Mass Spectrometry and Protein Analysis
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REVIEW

Mass Spectrometry and Protein Analysis

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Mass spectrometry is a central analytical technique for protein research and for the study of biomolecules in general. Driven by the need to identify, characterize, and quantify proteins at ever increasing sensitivity and in ever more complex samples, a wide range of new mass spectrometry–based analytical platforms and experimental strategies have emerged. Here we review recent advances in mass spectrometry instrumentation in the context of current and emerging research strategies in protein science.

The ability to identify proteins and to determine their covalent structures has been central to the life sciences. The amino acid sequence of proteins provides a link between proteins and their coding genes via the genetic code, and, in principle, a link between cell physiology and genetics. The identification of proteins provides a window into complex cellular regulatory networks.

Before the genomics revolution, chemical or enzymatic methods were used to probe the covalent structure of single, highly purified proteins, and typically, the products of such reactions were detected by ultraviolet (UV) absorbance or fluorescent spectroscopy. For example, polypeptides were sequenced by stepwise chemical degradation from the N terminus to the C terminus (Edman degradation), with subsequent identification of the released amino acid derivatives by UV absorbance spectroscopy. Gradually over the past two decades, mass spectrometers were interfaced with a number of protein chemistry assays to create detectors providing superior information. With the increased performance and versatility of the instrumentation, new protein analytical strategies have emerged in which mass spectrometry is the central element. For example, by the mid-1990s, a variety of mass spectrometry–based strategies had essentially replaced the Edman degradation as the mainstream method for determining the amino acid sequences of polypeptides.

The trend toward mass spectrometry as the technique of choice for identifying and probing the covalent structure of proteins was accelerated by the genome project. Genomics demonstrated the power of high-throughput, comprehensive analyses of biological systems. Genomics also provides complete genomic sequences, which are a critical resource for identifying proteins quickly and robustly by the correlation of mass-spectrometric measurements of peptides with sequence databases. The systematic analysis of all the proteins in a tissue or cell was popularized under the name proteomics, with mass spectrometry central to most proteomic strategies.

The analysis of a full proteome presents a formidable task and, in spite of recent technical developments, remains to be achieved for any species. The task is challenging because proteomes have a large and unknown complexity. What is certain is that the number of proteins in a species' proteome exceeds by far the number of genes in the corresponding genome. This diversity arises from the fact that a particular gene can generate multiple distinct proteins as a result of alternative splicing of primary transcripts, the presence of sequence polymorphisms, post-translational modifications, and other protein-processing mechanisms. Moreover, proteins span a concentration range that exceeds the dynamic range of any single analytical method or instrument. For example, it has been estimated that the concentration range of serum proteins exceeds 10 orders of magnitude (*1*). Although these challenges are daunting, they have stimulated advances in technologies for the analysis of proteins and proteomes. Here we describe a range of mass-spectrometric techniques, discuss their utility for protein analysis, and assess their ability to support or interface with a range of proteomic strategies.

MS Instruments and Their Use

Mass spectrometry was restricted for a long time to small and thermostable compounds because of the lack of effective techniques to softly ionize and transfer the ionized molecules from the condensed phase into the gas phase without excessive fragmentation. The development in the late 1980s of two techniques for the routine and general formation of molecular ions of intact biomolecules—electrospray ionization (ESI) (*2*) and matrix assisted laser desorption/ionization (MALDI) (*3*)—dramatically changed this situation and made polypeptides accessible to mass-spectrometric analysis. This catalyzed the development of new mass analyzers and complex multistage instruments [for instance, hybrid quadrupole time-of-flight (Q-Q-ToF) and tandem time-of-flight (ToF-ToF) instruments] (Table 1) designed to tackle the challenges of protein and proteome analysis (*4*, *5*). Mass spectrometers are used either to measure simply the molecular mass of a polypeptide or to determine additional structural features including the amino acid sequence or the site of attachment and type of posttranslational modifications. In the former case, single-stage mass spectrometers are used, acting essentially as balances to weigh molecules. In the latter case, after the initial mass determination, specific ions are selected and subjected to fragmentation through collision. In such experiments, referred to as tandem mass spectrometry (MS/MS), detailed structural features of the peptides can be inferred from the analysis of the masses of the resulting fragments. The types of mass spectrometers described below are most commonly used to support a range of research strategies in the protein sciences. They differ in their physical principles, their performance standards, their mode of operation, and their ability to support specific analytical strategies.

Table 1. Characteristics and performances of commonly used types of mass spectrometers. Check marks indicate available, check marks in parentheses indicate optional. +, ++, and +++ indicate possible or moderate, good or high, and excellent or very high, respectively. Seq., sequential.

