Analytical platforms for activity-based protein profiling – exploiting the versatility of chemistry for functional proteomics

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The field of proteomics aims to develop and apply technologies for the characterization of protein function on a global scale. Toward this end, synthetic chemistry has played a major role by providing new reagents to profile segments of the proteome based on activity rather than abundance. Small molecule probes for activity-based protein profiling have been created for more than a dozen enzyme classes and used to discover several enzyme activities elevated in disease states. These innovations have inspired complementary advancements in analytical chemistry, where new platforms have been introduced to augment the information content achievable in chemical proteomics experiments. Here, we will review these analytical platforms and discuss how they have exploited the versatility of chemical probes to gain unprecedented insights into the function of proteins in biological samples of high complexity.

Introduction: the opportunities and challenges for proteomics

A principal goal of modern biomedical research is to discover, assemble, and experimentally manipulate molecular pathways in cells and organisms to reveal new disease mechanisms. Toward this end, complete genome sequences for numerous bacteria and higher organisms, including humans, have laid the fundamental groundwork for understanding the molecular basis of life in its many forms. However, the information content of DNA sequences is limited and, on its own, cannot describe most physiological and pathological processes. Considering that proteins are the major mediators of most biochemical events that define cell and organism physiology, investigation of protein expression and function on a global scale, or proteomics, has become a primary focal point of post-genomic research.[1–3] Unlike oligonucleotides, however, proteins are a very diverse group of biomolecules that display a wide range of chemical and biophysical features, including membrane-binding, hetero/homo-oligomerization, and post-translational modification.[4] The biochemical complexity intrinsic to protein science intimates that several complementary analytical strategies will be needed to achieve the ultimate goal of proteomics – a comprehensive characterization of the expression, modification state, interaction map, and activity of all proteins in cells and tissues.

The most mature method for proteome analysis is two-dimensional polyacrylamide gel electrophoresis (2DE)[5] in which proteins are separated based on their isoelectric point, or pI, (1st dimension) and molecular mass (2nd dimension). Protein spots on the gel are then detected and identified by staining and mass spectrometry (MS) techniques, respectively. This approach has been extensively used for the comparative proteomic investigations to identify, for example, proteins that are differentially expressed in normal and disease tissue.[6] Although the generation of new fluorescent dyes for protein staining has increased the sensitivity and dynamic range of 2DE,[7] this method still suffers from a lack of resolving power that hinders the detection of several important classes of proteins, including membrane-associated[8] and low abundance proteins.[9] To address these limitations, alternative "gel-free" methods for quantitative proteomics have emerged.

A powerful LC-MS strategy for proteomics involves the use of isotope-coded affinity tags (ICAT).[10] This approach enables the comparison of protein expression in proteomes by treating samples with isotopically distinct forms of a chemical labeling reagent. ICAT methods provide superior resolving power compared to gel-based methods and improve access to membrane-associated proteins.[11] More recently, isotope-free MS methods for quantitative proteomics have emerged that

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rely on the intrinsic signal intensities of proteins (*e.g.*, mass spectral counts[12,13] or peak areas[13]). These approaches, especially when combined with an upfront multi-dimensional LC separation step,[14] offer a versatile strategy to estimate the relative expression level of many proteins in parallel in samples of high biological complexity.

A common shortcoming of both gel- and LC-based proteomic methods is that these technologies rely on separate protein resolution/enrichment (gel, LC, and/or avidin purification) and identification (MS) steps, which inherently limits their throughput. Antibody microarrays offer a potential solution to this problem by achieving protein separation, identification, and quantification in a single step.[15,16] In this approach, arrays of antibodies with specificity for individual proteins (or modified forms of proteins) are incubated with proteomic samples and bound proteins measured by one of several detection methods, including direct detection by random labeling of protein antigens with fluorescent dyes or indirect detection with a secondary anti-protein antibody. Direct detection has the advantage of only requiring a single antibody reagent per protein, but is often hampered by the disruptive effects of protein labeling on antigenicity. Indirect detection requires two antibodies per protein, but benefits from enhanced sensitivity and specificity. Reverse protein microarrays have also been described in which proteomes themselves are arrayed and the antibodies used for detection in a format analogous to Western blotting.[16] In addition to increasing the throughput of proteomic experiments by integrating the protein separation and detection steps, microarrays also consume much less material than conventional proteomic methods. Still, the general application of microarrays for proteomics is currently limited by the availability of high-quality capture reagents (*e.g.*, antibodies, aptamers, *etc*).

