Published in final edited form as:

Mol Biosyst. 2017 May 02; 13(5): 816–829. doi:10.1039/c6mb00861e.

Quantitative Proteomic Characterization of Redox-dependent Post-translational Modifications on Protein Cysteines

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Abstract

Protein thiols play a crucial role in redox signaling, in the regulation of enzymatic activity and protein function, and in maintaining redox homeostasis in living systems. The unique chemical reactivity of the thiol group makes protein cysteines susceptible to reactions with reactive oxygen and nitrogen species that form various reversible and irreversible post-translational modifications (PTMs). The reversible PTMs in particular are major components of redox signaling and are involved in the regulation of various cellular processes under physiological and pathological conditions. The biological significance of these redox PTMs in both healthy and disease states has been increasingly recognized. Herein, we review recent advances in quantitative proteomic approaches for investigating redox PTMs in complex biological systems, including general considerations of sample processing, chemical or affinity enrichment strategies, and quantitative approaches. We also highlight a number of redox proteomic approaches that enable effective profiling of redox PTMs for specific biological applications. Although technical limitations remain, redox proteomics is paving the way to a better understanding of redox signaling and regulation in both healthy and disease states.

Graphical abstract

1. Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anions ($O_2^{\bullet-}$), and hydroxyl radicals ($HO\cdot$), are inevitable byproducts of cellular aerobic metabolism. These molecules play important roles in redox signaling of various biological processes in response to extracellular stimuli.^{1, 2} The concentration of cellular ROS is subtly controlled by a number of enzymatic and non-enzymatic processes in order to maintain redox homeostasis for normal cell functions. Low levels of ROS are necessary for physiological redox signaling, whereas high ROS levels are associated with oxidative stress and oxidative damage to cellular components, such as DNA, lipids, and proteins, and eventually promote cell death. Oxidative stress is implicated in many human diseases, such as cancer, neurodegenerative diseases, diabetes, and other aging-related diseases.^{3–5} However, the molecular mechanisms underlying oxidative stress and ROS-mediated redox signaling are still unclear.

One major mechanism of redox signaling involves ROS-mediated reaction on protein cysteines whose thiol group can be deprotonated under physiological conditions,⁶ leading to the formation of many reversible and irreversible redox-dependent post-translational modifications (PTMs) (Fig. 1). The formation of redox PTMs is a subtly controlled biological process, which is mediated either enzymatically or by reactions with reactive oxygen/nitrogen/sulfur species (ROS/RNS/RSS).^{6, 7} The reactivity of individual Cys residues is primarily governed by solvent accessibility, thiol pKa, subcellular localization, and other factors contributing to the local micro-environment.^{8, 9} The reversible redox PTMs include disulfide formation (S-S), S-glutathionylation (SSG), S-nitrosylation (SNO), S-sulfenylation (SOH), and S-sulfhydration (SSH), all of which can be reduced back to free thiols by the antioxidant defense system or converted to other stable oxidized forms. Over the last decade, reversible Cys redox PTMs have been increasingly recognized as a fundamental redox signaling and regulatory mechanism by which redox signals can be transformed into biological responses by modulating protein functions and activities.^{10–12} In

contrast, sulfinylation (SO₂H) and sulfonylation (SO₃H) are generally considered irreversible redox modifications. However, the reversible reduction of SO₂H was observed in peroxiredoxins (Prx) by ATP-dependent sulfiredoxin (Srx).^{13, 14} These irreversible modifications reflect over-oxidation and are considered pathological markers of oxidative stress.¹⁵ Therefore, identifying redox Cys PTMs and tracking their dynamic changes under aberrant conditions are essential for understanding redox signaling and regulation under both physiological and pathological conditions.

Among these modifications, irreversible modifications such as SO₂H/SO₃H typically have good stability under sample processing and LC-MS conditions. Thus, direct analysis of these modifications in a complex sample is conceptually feasible. Chromatography-based methods, such as ion exchange chromatography (IEX)^{16, 17} and hydrophilic interaction chromatography (HILIC)¹⁷, have been used to enhance the proteomic discovery of these modifications. In contrast, measurement of reversible PTMs in complex biological systems has been challenging for several reasons, including the labile nature of these modifications, artificial oxidation, and conversion between different forms of reversible PTMs during sample processing. Moreover, these PTMs typically exist at low abundance. Therefore, effective enrichment approaches are required to reduce sample complexity and to enhance detection levels, especially for measuring endogenous levels of redox PTMs. To date, antibodies against specific types of Cys PTMs do not provide sufficient specificity for selective enrichment of redox PTMs in complex samples.^{18–20} To date, the detection of reversible redox PTMs has been largely conducted by utilizing either indirect or direct chemical enrichment strategies.

In particular, mass spectrometry (MS) has recently become an indispensable tool for advancing redox proteomics, enabling site-specific identification and quantification of redox PTMs at the proteome level.^{21–24} To date, thousands of redox-sensitive Cys sites have been successfully identified and documented in a number of redox databases for different organisms.^{25–28} Herein, we mainly focus on recent advances of quantitative proteomic approaches for reversible redox PTMs of Cys and their biological applications. The general considerations of sample processing and enrichment strategies will be discussed. For an introduction to the fundamentals of the chemistry and biology of Cys PTMs and MS-based redox proteomics, we refer the readers to several excellent recent reviews.^{6, 7, 24, 29}

