Current Methods for the Identification of Sites of *S*-Glutathionylation and *S*-Nitrosylation in Proteins

Adam Faccenda* and Bulent Mutus

Department of Chemistry & Biochemistry, University of Windsor, 401 Sunset Ave. Windsor, Ontario, N9B 3P4, Canada

Abstract: Chemical modification of protein thiols has profound effects on the structure and catalytic activity of proteins. These post-translational modifications are often transient in nature, and therefore characterization of them can be elusive, especially in the case of *S*-nitrosothiols. Many of the methods discussed here involve a trapping mechanism where the modified thiol is selectively reduced and covalently labeled, thus enabling secondary detection or isolation via various techniques. Protocols have also been developed to detect and map sites of *S*-glutathionylation and are also discussed here. Throughout this review, '*S*-modification' will refer to both *S*-nitrosylation and *S*-glutathionylation collectively.

Herein, we provide a brief review of current methodology available for the empirical elucidation of sites susceptible to *S*-modification, histochemical visualization of cellular compartments susceptible to *S*-modification as well as an account of factors currently known to dictate *S*-modification susceptibility.

Keywords: Nitric oxide, *S*-nitrosylation, *S*-glutathionylation, biotin switch assay, nanoparticle.

INTRODUCTION

The physiological relevance of nitric oxide (·NO) was discovered in the late 80's, a finding which was awarded the Nobel Prize for Physiology and Medicine in 1998. Since then, ·NO has been implicated in a diverse array of cellular functions including DNA replication and transcription, neurotransmission, hemostasis and vasodilation.

S-nitrosylation of cysteine residues of proteins plays a significant role in a number of cellular processes including vasodilation, neurotransmission, cellular localization, and cell cycle regulation [1]. This transient post-translational modification serves to stabilize, store and transport ·NO in the form of ·NO-transition metal complexes, low-molecular weight thiols, such as *S*-nitrosoglutathione (GSNO) or alternatively as *S*-nitrosoproteins. Identification of sites of *S*-nitrosylation in proteins is imperative in the understanding of the location, stability and frequency of these modifications.

The SNO moiety is notoriously unstable which makes characterization of SNO sites rather elusive. In the past, identification of SNOs often required harsh chemical modification that may have resulted in misidentification of other NO_x derivatives as SNOs [2]. Many of the detection strategies utilized in recent years are variations of Jaffrey's biotin switch protocol [3].

·NO itself is known not to react with thiols under anaerobic conditions [4], counterintuitive to the idea that hypoxic conditions increase protein *S*-nitrosylation in endothelial cells [5]. This observation may be explained by the sudden release of ROS known to occur in endothelial cells exposed to acute hypoxia allowing formation of higher oxides of ·NO [6]. Alternatively, the reaction of ·NO with cysteine to yield *S*-nitrosocysteine (CSNO) in the presence of an electron acceptor such as NAD+ might explain these observations [7]. The rate constant for the reaction of ·NO with O_2 in aqueous media is ~6 x 10^6 M^{-2} s^{-1} and the reaction is second order with respect to ·NO [8, 9]. The same rate constant is increased to 8.8 x 10^7 M^{-2} s^{-1} upon addition of phospholipid vesicles, suggesting the sequestering of both ·NO and O_2 in the hydrophobic regions of the vesicles, thus increasing the apparent rate of reaction [9]. The same phenomenon is thought to occur in the hydrophobic core of bovine serum albumin (BSA) producing N_2O_3, a potent nitrosylating agent, with a $t_{1/2} \approx 7$ min in the hydrophobic interior, much larger than it would be in aqueous media [10].

The redox status of the cell is under a dynamic balance and depends on the prompt release of redox active molecules. RSNOs serve this function by storing and stabilizing ·NO, then releasing it upon interaction with metal centers, protein thiols or other RSHs to affect the previously mentioned processes implicated to ·NO biology. Low molecular weight RSNOs may serve to transport ·NO in the blood stream. It has been suggested by our lab that circulating RSNOs are denitrosylated by cell surface protein disulfide isomerase (csPDI), which concentrates ·NO within the hydrophobic membrane where it can react with O_2 to produce N_2O_3. Intracellular thiols may then be nitrosylated at the cytosol-membrane barrier, thus constituting a proposed mechanism for csPDI-mediated ·NO transport into the cell [11]. Thioredoxin (Trx), a member of the same superfamily as PDI, was also recently reported to have denitrosylase activity [12] and a number of Trx SNO substrates were subsequently identified [13]. An additional mechanism of

