the cocrystallized analyte/matrix sample occurs. The rapid expansion of the vaporized matrix in MALDI leads to the translational excitation of analyte molecules and the release of the analyte molecules from the surface of the condensed phase sample into vacuum. The analyte may be precharged (e.g., exist as a salt), and the intact analyte ion may simply be transferred as an ion from the solid to the vapor state upon laser irradiation of the matrix. Alternatively, a neutral analyte may be ionized through ion-molecule reactions (e.g., proton transfer reaction) occurring in the energized selfedge or interfacial region between the solid and gas phases.

MALDI has remarkable efficiency in producing intact molecular ions (often $[M + H]^+$, $[M + Na]^+$) of large biological compounds. MALDI ionization sensitivity is also extraordinary, and total amounts of sample loaded onto the target surface often are in the picomole to femtomole range. The method has tolerance to buffers and other additives and gives predominantly singly charged ions for large biomolecules [35]. TOF mass analyzers are ideal for use with this ionization technique because they are compatible with high-mass ions and pulsed-ion production [34,35]. TOF analyzers separate ions according to their m/z ratios by measuring the time it takes for ions, accelerated to the same kinetic energy, to travel through a field-free region known as the flight or drift tube. The heavier ions move slower than the lighter ones [6].

An important application of MALDI is chemical imaging, using a technique called matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) [16]. Imaging combines parallel, high-throughput molecular analysis with location-specific information for the characterization of protein distributions directly from thin sections of intact biological tissue [39,40] and offers complementary information to two-dimensional (2D) gel electrophoresis and to shotgun proteomics for investigating proteomic differences. It is covered in Chapter 5 by Reyzer and Caprioli in this volume. Figure 1.2 illustrates the typical experimental process for MALDI imaging. Frozen tissue specimens are sectioned on a cryostat into about 5- to 20- μ m thick sections. The sections are thaw-mounted onto conductive MALDI target plates. Matrix is applied to the sections, depending on the experiment to be performed: droplets (nL to pL) can be deposited in arrays or on discrete morphological areas, or a uniform coating of matrix can be applied to the entire tissue section.



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FIGURE 1.2 Typical experimental design for IMS. From [16]. Copyright permission was obtained from Elsevier. (See the color version of this figure in Color Plates section.)

Mass spectra are obtained from each spot or from across the entire tissue section in a defined raster pattern. The acquired spectra can then be examined and processed to form 2D molecule-specific ion images [16]. The power of MALDI-IMS technology is its capability to link reliably protein data with specific cellular regions within the tissue.

MALDI-IMS has been employed as an imaging technology in a wide variety of applications from the analysis of small molecules such as drugs and endogenous metabolites to high molecular weight proteins (e.g., MALDI-IMS of a mouse model of Parkinson's disease revealed a significant decrease in PEP-19 expression levels in the striatum after administration of the drug MPTP [41]).

1.2.2 Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization (AP-MALDI)

Atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) was first described by Laiko et al. [42]. In contrast to conventional vacuum MALDI, AP-MALDI can be operated at atmospheric pressure instead of high vacuum where ions are typically produced at 10 mTorr or less. During the ionization process, the solid-phase target material containing analyte sample and matrix is irradiated with a pulsed laser beam. The matrix absorbs the photon energy and undergoes fast heating and evaporation, which results in the formation of gaseous analyte ions [43]. Because the ionization of AP-MALDI occurs at atmospheric pressure, thermalization of the resulting ions takes place owing to collisions with the ambient gases used in AP-MALDI, accounting for the soft ionization nature of AP-MALDI [42].

The AP-MALDI source makes use of a high voltage potential that is applied between the target tip and the heated inlet transport capillary. The laser is focused onto the surface of the target plate. Ions are desorbed from the angled replaceable target tip and carried by the dry carrier nitrogen gas into a mass spectrometer [44]. The sensitivity of detection for AP-MALDI can be affected by the geometry of the target tip, and its position relative to the inlet orifice, the nitrogen gas flow rate, gas nozzle position, etc. [42]. Furthermore, when a Nd: YAG laser with high laser power rather than a nitrogen laser is used, the signal intensity can be improved [45].

Given that AP-MALDI and conventional vacuum MALDI share common ionization mechanisms, they have many similar features including simplicity of sample preparation and tolerance to interference from salts [42], which can be detrimental for biomolecule analysis. AP-MALDI is an extension of conventional MALDI, but it has some unique characteristics. First, samples are handled at atmospheric pressure. Second, AP-MALDI is a softer ionization technique than vacuum MALDI, which is favored for protein analysis. For example, the heavier peptides/glycopeptides from protein digestion are less likely to fragment by AP rather than vacuum MALDI; thus, more peptides can be detected (Figure 1.3) [42]. Third, AP-MALDI can employ liquid matrices to improve the ionization reproducibility, which otherwise results in source contamination in vacuum MALDI. Fourth, the AP-MALDI ion source is easy to exchange with other atmospheric ionization sources, allowing it to be easily coupled to different mass analyzers (e.g., quadrupole ion trap (QIT) [46], TOF [45],

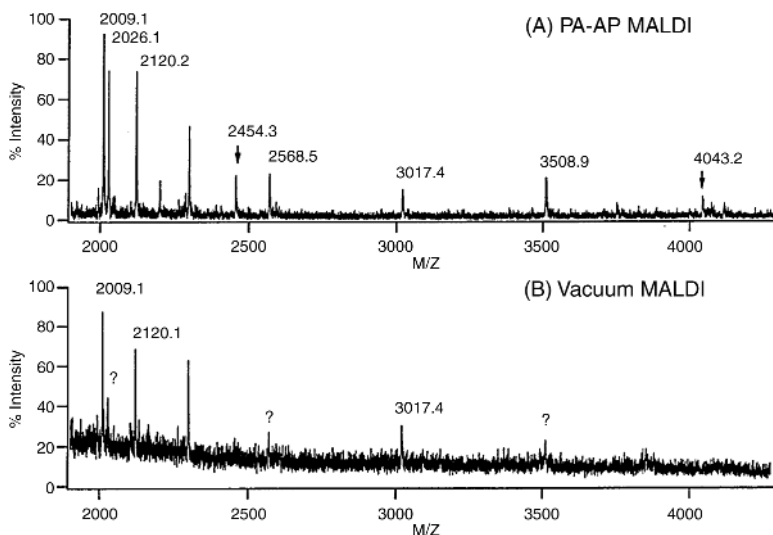


FIGURE 1.3 Partial mass spectra for tryptic digest of bovine fetuin. (A) AP-MALDI spectrum from 1.5-pmol deposition, showing eight identified peaks. (B) Vacuum MALDI spectrum for 1-pmol deposition, showing three identified peaks. From [42]. Copyright permission was obtained from ACS.

and Fourier transform ion cyclotron resonance (FT-ICR) [47]. Fifth, the analyte-matrix cluster ions in AP-MALDI caused by collisional cooling can be observed [43]. Nevertheless, the major disadvantage of AP-MALDI is the ion loss in atmospheric pressure interfaces, giving it lower sensitivity than conventional MALDI [46].

AP-MALDI MS has seen a variety of applications similar to those of conventional vacuum MALDI, including analysis in proteomics and determinations of oligosaccharides, DNA/RNA/PNA, lipids, bacteria, phosphopeptides, small molecules, and synthetic polymers [48]. The convenient and rapid exchange of the AP-MALDI source with other ionization sources and high throughput are attractive features. The major expected application for AP-MALDI is for the analysis of vacuum-incompatible samples like profiling of biological tissue samples, which requires the use of a wide range of liquid matrices at atmospheric pressure [43].

1.2.3 Surface-Enhanced Laser Desorption/Ionization (SELDI)

Surface-enhanced laser desorption/ionization (SELDI) as a prominent form of laser desorption/ionization (LDI) mass spectrometry was first described in 1993 by Hutchens and Yip [49]. It can be classified in three groups: surface-enhanced neat desorption (SEND), surface-enhanced affinity capture (SEAC), and surface-enhanced photolabile attachment and release (SEPAR) [50]. In SEND, analytes even for large molecules can be desorbed and ionized without adding matrix. This occurs because a compound with a chromophore to absorb laser energy is attached to the probe surface via physical adsorption or covalent modification [51]. In SEAC, the

probe surface plays an active role in the extraction, fractionation, cleanup, and/or amplification of the sample of interest. Common are chemical surfaces such as H50 (hydrophobic surface, similar to C_6-C_{12} reverse-phase chromatography materials), CM10 (weak-positive ion exchanger), Q10 (strong anion exchanger), IMAC30 (metal-binding surface), and biochemical surfaces containing antibodies, receptors, enzymes or DNA [50,52]. In SEPAR, an energy-absorbing molecule promotes analyte desorption and ionization, making this approach a hybrid of SECA and SEND [50]. Furthermore SELDI is commercially embodied in Ciphergen's ProteinChip® Array System (Ciphergen Biosystemes, Palo Alto, CA, USA), which simplifies the sample preparation with on-chip binding and detection [50]. SELDI is typically coupled with TOF mass spectrometers and is applied to detect proteins in tissue, urine, blood, and other clinical samples.

