Mass spectrometry-based methods for characterizing transient protein–protein interactions

Mass Spectrometry-based Methods for Characterizing Transient Protein-Protein Interactions.

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Abstract

The dynamic associations of transient protein-protein interactions (PPIs) are critical mediators of myriad biochemical processes. These specific, low-affinity interactions are often mediated by conserved amino acid sequences or short linear motifs (SLiMs) that interact with corresponding binding domains. The short-lived and dynamic nature of these interactions make their biophysical characterization a significant challenge. This review focuses on the development and future directions of mass spectrometry-based techniques for elucidating and characterizing SLiM-mediated PPIs. This includes the application of protein footprinting techniques for inferring the location of SLiM binding sites and the growing role of native MS for direct observation of protein-SLiM interactions, highlighting their potential for assessing small molecule modulation of transient PPIs and the identification of interfacial SLiMs.

Keywords

Transient Protein-Protein Interactions, SLiMs, Native Mass Spectrometry, Protein Footprinting, Protein Painting

Introduction

Common notions of biochemical processes often attribute specific biological pathways to the actions of a linear progression of individually operating proteins. However, most intra- and extracellular protein function is regulated by the formation of deterministic interfacial associations between partner proteins, which are generally referred to as protein-protein interactions (PPIs).[1,2] PPI dysregulation is implicated in numerous non-communicable disease[3,4] while microbial PPIs, including those between host and microbe proteins, play a central role in disease pathogenesis.[5] This has, therefore, led to the development of a plethora of approaches for detecting PPIs from a native biological environment.[6–8] However, unravelling the physiological role of PPIs in healthy and diseased cells, and ultimately their exploitation as non-canonical chemotherapeutic targets requires insight into the mechanisms of interfacial association and recognition derived from detailed interfacial structural information.[9]

Protein associations exist on a continuum, relating to their binding affinity, lifetime and stability, which are properties correlated with their buried surface area (**Figure 1**). Obligate PPIs refer to associations of proteins that are unstable in isolation and where protein function is obligate on complexation. Conversely, non-obligate PPIs are associations between independently stable proteins, whose association triggers specific biological responses.[10] Non-obligate PPIs can be generally classified into two broad categories, namely permanent or transient PPIs.[11,12] Permanent PPIs commonly involve large oligomeric complexes or antigen-antibody interactions. Once detected, the strength and stability of these enthalpically driven associations[13] are amenable to PPI interfacial characterization via atomic-resolution structural biology techniques such as x-ray crystallography, nuclear magnetic resonance (NMR) and more recently, cryo-electron microscopy (EM).[14–16] By contrast, the shorter-lived transient PPIs have smaller interfaces and are typically mediated by the interaction between a domain from one partner protein and a short linear motif (SLiM). These

SLiMs are often found either as linear hot segments or terminal peptides within conformationally flexible or intrinsically disordered regions (IDR) of the corresponding partner protein.[17,18]

During PPI formation, SLiMs undergo a disorder-to-order transition, resulting in a substantial reduction in conformational entropy, thus decreasing the strength of the interaction without necessarily sacrificing specificity.[18,19] The characteristic ease of association and dissociation of transient associations facilitates rapid responses to stimuli and is critical for mediating signaling networks and biochemical pathways.[20] However, this property also renders many transient protein complexes, particularly those involving SLiMs, too short-lived or unstable to be studied using many high-resolution structural biology techniques.[21]

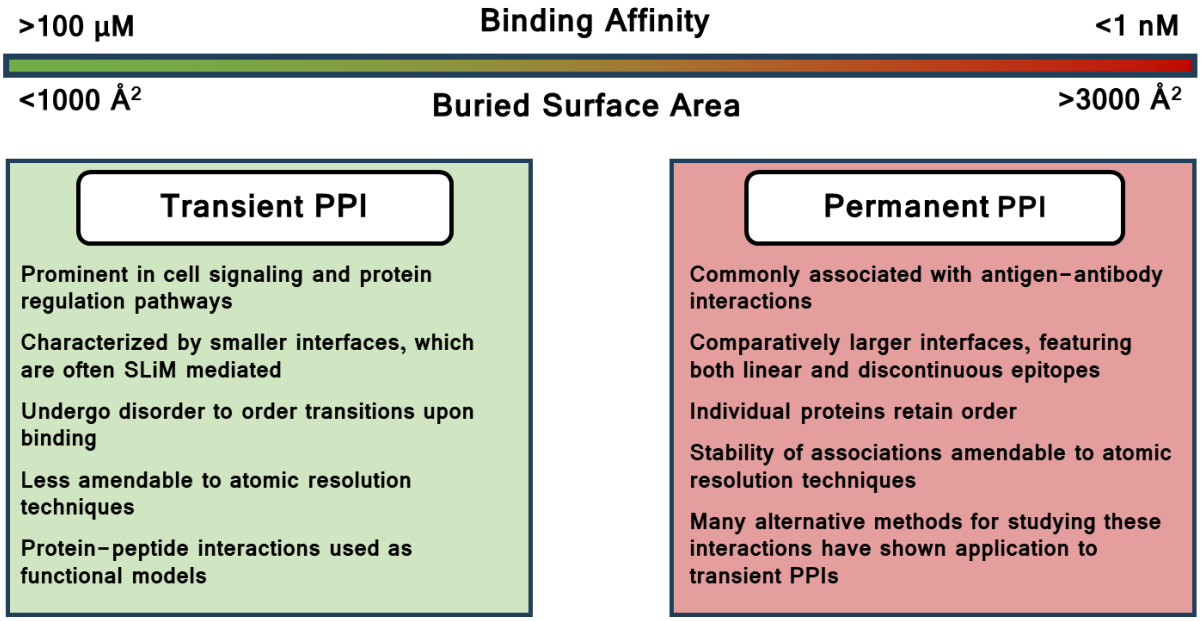

Figure 1. Classification of protein-protein interactions (PPIs) on the basis of binding affinities and buried surface area. Key characteristics of transient and permanent PPIs are highlighted.

