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Recent advances in mass spectrometry-based methods to investigate reversible cysteine oxidation

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Abstract

The post-translational modification of cysteine to diverse oxidative states is understood as a critical cellular mechanism to combat oxidative stress. To study the role of cysteine oxidation, cysteine enrichments and subsequent analysis via mass spectrometry are necessary. As such, technologies and methods are rapidly developing for sensitive and efficient enrichments of cysteines to further explore its role in signaling pathways. In this review, we analyze recent developments in methods to miniaturize cysteine enrichments, analyze the underexplored disulfide bound redoxome, and quantify site-specific cysteine oxidation. We predict that further development of these methods will improve cysteine coverage across more diverse organisms than those previously studied and elicit novel roles cysteines play in stress response.

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Introduction

Within the past decade, the role of cysteine oxidation in organismal stress response has been established as a fundamental regulatory mechanism [1–6]. With a redox-sensitive thiol side chain, cysteines can act as reversible oxidative switch and initiate signaling pathways in response to stress. They are commonly oxidized by reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), respectively, which accumulate in biological systems in stress states and can post translationally modify cysteines to affect and change protein and cellular function.

Cysteine oxidation can be characterized into two main categories: reversible oxidation and irreversible oxidation [7]. Reduced cysteines can be oxidized by ROS to form a relatively unstable and reactive sulfenic acid (R-SOH) and by RNS to form a nitrosothiol (R-SNO), which can then be hydrolyzed to form sulfenic acid. In the presence of proximal thiols, sulfenic acid can be stabilized by other cysteine containing molecules (e.g., proteins, glutathione) to form disulfide bonds (R_1-S-S-R_2). In addition, sulfenic acid can react with amines and amides to form sulfenamides (R_1-S-N-R_2), with cyclic sulfenamides forming to stabilize sulfenic acids through adjacent amides along the protein backbone. In the absence of proximal thiols or amides to form disulfide bonds/sulfenamides or in the presence of excess oxidant, sulfenic acid can be oxidized to sulfinic acid (R-SO_2H) and further oxidized to sulfonic acid (R-SO_3H). Although sulfinic acid can be reduced back to sulfenic acid in the presence of sulfiredoxin, these two increased oxidative states are often indicative of oxidative damage and can lead to protein dysfunctionality, degradation, and possibly cell death [8–10].

Interestingly, cysteines are one of the most uncommon amino acid residues within sequenced proteomes. In the reviewed Uniprot/Swissprot proteomes (accessed 20230601), nearly 83% of all proteins have a cysteine residue. However, cysteine residues only comprise 1.4% of all amino acid residues in these proteomes, making them difficult to study via traditional proteomic methods. Recent advances in mass spectrometry have furthered the ability to study low abundant species, such as cysteine-containing peptides. Traditional data dependent acquisition (DDA) methods operate by selecting the top n most abundant species within a sample for fragmentation. DDA methods are biased by abundance and can lead to data loss for low abundant species. In comparison, data independent acquisition (DIA) can be used to improve coverage of low abundant species [11]. DIA methods operate by fragmenting all coeluting species simultaneously within a specified mass-to-charge range. This leads to complex fragmentation spectra and until now required generation of detailed spectral libraries via DDA methods to ease downstream data analysis. Advancements in spectral

library generation [12], development of library-free DIA methods [13], and creation of DIA-specific software [14–16] are helping to mitigate the complexity of data analysis, but these nascent software/methods still require development and optimization for utility in the most complex proteomes. DIA-based proteomics has allowed for increased coverage of sub-stoichiometric post translational modifications and has been used to accurately examine cysteine oxidation [17]. In addition to advancements in DIA, the development of isobaric tags applied to study cysteine oxidation, such as tandem mass tags (TMT), allows samples across biological replicates and conditions to be multiplexed to increase the abundance of precursor peptide ions within a survey scan [18]. These isobaric tags can then be distinguished by distinct reporter ions in the fragmentation spectra such that peptide abundances across biological replicates and sample conditions can be accurately quantified.

