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Toward improvement of screening through mass spectrometry-based proteomics: ovarian cancer as a case study

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Abstract

Ovarian cancer is one of the leading causes of cancer related deaths affecting United States women. Early-stage detection of ovarian cancer has been linked to increased survival, however, current screening methods, such as biomarker testing, have proven to be ineffective in doing so. Therefore, further developments are necessary to be able to achieve positive patient prognosis. Ongoing efforts are being made in biomarker discovery towards clinical applications in screening for early-stage ovarian cancer. In this perspective, we discuss and provide examples for several workflows employing mass spectrometry-based proteomics towards protein biomarker discovery and characterization in the context of ovarian cancer; workflows include protein identification and characterization as well as intact protein profiling. We also discuss the opportunities to merge these workflows for a multiplexed approach for biomarkers. Lastly, we provide our insight as to future developments that may serve to enhance biomarker discovery workflows while also considering translational potential.

Introduction

Ovarian cancer is one of the leading causes of cancer deaths affecting women in the United States. Its relatively low five-year survival rate of 48.6% can be attributed to the fact that nearly two-thirds of women are not diagnosed until the cancer has metastasized to other areas of the body[1]. However, early detection of ovarian cancer has been shown to increase the five-year survival rate to over 90%[1]. Therefore, reliable methods of early stage ovarian cancer screening are of the utmost importance in positive patient prognosis.

Several screening methods currently exist for ovarian cancer, with one of the most common methods being biomarker testing[2]. Since the discovery of increased levels of carcinoembryonic antigen (CEA) in ovarian cancer patients, a variety of ovarian cancer biomarkers have been identified and approved for clinical diagnostic usage by the United States Food and Drug Administration (FDA)[3]. These biomarkers include single biomarkers, such as cancer antigen 125 (CA125) and human epididymis protein 4 (HE4), and more recently, biomarker panels such as OVA1® and Overa®[4–8].

Screening using the aforementioned biomarkers has been unable to reach desired levels for both sensitivity and specificity and require orthogonal screening methods and/or physician assessment. Dochez *et al.* compiled a subset of analyses to compare the diagnostic performance of CA125, HE4, and CA125 + HE4. Benchmarks showed sensitivity ranging from 58%–97% and specificity ranging from 53%–98%; high sensitivity was often accompanied by low specificity or vice versa[9–26]. OVA1® (Vermillion Inc), an FDA approved multiplexed index assay using CA125, transferrin, beta-2-microglobulin, transthyretin, and apolipoprotein A1 biomarkers, was developed as a new screening tool to be used in conjunction with physician assessment. Despite showing promising sensitivity of 82%–100% (dependent on the demographic tested), the assay was plagued by low specificity ranging from 26%–43%[6,27]. Later, a second generation multiplexed index assay was developed under the name Overa®, which replaced beta-2-microglobulin and transthyretin with HE4 and follicle stimulating hormone. With a sensitivity of 90%–96% and specificity of 47%–71%, there were notable improvements[8,28]. However, these tests require higher specificity to see broad, routine implementation for general women’s health screening purposes. These results have even led to news outlets, scientific authorities, and the FDA to caution against excessive use of these tests due to their penchant for false positives[29].

In recent years, efforts have been made to use various “-omics” technologies to identify novel, reliable ovarian cancer biomarkers to be used for early stage ovarian cancer screening purposes from DNA, RNA, proteins, and metabolites[30]. In the case of proteins and metabolites, mass spectrometry has proven to be an invaluable tool towards these efforts. Mass spectrometers are more readily available in clinical laboratories thanks to the growing number of *in vitro* diagnostic (IVD) assays. Here, we present an overview of different proteomics approaches compatible with mass spectrometry that have been developed over the years for ovarian cancer.

