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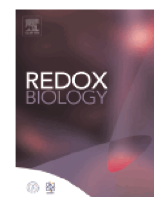

Differential alkylation-based redox proteomics – Lessons learnt

Katarzyna Wojdyla [1], Adelina Rogowska-Wrzesinska *

Protein Research Group, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

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ABSTRACT

Cysteine is one of the most reactive amino acids. This is due to the electronegativity of sulphur atom in the side chain of thiolate group. It results in cysteine being present in several distinct redox forms inside the cell. Amongst these, reversible oxidations, S-nitrosylation and S-sulfenylation are crucial mediators of intracellular redox signalling, with known associations to health and disease. Study of their functionalities has intensified thanks to the development of various analytical strategies, with particular contribution from differential alkylation-based proteomics methods. Presented here is a critical evaluation of differential alkylation-based strategies for the analysis of S-nitrosylation and S-sulfenylation. The aim is to assess the current status and to provide insights for future directions in the dynamically evolving field of redox proteomics. To achieve that we collected 35 original research articles published since 2010 and analysed them considering the following parameters, (i) resolution of modification site, (ii) quantitative information, including correction of modification levels by protein abundance changes and determination of modification site occupancy, (iii) throughput, including the amount of starting material required for analysis. The results of this meta-analysis are the core of this review, complemented by issues related to biological models and sample preparation in redox proteomics, including conditions for free thiol blocking and labelling of target cysteine oxoforms.

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* Corresponding author.
E-mail address: adelinar@bmb.sdu.dk (A. Rogowska-Wrzesinska).
[1] Present address: Cancer Institute, University College London, Paul O'Gorman Building, 72 Huntley Street, WC1E 6DD London, UK.

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1. Introduction

Cysteine is one of the least abundant amino acids in prokaryotic and eukaryotic organisms and it is also amongst the most oxidation-prone one [1]. Due to the strong electronegativity of the sulphur atom present in the side chain thiolate group cysteine can exist in many different redox forms inside the cell [1]. The majority of cysteine oxoforms are reversible and can be reduced to thiol (SH). These include disulphide bonds (S–S), S-glutathionylation (S-SG), S-nitrosylation (SNO) and S-sulfenylation (SOH). Sulfinic (SO_2H) and sulphonic acid (SO_3H) are the two oxoforms that are chemically irreversible. This is with the exception of eukaryotic 2-Cys peroxiredoxins, where SO_2H may be reduced by sulfiredoxin, in an ATP and Mg^{2+}-dependant reaction [2–4].

Reactivity of the thiolate extends the range of cysteine's modifications beyond redox chemistries, e.g. acylation by enzymatic attachment of lipids [5]. The complete spectrum of cysteine post-translational modifications (PTMs) was recently reviewed [6]. The focus of this review is on redox-based reversible oxidations, SNO and SOH in particular.

S-nitrosylation is the covalent addition of a nitroso group (NO) into cysteine's thiolate, directly by reactive nitrogen species (RNS), e.g. nitrosonium cation (NO^+) or peroxynitrite ($ONOO^-$) or indirectly by reactive oxygen species (ROS) via induction of NO production [7,8]. It may also result from trans-S-nitrosylation, transfer of a NO group from S-nitrosylated small molecules, e.g. S-nitrosoglutathione (GSNO) or S-nitrosylated proteins onto another protein-bound thiolate [7]. Although non-enzymatic, SNO is selective and transient modification which makes it an excellent signal transducer [9]. SNO of catalytic cysteines may be involved in regulation of enzyme's activity, e.g. inhibition of aldehyde dehydrogenase upon S-nitrosylation [10,11]. Additionally, SNO may also act as an antioxidant buffer by preventing formation of irreversible oxidations, as described in the heart during oxidative stress caused by ischaemia–reperfusion [9].

S-sulfenylation is the oxidation of thiolate to sulfenic acid which in vivo occurs mainly via reaction with hydrogen peroxide (H_2O_2) [12]. SOH is a non-enzymatic modification which may also result from conversion of other cysteine oxoforms, including SNO [7]. SOH is sub-stoichiometric and transient oxoform which, in an oxidising environment progresses to SO_2H and SO_3H. Therefore SOH has long been regarded as a marker of oxidative damage [13]. However, growing evidence suggests that SOH is also an important regulatory PTM. For instance, SOH of the active site cysteine in protein tyrosine phosphatases (PTP1B) reversibly inhibits their catalytic activity [14].

Both SNO and SOH are well recognised for their role in regulation of protein activity and protein–protein interaction thereby actively modulating intracellular signalling, as reviewed in Ref. [15]. Redox sensitive transcription factors exist in prokaryotes and eukaryotes and oxidative cysteine modifications are direct effectors of their functions [16,17]. Their role in maintenance of intracellular redox homoeostasis is equally important. Both SNO and SOH contribute to antioxidant capacity, in a similar manner to low-molecular weight thiols e.g. glutathione (GSH) [1]. There is also a growing body of evidence indicating their involvement in pathologies and disease conditions, as reviewed in Ref. [15,18].

All of the above make SNO and SOH key targets for basic and applied research. This was recognised over a decade ago and resulted in a dynamic development of analytical tools and their application to redox proteomes (redoxomes). The term redoxome was coined by Chiappetta et al. in 2010 [19]. It is defined as complete set of cysteine oxoforms within proteins of a given proteome. Study of the redoxome may be approached in multiple ways depending on its complexity and the required depth of analysis. Previously, global analysis was common. For instance, Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) reacts with SH groups resulting in a coloured product which may be measured spectrophotometrically at 412 nm. Using the extinction coefficient for TNB^{2-}, or a standard curve of cysteine, it is possible to calculate the amount of thiol groups present in a sample/protein [20]. Additionally, there exist modification specific methods, e.g. UV photolysis to assess global SNO levels [21]. In both cases the oxidative state of individual proteins and modification sites are unknown. The limited sensitivity of global methods prevents distinction of subtle changes caused by typical physiological levels of oxidative stress.

The above limitations are addressed by proteomics-based methods. Over the years numerous strategies were developed for both gel-based and gel-free analysis of redox proteomes. With the later generally more suited to resolve cysteine oxoforms [22]. The majority of those methods have been described in detail in several excellent reviews [1,6,23].

In recent years, the development of redox proteomics has been driven by application of a method/concept commonly referred to as differential alkylation. It is a tool to analyse reversible modifications of cysteine using specific reductants and alkylating reagents. Differential alkylation was introduced in 2001 as a biotin switch for analysis of SNO and has been evolving since [24]. In time, it was adapted to other cysteine oxoforms e.g. SOH [25], S-SG [26] and all chemically reducible cysteine modifications [27]. Regardless of the target oxoform and complexity, the principles remain unchanged. The initial step is blocking of SH groups. After removal of excess blocking/alkylating reagent, modification-specific reductants are added followed by blocking/alkylation of nascent thiols with a distinct blocking/alkylating reagent. Nascent thiols refer to the SH groups generated upon modification-specific reduction. As above, any excess reducing/alkylating reagent is removed prior to subsequent steps. Finally, the remainder of reversible cysteine modifications are reduced with a non-selective reductant and alkylated for stability. This final alkylation step is only applicable if the modification specific blocking/alkylation is irreversible. When reversible labels (e.g. those utilising a disulphide bond) are used, this step should be omitted as it prevents unambiguous assignment of modification site. The final step is also omitted where non-selective reducing agents e.g. dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) are used directly after free thiol blocking. Fig. 1 summarises the concept and main steps of differential alkylation-based redox proteomics experiments.

