

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry in Peptide and Protein Analysis

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Matrix-assisted Laser Desorption/Ionization Mass Spectrometry in Peptide and Protein Analysis

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDIMS), first introduced in 1988 by Hillenkamp and Karas, has become a widespread analytical tool for peptides, proteins and most other biomolecules. In MALDI (matrix-assisted laser desorption/ionization) analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, usually an ultraviolet (UV)-absorbing weak organic acid, after which laser radiation of this analyte–matrix mixture results in the vaporization of the matrix which carries the analyte with it. The matrix therefore plays a key role by strongly absorbing

the laser light energy and causing, indirectly, the analyte to vaporize. The matrix also serves as a proton donor and receptor, acting to ionize the analyte in both positive and negative ionization modes, respectively. The efficient and directed energy transfer during a matrix-assisted laser-induced desorption event provides high ion yields of the intact analyte and allows for the measurement of compounds with high accuracy and subpicomole sensitivity. The ability to generate such accurate information can be extremely useful for protein identification and characterization. For example, a protein can often be unambiguously identified by the accurate mass analysis of its constituent peptides (produced by either chemical or enzymatic treatment of the sample). This article discusses basic MALDI concepts and instrumentation and focuses on applications in the field of peptides and proteins, specifically on the utility of MALDI in protein identification, protein structural studies, and as a clinical assay.

1 INTRODUCTION TO MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

MALDIMS, first introduced in 1988 by Hillenkamp and Karas,⁽¹⁾ has become a widespread analytical tool for peptides, proteins, and most other biomolecules (oligonucleotides, carbohydrates, natural products, and lipids). The efficient and directed energy transfer during a matrix-assisted laser-induced desorption event provides high ion yields of the intact analyte, and allows for the measurement of compounds with high accuracy and subpicomole sensitivity.^(2–4) This article discusses basic MALDI concepts and instrumentation and focuses on applications in the field of peptides and proteins, specifically on the utility of MALDI in protein identification, protein structural studies, and as a clinical assay.

1.1 Mechanism of Matrix-assisted Laser Desorption/Ionization

MALDI provides for the nondestructive vaporization and ionization of both large and small biomolecules (Figure 1). In MALDI analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, usually a UV-absorbing weak organic acid, after which laser radiation of this analyte–matrix mixture results in the vaporization of the matrix which carries the analyte with it. The matrix therefore plays a key role by strongly absorbing the laser light energy and causing, indirectly, the analyte to vaporize. The matrix also serves as a proton donor and receptor, acting to ionize the

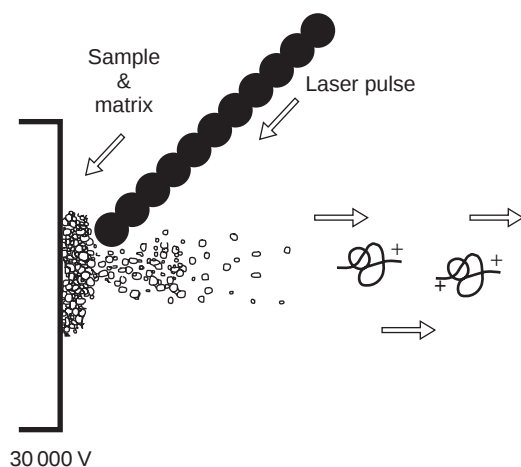


Figure 1 MALDI.

analyte in both positive and negative ionization modes, respectively.⁽⁵⁾

Several theories have been developed to explain desorption of large molecules by MALDI. The thermal-spike model⁽⁶⁾ proposes that the matrix molecules sublime from the surface as a result of local heating at low laser fluence, but above a certain laser intensity, a rapid rise in desorption efficiency is observed. The ejection of intact molecules is attributed to poor vibrational coupling between the matrix and analyte which leads to a bottleneck in the energy transfer from the matrix to the internal vibrational modes of the analyte molecule. The pressure pulse theory⁽⁷⁾ proposes that a pressure gradient is created normal to the surface and desorption of large molecules might be enhanced by momentum transfer from collisions with fast-moving matrix molecules.

It is generally thought that ionization occurs through proton transfer or cationization. The ionization depends critically on the matrix–analyte combination, but is not critically dependent on the number of acidic or basic groups of the analyte.⁽⁸⁾ This suggests that a more complex interaction of analyte and matrix, rather than simple acid–base chemistry, is responsible for ionization.

1.2 Matrix-assisted Laser Desorption/Ionization Mass Analyzers

There are three types of mass analyzers typically used with the MALDI ionization source: a linear time-of-flight (TOF), a TOF reflectron, and a Fourier transform mass analyzer (Figure 2). The linear TOF mass analyzer is the simplest of the three devices and has enjoyed a renaissance with the invention of MALDI. TOF analysis is based on accelerating a set of ions to a detector where all of the ions are given the same amount of

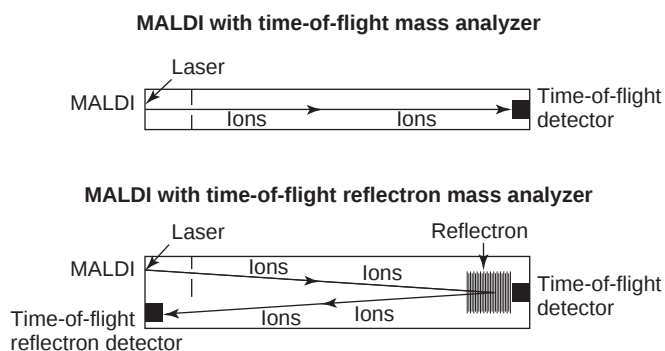


Figure 2 MALDI with TOF and TOF reflectron.

energy. Because the ions have the same energy, yet a different mass, the ions reach the detector at different times. The smaller ions reach the detector first because of their greater velocity while the larger ions take longer owing to their larger mass. Hence, the analyzer is called TOF because the mass is determined from the ions' time of flight. The arrival time at the detector is dependent upon the mass, charge, and kinetic energy (KE) of the ion. Since KE is equal to $1/2 mv^2$ or velocity $v = (2KE/m)^{1/2}$, ions will travel a given distance, d , within a time, t , where t is dependent upon their mass-to-charge ratio (m/z).

The TOF reflectron combines TOF technology with an electrostatic analyzer, the reflectron. The reflectron serves to increase the amount of time, t , ions need to reach the detector while reducing their KE distribution, thereby reducing the temporal distribution Δt . Since resolution is defined by the mass of a peak divided by the width of a peak or $m/\Delta m$ (or $t/\Delta t$ since m is related to t), increasing t and decreasing Δt results in higher resolution. This increased resolution, however, often comes at the expense of sensitivity and a relatively low mass range, typically $<10\,000\ m/z$. One innovation that has had a dramatic effect on increasing the resolving power of MALDI/TOF instruments has been delayed extraction (DE). DE is a relatively simple means of cooling the ions (and possibly focusing them) immediately after the MALDI ionization event. In traditional MALDI instruments the ions were accelerated out of the ionization source as soon as they were formed; however, with DE the ions are allowed to “cool” for $\sim 150\text{ ns}$ before being accelerated to the analyzer (Figure 3). This cooling period generates a set of ions with a much lower KE distribution, ultimately reducing the temporal spread of ions once they enter the TOF analyzer. Overall, this results in increased resolution and accuracy. Incidentally, the benefits of DE significantly diminish with higher-molecular-weight proteins ($>30\,000\text{ Da}$).