SELDI-TOF-MS is the extended form of MALDI-TOF-MS. The differences are the sample preparation and the software tools for interpreting the acquired data. In executing the SELDI process (Figure 1.4) [53], the first step is to select a chromatographic and preactivated ProteinChip array. Next, the protein sample solution is applied and incubated on the spots of the ProteinChip array. Third, by allowing the proteins to interact with the chromatographic array surface, on-spot contaminants and salts of the sample can be washed away to ensure efficient sample cleanup. This binding step to the SELDI surface can be viewed as a separation step, purifying the proteins bound to the surface. Fourth, matrices are added for the formation of a homogeneous layer of cocrystallized target proteins. After that, a laser beam raster is used to irradiate the spot, causing desorption and ionization of the proteins. The laser beam raster can be applied to cover selectively the entire spot surface, affording an output of the entire spot. Finally, multiple spectra are averaged to yield a final spectrum that displays the protein ions. Protein quantification is achieved by the correlation between the signal intensities and analyte concentrations of proteins in the sample.

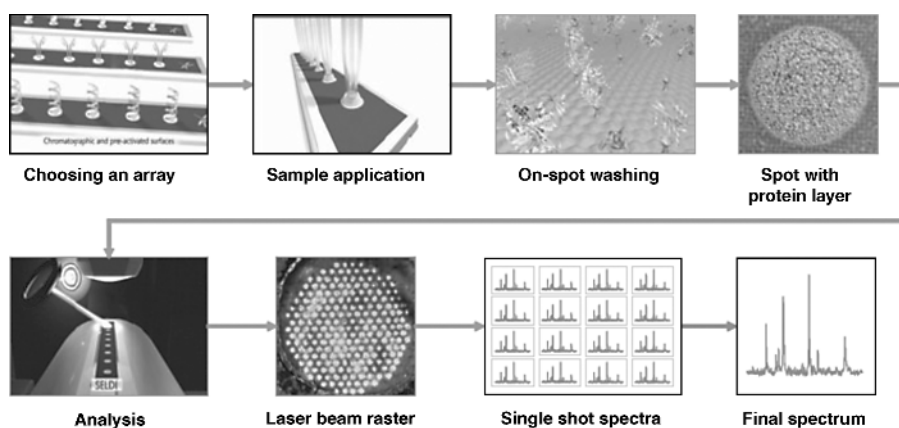


FIGURE 1.4 Experimental steps of SELDI-TOF-MS-based ProteinChip® System. From [53]. Copyright permission was obtained from Nature Publishing Group. (See the color version of this figure in Color Plates section.)

For the analysis of complicated biological systems containing hundreds of biological molecules together with salts (e.g., serum, blood, plasma, lymph, urine, whole cells, exudates) by MS, sample preparation and purification are necessary. Compared with some classic sample purification methods like liquid chromatography, electrophoresis, centrifugation, and immunoprecipitation, which are subject to losses of both analyte and minor components owing to nonspecific binding, SELDI can be directly and readily used to analyze the major and minor proteins in heterogeneous samples. This ionization method for analysis of macromolecules efficiently facilitates the investigation of biological molecules on-probe and simplifies sample purification and extraction steps in contrast to conventional LDI and MALDI [50]. Furthermore SELDI is rapid, highly reproducible, and offers good sensitivity for trace protein ($< \text{fmol/mL}$ using chemical arrays) analysis. It has had some impact in proteomics and drug discovery and can be used for discovery, analysis, and identification of post-translational modifications of disease-associated proteins [54].

1.2.4 Nanostructure-Initiator Mass Spectrometry (NIMS)

Nanostructure initiator mass spectrometry (NIMS) was introduced as a substitute to overcome typical limitations (sensitivity and spatial resolution) found with the use of matrices in laser methods such as MALDI. NIMS is a matrix-free, surface-based MS desorption/ionization technique that uses nanostructured surfaces or clathrates to trap liquid “initiator” materials (e.g., bis(tridecafluoro-1,1,2,2-tetrahydrooctyl)tetramethyl-disiloxane). These materials are released upon heating by laser irradiation, carrying with them absorbed analyte molecules (Figure 1.5A) [55–57]. The technique has been used in the characterization of proteolytic digests, single cells, tissues, biofluids (direct analysis of blood and urine), lipids, drugs, and carbohydrates. Imaging applications include peptide arrays, tissue (tissue/surface interface), and single cell. Some attributes of NIMS are minimal sample preparation, high-sensitivity/lateral resolution (ion-NIMS: 150 nm, as compared to MALDI and ESI), high salt tolerance, compatibility with standard laser based instruments, and reduced fragmentation (favored intact ion formation). The NIMS technique is also flexible, accommodating a variety of irradiation sources (laser or ion), surfaces, and initiator (depending on target analyte) compositions. In contrast to conventional MALDI, NIMS is capable of producing multiply charged proteins as ESI or cryo-infrared MALDI.

The nanostructured silicon surface in NIMS is composed of pores of approximately 10 nm in diameter (Figure 1.5C). Initiator molecules (Figure 1.5B), which are chosen depending on the target analyte, are trapped inside these pores. The initiator molecules are UV laser transparent (do not ionize) whereas the silicone nanostructure is an efficient UV absorber semiconductor. Analyte molecules are adsorbed on the initiator surface and desorbed upon initiator vaporization, caused by laser or ion irradiation. When ion irradiation is used, spatial resolution of approximately 150 nm can be achieved whereas laser-NIMS produces a spatial resolution of approximately 15 to 20 μm [55–57].

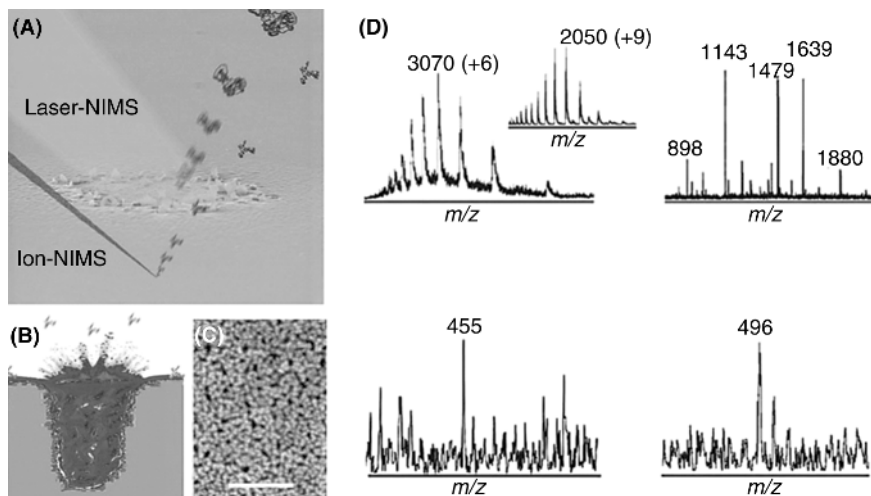


FIGURE 1.5 (A) Illustration superimposed on an SEM image of a NIMS surface after irradiation with a single laser shot (light grey), revealing localized surface distortion and destruction. By comparison, ion irradiation (dark grey red) allows a much higher lateral resolution. (B) Illustration of possible mechanism in which surface irradiation results in the vaporization or fragmentation of initiator (dark grey blue) trapped in a surface pore, triggering analyte desorption/ionization. (C) SEM image revealing that the NIMS surface is composed of 10-nm pores; scale bar, 100 nm. (D) Laser irradiation (wavelength 337 nm) of a NIMS surface. Upper left panel: detection of a multiply charged protein (50 nmol of β -lactoglobulin) in a similar manner to ESI (inset). Upper right panel: detection of a BSA tryptic digest (500 amol). Lower left panel: detection of the calcium antagonist verapamil (700 ymol). Lower right panel: detection of the endogenous metabolite 1-palmitoyllysophosphatidylcholine (50 amol). The initiator was bis(tridecafluoro-1,1,2,2-tetrahydrooctyl)tetramethyl-disiloxane; 0.5- μ l drops were used. From [57]. Copyright permission was obtained from Nature Publishing Group.

Protein ionization via laser-NIMS generates ESI-like spectra showing multiply charged states (Figure 1.5D). In this specific case, laser-NIMS showed lower charges than ESI for β -lactoglobulin, suggesting that the protein is less denatured by the NIMS ionization process. The superior sensitivity of laser-NIMS versus ESI or MALDI is also shown in Figure 1.5D for the detection of BSA peptide fingerprints at 500 amol (55% sequence coverage) [57]. Endogenous phospholipids can be detected from single metastatic breast cancer cells with less complexity than nano-ESI or MALDI [57]. Ion-NIMS, on the other hand, can be successfully used for high-resolving-power, label-free peptide array analysis, showing mass images and mass spectra collected for 1 fmol of peptide, representing a 1000-fold enhancement in sensitivity over TOF-SIMS strategies [57]. Typical laser energies for desorption/ionization with NIMS are approximately seven times lower than that of MALDI for analysis of a mixture of the tetrapeptide MRFA (50 fmol) and des-Arg⁹-bradikinin (25 fmol) when applying a laser energy of 110 mJ/cm² for MALDI and 15 mJ/cm² for NIMS. Better S/N ratios and less background ions are found for the collected NIMS mass spectra [58].