Proteomics Workflows

Instrumentation and workflows for mass spectrometry-based proteomics oftentimes fall into one of two categories: tandem mass spectrometry (MS/MS) based proteomics and intact protein profiling (also commonly referred to as protein fingerprinting and not to be confused with peptide mass fingerprinting). Both workflows can be applied for research use only (RUO) as well as clinical IVD applications. RUO workflows are often focused on biomarker discovery, involving protein identification followed by subsequent characterization to provide insight into protein structure, biological effects, and mechanism of action. Conversely, IVD workflows allow for accessible routine screening based on biomarker assays that have been developed, validated, and quality controlled for a given disease. Figure 1 provides an overview of workflows and usage, and Table 1 outlines several differences between the two. Swiatly *et al.* have written in-depth reviews on recent mass spectrometry-based proteomics and intact protein profiling approaches in the context of ovarian cancer[31]. Although matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) imaging mass spectrometry is not in the scope of this perspective, Kriegsmann *et al.* have written an excellent review regarding its use in the context of ovarian cancer[32].

MS/MS Based Proteomics

As the name implies, MS/MS based proteomics has traditionally involved the use of a separation method followed by MS/MS. This workflow can be further categorized into bottom-up or top-down approaches. Wither *et al.* and Donnelly *et al.* provide a more detailed overview of sample preparation and instrumentation used in bottom-up and top-down proteomics, respectively[33,34].

Bottom-up proteomics involves the use of digestion enzymes (i.e. trypsin or Lys-C) to digest proteins into peptides for MS/MS analysis. Peptides can be separated via offline (i.e. gel electrophoresis) or online (i.e. high performance liquid chromatography, HPLC; ultra-high performance liquid chromatography, UPLC; ion mobility) methods prior to detection via MS/MS or MSⁿ [35,36]. Common mass spectrometers used in bottom-up proteomics include 3D/linear ion traps, TOF/TOF, orbitrap, hybrid quadrupole-TOF (qTOF), and hybrid quadrupole-orbitrap (Q-Exactive) mass spectrometers. Following MS analysis, peptide sequences can then be queried against protein databases (i.e. MASCOT, UniProt) for peptide annotation[37,38]. Tyanova *et al.* provides a detailed protocol for protein annotation in MaxQuant using custom databases (including those found on UniProt)[39]. As an example, Kacirova *et al.* used this workflow to identify cadherin-1 (CDH1), vitronectin (VTN), and basement membrane specific-heparan sulphate proteoglycan core protein (HSPG2) as downregulated proteins in the pathogenesis of endometrial cancer[40]. Malaker *et al.* also used StcE, a mucin-selective bacterial protease derived from *Escherichia coli*, to further characterize the structure and function of mucins, which are known to be implicated in various cancers[41]. For example, mucin 16 (MUC16; aka CA125), is a well known protein associated with ovarian cancer. Protocols and instrumentation for this method of proteomics are very mature; however, information about proteoforms and post-translational modifications are not always attainable[42,43].

Top-down proteomics, on the other hand, involves the analysis of intact proteins, which addresses some of the limitations of bottom-up proteomics by allowing the determination of complete protein sequences and post-translational modifications. Separation methods are similar to those used in bottom-up proteomics, but unlike bottom-up proteomics and intact protein profiling, top-down proteomics workflows use mass spectrometers capable of analyzing high molecular weight proteins (tens or hundreds of kilodaltons) such as MALDI-TOF, Fourier-transform ion cyclotron resonance (FT-ICR), or hybrid FT-ICR mass spectrometers. This workflow also uses MS/MS or MSⁿ analysis followed by protein annotation via database searches. Ntai *et al.* were able to use immunoaffinity enrichment coupled to top-down proteomics on a Q-Exactive mass spectrometer to detect and quantify mutation specific post-translational modifications in the protein KRAS4b, which have been found to promote cancer progression in human cancers[44]. Delcourt *et al.* also used MALDI-TOF imaging mass spectrometry (IMS) followed by nanoLC-MS/MS on a Q-Exactive mass spectrometer to identify 11 putative biomarkers derived from the serous ovarian cancer microenvironment[45]. Advances in instrumentation and development of novel techniques in recent years have even allowed for analysis of intact protein complexes via native proteomics[46–51]. However, separation of intact proteins has been shown to be more difficult than peptide separation[52–61].