Although cysteines are uncommon, the functional diversity of post translational modifications that occur on cysteine residues point for a range of biological functions and pathways in which cysteines are involved. Yet the role of cysteine and how its different modification types play distinct roles in stress response is still being understood, especially in non-model and understudied organisms. As such, this review summarizes traditional methods that use mass spectrometry to interrogate the reversible redoxome and unveils new advances in investigating the cellular response to oxidative stress.

Cysteine enrichments: traditional indirect and direct methods

Reversible cysteine oxidation can be studied using a mode of enrichment strategies. Indirect methods to study cysteine oxidation include irreversibly blocking cysteines reduced *in vivo*, commonly with iodoacetamide (IAM) or N-ethylmaleimide (NEM), before using a strong reductant, such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP), to reduce all reversibly oxidized cysteines (Figure 1a). Furthermore, selective reductants, such as ascorbate to reduce sulfenylated cysteines [19] or nitrosothiols [20], or glutaredoxin to reduce S-gluathionylated cysteines [21], can be used to target specific oxidative modifications (Figure 1b). Regardless of the strength of reductant used, reduced cysteines can then be enriched using thiol-affinity resins using resin assisted capture (RAC) and subjected to on-resin digestion before label free quantitative LC-MS/MS analysis of cysteine oxidative states across stress conditions is performed [22–26]. These methods have been well established within the field and have been used across a range of biological systems. Recent approaches to improving these specific enrichments have focused on multiplexing resin assisted capture methods to increase sensitivity, improve accuracy, and decrease instrument run time, with multiplexing providing the ability to analyze multiple modification types by selective reductions from the same sample [27]. Furthermore, cysteine-specific isobaric tags, such as cysTMT, can be equipped to simultaneously multiplex and enrich for reversibly oxidized cysteines via an anti-TMT antibody [20,28,29].

