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Mass spectrometry in structural proteomics: The case for radical probe protein footprinting

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ABSTRACT

A critical assessment of the advantages and disadvantages of each of the solution-based structural proteomics mass spectrometry methods, known as hydrogen exchange, chemical cross-linking, limited proteolysis and radical probe protein footprinting, is presented. Separate consideration is given to each of the methodological steps involved in the chemical and enzymatic treatment of proteins and the subsequent analysis of modified or degraded proteins by mass spectrometry. A case is made to preference radical probe protein footprinting when all facets of the approaches are considered side-by-side. The RP-MS approach effects limited non-reversible oxidation at reactive amino acid side chains, the levels of which are easily resolved and measured. Furthermore, the RP-MS approach can be performed at the ion source interface with the mass spectrometer, can investigate the onset of oxidative damage, is amenable to on-plate deposition for high sample throughput, and can be applied to study otherwise intractable membrane proteins, and proteins *in vivo*.

1. Introduction

For decades X-ray crystallography and NMR spectroscopy have remained the most widely accepted approaches with which to characterise protein structures [1] and their complexes at atomic resolution. In the post-genomic era, limitations posed by genomic analysis [2] prompted the emergence of the field of proteomics [3]. This subsequently highlighted the role that mass spectrometry [4] was then playing in protein analysis and sequencing [5–7], that in turn led to its application in proteomics [8].

These advances led to the need to characterise the three-dimensional structures of previously uncharacterised proteins with higher sample throughput and at lower sample levels and purities than achievable by either X-ray crystallography or NMR spectroscopy. Today, fewer than 144,000 protein structures have been characterised at high resolution (X-ray and NMR structures, RCSB Protein Databank, August 2018) while some 120 million have been identified at the sequence level either directly or through the translation of gene sequences (Universal Protein (UniProt) Resource, August 2018). While computational approaches to build protein structures from sequence data *ab initio*, or using template or homology modelling, have improved in their reliability and performance [9], experimental based methods are still considered more reliable indicators of the physiological state of proteins.

The field of structural proteomics has emerged over the past 20 years [10], albeit the throughput that is currently achievable is lower than that for protein identification in proteomics. As in proteomics, mass spectrometry represents the only viable technology with which to tackle the structural characterisation of proteins at the molecular level, due to the speed of analysis and the sensitivity of modern instrumentation [11].

In contrast to what is now often described as “native mass spectrometry” [12,13], in which proteins are ionised, separated by mass [14] and/or mobility [15,16], and analysed in their gaseous state, solution based methods seek to emulate the conditions protein experience in a biological environment (Table 1), at least to the same or better extent to that achieved in X-ray crystallography and NMR spectroscopy experiments.

Solution based structural proteomics employing mass spectrometry involve four different approaches each requiring some modification, or fragmentation, of the protein or complex of interest ahead of analysis (Fig. 1). However, the level and specificity of each modification varies considerably across the four approaches. Limited proteolysis (LP) cleaves a protein or complex along the backbone adjacent to specific residues, an approach

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Table 1
Comparison of common mass spectrometry methods used in structural proteomics.

Method	Electrospray Ionization	Ion Mobility	Hydrogen Exchange	Radical Probe Mass Spectrometry	Chemical Cross-Linking	Limited Proteolysis
acronym	ESI-MS	IM-MS	HX-MS	RP-MS	CX-MS	LP-MS
ionization method	ESI	ESI	various	ESI[a]/various	various	various
analysis	direct	direct	post-treatment	post-treatment	post-treatment	post-treatment
solution state relevance	physiological relevance in question	physiological relevance in question	yes - D_2O replaces water	yes - HO• generated from water	subject to cross-linking chemistries	yes - site-specific endoproteinases
implementation aspect #1	difficult to ionise without careful handling and preparation	difficult to ionise without careful handling and preparation	slow exchange at amide hydrogen	HO• generated in microseconds and reacts in low milliseconds	cross-linking chemistry can be slow and incompatible with physiological conditions	endoproteinases digest proteins over many hours
implementation aspect #2	subject to gas phase structural perturbation during analysis	subject to gas phase structural perturbation during analysis	isotopic labelling susceptible to reversible chemistries H ⇔ D	fast and irreversible chemistry and small size of HO radical	irreversible but extensive/large modifications may perturb structure	can be accelerated with immobilized enzymes
structural resolution	low	low	mid	mid	mid	mid
resolution details	low structural resolution, protein complex stoichiometry	low structural resolution; average cross-sections and complex stoichiometry	measures solvent accessibility at backbone	measures solvent accessibility at oxidised side chains	measures solvent accessibility at modified residue side chains	measures solvent accessibility at oxidised side chains

[a] where oxidised protein is analysed directly by ESI-MS.

encumbered by the folded structure of a protein [17,18] and/or its interaction with some binding partner(s). Hydrogen exchange (HX or HDX) replaces labile hydrogen atoms throughout the protein or complex with deuterium (or sometimes tritium), where those at the amide backbone are retained after quenching [19,20]. Chemical cross-linking (XL or CX) modifies selective amino acid side chains and creates bridges where there were none between like and unlike residues across a protein's structure. Only residues in close proximity in a protein's structure or complex can be bridged if the cross-linker and the chemistry employed to effect the reaction does not alter that structure [21]. Finally, hydroxyl radical protein footprinting (HRF) using a radical probe (RP) reagent selectively oxidises the side chains of reactive residues, the extent of reaction impacted by protein structure constraints [22–24], or changes in structure due to folding or unfolding events [25].

