Trends in
Biochemical Sciences

CellPress
OPEN ACCESS

Review

Exploring protein conformations with limited proteolysis coupled to mass spectrometry

Chloé Van Leene [1,2], Laura Van Moortel [1,2], Karolien De Bosscher [1,2], and Kris Gevaert [1,2,*]

Limited proteolysis coupled to mass spectrometry (LiP-MS) has emerged as a powerful proteomic tool for studying protein conformations. Since its introduction in 2014, LiP-MS has expanded its scope to explore complex biological systems and shed light on disease mechanisms, and has been used for protein drug research. This review discusses the evolution of the technique, recent technical advances, including enhanced protocols and integration of machine learning, and diverse applications across various experimental models. Despite its achievements, challenges in protein extraction and conformotypic peptide identification remain. Ongoing methodological refinements will be crucial to overcome these challenges and enhance the capabilities of the technique. However, LiP-MS offers significant potential for future discoveries in structural proteomics and medical research.

Highlights

Significant improvements in limited proteolysis coupled to mass spectrometry (LiP-MS) protocols, including advanced digestion and data acquisition methods, have expanded its capabilities for structural proteomics.

LiP-MS has been used in diverse experimental models, including mammalian cells, microbial, fungal, and plant systems, and also biofluids, offering new insights into protein function and interactions.

LiP-MS can be used to investigate disease mechanisms, offering insights into protein dynamics in conditions such as cancer and neurodegenerative diseases.

Recent studies leverage LiP-MS for drug–target interaction profiling to aid the discovery and development of novel therapeutics.

LiP-MS is expected to advance structural proteomics and (bio)medical research, thereby aiding biomarker discovery and therapeutic target identification.

Marking accessible regions in protein structures using a MS-based read-out

The structure of a protein can be affected by several factors, including binding to other molecules, protein modifications, and fluctuations in its direct environment (e.g., cell temperature and osmolarity). Such structural changes may alter the functions of proteins, signal their degradation, or lead to their relocalization to cellular organelles or subcellular structures; these changes can also be hallmarks of protein-folding diseases [1].

MS remains the key technology for comprehensive and unbiased proteome analysis, and **bottom-up proteomics** (see Glossary) is the primary technology employed. Different MS-based methodologies were developed to study the structures and conformations of individual proteins, protein complexes, and entire proteomes (Box 1). Notably, in 2014, Picotti and colleagues introduced limited proteolysis coupled to mass spectrometry (LiP-MS) [2], a proteolytic approach that has become transformative for studying changes in protein structures on a proteome-wide scale. LiP-MS relies on the controlled proteolytic cleavage of proteins with a broad-specificity protease. By limiting the duration of this proteolysis step, the protease predominantly targets and marks surface-exposed and flexible protein regions because both surface accessibility and local structural flexibility influence digestion efficiency. Conditions that lead to structural changes in such protein regions result in altered proteolytic marks, which are revealed upon MS-based analysis (Figure 1). LiP-MS has been applied to various biological systems, including bacteria, yeast, mammalian cell lines, tissue samples, and cerebrospinal fluid (discussed later).

We first discuss seminal work on the development of the technique and then highlight recent advances and applications of LiP-MS, focusing on how it has been applied to non-complex matrices (e.g., single proteins) and used to explore human diseases for drug screening and biomarker discovery (Table 1). We also discuss examples of LiP-MS in non-human systems and describe current challenges and future perspectives.

[1]Vlaams Instituut voor Biotechnologie (VIB) Center for Medical Biotechnology, Ghent, Belgium
[2]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium

*Correspondence:
kris.gevaert@vib-ugent.be (K. Gevaert).

Box 1. Structural proteomic approaches involving the use of chemicals

In hydrogen–deuterium exchange MS (HDX-MS) studies, the rate at which protein-bound hydrogens (particularly the backbone hydrogens on the amides in peptide bonds) are exchanged for deuterium atoms present in the solution (deuterated water) is determined by measuring the mass shifts of peptides [48]. This exchange rate depends on the conformational state of the protein and is thus influenced by the involvement of protein backbone amides in protein secondary structures (e.g., α-helices and β-sheets), their exposure on the protein surface, and their involvement in protein–small-molecule or protein–protein binding interfaces, among others. As such, changes in HDX rates are proxies for changes in protein conformations and protein interactions with other (bio)molecules. Although HDX-MS was originally applied to individual proteins, larger protein complexes have also been analyzed (e.g., [49]). Aside from the rather limited structural resolution, a disadvantage of HDX-MS is that it has not yet been applied to complete proteomes.

A method related to HDX-MS is the stability of proteins from rates of oxidation (SPROX) method in which rates of chemically induced, peptide-bound methionine oxidation under increasing protein denaturing conditions are measured by quantitative MS [50]. SPROX-alike methods such as fast photochemical oxidation of proteins (FPOP) [51] and hydroxyl radical footprinting (HRF)-MS [52] lead to oxidation of many more amino acids than only the rare amino acid methionine, and thus lead to more refined structural protein analyses. All these oxidation-based methods can be applied to complete proteomes.

Chemical crosslinkers covalently freeze protein–protein interactions. Such crosslinked proteins are digested into peptides which are analyzed by MS. A plethora of crosslinkers that differ in the protein functional groups they target, and the spacer length between these groups, can be used for crosslinking MS (XL-MS) studies. Such studies have focused on individual protein complexes [53] and on complete proteomes [54]. XL-MS does not yet seem to have found widespread adaptation by the structural proteomics field, possibly because of difficulties in the identification of crosslinked peptides. However, progress is being made with the introduction of MS-cleavable crosslinkers and tailored database search algorithms that reduce the database complexity problem in XL-MS proteomic data, leading to more efficient identification of crosslinked peptides [55]. In addition, the introduction of crosslinkers holding handles that allow the enrichment of crosslinked peptides, such as the phosphonate-based PhoX crosslinker, is moving the XL-MS field forwards [56].

