DRUG DISCOVERY TODAY TARGETS

The continuing evolution of shotgun proteomics

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Shotgun proteomics has emerged as a powerful approach for the analysis of complex protein mixtures, including biofluids, tissues, cells, organelles or protein complexes. Having evolved from the integration of chromatography and mass spectrometry, innovations in sample preparation, multidimensional chromatography, mass spectrometry and proteomic informatics continually facilitate, enable and challenge shotgun proteomics. As a result, shotgun proteomics continues to evolve and enable new areas of biological research, and is beginning to impact human disease diagnosis and therapeutic intervention.

In shotgun sequencing of genomic DNA, fragments of a genome are sequenced and computationally reassembled to determine the genome of an organism. Analogous to this, in shotgun proteomics, complex protein mixtures are digested into peptides, analyzed by tandem mass spectrometry, and computer algorithms then map the peptides onto proteins to determine the original content of the mixture [1]. Shotgun proteomics is a gel-free approach based on multidimensional liquid chromatography separation of complex peptide mixtures coupled to mass spectrometry (MS). In a shotgun proteomics pipeline (Figure 1), a protein population of interest is prepared from a biological source using protein purification strategies including chromatographic or electrophoretic fractionation, or affinity purification. Enzymatic or chemical digestion then takes place to generate a mixture of peptides, which are subsequently separated using multidimensional chromatographic techniques before being introduced into a mass spectrometer. Peptides are then ionized by using electrospray ionization (ESI) [2] or matrix-assisted laser desorption/ionization (MALDI) [3] and analyzed by various mass spectrometers. Using a battery of sophisticated mathematical algorithms, the resultant peptide sequence data generated from tandem mass spectra (MS/MS) are compared against protein databases to identify proteins present in the original population. Effectively, there are four components to a shotgun proteomics approach: sample preparation, multidimensional chromatography, MS and proteomic informatics. This review introduces the components of this proteomics strategy and describes selected recent applications and emerging innovations in the continued evolution of shotgun proteomics.

Shotgun proteomics components

Sample preparation

A wide variety of samples can be analyzed using shotgun proteomics including biofluids, tissues, cells, organelles and protein complexes. In most cases, conventional methods, such as centrifugation of sucrose gradients for isolating organelles, are utilized for sample isolation. However, protein purification strategies have evolved substantially to make them more compatible to direct integration with proteomic approaches. For example, the tandem affinity purification approach is widely used to purify multiprotein complexes [4,5], and strategies involving chemical

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1359-6446/05/$ – see front matter PII: S1359-6446(05)03450-1