	IT-LIT	Q-Q-ToF	ToF-ToF	FT-ICR	Q-Q-Q	QQ-LIT
Mass accuracy	Low	Good	Good	Excellent	Medium	Medium
Resolving power	Low	Good	High	Very high	Low	Low
Sensitivity (LOD)	Good		High	Medium	High	High
Dynamic range	Low	Medium	Medium	Medium	High	High
ESI	✓	✓		✓	✓	✓
MALDI	(✓)	(✓)	✓			
MS/MS capabilities	✓	✓	✓	✓	✓	✓
Additional capabilities	Seq. MS/MS			Precursor, Neutral loss, MRM		
Identification	++	++	++	+++	+	+
Quantification	+	+++	++	++	+++	+++
Throughput	+++	++	+++	++	++	++
Detection of modifications	+	+	+	+		+++

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Time-of-flight (ToF) and hybrid ToF instruments. In ToF analyzers, the mass-to-charge ratio of an analyte ion is deduced from its flight time through a tube of specified length that is under vacuum. The performance of ToF analyzers has greatly improved, in particular in terms of resolution and mass accuracy (*6*). A resolving power exceeding 12,000 has become routine on many instruments, and with a proper mass calibration protocol, mass accuracies in the low–parts per million (ppm) range are achievable. ToF mass analyzers are the basis for analytical platforms operated with both ESI and MALDI. The Q-Q-ToF instruments exhibit high resolution and mass accuracy in MS and MS/MS mode. In the MS mode, the quadrupole acts as an ion guide to the ToF analyzer where the mass analysis takes place. In the MS/MS mode, the precursor ions (typically a multiply charged ion in ESI) are selected in the first quadrupole and undergo fragmentation through collision-induced dissociation in the second quadrupole. The product ions are analyzed in the ToF device. Spectra obtained in both full-scan and MS/MS modes exhibit good mass accuracy and high resolution, yielding an increased number of peptides detected and allowing for the determination of the charge state and unambiguous assignment of the mono-isotopic signal. All of these factors simplify the identification of peptides via database searches by tightening the search parameters and augmenting the confidence in the results. Finally, Q-Q-ToF instruments perform well for quantitative analyses and for the identification of posttranslational modifications.

