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Application of Mass Spectrometry in Proteomics

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Mass spectrometry has arguably become the core technology in proteomics. The application of mass spectrometry based techniques for the qualitative and quantitative analysis of global proteome samples derived from complex mixtures has had a big impact in the understanding of cellular function. Here, we give a brief introduction to principles of mass spectrometry and instrumentation currently used in proteomics experiments. In addition, recent developments in the application of mass spectrometry in proteomics are summarised. Strategies allowing high-throughput identification of proteins from highly complex mixtures include accurate mass measurement of peptides derived from total proteome digests and multidimensional peptide separations coupled with mass spectrometry. Mass spectrometric analysis of intact proteins permits the characterisation of protein isoforms. Recent developments in stable isotope labelling techniques and chemical tagging allow the mass spectrometry based differential display and quantitation of proteins, and newly established affinity procedures enable the targeted characterisation of post-translationally modified proteins. Finally, advances in mass spectrometric imaging allow the gathering of specific information on the local molecular composition, relative abundance and spatial distribution of peptides and proteins in thin tissue sections.

KEY WORDS: Mass spectrometry; proteomics; top-down and bottom-up approaches; relative and absolute protein quantitation.

INTRODUCTION

The term Proteomics refers to the analysis of all proteins in a living system, including the description of co- and post-translationally modified proteins and alternatively spliced variants. This includes their covalent and non-covalent associations, spatial and temporal distributions within cells, and how all these are affected by changes in the extracellular and intracellular conditions. Mass spectrometry (MS) is at the heart of virtually all proteomics experiments as it provides the key tools for the analysis of proteins. Developments of technology and methodology in the field of mass spectrometry and proteomics have been rapid over the last five years and are providing improved and novel strategies for global understanding of cellular

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function. In the following review, we provide a brief summary of currently used mass spectrometry-based methods in proteomics.

MASS SPECTROMETRY IN PROTEOMICS

Principles and Instrumentation

Mass spectrometers consist of three basic components: an ion source, a mass analyser, and an ion detector. MS measurements are carried out on ionised analytes in the gaseous phase, requiring a method to transfer molecules from solution or solid phase into this state. The two most commonly used techniques are matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) [1, 2]. Both MALDI and ESI are soft ionisation techniques in which ions are created with low internal energies and thus undergo little fragmentation. In MALDI samples are co-crystallised with an organic matrix on a metal target. A pulsed laser is used to excite the matrix, which causes rapid thermal heating of the molecules and eventually desorption of ions into the gas phase. Because of the usage of a pulsed laser, MALDI produces packets of ions rather than a continuous beam; it is therefore most often coupled to a mass analyser that can measure either a complete mass spectrum without scanning a mass range, or trap all the ions for subsequent mass analysis. This ionisation technique tolerates a reasonable amount of impurities in the sample to be analysed. Many different samples can be processed rapidly in an automated manner and these can be kept on the target for several days without compromising the quality of the analysis, allowing easy re-analysis when required.

ESI is based on spraying an electrically generated fine mist of ions into the inlet of a mass spectrometer at atmospheric pressure. This technique ionises molecules directly from solution, so it can easily be interfaced with liquid separation methods. Steady advances in the application of ESI to the analysis of peptides and proteins have been made, and the most notable improvements have come from reduction in the flow rate of the liquid used to create the electrospray, leading to more efficient creation of ions [3].

After ionisation, the sample reaches the mass analyser, which separates ions by their mass-to-charge (m/z) ratios. Ion motion in the mass analyser can be manipulated by electric or magnetic fields to direct ions to a detector, which registers the numbers of ions at each individual m/z value. In proteomics research, four basic kinds of mass analysers are currently being used: time-of-flight (TOF), ion trap, quadrupole, and Fourier transform ion cyclotron resonance (FTICR) analysers. All four differ considerably in sensitivity, resolution, mass accuracy and the possibility to fragment peptide ions. The latter results in mass spectra with an especially high content of information (MS/MS spectra) [4]. The combination of ion source, mass analyser and detector is usually determined by the application.

ESI is most frequently coupled to ion traps (three-dimensional and linear ion traps) and hybrid tandem mass spectrometers like quadrupole time-of-flight (Q/TOF) instruments. In the case of ion traps, the ions are first captured in the centre of the device for a certain time interval and are then scanned from the trap to the detector [3]. With this type of mass analyser it is not only possible to determine the

mass of a given peptide, but also its sequence. Ions with specific m/z ratios can be selected in the 'trap' for fragmentation, induced by collision of the ion with an inert gas or a surface in a process called collision induced fragmentation (CID). This energy causes the peptide ion to fragment at different points, commonly at the peptide bond. The recorded product ions represent the tandem mass spectrum (MS/MS or MS^2 spectrum) [5], which contains information on the amino acid sequence.

Three-dimensional ion traps are robust, relatively inexpensive and sensitive, but they exhibit low mass accuracy [6]. The two-dimensional ion trap is a recent development in which ions are stored in a cylindrical volume considerably larger than that of the three-dimensional ion traps, allowing increased sensitivity, resolution and mass accuracy [7, 8].

MALDI is usually coupled to TOF analysers, which separate ions according to their flight time down a field-free tube. Ions' time-of-flight is directly related to their m/z values and thus a mass spectrum can be acquired. The biggest drawback to TOF analysers is their inability to perform true MS/MS. However, recently developed TOF/TOF instruments have overcome this problem [9, 10]. TOF/TOF instruments incorporate a collision cell between two TOF sections. Ions of one m/z ratio are selected in the first TOF section, fragmented in the collision cell, and the fragments are separated in the second TOF section.

Hybrid Q/TOF instruments use a similar setup; here the collision cell is placed between a quadrupole mass filter and a TOF analyser [11, 12]. Ions of a particular m/z ratio are selected in the quadrupole mass filter, fragmented in the collison cell and the fragment ion masses are read out by a TOF analyser. Q/TOF instruments can be used interchangeably with a MALDI and an ESI source.

Both, TOF/TOF and Q/TOF instruments have high sensitivity, resolution and mass accuracy. The resulting fragment ion spectra are often more extensive and informative than those generated by trapping instruments.