Given sufficient biological and/or technical replicates, quantitative proteomics can be achieved using label-free quantification, which can be performed using software such as Proteome Discoverer (Thermo, Waltham, MA) or MaxQuant[39,62,63]. Alternatively, labeling methods such as the addition of tandem mass tags (TMTs) or isobaric tags for relative and absolute quantitation (iTRAQ) can be leveraged for quantitative proteomics[64,65]. Zhang *et al.* identified four differentially expressed proteins from epithelial ovarian cancer exosomes using TMTs, while Swiatly *et al.* identified five proteins using iTRAQ-tagging coupled to mass spectrometry from patient serum that were incorporated into existing diagnostic models to improve performance[66,67]. Relative and/or absolute quantitation can reveal differentially expressed proteins across disease states, allowing for the identification of putative biomarkers. From there, validation via immunohistochemistry and functional characterization of these putative biomarker proteins can provide the function of the protein in the context of the given disease, in this case ovarian cancer[68].

This workflow provides a wealth of information that can lead to an understanding of the biomarker(s) identified and studied. However, proteomics experiments often require a significant amount of sample preparation that varies depending on available equipment and instrumentation. For example, protein digestion in bottom-up proteomics may involve the use of materials that may be incompatible with LC-MS/MS, such as phosphate buffered saline (PBS) or sodium dodecyl sulfate (SDS), requiring additional cleanup steps for removal. Additionally, in a typical proteomics experiment using LC-MS/MS, each single run can take several minutes to hours. Quantitative experiments will also require multiple biological and/or technical replicates. Fractionation of samples, although not required, has also been reported to increase proteome coverage, and may be necessary if the biological sample used was not a rich source of proteins[52–61] can exponentially increase the run time for a single sample[52–61]. Previous studies have shown that sample pooling can be used to alleviate these run times, but caveats associated with doing so must be taken into consideration, as evidenced by Diz *et al.* and Oberg *et al.*[69,70]

While MS/MS based proteomics and metabolomics is more commonly used in RUO biomarker discovery applications, targeted IVD assays have also been developed due to the increasing presence of mass spectrometers in clinical laboratories in recent years[71,72]. For example, triple quadrupole (QQQ) and qTOF based LC-MS/MS analysis can be used for small molecule/peptide monitoring and quantitation. Macklin *et al.* have reviewed recent developments in clinical proteomics and examples of their usage[73]. Traditionally, enzyme-linked immunosorbent assays (ELISA) and biosensors have been used to detect protein biomarkers from clinical samples (i.e. blood, serum, tissue)[74,75]. However, mass spectrometry offers several large advantages over ELISA: higher throughput capabilities, broad applicability towards a large number of biomarkers and assays since compatible antibodies are not required, and the ability to distinguish between compounds that are indistinguishable via immunoassays such as proteoforms. Volmer *et al.* have noted that the latter capability applies to vitamin D; mass spectrometry is able to distinguish between different vitamin D compounds, whereas immunoassays lack that specificity[76].