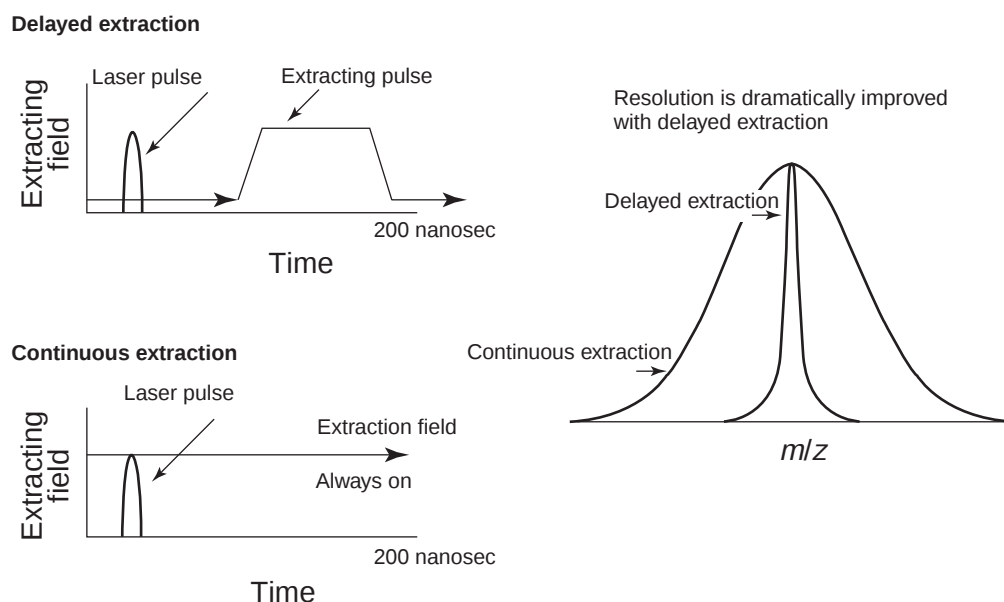


Figure 3 DE versus continuous extraction.

MALDIMS is most commonly combined with the TOF mass analyzers. However, their somewhat limited resolution (10^2 – 10^4) results in accuracy on the order of 0.2% to a high of 0.005% (with internal calibrant). Alternatively, MALDI instruments are also being coupled to ultrahigh-resolution ($>10^5$) mass analyzers such as the Fourier transform ion cyclotron resonance (ICR) mass analyzer. First introduced in 1974 by Comisarow and Marshall,⁽⁹⁾ Fourier transform mass spectrometry (FTMS) is based on the principle of a charged particle orbiting in the presence of a magnetic field. While the ions are orbiting, a radio frequency (RF) signal is used to excite them and as a result of this RF excitation, the ions produce a detectable image current. The time-dependent image current can then be Fourier-transformed to obtain the component frequencies of the

different ions which correspond to their m/z . FTMS has become an important research tool offering high accuracy with errors as low as $\pm 0.001\%$ (ppm accuracy). Different types of MALDI mass analyzers are compared in Table 1.

2 ANALYSIS OF PEPTIDES AND PROTEINS BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION

The utility of MALDI for protein and peptide analyses lies in its ability to provide highly accurate molecular-weight information on intact molecules. The ability to generate such accurate information can be extremely useful for protein identification and characterization. For

Table 1 General comparison of MALDI mass analyzers

	TOF	TOF reflectron	FTMS
Accuracy	0.05–0.2% (50–200 ppm)	0.03% (30 ppm)	0.005% (5 ppm)
Resolution	2000	10 000	100 000
m/z range	$>300\,000$	10 000	10 000
Tandem MS	MS	MS ²	MS ⁴
Scan speed	ms	ms	s
Comments	Highest mass range. Very fast scan speed. Simple design. Low cost.	Lower sensitivity than TOF. Good resolving power. Limited m/z range. Very fast scan speed. Simple design.	Capable of high resolution and exact mass measurements. Well-suited for tandem MS. Instrumentation is expensive, large. Requires high vacuum ($<10^{-7}$ torr). Requires superconducting magnet.

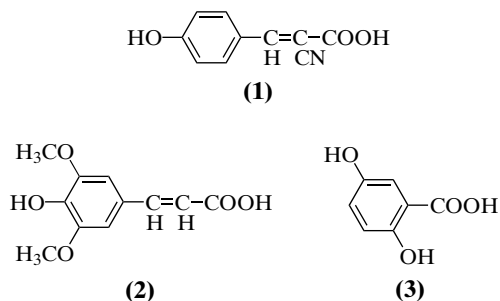
MS, mass spectrometry.

example, a protein can often be unambiguously identified by the accurate mass analysis of its constituent peptides (produced by either chemical or enzymatic treatment of the sample). Furthermore, protein identification can also be facilitated by analysis of the protein's proteolytic peptide fragments in the gas phase; fragment ions generated inside MALDI mass spectrometers via collision-induced dissociation (CID) yield information about the primary structure and modifications. Tandem mass spectrometry (n th series) (MS^n) experiments, previously allowed with quadrupole and ion trap mass spectrometers are now attainable with MALDI sources (known as postsource decay or PSD).

While the MALDI mass spectrometer is a powerful tool for the accurate mass determination of peptide mixtures, obtaining accurate mass measurements is highly dependent upon the sample and the sample preparation. Acquiring optimum MALDI data depends on the choice of suitable matrices and solvents, the functional and structural properties of the analyte, sample purity, and how the sample is prepared on the MALDI sample plate. Because the solvent or matrix is the medium by which the analyte will be transported to the gas phase and provides the conditions that make ionization possible, contamination of the sample with excessive salt (>10 mM) will affect these conditions and lead to reduced sensitivity. This is also true of chaotropic agents, including urea and guanidinium salts, and solvents like dimethyl sulfoxide and glycerol. Dialysis and reversed-phase liquid chromatography (RPLC), or exchange chromatography are useful methods for purifying samples of such contaminants prior to mass spectral analysis.

2.1 Sample Preparation

For peptides and proteins, the standard matrices^(1,10–13) are α -cyano-4-hydroxycinnamic acid (**1**) (α -cyano or CCA), 3,5-dimethoxy-4-hydroxycinnamic acid (**2**) (sinapinic acid or SA), and 2,5-dihydroxybenzoic acid (**3**) (DHB). CCA (**1**) is mainly used for peptides, glycopeptides and small proteins. SA (**2**) is commonly used for both peptide and protein analysis, and DHB (**3**) is



used for glycopeptides, glycoproteins, small proteins, and oligonucleotides (<10 bases). In the analysis of protein digests (peptide mapping), the choice of matrix plays an important role in the observation of the peptides produced. Some matrices can complement each other with respect to obtaining complete protein sequence coverage, for instance peptide mapping with CCA can yield more low-mass peptide ions (<2500 Da), while SA may provide better coverage for the higher-mass peptides (>2500 Da).

