EXPERT REVIEWS

Proteomics insights into deregulated protein *S*-nitrosylation and disease

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Nitric oxide (NO) can modulate cell function by the coupling of a nitroso moiety to a reactive cysteine in target proteins leading to the formation of a *S*-nitrosothiol (SNO), a process commonly known as *S*-nitrosylation. Aberrant *S*-nitrosylation of proteins, caused by altered production of NO and/or impaired SNO homeostasis, constitutes a mechanism that has been recently postulated in numerous pathophysiological settings. The thiol microenvironment, cellular redox environment, and activity of transnitrosylases and denitrosylases have been proposed as determinant factors for the specificity of *S*-nitrosylation. A number of methodological approaches have recently been developed for the proteomic identification of *S*-nitrosylated proteins and/or the identification of specific sites of nitrosylation. This review will consider novel aspects of SNO homeostasis and *S*-nitrosylation, the latest proteomic methods for the identification of *S*-nitrosylated cysteines in proteins, and how these novel technologies will impact our current knowledge of the role of deregulated *S*-nitrosylation in disease.

Keywords: biotin-switch technique • cysteine modification • denitrosylases • disease • nitric oxide • nitrosoproteome • nitrosylases • post-translational • proteomics

Nitric oxide (NO) is a diffusible gaseous molecule that is synthesized *in vivo* and acts as a signaling molecule in the body, playing a critical role in various physiological and pathophysiological processes. NO is synthesized by the catabolism of L-arginine to L-citrulline through a complex reaction catalyzed by nitric oxide synthase (NOS) enzymes, of which there are three distinct isoforms. NOS1 (nNOS) and NOS3 (eNOS) were initially cloned in neural cells and endothelial cells, respectively, are dependent on Ca^{2+}–calmodulin and are constitutively expressed. On the other hand, NOS2 (iNOS) was initially identified in macrophages, is independent of calcium and its induction leads to the production of high levels of NO during pathophysiological conditions [1,2]. Many of the physiological processes promoted by NO are mediated by the NO–cGMP signaling pathway. However, a large number of studies in recent years have provided ample evidence that NO can modulate cell function by the coupling of a nitroso moiety to a reactive cysteine (Cys) in target proteins, leading to the formation of an *S*-nitrosothiol (SNO), a process commonly known as *S*-nitrosylation [3].

Recent studies have uncovered enzymatic systems, termed nitrosylases and denitrosylases, that participate in the nitrosylation and denitrosylation of proteins and play an important role in maintaining SNO homeostasis in cells [4,5]. The altered production of NO and/or impaired SNO homeostasis may result in aberrant *S*-nitrosylation of proteins, a mechanism that has been recently postulated in numerous pathophysiological settings [3], including cardiovascular [6], respiratory [7], hepatic [8], neurodegenerative [9,10] and neoplastic [11] diseases.

Proteomic studies have been undertaken for the systematic identification and characterization in a particular organism, organ or cell type, of those proteins that undergo *S*-nitrosylation, namely, the *S*-nitrosoproteome. Recent improvements and advances in proteomic technologies have empowered such studies, providing researchers with better tools for exploring this post-translational modification (PTM). This review will consider novel aspects of SNO homeostasis and *S*-nitrosylation, the latest proteomic methods for the identification of *S*-nitrosylated Cys in proteins, and how these novel technologies

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will impact our current knowledge of the role of deregulated *S*-nitrosylation in disease.

S-nitrosylation & denitrosylation of proteins

Cys residues are known to be important for maintaining the native conformation of proteins; they are critical residues at the active sites of enzymes and are the most reactive residues to NO-derived reactive species at physiological pH. As long as NO itself is a poor nitrosylating agent, some mechanisms have been described for SNO formation within the biological environment [12]. Thus, dinitrogen trioxide (N_2O_3), which is formed from O_2 and NO, is the likely *S*-nitrosylating species in biological systems. However, a significant fraction of protein *S*-nitrosylation by NO may occur in the absence of O_2 [13] or through radical-based reaction mechanisms [14]. *S*-nitrosylation may also occur via the intermediacy of a ferric heme nitrosyl species. Importantly, the nitroso function can be transferred from one thiol to another in a transnitrosylation reaction that occurs via nucleophilic attack on the nitrogen atom of the SNO. Since the low-molecular-weight SNO pool (*S*-nitrosocysteine, *S*-nitrosocysteinylglycine and *S*-nitrosoglutathione) exists in equilibrium with the *S*-nitrosoprotein pool, transnitrosylation reactions have an important role in the maintenance of cellular SNO homeostasis (Figure 1).

Even though the chemical reactions involved in SNOs formation are relatively well understood, the exact mechanisms governing *S*-nitrosoprotein formation remain obscure. The types and levels of NO donors, the thiol microenvironment, the cellular redox environment, and the presence of transnitrosylases and denitrosylases have been proposed as factors that determine the specificity of *S*-nitrosylation (Figure 1) [15,16].

The 3D microenvironment of the reactive thiol has been claimed to play an important role in the prediction of enhanced susceptibility to *S*-nitrosylation. Thus, some studies have suggested that an acid–base motif or hydrophobic areas, which could be present either in the flanking primary sequence or emerge in tertiary structure, render low pKa and high sulfur atom exposure and promote specific *S*-nitrosylation of Cys residues [17,18]. However, apparently inert Cys residues are known to be specifically and selectively nitrosylated within proteins. A recent structural analysis of Cys *S*-nitrosylation suggest that, rather than being responsible for direct activation of Cys, the acid–base motif plays a role in protein–protein interactions that could facilitate transnitrosylation [19]. The authors further suggest the potential role of nitrosoglutathione as a transnitrosylating agent for proteins lacking the acid–base motif. Therefore, the specific interaction of other SNOs or nitrosoproteins (or other SNO–Cys sites within the same protein) with the particular Cys, may enhance its reactivity and specifically activate the residue for interaction with NO donors.

