

Chapter 1

Mass Spectrometry for Proteomics-Based Investigation

Alisa G. Woods, Izabela Sokolowska, Armand G. Ngounou Wetie,
Kelly Wormwood, Roshanak Aslebagh, Sapan Patel, and Costel C. Darie

Abstract Within the past years, we have witnessed a great improvement in mass spectrometry (MS) and proteomics approaches in terms of instrumentation, protein fractionation, and bioinformatics. With the current technology, protein identification alone is no longer sufficient. Both scientists and clinicians want not only to identify proteins but also to identify the protein's posttranslational modifications (PTMs), protein isoforms, protein truncation, protein–protein interaction (PPI), and protein quantitation. Here, we describe the principle of MS and proteomics and strategies to identify proteins, protein's PTMs, protein isoforms, protein truncation, PPIs, and protein quantitation. We also discuss the strengths and weaknesses within this field. Finally, in our concluding remarks we assess the role of mass spectrometry and proteomics in scientific and clinical settings in the near future. This chapter provides an introduction and overview for subsequent chapters that will discuss specific MS proteomic methodologies and their application to specific medical conditions. Other chapters will also touch upon areas that expand beyond proteomics, such as lipidomics and metabolomics.

A.G. Woods • I. Sokolowska • A.G. Ngounou Wetie • K. Wormwood
R. Aslebagh • C.C. Darie (✉)
Biochemistry & Proteomics Group, Department of Chemistry & Biomolecular Science,
Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5810, USA
e-mail: cdarie@clarkson.edu

S. Patel
Biochemistry & Proteomics Group, Department of Chemistry & Biomolecular Science,
Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5810, USA

Memorial Sloan Kettering Cancer Center, Molecular Pharmacology and Chemistry Program,
New York, NY USA

Abbreviations

BN-PAGE	Blue native PAGE
CI	Chemical ionization
CN-PAGE	Colorless native PAGE
DIGE	Differential gel electrophoresis
EI	Electron ionization
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
FT	Fourier transform
IT	Ion trap
LC–MS/MS	Liquid chromatography–mass spectrometry
<i>m/z</i>	Mass/charge
MALDI	Matrix-assisted laser desorption ionization
MALDI-MS	MALDI mass spectrometry
MS	Mass spectrometry
Mw	Molecular weight
Q	Quadrupole
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TIC	Total ion current/chromatogram
TOF	Time of flight

1.1 Introduction

Proteomics is the large scale study of the protein complement, also known as the proteome. Proteomics is studied through mass spectrometry (MS) [1–8]. MS can be used to investigate a large variety of chemical and biological molecules, including products of chemical synthesis or degradation, biological molecules such as proteins, nucleic acids, lipids, or glycans, or various natural compounds of either large or small molecular mass. Depending on what type of molecule is being analyzed, there are various types of MS focus, such as small-molecule MS, large-molecule MS, and biological MS (when the molecules investigated are biomolecules). Within biological MS, there are also different MS subfields, such as proteomics, lipidomics, glycomics, and metabolomics. The focus of proteomics is to analyze proteins and protein derivatives (such as glycoproteins), peptides, posttranslational modifications (PTMs) within proteins, or protein–protein interactions (PPIs).

The standard workflow in a proteomics experiment starts with sample fractionation, involving the separation of proteins prior to their analysis by MS [9–17]. This can be done by one or more biochemical fractionation methods. For example, a one-dimensional separation can be achieved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); a two-dimensional separation can be performed by two-dimensional electrophoresis or by affinity purification followed by

General proteomics experiment

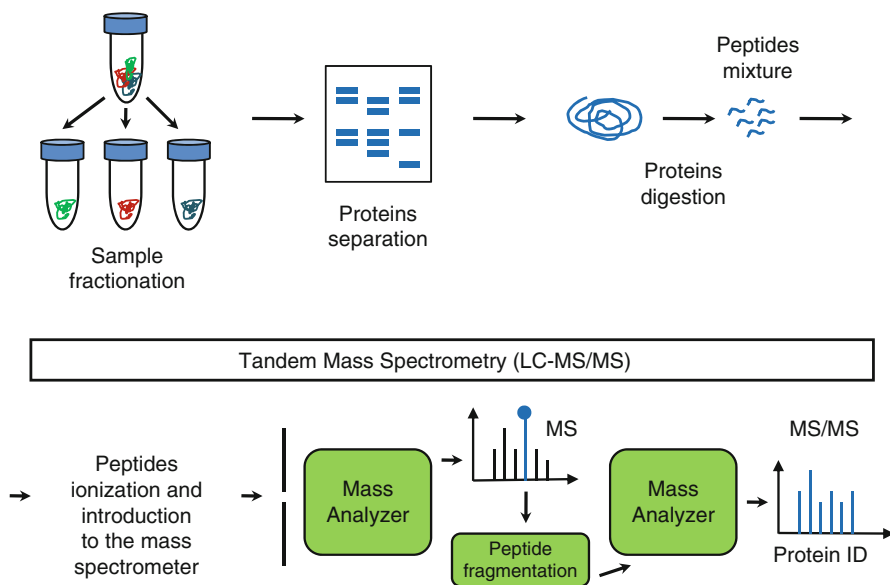


Fig. 1.1 General proteomic experiment workflow schematic. Reprinted and adapted with permission from the *Australian Journal of Chemistry* CSIRO Publishing <http://www.publish.csiro.au/?paper=CH13137> [15]

SDS-PAGE. Biochemical fractionation is then followed by enzymatic digestion (usually trypsin), peptide extraction, and peptide fractionation by HPLC and MS analysis [1]. Data analysis leads to identification of one or more proteins and further simultaneous investigation or re-investigation of the results can extract additional information from the same MS experiment, such as PTMs and interaction partners of some proteins (PPIs) [18–26]. A schematic of a proteomics workflow is shown in Fig. 1.1 and a schematic of a proteomics experiment is shown in Fig. 1.2a.

Proteomic analysis can be performed using samples from various sources such as supracellular, subcellular, intracellular, or extracellular, as well as at the peptide level (peptidomics), protein (regular proteomics), PTMs (“PTM-omics”), or protein complex level (interactomics). Proteomics can also be classified as classical or functional, when one analyzes protein samples from two different conditions (for example, normal and cancer), and targeted proteomics, when one focuses on a particular sub-proteome, such as phosphoproteomics or glycoproteomics. Proteomics can also be classified based on the protein complement from a set of samples that is being analyzed such as proteomes (i.e., all proteins) or sub-proteomes (i.e., just the nuclei or mitochondria). A schematic of such classification is shown in Fig. 1.2b.

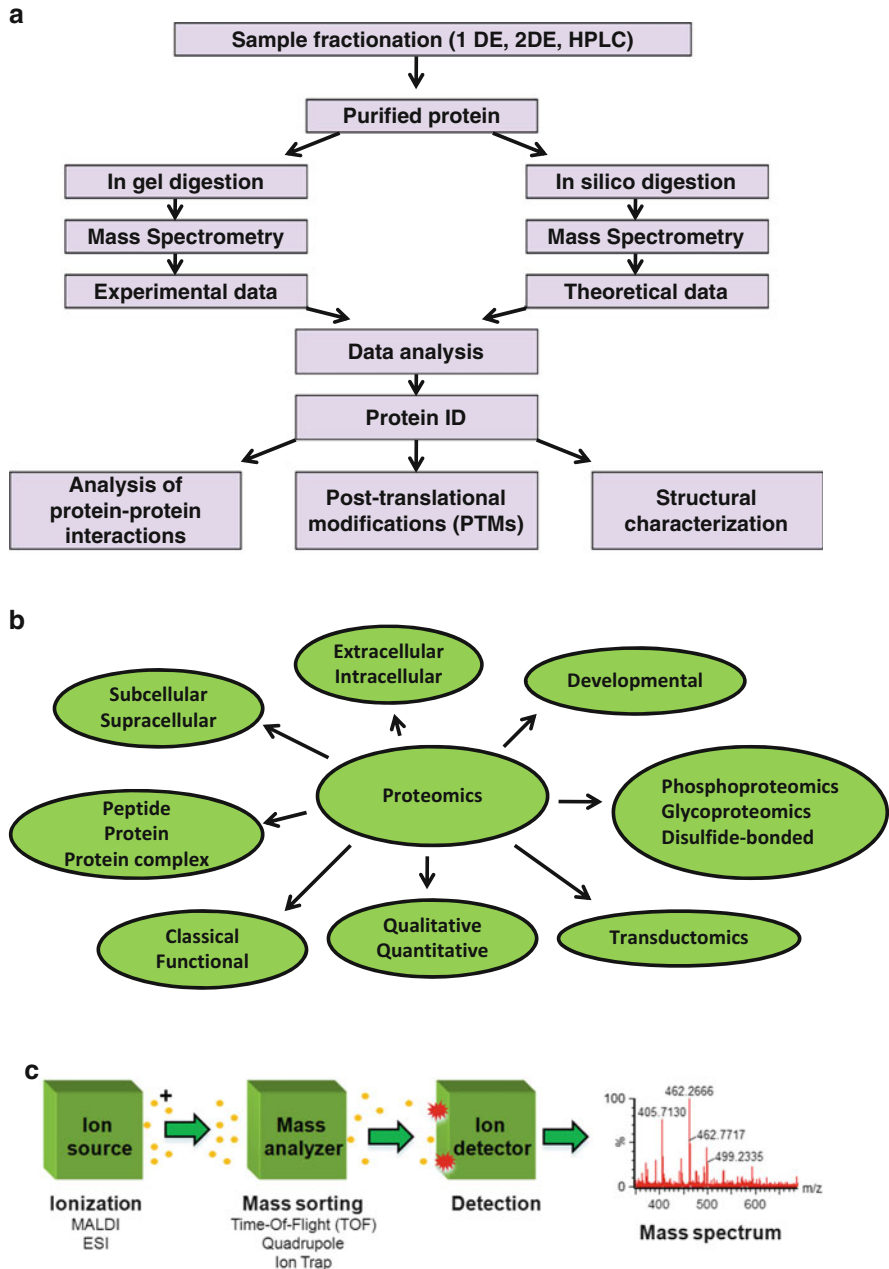


Fig. 1.2 General proteomics experiment. (a) Proteomics experiment workflow schematic. (b) Proteomics and applications schematic. (c) Mass spectrometer schematic. Reprinted and adapted with permission from the *Oxidative Stress: Diagnostics, Prevention, and Therapy*, S. Andreescu and M. Hepel, Editors. 2011, American Chemical Society: Washington, D.C [16]

Proteomic analysis can also focus on quality such as for protein identification, or the determination of protein amounts by quantitative proteomics. These analyses are usually performed using a mass spectrometer, the “workhorse” in a proteomics experiment. A mass spectrometer has three main components: the ionization source, a mass analyzer, and a detector (Fig. 1.2c). There are primarily two types of ionization sources on mass spectrometers: matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). The mass spectrometers are consequently named MALDI mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS). Here, we describe a proteomics experiment, specifically how proteins and peptides are analyzed by MS. We also describe the type of information that can be obtained from such an experiment.

1.2 Biochemical Fractionation

The first step in a proteomics experiment is biochemical fractionation, in which various proteins are separated from each other using their physicochemical properties. Biochemical fractionation usually depends on the goal of the experiment and it is perhaps the most important step in a proteomics experiment. A good sample fractionation usually leads to a good experimental outcome. A proteomics experiment can still be performed without biochemical fractionation, for example, when one analyzes the full proteome of a cell at once. However, without biochemical fractionation, the results in a proteomics experiment may not necessarily be optimal.

The physicochemical properties of proteins (or compounds of interest) that are used to achieve biochemical fractionation are, among others, molecular mass, isoelectric point, charge at various pH, and the protein’s affinity to other compounds. These properties of the proteins are well exploited by biochemical fractionations such as electrophoresis, centrifugation, and chromatography. Types of chromatography can include affinity chromatography, ion exchange chromatography, and size-exclusion chromatography.

