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Direct methods for detection of protein S-nitrosylation

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ABSTRACT

S-nitrosylation of protein cysteine residues is known to be an important mechanism for nitric oxide signaling. However, the detection of protein S-nitrosylation is still challenging due to technical limitations of current methods. This chapter provides a brief review on recent developments of methods, which directly target S-nitroso moieties for detection. We also describe in detail the protocol of an organophosphine-based biotin labeling of protein S-nitroso moieties.

1. Introduction

Nitric oxide (NO) is a cell signaling molecule involved in a number of physiological and pathophysiological processes. NO is synthesized by a family of enzymes known as nitric oxide synthases (NOS). Biological responses to NO are related to its in vivo reactions. NO can directly interact with some biomolecules such as heme centers and metalloproteins. NO can also undergo oxidation or other metabolic processes to form reactive nitrogen species (RNS). In particular, the reaction of RNS with protein cysteine residues (–SH) that results in S-nitrosylation (also known as S-nitrosation) has received a great deal of attention, because it represents an important post-translational modification (PTM) that transduces NO-dependent signals. To date, over 3000 peptides and proteins have been characterized and studied as S-nitrosylation targets. In many cases S-nitrosylation is believed to regulate protein function and activity with consequences reflected both physiologically and pathologically.

Although significant progress has been made to understand the biological importance of protein S-nitrosylation, the detection of S-nitrosylation in complex biological sample is still a challenge [1–11]. The products of S-nitrosylation are S-nitrosothiols (SNO). From a chemistry point-of-view, all of protein SNO belong to primary SNO compounds, which are unstable compounds due to the reactive/unstable nature of the S–N bond. It is known that SNO can easily undergo photolytic decomposition to form NO and thiyl radicals ($RS^{\cdot}$). Cytosolic reducing agents such as ascorbate, glutathione, and reduced metals, especially Cu(I), can also break S–N bonds. One should be aware of these possible side reactions when attempting to measure SNO concentrations in certain biological samples.

In the past years, many methods have been developed for the detection of SNO. These methods can be classified into two categories: (1) indirect detection methods and (2) direct detection methods. Indirect methods are widely used and very popular in the field. By definition, these methods are not targeting the SNO adducts as a whole to generate the detection signals. Instead, they usually break the unstable S–N bonds and capture either the sulfur or the nitrogen part for detection. Representative methods in this category include (1) chemiluminescence methods to analyze NO radicals generated from SNO; (2) Saville assay to analyze nitrite (NO_2^-) generated from SNO; (3) fluorescence detection (using 4,5-diaminofluorescein, for example) to analyze NO^+ generated from SNO; and (4) biotin-switch based methods to conjugate free thiols generated from SNO. These methods have made significant contributions to SNO research. In particular, a method reported two decades ago by Stamler and Loscalzo that involves the photolytic dissociation of the RS–NO bond followed by chemiluminescence, has been the landmark for quantitative analysis of protein S-nitrosylation [12,13]. The basis of this method stands on the ability of S–NO bond to undergo homolytic cleavage upon UV irradiation (~300–350 nm), to yield a thiyl radical (RS) and NO. Then NO reacts with ozone (O_3) and the light produced from the reaction can be detected by chemiluminescence. This method has been used to detect individual SNO-proteins following their purification [14,15]. A recent modification by Schoenfisch employs visible photolysis (500–550 nm) followed amperometric NO detection [16]. This strategy proved to be highly sensitive.

In this chapter, we will not review the progress in the category described above. Readers interested in indirect methods should

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read some review chapters published recently [1–11]. We will focus our attention to review recent developments in direct methods for SNO detection in proteins. We will also provide detailed protocols of a direct biotinylation method developed by this laboratory.

2. Direct methods for the detection of protein S-nitrosylation: non-derivatization based methods

Direct methods target intact SNO moieties in samples. These methods should be able to selectively recognize SNO from complex biological systems and generate sufficient signals for detection. Non-derivatization based methods in this category include mass spectrometry and anti-SNO antibody based methods.