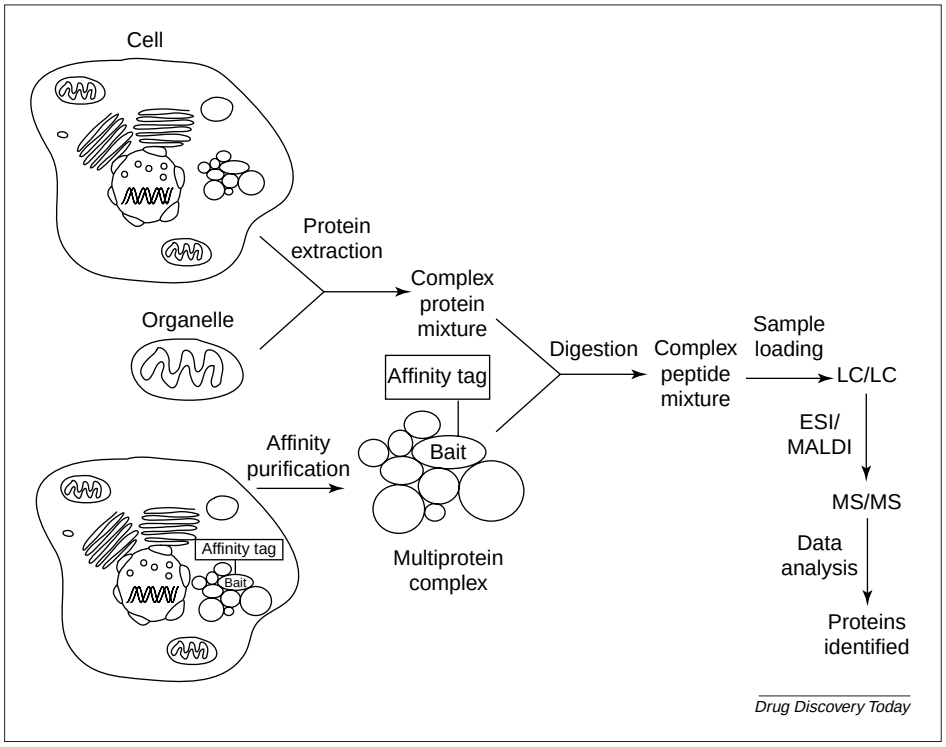

FIGURE 1

Pipeline for a shotgun proteomics experiment. In a shotgun proteomics experiment, proteins are extracted from any given sample and protein mixtures are digested into complex peptide mixtures. The resulting peptide mixtures are directly loaded onto two-dimensional liquid chromatographic systems (LC/LC) that are coupled to electrospray ionization (ESI) sources in a tandem mass spectrometer (MS/MS). With the research and development ongoing into placing LC eluates directly onto matrix-assisted laser desorption ionization (MALDI) plates [63–66], MALDI should soon be a viable alternative to ESI in a shotgun proteomics pipeline. Upon the generation of MS/MS datasets, data analysis with algorithms such as SEQUEST [11] or Mascot [13] facilitate the determination of the protein list of the original sample.

labeling, such as isotope coded affinity tag (ICAT) [6], have been utilized to reduce protein complexity for quantitative proteomics analyses. Because of the great dynamic range of protein abundance and the wide range of protein properties presents in a protein population, it is advantageous to work with purified organelles or other enriched subproteomes. In general, the probability of detecting low abundance components is maximized in less complex protein mixtures. To convert proteins into peptides, trypsin is typically the enzyme of choice because it is an aggressive, stable and cost-effective protease. Endoproteinase Asn-N, which cleaves at the N-terminus of asparagine, endoproteinase Glu-C, which cleaves at the C-terminus of glutamic acid, and proteinase K, which cleaves non-specifically, are examples of additional proteases that can be used as alternatives to trypsin to generate complementary peptides.

Multidimensional separations

When digesting a protein mixture to generate a peptide mixture, the complexity of the sample increases, and the resulting peptide mixtures must be separated in multiple dimensions before MS/MS analysis. The fundamental requirements for ideal multidimensional separation according to J.C. Giddings are that the mechanisms of separation must be orthogonal (i.e. via different chemical or physical properties) and that no resolution gained in the first dimension is lost in any subsequent dimension [7,8]. The ultimate goal is to maximize the peak capacity of a separation system, where peak capacity is defined as the maximum number of peaks that can consecutively stack into accessible separation space with a complete baseline separation between neighboring peaks [9]. Shotgun proteomics platforms can include a combination of chromatographic separation techniques including size exclusion (SE), anion exchange (AE), strong cation exchange (SCX), and reversed-phase (RP) (See Glossary) [9].