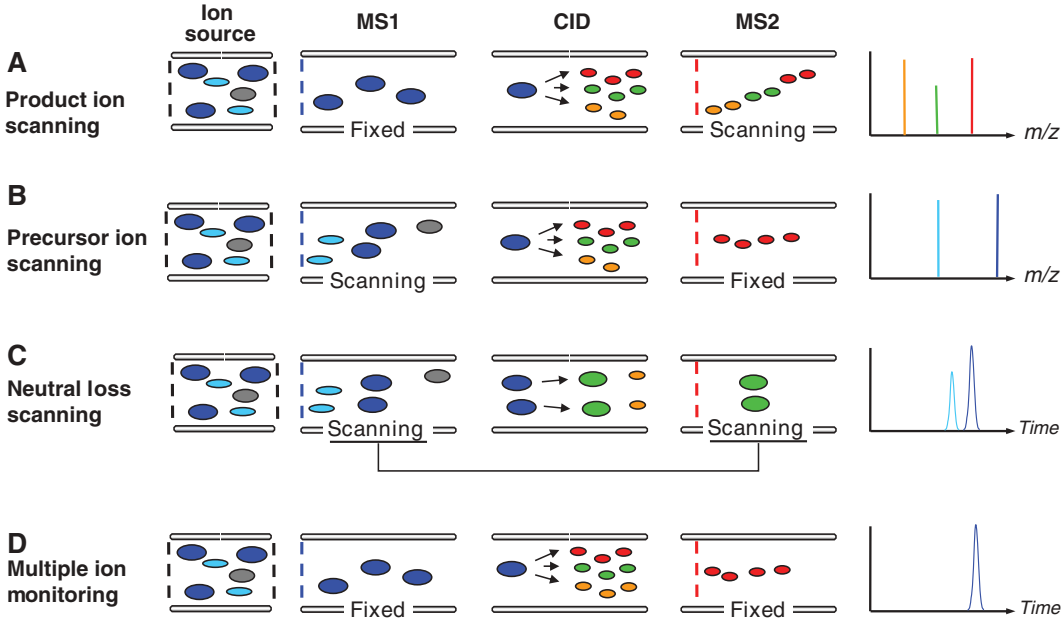

Fig. 1. Schematic representation of various types of tandem mass spectrometry experiments. (**A**) Product ion scanning is the most common MS/MS experiment in proteomics. Its purpose is the generation of fragment ion spectra for the identification of the amino acid sequence of specific peptides. In this experiment, the first analyzer (MS1) is set to a value that selects one specific precursor ion at a time. The selected ion undergoes CID in the collision cell, and the resulting fragments are analyzed by the second analyzer (MS2). This process is repeated for different precursors. (**B**) Precursor ion scanning sets the second analyzer (MS2) to transmit only one specific fragment ion to the detector. MS1 is scanned to detect all the precursor ions that generate this fragment. Typically, this method is used to detect a subset of peptides in a sample that contain a specific functional group, for instance a phosphate ester or a carbohydrate modification. (**C**) Neutral loss scanning scans both analyzers in a synchronized manner, so that the mass difference of ions passing through MS1 and MS2 remains constant. The mass difference corresponds to a neutral fragment that is lost from a peptide ion in the collision cell. The neutral loss scan is therefore used to detect those peptides in a sample that contain a specific functional group. A common application of this method is the detection of peptides phosphorylated at serine or threonine residues via a loss of phosphoric acid. (**D**) MRM consists of a series of short experiments in which one precursor ion and one specific fragment characteristic for that precursor are selected by MS1 and MS2, respectively. Typically, the instrument cycles through a series of transitions (precursor-fragment pair) and records the signal as a function of time (chromatographic elution). MRM is used for the detection of a specific analyte with known fragmentation properties in complex samples.

MALDI remains a valuable alternative ionization technique for peptides and proteins and is often used to complement results obtained by ESI MS. MALDI MS is very sensitive and more tolerant than ESI to the presence of contaminants such as salts or small amount of detergent. The MALDI technique has primarily been used in conjunction with ToF analyzers for molecular mass determination. It has been implemented on Q-Q-ToF or ToF-ToF mass spectrometers to provide true MS/MS capabilities. The resulting spectra characterized by singly charged precursor ions and those obtained on ToF-ToF instruments present high-energy collision fragments (cleavages of the peptidic bonds and side chains), which are readily interpretable (*7*).

Ion trap (IT) mass analyzers. In IT analyzers, ions are trapped and can therefore be accumulated over time in a physical device. The IT technology is characterized by MS/MS capabilities (*8*) with unmatched sensitivity and fast data acquisition. Used in conjunction with data-dependent acquisition (*9*), IT technology allows high-throughput analyses. However, IT analyzers have limited-resolution, low–ion trapping capacity, and space-charging effects result in mass measurements lacking accuracy. The development of linear ion trap (LIT) analyzers with higher ion-trapping capacities has expanded the dynamic range and the overall sensitivity of this technique, and LITs have been replacing classical quadrupole trapping devices. Typically, LIT instruments have an optional slow scanning function to increase resolution. They also have multiple-stage sequential MS/MS capabilities, in which fragment ions are iteratively isolated and further fragmented, a strategy that has proven to be very useful for the analysis of posttranslational modifications such as phosphorylation (*10*).

LIT devices have been implemented on triple quadrupole–type instruments (i.e., the second analyzer is substituted by a LIT) to offer a unique set of functionalities (*11*). The Q-Q-LIT geometry offers the scanning capabilities of a triple quadrupole instrument, including precursor ion and neutral loss scanning (Fig. 1, B and C), and increased sensitivity. These instruments therefore offer unique capabilities for the analysis of modifications. In addition, the multiple reaction monitoring (MRM) capability of Q-Q-LIT instruments (Fig. 1D) allows the detection of specific transitions between the precursor and one fragment of a given peptide. The selectivity resulting from two stages of analyzer combined with the high duty cycle results in quantitative analyses with unmatched sensitivity.

Ion cyclotron resonance and orbitrap mass analyzers. The development and commercialization of robust Fourier transform–ion cyclotron resonance (FT-ICR) mass spectrometers with external ion sources (*12*) represented a breakthrough in terms of resolving power and

mass accuracy. Measurements in the low ppm–sub ppm range can be achieved. The high resolution of this instrument not only accounts for better data quality, but it also increases the peak capacity and thus allows for the detection of more signals compared with instruments with lower resolving power. The development of hybrid FT-ICR instrument with an external LIT device has added robustness to this platform and allowed routine generation of low-resolution MS/MS spectra with accurate mass of the precursor ions. FT-MS performed on an LIT-ICR hybrid instrument allows true parallel full mass spectrum (MS1) and tandem mass spectrum (MS2) acquisition (not sequential), and it yields high-quality MS1 data that can be used for quantification. The only drawback of that approach is the relatively slow acquisition rate (several s per cycle) and the limited dynamic range of IT devices.

Very recently, a new type of mass analyzer called orbitrap (*13*, *14*) has emerged. It is the first analyzer introduced to the market in three decades that is based on a new physics principle (the separation of ions in an oscillating electric field). This instrument presents characteristics similar to an FT-ICR spectrometer in terms of resolution and mass accuracy but without the burden of an expensive superconducting magnet.