This basic workflow has been modified over the years to increase depth of analysis. Nowadays, differential alkylation combined with tandem mass spectrometry is able to characterise entire redox proteomes. This includes analysis of various cysteine oxoforms simultaneously, with site-specific resolution [28], estimation of relative abundances [29] and modification site occupancies [30].

The aim of this review is a critical evaluation of experimental setups, including both biological models and methods used for

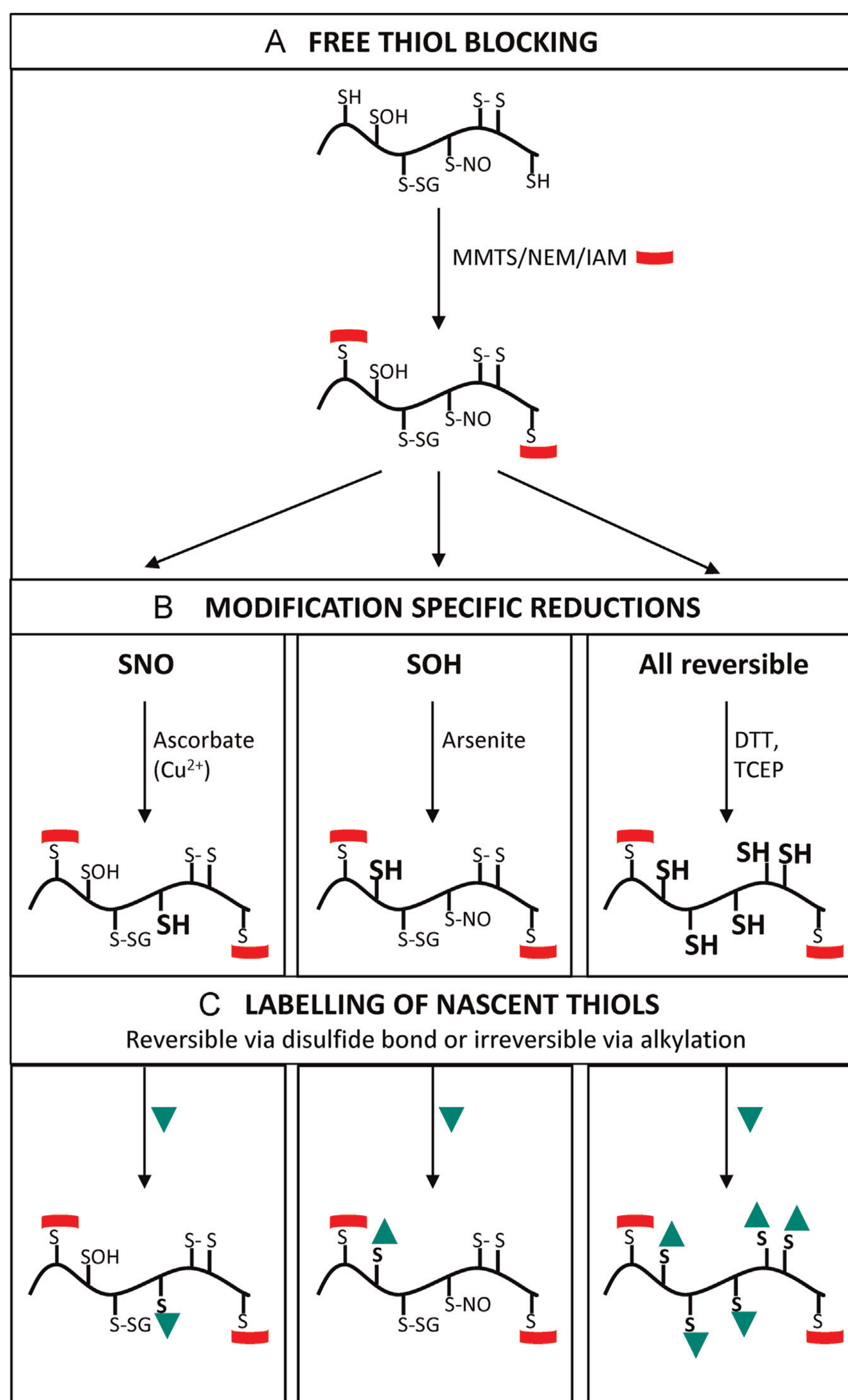

Fig. 1. Schematic summary of the principles of differential alkylation for analysis of reversible cysteine oxidations. Proteins are typically dissolved in denaturing buffers supplemented with metal ion chelators such as EDTA. A. Initially, reduced cysteine thiols (SH) are blocked for stability. Depending on the type of reagent (indicated by), blocking might be chemically reversible/reducible (MMTS) or irreversible (NEM, IAM). B. Subsequently, target cysteine oxoforms are reduced to SH groups. Ascorbate alone and in tandem with Cu^{2+} selectively reduces S-nitrosothiols. Arsenite selectively reduces S-sulfenylations. Strong reducing reagents like DTT and TCEP reduce all reversible modifications, which includes the oxoforms depicted here as well as non-redox modifications, e.g. acylation. C. Finally, nascent thiols are labelled for stability in reversible manner (via disulphide bond) or irreversible manner (via alkylation). Labelling reagents are often designed to provide quantitative information about the modification site. ▼ indicates: EDTA – ethylenediaminetetraacetic acid; MMTS – methyl methanethiosulfonate; NEM – N-ethylmaleimide; IAM – iodoacetamide; DTT – dithiothreitol; TCEP – tris(2-carboxyethyl)phosphine).

differential alkylation-based proteomic analysis of reversible cysteine oxidations, SNO and SOH in particular. We provide an overview of how differential alkylation-based methods have evolved over the years and the impact they have made on the depth and precision of SNO/SOH analysis. To facilitate that, we collected original research publications investigating SNO and SOH published since 2010. Those were analysed considering the following features: (i) assignment of modification type and site, (ii) quantitation of modification abundance, including correction of modification levels by protein abundance changes and modification site occupancy (iii) throughput and amount of starting material required. The results of this meta-analysis are the core of this review. We discuss future directions in the dynamically evolving field of redox proteomics.

Table 1
Selected proteomics/mass spectrometry-based studies (2010-to date) investigating various types of reversible cysteine modifications.