The aforementioned methods for quantitative proteomics provide important information on the relative levels of proteins (and their modification state) in cells, tissues, and fluids. However, these approaches, by measuring protein abundance provide, like genomics, only an indirect assessment of protein activity and may fail to detect important post-translational events that regulate protein function, such as protein–protein or protein–small-molecule interactions.[17] To address these limitations, complementary strategies for the functional analysis of proteins have been introduced. Prominent among these functional proteomic efforts is the use of chemistry for the design of active site-directed probes that measure enzyme activity in samples of high biological complexity.[18,19] The conceptual and experimental foundation for this chemical proteomic approach, referred to as activity-based protein profiling (ABPP), has been recently reviewed elsewhere[20–22] and will only be briefly discussed here. Instead, we will focus this article on recent advances in technologies for the acquisition and analysis of data generated in ABPP experiments. As should become apparent by the end of this review, we are rapidly learning that success in chemical proteomics experiments requires not only innovations in the design of small molecule probes, but also the creation of advanced analytical platforms for the in-depth characterization of probe-treated proteomes.

Activity-based protein profiling (ABPP)

The activity of enzymes is regulated by myriad post-translational events *in vivo.*[17] Common forms of post-translational regulation include production of inactive precursor enzymes (*e.g.*, protease zymogens), covalent modification (*e.g.*, glycosylation, phosphorylation), subcellular compartmentalization (*e.g.*, localization to the lysosome), and interaction with endogenous protein and small molecule inhibitors. Such widespread post-translational regulation of enzymes indicates that, for much of the proteome, protein abundance may not correlate directly with protein activity, and, as a consequence, the measurement of protein expression by conventional proteomic methods may fail to report on key changes in protein function that impact cell physiology and pathology.

Many post-translational modes of enzyme regulation share a common mechanistic foundation – they perturb the active site such that catalytic power and/or substrate recognition is impaired (Fig. 1).[17] Accordingly, it was hypothesised that chemical probes capable of reporting on the integrity of enzyme active sites directly in cells and tissues might serve as effective functional proteomic tools.[18,19,23] These activity-based protein profiling (ABPP) probes consist of at least two general elements: 1) a reactive group for binding and covalently modifying the active sites of many members of a given enzyme class or classes, and 2) a reporter tag for the detection, enrichment, and identification of probe-labeled proteins (Fig. 2). Typical reporter tags include fluorophores and/or biotin for in-gel detection and avidin-based enrichment of probe-labeled enzymes, respectively. To date, ABPP probes have been successfully developed for more than a dozen enzyme classes, including all major classes of proteases,[23–29] kinases,[30,31] phosphatases,[32,33] glycosidases,[34,35] GSTs,[36,37] and oxidoreductases.[36–39] Mechanistic studies have confirmed

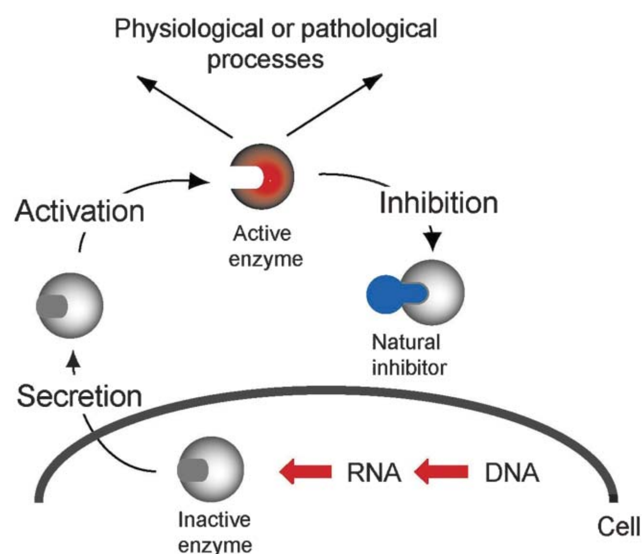

Fig. 1 Post-translational regulation of enzyme activity. Many enzymes are produced as inactive precursors, or zymogens, which require proteolytic processing for activation. Enzyme activity can be further regulated by interactions with endogenous protein inhibitors.

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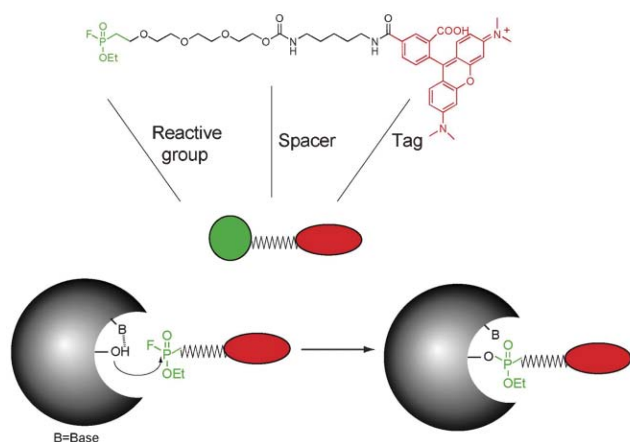