Conformational proteomics via LiP-MS

In 2005, pulse proteolysis was introduced to assess protein stability, as proxied by measuring the fraction of folded proteins under protein unfolding conditions [3]. It exploits the fact that unfolded proteins are more susceptible to proteolysis than folded proteins. Protein unfolding was introduced by adding increasing concentrations of a chaotrope, urea, to proteins. This was followed by a short incubation (a pulse) with a high concentration of a broad-specificity protease, thermolysin, which cleaves unfolded proteins with minimal cleavage of folded proteins. Using a quantitative gel-based readout, the rate of proteolysis was measured as a function of urea-induced protein denaturation, and the remaining fraction of folded proteins was determined. Its application was limited to individual proteins in non-complex systems, necessitating the development of novel approaches to study protein conformations on a proteome-wide scale.

Lomenick and coworkers introduced a similar method for profiling protein–drug interactions on a proteome-wide scale [4]. Their drug affinity responsive target stability (DARTS) method inferred that, upon binding a drug, a protein becomes structurally more stabilized, rendering it more resistant to proteolysis. In their work, protein mixtures were, among others, incubated with the antiproliferative agent rapamycin, the anticancer drug didemnin B, or the polyphenolic compound resveratrol, followed by proteolysis using broad-specificity proteases such as subtilisin or thermolysin. Proteins stabilized upon binding such molecules were resolved by SDS-PAGE and identified following protein band excision, in-gel digestion, and bottom-up proteomics. More recently, the DARTS method was exploited for measuring differences in protein thermostability introduced by external factors (Box 2).

Building on the pulse proteolysis and DARTS approaches, LiP-MS [2] allows more comprehensive, proteome-wide mapping of protein conformational changes (Figure 1). A cell lysate or a liquid biopsy sample is briefly incubated with a broad-specificity protease at a low protease-to-substrate ratio, which ensures that surface-accessible and flexible protein sites are primarily

Glossary

Bottom-up proteomics: a mixture of proteins is digested into peptides using a specific protease such as trypsin. The peptide mixture generated is then analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The peptides eluting from a chromatographic column are ionized in the mass spectrometer ion source and detected in what is referred to as an MS1 spectrum, where ion intensities are plotted as a function of their mass-over-charge (*m/z*) ratios. Depending on the acquisition method (DDA or DIA, described below), particular peptide ions are selected for fragmentation and detection in an MS2 or MS/MS spectrum that plots the intensity of peptide fragment ions. Although the intensities in MS1 spectra are typically used for peptide quantification, MS2 peptide fragmentation spectra are used to identify the peptide precursors and thus the proteins from which they originated.
Data-dependent acquisition (DDA): a bottom-up proteomic approach in which only the most abundant peptide ions in the MS1 spectrum, that fulfill predefined quality criteria, are selected for fragmentation. This type of analysis is stochastic, and therefore results in missing data and poor reproducibility between analyses.
Data-independent acquisition (DIA): this type of analysis overcomes the limitations of DDA by selecting all peptide precursor ions in a large, predefined MS1 mass-over-charge region rather than single peptide ions for fragmentation. As such, per MS2 event, different peptide precursor ions are fragmented together and algorithms are used to deconvolute the resulting chimeric MS2 spectra into those of the individual peptide precursors.
Glycoproteome: the set of glycosylated proteins in a cell, tissue, or organism.
N-terminomics: a set of proteomic workflows by which peptides covering the N terminus of a protein are enriched from proteome digests before their analysis by MS.
Selected reaction monitoring (SRM): a targeted proteomic technique in which only preselected peptides are sampled instead of stochastic sampling of all peptides. A mass spectrometer selects the peptide ions based on their LC column retention time and mass-