Sample–matrix preparation procedures greatly influence the quality of MALDI mass spectra of peptides/proteins. Among the variety of reported preparation methods, the dried-droplet method is the most frequently used. In this case, a saturated matrix solution is mixed with the analyte solution, giving a matrix-to-sample ratio of about 5000:1. An aliquot (0.5–2.0 μ L) of this mixture is then applied to the sample target where it is allowed to dry. Below is one specific example of the dried-droplet method:

- Pipet 0.5 μ L of sample to the sample plate (preferable concentration ~ 1.0 pmol μ L⁻¹).
- Pipet 0.5 μ L of matrix to the sample plate.
- Mix the sample and matrix by drawing in and out of the pipette.
- Allow to air dry.
- For peptides, small proteins, and most compounds a saturated solution of CCA (**1**) in 50:50 H₂O:acetonitrile (ACN) with 0.1% TFA (trifluoroacetic acid) is used.
- For glycopeptides/proteins and small compounds a saturated solution of DHB (**3**) in 50:50 H₂O:ACN with 0.1% TFA is used.
- For proteins and other large molecules a saturated solution of SA (**2**) in 50:50 H₂O:ACN with 0.1% TFA is used.

Alternatively, samples can be prepared in a stepwise manner. In the thin-layer method,^(14,15) a matrix homogeneous “film” is formed on the target first, and the sample is then applied and absorbed by the matrix. This method yields good sensitivity, resolving power, and mass accuracy. Similarly, in the thick-layer method,⁽¹⁶⁾ nitrocellulose (NC) is used as matrix additive and once a uniform NC-matrix layer is obtained on the target, the sample is applied. This preparation method suppresses alkali adduct formation and significantly increases the detection sensitivity, especially for peptides and proteins extracted from gels. The sandwich method⁽¹⁶⁾ is another variant in this category. A thin layer of matrix crystals is prepared as in the thin-layer method, followed by the subsequent addition of droplets of aqueous TFA, sample and matrix.

2.2 Protein Primary Sequence Analysis

Amino acid sequence information can be obtained by one of several methods using MALDIMS. The first is called protein mass mapping which consists of the site-specific enzymatic or chemical degradation of a protein followed by mass spectrometric analysis of the released peptides. Owing to the complexity of mixtures generated during proteolysis, MALDI/TOF MS is most ideally suited for such analyses. The traditional analytical methods used to characterize the released peptides consist of high-performance liquid chromatography (HPLC) or gel electrophoresis followed by N-terminal (Edman) sequencing and/or amino acid analysis. However, such methods are considerably more time-consuming and in some cases are not capable of separating individual peptides because of their low resolution. Consequently, high-resolution MALDI/TOF analysis can provide for a more rapid, accurate, and highly sensitive analysis of these complex peptide mixtures.

The purpose of protein mass mapping is not to sequence the entire protein but instead to generate information that can be used in protein database searches either to identify the protein or determine whether the protein is novel. Protein identification via database searching is facilitated by the accurate m/z values of the digest fragments, the specificity of the enzyme used, and the accurate m/z of the intact protein. Entire scientific conferences have been devoted to this approach which has been coined “proteomics.” Table 2 previews some of the protein databases available on the internet.

Typically, the type of protease used in the mass mapping approach is an endoprotease, a protease which cleaves internal residues. An extended version of this method follows the endoproteolytic digestion with an exoprotease, which cleaves successively from either the n-terminal or c-terminal side of a peptide or protein.

The result is a mass spectrum with peptides differing by one amino acid residue. The mass difference between ion signals determines the identity of the released amino acid and typically provides a sequence tag on the order of 1–12 amino acids. This method further increases the possibility of a database “hit” by providing one more piece of valuable information, a partial sequence tag. Figure 4 illustrates this approach.

Another MALDI-based approach that provides amino acid sequence information on peptides relies on the fragmentation of the ions in the field-free drift region (between the ion source and the detector) of the TOF mass analyzer. This fragmentation, referred to as PSD, is due to collisions between analyte ions and neutral matrix molecules or residual gas molecules during the desorption and acceleration stage. Initially these fragments have the same velocities as their precursor ion. Consequently, in a linear TOF mass analyzer these fragments arrive at the detector at the same time as their precursor ion and are therefore not represented in the mass spectrum. In a reflectron TOF mass analyzer, however, these fragments penetrate the reflectron at different depths and are therefore spatially differentiated, meaning that individual fragments arrive at the reflectron detector at different flight times. As a result, these fragments are represented in the mass spectrum and provide valuable structural information (Figure 4). Amino acid sequencing by PSD has become a popular method for sequencing peptides and is now available on most commercial MALDI reflectron instruments. Figure 5 shows the PSD spectrum of 10 pmol of angiotensin (m/z 1297 Da).

A third approach to obtaining peptide sequence information from MALDI is MS^n within an FTMS analyzer cell. One of the advantages of FTMS, in addition to its high resolution capabilities, is its ability to perform MS^n experiments. Dissociation of the precursor ion can be accomplished by the introduction of a collision gas or

Table 2 Protein databases available on the internet

NCBIInr	A nonredundant database compiled by the NCBI by combining most of the public domain databases (ESTs not included).
Swiss Prot	A curated protein sequence database which strives to provide a high level of annotation, such as the description of the function of a protein, its domain's structure, post-translational modifications, variants, etc. This database offers a minimal level of redundancy and high level of integration with other databases.
OWL	A nonredundant composite of four publicly available primary sources: SWISSPROT, PIR, (1-3), GenBank (translation) and NRL-3D. SWISSPROT is the highest priority source, all others being compared against it to eliminate identical and trivially different sequences.
Genpept	Protein translation of Genbank (ESTs not included).
Unknome	A theoretical database used in de novo MS/MS spectral interpretation that is created on-the-fly and contains all amino acid sequence permutations consistent with the parent mass and amino acid composition information contained in an MS/MS spectrum.

NCBI, National Center for Biotechnology Information; EST, expressed sequence tag; MS/MS, tandem mass spectrometry (2nd series).

