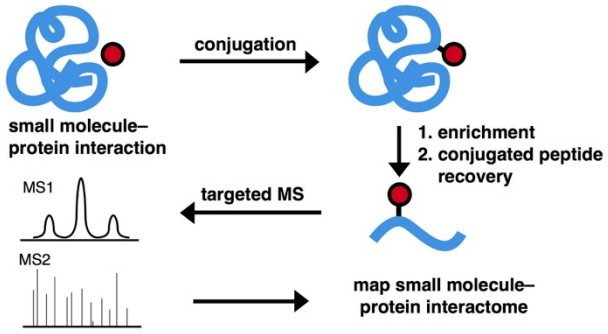
Mapping the Small Molecule Interactome by Mass Spectrometry

Mapping the small molecule interactome by mass spectrometry

Hope A. Flaxman[1], Christina M. Woo[1,*]

[1] Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford St, Cambridge, MA 02138
* Corresponding author: cwoo@chemistry.harvard.edu



Abstract

Mapping small molecule interactions throughout the proteome provides the critical structural basis for functional analysis of their impact on biochemistry. However, translation of mass spectrometry-based proteomics methods to directly profile the interaction between a small molecule and the whole proteome is challenging due to the substoichiometric nature of many interactions, the diversity of covalent and noncovalent interactions involved, and the subsequent computational complexity associated with their spectral assignment. Recent advances in chemical proteomics have begun fill this gap to provide a structural basis for the breadth of small molecule–protein interactions in the whole proteome. Innovations enabling direct characterization of the small molecule interactome have centered on faster, more sensitive instrumentation coupled to chemical conjugation, enrichment, and labeling methods that facilitate detection and assignment. These methods have started to measure molecular interaction hotspots due to inherent differences in local amino acid reactivity and binding affinity throughout the proteome. Measurement of the small molecule interactome is producing structural insights and methods to probe and engineer protein biochemistry. Direct structural characterization of the small molecule interactome is a rapidly emerging area pushing new frontiers in biochemistry at the interface of small molecules and the proteome.

Introduction

Small molecules are essential regulators of protein biochemistry as covalent modifications, noncovalent ligands, and essential cofactors.[1,2] These small molecule–protein interactions (SMPIs) modify protein function through a range of modes from irreversible covalent interactions[1] to non-covalent stable or transient binding events (Figure 1A).[2,3] Alterations in protein structure due to SMPIs result in diverse structural outcomes that change protein conformation and modulate protein–protein interactions, leading to broader functional consequences. These interaction sites hold important information about protein biochemistry, including proteomic activity, intermolecular associations, molecular recognition of protein surfaces, and local amino acid reactivity. However, understanding of the exact structural properties that comprise SMPIs remains limited, in part due to the lack of a robust technology to directly characterize these interactions in the whole proteome. Analytical methods like X-ray crystallography and NMR spectroscopy typically provide the transformative structural insights that

accelerate biochemical studies of protein behavior and the rational design of new small molecule ligands. While these methods have made significant impact in biochemistry, the strict requirements on protein compatibility limit the scope of X-ray and NMR analysis to measurement of stable interactions between a single protein and a small molecule *in vitro*. Methods to structurally characterize the global range of interactions that occur between a small molecule and the proteome within the context of physiologically relevant systems will be instrumental in illuminating the broader roles of SMPIs throughout biochemistry.

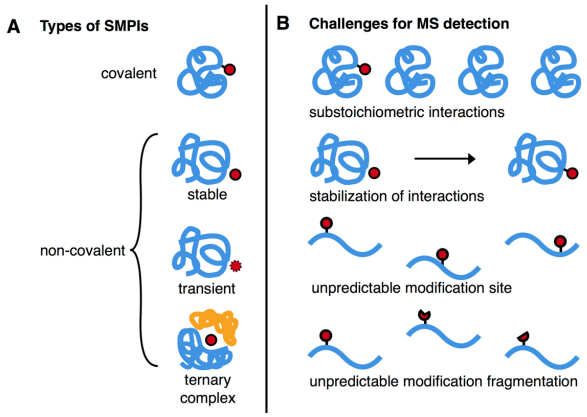


Figure 1. Overview of (A) major types of small molecule–protein interactions (SMPIs) and (B) challenges to directly detecting and characterizing SMPIs by MS**.**