Different systems based on modifications of the MALDI procedure have been developed to reduce the complexity of the sample on the surface used to immobilise proteins or protein digests prior to ionisation. One example is surface-enhanced laser desorption ionisation (SELDI) in which the sample undergoes a further purification step on the probe surface before MS analysis. The nature of this purification can be chemical (hydrophobic, coordination or ionic interaction between surface and sample) or biological (antibodies, DNA, enzymes interaction). The aim is to obtain the spectrum of a desired protein from a mixture of proteins. An additional advantage is that matrix is not necessary for ionisation and consequently spectra do not suffer from matrix signal interference [13]. Although many successful applications have been reported, particularly in the clinical field [14, 15], the lack of reproducibility is still limiting the contribution of SELDI-MS analysis in proteomic research [16].

A very promising alteration of the MALDI-TOF technique is desorption ionisation on silicon (DIOS), where the sample is loaded on a porous silicon surface and ionised without matrix assistance [17]. The most impressive applications are in enzymatic assay development and inhibitors screening, comprehensively reviewed in [18]. DIOS MS sensitivity has been stretched to yoctomols by differential adsorption of the sample on a chemically modified silicon surface [19].

FTICR analysers operate by trapping ions in a cell with a static magnetic field. Under the influence of the field, ions describe a circular cyclotron motion in the cell about the z-axis of the magnetic field. m/z can be determined by measuring the frequency of motion of the ions. This type of analyser is the most powerful currently available in terms of mass resolution ($M/\Delta M$) and mass measurement accuracy (MMA). MMA of these instruments is approximately 1 ppm, surpassing ion-traps by at least 2 orders of magnitude, and resolution can be routinely 100000. FTICR analysers are highly versatile and can be combined with both MALDI and ESI [20, 21] ion sources. The extremely high mass resolution/MMA makes FTICR-MS the instrument of choice for studying protein isoforms [22] (and see below).

A very recent technical development can provide an instrument that will probably create a huge impact in proteomics. The strategy was to combine a linear ion trap with a FTICR mass analyser. The linear ion trap is used to collect ions and then isolate and fragment them for injection into the ICR cell where accurate masses are determined [23–25]. This setup combines the high ion capacity and fast scan times of linear ion traps with the already mentioned advantages of the FTICR analyser. The first commercially available instruments of this kind are on the market and show already promising results, e.g. see [24, 26].

There are several other types of mass spectrometers in use in proteomics research undescribed here and the interested reader is referred to [3, 23, 27–30].

Protein Identification

The identification of proteins in proteomics is almost exclusively achieved by mass spectrometry [4]. Current MS methods can identify any protein for which a genome sequence is known. MS is highly sensitive: proteins present in femtomole quantities can be identified on a routine basis and extension to low zeptomole amounts has been achieved [19, 31]. Additionally, MS can in principle characterise the exact covalent structure of a protein, for example splice variants or post-translational modifications, without prior knowledge of the types of modifications or their locations.

The most common MS strategies analyse peptides rather than full-length proteins. MALDI-TOF mass spectrometry is relatively simple and robust to operate, has good mass accuracy, high resolution and sensitivity. It is widely used in proteomics to identify proteins from simple mixtures by a process called peptide mass fingerprinting [32]. In this approach, proteins of interest are digested with a sequence-specific enzyme such as trypsin and the generated peptides are analysed by MS. The determined masses, usually in the range of m/z 800–3000, are then compared against a database comprising peptide masses from a virtual digest of all proteins from a given organism with the same sequence-specific protease. This form of protein identification ideally requires an essentially purified target protein. The technique is therefore very often used in conjunction with two-dimensional gel electrophoresis (2D PAGE).

ESI ion trap and ESI Q/TOF MS are currently the most popular setups for the simultaneous analysis of large numbers of peptides derived from enzymatic digests of complex protein mixtures. This is mainly due to their ability to interface

chromatographic peptide fractionation methods online with the ESI based instruments and the possibility to obtain not only mass but also sequence information. The sequence information containing MS/MS spectra are compared against comprehensive protein sequence databases using one of several different algorithms [33–35]. This strategy is very similar to peptide mass fingerprinting and, in analogy, is called peptide fragmentation mass fingerprinting. The other main advantage of ESI over MALDI, is the production of multiply charged ions, which allows the measurement of high molecular weight peptides, often neglected in MALDI-TOF analysis.

THE BOTTOM-UP AND TOP-DOWN APPROACHES IN PROTEOMICS

Proteomics experiments most often invoke the simultaneous analysis of several thousands of protein species from complex biological samples. The separation of peptides and proteins is therefore a key element in proteomics analysis providing a method to simplify complex mixtures and deliver molecules to the ionisation source. Most of the existing separation methods (including gel electrophoresis and liquid chromatography based methods) are currently in use and have been recently reviewed [3, 36–38].

The choice of separation method used depends on the experimental question asked and, equally importantly, the level on which the proteins should be displayed. This is the branching point of two principle proteomics approaches, the bottom-up and the top-down approach.

In the bottom-up approach, complex protein mixtures are enzymatically digested into very complex peptide mixtures, which are then fractionated by multi-dimensional chromatography steps before they are subjected to tandem mass spectrometry. MS/MS spectra are recorded for as many peptides as possible, and the results used to search databases to identify the proteins in the original mixture. Such approaches are suitable for automation and high sample throughput can be achieved [39]. These procedures typically identify a very limited number of peptides per protein, but still enough to identify the gene from which the protein was transcribed. They work well in the study of microorganisms where the assumption that one gene codes for only one protein species is often true. However when higher eukaryotes are investigated, processes including alternative splicing, RNA editing and post-translational modification can lead to several different protein species from a single gene. Peptide-based identification strategies enable the identification of the genes from which these proteins are derived but do not adequately identify the different functionally important protein isoforms [40].

In the top-down approach, intact proteins are displayed and different isoforms can be isolated before MS identification and characterisation. This is especially useful to unravel complex patterns of splice variations or post-translational modifications [20, 22, 41, 42]. Difficulties in presenting intact proteins to the mass spectrometer, the complexity of the spectra and the lack of automation still restrict this application to time-consuming ‘one protein after the other’ studies. A more common top-down approach is to fractionate and display intact proteins, but to analyse them on the peptide level.