Fig. 2 General structure of an ABPP probe, shown for fluorophosphonate (FP) reagents that target the serine hydrolase enzyme family.[23,24] ABPP probes consist of a reactive group (green), a spacer (black) and a reporter tag, such as rhodamine (red). FP probes covalently modify the active site serine nucleophile of serine hydrolases as depicted.

that these probes can distinguish active enzyme from their zymogen[24] or inhibitor-bound[40] forms. Moreover, because ABPP probes label the active sites of their target enzymes, these reagents can be used as competitive profiling tools for inhibitor discovery in native proteomes, providing concomitant readouts of potency and selectivity.[41–44] Finally, advanced strategies for ABPP in which bulky reporter tags, such as fluorophores or biotin, are replaced with sterically benign azide or alkyne groups have enabled profiling of enzyme activities in living cells and animals.[45–47] In these experiments, downstream conjugation to reporter tags is accomplished by a bio-orthogonal reaction like the copper(I)-catalyzed azide–alkyne cycloaddition (click chemistry[45,46]) or the Staudinger ligation.[48] As we will discuss in the next section, tag-free versions of ABPP have fostered the development of high-content analytical platforms that could not otherwise have been achieved with conventional probe designs.

Biological applications of ABPP have already yielded several exciting discoveries. For example, profiling of cell and animal systems has identified enzyme activities elevated in a variety of disease models, including aggressive cancer cells and tumors,[36,40,49,50] invasive malaria parasites,[51] and obese livers.[37] Additionally, cell-based screening with libraries of ABPP probes has resulted in the discovery of a compound with anti-proliferative activity that inhibits the glycolytic enzyme phosphoglycerate mutase.[52] Certain ABPP probes also exhibit adequate cell permeability and target selectivity to serve as effective imaging tools to visualize enzyme activity in living systems.[50,53] Finally, ABPP methods have been implemented to characterize the proteome-wide selectivity of covalent enzyme inhibitors *in vivo*, facilitating the optimization of inhibitor dosing for pharmacological applications.[47]

The aforementioned studies have showcased several of the advantages of ABPP compared to conventional genomic and proteomic approaches. For example, ABPP has identified changes in protease activity that occur in the absence of alterations in protease expression,[40,49] as well as enzymes that are selectively active in living systems.[46,52] ABPP probes have also proven to be highly versatile proteomic tools, capable of profiling any fraction of cells and tissues, including traditionally challenging proteins like highly glycosylated and membrane-associated proteins.[40,49] Finally, by capturing manageable portions of "proteomic space" based on shared functional properties, rather than mere expression level, ABPP provides exceptional access to low-abundance proteins.[40] Indeed, based on this last feature, it could be argued that the theoretical information content of most ABPP experiments far exceeds the actual data procured due to limitations in downstream analytical technologies. Our own experiences would support this contention, as we have long recognized that the gel-based platforms originally utilized for ABPP display inherent resolution and sensitivity limits that preclude the comprehensive characterization of probe-labeled enzyme activities (and their sites of modification) present in individual proteomic samples. With the number of enzyme families addressable by ABPP continuing to increase at a remarkable pace, a pressing need for complementary innovations in methods for the analysis of probe-labeled proteomes is apparent. This new challenge is being met with great vigour by the chemical proteomics community, and several powerful "gel-free" technology platforms have recently been introduced that enhance the data acquisition and analysis phase of ABPP. Here, we will review these analytical platforms and discuss their respective strengths and limitations as pertains to the principal goal of augmenting the information content achievable in chemical proteomic experiments.

Advances in analytical platforms for ABPP – moving beyond the gel

As mentioned in the previous section, the most mature format for the characterization of probe-labeled proteomes in ABPP experiments is in-gel fluorescence scanning (IGFS) (Fig. 3).[54] IGFS has some excellent attributes, including its simplicity, robustness, throughput, and sample requirements. For example, our lab routinely analyzes more than 100 probe-labeled proteomes per day by one-dimensional IGFS. Modest quantities of proteome are required (10–30 μg of protein per sample), and the targets of fluorescent ABPP probes can be rapidly quantified by IGFS. However, the target portfolio of most ABPP probes far exceeds the number of proteins that can be resolved by 1D-SDS-PAGE. Additionally, many probe-labeled proteins are too low in abundance to visualize by this method. Finally, IGFS does not assign a molecular identity to probe-labeled proteins (or define sites of probe modification); this information must instead be garnered in a separate set of laborious studies using biotinylated probes, avidin enrichment, SDS-PAGE separation of avidin-enriched proteins, and MS analysis of in-gel trypsin-digested proteins.[24,40]

The limitations of IGFS have inspired efforts to move beyond gel-based formats for ABPP toward the creation of advanced analytical platforms that display superior sensitivity, resolution, and information capacity.