To give one example, proteins can be separated by electrophoresis, usually SDS-PAGE, reduced and denatured, and then separated according to their molecular mass. If the reduction step is not used, the disulfide bridges in a protein or between proteins remain intact, thus providing an additional fractionation principle: two proteins with low molecular mass (such as haptoglobin subunits) are kept together through disulfide bridges and are separated under SDS-PAGE under nonreducing conditions as a heterotetramer with a high molecular mass. In a different variant of SDS-PAGE, but not using the detergent (SDS), one may separate proteins under native conditions. Therefore, simply by adding one reagent (for example, SDS) or two (SDS and a reducing agent like dithiothreitol or DTT), separation of these proteins may have a totally different outcome. A variant of SDS-PAGE is tricine-PAGE [27, 28], which has a principle of separation similar to the SDS-PAGE, but it has the highest separating resolution in the low molecular weight (M_w) proteins and peptides (2–20 kDa), where SDS-PAGE has poor or very poor resolution. Therefore, SDS-PAGE and tricine-PAGE complement each other.

Other types of electrophoresis are blue native PAGE (BN-PAGE), colorless native PAGE (CN-PAGE), and detergent-less SDS-PAGE (native PAGE) [1, 4, 6, 18–22, 29–34], all native electrophoresis. BN-PAGE separates protein complexes by using the external charge induced by Coomassie dye; thus, the complexes will have the same charge and will separate according to their molecular weight. If the Coomassie dye is not used, the external charge is not induced and the separation does not take place according to the molecular weight of the complexes, but rather according to the internal charge of the protein complexes. This method, a variant on BN-PAGE, is named CN-PAGE. CN-PAGE is particularly useful when two protein complexes with identical mass must be separated from each other.

In addition to the techniques mentioned for biochemical fractionation, hyphenated techniques may also be used. The classical example is two-dimensional electrophoresis (2D-PAGE), which includes separation of proteins by isoelectric focusing and by SDS-PAGE [3, 7, 35–45], still used in some proteomics labs. In fact, a variant of 2D-PAGE is differential gel electrophoresis (DIGE), a powerful method for gel-based proteomics. Other fractionation methods such as pre-coated chips, centrifugal filters, and magnetic beads are also possible [46, 47].

1.3 Mass Spectrometry

A mass spectrometer has three main parts: an ion source, a mass analyzer, and a detector. Initially, the sample is ionized and the ions produced by MALDI or ESI source are separated in the mass analyzer based on their mass-to-charge (m/z) ratio. The ions are then detected by the detector. The end product is a mass spectrum, which is a plot of ion abundance versus m/z .

Ionization sources. Ionization of peptides is dependent on the electrical potential at the ion source and on the pH at which they are analyzed. At low pH, the peptides are protonated through the amino-containing amino acids such as Arg or Lys, while at high pH, the peptides are de-protonated through the carboxyl-containing amino acids such as Asp or Glu. When the electrical potential at the ion source is positive, ionization is in positive ion mode. Conversely, when the electrical potential is negative, ionization is in negative ion mode. Therefore, there are two types of ionization: positive, when peptides are analyzed at low pH and the Arg, Lys, and His are protonated, and negative ionization, when peptides are analyzed at high pH and the Asp and Glu are de-protonated. In the current chapter, we will focus only on positive ionization, because it is one of the most used ionization modes for analyzing peptides and proteins. In addition, the enzyme that is the most widely used in proteomics is trypsin which cleaves conveniently at the C-terminus of Arg and Lys and produces peptides that are, upon ionization, at least doubly charged (the peptide and the C-terminal amino acid) and produces a y product ion series upon collision-induced fragmentation (described later).

In addition to ESI and MALDI, there are several additional ionization methods, such as chemical ionization (CI), electron ionization (EI), or atmospheric pressure

chemical ionization (APCI) [48, 49]. EI is used for analysis of organic compounds and can be used for all volatile compounds with a mass smaller than 1,000 Da. EI provides good structural information derived from fragmentation. However, molecular mass determination is rather poor (poor signal or the absence of M^+ ions) [50]. Chemical ionization is the opposite: it is very good for the determination of the molecular mass of molecules, but it is not very good in providing structural information due to reduced fragmentation in comparison to EI. Therefore CI and EI could complement each other. In CI experiments, ionized species are formed when the gaseous molecules to be analyzed collide with primary ions present in the source under a high vacuum [51]. A variant of CI is negative CI used only for volatile analytes with a mass of less than 1,000 Da [52, 53]. Another ionization technique, APCI, is an alternative for analysis of compounds that do not ionize in ESI. During APCI, generally only singly charged ions are formed and it is usually applied to compounds with a molecular weight of less than 1,500 Da [54].

Mass analyzers. There are three main types of mass analyzers used for proteomics experiments: trapping type instruments (quadrupole ion trap—QIT, linear ion trap—LIT, Fourier transform ion cyclotron resonance—FT-ICR, and Orbitrap), quadrupole (Q), and time of flight (TOF) instruments.

Trapping type instruments first accumulate ions and then allow for mass measurement. The ion trap analyzers first capture ions in three-dimensional space (trap), and then electrostatic gate pulses to inject ions into the ion trap. The ion trap-based analyzers are relatively inexpensive, sensitive, and robust. They have been extensively used in proteomic analysis. However, a problem with these instruments is their accuracy for both precursor and product ions, partially overcome by an FT-ICR. Unfortunately, this instrument is not very often used in proteomics research because peptides do not fragment well and the instrument is expensive [55, 56].

In quadrupole mass analyzers, ions constantly enter the analyzers, which are separated based on their trajectory in the electric field applied to two pairs of charged cylindrical rods. There is an electric potential between each pair of rods drawing the ions towards one rod. These instruments provide good reproducibility and low cost, but their resolution and accuracy are limited [49, 57].

Instruments with TOF mass analyzers are popular for sample analysis in proteomics due to their high resolution and relatively low cost, speed of measurements, and high mass accuracy [49, 57]. In TOF mass analyzers, ions are accelerated by a known electric field and then travel from the ion source to the detector. The instrument measures the time it takes for ions with different masses to travel from the ion source to detector,

Mass spectrometers can have stand-alone analyzers or in combination, usually two or three analyzers within one instrument, thus taking advantage of the strength of all combined analyzers simultaneously. Examples of such instruments are Q-Trap, QQQ, Q-TOF, TOF-TOF, QQ-LIT; these instruments are also called hybrid mass spectrometers, and are highly sensitive and also have a high resolution [1, 57–59].

MS detectors. The MS detectors are usually electron multipliers, photodiode arrays, microchannel plates, or image current detectors.

1.4 MALDI-TOF MS

MALDI-TOF MS or MALDI-MS (Fig. 1.3a) is mostly used for determination of the mass of a peptide or protein and for identification of a protein using peptide mass fingerprinting. In MALDI-MS, the peptide mixture is co-crystallized under acidic conditions with a UV-absorbing matrix (for example, dihydrobenzoic acid, sinapinic acid, alpha-hydroxycinnamic acid) and spotted on a plate. A laser beam (usually nitrogen; 337 nm) then ionizes the matrix and peptides, which desorb and start to fly under an electrical field. The matrix molecules transfer a proton to peptides, which then become ionized, fly through the TOF tube, and are detected in the detector as a mass spectrum. Charged peptides fly through the mass analyzer as ions according to their mass-to-charge ratio (m/z) and to the formula: $[M + zH]/z$, where M is the mass of the peptide and z is the charge of the peptide; H is the mass of hydrogen (1.007825035 atomic mass units). In MALDI-MS analysis, the charge of peptides is almost always +1 and the peptides are mostly observed as singly charged; the formula is then $[M + 1 \times 1]/1$ or $[M + 1]/1$ or $[M + 1]$. Therefore, the peptides are mostly detected as singly charged peaks or $[MH]^+$ peaks (Fig. 1.3b).

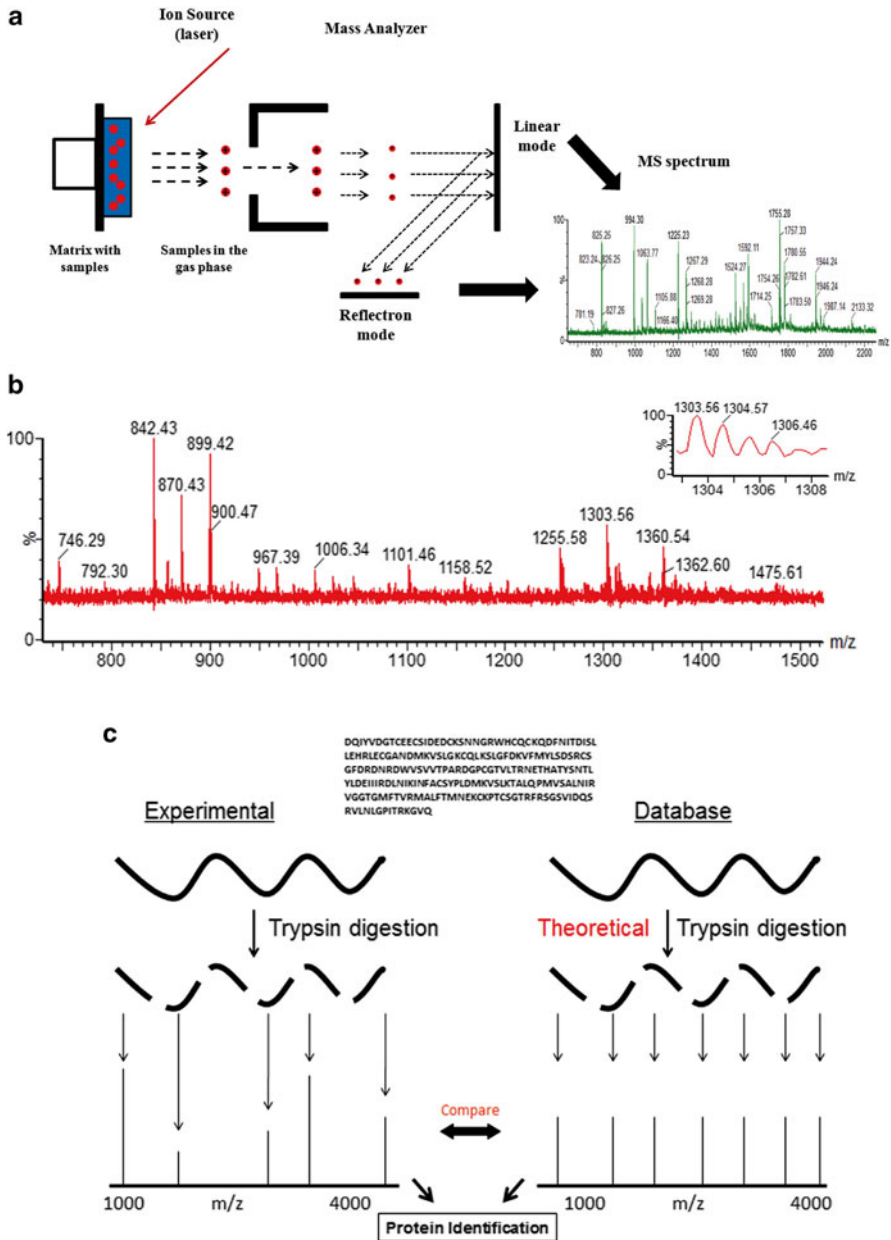
In the MALDI-MS mass spectrum, one peak corresponds to one peptide and many peaks correspond to many peptides, either from one protein or from more proteins. Database search of the MALDI-MS spectra usually identifies that single protein or those proteins through a process named peptide mass fingerprinting (Fig. 1.3c).