Intact Protein Profiling

Intact protein profiling is relatively newer compared to LC-MS/MS based proteomics experiments, *vide supra*. Profiling is most frequently accomplished using MALDI-TOF mass spectrometry. In the clinic, it comes in the form of benchtop instruments such as the BioTyper (Bruker Daltonics) and VITEK (bioMérieux). MALDI-TOF mass spectrometers are used to obtain a profile of a given biological sample, generally in the microprotein (≤ 30 kDa) range. Microproteins are ideal for profiling due to (1) the ease of ionization and subsequent detection compared to larger proteins and (2) the biological importance many microproteins exhibit; in microbiology, these are typically 16S rRNA proteins which in high copy number and have been used to identify microbes to the genus and species level[77–79]. With enough replicates for a given disease state, the profiles are inherently multidimensional and can be mined to select multiple putative biomarkers rather than a single biomarker. These putative biomarkers can then be used to develop models, databases, or scoring algorithms for IVDs to classify the measured profiles (i.e. benign or malignant in the case of tumors)[80–82]. Importantly, one of the main advantages to MALDI-TOF MS intact protein profiling is its high throughput capability (data acquisition for each biological sample only takes several seconds), which allows for large amounts of data to be generated in short order. Therefore, it is an extremely useful technique for data curation.

While MALDI-based profiling has shown promise in the clinic, it is also important to understand the current limitations of the technology. For example, although the microprotein range can be profiled relatively easily, detection of higher mass proteins proves to be more difficult, requiring specialized detectors[83]. Additionally, sample preparation is vitally important and can be the determining factor in accuracy, precision, and reproducibility when profiling for several reasons. Processing protocols for clinical samples need to ensure they are compatible with MALDI-TOF MS, as samples with incompatible materials (i.e. blood) may result in ion suppression and affect screening accuracy[84–86]. Another important factor in sample preparation is matrix selection and application. Like sample processing, matrix selection can easily be standardized once an ideal matrix has been chosen; matrix application, on the other hand, can be prone to variation. Figure 2 shows how differences in matrix application to the same sample can affect reproducibility, which in turn affects downstream statistical analysis.

Yet another consideration is that MALDI profiling only yields a putative mass-to-charge ratio, with limited information on identity. Therefore, for the purposes of biomarker discovery, intact protein profiling is often used prior to or in conjunction with LC-MS/MS based protein identification in order to generate biomarker leads. Lastly, early protein profiling studies using MALDI-TOF and surface-enhanced laser desorption/ionization TOF (SELDI-TOF) MS introduced doubts to the reproducibility of the technique; however, more recent studies have shown that intact protein profiling is precise enough for classification purposes when standard operating procedures (SOPs) are in place[77,87–97].

Despite the caveats mentioned above, a myriad of research and clinical studies have demonstrated that intact protein profiling has great potential as a widely available screening and diagnostic tool. Profiling is already used in the clinic for identification of pathogenic microorganisms via an FDA approved protocol. In a study by Wilson *et al.* the BioTyper

(Bruker Daltonics) was able to identify 98.4% of bacterial and yeast isolates, with 95.7% of those identification being of high confidence, proving that MALDI-based profiling in this mass range is a viable, accurate method of classification once the initial database and protocol have been developed and optimized[98]. Another particularly promising example is Mass-Fix[99,100]. Mass-Fix is an assay with accompanying software developed using MALDI-TOF MS designed to detect and quantify monoclonal proteins in plasma cell dyscrasias patients in a high throughput manner; a single technician was able to process hundreds of patient samples in an eight-hour shift. In collaboration with clinical colleagues, it has been used to screen thousands of patients and shows promise as a detection method for M-proteins[101,102]. Furthermore, availability of these assays as IVDs results in the collection of extremely large datasets; retrospective analysis of these intact protein profiling datasets can be used to further characterize putative biomarkers. Mellors *et al.* used the Mass-Fix assay to identify a post-translational modification in monoclonal proteins by showing that a small percentage of Mass-Fix positive patients displayed light chain N-glycosylation[103]. Eighteen other examples of studies using protein profiling as a diagnostic tool for ovarian cancer can be found in a meta-analysis performed by Li *et al.*[104]. Here, it was shown that based on this subset of studies, the pooled sensitivity and specificity were 77% and 72%, respectively, highlighting the profiling approach holds promise as a diagnostic tool but requires continued development for real world translation in ovarian cancer.

