Impact of Mass Spectrometry-Based Technologies and Strategies on Chemoproteomics as a Tool for Drug Discovery

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ABSTRACT: Chemoproteomics is an invaluable tool to discover protein targets from phenotypic assays and to understand on- and off-target engagement of potential therapeutic compounds. Highlighted in this technology perspective is our view on how improvements in mass spectrometry (MS)-based proteomics technology have dramatically impacted chemoproteomics. Improvements in sample preparation, MS instrumentation, data acquisition, and quantification strategies have enabled medicinal chemists, chemical biologists, and mass spectrometrists to develop new chemoproteomic experiments and improve existing methods. As a result of improvements in MS, we will detail how bead-based affinity capture and activity-based proteome profiling methods have been reduced from multiple LC–MS runs for samples and controls down to a single LC–MS run each for sample and control. With improvements in scan duty cycle and sensitivity, sufficient depth of proteome coverage can be obtained for capture-free methods, which do not utilize an enrichment step.

KEYWORDS: Mass spectrometry, chemoproteomics, drug discovery, target engagement

Pharmaceutical companies eagerly adopted mass spectrometry-based proteomics research as a technology to accelerate drug discovery at the dawn of the postgenomic era, ca. 2002. At the time, Figeys provided a review of the current proteomic activities being performed in drug discovery.¹ Four categories of proteomic experiments were highlighted: protein expression profiling, functional proteomics, phosphoproteomics, and chemoproteomics. The first three categories of proteomic experiments measure the effect of a small molecule on the state of the proteome in terms of changes in protein expression, protein–protein complexes, and signal transduction relative to a control treatment. In contrast, chemoproteomics is aimed at establishing direct binding of small molecules with protein targets. Not included in Figeys' assessment was the understanding that protein identification and quantitation by mass spectrometry was still in its formative stages. In the intervening years, significant improvements have occurred in all facets of chemoproteomics workflows. However, it can be argued that the profound improvements in mass spectrometry technologies were a key development to unleash chemoproteomics for drug discovery.

A variety of chemoproteomic success stories and detailed descriptions of the experiments have appeared in recent reviews.^{2,3} These reviews have focused on the chemoproteomic methods and their corresponding accomplishments, but have not emphasized the remarkable changes in sample preparation and mass spectrometry technologies. Herein, we describe the significant advances made in mass spectrometry technology and how these innovations have enabled chemoproteomics to become powerful tools in drug discovery pipelines.

■ DEVELOPMENT OF MASS SPECTROMETRY AND PROTEOMICS TECHNOLOGIES

Prior to 1996, in-gel protein identification required the use of N-terminal Edman sequencing. This situation quickly changed when in-gel tryptic digestion protocols were introduced to enable conversion of proteins separated in polyacrylamide gels into tryptic peptides amenable for MS analysis.^{4,5} Other new methodologies and technologies emerged including nanoliquid chromatography⁶ coupled to nanospray ionization sources,⁵ tandem mass spectrometers with high mass resolution,^{7–11} data-dependent MS/MS acquisition,^{12,13} and peptide sequencing algorithms with database searching.^{14,15} All these developments led to increasing the sensitivity, improving the breadth and depth of coverage, and aiding in identification of peptide fragments by matching to corresponding proteins.

■ IMPROVEMENTS IN DATA ACQUISITION AND INSTRUMENTATION

Bottom-up proteomics experiments are typically conducted using lengthy 60–120 min reversed-phase gradients on 75 μm $\times$ 150 mm columns with 250 nL/min flow delivered by a nano-LC system coupled to a sophisticated tandem mass spectrometer. This setup produces a complex total ion chromatogram consisting of mostly unresolved peaks ($>10^6$ peptides may be present in a single analysis of a full proteome). To combat the challenge of identifying 1 million peptides in a sample, an intelligent method of selecting ions for isolation and

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fragmenting them was developed, data-dependent acquisition (DDA) MS/MS. DDA was initially implemented as a user programmed instrument script for the TSQ-7000 triple quadrupole mass spectrometer (ca. 1996)¹² and as a fully programmed feature on the LCQ ca. 1998.¹⁶ DDA has been included on all tandem mass spectrometry systems ever since.

