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Activity-Based Protein Profiling: New Developments and Directions in Functional Proteomics

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Proteomic screening has become increasingly insightful with the availability of novel analytical tools and technologies. Detailed analysis and integration of the profound datasets attained from comprehensive profiling studies are enabling researchers to dig deeper into the foundations of genomic and proteomic networks, towards a clearer understanding of the intricate cellular circuitries they manifest. The major difficulty often lies in correlating the patho/physiological state presented with the underlying biological mechanisms; therefore, identification of causal variants as therapeutic targets is extremely important. Herein, we will describe methods that address this challenge through activity-based protein profiling, which applies chemical probes to the comparison and monitoring of protein dynamics across complex proteomes. Over recent years such activity-based probes have been creatively augmented with applications in gel-based separations, microarrays and in vivo imaging. These developments offer a newfound ability to characterise and differentiate cells, tissues and proteomes through activity-dependent signatures; this has expanded the scope and impact of activity-based probes in biomedical research.

Introduction

Advancement of methodologies in DNA sequencing will soon bring the $1000 genome to realisation.[1] This will hide little in terms of the genetic differences that set organisms or individuals apart. While these movements accelerate the pace by which complete genomes are unravelled (with over 680 genomes sequenced thus far),[2] there is a widening gap in deciphering the functions of the many proteins they encode.[3,4] The 24500 reading frames in the human genome[5] could alone generate up to a million diverse protein entities,[6] through processes like alternative gene splicing and post-translational modifications.[7] The field of proteomics strives to develop global methods for the analysis of protein expression and function at an organism/system-wide level, and dissect the complex dynamics of the protein ecosystem.[8] Platforms like two-dimensional gel electrophoresis[9] and mass spectrometry (enhanced with chromatographic separations—MudPIT,[10] or isotope coding—ICAT[11]) provide valuable insight by quantitatively resolving differences in protein abundance. They further enable biological samples, such as cellular extracts, tissues or even whole organisms, to be comparatively evaluated in high-throughput. These methods, however, lack the inherent ability to profile and distinguish proteins according to their actual biological activities or functional state, which has more important bearings on understanding the implications of these macromolecules in vivo.[12,13] This has prompted the development of alternative strategies for the discovery and characterisation of enzyme activities within highly complex biological samples.

Activity-based protein profiling presents great promise in attempting to address some of these issues by delivering a vital capacity to characterise the activities of enzymes en masse.[7,8,12–14] At the heart of this profiling strategy lies the activity-based probe (ABP) that is only responsive to catalytically active enzymes and not the inhibited or zymogen (precursor) forms. Since its introduction in the late 1990s, creative designs have expanded the arsenal of these small-molecule probes against diverse classes of enzymes, which range from proteases (serine,[15] cysteine,[16,17] aspartic,[18,19] metallo[20,21,22]) to glycosidases,[23] kinases,[24] phosphatases[25,26] and many others. ABPs rely on the principle of targeting the enzyme active site by using a specially designed "warhead". These magic bullets attach themselves covalently to the enzyme, and allow the enzyme adduct to be visualised or isolated and identified. ABPs can be tuned to desired levels of selectivity through modifications to the warhead; this allows a wide spectrum of broadly specific (mechanistically diverse) or narrowly specific (highly homologous) enzymes to be analysed rapidly in the presence of competing proteins or extracts. The majority of ABPs thus comprise: 1) a warhead (or reactive group) that targets the probe to the enzyme active site, 2) a flexible linker that is decorated with 3) a reporter tag—typically a dye for fluorescence-based visualisation or a tag (like biotin) for isolation, purification and characterisation (Figure 1A).

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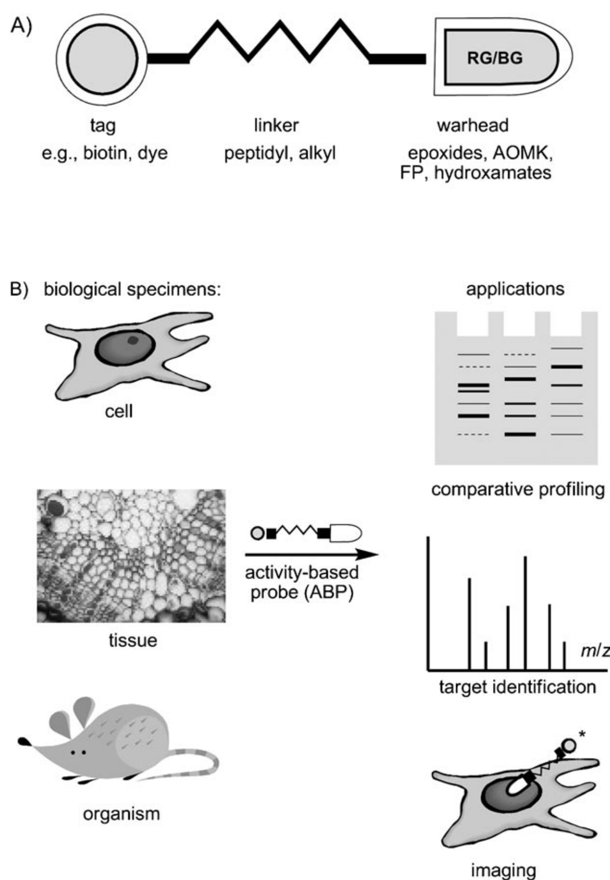