*Address correspondence to this author at the Department of Chemistry and Biochemistry, University of Windsor, 401 Sunset Ave, Windsor, Ontario, N9B 3P4, Canada; Tel: 519-253-3000 ext: 3533; Fax: 519-973-7098;
E-mail: afaccenda@gmail.com

1875-0427/11

·NO transport and uptake in murine macrophage RAW264.7 is thought to involve the amino acid transport system [14].

In addition to other oxidative modifications of cysteinyl side chains, the chemical addition of glutathione, or *S*-glutathionylation (SG), has also been shown to be a transient post-translational modification and is considered to be the most highly oxidized thiol modification with the exception of sulfinic and sulfonic acid derivatives [15]. It has been identified as a marker for oxidative stress [16] and has been implicated in a number of pathologies including viral infection, cancer, Alzheimer's, atherosclerosis, lung disease and diabetes [17]. Several proteins have been characterized as being targets of *S*-glutathionylation [18, 19, 20], however not much is known about the functional consequence of this modification.

PROTEOMICS APPROACHES FOR SNO CHARACTERIZATION

The pioneering work of Jaffrey *et al.* [3] resulted in the development of the Biotin Switch assay, which relies on the selective reduction of SNO by ascorbate and reaction of the resulting thiol with a thiol-specific biotinylating reagent allowing the characterization of *S*-nitrosylated proteins. In the Biotin Switch assay, a partially *S*-nitrosylated sample is first treated with alkylating agent to block any existing free thiols. Ascorbate is then added to reduce *S*-nitrosothiols to free thiols followed by reaction with a thiol-reactive biotinylating agent. At this point of the experiment, one can either pass the biotinylated protein sample over an immobilized streptavidin resin to isolate biotinylated proteins, or alternatively, the relative level of *S*-nitrosylated proteins can be inferred by SDS-PAGE (1 or 2 dimensional) and subsequent Western Blot analysis with streptavidin-HRP. Jaffrey's group has also since replaced the biotin-based thiol-reactive label with a highly sensitive ^{35}S-labeled reagent [21].

Since its development, there have been several articles published as methods for the improvement of the biotin switch assay including substitution of ascorbate with sinapinic acid, a non-disulfide reducing alternative [22], the use of ferricyanide to stabilize the SNO moiety and the inclusion of copper to increase sensitivity [23], however the latter may cause loss of ascorbate selectivity and is generally not recommended. One caution that must be noted when utilizing a biotin-based label for thiols is the potential for false positives given by endogenously biotinylated proteins. This is usually properly controlled for by performing the experiment excluding the thiol-reactive biotin labeling reagent such that any signal observed on the Western Blot is the result of endogenously biotinylated proteins. Alternative thiol-reactive labels could also be used including the ^{35}S-label previously mentioned.

Characterization of denitrosylation targets is arguably equally as important as the elucidation of *S*-nitrosylation targets, however there are few known denitrosylation substrates. The equilibrium of these opposing modifications plays a major role in determining the redox tone of the cell. In light of their discovery of thioredoxin as a denitrosylase [12], Benhar *et al.* provide a method to identify a number of denitrosylation substrates of thioredoxin1 (Trx1) [13] in an approach akin to that of Kozarova *et al.* [24] for identifying redox-sensitive thiols using heavy/light labeling technology. In this experiment, control Jurkat cells were cultured with a heavy isotope-labeled amino acid-containing medium whereas Trx-treated cells were treated in a light-(natural isotope)-containing medium in a methodology termed 'stable isotope labeling by amino acids in cell culture' or SILAC. Each culture was individually lysed and *S*-nitrosylated by treatment with CSNO, followed by treatment with either vehicle (control) or a recombinant Trx system. The lysates were then combined and a typical biotin switch assay was performed. The combined lysate was then separated on a liquid chromatograph and analyzed by mass spectrometry. Proteins were identified by comparison to *in silico* digests using Mascot v2.2 and SILAC light/heavy peptide pairs were elucidated using Rosetta Elucidator v3.2 software. Isotopic differences in the growth medium of control and Trx-treated samples allowed for the relative quantification of each peptide from relative peak intensities. In this experiment, heavy amino acid pairs represent control and light pairs represent Trx-treated lysate. Also, the relative abundance of the peak intensities in the mass spectrum infers the relative amount of light or heavy peptides. The premise of the method is that any peptides that are denitrosylated by Trx will be blocked by the thiol blocking agent, not be biotinylated, and therefore will not be bound by the streptavidin column. In other words, light-labeled peptides of Trx SNO-substrates will appear at much lower abundance in the mass spectrum than their heavy-labeled counterparts.