In this perspective, we highlight recent technological innovations to directly characterize a broad array of biochemical interactions between small molecules and proteins using mass spectrometry (MS). MS is now the primary analytical method to rapidly profile the proteome in an unbiased manner.[4] The advent of routine electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) technologies and faster, more sensitive MS instrumentation has enabled facile measurement of a range of biomolecules.[5,6] In a typical shotgun proteomics experiment, a cellular proteome is isolated and digested. The resulting peptides are analyzed by liquid chromatography–MS (LC–MS) and assigned by database searching. In this manner, entire proteomes are regularly profiled and quantified using stable isotope labeling (e.g., SILAC, TMT, iTRAQ), label free, or single ion monitoring methods within the span of a few hours.[7] Due to these improvements in analytical capability of proteomics, MS is similarly poised to broadly detect SMPI structural interfaces on samples ranging from single isolated proteins to the whole proteome. However, structural differences between a SMPI and an unmodified peptide requires integration of chemical tools with typical shotgun proteomics workflows (Figure 1B). Adaptation of MS workflows to broadly characterize SMPIs must address three hurdles: (1) a general covalent conjugation chemistry, (2) detection, and (3) database assignment. Herein, we highlight emerging strategies that use chemical tools coupled to MS that address each of these challenges and are beginning to realize a new era of direct global characterization of SMPIs. In addition to the strategic application of conjugation chemistry, enrichment methods, and targeted MS strategies that enable direct observation of SMPIs by MS, we also project how these insights will collectively advance new frontiers in biochemistry.

Chemical conjugation strategies for capture of covalent and noncovalent SMPIs

The choice of conjugation chemistry is critical for capturing specific types of SMPIs and for downstream detection and characterization by MS. Observation of a SMPI by MS requires a covalent bond between the small molecule and the protein surface that will withstand sample

processing prior to analysis and the MS ionization and fragmentation steps that are necessary for spectral assignment. Chemistry to covalently modify proteins with small molecules has long been used by the cell in the form of post-translational modifications. Likewise, covalent conjugation strategies for small molecules are commonly executed using reactive functional groups to capture a subset of reactive amino acids. Chemistry to conjugate small molecules to the proteome has been extensively reviewed.[8-10] Here, we primarily highlight examples of conjugation chemistry that have led to direct characterization of a SMPI from complex proteomes.

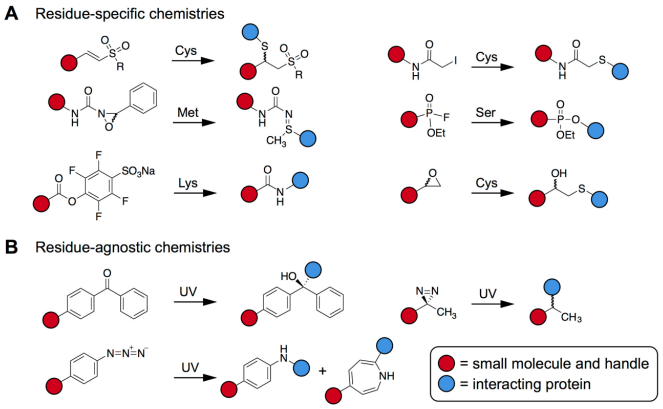


Figure 2. Examples of (A) residue-specific and (B) residue-agnostic strategies used to form covalent bonds between small molecules and proteins.