Mass spectrometry

MS is a tool to analyze molecules based on their mass-to-charge ratio. There are three main components in a mass spectrometer: an ion source, a mass analyzer and an ion detector. Molecules are generally converted into gas-phase ions by the so-called 'soft' ionization techniques such as ESI and MALDI. ESI is typically used to create ions from molecules in solution by spraying an electrically generated fine mist of ions into the inlet of a mass analyzer at atmospheric pressure [2], whereas MALDI is usually applied to solid phase ionization via short laser pulses [3]. Within a mass analyzer, an electric field or magnetic field is applied to the analytes, which manipulates them according to their mass-to-charge ratio. Finally, ions strike a detector and create a signal that allows for determination of their identities. In shotgun proteomics, a tandem mass spectrometer is required because of its capabilities to isolate ions, fragment them and then measure the mass-to-charge ratio of the fragments. It is in the interpretation of tandem mass spectra where peptides can be identified. There are many types of mass spectrometer that can be coupled to MALDI or ESI to generate tandem mass spectra that can be utilized in shotgun proteomic analysis, including quadrupole ion traps, linear ion traps, time-of-flight/time-of-flight and quadrupole time-of-flight (TOF) instruments (reviewed in [10]).

Proteomic informatics

Development of database search algorithms [11,12] in the 1990s enabled the translation of experimental MS/MS data into corresponding peptides. A typical shotgun proteomics experiment generates between 10 000 and 100 000 individual MS/MS spectra, which are commonly interpreted using search routines such as SEQUEST [11] or Mascot [13]. Both methods involve a comparison of experimental

GLOSSARY

Glossary of chromatographic separation techniques

Size exclusion (SE): peptides or proteins are separated based on their size in solution using non-interactive stationary phases with uniformly sized pores
Anion exchange (AE): peptides or proteins are separated as negatively charged species at high pH interacting with positively charged stationary phases, commonly with amino or ammonium groups, and competing with negatively charged counter ions, such as Cl^- or $HCOO^-$
Strong cation exchange (SCX): peptides or proteins are separated as positively charged species at low pH interacting with negatively charged stationary phases, commonly with phospho or sulfo groups, and competing with positively charged counter ions, such as Na^+, K^+, or NH_4^+
Reversed-phase (RP): peptides or proteins are separated based on hydrophobicity and their interactions with C4, C8 or C18 alkyl chains, for example, on the surface of a stationary phase, and eluted with low to high organic solvent gradients.

MS/MS spectral data with a set of theoretically predicted fragments for all peptides in a database. Validation of peptide hits using chemical properties of peptides and human expert knowledge is often required to gain a higher level of confidence in protein identification, which can be enormously time consuming. As a result, the rapid high-throughput production of shotgun proteomic data necessitated the development of statistical approaches to evaluate [14–17] and assemble [18–20] interpreted MS/MS results into protein identifications from a given sample. There is additional peptide information that can be used to improve identifications from shotgun proteomics analyses, including peptide retention time [21,22] and/or peptide isoelectric point [23,24].

An emerging issue has been how to compare datasets from different MS platforms given the unique features of each. In an attempt to address this issue, an open generic XML (extensible markup language) representation of MS data, named mzXML, was introduced by Pedrioli *et al.* to facilitate data management, interpretation and dissemination in proteomics research using different instrumentation platforms [25]. To harness the full potential of the shotgun proteomics approach, it is essential to develop an efficient and shared algorithm that can be automated and which allows systematic processing, analyses, validation and presentation of mass spectral data. One potential platform to fulfill this need is the open source approach for MS data analysis named the Global Proteome Machine [26,27].

Informatics approaches for the quantitative analysis of complex MS datasets enable novel applications of shotgun proteomics. A quantitative proteomics analysis containing a pairwise sample of isotopomers, such as $^{12}C/^{13}C$-ICAT or $^{14}N/^{15}N$ amino acids, requires informatics tools that convert MS-derived data of peptides into relative protein abundances by calculating the peptide ion current ratios from the MS-derived ion chromatograms [28,29]. In a similar approach, the ion chromatograms of individual samples can be analyzed to determine abundances of peptides in a single run for comparison against additional shotgun proteomics analyses using a common internal standard [30,31]. Differential MS combined with targeted MS/MS analysis of only identified differences might save both computation time and human effort compared with shotgun proteomics approaches.

