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Fast photochemical oxidation of proteins coupled with mass spectrometry

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ABSTRACT

Fast photochemical oxidation of proteins (FPOP) is a hydroxyl radical footprinting approach whereby radicals, produced by UV laser photolysis of hydrogen peroxide, induce oxidation of amino acid side-chains. Mass Spectrometry (MS) is employed to locate and quantify the resulting irreversible, covalent oxidations to use as a surrogate for side-chain solvent accessibility. Modulation of oxidation levels under different conditions allows for the characterisation of protein conformation, dynamics and binding epitopes. FPOP has been applied to structurally diverse and biopharmaceutically relevant systems from small, monomeric aggregation-prone proteins to proteome-wide analysis of whole organisms. This review evaluates the current state of FPOP, the progress needed to address data analysis bottlenecks, particularly for residue-level analysis, and highlights significant developments of the FPOP platform that have enabled its versatility and complementarity to other structural biology techniques.

1. Introduction

In order to gain a greater understanding of protein function, as well as protein-protein or protein-ligand interactions, a detailed understanding of protein structure and dynamics in-solution is critical. In recent years, development of a variety of protein structural analysis methods rooted heavily in mass spectrometry (MS) has highlighted MS as a valuable asset in structural biology. A remarkably diverse set of methodologies including hydrogen deuterium exchange (HDX) [1], chemical cross linking (XL) [2], and native ion mobility spectrometry (IMS) [3] are now routinely available. Often, these techniques are aimed at characterising large, highly dynamic, or heterogenous protein samples typically less amenable to study by higher resolution structural methods such as nuclear magnetic resonance (NMR), crystallography or, more recently, cryogenic electron microscopy (cryo-EM). Although not currently able to define structure to atomic resolution, protein structural analysis by MS requires only a small fraction of the sample needed by other methods in structural biology, offers a comparatively rapid analysis, and is often considerably more cost effective.

One particularly useful suite of techniques are the covalent footprinting methods, variations of which have been used in conjunction with mass spectrometry for many years. In these experiments, a chemical reagent is added to the sample to irreversibly label accessible regions of the protein, usually labelling a specific subset of amino acid side-chains. Some commonly used examples of these labelling reagents are glycine ethyl ester (GEE) which labels carboxylic acids [4], and dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (HNSB) which specifically targets tryptophan side-chains [5]. Typically, a bottom up liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach is then used to determine the location of the modified residues and assess the degree of modification, using a range of different quantification strategies [6–9] (Fig. 1). The rationale behind performing these experiments is that changes in protein structure caused by, for example, a change in conformation [10], protein-protein [11,12] or protein-ligand [13–15] interaction, will alter the solvent accessibility of certain residues, thus changing the extent to which these residues can be

Abbreviations: ApoE3, Apolipoprotein E3; CID, Collision Induced Dissociation; CTFR, Cystic Fibrosis Transmembrane Regulator; DDM, n-Dodecyl-beta-Maltoside; ETD, Electron Transfer Dissociation; FOX, Flash Oxidation; FPOP, Fast Photochemical Oxidation of Proteins; GEE, Glycine Ethyl Ester; HDX, Hydrogen Deuterium Exchange; HNSB, Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; HRFP, Hydroxy Radical Footprinting; IL-6R, Interleukin-6 receptor; IMS, Ion Mobility Spectrometry; KrF, Krypton Flouride; LC, Liquid Chromatography; LH2, Light Harvesting protein 2; MD, Molecular Dynamics; MRM, Multiple Reaction Monitoring; MS, Mass Spectrometry; nanoPOMP, Nanoparticle-promoted Photochemical Oxidation of Membrane Proteins; Nd:YAG, neodynium-doped yttritium aluminium garnet; NMR, Nuclear Magnetic Resonance; SASA, Solvent Accessible Surface Area; SOD1, Supoeroxide Dismutase 1; TFB, Trifluromethoxybenzyl; XL, Crosslinking.

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covalently modified by the added labelling reagents.

This strategy for identifying binding sites, or conformational differences between protein states, has many advantages. Firstly, the LC-MS/MS workflows used in the acquisition of this type of structural proteomics data can take full advantage of the recent advances in MS instrumentation. This includes greater sensitivity and faster MS/MS acquisition speed, prompted by the increased demands of more traditional, large scale proteomics workflows. Secondly, unlike footprinting methods such as HDX, the labelling is irreversible, and thus the quench conditions necessary to halt the labelling reaction need not limit the digest or LC-MS/MS analysis strategies. Indeed, once labelled, the sample can undergo extensive, optimised sample handling and LC separation before MS analysis without concern for losing structural information [16,17]. Similarly, standard ergodic fragmentation techniques, such as collision induced dissociation (CID), can be used to fragment modified peptides and attain residue level resolution, without the concern of label scrambling as with HDX. There are, however, limitations to these methods. Many of the labelling reagents which are commonly used only target specific types of amino acid, meaning the structural information obtainable from any one experiment can be limited. The long labelling reaction times mean the effect that labelling has on higher order structure must be considered to ensure that artefactual conformations are not being probed. Many of these reagents are considerably larger than the surrounding solvent molecules, effectively lowering structural resolution by only probing changes in the accessibility of residues to molecules of equivalent size to the labelling reagent, rather than solvent.

Hydroxyl radical footprinting (HRFP), which uses the highly reactive hydroxyl radical (•OH) as the labelling reagent, has emerged as an irreversible covalent labelling method that overcomes many of these issues. Most obviously, the small van der Waals radius of •OH is comparable to that of water, and thus can offer a more reliable assessment of changes in solvent accessibility than larger labelling reagents [11,18,19]. Although different residue types have different reactivities towards hydroxyl radicals, with sulphur-containing or large hydrophobic side-chains amongst those groups that are most reactive [20], fourteen of the 20 naturally occurring amino acids can be routinely labelled under standard FPOP conditions [21]. While only the most reactive amino acid side chains will be labelled in most experiments, this still makes the hydroxyl radical a *relatively* non-specific labelling reagent compared with other methods, increasing the available structural information from any one experiment [11]. The labelling chemistry is significantly more complex and varied than other covalent labelling methods. However, the side-chain modifications that form as a consequence of oxidation by •OH, the most common of which are +16 Da (incorporation of OH and abstraction of H) and + 14 Da (incorporation of O and abstraction of 2H) mass additions, are well understood. A complete list of the commonly observable mass differences, and the amino acids on which they are typically found, can be seen in Table 1, the mechanisms of formation of which have been reviewed extensively elsewhere [18,20].