2. Proteomic strategies for characterizing reversible Cys PTMs

2.1 General considerations of sample processing

One of the major challenges in characterizing reversible Cys PTMs is preservation of the endogenous redox state and minimizing both oxidative and reductive artifacts during sample preparation. In living cells, the majority of protein cysteines exist in a reduced state (80–90%).^{30–32} However, Cys thiols can be easily oxidized upon cell lysis and further processing. Currently, one common method to minimize artificial oxidation is *via* trichloroacetic acid (TCA) quenching followed by blocking free thiols with alkylating reagents under denaturing conditions.³³ TCA acts quickly to acid-trap free thiols by significantly lowering the pH. Alkylation reagents, such as N-ethylmaleimide (NEM) and iodoacetamide (IAM), are often included in the subsequent denaturing buffer to covalently

block free thiols, thus preserving cysteines in their native state for downstream processing and measurements.³⁴ NEM reacts with thiols *via* a Michael-type addition, which is more specific, faster, and less pH-dependent than the nucleophilic substitution reaction occurring with IAM, making NEM often the preferred blocking reagent for measuring Cys modifications.^{35–37} However, NEM may be less specific for thiols under alkaline conditions, where NEM may potentially react with the side chains of lysine and histidine.^{38, 39} In addition, denaturing conditions can enhance the blocking efficiency of NEM by reducing the effect of steric hindrance caused by buried thiols inaccessible in the proteins native state.⁴⁰ It should be noted that complete blocking of free thiols is a critical step, especially for indirect detection strategies, and is important in designing proper negative controls for identifying false positive signals arising from incomplete blocking.⁴¹ Moreover, existence of redox-active metal ions, such as Cu^{2+} and Fe^{3+} , even at trace amounts, can catalyze thiol oxidation^{42, 43} or decomposition of certain Cys PTMs, such as SNO and protein disulfides.⁴⁴ The inclusion of metal chelating reagents, such as diethylenetriaminepentaacetic acid (DTPA)^{45, 46} or neocuproine⁴⁷, into cell extracts or tissue homogenates is important for mitigating these side-reactions.

The stability of redox PTMs is another important factor for consideration. Many forms of Cys PTMs are labile and can be easily converted into alternate forms or lost completely, either enzymatically or non-enzymatically. For example, SNOs are UV-sensitive and not stable at high temperature.²¹ Therefore, SNO samples should be processed in the dark without significant heating. Chemical derivation approaches are often applied to convert unstable forms of Cys PTMs into stable and detectable products by taking advantage of their distinct chemical reactivity. Direct chemical labeling is another attractive strategy for detecting Cys modifications, which will potentially provide a straightforward approach compared to other methods. However, reaction efficiency and chemical specificity will be critical for direct labeling approaches.

2.2 Direct approaches

Direct analysis of Cys PTMs by MS is often preferred; however, due to their labile nature such detection has been challenging, although limited reports of direct detection for SSG, SNO and SOH have been published.^{48, 49} Alternatively, chemical labeling or derivatization approaches have been developed for directly targeting Cys PTMs based on specific reactions to each form of PTM. Subsequently, affinity or other chemical tags can be introduced at the redox-modified Cys residues to enable subsequent Western blotting or affinity enrichment experiments for MS analysis. For example, a number of dimedone-based reagents have been developed for specifically targeting SOH (Fig. 2a).⁵⁰ SNO can also be directly captured by organomercury resin^{51, 52} or selectively labeled with phosphine-based probes for affinity enrichment (Fig. 2b).^{53, 54} Chemical labeling approaches based on specific cross-reactions between SOH and SNO have also been applied for selective enrichment of protein SOHs and SNOs for proteomic analysis.^{55, 56} In addition to SOH and SNO, detection of SSH-modified proteins has been realized through a tag-switch approach.^{57, 58} Both protein persulfides and free protein thiols were first tagged with an electrophilic reagent, methylsulfonyl benzothiazole (MSBT), to generate benzothiazole-containing disulfides (Fig. 2c) and Cys-based disulfides. These mixed aromatic disulfides can be distinguished from Cys-based

disulfides by their increased reactivity toward certain carbon nucleophiles, such as cyanoacetate-based probes. Therefore, SSH-modified Cys sites can be selectively labeled with biotin-linked cyanoacetate probes for proteomic analysis.⁵⁹ Recently, a Biotin-Thiol-Assay (BTA) was also developed for direct analysis of SSH-modified proteins by using maleimide-PEG2-biotin (NM-Biotin) (Fig. 2c).⁶⁰ In this case, all free protein thiols will be irreversibly blocked by NM-Biotin, and only SSH-modified Cys-containing peptides or proteins can be further released through reduction by dithiothreitol (DTT).

Unlike as with reactive Cys PTMs, there is no method available for direct detection of SSG. However, *in vivo* metabolic labeling approaches have been reported for targeting SSG using biotinylated glutathione (GSH), including glutathione ethyl ester (biotin-GEE)⁶¹, N, N-biotinyl glutathione disulfide (biotin-GSSG)⁶² and glutathionylspermidine⁶³. Following labeling, the biotin-conjugated SSG proteins are enriched using avidin affinity chromatography. These approaches are quite useful in rapid and selective identification of SSG-modified proteins in cells. Nonetheless, the identified SSG sites may not reflect *in vivo* SSG modifications under physiological conditions.

2.3 Indirect approaches

Given the lack of effective methods for the direct detection of Cys PTMs, the majority of MS-based approaches for detecting reversible Cys PTMs are based on indirect strategies. The most commonly used indirect methods are the biotin-switch technique (BST)⁶⁴ and other types of switch strategies, such as resin-assisted capture (RAC)^{65, 66} and differential alkylation.^{67, 68} The indirect approaches typically consist of three major steps: (1) blocking free thiols; (2) selective reduction of the targeted form of Cys PTMs to free thiols; (3) chemical tagging or capture of nascent thiols. Effective blocking of free thiols by methyl methanethiosulfonate (MMTS), IAM or NEM is an essential step for most indirect approaches for their successful and accurate detection of oxidized thiols.