1.3 SPRAY-BASED IONIZATION METHODS FOR PROTEINS

1.3.1 Electrospray Ionization (ESI)

The principle of electrospray ionization was first described by Dole in 1968 [59] and coupled to MS in 1984 by Yamashita and Fenn [2]. ESI usually generates intact, multiply charged ions, generally in the form $[M + nH]^{n+}$ in both the positive (e.g., protonated) and negative (e.g., deprotonated) ion modes. In ESI-MS, “naked” ions form *via* progressive solvent evaporation from charged droplets of a liquid sample, sprayed in the presence of a strong electrical field. The formation of gaseous analyte ions by electrospray involves three steps: formation of charged droplets, shrinkage of the droplets owing to solvent evaporation, and transfer of ions to the gas phase. Although the macroscopic aspects of electrospray are generally well understood, the mechanisms for the final generation of desolvated (or nearly desolvated) ions from a charged droplet are not yet fully resolved. Two models describe this process. The charged residue model (CRM), conceived by Dole et al. [59], invokes successive cycles of solvent evaporation and coulombic fission at the Rayleigh limit until a droplet containing a single residual analyte ion remains. Complete evaporation of the solvent comprising this droplet eventually yields a “naked” analyte ion, the charged residue. The ion-evaporation model (IEM) proposed by Iribarne and Thomson [60] is based on transition-state theory and invokes, prior to complete desolvation of the droplet, sufficiently strong repulsion between the charged analyte ion and the other charges in the droplet that becomes to overcome solvation forces and the ion is ejected (field-desorbed) from the droplet surface into the gas phase [61].

With the advent of ESI, it became possible to study protein conformations. Different from traditional methods to investigate protein conformations such as circular dichroism (CD), NMR and X ray, ESI-MS offers several advantages for this purpose. First, ESI-MS is sensitive, requiring fmol and amol amounts of protein samples [12,62,63]. Second, ESI analysis makes use of a protein solution, which is important because most of biology and much of separations take place in solution. In traditional ESI experiments, organic compounds are often used as co-solvents; however, the use of highly organic solvents is no longer mandatory. This has lead to the birth of an emerging field in biomolecular MS, termed native ESI-MS [61,64–66]; the focus of this field is the analysis of intact proteins and protein complexes under near physiological conditions achieved by using neutral volatile buffer salts like ammonium acetate for protein sample preparation. The third is that gas-phase, multiply charged ions are generated from the protein sample [3]. This point plays a central role in protein studies, given that the charge-state distributions (CSDs) observable in protein ESI mass spectra are affected by the conformations that the protein held in solution at the moment of its transfer to the gas phase [12,67]. Typically, when a protein is in the folded structure, a narrow CSD in low-charge states is observed whereas the CSD is broadened and shifted to high-charge states after unfolding, probably because the unfolded protein has a greater capacity to accommodate charges on its surface because coulombic repulsions are reduced [62,68,69]. Therefore, information about the conformational states of the protein can often be

extracted based on the structural interpretation of CSDs in ESI-MS, upon controlling other experimental conditions [12]. Another MS-based approach to protein conformation study is to monitor protein hydrogen/deuterium exchange reactions, which are sensitive to the conformational structure; thus the exchange level determined by MS can be related to protein conformation [70–76], and this subject is covered in Chapter 7 by Kerfoot and Gross in this volume.

In 1994 Wilm and Mann introduced an important variant of conventional ESI, termed nanoelectrospray (nESI) [77]. While this technique uses the same fundamental sequence of charged-droplet generation followed by solvent evaporation, coulombic fission events, and finally ion formation, it is distinguished from regular ESI in several ways. First, nESI is typically performed using glass or quartz capillaries that are pulled to a fine tip ($\sim 1\text{-}\mu\text{m}$ inner diameter) and given a metallic (usually gold) coating to hold the electric potential; these are used instead of the metallic capillary used for conventional ESI. Approximately 1 to 3 μL of sample is injected into the glass capillary and electrosprayed at flow rates in the range of around 1 nL/min to several tens of nL/min [78,79]. The spray is driven primarily by the approximately 0.5 to 1.5-kV potential applied to the capillary, although it is often necessary to provide an auxiliary backing gas pressure to the sample to initiate and/or maintain a steady stream of the solution through the tip [61]. Second, in comparison to conventional ESI, a smaller initial droplet size in nESI leads to less nonspecific aggregation (both protein–protein and protein–salt), and its gentler interface conditions, while still allowing adequate desolvation, lead to less dissociation and disruption of oligomeric and higher order structures (Figure 1.6 shows the contrast between nESI and ESI for the ionization of a GroEL complex). The benefits of nESI analysis include high ionization efficiency, well-resolved peaks corresponding to the protein assembly, narrow charge-state distributions, reduced nonspecific adduct formation, and high salt tolerance.

1.3.2 Sonic Spray Ionization (SSI)

Besides ESI, another spray technique that can be successfully used for the analysis of proteins and peptides is sonic spray ionization (SSI) [80–83]. This soft atmospheric pressure ionization (API) method was first introduced by Hirabayashi et al. [84] in the early 1990s as a method for interfacing capillary electrophoresis and liquid chromatography instrumentation to mass spectrometers. The source works at room temperature (no heating applied to capillary) [85,86]. Ions and charged droplets are produced under atmospheric pressure, and their abundances depend on the nebulization gas flow rate. Optimal ion abundances are obtained at Mach numbers of approximately 1, which corresponds to sonic velocity [84,85].

In SSI, a solution is infused through a fused-silica capillary, which is fixed by an external stainless steel capillary, allowing its accurate positioning in the source body (Figure 1.7A). The fused-silica capillary is then inserted into an orifice from which it protrudes approximately 0.6 mm [84]. Nitrogen gas is then passed through the orifice, coaxial to the fused-silica capillary, nebulizing the eluent at gas flow rates that match sonic velocities. The generated spray, composed of charged droplets and ions at

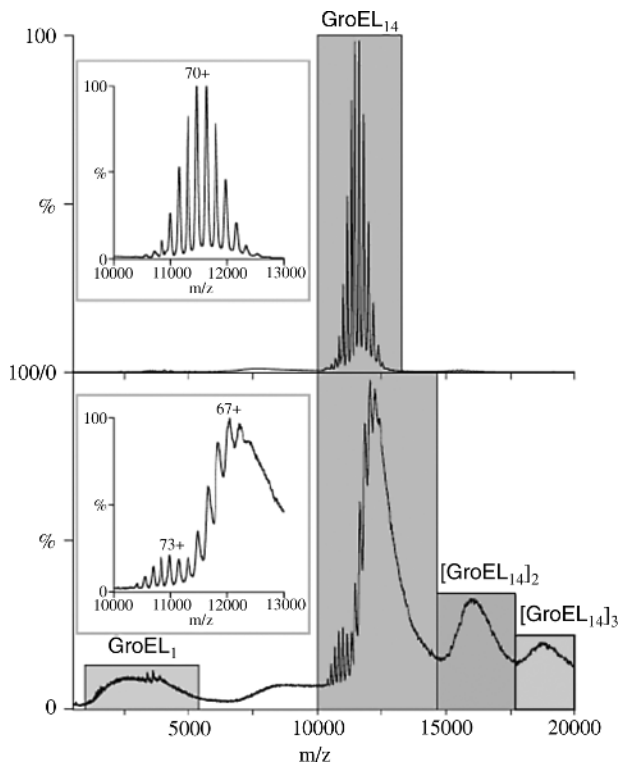


FIGURE 1.6 Conventional and nanoelectrospray MS of a protein complex. MS of the GroEL complex ionized by means of ESI (lower) and nESI (upper). Solution conditions were 200-mM ammonium acetate, pH 6.9, and a protein concentration of 2- μ M tetradecamer. The nESI spectrum displays a series of peaks around 11,500 m/z , which correspond to the 800 kDa tetradecamer. Conventional ESI of the same solution results in poorly resolved “humps” centered on 12,500, 16,000, and 18,500 m/z . These are assigned to the tetradecamer, a dimer of tetradecamers, and a trimer of tetradecamers, respectively. There is also a signal at low m/z that corresponds to the GroEL monomer. From [61]. Copyright permission was obtained from ACS.

atmospheric pressure, is then introduced through a sampling orifice into the mass spectrometer for mass analysis.

The mechanism of ion formation by SSI is not yet well understood. Early studies on the charged droplet formation mechanism suggest that the origin of the charged species cannot be ascribed to the traditional models of friction electrification (between the capillary surface and the solution), electrical double layer or statistical charging model [85,87]. Instead, a charged droplet formation mechanism occurs based on the non-uniformity of positive and negative-ion concentrations near the solution surface (at a gas boundary), determined by the surface potential [83]. The charged droplet formation in SSI may be based on the statistical charging model (sudden evaporation of liquid into smaller equally sized droplets, which are charged owing to microscopic