MS/MS modes of operation. Tandem mass spectrometry is commonly used in the product ion mode (Fig. 1A) to determine the amino acid sequence of a specific peptide. This technique is available on all instruments equipped with MS/MS capabilities. However, more specialized instruments (Table 1) allow other types of MS/MS experiments. Experiments to detect a subset of peptides that contain a specific functional group requiring precursor-ion or neutral-loss scans (Fig. 1, B and C, respectively) can only be performed effectively on triple quadrupole (Q-Q-Q) or quadrupole ion trap (Q-Q-LIT) instruments. For instance, phosphorylation and glycosylation can be detected very effectively in complex mixtures by generating specific reporter ions in the collision cell that can be detected by the specific scan functions (*15*–*17*). In a typical experiment, the precursor or neutral loss scan will detect the components of interest and then trigger a conventional MS/MS (product ion scan) to identify the amino sequence and localize the modifications.

Triple quadrupole–derived technologies also allow quantitative analyses with very high sensitivity in the MRM mode. Known (or suspected) analytes can be detected and quantified with a high degree of sensitivity and selectivity (Fig. 1D). The high selectivity results from monitoring one pair of precursor/fragment ions characteristic of a single peptide. In addition, the two levels of mass selection in MRM experiments result in a substantial increase in sensitivity, because the first mass filter only transmits a small ion population, and thus minimizes the overall chemical background. The collision-induced dissociation (CID) fragment ions derived from the precursor ions produce discrete signals, whereas chemical noise is randomly distributed. Finally, the nonscanning nature of this technique (high dwell time) increases in sensitivity by several orders of magnitude compared with the limit of detection (LOD) achieved by product ion scans.

Instrument performance. The pertinent characteristics of the various instrument types are summarized in Table 1. Instrument performance in terms of resolving power, LOD (sensitivity), and mass accuracy depends on the instrument type, the ionization method, and the scanning capabilities used. At this point, no instrument offers all capabilities simultaneously, and trade-offs need to be made based on the type of analysis to be performed.

The comparison of instrument performance is potentially a controversial subject, because specifications very much depend on the type of application, the sample analyzed, and the experimental setup. Very low LOD is often reported for individual peptides; however, for biological samples with high matrix background, the practical limits are often off by several orders of magnitude. Discrepancies in performance are often observed between an instrument performing under optimum conditions (typically above manufacturer specifications) and a routine, high-throughput operation. Whether the application focuses on identification or quantification will determine which platform and strategy is preferred. In the former case, resolving power (to improve separation of the various components) and mass accuracy are important factors, whereas in the latter case, the emphasis is on sensitivity, dynamic range, and MRM capabilities. Thus, biological questions to be addressed together with the experimental design should define the type of instrumental platform required.

The simultaneous collection of quantitative data (full scan mode) and qualitative data (MS/MS mode) is often difficult. Parallel data acquisition in some hybrid instruments (LIT-ICR) partially solves the problem. Precise quantification requires high-quality data characterized by intense signals and a high signal-to-noise ratio collected across the entire elution profile. Data quality is very much dependent on the data acquisition parameters (scanning time or dwell time for nonscanning instruments). Thus, usually trade-offs have to be made between data quality and throughput.

Latest developments. A number of recent developments are opening new opportunities for the characterization of biomolecules. Alternate fragmentation techniques to CID that are based on electron transfer of the ions present in the collision cell have been developed to improve peptide sequencing. In particular, electron capture dissociation (ECD) (*18*) and electron transfer dissociation (ETD) (*19*) have been implemented on FT-ICR and LIT instruments, respectively. These two techniques yield fragments that are complementary to the classical CID fragmentation. They tend to be more evenly distributed over the entire peptide backbone and are particularly useful in localizing posttranslational modifications. ECD and ETD are also applicable to large peptides and proteins. The ability to fragment and analyze intact proteins opens new possibilities for the direct analysis of intact proteins by mass spectrometry (called top-down approaches), which yield full amino acid coverage and precise identification and localization of modifications (*20*).

Classical and Emerging Proteomic Strategies

Although no proteomic strategies are currently capable of completely and routinely analyzing a proteome, the techniques are robust and their potential for complete proteome analysis is increasing rapidly. Moreover, the analysis of specific subproteomes, such as the proteins contained in organelles or subcellular fractions, has become routine. Proteomic studies also differ in their objectives. Many studies are descriptive, focusing on the identification of the proteins in a sample and the characterization of their post-translational modifications. More recently, quantitative measurements of either absolute protein quantities or quantitative changes of proteins between samples have been performed.

Virtually every mass spectrometry–based proteomic workflow consists of three distinct stages: (i) Protein samples are isolated from their biological source and optionally fractionated. The final protein sample is then digested and the resulting peptide sample is further fractionated. (ii) The peptides are subjected to qualitative and quantitative mass-spectrometric analysis. (iii) The large data sets generated are analyzed by suitable software tools to deduce the amino acid sequence and, if applicable, the quantity of the proteins in a sample. The peptide identity is assigned to the MS/MS spectra through database searching (*21*), which is performed according to established guidelines to generate consistent results (*22*). A subsequent statistical analysis of the search results is critical to ensure confidence in the identifications (*23*).

