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Direct Protein Detection from Biological Media through Electrospray-Assisted Laser Desorption Ionization/Mass Spectrometry

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We report here using a novel technology—electrospray-assisted laser desorption ionization (ELDI)/mass spectrometry—for the rapid and sensitive detection of the major proteins that exist in dried biological fluids (e.g., blood, tears, saliva, serum), bacterial cultures, and tissues (e.g., porcine liver and heart) under ambient conditions. This technique required essentially no sample pretreatment. The proteins in the samples were desorbed using a pulsed nitrogen laser without the assistance of an organic matrix. The desorbed protein molecules were then post-ionized through their fusion into the charged solvent droplets produced from the electrospray of an acidic methanol solution; electrospray ionization (ESI) proceeded from the newly formed droplets to generate the ESI-like protein ions. This new ionization approach combines some of the features of electrospray ionization with those of matrix-assisted laser desorption ionization (MALDI), that is, sampling of a solid surface with spatial resolution, generating ESI-like mass spectra of the desorbed proteins, and operating under ambient conditions.

Keywords: electrospray-assisted laser desorption/ionization • ELDI • post-ionization • laser desorption • two-step ESI • FD-ESI

Introduction

The two most promising ionization methods used in modern proteomics for the mass spectrometric characterization of proteins in biological samples are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI).[1−7] Since these two ionization methods sample proteins in different phases (the solution phase for ESI and the solid phase for MALDI), they are often used as complementary techniques. In this paper, we describe a new desorption/ionization method—electrospray-assisted laser desorption ionization (ELDI)—that combines some of the features of ESI with those of MALDI; this technique provides the advantage of allowing the direct, sensitive, and rapid characterization of major proteins in biological samples such as dried biological fluids, bacterial cultures, and tissues. When this technique is used, tedious sample pretreatment procedures, such as extraction, concentration, and filtration, can be avoided.

The principle of ELDI is based on one of our previous designs—fused-droplet electrospray ionization (FD-ESI; or two-step electrospray ionization)—which is a technique that combines aspects of both atmospheric pressure chemical ionization (APCI) and ESI.[8−10] In the FD-ESI source, gaseous analytes or neutral droplets containing the analytes (generated by a pyrolyzer or nebulizer) are conducted to the tip of an electrosprayer, where they are post-ionized through fusion or reaction with the charged solvent droplets or protonated solvent species generated through ESI; electrospray ionization processes then proceed from the newly formed droplet. The detection of multiply charged protein ions (i.e., obtaining ESI-like mass spectra) when using the FD-ESI approach supports the suggested ionization mechanism.[8−10]

One of the advantages of using FD-ESI for biological sample analysis is that the ionization and nebulization processes are separate events; this feature provides independent control over the condition of the sample solution and the composition of the ESI solvent. By varying the method of introducing the sample, unique applications have been demonstrated for liquid, gas, and solid sample analyses.[11−14] For example, nebulizing a protein solution through the use of an ultrasonic or numerical nebulizer can be used to produce neutral droplets containing protein molecules; after fusion, interference from small organic and inorganic compounds, such as NaCl, NH_4Cl, Tris, and SDS, can be eliminated successfully by using a less-polar solution, such as acidic methanol, for ESI.[11,12] In another example, the analysis of fatty acid mixtures was achieved through GC/ESI/MS, in which the gaseous molecules exiting the gas chromatograph (GC) were directed into the FD-ESI source.[13] A third example of the use of FD-ESI/MS involves the selective

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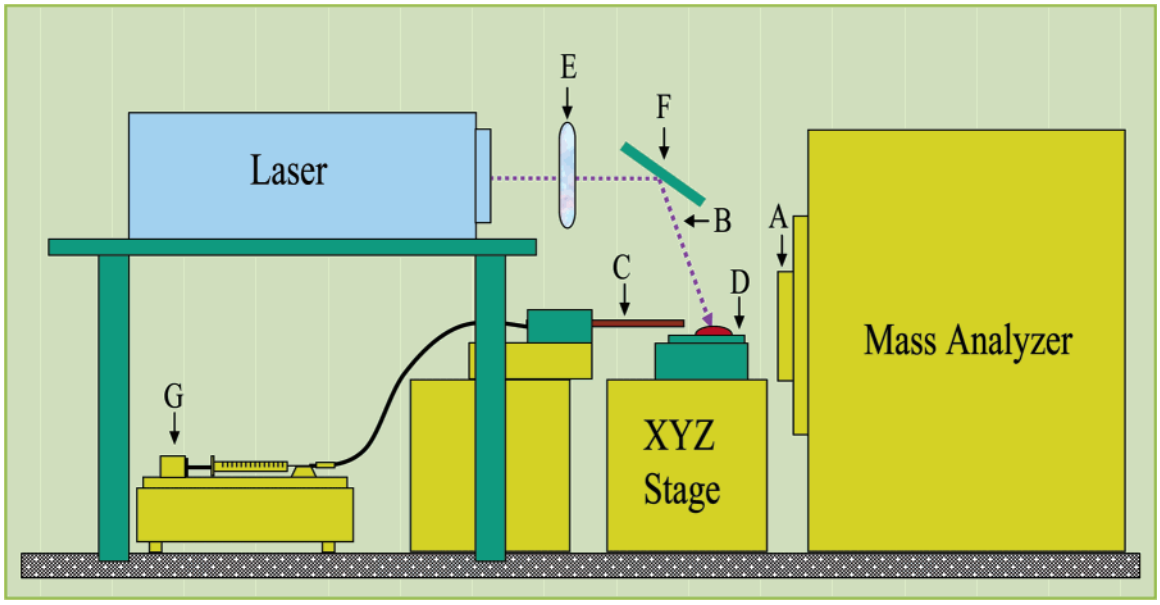

Figure 1. Detailed illustration of the ELDI MS setup. (A) Sampling skimmer of a mass analyzer; (B) nitrogen laser beam; (C) electrospray capillary; (D) sample plate; (E) focusing lens; (F) reflecting lens; (G) syringe pump.

ionization and rapid characterization of highly reactive ketenes and polar pyrolysates of the synthetic polymers passed through a flow pyrolyzer.[14,15]

An advantage of using FD-ESI combined with chromatography and pyrolysis for the analysis of the chemical compounds present in solid samples is that polar components in the gaseous pyrolysates can be ionized selectively through ESI; however, information regarding the spatial distribution of specific polar compounds in the solid sample is lost. This situation arises because the solid sample is heated rapidly at high temperature during pyrolysis, and therefore, the detected ion signals are an average of those from the whole sample. In addition, the technique cannot be used for protein analysis because proteins decompose at high temperature during pyrolysis. Solving these problems requires an energy source that is capable of providing spatial resolution and rapid energy input, that is, one that will generate gaseous intact protein molecules from a defined area on the biological sample's surface. An added bonus of developing such a technique is its potential ability to perform direct protein profiling or protein imaging of tissues.[16−20]

Laser desorption (LD) allows sampling over a small and defined area and, therefore, guarantees high spatial resolution of a solid sample. Unfortunately, only relatively small biological and chemical compounds (e.g., <3000 Da) can be detected through direct LD/MS analyses.[21−25] The failure to detect the signals of ions of larger molecules, such as proteins, after LD may be due to their low ionization efficiencies, their decomposition induced by the laser energy, or their rapid neutralization during the desorption/ionization processes. It has been reported, however, that a larger number of neutral molecules are produced during a laser desorption event, and consequently, several different approaches have been applied to post-ionize these neutral organic species.[26−30] Unfortunately, again, only small compounds are ionized and detected when using these approaches.