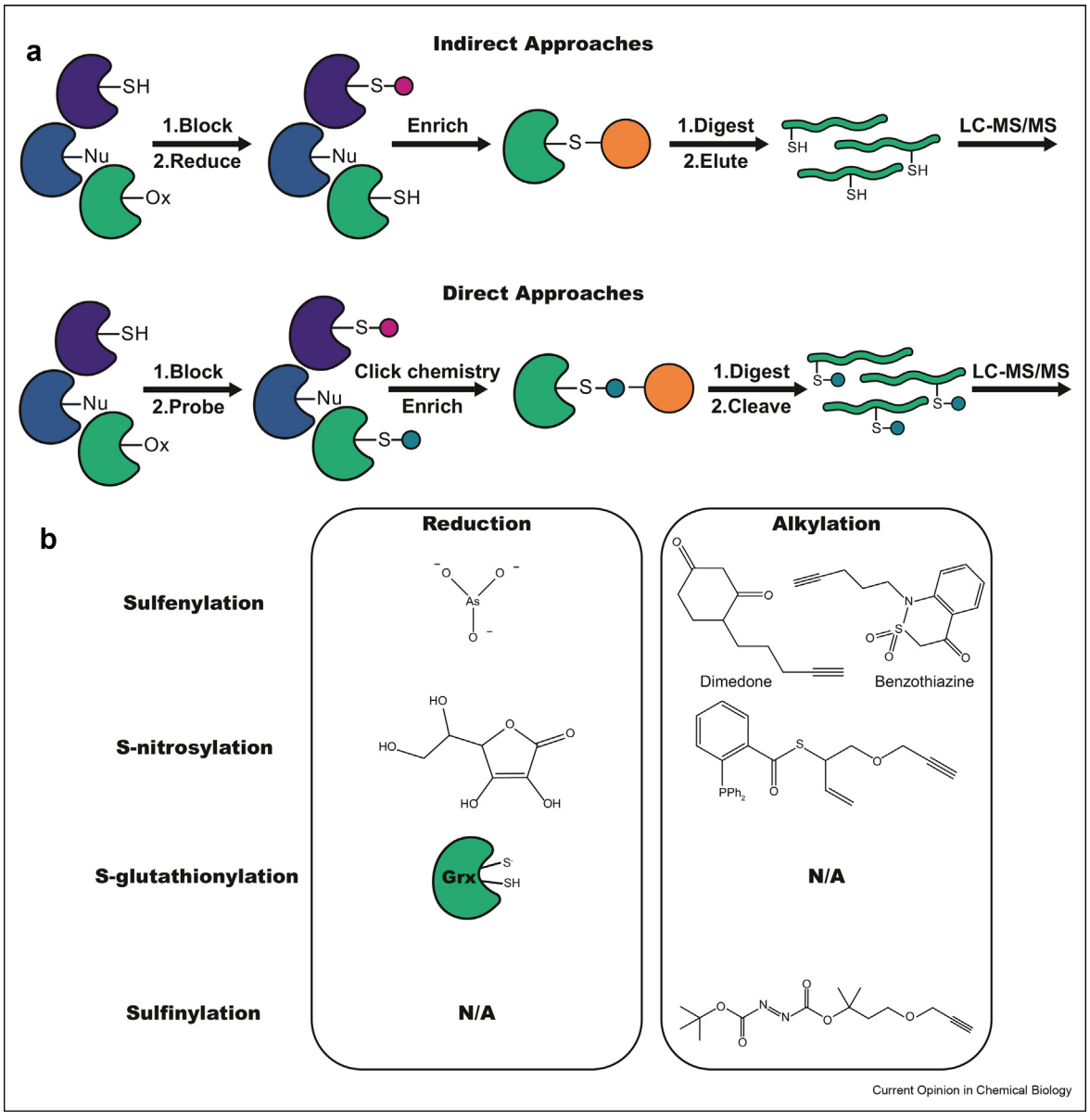
Direct methods to studying cysteine oxidation include using probes specific for cysteine oxidation types. These probes are often equipped with terminal alkyne groups available for click chemistry with a biotin azide derivative and subsequent enrichment via the affinity between biotin and streptavidin (Figure 1a). Dimedone [30] and benzothiazine-based probes [31] (Figure 1b) have been equipped to study sulfenylation and its role in signaling and stress response across a wide range of organisms, with dimedone switch techniques also being employed to study persulfide (R-S-S-H) oxidation states [32]. Sulfinylation can be probed using a synthesized probe, DiaAlk (Figure 1b), and has been implemented in *Caenorhabditis elegans* [33] and multiple human cell lines [34,35] among others. In addition, compounds to probe S-nitrosylation include triaryl phosphines conjugated to biotin via an ether spacer (Figure 1b) [36]. Through either indirect or direct approaches, the role of cysteine oxidation in biological signaling and stress response remains at the forefront of method development.

Recent developments in studying cysteine oxidation

Isotopic labeling and high pH fractionation

In lieu of traditional enrichment methods, high pH reversed phase offline fractionation offers a means to decrease the complexity of proteomic samples and increase the likelihood of detecting low abundant Cys-containing peptides [37–39]. A method for simultaneous protein expression and redox analysis (SPEAR) has been established by labeling reduced and reversibly oxidized cysteines with light (d_0) and heavy (d_5) isotopes of NEM, respectively, before high pH reversed phase fractionation [40]. In *Arabidopsis thaliana*, this method was able to identify nearly 5000 cysteine containing peptides representing 2700 proteins. In comparison, a recently published study using a traditional RAC method in *A. thaliana* was able to identify ~6900 oxidized cysteine containing peptides representing 3300 proteins [25]. SPEAR benefits from the ability to monitor translational and redox level changes within the same injection and allows for lower sample starting material when compared to traditional enrichment approaches (100 μg in SPEAR vs 500–1000 μg in RAC). However, offline fractionation increases instrument analysis time while not achieving the same cysteine coverage possible from traditional enrichment methods and therefore has limitations for in-depth studies of organismal cysteine oxidation.