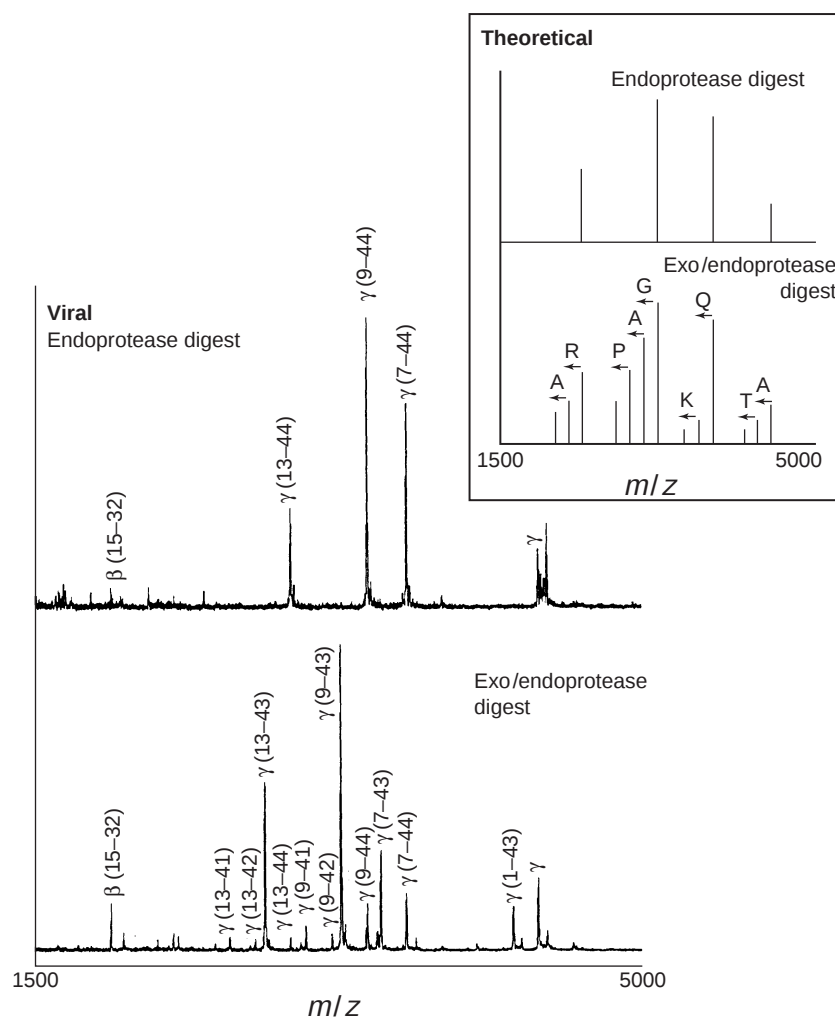


Figure 4 C-terminal ladder sequencing.

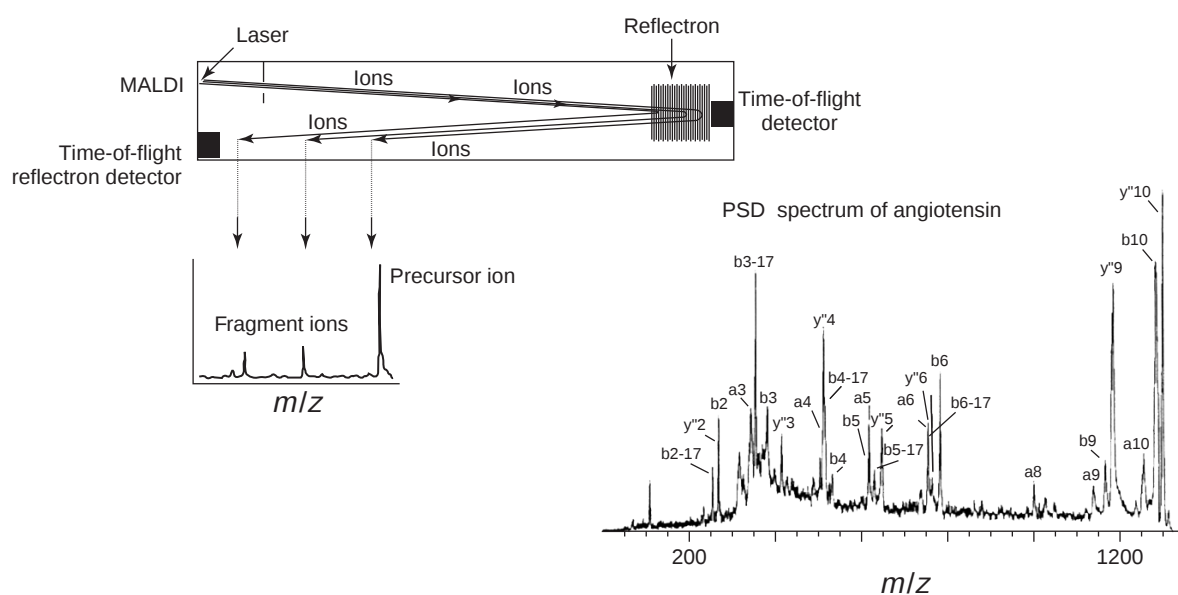


Figure 5 Postsource delay.

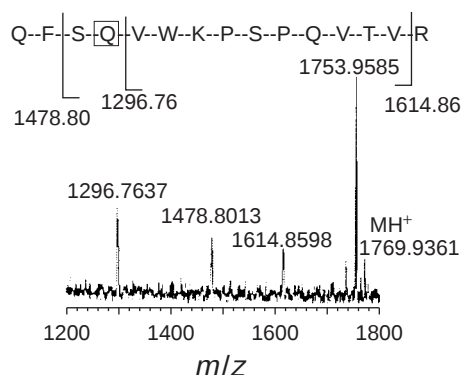


Figure 6 MS/MS with MALDI/FTMS.

by use of pulsed lasers to photodissociate selected ions. Other advantages associated with using FTMS include the simultaneous high mass resolving power of the fragments, the high sensitivity over a wide mass range and the high mass accuracy. The primary disadvantage is the lack of multiply charged species generated in MALDI/FTMS experiments, the lack of which reduces the amount of fragmentation. Figure 6 shows the MALDI/FTMS MS/MS spectrum of a viral peptide obtained from a mutant common cold (rhino) virus. Such analysis provided for the rapid (<20 min) identification of the mutated amino acid with <5 ppm mass accuracy.

2.3 Characterizing Protein Modifications (Co- and Posttranslational)

Protein modifications, including both co- and post-translational modifications (e.g. phosphorylation, sulfation, glycosylation, and N-terminal modifications), are recognized as important means of regulating the cellular distribution and modulating protein functions. For example, protein phosphorylation is known to play a critical role in cell signal transduction pathways.^(17,18) Carbohydrates provide various functional, immunological, and structural aspects of glycoprotein. Characterization of protein modifications is an important aspect for analyzing protein structure and function. The utility of MALDI for this purpose relies on its ability to perform accurate mass determination of peptide mixtures, its very high sensitivity and resolving power, and its buffer tolerance.

Typical MALDI protocols for protein modification characterization begin with the enzymatic digestion of the protein sample, resulting in a complex mixture of peptides. When a protein's primary structure is known, the molecular weight of a peptide can be used to corroborate the presence of any modification by its specific molecular-weight difference between the observed mass of peptide and that calculated on the basis of the sequence. This approach is straightforward

but has very little specificity and selectivity. The results can be ambiguous if a complicated digestion mixture is presented. This difficulty can be overcome by using MALDI PSD MS/MS to generate and record metastable fragment ions. The aid of a separation technique, usually HPLC, is sometimes needed before the MALDI analysis. This protein modification characterization approach has been employed by several groups to identify and sequence either glycoproteins^(19–22) or phosphoprotein.^(23,24)

2.4 Protein Structure Elucidation

Proteins in their native state are typically folded into well-defined, three-dimensional structures by relatively weak intramolecular forces (i.e. hydrogen bonds, electrostatic, or hydrophobic interactions). Because the function of the protein relies heavily on this structure, information on higher-order structure (i.e. tertiary protein conformation and quaternary protein–protein interactions) is of fundamental biological importance. There are a variety of spectroscopic methods available to study tertiary and quaternary protein structure, including X-ray crystallography and nuclear magnetic resonance (NMR), along with a variety of other spectroscopic techniques. It should be noted, however, that while these techniques constitute the state of the art as far as structural determination, they can be time-consuming and require deconvolution of complex data and a significant amount of material.