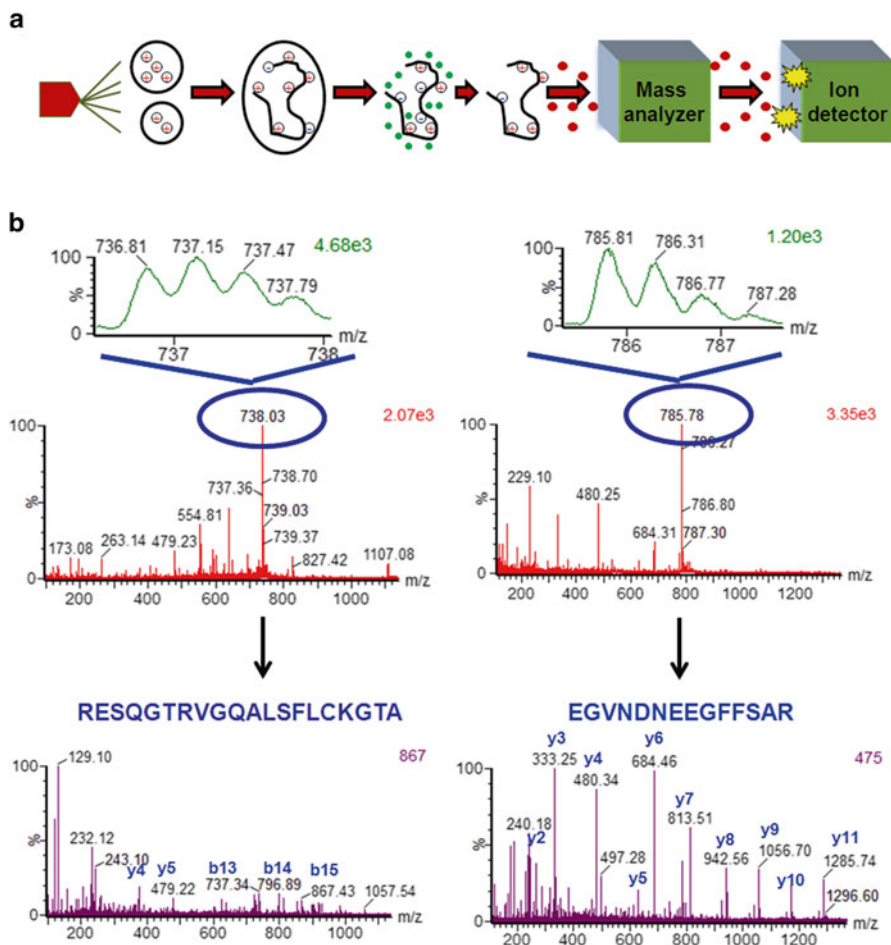
1.5 ESI-MS

In contrast to MALDI-MS, in which peptides are ionized with the help of a matrix (and are in the solid phase), in ESI-MS (Fig. 1.4a) peptides are ionized in the liquid phase, under high electrical current. Also, while in MALDI-MS peptides are mostly singly charged, in ESI-MS peptides are mostly double or multiple charged. Regarding the ionization method, peptides fly as ions according to m/z and calculation of the molecular mass of the peptide is performed according to the same $[M + z]/z$ formula, where z is again the charge (z is 2 for doubly charged peptides, 3 for triply charged peptides, etc.).

When a peptide mixture is injected into the mass spectrometer, all or most peptides that ionize under the experimental conditions are detected as ions in an MS

Fig. 1.3 (continued) peptide mixture is analyzed by MALDI-MS and a spectrum is collected. A similar experiment is performed in silico (a theoretical experiment in computer), but the cleavage is performed in all proteins from a database. During the database search, the best match between the theoretical and the experimental spectra then lead to identification of a protein. Reprinted and adapted with permission from the *Oxidative Stress: Diagnostics, Prevention, and Therapy*, S. Andreescu and M. Hepel, Editors. 2011, American Chemical Society: Washington, D.C [16]





spectrum in a process called direct infusion (ESI-MS mode). For example, if one has 10 peptides in an Eppendorf tube, one can identify all 10 peptides in one spectrum. However, in the MS one identifies only the masses of the peptides. In order to identify the sequence information about one particular peptide, one must isolate one peak that corresponds to one of the 10 peptides (precursor ion), fragment it in the collision cell using a neutral gas (for example, Argon gas), and record a spectrum (a sum of spectra) of the product ions that resulted from fragmentation of the precursor ion called MS/MS (ESI-MS/MS mode). Data analysis of the MS and MS/MS spectra usually leads to identification of the mass and sequence information about the peptide of interest. Examples of ESI-MS and ESI-MS/MS spectra are shown in Fig. 1.4b. As observed, the quality of the MS/MS spectra is directly dependent on the amino acid sequence, but more important, by the position of the proton-trapping amino acid (R, H, or K, in this case, R). For example, if the proton-trapping amino acid is on the N-terminus, low intensity b and y ions are observed (Fig. 1.4b, left). However, when the proton-trapping amino acid is located on the C-terminus, the fragments produced are almost always y ions of high quality. This is also the main reason for which most proteomics experiments use trypsin as an enzyme, since it cleaves the C-termini of R and K and produces peptides with an R or a K at the C-terminus.

Sometimes, when a peptide has more than one proton accepting amino acid such as Arg or Lys, the peptide may be protonated by more than two or three protons. Therefore, the same peptide may be identified with more than two or three charges. The advantage for these peptides is that if the precursor ion in a charge state of, e.g., 2+ does not fragment well in MS/MS, then the peak that corresponds to the same peptide but in a different charge state (e.g., 3+ or 4+) may fragment very well. One drawback for the multiply charged peptides is that they are usually longer (2,500–3,000 Da) than the regular peptides analyzed by MS (800–2,500 Da) and data analysis for these peptides may be more difficult than for regular peptides. However, overall, fragmentation of more than one peak corresponding to the same peptide but with different charge states may help in obtaining additional information about that peptide.

ESI-MS can be used not only for peptides but also for investigation of proteins and the information is particularly useful for determining the molecular mass of those proteins, of their potential PTMs, and of their conformation. In addition, the high molecular mass proteins can also be analyzed by ESI-MS in either positive mode (protonated) or negative mode (de-protonated), thus providing distinct, yet complementary, information regarding the distribution of charges on the surface of the protein investigated. Examples of MS spectra of a 16.9 kDa protein investigated by ESI-MS in both positive and negative mode are shown in Fig. 1.5.

1.6 LC-MS/MS

Analysis of peptide mixtures by ESI-MS for determination of the molecular mass of the peptides is usually a quick procedure. However if one wants to investigate the sequence information of more than one peptide, it is not the method of choice,

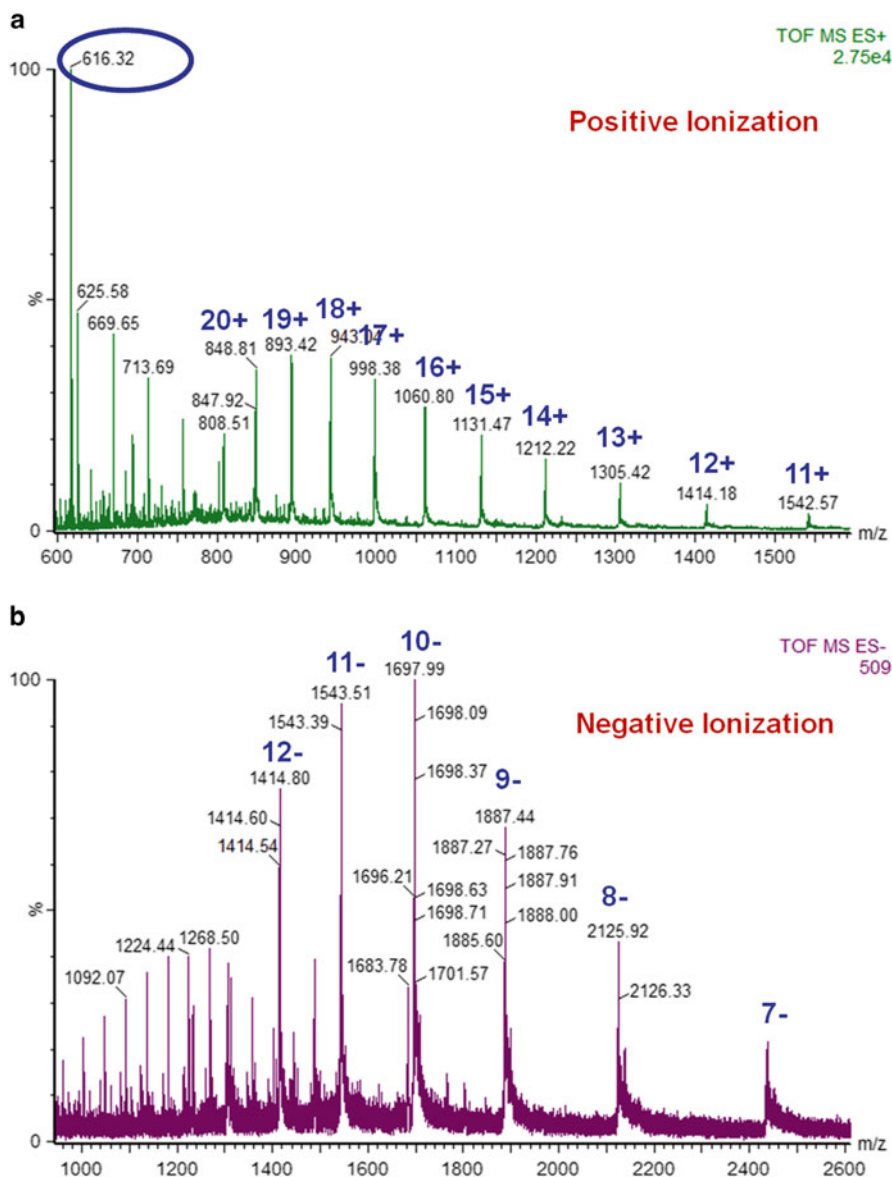


Fig. 1.5 ESI-MS proteins: ESI-MS spectra of intact 17 kDa protein, myoglobin, analyzed under acidic conditions (pH ~ 2). (a) MS spectrum in positive ionization; (b) MS spectrum analyzed in negative ionization. The positive (A) and negative (-) charges are indicated. The peak with m/z of 616.32 (1+) corresponds to the heme group, which is the prosthetic group of myoglobin. Reprinted and adapted with permission from the *Australian Journal of Chemistry CSIRO Publishing* <http://www.publish.csiro.au/?paper=CH13137> [15]

since fragmentation of the ions that correspond to peptides happens manually; one peptide at the time. For example, if one has 4 peptides in a mixture, we can determine the molecular mass of all peptides in minutes, but to determine their amino acid sequence, the peptides must be selected for fragmentation one at the time. Therefore, to automate this process, an alternative approach is necessary. One option is to fractionate the peptides by column chromatography coupled to an HPLC, i.e., reversed phase-based HPLC (reversed phase columns are particularly compatible with MS). The combination of HPLC and ESI-MS is named HPLC-ESI-MS or LC-MS. In this setting, the peptides are fractionated by HPLC prior to MS analysis. They can also be selected for fragmentation and then fragmented by MS/MS. In a process called data-dependent analysis (DDA), usually 3–4 precursor peaks (which correspond to peptides) are selected for fragmentation from one MS scan and fragmented by MS/MS in a process called LC-MS/MS. In LC-MS/MS, the mass spectrometer analyzes fewer peptides per unit of time as compared with ESI-MS, simply because the HPLC fractionates the peptide mixture over a longer period of time (such as a 60 min gradient) and gives the mass spectrometer more time to analyze more peptides. A schematic of the LC-MS/MS is shown in Fig. 1.6a.

Various types of improvements can be done to increase the number of MS/MS spectra with high quality data which can lead to identification of additional proteins. One is at the flow rate of the HPLC. On a high flow rate, the mass spectrometer will have less time to analyze the peptide mixtures, as compared with lower flow rate. On a longer HPLC gradient (such as 120 min), the mass spectrometer will have more time to analyze more peptides, as compared with a shorted gradient. The number of MS/MS may also influence the number of peptides fragmented per minute. For example, a mass spectrometer has usually one MS survey followed by several MS/MS, for example, between 3 and 10 channels for MS/MS (newer instruments can be up to 30 MS/MS). If the method is set to have one MS survey scan and then to do MS/MS of the two most intense peaks, then the instrument will work as follows: one second MS survey, one second MS/MS (Peak 1), one second MS/MS (Peak 2), and then again one second MS survey (Fig. 1.6a).

Assuming that a mass spectrometer has a cycle of one MS and two MS/MS (such as 0.1 s for an MS survey followed by selection of two precursor peaks for fragmentation by MS/MS; 3 s per MS/MS), this means that in 1 min, the MS instrument can perform ~ 30 MS/MS that can lead to identification of ~ 15 proteins. In a 120 min gradient, the possible number of proteins that can be identified is $\sim 15 \times 120 = 1,800$ proteins, but keeping in mind that the real length of a 120 min gradient is about 90 min (the rest of 30 min in washing with organic), this means that an MS run can identify $\sim 15 \times 90 = 1,350$ proteins. If the length of an MS/MS decreases from 3 to 1 s and the number of precursors selected within MS survey for MS/MS increases to 6, then the number of proteins identified increases by sixfold ($\sim 1,350 \times 6 = 8,100$ proteins). Assuming that these results are at a flow rate of $0.5 \mu\text{L}/\text{min}$, if we reduce the flow rate by $\frac{1}{2}$, the number of proteins that can be identified increases by a factor of 2 (i.e., $8,100 \times 2 = 16,200$).