Figure 1. A) The configuration of an activity-based probe (ABP). The warhead contains a reactive group or a binding group that targets the ABP to the active site of enzymes; FP: fluorophosphonate, AOMK: acyloxymethyl ketones. B) The diverse scales of proteomes that can be profiled and examples of applications of ABPs. This includes one dimensional SDS-PAGE separation and fluorescence visualisation, MS-based target discovery and microscopy to localise the target enzyme in vivo.

ABPs can be classified as either mechanism-based or affinity-based, depending on the mode by which the covalent linkage with the enzyme is established. Mechanism-based probes contain warheads that are chemically converted into reactive species by the cognate enzyme.[14] These probes do not require additional binding elements. In contrast, the warhead of affinity-based probes merely localises it to the enzyme active site; this necessitates a separate binding element (like a photo-cross-linking group) within the probe, to effect covalent labelling. There are two strategies in employing ABPs, which again draw from the design of the warhead, namely directed approaches, which target specific classes of enzymes, or nondirected approaches, which profile multiple enzyme classes simultaneously. Directed approaches exploit the mechanistic understanding of the enzyme for the controlled and predictable targeting of the desired protein class. Classical examples of such activity-based protein profiling approaches include the study of serine hydrolases by using fluorophosphonate probes[15,27] and cysteine proteases with epoxy-based derivatives.[16] The nondirected approach, on the other hand, involves the synthesis of structurally diverse candidate probe libraries that are screened against the proteome for multiple activity-dependent labelling events. Various ABPs have been developed for such whole proteome analysis, including early studies by Adam et al. that successfully differentiated various tissue samples by labelling with sulfonate ester probe libraries followed by fluorescence visualisation on gel.[28]

Using specific examples, we will showcase the range of available ABPs and their applications in functional proteomics (Figure 1 B). The creative use of ABPs reveals details of the mechanisms, active-site architecture, substrate/inhibitor selectivity and functional roles of enzymes within biological systems.[12,29] We shall begin by exploring the considerations that impact the design of these small-molecule ABPs, and illustrate the ways in which they have been exploited recently.[30]

Design Strategies for ABPs

The incorporation of a biologically active small-molecule warhead within a multifunctional probe involves several challenging tasks. Careful synthetic planning is required to incorporate functional components (like the tags or fluorophores) with potentially sensitive functional groups in the warhead. The size and cell permeability are also crucial considerations, especially for probes that are to be used in vivo for tracking or imaging. The design of the warhead, however, remains the most crucial and difficult part of the probe-design process; the various available approaches are highlighted in the following paragraphs.

One strategy in warhead design is to use a substrate mimic that contains an electrophilic group that can react covalently with a nucleophilic active-site residue that is essential for the catalytic activity of the enzyme (Figure 2). In a recent example of this approach, Cole and co-workers designed a mimic of acetyl CoA that functions as a general probe for labelling acetyltransferases—a class of enzymes with little structural homology across the family, but which collectively require acetyl CoA for activity.[31] Unlike a fluoroacetonyl CoA probe that cross reacted with kinases, the authors went on to design a sulfoxycarbonyl CoA variant that was found to more specifically target acetyltransferases (**1**). The transfer of a desthiobiotin affinity handle onto the active site cysteine residue allowed the enzymes to be selectively tagged and detected, even in cellular extracts. Zhang and co-workers synthesised and tested α-bromobenzylphosphonate-based probes that could form covalent adducts with protein tyrosine phosphatases (PTPs) but not other phosphatases (**2**).[26] The high specificity of the probe allowed the detection of PTPs in cell lysates and it was applied to the profiling of PTP expression across normal and cancer cell lines. Such comparative approaches could lead to the identification of therapeutic targets or diagnostic biomarkers. In a nondirected combinatorial approach to profiling by ABPs, Barglow and Cravatt identified a probe for the nitrilase superfamily (a class of enzymes that cleave C–N bonds by using a Cys-Lys-Glu catalytic triad). A chloroacetamide moiety (as the Cys-reactive group) linked with a Leu-Asp peptide motif were discovered to effectively target members of the nitrilase family, including nitrilase 2 and ureidoproprionase β (**3**).[32] A similar