Each of these approaches have been extensively applied and reviewed individually [26–36], or among two methods [37], but no review has yet comprehensively and systematically compared all four approaches side-by-side. A level of maturity has now been reached to justify such a comparison. This review compares the four approaches, within the page constraints of such a review, and makes a case for the benefits of radical probe protein footprinting.

2. Chemical and enzymatic probes; reaction times, specificity, and impact on structure

2.1. Deuterium

The least invasive structural probe is that employed in hydrogen exchange mass spectrometry (HX-MS). The replacement of hydrogen with its heavy isotope (deuterium or tritium), or the reverse in the

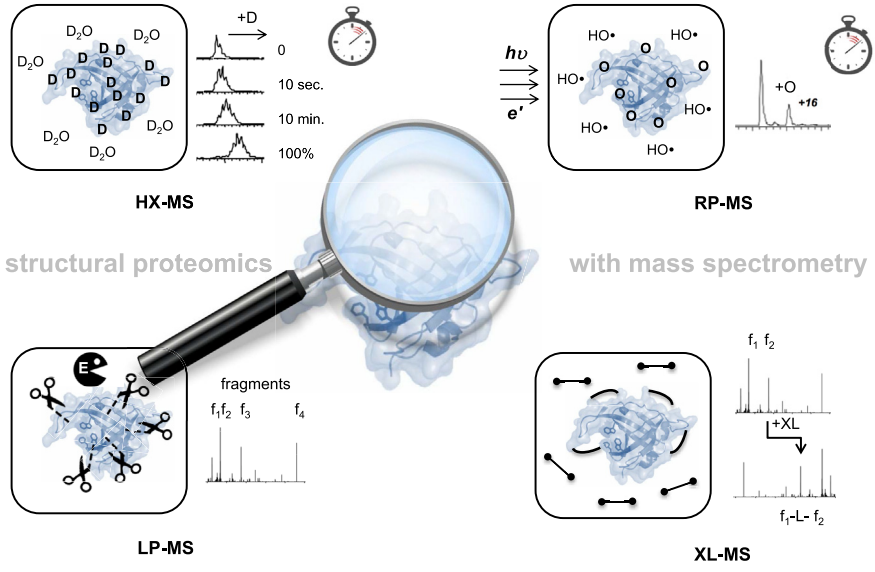


Fig. 1. Overview of the solution based structural proteomics approaches.

case of back-exchange reaction, has no appreciable impact on a protein's structure [38]. Hydrogen/deuterium exchange (HDX or just HX) has became widely used in conjunction with NMR spectroscopy [39] but increases in resolving power and mass accuracy, led to it being adopted with mass spectrometric detection [19].

In a typical HX-MS experiment [40] a protein or complex of interest is incubated in a solvent medium enriched with deuterium, usually D_2O. The exchange reaction is allowed to proceed for a specified period in time, typically ranging from a few seconds to several days (Table 2). Subsequently, the reaction is quenched by lowering the pH and temperature of the solution. This quenching step is required to retain the deuterium level at the slow exchanging amide hydrogen atoms of the backbone. In alternate pulse labelling experiments [41], to study protein folding dynamics and intermediates, exposure to deuterium is brief and an unfolded protein is triggered to refold, with the assumption that it readopts its native fold, by the addition of an appropriate buffer.

The deuterium content is then measured as rapidly as possible, either directly in terms of the global structure, or more often after proteolytic digestion and/or fragmentation within the mass spectrometer. Typically LC-MS is employed, using a relatively fast or steep elution solvent gradient and a temperature cooled column, both for desalting purposes and to minimise the number of overlapping isotopic peptide clusters in a mass spectrum. This is performed to allow the level of deuterium to be reliably measured [42] within these peptide segments. Note that the actual deuterium level at a single residue is not determined.

2.2. Hydroxyl radicals

Oxidation based, radical probe (RP) protein footprinting experiments introduces oxygen atoms rather than deuterium but does so in a more limited manner and exclusively at a set of reactive amino acid side chains [43]. Residues that react with hydroxyl radicals at rates of between 5×10^9 to 10^{10} $M^{-1}s^{-1}$ on millisecond timescales, without any appreciable to their side chain structures that can otherwise negatively impact a protein's structure and interactions, are Met, Cys > Trp, Tyr, Phe > His > Leu and Lys, shown in their order of reactivity (Table 2) [32,43].

Original X-ray synchrotron radiolysis, performed at a specifically-constructed beamline [43], and electrical discharge [22] experiments performed in an electrospray ion source of a mass spectrometer, generated hydroxyl radicals from the bulk solvent (according to Equation (1)) that reacted with protein molecules in the same solution. Both effect limited oxidation of the protein (typically 10%, but up to 30% globally) and preserve its structure where the exposure time was deliberately kept short (of between 10 and 30 msec). This radical exposure introduces up to a total of 8–10 oxygen atoms at reactive side chain residues for a modestly sized protein such as lysozyme, though this oxidation is distributed throughout the protein at a greater number of residues within the protein population in solution. Namely, in the mono-oxidised form not all oxidation occurs at a single reactive residue in all protein molecules in the population; it is distributed at different residues.

$$H_2O + h\upsilon/e^- \rightarrow H_2O^+\bullet + e^- (+H_2O) \rightarrow H_3O^+ + HO\bullet + e^- \quad (1)$$

Such experiments deliberately avoid slow Fenton chemistries [44] to prevent degradation and unpredictable protein denaturation in the presence of such chemical agents. Laser based photochemical oxidation approaches provide the ability to generate radicals on microsecond timescales [45], with the potential to study protein folding dynamics, albeit the oxidation reaction timescales are appreciably slower. However, the presence of hydrogen peroxide and other oxidants, not removed by quenching or other means post exposure, provide a means to extend reaction timescales beyond those intended. A recent investigation of this phenomenon, reported that laser-induced microsecond exposure of proteins in hydrogen peroxide results in reactions that extend over tens of milliseconds [46] consistent with synchrotron radiolysis and discharge studies. The laser-based approach also necessitates the introduction of scavengers to mop up unreacted radicals [47].