However, when we calculate the number of these proteins that can be identified, our assumption is that all the steps mentioned work perfectly. In practice, this is often

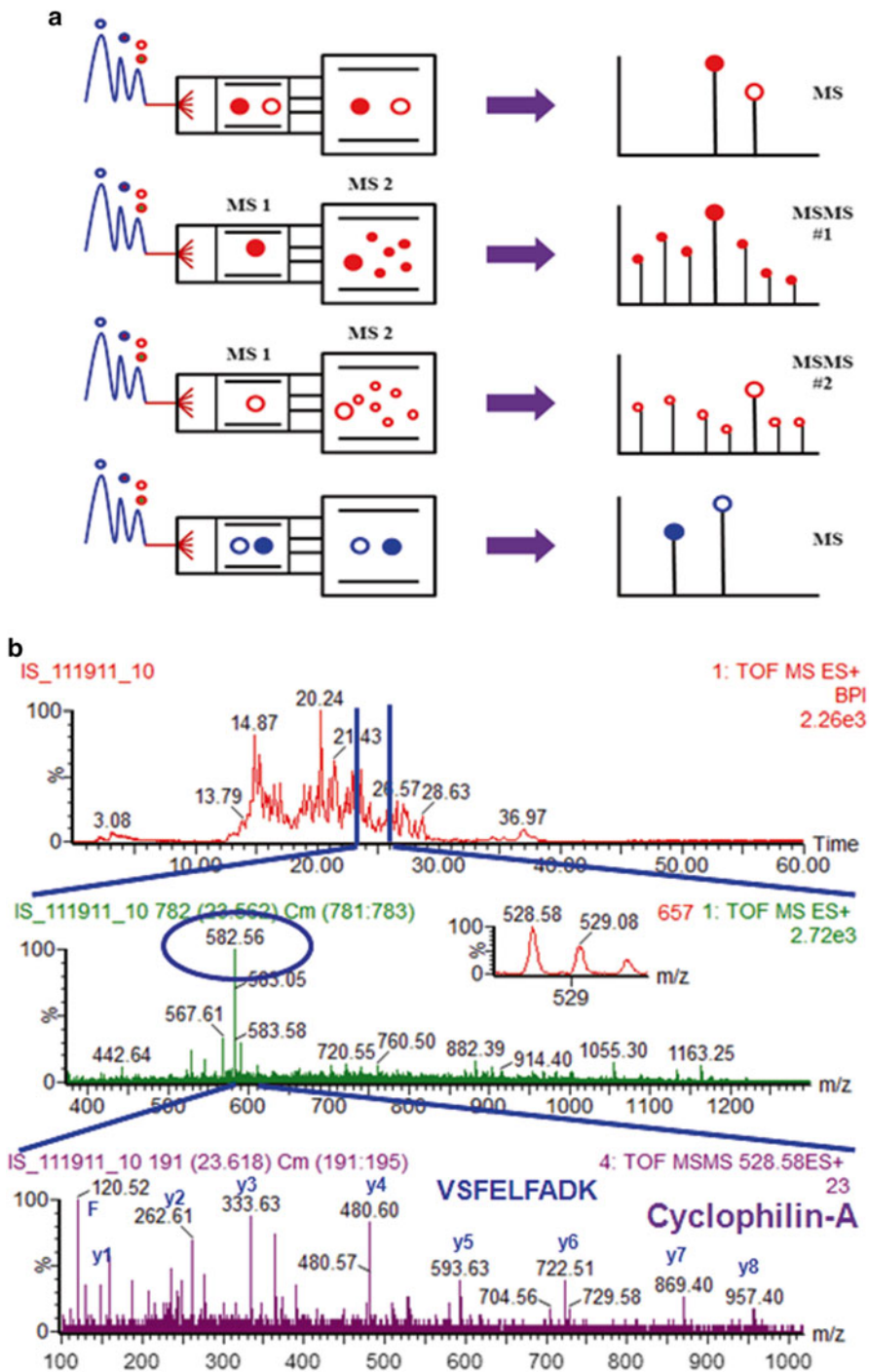


Fig. 1.6 LC-MS/MS experiment. (a) In each LC-MS/MS experiment, with elution of peptides from the HPLC gradually, the mass spectrometer analyzes corresponding ions via MS survey (recorded in an MS spectrum). Ions with highest intensity (typically 1–8 ions; two ions in this example) are selected for MS/MS fragmentation, fragmented, and then recorded as MS/MS #1 and MS/MS #2.

not the case. For example, the type and length of the gradient in HPLC (for example, sharp or shallow) do play an important role in peptide fractionation. An optimized versus a non-optimized nanospray will always play a role in the outcome of the proteomics experiment and the number of proteins identified. Obtaining a nanospray is just not good enough; “getting a good nanospray” is crucial to the success of a proteomics experiment. These and other known and/or unknown factors (not described here) that may influence the protein identification do indeed decrease the number of proteins identified in a proteomics experiment and in practice, a good LC–MS/MS run usually leads to identification of about 500–1,000 proteins. An example of a total ion current/chromatogram (TIC), MS, and MS/MS is shown in Fig. 1.6b.

1.7 Data Analysis

The raw data collected by a mass spectrometer are usually processed with software (for example, Protein Lynx Global Server, PLGS from Waters Corporation) and the output data (i.e., a peak list) is used for database search. There are many database search engines such as Sequest, X!Tandem, Mascot, or Phenyx. The results from the database search (such as from PLGS processing or Mascot search) can also be imported into a third-party software such as Scaffold (proteomesoftware.com) and further analyzed for protein modification, quantitation, and other factors.

MS may be not only qualitative but also quantitative and methods such as DIGE [60], isotope-coded affinity tag (ICAT) [5], stable isotope labeling by amino acids in cell culture (SILAC) [61], absolute quantitation (AQUA) [62], multiple reaction monitoring (MRM) [63], or spectral counting [64] have been successfully used in detection, identification, and quantification of proteins or peptides.

1.8 Protein Identification and Characterization

Determination of the molecular mass and amino acid sequence is the first step in protein identification. Once the protein is identified, then it is characterized. There are two methods for protein characterization using MS: a top-down approach when



Fig. 1.6 (continued) The mass spectrometer returns to the MS function at that point, recording an MS spectrum (MS survey). Once again ions with highest intensity are selected for fragmentation, fragmented, and recorded as MS/MS spectra. **(b)** An example of an LC–MS/MS experiment in which total ion current is recorded and at a specified time, an MS survey is recorded and one peak corresponding to a peptide (m/z of 582.56, doubly charged) is selected, and then fragmented in MS/MS. The fragmentation pattern (primarily b and y ions) from MS/MS provides sequence information regarding the peptide, leading to identification via database search. In this example, the peptide identified had the sequence VSFELFADK, identified as a component of human cyclophilin A. Reprinted and adapted with permission from the *Oxidative Stress: Diagnostics, Prevention, and Therapy*, S. Andreescu and M. Hepel, Editors. 2011, American Chemical Society: Washington, D.C [16]

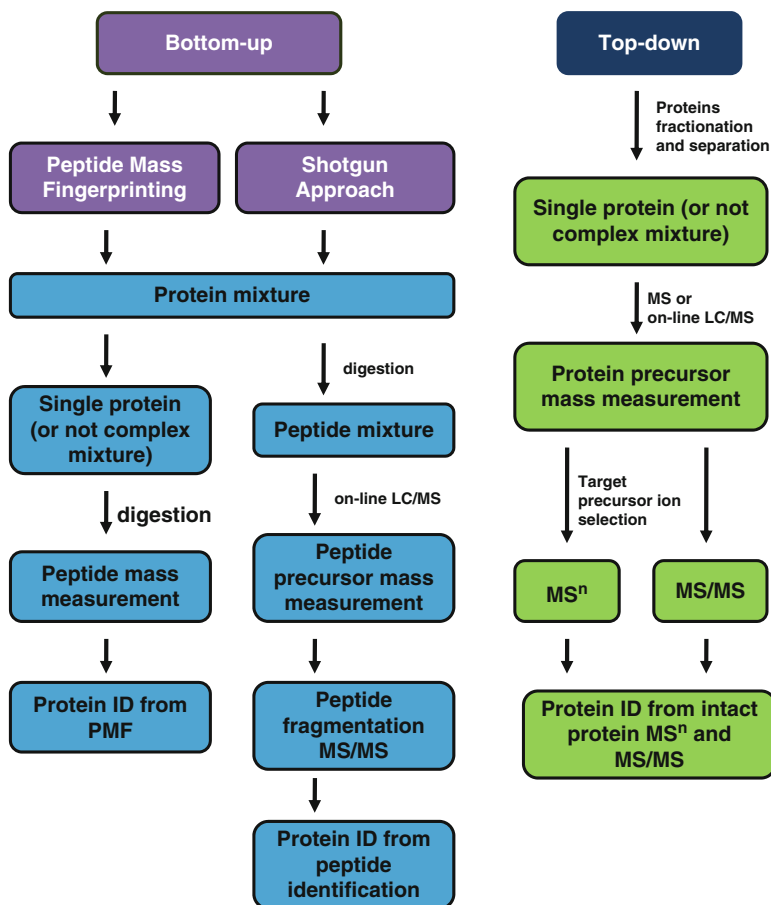


Fig. 1.7 Schematic workflow for bottom-up and top-down MS-based protein characterization and identification. Reprinted and adapted with permission from the *Australian Journal of Chemistry CSIRO Publishing* <http://www.publish.csiro.au/?paper=CH13137> [15]

intact proteins are investigated and bottom-up approach when proteins are digested and the peptide mixture is analyzed (Fig. 1.7).

A top-down approach allows for the identification of protein isoforms or any potential PTMs within proteins [65]. In bottom-up approach, digested proteins are subjected to MS analysis using on-line tandem mass spectrometry (MS/MS). In the same bottom-up approach, peptide mass fingerprinting for protein identification is also used, particularly in MALDI-MS analyses.

In a variation of bottom-up proteomics, known as shotgun proteomics, a large protein mixture is digested, and the resulting peptides are fractionated by one-dimensional or multidimensional chromatography and further analyzed by MS/MS [66]. For maximum protein identification and characterization, a combination of bottom-up and top-down proteomics is/can be used [67, 68].

Posttranslational modification identification strategies

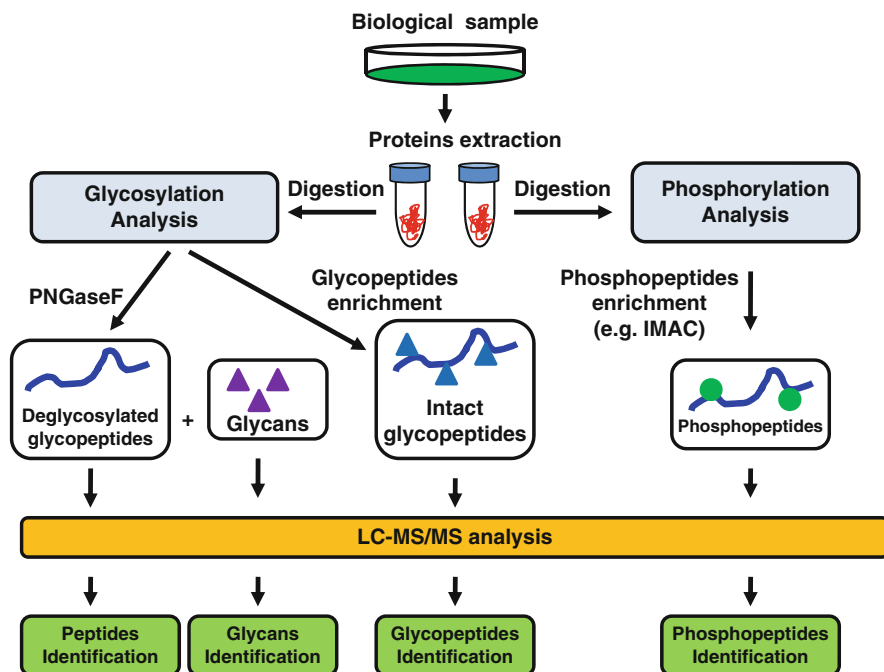


Fig. 1.8 MS-based characterization of protein PTMs (glycosylation and phosphorylation), general strategies. Reprinted and adapted with permission from the *Australian Journal of Chemistry* CSIRO Publishing <http://www.publish.csiro.au/?paper=CH13137> [15]

Characterization of proteins is not easy, but it becomes even more complicated due to the intensive PTMs of proteins. It is very difficult to fully identify PTMs at a particular time point in cells, tissues, or organisms and to derive a meaningful interpretation and biological significance from these identified PTMs. So far, the only method that is appropriate for large scale identification of PTMs is MS-based proteomics [69]. PTMs are time- and site-specific events and are important to all biological processes. However, for a meaningful characterization, special enrichment strategies must be used. These strategies are able to characterize most stable modifications in proteins which include glycosylation, phosphorylation, disulfide bridges, acetylation, ubiquitination, and methylation. MS approaches for identification and characterization of proteins and PTMs are shown in Fig. 1.8.