Examples for the Bottom-Up Approach

Multidimensional protein identification technology (MudPIT) is an automated bottom-up approach in which the complexity problem is addressed at the peptide level [43, 44]. Following enzymatic digestion of a total protein mixture, peptides are separated on a biphasic liquid chromatography column using a strong cation exchange support as the initial phase, and subsequently reversed-phase material. They are then delivered online to a tandem mass spectrometer, and MS/MS spectra are automatically detected for as many peptides as possible and those spectra used to search protein sequence databases. With this LC/LC/MS/MS procedure a high-separation of peptides is achieved. The complexity deconvolution takes place mainly in the chromatography step, but the ability of the mass spectrometer to handle several peptides at a time also contributes to the multiple dimensions [45]. In one attempt to demonstrate that the method provides the possibility for rapid and large-scale proteome analysis, 1484 proteins from a whole yeast cell lysate were identified by MudPIT [46].

Another strategy for dealing with complex peptide mixtures is the accurate mass and time tag (AMT) approach, which combines high-resolution capillary liquid chromatography separation with high-resolution accurate FTICR measurements [20]. An enzymatic digest of the entire proteome of an organism should theoretically contain a subset of peptides that have unique molecular masses. The mass of such a peptide can be calculated and, if its experimental mass determination is accurate enough, the parent protein is identified with high confidence, even without additional sequence information. Accurate mass tags are peptides which are unique within the annotated genomic database of the investigated organism, whose sequence is confirmed by MS/MS analysis and whose observed mass agrees with the theoretical mass within 1 ppm MMA [20]. To increase confidence of peptide identification, the LC elution times of mass tags are also determined and used as an additional criterion in the identification process. This concept of high-throughput protein identification with AMTs was applied to the proteome of *Deinococcus radiodurans*. The combined resolving power of capillary liquid chromatography and FTICR used together with the very high mass accuracy of the FTICR analyser to detect AMTs lead to the high confidence identification of proteins corresponding to more than 61% of the predicted genes [47].

One of the reasons why the AMT concept is not readily applicable to higher organisms is the unpredictable occurrence of post-translational modifications on proteins, which makes it difficult to find enough peptides that could serve as reliable AMTs to cover the proteome [48]. It is obvious that the method is also not able to distinguish between different splicing variants of the same protein.

Examples for the Top-Down Approach

An example of a top-down approach in which intact proteins are presented to the mass spectrometer is the analysis of small proteins from *Methanococcus jannaschii* [49]. In this approach, protein mixtures were separated off-line and then introduced in a FTICR mass spectrometer by infusion. Two other examples, also using FTICR MS and starting from intact and essentially pure carbonic anhydrase and bovine milk

protein PP3, respectively, used the top-down approach to characterise all post-translational modification sites in the heavily modified proteins to within one residue [22, 42].

A recent example using a top-down approach to display intact proteins and identify them on the peptide level illustrate the potential of this classical approach in proteomics. Nuclear proteins from rat fibroblasts cultured under normoxic or hypoxic conditions were compared [50]. Two-dimensional gel electrophoresis and MALDI-TOF MS showed large numbers of unexpected proteins in the nucleus, but more interestingly, for 30% of these proteins evidence was obtained that the nuclear protein was a previously unknown isoform and for 40% of the proteins post-translational isoforms were also discovered. The use of top-down display of intact, full proteins rather than peptides enabled the detection of these different isoforms.

QUANTITATIVE MASS SPECTROMETRY

In addition to the initial identification of phenotypic expression and protein characterisation, a key parameter in proteomics analysis, is the ability to quantitate proteins of interest. Quantitation is a vital tool towards an understanding of transcriptional, translational and post-translational effects that affect protein production and function.

In recent years quantitative proteomics by mass spectrometry has mainly focused on the differential quantitative determination of protein expression and not on absolute measurements, as many proteomic applications to drug target discovery or to track signalling events are concerned with relative rather than absolute abundances of proteins.

In mass spectrometry the amount of analyte in the sample does not correlate directly with the ion-current intensity of its mass spectrometric signal. Additional techniques have to be implemented to enable differential quantitation of proteins with mass spectrometry. In proteomics almost all of these additional methods involve the labelling of peptides with stable isotopes by either biosynthetic or chemical methods. Peptides can then not only be identified but isotope labelling also allows the measurement of differential amounts of the same peptide [51, 52].

Metabolic Isotopic Labelling

Metabolic labelling of proteins exploits the incorporation of isotopic labels during the process of cellular metabolism and protein synthesis. A simple and universal method for protein quantitation by mass spectrometry is whole cell stable isotope labelling. In the original approach, one experimental cell population is grown in an isotopically depleted medium enriched in ^{15}N, and the other in standard ^{14}N-rich medium. After extraction, the two protein samples are pooled. Proteins/peptides are then analysed using classic proteomics methods [53–55]. The MS measurement readily differentiates between peptides originating from the two pools because incorporation of a high abundance of ^{15}N shifts the mass of any given peptide upwards, which leads to a pair of peaks from each peptide. The ratios between the intensities or areas of the lower and upper mass components of these pairs of peaks directly reflect the difference in the amount of a given protein in the

two different cell pools. Accurate relative quantitation is possible because the MS intensity response to a given peptide is independent of the isotopic composition of the nitrogen atoms [56]. An additional advantage of this approach is the availability of multiple peptide pairs per protein allowing a good estimation of the error associated to the quantitation.

An increasingly popular method for protein quantitation by MS is the labelling of proteins by incorporation of isotopically modified amino acids. This technique was recently named SILAC, for stable isotope labelling by amino acids in cell culture [57]. In this approach, one population of cells is grown in medium containing the normal form of an essential amino acid; another population of cells is grown in medium supplemented with a stable isotope-labelled analogue. The two resulting protein samples are pooled and analysed essentially as described above. Different amino acids are suitable for SILAC, among others leucine (Leu-d0) and deuterated leucine (Leu-d3) [57], arginine ($^{12}C_6$-Arg) and fully substituted ^{13}C labelled arginine ($^{13}C_6$-Arg) [58], tyrosine ($^{12}C_9$-Tyr) and fully substituted ^{13}C labelled tyrosine ($^{13}C_9$-Tyr) [59] have been used.