The amino acid modifications generated from HRFP of proteins are thought to be largely independent of the methods utilised in •OH production, and thus a multitude of different approaches has arisen to generate hydroxyl radicals for protein footprinting, each with their own advantages and limitations. Perhaps the most widely accessible of these methods are the use of Fenton reactions, a chemical-based approach which uses iron(II) to generate hydroxyl radicals and hydroxide ions from hydrogen peroxide [22] or the use of high voltage electrical discharge during the electrospray ionisation process [23]. While these methods are both cost effective and convenient, needing little in the way of specialist equipment or reagents. Questions as to their ability to accurately report the native states of the proteins under investigation persist. Fenton reactions are slow, which can lead to overlabelling and often require the presence of chelating agents, such as EDTA, to increase the solubility of metal ions, which may disturb protein-protein or protein-ligand interactions [21]. Similarly, electrical discharge methods often require a convoluted workflow of collecting and condensing the

Table 1
Reactivities and common mass differences associated with hydroxyl radical oxidation of amino acid side-chains. Data from [20].

Side-Chain	Reactivity rate (M^{-1} s^{-1})	Δmass (Da)
Cys	3.5×10^{10}	+48, +32, −16
Trp	1.3×10^{10}	+16, +32
Tyr	1.3×10^{10}	+16, +32
Met	8.5×10^{9}	+16, +32, −32
Phe	6.9×10^{9}	+16, +32
His	4.8×10^{9}	+16, −22, −10, +5
Arg	3.5×10^{9}	−43, +16, +14
Ile	1.8×10^{9}	+16, +14
Leu	1.7×10^{9}	+16, +14
Val	8.5×10^{8}	+16, +14
Pro	6.5×10^{8}	+16, +14
Gln	5.4×10^{8}	+16, +14
Thr	5.1×10^{8}	+16
Lys	3.5×10^{8}	+16, +14
Ser	3.2×10^{8}	+16
Glu	2.3×10^{8}	−30, +16, +14
Ala	7.7×10^{7}	+16
Asp	7.5×10^{7}	−30, +16
Asn	4.9×10^{7}	+16

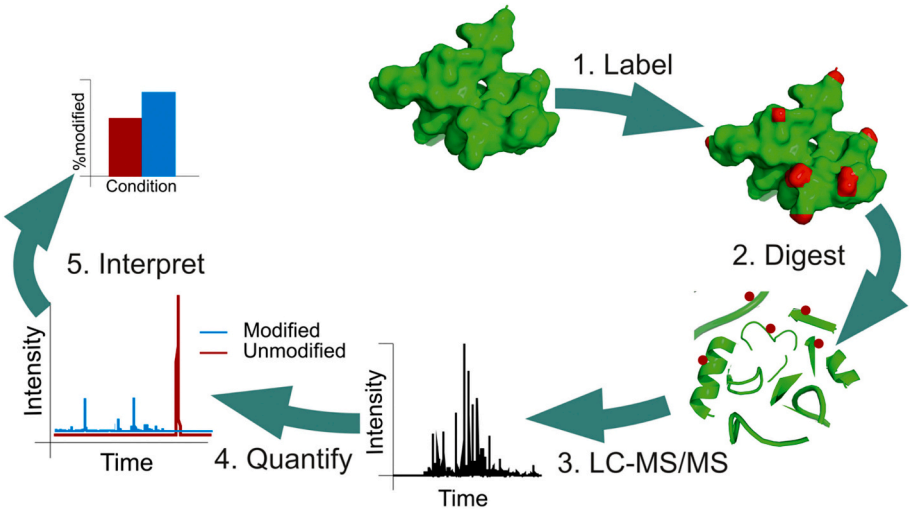

Fig. 1. General workflow for covalent footprinting methods. Covalent labelling with the chosen reagent (1) is followed by a bottom up proteomics approach including proteolytic digestion (2) and LC-MS/MS analysis (3) to determine the location of the chemical modification at the peptide or amino acid residue level. Quantification is performed, for example using a label-free approach for modified and unmodified species (4), and changes in labelling under different conditions can then be determined (5).

sample following electrospray [23,24], with questions also raised as to the extent to which proteins remain native during the transition to the gas phase under the severe electrochemical conditions necessary to generate significant oxidation [18]. Alternative methods such as radiolysis can be used to liberate hydroxyl radicals from water molecules by utilising either high energy electrons, in the case of electron pulse radiolysis [25], or high energy photons, in the case of X-ray synchrotron radiolysis [26]. Again, both of these techniques have been successfully utilised in HRFP for the study of protein structure [25,27], although a significant limitation of these methods is that they require specialist equipment, a high energy X-ray synchrotron beamline or a MeV Van Der Graaf generator, which make them somewhat less accessible to most laboratories.

UV photolysis of hydrogen peroxide as a method of generating hydroxyl radicals for HRFP of proteins was developed in the early 2000's [28]. Although more accessible than many of the radiolysis methods available at the time, these early UV based methods required up to several minutes of UV exposure before significant oxidation was observed [28]. Similarly long exposure times are often required for the generation of hydroxyl radicals using Fenton chemistry [17,21], and gamma ray mediated radiolysis [29,30]. These exposure lengths can be problematic, as longer exposure times increase the likelihood of probing artefactual conformations brought about by the labelling process itself. Although more recent 'fast Fenton' methods have seen this exposure length reduced to the ms timescale [30], even this can overlap with the timescales required for conformational changes and folding/unfolding events of many proteins [31–33]. Though more recent studies have shown that limited oxidation can be relatively benign to protein structure [34,35], others have shown significant conformational changes following HRFP [24,36], meaning the effect is likely protein dependent and, as such, a common goal in the generation of hydroxyl radicals for protein footprinting is to minimise the likelihood of probing artefactual conformations by generating a brief, but high dose exposure to the labelling radicals [25,26].

Fast photochemical oxidation of proteins (FPOP) [37], the focus of this review, is a HRFP method that generates hydroxyl radicals for oxidative labelling. FPOP has been achieved to-date most widely through the use of rapid, UV laser flash photolysis. This method uses relatively small, commercially available lasers and over recent years has been more widely accessible than other methods, as evidenced by the number of research groups adopting this technique [10,12,33,38–40]. This review focusses on five key elements of FPOP: the experimental apparatus, different types of protein analytes studied by FPOP, developments and expansions of the FPOP platform, methods of data analysis and quantification, and new developments in our understanding of the data, and its interpretation.

2. The FPOP experiment

In the most commonly implemented experimental format, samples to be analysed by FPOP require three main solution components: the protein analyte(s), usually at ~low μM concentration; a free amino acid known as the scavenger; and the hydrogen peroxide from which the hydroxyl radicals are generated. The lifetime of the radicals generated will depends on a number of factors, including the buffer composition, scavenger concentration and the concentration and reactivity of the analyte being studied. The scavenger amino acid, usually histidine [10,14,41] or glutamine [8,42–44], is therefore used to control the lifetime of the hydroxyl radicals and, subsequently, the extent of oxidative labelling [45]. In the absence of scavenger, the •OH lifetime is limited largely by the recombination reaction ($2\bullet OH \rightarrow H_2O_2$) which can extend the radical lifetime to ~100 μs [6]. As such, along with the rapid generation of •OH from photolysis, the presence of scavenger is critical to maintaining the short lifetime of the hydroxyl radicals, and fast labelling timescale of FPOP. Both the reactivity, and the concentration, of this scavenger, as well as the concentrations of hydrogen peroxide and the analyte, are solution components which can be tuned to change the level of sample oxidation and should be optimised for each study [10,39,42,45].