Briefly, a survey MS scan is performed from which the data-dependent algorithm selects the top *N* (1–10 originally, but now up to 200) abundant ions for MS/MS analysis. This cycle is repeated throughout the experiment. The data-dependent list is updated as new peptides elute from the nano-LC column and previously selected precursor ions are excluded or decrease in signal below the minimum signal intensity threshold. Exclusion criteria are set to eliminate the selection of singly charged ions and minimize the number of times a precursor ion is selected, in order to maximize the number of unique MS/MS spectra collected. The dynamic nature of precursor ions in data-dependent scans makes reproducibility between experiments difficult to obtain, especially for peptides from low abundance proteins. The data-dependent MS/MS duty cycle (MS survey scan + MS/MS scans) plays a critical role in determining how many proteins can be identified in a given experiment. Hebert et al.¹⁷ demonstrated that increased duty cycle time enabled reduction of time from days to 1 h to fully sequence the yeast proteome. The benefit of increased duty cycle time has been utilized to dramatically change the time spent and depth of coverage achieved in modern chemoproteomic experiments.

In a chemoproteomic workflow, pull-downs can contain anywhere from 200–1500 specific binding, nonspecific binding, and contaminant proteins, yielding ~4000–30 000 tryptic peptides. In the early 2000s linear ion traps and quadrupole time-of-flight (Q-TOF) instruments were being used for proteomic experiments. Linear ion traps could scan at ~5 Hz duty cycle, but achieved only nominal mass resolution, which added uncertainty in peptide molecular weight assignment and allowed singly charged ions (mostly small molecule contaminants) to be selected and produce unusable MS/MS spectra. Early Q-TOF instruments were able to select precursors based on charge states but, due to inefficient ion transmission, suffered from lower sensitivity. To overcome the sensitivity issues, the signal for many transient scans was summed, limiting the scan duty cycle to ~0.3–3 Hz.

By 2007, the Thermo Scientific LTQ-Orbitrap XL instrument was able to perform a single MS¹ scan at 30k resolution at *m/z* 400 and 10 MS/MS scans in 3.6 s (duty cycle = 2.7 Hz), which theoretically had the capacity to sequence ~15,000 peptides in a 90 min run. In practice, fewer peptides were identified from a single run due to insufficient signal in the MS/MS spectrum, mixed spectra due to a lack of chromatographic and mass resolution of isobaric species, or not registering a database hit. State-of-the-art instruments such as the Thermo Scientific Orbitrap Fusion LUMOS (30–60 Hz duty cycle) and ABSciex 6600 Q-TOF (50–200 Hz duty cycle) have the speed, sensitivity, and mass resolution to analyze an entire binding proteome in a single analysis. Optimum duty cycle on these instruments depends upon the concentration of the sample and the quality of spectra required.

As mass spectrometers improved over the past decade, the steps required to reduce sample complexity prior to LC–MS analysis also underwent a transformation. Ten years ago, a lane walking experiment for an affinity capture experiment (see

below) consisting of two gel lanes, 40 bands (20 test and 20 control bands), was performed to reduce sample complexity for MS analysis, but required 60 h to complete when using a 90 min LC method. Now, a pull-down experiment is likely to be prepared in the solution phase and analyzed as a single injection without any prefractionation, as modern mass spectrometers can theoretically sequence >150 000 peptides (with a 30 Hz duty cycle) in a 90 min run. When prefractionation of peptide samples is desired, the most common methods are off-line high pH RP-HPLC¹⁸ or strong cation exchange (SCX).¹⁹