Other likely determinants of specific *S*-nitrosylation are the interaction or close subcellular co-localization of protein targets with NOS isoforms. In endothelial cells and other cells overexpressing eNOS, *S*-nitrosylation is concentrated on the Golgi apparatus and in plasma membrane caveolae, which are the main sites of eNOS localization [20]. Also in this regard, during lymphocyte activation, eNOS selectively *S*-nitrosylates N-Ras on the Golgi apparatus, but not K-Ras, which is preferentially concentrated at the plasma membrane [21]. In epithelial cells, the *S*-nitrosylation and activation of cPLA2α, a key enzyme involved in the biosynthesis of prostanoids, is mediated by the COX-2-induced formation of a cPLA2α-iNOS-binding complex [22]. Scaffold or adaptor proteins facilitate the close association with nNOS and thereby the *S*-nitrosylation of NMDA receptor and Ras-like G protein during neuronal signaling. Therefore, the different compartmentalization in cells of putative *S*-nitrosylation protein targets,

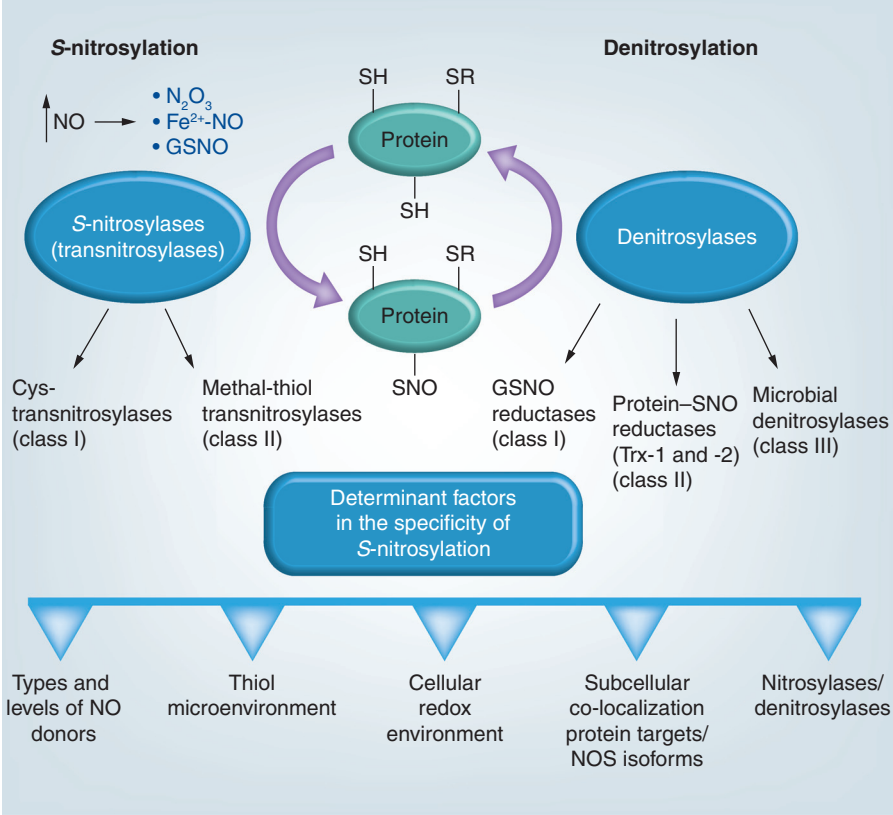

Figure 1. *S*-nitrosylation and denitrosylation of proteins. Mechanisms participating in *S*-nitrosylation and denitrosylation of proteins, and thereby maintaining SNO homeostasis in cells, are presented here. The altered production of NO and/or impaired SNO homeostasis, which may result in aberrant *S*-nitrosylation of proteins, has been implicated in the pathogenesis of several diseases. See text for further details.
GSNO: *S*-nitroglutathione; NO: Nitric oxide; NOS: Nitric oxide synthase; SNO: *S*-nitrosothiol; Trx: Thioredoxin.

protein complex formation or direct protein–protein interaction are critical for their specific biological activities and their regulation by NO-dependent mechanisms. The specificity of these mechanisms would thereby promote selective S-nitrosylation. Moreover, it has been suggested that some protein Cys residues may have evolved as 'NO sensors' and that these molecular adaptations made *S*-nitrosylation a pre-eminent form of NO signaling [23].

Apart from the direct interaction and/or subcellular compartmentalization of NOS isoforms with target proteins, other enzymes that may provide a basis for *S*-nitrosylation specificity are transnitrosylases. New research has highlighted the key role of enzyme-mediated processes in the nitrosylation and denitrosylation of proteins. In analogy with protein kinases, which bind target proteins and introduce phosphate groups, those proteins that directly introduce NO groups into target proteins may be considered protein nitrosylases [5,16]. Protein *S*-nitrosylases have been classified as Cys-transnitrosylases (class I) or methal-thiol transnitrosylases (class II) (Figure 1) [16]. While in class II nitrosylases, transfer of NO occurs from transition metal to thiol within and not between proteins, class I nitrosylases are *S*-nitrosylated proteins that transfer their NO group to target proteins through specific interactions. Thus, deoxygenation of SNO–hemoglobin (SNO-Hb) promotes its binding to the cytoplasmic chain of AE1, which is anchored to the erythrocyte membrane. Then, the transfer of the NO group from SNO-Hb to AE1 via transnitrosylation propagates a vasodilatory signal. Actually, the *ex vivo* storage of blood depletes SNO-Hb and reduces the ability of red blood cells to induce vasodilation, significantly accounting for their impaired ability to deliver oxygen [24]. In other cases, although *S*-nitrosylation of caspases is known to inhibit apoptotic activity, SNO-caspase-3 binds and transnitrosylates XIAP, inhibiting its ubiquitylating activity and propagating apoptotic signals in neurons [25]. The nitrosylated form of thioredoxin 1 (SNO-Trx1) has also been reported to participate in the transnitrosylation of proteins, with procaspase-3 being the most well characterized target [15]. Hence, it has been reported that nitrosylation of the Cys73 of Trx1 confers affinity of SNO-Trx1 for procaspase-3, promotes the transnitrosylation of its catalytic Cys and prevents apoptosis in Jurkat T cells [26].