In a typical laser-based FPOP experiment, a solution of protein, an amino acid scavenger and hydrogen peroxide is pumped at a fixed flow rate through a coated silica capillary which intersects the rapidly pulsed beam of the UV laser. The extremely short pulse width of the laser (~17 ns) generates hydroxyl radicals on the nanosecond timescale [37] which, depending on solution conditions, can have a lifetime as short as 0.1 μs [37,45]. Despite some concern regarding the generation of secondary radicals which may go on to label the protein after the hydroxyl radicals themselves have disappeared [33], FPOP has been reported to label on a time scale faster than protein unfolding [36,37], generating significant protein oxidation after a single radiation exposure [11,37,46]. While some experimental setups have used Nd:YAG lasers operating at 266 nm [46], more commonly, FPOP experiments use KrF excimer lasers operating at 248 nm [8,10,37,41,47]. This wavelength is chosen as it triggers photolysis of hydrogen peroxide while ensuring minimal absorbance by water or the protein molecules [37]. To minimise hydrogen peroxide induced background oxidation, the hydrogen peroxide is typically added immediately prior to the sample being loaded in to the syringe pump [10,41–43], some setups use online mixing with multiple syringe pumps to further minimise this effect [16,48]. The UV beam used to initiate photolysis of the hydrogen peroxide is typically orientated orthogonally to the capillary, and focussed onto a transparent window in the silica capillary [43], through which the sample is irradiated (Fig. 2). In this arrangement, the degree to which the sample is oxidised is dependent on three main factors: the firing frequency of the laser, the width of the transparent window, and the flow rate of the sample. In an ideal scenario, each protein molecule in the sample would be irradiated no more than once, thus minimising the risk of labelling artefactual conformations generated by repeated oxidation of a single molecule. Many studies achieve this by incorporating an exclusion volume of unirradiated sample between each shot of the laser. Assuming a 'plug flow' model (i.e. solvent at every radius of the capillary flows at the same rate) ensures the irradiated volume per unit time is less than the flowrate of the sample [49,50].

However, detailed studies using laminar flow modelling, where solvent flows more quickly at the centre of the capillary than at the capillary walls, have shown that, due to the slower moving proteins furthest from the capillary centre, having 100% of the sample experience a single UV exposure is not feasible under typical FPOP experimental conditions [47]. That said, Konermann et al. demonstrated that the interplay between the firing frequency, the width of the irradiation window, the flowrate of the sample, and their effects on sample oxidation, could be collapsed into a single parameter: c – a measure of the overall labelling intensity [47]. These authors then suggested that maintaining a c value of ~0.7, where 90% of the sample is irradiated only once, offers a reasonable compromise between the degree of labelling and the desired single exposure conditions [47].

Following irradiation, the sample flows immediately into a quench solution, typically containing methionine and catalase, to quench any remaining radicals and to remove excess peroxide from the solution respectively [10,41,44].

Although the global extent of protein oxidation can be assessed from intact MS analysis [43], the most commonly used method of data analysis is a bottom up LC-MS/MS approach, followed by database searching of the most common oxidative mass changes (see Table 1) to identify modified peptides. The extent of oxidation is then typically quantified to either peptide [12,43], or residue [10,12,41,51] level resolution, usually using a label free, area-under-the-curve quantification strategy [7,10,41,43], although other methods such as multiple reaction monitoring (MRM) quantification of the remaining unmodified peptides have been used [8,52].

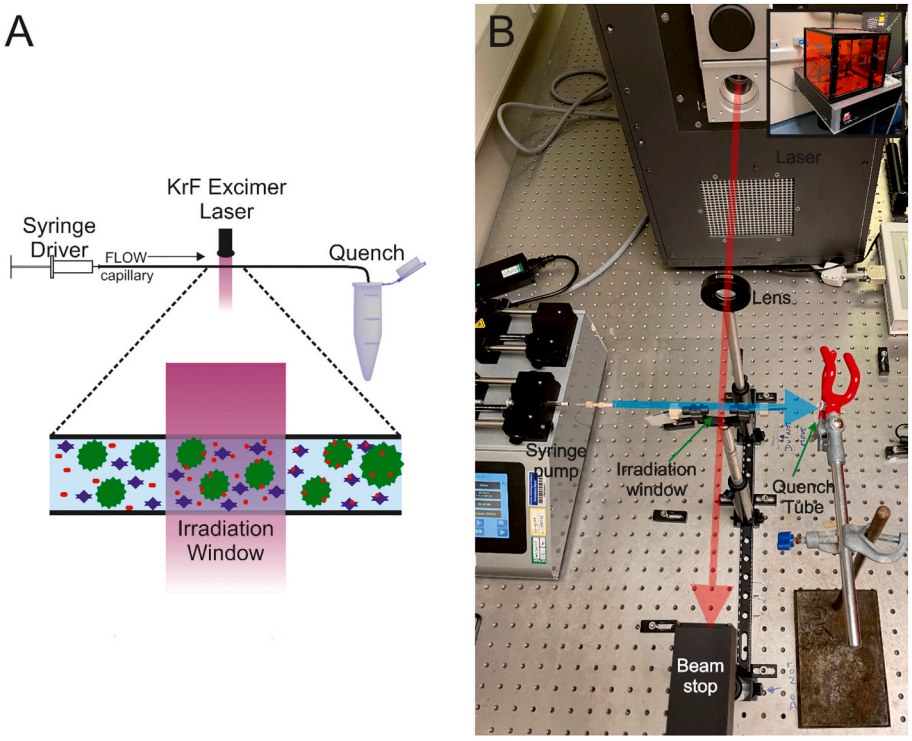

Fig. 2. Schematic representation (A) and practical example (B) of a basic FPOP setup using an excimer laser. The sample under investigation, containing protein, hydrogen peroxide and scavenger (green, red and blue respectively in A), is pumped through a polyimide-coated borosilicate capillary. Where the capillary intersects with the laser beam, the polyimide coating has been removed to create a UV transparent irradiation window at which the pumped solution is exposed to the laser beam. After exposure the sample is collected in a tube containing methionine, which quenches any remaining radicals, and catalase to breakdown any remaining hydrogen peroxide. Under normal operation, in this example, an amber Perspex box enclosure with interlocks is used to guard against radiation exposure (B, inset). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Applications and analytes studied using FPOP

Since the early pioneering developments of FPOP by Hambly and Gross in 2005 [37], the technique has been developed to study a wide variety of different protein analytes, in numerous fields of inquiry. Below are a selection of some of the main areas of study and types of analyte for which FPOP has thus far been utilised as a structural MS tool.