Two common PTMs in proteins are glycosylation and phosphorylation. Glycosylation is commonly found in extracellular proteins or in the proteins that form the extracellular side and are responsible for biological processes such as cell–cell communication or ligand–lectin interaction [70, 71]. In the pharmaceutical and

biotechnology industry that focus on biotherapeutics, glycosylation is a critical modification of recombinant proteins, which influences their stability and solubility [72, 73]. Therefore, characterization of glycoproteins is difficult because the glycosylation is not uniform and usually more glycoforms are simultaneously produced by the cells and the accuracy in the MS-based identification and characterization of the glycoprotein isoforms is crucial [74].

Analysis of glycoproteins may be accomplished by LC–MS/MS analysis of tryptic digests. This method allows for identification of saccharide diagnostic fragments (i.e., hexoses), but its detection efficiency for glycoproteins is rather poor [75–77]. A better strategy involves glycoprotein enrichment by affinity chromatography (lectins), which facilitates its identification in subsequent LC–MS/MS analysis [78]. Another strategy involves the release of the glycans from glycopeptides, followed by targeted analysis of the glycans. N-linked glycans can be digested using peptide-*N*-glycosidase F (PNGase F), while O-linked glycans can be released by β -elimination. Change in mass units for peptides upon glycan removal allows for identification of the types of glycosylation (N- or O-linked), as well as the sites of glycosylation. For N-linked glycans, PNGase F treatment leads to an asparagine-to-aspartate conversion, with a net increase of 1 mass unit [14].

For O-glycans, conversion of serine to alanine and of threonine to aminobutyric acid results in a net loss of 16 mass units [79]. While the currently developed methods allow for fast and reliable identification of glycosylation sites, their characterization is still a great challenge, mostly due to the presence of glycan positional isomers [74].

Phosphorylation is a common and reversible PTM that plays a role in modulating many cellular processes [80]. Abnormal phosphorylation in various proteins and the phosphorylation patterns in the proteomes have been connected to various diseases [81, 82]. Therefore identification of protein phosphorylation will allow us to understand many physiological processes such as the phosphorylation-based signal transduction pathways and hopefully may lead to the discovery of new therapeutic targets [83–85].

Identification and characterization of phosphorylation on peptides are usually accomplished by MS and scanning for neutral loss of HPO_3 (80 mass units) from phosphotyrosine and H_3PO_4 (98 mass units) from phosphoserine and phosphothreonine residues [86, 87] usually allows the identification of phosphopeptides and the amino acid that is phosphorylated. Complete methylation of peptide sample followed by MALDI-MS analysis in both positive and negative ionization modes was also successfully employed [88]. However, since phosphorylation is a transient event and phosphorylation–dephosphorylation events may have opposite biological effects, data verification, data validation, and data interpretation may be difficult. Therefore, enrichment of phosphopeptides using TiO_2 , metal-oxide-based resins (MOAC), a combination of TiO_2 and IMAC (TiMAC), and antibody affinity purifications [89, 90] is advised.

Another important protein PTM is disulfide bridges [11, 12, 15, 91], formed through the oxidation of cysteine residues, with an important role in maintaining the

three-dimensional conformation of proteins and inherently their physiological function. Disulfide bridges are usually found in extracellular and membrane-bound proteins, both as homodimers and homopolymers, but also as heterodimers and heteropolymers. Correct disulfide bridge formation is essential for proteins in adopting their optimal three-dimensional structure and assignment of the disulfide connectivities allows researchers to understand the structure and function of these proteins under physiological conditions and to predict problems in normal functioning of proteins when the disulfide bridges are scrambled or misconnected [92, 93].

Assignment of disulfide bridges in proteins may be accomplished by many approaches. For example, separation of disulfide-linked proteins or peptides by SDS-PAGE or tricine-PAGE under nonreducing and reducing conditions, followed by Coomassie staining and MS analysis, is one option. This MS analysis involves digestion of reduced and non-reduced aliquots of the same peptide mixture, followed by comparison of the masses of peptides that contain one cysteine, versus the disulfide-linked peptides in their oxidized form using MALDI-MS or ESI-MS [94, 95]. This task (assignment of disulfide bridges) is difficult when only one protein is analyzed and it is even more difficult when there are more cysteine residues per protein. In addition, there is no particular approach that allows one to identify disulfide bridges on a large scale. While mass spectrometry is capable of simultaneously analyzing many disulfide bridges, there are no bioinformatics means to interpret the MS data. Here we discussed only three types of PTMs, but there are many additional PTMs with biological significance and a similar number of challenges that are yet to be solved for each PTM and for automation of high-throughput identification and characterization of PTMs. However, it is clear to us that MS-based proteomics is perhaps the best and only option to accomplish both identification and characterization of PTMs.

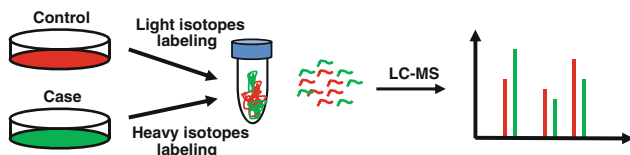
1.9 Mass Spectrometry-Based Peptide and Protein Profiling and Quantitation

In addition to qualitative proteomics, another dimension in a proteomics experiment is quantitative analysis of proteins from the samples analyzed. Changes in protein expression between different physiological states or between physiological and pathological ones are common and qualitative proteomics without quantitative interrogation is at its best, partial proteomics. Therefore adding an extra dimension (quantitative) to MS-based proteomics expands its capabilities and advantages [96].

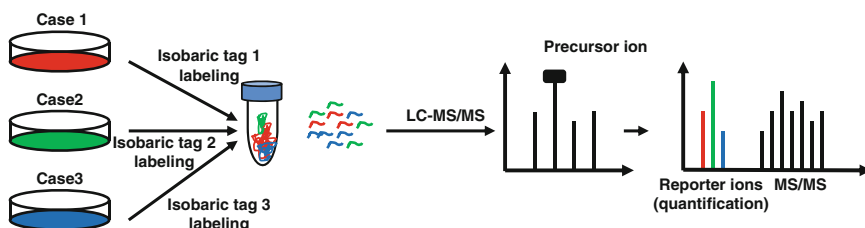
There are many workflows that have been developed and optimized to interrogate two or more proteomes or particular proteins from these proteomes using quantitative analysis, some of which are depicted in Fig. 1.9. Traditional quantitative analysis compares two sets of proteomes or a proteome from two different physiological states or physiological and pathological states and is a gel-based protein profiling technology which employs 2D-PAGE [97]. The protein spots that have different

Protein quantification

Metabolic labeling –pairwise comparison (e.g.SILAC, ICAT)



Metabolic labeling –multiple comparison (e.g.iTRAQ, TMT)



Chemical labeling –large-scale comparison (e.g.AQUA)

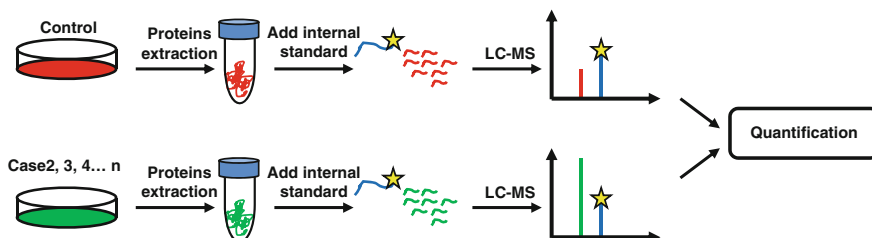


Fig. 1.9 MS-based protein quantification workflow strategies via stable isotope labeling. Reprinted and adapted with permission from the *Australian Journal of Chemistry CSIRO Publishing* <http://www.publish.csiro.au/?paper=CH13137> [15]

intensities are usually excised, digested, and analyzed by MS, usually accomplished by instruments capable of MS/MS fragmentation (i.e., triple quadrupole or ion trap mass spectrometers), simply because many compounds have similar masses and it may be difficult to monitor them in complex matrices. In addition, combination of MS and MS/MS allows one to use a combination of precursor ion for MS and fragment ions for MS/MS, thus providing a more selective monitoring of peptide/protein quantity [98–100].

Protein quantitation can be made using label-based or label-free techniques. Label-free methods are often used in many proteomic measurements because they

are simple and the cost is low [99]. Proteins also do not require special handling, such as tag or isotope labeling. With current advancements in software technology, there is no limit to the number of samples that can be analyzed.

Among the approaches used for label-free protein quantification are spectral counting and measurement of MS precursor ion intensity (or chromatographic peak area) [101, 102]. In spectral counting, one measures the number of spectra that correspond with peptides that are part of one protein [64, 103], while MS precursor ion intensity approach interrogates the chromatographic peaks corresponding to particular peptides at a normalized elution time. The protein or peptide quantity is then calculated using a standard curve or area under the curve as compared with another sample. All these label-free methods, although fast and cheap, also have disadvantages: they depend on analytical and biological reproducibility and any variation in sample preparation or sample analysis can lead to technical and instrumental errors, well reflected in the quantitation outcome [104, 105].

In addition to label-free quantitation, label-based quantitation strategies have emerged that use stable isotopes (^{13}C , ^{15}N , ^{18}O , or ^2H) [106], in which native and labeled samples are combined and analyzed simultaneously. This isotope-based quantitation is also called absolute quantitation, as opposed to label-free, relative quantitation.

Using the absolute quantification method, synthetic peptides or proteins are used, which are labeled with stable isotopes on one or more amino acids [6, 107]. The peptides are used as internal labeled standards, which are added directly to the samples to be analyzed. Due to the difference in the isotope pattern (and the mass difference) during MS analysis, quantitation can be performed [108–110]. One label-based quantitation method is stable isotope labeling of select amino acids (usually arginine or lysine) in cell culture (SILAC), used for metabolic labeling [61]. This method can be used in many applications such as investigation of signaling pathways [8, 22, 111–119], but it is mostly restricted to cell culture and it cannot be used to investigate biological fluids (i.e., blood, urine, saliva) [120]. However, recently, Matthias Mann's group created labeled mice. Animals were fed with a ^{13}C arginine- and ^{13}C lysine-infused diet and then can be used to investigate biological fluids [121, 122]. Another method for stable isotope labeling is the incorporation of labeled/modified tags on specific amino acids. In one such method, cysteine residues may be labeled using an ICAT, in which two conditions (i.e., two proteomes) are investigated [5]. For analysis of more than two conditions, such as for time-course experiments, or for three or more different biological samples, labeling strategies using isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT) have been successfully developed [123–125]. Although these methods have improved quantification capabilities, there are still limitations such as lack of reproducibility between individual analyses. Some of these issues are addressed through targeted quantification using approaches such as selected reaction monitoring (SRM) [126] and MRM [127], which have shown excellent reproducibility when used with stable isotope-labeled internal standards [128, 129].

With fast advancement in current proteomic methodologies and technologies, protein quantification and profiling will become a standard for use in clinical

diagnostic laboratories. However, before large scale proteomics experiments can be performed for clinical use, the reproducibility of large scale quantitation must be addressed and new, improved quantitation platforms developed and tested. Nevertheless, these new technologies indeed have the potential to be easily integrated in the set of tools that can perform disease- or disorder-specific protein profiling and that future is close to becoming a reality.