Several proteins with denitrosylase activity are now recognized, and their classification as GSNO reductases (class I), protein–SNO reductases (class II) and microbial denitrosylases (class III) have been suggested (Figure 1) [16]. GSNO reductase (GSNOR), previously recognized as alcohol dehydrogenase III, is an important enzyme involved in SNO homeostasis, and the genetic knockout of GSNOR in mice increases the levels of both GSNO and protein SNO, which demonstrates that GSNO is in equilibrium with a pool of SNO proteins [27,28]. In an experimental model of asthma, the genetic deletion of GSNOR in mice increased lung SNOs and exerted protection from airway hyper-responsivity [29]. Furthermore, the treatment of human hepatocytes with L-nitrosocysteine (CSNO) caused a rapid increase in *S*-nitrosoprotein content [30], later returning to basal levels due to an increase in GSNOR expression and activity [31]. Recently, it has been demonstrated that inhibition of NO production during cholestasis ameliorates hepatocellular injury and improves *S*-nitrosothiol homeostasis by reverting the reduction in GSNOR expression and activity, thereby reducing the *S*-nitrosylation of hepatic proteins [8]. In addition, a recent study reported that GSNOR$^{-/-}$ mice exhibited substantial *S*-nitrosylation and proteasomal degradation of a key DNA repair enzyme, and were found to have a tenfold higher incidence of spontaneous hepatocellular carcinoma (HCC). Moreover, the authors found that GSNOR abundance and activity was significantly decreased in 50% of patients with HCC [11]. Human carbonyl reductase 1 (hCBR1) has been reported to possess GSNO-reducing activity and seems to be responsible for a substantial fraction of the GSNOR activity in lung adenocarcinoma cells [32]. Therefore, this enzyme may be also a class I denitrosylase partially responsible for regulation of GSNO levels in tissues.

Class II denitrosylases, such as thioredoxins 1 and 2, are protein–SNO reductases. The thioredoxin/thioredoxin reductase (Trx/TrxR) system, a well-known reductant of oxidized thiols, has been reported as a specific enzymatic mechanism of regulating basal and stimulus-induced protein denitrosylation in distinct cellular compartments [33,34]. The Trx/TrxR system denitrosylates low-molecular-weight SNOs as well as SNO proteins, particularly caspases [34]. When cells are treated with auranofin, a highly specific TrxR inhibitor, increased protein nitrosylation levels are observed [34–36], suggesting that when the protein reduction activity of Trx1 is attenuated, it may either lose its ability to denitrosylate or increase its ability to transnitrosylate. A comparison of proteins that are transnitrosylated but not denitrosylated by Trx might provide insight into the exact molecular mechanism of Trx-mediated protein denitrosylation and target specificity [15]. The cellular redox environment constitutes a key determinant factor for the *S*-nitrosylation and denitrosylation of proteins, and may contribute to the specificity of this PTM. Thus, transnitrosylation via SNO-Trx1 has been considered as a preventive measure against irreversible oxidative modifications of proteins in the short term and as a signaling mechanism contributing to the cellular stress response [15].

Our knowledge of the cellular mechanisms governing nitrosylation and denitrosylation of proteins, and thereby maintaining SNO homeostasis in cells, has recently been increased. The alteration or manipulation of the levels of the enzymatic systems involved, through overexpression or specific gene silencing, may be easily carried out. However the use of a number of alternative endogenous regulators or pharmacological treatments can be more physiologically relevant. Furthermore, coupling of these studies to current powerful proteomic methodologies undoubtedly constitutes a promising approach to investigate *S*-nitrosylation target specificity.

The detection of *S*-nitrosylated proteins

A number of factors, including the sensitivity of SNOs to light leading to homolytic cleavage of the S–NO bond, and their ready reduction by agents such as ascorbate and transition metals, complicates the detection of *S*-nitrosylated proteins. Additionally, SNO proteins are usually present at low levels in cells and tissues, and sensitive and specific methods are needed for their efficient

detection. The development by Jaffrey and coworkers [37] of the biotin-switch technique (BST), an original approach that permitted the selective tagging of SNO proteins with biotin, made the proteomic analysis of protein *S*-nitrosylation feasible. The BST consists of three steps (Figure 2). In the first step, free protein thiols are specifically blocked with the methylating agent methyl methanethiosulfonate (MMTS). The second step involves the selective reduction of protein SNO groups to thiols with ascorbate, and in the final step these newly formed thiols are reacted with the thiol-specific biotinylating reagent N-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). The biotin-labeled proteins can be detected on Western blots following incubation with anti-biotin antibodies or recognition via streptavidin, or alternatively they can be captured on streptavidin matrices. Several issues have been raised regarding the specificity of the BST, including the possibility that the ascorbate treatment may reduce other Cys oxidation-derived modifications, such as *S*-glutathionylation or *S*-oxidations. However, it has been demonstrated that a transnitrosylation reaction occurs between ascorbate and SNO to yield the semidehydroascorbate radical and NO [38].