Reference	Cysteine modification	Method	Metal ion chelators (in lysis buffer)	Free thiol blocking/alkylating reagent	Reductant	Labelling type	Inherent quantitation	Unambiguous modification site assignment	Correction of modification levels by protein abundance changes	Model system	Number of modified proteins/peptides/sites	Amount of starting material/condition [mg]
[29]	SNO	iodoTMT™	1 mM EDTA, 0.1 mM neocuproine	MMTS	5 mM ascorbate	Indirect, irreversible	TMT™	Yes	No	CysNO-treated BV-2 cells; LPS-stimulated BV-2 cells; LPS + SAC stimulated BV-2 cells	134 Sites 101 sites 115 sites	0.4
[52]	SNO	cysTMT™	1 mM EDTA, 0.1 mM neocuproine	NEM	1 mM ascorbate + 1 mM $CuSO_4$	Indirect, reversible	TMT™	™No	No	GSNO-treated HPAEC cells; CysNo-treated HPAEC cells	220 Sites; 11-25 sites	0.2; N/a
[34]	SNO	Phenylmercury resin	1 mM DTPA, 0.1 mM neocuproine	MMTS	–	Direct, reversible	N/a	No	+/−	GSNO-treated mouse liver homogenates	150 Peptides	3
[64]	SNO	cysTMT™	1 mM EDTA, 0.1 mM neocuproine	cysTMT	20 mM ascorbate	Indirect, reversible	TMT™	No	+/−	Mouse hearts after ischaemic insult	275 Peptides after ischaemic insult	1
[35]	SNO	Thiopropyl sepharose	1 mM EDTA, 0.1 mM neocuproine	NEM	5 mM ascorbate + 5 μM CuCl	Indirect, reversible	iTRAQ	+/−	No	GSNO-treated mouse skeletal muscle homogenates	488 Sites	0.5
[82]	SNO	Thiopropyl sepharose	1 mM EDTA, and 0.1 mM neocuproine	NEM	20 mM ascorbate	Indirect, reversible	Label-free using in-house software	+/−	No	GSNO-treated, perfused mouse heart homogenates	> 2000 Sites	1
[45]	SNO	Organomercury resin	1 mM DTPA, 0.1 mM neocuproine	MMTS	–	Direct, reversible	N/a	No	No	Non-stimulated mouse liver homogenates	328 Peptides	3 and 30
[92]	SNO	ICAT	1 mM EDTA, 0.1 mM neocuproine	MMTS	10 mM ascorbate	Indirect, irreversible	Light and heavy ICAT	Yes	No	SNO-Trx treated SH-SY5Y cell lysates	N/a	0.3
[93]	SNO	PEO-iodoacetyl-biotin	1 mM EDTA, 0.1 mM neocuproine	IAM	5 mM ascorbate	Indirect, irreversible	N/a	Yes	No	SNAP/L-cysteine-treated MS-1 cells	586 Sites	1.5
[94]	SNO	Thioredoxin trap mutant	1 mM EDTA, 0.1 mM DTPA;	NEM	–	Direct, reversible	N/a	No	No	CysNO-treated THP1 cells; LPS/IFN-γ stimulated RAW264.7 cells	~400 Proteins; ~200 Proteins	3
[95]	SNO	ICAT	1 mM EDTA, 0.1 mM neocuproine	MMTS	10 mM ascorbate	Indirect, irreversible	Light and heavy ICAT	Yes	No	SNO-Trx 1-treated SH-SY5Y cell lysate	50–76 Sites	0.3
[96]	SNO	HPDP-biotin	1 mM EDTA, 0.1 mM neocuproine	MMTS	50 mM ascorbate	Indirect, reversible	N/a	No	No	GSNO-treated recombinant human proteins on chip	834 Proteins	N/a
[53]	SNO	HPDP-biotin	100 μM neocuproine	NEM	1 mM ascorbate + 10 μM $CuSO_4$	Indirect, reversible	N/a	+/−	No	mouse with spared nerve injury	161 Peptides	0.01
[46]	SNO	Organomercury resin	1 mM DTPA, 0.1 mM neocuproine	MMTS	–	Direct, reversible	N/a	No	No	6 Different mouse tissues	1011 Sites	N/a
[28]	SNO	ICAT	5 mM EDTA, 0.5 mM neocuproine	MMTS	5 mM ascorbate + 1 mM $CuCl_2$	Indirect, irreversible	Light and heavy version of ICAT	Yes	No	NaCl-treated *Arabidopsis* cells	123 Peptides	2
[97]	SNO	HPDP-biotin	1 mM EDTA, 0.1 mM neocuproine	MMTS	10 mM ascorbate	Indirect, reversible	N/a	+/−	No	CysNO-treated NPrEC cells	82 Sites	1
[98]	SNO	HPDP-biotin	1 mM EDTA, 0.1 mM	NEM	20 mM ascorbate	Indirect, reversible	SILAC	+/−	No	LPS and IFN- γ-treated RAW 264.7 cells	156 Proteins	1

Table 1 (*continued*)

Reference	Cysteine modification	Method	Metal ion chelators (in lysis buffer)	Free thiol blocking/alkylating reagent	Reductant	Labelling type	Inherent quantitation	Unambiguous modification site assignment	Correction of modification levels by protein abundance changes	Model system	Number of modified proteins/peptides/sites	Amount of starting material/condition [mg]
			neocuproine									
[54]	SNO	cysTMT™	1 mM EDTA, 0.1 mM neocuproine	NEM	1 mM ascorbate + 1 mM $CuSO_4$	Indirect, reversible	TMT™	No	No	NO donor-treated cardiac mitochondria	N/a	0.2–0.3
[99]	SNO	ICAT	5 mM EDTA, 0.5 mM neocuproine	MMTS	5 mM ascorbate	Indirect, irreversible	Light and heavy version of ICAT	Yes	No	Control *Arabidopsis* cells and NaCl treated	53 Sites 5 sites	2
[77]	SNO	d5-NEM	1 mM EDTA, 0.1 mM neocuproine	NEM	5 mM ascorbate + 1 μM CuCl	Indirect, irreversible	Isotopomers of NEM	Yes	No	CysNO-treated SH-SY5Y cells	8 Peptides	N/a
[57]	SNO	Biotin maleimide	–	NEM	30 mM sinapinic acid	Indirect, irreversible	N/a	No	No	CysNO and LPS-treated RAW 264.7 cells	N/a	N/a
[87]	SNO + S-SG	Gold nanoparticles	–	IAM	–	Direct, reversible	N/a	+/−	No	GSNO-treated PDI and DUSP12 recombinant proteins	3 SNO and 5 S-SG peptides respectively	N/a
[65]	SNO– > -AR[a]	IodoTMT™	1 mM EDTA, 0.1 mM neocuproine	IAM	20 mM ascorbate– > 5 mM TCEP	Indirect, irreversible	TMT™	Yes	No	GSNO-treated H9c2 cells under hypoxia	266 sites	0.3
[79]	SNO; AR	Thiopropyl sepharose	10 mM EDTA, 0.1 mM neocuproine; TCA	NEM	5 mM ascorbate + 5 μM CuCl; 10 mM DTT	Indirect, reversible	iTRAQ	No	No	GSNO-treated mouse muscle; RAW 264.7cells	488 SNO sites	0.5; 0.1
[82]	SNO + AR	Thiopropyl sepharose	1 mM EDTA, 0.1 mM neocuproine	NEM	20 mM ascorbate; 10 mM DTT	Indirect, reversible	N/a	No	No	Mouse hearts subjected to various perfusion/ischaemia protocols	47 SNO sites	1
[78]	SNO + AR	NEM	1 mM EDTA, 0.1 mM neocuproine	NEM	5 mM ascorbate + 1 μM CuCl; 50 mM TCEP	Indirect, irreversible	Light and heavy version of NEM	Yes	No	CysNO-treated SH-SY5Y	Targeted analysis of specific proteins, 11 sites	N/a
[27]	S-S/AR	ICAT	10% TCA	IAM	10 mM TCEP	Indirect, irreversible	Light and heavy cleavable ICAT	Yes	Yes	H_2O_2 treated *Schizosaccharomyces pombe*	1195 Peptides	2.1
[85]	AR	GELSILOX	1 mM EDTA	NEM	10 mM DTT	Indirect, irreversible	O^{18}	+/−	Yes	Proof of principle: diamine-treated EA. Hy296 cells	254 Sites	0.5
[33]	AR	ICAT	10% TCA	IAM	20 mM TCEP	Indirect, irreversible	Light and heavy ICAT	Yes	Yes	H_2O_2-treated *wt* yeast/ Trx1 and Trr1 mutant yeast	~500 Peptides	2
[44]	AR	Thiopropyl sepharose	2 mM DTPA	NEM	10 mM DTT	Indirect, reversible	Label-free	No	No	Perfused rat heart	6559 peptides	N/a
[58]	SOH	Biotin maleimide	1 mM EDTA, 0.1 mM neocuproine	Maleimide	20 mM arsenite	Indirect, irreversible	N/a	No	No	Kidney medula of spontaneously hypertensive rats	32 Proteins	5
[43]	SOH	1,3- Cyclohexadione derivatives	100 μM DTPA	NEM, IAA	–	Direct, irreversible	N/a	+/−	No	Example labelling of purified proteins and cells	N/a	N/a
[32]	SOH	DAz-2	–	–	–	Direct, irreversible	Light and heavy DAz-2	Yes	No	H_2O_2-treated C64S C82S Gpx3	1 Site	N/a
[69]	SOH	Dimedone	–	Iododimedone	–	Direct, irreversible	Light and heavy dimedone/ iododimedone	Yes	No	H_2O_2-treated C64S C82S Gpx3 and GAPDH	1 Site/protein	N/a
[100]	SOH	Yap1-cCRD	20% TCA	IAA	–	Direct, reversible	N/a	No	No	H_2O_2-treated *Saccharomyces cerevisiae*	42 Proteins	N/a

2. Model systems for studying reversible cysteine oxidations

The choice of model system in redox proteomics is very important, because they can vary dramatically in the way they resemble physiological redox homoeostasis. Therefore both the choice of biological system and oxidative stimulus are considered in the following section.