Like HX-MS, all RP-MS protein footprinting experiments assess the levels and site of oxidation after proteolysis and/or fragmentation within the mass spectrometer. Levels of oxidation are more easily measured and quantified in the peptide constituents or protein fragments in this regard, since oxygen introduces a more easily resolved, larger mass increase (nominally 16 Da per atom). Where a protein structure has been predicted or modelled, the RP-MS oxidation data can be used to confirm that structure or generate model for a complex [48,49] using software designed for that purpose. The Protein Oxidation Interface Modeller (PROMIXO) algorithm [50] can be used to assist with this process.

Only in the case of the electrical discharge experiments, has direct evidence been obtained for the preservation of a protein's structure using ion mobility mass spectrometry [51]. Here, the

Table 2
Chemical and enzymatic reagents, levels, reaction times, and specificity of structural proteomics MS approaches.

Approach	chemical or enzymatic reagent(s)[a]	levels	reaction times	specificity
HX-MS	D_2O	100%	seconds to days	none
RP-MS	H_2O (H_2O_2[b])	n/a (30% or mM levels[b])	milliseconds	Met, Cys > Trp, Tyr, Phe > His, Pro > Leu (>Lys[c])
XL-MS	N-hydroxysuccinimide ester (NHS) imidoester maleimide alkylhalide carbodiimide phenylazide	20–50 fold molar excess over protein[d]	hours	Lys Lys Cys Cys Asp and Glu non-specific
LP-MS	trypsin Lys-C Arg-C Glu-C (name) Asp-N chymotrypsin	(protease) 1:5000 (protein) 1:50 1:50 1:200 1:200 1:2000	hours to day	Arg and Lys Lys Arg Glu (Gln[e]) Asp (Asn[e]) aromatics (Tyr, Phe, Trp)

[a] Only the most common chemical and enzyme reagents are listed for XL-MS and LP-MS.
[b] The use of hydrogen peroxide removes physiological-like conditions, can substantially increase reaction time, and requires reagent scavenger.
[c] Other less reactive residues that substantially modify residue side chains that can impact on their stabilising interactions are not shown.
[d] Optimal cross-linker to protein molar ratios for reactions need to be determined for each reagent.
[e] Subject to pH of solution at which reaction is performed.

limited uptake of oxygen was found to have no impact on the mobility of proteins with their structural cross-sections unchanged across all oxidised and unoxidised forms (Fig. 2).

2.3. Cross-linkers

The structural proteomics MS approach which causes the greatest modification to proteins and their complexes is cross-linking mass spectrometry (known variably as XL-MS, CL-MS or CX-MS). A wide range of cross-linkers has been employed to effect the irreversible modifications to proteins (Table 2) that have been reviewed elsewhere [29,30]. The original bifunctional cross-linkers were coupled to cysteine residues and took advantage of the reduction of disulphide bridges back to a thiol form [52,53].

The majority of cross-linkers currently used today involve reaction at the primary amine of lysine residue side chains and include N-hydroxysuccinimide (NHS) [54] and sulfosuccinimidyl esters, imidoesters or imidates, and carbamates (Fig. 3A–C respectively). Carbodiimide cross-linkers that couple with the carboxylic acid groups on the side chains of aspartic and glutamic acid residues have also been developed to provide a greater number of cross-link targets and these include 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Fig. 3D) and acid dihydrazides [55,56]. Dicarbonyl reagents that respectively couple arginine and lysine residues have also been developed [57,58] while the development of genetically encoded p-benzoyl-L-phenylalanine residue allows for the incorporation of a photo-activated cross-linking reagent with the advantage that it enables the investigation of protein interactions within the cell [59–61].

Irrespective of the cross-linker, the conditions to effect the reactions are necessarily restricted in terms of pH, temperature, and solvent/buffer composition in order to preserve the structure of the proteins under investigation. For the most part, cross-linking chemistries are very far removed from the solution environments proteins are subjected to *in vivo*, and this poses a serious challenge to the method [60]. The ratio of cross-linking reagent to protein must also be carefully controlled to prevent undesirable side reactions [62,63] and protein denaturation [64,65] and is generally kept at a 20–50 mol excess over protein (Table 2).