So far, metabolic isotope labelling has been used to compare two cellular states, such as diseased versus normal. Very recently the SILAC method has been extended to allow simultaneous quantitation of proteins originating from three cellular states [60]. Three different populations of cells were grown in media containing different versions of arginine, $^{12}C_6^{14}N_4$-Arg, $^{13}C_6^{14}N_4$-Arg and $^{13}C_6^{15}N_4$-Arg. After protein extraction and pooling of the samples, arginine-containing peptides generated by proteolytic cleavage occur as triplets in the mass spectra showing a mass separation by 6 or 10 Da. Again, the intensity of each peak directly indicates the relative amount of the respective protein from the corresponding cell pool.

Until very recently, the use of metabolic labelling was limited to samples derived from cell culture. The applicability of the method has now been extended by quantitative ^{15}N metabolic labelling of the multi-cellular organisms *Caenorhabditis elegans* and *Drosophila melanogaster* [61]. After labelling the organisms by feeding them on ^{15}N-labelled *E. coli* and yeast, respectively, the relative abundance of individual proteins was determined by mass spectrometry.

Chemical Labelling

When metabolic labelling of proteins is not possible or not desirable, chemical-labelling techniques can be used as an alternative quantitative tool. A widely used chemical labelling technique for quantitative proteomics is the isotope-coded affinity tag (ICAT) method [62] and modified versions of it [63–65]. The ICAT reagents include a cysteine-reactive group, an isotopically light or heavy linker and a biotin affinity tag. The light and the heavy reagents are used to label the cysteine residues from proteins of two different sources. The two protein samples are then combined and enzymatically cleaved into peptide fragments. Cysteine-containing peptides are isolated using avidin affinity chromatography and subsequently identified, and quantitated by microcapillary LC/MS/MS. ICAT-labelled peptides elute as pairs from a reverse-phase column. By calculating the ratio of the areas under the curve for identical peptide peaks labelled with the light and the heavy ICAT reagent, the relative abundance of that peptide in each sample can be determined, which is

directly related to the abundance of the corresponding protein. The complexity of the peptide mixture is greatly reduced because only the cysteine-containing peptides are analysed in the mass spectrometer. This directly translates into more identified proteins per analysis time and into the identification of lower abundance proteins [66].

Recently, a second generation of ICAT reagents have become commercially available (for an example see [67]), making the technology widely available.

Another popular way of labelling peptides with isotopes is the incorporation of oxygen-18 during enzymatic cleavage of the respective protein. Proteolytic enzymes incorporate oxygen atoms from the solvent into the C-termini of the resulting peptides. In a basic approach one set of proteins is cleaved in heavy water ($H_2^{18}O$), and the other set is cleaved similarly in light water ($H_2^{16}O$). The samples are pooled after the digestion step and identification and quantitation by mass spectrometry are the final steps of the analysis. Again, quantitation is based on isotope ratios [68]. An elegant method for global differential proteomics using the concept of proteolytic enzyme catalysed oxygen-18 incorporation was described very recently [69]. The method is based on the assumption that one unique peptide is sufficient to unambiguously identify each parent protein. Because each protein contains precisely one *N*-terminus, the method targets *N*-terminal peptides in the attempt to reduce sample complexity in a proteome wide profiling experiment enabling simultaneous relative quantitation. In a first step, two different proteins pools are separately digested with trypsin and subsequently, one is labelled with ^{18}O and the other is labelled with ^{16}O isotopes by post-cleavage trypsin catalysed oxygen-exchange. The two samples are mixed and separated by two identical chromatographic steps. Fractions collected from the first run are treated in a way that alters the retention time of *N*-terminal peptides only, while other peptides remain unaffected. Each fraction is then analysed again in a second run. Consequently, all modified *N*-terminal peptides exhibit altered retention times whereas the remaining peptides elute from the column with the same retention time as in the first run. The introduced difference in retention time is large enough to allow the separation and collection of the *N*-terminal peptides [70]. Collected peptides derived from protein *N*-termini are then identified and quantitated by MS [69].

A very promising new development in quantitative mass spectrometry is a group of four isobaric mass tags that allow the simultaneous quantitation of peptides/proteins from up to four samples. These reagents contain an amine reactive group, ensuring the covalent labelling of all peptides in a given sample digest, a balance group and a reporter group. Four different reporter groups with masses between m/z 114–117 are mass matched with respective balance groups (m/z 31–28) resulting in four different reagents with identical masses. Proteins from four different samples, e.g. from a cell stimulation experiment with four time points, are separately extracted and digested. Resulting peptide pools are labelled with the different isobaric mass tags, combined and analysed by LC/MS/MS. Identical peptides derived from different samples and therefore labelled with different mass tags are nonetheless identical in mass and hence the MS of the mixture resembles the MS of an individual sample, provided that the same peptides are present in all samples. The actual quantitation takes place in MS/MS mode. As a result of fragmentation, there is neutral loss of the balance group and the reporter groups are generated, displaying

diagnostic ions in a low-mass region that is essentially free of other common ions. Quantitating the peak areas of these resultant ions represents the relative amount of a given peptide in the respective sample. The simultaneous identification of the peptides is not exacerbated by the mixing of multiple differently tagged proteome samples because fragmentation patterns of identical peptides from different samples are identical apart from the generated reporter ions. The described isobaric mass tags are known as iTRAQ reagents [71]. A very similar approach was introduced recently based on so called Tandem Mass Tags [72, 73].

Several other techniques that use chemical labelling of proteins/peptides with different isotopes and reagents have been developed and are in widespread use. For a comprehensive overview, the interested reader is referred to [51, 74].