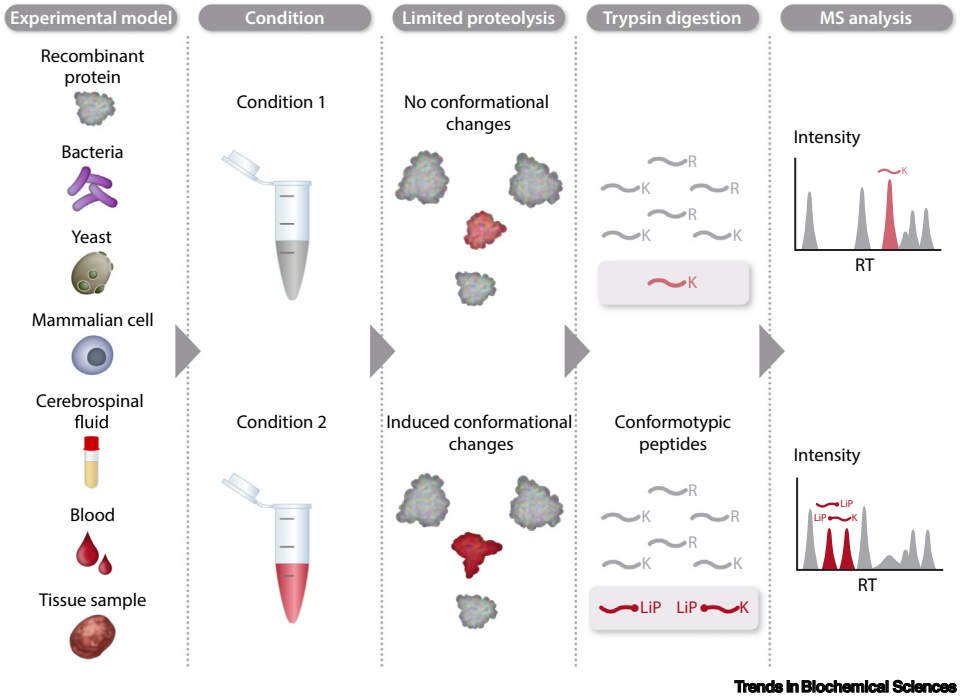

Figure 1. Schematic of the limited proteolysis coupled to mass spectrometry (LiP-MS) workflow. Proteins are extracted from an experimental model system, such as purified recombinant proteins, bacteria, yeast, mammalian cells, biofluids, or tissue, under native(-like) conditions. Each proteome extract is split into a control sample and a sample to be subjected to limited proteolysis with a broad-specificity protease (e.g., proteinase K, subtilisin, thermolysin, pepsin, papain, or elastase) that cleaves solvent-exposed, accessible, and flexible regions, thus generating structure-specific protein fragments that differ between conditions. These protein fragments are then denatured and fully digested with trypsin to generate peptides that are measurable by bottom-up proteomics. The peptide mixture includes conformotypic peptides that can be either fully tryptic or semi-tryptic. Lastly, all samples are subjected to an unbiased MS analysis. K and R indicate tryptic termini. N- and C-terminal semi-tryptic peptides are indicated by ~LiP and LiP~K respectively. Abbreviations; K, lysine; R, arginine; RT, retention time.

cleaved, thereby marking them. Next, the protease is inactivated by heating, which also induces protein denaturation, followed by full protein digestion with trypsin, which cleaves at the C-terminal side of arginines and lysines. A control sample not treated with the broad-specificity protease undergoes full trypsin digestion. Combining limited proteolysis with full trypsin digestion creates fully tryptic and semi-tryptic peptides. In the LiP samples, fully tryptic peptides originate from buried protein regions inaccessible to the broad-specificity protease and from protease-accessible regions that are structurally rigid and limit proteolysis. Comparing the levels of fully tryptic peptides between control and LiP samples may reveal peptides from accessible and/or flexible regions because their levels are expected to decrease in LiP samples (Figure 1). Semi-tryptic peptides predominantly result from proteolysis at surface-exposed and/or flexible regions by the broad-specificity protease, and are uniquely present or are found at highly increased levels in LiP samples. Peptides whose levels differ between control and LiP samples are proxies for the conformation of the proteins they originated from and are therefore termed conformotypic peptides (Figure 1). In the original LiP-MS publication [2], targeted, **selected reaction monitoring (SRM)**-based assays were used to quantify such peptides.

Detailed LiP-MS protocols have been published by Picotti and colleagues in 2017 and 2023, and we refer to these for full technical descriptions [5,6]. Several key improvements were introduced [6], including digesting samples with a combination of endoproteinase-LysC and trypsin, rather

over-charge value as observed in an MS1 spectrum. When triggered, the selected peptide ions are fragmented and their most intense fragment ions are read out. The combination of the peptide characteristics and fragment ion datapoints must be unique for the studied peptides. Furthermore, by spiking the analyte mixtures with known amounts of stably isotopically labeled variants of the selected peptides, accurate peptide quantification is achieved.

Structural barcodes: these barcodes allow the LiP-MS data of a single protein to be visualized in an intuitive manner. Using the primary amino acid sequence, a barcode presentation is created in which individual colored bars represent fully tryptic, semi-tryptic, and conformotypic peptides. In this way, a visual is created that shows the LiP-affected protein regions.