Strategies and Challenges in Merging Identification and Characterization with Profiling Workflows

While protein identification and characterization and intact protein profiling both have their respective strengths and weaknesses, both workflows are not mutually exclusive. As alluded to above, these two workflows are often used as orthogonal methods to provide a wealth of information on disease states. In doing so, comprehensive datasets containing information, such as mechanism of action and progression of disease, and protocols can be generated that pave the way for biomarkers to break into the clinic.

With differentially regulated peaks that are consistently detected and identified by intact protein profiling, further exploration can lead to the identification and characterization of key protein biomarkers. Timms *et al.* also used this approach when identifying potential diagnostic MALDI-TOF MS peaks that could be combined with CA125 testing to more effectively detect early stage ovarian cancer[81]. Here, patient serum samples were profiled to detect differentially regulated protein peaks, leading to the identification of connective tissue-activating peptide III (CTAPIII) and platelet factor 4 (PE4) using MS/MS on digested peptides and literature searches, respectively. The combination of diagnostic peaks for CTAPIII and PE4 with CA125 allowed for classification of cases 11–15 months earlier than with CA125 alone. Interestingly, CTAPIII had been one of the biomarkers originally considered during OVA1® development, but was presumably dropped due to lack of immunoassay availability[105]. As previously mentioned above, this highlights the utility of mass spectrometry in the clinic as it allows for the development of assays that may otherwise be unavailable via immunoassays alone.

The main concern when following a profiling and identification workflow is to ensure that: (1) samples are properly handled or aliquoted since sample preparation steps for intact protein profiling and protein identification are not always compatible, and (2) there is enough sample to perform both workflows. The first concern simply requires proper experimental planning and design. The latter concern, however, may be harder to address. For example, clinical studies may result in less than ideal sample quantities, and it may not always be feasible to acquire more patient-derived samples. Therefore, it is important to consider these practical limitations when attempting to take advantage of multiple orthogonal discovery workflows.

Conversely, protein biomarkers identified through LC-MS/MS based proteomics can be used to inform classification models, databases, and scoring algorithms. Here, the use of intact protein profiling is pivoted from a discovery role into a detection role. Normally, distinguishing peaks of interest picked from thousands to tens of thousands of signals in an untargeted manner are used to differentiate between conditions. However, it is possible to interrogate the data in a targeted manner from a panel of proteins identified through other proteomics experiments and literature searches, allowing MALDI-TOF MS to act as a means of detection as opposed to a means of discovery. Hernandez *et al.* used this approach by taking 19 proteins with known relation to ovarian and breast cancer to inform a classification model for data collected via MALDI-TOF imaging mass spectrometry[106].

However, the use of intact protein profiling as a detection strategy is dependent on the ability of MALDI-TOF MS to detect these proteins; if these peaks are not detectable via MALDI-TOF MS, they cannot be reliably used in intact protein profiling for screening purposes. Furthermore, it must be known whether the chosen panel of proteins is up or downregulated in diseased vs healthy conditions and to what degree. While some biomarkers show similar expression across multiple cancers (i.e. BRCA1/2 downregulation in ovarian and breast cancer), others can display differential expression in different cancers (i.e. cystatin A)[107–109]. Although quantitation has traditionally been performed using LC-MS/MS workflows, Källback *et al.* has shown MALDI-TOF and MALDI-FT-ICR MS can provide comparable results[110]. With that being said, given these criteria are met, panels can be compiled from identified potential biomarkers and narrowed while optimizing data acquisition parameters to ensure high quality data can be collected on these select peaks.