The next critical step is selective reduction of the Cys PTMs to free thiols. A number of reducing reagents or antioxidant enzymes have been used for the selective reduction of alternate forms of Cys PTMs. For example, DTT and tris(2-carboxyethyl)phosphine (TCEP) can reduce all reversible PTMs. The resulting levels of free thiols after DTT and TCEP reduction represent the total number of reversibly modified cysteines and may be used as an indicator of overall *in vivo* redox state (Fig. 3a).^{31, 34, 69–71} Ascorbate in combination with $\text{Cu}^+/\text{Cu}^{2+}$ have been widely used to selectively reduce SNOs (Fig. 3b),^{64, 72–74} while sodium arsenite was reported for selectively reducing SOHs (Fig. 3c).^{75–77} A cocktail of reagents consisting of a mutant form of glutaredoxin 1 (Grx1), glutathione, NADPH, and glutathione reductase allows selective removal of glutathione moieties from SSG-modified proteins (Fig. 3d) to enable quantitative measurements of SSG modifications in cell and tissue samples.^{11, 78–80} While the success of indirect approaches relies heavily on the specificity of the reducing reaction, the selective reduction strategies used in most indirect approaches are still not perfect in terms of specificity toward the target form of Cys PTMs. For example, ascorbate not only reduces SNO but also mixed disulfides^{41, 81, 82} and sulfenic acids⁸³ to a certain degree. Similarly, Grx1 is specific for deglutathionylation of proteins, but it also can have a small degree of non-specific reduction of protein disulfides.⁸⁴ Therefore, employing

proper negative and positive controls will be crucial to ensure confident detection of endogenous levels of redox modifications.

After selective reduction, the nascent protein thiols can be directly captured using thiol-affinity resin or chemically labeled with affinity or chemical tags for enrichment and subsequent quantification prior to LC-MS/MS analysis. There are a number of thiol reactive reagents available for labeling free cysteines. Pyridyldithiol-based reagents contain an active aromatic disulfide that can efficiently react with free thiols to form a stable mixed disulfide by thiol-disulfide exchange. The resulting product is cleavable by reducing agents, such as DTT or TCEP. This reversible labeling technique is very useful for peptide enrichment and MS analysis with both BST and RAC approaches. For example, in the BST assay pyridyldithiol-biotin (biotin-HPDP) has been used to reversibly label SNO-modified cysteines after ascorbate reduction, followed by enrichment on a streptavidin column.⁶⁴ The captured peptides can be easily eluted by using DTT under mild reducing conditions.⁸⁵ In addition, removal of affinity tags before MS also enhances the sensitivity of detection. The RAC approaches also take advantage of pyridyldithiol chemistry using Thiopropyl Sepharose 6B resin.^{65, 66} The reduced cysteines are directly captured on the resin by disulfide bond exchange. The captured proteins or peptides are then eluted by DTT for MS analysis. As an improved method for analyzing Cys PTMs, RAC offers a simpler workflow and better specificity in capturing Cys-containing peptides (>95%) compared to biotin-based enrichment.^{66, 69, 73, 79, 80}

Alternatively, the free cysteines can be irreversibly trapped by using alkylation reagents, such as iodoacetyl- or maleimide derivatives (Fig. 3). A variety of fluorophores, epitope labels, and stable isotope-code tags can be conjugated with these reagents for selective enrichment or detection of redox modified cysteines. For instance, the iodoacetyl-biotin^{86, 87}, iodoacetyl-His⁶⁸ and isotope-coded affinity tags (ICAT)^{89, 90} have been used as the alternatives of biotin-HPDP for analysis of SNOs. The irreversible tags could enhance the unambiguous identification of modified Cys sites by the mass shifts in MS detection.

2.4 Quantitative strategies

Quantitative strategies have been coupled with various enrichment methods for global identification and quantification of Cys PTMs.^{22–24, 29} While label-free quantification is conceptually feasible for redox PTMs, the majority of quantitative redox proteomics studies have been based on stable isotope labeling approaches. More recently, targeted quantification has emerged as a promising strategy for precise quantification of redox modifications.

2.4.1 Isotope labeling approaches—Several common stable isotope labeling strategies have been incorporated into redox proteomics workflows as illustrated in Fig. 4. For relative quantification following the blocking of free thiols and selective reduction, light and heavy isotope-coded reagents are used to label the newly derived free cysteines from two samples, respectively. (Figure 4a) Then the labeled samples are combined (1:1) and typically subjected to digestion/enrichment and LC-MS/MS analysis. The ratio of signal intensity

from heavy- and light-labeled peptide pairs in the MS1 detection provide the relative amounts of oxidized protein cysteines.

Stable isotopes can also be incorporated into proteins through metabolic approaches, such as stable isotope labeling with amino acids in cell culture (SILAC). SILAC-based relative quantification can be easily adapted to the workflow of most redox proteomics approaches, such as BST and RAC^{91, 92}, although the applications are mostly limited to cell culture. Moreover, the isotopic tags can be introduced through *in vivo* chemical labeling for specific types of Cys PTMs. For example, cell-permeable isotope-coded dimedone probes have been developed for quantitative analysis of protein SOHs in living cells.^{93–95} The probe consists of a dimedone moiety for specific trapping of SOHs, an isotope-coded linker for MS-based quantification and a clickable functional group (azide or alkyne) for biorthogonal reactions after cell lysis. After labeling, an acid-cleavable biotinylated tag can be conjugated to proteins or peptides *via* click chemistry, which facilitates the elution of biomolecules from avidin and subsequent MS quantification.