An alternative approach to investigate higher-order protein structure using MS is to probe the surface availability of regions of the molecule using the protein mass mapping method. Proteolytic digestion is dependent on the availability of cleavage sites which is a reflection of the protein structure. Therefore, proteolysis performed in modified solution conditions can sample different protein conformations, and relative differences in these conformations can then be discerned through direct comparison of the resulting mass spectrometric data. For example, digestion of a protein in its denatured or unfolded state will most likely result in the release of additional digest fragments when compared to the digest of the protein in its native or folded state. In the unfolded state, more cleavage sites will be accessible to the protease owing to lack of tertiary structure.

Using this method, Lewis was able to recognize conformational differences (i.e. changes in tertiary structure) in regulatory proteins brought on by the interaction with an effector molecule.⁽²⁵⁾ The allosteric protein calmodulin (CaM) is known to take on different conformational states depending on the presence or absence of its effector, calcium. Different proteolytic mass maps were observed for CaM, indicating structural differences in the protein depending on the presence of calcium.

The overall tertiary structure of CaM reveals a dumbbell shaped molecule (148 amino acids) with two

globular domains separated by a single long central α -helix (amino acids 65–92). It has been proposed that calcium activates CaM by exposing hydrophobic residues (near the two ends of the central helix) which interact with CaM target proteins. Small-angle X-ray scattering analysis has shown that the central helix acts as a flexible “tether” which, upon binding calcium, allows the two globular domains to come together and in effect “hug” the target protein, via the hydrophobic regions. Mass maps resulting from digests by trypsin, chymotrypsin, and pepsin all indicated that the protein had undergone a tertiary structural change in the presence of calcium and in doing so hindered the activity of the enzyme in the central helical region. Presumably, the coming together of the two globular domains places certain residues of the central helix, normally cleaved in a calcium-free environment, in a conformation which does not allow the enzyme accessibility.

Thus, in observing qualitative differences between spectra obtained in the absence and in the presence of an effector, the mass mapping approach allows different solution conformations (differences in tertiary structure) of a protein to be identified and which regions of a protein are effected by such conformational changes to be determined. Figure 7 shows the trypsin digests of CaM in the presence and absence of calcium. Comparison of the two mass spectra reveals obvious qualitative differences corresponding to cleavages in the central helical region of the protein. Digestion fragments resulting from cleavages in the central region (residues 65–92) of the molecule are not present in the calcium-free environment.

Investigating protein/protein interactions (quaternary protein structure) has also been pursued with limited

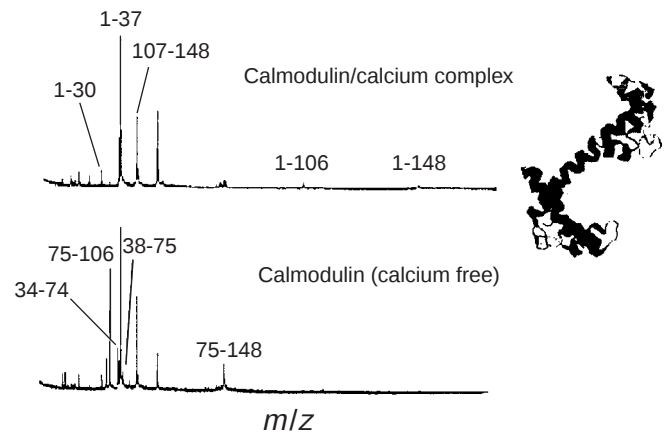
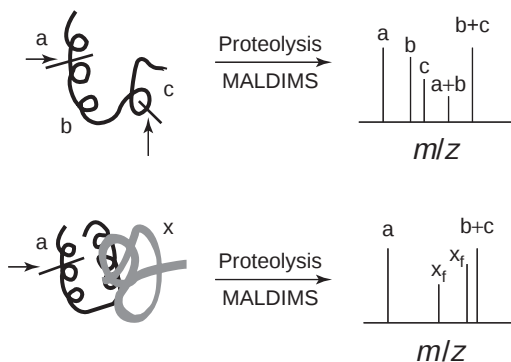


Figure 7 Protein mass mapping.

proteolysis combined with MS, the goal being to determine the topographical regions involved in their interaction.⁽²⁶⁾ Again, since enzymatic digestion of a protein is dependent on the availability of cleavage sites, it can be assumed that digestion of a protein complex should hinder access of the protease to cleavage sites normally accessible in the digestion of the individual proteins. Toward this end, comparative mass mapping should reveal regions of the protein(s) involved in the formation of the complex. Changes in the susceptibility of cleavage sites toward proteases can arise from several physical effects as a result of the complex formation. One is a direct consequence of the formation of the complex, the steric hindrance, and another is an indirect result of the formation of the complex, a conformational change, that may alter the accessibility of not only

In theory - proteolysis of a protein and a protein/protein complex



In practice - limited proteolysis of a p21-B/Cdk2 complex

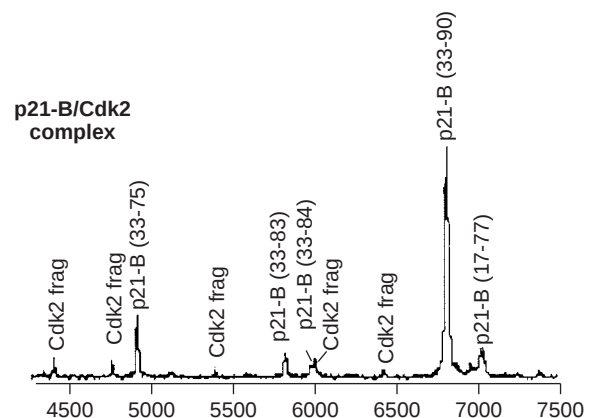


Figure 8 Protein–protein interactions with mass mapping. The symbols represent a library of potential ligands for the receptor IgG.

the sites involved in the interaction, but remote sites as well.

Siuzdak et al. performed MALDI analysis on the digests of the kinase inhibitory domain of the cell cycle regulatory protein, p21-B in both the free solution state and in the 1:1 complex of p21-B and cyclin-dependent kinase 2 (Cdk2) as shown in Figure 8.⁽²⁶⁾ Mass mapping of the trypsin digests of the free p21-B revealed peaks corresponding to all of the 12 potential trypsin cleavage sites (amino acids 9, 16, 19, 20, 32, 46, 48, 67, 69, 75, 83, and 84). However, MALDI mass spectra for the trypsin digests of p21-B in the presence of Cdk2 showed that several p21-B fragments that were produced in the absence of Cdk2 disappear in the presence of Cdk2. Specifically, Siuzdak et al. found that amino acids 46, 48, 67 were protected from trypsin cleavage in the presence of Cdk2. This data was later crystallographically supported by the crystal structure data on a homologous system using a p21 homolog p27.⁽²⁷⁾

Other methods of probing higher-order structure include investigating the chemical reactivity of individual amino acids in a protein and chemical cross-linking studies. Both approaches stem from the fact that MALDI has proved to be a powerful method

with which to characterize covalent post-translational modifications.⁽²⁸⁾ Using simple modification chemistries such as acylation or succinylation, Glocker et al. have shown that there is a clear correlation between the relative reactivity of specific amino acids and their surface (accessibility) topology in a protein.⁽²⁹⁾ Chemical cross-linking studies consist of treating proteins with cross-linkers prior to digestion. Cross-linkers covalently attach adjacent protein regions or protein subunits, therefore the resulting proteolytic fragments are good indications of the overall tertiary and/or quaternary protein structure.