1.10 Identification of Protein–Protein Interactions (PPIs) Using MS

The molecules within a cell are not static, but rather dynamic. They form various types of interactions. These interactions can be static, as in protein complexes, or dynamic, as in transient protein interactions such as hormone–receptor interactions or substrate–enzyme interactions. All of these interactions within a cell form the interactomics network or interactome, and the proteins are a major component [130], modifying and controlling their own or other proteins' functions [131]. The dysregulation of these interactions, particularly PPIs, usually leads to a pathological state such as diseases or disorders and their investigation is essential to the current efforts to understand these diseases or disorders.

There are many methods for identifying PPIs such as size-exclusion chromatography (SEC) [132–134], sucrose gradient ultracentrifugation [135, 136], the yeast two-hybrid system (Y2H), or affinity purification MS (AP-MS) [3, 130, 137–140]. These methods allow identification of stable PPIs (i.e., by sucrose gradient, SEC, or AP-MS), of binary interactions (Y2H), or of transient PPIs (AP-MS). However, these current methods have limitations. Sucrose gradient ultracentrifugation and SEC are time-consuming and not suitable for automation, while Y2H and AP-MS can be automated, but have high rate of false positive identifications of PPIs [141–143]. Therefore, efforts are being made to reduce these limitations.

Native gel electrophoresis (clear native PAGE or CN-PAGE and BN-PAGE) are an alternative option and separate protein complexes according to their molecular mass (BN-PAGE) or according to their internal charge and independent of their mass (CN-PAGE) [21, 144]. The advantage of these methods is that they can separate all protein complexes from the whole proteome in one single experiment and can be combined with MS to identify protein complexes [145, 146]. However, the problem with these methods is that the gels are usually “home-made,” are not always reproducible, and require extensive work and bioinformatics expertise.

As an alternative option, one may also use ESI-MS for direct measurement of stable and transient PPIs in a solution [147, 148]. However, we still do not have capability to fully comprehend and the means to fully investigate the PPIs, in particular transient PPIs and even more complicated transient PPIs that have transient or reversible PTMs. This field will perhaps be called something like PTM-ed-PPI-omics.

1.11 Recent Advances

Currently we have the means to create disease animal models or control animal models (such as the SILAC mouse for absolute quantitation) [149, 150]. Capabilities of mass spectrometers have recently increased and currently MS-based technology allows us to identify thousands of proteins. Current machines are rich in additional technology that allows one to identify not only proteins and their PTMs and PPIs but also their shape and configuration. Such instruments are commercially available [151], thus filling the need for analysis of proteins that are not easy to investigate using classical approaches such as X-ray and NMR [152]. Therefore, these and other methods not listed here not only provide a solution for analysis of challenging proteins but have also opened the doors to new fields such as structural proteomics [153, 154].

1.12 Challenges and Perspectives

Many genomes have been sequenced. Many proteins from various sources have been identified. However, it will be an enormous mistake to state that we have identified a full proteome of a whole cell. This milestone has not yet been achieved. We have identified many or most of proteins in specific cells, such as bacteria, expressed at a particular time point, under particular growing conditions. However, while we are close to identification of most proteins in a cell, we are far from identification of the full proteome, including all proteins, isoproteins, modified proteins (PTMs), and truncated proteins. After the sequencing of the human genome, humans realized that our genome contains only ~30,000 genes, but encodes for about 100,000 unique protein sequences [155, 156]. Adding the truncated proteins, splice isoforms, and mutated proteins, PTMs will give us a number of, between 1 and 2,000,000, proteins, many of them expressed either transiently or in a very low concentration, thus making the cell's proteome complexity more difficult to analyze and interpret [157]. To partially overcome some of these challenges, more and more advanced MS-based technologies can be combined with many fractionation, separation, and identification methods within one experiment, such as combining immunoaffinity with gel electrophoresis, liquid chromatography (LC), and MS, to increase sensitivity and dynamic range. Furthermore, there is plenty of room for optimization of MS-based methods to be successfully used in high-throughput analysis.

Therapeutic proteins are frequently membrane proteins [158–161]. However, the membrane proteomics (membranomics) is the most difficult task that can be achieved in proteomics. In addition, transmembrane proteins are the most modified proteins by PTMs and are simply very difficult to investigate by MS, although some progress has been made in this direction [162, 163].

1.13 Conclusions

Despite the many challenges that the MS and proteomics fields face, the impact of MS is stronger and stronger, year after year. The number of unknown proteins decreases and protein databases become more comprehensive over time. With new MS technology and with combinatorial approaches towards the simultaneous identification of proteins, isoproteins, and truncated PTMs, PPIs will hopefully allow us to completely characterize proteomes at both a qualitative and quantitative level.

Acknowledgements We would like to thank Ms. Laura Mulderig, Scott Nichols, and their colleagues (Waters Corporation) for their generous support in setting up the Proteomics Center at Clarkson University. CCD thanks Drs. Thomas A. Neubert (New York University), Belinda Willard (Cleveland Clinic), and Gregory Wolber and David McLaughlin (Eastman Kodak Company) for donation of a ToFSpec2E MALDI-MS (each). CCD thanks his advisors, Vlad Artenie, Wolfgang Haehnel, Paul M. Wassarman, and Thomas A. Neubert, for their advice and support. This work was supported in part by the Keep a Breast Foundation (KEABF-375-35054), the Redcay Foundation (SUNY Plattsburgh), the Alexander von Humboldt Foundation, SciFund Challenge, private donations (Ms. Mary Stewart Joyce and Mr. Kenneth Sandler), the David A. Walsh fellowship, and by the U.S. Army research office (DURIP grant #W911NF-11-1-0304).

References

1. Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. *Nature* 422:198–207
2. Aivaliotis M, Karas M, Tsiotis G (2006) High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of cytoplasmatic protein complexes from *Chlorobium tepidum*. *Photosynth Res* 88:143–157
3. Blagoev B, Kratchmarova I, Ong SE, Nielsen M, Foster LJ, Mann M (2003) A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol* 21:315–318
4. Camacho-Carvajal MM, Wollscheid B, Aebersold R, Steimle V, Schamel WW (2004) Two-dimensional Blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: a proteomics approach. *Mol Cell Proteomics* 3:176–182
5. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17:994–999
6. Ong SE, Foster LJ, Mann M (2003) Mass spectrometric-based approaches in quantitative proteomics. *Methods* 29:124–130
7. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 68:850–858
8. Zhang G, Spellman DS, Skolnik EY, Neubert TA (2006) Quantitative phosphotyrosine proteomics of EphB2 signaling by stable isotope labeling with amino acids in cell culture (SILAC). *J Proteome Res* 5:581–588
9. Darie C (2013) Mass spectrometry and proteomics: principle, workflow, challenges and perspectives. *Mod Chem Appl* 1:e105
10. Darie CC (2013) Mass spectrometry and its application in life sciences. *Aust J Chem* 66:1–2
11. Ngounou Wetie AG, Sokolowska I, Woods AG, Darie CC (2013) Identification of post-translational modifications by mass spectrometry. *Aust J Chem* 66:734–748
12. Ngounou Wetie AG, Sokolowska I, Woods AG, Roy U, Deinhardt K, Darie CC (2014) Protein-protein interactions: switch from classical methods to proteomics and bioinformatics-based approaches. *Cell Mol Life Sci* 71(2):205–228

13. Ngounou Wetie AG, Sokolowska I, Wormwood K, Michel TM, Thome J, Darie CC, Woods AG (2013) Mass spectrometry for the detection of potential psychiatric biomarkers. *J Mol Psychiatry* 1:8
14. Sokolowska I, Ngounou Wetie AG, Roy U, Woods AG, Darie CC (2013) Mass spectrometry investigation of glycosylation on the NXS/T sites in recombinant glycoproteins. *Biochim Biophys Acta* 1834:1474–1483
15. Sokolowska I, Ngounou Wetie AG, Woods AG, Darie CC (2013) Applications of mass spectrometry in proteomics. *Aust J Chem* 66:721–733
16. Sokolowska I, Woods AG, Wagner J, Dorler J, Wormwood K, Thome J, Darie CC (2011) Mass spectrometry for proteomics-based investigation of oxidative stress and heat shock proteins. In: Andreescu S, Hepel M (eds) *Oxidative stress: diagnostics, prevention, and therapy*. American Chemical Society, Washington, DC
17. Woods AG, Ngounou Wetie AG, Sokolowska I, Russell S, Ryan JP, Michel TM, Thome J, Darie CC (2013) Mass spectrometry as a tool for studying autism spectrum disorder. *J Mol Psychiatry* 1:6
18. Darie CC, Biniossek ML, Winter V, Mutschler B, Haehnel W (2005) Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays*. *Febs J* 272:2705–2716
19. Darie CC, Janssen WG, Litscher ES, Wassarman PM (2008) Purified trout egg vitelline envelope proteins VE β and VE γ polymerize into homomeric fibrils from dimers in vitro. *Biochim Biophys Acta* 1784:385–392
20. Schagger H, Cramer WA, von Jagow G (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal Biochem* 217:220–230
21. Schagger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal Biochem* 199:223–231
22. Spellman DS, Deinhardt K, Darie CC, Chao MV, Neubert TA (2008) Stable isotopic labeling by amino acids in cultured primary neurons: application to brain-derived neurotrophic factor-dependent phosphotyrosine-associated signaling. *Mol Cell Proteomics* 7:1067–1076
23. Darie CC, Shetty V, Spellman DS, Zhang G, Xu C, Cardasis HL, Blais S, Fenyó D, Neubert TA (2008) Blue Native PAGE and mass spectrometry analysis of the ephrin stimulation-dependent protein-protein interactions in NG108-EphB2 cells. Springer, Düsseldorf, Germany
24. Darie CC, Litscher ES, Wassarman PM (2008) Structure, processing, and polymerization of rainbow trout egg vitelline envelope proteins. Springer, Düsseldorf, Germany
25. Darie CC, Biniossek ML, Gawinowicz MA, Milgrom Y, Thumfart JO, Jovine L, Litscher ES, Wassarman PM (2005) Mass spectrometric evidence that proteolytic processing of rainbow trout egg vitelline envelope proteins takes place on the egg. *J Biol Chem* 280:37585–37598
26. Darie CC, Biniossek ML, Jovine L, Litscher ES, Wassarman PM (2004) Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. *Biochemistry* 43:7459–7478
27. Schagger H (2006) Tricine-SDS-PAGE. *Nat Protoc* 1:16–22
28. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379
29. Jovine L, Darie CC, Litscher ES, Wassarman PM (2005) Zona pellucida domain proteins. *Annu Rev Biochem* 74:83–114
30. Litscher ES, Janssen WG, Darie CC, Wassarman PM (2008) Purified mouse egg zona pellucida glycoproteins polymerize into homomeric fibrils under non-denaturing conditions. *J Cell Physiol* 214:153–157
31. Wassarman PM, Jovine L, Qi H, Williams Z, Darie C, Litscher ES (2005) Recent aspects of mammalian fertilization research. *Mol Cell Endocrinol* 234:95–103
32. Darie C (2013) Investigation of protein-protein interactions by blue native-PAGE & mass spectrometry. *Mod Chem Appl* 1:e111