Shotgun proteomics systems and applications

SCX, RP and MS/MS

Initially, shotgun proteomics was thought of in general terms as a process whereby a protein complex, for example, would be digested into peptides and analyzed via a single dimension RP into an MS/MS ESI system [1]. Not surprisingly, it quickly became clear that an RP column alone lacked the separation to resolve the peptide mixture from a protein complex. This need led to the initial development of a method now named multidimensional protein identification technology (MudPIT; previously called DALPC), in which SCX was coupled to RP/MS/MS using non-volatile salts to identify proteins from *Saccharomyces cerevisiae* ribosomes and a soluble lysate [32]. A series of methodological improvements, including the use of the volatile salt ammonium acetate, led to the development of MudPIT [32–34].

The most commonly applied shotgun proteomics approach incorporates SCX and RP chromatography coupled to MS/MS spectrometry. There are three approaches that have been described in the literature to carry out an SCX/RP/MS/MS analysis of a proteome (Figure 2). SCX can either be run off-line or on-line (Figure 2): off-line meaning that SCX fractions do not directly elute onto RP material, but are instead collected first by a fraction collector (Figure 2a); on-line meaning that SCX fractions are directly eluted onto an RP trap (Figure 2b) or bumped onto the RP portion of a biphasic column (Figure 2c).

MudPIT is a shotgun proteomics approach that incorporates SCX, RP and MS/MS in a fully online fashion (Figure 2c) [32–34]. In this approach, a biphasic microcapillary column is first packed with RP and SCX high performance liquid chromatography (HPLC) grade materials and loaded with a complex peptide mixture generated from a biological sample (Figure 2c). Next, the packed and loaded column is interfaced with a quaternary HPLC pump that acts as the ion source for a tandem mass spectrometer. In the chromatographic step, peptides are directly eluted off of the biphasic microcapillary column, ionized and then analyzed in the tandem mass spectrometer. The resulting MS/MS data from a MudPIT analysis are searched using the SEQUEST algorithm [11]. MudPIT analyses can easily result in thousands of peptide identifications for any given sample. Manually inspecting and validating spectra, as well as organizing the results to determine which proteins were present in the original sample, are labor intensive and daunting tasks. Dedicated computer programs have been developed to identify proteins, present the data and allow comparison of MudPIT data files.