Desorption electrospray ionization (DESI) mass spectrometry, recently reported by Cooks et al., is capable of providing signals for protein ions at the surfaces of soft solids, but this technique lacks high spatial resolution; in addition, the desorption energy upon bombarding the sample surface with the charged droplets may not be sufficient to desorb the proteins tightly bound in tissues.[31−33] Because ESI can be used to generate protons and multiply charged droplets for the post-ionization of gaseous molecules in FD-ESI, we turned our attention to using a UV pulse from a nitrogen laser to desorb intact protein molecules from solid biological samples and then to post-ionize the gaseous protein molecules through ESI; we call this approach "electrospray-assisted laser desorption ionization" (ELDI).[34] In this paper, we reported the results of using ELDI to qualitatively analyze the predominant proteins in various biological media including dried biological fluids (blood, tear, saliva, serum), bacterial cultures, and tissues (porcine liver, and heart) under ambient conditions.

Experimental Section

Sample Preparation. The protein standards, chemicals, and organic solvents (HPLC grade) were purchased commercially (Sigma or Aldrich, St. Louis, MO) and used without further purification. To use ELDI to characterize protein in the solution, the sample solution (4 μL of protein solution or biological fluid) was spread uniformly by pipetting it over a ca. 0.2-cm^2 surface area of a stainless steel sample plate. The sample solution was then air-dried. The selected bacterial colony (diameter: ca. 2 mm) was scratched from the culture media in a Petri dish and applied evenly on the sample plate. For tissue analysis, a randomly selected tissue (ca. 10 × 2 × 2 mm) was cut by a razor and placed directly on the acrylic sample plate.

Instrumental Setup for ELDI. The setup of the ELDI instrument is displayed in Figure 1. The sample plate was first positioned on an XYZ-stage in front of the sampling capillary of an ion trap or Q-TOF mass analyzer. The sample area was then exposed to a pulsed nitrogen laser (337 nm) operated at 10 Hz (controlled by a sweep function generator), a pulsed energy of ca. 150 μJ, and length of 4 ns. The laser beam (spot size: ca. 100 μm × 150 μm) was focused through an objective lens. The laser-ablated materials were post-ionized in the electrospray solvent plume [methanol/water solution (1:1, v/v)

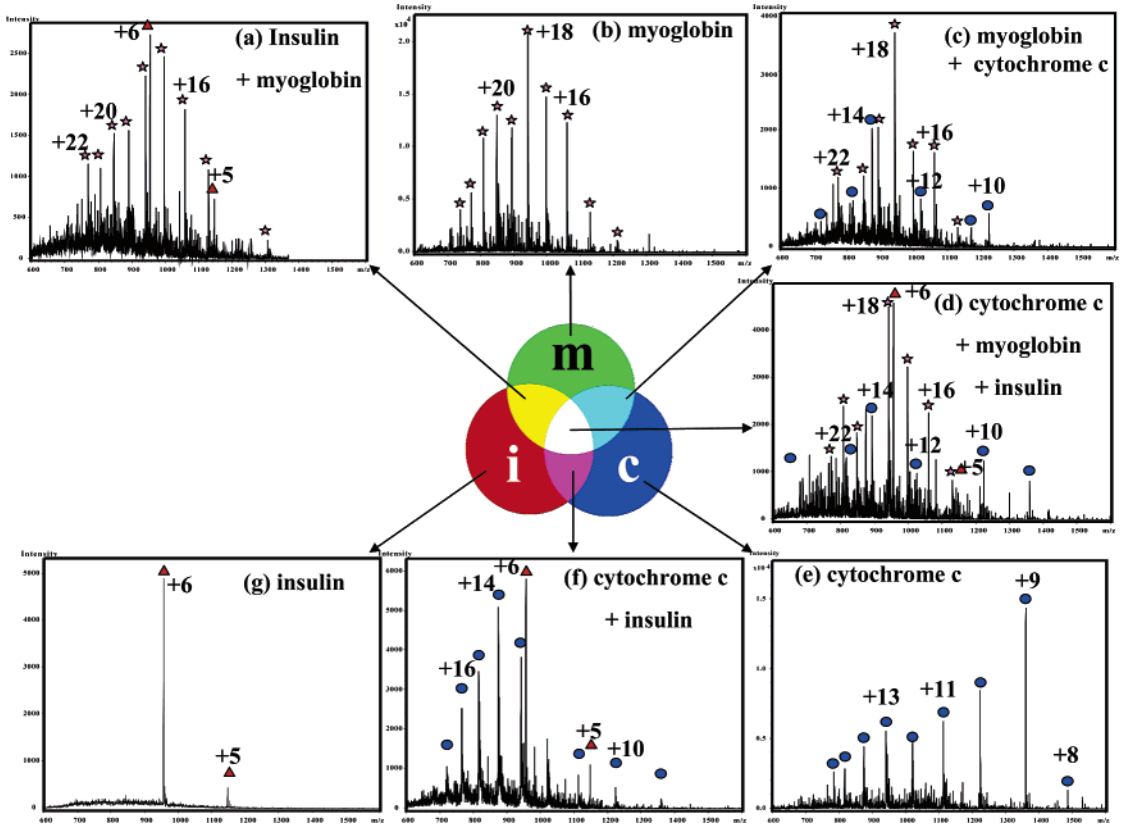

Figure 2. Positive ELDI mass spectra recorded from dried samples spots containing protein standards; the concentration of each protein was 10^{-4} M. (a) A mixture of insulin and myoglobin; (b) myoglobin; (c) a mixture of myoglobin and cytochrome *c*; (d) a mixture of cytochrome *c*, myoglobin, and insulin; (e) cytochrome *c*; (f) a mixture of cytochrome *c* and insulin; (g) insulin.

containing 0.1% acetic acid]. The solvent was delivered through the electrospray capillary at a flow rate of 150 μL/h. A nebulizing gas, commonly used in conventional ESI, was not used during ELDI. The ESI plume was directed toward the ion sampling orifice (i.e., parallel to the sample plate). The resulting ions were sampled into the mass analyzer through the ion sampling capillary. The electrospray needle and the sample plate voltages were both held at 0 V (grounded), and the sampling cone voltage in the ion trap and Q-TOF mass analyzer was maintained at −4.5 kV. The parameters that influence the ion sensitivity in ELDI include the nitrogen laser's energy and frequency; the incident laser angle relative to the sample plate; the composition of the ESI solution and flow rate; the size of the ESI plume; and the relative distances between the ESI capillary tip and laser spot, the ESI tip and the entrance of the sampling skimmer, and the laser spot and the entrance of the sampling skimmer. In this study, however, because of the limited availability of equipment in our laboratory, we adjusted only a few of these parameters (e.g., the incident laser angle and the relative distances of each part) in an effort to maximize the ion signal. The strongest ion signal was obtained at an incident laser angle of ca. 45° and a focal length of 15 cm. The sample plate was moved during data acquisition to ensure that a fresh sample area was probed.