33. Darie C (2013) Post-translational modification (PTM) proteomics: challenges and perspectives. *Mod Chem appl* 1:e114
34. Woods AG, Sokolowska I, Yakubu R, Butkiewicz M, LaFleur M, Talbot C, Darie CC (2011) Blue native page and mass spectrometry as an approach for the investigation of stable and transient protein-protein interactions. In: Andreescu S, Hepel M (eds) *Oxidative stress: diagnostics, prevention, and therapy*. American Chemical Society, Washington, DC
35. Ghezzi P, Bonetto V (2003) Redox proteomics: identification of oxidatively modified proteins. *Proteomics* 3:1145–1153
36. Li X, Pan W, Yang GZ, Di YN, Zhao F, Zhu LY, Jiang ZH (2011) Proteome analysis of differential protein expression in brain of rats with type 1 diabetes mellitus. *Exp Clin Endocrinol Diabetes* 119:265–270
37. Muroi M, Kazami S, Noda K, Kondo H, Takayama H, Kawatani M, Usui T, Osada H (2010) Application of proteomic profiling based on 2D-DIGE for classification of compounds according to the mechanism of action. *Chem Biol* 17:460–470
38. Polden J, McManus CA, Remedios CD, Dunn MJ (2011) A 2-D gel reference map of the basic human heart proteome. *Proteomics* 11(17):3582–3586
39. Stefanescu R, Iacob RE, Damoc EN, Marquardt A, Amstalden E, Manea M, Perdivara I, Maftai M, Paraschiv G, Przybylski M (2007) Mass spectrometric approaches for elucidation of antigen-antibody recognition structures in molecular immunology. *Eur J Mass Spectrom (Chichester, Eng)* 13:69–75
40. Sun X, Jia HL, Xiao CL, Yin XF, Yang XY, Lu J, He X, Li N, Li H, He QY (2011) Bacterial proteome of *Streptococcus pneumoniae* through multidimensional separations coupled with LC-MS/MS. *OMICS* 15(7–8):477–482
41. Wang Y, Li R, Du D, Zhang C, Yuan H, Zeng R, Chen Z (2006) Proteomic analysis reveals novel molecules involved in insulin signaling pathway. *J Proteome Res* 5:846–855
42. Bauw G, Rasmussen HH, van den Bulcke M, van Damme J, Puype M, Gesser B, Celis JE, Vandekerckhove J (1990) Two-dimensional gel electrophoresis, protein electrophoresis and microsequencing: a direct link between proteins and genes. *Electrophoresis* 11:528–536
43. Celis JE, Gromov P (1999) 2D protein electrophoresis: can it be perfected? *Curr Opin Biotechnol* 10:16–21
44. Celis JE, Gromov P, Ostergaard M, Madsen P, Honore B, Dejgaard K, Olsen E, Vorum H, Kristensen DB, Gromova I, Haunso A, Van Damme J, Puype M, Vandekerckhove J, Rasmussen HH (1996) Human 2-D PAGE databases for proteome analysis in health and disease: <http://biobase.dk/cgi-bin/celis>. *FEBS Lett* 398:129–134
45. Celis JE, Gromova I, Moreira JM, Cabezon T, Gromov P (2004) Impact of proteomics on bladder cancer research. *Pharmacogenomics* 5:381–394
46. Taurines R, Dudley E, Conner AC, Grassl J, Jans T, Guderian F, Mehler-Wex C, Warnke A, Gerlach M, Thome J (2010) Serum protein profiling and proteomics in autistic spectrum disorder using magnetic bead-assisted mass spectrometry. *Eur Arch Psychiatry Clin Neurosci* 260:249–255
47. Taurines R, Dudley E, Grassl J, Warnke A, Gerlach M, Coogan AN, Thome J (2011) Proteomic research in psychiatry. *J Psychopharmacol* 25:151–196
48. Dass C (2007) *Fundamentals of contemporary mass spectrometry*. Wiley-Interscience, Hoboken, NJ
49. Hoffmann ED, Stroobant V (2007) *Mass spectrometry: principles and applications*, 3rd edn. J. Wiley, Chichester, West Sussex, England
50. Abate S, Ahn YG, Kind T, Cataldi TR, Fiehn O (2010) Determination of elemental compositions by gas chromatography/time-of-flight mass spectrometry using chemical and electron ionization. *Rapid Commun Mass Spectrom* 24:1172–1180
51. Harrison AG (1992) *Chemical ionization mass spectrometry*, 2nd edn. CRC Press, Boca Raton, FL
52. Rivera-Rodriguez LB, Rodriguez-Estrella R, Ellington JJ, Evans JJ (2007) Quantification of low levels of organochlorine pesticides using small volumes ($\leq 100\ \mu\text{mol}$) of plasma of

- wild birds through gas chromatography negative chemical ionization mass spectrometry. *Environ Pollut* 148:654–662
53. Dougherty RC (1981) Negative chemical ionization mass spectrometry: applications in environmental analytical chemistry. *Biomed Mass Spectrom* 8:283–292
 54. Zaikin VG, Halket JM (2006) Derivatization in mass spectrometry—8. Soft ionization mass spectrometry of small molecules. *Eur J Mass Spectrom (Chichester, Eng)* 12:79–115
 55. Marshall AG, Hendrickson CL, Jackson GS (1998) Fourier transform ion cyclotron resonance mass spectrometry: a primer. *Mass Spectrom Rev* 17:1–35
 56. Martin SE, Shabanowitz J, Hunt DF, Marto JA (2000) Subfemtomole MS and MS/MS peptide sequence analysis using nano-HPLC micro-ESI fourier transform ion cyclotron resonance mass spectrometry. *Anal Chem* 72:4266–4274
 57. Parker CE, Warren MR, Mocanu V (2010) Mass spectrometry for proteomics
 58. Yates JR, Ruse CI, Nakorchevsky A (2009) Proteomics by mass spectrometry: approaches, advances, and applications. *Annu Rev Biomed Eng* 11:49–79
 59. Domon B, Aebersold R (2006) Mass spectrometry and protein analysis. *Science* 312:212–217
 60. Viswanathan S, Unlu M, Minden JS (2006) Two-dimensional difference gel electrophoresis. *Nat Protoc* 1:1351–1358
 61. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 1:376–386
 62. Stemmann O, Zou H, Gerber SA, Gygi SP, Kirschner MW (2001) Dual inhibition of sister chromatid separation at metaphase. *Cell* 107:715–726
 63. Anderson L, Hunter CL (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics* 5:573–588
 64. Liu H, Sadygov RG, Yates JR III (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem* 76:4193–4201
 65. McLafferty FW, Breuker K, Jin M, Han X, Infusini G, Jiang H, Kong X, Begley TP (2007) Top-down MS, a powerful complement to the high capabilities of proteolysis proteomics. *FEBS J* 274:6256–6268
 66. McDonald WH, Yates JR III (2003) Shotgun proteomics: integrating technologies to answer biological questions. *Curr Opin Mol Ther* 5:302–309
 67. Wu S, Lourette NM, Tolic N, Zhao R, Robinson EW, Tolmachev AV, Smith RD, Pasa-Tolic L (2009) An integrated top-down and bottom-up strategy for broadly characterizing protein isoforms and modifications. *J Proteome Res* 8:1347–1357
 68. Han X, Aslanian A, Yates JR III (2008) Mass spectrometry for proteomics. *Curr Opin Chem Biol* 12:483–490
 69. Savitski MF, Savitski MM (2010) Unbiased detection of posttranslational modifications using mass spectrometry. *Methods Mol Biol* 673:203–210
 70. Spiro RG (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12:43R–56R
 71. Marino K, Bones J, Kattla JJ, Rudd PM (2010) A systematic approach to protein glycosylation analysis: a path through the maze. *Nat Chem Biol* 6:713–723
 72. Read EK, Park JT, Brorson KA (2011) Industry and regulatory experience of the glycosylation of monoclonal antibodies. *Biotechnol Appl Biochem* 58:213–219
 73. Kamoda S, Kakehi K (2008) Evaluation of glycosylation for quality assurance of antibody pharmaceuticals by capillary electrophoresis. *Electrophoresis* 29:3595–3604
 74. Leymarie N, Zaia J (2012) Effective use of mass spectrometry for glycan and glycopeptide structural analysis. *Anal Chem* 84:3040–3048
 75. Pan S, Chen R, Aebersold R, Brentnall TA (2011) Mass spectrometry based glycoproteomics—from a proteomics perspective. *Mol Cell Proteomics* 10(R110):003251
 76. Morelle W, Michalski JC (2007) Analysis of protein glycosylation by mass spectrometry. *Nat Protoc* 2:1585–1602

77. Wuhrer M, Catalina MI, Deelder AM, Hokke CH (2007) Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J Chromatogr B Analyt Technol Biomed Life Sci* 849:115–128
78. Mechref Y, Madera M, Novotny MV (2008) Glycoprotein enrichment through lectin affinity techniques. *Methods Mol Biol* 424:373–396
79. Bond MR, Kohler JJ (2007) Chemical methods for glycoprotein discovery. *Curr Opin Chem Biol* 11:52–58
80. Tarrant MK, Cole PA (2009) The chemical biology of protein phosphorylation. *Annu Rev Biochem* 78:797–825
81. Blume-Jensen P, Hunter T (2001) Oncogenic kinase signalling. *Nature* 411:355–365
82. Cohen P (2001) The role of protein phosphorylation in human health and disease. The Sir Hans Krebs Medal Lecture. *Eur J Biochem* 268:5001–5010
83. Badiola N, Suarez-Calvet M, Lleo A (2010) Tau phosphorylation and aggregation as a therapeutic target in tauopathies. *CNS Neurol Disord Drug Targets* 9:727–740
84. Cohen P (2002) Protein kinases—the major drug targets of the twenty-first century? *Nat Rev Drug Discov* 1:309–315
85. Strebhardt K (2010) Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. *Nat Rev Drug Discov* 9:643–660
86. Le Blanc JC, Hager JW, Ilisiu AM, Hunter C, Zhong F, Chu I (2003) Unique scanning capabilities of a new hybrid linear ion trap mass spectrometer (Q TRAP) used for high sensitivity proteomics applications. *Proteomics* 3:859–869
87. Unwin RD, Griffiths JR, Leverenz MK, Grallert A, Hagan IM, Whetton AD (2005) Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. *Mol Cell Proteomics* 4:1134–1144
88. Xu CF, Lu Y, Ma J, Mohammadi M, Neubert TA (2005) Identification of phosphopeptides by MALDI Q-TOF MS in positive and negative ion modes after methyl esterification. *Mol Cell Proteomics* 4:809–818
89. Beltran L, Cutillas PR (2012) Advances in phosphopeptide enrichment techniques for phosphoproteomics. *Amino Acids* 43:1009–1024
90. Corthals GL, Aebersold R, Goodlett DR (2005) Identification of phosphorylation sites using microimmobilized metal affinity chromatography. *Methods Enzymol* 405:66–81
91. Ngounou Wetie AG, Sokolowska I, Woods AG, Wormwood KL, Dao S, Patel S, Clarkson BD, Darie CC (2013) Automated mass spectrometry-based functional assay for the routine analysis of the secretome. *J Lab Autom* 18:19–29
92. Gorman JJ, Wallis TP, Pitt JJ (2002) Protein disulfide bond determination by mass spectrometry. *Mass Spectrom Rev* 21:183–216
93. McAuley A, Jacob J, Kolvenbach CG, Westland K, Lee HJ, Brych SR, Rehder D, Kleemann GR, Brems DN, Matsumura M (2008) Contributions of a disulfide bond to the structure, stability, and dimerization of human IgG1 antibody CH3 domain. *Protein Sci* 17:95–106
94. Sokolowska I, Gawinowicz MA, Ngounou Wetie AG, Darie CC (2012) Disulfide proteomics for identification of extracellular or secreted proteins. *Electrophoresis* 33:2527–2536
95. Sokolowska I, Ngounou Wetie AG, Woods AG, Darie CC (2012) Automatic determination of disulfide bridges in proteins. *J Lab Autom* 17:408–416
96. Panchaud A, Affolter M, Moreillon P, Kussmann M (2008) Experimental and computational approaches to quantitative proteomics: status quo and outlook. *J Proteomics* 71:19–33
97. Berkelman T (2008) Quantitation of protein in samples prepared for 2-D electrophoresis. *Methods Mol Biol* 424:43–49
98. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B (2007) Quantitative mass spectrometry in proteomics: a critical review. *Anal Bioanal Chem* 389:1017–1031
99. Xie F, Liu T, Qian WJ, Petyuk VA, Smith RD (2011) Liquid chromatography-mass spectrometry-based quantitative proteomics. *J Biol Chem* 286:25443–25449
100. Pan S, Aebersold R, Chen R, Rush J, Goodlett DR, McIntosh MW, Zhang J, Brentnall TA (2009) Mass spectrometry based targeted protein quantification: methods and applications. *J Proteome Res* 8:787–797

