Bottom-Up Mass Spectrometry–Based Proteomics as an Investigative Analytical Tool for Discovery and Quantification of Proteins in Biological Samples

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Objective: The objective of this overview is to introduce bottom-up mass spectrometry (MS)–based proteomics approaches and strategies, widely used in other biomedical research fields, to the wound-healing research community.
Approaches: Two major proteomics workflows are discussed: gel-based and gel-free chromatographic separation to reduce the complexity of the sample at protein and peptide level, respectively, prior to nano–liquid chromatography–tandem mass spectrometry analysis. Other strategies to discover less abundant proteins present in the sample, are also briefly discussed along with label-free and label-incorporated methods for protein quantification. Overall, the experimental workflows are designed and continually improved to increase the number of proteins identifiable and quantifiable.
Discussion: Recent advances and improvements in all areas of proteomics workflow from sample preparation, to acquisition of massive amounts of data, to bioinformatics analysis have made this technology an indispensable tool for in-depth large-scale characterization of complex proteomes. This technology has been successfully applied in studies focusing on biomarker discovery, differential protein expression, protein–protein interactions, and post-translational modifications in complex biological samples such as cerebrospinal fluid, serum and plasma, and urine from patients. The publications from these studies have reported greater number of identified proteins, novel biomarker candidates, and post-translational modifications previously unknown.
Conclusions: The qualitative and quantitative protein analysis of the protein population of wound tissues or fluids at different stages is important in wound healing research. Given the complexities and analytical challenges of these samples, MS-based proteomic workflows further improved with recent advances offer a powerful and attractive technology for this purpose.

Abbreviations and Acronyms

AQUA = absolute quantification
DDA = data-dependent acquisition
DIA = data-independent acquisition
Hex = hexose
HexNAc = *N*-acetyl hexosamine
ICAT = isotope-coded affinity tag
iTRAQ = isobaric tags for relative and absolute quantification
LC = liquid chromatography
MS = mass spectrometry
MS/MS = tandem MS
MudPIT = multidimensional protein identification technology
MW = molecular weight
NeuNAc = *N*-acetyl neuraminic acid

(*continued*)

INTRODUCTION

Wound healing involves a complex series of processes encompassing repair, regeneration, and remodeling of damaged tissues eventually culminating in restoration of tissue integrity. Both the tissues surrounding and the fluid bathing the wound site are important modulators of the wound environment. These matrices are heterogeneous complex mixtures of small molecules as well as large molecules, especially proteins with potentially varying degrees of dynamic ranges reflecting the overall healing status of wounds and their potential to heal or otherwise. As protein and small molecules such as cytokines, growth factors, proteinases, and extracellular matrix components synergistically work to heal wounds slight deviations to any of these may result in prolonged abnormal healing. Discovery of novel

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Abbreviations and Acronyms (*continued*)

PTM = posttranslational modification

Q-TOF = quadrupole time-of-flight

RP = reversed-phase

SCX = strong cation-exchange

SDS-PAGE = sodium dodecyl sulfate poly-acrylamide gel electrophoresis

SILAC = stable isotope labeling with amino acids in cell culture

SpC = spectral counts

TMT = tandem mass tags

protein drug targets, regulators, and markers is crucial to development of novel therapies, clinical diagnostic and prognostic technologies. Hence, the discovery and profiling of the protein complement in wound tissues or fluids at different stages is an essential part of wound healing research.

Among many different technologies used for proteomics analyses, including two-dimensional gel electrophoresis[1,2] and protein- and antibody-based microarrays,[3–5] bottom-up mass spectrometry (MS)–based proteomics has become the most widely used approach for characterization and quantification of proteins present in a biological sample or system such as biological fluids and disease tissues. Based on the review articles that have been published recently and the references therein, MS-based proteomics has become an indispensable analytical tool in many research fields such as breast cancer,[6,7] cardiovascular disease,[8,9] multiple myeloma,[10] and clinical research.[11,12] However, this technology has been vastly underutilized in wound healing research.* The scope of this overview is to briefly introduce bottom-up MS-based proteomic approaches to wound healing research.