Protein Analysis through ELDI MS. The sample plate was positioned in front of the sampling capillary of the mass analyzer (Bruker Dalton Esquire 3000 plus ion trap or Bruker Dalton TOF Q), and the sample surface was irradiated with the pulsed nitrogen laser. To prevent the surface of the tissue sample from charring or depleting by the laser, the sample plate was manually moved (ca. 0.2 mm/s) during data acquisition to ensure that a fresh sample area was probed. Each mass spectrum was averaged over ca. 200 laser pulses. The laser-ablated materials were post-ionized through their interactions with the charged droplet plume generated upon electrospraying the methanol/water solution. The ESI plume was directed toward the ion sampling orifice (i.e., parallel to the sample plate). The ions generated in the ELDI source were directed toward the mass analyzer; the mass spectra were recorded at a scan rate of ca. 2 s/scan.

Protein Analysis through Conventional ESI−MS and MALDI MS. The proteins in the tissue were extracted with methanol; after concentration, the sample was subjected to conventional ESI (using a Bruker Dalton TOF Q mass spectrometer equipped with an ESI source) and MALDI (using a Bruker Dalton Autoflex MALDI/TOF) analyses. In the conventional ESI−MS analysis, the sample solution was transferred through a electrospray capillary (100 μm i.d.) at a flow rate of 150 μL/h. For MALDI MS analysis, an aqueous solution containing the protein extracts was mixed with an equal volume of saturated α-cyano-4-hydroxycinnamic acid solution (1 μL each); this solution was then applied to the MALDI sample target and dried under ambient conditions.

Results and Discussion

To perform protein analysis with ELDI under ambient conditions, a few drops of a solution containing the protein standard (10^{-4} M) were applied to a sample plate possessing a stainless steel surface; after drying, we analyzed the surface of

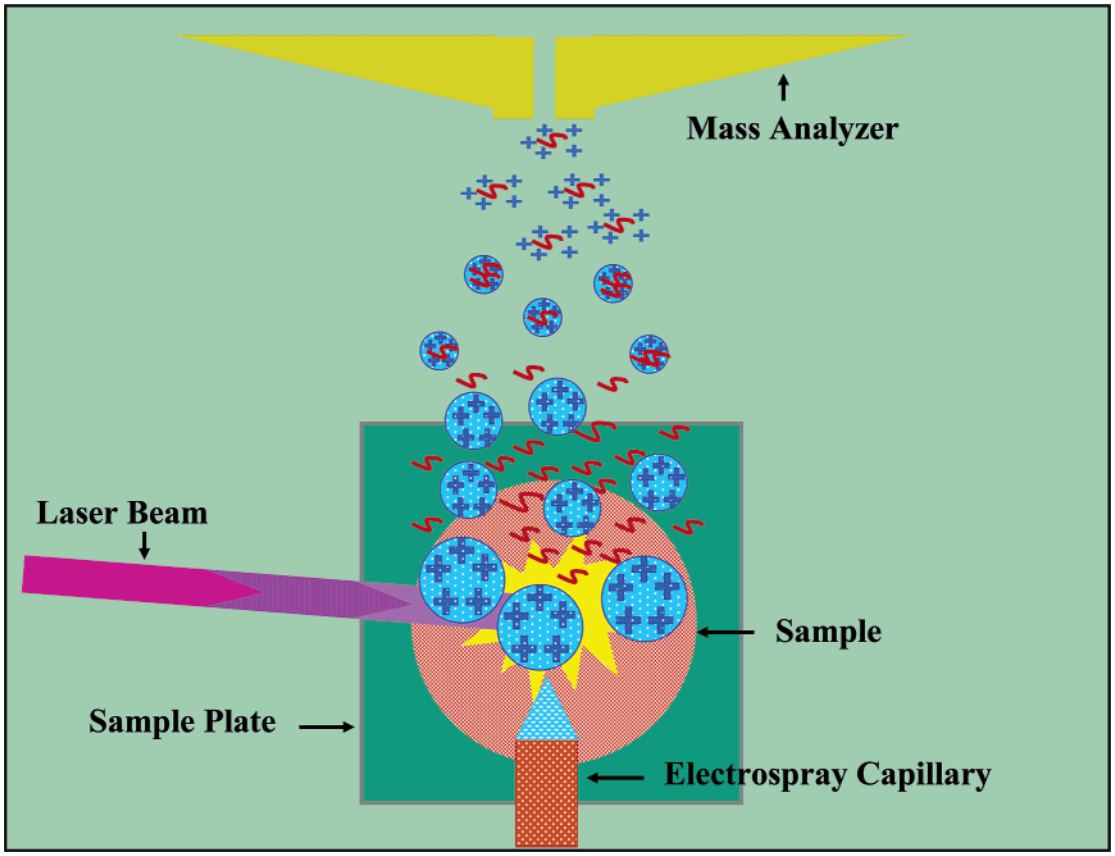

Figure 3. Schematic illustration (top view) of the ELDI MS technique for desorption and ionization of proteins from the surface of a solid. Protein molecules on the sample surface are desorbed upon irradiation with a nitrogen laser. The gaseous protein molecules fuse into the charged solvent droplets generated from an electrospray. Electrospray ionization proceeds from the charged droplets containing the sample molecule.

the sample spot through ELDI MS. No protein ion signal was observed when performing only the laser pulse or only ESI analysis.[34] In contrast, we obtained ESI-like protein mass spectra when the protein molecules were desorbed by the pulsed nitrogen laser and post-ionized by the charged droplets produced through ESI (Figure 2). The lack of protein signal when the ESI mode was applied alone indicates that DESI-like ion formation processes did not occur during ELDI. The detection of the protein ion signals when using ELDI indicates that intact protein molecules survive the LD event. That is, the complete decomposition of the protein molecule during LD did not occur. Figure 2 displays the ELDI mass spectra obtained from dried sample spots containing pure protein standards (myoglobin, cytochrome *c*, and insulin, respectively) and protein mixtures. The intensities of each protein's ion signals are nearly the same for those containing the insulin/myoglobin and insulin/cytochrome *c* mixtures (Figure 2a,f). This is rational since the concentration of the protein standard solutions is the same (i.e. ,10^{-4} M). But the presence of myoglobin ion suppressed the signal of the cytochrome *c* ion by ca. 50% (Figure 2c,d). These findings are similar to those of conventional ESI−MS analyses (data not shown).