The Future of Biomarker Discovery and Clinical Screening

As the use of mass spectrometry-based proteomics continues to increase and knowledge on biomarkers continues to grow, it is important to consider the ultimate goals of each study regardless of the workflow(s) used. Discovery workflows continue to benefit from advances in instrumentation. Technical improvements such as the emergence of dual source instruments show promise by allowing labs to perform both LC-MS/MS based proteomics and MALDI-TOF MS based intact protein profiling on single instruments as opposed to purchasing two separate instruments. Considerations must also be given as to whether a study can be translated to the clinic, and if so, how that may be done. Unfortunately, clinical implementation of mass spectrometry-based instrumentation has been slow, though great strides have been made in recent years. Assays must be developed, validated, and

in certain cases approved by regulating bodies (i.e. FDA) in order to be used in the clinic, and proper instrumentation to perform these assays must be available. However, availability of mass spectrometers in clinical laboratories is limited due to the high amounts of capital required for their acquisition. Genzen has noted that the cost associated with analytical instruments causes clinical laboratories to favor those that are compatible with a large number of their laboratory developed tests (LDTs), which could number in the dozens to hundreds[111]. This results in instruments with more niche uses, such as mass spectrometers, having lower purchasing priority compared to other more commonly used instruments. At present, MALDI-TOF MS based intact protein profiling has three main barriers to mainstream adoption as a screening method for early stage ovarian cancer: (1) a lack of standardized sample preparation protocols, (2) low performance instrumentation in comparison to their research laboratory counterparts, and (3) obtaining regulatory approval. Before standardized sample preparation protocols can be developed, easily accessible methods of sample collection must first be standardized. Costas *et al.* have previously reviewed potential sample collection methodologies to be adopted for screening purposes in the context of early-stage ovarian cancer[112]. Boylan *et al.* have used LC-MS/MS based bottom-up proteomics to compare one of these methodologies, Pap tests, to more traditional sample collection methods (i.e. tissue collection) in a patient diagnosed with high grade serous ovarian cancer.[113] Here, 4934 proteins were identified from tumor tissue, Pap test fluid, or cervical swabs, with 2293 proteins being found in samples from all three collection methods (i.e. CA125, HE4, leucine rich alpha 2 glycoprotein). While MALDI-TOF MS can be found in clinical laboratory settings, compared to RUO instruments, clinical laboratory based instrumentation is often less advanced; features such as increased mass resolving power or tandem mass spectrometry capabilities via TOF/TOF mass analyzers are absent. But despite the basic feature set in clinical MALDI-TOF MS, previous efforts in intact protein profiling (i.e. clinical microbial identification, Mass-Fix) has shown that they are more than capable. Currently, clinical laboratory instrumentation can only be used for applications that have obtained regulatory approval. Interestingly, proposed legislation such as the Verifying Accurate Leading-edge IVCT Development (VALID) Act of 2020/2021 could change the way IVD assays are regulated, which could allow mass spectrometry-based tests and assays to be more easily approved. Genzen and Johnson and Marchant have discussed the VALID Act in more detail and its implications in the future of *in vitro* clinical tests[111][114]. Lastly, as evidenced by Mass-Fix, significant amounts of collaboration between academic and clinical institutions is necessary for the development of clinical assays utilizing MALDI-TOF protein profiling. It also had the benefit of a large serum database from which biological material could be sourced to build a spectral database for analysis. Unfortunately, ovarian cancer currently lacks the large collaborations and resources that were available for the development of Mass-Fix, and reliable identification and detection of early stage biomarkers has not been established. With that being said, Mass-Fix has demonstrated a promising example of a pipeline for mass spectrometry-based screening. Therefore, we believe that with the establishment of standardized sample collection methods and discovery of reliable biomarkers, a similar pipeline can be developed for ovarian cancer with a large, diverse sample size that can be used to develop spectral databases and appropriate collaborations in place. Overall, we have

seen exciting developments in mass spectrometry-based biomarker discovery and screening in recent years, and we await further discoveries in the coming years.