Integration of a covalent modification chemistry coupled to MS analysis in a complex proteome was first advanced in the context of activity-based protein profiling (ABPP), developed by Cravatt and coworkers.[11] ABPP uses covalent modifiers of enzyme catalytic sites to identify active enzymes by taking advantage of small molecule probes containing electrophiles that form covalent bonds to specific amino acid residues.[12] Initial applications of ABPP used chemical probes that target serine residues.[11] The chemistry has now been extended to form conjugates to cysteine and lysine residues.[13-16] These reactive electrophiles (e.g., fluorophosphonates, epoxides, alkylchlorides) are integrated with small molecules that selectively interact with a subset of the proteome and a cleavable enrichment handle for direct identification by MS (Figure 2A).[12] Applications of ABPP have revealed a range of biochemical reactivity of several major classes of proteins, including methyltransferases and serine hydrolases[17,18] and have led to the discovery of new chemical probes, such as aza-β-lactams that target serine hydrolases.[19] ABPP has been extended to identify new inhibitors via competition. In competitive ABPP, the proteome is treated with an inhibitor library that may block a subset of enzyme active sites. The ABPP probes will only form covalent marks at sites unoccupied by the inhibitor; these links are then detected by MS.[12] While competitive ABPP primarily identifies competitive interaction sites that are marked by the covalent probe at the expense of broader SMPIs that may occur, the approach has been extensively employed with applications ranging from identification of inhibitors of a target protein from a pool of small molecules[20] to understanding prodrug activation.[21]

Redox-activated chemistry is a growing area for SMPI detection that expands the amino acid residues available for selective chemical conjugation (Figure 2A). Chemical conjugation through redox activity has previously been employed by hydroxyl-radical footprinting on isolated proteins to map exposed protein surfaces[22] or by using reporter proteins like ascorbate peroxidase (APEX) to map protein compartmentalization[23] or protein–protein interactions.[24-26] APEX oxidatively couples a phenol in the reporter probe, which carries an enrichment handle for

downstream MS analysis, to proteomic tyrosine residues.[23] Recently, innovative selective redox chemistries have been embedded into chemical probes for SMPI profiling.[27,28] Aye and coworkers developed the targetable reactive electrophiles and oxidants (T-REX) system for *in vivo*, light-triggered redox labelling events on specific target proteins. To achieve spatiotemporal redox reactivity, a small molecule carrying a photo-caged Michael acceptor and an alkyne handle is targeted to an epitope-tagged protein. A covalent labelling event between cysteine and the small molecule is then triggered by light.[28] This system has been used to study the effects of lipid-derived electrophiles (LDEs) on cell signaling in several redox-sensitive pathways.[29,30] Separately, Chang and coworkers developed a redox-activated chemical targeting (ReACT) system for profiling reactive methionine residues. ReACT uses an oxaziridine-based reagent to selectively label methionine residues by redox chemistry in the presence of more nucleophilic amino acids like cysteine. The ability to profile reactive methionine residues enables broader measurement of redox reactivity in protein biochemistry.[27] Development of new controlled redox chemistries will continue to expand the range of protein surfaces that are accessible for chemical conjugation and thus reveal new protein biochemistry through SMPI profiling.

Chemical strategies to capture non-covalent binding interactions of small molecules within the cell involve the triggered formation of an amino acid-agnostic covalent conjugation event by an external stimulus. A general conjugation chemistry that marks any amino acid is preferred in order to capture the greatest diversity of non-covalent molecular interactions. Photochemistry has emerged as the primary means to form such amino acid-agnostic marks to the proteome.[31] Early efforts to study protein reactivity using small molecules, performed by Westheimer and coworkers, used photochemistry to capture the interactions between isolated yeast alcohol dehydrogenase and a diazoacetate.[32] More reactive photochemical functional groups, such as the diazirine, benzophenone, or arylazide, are now readily incorporated into small molecules and conjugated to a protein or proteome by photoirradiation (Figure 2B).[33-35] On activation with light, these functional groups form reactive carbenes or nitrenes that insert locally into the interacting protein interface. The diazirine is commonly employed for SMPI capture due to its minimally perturbative size and its ability to be readily installed on ketones within the small molecule of interest[36] or incorporated in the form of a modular tag, such as the “minimalist tag” developed by Yao and coworkers.[37] The “minimalist tag” is functionalized with a diazirine and a terminal alkyne for photocrosslinking and enrichment, respectively.[37] Photochemistry has been used to determine the mechanism of action of several natural products,[38,39] in ABPP targeting methyltransferases,[17] and in fragment-based screening profiles.[40] Although photochemistry generates the greatest breadth of conjugates within the protein binding site, the concentration-dependent balance between specific and non-specific SMPI capture is not well understood. As direct modification site profiles from photochemically produced SMPIs are increasingly measured, the underlying chemistry of carbene insertion to the proteome is being increasingly characterized.[41]