Utilizing the SILAC method, ~280 proteins were identified as being *S*-nitrosylated, of which 145 were selected for further analysis. Of these, 46 proteins were identified as potential Trx substrates, belonging to diverse functional categories including cytoskeletal organization, metabolism, signaling and stress responses, demonstrating for the first time, a method for the large-scale identification of denitrosylation substrates.

SNO AND SG HISTOCHEMICAL TECHNIQUES

As mentioned above, endogenously produced ·NO is relatively short-lived with a half-life of only a few seconds[25]. For this reason, the locale of ·NO production is also an important determinant of which proteins it will target. *S*-nitrosylation targets have been shown to localize in certain organelles including the mitochondria, the Golgi complex and the nucleus [26, 27]. Existing methods for the visualization of *S*-nitrosylation target compartmentalization in intact tissue include the use of fluoro-conjugated antibodies to SNOs. However, due to the lability of the SNO moiety the specificity of these antibodies is questionable [28]. As an alternative, Ckless *et al.* [28] developed a modified biotin switch method for use with intact cells with minimal contribution from endogenously biotinylated proteins. Cell cultures were fixed and permeabilized on glass cover slips followed by the prototypical biotin switch protocol involving thiol blocking (MMTS), selective denitrosylation (ascorbate) and biotin labeling. Biotinylated proteins were labeled with a streptavidin-FITC conjugate and visualized by fluorescence confocal microscopy. An overall increase in nitrosylated proteins in a murine lung cell line upon treatment with cytokines TNFα and IFNγ was demonstrated. This method was also found amenable for use

with frozen tissues as well to assess *S*-nitrosylation levels in the nucleus.

In a similar study to assess levels of *S*-glutathionylation, Aesif *et al.* [29, 30] employed glutaredoxin1 (Grx1) to selectively reduce and visualize protein mixed disulfides in a paraffin-embedded lung tissue. To this end, all free thiols were alkylated with *N*-ethylmaleimide (NEM). Grx1 was then added to reduce *S*-glutathionylated proteins and the newly generated thiols were then labeled with *N*-(3-maleimidylpropionyl) biocytin, a biotinylated thiol-reactive reagent. The tissues were then labeled with a fluoro-conjugated streptavidin and visualized via fluorescence confocal microscopy. Using this method, it was found that protein *S*-glutathionylation was most prevalent in the epithelium of the conducting airway and was found to increase upon exposure to oxidants, a finding consistent with the literature. Although valuable information may be drawn from these experiments, it must be noted that the incubation of the tissue with Grx1 requires a concomitant incubation of reduced glutathione (GSH) in order to maintain the Grx1 in its reduced (active) state. This brings to question the integrity of any inter- and intramolecular protein disulfides since GSH may reduce these disulfide bonds and cause exaggerated biotin-labeling of the tissue.

FACTORS DETERMINING S-MODIFICATION SUSCEPTIBILITY

It is well known that the cysteine content of a protein is an inadequate measure of its susceptibility to *S*-modification. This is well demonstrated in the case of skeletal muscle Ca^{2+} release/ryanodine receptor, which contains fifty cysteine residues, only one of which has been shown to be susceptible to *S*-nitrosylation [31]. The microenvironment surrounding the thiol, thiol pKa and solvent accessibility all play significant roles in determining thiol susceptibility to nitrosylation [21]. Additionally, evidence has shown that an acidic and/or basic amino acid-rich sequence flanking a cysteine residue effectively lowers its pKa, thus making it more susceptible to *S*-nitrosylation [1, 32].