Our meta-analysis had shown that since 2010, there were 12 studies on mammalian tissues/organs, 17 studies on cell lines, 3 studies in yeast, and 5 on purified proteins (Table 1). Importantly, only 13 of the 35 studies collected measured endogenous levels of SNO/SOH. Endogenous refers to physiological (basal) or pathological (stimuli-induced) levels of oxidation that do not involve direct treatment with ROS/RNS donors. Use of ROS/RNS donors in vitro is common practise in these studies. GSNO and S-nitrosocysteine (CysNO) are typically used to induce SNO. Hydrogen peroxide (H_2O_2) is commonly used to generate SOH (Table 1). Although these donors exist in vivo their concentrations used for SNO/SOH induction in vitro are higher than physiological. To give a specific example, intracellular levels of H_2O_2 vary between 1 and 700 nM in aerobic organisms [31]. The studies we have collected use between 3.5 and 300 times this concentration [27,32,33]. Such high concentrations seem excessive considering the excellent membrane permeability of H_2O_2 [31]. This is especially true, for studies that aim to measure cysteine oxidation in vivo [33].

The routine use of harsh oxidising conditions may be justified when the sensitivity of detection is insufficient and in cases where ROS/RNS donors are of limited membrane permeability [15]. However, the physiological relevance of such conditions should also be considered.

Membrane impermeability to common oxidants may be circumvented by direct oxidation of protein lysates such as was the case for 7 of the studies (20%) collected in Table 1, e.g. in Ref. [34,35]. However, these models are a poor representation of intracellular redox homoeostasis for several reasons. Firstly, cell lysis abolishes organelle redox microenvironments, altering the redox status of compartment specific cysteine oxoforms and introducing artefactual modifications. Moreover, small molecule antioxidants such as GSH may be lost during protein precipitation in acid e.g. TCA which may affect cysteine's susceptibility to oxidation [36]. Denaturing conditions disregard the role of protein structure and protein–protein interactions in the susceptibility to oxidation. They also destabilise labile oxoforms e.g. SOH, creating artefacts as reviewed in Ref. [37]. All the above makes such in vitro studies inadequate for analysis of even the most severe physiological insult, however they can be successfully used as models in mechanistic studies e.g. when new oxidants are evaluated.

3. Sample preparation for redox proteomics

Due to the high reactivity and instability of cysteine oxoforms as well as their sub-stoichiometric abundances the conditions used for protein extraction prior to redox proteomics are extremely important. Therefore, the following sections are dedicated to general and modification-specific issues related to sample preparation.

Cells are heterogeneous redox entities with compartment-specific redox activities. For example, the mitochondrial electron transport chain (ETC) produces large quantities of ROS which do not appear to the same extent in the cytoplasm [38]. Such compartmentalisation is crucial for maintaining redox homoeostasis and allows the cell to deal with redox stressors at the source. For instance, rotenone (a membrane permeable pesticide) inhibits electron flow from Complex I of ETC which increases ROS/RNS production and oxidative stress in mitochondria. However, as long as the redox capacity of mitochondria is able to withstand this toxic insult other cellular compartments remain unaffected [39].

3.1. Cell lysis and protein extraction

The initial step of sample preparation disrupts cellular compartmentalisation and creates a homogenous mixture of biomolecules. Considering the rotenone example, cell lysis would mix the more highly oxidising environment of mitochondria with compartments in steady-state e.g. nucleus and cytoplasm. ROS/RNS from mitochondria increase levels of reactive species in the lysate leading to artificial changes in redox status of other cellular cysteines. Although it is not possible to remove these artefacts completely, they may be minimised by appropriate sample handling.

There are several points of general importance. Oxygen (O_2), its reactive species and transition metal ions together are a potent source of hydroxyl radicals ($HO^\bullet$) generated via the Fenton reaction [40]. Therefore it is highly recommended that buffers and working solutions are depleted of O_2 and/or contain metal ion chelators e.g. ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA) in sufficient concentrations [41]. Neocuproine is a Cu^+ chelator, used to prevent Cu^+-catalysed displacement of NO groups from SNO proteins [42]. However, neocuproine should be avoided when Cu^+/Cu^{2+} is used in tandem with ascorbate for selective reduction of SNO (discussed further below). According to our meta-analysis, the most common metal ion chelators used are EDTA and neocuproine which were used concomitantly in 20 studies (57%) (Table 1). DTPA was also used, either alone [43,44], in combination with neocuproine [34,45,46] or EDTA [47].

Due to the reactivity and transient nature of cysteine oxoforms, sample processing time should be minimised. Processing time refers to the period from sample stimulation until labelling of target oxoforms. Continuous analysis with no intermediate storage is preferred for maximal sensitivity. To give specific example, we observed that shortening protein precipitation time from overnight to 1 h improved SNO signal intensity considerably (data not shown). This is also in line with Burgoyne and Eaton who recommend replacement of lengthy and loss-prone precipitations with size exclusion chromatography for desalting [15]. Finally, the majority of cysteine oxoforms, SNO in particular, are light sensitive; therefore all steps from cell disruption until labelling of nascent thiols should be performed under minimal light exposure [21].

3.2. Blocking of free thiols

The following paragraphs are dedicated to technical aspects of sample preparation prior to differential alkylation. This includes timely and effective blocking of SH groups in order to preserve in vivo redox status and minimise artefactual modifications. This is typically achieved by acid precipitation or by chemical blocking/alkylation [36]. Each method has certain advantages and limitations as extensively discussed by Hansen and Winther [36]. A combination of the two, might be the most effective, especially in analysis of S-nitrosoproteome as SNO groups remain reactive even under acidic conditions [36].

Typically, samples are lysed in a denaturing buffer, e.g. HENS buffer (HEPES, EDTA, neocuproine, SDS) containing a blocking (S-methyl methanethiosulfonate, MMTS) or alkylating reagent (N-ethylmaleimide, NEM; iodoacetamide, IAM) [36]. Our meta-analysis revealed that NEM is the most frequently used for blocking of SH groups (14 studies), followed by MMTS (10 studies) and IAM (6 studies). Each of those reagents poses certain advantages and limitations and there is no consensus as to which of them to use

[36,48]. For instance, NEM is a cell permeable alkylating reagent which irreversibly binds to GSH thereby altering cellular redox equilibrium [36]. Importantly, despite the controversy, no systematic study was conducted to offer direct comparison of MMTS, NEM and IAM in terms of effectiveness, pH dependence and selectivity. Perhaps, such systematic study would put an end to the long standing dispute over SH blocking. Regardless, SH blocking/alkylation is critical as any failure or incompleteness at this step artificially increases target modification levels leading to false positive results. Therefore it should be thoroughly evaluated by western blot or MS-based analysis prior to continuation of an experiment, for every type of sample and experimental condition (discussed below). Finally, it is crucial to remove any excess blocking/alkylating reagent prior to subsequent steps. This may be achieved by protein precipitation or size exclusion chromatography as reviewed in [36].