MS analysis of substantially purified proteins. This approach is exemplified by the original proteomic approach: two-dimensional (2D) gel electrophoresis followed by the mass-spectrometric identification of the protein(s) in a single gel spot (Fig. 2A). The targeted proteins are digested and identified by mass spectrometry, usually peptide mass fingerprinting using a MALDI-ToF instrument. More recently, variants of this approach have been developed in which various combinations of sequential electrophoretic or chromatographic separation methods are combined to achieve sufficient peak capacity to resolve complex samples (*24*, *25*). Quantification is achieved at the protein level by comparing the signal intensities of identical proteins in different

samples. The strength of these methods is their ability to resolve related proteins, such as differentially modified forms, and the low degree of complexity of the samples generated for mass spectrometry analysis. The methods suffer from limited dynamic range, insufficient power to resolve proteomes, and limited sample throughput. Furthermore, important classes of proteins, including membrane proteins, are difficult to analyze by these methods, which are best suited for the analysis of protein samples of limited complexity and for studies where specific proteins need to be extensively characterized.

MS analysis of complex peptide mixtures. In this method, also referred to as shotgun proteomics, complex protein samples are digested, and the resulting peptide samples are extensively fractionated and analyzed by automated MS/MS, typically using rapidly scanning analyzers such as IT mass spectrometers (Fig. 2B). Protein samples analyzed by this method include complete cell lysates or tissue extracts, subcellular fractions, isolated organelles, or other subproteomes.

If samples are labeled with stable isotopes, the ratio of signal intensities of differentially labeled but chemically identical analytes can be used to determine accurately their relative abundance in different samples (Fig. 3A). Multiple analyses can be performed concomitantly by using tandem mass tags (Fig. 3B). Alternately, the absolute quantity of peptide can be determined by adding calibrated amounts of isotopically labeled peptides into the sample before the MS analysis (Fig. 3C).

The strength of the shotgun approach is its conceptual and experimental simplicity, increased proteomic coverage compared with the method described above, and accurate quantification. The shotgun method suffers from limited dynamic range, informatics challenges related to inferring peptide and protein sequence identities from the large number of acquired mass spectra, a high redundancy, and the enormous complexity of the generated peptide samples. These limitations have been addressed, in part, by the use of fractionation that reduces the complexity of the peptide sample. Popular fractionation methods target information-rich subsets of the proteome, such as the cysteine-containing peptides (*26*), phosphorylated peptides (*27*, *28*), or glycosylated peptides (*29*). Shotgun proteomics is most suitable for the rapid identification of the components of complex sample mixtures and for the comparative quantitative analysis of the proteins contained in different samples. Because the connection between the peptides that are analyzed in the mass spectrometer and the protein(s) from which the peptides originate is lost during proteolysis, this approach is less well suited for the extensive characterization of proteins with multiple modifications.

Comparative pattern analysis. Comparative peptide pattern analysis (Fig. 2C) is conceptually