We believe that the ionization of protein molecules in ELDI is similar to the process that occurs during fused-droplet electrospray ionization (FD-ESI), where gaseous neutral protein molecules desorbed through laser desorption are post-ionized by their fusion with charged solvent droplets and the subsequent ESI processes from the newly formed droplet. Detection of the multiply charged protein ions (i.e., an ESI-like mass spectrum) supports this suggested ionization process. Figure 3 illustrates our suggested mechanisms for the desorption/ionization of proteins through ELDI.

The detection limit of ELDI toward the analysis of a dried protein standard was estimated, using cytochrome *c* standards with different solution concentrations (from 10^{-4} to 10^{-9} M). We note that the detection limit of the ELDI approach was 10^{-8} M. This is similar to that of conventional ESI. However, rather than obtaining a complete multicharge protein ion series at low concentrations (e.g., 10^{-8} M), we observed only partial cytochrome *c* ion peaks on the ELDI mass spectra (see Figure 4c−e). In a typical ELDI experiment, 2 μL of the sample solution was applied to the sample plate; after drying, a circle was created having a diameter of ca. 2 mm. The desorption area for each laser pulse was focused to ca. 100−200 μm; assuming that all of the protein molecules were desorbed in this area, this process will provide ca. 50 amol of protein molecules for each ELDI mass spectrum. This detection limit also seems to be competitive with that of MALDI MS analysis. The decrease of cytochrom *c* ion signals with its solution concentration also suggests that directly semiquantitative protein analysis from solid by ELDI MS may be possible.

The ability to obtain protein ion signals directly from solids through ELDI suggests that protein molecules do not require the assistance of an organic matrix to desorb efficiently from the solids during LD. In fact, mixing the protein standard with a UV-absorbing organic acid (such as α-cyano-4-hydroxycinnamic acid, a common matrix used in MALDI) did not change the charge distributions of the cytochrome *c* ions in

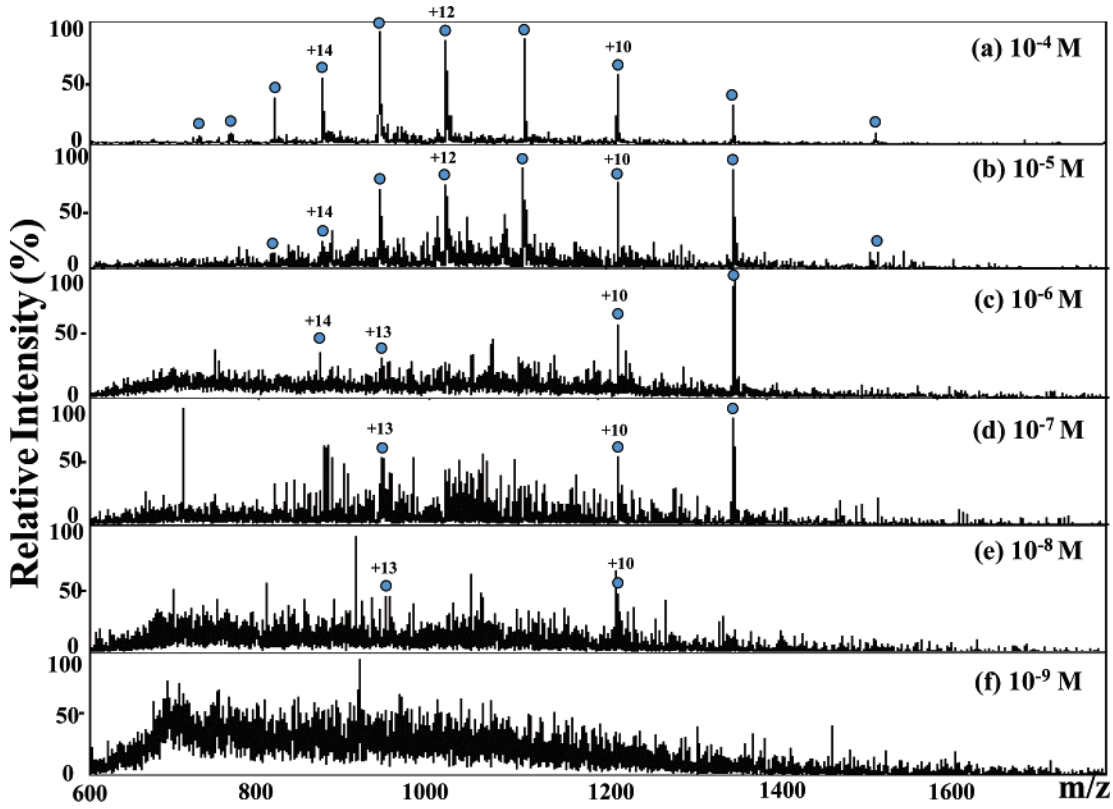

Figure 4. Positive-mode ELDI mass spectra recorded from dried sample spots obtained from cytochrome *c* standards of different solution concentrations: (a) 10^{-4}, (b) 10^{-5}, (c) 10^{-6}, (d) 10^{-7}, (e) 10^{-8}, and (f) 10^{-9} M.