Chemical enrichment strategies coupled to MS

Shotgun proteomics workflows typically collect a full scan mass spectrum (MS1) followed by an iterative selection of the most abundant species from MS1 for tandem MS sequencing (MS2) in a process termed data dependent acquisition (DDA).[42] The depth of selection is restricted by instrument speed and analyte complexity. Challenges in direct detection of SMPIs by MS stem from the unpredictable ionization efficiencies of the small molecule-conjugated peptide and the substoichiometric nature of most SMPIs (*i.e.* only a fraction of the total protein is complexed with the small molecule). Both factors reduce the observed MS abundance of the conjugated peptide relative to unmodified peptides. Low abundance species may go unselected for tandem MS sequencing, preventing assignment of the conjugated peptide. Therefore, structural mapping of the small molecule interactome typically requires a strategy to selectively enrich and recover SMPIs.

Affinity enrichment strategies that isolate SMPIs or the conjugated peptide(s) are typically performed by selection for the small molecule. Selection using the small molecule is enabled by functionalization with a reporter group which is used as a handle for detection and enrichment of the SMPI. To minimally affect the small molecule's native interactions, enrichment handles are often attached after chemical conjugation of the SMPI using a biocompatible chemistry (e.g., click chemistry).[37,43] Following affinity enrichment (e.g., biotin–streptavidin), enzymatic digestion of the enriched proteins will release non-conjugated peptides for protein identification by MS, often leaving the enrichment handle and the conjugated peptide behind.[44] Selection of the chemistry for the reporter group and enrichment strategy has been widely reviewed.[45,46]

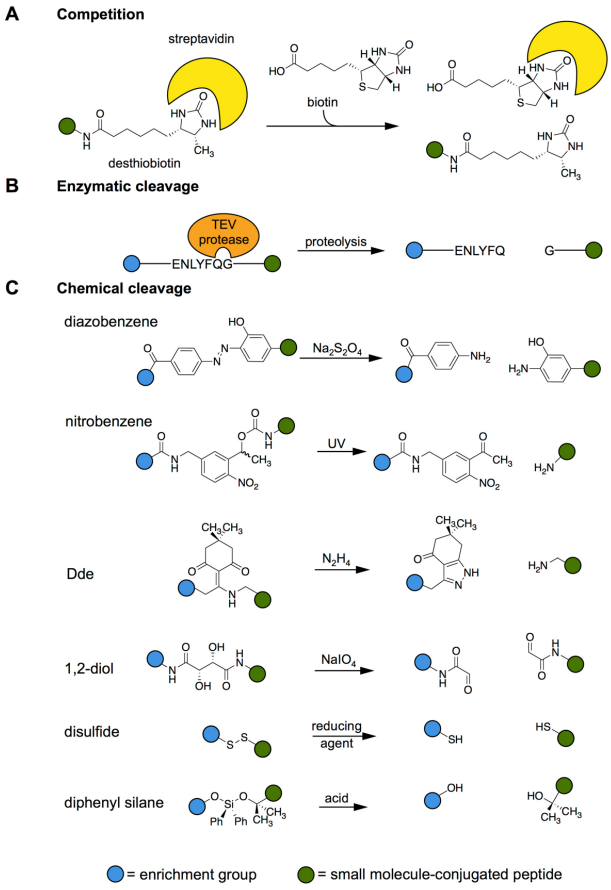


Figure 3. Strategies for recovery of small molecule-conjugated peptide by (A) competition of the enrichment group, (B) enzymatic cleavage of a linker, and (C) chemical cleavage of a linker.