3.1. Biopharmaceuticals

Perhaps the most widely explored application of FPOP is for the analysis of the structure, dynamics and the protein-protein/protein-ligand binding interactions of biopharmaceuticals. Indeed, many characteristics of FPOP make this technique well suited for this purpose. Firstly, buffers used in FPOP and the formulation buffers used for biopharmaceuticals are often highly compatible. Free amino acids, used as •OH scavengers in FPOP experiments, are often already present as excipients in formulation buffers [10,53]. Indeed, FPOP has been used to

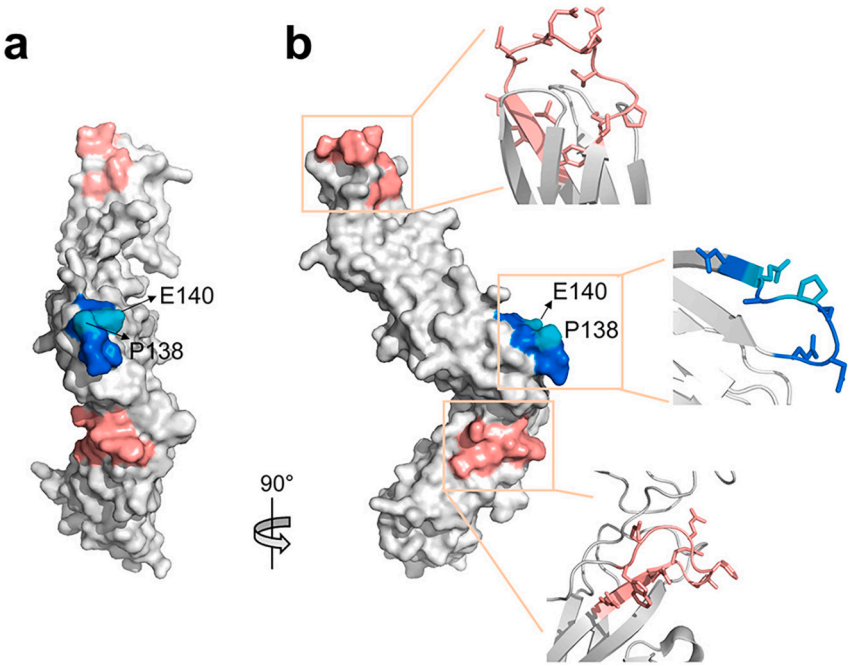

Fig. 3. Structural changes of IL-6R (PDB: 1N26) upon adnectin binding (a) front view and (b) side view. Changes in deuterium uptake or FPOP labelling upon adnectin binding are highlighted. Region 135–141 highlighted in blue and cyan undergo changes in both deuterium uptake and FPOP labelling in the presence of the adnectins. FPOP highlights a region of reduced solvent accessibility, colored coral in (b, upper inset) that were not observed by HDX likely due to fast dynamics in this loop region. Reprinted with permission Li KS, Chen G, Mo J, et al. Orthogonal Mass Spectrometry-Based Footprinting for Epitope Mapping and Structural Characterisation: The IL-6 Receptor upon Binding of Protein Therapeutics. *Anal Chem.* 2017;89(14):7742–7749. Copyright (2017) American Chemical Society [7]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assess conformational changes in mAbs resulting from different formulation buffer conditions, and the stabilising effects of various excipients, such as polysorbates [49].

FPOP has been highlighted in numerous studies as being highly complementary to other structural MS methods, such as HDX, commonly used in the biopharma sector. This is both in terms of labelling timescale and labelling targets (i.e. side-chain vs backbone) [7,41,54] and a number of studies have utilised both FPOP and HDX to characterise protein structure [7,15,41,51,54,55]. Li et al. used both HDX and FPOP to study the extracellular region of interleukin-6 receptor α-chain (IL-6R), a human transmembrane receptor implicated in a variety of autoimmune diseases, upon binding with adnectins, a class of therapeutic proteins designed from fibronectin domains [7]. HDX and FPOP together narrowed the critical binding epitope on IL-6R to a six amino acid sequence in the DII domain of the receptor, where residue level inspection of the FPOP data highlighted two possible binding residues within this region [7] (Fig. 3). Interestingly, several regions were found to undergo changes in loop dynamics, as well as various side-chain rearrangements which were observed using FPOP in the adnectin bound state, but were not observed using HDX, further highlighting the utility of using both of these methods in parallel [7].

As well as successful application to epitope mapping [7,12,51,56], FPOP can be used to probe local and long-range conformational changes in mAbs associated with reversible self-association [10] and with Fc binding [55]. Shi et al [55]. studied the Fc binding interaction between an IgG1 mAb and its Fc receptor, FcγRIII, using FPOP to identify six residues on FcγRIII, and three residues on IgG1 that were critical for the Fc binding interaction, which was found to be consistent with the crystal structure of the IgG1/FcγRIII Fc complex [55]. Interestingly, Fc binding was associated with changes in oxidative labelling showing both protection and exposure on seven residues in the Fab domain, distal from the Fc binding interface [55]. Long-range conformational changes have also been observed by FPOP in aggregation-prone mAbs [10] and could indicate a sensitivity of FPOP to probe subtle conformational changes challenging to identify using other structural and footprinting approaches [55].

FPOP has also been used to characterise common biopharmaceutical targets including the soluble regions of viral membrane proteins [57,58]. Poor et al [57]. used FPOP to track conformational changes of the PIV5 F protein, which is involved in the fusion of viral and host cell membranes during infection by viruses of the *Paramyxoviridae* family [57]. This protein was proposed to undergo significant refolding during activation and the subsequent membrane fusion process, although little was known about the nature of the refolding event involved. Using increasing temperatures as a surrogate for this refolding event, the authors employed FPOP to study the intermediate structures between the known pre- and post-fusion assemblies, proposing a mechanism whereby portions of the protein refold to form an extended α-helix, inserting a hydrophobic N-terminal 'fusion peptide' into the target membrane, before further conformational rearrangements bring the virus and host cell closer together, allowing membrane fusion [57]. This study highlights the utility of FPOP to characterise potential biotherapeutic targets, as well as transient conformational states, with the authors suggesting that FPOP could provide valuable guidance for developing more targeted antiviral treatments [57].

3.2. Membrane proteins

Despite being of significant biopharmaceutical interest, membrane proteins are notorious for being amongst the most challenging class of analyte to characterise by higher resolution methods, such as crystallography and cryo-EM [59]. Frequently encountered issues relate to their expression, purification, and reconstitution into suitable membrane surrogates for analysis. Structural mass spectrometry's ability to characterise complex mixtures and different conformational states make it well-suited for the characterisation of membrane proteins. Amongst the range of structural MS methods being increasingly utilised to study membrane proteins [59], FPOP has a distinct advantage due to the irreversible nature of the footprinting. This allows extensive sample preparation after labelling to remove detergents used in membrane protein solubilisation that would otherwise impact the sensitivity and quality of the subsequent LC-MS analysis [59]. Although, to date, relatively few studies have been conducted on membrane proteins using FPOP, it has been successfully employed in a variety of different solubilising agents including amphipols [42], detergent micelles [42], and nanodiscs [60]. Lu et al. studied the conformation of the model protein light harvesting complex 2 (LH2) from *Rhodobacter sphaeroides* in both detergent micelles and nanodiscs [60]. The ~18 transmembrane helices of this protein were found to be significantly protected from FPOP induced oxidation in the transmembrane region, with more protection generally found when using nanodiscs [60], an important observation as nanodiscs have been shown to be a better mimic of the lipid bilayer environment compared to the more widely used detergents [61]. Other studies have similarly identified that, while residues in the transmembrane region of membrane proteins can be oxidatively labelled [62], these regions are often heavily protected from oxidation [49,62,64].