4. Application of gel-based strategies in redox proteomics

There exist numerous gel-based strategies for cysteine oxidation analysis, the majority of which are based on differential alkylation [1]. Some make use of fluorescently conjugated alkylating reagents [49,50] others antibody-based detection by western blot, as reviewed in Ref. [15]. However, in redox proteomics similar to expression proteomics, gel-free strategies offer improvements in terms of sensitivity, resolution, quantitation and throughput [1]. We direct the interested reader to reviews where the advantages and limitations of gel-based approaches to redox proteomics are described in detail [1,51].

Despite serious limitations, we believe that gel-based strategies still have a niche in redox proteomics. Paradoxically, one of their advantages may be their global character which makes them excellent tools for quality control e.g. to evaluate efficiency of SH blocking. Additionally, they may be used for assessment of global modification levels between experimental conditions, e.g. SNO levels between control and oxidative stimuli [30]. Such evaluation saves often expensive reagents and mass spectrometry (MS) analysis time until all experimental steps and biological models are fully optimised.

5. Modification specific reduction and labelling of nascent thiols

In a typical differential alkylation protocol, after initial SH blocking target cysteine oxoforms are labelled (Fig. 1). This may be effected by direct reaction of a modified cysteine with a labelling reagent or indirectly. In the latter, modified cysteines are first chemically reduced to SH and those are subsequently labelled in a reversible (by disulphide bond) or irreversible (by alkylation) manner. The focus of the following sections is on indirect methods however due to an increasing number of direct methods a comparison of the two approaches is also provided in subsequent section.

There are few general considerations for how indirect methods should be performed, regardless of target oxoform. This includes the reaction conditions. Denaturing buffers are necessary for effective reduction and alkylation of those cysteines buried within the tertiary structure of a protein. This is preferred despite the fact that denaturing conditions might destabilise labile cysteine oxoforms [15]. Another decision is whether to label at the protein or peptide level, which is a dilemma in many modificomics studies and is of utmost importance in studies of transient and highly reactive modifications, such as oxidations. It is the preference of us and others that labelling is performed at the protein level [30,35]. This reduces the time between cell disruption and labelling of target cysteines, minimising sample preparation artefacts. Peptide level labelling, although preferable for improved accessibility to modified residues, is not desired due to significantly increased sample processing time caused by proteolytic digestion. Additionally, digestion is typically preceded by DTT/TCEP reduction and alkylation of cysteines, a step that is not compatible with specific SNO/SOH analysis.

After blocking of SH groups, labelling of target oxoforms is achieved by selective reduction and alkylation. Strong reducing agents like DTT/TCEP reduce all reversibly oxidised cysteines and other non-redox modifications, e.g. acylation. Sodium ascorbate and sodium arsenite are more selective, reducing SNO and SOH, respectively. However their actual selectivity is controversial.

5.1. Selective reduction of SNO – ascorbate based

Ascorbate remains the most commonly used reductant of S-nitrosothiols, even though its selectivity is not clearly defined. For this reason, over the years of ascorbate usage, variations in its concentration were used, alone or in tandem with Cu^{2+} see Table 1 [52–54]. The former is more common as it was used in 12 recent studies in the concentration range 5–50 mM. Ascorbate in combination with Cu^{+}/Cu^{2+} was applied in 8 studies and its concentration ranged between 1 and 5 mM. However, no systematic study evaluated the concentration dependant selectivity of SNO reduction and there remains no consensus as to which reduction conditions provide superior selectivity and sensitivity of SNO detection. This is bizarre as the chemistries behind the different approaches are known. Ascorbate in higher concentrations (e.g. 20 mM) reduces SNO directly, on the basis of nucleophilic attack [55]. Lower concentrations of ascorbate (e.g. 1 mM) act as reducing agent only in combination with Cu^{2+}. Here ascorbate reduces Cu^{2+} to Cu^{+} which in turns reduces SNO [55]. Ascorbate/Cu^{2+} combination was shown to increase sensitivity of SNO detection [56] but with no concomitant improvement in selectivity, as discussed in Ref. [15]. Due to the differences in chemistry, it is crucial not mix the two approaches and use high ascorbate concentrations in tandem with Cu^{2+} ions. Although ascorbate is the most common, other reductants, such as sinapinic acid have also been used for SNO reduction [57].

5.2. Selective reduction of SOH–arsenite based

Arsenite-based reduction of SOH is less frequent and is typically replaced by direct labelling with dimedone (discussed in a subsequent section). According to Table 1 there is only one recent study where arsenite-based reduction was used to identify SOH sites from kidney medula of spontaneously hypertensive rats [58].

Due to discrepancies in ascorbate/arsenite-based protocols, reduction efficiency must be evaluated in each experiment including appropriate controls. For instance, a control for selectivity of ascorbate reduction (negative control) would be to irreversibly block free thiols and UV photolysed SNO groups with one alkylating reagent, followed by reduction with ascorbate and alkylation with an alternative alkylating reagent, such as it was suggested by Forrester et al. [59].

5.3. Non-selective reduction of all reversibly modified cysteines

Strong reducing agents such as DTT and TCEP are typically used for reduction of all reversible cysteine modifications. This includes reversible oxoforms (disulphides, S-glutathionylation, S-nitrosylation and S-sulfenylation) as well as non-redox modifications, e.g. acylation. According to our analysis DTT and TCEP are used equally frequent in the concentration range 5–50 mM. When, using DTT it

is important to remove its excess prior to the labelling/alkylation. Otherwise SH groups of DTT might compete with SH groups of target proteins thereby hampering completeness of the labelling. In case of TCEP, which is a non-thiol based reductant, reduction and labelling/alkylation might be performed simultaneously. This is providing that iodoacetamide or maleimide analogues are not used for labelling/alkylation because they do react with TCEP [36].

Following reduction, labelling of nascent thiols is equally important (Fig. 1C). Whether provided by manufacturers or determined empirically the key is to establish optimal labelling conditions. This includes reagent concentration, pH, reaction time, temperature and other details, e.g. simultaneous versus sequential reduction and alkylation. Selected aspects of this crucial process are discussed further in subsequent sections (see Sections 6 and 7). Quenching of the labelling reaction is also important. As we have recently shown it is necessary to neutralise and remove unbound excess tag in order to minimise false positive results [30]. We highly recommend, that all features discussed are empirically validated in pilot experiments [30].

Overall, perhaps the most pragmatic view on modification-specific reduction is that inconsistency in reduction conditions and its controversial selectivity would not be an issue if differential alkylation-based studies were used exclusively as screening approaches. Subsequently these data could be verified with orthogonal strategies including use of anti-SNO-antibody [60] or OxMRM [61]. These however are still rarely implemented.