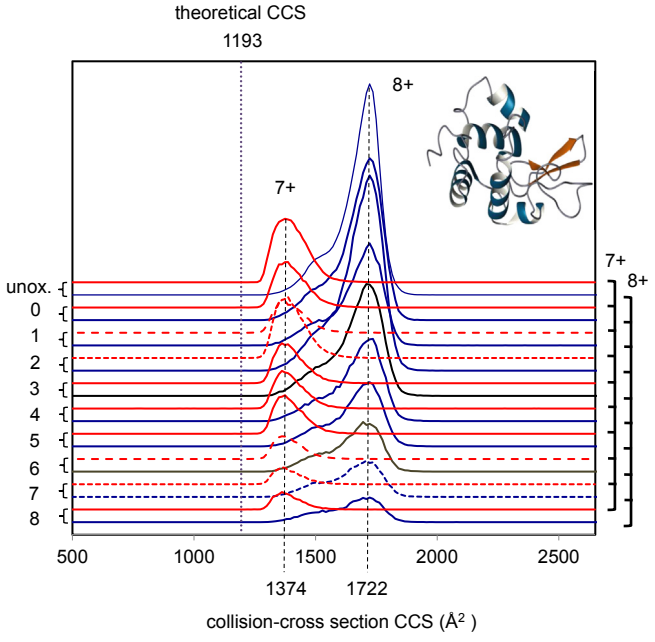


Fig. 2. Collision cross sections of unoxidised (0) and oxidised (1–8) forms of the protein lysozyme of two different charge states (7+ and 8+).

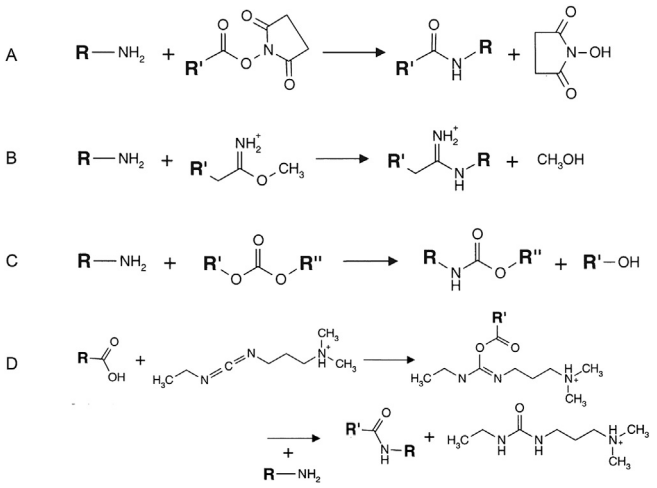


Fig. 3. Common cross-linking reagents involve reaction at the primary amine of lysine residue side chains that include: (A) N-hydroxysuccinimide and sulfosuccinimidyl esters, (B) imidoesters or imidates, and (C) carbamates (Fig. 3A–C respectively), and at the carboxylic acid groups of aspartic and glutamic acid residues that include (D) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

By using differential peptide mapping, where the spectrum of the protein sample post-digestion is compared before and after chemical treatment, it is possible to determine those peptides that are interconnected during a cross-linking reaction thereby identifying sites within reach, by the length of the cross-linking reagent, in the protein structure or complex. The preparation of protein samples for digestion is a critical aspect of XL-MS in order to maximize the coverage of the cross-linked protein that requires optimal proteolytic cleavage, and recovery and detection of the conjugated peptide components.

The probability that the cross-linked products reflect the actual proximity of the segments of a protein or binding partners must carefully be considered given the possibility that the cross-linking chemistries and conditions might perturb a protein's structure. Another common misconception is that the absence of a cross-linker demonstrates that residues are not in close proximity or do not interact. This outcome may reflect the incompatibility of the cross-linking reagent and that its geometry prevents a reaction from occurring.

2.4. Endoproteinases

Limited proteolysis has an important role in the regulation of many biological pathways, ranging from blood coagulation, cell proliferation, to cell death [66]. The application of limited proteolysis to study protein structures and their complexes has been in widespread use for a number of decades [17]. Endoproteases have a range of specificities in terms of the residues they cleave [67] typically with reaction times over a period several hours (Table 1).

Site-specific endoproteinases such as trypsin, Glu-C, Asp-N etc. reproducibly cleave at a limited set of residues such that the peptides produced contain common residues at their N or C-termini. Trypsin, in particular, cleaves a protein on the C-terminal side of arginine and lysine residues, the cleavage only encumbered when a proline residue is N-terminal to these residues [68]. Proline contains no hydrogen at its nitrogen atom by virtue of its cyclic side chain. Key to the success of the approach is the ratio of endoproteinase to protein and the reaction time (Table 2).

Limited proteolysis of protein complexes purified by native gel electrophoresis can aid in their study [69], while recovery methods to extract such proteins and complexes for proteolytic

treatment to investigate interaction domains have also been developed [70].

It is frequently necessary to quench or stall the proteolysis reaction by changing the solution conditions. Quenching is often achieved by changing the pH to that which the enzyme is inactive or adding a denaturant. The addition of trifluoroacetic acid or acetonitrile is common. Consideration to the impact of such agents on subsequent MS analysis is thus important.

3. Mass analysis of products

The mass analysis of the products of each of the structural proteomics approaches is an important consideration for the successful determination of protein structure (Table 3).

3.1. Mass analysis of deuterated peptides in HX-MS

While in the case of HX-MS little chemical change is imparted to the protein, the minimal mass change resulting from the replacement of multiple hydrogen atoms with deuterium poses a challenge in terms of measuring levels of incorporation and their ultimate site in a population of protein molecules or their peptide components. A change in deuterium content is measured by the incremental change in mass of the undeuterated forms of the protein (by approximately 1 Da per deuterium incorporated) and their peptide constituents. The overall level of deuterium incorporated is measured according to:

$$\%D = [(m_t - m_0)/m_{100} - m_0] \times N \quad (2)$$

where %D is the deuterium content of a peptide with N amide hydrogens and m_0, m_t and m_{100} represent the masses of the peptide at 0 and t minutes, and fully exchanged.