DISCUSSION OF METHODS

Fractionation at protein and peptide levels

Due to the complexity and dynamic range of proteins in biological samples including wound tissue and fluid, fractionation is essential to reduce the complexity to maximize the number of proteins returning identification and quantitation in the overall analysis. The two major approaches widely used in proteomics are gel-based and gel-free. Gel-based approaches employ two-dimensional gel-electrophoresis or one-dimensional sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) to fractionate at protein level. In the workflow we outline here, SDS-PAGE is used to separate proteins according to their molecular weight (MW) and the whole lane is excised into equally sized segments. Each segment is treated as a fraction of the original sample. The length that the gel is run and number of segments the lane is excised depend on the complexity of the protein mixture. The proteins in these gel segments are then in-gel digested with a suitable protease (typically with trypsin). The peptides are extracted prior to MS/MS analysis. Each in-gel digestion is acquired in an independent liquid chromatography-tandem mass spectrometry (LC-MS/MS) data acquisition. This SDS-PAGE prefractionation followed by LC-MS/MS technique is known as GeLC-MS or GeLC-MS/MS. Gel-free approaches, on the other hand, employ an initial proteolytic digestion of the complex mixture with fractionation at the peptide level using multidimensional liquid chromatography. Among several multidimensional liquid chromatographic methods,[13] the most extensively used technique separates the complex peptides mixture using strong cation-exchange (SCX) chromatography, and then reversed-phase (RP) LC-MS/MS. The principle behind this is that peptides are first separated on the basis of their charge, and then on the basis of their hydrophobicity. SCX chromatography can be directly coupled to RP chromatography or offline. This technique is known as multidimensional protein identification technology (MudPIT).[14] Gel-based fractionation offers a number of advantages compared to gel-free approach. Gel electrophoresis removes

*For instance, at the time of writing, a literature search with "Proteomics + Mass Spectrometry + Cancer" in ISI Web of Science scientific citation index (www.webofknowledge) returned 3,190 publications for the time period from January 2000 to July 2013, whereas "Proteomics + Mass Spectrometry + Wound Healing" only returned 34 publications.

low MW impurities including detergents and buffer constituents, which are often detrimental to the mass spectrometer and reverse phase columns in the LC-MS/MS system. Both GeLC-MS and MudPIT are in widespread use among the researchers using MS-based proteomics approaches for studies in their respective fields. One major issue common to both gel-based and gel-free approaches is the reproducibility of the identified protein lists for repeat or replicate analysis. The reproducibility can be impacted by each experimental step in the workflow including the mass spectrometer itself. The degree of these contributions to the reproducibility can be, however, minimized by careful experimental design and sample preparation procedures.[15] It is worth noting that reproducibility of the data generated by GeLC-MS/MS approach should be higher because the complexity of the sample is reduced owing to the protein separation by MW on the gel essentially partitioning high and low abundance proteins into different fractions. The gel-free approach on the other hand, results in a greatly increased complexity of the generated peptide mixture not only effectively losing the MW relation information but also allowing high abundance proteins to dominate the available analytical space in both chromatographic dimensions as well. The ultimate goal of both approaches is to maximize the number of protein identifications, which greatly depends on how long the data acquisition is performed by the mass spectrometer. There have been numerous studies using either one or both approaches reported in the past. For a performance comparison of the two approaches, a reader may refer to a publication on proteomics analysis of amniotic fluid[16] and opportunistic pathogen *Burkholderia vietnamiensis*.[17] Mann and co-workers reported three studies employing GeLC-MS to analyze the cerebrospinal fluid,[18] tear fluid,[19] and urine[20] proteomes, each identifying highest number of proteins reported per sample type at the time of publication. Irrespective of pros and cons of GeLC-MS and MudPIT, it should be noted that analyzing the sample with both approaches can yield a greater number of protein identifications than each individual approach.

nanoLC-MS/MS data, database search, and data mining

MS/MS data are more often acquired in data-dependent acquisition (DDA) mode than data-independent acquisition (DIA) mode, dynamically choosing the most abundant precursor ions from the surveyor scan (MS). The faster the scanning speed of the mass spectrometer, the greater the number of MS/MS spectra acquired per second. LC-MS/MS data therefore contain, in addition to the exact masses of the peptides (MS) present in the sample, their product ion spectra (MS/MS or tandem) fragmented along the peptide's backbone, usually by collision-induced dissociation. These product ions are unique to the amino acid sequence of the peptide. Since one LC-MS/MS experiment data file contains several thousands of such spectra, it is much easier and more common to match the measured product ion spectra and peptide masses against a protein database with a search algorithm. Two review articles[21,22] are referenced for more information on commercially and publicly available search algorithms. Peptide sequences interpreted by the algorithm will lead to highly confident identification of the protein.[23,24] Despite the automated nature of searching LC-MS/MS data against database, one should keep a critical attitude toward search results and verify key identifications using the underlying raw data.[25]