Table 1. Overview of LiP-MS applications discussed in this review

Key findings	Species	Material	Refs
Individual proteins			
Potential polymer alternatives for PEG for protein bioconjugation	Human	Recombinant interferon-α2a protein	[15]
Interactions between respiratory syncytial virus F glycoprotein and antibodies, adenylyl cyclase 8, and calmodulin; differential interactomes of RAB GTPases and α-synuclein in different structural states	Human, bovine	Recombinant proteins, HEK-293T cells	[16]
Contact sites and conformational changes between AC8 and its regulators	Bovine	Recombinant adenylyl cyclase AC8	[17]
Human disease mechanisms and protein drug discovery			
Significant changes in protein abundance and structure due to *CALR* mutations	Human	Primary granulocytes (isolated from peripheral blood)	[18]
251 proteins with altered conformations due to dephosphorylation	Human	MCF-7 cells	[19]
HSP60 as the most significantly altered protein upon LPS stimulation	Mouse	RAW264.7 cells	[20]
Insights into compound 1 binding to the ATP pocket of CDK family members	Human	U2OS cells	[21]
Crellastatin A as a potent PARP-1 inhibitor; potential probe for new PARP-1 inhibitors	Human	HeLa cells	[22]
Binding of gracilioether A revealed USP5 regions protected from proteolysis; insights into molecular interactions	Human	HeLa cells	[23]
Mortalin as primary target of mycalin A; disrupted mortalin–p53 complex promotes apoptosis in tumor cells	Human	HeLa cells	[24]
PP2A as a direct target of lomitapide; induced AMPK-regulated autophagy and CRC cell death	Human	HCT116 and HT29 cells	[25]
RANBP3 as a direct target of NU2058; increased RANBP3 binding to β-catenin, thus promoting nuclear export and inhibiting c-MYC	Human	DLD1, HCT15, RKO, and SW620 cells	[26]
IQGAP2 as a key target of isoliquiritigenin; lipid-lowering effects mediated through the IQGAP2–CREB–SIRT1 axis	Human	HepG2 cells	[27]
DLAT as a key target of hyperforin; enhanced mitochondrial function and promoted weight loss	Mouse	C3H10T1/2-derived adipocyte cells	[28]
Concentration-dependent interactions of α-amanitin affecting protein synthesis pathways	Human, mouse	Huh7 and AML12 cells	[29]
TXNRD1 isoforms 2 and 3 as targets of elovanoid-N34; induced structural changes in the FAD interface domain	Human	Retinal pigment epithelial (RPE) cells	[30]
MAPK3 as target of trihydroxy-phenolacetone (THP); THP counteracted PS-NP-induced damage by inhibiting nerve cell apoptosis	Rat	Primary cell cultured by hippocampal neurons	[31]
ACE2 as a direct binding target of benzoylaconitine	Human	HEK-293T cells	[32]
Biomarker discovery in neurodegenerative disease			
LiP peptides have higher discriminatory power than protein levels of α-synuclein; identified 76 proteins with conformational changes in CSF from PD patients	Human	CSF	[1]
38 proteins with altered abundance; CD5L formed covalent complexes with IgM in elder mice	Mouse	CSF	[35]
53 regions in 12 proteins with conformational changes	Human	CSF	[36]

Table 1. (continued)

Key findings	Species	Material	Refs
23 proteins with structural alterations; highlighted gender-specific changes and elevated complement protein levels	Human	CSF	[37]
Protein conformational studies in bacteria, yeast, and plants			
Fructose-1,6-bisphosphatase (FBP)-mediated regulatory mechanism in glucose uptake	*Escherichia coli* BW25113	Cell lysate	[7]
Species-specific enzyme interaction patterns with different metabolites	Different bacterial species	Cell lysate	[38]
Molecular events during osmotic stress and heat shock	*Saccharomyces cerevisiae* BY4742	Cell lysate	[7]
Features of reversible protein aggregation	*S. cerevisiae* BY4742	Yeast LiP data from Cappelletti *et al.*	[39]
Age-related alterations in protein translation and folding; identified GLT1 polymerization as affecting amino acid homeostasis	*S. cerevisiae* BY4741	Cell lysates	[40]
Xenon-induced conformational changes in 60 proteins involved in ATP-driven and glycolytic processes	*S. cerevisiae* S288c	Cell lysates	[41]
Optimized LiP protocol for plant material with a focus on metabolite–protein interactions	*Arabidopsis* and *Marchantia*	Roots, seedlings, liverworts	[42]

than trypsin alone, thus reducing the overall digestion time and increasing the digestion reproducibility [7]. Furthermore, the overall proteome depth and reproducibility (because of fewer missing values) increased by using MS-based **data-independent acquisition (DIA)** rather than **data-dependent acquisition (DDA)** [8]. To analyze such DIA data, a spectral library based on identified DDA MS/MS spectra was used.

Further innovations emerged that have increased our understanding of drug–target interactions, notably through LiP small-molecule mapping (LiP-SMap), which systematically detects proteins that become differentially susceptible to protease cleavage upon binding a small molecule [8]. Such altered susceptibility is either a direct result of small-molecule binding, which shields particular protein regions from digestion, or secondary to drug-induced changes in protein conformation. Complementing this approach, LiP-Quant incorporates machine learning to improve target identification accuracy, thus enabling drug-binding affinity estimations in complex biological mixtures [9]. For detailed analysis of LiP-MS data, a new R package was introduced, MSstatsLiP [6]. In addition to providing different types of data quality measures, MSstatsLiP performs a statistical analysis on peptide LiP-MS data to identify conformotypic peptides and, among others, plots LiP-MS data as **structural barcodes** on the primary protein sequence to reveal those protein regions that were surface-exposed, structurally flexible, or underwent induced conformational changes. Recently, FragPipe LiP processor (FLiPPR) was created to further improve LiP-MS data analysis by more effectively dealing with missing data, which remains an inherent problem in MS-based proteomics [10,11]. The way in which FLiPPR fills in missing data increases the statistical power and helps to identify structural changes. In addition, FLiPPR makes statistical corrections more organized and implements data merging at three levels – precursor ions, peptides, and cut-sites – enabling detailed data integration and more consistent and straightforward LiP-MS data analysis [12].

LiP-MS applications to individual proteins

LiP-MS started as a technique to study single purified proteins [13,14] and has since evolved to a powerful tool for exploring protein conformational changes in complex biological samples.