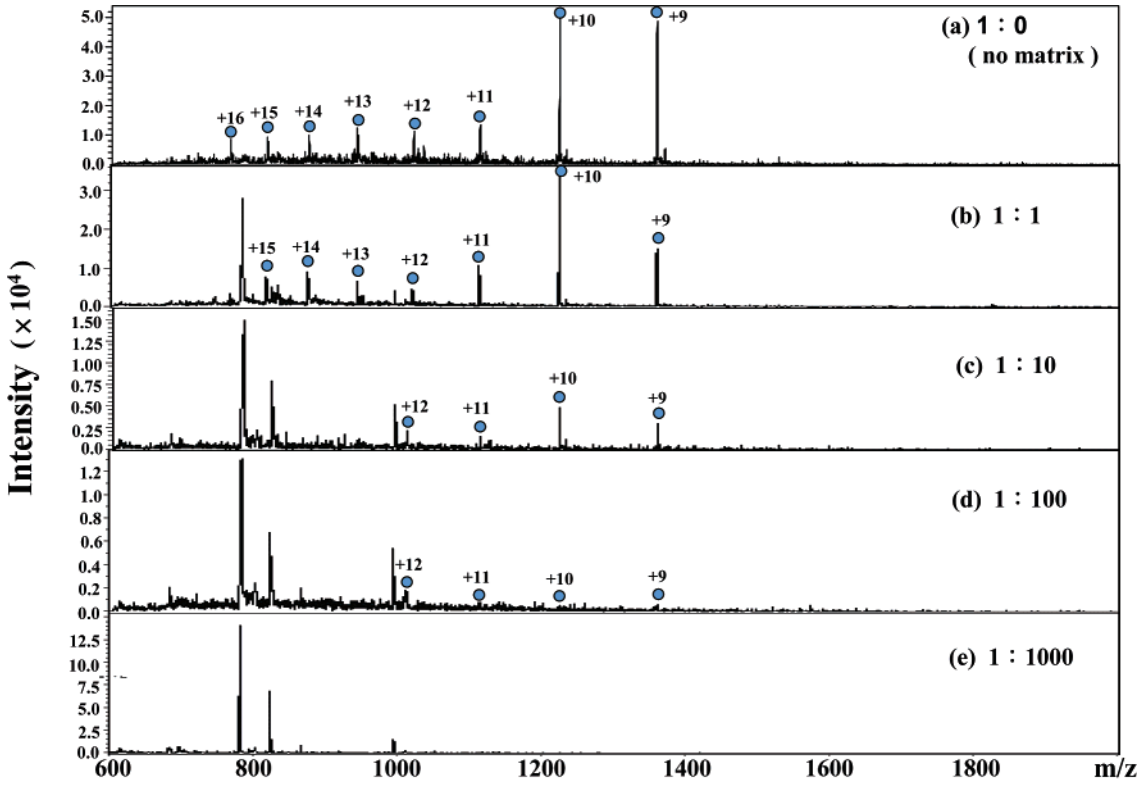

Figure 5. Positive-mode ELDI mass spectra of dried cytochrome *c* solutions (10^{-4} M). The sample solutions were prepared (a) in pure water and (b−e) after mixing the solution with saturated α-cyano-4-hydroxycinnamic acid solution at volume ratios of (b) 1:1, (c) 1:10, (d) 1:100, and (e) 1:1000.

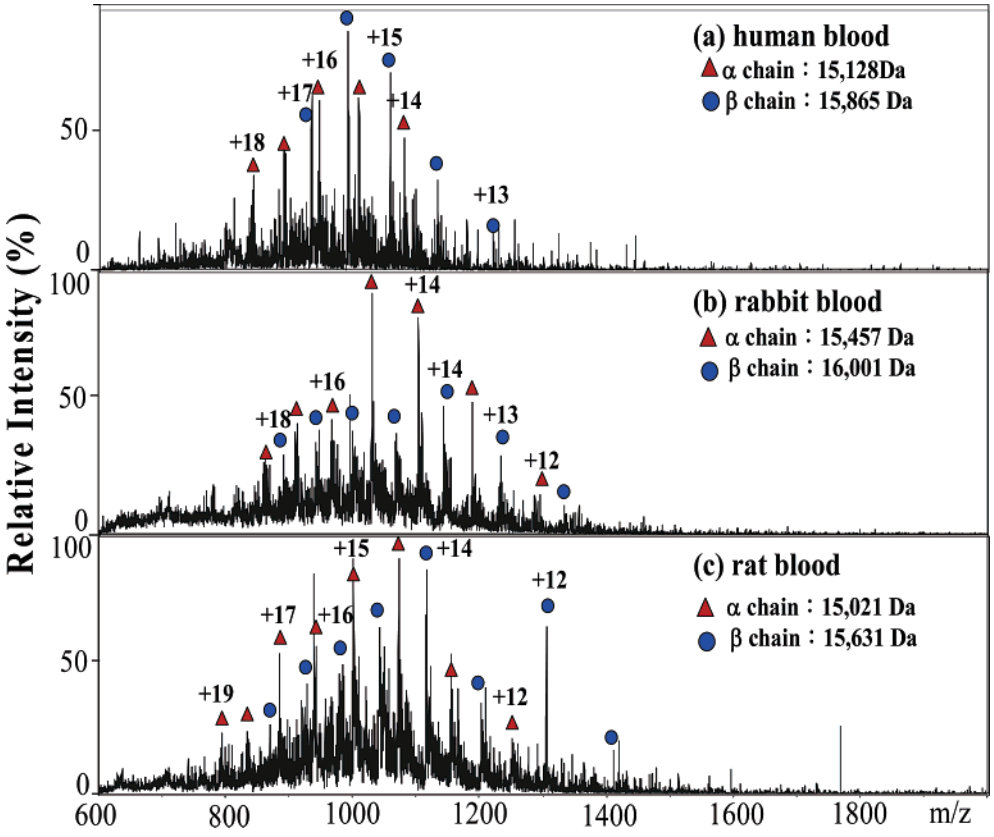

Figure 6. Positive-mode ELDI mass spectra of whole blood samples from (a) human, (b) rabbit, and (c) rat sources. Each sample was applied to the sample plate, and after drying it, the sample spot was analyzed through ELDI MS.

the ELDI mass spectra; the detection limit of cytochrome *c* was increased, however, upon increasing the amount of MALDI matrix in the sample solution (Figure 5). For example, the intensity of cytochrome *c* ion decreased ca. 100-fold when the protein standard was diluted by the same factor with α-cyano-4-hydroxycinnamic acid as the MALDI matrix (Figure 5d). The decrease in the intensity of the protein ions' signals upon addition of a MALDI matrix may be due simply to the diluting effect of the matrix molecules; that is, more molecules compete for the charge. In MALDI, the intact protein ion is both desorbed and ionized from the desorption area; thus, to enhance its ionization and prevent its decomposition, as a result of directly absorbing the laser energy, it is necessary to surround the protein molecules within a large amount of organic matrix during MALDI analysis. In ELDI, however, the laser energy is used only for desorption—the ionization of the protein molecule is performed after redissolving the molecule in the charged solvent droplet prior to subjecting it to further ESI processes; thus, it appears that the presence of the organic matrix in the sample is not as crucial as it is for MALDI. In fact, the results of our ELDI MS analyses indicate that gaseous intact protein molecules are generated during laser desorption in the absence of any protecting UV-absorbing organic matter.