6. Reversible versus irreversible methods for labelling of nascent thiols

As mentioned previously, modification-specific reduction is typically followed by labelling of nascent thiols for stability. Depending on the chemistry of the labelling reagent this may result in either a chemically reversible/reducible or irreversible modification of the reduced thiol. Our meta-analysis shows that irreversible labelling is slightly more common as it has been applied to 14 recent studies, as opposed to the 12 studies where reversible labelling was used. Reversible binding is typically effected by thiol-disulphide exchange between nascent thiol and disulphide within the labelling reagent. An example of this is thiopropyl sepharose resin which provides a fast and convenient method combining both labelling and selective enrichment of target SH groups prior to MS-based analysis [62]. Coupling to the resin and enrichment might be performed at protein or peptide level and according to Su et al. both approaches provide equally high enrichment efficiencies of over 95% [35]. This allowed the authors to identify 197 SNO proteins, corresponding to 488 SNO sites from GSNO-treated mouse muscle homogenates [35]. The above example illustrates that strategies utilising reversible label may prove informative, when carefully optimised. Generally, however, they do possess several limitations. The lack of unambiguous modification site assignment is critical, especially when multiple cysteines occupy the same peptide. Additionally, the generated disulphide bond prohibits use of reducing agents, e.g. DTT/TCEP prior to proteolytic digestion which might diminish digestion efficiency and ultimately result in lower numbers of identified peptides.

Much more effective are alkylating reagents (e.g. variants of iodoacetamide) that irreversibly label nascent SH groups. This provides a unique mass increase for the modified peptide which is unambiguously identified upon mass measurement (MS spectrum). Following that, the site of the modified cysteine is assigned, based on the peptide fragmentation pattern (MSMS spectrum). The prerequisite for a tag of this nature being that it remains intact upon fragmentation during tandem mass spectrometry.

Isotope-Coded Affinity Tag (ICAT) reagents were amongst the pioneering irreversible labels, adapted to the redox field from quantitative proteomics [63]. In the recently commercialised cysteine reactive Tandem Mass Tag (TMT™) isobaric labels we observe the tendency to move from reversible to irreversible tags where the disulphide-based cysTMT™ [52,54,64] was quickly replaced with iodoacetyl-based iodoTMT™ [29,30,65]. Considering their advantages and increasing availability, we believe that irreversible tags will dominate redox proteomics studies in the future.

7. Indirect versus direct methods of identifying target cysteine oxoforms

Differential alkylation-based proteomics methods, such as the many variations of the biotin switch, rely on replacement of modified groups with a stable, identifiable molecule. These methods are therefore indirect and they require appropriate controls and orthogonal validation in order that the results can be fully relied upon. In contrast, direct methods, aim to preserve oxidative modifications in their native form which eliminates many of the issues with differential alkylation raised above.

Enrichment utilising phenylmercury resin is an example of a direct method for SNO detection [34,45,46]. It is based on the affinity between sulphur and the mercury cation (Hg^{2+}) where upon binding of SNO-containing proteins/peptides NO is hydrolysed, stabilising the bond between mercury and the nascent thiol [66]. Despite the direct binding, the actual site assignment is indirect whereby performic acid is used to release bound thiols by their oxidation to SO_3H [45,67]. Although reported complete, such untargeted oxidation, may still result in ambiguities when multiple reversible cysteine modifications or endogenous sulphonic acids are present on the same peptide. Additionally, quantitative analysis is limited [34], which in part might be due to the complicated sample processing scheme. Therefore, the advancement of this direct strategy is still far behind differential alkylation-based proteomics workflows.

In contrast to this, direct labelling utilising 5,5-dimethyl-1,3-cyclohexanedione (dimedone)-based probes is the most reliable chemistry for detection of intracellular SOH levels [32,43,68,69], see also Table 1. These reagents label SOH irreversibly without prior reduction. The newest, cell permeable derivatives of dimedone allow trapping of SOH groups in vivo, which significantly reduces artefact rates [32,70,71]. Introduced in 2011, isotopically labelled dimedone and iododimedone analogues allow direct quantitation of SOH sites, including quantitation of relative SOH occupancy [69]. Despite these clear advantages, the limitation of dimedone-based probes is low throughput. Since their introduction in 2009, dimedone analogues Daz-1 and DAz-2 have been applied to samples of moderate complexity, e.g. model proteins [72,73]. In fact, none of the recent dimedone-based studies, reported as many SOH proteins as have been reported from differential alkylation-based studies, see Table 1. In one of them Tyther et al. identified 32 SOH-modified proteins from kidney medulla of spontaneously hypertensive rats [58] (Table 1).

The key advantage of differential alkylation-based methods for analysis of cysteine oxidation is that the same affinity tag can be incorporated into different oxoforms. This allows for simultaneous analysis of multiple cysteine oxidations [30]. Using this principle we developed the SNO/SOH TMT strategy and quantified 475 SOH and 479 SNO sites (from 311 proteins) in *Escherichia coli* under low and mild oxidative stress [30]. In our most recent study we quantified 710 SOH and 986 SNO sites from 569 proteins in human hepatocarcinoma-derived C3A spheroids under acetaminophen (APAP) treatment [74]. Such simultaneous analysis allows investigation of possible cross-talk/interplay between oxidative PTMs which will be discussed in greater detail in the following

section.

It is likely that the decreased ambiguity of direct methods combined with the flexibility of indirect, differential alkylation-based approaches will be used in concert in the future to achieve a more complete and accurate coverage of redox proteomes.

8. Determination of modification site and type

The Human proteome contains over 21,0000 cysteines which vary in their susceptibility to oxidation. This is also true even within a single protein where a multitude of factors contribute to spatio-temporal redox transitions of individual cysteines. These are neighbouring amino acids, local pH and the higher order structure of the protein, just to name few [1,22]. Therefore, site resolution of the redox proteome is necessary.

There are several requirements to resolve modification sites. Firstly, sites should be irreversibly labelled with a unique tag. Such a tag provides a characteristic mass increase for the target cysteine-containing peptide upon MS analysis. The formula and final mass of the tag is critical because too small tags might not be easily distinguishable upon MS whereas too large tags might hamper ionisation of modified peptides or simply fall outside the typical m/z range of peptide analysis. Additionally, tags should remain intact upon both ionisation and fragmentation. Otherwise, it may complicate MSMS spectrum thereby impeding peptide sequencing and assignment of modification site, as it is known for the biotin-based tags [75].

The meta-analysis reveals that 13 recent studies provide unambiguous assignment of modification site. In a further 8 studies resolution of modification site may be possible if the target peptide contains only one cysteine residue. Altogether these data indicate clear trend toward quantitative analysis of cysteine modifications.

Iodoacetyl Tandem Mass Tags (iodoTMT™) are an example of a cysteine-reactive tag that facilitates resolution of modification site. This is due to its covalent binding to nascent SH groups which result in addition of 324.2 Da (iodoTMT™zero) or 329.2 Da (iodoTMT™-6plex) to a peptide containing a reduced cysteine residue [65]. The chemical formula of the iodoTMT tag can be easily implemented into standard proteomic database searches, allowing for automated modification site assignment. Once incorporated, the tag is inert and remains intact during ionisation and MS analysis. It does, however, dissociate upon fragmentation, providing a signature reporter ion in low *m*/*z* region of MSMS spectrum which is used for relative quantitation (discussed in more detail below). Furthermore, the reporter region is recognised by anti-TMT™ antibody which may be used for selective enrichment of iodoTMT™-containing peptides from complex peptide mixtures.

Amongst its limitations is a non-specific labelling of primary amines when suboptimal conditions for iodoalkylation are used [76]. This, however, is a common issue of tags containing iodoacetyl groups.