A similar study by Ashcroft and co-workers utilised FPOP to study the effects of amphipols as solubilising agents on the structure of OmpT, a ~ 35 kDa 10-stranded β-barrel outer membrane protein [42]. Their results identified increased protection in the extramembrane domain of the protein when using amphipols compared with detergent micelles, rationalising this as the result of additional intermolecular contacts in these regions when using amphipols. In contrast, higher association of the DDM micelle with the lower trans-membrane domain could explain the decreased solvent accessibility observed with the use of detergents (Fig. 4) [42]. These studies highlight the importance of considering the role of the proxy for the lipid bilayer environment in the FPOP experiment. Indeed a recent study by Joshua Sharp's group [63] used a series of elegant experiments including membrane tethered hydroxyl radical dosimeters, to probe the scavenging effects of free and self-organized detergents. Their results show that while self-organized amphiphiles are not effective scavengers of bulk hydroxyl radicals, the non-random distribution of the membrane protein and scavenger in the vicinity of the membrane could lead to a high rate of scavenging at the membrane [63]. In light of this work the differences in oxidation of OmpT seen between solubilisation in DDM and amphipols may be as a result of different local scavenging capabilities of amphipol versus detergent and local concentrations of analyte and scavenger. Despite this there is good evidence that FPOP of proteins in or at the membrane is entirely possible and is supported by more established methods such as NMR [64]. Another recent development, nanoparticle-promoted photochemical oxidation of membrane proteins (nanoPOMP) further addresses this non-random distribution in the membrane vicinity [65]. Titanium dioxide nanoparticles interact with phosphate at the lipid surface and upon laser exposure produce excited electron states that can react with surface water molecules to give elevated local concentrations hydroxyl radicals as well as other radical species via other mechanisms. Further reaction of added acetone with the lipid bilayer allows the radicals to better penetrate the membrane region to increase the oxidation coverage of transmembrane region of the protein. Using this nanoPOMP approach the authors probed the conformational changes of a membrane protein hGLUT1 which is known to adopt inward and outward facing conformations upon binding with maltose and cytochalasin b respectively They showed distinct groups of residues in transmembrane regions underwent differential oxidation. This included two residues whose oxidation levels reflected the changes in SASA following conformational changes upon binding the substrates as well as residues showing reduced oxidation in both bound states supporting their interaction with the substrates in the binding pocket [65].

Rather than using membrane mimetics, several other studies have utilised FPOP to characterise the structure of membrane proteins in their native lipid or cellular environments [38,39,62]. Farrokhi and co-

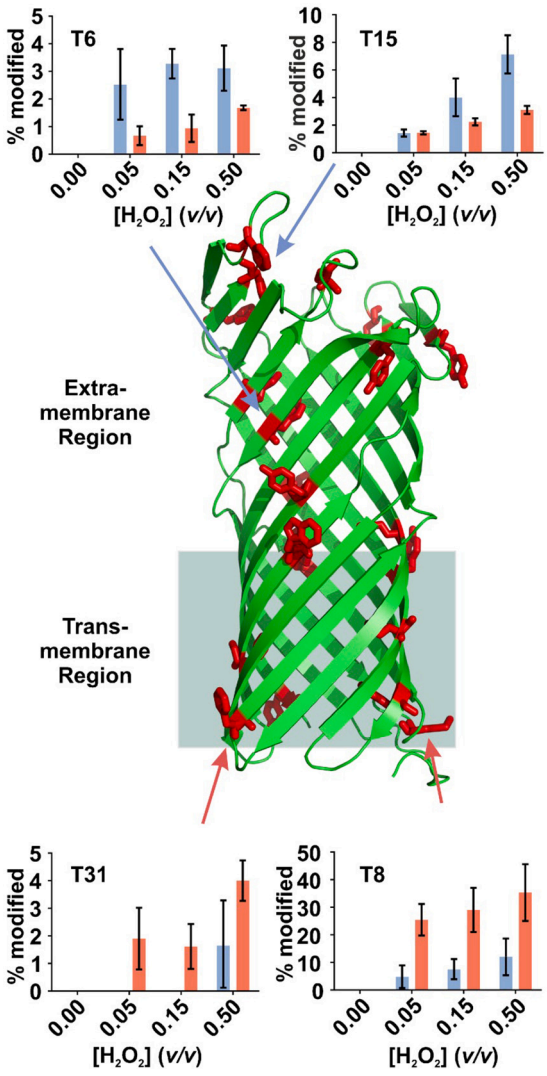

Fig. 4. Structure of OmpT (PDB 1I78) which was solubilized in DDM detergent micelles or in the amphipol A8–35. Graphs show quantification of oxidation levels in DDM (blue) or in A8–35 (red) for four tryptic peptides of particular interest, and arrows (red and blue) indicate the respective residues that are modified in each peptide. Aromatic amino acid residues are shown in stick form and colored red on the structure. Residues towards the lower boundary of the transmembrane region are less readily labelled in DDM, whereas residues in the extra membrane region are shown to be less readily labelled in A8–35. Reprinted with permission from Watkinson TG, Calabrese AN, Ault JR, Radford SE, Ashcroft AE. FPOP-LC-MS/MS Suggests Differences in Interaction Sites of Amphipols and Detergents with Outer Membrane Proteins. *J Am Soc Mass Spectrom*. 2017;28(1):50–55. Copyright (2018) American Chemical Society [42]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

workers [39] used FPOP labelling in a "reversed-footprinting" approach to study the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In this 'revesed' approach, the signal of the unoxidized peptides are quantified and compared between samples. This assumption here is that if a peptide/residue becomes more exposed then it will be more susceptible to oxidation and the intensity of the oxidised precursor with therefore decrease. This removes the caveats of considering both oxidised and unoxidized peptides, see data analysis section below. CTFR is a primary therapeutic target for the treatment of cystic fibrosis, and FPOP was carried out within saponin semi-permeabilized baby hamster kidney cell membranes. Upon examination of the FPOP data, the authors were able to identify 'structural marker' peptides; peptides at key positions of inter domain contacts, one of which, located in the ATP binding pocket, was speculated to report on the different 'gating states' of the protein, and the presence of both open and closed conformations [39].