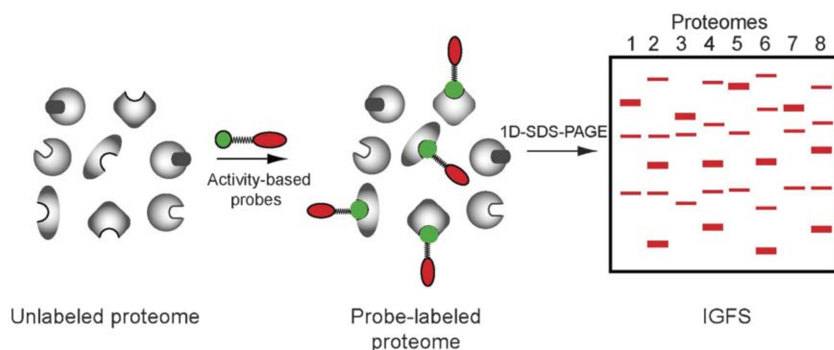

Fig. 3 Analysis of ABPP experiments by in-gel fluorescence scanning (IGFS). Proteomes are treated with fluorophore-conjugated ABPP probes, separated by 1D-SDS-PAGE, and probe-labeled enzymes visualized by in-gel fluorescence scanning (IGFS). Active and inactive enzymes depicted with open and blocked active sites, respectively.

LC-MS platforms for ABPP

Active site-peptide profiling – consolidated identification of probe targets and sites of probe modification

Adam and colleagues have described an LC-MS strategy for ABPP that consolidates into a single step the identification of protein targets of chemical probes and the specific residues labeled by these reagents.[55] In this approach, probe-treated proteomes are digested in solution with trypsin and incubated with an affinity capture matrix (*e.g.*, agarose beads conjugated with avidin or anti-rhodamine antibodies for biotinylated and rhodamine-tagged probes, respectively) to specifically isolate probe-labeled peptides (Fig. 4a). These peptides are then eluted from the affinity matrix and analyzed by LC-MS/MS to concurrently identify the protein targets of ABPP probes and their site of probe modification.

This LC-MS platform was applied to characterize the proteome-wide reactivity of a rhodamine-tagged phenyl sulfonate ester (PS-rhodamine) probe. PS probes had previously been shown by gel-based ABPP to target several mechanistically distinct enzymes in an active site-directed manner.[36,38] However, the active site residues labeled by PS-rhodamine remained unknown. Using LC-MS methods, Adam and colleagues identified the sites of probe labeling for five enzyme targets of PS-rhodamine in cell and tissues proteomes. In each instance, probe labeling was found to occur on a conserved active site residue, including catalytic nucleophiles and residues of unassigned mechanistic function. These results suggest that ABPP may prove useful for mapping novel catalytic residues in enzyme active sites.

Several advantages of active site-peptide profiling by LC-MS are apparent when compared to whole protein profiling by IGFS. The consolidated identification of both protein targets and sites of probe modification is particularly valuable for screening new probes with uncharacterized protein reactivity, enabling the discrimination of probes with high selectivity for enzyme active sites from those that show unacceptable levels of non-specific (*e.g.*, surface residue) labeling. LC-MS also offers an orthogonal separation platform to resolve probe-labeled enzymes of simliar molecular mass that co-migrate by SDS-PAGE. As long as these enzymes contain distinct active site sequences, their probe-labeled tryptic peptides should be resolvable by LC. Finally, by introducing an affinity enrichment step prior to LC-MS analysis, active site-peptide profiling facilitates the characterization of low abundance targets of ABPP probes.

On the negative side, by exclusively isolating probe-labeled peptides, active site-peptide profiling discards a large quantity of potentially useful proteomic information. For example, certain proteins targeted by ABPP probes may possess post-translational modifications that regulate their activity. Unless these modifications happen to reside on the probe-labeled peptide itself, they will not be detected. Additionally, access to the entire tryptic digest of probe-labeled proteins can increase the statistical confidence of protein assignments and assist in quantifying the relative levels of these proteins in comparative proteomic experiments.

ABPP-MudPIT – an LC-MS platform for comparative profiling of enzyme activities in any proteomic sample

Seeking an LC-MS platform to perform comparative ABPP experiments in a quantitative mode, Jessani and colleagues have combined this chemical proteomic method with the multidimensional protein identification technology (MudPIT[14]).[56] The resulting ABPP-MudPIT approach involves, first, treatment of proteomes with biotinylated probes, and then enrichment of probe-labeled proteins using avidin-conjugated beads, on-bead trypsin digestion, and multidimensional LC-MS/MS analysis of the resulting tryptic peptide mixture (Fig. 4c). ABPP-MudPIT was applied to profile enzyme activities in a panel of primary human breast tumors, resulting in the identification of more than 50 probe-labeled enzymes in a single experiment.[56] Moreover, the relative quantity of enzyme activities could be estimated using spectral counting methods,[12,13] enabling the discovery of a set of enzymes selectively elevated in aggressive (*i.e.*, estrogen receptor-negative) breast tumors.