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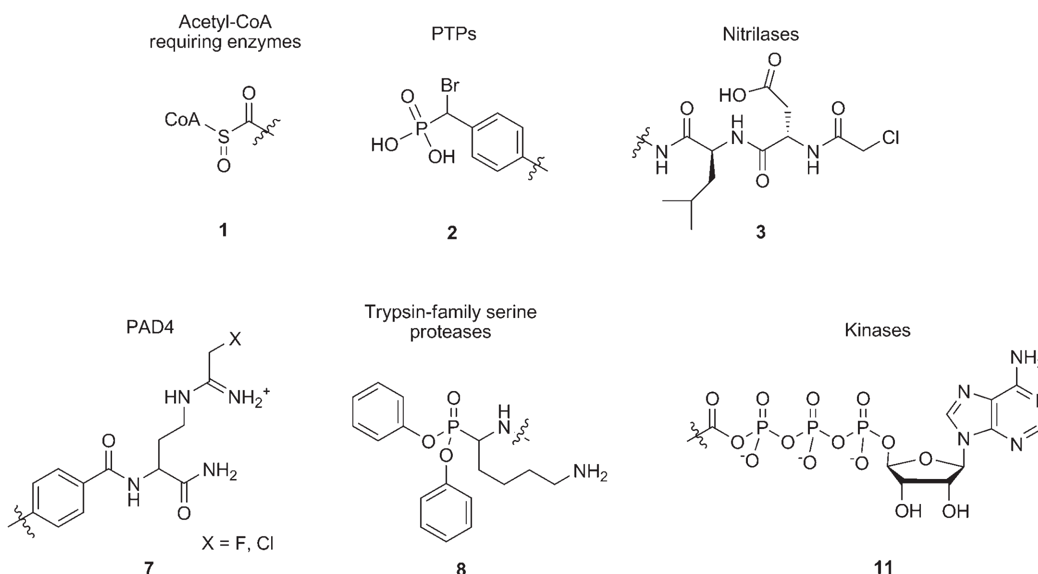

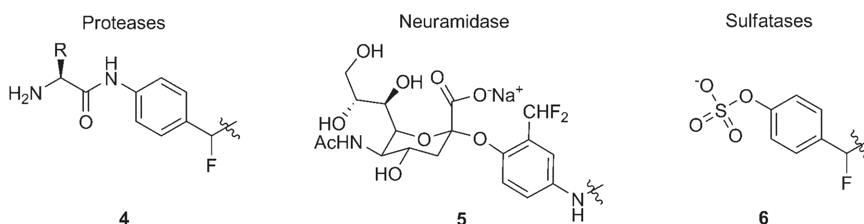

Figure 2. Warhead design of activity-based probes (ABPs). A variety of electrophilic and quinone or quinolimine methide-based warheads have been designed against different classes of enzymes.

strategy has been adopted in the design of ABPs against ubiquitin-specific proteases, and for the study of their specificity, activity and localisation.[33]

There are other classes of ABPs that are also designed to react covalently with enzymes by mimicking substrate design. The target enzymes act on these probes and form a reactive intermediate, which goes on to react with a proximal nucleophilic residue in the enzyme active site and covalently links the probe and enzyme. A number of probes based on the quinolimine or quinine methide reactive intermediate have found interesting applications in protein profiling and detection (Figure 2). Our group has demonstrated the use of a panel of such probes that target the major classes of proteases in protease fingerprinting experiments.[34,35] Sixteen probes containing different P_1 amino acid residues[36] were assembled and assayed against a variety of proteases, including serine, cysteine, aspartic and metalloproteases (**4**). By quantifying the different labelling intensities with each probe, we generated unique substrate fingerprints for each enzyme (Figure 3 A).[34] Lu et al. have also applied quinone methides in the development of probes against neuramidase—the exoglycosidase displayed on the surface of influenza viruses that promotes virulence (**5**).[37] The probes were shown to be able to label whole virus particles and even capture the virus particles on 96-well ELISA plates. A similar design was used in ABPs developed against sulfatases (**6**).[38]

Another concept frequently adopted in the design of ABPs is the modification of irreversible inhibitors with the addition of linkers and relevant fluorescent or affinity tags. Known irreversible inhibitors have the advantage of serving both as the reactive group and the recognition element, and confer the required selectively to the ABP. In one such example ABPs for protein arginine deiminase 4 (PAD4, an enzyme implicated in rheumatoid arthritis) were synthesised by tagging known haloacetamidine-based inactivators of PAD4 with a rhodamine dye (**7**).[39] This modification of the original fluoro- and chloroamidine inhibitors did not compromise their potency. It was also found that the fluoroamidine probe was able to target only the active form of the enzyme with high selectivity.