3 APPLICATIONS

3.1 Diagnostic

As mentioned previously, the power of MALDI lies in its ability to analyze complex mixtures, making MALDI a promising method for the diagnostic screening of biological fluids (serum, cerebral spinal fluid, urine). However, low analyte concentrations and signal suppression due to large amounts of host protein (e.g. hemoglobin in blood) often hinder the screening of such complex

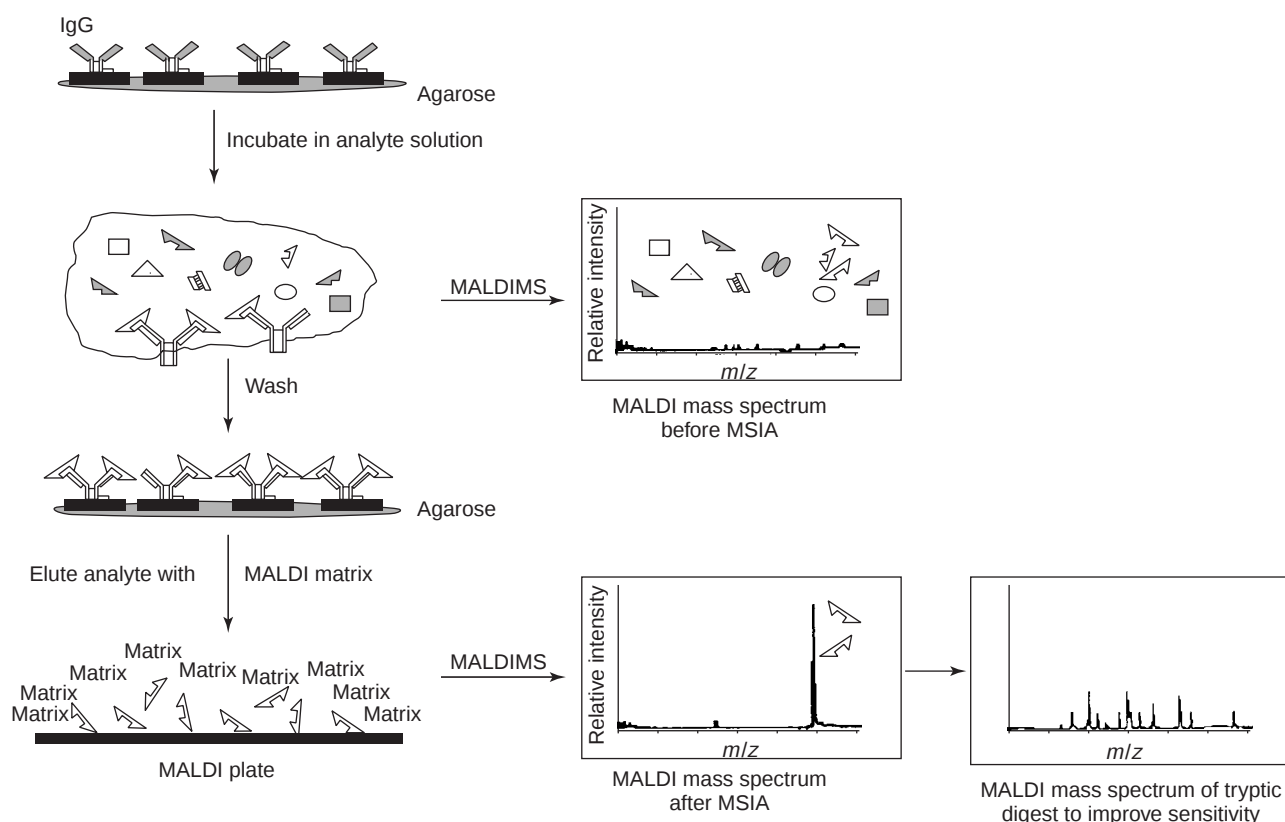


Figure 9 MSIA.

natural fluids. It therefore becomes necessary to isolate the analyte from the fluids prior to MALDI analysis. Mass spectrometric immunoassay (MSIA) (Figure 9), developed by Nelson et al., relies on the affinity capture (antibody–antigen recognition) of the analyte from the biological matrix prior to mass spectrometric analysis.⁽³⁰⁾ This method not only preconcentrates the analyte, but also provides for the most selective form of analyte isolation, making this an extremely powerful technique for screening biological fluids. Nelson et al., for example, were able to detect unambiguously femtomole amounts (nanomolar concentrations) of a snake toxin doped into human whole blood.

Similar to the enzyme-linked immunosorbent assay (ELISA), MSIA consists of the nanoscale immunoaffinity purification of the analyte of interest. Isolation is performed by incubating the biological matrix with antibodies immobilized on a solid support, such as resin beads. During incubation, the analyte is captured on the immobilized antibody and, after a series of washes, the antibody–antigen interaction is disrupted by the addition of the acidic MALDI matrix. Subsequent mass spectrometric analysis of the eluant confirms the presence of the antigen (analyte) at a specific m/z value. Unlike the ELISA, however, any nonspecifically bound molecules can be unambiguously differentiated from the analyte of interest owing to unique m/z values measured. In addition, incubation times are on the order of 15–20 min, significantly shorter than with the conventional ELISA.

In preliminary experiments aimed at identifying cardiac troponin T (cTnT) present in the urine of chronic renal failure patients, Fitzgerald et al. used biotinylated-anti-troponin mAb immobilized on streptavidin beads to affinity capture $0.15\ \mu\text{g mL}^{-1}$ ($\sim 44\ \text{pmol}$) cTnT from Tris buffer.⁽³¹⁾ In the MALDI mass spectrum of $9\ \mu\text{g mL}^{-1}$ cTnT in 100-mM Tris buffer (Figure 10b), no ion signals of cTnT are observed. Figure 10(a) shows the mass spectrum obtained after the MSIA isolation and elution, where cTnT is observed at $m/z \sim 34\ \text{kDa}$. Thus, the combination

of MS with immunoaffinity capture serves as a rapid, sensitive, and selective means of detecting biologically relevant compounds.