Differential alkylation is another commonly applied strategy in redox proteomics designed to obtain the stoichiometric ratio (or occupancy) of thiol oxidation for a given sample (Fig. 4b). Typically, a pair of different isotope-coded alkylation reagents is used to label reduced and oxidized cysteines within the same sample. The light version of reagent is used to label the reduced cysteines first, followed by reduction and subsequent alkylation of the oxidized cysteines using the heavy isotopic version. LC-MS/MS data on the isotope-coded peptide pairs will provide the relative abundance of reduced versus oxidized cysteines as light/heavy ratios. The percentage (or occupancy) of oxidation at a given Cys site can be calculated by the ratio of the oxidized signal to the total signal (sum of reduced and oxidized). This differential alkylation strategy has been measured in the redox state of Cys and occupancy of Cys oxidation in a number of redox proteomic approaches, such as OxICAT^{31, 32, 34, 70, 96–99} and α -switch methods^{67, 100, 101}. However, since there are no specific enrichment approaches available for NEM-alkylated peptides, the α -switch approach still falls short of the desired level of proteome coverage and sensitivity.¹⁰² Recently, a differential labeling strategy for quantifying protein SOHs was also proposed by using isotope-coded dimedone and iododimedone (ICDID), where sulfenic acids are trapped with d_6 -coded dimedone followed by alkylation of free cysteines with 2-iododimedone.¹⁰³ MS-based quantification is based on the relative abundance of heavy to light isotope-coded peptides, allowing the determination of the ratio of SOH to reduced thiols in proteins.

2.4.2 Isobaric labeling approaches—Among the stable isotope labeling approaches, isobaric labeling has become increasingly popular for quantitative proteomics due to its ability to enable multiplexing for simultaneous quantification of complex samples. Isobaric labeling reagents incorporate multiple versions of heavy isotopes in a reporter group while using the balance group to counter the variation in mass of the reporter ions. The isobaric equivalents remain indistinguishable in LC separations and MS1 scans but are fragmented into reporter ions with different masses in the tandem MS scan (MS/MS), thus enabling simultaneous quantification of peptides/PTMs from multiple biological samples in a single MS/MS run. The most commonly used isobaric labeling reagents include isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT). Several thiol-

reactive isobaric labeling reagents, such as cysTMT and iodoTMT, have been introduced as tag-switch reagents similar to the BST-based approaches. In general, iodoTMT is the preferred reagent due to its irreversible labeling nature, which offers better compatibility under reducing conditions than cysTMT reagents.¹⁰⁴ In this workflow, a set of isobaric TMT reagents (e.g., six-plex TMT) can be used to specifically label protein thiols reduced from oxidized cysteines of different samples. After enzymatic digestion, TMT-labeled peptides are selectively enriched by using anti-TMT resin.⁷⁴ Multiplexed quantification is achieved based on the isobaric reporter ion intensities of MS/MS spectra of TMT-labeled peptides. (Fig. 5a) Combined with differential labeling strategy, thiol-reactive TMT reagents allow stoichiometric measurements (or occupancy) of Cys PTMs.^{104, 105} In this case, two versions of TMT reagents are used to label the reduced cysteines and the oxidized cysteines (after reduction) in the same sample, respectively. In principle, multiple types of Cys PTMs can be simultaneously measured in parallel using different channels of TMT reagents.^{75, 10675}

Recently, we and others have developed a RAC approach combined with standard isobaric labeling reagents as a general strategy for facilitating sensitive identification and quantification of redox PTMs at a proteome-wide level (Fig. 5b).^{65, 66, 69, 73, 79, 80, 107} The RAC approach offers superior flexibility so that all steps of capture of thiol-containing proteins, protein digestion, and isobaric labeling are performed on-resin. It is worthy to note that the on-resin operation also facilitates multiplex labeling. By combining isotopic and isobaric labeling, highly multiplexed quantification can be achieved with RAC for redox proteomics.^{108–110} The covalent capture and immobilization of free thiol-containing protein/peptides using the RAC approach allows for more stringent washing to further reduce contaminants and non-specific binding. Isobaric labeling coupled with RAC allows for multiplexed quantification and stoichiometric measurement for multiple Cys redox PTMs, including SNO, SSG and total thiol oxidation^{66, 69, 73, 79, 80}.

2.4.3 Targeted quantification—While global quantitative redox proteomics serves as a discovery tool for novel Cys PTMs, MS-based targeted quantification has emerged as a promising method for more precise quantification of known target proteins and PTMs. With the addition of a known concentration of synthetic peptide serving as an internal standard, targeted approaches allow precise and accurate quantification of PTMs (Fig. 6). Targeted proteomics is typically carried out using selective reaction monitoring (SRM) (also called multiple reaction monitoring) on a triple quadrupole (QQQ) or parallel reaction monitoring (PRM) on a high resolution mass spectrometer (e.g., Q-Exactive).^{111, 112}

Recently, several groups have reported different strategies of targeted quantification applied to redox PTMs. For example, targeted approaches have been applied to direct label-free analysis of stable Cys modifications, such as Cys-SO₂H/SO₃H.^{20, 67, 113, 114} Due to the high sample complexity, selective enrichment of targeted proteins from a complex biological sample can significantly increase the sensitivity of SRM-based detection and quantification (Fig. 6).^{20, 67} Targeted quantification has also been applied for stoichiometric measurement of Cys oxidation by combining differential isotopic alkylation with NEM and deuterated NEM, which allows for analysis of low-abundance target proteins that are undetectable by global MS-based proteomic approaches.^{67, 115} In principle, targeted quantification strategies

can easily be coupled with most indirect redox proteomics approaches, such as BST and RAC, to achieve more precise quantification of the dynamics or occupancy of Cys PTMs.