With the improvements in proteomics workflows and development of new instruments, the rapid growth of large-scale MS-based proteomics experiments generates colossal amount of data producing longer lists of proteins confidently identified. Most of the proteins found in these lists are housekeeping proteins while only a low percentage is often found to be interesting and clinically relevant proteins such as kinases, phosphatases, and scaffold proteins. To interpret the data correctly and obtain a deeper understanding of the biological systems, novel bioinformatics tools are required to "mine" the proteomics data. In addition to commercially available tools such as GeneGo and Ingenuity Pathway Analysis, as pointed out in a recent article,[26] freely available software tools such as Gene Ontology and Kyoto Encyclopedia of Genes and Genomes are currently widely used to extract biologically relevant information on signaling pathways, biological networks, and protein–protein interactions from these large data sets.

Strategies for increasing the depth in proteomes

Even after the fractionation of the sample, at both protein and peptide level prior to the MS analysis, discovering the proteins of interest may still be challenging. This is mainly due to the dynamic range of concentrations of proteins present in the sample and often the proteins of interest are very low in abundance. Because of the lower dynamic range of detection of the mass spectrometer versus the inherent dynamic range of the sample, these less abundant proteins are often not detected. To overcome this dynamic range issue, over

the last decade, some strategies have been devised. One such strategy is depletion methods to remove high abundant proteins using immunoaffinity columns. Though this has enhanced the detection of proteins it has also resulted in loss of proteins that are bound to these depleted proteins. Another strategy is immunoprecipitation, which is ideal for isolation of protein complexes. A protein and its interacting partners from a complex environment can be isolated using affinity enrichment. The resulting sample is often less complex and provides information about the composition of the interaction network, if all proteins are identified. The GeLC-MS/MS analysis allows an unbiased discovery of almost all the binding partners at once as opposed to immunoblot analysis, which requires a hypothesis about interactors and focuses on the identification of one protein at a time. Because of the high sensitivity of the mass spectrometers, good controls, stringent wash conditions together with careful interpretation of results are required to obtain accurate information from an experiment like this. Another important factor often overlooked is digestion efficiency of the proteases used, which plays a crucial role for the success of every LC-MS/MS experiment. Owing to its very high specificity, relatively greater efficiency, and ability to generate peptides leading to better quality MS/MS data, trypsin is the most widely used protease in bottom-up proteomics. This efficiency can be further improved as revealed by a recent study[27] showing that in-solution digestion of a sample by a combination of Lys-C/Trypsin is more efficient than that by trypsin alone.

Post-translational modifications analysis

Post-translational modifications[28] (PTMs) are chemical modification of amino acid side chains as well as the amine and carboxy termini of the protein generating a large diversity of gene products. These modifications are required for normal cellular function and alterations in the regulation of these modifications can lead to diseases. The analysis of post-translational modifications using MS has a unique advantage since the mass spectrometer directly measures the mass of the modified peptide ion detecting the mass shift due to chemical modification. In a large-scale analysis, LC-MS/MS data need to be searched with the PTM modifications of interest to the user. Two main obstacles persist for analyzing modified peptides from complex mixtures using MS/MS data. First, the stoichiometry of the modified to unmodified protein is often low requiring enrichment strategies to detect and identify them using MS. Various strategies have been reported for enriching peptides containing PTMs such as phosphorylation[29] and glycosylation.[30] Second, PTMs, especially larger modification like glycosylation, can often cause the fragmentation pattern (MS/MS data) to be less informative with regard to peptide sequence, than the unmodified peptides. This makes the modified peptide identification and/or explicit identification of the modification site difficult. For instance, Figure 1 shows the MS/MS data of *N*-glycosylated-tryptic peptide from carcinoembryonic antigen protein marker. MS/MS spectrum is dominated by the neutral losses of carbohydrate moieties from the *N*-glycan without a single sequence specific ion from the peptide itself. Even though the spectrum is rich with information about the composition of glycan, lack of peptide backbone fragment ions places this MS/MS spectrum in the unmatched list on a database search. This is, however, less problematic for smaller modifications such as phosphorylation, glycation, acetylation, and oxidation. Different activation methods[31,32] like electron transfer dissociation and electron capture dissociation are more effective leaving the PTMs with peptide backbone fragment ions, thus increasing the chances of identifying them.