3.2 Quantitative Aspects of Matrix-assisted Laser Desorption/Ionization

The ability of MALDI to analyze complex heterogeneous mixtures has made it a common and valuable technique in the analysis of biological fluids, analysis commonly performed by immunoassays and HPLC. Immunoassays generally have low reproducibility and reliability and provide little or no selectivity between a drug and its metabolites. This lack of specificity is a significant limitation since metabolites, although structurally similar to the parent compound, often have different biological activity. Mass spectrometric analysis, on the other hand, allows for co-extracted metabolites to be identified and quantitatively monitored (unless they have the same molecular weight). Although HPLC is relatively selective and accurate, the sensitivity is compound dependent and method development can be time-consuming. Thus, while both techniques are useful, both suffer when compared to the speed, sensitivity, and accuracy offered by MS.

In the following example, an automated MALDI MS procedure was used to improve the clinical analysis of a cyclic peptide (the immunosuppressant drug cyclosporin A (CsA)) (**4**) from whole blood extracts.⁽³²⁾ A MALDI mass spectrometer equipped with automated multisampling capabilities was used to facilitate data collection and analysis of CsA. Extraction optimization was performed by generating an array of solvent systems to identify successful extractions. The first generation of experiments revealed four effective binary solvent systems (hexane/EtOH, ACN/H₂O, ACN/MeOH, hexane/CHCl₃). A new array based on these solvent systems was generated and in a second iteration of these experiments, hexane/CHCl₃ (70:30) was found to provide the most effective single-step extraction for cyclosporin and its metabolites (Figure 11). In order to determine the efficiency of the new extraction

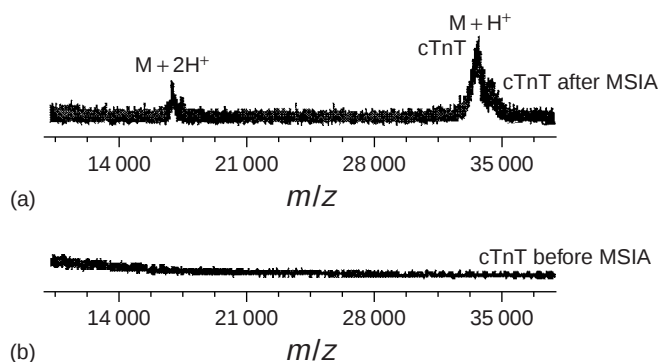
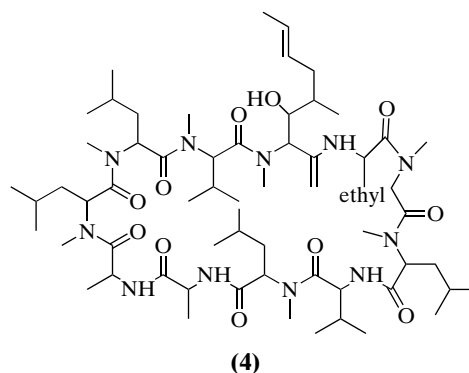


Figure 10 MSIA example.



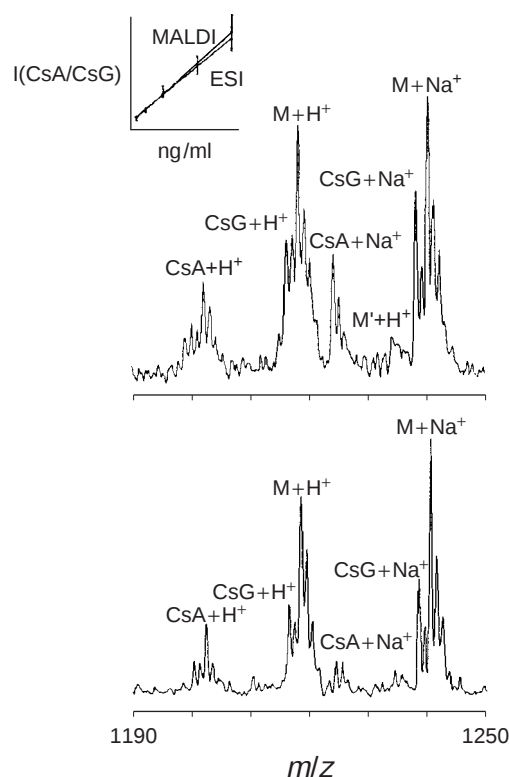


Figure 11 MALDI pharmacokinetics.

procedure it was compared to the previously developed “ether” extraction.

Initially, calibration curves were obtained using the “ether” extraction (a plot of the ratio of $\sum \text{int.}[\text{CsAH}^+ + \text{CsANa}^+]/[\text{CsGH}^+ + \text{CsGNa}^+]$ vs CsA concentration) with both the ESI (electrospray ionization) and MALDI mass spectrometers (cyclosporin G, CsG, was used as the internal standard). The data exhibited a linear relationship in the concentration range 0–1500 ng mL⁻¹ (Table 3) with an excellent correlation coefficient ($R = 0.999$) for both electrospray and MALDI. The new extraction generated standard curves for CsA that were very similar to those from the “ether” extraction method.

Table 3 Results obtained from the ESI/MS and MALDIMS analysis of extracted SA from standard blood samples using the 70/30 hexane/CHCl₃ extraction

Standard ng mL ⁻¹	ESI ng mL ⁻¹ (error)	MALDI ng mL ⁻¹ (error)
100	98 (2%)	94 (6%)
250	231 (8%)	238 (5%)
500	523 (5%)	543 (9%)
1000	1009 (1%)	961 (4%)
1500	1404 (6%)	1514 (1%)

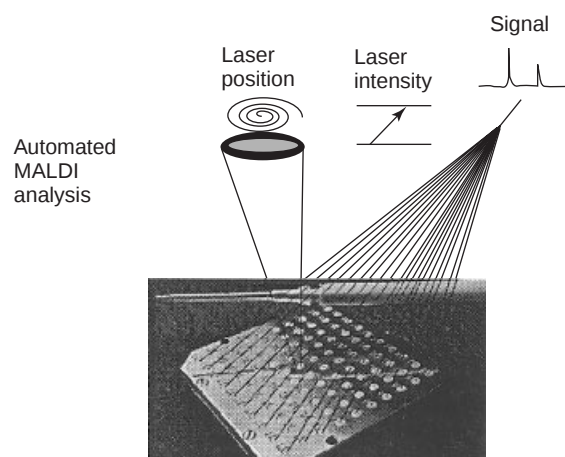


Figure 12 MALDI automation.

The automation of MALDI analyses, such as that used in the cyclosporin study, is becoming increasingly important in proteomics and combinatorial chemistry. These analyses are driven by a computer-controlled procedure to monitor the ion signal as a function of laser position and laser intensity (Figure 12). To accomplish this, the computer workstation automatically adjusts laser intensity and searches the sample well until a signal (within the specified mass range and intensity threshold) is obtained. Based on a careful preselection of autosampler options, each parameter (laser intensity, search pattern and step size in well, signal within a specified m/z range, and m/z range) is adjusted to minimize time of analysis and maximize signal quality.