Absolute Quantitation

Although this chapter focuses mainly on the relative comparison and quantitation of proteins, in many instances it is desirable to quantitate the absolute amount of a given protein. The measurement of exact protein or peptide amounts in a given system often, and in contrast to comparative quantitation analysis, involves a qualitative analysis prior to the quantitative analysis, such that the entity to be measured is already well defined.

Many approaches for absolute quantitation of proteins and peptides involve the use of a standard curve, which is developed with a stable isotope-incorporated peptide. This peptide is then used as an internal standard by spiking the analytical sample with a known amount. The ratio between the synthetic and endogenous peptide is determined by MS, and the absolute amount of this peptide can be calculated [53, 75]. If a particular protein in a complex biological sample is to be quantitated in absolute terms by stable isotope dilution combined with MS, the search for the corresponding peptide pair resembles the search for a needle in a haystack and therefore a dramatic reduction of sample complexity is needed before the actual quantitation step. Often this is achieved by purification procedures that have to be specifically designed for the protein/peptide of interest (e.g. see [53, 75]). A new, generally applicable procedure allowing the absolute quantitation of specific proteins in complex mixtures was introduced recently [76, 77]. It is based on visible isotope-coded affinity tags (VICAT), which are based on the earlier described ICAT reagents, containing a thiol reactive group, a biotin affinity handle, a photo-cleavable linker for removing a part of the tag after biotin affinity purification of tagged peptides, an isotope tag for distinguishing sample and internal standard peptides and a ^{14}C-marked (visible) moiety for tracking the chromatographic location of the target peptide by a method other than mass spectrometry (scintillation counting). Isolated proteins are mixed with a specific amount of synthetic internal standard peptide (unique to the protein to be quantitated) already tagged with the 'heavy' VICAT reagent, and labelled with the 'light' VICAT form. After tryptic digestion, the peptides are separated by gel based isoelectric focusing. After tracking down the location of the peptides of interest by scintillation counting, eluted fractions from the gel are subjected to biotin affinity chromatography and combined microliquid chromatography/electrospray ionisation mass spectrometry operating in selected reaction monitoring (SRM) mode

(see Fig. 1). Several fragment ions from the lightly tagged peptide and the respective fragment ions from the heavily tagged internal standard are monitored, and the light-to-heavy peak areas are averaged to obtain the ratio of protein-derived peptide to internal standard. This method should be amenable to multiplexing, i.e. for the analysis of several proteins in a single sample.

There is another, related strategy for the absolute quantitation (AQUA) of proteins [78]. In this approach total proteins from whole cell-lysates are fractionated by 1D PAGE, and regions of the gel consistent with protein migration are excised. This process is followed by in-gel digestion in the presence of the respective isotopically labelled standard peptides. After extraction of peptides, quantitation again is achieved by LC/MS/MS in SRM mode as described above.

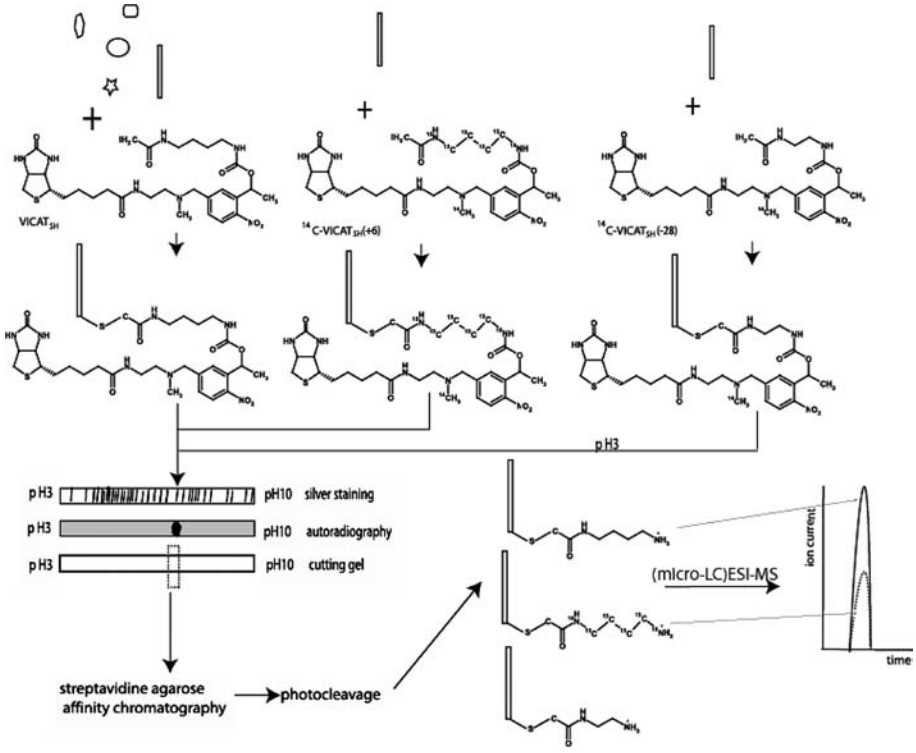

Fig. 1. Schematic representation of the absolute quantitation of a target protein using VICAT labelling. Cysteine containing peptides from a complex protein mixture are tagged with VICAT$_{SH}$. A unique peptide belonging to the protein to be quantitated is tagged with $(+6)^{14}$C-VICAT$_{SH}$, which is 6 Da heavier than VICAT$_{SH}$, and a separate pool with $(-28)^{14}$C-VICAT$_{SH}$, which is 28 Da lighter than VICAT$_{SH}$. The tagged peptides from the sample are mixed with the tagged standard peptides, and separated on immobilised pH gradient gel strips by IEF. The position of the tagged peptides is localised by ^{14}C-autoradiography; the signal is mainly derived from the $(-28)^{14}$C-VICAT$_{SH}$ modified standard peptide added in excess for this purpose (also called IEF marker). Peptides from the indicated region are eluted from the gel and the tagged peptides are captured on streptavidin-agarose beads. After photocleavage, the isolated peptides are analysed by (micro-LC)/ESI MS using selected reaction monitoring (SRM). Absolute quantitation is achieved by obtaining the light-to-heavy ratios of the ion peak areas for the specified fragments ions.