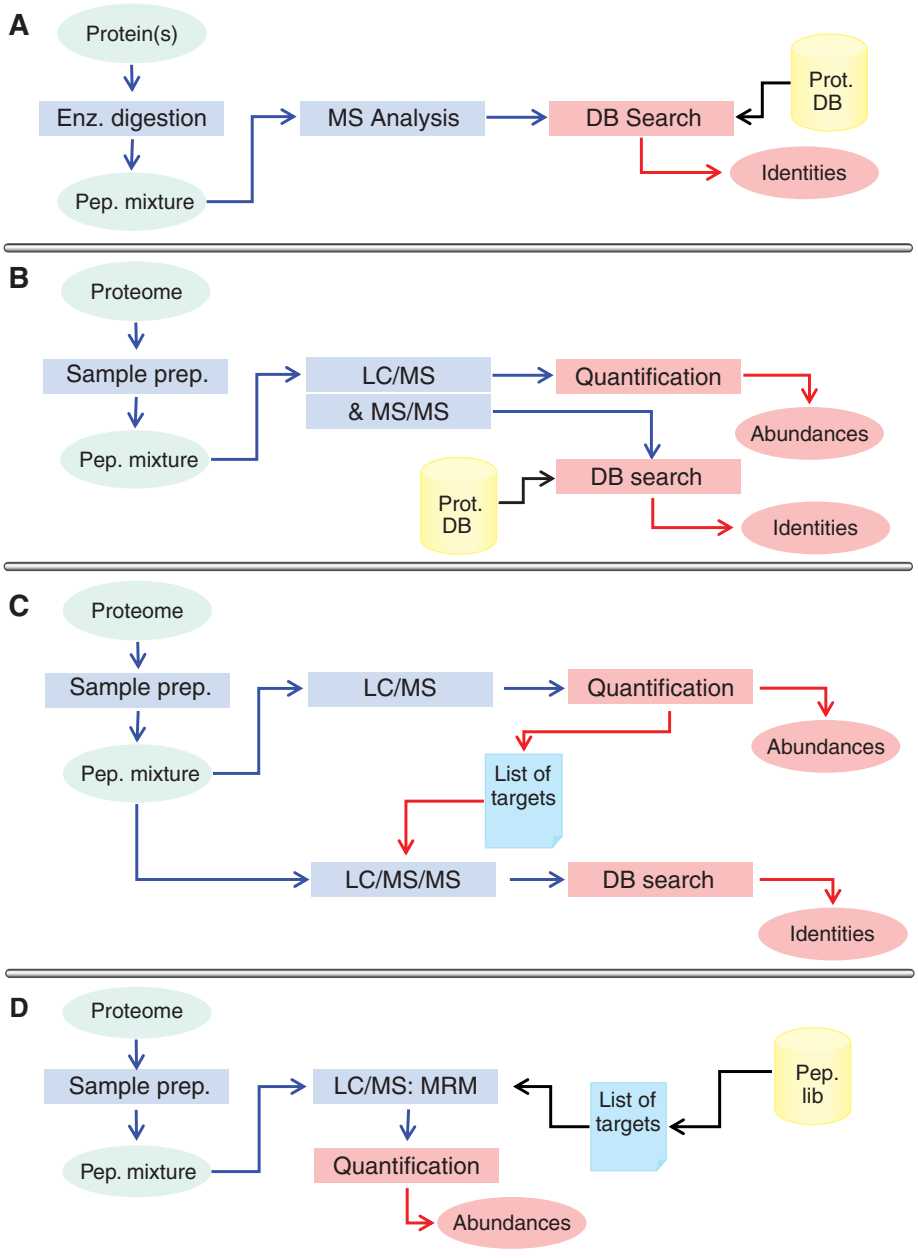

Fig. 2. Proteomics strategies. (**A**) Identification of simple protein (prot.) mixtures from 2D gel electrophoresis or pull-down experiments is carried out by enzymatic (enz.) digestion and by mass spectrometry analysis of the resulting peptides (pep.) (in ESI or MALDI mode). Peptide masses allows their identification (and that of the parent proteins) using peptide mass fingerprinting (PMF). Additional MS/MS data are also used for the peptide identification. (**B**) Random protein identification and quantification, also referred to as shotgun proteomics, couples identification and quantification of specific peptides in a sample. Selected peptides are subjected to product ion scanning (Fig. 1A) in a tandem mass spectrometer. The precursor ions are selected randomly, and typically only a fraction of the precursor ions detected are selected (undersampling). The ion intensities in MS1 are used to quantify the analytes by relating the signal intensity of the selected analyte to the signal intensity of a suitable reference molecule (frequently, a reference peptide labeled with heavy stable isotopes). (**C**) Quantification-driven identification decouples quantification and identification of peptides. In a first step, peptides that quantitatively differ between samples are detected by comparing the MS1 peptide patterns (mass versus chromatographic retention time) between samples, allowing for a more extensive analysis of the peptide patterns. Candidate peptides that show interesting quantitative properties are subjected to MS/MS sequencing in a second step using an inclusion list resulting from the primary analysis. (**D**) Hypothesis-driven peptide identification measures with high precision the abundance of a series of predetermined peptides. The targeted peptides, usually identified from previous experiments, are subjected to MRM (Fig. 1D). Accurate quantification is achieved by adding a suitable calibrated reference peptide.

similar to the 2D gel electrophoresis method in that 2D patterns of features are generated for each sample, and the patterns are compared to identify quantitative or qualitative changes. Such features are then further characterized, for example, by sequencing or by determining their posttranslationally modified state. However, in MS-based pattern analysis methods, protein samples are proteolyzed, fractionated, and the resulting peptides are analyzed by liquid chromatography (LC)/MS. The two dimensions to describe a peptide ion are chromatographic elution time and mass. Quantification of the detected features is achieved by integrating the ion counts of each signal. The main advantage of this method is that all the features that are detectable by MS can be quantified. This is in contrast with the shotgun methods, in which only identified peptides are quantified. However, in practice, it is extraordinarily challenging to generate highly reproducible patterns and to develop software tools that reliably match related patterns (*30*, *31*). Such analyses result in a list of features that represents putative peptide ions, with the following attributes: mass-to-charge ratio (*m*/*z*), charge state, elution time, and ion intensity. The peptides that need to be sequenced (for instance, features indicating different expression between two samples) are included in a list and then submitted to a new, directed mass-spectrometric experiment to collect MS/MS spectra of these features exclusively. This type of analysis is also well suited for the MALDI/MS/MS platform, because the samples are "immobilized" on the sample plate and can therefore be interrogated sequentially and without any time constraints.