ABPP-MudPIT exhibits several beneficial features for in-depth proteome analysis, including exceptional resolution (owing to multidimensional separation) and sensitivity (owing to affinity enrichment of probe-labeled targets), as well as coupled target detection and identification. These attributes

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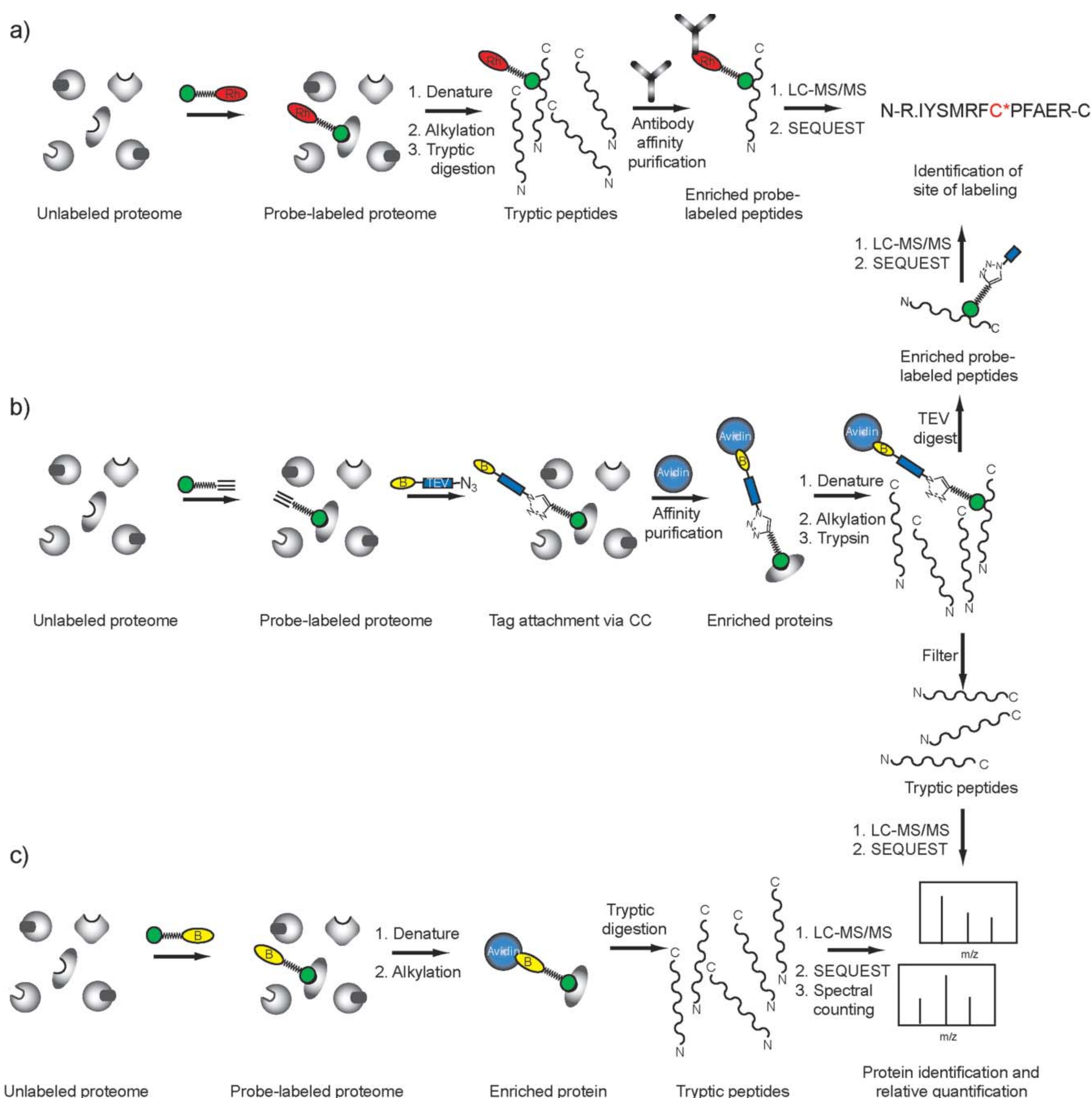