The levels of deuterium incorporation vary within each peptide or protein molecule, a consequence of the different individual rate constants of exchange. These products have similar properties and size to their unlabelled counterparts, resulting in many overlapping peaks in a mass spectrum; even one obtained with high mass resolution. This is compounded by the use of pepsin as a general, non-residue specific protease (see Table 2) employed to digest any protein. As a result, the separation of peptide products (designated P + nD in Fig. 4) in time requires the use of LC-MS, using a short steep gradient, preferably supplemented by ion mobility where such capability is available [71].

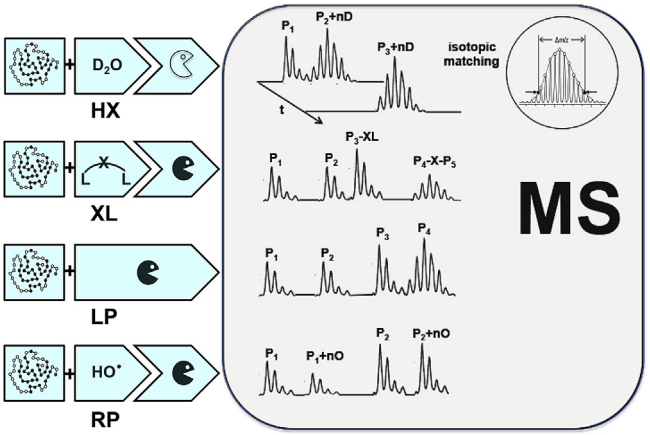


Fig. 4. Nature of solution reactions (in blue) and peptide products and their MS analysis (in grey) for each of the structural proteomics approaches. Pepsin and site-specific endoproteases are represented by white and black symbols respectively.

Most HX-MS software applications utilize selected ion extraction from the total ion chromatogram and curve fitting of the isotopic profile for each peptide to a theoretically predicted one (Fig. 4) to determine the deuterium levels incorporated within each peptide. Various computational approaches and algorithms [72–74] have been developed to help quantify and visualize the amount of deuterium across a protein's backbone to subsequently predict and model the nature of the protein or its complex. Statistical and logical analysis of the deuterium content within overlapping peptides can help to provide higher spatial resolution than that obtained at the peptide level, but this is not fool proof.

Despite efforts to digest and detect peptides quickly, back exchange is a consistent issue and methods to correct for it [75] are not without simplifications or assumptions. HX-MS experiments must be carefully designed and executed, with appropriate controls and repeat runs, to minimise errors reported for deuterium content, exchange rates, and solvent protection factors [76].

3.2. Mass analysis of cross-linked peptides in XL-MS

In addition to crosslinking between residues within a single protein, numerous single modified residues (without bridging) (designated P-XL in Fig. 4) occur as well. Furthermore, the

Table 3
Analysis of peptide products in structural proteomics mass spectrometry based approaches.

Method	Hydrogen Exchange	Radical Probe Mass Spectrometry	Chemical Cross-Linking	Limited Proteolysis
acronym	HX-MS	RP-MS	XL-MS	LP-MS
nature of products	isotopically-labelled protein and peptides	oxidised protein and peptides	chemically cross-linked protein and peptides	proteolytic peptides
analysis approach	LC-MS	direct ESI, LC-MS or MALDI-MS	LC-MS	direct ESI, LC-MS or MALDI-MS
mass analysis consideration #1	many overlapping peptides ($+n$ x 1) where isotopic enrichment varies within each peptide	easily resolved peptides ($+n$ +16)	many cross-linked peptide products	easily resolved proteolytic peptides
mass analysis consideration #2	peptide separation can be assisted by ion mobility	easy determination of oxidation levels	non-specific interactions and unexpected side products of unpredicted mass	in-complete digestion can limit structural resolution
mass analysis consideration #3	% deuterium incorporation requires specialised software/ approaches	quantitation most reliable when peptide fragments contain a single oxidation site	cross-linked peptides are a small fraction of total peptide pool	autolysis or enzyme inactivity may limit peptide generation/ coverage

cross-linked peptides and “dead-end” or “mono-linked” peptide products produced after proteolysis typically represent only a small fraction of the total peptide pool. Thus, to improve their detection, some enrichment of the modified products by size exclusion, ion exchange, or affinity chromatography is often required prior to LC-MS and MS/MS analysis.

Specialised software is needed to aid the analysis of the products and a number of algorithms have been developed [77–79]. Employing mass maps and database searching is challenging in this regard, since all possible cross-linked peptides must be predicted by in silico digestion of all proteins followed the creation of all possible pair-wise combinations of peptides [78] that contain a residue that is capable of cross-linking in the actual experiment. If the proteins in complex are known, cross-linked peptides can be identified with reasonable confidence [77], but for unknown interactions a range of approaches and algorithms have been employed to determine the confidence of a match and reduce false positives. These include the application of data filters, probabilistic approaches [79], machine learning, and target-decoy approaches [28]. These are employed together with methods which attempt to visualize the complexes [80,81].

Most cross-linkers are not cleaved during fragmentation in MS/MS experiments, thus generating large fragments in which both interconnected peptide chains (designated P-X-P in Fig. 4) contribute fragment ion profile. Cleavable linkers, however, have been designed [82] in an effort to simplify the identification of different cross-linked species, and reduce the potential of identifying false-positive cross-linked products.