For structural characterization by MS, the peptide directly conjugated to the small molecule must be recovered. Recovery of the conjugated peptide is performed by competition with a higher affinity small molecule, cleavage of the affinity handle with a protease (e.g., TEV protease), or chemical cleavage of specific functional groups (Figure 3). The most common applications of competition strategies involve reducing the inherent biotin–streptavidin affinity, followed by displacement and recovery of the conjugated peptide (Figure 3A). Attenuation of the enrichment affinity is achieved by use of monomeric streptavidin to weaken the interaction, by changing pH sensitivity, or, most commonly, by use of biotin analogs like desthiobiotin that can be eluted by competition.[47] The desthiobiotin–streptavidin interaction (K_d = 50 pM) is displaced by

the several orders of magnitude stronger biotin–streptavidin interaction (K_d = 1 fM).[48] Enrichment of desthiobiotin-carrying SMPIs is followed by competitive elution from streptavidin with biotin. The competitive displacement strategy benefits from the gentle elution conditions, but leaves an excess of biotin in the eluent that must be removed by other means. In addition, weakening the initial enrichment affinity may decrease the enrichment efficiency by requiring longer incubation times for association with streptavidin and reducing stringency of the subsequent wash steps prior to elution. Nonetheless, the competitive displacement strategy has been used to identify kinases and their nucleotide binding sites with ATP-desthiobiotin and ADP-desthiobiotin probes[49] and interacting protein–protein binding sites with APEX.[50]

Protease-based cleavage strategies are widely used due to their high compatibility with traditional MS-based proteomics. Of the available proteases, the tobacco etch virus (TEV) protease has been the most widely used. The TEV protease recognizes a short 7-amino acid peptide sequence with high cleavage specificity (Figure 3B). Inclusion of the peptide sequence in the enrichment handle allows for selective cleavage with TEV, leaving a mark on the small molecule-conjugated peptide after recovery. The tandem orthogonal protease strategy introduced by Cravatt and coworkers uses an azide-TEV-biotin tag in combination with ABPP to recover cysteine-conjugated peptides for quantitative site-specific assignment of covalent cysteine modifiers.[13,51] The TEV protease strategy was also used by Nomura and coworkers to directly map reactive cysteines *in vivo*.[52] Although the protease cleavage of TEV is compatible with MS-based proteomics, the large size of the probe may inhibit initial tagging and capture of the SMPI. Proteolysis is additionally a relatively slow cleavage mechanism that requires removal of the protease prior to analysis of the recovered conjugated peptides.

Cleavable chemical functional groups have been widely used to improve SMPI enrichment efficiency and to recover the small molecule-conjugated peptide. Chemical tags are also readily modified with additional markers (i.e., isotopic codes) to facilitate MS identification of tagged peptides. The diazobenzene was first developed by Bogyo and coworkers as a cleavable functional group used for MS-based proteome profiling.[53] The diazobenzene is reductively cleaved by sodium dithionite, which allows for recovery of the conjugated peptide or protein and leaves only salts that are removed prior to MS analysis. Weerapana and coworkers used diazobenzene probes to site-specifically identify reactive cysteines in the whole proteome.[54] A number of other cleavable probe chemistries have also been developed, including photo-cleavable nitrobenzene, hydrazine-cleavable Dde, sodium periodate-cleavable 1,2-diol, reductively-cleaved disulfide or diazobenzene, and acid-cleavable diphenyl silane or acetal functional groups (Figure 3C).[55-57] As acid is generally used to assist with MS ionization and the excess reagent is readily removed by evaporation, the silane strategy has proven especially compatible with downstream MS analysis.[56,58] The choice of cleavage chemistry is a critical aspect of recovering conjugated peptides, as it affects the quality of downstream MS analysis.