Recent advances in machine learning technology has seen computational methods emerge as a powerful alternative for predicting PPI interfaces.[22] However, while showing potential to provide significant new biological insights,[23] high confidence predictions still tend to be reproductions of

already resolved, more stable PPIs, whereas PPI interactions involving SLiMs are less reliable.[24]

Therefore, competent exploration of transient PPI chemical space has necessitated the development of numerous innovative *in vitro* approaches for gaining structural insight into SLiM-mediated interfacial interactions. Here we focus our discussion on recent developments in mass spectrometry-based methods for structurally characterizing transient PPIs and the identification of interfacial SLiMs either by inferring the interfacial regions or by directly observing SLiM interaction (**Figure 2**). Most of the methodology discussed below traces their roots to approaches for studying higher-affinity permanent PPIs. We have therefore focused on methodological innovations that have facilitated transition into the transient PPI space.

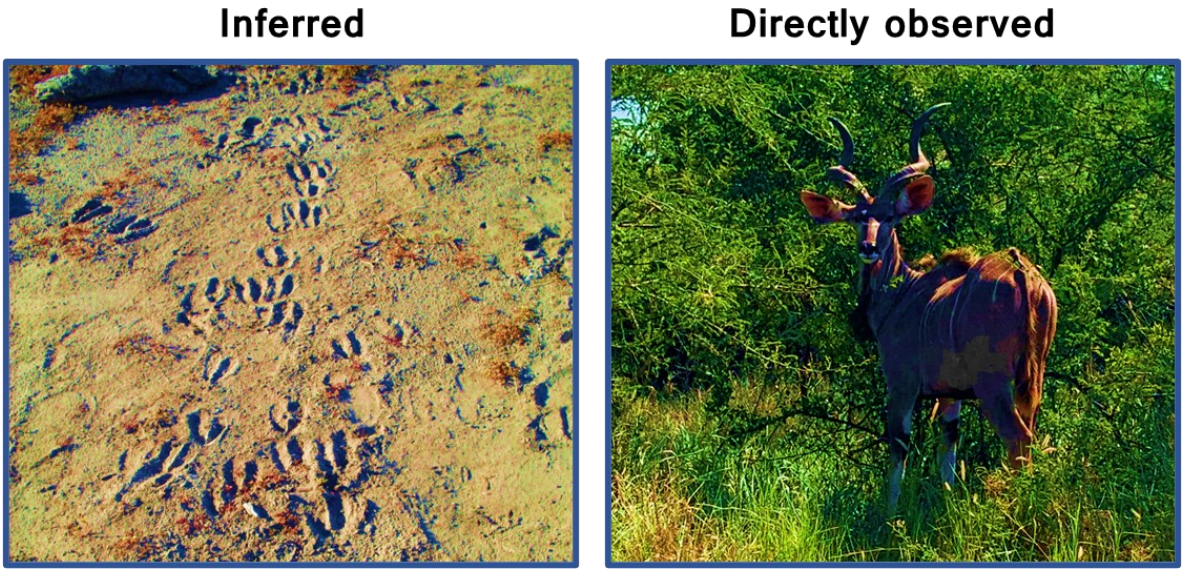

Figure 2. Inferred observation vs direct observation. The images on the right and left give different yet complementary information. On the left, we can conclude that an animal was recently present and what animal it likely was. We cannot infer specific information, including height and horn length. All this information is obtained from the image on the right and is less ambiguous, but we cannot infer that the animal had previously been in a riverbed before entering the bush. Images obtained with permission from the personal collection of CGLV.

Inferring SLiM interactions – The emergence of protein footprinting.

Protein footprinting is a general term referring to experimental techniques that rely on covalent chemical modification of a solvent-accessible protein surface.[25] PPI formation most commonly occurs via electrostatic interaction between 'hot-spot' residues on opposing interfaces, with a subsequent exclusion of bulk solvent.[26] With careful control of the *in vitro* conditions, protein footprinting methods are amenable to preserving transient PPIs, and the subsequent ''masking'' of buried interfacial contact areas excludes their participation in solvent-mediated chemistry. This allows for the interfacial interacting regions to be inferred by determining the extent of chemical modification throughout the protein sequence both in the absence and presence of the protein binding partner. This is typically achieved via MS analysis under denaturing conditions (**Figure 3**).[27]

Dating back to the 1950's, hydrogen/deuterium exchange (HDX) methodologies, which relates the rate of exchange between backbone amide hydrogens to protein structure, dynamics and solvent exposure, represented the first commonly accessed footprinting methodology.[28] More recently, the hyphenation of HDX with MS has proven a useful, broad-spectrum methodology that is widely applicable for a range of protein biochemical studies.[29]

However, the inherent reversibility of the exchange process and the risk of deuterium scrambling are confounding factors that must be carefully controlled. To that end, increased use of automation for liquid handling, commercially available HDX-MS platforms, and data analysis workflows have substantially increased reproducibility in HDX-MS experiments. In addition, recent advances in the standardization of best practices in data collection, analysis, and interpretation of HDX MS data has increased the robustness of HDX, its accessibility to non-specialists and its widespread adoption for structural biology.[30]

Deuterium lability resulted in the emergence of first-generation non-reversible covalent modifiers, which relied on the specific chemical reactivity of side chain residues.[25,27] However, in addition to relatively slow rates of reaction, spatial resolution is entirely reliant on the abundance of the reactive residue.