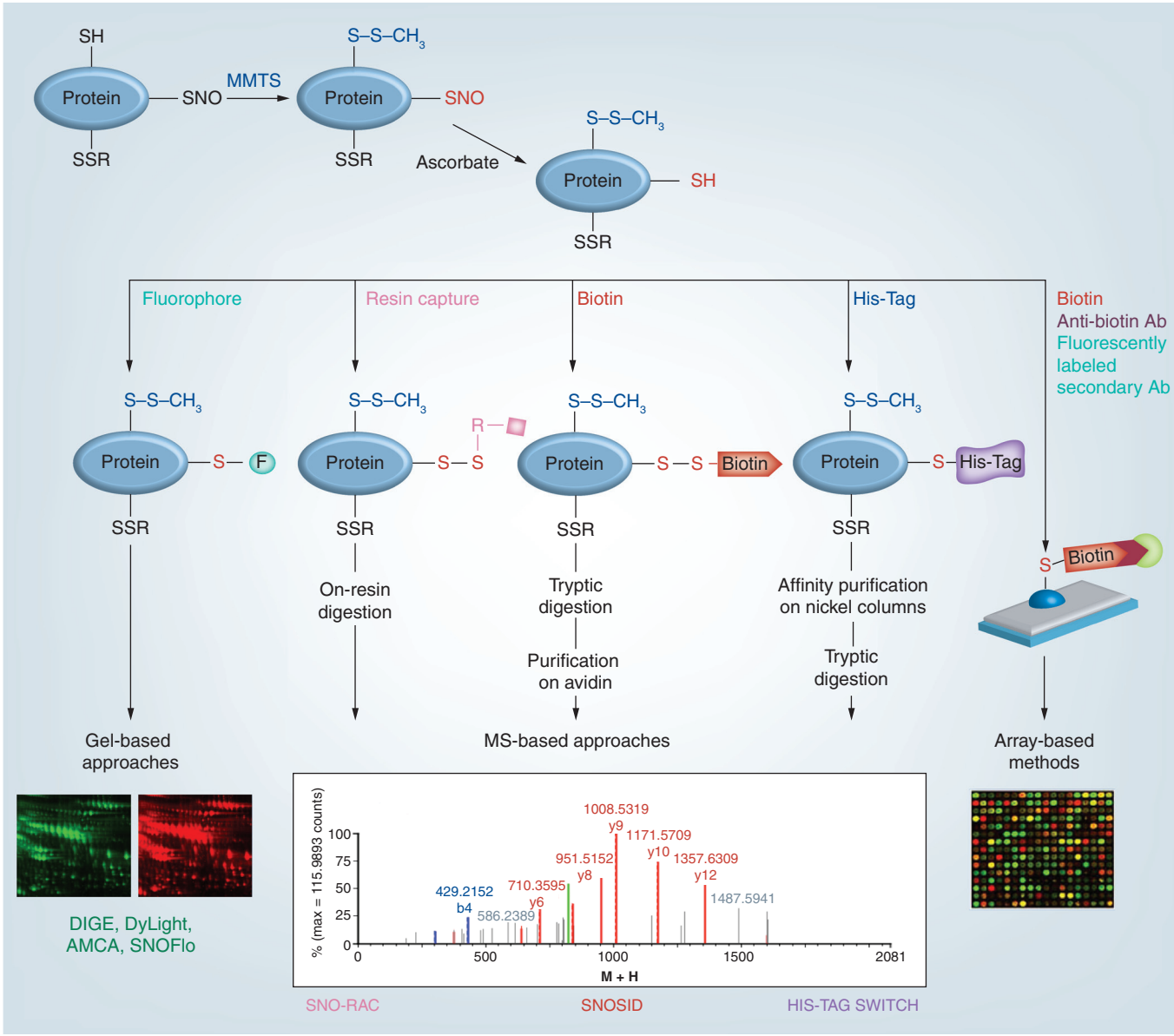

Figure 2. Biotin-switch technique-based methods for the proteomic analysis of *S*-nitrosylated proteins. Using the biotin-switch technique assay, previously *S*-nitrosylated proteins are tagged with biotin, allowing their immunodetection or alternatively their purification on avidin/streptavidin resins for proteomic identification. Some of the recently developed enhancements of the biotin-switch technique for the proteomic identification of *S*-nitrosylated proteins and/or the identification of specific sites of nitrosylation are represented in the figure. See text for further details.
Ab: Antibody; AMCA: 7-amino-4-methyl coumarin-3-acetic-acid; M + H: Molecular mass of protonated molecular ions; MMTS: Methyl methanethiosulfonate; SNO-RAC: *S*-nitrosothiol resin-assisted capture; SNO: *S*-nitrosothiol; SNOFlo: *S*-nitrosothiol fluorescence saturation; SNOSID: SNO site identification; SSR: Disulfide.

Since ascorbate does not directly reduce the S–NO bond and the transnitrosylation reaction is distinctive among Cys oxidation products, the ascorbate step confers specificity to the BST. Since UV light efficiently cleaves S–NO bonds, SNO photolysis prior to the assay has been suggested be useful as a separate control for ascorbate specificity or incomplete blocking of thiols [39]. On the other hand, to definitively determine whether the S–NO modification of a particular protein is biologically or physiologically relevant, additional controls, including site-directed mutagenesis and biochemical and pharmacological characterization, might be needed [40].

Proteomic methods: BST improvements

Soon after its development, the BST was promptly applied to the proteomic analysis of *S*-nitrosylated proteins in a variety of organs and cell types [41]. Recently, a number of significant modifications and enhancements of the BST have been developed to perform proteomic identification of SNO proteins and/or the identification of specific sites of nitrosylation (Figure 2).