9. Qualitative versus quantitative analysis

Quantitation is nowadays a mandatory component of proteomics analysis. It is also becoming a commonplace in studies of redox proteomes, as 21 recent studies were conducted in quantitative manner (Table 1). Quantitative information is essential to characterise dynamic and transient redox proteomes. Amongst all putative sites, a distinction can be made between those which are "susceptible" or "sensitive" to the state of the redox environment. Susceptible sites are those whose redox environment make them highly oxidised under physiological conditions in the cell. Sensitive sites are those, which occupy a redox environment in which they are typically not oxidised but can become modified under conditions of oxidative stress. We observed this in a study of the *E. coli* redox proteome under low and mild oxidative stress. From 540 SNO/SOH sites identified in total only for 6 sites SNO/SOH levels changed significantly between the two conditions [30]. Information about the relative abundance of modified sites is crucial to distinguish between susceptible and sensitive sites.

In a typical quantitative experiment, abundance of a modified peptide is measured under control and stress conditions and these values are used to estimate relative fold change of the modification. Such relative quantitation may be delivered in numerous ways. It may be inherent to the differential alkylation, where quantitative information is typically derived from the cysteine alkylating reagent e.g. isotopomers of NEM and iodoTMT™ [29,65,77,78]. This was the case for 15 studies collected in Table 1. It may also be separate from the identification of the modification site, for example using dimethyl or iTRAQ-based quantitation [35,79]. This approach was implemented in the 6 recent studies (Table 1). Each of these quantitative strategies poses advantages and limitations which were discussed in detail previously [6,37].

D-Switch was among the first quantitative approaches [77]. It utilises isotopically labelled NEM to quantify target cysteines. In this method, the pool of SH containing proteins is labelled with isotopically "light" version of NEM whereas SNO proteins after selective reduction are labelled with an isotopically "heavy" version of NEM. Unfortunately, application of non-isobaric isotopic labels doubles sample complexity for MS analysis, which decreases the overall identification/quantitation rate. In the original study 11 SNO-containing peptides corresponding to 8 proteins were quantified from CysNO-treated human neuroblastoma cells [77]. Furthermore, the multiplexing capability is limited to only two channels.

Both analysis throughput and quantitation depth improved with introduction of cysteine reactive isobaric labels. A pioneering study, by Murray et al., reports 25 SNO sites from human pulmonary artery endothelial (HPAEC) cells after an in-culture stimulation with 200 μM CysNO using the now unavailable reversible cysTMT™ tags [52]. Development continued with the introduction of irreversible iodoacetyl Tandem Mass Tags (iodoTMT™). Pan et al. applied those for sequential quantitation of SNO and all reversible cysteine modifications. In this way, 266 redox sensitive sites were quantified in GSNO-treated H9c2 cells under hypoxia [65].

In one of the most recent studies utilising iodoTMT™, not only hundreds of modification sites were quantified under near physiological levels of oxidative stress, but also, for the first time, simultaneous analysis of SNO and SOH was undertaken [30].

Advances in label-free quantitation make it an attractive alternative to chemical labelling-based strategies, due to the reduced costs, simplified protocols and a theoretically unlimited level of multiplexing. Here quantitative information is extracted from the chromatographic peak area instead of from the relative abundance of differentially labelled species. The technical details of label-free quantitation have been described in numerous reviews e.g. in Ref. [80,81].

Interestingly, label-free quantitation is still rare in redox proteomics. According to meta-analysis, it was used in only 2 out of 20 recent quantitative studies (Table 1). Kohr et al. applied peak area based quantitation to measure SNO levels in GSNO-treated mouse heart homogenates [82]. For the quantitative ratio to be calculated, the same peptide has to be identified post enrichment in both samples. This might be challenging, considering the poor reproducibility of affinity-based enrichment strategies and the stochastic nature of data-dependant acquisition (DDA). Furthermore, isobaric labelling-based approaches have the advantage that

samples are pooled prior to analysis which aids in the detection of low abundance species. These may be below the limit of detection in a label-free approach. Considering all the above, we believe that isobaric labelling will remain a key tool in quantitative redox proteomics.

10. Correction of modification levels by protein abundance changes

For the PTM to be unambiguously quantified modification abundances should be corrected by respective changes in protein abundances [1]. However, such correction was implemented with moderate success into modificomics workflows [83] and it is a rare practise in redox proteomics.

The approaches with the potential for such analysis were OxICAT (ICAT – Isotope Coded Affinity Tag) and OxMRM (MRM – Multiple Reaction Monitoring) [61,84]. This is thanks to the unique design of the two strategies, which quantify both reduced (SH) and all reversibly oxidised cysteine oxoforms [61,84]. However neither of the methods actually applied quantitative information with intent to correct oxidation abundances by changes in protein abundances. Instead, they focus on the proportion of oxidised and non-oxidised cysteines.

Over the years of method development the focus was on more immediate goals, e.g. alternatives to error-prone modification specific reduction, implementation of quantitative workflows as well as increasing the overall sensitivity and throughput of the methods. This trend is evident from our meta-analysis as there are only 5 recent studies that attempted to correct modification levels by changes in protein abundance [27,33,34,64,85]. In most cases, it required additional experiments to estimate protein abundance changes [27,33,85].

Iodoacetyl Tandem Mass Tags (iodoTMT™) allow for simultaneous analysis of up to 6 samples/experimental conditions. Alternatively, they allow analysis of distinct cysteine subsets between fewer experimental conditions [30,65]. We showed that labelling all DTT/TCEP reversible cysteine modifications with iodoTMT™ allows for sufficient estimation of protein abundance changes [30]. This correction should be more accurate, than using a separate experiment in parallel, as it guarantees identification of the fully reduced form and quantitation is derived from the same scan as the modified species. However, it is important to mention that this approach does not include the contribution of irreversible oxidations (SO_2H and SO_3H) to the pool of cysteine modifications. Therefore, it is more accurate in the models of moderate oxidative stress.

To further emphasise the importance of correcting modification levels by protein abundance changes we have performed analysis of the results from our *E. coli* study [30]. Fig. 2 shows the average fold change of SNO/SOH peptides differentially modified between low and mild oxidative stress, with and without correction for protein abundance alterations. It is clear that with no correction only one site would qualify as differentially regulated.

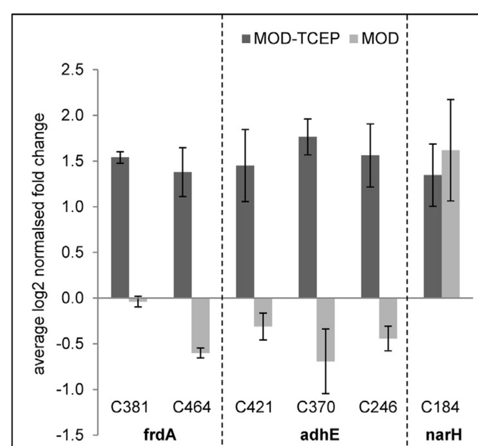

Fig. 2. Correction of modification levels by protein abundance changes is necessary to determine the true direction of regulation. Presented are SNO/SOH modification sites from *Escherichia coli* proteins. Those 6 sites were found differentially regulated between low and mild oxidative stress according to 2 sigma significance analysis performed after correction of modification levels by protein abundance changes (dark grey bars) [30]. The light grey bars represent regulation of those sites, excluding the correction for change in protein abundance. fraA-flavoprotein subunit of fumarate reductase FrdA, adhE-aldehyde-alcohol dehydrogenase; narH-respiratory nitrate reductase 1 beta chain.

11. New face of quantitation–modification site occupancy

In addition to the relative abundance change, quantitative information may be used to calculate modification site occupancy. Modification site occupancy is the fraction of a residue occupied by the target modification. Occupancy analysis requires that the total abundance of the site accessible for modification is known. Ideally, this should be the sum of all reversible and irreversible modifications of the given cysteine under given conditions. This however is difficult to achieve practically, as tools for analysis of SO_2H and SO_3H are lacking [61].