A different approach to relative and absolute protein quantitation using multi-photon detection has been recently described [40]. Proteins from whole cell lysates are radio-iodinated with either ^{125}I or ^{131}I and subsequently separated on 2D gels. Gels are then imaged and the protein spots quantitated by multi-photon detection (MPD), a method based on measurement of radioisotopes which decay by the electron capture mechanism and hence emit multiple photons/particles of defined energies simultaneously. Background radioactivity very rarely provides coincident emissions of defined energies, allowing the supersensitive detection of appropriate radiolabels by MPD. The technology is linear over 7 orders of magnitude down to low zeptomole levels for ^{125}I. MPD is able to discriminate between different radio-isotopes by virtue of the different energies of their emissions, thereby enabling the analysis of differently radio-iodinated protein pools on a single 2D gel. More than 60 different isotopes are suitable for MPD measurements, enabling theoretically very high levels of multiplexing on single 2D gels. The described MPD/2D PAGE approach cannot only be used in top-down differential display analyses, but also for absolute measurements of protein amounts in a high throughput context.

CHARACTERISATION OF POST-TRANSLATIONAL MODIFICATIONS BY MASS SPECTROMETRY

Post-translational modifications (PTMs) are processing events that change the properties of a protein by proteolytic cleavage or by covalent addition of a modifying group to one ore more amino acids. Delineation of protein function solely on the basis of changes in abundance provides a very limited view of the proteome since numerous protein activities are modulated by PTMs that may not be accurately reflected by changes in protein abundance. This is the reason why many of the mass spectrometry driven techniques used to characterise and quantitate proteins and peptides on a proteome-wide scale have been applied, to the characterisation of post-translationally modified proteins.

A protein that has experienced some form of post-translational modification exhibits a mass increase or decrease relative to the molecular weight calculated from its amino acid sequence. Phosphorylation of a tyrosine residue, e.g., leads to a mass increment of 80 Da, which could, in principle, be detected by a very accurate mass determination by mass spectrometry of the intact protein. Usually, modified proteins are further characterised by enzymatic digestion and subsequent peptide mass mapping. In case the mass of the modified peptide is not sufficient to determine the nature of the modification and its location, the respective peptides are analysed by tandem mass spectrometry (an example is given in Fig. 2). This procedure is very often the last experimental step in the characterisation of a modified protein or peptide. Problems may arise from the complexity of the post-translationally modified protein sample and the characteristics of the modified peptides [79, 80]. The size and the physicochemical properties of the peptides influence their ionisation and detection efficiency and consequently make them difficult to find in a high background of other ions. In addition, determination of the modified amino acid is sometimes not possible due to incomplete ion series in the tandem MS experiment.

Many of the currently used procedures to identify and characterise post-translationally modified proteins on a global scale employ some form of specific

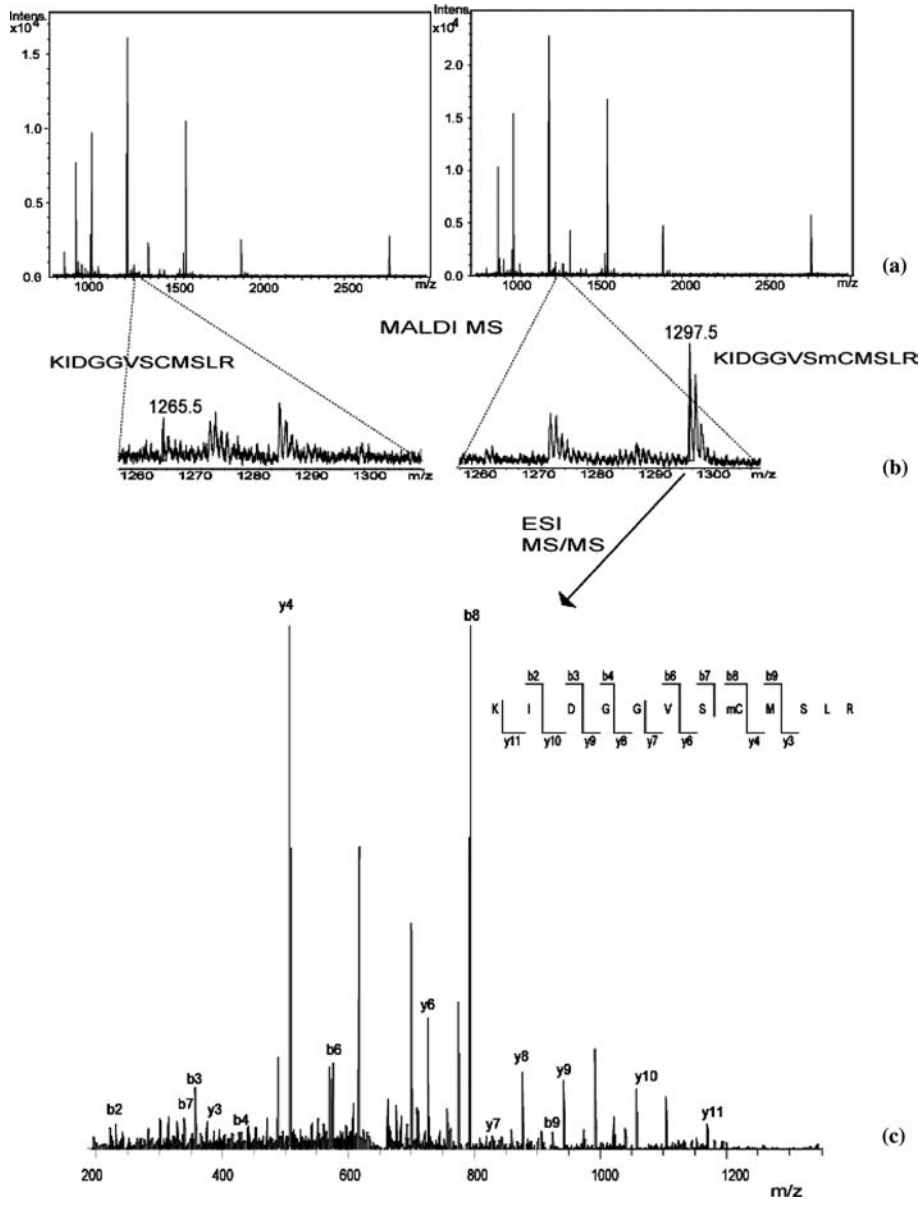

Fig. 2. Example of PMT identification. (a) MALDI spectra of a standard protein, which is unmodified (left) and *in vitro* oxidised (right). (b) Enlargements of the spectra in the m/z 1250–1350 region show differences in the peptide profiles. The peak at m/z 1265.5 is attributed to the peptide KIDGGVSCMSLR, and m/z 1297.5 is thought to be the same peptide with a modification of +32, likely to be the result of a R-SH to a R-SO_2H conversion. (c) The identification of the modification on cysteine (mC) is confirmed by static ESI MS/MS sequencing of the m/z 1297.5 ion. Fragment ions from the y and b series are highlighted in the spectrum and on the sequence.