In this study, we demonstrate several applications of ELDI MS for the direct characterization of proteins from complex biological matrixes. The major proteins present in dry biological fluids, such as blood, saliva, tears, and serum, can be characterized rapidly with only a minimal degree of sample pretreatment. One drop of the biological fluid was applied to the sample plate, and after drying it, the surface of the sample spot was analyzed using ELDI MS. The identification of the proteins is based on the previous literature reporting and the results of Swiss-Prot database searching. We observed two series of the protein ion signals from whole blood samples collected from human, rabbit, and rat sources, respectively (Figure 6); the molecular weights of these proteins matched well with each mammal's α and *β* hemoglobin chains.[32] Lysozyme was the predominant protein that we observed in both human tear and saliva (calculated MW: 14 682; Figure 7a and b).[35,36] We detected several intact protein ions, including albumin (calculated MW: 66 441), in human serum without subjecting it to any prior chromatographic separation (Figure 7c).[37]

Second, we used ELDI MS to rapidly characterize the major proteins present in different bacterial cultures. In these experiments, the selected bacterial colony in a Petri dish was scratched and applied to the sample plate and then subjected to ELDI MS analysis. Figure 8 displays the ELDI mass spectra of three different bacterial cultures: *Vibrio cholerae*, *Salmonella*, and *Streptococcus pyogenes*. It is clear that different bacterial colonies can be rapidly distinguished based on the differences in the molecular weights of their respective predominant proteins [hypothetical protein VCA0797 (theoretical MW, 11 090) for *V. cholerae*; PhoP regulated protein (theoretical MW, 12 072) for *Salmonella*; and copper chaperone (theoretical MW, 6481) for *S. pyogenes*]. Again, the identification of the bacterial proteins was based on the library search results through the Swiss-Prot database.

Finally, we used ELDI MS to rapidly characterize the major proteins present in two porcine tissues. We applied a piece of tissue, randomly selected and cut by a razor, onto the sample plate and then used ELDI MS to analyze the predominant proteins present on the surface of the tissue under ambient

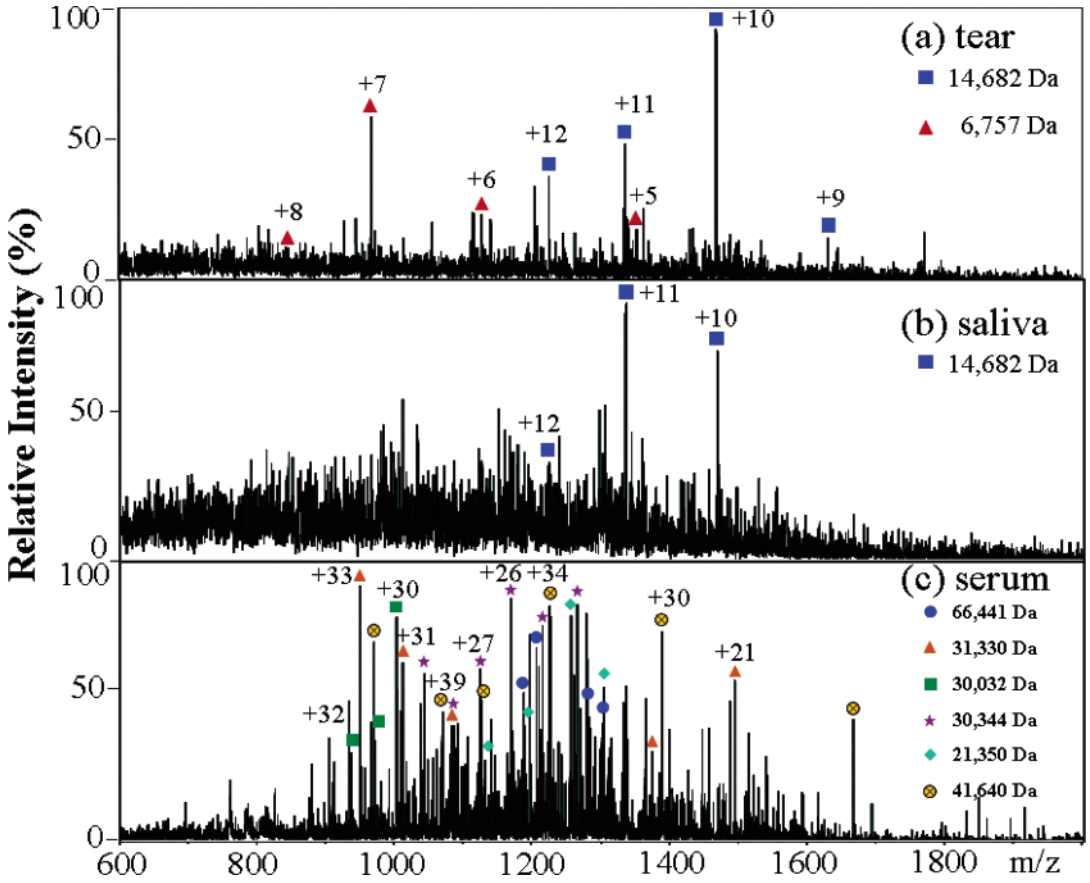

Figure 7. Positive-mode ELDI mass spectra of human biological fluids: (a) tear, (b) saliva, and (e) serum. Each sample was applied to the sample plate, and after drying it, the sample spot was analyzed through ELDI MS.

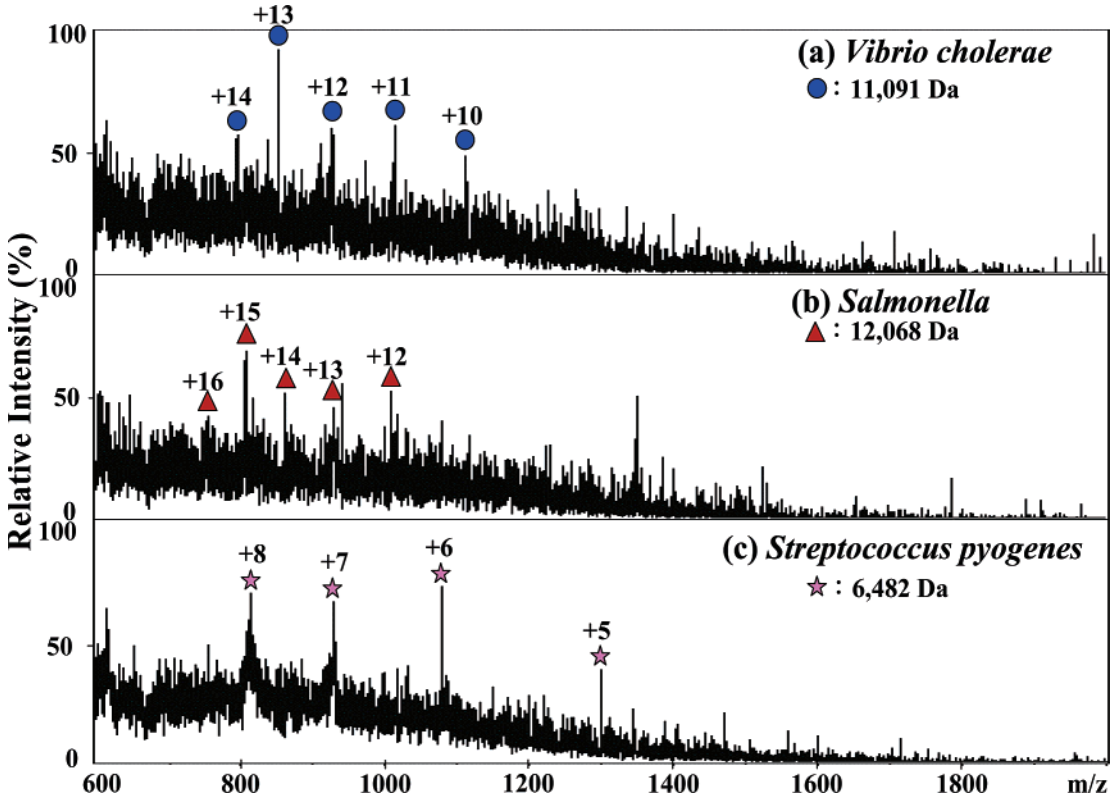

Figure 8. Positive-mode ELDI mass spectra of bacterial cultures: (a) *V. cholerae*, (b) *Salmonella*, and (c) *S. pyogenes*. The bacterial colony was scratched from its medium and spread on the sample plate prior to ELDI MS analysis.