Targeted MS strategies for confident assignment of the small molecule-conjugated peptide

Recovery of a small molecule-conjugated peptide enables its direct characterization and ultimately structural definition of the SMPI, but confident assignment of the conjugated peptide from MS data is not without challenges. Shotgun proteomics data are commonly assigned by database searching against the predicted peptides from the relevant species proteome. Database searching is capable of identifying conjugated peptides carrying predictable modifications with a uniform, defined mass that do not produce unexpected fragments and occur on specific amino acid residues.[59] Direct characterization of small molecule conjugated peptides has been performed in the context of residue-specific covalent modification[60,61] and in select other examples, such as identification of biosynthetic proteins based on high-occupancy cofactor binding.[62]

However, the experimental and computational challenges are enhanced when attempting to profile more diverse modifications, such as complex small molecules that possess their own

fragmentation pathways or small molecule-conjugated proteins formed by amino acid-agnostic photocrosslinking chemistry.[41] The diversity of modifications photochemistry can produce is beneficial for generality of the types of SMPIs captured, but diverse modifications pose a significant bottleneck during characterization of modified peptides by database searching. The addition of a single modification to any amino acid on peptides from the human proteome (2.6 million tryptic peptides) increases the database search space by 60-fold. The search space increases further with the addition of two modifications (1000-fold) or three modifications (28,000-fold) per peptide.[58] Additionally, more complex small molecule structures generate their own fragments that are unaccounted for, or potentially misassigned, by the database search algorithm. Unpredictable fragmentation of a small molecule at an unknown conjugation site in the whole proteome produces an unmanageable search space, yet without accounting for all of the potential diversity, some conjugated peptide assignments will be missed.