enrichment of the molecules to be identified prior to microcharacterisation by MS. This enrichment step can take place on the intact protein level as well as on the peptide level.

Anti-phosphotyrosine antibodies are widely used for immunoprecipitation of intact phosphoproteins in mass spectrometry driven proteomic studies of cell signalling [60, 81, 82]. However less specific, anti-phosphoserine and anti-phosphothreonine antibodies are used for the same purpose [83]. The availability of novel antibodies specific for mono- and asymmetrically dimethylated arginine residues make this modification amenable for global proteomics studies as well [84].

A popular affinity-enrichment strategy for phosphopeptides is immobilised metal affinity chromatography (IMAC). Phosphate groups of phosphopeptides show a strong affinity towards immobilised Fe^{3+}- and Ga^{3+}-ions and this method is therefore used to enrich phosphopeptides from crude peptide mixtures prior to LC/MS/MS analysis in large scale phosphoproteomics experiments [85, 86]. Glycoproteins and glycopeptides can be enriched with the help of lectins, carbohydrate binding proteins that recognise specific carbohydrate structures. Lectin-based affinity enrichment of glycopeptides is used in the large-scale identification of glycosylated proteins from complex biological samples [87, 88]. For a detailed overview of mass spectrometric methods to analyse not only glycopeptides but also the respective oligosaccharides, the reader is referred to [89].

A technology that is increasingly used in the characterisation of PTMs is the chemical derivatisation of the modifying group which allows the subsequent introduction of a tag for affinity purification. The phosphate group can be converted to an affinity tag by a β-elimination-Michael addition reaction or by phosphoamidate chemistry [90–92]. This approach has also been used on phosphopeptides to introduce reporter groups that generate diagnostic fragment ion signals for use in MS/MS precursor ion scanning [93]. *O*-glycosylated amino acids also undergo β-elimination and are therefore amenable for affinity tag introduction [94]. A recent proteome-wide screen for *N*-glycosylated proteins employed hydrazide chemistry to selectively immobilise this type of glycoproteins to a solid support for subsequent analysis [95]. Tyrosine nitration and cysteine nitrosylation can also be followed by introducing an affinity tag. Nitrotyrosine can be converted into amino-tyrosine which then in turn can be biotinylated [96]. A methodology known as the 'biotin-switch' selectively introduces a biotin tag in place of the S-nitroso group and allows the study of this PTM on a proteome wide scale [97, 98].

Quantitation of post-translationally modified proteins comprises not only the determination of protein abundance but also the relative or absolute determination of the extent of modification at each amino acid, which makes it a difficult task to achieve. A majority of the methods developed for quantitative mass spectrometry have so far been used in the quantitation of post-translationally modified proteins as well. Metabolic labelling of proteins by growing cells on ^{15}N containing medium enabled the accurate quantitation of changes in the levels of modifications at specific sites in individual yeast proteins [53]. The already described β-elimination/michael addition reaction together with isotope coded affinity tags has been applied for differential determination of Ser/Thr phosphorylation [90, 91]. A quantitative differential display analysis of *N*-linked glycoproteins has been performed by combining a selective conjugating of these proteins to a solid support and a

subsequent labelling of peptides with *N*-linked carbohydrates with isotope coded affinity tags [95]. Very recently, the stable isotope labelling in cell culture method was combined with enrichment of phosphotryrosine containing proteins in a quantitative study of EGF signalling networks. In this study the use of SILAC not only enabled quantitation of proteins but also allowed to perform the experiment in a time resolved manner [60]. Absolute quantitation of modified proteins and peptides has also been done using the previously mentioned AQUA method [78].

A comprehensive overview on modification specific proteomics including quantitative aspects can be found in [74, 99–101].

A recent technical development turned out to be extremely helpful in the characterisation of post-translationally modified proteins. Electron capture dissociation (ECD) was introduced in 1998 as a new tandem mass spectrometry technique for studying polypeptides and proteins [102]. A few of the most important differences between ECD and collision induced dissociation (CID) are that ECD results in extensive fragmentation of the backbone of small proteins, that it does not generate many internal fragments, and especially that it cleaves the peptide back-bone while leaving potential modifications on amino acids intact [21]. The combination of ECD with the very high mass accuracy and resolving power of FTICR makes the efficient sequencing of glycopeptides, phosphopeptides and other types of modified peptides possible and has also been applied to the analysis of modified intact proteins [22, 42, 103–105].

IMAGING MASS SPECTROMETRY

Another development in the application of mass spectrometry in proteomics is the use of MALDI-TOF MS in profiling and imaging proteins directly from thin tissue sections, a technology known as protein profiling and imaging mass spectrometry (IMS) [106]. IMS provides specific information on the local molecular composition, relative abundance and spatial distribution of peptides and proteins in the analysed section.

The choice between profiling and imaging proteins depends on the overall goal of the experiment. Using profiling, the matrix is deposited in discrete spots on specific regions of interest on a tissue section. To obtain significant statistics, the signals from 100 to 1000 laser shots per spot are averaged; typically 300–500 distinct mass signals are detected in a m/z range from 2000 to 70000. This approach allows protein profiles to be obtained from defined areas of the tissue and facilitates comparisons between and within tissues. Relatively large numbers of samples can be processed in several hours and the generated data is analysed by software packages to identify markers indicative of ongoing biological processes in the tissue. This approach is nicely illustrated in a recent study in which protein mass profiles were generated from sections of human lung cancer tissue. Biostatistical selection of differentially expressed peaks used together with a computerised class-prediction tool allowed not only the discrimination between normal and malignant tissue, but also subclassification of primary tumours and prediction of patient survival [107].