3.3. Aggregation, protein/protein and protein/ligand interactions

FPOP has been used in a variety of studies to probe the interaction interfaces and conformational changes associated with both protein/ligand [13,14,37] and protein/protein [4,11] interactions. Two notable studies have focussed on tracking conformational changes associated with the metal-ion [13] or peptide ligand [14] binding to calmodulin, a small calcium binding protein known to act as part of a calcium signal transduction pathway. By titrating the calcium ion concentration, Liu et al. [14] used FPOP to track conformation changes and the order of successive calcium binding to calmodulin even calculating their binding affinities [14]. The authors were able to track the changes associated with melittin binding and, with residue level quantification, distinguish potential roles for three residues on the same proteolytic peptide as either undergoing conformational change or participating in direct interaction with the peptide ligand. Furthermore six hydrophobic residues located either in the N- or C-terminal hydrophobic clefts were found to be critical for the biding of the peptide ligand [14]. This same principle has been used to study protein/protein interactions. In the case of one study by Gau et al. [4] focussing on apolipoprotein E3 (ApoE3), FPOP revealed that the C-terminal helix, and a flexible region preceding it, had significantly lower access to solvent in the tetramer than in monomeric form, consistent with the hypothesis that this region constituted the primary self-association interface [4].

Interestingly, there are many examples of utilising FPOP in the study of aberrant protein/protein interactions and aggregation. Indeed, given that many protein aggregation events are thought to be driven by partial unfolding and solvent exposure of hydrophobic side-chains [40,41], HRFP techniques such as FPOP, with their proclivity for labelling these groups in particular, are perhaps uniquely well placed to study conformational changes associated with protein aggregation, and even the aggregation pathways themselves. To date, FPOP has been used to study a variety of aggregation-prone proteins [4,10,40,66] including some amyloid systems [41,54,67] and would seem particularly applicable to these types of studies. The irreversible nature of the oxidative labelling meaning that disaggregation with strong chemical denaturants usually used in the study of protein aggregates and fibrils can be employed without sacrificing the integrity of the label.

Sheng et al. [40] used FPOP to characterise human copper-zinc superoxide dismutase (SOD1), a protein implicated in the formation of toxic aggregates in amyotrophic lateral sclerosis. The authors were able to identify two hydrophobic side-chains, Phe20 and Ile112, which were susceptible to oxidation by FPOP, despite being predicted to have little to no access to solvent [40]. They suggest a possible explanation for these findings is that partial, transient unfolding of the β-barrel structure exposes these hydrophobic groups, generating an increased non-polar accessible surface area, which could contribute to the early aggregation events in this protein. Although the authors note the fact that O_2 and H_2O_2 are the products of SOD1 catalysis and that presence of OH radicals generated inside the active site channel complicates the interpretation based solely on solvent accessibility.

In another amyloid study by Li et al. [67], FPOP was used to probe the aggregation of $A\beta_{1–42}$, generally considered to be the most pathogenic Aβ peptide isoform involved in Alzheimer's disease [67]. By subjecting the $A\beta_{1–42}$ peptide to FPOP at different points during aggregation, the authors were able to take full advantage of the fast timescale and irreversible nature of FPOP labelling to track conformational changes within the peptide, at residue level resolution, during the aggregation process [67]. Their data revealed five distinct stages of $A\beta_{1–42}$ aggregation, identifying residues in both the central and C-terminal domains which displayed significantly increased protection from

labelling later in the aggregation process, suggesting drastically decreased access to solvent. Interestingly, their data also revealed that residues in the N-terminal region remained largely consistent in their degree of oxidative labelling throughout aggregation, suggesting this region remains solvent exposed, consistent with solid state NMR data [67].

4. Adaptations and improvements to the FPOP platform

Owing to the relatively simple syringe pump arrangement, and fast labelling timescale of the technique, the FPOP experimental setup lends itself well to adaptation for the study of fast conformational changes and protein folding, as was quickly recognised by many in the structural MS field. One early study from Konermann's group conducted on the model protein myoglobin used a novel triple syringe pump FPOP arrangement to initiate time resolved pH jumps prior to oxidative labelling, allowing the authors to characterise both early (50 ms) and late (500 ms) structural intermediates in the acid-induced unfolding pathway [8]. Subsequent studies from the same group later expanded on this work by using similar pH jump methods to study the folding pathways of other model proteins [68], and the folding and subunit assembly of homodimers [69], eventually progressing to fast laminar flow mixing strategies to achieve sub millisecond time resolution [70].

Laser induced temperature jump experiments, coupled with FPOP, can be used to probe protein folding in the sub millisecond time range and at the residue level, something that is difficult to probe by other structural biology approaches [44,71]. Gross and co-workers utilised a dual laser setup for this purpose, where the sample was first irradiated using an Nd:YAG laser to initiate the temperature jump, prior to irradiation with an excimer laser to generate the hydroxyl radicals [44]. These two systems were coupled via a delay circuit, allowing variable time delays between unfolding and oxidative labelling. This allowed the authors to characterise their test protein, barstar, to residue level resolution, finding evidence consistent with a nucleation-condensation folding mechanism centred around $helix_1$ of the protein [71].

Other adaptations and improvements to FPOP have focussed on more accurate measurement of the radical dose to ensure reproducibility and consistency. For example, Sharp and co-workers have developed strategies to more precisely measure the hydroxyl radical dose experienced by the protein analyte by inline dosimetry, measuring changes in the UV absorbance of either adenine [72] or TRIS buffer [73]. Consequently, this allows FPOP comparisons between different solution conditions where the •OH scavenging ability of the buffer may vary [49,73,74]. Alternatively, Gross and co-workers have shown that the incorporation of a reporter peptide, usually leucine enkephalin, into the FPOP analyte solution can serve a similar purpose. Using oxidation on this peptide as an internal standard to correct for changes in the scavenging ability of the buffer [75], this approach has been used to ensure equivalent 'scavenging potentials' when comparing multiple biotherapeutic samples [76]. Multiple studies have since utilised this method to perform time-dependent FPOP experiments, where the concentration of scavenger in the buffer is titrated and the increasing modification on the reporter peptide is plotted against the modification of the analyte protein [15,55,75]. This generates oxidation response curves, somewhat analogous to deuterium uptake plots in HDX.