■ IMPROVEMENTS TO MS-BASED QUANTITATIVE STRATEGIES

Most profiling chemoproteomics experiments rely upon relative quantitation methods to enable comparison of test and control samples. Three different relative quantitation strategies can be deployed: label-free quantitation (LFQ), stable isotope labeling of amino acids in cell culture (SILAC), and isotope mass tagging of peptides. These approaches are summarized in Figure 1. LFQ measures the total signal differences between individual test and control samples, and

Figure 1. Depiction of three quantitative strategies for chemoproteomics experiments. Label-free quantitation (green) relies on the comparison of areas under the curve for peptide ions in MS¹ across multiple conditions. SILAC (orange) utilizes heavy- and light-labeled amino acids in the media of cells. The heavy and light samples are combined and the heavy to light ratios of isotopic pairs of molecular ions in the MS¹ experiment are compared. Tandem mass tags (purple) are used to label peptides on the N-terminus with isotope encoded tags. Differentially labeled samples are combined, and the intensities of reporter ions in the MS² (or MS³) are compared.

requires no special labeling of protein or digested peptides. LFQ identifies peptides that occur across multiple samples by searching for matching m/z (with high accuracy) and retention times (within a varying window).^{20,21} Early LFQ efforts measured spectral counts (i.e., how many times a peptide precursor ion was selected for MS/MS), but with the development of more sophisticated processing tools, area-under-the-curve (AUC) measurements are now performed. Quantitation by spectral counts as a proxy for protein abundance is highly discouraged.²² LFQ requires careful control and reproducibility throughout the entire experiment to obtain accurate results. This is achieved by performing protein and peptide concentration measurements during sample processing and sometimes spiking in internal standard peptides before MS analysis. LFQ can be computationally expensive as the matching of peptides between samples needs to allow for slight variations in retention time. Test and control samples must be analyzed by MS separately (with multiple replicates), and therefore, LFQ experiments can become time intensive.

Quantitation using SILAC is accomplished by growing control cells with standard media (referred to as “light”) and test cells with media containing “heavy” amino acids (i.e., usually $^{13}\text{C}_6$ -Lys and $^{13}\text{C}_6$ $^{15}\text{N}_4$ -Arg).^{23,24} After sample processing and protein normalization, the samples are combined and prepared for MS analysis. Relative quantitation is done by examining the heavy to light ratios of isotopic pairs of molecular ions in the MS¹ experiment. SILAC is one of the most popular methods for measuring protein abundance with the primary advantage that variation arising from sample preparation is controlled by normalizing total protein amount before combining the test and control cell lysates. A “flip” experiment can be performed using light-labeled cell lysate as the test and heavy-labeled cell lysate as the control to eliminate false positives. SILAC experiments need to be well coordinated with cell culture activities since unlabeled and SILAC labeled cells need to be generated. The SILAC strategy is not commonly performed using tissue or primary cell lines.

Isotope mass tagging enables sample multiplexing, which can be useful to profile multiple conditions in a single MS experiment.^{25–27} Isotope tags for relative and absolute quantitation, ITRAQ (4- and 8-plex), and tandem mass tags, TMT (6- and 10-plex), are the most common mass tags. They contain a reagent that consists of an amine reactive NHS ester, a mass balance moiety, and a mass reporter group. Each tag adds the same isobaric mass to the N-termini (and lysine side chains) of tryptic peptides. For a TMT10-plex experiment, each condition is labeled with a distinct TMT reagent. The 10 sets of labeled peptides are combined into a single sample and analyzed by MS. Peptides that occur across the samples appear as a single molecular ion in the MS¹ spectrum and exhibit identical retention times. When fragmented by collisional activated dissociation, peptide specific fragment ions and mass encoded fragment ions are detected. In the MS/MS spectrum, the peptide specific fragment ions are used for identification, while the ratios of the mass encoded fragment ions provide the relative quantitation of the individual samples. Co-isolation of labeled peptides with similar m/z can distort the ratio of mass encoded fragment ions and therefore provide poor quantitative information. As a solution to coisolation issues, an MS³ method was developed by Gygi et al.²⁸ that provides more accurate results but at the potential cost of reduced protein identifications and precision.²⁹ Isotope mass tagging is a key

technology for proteome-wide biophysical experiments such as CETSA and SPROX (see below).