Fig. 4 Analysis of ABPP experiments by various LC-MS platforms. a) Active site-peptide profiling. Probe-treated proteomes are digested with trypsin and the labeled peptides enriched with anti-rhodamine (Rh) antibodies (or avidin for biotinylated probes). LC-MS/MS analysis of the enriched peptide mixture reveals the identity of probe-labeled proteins and sites of probe modification. b) TOP-ABPP. Here, proteomes are treated with alkynylated probes and then subjected to CC to attach a biotin (B)-azide tag containing a TEV protease cleavage site. Biotinylated proteins are enriched with avidin beads and subjected to on-bead trypsin digestion. Filtration provides tryptic peptides that are analyzed by LC-MS/MS to provide information on entire protein sequences. Probe-labeled peptides are then released from avidin beads by TEV protease and analyzed by LC-MS/MS to provide information on probe labeling sites. c) ABPP-MudPIT. Proteomes are treated with biotinylated probes and probe-labeled proteins enriched with avidin beads, digested on bead with trypsin, and analyzed by MudPIT to provide protein identities and relative activity level (based on spectral counts).

suggest that ABPP-MudPIT could be applied to any proteomic sample, even those that are highly complex in molecular and cellular composition (such as primary human tumors). Additionally, by capturing intact probe-labeled proteins, ABPP-MudPIT provided much greater sequence coverage of these proteins compared to active site-peptide profiling, thus permitting estimation of their relative levels by spectral counting. On the other hand, ABPP-MudPIT is not well-suited for the characterization of probe labeling sites, as, in this method, probe-modified peptides either co-elute in a background of excess unlabeled tryptic peptides or remain attached to the affinity support.

A tandem orthogonal proteolysis (TOP) strategy for high-content ABPP

The complementary virtues and limitations of active site-peptide profiling and ABPP-MudPIT suggest that an ideal LC-MS platform might embody a unification of these methods. Toward this goal, Speers and colleagues recently introduced a tandem orthogonal proteolysis (TOP) strategy for the parallel characterization of probe-labeled proteins and sites of probe modification.[57] The TOP method was combined with ABPP by exploiting click chemistry (CC) techniques,[45,46] as outlined in Fig. 4b. In this approach, proteomes are first labeled with an alkynyl ABPP probe, after which a CC reaction is used to introduce a biotin tag with a tobacco etch virus protease (TEV) cleavage site. Tagged proteins are then subject to avidin/streptavidin enrichment and on-bead trypsin digestion. The supernatant, which contains unlabeled peptide fragments of the enriched proteins, is isolated by filtration and then the probe-labeled peptides eluted from the beads by incubation with TEV. The trypsin and TEV digests are then analyzed in sequential LC-MS/MS experiments to characterize probe-labeled proteins and site(s) of probe modification, respectively. Notably, because the azide-TEV-biotin portion of the probe is added following proteome labeling, the TOP-ABPP method circumvents the potential negative impact that this sterically cumbersome reporter tag might exert on specific probe–protein interactions.

Using TOP-ABPP, Speers and colleagues identified more than 30 targets of a PS-alkyne probe in mouse heart proteome.[57] Multiple types of specific labeling events were observed, including those that occurred on active site residues, on ''exo-site'' residues that correspond to sites of endogenous regulation by glutathione and nitric oxide, and on conserved residues of unknown function. For the last two classes of ''unanticipated'' targets of PS probes, their parallel identification in both the trypsin and TEV phases greatly increased confidence in the accuracy of their assignment. Overall, these findings indicate that a wide range of functional residues can be targeted by the PS class of ABPP probes. More generally, by combining the attributes of active site-peptide profiling, MudPIT, and CC technologies, TOP-ABPP should offer a powerful strategy to perform high-content comparative proteomics experiments *in vitro* or *in vivo*.

Summary

Advances in LC-MS platforms for ABPP have significantly increased the information content achievable in chemical proteomic experiments. Indeed, it is now possible to perform ABPP experiments in an entirely gel-free mode, where the relative levels of enzyme activities can be determined in two or more proteomes in parallel with a full characterization of their modification state (probe-labeling site and other modifications). In spite of these advantages, LC-MS platforms for ABPP are not without some significant limitations. Most notably, these approaches are, in general, quite time-consuming (many hours per sample), difficult to perform in parallel, and require large quantities of proteome (~ 0.5 mg or more). Thus, a researcher who is confronted with the challenge of rapidly analyzing numerous proteomic samples of limited quantity (not an uncommon situation, especially in clinical labs working with primary human specimens) is left with an unsatisfactory set of options – either low-resolution ABPP of many samples by IGFS or high-resolution analysis of a handful of samples (possibly pooled to provide sufficient material) by LC-MS methods. Fortunately, additional gel-free platforms for ABPP have begun to emerge that address the problem of balancing breadth and depth in functional proteomic experiments.

A capillary electrophoresis platform for ABPP

The relatively low-throughput and high sample demands of LC-MS methods have inspired the creation of a capillary electrophoresis (CE) platform for ABPP (Fig. 5).[58] This CE strategy, which was developed by Okerberg and colleagues, also exploits the fact that ABPP probes generally label single residues in enzyme active sites, thus making it possible to analyze proteomes at the level of probe-modified active site peptides (*e.g.*, following trypsin digestion). Active site-peptide profiling by CE was accomplished using low-run buffer pH in coated capillaries, which minimized electroosmotic flow and absorption of peptides to the capillary surface. This technology affords very high-resolution and reproducible separations of probe-labeled peptides, which can be detected and quantified by laser-induced fluorescence (LIF).