Development work has also shown that the radical labelling chemistries of the FPOP platform can be significantly expanded to include more than just •OH labelling, with the goal of attaining greater structural information by targeting different types of amino acids requiring only a change in the reagents used in the experiment and these can be chosen to suit the system under study. Both carbonate ($CO_3^{\bullet}$) [77] and sulfate ($SO_4^{\bullet}$) [19] are just two examples of radical anions that can be generated using the FPOP platform. The radicals are generated either by direct photolysis of a reagent using the standard 248 nm KrF excimer laser, or by secondary radical generation from the interaction of •OH with an added reagent or buffer. Hydroxyl radicals from FPOP have been used to initiate radical trifluoromethylation ($\bullet CF_3$) of proteins through secondary radical generation. The footprinting specificity of radical trifluoromethylation encompasses 18 of the 20 naturally occurring amino acids, but is radically different to that of •OH, more frequently labelling side-chains less amenable to oxidation by the standard FPOP approach, such as glycine and alanine [78]. In another example of the flexibility of the laser-induced FPOP approach, the production of positively charged labelling reagents is also possible [79]. For example, the trifluomethoxy benzyl (TFB) carbocation is produced from trifluomethoxy benzyl bromide upon exposure to laser irradiation at 248 nm and mainly targets nucleophilic residues. The increased hydrophobicity of the resultant TFB adduct peptides also helps to improve separation of isomeric peptides [79].

Some refinements have forgone the typical syringe pump format entirely, in favour of static, single shot experiments. Riaz et al. developed a novel FPOP setup utilising a beam mirror to direct the UV irradiation into a 96-well plate [80]. While this arrangement may be less well suited to the protein folding experiments described above, this method does remove the complication of laminar flow on single exposure conditions [47], with the authors suggesting that further development would make FPOP in this setup a good candidate for automation, with possible applications for screening [80].

Jones and co-workers, have utilised both static [81], and flow based methods [38] in the development of *in vivo*l FPOP (IV-FPOP). This remarkable expansion in the potential applications of FPOP has allowed direct structural characterisation of proteins in various different cellular compartments, as well as the cell membrane and various organelle membranes, to residue level resolution in their native cellular environment [38]. One early study was able to identify more than 100 different proteins that had been oxidatively modified following IV-FPOP, that were located in 10 different cellular compartments [38]. Further development of the IV-FPOP method, focussed on reducing cell aggregation in the flow system, later improved the number of identified proteins by more than an order of magnitude [82]. The same research group later expanded on this idea to develop FPOP in higher organisms, demonstrating that this method could characterise protein structure to residue level resolution in the routinely used model organism, *Caenorhabditis elegans*, identifying more than 500 different labelled proteins in six different organs of the worm [48]. This number was increased further, and multiple oxidation sites per protein were characterised, by the use of chemical penetration enhancers to increase hydrogen peroxide uptake in *C. elegans* [83].

Very recently alternative FPOP approaches have also emerged as well as the first commercialised, benchtop FPOP platforms. The FOX (Flash OXidation) Protein Footprinting System [84] from GenNext Technologies dispenses with the requirement for the laser for photolysis instead employs a Xenon flash lamp. This highly automated system also incorporates an inline radical dosimeter allowing for in-experiment control of changes in radical scavenging, for example by changes in buffer composition between samples. The instrument will likely provide a lower entry barrier for groups employing FPOP owing to its ease of operation, compact size, and reduced safety and maintenance burden by removing the need for a class 4 laser.

5. LC-MS/MS and data analysis strategies

Despite the many advantages of using FPOP for the structural characterisation of proteins, perhaps the most significant and widely recognised caveat across all implementations of FPOP is the complexity of the resulting data [8,85].

By far the most routinely adopted strategy for FPOP data acquisition and analysis, is a bottom up, reverse phase LC-MS/MS approach. Oxidations are quantified by label-free, area-under-the-curve integration comparing the oxidised forms of each peptide to their corresponding unlabelled variants. Indeed, this method carries with it several advantages. Firstly, modified peptide variants typically separate from both

each other, depending on the modified residue within the peptide, and the unmodified peptide variant. The modified variant typically elutes at a shorter retention time than the unmodified peptides due to the increased hydrophobicity conferred by the addition of oxygen. Not only does this allow a distinction to be drawn between genuine FPOP oxidations, and oxidations introduced after LC separation by the electrospray process [10], but fragmentation spectra taken of each peak in the LC trace allows residue level identification and quantification of oxidations, and thus higher structural resolution. This method has been shown to reliably quantify modified peptides at residue level resolution, even for those ~10,000 times less abundant than the unmodified variant [86]. Additionally, separation of peptides modified on the same residue, but with different positional isomers (i.e *ortho, meta, para* positions on Phe side-chains) has been highlighted by studies using FPOP (Fig. 5) [10,41,54]. as a source of a further increase in structural resolution to the sub-residue level. Some studies have begun to utilise the changes in LC retention time for modified peptides as a potential aid to MS/MS in identifying the modified residues within those peptides [10]. Although, historically much of the analysis, in terms of assignment and quantification, was done manually, the popularity of this approach and the increasing use of FPOP and covalent labelling methods generally has led to the development of several automated analysis methods [87,88].

However, the above method is also associated with many shortcomings. Firstly, missed proteolytic cleavages generated by oxidations at Arg and Lys side chains can complicate the quantification of modifications on these residues [8], especially given the prominence of trypsin and lysC as the proteolytic enzymes of choice for bottom-up digest procedures. Secondly, this quantification method implicitly assumes that all peptide variants have the same ionisation efficiencies [8,39] which is unlikely given the effect some oxidative modifications are known to have on the charge state distribution of some peptides [10]. However the the use of a comparative, or differential, experimental design through the incorporation of control samples can overcome these issues where appropriate. Lastly, partial separation of oxidised products of the same *m/z* can make both quantification and assignment challenging, as the resulting fragmentation spectra used for assignment can be chimeric.

Alternative quantification and analysis strategies have been developed which rely on the *co-elution* of different oxidised products of the same peptide, utilising either hydrophilic interaction chromatography

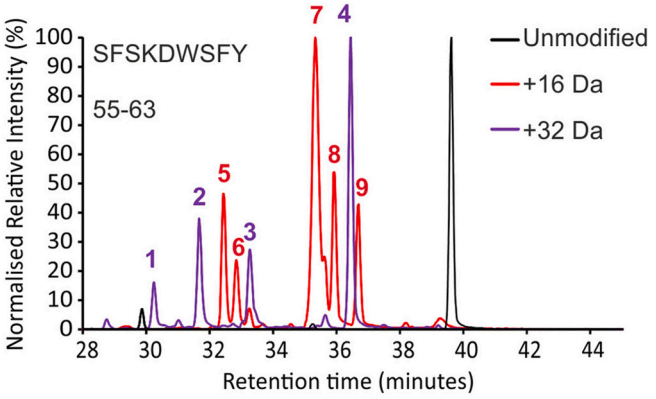

Fig. 5. XICs for unmodified (black), +16 Da (red) and + 32 Da (purple) modifications for a peptide, SFSKDWSFY from β_2-microglobulin. MS/MS showed that the modified residue in peaks 1–9 was the C-terminal tryptophan. Each peak corresponding to different structural isomers if either +16 Da or + 32 Da oxidation. Reprinted from Cornwell O, Radford SE, Ashcroft AE, Ault JR. Comparing Hydrogen Deuterium Exchange and Fast Photochemical Oxidation of Proteins: a Structural Characterisation of Wild-Type and ΔN6 β2-Microglobulin. *J Am Soc Mass Spectrom*. 2018;29(12):2413–2426. Copyright (2017) American Chemical Society [41]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[85] or size exclusion chromatography [89]. The relative proportion of oxidised products can then be quantified to residue level resolution by MS/MS, rather than using extracted ion chromatograms, by comparing modified and unmodified fragment ion intensities [85,89]. Although this simplifies the data analysis and quantification of FPOP data considerably, this method, too, comes with its own complications. For example, the fragmentation ion intensities for each oxidised position must reflect the true extent of oxidation at each residue. This is relatively uncommon when using the most popular fragmentation approach, CID, given the varying dissociation chemistries of each oxidised isomer [90] and, as such, these methods rely on electron transfer dissociation (ETD). While ETD has been shown to accurately report on the relative degrees of oxidation at specific sites when quantifying using MS/MS data [90], ETD is known for its charge dependent, and often poor, fragmentation efficiency.