Unpredicted side reactions generated in the cross-linking chemistries [62,63] contribute to complications, and an essential step in the data analysis is to assess the quality of product identification and to consider the possibility of errors from miss-assigned products and their consequences to the prediction of protein structures.

3.3. Mass analysis of unmodified and oxidised peptides in LP-MS and RP-MS

The mass analysis of peptide products in limited proteolysis and RP-MS experiments is considerably more straightforward than for the other two approaches. In LP-MS (limited proteolysis mass spectrometry), the use of site-specific endoproteases (Table 2) allows most, if not all, of the peptides (P_n) to be confidentally assigned based upon their mass alone, even accounting for possible missed-cleaved products. Unlike all other approaches, the site-specific protease used to assess a protein's structure itself generates the peptide products in a single step for subsequent MS analysis, and no separate proteolysis typically using pepsin in HX-MS experiments or a site-specific protease for XL-MS and RP-MS experiments (see Fig. 4) is required. MS/MS experiments can be employed to identify peptides where ambiguities exist.

The analysis of RP-MS products also proceeds after site-specific proteolysis of the unoxidised and oxidised protein. The oxidised peptides are easily resolved from their unoxidised counterparts having masses which are increased by +16 units (designated P + nO in Fig. 4) associated with the addition of oxygen to the reactive side chains, without any or appreciable structural change to the side chains [32,43]. As such the presence of oxidation in each peptide can be determined on mass spectrometers with modest mass resolution, without additional LC separation, on either ESI or MALDI (matrix-assisted laser desorption ionization) based instruments. The levels of oxidation are quantified based upon the ratio of the summed area of all peaks for each oxidised peptide versus its unoxidised counterpart. Tandem MS/MS experiments can be employed to determine the site of oxidation where this is undecided from the mass map, although mass measurements for peptides that contain only one site of oxidation are preferred for reliable quantitation [31,32,35]. A second protease can help narrow the sites of oxidation where ambiguities exist.

4. The case for radical probe protein footprinting

Beyond the advantages described above, and summarized in Tables 1 and 3, radical probe protein footprinting affords several additional benefits for protein structure analysis with mass spectrometry.

4.1. “On-the-fly” reaction and analysis

An advantage of RP-MS and LP-MS approaches over cross-linking is the ability to perform the reaction chemistries directly ahead of analysis by LC-MS or related approaches. The latter can be achieved using immobilized enzyme columns [83] that accelerate proteolytic digestion by substantially increasing the protease to substrate ratio without complications posed by autolysis when the protease is free in solution.

Some efforts have been directed at effecting HX-MS at the interface with the mass spectrometer to allow for a completely online analysis [84], though only after labelling of the protein with deuterium. Low temperature chambers have been developed to enclose the entire HPLC unit and commercial systems have been produced. The challenge of such systems is to generate proteolytic fragments with close to 100% sequence coverage. This can be aided by the use of alternate acid-resistant proteases with complementary digestion profiles to pepsin, and/or the use of alternate fragmentation approaches including electron capture (ECD) [85] and electron transfer dissociation (ETD). Improving the efficiency of pepsin has also been studied by employing high pressure digestion approach [86].

The RP-MS approach offers the opportunity to conduct the oxidation reactions through synchrotron radiolysis or laser-based photolysis of a solution passed directly into a mass spectrometer for analysis. However, use of a synchrotron beamline is physically cumbersome for any such routine application, and there are practical issues with the actual reaction timescales when lasers are employed [46].

The discharge approach, in contrast, effects limited oxidation at the solution interface of a conventional ESI source. This provides for a simple on-the-fly analysis of the products as demonstrated in the original RP-MS experiments (Fig. 5) [22]. Subsequent coupling of the approach to ion mobility mass spectrometry revealed that limited protein oxidation had no impact upon the collision cross section of the protein molecules (Fig. 2). This provided direct evidence for the validity of the RP-MS method [51] beyond earlier indirect evidence based on a correlation of oxidation levels measured post-digestion at reactive side chains and their theoretical solvent accessibilities [23,31–33,35]. To establish the site and levels of oxidation in a direct approach, a protein oxidised in this manner could be fragmented in a so-called top-down approach [87], thereby avoiding the need for proteolytic digestion altogether.

4.2. On plate deposition aids high-sample throughput

To help facilitate high-sample throughput, the electrical discharge based RP-MS approach has also been coupled to on-plate deposition for subsequent MALDI based analyses [88]. Instrument manufacturers have developed MALDI based instruments that can operate unaided, even with the use of an automated plate loader, to analyse several hundred samples in quick succession following their deposition on a multi-sample plate. Such deposition can also help reduce sample consumption by localizing small amounts of oxidised

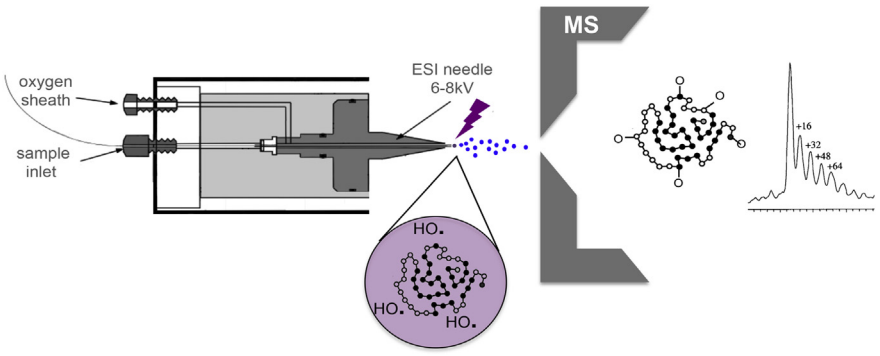


Fig. 5. "On-the-fly" oxidation of protein by electrical discharge with an ESI source that is detected directly in the mass spectrometer.

protein directly onto the MALDI target. These platforms have also recently been investigated in terms of developing a high throughput HX-MALDI-MS workflow [89] and have been paired with LP-MS [90].