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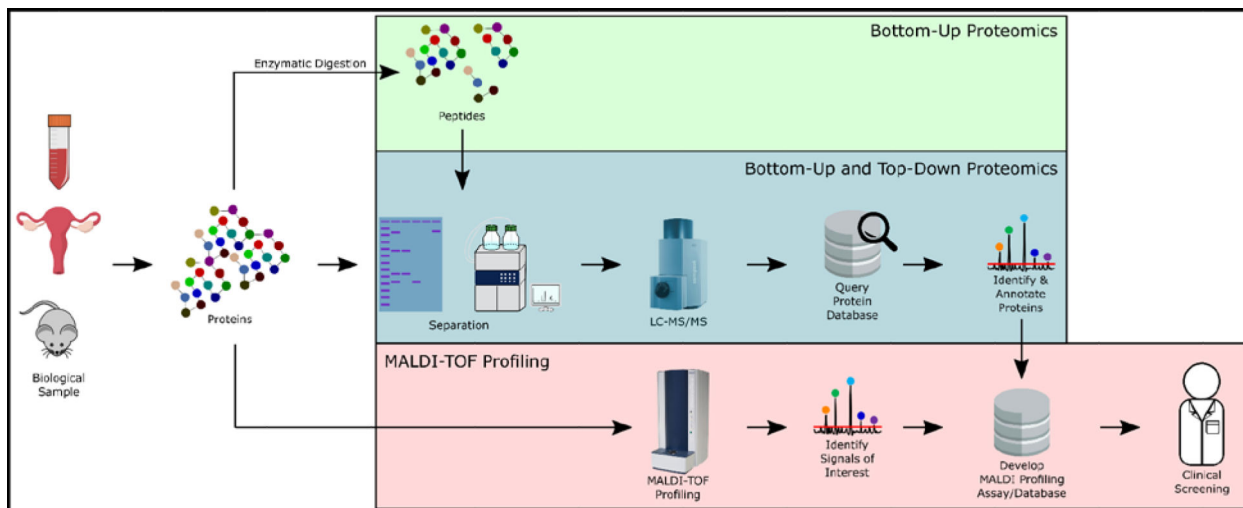


Figure 1: Workflow from the bench to the clinic. Proteins can be extracted from fluid or tissue samples. Under the protein identification/characterization workflow, researchers have the option of digesting proteins into peptides using various methods prior to separation and MS/MS (bottom-up proteomics; green box). Otherwise, intact proteins can be maintained (top-down proteomics; blue box). In bottom-up and top-down proteomics, separation can be performed using gel electrophoresis or liquid chromatography, and is followed by collection MS/MS spectra. These spectra can be queried against peptide sequencing databases to ascertain their identities (blue box). To use this information for clinical screening purposes, development of a means of detection is still required. Under the intact protein profiling workflow (red), researchers collect protein profiles from intact proteins using MALDI-TOF mass spectrometers. Signals of interest can be putatively identified from these spectra and a database can then be compiled using protein profiles from different disease states, which can then be used in clinical settings to screen patient samples (i.e. benign or malignant).