Given the success of using well-characterised inhibitors as reactive groups in proteolytic enzymes, researchers now endeavour to develop probes that are selective for subfamilies of proteins. This could provide a useful tool in pointed assessments of a desired panel of enzymes for imaging, functional

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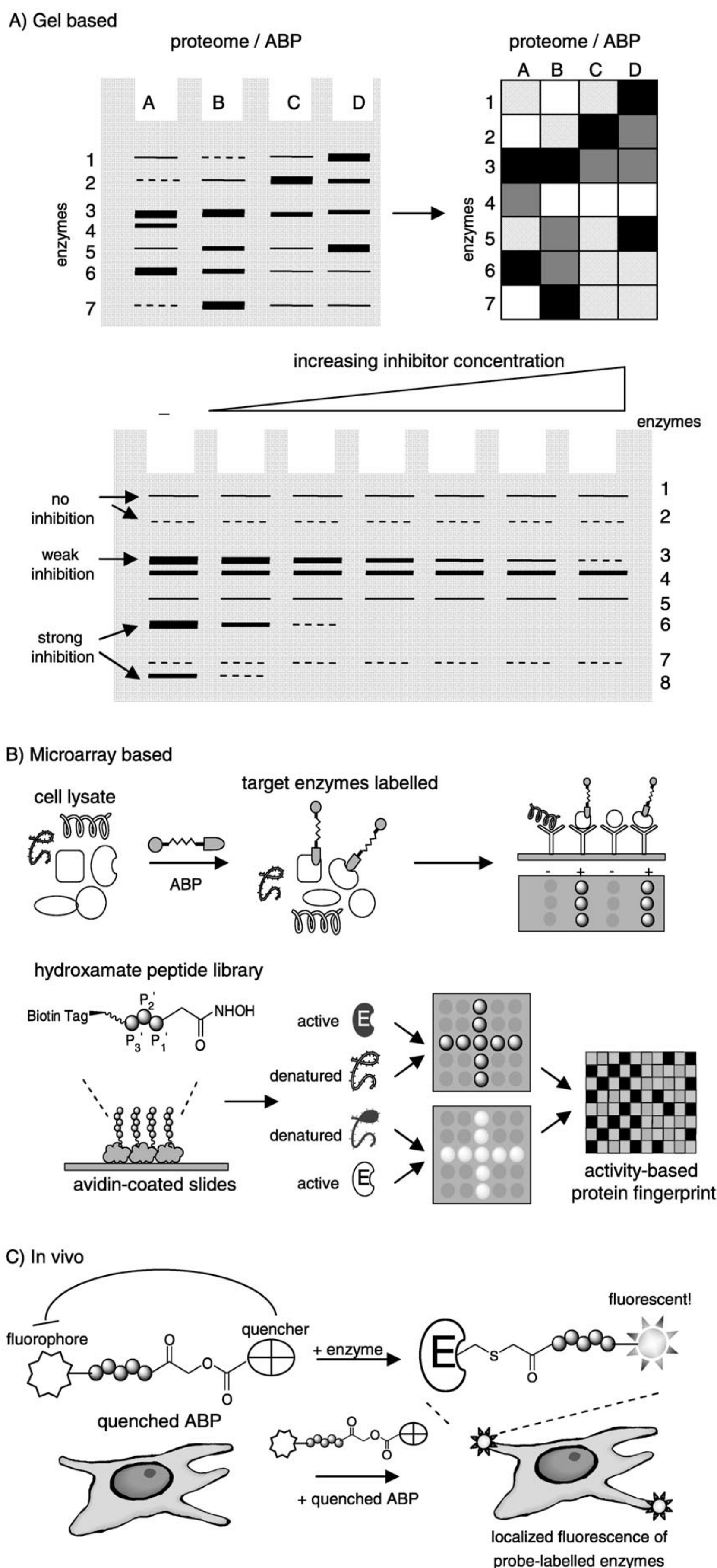