MS-based quantitative proteomics approaches

Bottom-up MS-based proteomics, GeLC-MS/MS and MudPIT, can also be employed to quantify differences in protein expression levels across samples. In large-scale analyses, relative quantification of peptides usually involves either label-free or label-incorporated approaches to discern differences in protein abundances among different biological conditions, and results are usually expressed with an accompanying significance and fold change. In comparison to quantitation using label-incorporated methods, label-free quantitation is inexpensive, applicable to any organism, and does not require special sample preparation. A label-free method is based on spectral count (SpC) analysis.[33] The SpC for a protein is the number of MS/MS spectra assigned to a given protein sequence and represents a sum of the SpC of all the contributing peptides. SpCs correlate to the abundance of that protein and allow it to be compared across multiple samples. Since SpC is not a direct measurement and is only correlated with protein abundance, this strategy is semi-quantitative. The disadvantage of label-free quantification is that variations due to sample handling and/or peptide ionization cannot be accounted for. Some normalization methods were recently evaluated to correct these variations.[34] Label-incorporation methods,[35] on other hand, enable relatively more accurate

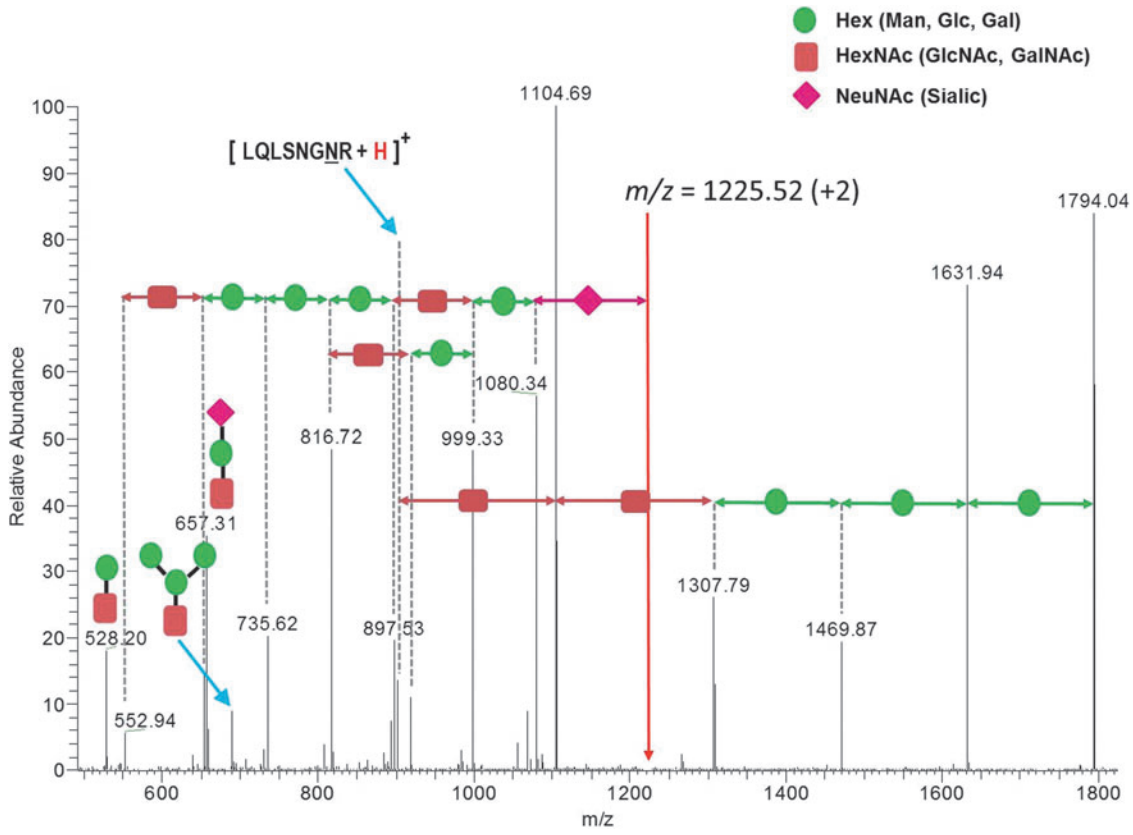

Figure 1. Annotated MS/MS spectrum of *m/z* = 1224.52 (+2) precursor ion corresponding to LQLSNGNR-$(GlcNAc)_2(Man)_3(HexNAc)(Hex)(NeuNAc)$ *N*-glycan from carcinoembryonic antigen (CEA) marker. MS/MS spectrum is dominated by neutral losses of carbohydrate moieties without any sequence-specific ions from the peptide LQLSNGNR (glycan is attached to the underlined Asp N). MS/MS, tandem mass spectrometry; NeuNAc, *N*-acetyl neuraminic acid. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