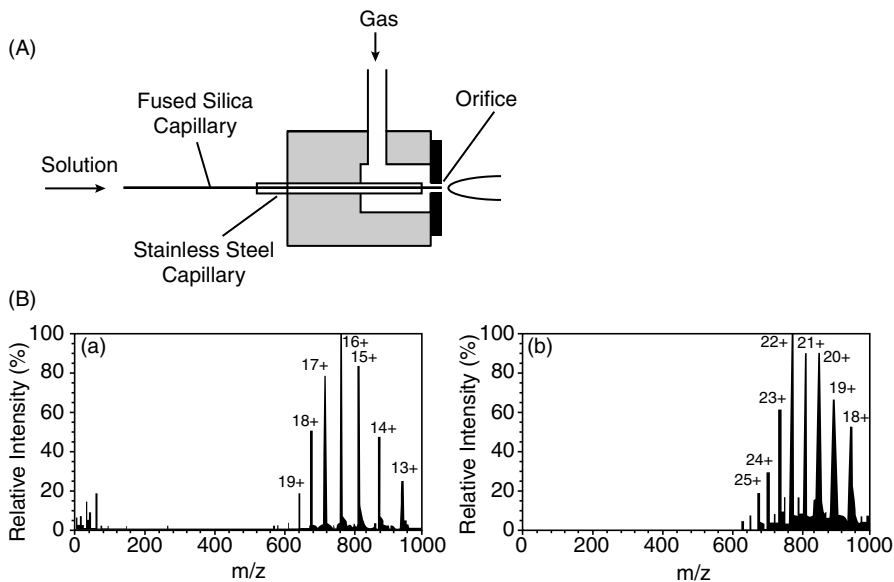


FIGURE 1.7 (A) Typical schematic of an SSI. From [84]. Copyright permission was obtained from ACS. (B) Mass spectra obtained from methanol/water/acetic acid (47.5/47.5/5.0%, v/v/v) solutions of (a) cytochrome *c* from sheep heart (MW 12,300) and (b) myoglobin from horse skeletal muscle (MW 17,000 Da). A high voltage of 1 kV was applied to the source housing and the gas-flow rate was 3.0 L/min. From [83]. Copyright permission was obtained from Wiley.

fluctuations in the ion concentration in a bulk liquid). Gaseous ions are formed as a result of the charge residue model (continuous evaporation and fission cycles leading to droplets that contain on average one analyte or less); gas-phase ions are then formed after the remaining solvent has evaporated [82,84,85,88–92]. Given that no electrical field is applied to the solution in SSI, low charge-state ions are produced [83]. When one applies a high voltage to the source housing (solution surface), one can see increased charge density on the droplets and improved ion formation efficiencies [83].

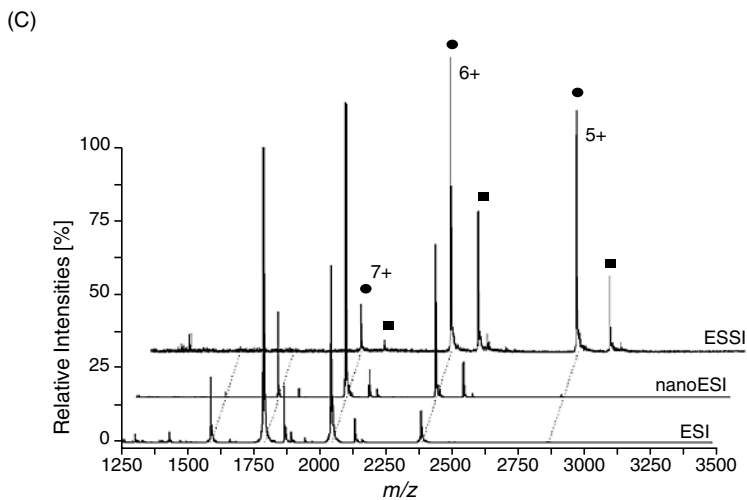
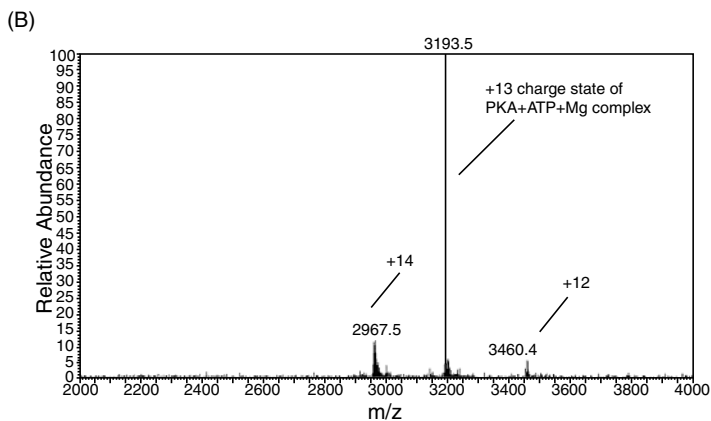
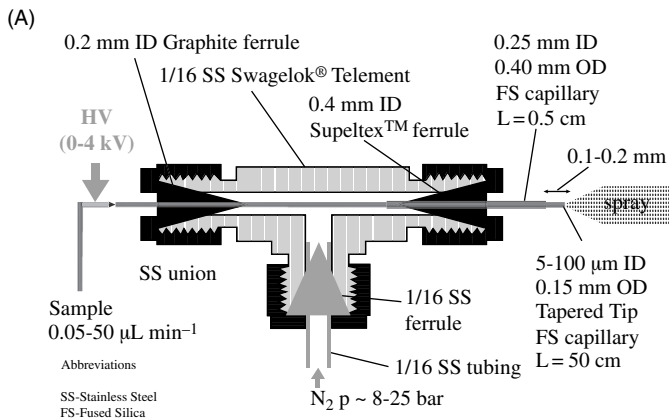
An attribute of SSI is its simplicity because no high voltage or heating are used in ion formation. Ions are typically formed with low internal energies, making this technique promising for the study of thermal labile molecules, cluster ions, and fragile complexes (i.e., loosely bound metal-assembled cages) [83,93–95]. This attribute may be a disadvantage because excessive clustering makes data interpretation difficult. Given that low charge-state ions are typically generated by SSI, high voltages must be applied to the source housing to increase charge density on the droplets [83]. As an example of cluster formation and SSI gentle ionization character, the abundance for the protonated L-serine octamer is approximately 10–15 times higher when formed via SSI than by ESI, with virtually no oligomeric species, primarily attributed to the lower average internal energies of the ions produced as compared to ESI [91].

There are reported protein and peptide applications using SSI, and they include the analysis of RNase A, lysozyme, bovine serum albumin (BSA), myoglobin, cytochrome *c*, and carbonic anhydrase II [80–82]. Applications in other areas (e.g., drugs [96,97], oligosaccharides [98,99], phenolic compounds [100], oligonucleotides [101,102], and neurotransmitters [85]) have been reported, but these are beyond the scope of this chapter. The first spectra of proteins and the formation of multiply charged ions (Figure 1.7*B*) [83] by SSI were collected with a quadrupole mass spectrometer. The spectra show charge state distributions ranging from 13^+ to 19^+ for cytochrome *c* ($MW \approx 12,300$) and from 18^+ to 25^+ for myoglobin ($MW \approx 17,000$).

Capillary isoelectric focusing (CIEF) can be coupled with MS by using SSI for the analysis of proteins, as reported by Hirabayashi et al. [80,81]. An SSI interface setup with a buffer reservoir placed in between the sample introduction capillary of the ion source and the electrophoresis-separation capillary is required. This allows for online and one-step CIEF/MS analysis. Given that SSI uses a high-velocity gas to generate the spray, one can use a wide range of buffers solutions, solution flow rates, and high-polymer ampholytes (used in CIEF) without clogging the spray nozzle of the interface. Filling the buffer reservoir with acetic acid and introducing it through a pinhole into the sample introduction capillary of the SSI source reduces ion suppression caused by the ampholytes used in CIEF. Using this approach, one can detect down to 160 fmol of myoglobin and cytochrome *c* and separate the acidic and basic bands of myoglobin. In a recent application, SSI was used to obtain spectra of proteins with low charge states (as compared to ESI), hence decreasing overlap of peaks obtained from protein mixtures and facilitating mass spectral interpretation [82]. When contrasting SSI and ESI analysis of RNase A and lysozyme, one sees a dramatic reduction in charge states with SSI as the ionization method for these two proteins.

1.3.3 Electrosonic Spray Ionization (ESSI)

Electrosonic spray ionization (ESSI) is a hybrid between ESI and SSI; it uses a traditional micro ESI source and a supersonic gas jet similar to SSI [103]. The method can be used to study protein–ligand complexes owing to its gentle ionization character that allows formation of cold ions (low internal energies) [103–106]. Not only polymers [107] can be analyzed, also gas-phase basicities of proteins and peptides can be measured by this method [108–112]. The most distinctive characteristics of the ESSI method are the narrow charge-state distributions and narrow peak widths (efficient desolvation) as compared to those of ESI and nano-spray [103]. The method can preserve solution protein and protein complex structures at physiological pH values, ionizing the systems with a charge-state distribution characteristic of its conformation in solution [103,104]. The formation of broader charge-state distributions is typically associated with unfolding of proteins during ionization whereas narrow and lower charge-states (as observed in ESSI) are associated with native-like or folded (which defines their biological role) ion structures in the gas phase [113]. Tolerance to high salt concentrations, tunable source potential, lack of arcing, and weak dependence on temperature are



other attributes of the technique [103]. ESSI can be successfully coupled to several mass analyzers, including hybrid quadrupole time of flight [105], triple quadrupoles, and linear ion traps [103,112], thus demonstrating that it can be used in any instrument that has an API interface.

The general design of an ESSI source consists of a gas nebulizer made of inner and outer deactivated fused silica capillaries (Figure 1.8A). Nitrogen (N_2) is used as the nebulizing gas at a typical flow rate of 3 L/min (sonic velocity). The solvent is sprayed under the influence of an applied high voltage, typically in the range 0 to 4 kV (0 kV would be “pure SSI”). The voltage can be applied to the liquid sample through a copper alligator clip that attaches to the stainless steel tip of the infusion syringe. The gas jet composed of electrosprayed aqueous microdroplets and free gas-phase ions is directed to the inlet of an atmospheric interface of the mass spectrometer [91].