Box 2. Protein thermostability measurements for functional proteome studies

In 2014, Savitski and colleagues took the cellular thermal shift assay (CETSA) [57] to the next level by introducing an MS-based read-out in their thermal proteome profiling (TPP) method [58]. CETSA and TPP are based on the same principle: the thermal stability of a protein will change upon binding a small molecule or drug. In these methods, equal amounts of purified proteins, a cell or tissue lysate, or even intact cells and tissues, are divided over several fractions which are all incubated with a small molecule or drug of interest. Each fraction is then brought to a set higher temperature, creating a temperature gradient across the different fractions. By increasing the temperature, proteins will start to unfold, aggregate, and precipitate. These precipitated proteins can be removed from a protein mixture by a simple centrifugation step, allowing sampling of the remaining soluble proteins. The latter can be analyzed by SDS-PAGE and western blotting (CETSA) or by MS (TPP) to generate protein melting curves in which the fraction of soluble proteins is plotted as a function of time. The temperature at which half of a studied, individual protein remains soluble is called the protein melting temperature, and this melting temperature depends on the initial stability of that particular protein. TPP studies focused initially on identifying the targets and off-targets of small molecules and drugs or drug candidates [59,60]. More recently, TPP has been applied to establish a so-called meltome atlas of the proteomes of different model species [61] to identify aggregation-prone proteins in human cells [62] and to better understand the functional roles of proteoforms [63]. Interestingly, Zhang *et al*. showed that drug-bound proteins precipitate in buffers with a higher concentration of organic solvents as compared to their drug-free counterparts, and that their solvent-induced protein precipitation (SIP) approach can also be used to identify drug targets [64]. Most recently, a thermostability-assisted limited proteolysis-coupled MS (TALiP-MS) approach was developed to aid drug target discovery. It complements TPP and LiP-Quant for target identification and increased the number of target peptides detected in LiP-MS experiments by up to eightfold [65].

Applied to purified proteins, LiP-MS allows one to probe structural features under highly controlled conditions, thereby providing insights into structural dynamics, bioconjugation behavior, and interaction specificity. Such earlier studies paved the way for LiP-MS applications in more complex systems such as cell lysates, which will be discussed later (Table 1).

In a bioconjugation study, non-covalent polymer interactions with interferon α2a (IFN-α2a) were evaluated to find new alternatives to the gold standard polyethylene glycol (PEG). The proteolytic cleavage patterns of IFN-α2a interacting with different polymers were comparable to those observed when PEG was used, and indicated weak transient interactions between the polymers and surface of IFN-α. Both poly(2-ethyl-2-oxazoline) (PEtOx) and linear polyglycerol (LPG) bioconjugates produced a similar biological activity, that was monitored using a secreted embryonic alkaline phosphatase (SEAP) reporter gene assay, suggesting their potential as PEG alternatives for bioconjugation [15].

LiP-MS was also used to evaluate different conformation-specific protein–protein interactions [16]. For example, applied to RAB GTPases, differential interactomes of their conformationally similar GDP- and GTP-bound forms were mapped, but detecting low-abundance interactors was cumbersome. In addition, the ability of LiP-MS to systematically compare the interactions of α-synuclein in its monomeric and amyloid fibril conformational states within complex cellular extracts provided a valuable dataset of putative interactors for future research. Further, known interactions between the respiratory syncytial virus F glycoprotein and site-specific antibodies were identified, and interactions between adenylyl cyclase 8 (AC8) and calmodulin (CaM) were detected, demonstrating its applicability to integral membrane proteins [16]. Building on these latter findings, another study determined the structure of purified bovine AC8 by cryo-electron microscopy (cryo-EM), followed by LiP-MS to identify contact sites and conformational changes between AC8 and its regulators [CaM, stimulatory G protein α (Gαs), and the G protein β–γ complex (Gβγ)], and to detect binding interfaces and regions of altered surface accessibility. Cross-validation with the LiP-MS datasets [16] offered deeper insights into the regulation of AC8 and the roles of its structured and flexible domains [17].

Proteome-wide LiP-MS to study human disease mechanisms and drug discovery

The advances in LiP-MS described above paved the way for its application to complex systems such as cell lysates, enabling the exploration of protein interactions, structural dynamics, and

conformational changes on a proteome-wide scale. Recent LiP-MS studies have deepened our understanding of human disease pathology and identified potential therapeutic strategies (Table 1), as discussed below.

LiP-MS uncovers disease mechanisms

In the context of myeloproliferative neoplasms, calreticulin (*CALR*) mutations can lead to these rare blood cancers that are characterized by excessive production of red blood cells, white blood cells, or platelets. LiP-MS analysis of primary neutrophils (white blood cells) from patients with *CALR* mutations revealed that homozygous mutations significantly impacted on the **glycoproteome**, leading to chaperone defects and misfolding of myeloperoxidase. By contrast, the number of proteins with altered levels and/or structural changes in patients with heterozygous *CALR* mutations was low, suggesting that some cellular processes are uniquely affected by homozygous *CALR* mutations [18].

In breast cancer research, one LiP-MS study examined phosphorylation-induced protein conformational changes in MCF-7 cell lysates and revealed 251 proteins with altered conformations following treatment with alkaline phosphatase, showing the impact of phosphorylation on protein structures and/or protein–protein interactions. Notably, there was little overlap between the hits obtained by LiP-MS and a complementary approach, stability of proteins from rates of oxidation (SPROX) (Box 1), emphasizing the unique capabilities of each technique in detecting conformational changes [19].

To better understand the molecular dynamics of acute inflammation, lipopolysaccharide (LPS)-treated macrophages were used to study protein structural changes. HSP60 was identified as the most significantly altered protein. Cellular thermal shift assays further validated these findings, demonstrating that HSP60 exhibits enhanced thermal stability in activated macrophages and forms a complex lacking HSP10 [20].