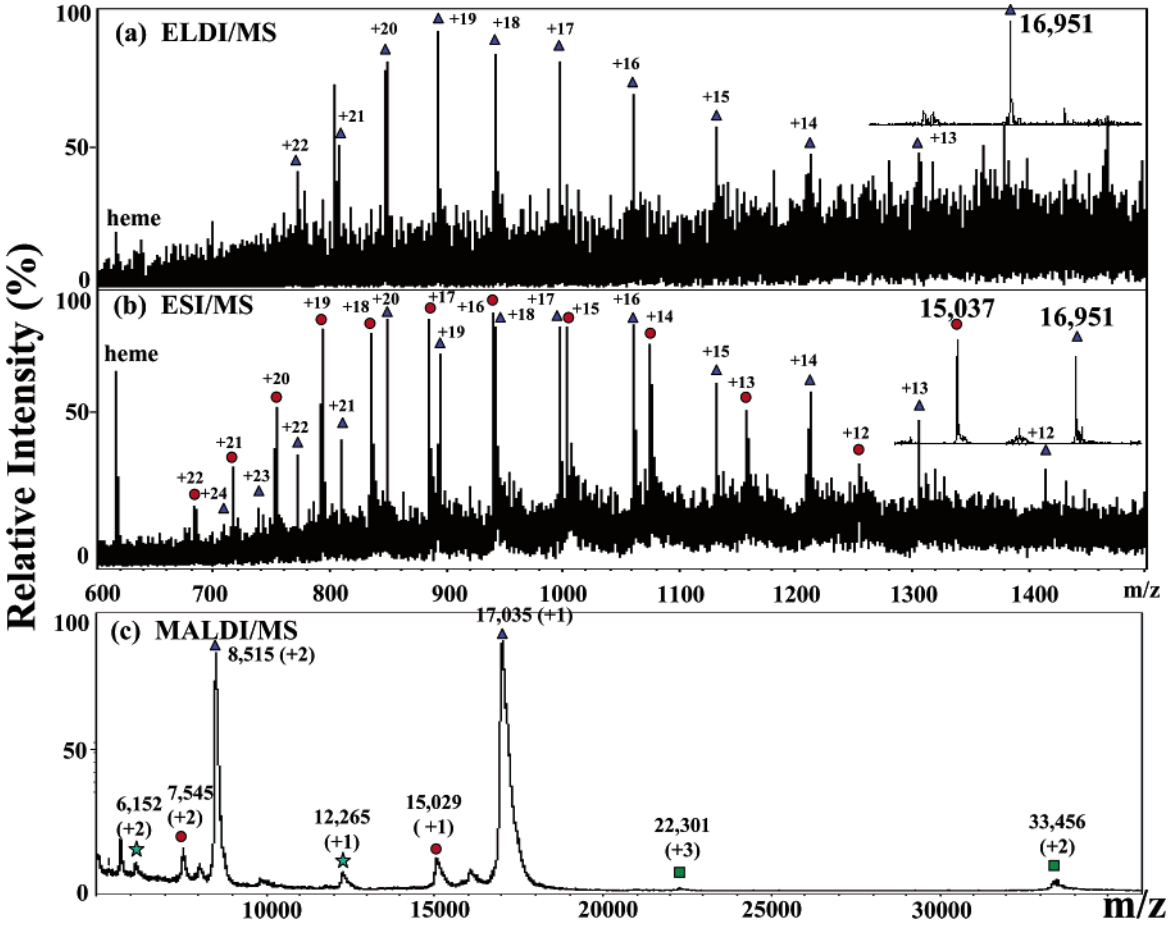

Figure 9. (a) Positive-mode ELDI mass spectrum of the proteins in a sample of porcine heart tissue. Protein extracts from the porcine heart tissue as analyzed using (b) ESI−MS and (c) MALDI MS. The insets in panels a and b present the deconvoluted protein ion signals from the ELDI and ESI mass spectra.

conditions. Figure 9 display the ELDI, ESI, and MALDI mass spectra of porcine heart tissues, respectively. Generally speaking, the predominant heart tissue protein (i.e., myoglobin) detected by the three methods is the same except the signal of heme (m/z 616) released from native myoglobin appeared only in the ELDI and ESI mass spectra. As it is showing in the deconvoluted mass spectra in Figure 9a,b, very accurate myoglobin ion mass (both calculated and theoretical MW, 16 951) was obtained by ELDI and ESI. However, due to a linear-TOF mode which was used to detect the protein ion signal in MALDI, the resolution (and the accuracy of the detected protein ion mass) of the myoglobin ion signal in MALDI is much lower than that in ELDI and ESI (Figure 9c). Additional strong protein ion signal having the calculated mass of 15 037 Da was detected on ESI mass spectra. Although this protein was also detected by MALDI, the ion intensity was quite low (Figure 9a,c). Very weak ion signals of albumin [m/z 33 456 (+2) and 22 301 (+3)] and two other proteins [m/z 16 024 (+1) and 8030 (+2); m/z 12 265 (+1) and 6152 (+2)] were detected only on the MALDI spectrum (Figure 9c).

The results of the porcine liver analysis demonstrate that three prominent protein ion signals (having calculated masses of 16 036, 15 042, and 14 177 Da) were detected in the ELDI spectrum, two of them (16 036 and 15 042 Da) were detected in the MALDI spectrum, and one of the proteins (15 042 Da) was detected in the ESI mass spectrum (Figure 10). Although no signals of the myoglobin ion were observed, the signal of the ion from heme was still detected in both the ELDI and ESI mass spectra. It is evident that, based on the calculated molecular masses of the detected protein ions, the prominent protein ions detected from porcine liver tissue by ELDI MS are similar to those obtained in conventional ESI and MALDI MS analyses.

Obviously, the mass accuracy for determining the molecular weights of proteins is improved dramatically when using ELDI, relative to the low resolution of the ion signals obtained when using MALDI/time-of-flight (TOF) mass spectrometry (where TOF must be operated in the linear mode to detect intact protein ions). The presence of signals of unknown ions in the ELDI mass spectra of the porcine heart and liver tissue samples may have arisen from incomplete protein ion series (particularly at low concentrations) and the presence of such species as lipids, peptides, decomposition products, and adduct ions.