Figure 3. Applications of activity-based protein profiling. A) Gel-based applications. Top: comparative proteomics across different tissues or proteome sources,[60] or comparison of enzyme properties through screening against a panel of probes;[34] bottom: concentration-dependent application of inhibitors allows the determination of relative potency against a range of enzymes simultaneously (provided that a broad spectrum ABP is used for labelling).[61,62] B) Microarray-based applications. Top: application of an antibody microarray to detect positively labelled enzymes in cell lysates;[67] bottom: the use of a reciprocal labelling strategy to elucidate activity-based protein fingerprints.[72] C) In vivo applications: the use of a quenched acyloxymethyl ketone warhead (that targets cysteine proteases) in localising enzymatic activities in live cells.[74]

annotation or comparative proteomics. With this objective, Baruch et al. have developed ABPs specific for the trypsin subfamily of serine proteases, by taking advantage of the diphenylphosphonate scaffold (**8**).[40] In other examples, ABPs with added peptide-recognition elements, which contribute additional binding interactions with the protease active site, have been found to be effective in selectively targeting cysteine proteases like cathepsin C,[41] the broader papain family[42] as well as caspases.[43,44] The same success has been achieved by using zinc-binding hydroxamate-based derivatives against matrix metalloproteases, by modifying the neighbouring prime sites with natural or unnatural amino acids[45] and similarly so with γ-secretases.[46] These accomplishments herald the growing ability to design selective ABPs against almost any class of enzyme with controlled targeting precision.

Affinity-based ABPs that utilise reversible inhibitors in the design of the probe warhead have also been developed. For enzymes like metalloproteases, for which the hydrolysis of substrates does not involve inter-

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mediates that are transiently linked with the enzyme, it is difficult to design probes based on substrate mimics. As described earlier, cross-linking groups have to be incorporated into such ABPs to provide covalent attachment to the enzyme by UV irradiation. These ABPs typically employ a modified reversible inhibitor as the recognition element to deliver the probe to the active site. Several groups, including ours, have successfully developed such ABPs for metalloproteases[20,21,22] and aspartic proteases.[19] Recently, similar probes for histone deacetylases[47] and methionine aminopeptidases[48] have been reported.