Okerberg and colleagues demonstrated the value of CE-based ABPP by evaluating the proteome-wide target selectivity of the serine protease inhibitor nafamostat. Several members of the kallikrein family of serine proteases were identified as targets of nafamostat. Importantly, these enzymes all share approximately the same molecular mass (25–30 kDa) and thus are difficult to characterize by gel-based ABPP due to co-migration. These results highlight one of major advantages of CE-based ABPP, which provides a protein-size independent,

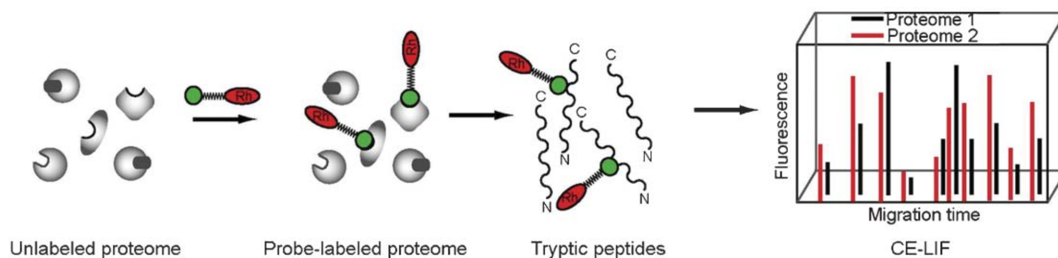

Fig. 5 Analysis of ABPP experiments by capillary electrophoresis (CE). Proteomes are treated with fluorophore-tagged probes, digested with trypsin, and the probe-labeled peptides separated by CE and detected by laser-induced fluorescence (LIF).

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high-resolution platform for comparative proteomics. Other attributes of this approach include high-throughput and low sample requirements. Indeed, 96-channel CE instruments are commercially available, which could afford a remarkable multiplexing capacity to ABPP experiments. Moreover, because CE runs require negligible quantities of proteome (10–20 nL at 1–10 ng protein/nL), this method is particularly well-suited for samples of trace amount (*e.g.*, tumor biopsies). One potential shortcoming of CE-based ABPP is that, like gel-based methods, the identities of probe-labeled proteins are not immediately apparent. However, this deficiency can be addressed by running parallel LC-MS experiments and cross-correlating the data sets to assign protein identities to specific peaks on the CE.[58]

A microarray platform for ABPP

DNA microarrays have revolutionized our ability to characterize genes and transcripts on a global scale.[59] Complementary studies with protein microarrays have also proven valuable for proteomics.[4,15,60] In particular, much effort has been put forth to develop antibody microarrays for the parallel analysis of protein expression and modification state. Recently, a microarray platform for ABPP was introduced by Sieber and colleagues.[61] In this approach, proteomes are first treated with ABPP probes in solution, and then the labeled enzymes are captured and visualized on glass slides displaying an array of anti-enzyme antibodies (Fig. 6). ABPP microarrays were found to exhibit superior sensitivity and resolution compared to gel-based methods, permitting the parallel analysis of several protease activities in proteomes. For example, ABPP microarrays were capable of detecting 2–8 ng/mL of the clinical prostate cancer biomarker prostate specific antigen (PSA), which was a $\sim$ 50-fold increase in sensitivity compared to gel-based ABPP and in the range of endogenous serum levels for this serine protease. These results suggest that ABPP microarrays could provide a strategy to measure free (active) *versus* total PSA to assist in the diagnosis of prostate cancer.

ABPP microarrays display many of the advantages of both LC-MS and CE-based methods. Most notably, by incorporating orthogonal strategies for protein labeling (ABPP) and capture (antibody), ABPP microarrays consolidate into a single step the isolation, detection, and identification of probe-labeled enzymes. Thus, like LC-MS, ABPP microarrays assign a molecular identity to probe-labeled proteins, but do so with minimal sample requirements and exceptional throughput (akin to CE). ABPP microarrays also address some key limitations of conventional antibody microarrays by eliminating the need for random protein labeling and/or secondary antibodies. ABPP microarrays currently possess two major limitations. First and foremost, only a limited number of high-quality antibodies are available for incorporation into ABPP microarrays. Although efforts are underway to increase the repertoire of microarray-compatible antibodies,[15] this potentially finite technical problem is still likely many years away from a complete solution. The advent of additional high-specificity capture reagents, including oligonucleotide-tagged small molecule probes[62] and aptamers,[63] may offer complementary formats for ABPP microarrays. A second shortcoming of ABPP microarrays is that this approach requires prior knowledge of the protein targets of probes and is therefore not well-suited for the discovery of unanticipated sites of probe labeling in proteomes. Nonetheless, if a proteome-wide collection of microarray-compatible antibodies eventually becomes available, it is conceivable that ABPP microarray experiments could be performed in the "target discovery" mode (*i.e.*, without the need to anticipate which antibodies should be included on the microarray).