3. Highlights of redox proteomics approaches in biological applications

In the following section, we briefly highlight several common redox proteomic approaches in the context of biological applications and their performance in terms of sensitivity or proteome coverage for characterizing the redox proteome in biological systems (Table 1).

3.1 Global redox proteomics

The OxICAT approach is one of the first redox proteomic methods described. It utilizes IAM-based ICAT reagents to specifically react with reduced and reversible oxidized thiols in a single sample using light and heavy versions of the ICAT reagents.³⁴ To date, the oxICAT approaches have been commonly used for global profiling the redox state of protein thiols in a number of model organisms, including *S. cerevisiae*,^{31, 96} *C. elegans*,^{70, 97} *E. coli*,³⁴ *D. melanogaster*⁹⁸ and marine diatoms.³² Collectively, these results have led to several important conclusions: (1) the majority of protein cysteines exist in the reduced state *in vivo* (~90%); (2) the occupancy of Cys oxidation is important for identification of redox-regulated Cys sites. While successful, the OxICAT method has several limitations. First, the botin-based enrichment often results in nonspecific binding of non-Cys peptides, limiting the overall proteome coverage (Table 1). Second, the paired differential labeling strategy only allows one sample to be analyzed by LC-MS/MS at a time. Missing data (i.e., inconsistency between separate sample analyses) is a significant issue when many samples require analysis due to the stochastic sample nature of tandem MS/MS.

To address the lack of ability for sample multiplexing using the oxICAT method, isobaric labeling strategies were introduced to enable 6-plex and 10-plex labeling by TMT reagents. The use of thiol-reactive cystTMT and iodoTMT labeling reagents in conjunction with anti-TMT resin enables specific enrichment and multiplexed quantification of TMT-labeled Cys-peptides. In an early study, Murray et al. reported the use of the TMT switch assay for measuring SNO-modified proteins in human pulmonary arterial endothelial cells *in vitro* and *in vivo* using the cystTMT6 method.⁷⁴ In S-nitrosoglutathione (GSNO)-treated cell lysates, 220 SNO-modified Cys sites on 179 proteins were successfully identified. Later, the cystTMT labeling approach was used for measuring SNO occupancy in the myocardium using a differential labeling strategy, revealing the protective role of SNO against damaging oxidation of cysteines during ischemia-reperfusion (IR) injury.¹⁰⁵ With the improved iodoTMT tagging, Pan et al. developed a TMT switch assay for sequential quantification of SNO and total reversibly oxidized cysteines, leading to identification of 266 redox sensitive sites in GSNO-treated H9c2 cardiomyocytes under hypoxia.¹⁰⁴ Using a similar approach, Behring et al. investigated the link between the Western diet and cardiac dysfunction by global measurements of oxidized cysteines in the left ventricle of mouse hearts.¹⁰⁶ The differential labeling approach with iodoTMT reagents allowed quantification of Cys occupancy on 173 proteins. The results showed a high-fat and high-sucrose (HFHS) diet can lead to significant alteration of the oxidation state of 112 Cys-containing peptides. Moreover, the iodoTMT-based approach can be applied to simultaneous analysis of SNO,

SOH and total reversibly oxidized cysteines in *E. coli* under low and mild oxidative stress conditions, leading to the quantification of 114 SOH and SNO modified peptides.⁷⁵ In many instances, SNO and SOH occupy the same Cys site, but the relative occupancies of these two modifications could be quite different.

Another major advancement in redox proteomics is the development of resin-assisted enrichment/capture approaches due to their increased specificity, sensitivity, and proteome coverage.^{65, 66} Global quantitative profiling of protein SNOs has been demonstrated using the RAC approach in combination with iTRAQ quantification in different types of cells induced with NO-donors, leading to identification of hundreds of redox-sensitive sites.^{65, 66, 73} Recently, global quantitative profiling of SSG was demonstrated using the RAC/TMT approach in mouse macrophages under nanoparticle-induced oxidative stress.⁸⁰ A total of 2494 unique SSG-modified Cys sites from 1276 proteins were identified and quantified across all treatment conditions. The results highlighted a broad set of redox sensitive proteins and specific Cys residues associated with cellular redox stress and impairment of macrophage phagocytic function. Similarly, Guo et al. adapted RAC/TMT for proteome-wide quantification of Cys redox status in cyanobacteria under light/dark modulation.⁶⁹ The changes of oxidation levels for approximately 2100 Cys sites on 1060 proteins under light and dark conditions were quantified. Moreover, the level of sensitivity achieved with this method, combined with stoichiometry data, facilitated prediction of potential functional Cys-sites for novel redox proteins.

Alternatively, Hg-resin-based enrichment approaches have emerged as a promising strategy for studying redox proteomics, especially for SNO, since phenylmercury-modified agarose resins can specifically capture SNOs by forming stable thiol-Hg bonds.⁵¹ However, initial blocking of free thiols prior to enrichment is still required due to the high reactivity of thiols to Hg moieties. The direct reaction with SNOs to form stable Hg-thiol bonds improves the specificity of overall detection compared to BST or RAC approaches, which rely on ascorbate reduction. In addition, the capture of SNO with Hg-resin can be performed under relatively acidic conditions, which are favorable for stabilizing SNOs during sample preparation.¹¹⁸ For MS-based analysis, the captured proteins can be digested on-resin, followed by elution with performic acid. Mercury-resin based approaches have enabled the detection of endogenous levels of protein SNOs in multiple types of tissues, leading to identification of >1000 SNO sites.^{51, 52, 116} Moreover, Hg-resin can be used in other indirect approaches to capture multiple forms of oxidized cysteines after selective reduction. Recently, the Hg-resin-based approach has been applied to simultaneous analysis of multiple types of redox PTMs in mouse liver, including SNO, SSG, SOH and S-acylation (SAc), which ultimately revealed endogenous redox signaling networks utilizing Cys PTMs.¹¹⁷