Hypothesis-driven strategies. It can be expected that incremental improvements in instrument performance will continue to translate into more-sensitive, faster, and more-reliable proteomic analyses. However, it is not clear whether such advances will be sufficient to eliminate the major bottlenecks encountered in the current proteomics approaches. We have argued before (*32*) that proteomics needs to undergo a paradigm shift to reach the goal of robustly and globally analyzing proteomes. The essence of this shift is the transformation of proteomics from a mode where in every experiment, the proteome is rediscovered, to a mode in which the information from prior proteomic experiments is used to guide the present experiments. Specifically, it can be anticipated that extensive (complete) proteome maps containing all the peptides of a species that are observable by mass spectrometry will be generated and that future strategies will aim at the targeted, nonredundant analysis of information-rich peptides. For mass spectrometry instrumentation and strategy, this shift of paradigm requires the development of instruments and data acquisition protocols that support the fast, sensitive, and robust analysis of previously generated lists of target peptides. Databases that allow the extraction of peptides that uniquely identify a specific protein or a specific modified form of a protein and that are easily detectable by

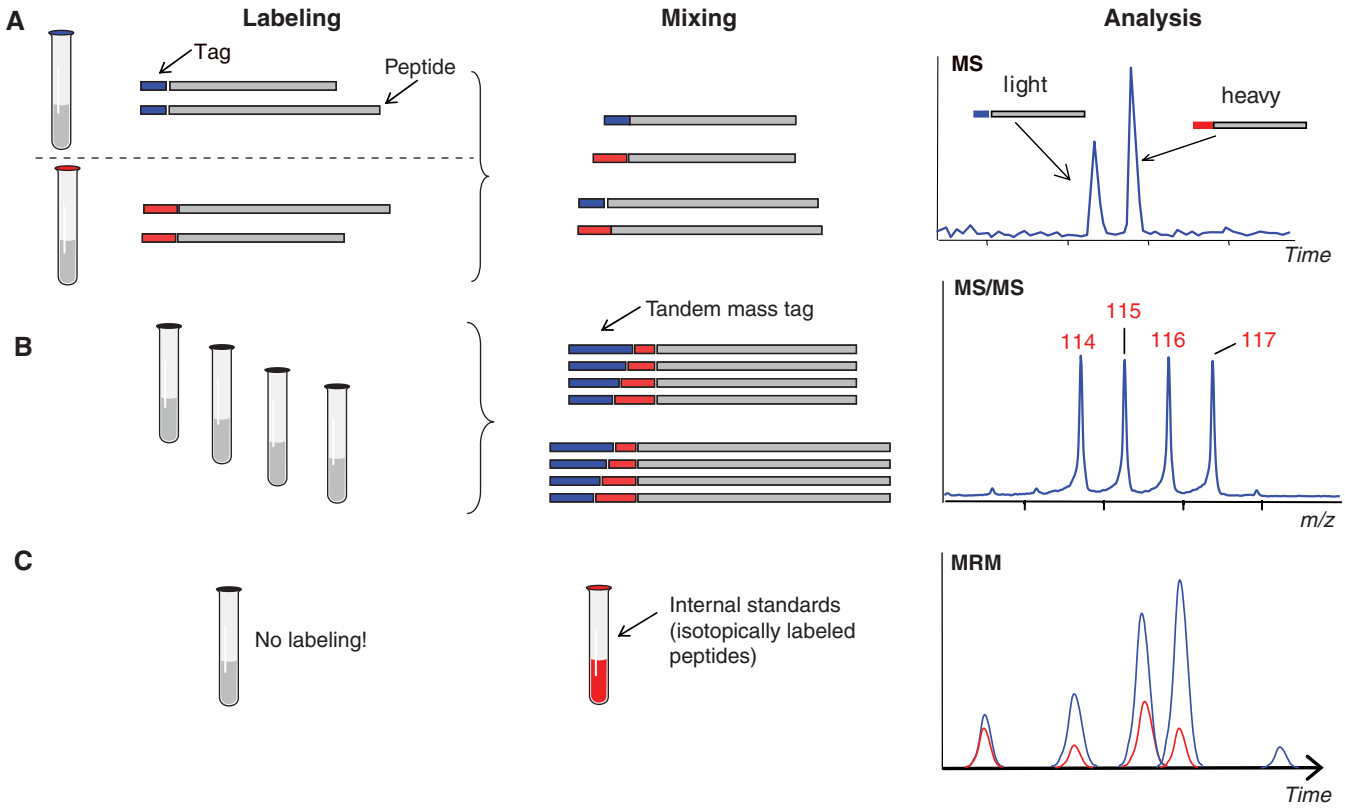

Fig. 3. Strategies for quantitative peptide analyses. (**A**) Quantification using isotope dilution is widely used and accepted in the proteomics community. It is based on the incorporation of a stable isotope signature into all of the proteins of one sample and the incorporation of a different stable isotope signature in all proteins of a second sample. The samples are then combined to serve as mutual references. Stable isotope incorporation has been achieved by chemical modification of proteins using suitable isotope coded labeling reagents (*26*), metabolic labeling (*35*), or by enzyme reactions (*36*). The method is schematically illustrated here. (**B**) Quantification using tandem mass tags relies on variants of stable isotope labeling reagents (*37*, *38*). They consist of two isotopically labeled elements, which have an overall constant mass. Currently, these reagents can be multiplexed to four channels. Quantification is performed in the MS/MS mode by measuring the relative intensity of the reporter group attached at the N terminus and observed in low mass range of the CID spectrum. (**C**) Quantification using internal standards is a variant of isotopic dilution in which a subset of isotopically labeled peptides is added to the sample at defined concentrations to perform precise quantification using calibration curves. Although it is more demanding in terms of sample preparation, this method is likely to gain importance in the future in the more directed approach indicated above for quantifying proteins in a larger number of samples. It may also be a more effective way to perform hypothesis-driven studies by screening for known or putative proteins (i.e., peptides) present in samples.

mass spectrometry are just emerging (*33*). It can be anticipated that biological hypotheses will generate lists of proteins that need to be characterized and quantified in a particular study. Such lists of proteins can then be submitted to the database to produce the minimal set of peptides required to test the hypothesis. This set of peptides can then be measured by targeted methods, including MRM (Fig. 2D). The directed nature of this approach allows the mass spectrometer to focus on a nonredundant set of targets and therefore leads to a substantial gain in throughput and sensitivity. By adding calibrated, isotopically labeled reference peptides, precise quantitative information can be obtained.