When imaging is used, a tissue section is uniformly coated with matrix solution by air spraying so that protein profiles can be acquired over the entire area. Mass spectrometric data is acquired using a discrete Cartesian pattern of spots. The

distance between spots is fixed and depends on the chosen resolution, typically ranging from 25 to 200 μm. The number of laser shots per spot is predetermined und usually 20–50 shots are recorded. From the intensity of a given m/z value monitored in each spectrum, a two dimensional ion density map or image can be reconstructed using specialised software [108–110]. From a single imaging experiment, several hundred images, each at a specific molecular weight, can be made. The described imaging approach provides high-resolution protein distribution information within a tissue section and can be used as a tool for the investigation of cellular processes in both healthy and diseased tissues [111].

A profiling or imaging experiment can be followed by a protein identification step. Detected proteins of interest are extracted from the tissue section, separated by HPLC and the different fractions are then analysed by MALDI MS. Fractions containing the proteins of interest are subjected to tryptic digestion and the peptides are identified by tandem mass spectrometry [106].

IMS is a very promising discovery tool in research, because proteins recorded don't have to be known in advance. In addition the cellular origins and relative concentrations of the markers across the section can be assessed. However, there are drawbacks to the technique. The resolution of the images is limited by the laser spot size on the tissue, currently about 30 μm in diameter [106, 112]. For subcellular studies, spot sizes well below 20 μm are needed, and therefore such analyses are not yet applicable. Furthermore, the commonly used sample preparation procedures favour primarily hydrophilic proteins to be accessible for MALDI MS analysis. Finally, as IMS is based on MALDI-TOF MS, all the physical limitations linked to this technique apply, leading to poor mass resolution of signals above m/z 50,000 and to the fact that approximately 90% of the observed signals are below m/z 30,000 [111].

Very recently, a novel approach to MALDI molecular imaging using an ion microscope was introduced [113]. Single analytes are co-crystallised with matrix and desorbed/ionised with laser pulses. The laser spot used is ~200 μm in diameter and lies well within the area amenable to analysis in the ion microscope. The image formation is obtained very similarly to wide-field optical microscopy. The ions produced by a single laser shot pass through the time-of-flight mass spectrometer forming an ion-optical image on a position sensitive detector. In this way, a series of molecular images separated by mass-to-charge ratio (m/z) is generated. These molecular images reveal spatial detail from within the laser spot.

The big advantage of the mass spectrometric microscope is the fact that the spatial resolution is independent of the spot size and the ionisation beam [113, 114], which allows a large area to be examined without having to move the sample or the laser spot. The instrument's ion optics allow an overall magnification of 10× and 20× and the experimental resolution for both overall magnifications is ~4 μm.

Compared to the IMS technique described above, the MALDI ion microscope is able to produce images with higher spatial resolution with a considerably lower number of imaging steps, which significantly reduces the analysis time. Due to the decoupling of spatial resolution from the source conditions, additional ionisation methodologies like infrared laser sources can be used for imaging purposes. It must be said that the here described MALDI MS microscope is still in its infancy as it is capable of recording single-analyte images only. A parallel detection of ions in both

space and time is required to obtain high-resolution images for all masses with each laser shot. Specialised detectors are currently under development [113, 115].

CONCLUSIONS

The developments based on mass spectrometry in proteomics in the last few years have been enormous. High-throughput identification of proteins together with relative quantitation is now possible, absolute quantitation of single peptides in complex mixtures has been established, the large scale characterisation of post-translational modifications is on its way (e.g. [116, 117]) and intact, highly modified proteins can be entirely analysed in the mass spectrometer [105]. However, there are still many challenges to be resolved in order to push proteomics research forward.

The first deals with analytical sensitivity. One of the major necessities for proteomics in higher eukaryotes is the capacity to analyse small numbers of cells sampled directly from physiologically relevant sources, i.e. tissue, as it is increasingly well known that cell lines, as a major model for proteomics analysis of cellular function, are of limited relevance to tissue physiology in higher eukaryotes [118]. The limited analytical sensitivity of both the detection level of proteins and the intrinsic sensitivity of MS protein identification methods have limited the analysis of small amounts of cells derived directly from tissue so far. Yet there are already indications that this problem might be overcome in the near future. Protein identifications from as little as 0.5 pg of whole proteome extracts have been reported [31] and the absolute sensitivity limit for the MS identification of a single peptide has been pushed below the zepomole border [19].

The second major challenge is the need for new methods for high-throughput and accurate measurement and identification of intact proteins. Recent developments in FTICR mass spectrometry together with ECD based protein fragmentation tackle the problem of accurate mass determination and identification/characterisation of intact proteins [20, 105]. However, before a top-down approach to study intact proteins will have a broad impact on proteomics, the separation and delivery of proteins to the mass spectrometer in a large-scale manner needs to be improved and automation of the necessary mass spectrometry steps needs to be developed.

The third area that needs to be developed is the dynamic range of display and detection of proteins. In typical human cells, proteins differ in abundance over six orders of magnitude; this dynamic range extends to 9 orders of magnitude in samples like serum [36]. Even with a combination of high-performance chromatographic separation techniques and mass spectrometry, the currently achievable dynamic range of peptide identification in a mass spectrometry experiment does not exceed 10^4–10^5 [48], which means that the current techniques are neither suitable to even theoretically detect all proteins derived from a human cell lysate nor able to measure absolute amounts of many different proteins simultaneously. However, the very high dynamic range and sensitivity of multi-photon detection of gel separated proteins together with the ability of absolute quantitation holds many expectations [40]. In fact, MPD combined with described super-sensitive mass spectrometry approaches [19, 31] could develop into a powerful tool to overcome many present limitations.

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