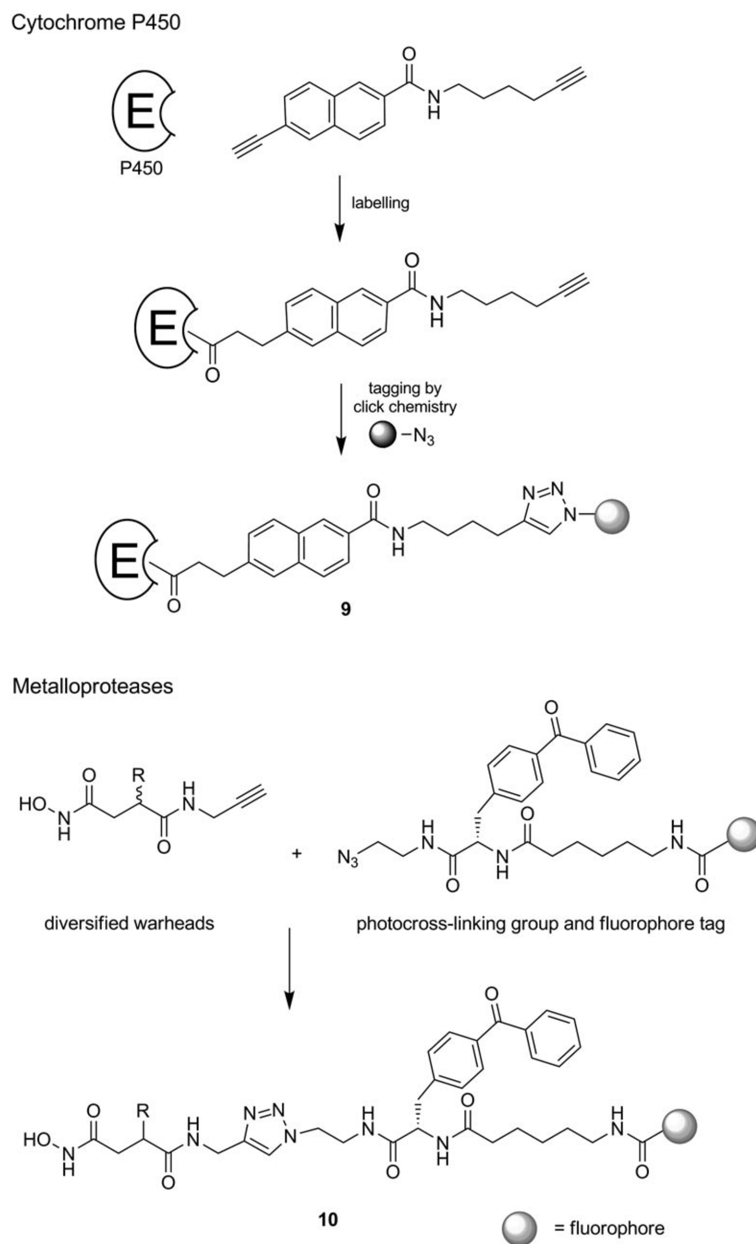
Click chemistry based design concepts

To increase the versatility and synthetic convenience of deploying ABPs in both protein visualisation and/or isolation, several groups have conceived the use of "clickable" handles to incorporate reporter groups postlabelling. In these cases, the probe and tag are each engineered with a complementary pair of small, orthogonally reactive chemical entities (Figure 2). Staudinger ligation, which covalently links a phosphane and azido groups, or Huisgen's 1,3-dipolar cycloaddition between an alkyne and azide, are examples of such click-chemistry approaches. A desirable feature of this method is that it reduces the size of the ABP—the incorporation of large fluorophores or tags at the outset can inhibit the delivery of the probe into the cell. Such a two-step labelling approach could thus provide the desirable utility for studying biologically relevant systems in their native state.

Speers et al. applied this strategy to compare differences in enzyme activities between invasive and noninvasive breast cancer cells.[49] It was found that the placement of the alkyne or azide on either the probe or reporter contributed varying effects to the selectivity, binding kinetics and signal-to-noise (background binding). The alkyne phenylsulfonate ester probe was used in vivo because of its higher sensitivity. It was able to discern and quantify candidates like protein disulfide isomerase and enoyl CoA hydratase that were down-regulated in invasive cell types. A trifunctional tag containing the complementary azide handle as well as both rhodamine fluorophore and biotin group was used for chromatographic mass spectrometry based identification of the enzymes of interest. Ovaa and colleagues developed click-ABPs against the proteasome by using a cell permeable irreversible inhibitor $AdaAhx_3L_3VS$, modified with an azide handle.[50] The probe was shown to successfully label several active proteasome subunits in living cells.

In a recent example, Wright and Cravatt devised ABPs against the cytochrome P450 superfamily—a diverse group of enzymes with broad substrate specificities.[51] These proteins are of particular interest for pharmaceutical purposes due to their important roles in metabolism and drug clearance in vivo. The probe design utilised a P450 mechanism-based inhibitor, 2-ethynylnapthalene, conjugated with an alkyne tag (Scheme 1). The probe was catalytically oxidised by P450 into a reactive ketene, which then reacted with a nucleophilic residue in the enzyme (**9**). It was subsequently tagged with rhodamine or biotin reporters that contained the complementary azide handle. The strategy was shown to successfully profile drug–P450 interactions both in vitro and in vivo; this paves the way for evaluating drug metabolism in living systems, effective dosing and personalised medicine.

Apart from the two-step labelling approach, we utilised click chemistry for the modular synthesis of ABPs against metalloproteases, which are otherwise challenging to synthesise individually (Scheme 1).[22] This enabled the rapid assembly of a panel of hydroxamate-based trifunctional probes, that each



Scheme 1. Click chemistry based strategies in activity-based protein profiling. The use of alkynes and azide derivatives for targeting cytochrome P450[51] and metalloproteases.[22]

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contained a benzophenone photocross-linker and a rhodamine fluorophore (**10**). The P_1' position of the ABPs was diversified with twelve natural and unnatural amino acids, which rendered characteristic fingerprints when profiled against a panel of seven metalloenzymes.

Bogyo and colleagues have interestingly reciprocated the idea of click ABPs in the development of chemically cleavable probes that facilitate tag release.[52] Here, the goal was to be able to release the probe from the enzymes before MS-based protein deconvolution. The authors incorporated a diazobenzene linker that could be cleaved under mild reducing conditions. This could aid in the identification of proteins isolated in activity-based protein profiling studies in which pull-downs are used to enrich for proteins of interest. The streptavidin–biotin interaction that is commonly used in ABPs is not easily disrupted. The release strategy developed provided more reliable MS data compared to usual on-bead tryptic digestion.