Gel-based approaches

Following BST, the streptavidin-captured proteins can be separated by 2DE. After adequate staining of protein spots, MS/MS can be used to identify *S*-nitrosylated proteins (Figure 2). A significant improvement of this approach is the coupling of DIGE to the BST [42,43]. The thiols formed after the ascorbate reduction step are labeled with maleimide conjugated to fluorescent cyanine dyes (Cy3 or Cy5). After labeling, equal amounts of control and SNO-treated samples are mixed and separated on the same 2D gel (Figure 2). In this manner, those protein thiols that have been selectively modified by the SNO treatment can be detected as spots showing differential fluorescent intensity of the two dyes in the gel. Other DIGE approach use the newly developed DyLight maleimide sulfhydryl reactive fluors (Pierce–Thermo Fisher Scientific) instead of the biotin-HPDP in the BST [44,45]. After labeling, each of the individual samples can be visualized independently on the same gel by selecting appropriate excitation and emission wavelengths. Furthermore, DyLight labeling also causes an acidic shift and a minor upwards shift in every single spot (each DyLight fluor molecule contains three to four negative charges and an approximate 1 kDa mass) and protein spots with a shifted DyLight pattern can be directly picked from the gel for mass spectrometry (MS) identification. DIGE-based methodologies will also be useful to address other outstanding questions. For instance, there is a paucity of information regarding the amount of a particular protein that is *S*-nitrosylated under different physiologically relevant conditions. Significantly, by using these DIGE approaches, a relative *S*-nitrosylation level for each protein may be obtained from the direct comparison of fluorescent intensity from each fluorophore in a single spot.

Other fluorescence-based modification of the BST has been reported [46], in which the fluorophore 7-amino-4-methyl coumarin-3-acetic-acid (AMCA)-HPDP is used instead of biotin-HPDP, and the previously *S*-nitrosylated proteins can be directly visualized on gels after non-reducing SDS-PAGE and UV exposure. Furthermore, after in-gel tryptic digestion of the fluorescent band and liquid chromatography (LC)-MS/MS analysis, the recognizable AMCA tag in the MS spectra ensures the accurate site identification of the *S*-nitrosylated Cys residues. Other methodology with enhanced sensitivity compared with the commonly used BST coupled to 2DE is the 'fluorescence switch' technique developed by Tello and colleagues [35]. This approach uses fluorescent maleimide reagents instead of biotin-HPDP and, coupled with 2DE separation of proteins, enables specific detection of the fluorescence substituting *S*-nitrosylation, while allowing for fluorescent total protein.

A novel quantitative BST-based approach for measuring changes in protein *S*-nitrosylation has recently been reported [47]. In this method, called fluorescence saturation (SNOFlo), after denaturation and division of the cell extract into two equal fractions, one fraction is labeled with a 60-fold excess of BODIPY FL-maleimide (BD), an uncharged Cys-specific fluorescent dye, and the second fraction is treated with ascorbate to reverse the SNO modification, and is likewise labeled with BD. The uncharged nature of the dye and its lack of influence on the modified protein isoelectric points permits spot matching between ascorbate-treated and untreated samples in subsequent 2DE with fluorescence quantification. This permits the determination of protein *S*-nitrosylation regulation between control and experimental samples.

MS-based approaches

Although DIGE techniques coupled to the BST can be used to assess the extent of the *S*-nitrosylation in each particular protein, these approaches are hardly informative about the modified Cys(s) on the protein. In addition, hydrophobic membrane proteins are under-represented in 2DE gels because of their relative incompatibility with the necessary isoelectric focusing step. Furthermore, all gel-based approaches tend to favor the identification of abundant proteins. To address these issues, several research groups have dedicated efforts to developing robust gel-free methods for proteomic analysis of *S*-nitrosylation.

The introduction of a tryptic digestion step before avidin capture was one of the first proteomic-oriented modifications of the original BST. This improved method, known as SNO site identification (SNOSID) [48], allows the selective isolation of previously *S*-nitrosylated peptides for their sequencing by LC-MS/MS and the identification of modified Cys residues (Figure 2). In another variation, a conjugate of iodoacetamide and a His-tagged peptide that irreversibly binds to Cys residues is used instead of biotin-HPDP. This approach, known as the HIS-TAG switch method [49], ensures that proteins are covalently labeled through all purification steps (Figure 2). In addition, the His-Tag reagent is partially cleaved after tryptic digestion, producing a reporter ion and a final mass shift in MS spectra, which are indicative of previously *S*-nitrosylated peptides.

A recent adaptation of the BST takes advantage of the use of a thiol-reactive resin instead of biotin-HPDP, thus integrating the specific labeling and 'pulldown' steps into a single step (Figure 2) [50]. This technique, which has been named resin-assisted

capture (SNO-RAC), was combined with isobaric tags for relative and absolute quantitation (iTRAQ) to detect the kinetics of *S*-nitrosylation/denitrosylation. In another recent study, Benhar and colleagues coupled SILAC with BST and LC-MS/MS analysis to identify new substrates of denitrosylase Trx1 [51]. Also recently, Liu and colleagues used this thiol-reactive resin to selectively enrich SNO peptides reduced by ascorbate followed by nanoscale LC-MS/MS [52]. Furthermore, in this study, two alkylation agents with different added masses were employed to differentiate the S–NO sites from the non-S–NO sites. In addition, a recent complementary proteomic MS approach adapted the BST using deuterated labeled *N*-ethylmaleimide in order to quantitate free thiol versus nitrosothiol for each modified Cys residue [53].