AFFINITY-BASED PROFILING METHODOLOGIES

The most frequently used strategy for chemoproteomics relies upon the affinity of a small molecule that is chemically modified to capture proteins of interest from cell lysates or tissues through noncovalent or covalent binding. Coupled with competition experiments using free compound, specific binders can be determined. Figure 2 summarizes the various noncovalent and covalent affinity-based profiling methods that are commonly performed in the context of a kinase inhibitor.

Figure 2. Common strategies for assessing target binding profiles of a kinase inhibitor. (A) Affinity capture-mass spectrometry: in a competition experiment from cell lysate, specific binders can be competed from the pull-down with a free inhibitor leading to a decrease in signal compared to the vehicle incubated pull-down. (B) Activity-based protein profiling: in a competition experiment from cells or cell lysate specific binders can be competed from the pull-down with a free inhibitor leading to a decrease in signal compared to the vehicle incubated pull-down. (C) CETSA-MS: binding of a compound to a protein can result in melting curve shifts from stabilized or destabilized proteins in cells or lysate.

There are multiple ways to enable noncovalent capture of a protein target, including attaching a small molecule bait with a PEG linker, to a solid support^{30,31} or a soluble recognition element such as a biotin or chloroalkane tag³² for enrichment with streptavidin or HaloTag, respectively. Competition experiments are performed with test and control samples processed in parallel. In the control experiment, target proteins are captured using the immobilized bait and then eluted with DMSO.³¹ In the test experiment, the target proteins are also captured with the immobilized bait and removed using a free, active compound with an analogous chemical structure as the

bait. The differentiated proteins eluted by DMSO compared to test compound reveals the target proteins of the bait. Competition experiments also can be conducted with free, active compound and a structurally similar, inactive analog compound to serve as more specific control (Figure 2A). The bait (probe) molecule can be designed for selectivity screening for broader protein classes such as kinases or histone deacetylases (HDACs).^{33,34}

Issues with capturing binders with slow on-rates/fast off-rates and performing bead-based in-cell experiments still exist. Biotin-based probe constructs can have poorer cell permeability than the ligand, which can make it difficult to recapitulate phenotypic activity. Moreover, biotin pull-downs can also result in more nonspecific binding than bead-based affinity capture experiments. HaloTag probes can retain good cell permeability and, coupled with the rapid binding kinetics, have demonstrated in-cell target engagement of HDACs.³⁵

Covalent capture technologies are convenient methods for identifying binding partners of a compound to enable assessment of on- and off-target engagement in cell lysates and in live cells. One of the most developed and utilized strategies for covalent capture is activity-based proteome profiling (ABPP), which was pioneered by Cravatt and co-workers (Figure 2B).³⁶ A simple implementation utilizes a directed probe molecule that covalently reacts in or near the binding pocket for a specific class of enzymes. The reactive probe can be linked to either a fluorophore (for gel visualization) or soluble binder such as biotin or HaloTag (to generate pull-downs for MS analysis). Many useful directed and nondirected probes have been developed, covering a wide variety of enzymes. Competition experiments can be conducted with free noncovalent molecules to identify its specific enzyme target. These experiments can be interrogated in one or two LC–MS analyses (depending upon the chosen quantitation strategy). Thus, the specific activity of a compound against target proteins within a class of enzyme(s) can be probed.

The commercialized KiNativ screening assay uses desthiobiotinylated-ATP as a probe to react with lysine at or near the ATP binding pocket in kinases in order to identify on- and off-target kinase activity using competition experiments.³⁷ Analysis using targeted multiple reaction monitoring (MRM) mass spectrometry experiments can be performed since modified tryptic peptides can be predicted for all peptide species and standards can be synthesized. MRM experiments, performed on a low resolution MS such as a triple quadrupole, or precursor reaction monitoring (PRM) experiments, performed on a high-resolution MS such as a Q-Exactive or Q-TOF, can be developed to detect and quantitate hundreds of peptides with exquisite specificity during a single LC–MS analysis. The mass spectrometer is programmed to measure unique precursor and product ion pairs for each modified tryptic peptide at its expected LC elution time determined from the corresponding synthetic standards.