Because an additional organic matrix is not used in ELDI, it is not necessary to obtain a thin slice of tissue sample for such protein analyses, unlike the case of conventional protein image analyses performed using MALDI.[16−19] This feature may greatly simplify the sample preparation procedures required for direct protein analyses of tissues. However, since the ion signals of the proteins in trace amount may still be suppressed by the presence of the protein in large quantity (e.g., myoglobin in porcine heart), applications of ELDI MS for protein profiling or imaging directly on tissue remains a future research topic.

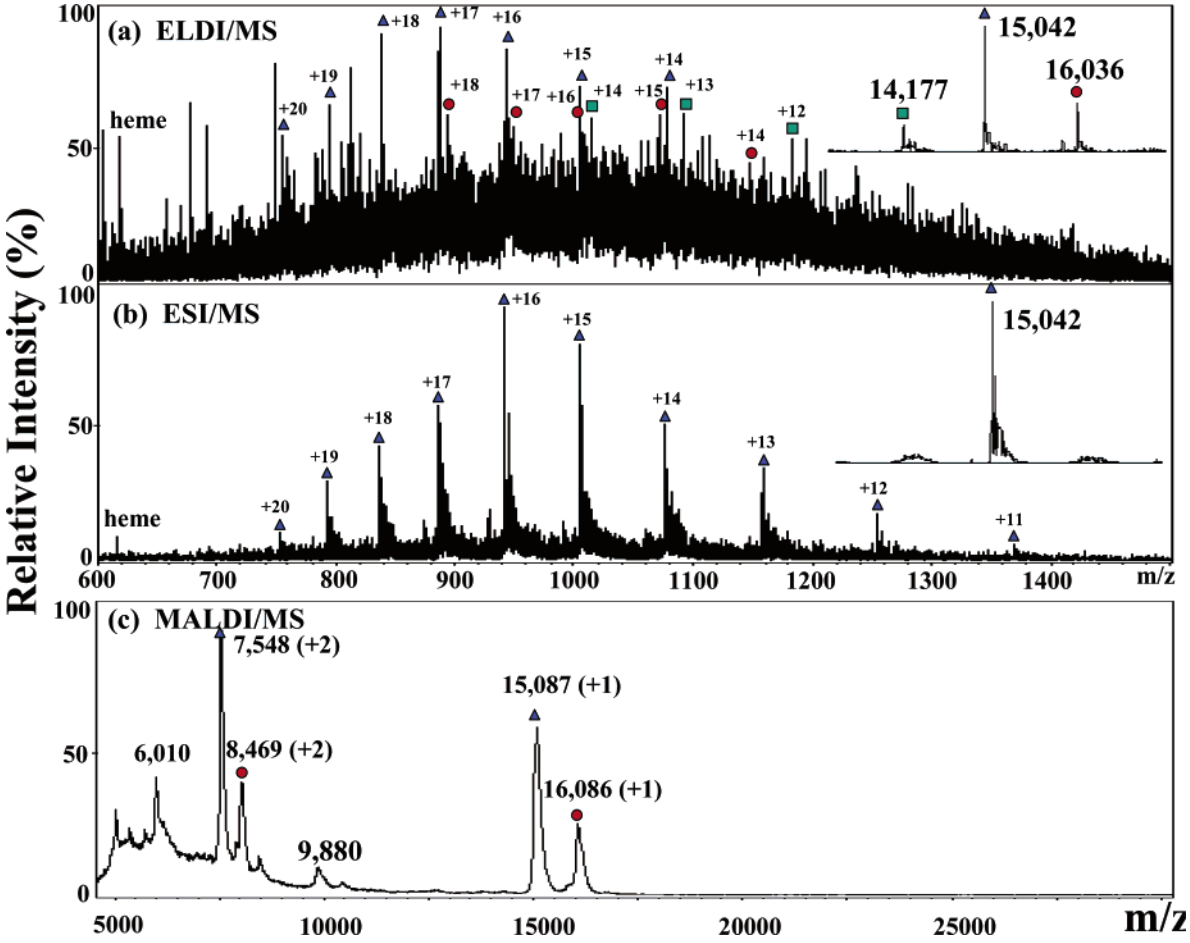

Figure 10. (a) Positive-mode ELDI mass spectrum of the proteins in a sample of porcine liver tissue. Protein extracts of the porcine liver tissue as analyzed using (b) ESI−MS and (c) MALDI MS. The insets in panels a and b display the deconvoluted protein ion signals from the ELDI and ESI mass spectra.

Conclusion

In this paper, we report direct characterization of protein from various solids through a new desorption/ionization source for mass spectrometry, ELDI, which incorporates some features of both ESI and MALDI. The results we obtained from ELDI MS indicate the feasibility of using this approach for qualitative protein analysis; indeed, it exhibits a number of advantages: (1) Proteins in the solid are desorbed and ionized efficiently under ambient conditions. This feature allows us to avoid many of the inconvenient limitations provided by the vacuum, which is required for operation of a MALDI system. Changing the sample is also easier. In addition, sampling under ambient conditions allows the examination of vacuum-sensitive samples, such as tissues, without adverse effects. (2) Although we have not demonstrated it in this paper, with the assistance of the precise movement of a stepper motor and using LD for sampling at high spatial resolution, it is possible to obtain predominant protein information on a particular surface area of a tissue. The restrictions of laser-focusing optics, which are imposed when using va acuum system, are also removed under the ambient conditions of ELDI; thereby, ELDI allows the use of near-field optical probes that can greatly reduce the laser spot size. (3) The use of organic or inorganic matrixes is unnecessary for successful protein analysis, unlike the situation when using MALDI. This feature greatly simplifies the sample preparation procedure and allows the rapid characterization of proteins from solid samples. (4) ESI-like protein mass spectra are obtained. The detected mass range is extended through the formation of multiply charged protein molecules. The molecular weights of intact proteins can be measured more accurately for top-down proteomics analyses, and the prospects of sequencing peptides and proteins directly from biological media using ELDI and tandem mass spectrometry is promising.

The ELDI desorption/ionization process is performed at atmospheric pressure, and thus, it is readily compatible with most mass analyzers. For example, we have successfully coupled the ELDI source with various mass analyzers, including ion trap, Q-TOF, and triple quadrupole mass analyzers possessing heated capillary or skimmer inlet systems. The installation of the ELDI source onto an FT mass spectrometer is under investigation in our laboratory. It seems that there are no particular requirements for interfacing the ELDI source to the different types of mass analyzers, and the assembly is quite simple; therefore, it appears this new ionization source can be implanted in nearly all of the mass spectrometers that are used in general chemistry or biochemistry laboratories.

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