2.1. Mass spectrometry

Identification of protein SNO sites by mass spectrometry is usually challenging because of the lability of the SNO groups. Commonly employed mass ionization sources, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), under typical conditions can induce dissociation of the S–N bonds [17–19]. ESI is more suitable than MALDI for qualitative analysis because ESI conditions are milder and may preserve the labile SNO groups. It was reported that, under very strict ionization conditions, S-nitrosylated proteins can be analyzed by mass spectrometry. One example is the characterization of SNO-thioredoxin-1 by Wang et al. [20]. In this example, thioredoxin-1 was subjected to S-nitrosylation, precipitation, digestion, and finally analyzed by LC/MS/MS using ESI quadrupole time of flight (QTOF) mass spectrometry. The authors stated that the optimization of voltage and collision energy was critical to achieve reliable signal/noise ratios. Buffer composition was also carefully controlled to minimize S–NO depletion. Although thioredoxin-1 was effectively analyzed by this technique, the application of it to other proteins will require extensive adjustment of experimental parameters. The stability of SNO in peptides is dependent upon the peptide sequence. Therefore the optimal voltage of ionization for any given peptide should be determined separately. In addition, analysis of mass results may not be trivial. Under some circumstances 3-nitroso (3-NO) tyrosine could be formed as the nitrosylation product, [21] and this could be misidentified as an SNO moiety. Therefore, control experiments must be performed to rule out possible false positive results regarding this post-translational modification. Up to date, the expansion of direct mass spectrometry-based methods for SNO proteomic studies is still limited and very challenging.

2.2. Anti-S-nitrosocysteine antibody: Immunohistochemical approach

Detection of protein S-nitrosylation using traditional methods like immunoprecipitation or Western blotting is not typically performed because the unstable S–N bonds may be broken during SDS–PAGE separation. Nevertheless the antibody can be used on non-reducing gels and to immunoprecipate SNO-proteins [22,23]. There has been some progress using anti-S-nitrosocysteine antibodies as a detection tool. Both monoclonal [24] and polyclonal antibodies [25] have been produced which bind the SNO functionality. This method consists of labeling SNO-protein with a primary antibody, anti-S-nitrosocysteine, followed by detection with a secondary antibody labeled with a chemical or biochemical reporter (horseradish peroxidase, ^{125}I, biotinylated secondary antibody or fluorescein isothiocyanate). The anti-SNO antibody method has been applied for immunohistochemical detection of S-nitrosylated proteins in cells and in lung [25] and vascular [24,26] tissues. Both positive and negative controls are required and should be conducted on serial sections in which the test section is flanked by both a positive and a negative control.

3. Direct methods for the detection of protein S-nitrosylation: derivatization based methods

Methods in this category usually rely on chemical approaches using reagents which can directly react with SNO to form stable adducts. Reagents based on these reactions can be used to enrich SNO proteins and analyze SNO components.

3.1. Gold nanoparticles

Some transition metals like gold have high affinity toward sulfur. Recent studies found that gold nanoparticles (AuNP) can react with SNO to form AuNP-protein thiolate conjugates [27]. Based on this reactivity, Mutus et al. developed a AuNP enrichment method for identifying protein SNO sites [28]. This method is described in Scheme 1. Free thiols in protein are first blocked by a SH-alkylation reagent-iodoacetamide (IAM). The protein is then proteolyzed and the digested fragments are treated with AuNP. SNO containing peptides are expected to react with AuNP to form AuNP-thiolate conjugates. The AuNP-bound peptides can then be harvested, released from AuNP surface, and finally identified by mass spectrometry.

This method is quite sensitive and easy to use. However, like the biotin switch method, this method is still a subtractive method. Since AuNP can also react with thiols to form AuNP-thiolate conjugates, all free thiols in protein have to be blocked. Given the fact that SNO concentrations are very low compared to free thiols in real biological systems, incomplete free thiol blocking could lead to false positive results. Another problem is that the target protein was subjected to proteolysis to generate SNO-peptides before AuNP was introduced. It is unclear how much SNO can be retained in peptide fragments. The environment/structure change on SNO adducts could dramatically affect the stability of SNO. Therefore some SNO peptides could decompose under the proteolysis step to form other products like disulfides. Finally, the authors stated that bulky disulfide containing peptides should not interact with AuNP, while less bulky disulfide peptides may react with AuNP to form Au-thiolate conjugates. This may also lead to false positive signals with some proteins.

3.2. Organomercury resin capture (MRC)

Organomercury complexes are known to react with S-nitrosothiols to form stable mercury-thiolate conjugates [29]. Ischiropoulos et al. applied this reaction in their proteomic studies of endogenous S-nitrosylation [30]. As shown in Scheme 2, this method consists of three steps: (1) free thiols are blocked with reagents like methyl methanethiosulfonate (MMTS); (2) SNO proteins are captured by organomercury reagents; (3) after enrichment, SNO proteins are released from the reagents and subjected to LC-MS/MS analysis. This method was adapted to both solid (method A) and solution phase (method B) sample enrichments by conjugating phenylmercury moiety with agarose beads or with a biotin tag. Performic acid was used to release bound protein or peptide fragments from the mercury surface. Interestingly, performic acid can oxidize the resulting thiols to sulfonic acid, producing a unique MS signature for site-specific identification.

This mercury based method has been used to identify a large library of SNO proteins ranging from 15 to 270 kDa. In addition, the organo-mercury reagents seem to be highly specific for SNO and do not react with disulfides to form mercury-thiolate conjugates [31]. However, organo-mercurial can react with free thiols and therefore