Another covalent probe strategy suited for protein target identification is to use a photoaffinity probe.³⁸ The trifunctional probe consists of a small molecule ligand bait to recognize the protein target binding pocket, a short linker that contains an alkyne or strained cyclooctyne that enables click chemistry mediated detection (fluorescence visualization or enrichment for MS analysis), and a photo-cross-linking moiety such as a phenyl azide, diazirine, benzophenone, or tetrazole.³⁸ Upon activation, the photoreactive moiety reacts with residues

near or at the binding pocket, cross-linking the probe and the protein. The cross-linked probe–protein can be visualized using fluorescence or pulled down for analysis by MS. Photoaffinity probes exhibit similar disadvantages as ABPP, in addition to suffering from nonspecific labeling and short lifetimes of the reactive intermediate, which can lead to poor cross-linking yields. Well-designed photoaffinity probes are capable of surveying cells lysates and live cells, a major advantage over affinity capture techniques. Weaker binders and membrane proteins can be captured since more stringent washing conditions can be utilized than noncovalent capture methods. For example, Parker et al. recently demonstrated a fragment-based screening approach based upon photoaffinity labeling.³⁹

■ CAPTURE-FREE CHEMOPROTEOMICS

Protein structural conformation changes are often induced by ligand binding. In general, binding with the ligand stabilizes the protein structure resulting in increased melting temperature, decreased susceptibility to denaturants such as guanine or urea, and alteration of the peptides detected when digested under limited proteolysis conditions. Three proteomics experiments have been developed, which utilize ligand-induced stabilization/destabilization of protein structure: cellular thermal shift assay (CETSA),^{40,41} stability of proteins from rates of oxidation (SPROX),⁴² and drug affinity responsive target stability/limited proteolysis–selective reaction monitoring (DARTS/LiP-SRM).⁴³ Each of these experiments is a capture-free strategy for assessing target identification, i.e., no capturing, pull-down, or tagging element on the probe is required, but for mass spectrometry, this analysis is more challenging because the entire proteome is analyzed in these experiments.

The thermal shift assay is based on the change in melting temperature and shift in melting curve that can occur when a ligand binds to a protein (stabilizing or destabilizing the protein structure).^{40,41,44–46} It is a highly sensitive technique for finding weak and strong binders and has been widely used for *in vitro* compound screening with recombinant protein. In 2013, the Nordlund group established the cellular thermal shift assay (CETSA), which extended the capabilities of the assay to include cellular/tissue lysates and more importantly, intact cells.⁴⁰ The ability of CETSA experiments to use live cells is a unique property (compared to other capture-free experiments), and enables the assessment of target engagement and differentiation between direct and indirect binders. In 2014, CETSA-MS (or thermal proteome profiling) was described, which allows for a proteome-wide analysis with protein quantitation performed using an isotopic labeling strategy (TMT).⁴¹

In the CETSA-MS experiment, multiple (6–10) aliquots of test and control-treated material are generated (Figure 2C). Each test and vehicle-treated pair are briefly incubated at a specific temperature within a typical range of 37–63 °C (the temperature range can be varied). As the incubation temperature increases, individual proteins begin to denature and aggregate to form insoluble proteins within the cellular or lysate material. After heat treatment, the cells are lysed by several freeze–thaw cycles in the presence of a gentle detergent (e.g., NP-40 or Triton X-100). Harsher lysis conditions must be avoided to prevent the resolubilization of denatured proteins. The insoluble proteins and cellular debris are removed by centrifugation. The remaining soluble proteins

are subjected to traditional bottom-up proteomics workflows and are relatively quantified using TMT-enabled MS to develop thermal melting curves for each identified protein under the test and control conditions. The melting temperature shift (T_m) between test and control curves for each protein is calculated to determine if stabilization (i.e., positive T_m temperature shift) or destabilization (i.e., a negative T_m temperature shift) occurs due to interaction with the test compound.