quantification based on a stable isotope dilution concept. Because a stable isotope-labeled peptide has the same chemical properties as its native counterpart, the two peptides within a mixture should exhibit identical behavior during chromatographic separation and electrospray ionization processes. Isotope-labeled (heavy) peptides and their native (light) peptides can be easily distinguished in a mass spectrometer by their differences in mass, thus enabling accurate peptide, hence protein, quantification. Most commonly used stable isotopes are ^{13}C, ^{15}N, ^{2}H, and ^{18}O and these can be metabolically, enzymatically, or chemically incorporated into proteins or peptides. In label-incorporated methods, samples are combined after the labeling and analyzed by nanoLC-MS/MS, which essentially avoids differences induced by variations in instrument performance between measurements. As a result, in comparison with label-free approaches, quantification precision is markedly improved. Popular label-incorporated methods[36] include stable isotope labeling with amino acids (SILAC), Absolute QUAntification (AQUA), isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantification (iTRAQ), and tandem mass tags (TMT).

DISCUSSION OF RELEVANT LITERATURE

Published wounds-related studies using proteomics approaches

Adaptations of this technology in wounds-related analysis, poses some specific analytical challenges. As discussed in one review,[37] these challenges range from sample collection, to controls, to dynamic range issue of the proteins present in samples originating from wound fluids and tissues. Further, besides the potential bacterial contamination, wound fluids and tissues can also be contaminated with serum or plasma and multiple keratins from skin composition, respectively, making the detection and analysis of less abundant proteins very challenging. Based on the small body of reported studies, proteomics analyses have been performed on both wound fluids and biopsied tissues. One such study[38] reported changes in hemoglobin level with time in tissues from experimentally inflicted skin wounds on rats. Oh *et al.*[39]

evaluated collagen and related proteins present in a line of human skin fibroblasts identifying 80 proteins including isoforms. After depleting the most abundant proteins, MudPIT proteomic analysis was reported[40] to have been performed on acute and chronic wound fluids elucidating low-abundant regulators in wound healing. Identifying a total of 149 proteins, a GeLC-MS/MS proteomic study[41] reported the first comparative proteome analysis of wound exudates from human normal healing and non-healing skin wounds indicating differential distribution of specific proteins among the two different healing phenotypes. In another study,[42] using an enrichment strategy and nano LC-MS/MS, a total of 104 glycoproteins were identified in wound fluids from diabetes mellitus patients. The characterization of the glycosylation site and glycosylation forms from MS/MS data as illustrated in Figure 1 was not part of the objective of this study, however. One interesting fact in these studies is the very low number of protein identifications reported. Given the current capabilities of proteomics approaches outlined here, it can only be suggested that these numbers can dramatically be improved and a great deal of information from wounds-related samples can hence be obtained.

INNOVATION

Recent advances in MS platforms

A typical bottom-up MS-based proteomics workflow is illustrated in Figure 2. This workflow can be applied to samples as simple as protein identification from gel bands, to more complex samples like protein complexes (e.g., immunoprecipitations), and large-scale quantitative protein profiling across samples from different stages of the chronic or normal wound healing process. Mass spectrometers that are hybrid and tandem in space or time are the most critical component of this workflow. Among many different mass spectrometers,[31,32,43,44] two configurations are widely used in nanoLC-MS/MS platforms for discovery proteomics: quadrupole time-of-flight (Q-TOF) and Orbitrap[45] technology-based instruments. These mass spectrometers are often operated in DDA mode. Recently, however, new strategies[46–48] have been developed for their operation in DIA modes to maximize the percentage of peptide identification. The advances made in these instruments, especially over the last few years, include improved sensitivity, higher scanning speed, mass resolution, and mass accuracy. Modern Q-TOF instruments, for instance, now have resolution in excess of 20,000 while that of Orbitrap-based instruments has exceeded 200,000, at $m/z = 400$. These mass spectrometers can produce many more MS and MS/MS spectra per second ($\sim$15–20 Hz) and routinely make high resolution and accuracy measurements without sacrificing robustness, speed, or sensitivity. Mass accuracies in both MS and MS/MS spectra, are in the very low parts-per-million range significantly improving the percentage of peptides that can be identified. Directly coupling these mass