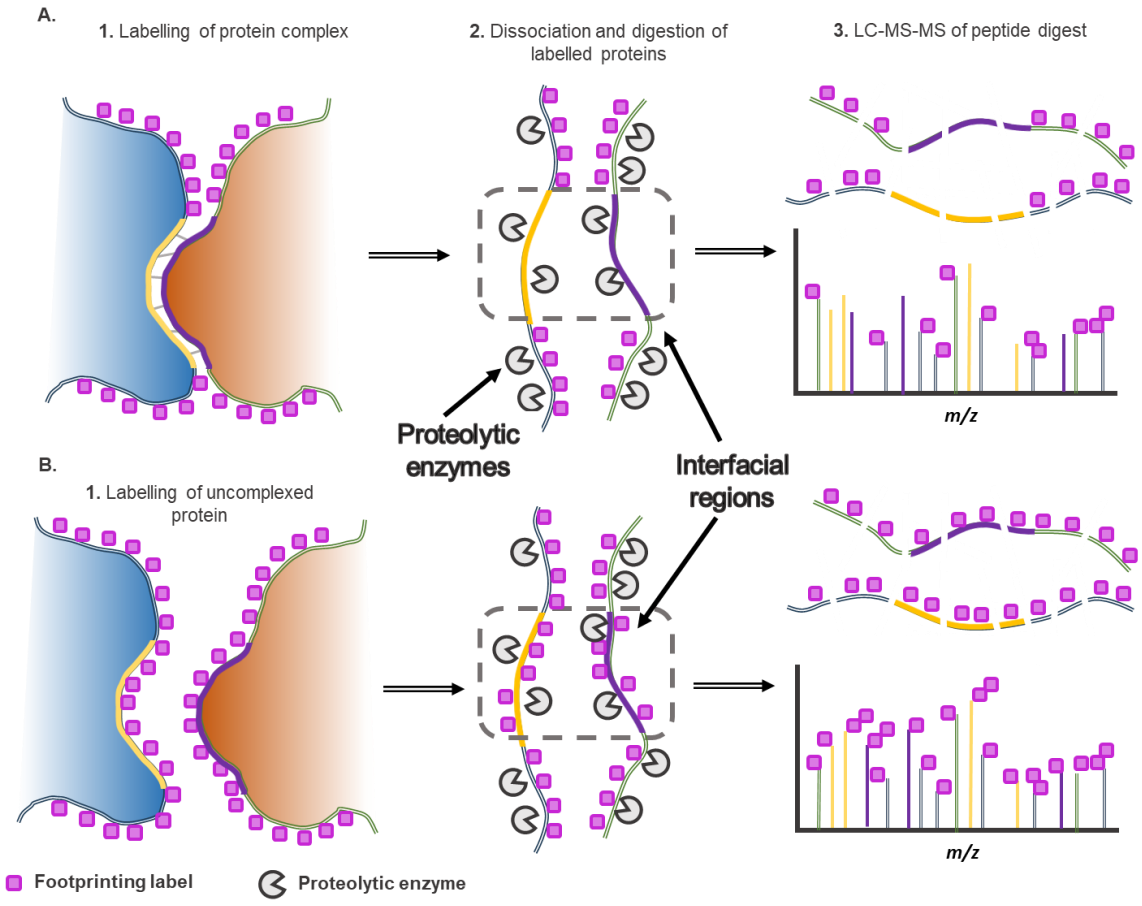

Figure 3. An illustrative description of protein footprinting, which occurs in two separate experiments (**A** and **B**). **A.** PPI complexation excludes the interfacial regions from participating in the labeling reaction. **B.** Uncomplexed protein will not exclude labelling of the interfacial region. Subsequent protein denaturing and proteolytic digestion will generate a suite of differently labelled peptides. Head-to-head comparison of the peptides from experiments **A** and **B** via mass spectrometry can allow the solvent-excluded region, including the interfacial SLiM, to be inferred.

The incorporation of radical chemistry and fast photochemical oxidation of proteins (FPOP),[31,32] in particular, proved a step-change in the capabilities of covalent protein footprinting for determining higher-order protein structure.[33] During FPOP, hydroxy radicals, generated in situ via photolysis of hydrogen peroxide, modify solvent-accessible amino acid side chains. In addition to rapid reaction, FPOP reliably labels 14 of the 20 unique side chains.[34] With respect to biomacromolecule interfaces, FPOP has proven particularly useful for mapping antibody-antigen interactions[35,36] and has made significant strides in footprinting transmembrane proteins.[37,38] In addition, the FPOP approach has been used to characterize the interface of transcription factor FOX04 with double-

stranded DNA.[39] Most significantly, these studies incorporated top-down MS techniques, including the use of isotope-depleted proteins to improve the spatial resolution of footprinting experiments.[39,40]

Carbene footprinting – the current state of the art

The advantages of rapid global footprinting brought on by FPOP were further enhanced through the development of carbene footprinting methodology using diazirine chemistry (**1 – 5, Figure 4**). Unlike in situ peroxides, diazirine precursors are unreactive until the commencement of photolysis and the in-situ formation of reactive carbene radicals, capable of reacting with all amino acids and inserting at different atoms of the same residue. In addition, the rate of carbene – amino acid reactions are an order of magnitude faster than hydroxyl radical reactions and, seemingly, do not share the same sensitivity to protein concentration. Furthermore, the nanosecond half-lives of carbene radicals limits their diffusion as reactive entities, thus restricting footprinting to the protein surface.[41,42]