The mechanism of ESSI ion formation is likely to be the charge residue model [88,114,115]. The main difference between ESSI and ESI or nano-ESI is that ESSI is a more efficient desolvation process, attributed to the production of initial ultrafine droplets (generated by the supersonic nebulizing gas). These droplets are easily desolvated in a short time [88,103]. The faster desolvation and low temperatures of the spray, caused by adiabatic expansion of the nebulizing gas, leads to the formation of ions with low internal energies (lower than those produced by ESI or nano-ESI), giving ESSI the required “softness” for the analysis of noncovalent interactions.

Electrosonic Spray Ionization for Protein Analysis ESSI can be a useful tool for the study of noncovalent interactions owing to its soft ionization character (a comparison of ESSI and nano-spray generated spectra recorded for trypsin was carried out, showing narrower peaks and lower charge states for ESSI) [103]. The ESSI spectrum is dominated by a single charged state, whereas the other charge states do not contribute to more than 5% relative abundance. Efficient transfer by ESSI of intact complexes to the mass spectrometer can be achieved (Figure 1.8B for kinase A after conversion to its ATP/Mg adduct by addition of excess ATP Mg salt) [103]. Other applications include the use of deprotonation reactions in an evaluation of gas-phase basicities of globular and denatured proteins [110] and the analysis of enzyme-substrate and enzyme-substrate inhibitor complexes [104]. ESSI can be utilized to measure dissociation constants (K_D) for protein–ligand systems, showing good agreement with solution results [105]. A comparison of K_D values obtained by



FIGURE 1.8 (A) Schematic of an ESSI source. From [103]. Copyright permission was obtained from ACS. (B) ESSI spectrum of bovine protein kinase A catalytic subunit (200 nM in 10-mM aqueous ammonium acetate, pH 7.8) in the presence of 100- μ M ATP Mg salt. From [103]. Copyright permission was obtained from ACS. (C) Representative mass spectra of the noncovalent HEWL-NAG₃ complex (HEWL 10 μ M, NAG₃ 60 μ M in 20-mM ammonium bicarbonate buffer) using different ionization techniques. The charge states of free protein signals (filled circle) and HEWL-NAG₃ complex signals (filled square) are given for ESSI. From [56]. Copyright permission was obtained from Elsevier.

ESSI with those from ESI or nano-ESI shows that ESSI can give K_D values that are most similar to those determined by solution methods. One reason may be that ESSI ion complexes are less prone to dissociation as compared to those formed by ESI or nano-ESI. An example is the HEWL-NAG3 complex (Figure 1.8C), demonstrating the softness of the method [105].

1.4 AMBIENT IONIZATION METHODS

In most applications, MS required moderate to extensive sample preparation followed by introduction of the sample into the high vacuum conditions prior to analysis, limiting in-situ analysis and increasing the possibility of contamination during sample handling. These drawbacks are overcome with the introduction of desorption electrospray ionization (DESI) and direct analysis in real time (DART), which can be viewed as ambient ionization methods. Samples can be examined in the open environment (natural or in the laboratory), and typically no sample preparation is required, allowing for in-situ analysis while preserving all attributes associated with MS analysis. These approaches should open a new era in mass spectrometry.

After the first reported applications using DESI and DART [116,117], a whole new family of ambient methods and variants emerged. DESI variants such as reactive-DESI (reactions accompanying desorption), nonproximate detection DESI (transport of sample ions at long distances), geometry-independent DESI, transmission-mode DESI, and liquid sample DESI were soon introduced either to increase selectivity and sensitivity for trace analysis or to facilitate direct sample analysis [118–124]. Another ionization method termed desorption atmospheric pressure chemical ionization (DAPCI) was also developed to study ionization mechanism for explosive compounds [118]. Other established ambient ionization methods include electrospray-assisted laser desorption/ionization (ELDI) [125], matrix-assisted laser desorption electrospray ionization (MALDESI) [126], extractive electrospray ionization (EESI) [127], atmospheric solid analysis probe (ASAP) [128], jet-desorption ionization (JeDI) [129], desorption sonic-spray ionization (DeSSI) [130], field-induced droplet ionization (FIDI) [131], desorption atmospheric pressure photoionization (DAPPI) [132], plasma-assisted desorption ionization (PADI) [133], dielectric barrier discharge ionization (DBDI) [134], liquid microjunction surface sampling (LMJ-SSP) [135], atmospheric pressure thermal desorption ionization (APTDI) [136], surface-sampling probe (SSP) [137], fused-droplet electrospray ionization (FD-ESI) [138], helium atmospheric pressure glow discharge ionization (HAPGDI) [139], neutral desorption extractive electrospray ionization (ND-EESI) [140], laser ablation electrospray ionization (LAESI) [141], low-temperature plasma (LTP) [82], and laser spray ionization (LSI) [142]. Although these methods can be used for ambient analysis, protein or peptide analysis has been achieved in a few cases owing to the ionization process involved (i.e., the amount of internal energy deposited into a protein). In the following subsection, we will focus on instrumentation, ionization mechanisms, and the successful applications on protein analysis of various ambient methods.

1.4.1 Desorption Electrospray Ionization (DESI)

DESI allows to record spectra of condensed-phase samples (pure, mixtures, or tissue) under ambient conditions, making the samples accessible during analysis for manipulation by ordinary physical or chemical means [118,143–146]. Analysis of small and large molecules, very short analysis time (high-throughput), high selectivity (reactive-DESI and MS/MS), and sensitivity are other attributes of this method. The DESI method is based on directing a pneumatically-assisted electrospray onto a surface (e.g., paper, metal, plastic, glass, and biological tissue), from which small organics and large biomolecules are picked up, ionized, and delivered as desolvated ions into the mass spectrometer. Ions are generated by the interaction of charged microdroplets or gas-phase ions derived from the electrospray with neutral molecules of analyte present on the surface [116,118]. DESI is a soft ionization method and shows ESI-like spectra of proteins, primarily attributed to some common features of the ionization process that produces low-energy intact molecular ions through fast collisional cooling under atmospheric conditions [145]. The method can be used for many types of compounds (polar/nonpolar, and low/high molecular weight) in forensics and homeland security (e.g., explosives, chemical warfare agents, bacteria) [118,120,121,146–148], biomedical (e.g., tissue imaging, proteomics, lipidomics, pathology) [26,149–153], pharmaceutical/industrial (e.g., drug analysis, pharmacokinetics, polymers, process monitoring, metabolomics, environmental analysis) [107,154–158], and other fields. Many of these applications can be implemented with various mass spectrometers, including triple quadrupoles [159], linear ion traps [160], Orbitrap [161], quadrupole time of flight (QTOF) [162], ion-mobility/TOF, and ion-mobility/QTOF hybrids [162], Qtraps [122], Fourier transform ion cyclotron resonance (FTICR) instruments [163], and miniature ion trap mass spectrometers [164].

DESI Ionization Source In a typical DESI setup (Figure 1.9A) the source consists of a solvent nebulizer made of deactivated fused-silica capillary, similar to the one used in ESSI [103]. Nitrogen (N_2) is used as the nebulizing gas at a linear velocity of approximately 350 m/s. The solvent (typically mixtures of methanol, water, and small amount of acetic acid) is sprayed under the influence of an applied high voltage (typically in the range 3–6 kV). The gas jet composed of electrosprayed aqueous microdroplets and free gas-phase ions is directed onto the analyte-containing surface at various incident angles (usually from as low as 25° up to 80° depending on the analyte) to the normal. The resulting droplets, ions, and neutrals are collected at a shallow angle from the surface. The ions are then transferred as a result of electrostatic and pneumatic forces to a mass spectrometer equipped with an atmospheric pressure interface. The source is typically mounted on an *xyz*-moving stage, allowing it to be positioned at any chosen point with respect to the sample. The moving stage also has a tangent arm drive miniature stage that allows precise angular adjustment from 0 to 90° (Figure 1.9A).

DESI Ionization Mechanisms Droplet pickup has been suggested as the primary ionization mechanism in DESI, although there is evidence for chemical