To date, specific detection of SOH was primarily based on the reaction between dimedone and SOH, which encouraged the development of dimedone-based probes for SOH detection in living cells.^{50, 93, 94, 119} Recently, Yang *et al.* developed isotope-coded alkyne-functionalized dimedones (DYn-2 and *d*₆-DYn-2) for site-specific mapping and quantification of protein SOHs in human RKO cells in response to exogenous H₂O₂ treatment and endogenous EGF stimulation.⁹³ Roughly 1,000 SOH-modified Cys sites from more than 700 proteins were identified and more than 300 sites were quantified. The results

demonstrated a potential for the method to enable systematic study of biologically relevant SOH events under oxidative stress,⁹³ however, large amounts of starting material are required (e.g., ~30 mg proteins per condition) due to the low abundance of SOH.

Finally, the significance of hydrogen sulfide (H₂S) and protein sulfhydrylation (SSH) has been increasingly recognized in redox regulation.^{120, 121} Recently, a Biotin-Thiol-Assay (BTA) has been developed for global profiling of SSH-modified proteins.⁶⁰ Low concentrations of maleimide-PEG2-biotin (NM-Biotin) were used to selectively label both free cysteines and sulfhydrylated cysteines in a biological sample, followed by avidin enrichment. Since NM-Biotin reacts with persulfide groups (-SSH) to form biotinylated disulfides, the SSH-modified proteins can be selectively eluted from avidin column by using DTT. Recently, the BTA assay has been successfully applied for the measurement of SSH-modified proteins in mouse pancreatic β cells in response to thapsigargin-induced endoplasmic reticulum (ER) stress, leading to quantification of ~820 SSH sites from more than 500 proteins.

3.2 Targeted redox proteomics

Besides global profiling, targeted quantification of redox PTMs is a promising direction for more precise measurements of specific redox PTMs from previously identified protein candidates or pathways. Held et al. first reported a MRM-based *d*-switch approach, termed OxMRM, for targeted quantification of both reversible and irreversible Cys oxidation in low-abundance endogenous proteins, such as p53 and protein tyrosine phosphatase-1B (PTP1B).⁶⁷ Isotope-coded NEMs, *d*₀-NEM and *d*₅-NEM, were used to sequentially alkylate free and oxidized cysteines in the sample. To acquire maximum sensitivity, antibody-based enrichment approaches were applied for enrichment of the targeted proteins, followed by on-resin digestion and LC-MS/MS analysis. In this work, the oxidation levels of 7 of 10 cysteines in p53 and 8 of 10 cysteines in PTP1B were quantified. The OxMRM approach also enabled direct quantification of irreversible Cys oxidations, such as SO₂H and SO₃H, because of their stability during sample preparation. The results showed the increased levels of SO₂H and SO₃H oxidations at Cys215 in PTP1B with Adriamycin-induced DNA damage. Later, the same group adapted the OxMRM method to determine the oxidation levels of protein cysteines in Mitochondrial Complex I in a Parkinson's disease mouse model.¹¹⁵ The OxMRM approach allowed quantification of the stoichiometry of reversible oxidation for 34 unique Cys residues out of a total 130 Cys residues within Complex I isolated from control *versus* glutathione-depleted mouse brains.

Another recently reported approach focused on targeted quantification of Cys oxidation in protein tyrosine phosphatases (PTPs), a class of protein important in cellular signaling.²⁰ Reversible oxidation of active Cys sites of PTPs is emerging as an important cellular regulatory mechanism associated with human diseases. In the work by Karisch et al., an antibody-based targeted quantitative approach for global measurement of Cys oxidation (q-oxPTPome) and expression level of PTPs (qPTPome) in different cell lines and tissues was reported.²⁰ A monoclonal antibody (oxPTP Ab) against the SO₃H-modified signature motif of PTPN1 (VHC_{SO₃H}SAG) was used to specifically capture "hyperoxidized" PTPs (SO₃H-modified PTPs) in proteomic samples. Both free and oxidized cysteines were converted to SO₃H-modified species for immune-purification and targeted quantification. The method

allowed identification and quantification of most classical PTPs (17 non-receptor PTPs, 19 out of 21 receptor PTPs) and their oxidation levels in various cell lines and tissues under exogenous and endogenous stimuli. A number of specific PTP substrates can be identified by combining qPTPome with phosphotyrosine (pY) proteomics.

4. Perspective and outlook

Quantitative proteomics approaches for characterizing Cys-based redox PTMs has emerged as an indispensable tool for redox biology. It enables the discovery of novel redox modified proteins, redox-regulated signaling pathways or networks, and the identification of functional Cys sites of specific proteins. These findings can provide great insight into the redox regulation and signaling mechanisms involved in both physiological and pathophysiological conditions. Despite significant advances over the last decade, the field of proteomics of Cys redox PTMs is still relatively immature, primarily due to factors associated with the complexity, lability, and the dynamic nature of redox PTMs and to the technical challenges that arise from their detection.