Applications of ABPs in Functional Proteomics

Beyond expanding the repertoire of enzymes that can be targeted by ABPs, researchers have made much progress in applying activity-based protein profiling towards dissecting the functional architectures of living systems. In addition to SDS-PAGE based gel separations to functionally differentiate complex tissues and lysates labelled with ABPs,[9,53] liquid chromatography-tandem mass spectrometry (LC-MS/MS) based approaches are accelerating the screening and identification of proteins targeted by ABPs.[10,14b] The platform can further resolve the specific residues modified by the ABP at the enzyme active site.[54] Platforms like microarrays and fluorescence microscopy have also been exploited in diverse applications, which meaningfully extend the contributions of activity-based protein profiling.

Biological discoveries with ABPs

ABPs have been applied in protein annotation and to better understand their roles in biological pathways. Jessani et al. have shown that despite low sequence homology, 9-*O*-acetylesterase (SAE) is labelled with the fluorophosphonate ABP, which typically targets only serine hydrolases; this has identified SAEs as a potentially cryptic class of this enzyme superfamily.[55] The ABP binding site was confirmed to contain a catalytic serine residue by using site-directed mutagenesis. Classification based on sequence homology might never have predicted this functional relationship; this highlights the useful insight that ABPs can provide. Evans and colleagues used a natural-product inspired ABP library to identify a compound that inhibited breast cancer cell proliferation.[56] The target of this probe was identified as brain-type phosphoglycerate mutase 1, which converts 3-phosphoglycerate to 2-phosphoglycerate during glycolysis; this result reinforces the hypothesis that inhibitors of this metabolic pathway can be applied to cancer therapy. Barglow and Cravatt differentiated the activities of enzymes in lean and obese mice by using a peptidyl α-chloroacetamide probe library.[57] This led to the identification of hydroxypyruvate reductase, an enzyme that converts serine to glucose and was up-regulated sixfold in the livers of obese mice. A study by Quigly and colleagues identified urokinase, a secreted serine hydrolase, as one of the potential enzymes implicated in the metastatic intravasation of human fibrosarcoma cells.[58] The same enzyme has also been identified in prior activity-based protein profiling of breast and melanoma cell lines.[59]

Patricelli et al. developed probes for kinases and ATP-dependent enzymes by using biotinylated acyl phosphate derivatives of ATP or ADP (**11**). The probes were designed to bind covalently to the active site lysine residue in kinases.[60] In order to test the coverage of the kinome that could be probed by these ABPs, the authors tested 100 human, mouse, rat and dog proteomes in 4000 MS runs, and identified 247 kinases, which represents 77% of the total characterised kinase repertoire (with an assignment cut-off probability of 95%). The ABP was also used to distinguish the kinase profiles in ten cancer cell lines (Figure 3 A, top) as well as to determine the IC_{50} values of ATP competitive kinase inhibitors by quantitative MS. In-gel methods for inhibitor screening have also been developed by Cravatt et al.[61] The principle is based on the reduction in fluorescence intensity of ABP-labelled protein bands upon introduction of inhibitors (Figure 3 A, bottom). This can be used at a proteomic scale by addition of inhibitors in vivo or in lysates to compete with ABP labelling; this allows multiple enzymes to be profiled simultaneously. Concentration dependent inhibition can further facilitate IC_{50} determination. In one such example, low nanomolar carbamate inhibitors were discovered against a serine hydrolase, α/β-hydrolase domain six.[62] A similar strategy has been reported for uncovering selective inhibitors against the proteosome.[63]

Activity-based protein profiling with microarrays

Our group was one of the first to demonstrate the utility of ABPs in high-throughput enzyme profiling on protein microarrays. Using a series of probes that target phosphatases as well as serine and cysteine proteases, we showed that proteins could be positively labelled and thereby annotated on high-density arrays.[64] Miyake and colleagues established a more quantitative approach by using ABPs to reveal both the probe-binding kinetic constants as well as inhibition constants of competing inhibitors on microarrays.[65] This facilitates rapid inhibitor screening—in a similar way to that developed by Cravatt et al. with the in-gel system—by monitoring the reduction in ABP labelling upon enzyme inhibition. Recently, we demonstrated that the ABP approach could be applied to protein fingerprinting to obtain functional fingerprints with a panel of focussed ABPs.[66] Vinyl sulfone based probes were designed with 20 natural and unnatural P_1 amino acid residues. The ABP panel was profiled against four cysteine proteases to reveal fingerprints that closely matched those obtained with gel-based separation.