Early iterations utilized diazirine gas (**1**) as a source of reactive methylene proved capable of global labeling of solvent-exposed protein surfaces.[43,44] However, despite its obvious potential, low water solubility and general safety concerns have limited wider its application. Rejuvenation of carbene footprinting was initiated through the development of diazirine-modified amino acids or alkyl acids (**2** and **3**) as stable, water-soluble carbene sources whose activation wavelengths were outside the amino acid absorbance range. Subsequent labelling was sufficiently sensitive to detect labelling differences resulting from conformational changes in calmodulin.[42,45] Incorporation of tandem MS techniques further enhanced the temporal resolution to include site-specific information of the interaction between calmodulin and the M13 peptide. However, neutral mass losses from gas-phase fragmentation and evidence for reactivity biases at certain residues were early limitations.[42,45] Through reinterrogation of early reports of diazirine photolabeling,[46,47] Manzi et al. developed a new footprinting agent, trifluoromethylaryl diazirine (TFMAD, **4**), whose balanced chemical functionality allowed interaction with hydrophobic and polar regions of the protein surface.[48]

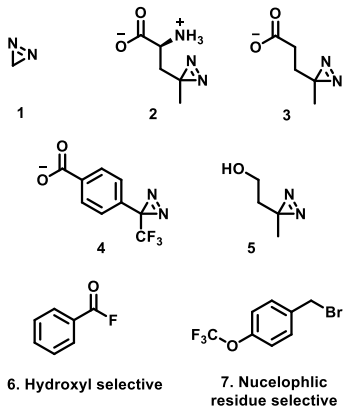

Figure 4. Examples of protein footprinting reagents including diazirene containing small molecules (**1 – 5**)[42–48] as sources of carbene labels, and modern residue-specific labels **6** and **7**.[37,49]

While TFMAD had a preference for basic and hydrophobic residues, the overall improvement in surface coverage provided previously unknown insight into the dynamic interactions between USP5, a 100kDa deubiquitinating protease, and di-ubiquitin identifying SLiMs in both the Zn-finger ubiquitin-binding and catalytic domains.[48] Furthermore, TFMAD labeling proved more stable when exposed to MS/MS fragmentation, facilitating residue level insight into some regions of the HEWL-NAG_5 interaction, which were in agreement with x-ray crystal data.[48] TFMAD-mediated footprinting has since been applied to study several interacting interfaces, including protein-ligand, soluble protein-protein and membrane protein-protein interactions, providing new structural and mechanistic insight not previously accessible by conventional structural biology methodologies.[50–52]

By introducing ion mobility into a typical LCMS workflow, Lu et al. were able to separate isobaric peptide isomers that had been labelled at different sites of the same residue (**Figure 5**) and through this, obtain sub-residue resolution, which revealed nuances between the binding of estrogen-related receptor α, with agonists and inverse agonists.[53]

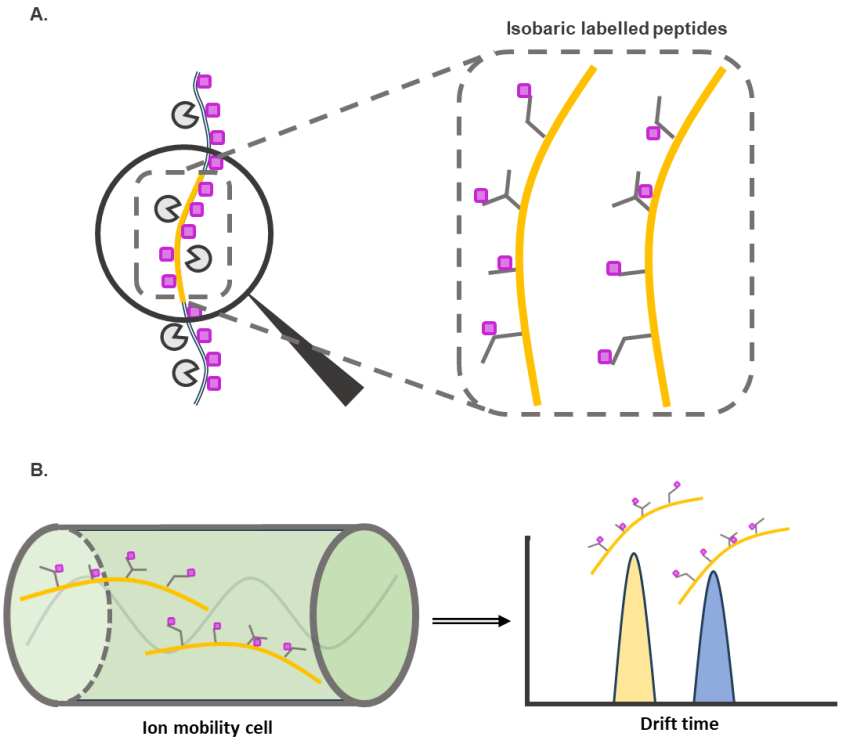

Figure 5A. The non-specificity of carbene labeling can result in covalent labels being inserted into different regions of the same amino acid, leading to isobaric (same *m/z*) peaks in the mass spectrum. **B.** Gas-phase separation using ion mobility allows for these isomers to be individually analyzed by mass spectrometry, thus providing sub-residue resolution.

Future directions – residue-specific labelling and label blending

Advances in carbene footprinting has precipitated interest in understanding labeling preferences of diazine probes and what structural information labeling preferences may be able to reveal.[54] This has further seen the reemergence of site-specific labelling agents for uncovering residue-specific information. However, this specificity is currently limited to nucleophilic amino acids (**6** and **7, Figure 4**).[37,49]

Combining or blending labeling reagents has been explored as a means of improving consistency and magnitude of surface modification.[55] There is, therefore, substantial scope for developing arrays of site-selective or specific labels, whose tuned reactivity gives insight into the local chemical environment in which it reacted. Furthermore, the application of established MS adjacent technologies, including top-down fragmentation[56,57] and ion mobility,[58,59] facilitates discernment between labels at a residue or even sub-residue level.