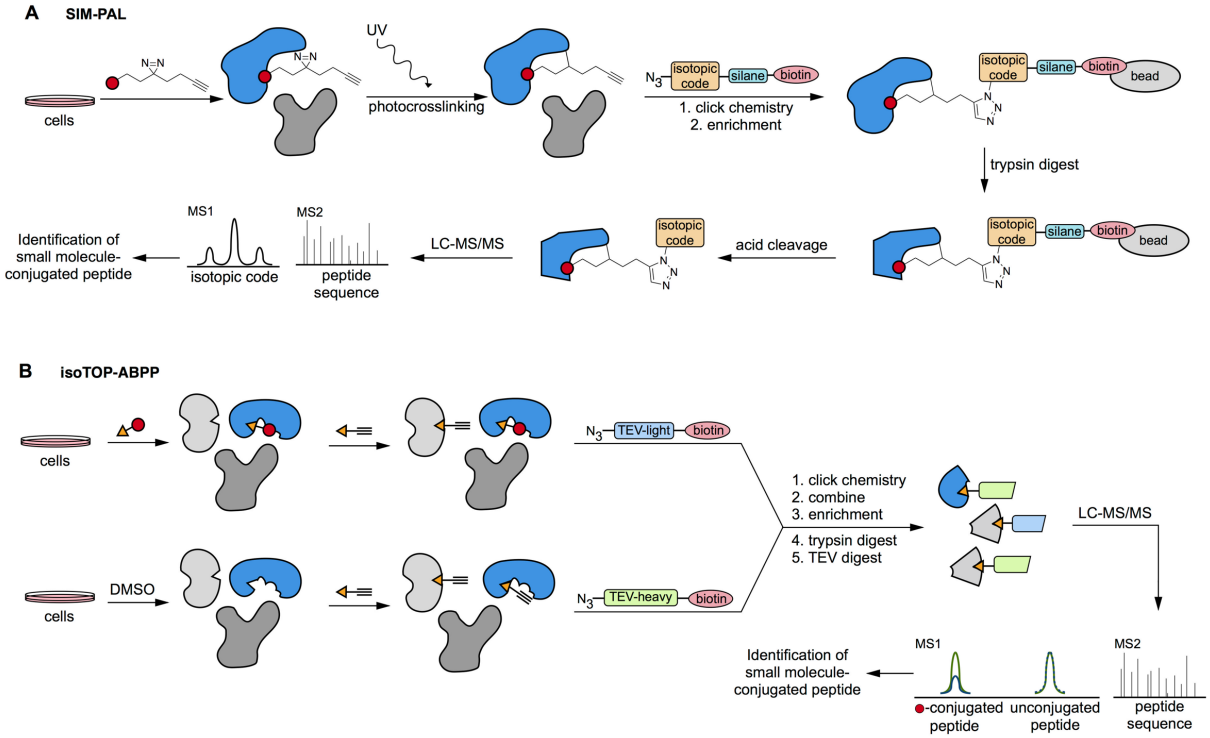


Figure 4. Strategies for isolating and detecting peptides using targeted MS include (A) small molecule interactome mapping by photo-affinity labeling (SIM-PAL) and (B) isotopic tandem orthogonal proteolysis– activity-based protein profiling (isoTOP-ABPP).

To circumvent challenges to database searching of diverse SMPIs, common strategies include installation of a specific marker for conjugated peptides by strategic installation of isotopes in the enrichment tag. Our lab developed a platform, termed small molecule interactome mapping by photo-affinity labeling (SIM-PAL), to incorporate an isotopic code exclusively into the small molecule-conjugated peptide for detection by mass-independent MS (Figure 4A). Mass-independent MS uses the imprinted isotopic code to provide an orthogonal handle for detection and validation of the conjugated peptide from the complex proteome, increasing the confidence of modified peptide identifications. In general, this strategy involves incorporating a known ratio of stable isotopes or heteroatoms with distinct isotopic distributions (e.g., $^{79/81}$Br, $^{35/37}$Cl) into either

the small molecule or the enrichment handle, leading to a unique, identifiable isotopic pattern in modified peptides that is predictable and detectible by MS.[63] Species assigned as conjugated peptides by database searching are cross-validated using a pattern matching algorithm for the isotopic code.[63,64] Mass-independent MS was recently applied to structurally map the broader proteomic interactions of several NSAIDs.[58] Isotopic coding has been applied to other unpredictable modifications to the proteome[64] and to identify new natural products,[65] illustrating the broad utility of isotopic coding to enable targeted MS analysis.

A technique termed isoTOP-ABPP was developed by Cravatt and coworkers that uses isotope labeling to validate SMPI identification and specificity (Figure 4B). In this strategy, two samples are prepared: one is treated with the small molecule of interest followed by a cysteine-reactive, alkyne-containing probe to fill unreacted sites; the other sample is treated only with the cysteine-reactive alkyne probe. Click chemistry is used to attach an isotopically-labelled, TEV-cleavable, biotin-azide to the alkyne-conjugated peptides. Upon enrichment and cleavage, the released peptides with a significant change in abundance between samples are identified as those that bound the small molecule.[66] This strategy requires the SMPI to occur on an amino acid that reacts with a known highly reactive probe. Alternatively, directly labeling the small molecule-conjugated peptide with the isotopically-labeled azide-TEV-biotin probe yields assignment of the conjugated peptide with fragment-based molecules.[40] Isobaric tags have also been used to isotopically code conjugated peptides for confident structural assignment by MS.[67] A systematic evaluation of the extent to which isotope coding and labelling supplements database searching of conjugated peptides will quantify the degree of conjugated peptide complexity that benefits from an isotope-targeted MS strategy.