Another modified BST for the site-specific identification of SNO proteins has recently been reported [54]. In this method, free Cys thiols are *S*-alkylated with iodoacetamide (IAM) and specific labeling of Cys–SNO is performed by reduction with ascorbate followed by irreversible biotinylation with PEO-iodoacetyl-biotin. The disulfide bonds are further reduced by tris (2-carboxyethyl)-phosphine and *S*-alkylated by IAM. After tryptic digestion, the biotinylated peptides are enriched by avidin affinity purification and analyzed by LC-MS/MS. Using this approach, the *S*-nitrosylation sites can be unambiguously determined based on the characteristic mass shift of the fragment ion from a biotinylated Cys residue in the MS/MS spectra.

The BST is influenced by the abundance of the SNO protein, and low-abundance SNO proteins may be overlooked. To address this limitation, a modified BST for detecting SNO proteins on protein microarrays (Figure 2) has recently been developed [55]. This method uses an anti-biotin antibody and fluorescently labeled secondary antibody for detection of SNO proteins, and was used to screen a yeast protein microarray containing approximately 4000 glutathione *S*-transferase-tagged open reading frames after SNO treatment. This high-throughput screening with unbiased proteome coverage revealed some determinants of *S*-nitrosylation, which may be overlooked in alternative proteomic analyses. Thus, this study identified large sets of target proteins, among which those with active-site Cys thiols residing at the N-termini of α-helices or within catalytic loops were particularly prominent [55].

Another recent study used the BST in conjunction with antibody array screening for the identification of previously undescribed SNO proteins in neuronal systems [56]. In this study, SNO proteins from human neuroblastoma cells, in which nNOS and eNOS were activated to produce physiological concentrations of NO, were converted to their biotinylated form by using the BST and subjected to antibody array with fluorescence detection. In total, 25 candidates were identified and, among them, PTEN was found to be preferentially *S*-nitrosylated by low concentrations of NO [56].

Novel tools & strategies

The potential pitfalls of BST, namely the incomplete blocking of reduced Cys residues and the efficiency/specificity of ascorbate reduction, have stimulated the development of alternative approaches for exploring the *S*-nitrosoproteome (Table 1).

Direct detection of SNO proteins by MS

It is obvious that direct detection of Cys–SNO in proteins by MS could avoid many of the specificity and sensitivity concerns raised by the BST or similar chemical derivatization methods. Unfortunately, the labile nature of the SNO bond represents an obstacle against the progress of the study of *S*-nitrosylation by direct MS detection. For example, in MALDI-TOF MS, the laser energy required for peptide ionization causes the loss of NO from the Cys residue. However, it has been reported that under gentler conditions, as in ESI-MS, *S*-nitrosylated peptides can be observed with a +29 Da difference with respect to the unmodified ions. Thus, Wang and colleagues [57] developed an LC/MS/MS strategy that enables us to directly identify *S*-nitrosylation sites in proteins using ESI quadrupole TOF (ESI-QTOF) MS. In this study, both cone and collision energy voltages in QTOF-MS were fine-tuned to preserve the S–NO bonds, and the authors found that Cys73 in human Trx1 was specifically *S*-nitrosylated after GSNO treatment. Another group applied a similar approach to study *S*-nitrosylation in plant Cys-rich metal-binding proteins [58]. Recently, a new type of dissociation method using metastable atoms as an energy source has been explored for the characterization of *S*-nitrosylated peptides in quadrupole ion-trapping instruments [59]. In this method, termed metastable atom activated dissociation (MAD), isolated precursor ions interact with a high kinetic energy beam of helium metastable atoms, producing a high degree of peptide backbone cleavages, resulting in a-, b-, c-, x-, y- and z-type ions while retaining Cys–SNO modification. Compared with collision-induced dissociation, which is a dissociation method traditionally employed in ESI MS/MS, MAD produced between 66 and 86% more fragment ions, which preserved the labile NO modification. However, such approaches were limited to synthetic peptides or purified proteins, or were able to identify only a few Cys–SNO sites from complex mixtures, and therefore their suitability to global *S*-nitrosoproteomic studies remains to be explored.

New chemical derivatization methods for SNO

A recently demonstrated property of gold nanoparticles (AuNPs) is their capacity to react with SNO proteins to yield NO and AuNP–protein thiolates [60]. Based on these findings, Faccenda and colleagues have reported an AuNP-based method for the enrichment and identification of SNO sites [61]. In this approach, free Cys thiols are blocked with IAM and subjected to proteolysis and incubation with AuNPs. The SNO peptides react with AuNPs and release free NO and AuNP-bound peptides, which are incubated with DTT to release the bound peptides, which are analyzed by MS to identify the SNO protein sites. However, this method poses a problematic shortcoming, since AuNPs react with both *S*-nitrosylated and *S*-glutathionylated Cys residues.

To overcome the problems and limitations of existing methods, particularly their dependence on the complete blocking of reduced Cys residues, other approaches to directly label Cys–SNO are desirable. The unique functionality of the SNO group has led researchers to explore new reactions that could specifically target

SNO moieties and convert unstable SNO to stable and detectable products. One of these approaches takes advantage of the reaction between phenylmercury compounds with Cys–SNO to form relatively stable thiol–mercury bonds. Doulias and colleagues [18] used an organomercury resin synthesized by conjugation of ρ-aminophenylmercuric acetate to agarose beads to capture SNO proteins and peptides followed by on-column tryptic digestion and LC-MS/MS to identify Cys–SNO residues. They also used a phenylmercury–polyethyleneglycol–biotin compound for in-solution affinity capture.