LiP-MS applied to drug discovery studies

CDK9 inhibitors have emerged as promising agents that disrupt transcriptional processes essential for tumor cell survival. Among these, compound 1 demonstrated strong affinity and selectivity for several CDK family members, namely CDK1, CDK4, CDK6, CDK9, and CDK11A. The peptide-level resolution offered by LiP-Quant analysis on U2OS lysates provided valuable insights into the binding of compound 1 in the ATP-binding pocket of these kinases. This experimental finding aligned with predictions made from molecular modeling, reinforcing the accuracy of both approaches [21]. Similarly, PARP-1 inhibitors are notable cancer therapeutics. Using DARTS followed by targeted LiP multiple reaction monitoring (MRM), the sulfated bis-steroid metabolite crellastatin A (CreA) was identified as a potent inhibitor of PARP-1. Molecular docking studies supported its binding, suggesting the potential of CreA as a probe for developing new PARP-1 inhibitors [22].

Focusing on protein ubiquitination, increased activity of ubiquitin carboxyl-terminal hydrolase 5 (USP5) is associated with various cancers such as breast and lung cancer. By comparing untreated HeLa cell lysates to lysates treated with the marine polyketide gracilioether A (GeA), USP5 regions that were protected from proteolysis were identified, revealing insights into the molecular interaction between GeA and USP5, and such structural information may open the way to develop novel USP5 inhibitors [23]. In a related line of research, interactome characterization of the sponge metabolite mycalin A (MA) identified mortalin, a mitochondrial heat shock protein enriched in some types of cancer, as its primary target. This interaction, validated through targeted LiP-MS and molecular docking, suggested that MA plays a role in disrupting the mortalin–p53 complex,

thereby influencing p53 transcriptional activity. Indeed, MA treatment led to the overexpression of p53 target genes in MA-responsive cells, thereby promoting apoptosis [24].

In colorectal cancer (CRC), LiP-SMap was used to uncover a new application for the lipid-lowering drug lomitapide. Protein phosphatase 2 (PP2A) was identified as a direct target of lomitapide, leading to autophagy and subsequent CRC cell death [25]. Surprisingly, also in CRC, RAN-binding protein 3 (RANBP3) was found to be the direct target of the CDK2 inhibitor NU2058. This interaction increased RANBP3 binding to β-catenin and promoted its nuclear export, and inhibited the transcription of *MYC* and cyclin D1 (*CCND1)*, leading to cell senescence. Given that RANBP3 significantly regulates CRC tumorigenesis, targeting it with NU2058 could be a potential therapeutic strategy [26].

Shifting focus from cancer to liver disease, LiP-SMap profiling of the flavonoid isoliquiritigenin (ISO) in HepG2 cell lysates identified IQGAP2 as a key target, and the lipid-lowering effects of ISO were mediated through the IQGAP2–CREB–SIRT1 axis [27]. Hyperforin (HPF) was similarly studied, and identified DLAT, a component of the pyruvate dehydrogenase complex, as its key target, underscoring the potential of HFP as a therapeutic agent for obesity by enhancing mitochondrial function and promoting weight loss [28]. In addition, LiP-MS was used to investigate α-amanitin (α-AMA)-induced hepatotoxicity in human- and mouse-derived hepatocytes, and revealed α-AMA effector proteins and their concentration-dependent interactions that affect protein synthesis pathways, including binding to RNA polymerase II [29].

In the realm of neurological diseases, thioredoxin reductase 1 (TXNRD1) isoforms 2 and 3 were identified as targets of elovanoid (ELV)-N34, an endogenously formed lipid mediator in neural cells. ELV-N34 was found to induce structural changes in the oxidoreductase FAD-binding domain of TXNRD1, reducing its activity and suggesting novel regulatory mechanisms that could impact on protection against oxidative stress-related diseases and promote extended lifespan [30]. Another study focused on the effects of developmental exposure to polystyrene nanoplastics (PS-NPs) on cognitive function. Trihydroxy-phenolacetone (THP) was identified as a potential mitigator, and LiP-SMap identified MAPK3 as its main interactor. Further analyses revealed the impact of THP on processes related to environmental stimuli, synaptic and nervous system functions, and apoptosis. Network analysis highlighted significant interactions involving MAPK3 and showed that THP counteracts PS-NP-induced damage by inhibiting nerve cell apoptosis through downregulation of MAPK3 signaling pathways [31].

In cardiovascular research, LiP-MS was used to identify angiotensin-converting enzyme 2 (ACE2) as a potential target of benzoylaconitine (BAC), a plant-derived molecule used for the treatment of heart failure. Direct binding to and activation of ACE2 by BAC were confirmed with surface plasmon resonance and an ACE2-dependent reporter gene assay [32].

Protein conformations as biomarkers of neurodegenerative diseases

LiP-MS has been used to map structural changes in proteins associated with neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD). In PD research, LiP-MS data from cerebrospinal fluid (CSF) samples distinguished protein structural alterations between healthy individuals and PD patients, surpassing traditional quantitative proteomic data used for biomarker discovery [33,34]. Notably, changes in LiP peptides exhibited higher discriminatory power than overall changes in the protein levels of α-synuclein, a known PD biomarker. Furthermore, integrating LiP findings with α-synuclein data enhanced disease classification accuracy. In total, 76 proteins with structural changes were identified in CSF samples of PD patients, including the α1 (III) chain of collagen (COL3A1) and peptidyl-glycine α-amidating monooxygenase

(PAM), both of which have been linked to PD in genome-wide association studies. Although powerful, limitations of this study included a small dataset, sparse detection of α-synuclein, and no downstream validations of the identified conformotypic changes [1].