Such strategies are best implemented on mass spectrometers with Q-Q-LIT geometry related to the triple quadrupole instrument that has been used for decades to quantify small molecules drugs and their metabolites in serum. The same type of protocols can be applied to proteomics studies.

As a variant of this approach, Smith developed the concept of using accurate mass tags to identify peptides by matching accurately measured peptide masses with those calculated for peptides present in a database (*34*), thus obviating the need to sequence each peptide in each sample. With the rapid increase in accessible data from prior proteomic experiments and the development of mass spectrometer control software that supports large inclusion lists for targeted analyses, the use of the hypothesis-driven strategies can be expected to increase.

Outlook and Conclusion

Protein analysis and, more specifically, proteomics have driven the development of mass spectrometry for the past decade. Technological advances have translated into major improvements in mass accuracy, resolving power, LOD, and accuracy of quantification and new experimental strategies aimed at the routine and comprehensive analysis of whole proteomes. New mass-spectrometric strategies to analyze intact proteins, protein complexes, and low-redundancy target workflows are emerging. Although these mass spectrometry technologies have been driven by protein research; once developed, they will equally effect the analysis of other types of biomolecules, including metabolites, lipids, and carbohydrates. It can therefore be anticipated that the use of mass spectrometry in the life sciences will become even more prevalent and diversified.

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REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

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Advances in molecular biology, organic chemistry, and materials science have recently created several new classes of fluorescent probes for imaging in cell biology. Here we review the characteristic benefits and limitations of fluorescent probes to study proteins. The focus is on protein detection in live versus fixed cells: determination of protein expression, localization, activity state, and the possibility for combination of fluorescent light microscopy with electron microscopy. Small organic fluorescent dyes, nanocrystals ("quantum dots"), autofluorescent proteins, small genetic encoded tags that can be complexed with fluorochromes, and combinations of these probes are highlighted.

Fluorescence has long been used to visualize cell biology at many levels, from molecules to complete organisms. Originally, fluorescence was mainly observed from small organic dyes attached by means of antibodies to the protein of interest. However, antibody targeting of intracellular proteins normally requires cell fixation and permeabilization. Later, fluorophores could directly recognize organelles, nucleic acids, and certain important ions in living cells. In the past decade, fluorescent proteins have enabled noninvasive imaging in living cells and organisms of reporter gene expression, protein trafficking, and many dynamic biochemical signals. Hybrid systems in which small organic fluorophores are genetically targeted are filling other useful niches including determination of protein age, correlative electron-microscopic localization, and rapid photoinactivation of selected proteins. Meanwhile, semiconductor nanocrystals have been developed with higher brightness and photostability than previous fluorophores, but their targeting currently remains challenging. This review will discuss recent developments in fluores-

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