Figure 1

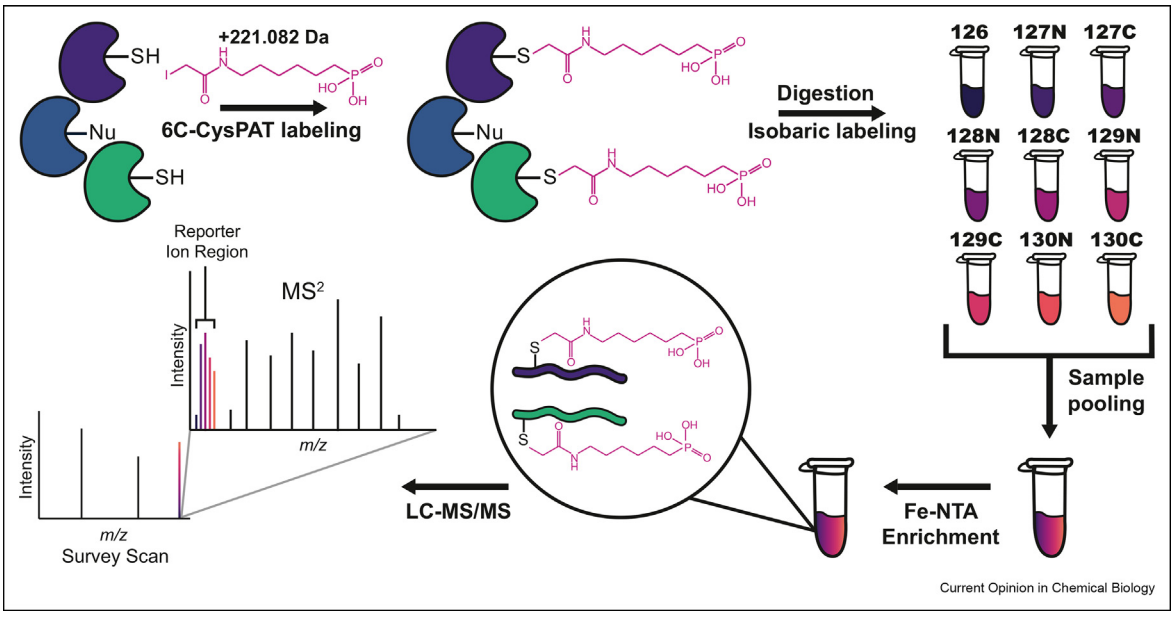


Mass spectrometry-based strategies to quantify reversible cysteine oxidation. **a)** Indirect approaches enrich for reduced cysteines prior to LC-MS/MS analysis. Direct approaches use alkylating probes for a specific modification. These probes are often equipped with a terminal alkyne available for click chemistry to biotin and subsequent streptavidin enrichment prior to LC-MS/MS analysis. **b**) Selective reductants and alkylating probes can be used for specific cysteine oxidation modifications of interest.

Developing novel enrichment techniques to quantify cysteine oxidation

As enrichments typically require 500−1000 μg of starting material, necessary advances in cysteine enrichment strategies include optimization and miniaturization. Recently, a cysteine-specific phosphonate adaptable tag (CysPAT) has been synthesized to selectively label reduced cysteines [41,42]. With a free iodo-moiety on one end and a phosphonic group on the other (Figure 2), CysPAT can irreversibly alkylate free thiols prior to selective enrichment via the terminal phosphate group by metal affinity chromatography. This approach has the advantage of providing coenrichment of phosphopeptides, giving PTM multiplexing capabilities. In studies where selective enrichment of cysteine-containing peptides is desirable, samples can be incubated with a phosphatase prior to incubation with CysPAT to decrease phosphopeptide interferences. CysPAT has been used to identify >10,000 oxidized cysteine containing peptides representing 3800 proteins from

Figure 2



Experimental workflow using 6C-CysPAT and isobaric labeling for analysis of cysteine containing peptides. Reduced cysteines are labeled with 6C-CysPAT before subsequent trypsin digestion and isobaric labeling. Cysteine-containing peptides are then enriched for and analyzed via LC-MS/MS. Peptides within each condition are quantified based on the intensity of a condition's respective reporter ion in the fragmentation spectra.