In AD studies, LiP-MS elucidated protein structural dynamics linked to aging and neurodegeneration. One study profiled the CSF proteomes of young and old mice, and identified 38 protein groups with altered abundance, particularly IgM immunoglobulins [35]. Six high-confidence candidates showed structural changes associated with aging, including proteins such as KNG1, ITIH2, Lp-PLA2, and 14-3-3 that are known to associate with AD. Another LiP hit, the CD5 antigen-like protein (CD5L), was found to form covalent complexes with IgM, as validated by western blot analysis, and this interaction increased with age. Such findings point to potential biomarkers and mechanisms relevant to aging and neurodegenerative diseases, and these await validation in larger (human) cohorts [35]. Another AD study analyzed human CSF samples across disease stages – healthy controls, mild cognitive impairment (MCI), and AD. Significant conformational changes were identified in 53 regions of 12 proteins when comparing controls to disease groups, pointing to a possible functional relevance of these proteins in AD progression [36]. A third study combined *N*,*N*-dimethyl leucine (DiLeu) isobaric tag labeling with LiP-MS (DiLeu-LiP-MS) to quantify structural changes in proteins from AD serum samples. High-pH peptide fractionation before liquid chromatography and tandem MS (LC-MS/MS) enhanced proteome coverage, enabling the detection of 23 proteins with structural alterations, seven of which are implicated in AD pathogenesis. The ability of the method to identify gender-specific protein changes and elevated complement protein levels highlights its potential for high-throughput structural protein analysis across various biological systems [37].

LiP-MS applications beyond human health and disease: insights from bacterial, yeast, and plant systems

Although LiP-MS has led to significant strides in studying human health and disease, its applications extend far beyond these contexts and it has been effectively employed in other biological systems, including bacteria, yeast, and plants. In this section we delve into LiP-MS studies across these model systems (Table 1), showing the versatility and impact of LiP-MS in non-human systems.

Bacteria

LiP-MS has been instrumental in understanding bacterial protein function and adaptation to environmental changes. For example, LiP-MS was used to study bacterial nutrient adaptation by growing *Escherichia coli* on eight different carbon sources. This revealed distinct metabolite–protein interactions and identified fructose-1,6-bisphosphatase as a regulator of glucose uptake [7]. In addition, LiP-SMap was employed to study metabolite-level regulation of enzymes of the Calvin cycle and central carbon metabolism in four autotrophic bacteria. This study uncovered extensive enzyme interactions with metabolites such as ATP, GTP, GAP, acetyl-CoA, and citrate, particularly at higher concentrations, and highlighted species-specific interaction patterns [38].

Yeast

Yeast, a model organism for cellular and molecular biology, has also been studied by LiP-MS techniques. Cappelletti *et al.* utilized LiP-MS to capture a range of molecular events during the yeast response to acute osmotic stress, including allostery, altered enzyme activity, site occupancy, and phosphorylation, with single functional site resolution. Their analysis identified not only proteins that were phosphorylated upon osmotic shock, as detected by phosphoproteomics, but also revealed changes in additional proteins, demonstrating the complementary nature of structural and phosphoproteome analyses. Furthermore, in heat-shocked yeast, LiP-MS provided insights into protein aggregation, chaperone–client interactions, and potential allosteric regulation of

chaperones, showcasing the ability of the technique to detect complex protein dynamics under stress conditions [7].

Similarly, using LiP-MS, 96 yeast proteins undergoing significant structural changes during heat shock were identified, revealing both pronounced and subtle alterations in protein structures. Furthermore, this study shed light on the sequence features that may help to predict whether proteins will aggregate reversibly, demonstrating that both disordered and structured regions contribute to protein phase separation under stress conditions [39].

Another study examined aging-related protein structural changes in budding yeast and focused on abundant proteins. It revealed age-related alterations of proteins involved in translation, folding, and metabolism, and identified glutamate synthase GLT1 polymerization as a key factor in disrupting amino acid homeostasis during aging. Inhibiting Glt1 polymerization restored amino acid balance, enhanced mitochondrial function, and extended lifespan in aged cells, illustrating the potential of Glt1-polymerization-based control of yeast longevity. Despite focusing on early aging processes and abundant proteins, LiP-MS provided insights into subtle age-related changes in protein activity. However, the need for more comprehensive temporal mapping was noted [40].

Beyond the classical stress-response studies, protein–gas interactions in yeast lysates have been explored. Incubation of yeast lysates with xenon (Xe) revealed Xe-induced conformational changes in 60 proteins, none previously known to interact with Xe. These Xe-interacting proteins were primarily involved in ATP-driven and glycolytic processes [41].

Plants

Building on the successes observed in bacterial and yeast systems, the LiP protocol has recently been optimized for use with plant material, focusing on metabolite–protein interactions. It has been successfully applied to tomato roots, *Arabidopsis* seedlings, and *Marchantia* liverworts, demonstrating the general applicability of this technique to a wide range of plant species. This adaptability opens new avenues for exploring plant protein dynamics and their interactions with metabolites in various physiological contexts [42].

Challenges of LiP technologies for studying protein conformations

As the previous sections illustrate, LiP technologies have greatly contributed to our understanding of how proteins conformationally act when confronted with different types of molecular perturbators or cellular stressors. Nevertheless, in our opinion, the current LiP technologies face several challenges.

Challenges associated with cell lysis

First, most LiP studies have been performed on cells or tissues that were gently lysed, avoiding the use of strong detergents in high concentrations because these are likely to destroy tertiary and quaternary protein structures. However, under such gentle lysis conditions, not all types of proteins will be efficiently extracted and especially membrane proteins will be under-represented in the lysates. Note that, for this reason, the thermal proteome profiling method was adapted to allow thermal profiling of membrane proteins by including mild detergents during protein extraction [43] or by including biotinylation of cell-surface proteins to affinity-capture these proteins [44].