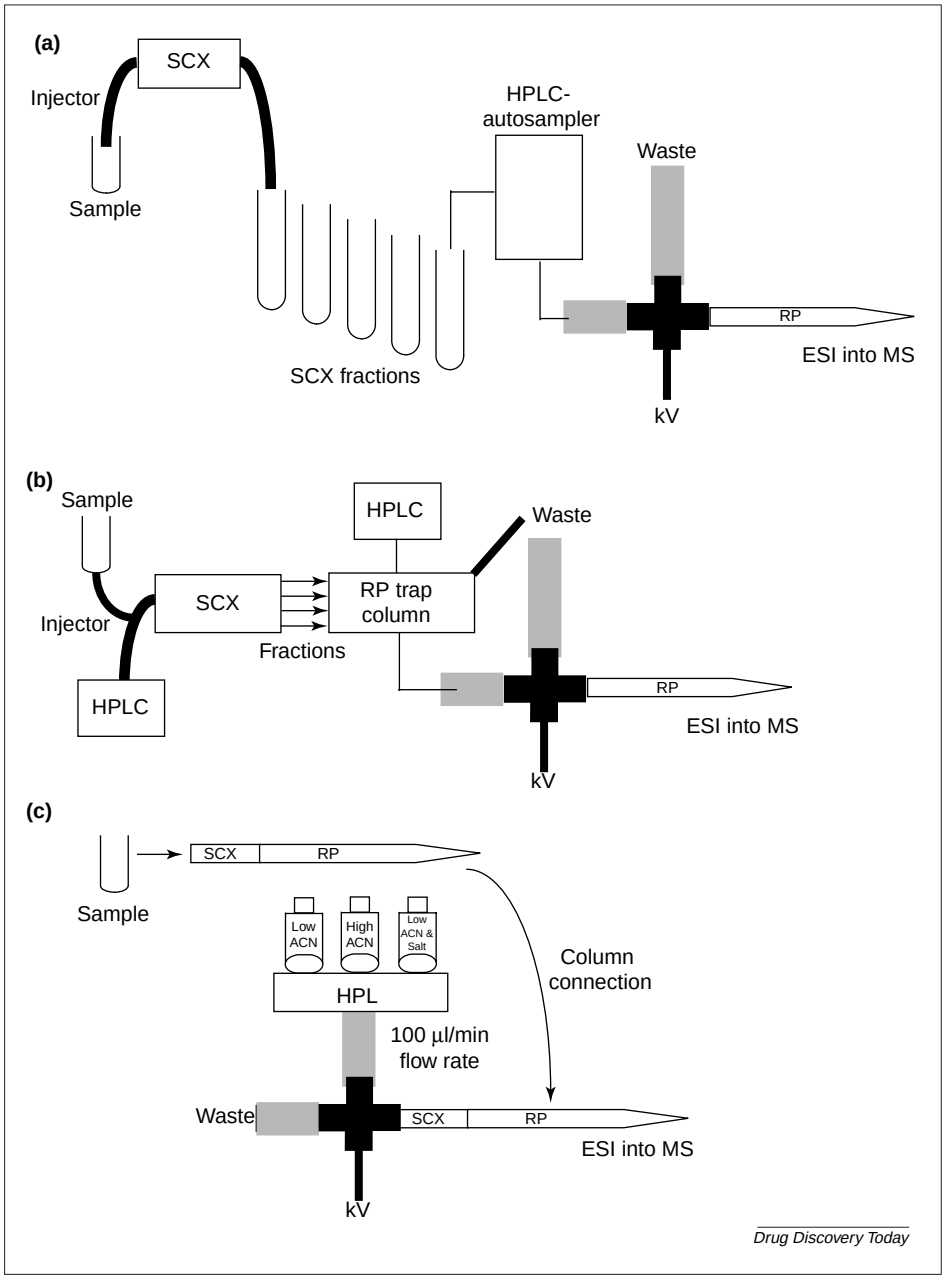

FIGURE 2

Off-line and on-line configurations of a SCX/RP/MS/MS experimental setup. In general, three configurations can be used to carry out an two-dimensional liquid chromatographic MS/MS experiment. In this figure, the example is SCX, RP and MS/MS. **(a)** In an off-line configuration, peptides or proteins can first be purified by SCX chromatography and fractions collected. If proteins are used, the samples must be digested before analysis via RP and MS/MS. Each fraction containing peptides can be loaded via an autosampler onto an ESI interface with a RP microcapillary column. **(b)** In one on-line configuration, peptides can be loaded onto a SCX column and SCX fractions eluted onto a RP trap in an HPLC configuration to wash salts out of the system before RP and MS/MS analysis. In this configuration, potassium chloride can be used to elute peptides from the SCX column. However, there is usually a significant dead volume between the two RP columns, which can affect performance. **(c)** In the MudPIT system, a biphasic microcapillary column with both SCX and RP packing materials is prepared and loaded off-line. Upon insertion of the system, a fully automated analysis can be run with salt bumps moving fractions from the SCX to the RP, and RP gradients eluting peptides into the mass spectrometer. In this approach, a volatile salt must be used, such as ammonium acetate or ammonium formate.

The DTASelect program allows selection criteria to be applied to the determination of peptide matches and provides an output file where data from SEQUEST and DTASelect is displayed in table format and peptide matches are grouped according to their originating protein [18]. The Contrast program allows for the comparison of multiple MudPIT analyses to determine the protein content of multiply related samples [18]. Recent examples of the analysis of proteomes via MudPIT include the analysis of sumoylated proteins from *S. cerevisiae* [35], protein modifications in the Golgi [36], the lung microvascular endothelial cell surface proteome [37], the protein expression profile of hyper- or hypo-contractile hearts from phospholamban mutant mice [38], and the multiprotein transcriptional regulatory complex mediator [39].