To achieve RP-MS experiments, a grounded MALDI sample plate was placed approximately 5 mm below the tip of the electrospray needle operated at a needle voltage of some 6 kV (Fig. 6). Solutions of buffered protein where then passed through the needle, with oxidation levels controlled by adjusting the flow rate from 1 to 5 mL/min of protein solutions. Demonstrated for the model protein lysozyme, deposition of the partially oxidised protein was followed by its on-plate digestion. This resulted in the formation and tryptic peptides in their unoxidised and oxidised form that were subsequently detected when ionised from the plate [88]. Consistent with the initial radiolysis [24] and discharge studies [22], the same residues and oxidation levels were observed when this deposition approach was employed. These were in accord with the accessibility of such residue side chains in the intact protein structure [88].

By increasing the electrospray needle voltage from 6 to 9 kV above the grounded plate, the level of oxidation across the tryptic peptides increased accordingly. A deviation in the relationship between the oxidation levels and side chain accessibilities was observed consistent with some denaturation of the protein (Fig. 6). To avoid such denaturation, the lower (6 kV) needle voltage is used to maintain oxidation levels that equate with the accessibility of the

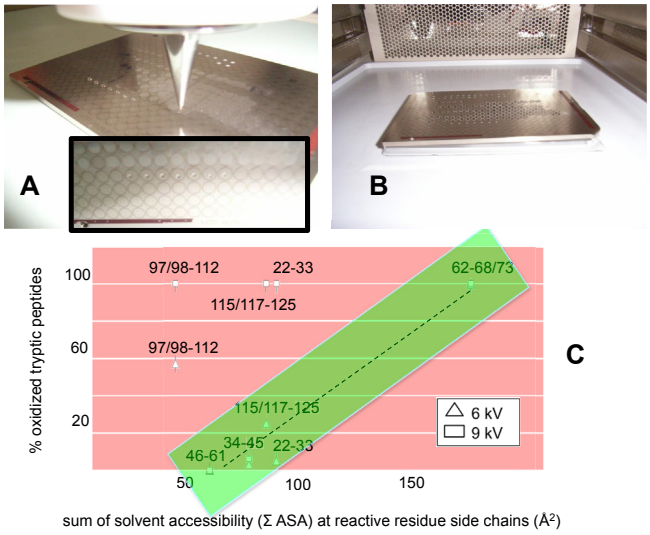


Fig. 6. Photographs showing (A) simultaneous oxidation and deposition employing discharge RP-MS approach (insert shows deposited droplets), (B) subsequent on-plate proteolytic digestion, and (C) correlation of oxidation levels and solvent accessibility of side chains when 6 kV (versus 9 kV) is applied to the needle.

side chains (green coloured zone in Fig. 6). Only the high reactivity of methionine (Met-105) with oxygen in segment 97/98–112 defies this relationship [88]. The ability to adjust the degree of denaturation extends the capability of RP-MS to study the onset of protein damage.

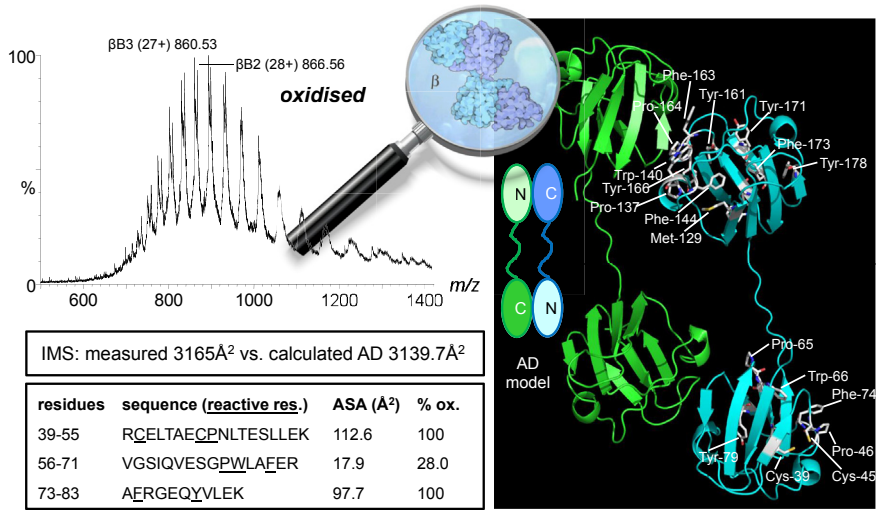
4.3. Studies of the onset of protein damage

Hydroxyl radicals, and other reactive oxygen species (ROS), are a normal consequence of aerobic cellular metabolism. The exposure of living systems to radicals induced by environmental conditions, such as their exposure to UV light, can also contribute to the oxidative damage of biological molecules. Protein oxidative damage and degradation, in particular, have been associated with a range of human diseases and ageing processes [91].