Ideally, detection of redox PTMs would be carried out by utilizing direct chemical tagging methods, enabling highly specific detection. However, due to the lack of direct tagging chemistry available for most forms of redox PTMs, the most successful quantitative redox proteomics approaches so far have been based on indirect methods coupled with isotope labeling (e.g., OxICAT, RAC/TMT). However, the major limitation of all the indirect strategies is the specificity for the detection of redox PTMs. In general, such indirect detection strategies are based on the selective reduction of a specific form of redox PTM (e.g., SNO) to a free thiol and subsequent enrichment and LC-MS/MS. However, the selective reduction of oxidized thiols typically results in some level of non-specific reaction/reduction of other forms of redox PTMs, such as protein disulfides. This limitation presents a significant challenge for the detection of low-abundant PTMs such as SNO and SOH, especially at physiological/endogenous levels. Indeed, most data obtained on SNO or SOH modifications under physiological conditions based on ascorbate or arsenite reduction and enrichment were not reliable.^{41, 76} Typically, NO donors or H₂O₂ treatment of cells to increase levels of SNO and SOH significantly above that of endogenous levels are required for discovery of modifications using these indirect strategies. Moreover, proper negative and positive controls are crucial for addressing such limitations. It is notable that the most successful indirect strategies employed to study redox proteomics have focused on the relative abundance of multiple abundant forms of redox PTMs, including total Cys oxidation and SSG using approaches such as OxICAT or RAC/TMT.^{32, 7080, 122} However, even with these relatively abundant modifications, a fairly large amount of starting materials is required for effective proteome profiling.¹²³ Therefore, further advances will be necessary to enhance the overall sensitivity and proteome coverage for profiling redox PTMs in cells and tissues. Multi-dimensional fractionation and high-resolution separation strategies could be useful in reducing sample complexity and enhancing the proteome coverage and sensitivity of detection.^{17, 113, 124}

Despite the advances made with regard to the detection of total oxidation and SSG-modifications using indirect strategies, it is still extremely challenging to globally profile

SOH and SNO, two PTMs that play important roles in redox signaling, especially under physiological conditions. To address these limitations, approaches built on the direct chemical reactivity of SNO and SOH moieties have shown some promising aspects. For example, the detection of endogenous levels of protein SNOs in multiple types of tissues has been demonstrated by the mercury-resin-based approach;^{51, 52, 116} however, the overall sensitivity of this approach is still far from being employed for use in broad applications since it often requires >10 mg of starting protein for each sample. Therefore, there is still a great need to develop novel chemo-selective SNO labeling reagents to facilitate specific labeling and for more effective enrichment and profiling of SNOs in complex samples.^{125, 126} Similarly, the development of dimedone-based probes is another example of a method which enables specific detection of SOH-modified proteins even at the proteome level.^{50, 93} As with the case for SNO, the overall sensitivity for detecting endogenous levels of SOH modifications is still quite low and a large amount of starting material (e.g., 30 mg) is necessary.⁹³ In addition to the low-abundance of SOH, the transient nature of the modification requires chemical probes that offer both high specificity and reactivity. The continued development of novel probes that offer higher reactivity may enable more effective detection of SOHs.^{127, 128}

Relative quantification of redox PTMs is very useful; however, it is extremely important to be able to measure the site-occupancy or stoichiometry of each form of Cys PTM.¹²⁹ The degree of modification on each Cys residue, along with its redox sensitivity data, is critical for predicting the functional importance of the redox modified Cys sites.^{34, 69} Therefore, occupancy analysis is critical for redox proteomics; it has been suggested that occupancy analysis should be performed routinely in all studies for redox proteomics. In occupancy analysis, the levels of total cysteines and of each form of oxidized cysteines need to be simultaneously measured in a given biological sample. However, irreversible oxidation, such as SO₂H and SO₃H, are typically excluded in such measurements since the contribution of irreversible oxidation is often minor in most conditions.¹⁷ In addition, occupancy analysis is not amenable to peptides containing multiple Cys residues due to the existence of multiple combinations of oxidized cysteines in a given sequence. Despite these limitations, a number of reported approaches have proved to be quite effective in measuring site occupancy for different forms of PTMs and for identifying functional Cys sites.^{34, 69, 129, 130} However, the complexity of redox PTMs still presents a significant challenge due to their large diversity under physiological conditions. Advances in redox proteomics have made it possible to simultaneously measure several forms of redox PTMs. For example, both SNO and total reversible oxidation were globally measured in the same experiment with RAC-based approaches^{66, 131} and tag-switch approaches^{101, 104}. Recently, simultaneous quantitative analysis of three distinct cysteine subsets, including SNO, SOH, and total reversible oxidation, have been reported.⁷⁵ It will be extremely interesting to decipher the redox proteome for each form of modified cysteine in terms of its dynamic changes and site occupancy under physiological or pathological conditions. Such integrative quantification of multiple forms of redox PTMs would shed significant light on the biological roles of these redox PTMs and on the complexity of redox signaling and regulation in biological systems. Another area of interest is the interplay between redox PTMs and other types of non-redox PTMs such as phosphorylation. There is increasing evidence that Cys oxidations are

involved in modulation of phosphorylation, acetylation, and ubiquitination. For example, SNO has been linked to the competitive inhibition of the phosphorylation of eNOS¹³² and to the global decrease of ubiquitination^{133, 134}. A recent proteomic study also suggested that redox-sensitive cysteines are important players in regulation of PTPs and phosphorylation signaling.²⁰ Therefore, large-scale integrative analysis of redox PTMs and other PTMs would help us understand the complexity of intracellular signaling and regulation in both healthy and disease states.

We envision that future advancements in the field of redox proteomics will continue to enhance the overall sensitivity, specificity, and coverage of the redox proteomes of protein cysteines. Quantitative analysis of redox PTMs with significant coverage (i.e., identifying >10,000 Cys sites), similar to those achieved with phosphoproteome, may soon become a reality.¹³⁵ The development of novel approaches to enable broad quantification of both relative changes and site occupancy of multiple types of redox PTMs will significantly enhance our understanding of the role of protein thiols in redox signaling and regulation. The simultaneous measurement of multiple redox PTMs and other PTMs, such as phosphorylation or acetylation by either global or targeted quantification methods, may reveal significant insight into cell signaling through “cross-talk” between different types of PTMs.