Using antibody-mediated capture, Sieber et al. have developed a two-step method for profiling enzymes in complex proteomes on microarrays (Figure 3 B, top). Lysates were first la-

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belled in solution with fluorescent ABPs and then captured on slides arrayed with the enzyme-specific antibodies.[67] The strategy was tested against four proteases that were up-regulated in cancer. We developed another approach, termed "expression display", which also took advantage of the capacity and throughput of microarrays as a deconvolution tool.[68] Repertoires of proteins were first linked to their coding mRNA by ribosome display. Rounds of selection were then imposed by using biotinylated ABPs to enrich for enzymes with relevant activities, followed by deconvolution and identification of these proteins by using DNA microarrays. This methodology could be applied to protein evolution and engineering to isolate novel enzyme variants with desired functionalities. Schultz and co-workers developed a series of fluorescent peptide acrylate probes conjugated with peptide nucleic acid (PNA) linkers against caspases, which could be similarly decoded by using DNA microarrays.[69] The probes were used to detect caspase activation upon the induction of apoptosis in cell lysates. PNA-encoded libraries were also applied in the discovery of cathepsin inhibitors[70] and in the functional profiling of dust-mite extracts.[71]

To further exploit the throughput of microarrays in activity-based profiling, our group has developed a small-molecule microarray platform to study and compare the activities of proteins in vitro.[72] A library of 1400 biotinylated hydroxamate peptides were immobilised and screened against fluorescently labelled enzymes. The strategy allowed the comparison of activity-dependent differences amongst closely related enzymes by minimising unspecific binding effects, which confound microarray analysis, through a novel two-colour, reciprocal labelling and application system (Figure 3B, bottom). Screens against four representative metalloproteases furnished highly resolved protein fingerprints that proved useful in the design of selective probes or inhibitors.

Towards profiling of enzyme activities in vivo

The ability to visualise and monitor the activity of enzymes inside living cells presents vital opportunities for the use of ABP in bioimaging. Hang and colleagues applied a mechanism based E-64 probe with an azide handle that targeted cathepsin B in living cells.[73] Following ABP application, the cells were fixed and treated with phosphine–biotin, followed by visualisation by using AlexaFluor647-conjugated streptavidin. The ABPs were used to monitor *Salmonella typhimurium* infections of primary macrophages, and showed that cathepsin B was excluded from the intracellular bacteria-containing vacuoles. In another interesting development Bogyo et al. have created a series of quenched ABPs that only turn fluorescent upon positive reaction and labelling with their target enzymes (Figure 3C). The strategy was tested successfully against the papain family of cysteine proteases by using probes with an acyloxymethyl ketone warhead.[74] This provides a method to monitor, in real-time, the activities of proteases inside living cells.

Outlook

ABPs have opened up a niche in functional profiling that has not been easily accessible by many other strategies. Various groups have diligently pursued this field and have developed many versions and generations of ABPs to target a wide repertoire of enzymes. This has given rise to many important implications that stem from activity-based protein profiling, as has been highlighted herein. Boasting applications from functional annotation to in vivo imaging, activity-based protein profiling represents an indispensable postgenomic tool with which to quickly tease apart proteomes for biologically-relevant activities. The coming years will see a further expansion of the ABP arsenal, not only in the development of probes against novel enzyme classes, but also in tuning the selectivity of existing probes towards a narrower spectrum of proteins. Such advances could empower researchers to explore the functional localisations and distributions of active enzymes within live cells and tissues, and provide a useful alternative to antibody-based in situ hybridisations approaches, which only indicate abundance (not activity). Ideally the goal would be to develop a specific ABP for every available enzyme to completely cover the catalome, analogous to the expansive way in which antibodies are available today.

Looking forward, the maturing mass-spectrometry arena is likely to lead to methods that will improve the sensitivity as well as the speed by which labelled proteins can be identified. Coupling MS tools (like isotope-coded affinity tag; ICAT) with ABPs would also enable quantitative, functional assessments across proteomes. This method has already been tested by Overkleeft and colleagues in a proteomic study of cathepsins.[75] Everley and colleagues have also reported a similar approach with cleavable ABPs.[76] Such comparative proteomic strategies, which take advantage of ABPs to home in on functional differences, are likely to yield important results that can be vital to biomarker discovery (for use in prognostication) and/or target identification for therapeutic development. Platforms like microarrays also offer a rapid ability in deconvolution that could be further exploited in future activity-based protein-profiling studies.

In summary, future developments should further streamline applications in activity-based protein profiling. Better integration of the information obtained from large-scale "omics" studies could further enable researchers to dig deeper into the mechanistic basis and functional significance of yet uncharacterised biomolecules. Together with bioinformatics strategies (like the ENCODE project[3] and structure–function predictions[4]), activity-based protein profiling could significantly contribute to the first catalogue of annotated human proteins that may be established over the next decade. These are exciting times ahead for all of us.

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