101. Negishi A, Ono M, Handa Y, Kato H, Yamashita K, Honda K, Shitashige M, Satow R, Sakuma T, Kuwabara H, Omura K, Hirohashi S, Yamada T (2009) Large-scale quantitative clinical proteomics by label-free liquid chromatography and mass spectrometry. *Cancer Sci* 100:514–519
102. Ono M, Shitashige M, Honda K, Isobe T, Kuwabara H, Matsuzuki H, Hirohashi S, Yamada T (2006) Label-free quantitative proteomics using large peptide data sets generated by nano-flow liquid chromatography and mass spectrometry. *Mol Cell Proteomics* 5:1338–1347
103. Qian WJ, Jacobs JM, Camp DG II, Monroe ME, Moore RJ, Gritsenko MA, Calvano SE, Lowry SF, Xiao W, Moldawer LL, Davis RW, Tompkins RG, Smith RD (2005) Comparative proteome analyses of human plasma following in vivo lipopolysaccharide administration using multidimensional separations coupled with tandem mass spectrometry. *Proteomics* 5:572–584
104. Petyuk VA, Jaitly N, Moore RJ, Ding J, Metz TO, Tang K, Monroe ME, Tolmachev AV, Adkins JN, Belov ME, Dabney AR, Qian WJ, Camp DG II, Smith RD (2008) Elimination of systematic mass measurement errors in liquid chromatography-mass spectrometry based proteomics using regression models and a priori partial knowledge of the sample content. *Anal Chem* 80:693–706
105. Strittmatter EF, Ferguson PL, Tang K, Smith RD (2003) Proteome analyses using accurate mass and elution time peptide tags with capillary LC time-of-flight mass spectrometry. *J Am Soc Mass Spectrom* 14:980–991
106. Zhang R, Regnier FE (2002) Minimizing resolution of isotopically coded peptides in comparative proteomics. *J Proteome Res* 1:139–147
107. Julka S, Regnier F (2004) Quantification in proteomics through stable isotope coding: a review. *J Proteome Res* 3:350–363
108. Bronstrup M (2004) Absolute quantification strategies in proteomics based on mass spectrometry. *Expert Rev Proteomics* 1:503–512
109. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci U S A* 100:6940–6945
110. Kirkpatrick DS, Gerber SA, Gygi SP (2005) The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods* 35:265–273
111. Zhang G, Neubert TA (2011) Comparison of three quantitative phosphoproteomic strategies to study receptor tyrosine kinase signaling. *J Proteome Res* 10:5454–5462
112. Zhang G, Deinhardt K, Chao MV, Neubert TA (2011) Study of neurotrophin-3 signaling in primary cultured neurons using multiplex stable isotope labeling with amino acids in cell culture. *J Proteome Res* 10:2546–2554
113. Deinhardt K, Kim T, Spellman DS, Mains RE, Eipper BA, Neubert TA, Chao MV, Hempstead BL (2011) Neuronal growth cone retraction relies on proneurotrophin receptor signaling through Rac. *Sci Signal* 4:ra82
114. Zhang G, Ueberheide BM, Waldemarson S, Myung S, Molloy K, Eriksson J, Chait BT, Neubert TA, Fenyo D (2010) Protein quantitation using mass spectrometry. *Methods Mol Biol* 673:211–222
115. Neubert TA, Tempst P (2010) Super-SILAC for tumors and tissues. *Nat Methods* 7:361–362
116. Zhang G, Neubert TA (2009) Use of stable isotope labeling by amino acids in cell culture (SILAC) for phosphotyrosine protein identification and quantitation. *Methods Mol Biol* 527:79–92, xi
117. Zhang G, Fenyo D, Neubert TA (2009) Evaluation of the variation in sample preparation for comparative proteomics using stable isotope labeling by amino acids in cell culture. *J Proteome Res* 8:1285–1292
118. Zhang G, Fenyo D, Neubert TA (2008) Screening for EphB signaling effectors using SILAC with a linear ion trap-orbitrap mass spectrometer. *J Proteome Res* 7:4715–4726
119. Zhang G, Neubert TA (2006) Automated comparative proteomics based on multiplex tandem mass spectrometry and stable isotope labeling. *Mol Cell Proteomics* 5:401–411

120. Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, Wang Y, MacNeill J, Mitchell J, Gygi SP, Rush J, Polakiewicz RD, Comb MJ (2008) Signaling networks assembled by oncogenic EGFR and c-Met. *Proc Natl Acad Sci U S A* 105:692–697
121. Kruger M, Moser M, Ussar S, Thievensen I, Lubner CA, Forner F, Schmidt S, Zanivan S, Fassler R, Mann M (2008) SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* 134:353–364
122. Zanivan S, Krueger M, Mann M (2012) In vivo quantitative proteomics: the SILAC mouse. *Methods Mol Biol* 757:435–450
123. Muth T, Keller D, Puetz SM, Martens L, Sickmann A, Boehm AM (2010) jTraQX: a free, platform independent tool for isobaric tag quantitation at the protein level. *Proteomics* 10:1223–1225
124. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 3:1154–1169
125. Dayon L, Hainard A, Licker V, Turck N, Kuhn K, Hochstrasser DF, Burkhard PR, Sanchez JC (2008) Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal Chem* 80:2921–2931
126. Holman SW, Sims PF, Evers CE (2012) The use of selected reaction monitoring in quantitative proteomics. *Bioanalysis* 4:1763–1786
127. Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM (2007) Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc Natl Acad Sci U S A* 104:5860–5865
128. Duncan MW, Aebersold R, Caprioli RM (2010) The pros and cons of peptide-centric proteomics. *Nat Biotechnol* 28:659–664
129. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, Spiegelman CH, Zimmerman LJ, Ham AJ, Keshishian H, Hall SC, Allen S, Blackman RK, Borchers CH, Buck C, Cardasis HL, Cusack MP, Dodder NG, Gibson BW, Held JM, Hiltke T, Jackson A, Johansen EB, Kinsinger CR, Li J, Mesri M, Neubert TA, Niles RK, Pulsipher TC, Ransohoff D, Rodriguez H, Rudnick PA, Smith D, Tabb DL, Tegeler TJ, Variyath AM, Vega-Montoto LJ, Wahlander A, Waldemarson S, Wang M, Whiteaker JR, Zhao L, Anderson NL, Fisher SJ, Liebler DC, Paulovich AG, Regnier FE, Tempst P, Carr SA (2009) Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol* 27:633–641
130. Koh GC, Porras P, Aranda B, Hermjakob H, Orchard SE (2012) Analyzing protein-protein interaction networks. *J Proteome Res* 11:2014–2031
131. De Las Rivas J, Fontanillo C (2010) Protein-protein interactions essentials: key concepts to building and analyzing interactome networks. *PLoS Comput Biol* 6:e1000807
132. Cavanagh J, Thompson R, Bobay B, Benson LM, Naylor S (2002) Stoichiometries of protein-protein/DNA binding and conformational changes for the transition-state regulator AbrB measured by pseudo cell-size exclusion chromatography-mass spectrometry. *Biochemistry* 41:7859–7865
133. Wen J, Arakawa T, Philo JS (1996) Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. *Anal Biochem* 240:155–166
134. Mayer CL, Snyder WK, Swietlicka MA, Vanschoiack AD, Austin CR, McFarland BJ (2009) Size-exclusion chromatography can identify faster-associating protein complexes and evaluate design strategies. *BMC Res Notes* 2:135
135. Berkowitz SA (2006) Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. *AAPS J* 8:E590–E605
136. Phizicky EM, Fields S (1995) Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* 59:94–123

137. Sokolowska I, Woods AG, Gawinowicz MA, Roy U, Darie CC (2012) Identification of potential tumor differentiation factor (TDF) receptor from steroid-responsive and steroid-resistant breast cancer cells. *J Biol Chem* 287:1719–1733
138. Ngounou Wetie AG, Sokolowska I, Woods AG, Roy U, Loo JA, Darie CC (2013) Investigation of stable and transient protein-protein interactions: past, present, and future. *Proteomics* 13(3–4):538–557
139. Sokolowska I, Woods AG, Gawinowicz MA, Roy U, Darie CC (2012) Identification of a potential tumor differentiation factor receptor candidate in prostate cancer cells. *FEBS J* 279:2579–2594
140. Chautard E, Fatoux-Ardore M, Ballut L, Thierry-Mieg N, Ricard-Blum S (2011) MatrixDB, the extracellular matrix interaction database. *Nucleic Acids Res* 39:D235–D240
141. Pflieger D, Gonnet F, de la Fuente van Bentem S, Hirt H, de la Fuente A (2011) Linking the proteins—elucidation of proteome-scale networks using mass spectrometry. *Mass Spectrom Rev* 30:268–297
142. Suter B, Kittanakom S, Stagljar I (2008) Two-hybrid technologies in proteomics research. *Curr Opin Biotechnol* 19:316–323
143. Suter B, Kittanakom S, Stagljar I (2008) Interactive proteomics: what lies ahead? *Biotechniques* 44:681–691
144. Krause F (2006) Detection and analysis of protein-protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (Membrane) protein complexes and super-complexes. *Electrophoresis* 27:2759–2781
145. Sokolova L, Wittig I, Barth HD, Schagger H, Brutschy B, Brandt U (2010) Laser-induced liquid bead ion desorption-MS of protein complexes from blue-native gels, a sensitive top-down proteomic approach. *Proteomics* 10:1401–1407
146. Darie CC, Deinhardt K, Zhang G, Cardasis HS, Chao MV, Neubert TA (2011) Identifying transient protein-protein interactions in EphB2 signaling by blue native PAGE and mass spectrometry. *Proteomics* 11:4514–4528
147. Heck AJ, Van Den Heuvel RH (2004) Investigation of intact protein complexes by mass spectrometry. *Mass Spectrom Rev* 23:368–389
148. Kaddis CS, Lomeli SH, Yin S, Berhane B, Apostol MI, Kickhoefer VA, Rome LH, Loo JA (2007) Sizing large proteins and protein complexes by electrospray ionization mass spectrometry and ion mobility. *J Am Soc Mass Spectrom* 18:1206–1216
149. Petyuk VA, Qian WJ, Chin MH, Wang H, Livesay EA, Monroe ME, Adkins JN, Jaitly N, Anderson DJ, Camp DG II, Smith DJ, Smith RD (2007) Spatial mapping of protein abundances in the mouse brain by voxelation integrated with high-throughput liquid chromatography-mass spectrometry. *Genome Res* 17:328–336
150. Qian WJ, Petritis BO, Kaushal A, Finnerty CC, Jeschke MG, Monroe ME, Moore RJ, Schepmoes AA, Xiao W, Moldawer LL, Davis RW, Tompkins RG, Herndon DN, Camp DG II, Smith RD (2010) Plasma proteome response to severe burn injury revealed by 18O-labeled “universal” reference-based quantitative proteomics. *J Proteome Res* 9:4779–4789
151. Zhong Y, Hyung SJ, Ruotolo BT (2012) Ion mobility-mass spectrometry for structural proteomics. *Expert Rev Proteomics* 9:47–58
152. Sali A, Glaeser R, Earnest T, Baumeister W (2003) From words to literature in structural proteomics. *Nature* 422:216–225
153. Jurczek E, Barran PE (2011) How useful is ion mobility mass spectrometry for structural biology? The relationship between protein crystal structures and their collision cross sections in the gas phase. *Analyst* 136:20–28
154. Scarff CA, Thalassinou K, Hilton GR, Scrivens JH (2008) Travelling wave ion mobility mass spectrometry studies of protein structure: biological significance and comparison with X-ray crystallography and nuclear magnetic resonance spectroscopy measurements. *Rapid Commun Mass Spectrom* 22:3297–3304
155. Roberts GC, Smith CW (2002) Alternative splicing: combinatorial output from the genome. *Curr Opin Chem Biol* 6:375–383

156. Paulo JA, Kadiyala V, Banks PA, Steen H, Conwell DL (2012) Mass spectrometry-based proteomics for translational research: a technical overview. *Yale J Biol Med* 85:59–73
157. Angel TE, Aryal UK, Hengel SM, Baker ES, Kelly RT, Robinson EW, Smith RD (2012) Mass spectrometry-based proteomics: existing capabilities and future directions. *Chem Soc Rev* 41:3912–3928
158. Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3:639–650
159. Das N, Biswas B, Khera R (2013) Membrane-bound complement regulatory proteins as biomarkers and potential therapeutic targets for SLE. *Adv Exp Med Biol* 734:55–81
160. McMahon G (2000) VEGF receptor signaling in tumor angiogenesis. *Oncologist* 5(Suppl 1): 3–10
161. Zwick E, Bange J, Ullrich A (2001) Receptor tyrosine kinase signalling as a target for cancer intervention strategies. *Endocr Relat Cancer* 8:161–173
162. Whitelegge J, Halgand F, Souda P, Zabrouskov V (2006) Top-down mass spectrometry of integral membrane proteins. *Expert Rev Proteomics* 3:585–596
163. Souda P, Ryan CM, Cramer WA, Whitelegge J (2011) Profiling of integral membrane proteins and their post translational modifications using high-resolution mass spectrometry. *Methods* 55:330–336