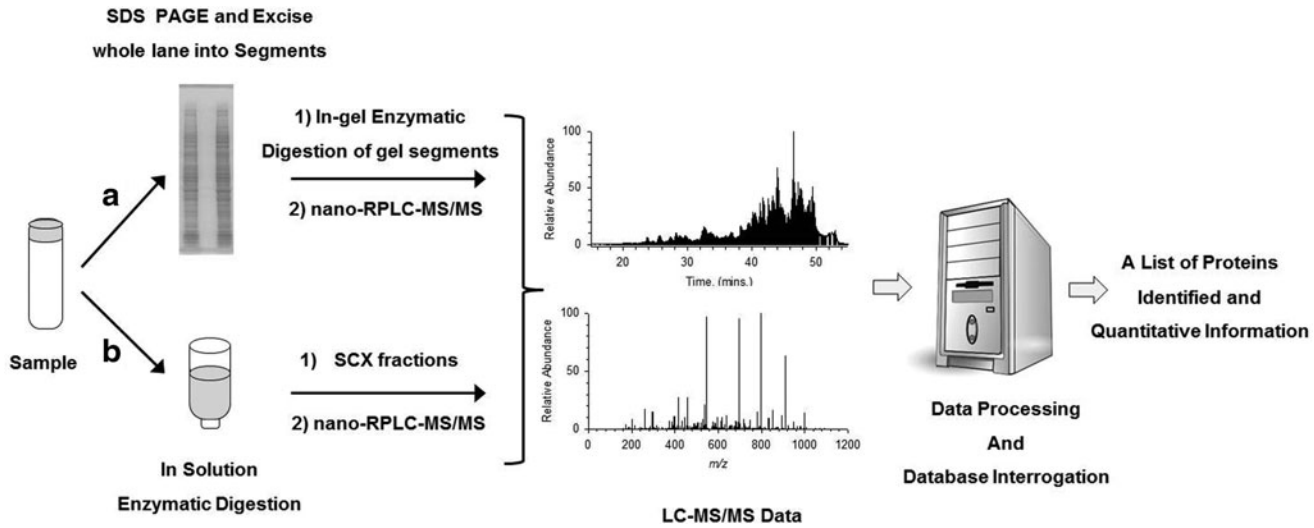

Figure 2. Typical workflow for bottom-up MS-based proteomics experiment. Sample is proteins extracted from biological fluids or tissue. Two approaches: **(a)** Proteins are separated on SDS-PAGE gel in-gel digestion of excised gel segments and rpChromatography (GeLC-MSM type analysis); **(b)** In-solution digestion of proteins, fractionated by SCX and rpChromatography (MudPIT type analysis). MS, mass spectrometry; SCX, strong cation-exchange; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis.

spectrometers to the latest very low and narrow-bore high pressure liquid chromatography via electrospray (nanoLC-MS/MS), complex peptide mixtures can be continuously analyzed as the peptides elute from the RP column. One compelling example for rapid evolution of these instruments and workflows over the last 2–3 years is the study that Mann's group reported[49] in which nearly complete coverage of the yeast proteome was obtained in 4–6 h of data acquisition on a QExactive (Thermo Scientific) mass spectrometer. According to a recent interview,[50] however, it took 3 months for them to generate the yeast proteome map with almost 4,000 proteins using the same procedure with 40 h of data acquisition on a different Orbitrap-based mass spectrometer in 2008.[51]

REMAINING CHALLENGES AND OUTLOOK

Recent advances and improvements of bottom-up MS-based proteomics technology have contributed to faster and better quality of data acquisition. Aimed at increasing protein identifications, certain experimental strategies utilized have moved biomedical research to new horizons. A recent article[52] reported attempts made to cut down starting material and measurement time required for the analysis by eliminating the fractionation steps. Despite these advances, certain limitations, however, still remain to be resolved. Foremost, the dynamic ranges of protein concentrations within certain biological matrices are beyond the intrinsic capability of any current mass spectrometer. Another related issue is under sampling by the mass spectrometer. As a consequence of the nature of DDA, as recently reported,[53] only a fraction of the peptides detectable by a mass spectrometer are in fact selected for MS/MS and hence even lower fraction is identified. Further, the identification of peptides by database-searching algorithms is limited due to alternative splicing and PTMs. If these alternative spliced products are not in the database or if these PTMs are not directly searched for, a considerable percentage of MS/MS spectra will be unidentified. All these give rise to a lower percentage of overlap of identified proteins from repeat analyses of the same or similar biological samples making sample-to-sample comparison more difficult. MS-based quantitative proteomics is making large strides toward a better understanding of biological systems although its full potential is yet to be realized. However, it continues to be an indispensable discovery tool for large-scale analysis of proteins in biomedical research, including wound healing.

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