Coupling this to recent developments in isotope depletion MS methodology[60–62] can be used to further increase experimental sensitivity and reduce spectral complexity following gas-phase fragmentation. In this context, cocktails of site selective or specific labels will add an additional physicochemical dimension to PPI footprinting.

Protein painting – the return of non-covalent footprinting

While an extension of the notion of solvent exclusion as a proxy for interfacial interaction, protein painting represents a departure from covalent labelling methods. This approach is based on the notion of protein staining (e.g. Coomassie brilliant blue, **8**, **Figure 6**) and the tendency of pigments to interact with charged and polar amino acid residues.[63–66]

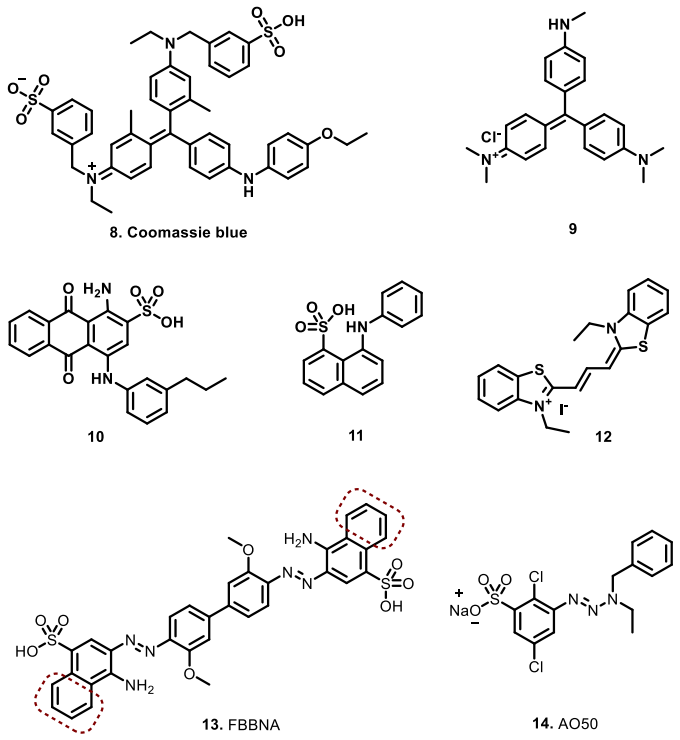

Figure 6. Coomassie blue (**8**) and four pigments (**9 – 12**) used by Liotta et al. for initial protein painting experiments.[63] Pigment **13**, developed through an optimization campaign is a conjugate between fast Blue B and naphthionic acid (FBBNA). Highlighted regions on **13** indicate the putative hydrophobic clamp regions. Pigment **13** was used alongside pigment AO50 to improve surface coverage.[64]

During protein painting, small molecule pigments are incubated with a native protein assembly, where solvent-accessible regions are non-covalently 'coated', including potential proteolytic recognition sequences (**Figure 7**). This process then requires excess dye to be removed, followed by protein denaturation and proteolysis.