CETSA-MS has shown great promise as a method to support target validation and has caught the interest of many in the drug discovery field. However, like all methods, it is not without its limitations. While few false positives have been found in data sets (because observing positive shift in T_m is well correlated with a binding event having occurred), not all binders exhibit a shift in T_m and can be missed by this analysis. In such instances, traditional bait and capture can be used to provide a more informative picture regarding on- and off-targets.

SPROX is another capture-free method for evaluating target occupancy of a compound.⁴² In the SPROX workflow, the extent of unfolding for apo and liganded proteins in a cell lysate is compared after treatment with a denaturant. The basis of the methodology is an extension of Fitzgerald's pioneering work on characterizing the thermodynamic properties of native and protein–ligand complexes using SUPREX (stability of unpurified proteins from rates of H/D exchange).^{47,48} To overcome the deficiencies associated with H/D exchange, SPROX measures the extent of unfolding mediated by methionine oxidation after addition of H_2O_2 . Proteins under each test condition (i.e., increasing concentration of denaturant) are digested into peptides and mass-tagged with TMT or ITRAQ labels to provide relative quantitation. Test and control sample sets are analyzed in separate runs, processed to identify proteins, and quantified by monitoring the relative change in concentration of oxidized methionine on peptides as a function of denaturant concentration. Unlike CETSA, SPROX requires specific amino acids to be present on the peptide being measured and can only be performed using cell lysates.

The basic principle of DARTS draws from the experiments on a single protein to detect structural changes in an apo and liganded protein when subjected to limited proteolysis.⁴³ Besides the ligand blocking the binding pocket, other changes can occur in the protein structure, which tends to (but not always) reduce the extent of proteolysis. Lomenick et al. expanded this methodology to enable this measurement to be done proteome-wide from cell lysates using MS or immunoblot readouts. The technique has been applied to mid micromolar binders but has not taken off in popularity due to requiring extensive optimization of proteolysis conditions, which is difficult to perform for unknown protein targets.

LiP-SRM is similar to DARTS as it also relies upon limited proteolysis for detection of ligand–protein interactions.⁴⁹ However, in this workflow, the cell lysate treated with control or ligand is subjected to a two-step digestion process: limited proteolysis (low enzyme concentration and short time) in the native state followed by a tryptic digestion on the denatured material. Ligand-bound (or “protected”) regions exhibit tryptic peptides by MS, while unbound (or “unprotected”) regions are cleaved by both enzymes to form “half-tryptic” peptides.⁴⁹ Both DARTS and LiP-SRM are suited for validating discovery findings and providing additional insight into data sets

obtained by other chemoproteomic technologies, rather than being used as a primary tool for finding new drug targets.

SUMMARY

Chemoproteomics encompasses a broad set of strategies to enable target discovery and understand both on- and off-target engagement. The choice of which strategy (or combination of) to perform, whether it is bead-based, covalent attachment, or biophysical, will vary depending upon the question to be answered and capabilities available to a project. All probe-based strategies require additional chemical synthesis of the ligand and further experimentation to optimize protein capture or tagging elements. Biophysical experiments require no additional chemistry but are labor intensive and require meticulous sample testing conditions and sample preparation. No matter which strategy is chosen, all global chemoproteomic experiments depend upon sample preparation, high-end MS instrumentation, and data processing. Over the past decade, the sensitivity of MS instrumentation has significantly improved yielding more depth and breadth of protein coverage, yet more reduction in MS analysis time is needed. Further increases in the proteome coverage depth may enable reliable determination of binding sites when using covalent labeled probes. New workflows utilizing top-down proteomics,⁵⁰ sequencing of intact proteins, may emerge to further streamline sample preparation workflows and differentiate between active and inactive proteoforms of the same protein.

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Notes

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