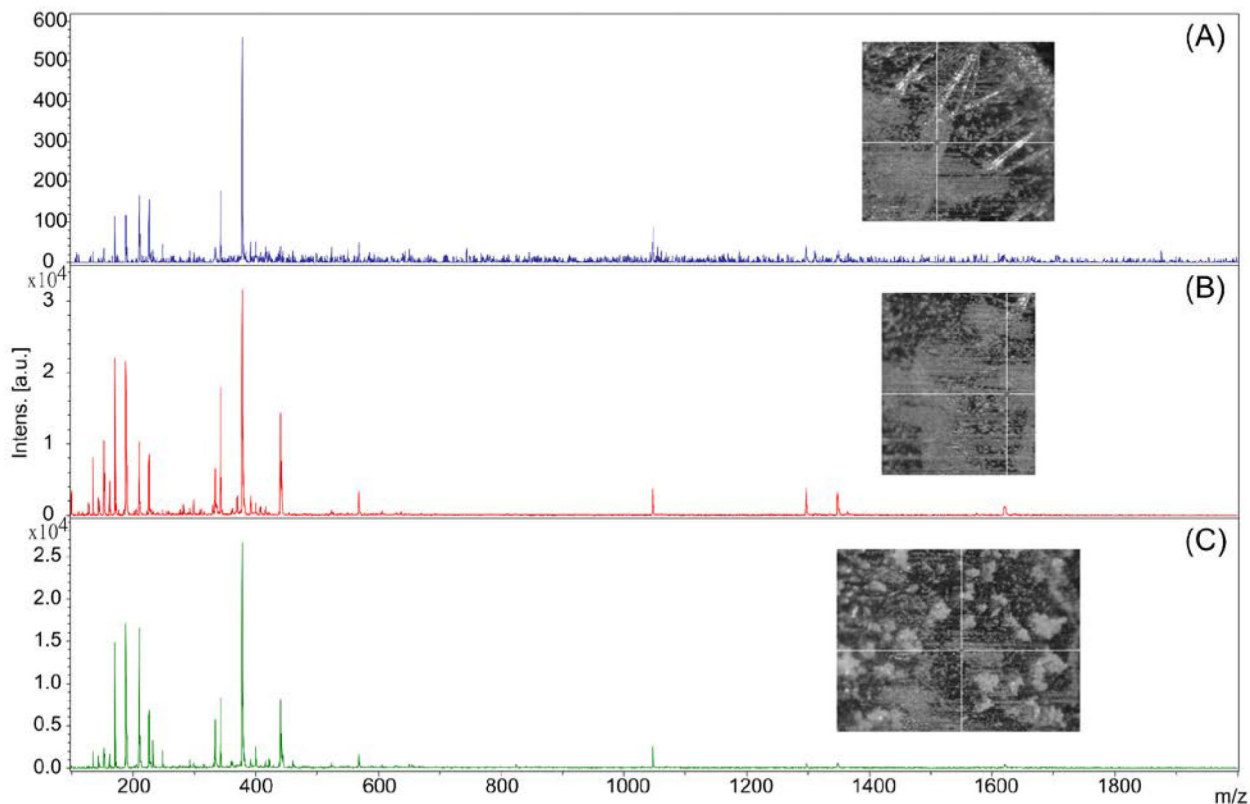


Figure 2:

Example of how differences in matrix application and crystallization can affect MALDI-TOF mass spectra. Three spots of Peptide Calibration Standard (Bruker) were co-crystallized with 50:50 alpha-cyano-4-hydroxy-cinnamic acid: 2,5-dihydroxybenzoic acid in 70:30 acetonitrile:MilliQ water w/ 0.1% trifluoroacetic acid. All spectra were acquired with the same calibrated method (Laser Power: 15%; Detector Gain: 6.2x; Shots: 200; Frequency: 60Hz; Mass Range: 100–2000 Da). (A) The first spot resulted in co-crystallization that produced large, slender crystals. These crystals resulted in weaker ionization producing low intensity spectra. (B) The second spot had much smoother co-crystallization. This resulted in higher intensity ionization across our entire mass range. (C) The third spot produced large, rounder crystals. Smaller mass peaks (< 300 Da) were able to ionize well, but larger mass peaks (> 1200 Da) were not able to ionize well.

Table 1:

Comparison of MS/MS based proteomics and MALDI-TOF protein profiling workflows.

MS/MS Based Proteomics	MALDI-TOF Protein Profiling
Allows for analysis of intact proteins or peptides	Used for analysis of intact proteins
Requires more extensive sample preparation/cleanup	Minimal sample preparation required
Often coupled to online or offline separation/fractionation method	Separation/fractionation is not required
Allows for identification and characterization of proteins/peptides	Only provides putative mass value
Can perform absolute and/or relative quantitation	Can only perform relative quantitation
Sample preparation, cleanup, and separation/fractionation result in lower throughput workflow	Minimal sample preparation results in higher throughput analysis

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