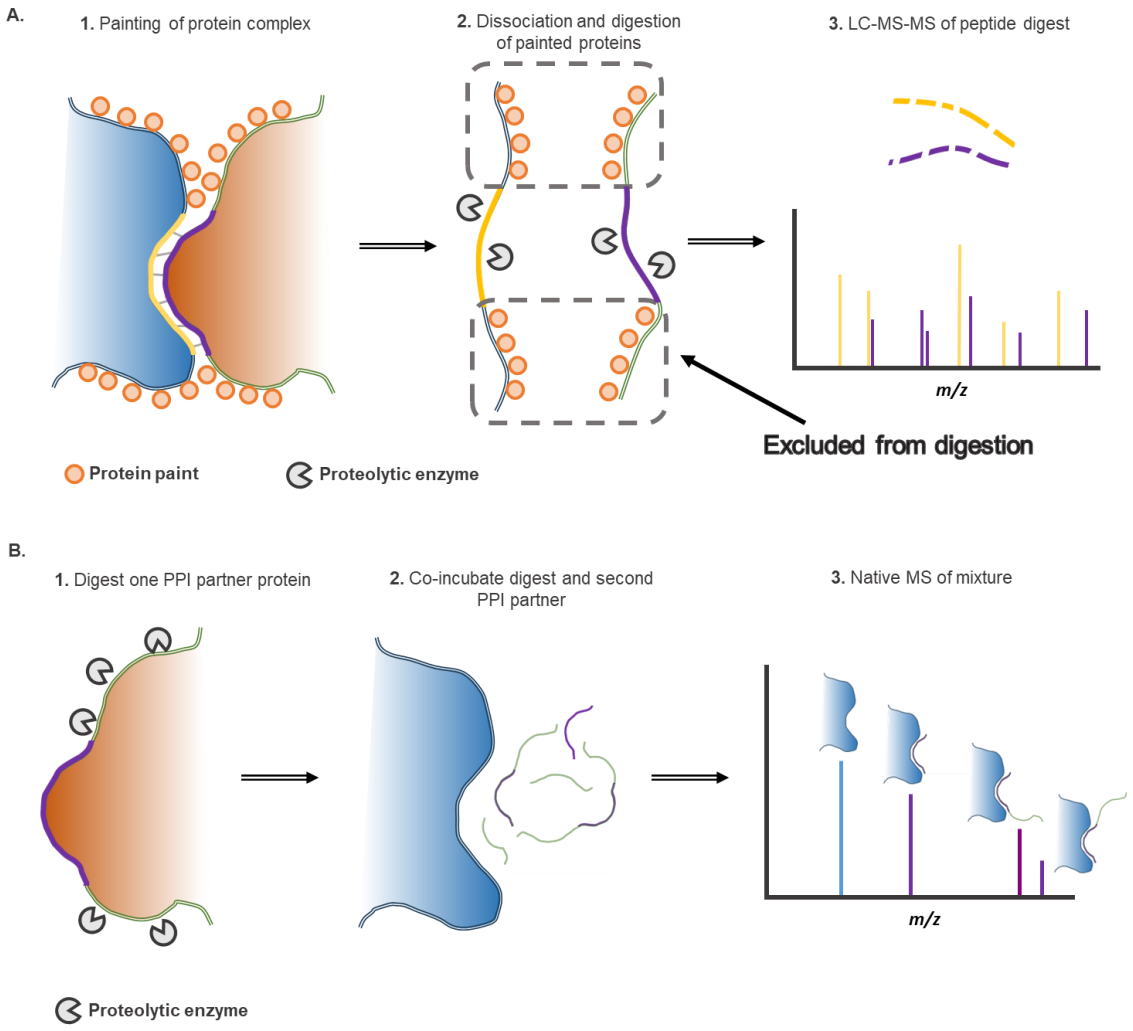

Figure 7A. Illustrative description of protein painting, whose workflow somewhat mirrors that of protein footprinting (**Figure 3**). The primary difference is the painted regions are protected from proteolytic digestion. Subsequent mass spectrometry analysis will only involve peptides found in unpainted regions, allowing the interfacial regions to be inferred. **B.** Identification of interacting SLiMs can be achieved by combining limited proteolysis with native mass spectrometry. Here, one of the interacting partner proteins is subjected to proteolysis to generate a suite of overlapping peptides representative of the protein sequence. Incubation of these peptides, with the corresponding interacting partner protein, followed by native mass spectrometry, allows for binding peptides to be directly observed. Analysis of common binding motifs can further narrow down the likely interfacial SLiM.[67]

Occlusion of proteolysis labile residues results in a suite of proteolytic peptides drawn only from 'unpainted' regions. This will include the previously buried PPI interface and its interacting SLiM, allowing interfacial associations to be inferred.[63] In contrast to footprinting experiments, the number of peptide fragments in an analyte is likely substantially reduced. However, the kinetics of pigments deemed suitable for protein painting require unusually rapid on rates combined with off rates an order of magnitude slower than PPI dissociation rates. Furthermore, while non-covalently bound, pigments need to remain associated during dye removal, denaturing and proteolysis steps,[63] which together makes sample handling potentially complex.

In its first reported application, a small cohort of pigments were screened against carbonic anhydrase II for candidates which conformed to the requisite kinetic properties.[63] This trial yielded four pigments (**9 – 12, Figure 6**), which were used to resolve interfacial information relating to the proinflammatory interleukin 1β complex. In addition to concordance with the reported x-ray crystallography data for the IL1β – IL1RI PPI, the authors were also able to identify unreported contact points in the IL1β – IL1RI – IL1RAcP ternary complex. This led to the identification of a synthetic linear peptide motif derived from IL1RAcP, which was capable of disrupting complex formation.[63] The minimal interfacial region represented by the IL1RAcP peptide was used to identify druggable hotspots from which anti-osteoarthritis complex disrupting compounds could be derived.[68]

While general principles of pigment – amino acid interactions were known,[63–66] an in-depth understanding of the structural characteristics required for broad spectrum protein association was lacking. Therefore, analysis of any new PPI system would likely require pigment screening and the use of multiple pigments to ensure suitable surface coverage. In a follow-up, akin to an SAR analysis, Haymond et al. developed an optimized azo dye conjugate between fast Blue B and naphthionic acid (FBBNA, **13, Figure 6**), whose balance of physicochemical properties was capable of high levels of protein coverage against several proteins. They determined that the improved coverage correlated with the presence of

hydrophobic aryl regions on the outside of the molecule, referred to as a 'hydrophobic clamp' which they hypothesized could enhance interaction with hydrophobic pockets or aromatic amino acid residues.[64] Following this optimization study, protein painting using FBBNA in conjunction with a second paint (AO50, **14, Figure 6**) was used to investigate the pro-apoptotic YAP–ZO-1 interaction. Here, in addition to confirming available interfacial information,[69,70] the authors identified functionally relevant, druggable hot spot regions of this PPI.[64] The same combination of pigments were also used to map the transcriptional checkpoint interaction between PD-1 and PD-L1. Here again, interfacial characterization correlated closely with previous structural reports[71] as well as identifying a specific functionally relevant hot spot of PD-1, from which they elucidated a PPI inhibitory interfacial SLiM from PD-L1.[64] Further optimization of the PPI modulatory SLiM resulted in a lead peptide whose interaction with PD-1 initiated PD-1 signaling.[72]

Future directions – Combining proteolytic enzymes and increased spatial resolution.

To date, protein painting experiments have only exploited tryptic digestion for generating interfacial peptides. However, in addition to trypsin-recognition motifs, FBBNA was found to interact strongly with chymotrypsin-recognizing tyrosines. Furthermore, FBBNA remained protein-bound under both thermal and chemical denaturing conditions.[64] Accordingly, the development of new paints, whose properties facilitate the incorporation of multiple proteolytic enzymes, including those which operate at different pHs will increase the spatial resolution of this protein painting. While there are only a handful of reports using this technique for the elucidation of PPI interfaces, the reported data has proven sufficiently robust to facilitate transitional applications. The inherent simplification afforded by narrowing the suite of proteolytic peptides, coupled to reduced analytical complexity afforded by no additional labels, renders this approach amendable to complex mixtures of interacting proteins.

Directly Observed SLiM interaction.