In another of the two possible on-line configurations, the experimental setup elutes SCX fractions onto a RP trap before a RP column that elutes into the mass spectrometer (Figure 2b). By using such a set up, researchers are able to use salts incompatible with MS, such as sodium chloride or potassium chloride, to elute peptides off of the SCX column as these salts can be washed away from peptides on the RP trap column [40–42]. As of yet, this type of platform has not been widely utilized in the literature, possibly owing to the complexity of the valve architecture needed to control such a system. A variation of this on-line configuration that does not include a trapping column can be used with MS-compatible salts such as ammonium acetate and ammonium formate. For example, Xiang *et al.* analyzed membrane proteins from two different breast cancer cell lines using on-line SCX, RP and MS/MS, where the SCX and RP columns were physically separated by a valve but ammonium formate was used, negating the need for an RP trapping column [43].

In an off-line configuration, SCX fractions are generated and then each fraction is individually analyzed by RP chromatography into a mass spectrometer [32] (Figure 2a). Using this approach, either proteins or peptides can be separated by SCX; however, if proteins are separated, the fractions must be digested before RP and MS/MS. Because fractions are collected and can be buffer exchanged, sodium chloride or potassium chloride can be used for the SCX fractionation, despite the incompatibility of these salts with mass spectrometers. Recent applications of this approach include analyses of a human blood serum proteome [44], a human

blood plasma proteome [45], a human saliva proteome [46] and a cortical neuron proteome [47]. These efforts are largely for the detection and development of biomarkers for human disease [48,49]. The flexibility of the off-line approach allows for the incorporation of additional separation techniques to undertake, in effect, three-dimensional analyses. For example, Jacobs *et al*. detected and identified 1574 unique proteins from 5838 peptides by separating intact proteins from human mammary epithelial cells using size exclusion chromatography, collecting fractions, digesting proteins, separating peptides by off-line SCX followed by fraction collection, and analyzing each fraction by RP and MS/MS [50]. In a different approach for the analysis of phosphopeptides, protein samples were run on one-dimensional gels, extracted from gel slices, enzymatically digested, and analyzed by off-line SCX and RP/MS/MS; hundreds of phosphorylation sites were identified in a nuclear HeLa cell lysate [51] and 16.5-day-old mice embryo forebrains and midbrains [52]. The main limitation of the off-line SCX, on-line RP/MS/MS approach is the need to transfer each SCX fraction to an autosampler for RP and MS/MS.

Quantitative proteomics

The goal of a quantitative proteomics analysis is to determine the changes in protein expression in a given cell from a given organism when subjected to a stimulus. To achieve this, the mass of proteins from an organism must be modified in one cellular condition so that it is unique from the mass of proteins from another cellular condition. A mixture of 'heavy' and 'light' proteins from each condition can then be analyzed via shotgun proteomics to determine the protein expression profiles of the original samples. Using an approach such as ICAT, protein mixtures are covalently modified after isolation from cells [6]. Subsequently, cells are grown in 'heavy' and 'light' media using an approach such as metabolic labeling [53], thereby differentiating the mass of all proteins. Quantitative proteomic applications commonly use shotgun proteomics strategies incorporating SCX, RP and MS/MS. For example, recent analyses using the ICAT reagent include SCX fractionation followed by avidin-affinity chromatography and RP/MS/MS [54,55]. Complete metabolic labeling for quantitative proteomics analyses coupled to MudPIT has been demonstrated in *S. cerevisiae* [53] and live rats [56], and metabolic labeling with selected amino acids coupled to two-dimensional chromatography and MS/MS spectrometry was used to analyze apoptosis [57].