Therefore, those irreversible modifications are often excluded from the pool, justified by the fact that their contribution is negligible in most conditions. For instance, Kohr et al. expressed SNO occupancy in vitro as proportion of SNO to the sum of SH and SNO abundance [64]. This, however, neglects the contribution of other abundant cysteine oxoforms, e.g. disulphides [64]. Regardless, this pioneering study initiated a new trend in quantitative data analysis and has been actively followed since [30,34,86].

The recently developed SNO/SOH TMT approach allows determination of both SNO and SOH occupancy of any given site which is calculated in proportion to total reversible cysteine modifications. Such simultaneous analysis allowed us to observe that co-occurring SNO and SOH have distinct occupancies [30]. Additionally, we observed that sites with the highest modification occupancy under treatment/stress conditions are seldom differentially modified [30]. Finally, we also observed in both bacterial and mammalian redox proteomes that the average SOH occupancy is typically lower than the average SNO occupancy [30,74]. This, however, might be solely a technical issue, related to difficulties in SOH detection [12]. In fact, our observations are contrary to the findings of Wang et al. who reported S-oxidation to be more abundant than S-nitrosylation in CysNO-treated neuroblastoma cells [78]. Thanks to the above studies, the relevance of in-depth quantitation of modification sites is becoming realised. We support the view of Murray and Van Eyk, that with available tools occupancy analysis should be performed routinely in all studies of redox modifications [86]. This would allow construction of repositories of basal modification levels which could be used as a reference when pathological modification levels are measured. We believe that with future expansion of site occupancy analysis we will soon be able to determine the key regulatory features of redox PTMs, e.g. what proportions of modification are indicative of signalling, protection or oxidative damage.

12. Analysis of possible cross-talk between cysteine oxoforms

Previously, when each individual modification was analysed independently, often with conceptually distinct methods, cross-

talk between cysteine oxoforms was not visible [71]. Technological advances of recent years, cysteine-reactive isobaric labels in particular, allow for investigation of potential interplay between cysteine oxoforms.

It was again the work of Kohr et al. who were the first to analyse both SNO and a pool of all reversible cysteine modifications in the same experiment [82]. The analysis is parallel – SNO proteins are enriched by S-nitrosylation-Resin Assisted Capture (SNO-RAC) whereas DTT/TCEP reducible oxoforms (Ox) are enriched by Ox-RAC. MS analysis is performed separately and the two subsets are combined afterwards [82]. This elegantly designed approach, might however suffer from the stochastic nature of data-dependant acquisition which may not always provide a pair of SNO and oxidatively modified peptides.

Nevertheless, the study was critical to set the direction for concurrent analysis of cysteine oxoforms. Indeed, more studies followed, which attempted to analyse the interplay of SNO and other reversible oxoforms in either a simultaneous [82], parallel [78,79] or sequential manner [65]. In all of these examples, quantitation is effected by isobaric labelling which facilitates relative abundance comparison between target cysteine subsets. In particular, the approach by Pan et al. was cleverly designed. They took advantage of iodoTMT™-6plex multiplexing capability which allowed quantitative profiling of both SNO and the remainder of reversible cysteine modifications between 3 experimental conditions. Sequential iodoTMT™-6plex labelling not only reduces the amount of biological material required but also increases throughput of the analysis [65]. Combined analysis of distinct cysteine oxoforms provided insights into the SNO-guided protection of cardiomyocytes against oxidative stress upon hypoxia [65].

We intended to take such analysis further with our SNO/SOH TMT strategy which allows simultaneous analysis of 3 distinct cysteine subsets: all reversible, SNO and SOH modifications [30]. Apart from the study by Faccenda et al., this is the first approach allowing quantitative analysis of different cysteine oxoforms simultaneously [87]. Due to the fact, that both SNO and SOH are quantified from the same MSMS spectrum the co-occurrence is more apparent and more easily verified [30]. For instance, we observed in both bacterial and mammalian models, that majority of SNO and SOH sites co-occur [30,74].

As reviewed by Evangelista et al. cross-talk between SNO and SOH seems likely [7]. However, there is certainly room for expansion in this area, now that quantitative methodologies allow such simultaneous analysis. We believe that combinatorial analysis should be amongst future directions in redox proteomics and that it will shed light on the as yet, largely unexplored role of redox processes in physiology and pathology.

13. Remaining challenges

Despite tremendous improvements of recent years, there remain several challenges in redox proteomics. Amongst the most critical is the amount of starting material required for analysis. Our meta-analysis revealed that the vast majority of studies require milligrams of starting material (15 out of 26). A further 10 were based on higher micrograms (100–500 μg) of cell/tissue samples (Table 1). In fact there was only one study that used 10 μg of murine protein extracts to analyse SNO levels in spinal cord after sciatic nerve injury [53]. High amounts of starting material are necessary due to sub-stoichiometric and transient nature of cysteine oxoforms, loss-prone differential alkylation-based strategies and limited selectivity of enrichment.

Therefore, we should maximise our efforts to refine labelling and enrichment protocols in a way that will allow analysis of human tissue samples and body fluids in the future. We believe that the key to this will be sequential analysis and orthogonal fractionation/purification strategies that will maximise efficiency and throughput of analysis and will bring us closer to clinical scale research. This is similar to the technological progression in the analysis of other PTMs such as phosphorylation [88].

Additional challenges include analysis of reversible cysteine modifications from target subcellular locations. Effective organelle enrichment is often a lengthy process which is contrary to what is required for preservation of oxidative PTMs. This might be overcome if more membrane permeable probes like DAz-1, DAz-2 are developed, allowing for stabilisation of target modification prior to organelle specific enrichment [71,89].

14. Future directions

Having the proper tools in form of e.g. SNO/SOH TMT workflow the interplay between cysteine oxoforms should be investigated in various biological systems. This may include calculation of the likelihood of interdependence between distinct cysteine oxoforms, as it is applied for analysis of histone modifications [90].

The integration of quantitative workflows that would allow the study of the interplay between redox and non-redox PTMs such as phosphorylation and acetylation would also be of importance. There already exists evidence to support a tight regulatory connection between these two seemingly different classes of PTMs. For instance it was mentioned by Pan et al. that in the heart, cysteine oxoforms might be the triggers and modulators of signalling cascades exerting cardioprotection [65].

Interplay between SNO and other non-redox PTMs, e.g. palmitoylation and ubiquitination was discussed in an excellent review by Evangelista et al. [7]. We fully support the view and believe that global, systemic analysis of redox and non-redox PTMs will broaden our understanding of the intracellular signalling complexity in health and disease [7].

Amongst the future goals in redox proteomics we see absolute quantitation as discussed in Ref. [91]. The first attempts at “absolute” quantitation were performed using oxICAT and OxMRM approaches [61,84]. Unfortunately, only the proportion of oxidised to all reversible cysteine oxoforms can be determined, without taking into account contribution of irreversible modifications. Additionally, difficulties in the production of synthetic standards currently prohibit determination of the actual amounts of modified residues.

15. Conclusions

Redox proteomics is a dynamically evolving field and differential alkylation-based strategies are contributing greatly to its development. These are ubiquitous in the analysis of virtually any reversible cysteine modification, identifying not only modified proteins, but also individual modification sites. The majority of differential alkylation-based proteomics strategies are quantitative, providing information about relative abundance change, although, the tendency is now also toward analysis of modification site occupancy which adds a new dimension to quantitative studies. We believe that the key for further improvements is the unification of biological models and sample preparation schemes. This combined with in-depth analysis provided by differential alkylation-based proteomics might be sufficient for analysis of endogenous redoxomes in the future.

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