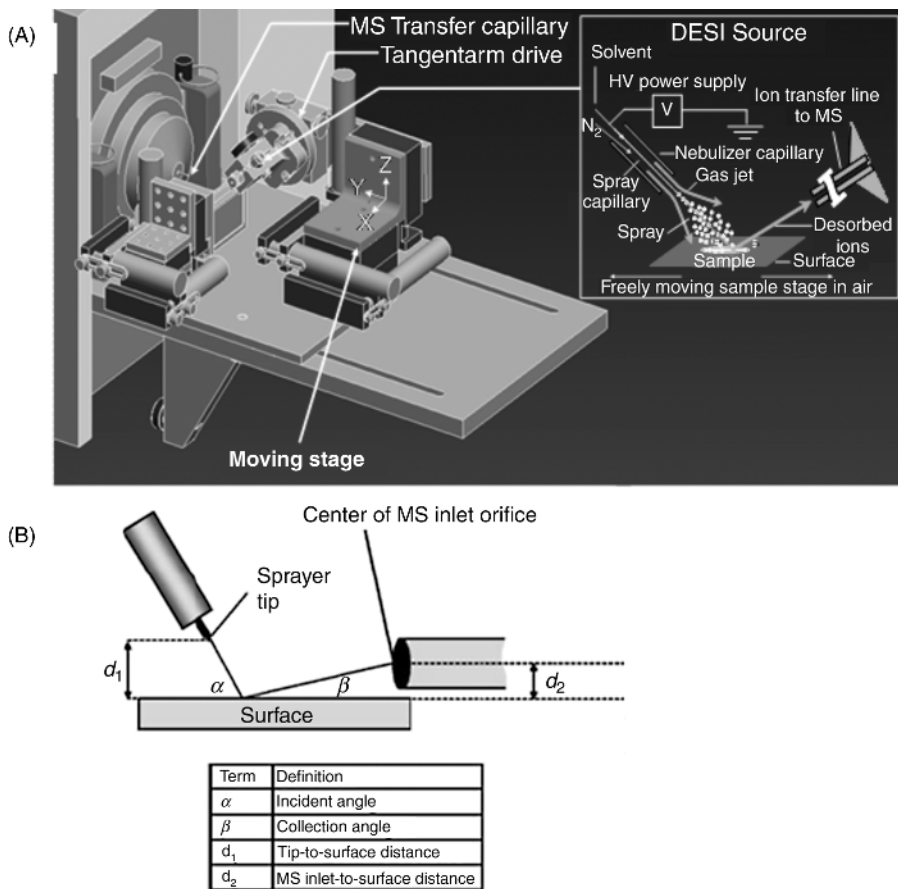


FIGURE 1.9 (A) DESI source and moving stage used to position the source; an early prototype of the OmniSpray source of Prosolia, Inc. The source is fitted with an ion-transfer capillary. From [118]. Copyright permission was obtained from ACS. (B) Definitions of terms used in conjunction with DESI. From [144]. Copyright permission was obtained from Wiley.

sputtering (reactive ion–surface collisions) and gas-phase ionization processes (e.g., charge transfer, ion–molecule reactions, volatilization/desorption of neutrals followed by ionization) [116,118,144,165,166]. According to the droplet pickup mechanism, the surface is pre-wetted by initial droplets (velocities in excess of 100 m/s and diameters of less than 10 μm), forming a solvent layer that helps surface analytes become dissolved. These dissolved analytes are picked up by later arriving droplets that are impacting the surface, creating secondary droplets containing the dissolved analytes. Gas-phase ions are then formed from these secondary droplets by ESI-like mechanisms [144,165,166]. The resulting gas-phase ions have internal energy values similar to those in ESI and ESSI [167]. The formation of cold ions gives DESI its soft

ionization character that affords ESI-like spectra, especially for proteins and polypeptides.

DESI Analytical Performance Signal intensity in DESI spectra depends on incident angle (β), collection angle (α), tip-to-surface distance (d_1), MS inlet-to-surface distance (d_2), and other geometric parameters, as defined in Figure 1.9B. Nebulization gas velocity, spray solvent flow rate, and spray potential also affect performance. The type of surface analyzed (its texture and electrical conductivity) is also a factor that affects the ionization process. The limits of detection (LODs) are in the low picogram to femtogram range for small molecules and some biopolymers [116,168]. The dynamic range is five orders of magnitude, and relative standard deviations (RSD) of 5% for quantitation (lower if using an internal standard) can be achieved [144]. For imaging applications, spatial resolution approaching 40 μm can be obtained [169]. Accuracies in the range of $\pm 7\%$ relative errors are possible [116,170].

DESI for Protein Analysis Protein and peptides show ESI-like spectra when analyzed by DESI, which is in part due to the ionization mechanism that takes place in DESI (droplet pickup or analyte microextraction into solution). Since the first reported applications of DESI for protein and peptide analysis [116,144], various research groups implemented applications ranging from solid-sample analysis (from surfaces) to direct analysis of liquid samples or liquid films [122,123,171–174]. An additional feature of liquid DESI is that it is easy to desorb large proteins directly from solution (Figure 1.10) [122]. For example, high mass proteins (e.g., BSA with MW of 66 kDa) appear to be relatively easily desorbed and ionized from solution than from dried samples on surface, probably due to less aggregation in solution than in the solid form [122]. Low detection limits and minimal sample preparation can apply to the

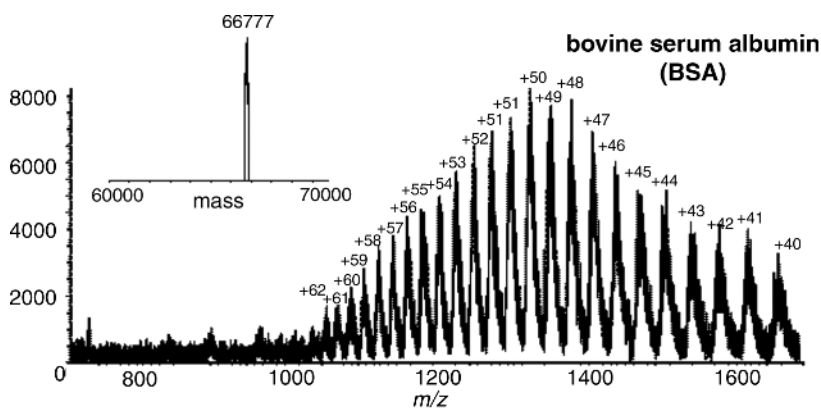


FIGURE 1.10 MS spectra showing the direct DESI-MS analysis of solutions containing bovine serum albumin (BSA). The insets show the corresponding deconvoluted spectra. From [122]. Copyright permission was obtained from Elsevier.

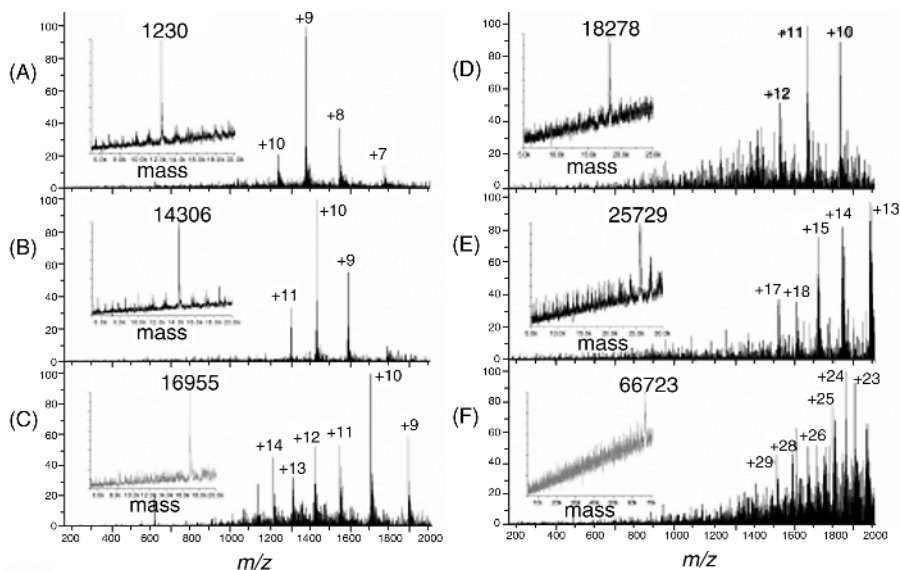


FIGURE 1.11 DESI-mass spectra and corresponding deconvoluted mass of intact proteins: (A) cytochrome *c* ($> 10 \text{ ng/mm}^2$), (B) lysozyme ($> 10 \text{ ng/mm}^2$), (C) apomyoglobin ($> 50 \text{ ng/mm}^2$), (D) α -lactoglobulin B ($> 100 \text{ ng/mm}^2$), (E) chymotrypsinogen A ($> 100 \text{ ng/mm}^2$), and (F) BSA ($> 4000 \text{ ng/mm}^2$). Proteins were deposited on a Plexiglas surface. From [159]. Copyright permission was obtained from ACS.

analysis of proteins from solid surfaces [159]. Basile et al. [159] evaluated the DESI response for the detection of proteins ranging in molecular mass from 12 to 66 kDa (Figure 1.11) and found detection limits that decrease with decreasing protein molecular mass. High mass resolving power can be obtained in protein and peptide identification by coupling DESI with Fourier transform ion cyclotron resonance mass spectrometry [163]. Other applications of peptide analysis can be envisioned for the direct identification of tryptic digests; examples are cytochrome *c* and myoglobin deposited on HPTL plates. After separation on the HPTL plates, the resulting bands are exposed to the DESI sprayer for peptide identification.