Furthermore, all lysis methods inevitably dilute proteins in buffers that do not resemble intracellular conditions. Such protein dilutions also affect equilibria in protein complexes and protein conformations in general, which implies that lysates may poorly represent cellular conditions.

Finally, if lysis conditions cause partial disruption of cellular organelles, compartmentalized proteins may be released and engage into aberrant, non-physiological complexes with proteins that they would not normally encounter. In the case of enzymes, this can lead to altered protein modifications, thereby leading to aberrant protein conformations. Circumventing the actual lysis step in LiP studies would solve this problem, meaning that, ideally, the LiP step should be performed in living cells.

Challenges associated with peptide identification

A second challenge is the identification of semi-tryptic conformotypic peptides, especially those that lack the C-terminal lysine or arginine residues that are generated by trypsin. By their very nature, semi-tryptic peptides are shorter than tryptic peptides and are therefore often more hydrophilic and thus less well retained on the chromatographic column used before MS analysis. Because they have fewer peptide bonds, they also generate fewer fragment ions, resulting in less informative peptide fragmentation spectra, which in turn hampers unambiguous identification. Furthermore, semi-tryptic peptides that do not end with lysine or arginine are less basic than the majority of the other peptides present in the mixture. They will thus suffer from ionization suppression, implying that larger amounts of such peptides are needed to reach a given sensitivity threshold.

Several solutions have been described that, when implemented in LiP-MS workflows, tackle some of the issues described here. For instance, following incubation of a protein mixture with a broad-specificity protease, an **N-terminomics** workflow could be used to label the novel N-termini generated in a protein by proteolytic digestion, which allows further enrichment of the semi-tryptic conformotypic peptides carrying these novel N-termini. Several N-terminomics workflows are available (reviewed in [45]); however, all of these consume time and sample, but yield analyte mixtures that are largely devoid of confounding tryptic peptides, thereby making the identification of semi-tryptic conformotypic peptides more efficient. At the level of peptide identification, machine learning-based algorithms have been applied to boost the identification of non-tryptic peptides such as immunopeptides [46,47]. Therefore, one may expect that, when applied to LiP-MS data, such machine learning-based algorithms will also boost the identification of semi-tryptic conformotypic peptides.

Another challenge of LiP-MS is the difficulty in detecting peptides from low-abundance proteins and low-abundance peptides in general. Failure to detect these may obscure valuable structural insights regarding, among others, (low-abundance) proteins that play crucial roles in cellular function. Peptide enrichment strategies or advanced data-acquisition methods may mitigate some of these challenges, but they are not universally effective across all sample types or protein abundance levels.

Challenges associated with interpreting LiP-MS data

Interpreting LiP-MS data presents another challenge. Significant changes in the levels of (semi-) tryptic peptides can arise from various molecular events, such as alterations in protein folding, conformation, or binding to small molecules and other proteins. For example, reduced levels of a semi-tryptic peptide might reflect a conformational change but could equally indicate a bound ligand that shields specific protein regions from proteolysis. This ambiguity complicates the interpretation of data at a protein structure level, and often necessitates additional techniques to accurately attribute observed proteolytic changes to specific structural changes.

Concluding remarks

In summary, LiP-MS has established itself as a versatile and powerful proteomic tool that can offer novel insights into protein conformations and dynamics, and has clearly evolved to encompass a broad range of applications. These include studying different, complex biological systems,

Outstanding questions

Given the current technical limitations of the LiP-MS technology, how many protein conformational changes are not captured by this technology?

Because LiP currently cannot be directly applied to living cells, are the conformational protein changes captured via LiP in cell lysates identical to those occurring in the endogenous environment of the protein, namely the cellular context?

Because different conformotypic peptides can be identified from a single protein, how can one correctly distinguish between the peptides that have captured the relevant conformational changes directly induced by an added compound or a stressor and those that report irrelevant indirect, bystander, or longer-distance effects?

Especially in higher eukaryotes, one protein-coding gene does not give rise to a single protein, but instead to a collection of proteins. These chemically different protein variants, or proteoforms, among others, originate from differential splicing of immature transcripts and a staggering number of protein modifications, often with crosstalk between these modifications. At the very least, such proteoforms are present at different levels in a population of cells and may well adopt different conformations. How can we tweak conformational proteomic technologies in such a way that proteoform-specific conformations can be distinguished?

At a given point in time, in how many different conformations is a single proteoform present in a living cell, and how many of these are important for that proteoform to exert its function?

understanding disease mechanisms, aiding drug discovery, identifying disease biomarkers, and characterizing protein interactions with various (bio)molecules, as well as the structural impact of protein modifications and environmental stress. Despite its successes, challenges remain, particularly in protein extraction and peptide identification. Future directions may include in-cell approaches for native protein studies, advancing peptide enrichment strategies for low-abundance conformotypic peptides, and integrating machine learning for more accurate peptide identification and structural interpretation. Such advances will be crucial to further enhance the capabilities of the technique (see Outstanding questions) and promise to unlock further insights into protein dynamics and contribute to advances in health and disease research.

Acknowledgments

K.G. acknowledges support from the Flanders Research Foundation (FWO), project G002721N, and from the Special Research Fund of Ghent University, project BOF/24J/2023/152. K.D.B. acknowledges support from the FWO, project G0A5124N, and from the Special Research Fund of Ghent University, project BOF/24J/2021/374.

Declaration of interests

The authors declare no competing interests.

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