By extending the reaction timescale, from several milliseconds to tens and hundreds of milliseconds, the onset and effect of extended radical exposure on proteins and their complexes can be directly studied by RP-MS [92]. For radiolysis or photolysis based experiments, the reaction timescale is controlled by use of a shutter or the length of the laser pulse. When the electrical discharge approach is used, the reaction time can be adjusted by controlling the flow rate of protein solutions through the ESI discharge needle or by an increase to the needle voltage. The lower the solution flow rate, the greater the time proteins are exposed to radicals at the needle tip. When the needle voltage is raised (from 6 kV to 8–9 kV), more radicals are produced at the tip that can overcome the greater acceleration of ions, and thus the reduced time they spend, travelling from the needle to the entrance to the mass spectrometer or a sample collection device [20,26,27,30,32] or plate [88]. This results in a deviation in the reaction levels at reactive residues from those predicted based upon their accessibility to solvent within a protein's structure [70]. Sustained radical exposure can result in structural perturbation in part or across a protein, greater degradation through cleavage at the protein backbone, or promote cross-linking. In practice, all changes may occur simultaneously [31].

Crystallins represent good models to study such effects. Hydroxyl radicals and other reactive oxygen species (ROS) have been directly implicated in cataract formation in mammals, through damage to the lens of the eye. Crystallins have low turn over in the lens of the eye so that accumulated oxidative radical damage impacts on their ability to interact and aggregate. Hydroxyl radicals promote uncontrolled cross-linked products and degradation which reduce the transparency of the lens leading to blindness.

The onset of oxidative damage has been examined in crystallins alone, and in complex in terms of several α, β and γ-crystallins [92–97]. In the case of the α-crystallins [92,93], the N-terminal domains were found to be more susceptible to oxidative damage with earlier onset times than for the more protected central



residues	sequence (reactive res.)	ASA (Å²)	% ox.
39-55	RCELTAECPNLTESLLEK	112.6	100
56-71	VGSIQVESGPWLAFER	17.9	28.0
73-83	AERGEQYVLEK	97.7	100

Fig. 7. ESI mass spectrum of the RP-MS oxidised βB2B3-crystallin heterodimer, its measured collision cross section by IMS, and a model (AD) with a comparable cross section and oxidation levels which best match residue accessible solvent areas (ASA).

domains. The ability of antioxidants to prevent this damage were also studied [96]. A structure for the β2β3-crystallin heterodimer, detected by mass spectrometry [97], was modelled based on its collision cross section and closest match of the levels of oxidation within the β3-subunit compared with the side chain accessible solvent areas (ASA) at the reactive residues (Fig. 7) [94]. The stability of this complex to increased oxidation was also explored [95]. In a taxon-specific upsilon(υ)-α-crystallin complex (1:2) segments of the former protein were found to be more protected than others from radical damage thereby directly identifying the interaction interface(s) [93].

In such RP-MS experiments, the oxidation levels in segments of the proteins, released after proteolysis, are measured as a function of reaction time. Oxidation within different segments is found to rise and fall, the fall associated with the increased degradation of the protein in these segments such that their oxidised forms diminish [31,92,93]. The formation of complexes among common or different protein subunits can stabilise protein structures and prevent their degradation [16,96], allowing these complexes to be studied by RP-MS.

5. Conclusions

A thorough assessment of the merits and pitfalls of the major solution-based structural proteomics mass spectrometry methods by considering each of the steps in the chemical and enzymatic treatment and analysis of modified or degraded proteins. A strong case to preference radical probe protein footprinting has been made when all experimental facets of the protein's treatment, modification and analysis are considered.

The strongest competitor to RP-MS experiments is undoubtably HX-MS; the most mature of the mass spectrometry based structural proteomics approaches. It offers benefits in terms of a lack of residue specificity, but is considerably hampered by reversible back exchange and the complexity of product analysis. Irreproducibility, impacted by the need to control a wide range of variable parameters, difficulties with identifying pepsin products after digestion due to the enzymes lack of specificity, and the time required for the entire experimental workflow are all very real problems that have been identified [98].

In contrast, radical probe protein footprinting reproducibly generates oxidised products at a range of reactive residues, most involving the simple addition of one or more oxygen atoms. The chemistry is rapid and irreversible, and the analysis relatively straightforward. The ability to perform such chemistries from the radiation or electrical discharge of water, without the addition of reagents, also provides an environment to study proteins in their native, or near native, state. The discharge approach in particular enables the proteins to be oxidised and passed directly into the mass spectrometer or deposited onto a MALDI sample plate as desired.

While currently less practiced than the earlier described structural proteomics methods, RP-MS techniques are likely to find an increasingly wider application by both mass spectrometry specialists and the wider biology research community in the not to distant future. Interesting future applications and opportunities include in the study of membrane proteins [99], the impact of oxidation of protein fibril formation [100], and footprinting of proteins and complexes *in vivo* [101].

Integrating the mass spectrometry based structural proteomics approaches, described in this review, with the more traditional high-resolution atomic spectroscopic techniques such as X-ray crystallography and nuclear magnetic resonance (NMR), can help overcome limitations of the latter in terms of the higher sample quantities and purities required. Coupled with emerging methods such as cryo-electron microscopy (cryo-EM), and combined with computational models, the structural proteomics approaches can help overcome technical limitations of each of the individual techniques so as to allow proteins and complexes across a wider range of structural complexities to be solved. Although an increasing number of protein structures and complexes have been steadily revealed, many have proved recalcitrant using any single structural method, especially where sample quantities are limited and structural heterogeneity occurs.

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