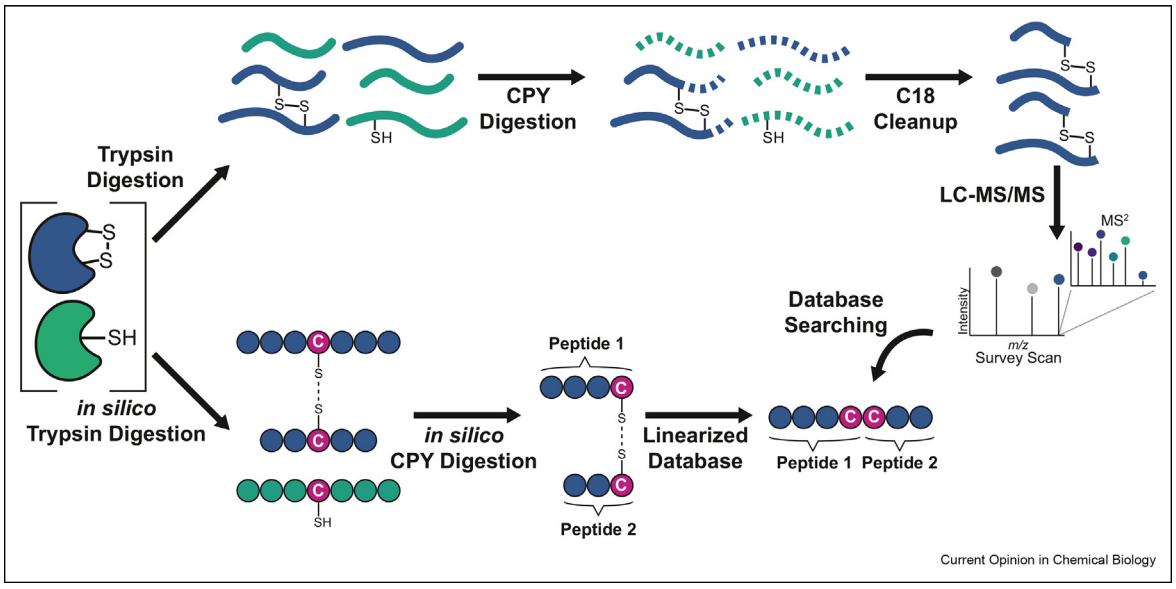
250 μg of protein from HeLa cells. However, it is not commercially available and requires synthesis, making it not as accessible as alternative probes. Recently, Lie et al. established a method using a commercially available CysPAT residue with a six-carbon linker between either functional end of the compound, termed 6C-CysPAT (Figure 2) [43]. When implemented in tandem with TMT technology, they report >17,000 unique cysteine-containing peptides representing 9900 proteins from only 25 μg of protein from SH-SY5Y human neuroblastoma cells. However, it must be noted that this study enriches for cysteines both reduced and reversibly oxidized *in vivo*, not just those that are reversibly oxidized cysteine residues as the initial CysPAT study analyzed. Regardless, this method displays a highly selective and efficient enrichment method for studying cysteine oxidation and may be used as a sensitive alternative to traditional RAC methods, especially in sample limited applications.

Investigating the disulfide bound redoxome

Approaches to investigating disulfide bonds aim to use these covalent linkages between cysteines as a native crosslinker to determine peptides bound to each other. These methods have been used in the identification of antimicrobial peptides in non-complex samples and have recently been adapted to investigate disulfide bonds at a proteome level [44,45]. Similar to indirect approaches, free thiols are first blocked with an alkylating agent before proteolysis without reduction, leaving disulfide bound peptides intact. Strong cation exchange chromatography is then utilized to enrich the disulfide bound peptides, as they will be larger in size and carry higher charge states, before analysis via LC-MS/MS. Although data analysis from this method can be complex and require manual confirmation of disulfide linkages, software such as pLink-SS aids in the delineation of disulfide bound proteins and has been used to study disulfide bonds in *Escherichia coli* [45], human endothelial cells [45], rat renal cells [46], and whey protein extracts [47]. Advancements in this method focus on utilizing multiple proteases to decrease the likelihood of single peptides containing multiple disulfide bonds, which have decreased MS/MS fragmentation efficiencies, and increase coverage of disulfide containing peptides [48]. Other advancements include increasing fragmentation efficiencies of these higher molecular weight compounds by utilizing HCD to increase the coverage of the disulfide bound redoxome [46–48].