In contrast to inferring interaction regions, direct observation of PPIs is less interpretative and is thus less susceptible to false positives. Accordingly, high-resolution approaches such as x-ray crystallography and cryo-EM are the methods of choice for studying PPIs.[73] However, the low stability of transient SLiM-mediated PPIs, in many cases, makes high-resolution methods unsuitable.[74] Fortuitously, in many cases the SLiM–domain interaction is sufficiently dominant that a short peptide sequence containing the SLiM motif can be exploited as a functional proxy of a full-length PPI (**Figure 8**).

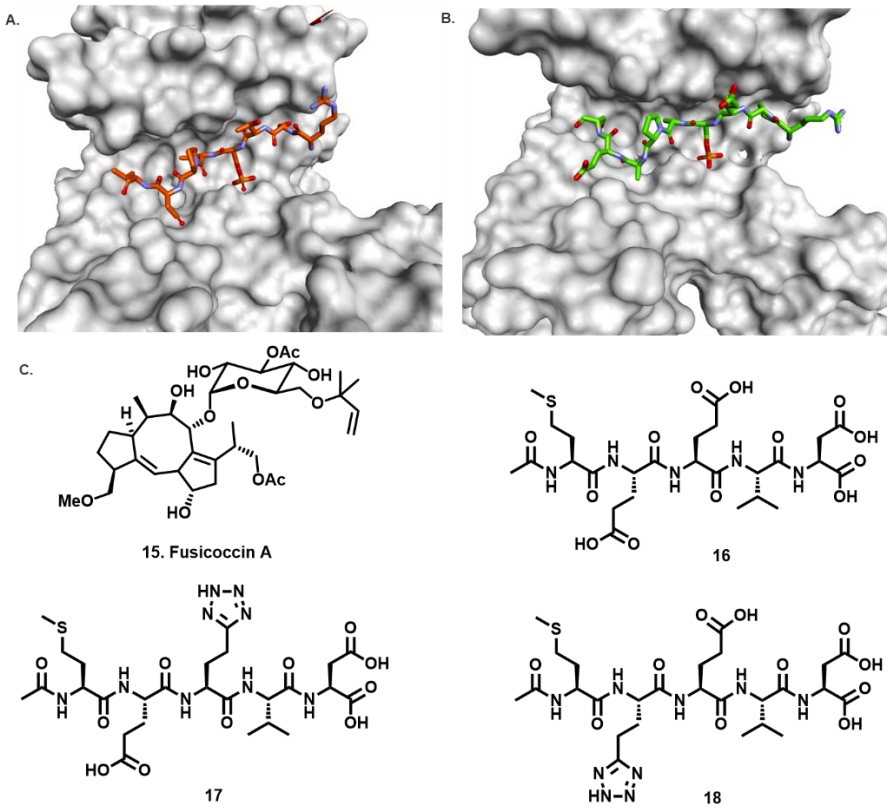

Figure 8. X-ray cocrystal structure of eukaryotic regulatory protein 14-3-3σ complexed to phosphorylated interfacial SLiMs from C-Raf (panel **A**, PDB 4FJ3)[75] and SOS-1 (panel **B,** PDB 6Y44)[76] partner proteins. Both SLiM-protein interactions are used as proxies for studying the interface of the full transient protein-protein interaction. **C.** Protein-protein interaction modulatory compounds, whose efficacy was demonstrated through native mass spectrometry.[77,78]

This simplified interaction overcomes several stability challenges associated with full-length transient PPIs and can be applied for interfacial characterization and modulator design, while the smaller interface search area simplifies hot-spot identification.[79]

Native mass spectrometry and PPI modulation

Native mass spectrometry (MS) is an expanding branch of biomolecular MS in which solution-phase structural information of biomolecules is retained during the ionization process into the gas phase.[80,81] Using this technique, intact protein ions which retain the non-covalent interactions that dictate structural folds can be detected, and non-covalent protein-protein and protein-ligand interactions can be directly observed. Moreover, MS is replete with advantages in speed, sensitivity, experimental setup and sample consumption. Accordingly, there are numerous examples of the application of native MS for studying full-length PPIs in the gas phase.[82] In addition, native MS has recently been used as a tool for directly observing SLiM–domain interactions in the gas phase. Leney and co-workers observed the association between the eukaryotic regulatory protein 14-3-3σ with phosphorylated SLiMs from three PPI partner domains, namely p53 LRRK2 and ERα, all of which are pharmacologically relevant transient PPIs. Here, the relative abundance of apo- to peptide bound 14-3-3σ was used to assess binding sociometry, which could be modulated in the presence of fusicoccin A (**15, Figure 8**) as a known, small molecule PPI stabilizer. They further demonstrated that the unique *Δm/z* afforded by dual peptide and small molecule binding meant that their PPI stabilizer could be identified out of a cocktail of small molecules. This latter result was significant since it demonstrated the possibility of larger-scale native MS-based PPI modulator screening using mass-curated libraries.[77] This approach has further been applied to help elucidate the mechanism of molecular glue-mediated PPI stabilization.[83]

Though our own interest in the pharmacological potential of transient PPIs, we used native MS to observe the gas phase association between an acetylated MEEVD SLiM (**16**) as a mimic of the PPI mediating pentapeptide found in the HSP90 C-terminal region, and the TPR2A domain of HOP.[78,84] In a similar

approach to Leney and co-workers, we utilized alterations in the relative abundance of apo and bound species, to determine that non-natural SLiM mimicking peptides (**17** and **18, Figure 8**) disrupted the TPR2A-MEEVD interaction. Critically, this translated into the ability to disrupt the interaction between full domains in an ELISA style assay,[78] which have further been used as probes for investigating the HOP-HSP90 PPI as a target for Kaposi's sarcoma-associated herpesvirus (KSHV).[85]