1.4.2 Fused-Droplet Electrospray Ionization (FD-ESI)

Fused-droplet electrospray ionization (FD-ESI) [138,175], a two-step electrospray ionization method [176,177], evolved from multiple-channel electrospray ionization (MC-ESI) [113,178–180]. In the multiple channel experiment, the analyte sample is introduced into one spray channel while other surrounding channels are used to generate the charged droplets that are fused with the analyte sample spray to form newly created droplets containing the analyte. Separating the ionization and nebulization process, Shiea and coworkers developed the newer ionization source, FD-ESI, in 2002 [175]. In the first step, the sample solution is ultrasonically nebulized to form fine aerosols that are transported to the skimmer of the mass spectrometer. These neutral

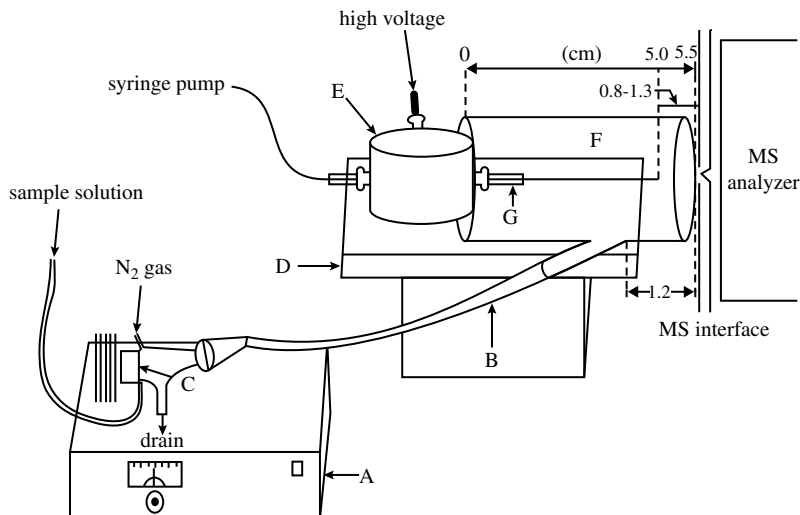


FIGURE 1.12 Schematic diagram of fused-droplet electrospray ionization mass spectrometry (FD-ESI-MS): (A) ultrasonic nebulizer, (B) Teflon tube, (C) piezoelectric transducer, (D) acrylic plate, (E) three way tee, (F) glass reaction chamber, (G) electro spray capillary in a Teflon tube. From [175]. Copyright permission was obtained from ACS.

aerosols are then fused in a reaction chamber with charged methanol droplets generated by electrospray. In the second step of a two-step process, ESI occurs for the newly created droplets, leading to the production of analyte ions [138,175].

FD-ESI Ionization Source A typical setup for FD-ESI (Figure 1.12) [175] consists of four parts, a traditional ESI source, a sample nebulizer assembly, a reaction chamber, and a mass spectrometer. The aqueous protein sample solution is pumped at an adjustable flow rate onto the surface of the piezoelectric transducer of an ultrasonic nebulizer to generate fine aerosols, which are subsequently transported with carrier nitrogen gas through the sidearm, a Teflon tube, into the reaction chamber. The end of the glass reaction chamber is positioned directly in front of the sampling skimmer of a quadrupole mass spectrometer. The solvent such as methanol containing 1% acetic acid is electrosprayed continuously from a fused-silica capillary that is located at the center of the glass reaction chamber.

A modified FD-ESI apparatus [138] has advantages in providing salt tolerance for biological analysis. By replacing the ultrasonic nebulizer that generates the analyte aerosols with a pneumatic nebulizer from a commercial atmospheric pressure chemical ionization (APCI) probe [138], one can reduce sample consumption by 10 times compared to unmodified FD-ESI. To prevent the buildup of air pollutants in the methanol and fine acidic aerosols in the open air, an exhaust extractor is used in the fusion area.

FD-ESI for Protein Analysis FD-ESI can successfully ionize peptides and proteins dissolved in pure water [176,178]. Extremely high salt tolerance appears

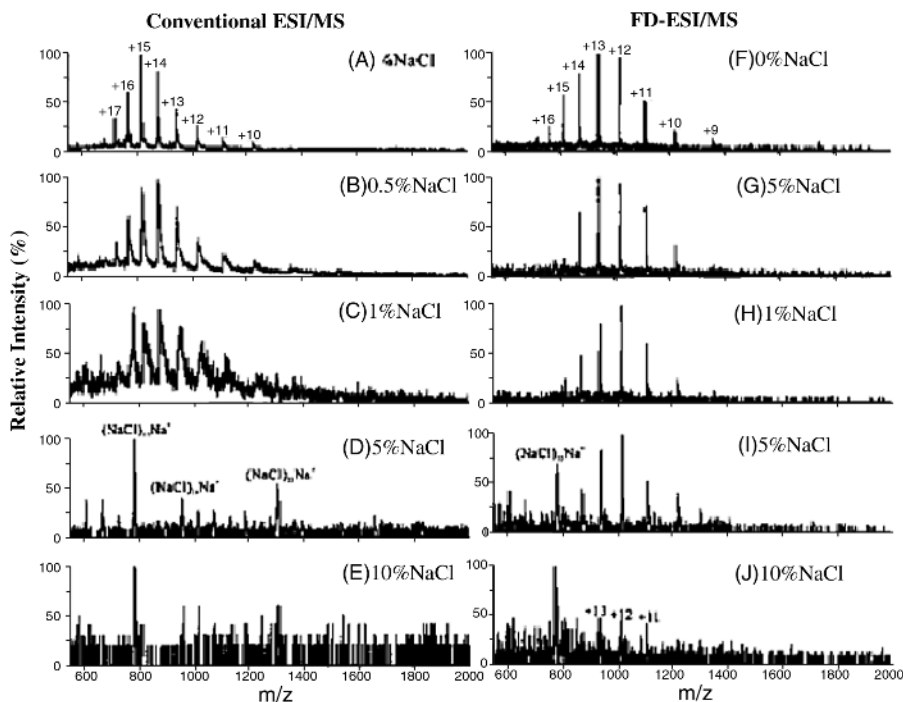


FIGURE 1.13 Positive ESI mass spectra of cytochrome *c* (10^{-6} M) that was dissolved in the aqueous solutions, which contained various amounts of NaCl (from 0 to 10% by weight). The mass spectra were obtained by conventional ESI-MS (A–E) and FD-ESI-MS (F–J). From [175]. Copyright permission was obtained from ACS.

to be one important advantage of FD-ESI compared to the traditional ESI in biological molecule analysis. As mentioned above, in ESI the moderate to large amounts of inorganic salts in the sample solution decrease the electrospray stability and sensitivity, owing to the formation of the various salt-protein adduct ions and the effect of ion suppression [138].

An example (Figure 1.13) is a comparison of conventional ESI and FD-ESI for the analysis of cytochrome *c* solution that contains NaCl at various concentrations. Using conventional ESI-MS, one finds that the mass spectra degrade with increasing NaCl concentration. When FD-ESI-MS is used to analyze these solutions, protonated proteins with nearly unchanged peak widths can still be observed, even when the NaCl concentration reaches 10% [175]. A similar desalting effect using organic spray solvent was seen in the liquid sample DESI experiment [181] whereby the liquid sample could be directly injected with no need of nebulization. The low solubility of inorganic salts in methanol spray solvent appears to exclude salt from the newly created fused droplets. Therefore, in FD-ESI the composition of the electrospray solvent is more important than that of sample solution in determining the ionization efficiency, and by adjusting the composition of the electrospray solvent, good quality

mass spectra can be obtained [138]. The disadvantage for FD-ESI, however, is that sample consumption exceeds that of the conventional ESI experiment.

1.4.3 Electrospray-Assisted Laser Desorption Ionization (ELDI)

Electrospray-assisted laser desorption ionization (ELDI) [125,161,182–188], another ambient ionization method, can be used to analyze protein samples both in the solid phase and in solution. Originating from the principle that the protein ionization can be achieved by mixing protein solutions with the ESI plume in FD-ESI, laser ablation was applied to desorb the protein samples. The created neutral proteins undergo post-ionization when merged with the ESI plume. Thus desorption and ionization are separate processes for protein analysis in ELDI.

Solid ELDI in Protein Analysis In the original setup for solid ELDI (Figure 1.14A) [125], the solid protein sample, from deposition on the mobile support plate, is desorbed by a laser beam. The resulting neutral protein droplets are ionized by the charged droplets generated from the ESI, giving multiply charged proteins that are detected by a mass spectrometer. By optimizing different distances and angles as well as the electrospray solvent composition, multiply charged cytochrome *c* ions can be successfully detected (Figure 1.14B).

Liquid ELDI in Protein Analysis Liquid ELDI allows the desorption and ionization of proteins from their native biological environment under ambient conditions [183]. In the liquid ELDI experiments, a small amount of protein solution, deposited onto the sample plate and mixed with the inert particles that serve as the matrix, is submitted to laser ablation. The laser energy is adsorbed by the inert particles and transferred to the surrounding solvent and analyte molecules for desorption. The desorbed neutral proteins are post-ionized by an ESI plume, producing multiply charged proteins. Given the high salt tolerance of ELDI and the effect of the ESI solvent, better-quality protein mass spectra (Figure 1.15) can be obtained in the analysis of proteins from human blood, tears, and bacteria extract than with traditional ESI and MALDI [183].

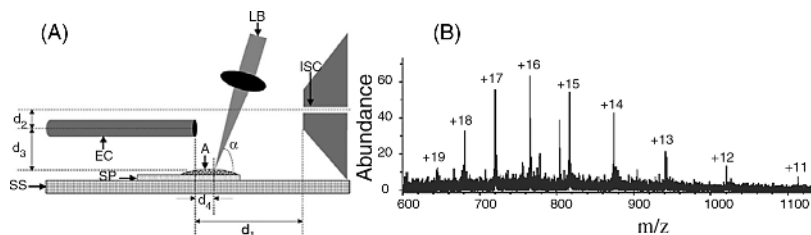


FIGURE 1.14 (A) Graphic representation of the geometry of the ELDI setup. analyte sample (A), sample support plate (SP), mobile sample stage (SS), laser beam (LB), electrospray capillary (EC), ion sampling capillary (ISC); (B) Solid ELDI mass spectrum of cytochrome *c*. From [125]. Copyright permission was obtained from Wiley.