Conclusions and future perspectives

Here, we have attempted to highlight a key, but often overlooked feature of chemical proteomics experiments – the analytical platforms utilized for data acquisition and analysis. ABPP experiments originally relied on 1D or 2D gels for the separation and visualization of probe-labeled proteins. The limited resolving power and sensitivity of these methods, coupled with their inability to directly assign molecular identities to probe targets, have inspired the advancement of gel-free technologies for evaluating probe–proteome reactions. These advanced analytical platforms utilize a range of separation and detection strategies, including LC-MS, CE-LIF, and antibody microarrays, to achieve an unprecedented breadth and depth of proteome coverage in ABPP investigations. The complementary strengths and weaknesses of each of these methods (Table 1) suggest that the selection of an appropriate analytical platform should be guided by the specific experimental question being addressed. For example, the *in-depth* comparative characterization of a limited number

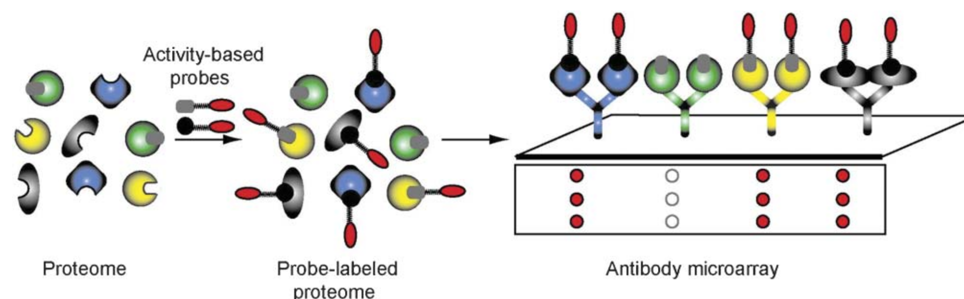

Fig. 6 Analysis of ABPP experiments by antibody microarrays. Proteomes are treated with fluorophore-tagged probes and probe-labeled enzymes captured and visualized on glass slides arrayed with anti-enzyme antibodies.

Table 1 A comparison of the properties of analytical platforms for ABPP

Analytical platform	Sensitivity limit (pmol/mg proteome)	Consumption[a] (μg)	Throughput[b] (per day)	Protein identity	Site of labeling
IGFS	1–10	10–30	100	No[c]	No
LC-MS					
Active-site peptide profiling	0.1–1	> 500	10	Yes	Yes
ABPP-MudPIT	0.1–1	> 500	2	Yes	No
TOP-ABPP	0.1–1	> 500	2	Yes	Yes
CE-LIF	0.05–0.1	0.01–0.1	1000	No[c]	No
Microarray	0.1–1	1–3	1000	Yes	No

[a] Estimated proteome material required to achieve maximal information content. [b] Theoretical throughput based on instrument capacity. [c] Identification possible in combination with an additional LC-MS step.

of plentiful proteomes (*e.g.*, cancer cell lines or tissues from genetically engineered mice) may best be accomplished by LC-MS techniques (*e.g.*, ABPP-MudPIT). In contrast, the targeted analysis of a set of enzyme activities across a large number of proteomic samples of limited quantity (*e.g.*, tumor biopsies) would be more effectively addressed by CE-LIF or antibody microarray methods. When looking forward, ABPP microarrays perhaps hold the greatest potential to mature into an optimal platform for functional proteomic experiments, as this technology exhibits exceptional sensitivity and throughput, while consuming minimal amounts of sample. Of course, the extent to which ABPP microarrays find wide use in the field of functional proteomics will depend on the ease with which a proteome-wide set of high-specificity capture reagents can be created.

Finally, it merits emphasis that the successful development of each of the analytical platforms described in this review was only possible because of the versatility afforded by small-molecule probes, which constitute the basic currency of ABPP. By synthesizing and applying chemical probes to interrogate the proteome, ABPP researchers have opened up new possibilities for downstream analysis, including tools for protein enrichment (*e.g.*, biotinylated probes for ABPP-MudPIT), detection (*e.g.*, fluorescent probes for CE-LIF and ABPP microarrays), and functional characterization (*e.g.*, TEV-cleavable probes for active site-peptide profiling). Future advances in ABPP, and chemical proteomics in general, will likely require the continued integration of synthetic and analytical chemistry efforts toward the ultimate goal of creating a universal strategy for the comprehensive analysis of enzyme activities in any proteomic sample.

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