Recent work has begun on attempting to enrich for disulfide bound peptides by utilizing their innate folding to prevent interaction with a non-specific protease [49]. Carboxypeptidase Y (CPY) assisted disulfide bond identification (CADI) uses CPY to selectively cleave at the carboxyl end of linear peptides (Figure 3). However, disulfide bound cysteine residues are unable to bind to the enzymatic region of CPY and are therefore unable to undergo digestion. Thus, disulfide bound peptides remain intact, and disulfide bonds are often found at the

Figure 3



Experimental workflow using CADI for analysis of disulfide containing peptides. Proteins undergo trypsin digestion without reduction and alkylation, leaving disulfide bonds intact. Peptides are then further digested with carboxypeptidase Y (CPY), leaving only disulfide bound dipeptides for LC-MS/MS analysis. Database searching is conducted by predicting all intramolecular disulfide bonds within a protein before *in silico* trypsin and CPY digestion. These peptides are then linearized by peptide 1 becoming the N-term while peptide 2 becomes the C-term on the resulting linearized peptide. This ensures accurate identification of disulfide bound peptides if fragmentation occurs at the peptide bond or the disulfide bond.

C-term of peptides when analyzed via LC-MS/MS. Database searching is performed against a manually curated database. For each protein, all possible intramolecular disulfide bonds are predicted, and the proteins undergo *in silico* trypsin and subsequent CPY digestion, leaving all predicted intramolecular dipeptides (Figure 3). These disulfide bound dipeptides are then linearized, where peptide 1 becomes the N-term and peptide 2 becomes the C-term of the newly pseudo-linearized peptide. Linearization ensures identification of a peptide even if fragmentation occurs at both peptide bonds and the disulfide bond in MS/MS acquisition. CADI was able to identify 76% of disulfide bonds compared to 80% via pLink-SS from ten disulfide bound dipeptide standards within a complex HEK293T cell lysate, showing selectivity and comparability to already established methods. CADI is limited by its database curation. Prediction of all intermolecular disulfide bonds within a proteome would exponentially expand the search space and thus cannot be identified using this method. Although still developing and not yet applied to whole cell lysates, CADI may offer a method to selectively enrich for and identify disulfide bound proteins.

Methods to quantify site-specific cysteine occupancy and reactivity via chemoproteomics

Development of direct methods through chemoproteomic probes continues to improve the understanding of site-specific cysteine oxidative occupancy [50–53]. A triphenylphosphonium ylide, also known as a Wittig reagent, has been recently developed with a terminal alkyne for click chemistry to probe sulfenylated cysteines in both cellular lysates and live cells [50]. Under steady state-redox conditions in intact A549 lung carcinoma epithelial cells, the probe was able to identify >2000 sulfenylated cysteines, greatly outperforming a previously established benzothiazine probe (BTD) [54], which identified 126 sulfenylated sites. The authors hypothesized that a differential labeling method with the Wittig probe and an iodoacetamide-alkyne probe (IPM) could be used to determine sulfenic acid occupancy. By labeling intact cells with a ^{13}C labeled Wittig reagent before lysing, reducing reversibly oxidized cysteines, and then labeling subsequently reduced thiols with IPM, different oxidative modifications occurring on the same cysteine could be found. Based on comparison of the MS1 abundance of the same cysteine being labeled by the two different probes, the percent of that cysteine being sulfenylated could be determined. From this experiment they were able to quantify >7300 cysteine sites and >6600 sulfenylated sites on nearly 3400 proteins. From extracted ion chromatograms of cysteines labeled by both probes, they were able to calculate site-specific ratios of sulfenic acid occupancy, giving the first stoichiometric understanding of the sulfenome.