What follows is a brief description of a MALDI automation procedure performed for the analysis of peptides. Here, the laser intensity was initially set at a minimum energy setting and then allowed to increase up to a maximum ($\sim 2\text{--}50\ \mu\text{J/pulse}$, as controlled by a variable neutral density filter). Step sizes were made in five increments, resulting in an increase in laser intensity of approximately $10\ \mu\text{J/pulse}$ per step. The laser intensity was increased until an acceptable data signal was acquired, whereby if no signal was observed the laser beam was repositioned on the well and MALDI analysis resumed at the lower laser power. To adjust the laser position on the MALDI sample plate, a preprogrammed spiral search pattern was used which began in the center of each circular well and spiraled outward in 0.2 mm increments. For each sample well analysis, only signals that reached a specified intensity were saved and once this signal was observed the analyses would automatically move to the next well. On average the total time spent for each sample well analysis was 140 s. This included acquisition time for averaging 64 scans and a delay for adjusting laser intensity and repositioning the sample plate.

3.3 Characterizing Peptides and Reactions Directly from the Solid Phase

Several reports have shown that MS can be very useful in characterizing compounds covalently bound to solid polymeric supports subsequent to their chemical cleavage from the resin. More specifically, the characterization of peptides and carbohydrates covalently linked to a polymeric support through a photolabile linker is feasible using MALDIMS. The scheme outlined in Figure 13(a) permits the characterization of resin-bound analytes in a single step and requires no pretreatment of the sample to induce cleavage from the support. In addition, this approach also shows that the technique is suitable for monitoring chemical reactions on the solid phase.^(33,34)

Figure 13(b) shows the mass spectrum of the unprotected resin-bound peptide. MALDI analysis yielded a characteristic $[M+H]^+$ signal at m/z 1247 which is consistent with the expected mass of the free peptide (MW = 1245.5 Da). The spectrum in Figure 13(b) was acquired from a single bead. The peptide (in a chemical cleavage step) was recovered from resin samples and then subjected to MALDI analysis, indicating that the sample was not completely photolyzed in the experiment. This is not surprising, as only a small amount

of material (<femtomoles) is typically consumed during such analysis. Figure 13(c) shows the MALDI analysis of resin-bound carbohydrate, illustrating the application of this technique to another class of compounds.

The one-step MALDI procedure for the direct analysis of resin-bound molecules described above is also well suited to studying chemical reactions on the solid phase. As a demonstration of this utility, the coupling reaction of a Boc-Arg(Tos) residue (preactivated as the hydroxybenzotriazole ester) to the protected 11 amino acid peptide (described above) was monitored as a function of time. Interestingly, the Fmoc protected peptide was not amenable to MALDI analysis; the absence of appropriate protonation sites in this sample probably prevents ionization. Therefore, prior to analysis resin samples were treated with 20% (v/v) piperidine in dimethylformamide (DMF) in order to remove the Fmoc protecting groups, after which the reaction proceeded quickly and coupling appeared nearly complete in just 6 min.

The direct analysis of resin-bound molecules by MALDI offers several important advantages. The first is the lack of an additional cleavage step prior to mass analysis, which is often required by other methods of characterization. Performing MALDI directly on the solid phase requires less sample handling and more efficient management since the resin-bound compound can be easily recovered for subsequent manipulations (again, <femtomoles of material is consumed in typical MALDI analyses). The most significant advantage is that it can be used to monitor chemical reactions on the solid phase in real time, in much the same way that thin-layer chromatography is used to monitor reactions in solution. Furthermore, all analytes amenable to MALDI ionization should prove suitable for routine analysis by this procedure.

4 CONCLUSION

Since the late 1970s, tremendous progress has been made in the analysis of peptides and proteins by MS. Much of this enormous growth can be attributed to advances in desorption/ionization techniques such as MALDI, namely in resolution and accuracy. In addition, it is now routine to analyze peptides and proteins at femtomole levels which makes it well suited for protein mass mapping and structural determination. We are all familiar with the prominence of gel electrophoresis methods in molecular biology studies; we posit that MS methods will capture this position as a primary research tool in coming years owing to its superior sensitivity, precision, accuracy, and throughput. The applications discussed here are just a few examples of current approaches that will see expanded use in the future.

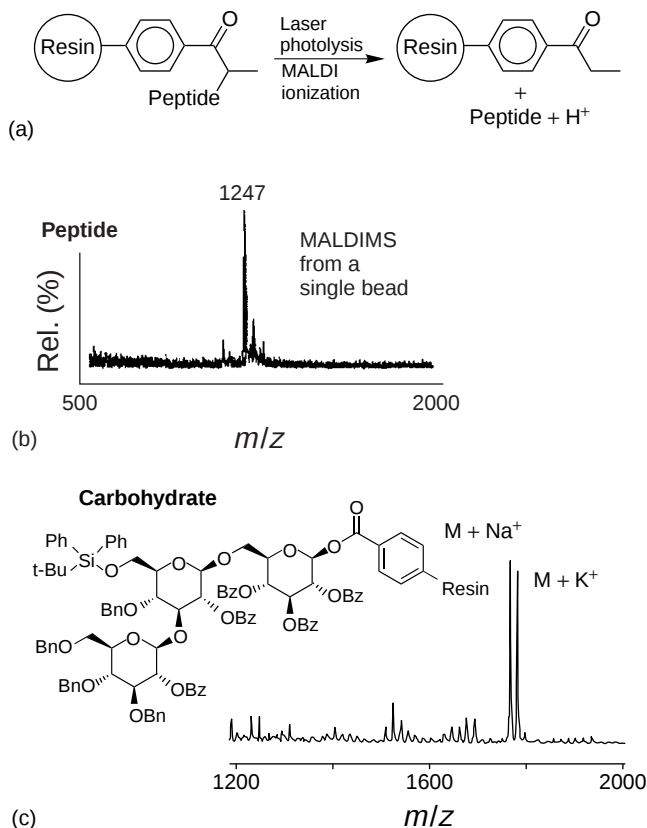


Figure 13 MALDI analysis from the solid phase.

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ABBREVIATIONS AND ACRONYMS

ACN	Acetonitrile
CaM	Calmodulin
CCA	α -Cyano-4-hydroxycinnamic acid
Cdk2	Cyclin-dependent Kinase 2
CID	Collision-induced Dissociation
CsA	Cyclosporin A
CsG	Cyclosporin G
cTnT	Cardiac troponin T
DE	Delayed Extraction
DHB	2,5-Dihydroxybenzoic acid
DMF	Dimethylformamide
ELISA	Enzyme-linked Immunosorbent Assay
ESI	Electrospray Ionization
EST	Expressed Sequence Tag
FTMS	Fourier Transform Mass Spectrometry
HPLC	High-performance Liquid Chromatography
ICR	Ion Cyclotron Resonance
KE	Kinetic Energy
MALDI	Matrix-assisted Laser Desorption/Ionization
MALDIMS	Matrix-assisted Laser Desorption/Ionization Mass Spectrometry
MS	Mass Spectrometry
MS ⁿ	Tandem Mass Spectrometry (<i>n</i> th series)
MSIA	Mass Spectrometric Immunoassay
MS/MS	Tandem Mass Spectrometry
NC	Nitrocellulose
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance
PSD	Postsource Decay
RF	Radio Frequency
RPLC	Reversed-phase Liquid Chromatography
SA	3,5-Dimethoxy-4-hydroxycinnamic acid
TFA	Trifluoroacetic Acid
TOF	Time-of-flight
UV	Ultraviolet

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