Alternatives to SCX for shotgun proteomics

Several alternatives to SCX, RP and MS/MS for shotgun proteomics, including coupling liquid chromatography and capillary electrophoresis, have been described (reviewed in [9]). However, head to head comparisons on real samples to determine the advantages and disadvantages of one system over another are rare. An exception to this is the non-chromatographic approach using isoelectric focusing of peptides in the first dimension of a shotgun proteomics analysis [58]. In this analysis, isoelectric focusing was compared with SCX as the first dimension using an off-line configuration; 13% more proteins were identified using narrow range immobilized pH gradients than when using SCX [58]. A potential additional advantage with this approach is the use of the pI of peptides in protein identification [23,24].

In an alternative chromatographic approach to SCX, RP and MS/MS, one could use AE in a similar fashion. In a configuration similar to Figure 2b, but with the SCX column exchanged for an AE column, Mawuenyega *et al*. performed a large-scale protein identification of *Caenorhabditis elegans* and identified 1616 proteins from both soluble and insoluble fractions, including 242 membrane proteins [59]. By using a configuration as shown in Figure 2b, they were able to use sodium chloride to elute peptides from the AE column onto an RP trap before RP and MS/MS using a quadrupole TOF hybrid mass spectrometer [59]. Isobe and colleagues have further demonstrated the utility of the AE, RP and MS/MS platform in an analysis of the *Escherichia coli* proteome, where they detected and identified 1480 proteins [60].

Emerging innovations

Innovations that facilitate sample preparation, multidimensional separations, MS and proteomic informatics for use in shotgun proteomics continually occur. In multidimensional separations, for example, effort is underway to dramatically increase the peak capacity of liquid chromatography by using smaller particles and ultrahigh pressure liquid chromatography (greater than 50 000 psi) [61,62]. This work will permit far more complex peptide mixtures to be analyzed in a given time period with a shotgun proteomics system.

ESI has dominated shotgun proteomics as the method of coupling multidimensional chromatography to MS/MS spectrometry. In theory, there is no reason why MALDI cannot be incorporated into shotgun proteomics, but there have been technical problems with this set up. However, several recent publications have described the deposition of liquid chromatography eluates onto MALDI plates [63–66]. Indeed, Pan *et al*. recently described the use of a system for biomarker detection in which a serum isolate was separated by RP, fractionated directly onto a MALDI plate, and analyzed via MALDI/TOF–TOF MS [67]. An additional MS innovation for shotgun proteomics is the recent development of a linear quadrupole ion trap fourier transform mass spectrometer (FTMS) [68]. This instrument couples ion trap technology, which is the instrument used in all MudPIT publications, to the accurate mass and high resolution capabilities of a FTMS. The ability to accumulate ions via trap technology and then accurately distinguish small differences in mass will permit improved proteomic informatics.

Shotgun proteomics and drug discovery

Because of the only recent emergence of shotgun proteomics, there are relatively few descriptions of its use in human therapeutic intervention and diagnostics. The application of shotgun proteomics to the analysis of biofluids with the aim of discovering novel biomarkers of human disease is of particular interest for drug discovery [44–47]. In vaccine development, the analysis of the surface proteins of infectious agents can be analyzed by shotgun proteomics to provide potential antigens. For example, a MudPIT analysis of the proteome of the causative agent of human malaria, *Plasmodium falciparum* [69], provided a large number of potential vaccine candidates, the antigenicity of which has been evaluated [70]. Shotgun proteomics has yet to be described in the literature in a drug target identification study. However, this will probably occur in the near future, as a more classical approach – two-dimensional gel electrophoresis – was used to successfully identify methionine aminopeptidases as a molecular target of bengamides, a class of tumor growth inhibitory agents [71]. As shotgun proteomic technology is further adapted by scientists in academia and industry, novel applications for this technology in drug discovery and human disease therapeutic intervention will emerge.

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