Acknowledgments

Portions of the work were supported by NIH Grants UC4DK104167, P41GM103493, and U01ES027292. The experimental work described herein was performed in the Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, a national scientific user facility sponsored by the DOE under Contract DE-AC05-76RL0 1830.

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Fig. 1.
Redox-dependent modifications on protein cysteines.

Fig. 2.
 Direct approaches for reversible Cys PTMs. (a) Dimedone-based chemical probes for SOH. (b) Organomercury and phosphine-based probes for SNO. (c) Tag-switch method and Biotin-Thiol-Assay for SSH.

Fig. 3.
Indirect strategies for reversible Cys modifications.

a. Relative quantification **b. Differential alkylation approaches**

Fig. 4. Stable isotope labeling approaches for redox PTMs. (a) Relative quantification; (b) Stiochiometric measurement for redox PTMs with differential alkylation approaches.

a. TMT-based tag-switch approaches for relative quantification

b. RAC approaches for relative quantification

Fig. 5.
Isobaric labeling approaches for Cys PTMs.

Fig. 6.
Targeted quantification of Cys PTMs.

Table 1

A summary of selected redox proteomic approaches in the context of biological applications.

Strategy	Approach	Enrichment	Quantification	PTMs	Performance Matrices	Refs
Global	Dimedone probes	Biotin-avidin affinity	Isotopic labeling, MS1	SOH	BM ^a : human cells with either exogenous H ₂ O ₂ or growth factor stimulation, <i>in situ</i> labeling SM ^b : ~30 mg protein/condition PC ^c : quantified > 1000 Cys sites from 700 proteins;	93
					BM: <i>S. cerevisiae</i> and <i>C. elegans</i> during different lifespans SM: ~200 µg protein/condition PC: < 300 Cys sites	70, 96
	OxICAT	Biotin-avidin affinity	Differential alkylation, MS1	Total oxidation	BM: <i>D. melanogaster</i> during aging and fasting SM: ~30–40 µg protein/condition PC: identified ~1191 Cys sites from 424 proteins; quantified 537 peptides from 214 proteins	98
					BM: marine diatoms treated with 150 µM H ₂ O ₂ SM: 100–200 µg protein/condition PC: identified 3845 Cys sites from 1744 proteins; ~300 redox-sensitive proteins using 2D-LC-MS/MS	32
					BM: HEK293 cells treated with 0.5 mM CysNO SM: ~1 mg protein/condition PC: quantified ~400 peptides	65
	RAC with isobaric labeling	Disulfide exchange	Isobaric labeling, MS2	SSG	BM: mouse macrophages with nanoparticle-induced immune dysfunction SM: ~500 µg protein/condition PC: quantified 2494 Cys sites from 1276 proteins	80
					BM: cyanobacteria under light/dark modulation SM: ~500 µg protein/condition PC: quantified ~2100 Cys sites from 1060 proteins	69
	TMT-tag switch	Anti-TMT resin	Isobaric labeling, MS2	Total oxidation	BM: mouse hearts with ischemia-reperfusion injury SM: ~1 mg homogenate/condition PC: quantified 275 peptides	105
					BM: Rat cardiomyocyte Under Hypoxia SM: ~300 µg protein/condition PC: quantified 266 Cys sites from 169 proteins	104
					BM: hearts from mice with diastolic dysfunction SM: ~100 µg protein/condition PC: quantified 270 Cys sites from 173 proteins	106
Hg-resin-based approaches	Hg-thiol affinity	Label free	SNO	SNO, SOH, total oxidation	BM: <i>E. Coli</i> treated with 100 µM H ₂ O ₂ SM: ~100 µg protein/condition PC: quantified 114 redox-modified peptides	75
					BM: mouse liver under physiological condition	51

Strategy	Approach	Enrichment	Quantification	PTMs	Performance Matrices	Refs
Targeted	BTA assay	Biotin-avidin affinity	Isotopic labeling, MSI	SSH	SM: up to 30 mg protein/condition PC: identified 328 peptides from 192 proteins	52
					BM: wild-type mouse brain, heart, kidney, liver, lung and thymus SM: up to 30 mg protein/condition PC: identified 1011 Cys sites from 647 proteins	
					BM: wild-type mouse brain SM: up to 30 mg protein/condition PC: identified 269 Cys sites in 136 proteins	
	OxMIRM	Antibody-based enrichment	Differential alkylation with Isotope-coded NEMs, SRM	Total oxidation, SO ₂ H/SO ₃ H	BM: wild-type mouse liver SM: up to 30mg protein/condition PC: identified 2596 Cys sites from 1319 proteins	117
					BM: mouse pancreatic β cells with drug-induced endoplasmic reticulum stress SM: ~4 mg cell lysates/condition PC: identified >1000 Cys sites from 820 proteins; quantified 820 peptides from > 500 proteins	
Targeted	q-oxPTPome	Antibody-based enrichment	Differential oxidation, SRM	Total oxidation, SOH, SO ₂ H, SO ₃ H	BM: cysteine oxidation status of endogenous p53 and PTP1B in cancer cell lines SM: ~10 ⁷ cells in 15-cm culture plates Targets: 15 Cys sites	67
					BM: classical protein-tyrosine phosphatome in cells and tissues SM: not described Targets: 49 Cys sites from 36 PTPs	

^aBM: Biological model;
^bSM: Starting material;
^cPC: Proteome coverage.