Equipping these chemoproteomic probes to perform quantitative thiol reactivity profiling (QTRP) or activity-

based protein profiling (ABPP) has become a large focus in redox proteomics. QTRP and ABPP methods aim to investigate the reactivity of specific cysteine residues and determine the role individual cysteines have in protein and cellular response to oxidative stress. By incubating protein lysates with different concentrations of the same probe, a cysteine's innate reactivity can be determined, with the most reactive cysteines often getting labeled even at low probe concentrations. Similar to direct methods to probe cysteine oxidation, maleimide-based probes and isotopically labeled iodoacetamide (IA)-alkyne probes have been developed to multiplex samples while investigating cysteine reactivity [55—60]. With a free alkyne available for click chemistry to biotin and subsequent enrichment via streptavidin, these methods are sensitive ways to further investigate the role of specific cysteine residues in a protein's function. However, these methods are limited by the small commercial availability of these probes and the high cost of single experiments, with isobaric tags often costing thousands of dollars for a two-condition quantitative comparison. Thus, Yang et al. removed the multiplexing capabilities to decrease cost and instead opted to use an IA-alkyne probe in tandem with DIA to increase coverage of the cysteineome and improve biological assessment of cysteine oxidative states [17]. In K562 cells, they identified >9000 cysteine-containing peptides, a 40% increase compared to the same sample using the original published ABPP method [61] and were also able to apply this method to study circadian rhythms in mice liver to quantify the oxidation of 4200 cysteine sites. Although this method increases instrument time by approximately 50% when compared to the isotopically labeled studies, it and similar methods using DIA may prove fruitful to investigating low abundant cysteines and their innate reactivities. In addition, curation of the CysDB, a database combining information of cysteine oxidation states and reactivity from a variety of cysteine redox studies, provides a basis for cross-comparison of cysteine oxidation studies [62]. Through the continued development of this and similar databases, further understanding of the role of cysteine oxidation in protein and cellular function will be achieved.

Conclusion and future outlooks

With advancements in mass spectrometry methods, investigations into biological systems become more capable of answering complex and pertinent research questions. The past two decades of research have established methods to delineate cysteine oxidative states and the role they play across conserved signaling pathways in model organisms. Approaches to detect specific cysteine modifications, including the disulfide bound proteome, will provide insight into not only oxidative-dependent signaling pathways but also how cysteine oxidation may affect a protein's structure, its interactors, and give further insight into its function within a signaling pathway. In addition, investigations into non-model systems have only just begun. Miniaturization of cysteine enrichment strategies while still maintaining adequate coverage of the reversible redoxome will push the field forward as novel biological pathways for stress response are discovered in these non-model organisms. Chemoproteomics also continues to broaden the understanding of the role cysteine oxidation plays within a cellular context. Organelle localized probes and combination of chemoproteomics with proximity-labeling methods may begin to provide a larger insight into sub-cellular cysteine oxidation and how it affects the larger interactome [63]. Site specific quantitation across oxidative states will provide functional insight into the reactivity of specific cysteines and improve knowledge cellular response to oxidation. Through continued development of techniques to study cysteine oxidation, the role it plays in a larger biological framework will only be further and further understood and drive innovative research in the field of biological stress response.

Declaration of competing interest

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

Data availability

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

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* of special interest
** of outstanding interest

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By combining TurboID and cysteine enrichment, this study equipped local cysteine oxdiation (Cys-LOx) and was able to identify and quantify mitochondrial-specific cysteine oxidation and cysteine reactivity. Across a panel of sub-cellular compartments, this study identified >3500 cysteines not previously identified in whole-cell cysteine enrichment studies in HEK293T cells and murine bone marrow-derived macrophages, providing a platform to studying sub-cellular cysteine oxidation states.