Outlook

Direct structural characterization of SMPIs opens new opportunities for a broad range of biochemical measurements and applications. The described advances in conjugation chemistry, chemical enrichment strategies, and targeted MS methods have revealed new frontiers in the types and breadth of SMPIs that can now be readily measured. Relevant cellular SMPIs take place at a range of binding constants,[68] yet to date only the strongest of these interactions have been observable on a whole proteome scale relying on comparative proteomics, which does not provide direct evidence for the SMPI. Advances in chemical techniques now allow for direct characterization of small molecule-conjugated peptides, enabling measurement of transient interactions and protein ternary complex formation. Further characterization of the conjugation chemistry may reveal methods to quantitatively measure structural properties including dissociation constants, binding site size, and differences in small molecule binding affinity in simple and complex protein mixtures. For example, Sinz and coworkers have used MS to obtain structural insight to the conformational changes induced by a small molecule ligand to a single protein;[69] in the future such insights could be gained across the entire small molecule interactome. As MS sensitivity increases and chemical techniques improve, direct SMPI profiles of small molecule activity in rare cell types may also become a reality.

Direct observation of SMPIs is poised to transform target identification of small molecules in several capacities. For example, multiple small molecules may be competitively profiled for protein interactions.[40] Targeted MS for direct structural characterization of a SMPI provides immediate validation of the interaction site and may further reveal the effects of the small molecule and metabolic byproducts that generate independent proteomic interactions and biological functions. The application of these techniques to discover novel protein interactions and thus potential pharmaceutical uses for uncharacterized natural products is a particularly ripe area. As a majority of FDA-approved drugs are derived from natural products[70] and the target(s) of many natural products remain uncharacterized, it is likely that novel, undefined molecular interactions exist among these privileged scaffolds. Additionally, the complete molecular mechanisms of action, including off-target interactions and their effects, are not known for many clinical

therapeutics. By using chemoinformatics to focus on proteins that bind similar ligands, Roth and coworkers predicted thousands of new drug–protein interactions and experimentally verified 23 previously unknown interactions.[71] Additional insights from the strategies highlighted here will both validate the broad range of drug–protein interactions and provide new data to predict proteomic recognition of small molecule ligands. These results will reveal binding to global targets, molecular mechanisms of off-target effects, and supply new biological targets for additional therapeutic indications.

While not all molecular interactions observed by direct SMPI profiling will yield a direct biological function, new protein degradation strategies are poised to take advantage of the observed interactions.[72] Small molecule ligands that form specific proteomic interactions, regardless of function, may be further functionalized to recruit the ubiquitin proteasome system and thus target the protein for degradation. Furthermore, the photochemistry used to capture binding site interactions may be repurposed to stabilize small molecule protein interactions. Thus, probes for SMPIs may be further applied to strengthen weak binding interactions on "undruggable" proteins[73] or as a novel conjugation chemistry for binding site-specific attachment of a small molecule therapeutic to a protein or delivery vehicle.[74]

Conclusion

In this perspective, we highlighted applications of chemical tools and MS that advance efforts to profile the small molecule interactome beyond protein identification and to directly characterize SMPI interfaces. Major advances in MS instrumentation that yield rapid proteomic profiles are increasingly integrated with chemical strategies to structurally assign small molecule-conjugated peptides from complex mixtures. Conjugation chemistry, either amino acid-specific nucleophilic or redox chemistry, or amino acid-agnostic photochemistry, reports on local proteomic reactivity and binding site ligandability. These conjugation chemistries mark the proteome for subsequent enrichment. Small molecule-selective enrichment strategies are commonly required for protein identification; recovery of the conjugated peptide by competitive displacement or a cleavable enrichment handle allows for direct characterization by MS. Finally, by embedding the small molecule-conjugated peptide with specific isotopic codes or labels, either directly imprinted on the small molecule or probe, or through isobaric tagging (e.g., TMT), targeted MS strategies yield confident assignment of the structural underpinnings of SMPIs. The measurement of these forms of structural data have broad implications for the future of biochemistry. With the measurement of a broader range of interactions, structure–function and mechanism of action studies will be accelerated and molecular recognition and protein conformation globally measured. Eventually prediction and engineering of new interactions will be possible. Continued innovations in both chemical tools and MS technology will ensure further exciting developments in biochemistry at the interface of small molecules and the proteome.

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