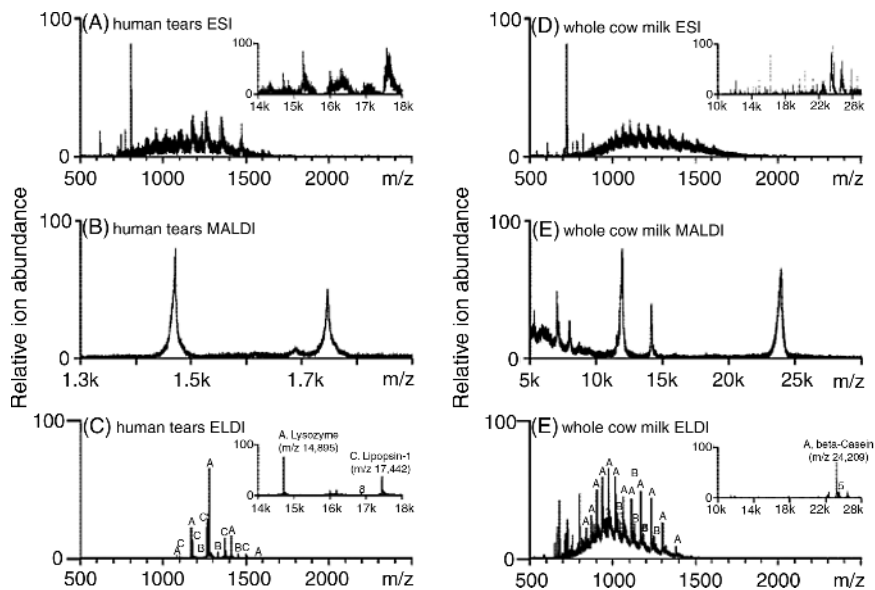


FIGURE 1.15 Positive ELDI mass spectra of human tears and whole cow milk; for comparison, conventional ESI and MALDI mass spectra of these biological fluids are also presented. From [183]. Copyright permission was obtained from ACS.

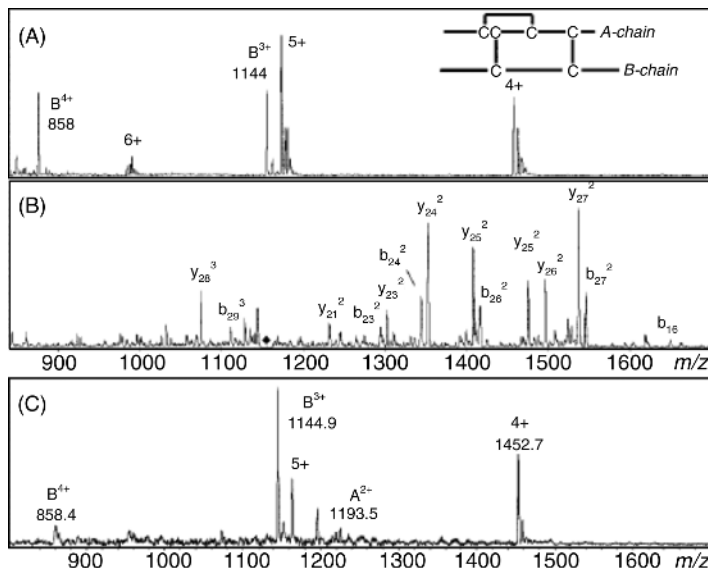


FIGURE 1.16 Reactive-ELDI experiments for online disulfide reduction of insulin with DTT. (A) Reactive-ELDI for disulfide reduction of insulin with DTT in the ESI solution yield new peaks at m/z 858 (insulin B-chain, 4^+) and 1144 (insulin B-chain, 3^+); (B) subsequent CADMS/MS of m/z 1144 confirmed the B-chain identity; (C) a solution of DTT was deposited onto the sample plate and desorbed by laser irradiation while insulin was electrosprayed. From [188]. Copyright permission was obtained from ACS.

Reactive-ELDI Similar to reactive DESI, some gas-phase reactions can be integrated in the ionization process of ELDI [187,188]. For reactive ELDI, the reactant ions are generated from either the ESI plume or the desorbed solution sample. Online disulfide bond cleavage of insulin can be achieved successfully via reactive ELDI in which the protein samples are either electrosprayed to react with laser-desorbed DTT or desorbed by laser irradiation followed by the reaction with sprayed DTT (Figure 1.16) [188]. Reactive ELDI can also be used to monitor other reactions including small-molecule reactions [187].

There are some interesting applications using ELDI, including coupling MS analysis with TLC [182] and chemically imaging different solid surfaces [189]. By

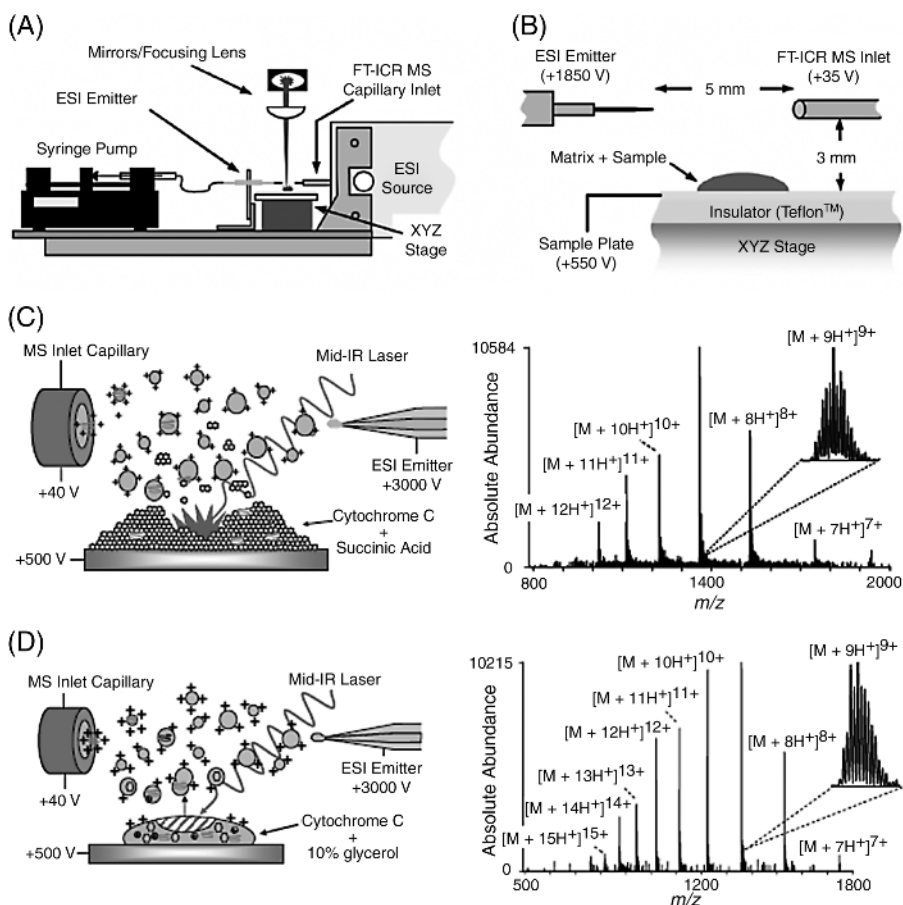


FIGURE 1.17 (A) Front and (B) side detailed views of the MALDESI Source. From [191]. Copyright permission was obtained from Elsevier. Schematic of (C) solid-state IR-MALDESI with ESI post-ionization and representative mass spectrum of bovine cytochrome *c* mixed with succinic acid. (D) liquid-state IR-MALDESI with ESI post-ionization and representative mass spectrum of bovine cytochrome *c* mixed with 10% glycerol. From [192]. Copyright permission was obtained from Elsevier.

optimizing the laser desorption energy, the composition of the electrospray solvent, and the matrix, intact protein ions can be observed with high sensitivity [184,185].

1.4.4 Matrix-Assisted Laser Desorption Electrospray Ionization (MALDESI)

MALDESI [126,190–193], a hybrid atmospheric pressure ionization method, combines the desirable attributes of ESI, MALDI, and ELDI into an integrated pulsed ionization source that generates multiply charged ions [191]. The MALDESI ion source is similar to the ELDI setup (Figure 1.17A and B), and the key to distinguishing between the two is that matrix is not necessary for ELDI whereas it is required for MALDESI [191]. For the latter, the analyte is deposited on the surface that is to be exposed to laser ablation, and the desorbed analyte then undergoes post-ionization by an ESI plume to generate multiply charged ions for MS detection.

MALDESI for Protein Analysis With minimal preparing of biological samples and by avoiding subjecting sensitive samples (i.e., tissue) to high vacuum, one can directly ionize samples in the solid or liquid states by using MALDESI (Figure 1.17C and D). For example, one can deposit a cytochrome *c* solution on a stainless steel target, dry it under open air, and analyze the sample by MALDESI to obtain multiply charged cytochrome *c* molecules.

1.5 CONCLUSIONS

One can see that a variety of protein ionization techniques based on MALDI or ESI are evolving. Because this field is rapidly developing, it is not possible to cover all protein ionization methods, and the authors apologize for any omissions. As the performance of the current MS ionization technologies, although highly effective, cannot meet all real-world demands in biochemistry and molecular biology, we can expect protein ionization methods to undergo further development; the only limitation seems to be our imagination [36].

ACKNOWLEDGMENTS

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