Another recently developed method takes advantage of the phosphine-mediated reactions of SNO [62]. In this approach, SNO groups rapidly react with a biotin-labeled phosphine substrate, providing a stable disulfide product conjugated to biotin at the formerly Cys–SNO residues. In another recent report, Bechtold and coworkers [63] described another phosphine-based method for the specific labeling of Cys–SNO. In this case, the reaction with the water-soluble phosphine tris(4,6-dimethyl-3-sulfonatomethyl)-phosphine trisodium salt hydrate reacts with SNO to form covalent *S*-alkylphosphonium adducts, which may be amenable to mass detection in MS. The use of these promising phosphine-based compounds in complex protein samples will help to define their reliability as SNO proteomic tools. Undoubtedly, the development of such direct methods that only target SNO moieties will be a breakthrough for future studies of the *S*-nitrosoproteome.

Table 1. Novel proteomic methods for the identification of *S*-nitrosylated cysteines in proteins.

Method	Principles
Direct detection of SNO proteins by MS	
ESI-QTOF [57,58]	Preserve the S–NO bonds Direct detection of *S*-nitrosylation sites in proteins
MAD-MS [59]	Use of a beam of helium metastable atoms Produce more fragment ions, which preserve the S–NO modification
New chemical derivatization methods	
Gold nanoparticles [60,61]	Blockage of Cys thiols with IAM Proteolysis and incubation with gold nanoparticles, which react with SNO peptides Incubation with DTT to release the bound peptides LC-MS/MS to identify the SNO protein sites
Phenylmercury-based methods [18]	Reaction with Cys–SNO to form relatively stable thiol–mercury bonds Use of an organomercury resin to capture SNO proteins and peptides, on-column tryptic digestion and LC-MS/MS
Phosphine-based methods [62,63]	Phosphine-based compounds (biotin-labeled phosphine substrate [62], water-soluble phosphine TXPTS [63]) Convert unstable SNO to stable and detectable products Mass detection by MS
Computational methods for the identification of Cys–SNO sites [64,65]	GPS 3.0 algorithm [64] Software GPS-SNO 1.0 [64] Software CPR-SNO [65]

Cys: Cysteine; DTT: Dithiothreitol; IAM: Iodoacetamide; LC: Liquid chromatography; MAD-MS: Metastable atom-activation dissociation mass spectrometry; MS: Mass spectrometry; QTOF: Quadrupole time of flight; SNO: *S*-nitrosothiol; TXPTS: Tris(4,6-dimethyl-3-sulfonatomethyl)-phosphine trisodium salt hydrate.

Computational methods for the identification of Cys–SNO sites

Computational studies have been shown to be able to rapidly generate helpful information about PTMs for further experimental verification. Therefore, computational approaches for the prediction of PTM sites are receiving considerable attention, and a great number of databases and computational tools have been developed for PTM analyses. Although computational prediction of SNO sites in proteins remains a challenge, some recently developed methods represent a major advance in this direction. Thus, Xue and colleagues have reported the improvement and release of the GPS 3.0 algorithm and the development of the novel computational software GPS-SNO 1.0 [101] for prediction of SNO sites [64]. In their work, they used data from 504 experimentally verified SNO sites in 327 unique proteins, which were obtained from the scientific literature and public databases. Then, they also collected 485 potentially *S*-nitrosylated substrates from large- or small-scale studies, in which the exact SNO sites had not been experimentally determined. After the application of GPS-SNO 1.0, they predicted 359 (74%) of these targets with at least one potential SNO site. The incorporation of a set of supervised methods algorithm (support vector machine) have been recently used to develop a new efficient predictor, CPR-SNO [65]. The developers claim that CPR-SNO has a better performance that GPS-SNO 1.0. The predictions from these and other computational methods might be of use for annotating large-scale potential SNO sites and for further experimental verification.

The study of the *S*-nitrosoproteome in disease

The new tools available for the analysis of protein *S*-nitrosylation allow us to study the *S*-nitrosoproteome on a global scale. Although methodological issues remain, the improvement of existing methods, along with their fine-tuning, is now encouraging the use of proteomic approaches to decipher the role of protein *S*-nitrosylation in the context of different pathologies. Certainly, the use of such nitrosoproteomic methods will suggest novel mechanisms for the dysregulation of protein *S*-nitrosylation in disease and add new potential therapeutic targets. Many *S*-nitrosylated peptides and proteins have been implicated in mammalian pathophysiology and the number is growing steadily [3]. However, the number of published studies dealing with the *S*-nitrosoproteome in a pathological setting is still limited.

Recent research has uncovered a connection between deregulated *S*-nitrosylation of proteins and neurodegeneration [66]. However, to date there are no comprehensive proteomic studies of *S*-nitrosylation in neurodegenerative diseases. Nevertheless, Bizzozero and Zheng have reported the identification of major *S*-nitrosylated proteins in murine experimental autoimmune encephalomyelitis (EAE), which serves as a model for the human disease multiple sclerosis [67]. In this study, SNO proteins in the spinal cord from control and EAE mice at various disease stages were biotin-labeled using the BST, and streptavidin-bound fractions were analyzed by blotting using antibodies against previously identified major *S*-nitrosylated substrates of spinal cord tissue. The authors found that the *S*-nitrosylation state of several proteins, particularly neuronal specific enolase and glyceraldehyde-3-phosphate dehydrogenase, increased in EAE. However, is still unclear whether increased *S*-nitrosylation of these or other proteins in the course of the disease is indeed deleterious.