Identifying interacting SLiMs through native MS

While native MS methods for the direct observation of SLiM–domain interactions are showing growing promise, examples to date have all relied on available interfacial structural information. The identification of interfacial interacting SLiMs is far from trivial, requiring a combined ability to generate peptides representative of an interacting protein and assess their ability to interact with the corresponding partner. Methods originally developed for mapping antibody–antigen interactions, including synthetic peptide scanning,[86] phage display[87,88] and more recently affimers,[89] have proven useful for the identification of SLiMs. However, while powerful, these approaches have idiosyncratic technical drawbacks, including the requirement either for large-scale synthesis of overlapping peptides, or the construction of custom phage libraries representative of the PPI partner proteins as well as the identification of binders through sequencing.[90–92]

Lu et al. had previously shown that proteolytic excision of an 11 amino acid linear motif from a 40 amino acid segment of Amyloid β-Protein (Aβ) followed by incubation with the anti-Aβ antibody and analysis by native MS was sufficient to observe an interfacial protein-peptide interaction representing the antibody-antigen interaction.[93]

We have subsequently shown that this approach is transferable to SLiM-mediated transient PPIs (**Figure 7B**). In the first of two proof of concept studies, limited proteolysis of the 119 amino acid C-terminal domain of HSP90 followed by incubation with TPR2A and native MS analysis showed interactions between TPR2A and three peptides, all of which contained the known MEEVD SLiM, were observed. In addition, binding was observed for one non-MEEVD-containing

peptide, which we have hypothesized is a previously unknown secondary interacting region required for PPI stability.[67] In the second study, we investigated the Mycobacterial chaperone PPI between DnaK and GrpE, where we identified two overlapping binding peptides, both of which originated in the primary PPI interface.[67] Importantly, Li et al. had previously shown that hot-spot mutation in this region prevented PPI formation, as well as DnaK chaperone activity.[94]

Future directions

The key advance of native MS approaches is the simplification they offer in terms of speed and sensitivity, not only for detecting SLiM–domain interactions but also for quantifying changes to these interactions in the presence of PPI modulators, stabilizers and disruptors. The relationship between SLiM association in the gas phase and full-length PPI formation is critical for translational PPI drug discovery. This relationship also presents an interesting opportunity to investigate the impact of domain mutation on SLiM interaction as a proxy for changes in PPI formation.

The addition of native MS to the toolbox of methods for identifying interfacial SLiMs further enhances its utility as a multimodal approach for investigating transient PPIs. In addition, once an interacting SLiM has been identified, top-down MS methodologies, particularly those which utilize electron and photon-mediated fragmentation, can be used to map sites of non-covalent interaction.[95] This, in turn, brings in opportunity for the incorporation of isotope-depleted proteins for increased top-down fragmentation sequence coverage and spatial resolution of peptide binding sites.[60,61]

Concluding remarks

SLiM-mediated PPIs represent an important class of biological interfacial associations whose investigation will not only enhance our understanding of complex biological networks but is also a significant opportunity to drug new chemical space. Effective drugging of transient PPIs necessitates developing a comprehensive structural framework, which underpins PPI interfacial association, including the identification of SLiMs, their binding sites and interfacial hot spots. This information is essential not only for rationalising PPI formation but also for the

rational design of PPI modulatory compounds, which can act as probes for pharmacologically validating promising PPIs or indeed as scaffolds for developing novel chemotherapeutics. The scope of transient PPIs extends to their role in microbial pathogenesis, either through exploitation of host PPIs or hijacking host-based factors through the interaction of host and microbial proteins. This offers exciting opportunities to begin developing host-based antimicrobial therapies, which are less susceptible to the emergence of drug resistance (see outstanding questions).[96]

The substantial challenges associated with studying full-length transient PPIs often renders the investigation of full-length PPIs through powerful high-resolution methods such as x-ray crystallography and cryo-EM unsuitable. This has precipitated numerous innovative methods and techniques which seek to circumvent low stability whilst increasing experimental sensitivity and resolution.

In this review, we have focused on several approaches that have evolved from alternative methods for studying antibody–antigen interactions and, in our view, hold significant promise for the identification of interfacial SLiMs, as well as the characterization of their interaction sites. These approaches are still in their relative infancy, and as such, the handful of examples discussed here have primarily been applied as proof-of-concept studies against known PPIs. Given the low stability and short lifetimes of transient PPIs, the approaches reviewed here, still experience limitations associated with PPI stability. Therefore, there remains substantial scope for collaborative efforts between chemical biologists, synthetic chemists and biochemists to develop new footprinting and painting chemistry, which can enhance the resolution and general applicability of these approaches. Similarly, the ability to identify interfacial epitopes and use this information for developing PPI modulators in the absence of atomic resolution structural information is a tantalizing prospect. Furthermore, directly observed methods are an efficient means of assessing alterations in protein-SLiM associations. While this has been effective in assessing the impact of PPI modulatory compounds, it may well prove useful as a tool for rapidly assessing the impact of interfacial mutations on PPI formation (see outstanding questions)

The selected approaches are complementary in nature, making their orthogonal use a source of important data which can be used to explore new and unresolved PPIs. This is particularly relevant, given the significant recent advances in other MS-based methods, such as cross-linking MS and limited proteolysis MS (LiP-MS), which aim to streamline the identification of PPIs from the intracellular environment.[6–8] There are already examples of crossover experiments incorporating techniques discussed above, including modification of interacting SLiMs with non-specific photoactivatable diazirines,[97,98] or residue-specific covalent warheads,[99] to restrict the surface modification to residues located around the binding site, either to optimize PPI modulation or to streamline interfacial characterization.

In conclusion, the improved access to the chemical space of transient PPIs offered by these emerging techniques has substantial promise for developing new understanding of their role in cellular biology, disease progression and the translation into new drug therapies. This is particularly relevant for addressing unmet medical needs for the plethora of neglected communicable and infectious diseases for which there are no current well-defined target, or selective therapies.

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