Instead of quantifying the degree of oxidation separately for each oxidised variant of a peptide, several studies have instead simply quantified the loss in intensity of unmodified peptide [8,39,40,52], often by comparisons to internal standards [8,39], a quantification method sometimes referred to as reverse FPOP [39]. This strategy avoids many of the pitfalls of other methods, such as differential cleavage at oxidised Arg and Lys side-chains, as well as different ionisation efficiencies or partial LC separation of oxidised products. Additionally, this quantification strategy inherently also quantifies •OH induced backbone cleavages which, although uncommon under normal FPOP conditions [35], are not quantified by other methods. Although this is perhaps the simplest quantification strategy for FPOP data, it is fundamentally limited to peptide level resolution. This can be problematic, as the degree of oxidation on the most •OH reactive residues in the peptide can often mask small but significant changes in oxidation levels on less reactive side-chains [86].

Clearly, each of these methods have their own advantages and limitations, and the choice of which method is optimal will depend on the priorities of each individual experiment (i.e. structural resolution, sensitivity, or precision/accuracy of quantification). Indeed, there is significant space for further development and optimisation in this area. More powerful automated software platforms are being developed and commercialised such as by Protein Metrics. Through examining the utility of alternative MS/MS acquisition methods or additional/alternative separation approaches may be employed. 2D-LC has already been shown to drastically increase the number of identifiable and quantifiable peptides in FPOP through improved separation [91]. A multiplexed isotopic tagging and labelling approach, cPILOT has recently been shown to increase throughput of FPOP experiments in IV-FPOP of *C. elegans* [92]. Given the recent developments in commercially available, high resolution ion mobility spectrometry instruments [93] IMS can offer an extra dimension of resolution for improved identification and quantification of oxidations. IMS separation coupled with covalent labelling methods was recently highlighted as having significant implications for drug design, where IMS was used to differentiate sub-residue peptide isomers from carbene labelling experiments, revealing interaction nuances between differential ligand binding [94].

6. Data interpretation

Changes in the degree of oxidation of amino acid side-chains in FPOP can report on structural and accessibility changes in proteins. Many indicate a clear positive correlation between the degree of oxidative labelling and the expected solvent accessibility of the residues [91,95,96]. However, recent studies have been directed towards probing the nuances involved in the relationship between solvent accessibility and the degree of oxidation observed, allowing more detailed and in-depth interpretations of the data.

Numerous studies have now highlighted the importance of sequence context [96,97] and local microenvironment [10,41,50] on the extent of oxidative labelling. Charvatova et al. [11] identified varying

relationships between SASA and the extent of labelling for different amino acid types [11]. Moreover, it was found that, for some side-chains, a varying minimum level of solvent exposure was required before any labelling was observed, although the extent to which these relationships remain true across different protein analytes remains to be seen [11]. More recent studies have also suggested reactive side-chains can compete for hydroxyl radicals with nearby groups, after identifying that less reactive residues could be less oxidised in unfolded proteins, relative to their folded native states, despite the presumed increase in solvent accessibility [96]. Additionally, it has been suggested that preferential hydrogen bonding between hydrogen peroxide and certain amino acids, such as histidine and arginine, can give rise to high local concentrations of •OH following photolysis [50]. This is, perhaps, one of the more notable caveats of utilising FPOP as opposed to radiolysis HRFP methods, where •OH generation from water is likely to give rise to a more uniform distribution of labelling radicals. Other possible factors regarding local microenvironment and the relationship between SASA and the extent of oxidative labelling have also been raised, involving the differential exposure of regions of the protein to hydrogen peroxide and scavenger [40], as well as electron tunnelling effects [40], although these have not widely been explored in the context of the FPOP literature.

While the analysis and interpretation of FPOP data is far from being able to accurately predict SASA for side-chains under all conditions, early attempts have already been made to establish protection factors for FPOP data [95]. Our current understanding of the technique has thus far enabled SASA calculations derived from FPOP labelling to assess the quality of protein structural models [96], drive MD simulations to identify folding intermediates [98] and even predict protein structure [99,100].

7. Conclusions

The utilisation of FPOP to provide insights into the structure of proteins, and their complexes, has seen broad applicability across the field of structural biology, including in areas that are of high biopharmaceutical relevance. This has been aided by its complementarity to other footprinting methodologies, as well as the relatively low setup threshold compared to other HRFP techniques. Continued modification and adaption of the technology with alternative reagents, laser regimes and data quantification strategies have greatly amplified the flexibility of the technique, allowing examination of biological systems from single proteins to whole, live organisms.

Although the number of research groups using FPOP has steadily increased, in comparison to more well-established MS based footprinting methods, such as HDX, FPOP is still in its relative infancy, where the capabilities and limitations of this method are still being explored. For the time being, data analysis remains complex and time-consuming, particularly for residue level quantification, hindering, wider uptake by industry and biopharma. The need to use a class 4 laser is also a barrier to wide adoption due to the cost of health and safety precautions required preventing their use as bench top devices in conventional molecular biology laboratories. Though recent development of bench top, laser free platforms will reduce the entry barrier. Furthermore, the nuances of the radical labelling processes involved are still not well understood. How the modulation of an amino acid side-chain's oxidation is affected by the reactivity and solvent exposure of other side-chains in close proximity being one area that requires further investigation before oxidation changes can be fully interpreted.

That being said, the rate of advancement and increased understanding of this method in recent years has rapidly brought FPOP in line with other structural MS approaches, such as chemical cross linking and HDX, as an integrative structural biology tool to complement higher resolution techniques such as cryo EM, x-ray crystallography and NMR. Together, this suggests a bright future for FPOP in structural biology, where further development will no doubt increase the utility of FPOP and catalyse broader use of this powerful and rapidly evolving, structural MS approach.

Author contributions

O.C. was responsible for writing the original draft. J.R.A was responsible for original figures. O.C. and J.R.A. contributed equally to review and editing of the draft.

CRediT authorship contribution statement

Owen Cornwell: Conceptualization, Writing – original draft, Visualization, Writing – review & editing. **James R. Ault:** Conceptualization, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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