NO has long been recognized as a key mediator in liver physio(patho)logy, and a number of recent studies suggest that deregulation of *S*-nitrosylation may be the cause of a number of liver disorders. Our group has recently reported that the deregulation of GSNOR during induced cholestatic liver injury enhances *S*-nitrosylation of hepatic proteins in rats [8]. The BST-based proteomic analysis of *S*-nitrosylated proteins in cholestatic livers revealed important enzymes responsible for energy production and metabolism, as well as molecular chaperones and proteins involved in the structural integrity of the cells. Furthermore, the *S*-nitrosylation during cholestasis of methionine synthetase and betaine-homocysteine *S*-methyltransferase, which are important enzymes involved in the methionine cycle, suggest novel therapeutic targets in cholestatic liver injury [8]. Notably, GSNOR deficiency in the liver has recently been shown to promote hepatocarcinogenesis via *S*-nitrosylation and proteasomal degradation of the key DNA repair enzyme O(6)-alkylguanine-DNA alkyltransferase [11]. These and other studies [31] highlight the importance of GSNOR in maintaining *S*-nitrosothiol homeostasis in the liver and encourage further proteomics research on hepatic *S*-nitrosylation/denitrosylation and their role in liver disease.

NO plays an important role in virtually all aspects of cardiovascular physiology and the emergence of SNO signaling portends a new era in cardiovascular biology [6]. Therefore, some recent studies have addressed the *S*-nitrosoproteome in several cardiovascular conditions. Kohr and coworkers have described a new protocol based on the SNO-RAC method, for the measurement and site determination of protein Cys oxidation (Ox-RAC) [68]. They used this new method in tandem with the SNO-RAC technique to demonstrate that ischemia preconditioning (IPC)-induced protein *S*-nitrosylation shields critical Cys residues against oxidation. IPC transiently increased protein *S*-nitrosylation in mice hearts and a total of 47 SNO sites in 33 unique SNO proteins were identified. Interestingly, a high proportion (76%) of these proteins exhibited reduced or no oxidation at the same site following ischemia and early reperfusion, suggesting that *S*-nitrosylation provides a direct protective effect against Cys oxidation following ischemia reperfusion injury. On the other hand, *S*-nitrosylation has been implicated in ischemic brain injury, and Wiktorowicz and colleagues have recently studied endogenous brain SNO proteins in a rat hypoxia-ischemia/reperfusion model [47]. Using the SNOFlo method, they identified 41 proteins showing differential *S*-nitrosylation status. Among them, 21 proteins showed the greatest changes upon hypoxia induction, while 19 showed the greatest changes upon reperfusion. The functional analysis of differentially *S*-nitrosylated proteins indicated their involvement in apoptosis, branching morphogenesis of axons, cortical neurons and sympathetic neurites, neurogenesis and calcium signaling.

NO plays a crucial role in lowering vascular resistance of the uterus and placenta unit throughout pregnancy, and deregulated *S*-nitrosylation may participate in the pathogenesis of preeclampsia, a major complication of pregnancy causing maternal, fetal and neonatal morbidity and mortality. A recent study used the BST coupled with the CyDye/2D-DIGE method to compare the nitrosoproteomes in normotensive and preeclamptic human placentas [69]. The authors identified 41 human placental SNO proteins, and among them the levels of 15 SNO proteins were lowered and that of six other SNO proteins were increased by preeclampsia. Many of these SNO proteins were enzymes critical to protein synthesis, folding, PTM and degradation.

Expert commentary & five-year view

Since its discovery in 1987, many biological roles have been identified for NO, and SNO-mediated mechanisms are now recognized as key mediators of NO bioactivity. Over the past decade, hundreds of proteins [16] have been shown to become *S*-nitrosylated, and in many cases this modification is accompanied by altered function. Recent research has broadened our knowledge of SNO homeostasis, revealing some of the molecular mechanisms governing *S*-nitrosylation/denitrosylation of proteins. Thus, contributing factors for specificity of nitrosylation, including nytrosylase and denitrosylase enzymes, have recently been uncovered. In addition, recent advances in methods of detection and identification of SNO proteins are enabling the study of the nitrosoproteome under physiological and pathophysiological conditions. These global proteomic methods are further enabling the structural and functional characterization of the *in vivo* nitrosoproteome and are greatly contributing to uncovering structural features that can accommodate multiple mechanisms for *S*-nitrosylation *in vivo* [18,48,55]. Aberrant *S*-nitrosylation of proteins has been repeatedly reported to be associated with disease, and the analysis of nitrosoproteomes in pathological conditions is beginning to be reported. We can expect an increase in this type of studies in the immediate future, since diseased nitrosoproteomes constitute a promising foundation for the development of novel therapeutic targets. An additional logical step forward is the use of the available or further developed methods to quantify SNO proteins from biological fluids or tissues, with the aim of developing new diagnostic or prognostic biomarkers of disease. In the near future, the burgeoning evolution in proteomics will undoubtedly boost our knowledge concerning deregulated protein *S*-nitrosylation and disease.

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Key issues

- Nitric oxide (NO) can modulate cell function by the coupling of a nitroso moiety to a reactive cysteine in target proteins, leading to the formation of a *S*-nitrosothiol (SNO), a process commonly known as *S*-nitrosylation. There is increasing evidence of dysregulated *S*-nitrosylation in a wide spectrum of human diseases.
- The types and levels of NO donors, the thiol microenvironment, the cellular redox environment, and the presence of transnitrosylases and denitrosylases have been proposed as factors that determine the specificity of *S*-nitrosylation.
- The development of the biotin-switch technique, an original approach that permitted the selective tagging of SNO proteins with biotin, made the proteomic analysis of protein *S*-nitrosylation feasible. Recently, a number of significant modifications and enhancements of the biotin-switch technique have been developed to perform proteomic identification of SNO proteins and/or the identification of specific sites of nitrosylation.
- Array-based methods, direct detection by mass spectrometry, new chemical derivatization strategies and computational methods for the identification of cysteine–SNO sites are emerging as novel and promising tools for the study of the nitrosoproteome.
- Although methodological issues remain, the improvement of existing methods along with their fine-tuning is now encouraging the use of proteomic approaches to decipher the role of protein *S*-nitrosylation in the context of different pathologies.

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