Under anoxic conditions, ·NO is a poor nitrosating agent suggesting that it is the oxidized products of ·NO that are the predominant nitrosylating agents. Further, both ·NO and O_2 are known to be hydrophobic and, consequently, they both partition into the hydrophobic regions of both the cell and of individual proteins. Indeed, it has been suggested that proteins can catalyze their own nitrosylation in this manner, thus implicating three-dimensional protein structure as a factor for determining susceptibility to *S*-nitrosylation [31, 33]. It has also been suggested that the hydrophobic core of bovine serum albumin acts as a nitrosylation catalyst by promoting the oxidation of ·NO to nitrosonium (NO^+) or nitrous anhydride (N_2O_3) which results in the formation of low molecular weight RSNOs [10]. Additionally, in human vascular smooth muscle cells, cysteine residues susceptible to *S*-nitrosylation were found to reside in hydrophobic pockets in several proteins [1].

In a modified biotin switch method, Hamnell-Pamment *et al.* [34] developed a method for the determination of *S*-glutathionylation sites from ECV304 (human urinary bladder carcinoma) lysates. In this experiment, intact cells were treated with diamide which oxidizes any free thiols, then with NEM to alkylate free thiols. The cells were then lysed and selectively deglutathionylated by treatment with a mutant glutathione reductase in the presence of NADPH and wild type glutathione reductase. Previously glutathionylated thiols, now free, were subsequently labeled with either biotin-maleimide or biotin-(polyethylene oxide)-maleimide and proteolyzed. Biotinylated peptides were isolated by passage over an immobilized streptavidin-resin and analyzed by mass spectrometry.

The biotin-(polyethylene oxide)-maleimide derivative was used to simply increase the size of the label in an attempt to improve binding to the streptavidin-resin. It was found that inclusion of the polyethylene oxide spacer resulted in the identification of a single biotinylated peptide of γ-actin, a peptide that was not identified using the biotin label without spacer. Furthermore, it was observed that inclusion of the polyethylene spacer affected the metastable fragmentation of tryptic peptides, which leads to the caveat that fragmentation patterns may depend on the specific biotin label that is used, an affect that is not well documented. Nonetheless, three different proteins were identified as being *S*-glutathionylated, two of which were previously shown to be *S*-glutathionylated by the same group using a similar method [35].

NANOPARTICLE-BASED METHODS FOR ENRICHMENT OF CYSTEINYL PEPTIDES

Nanoparticles (NPs) are gaining much attention as a potential tool in many areas of research owing to their distinctive optical properties, and ease of isolation, high selectivity and high surface-to-volume ratios. Palani *et al.* [36] report the use of iron-based nanoparticles to selectively pull down cysteine-containing peptides from complex mixtures. In this study, the Fe_3O_4·SiO_2 NPs were first functionalized with phosphonate-(stabilizer)-terminated and amino-terminated functional groups. A thiol-binding moiety, *N*-succinimidyl 3-2(pyridyldithio) propionate (SPDP) was then covalently linked to the amino groups and the Fe_3O_4·SiO_2 NPs were applied to complex mixtures of tryptic peptides. Although the Fe_3O_4·SiO_2 NPs showed high specificity for Cys-peptides and their magnetic character allowed facile isolation, their affinity for modified Cys-peptides remains to be determined.

In a similar study, Susumu *et al.*[37] functionalize gold nanoparticles (AuNPs) with an *N*-ethylmaleimide-(NEM)-terminated ligand, which can be used to selectively and covalently bind thiol-terminated peptides. An amine-reactive chromophore, Cy5, is then conjugated to the terminal amino group of the peptide, thus allowing full quantitation of thiol-peptides per AuNP surface by monitoring changes in absorption intensity of the chromophore. One significant advantage of these AuNP-conjugates is their stability in solutions of high ionic strength and thiol concentration, conditions that cause the rapid aggregation of bare or citrate-capped AuNPs. This quality makes these AuNP-conjugates amenable to fully quantitative biological assays, one potential use being similar to that of the abovementioned SILAC method to quantitate thiol-peptides in a complex mixture.

AuNPs are of particular interest in the characterization of the *S*-nitroso- and *S*-glutathioproteome due to their well-

established near-covalent interaction with thiols. In a recent publication from our lab [38], citrate-capped AuNPs were employed to selectively bind and isolate for thiol-containing peptides of protein digests. Owing to the relatively low abundance of sulfur-containing amino acids in proteins, this method has the potential to significantly 'clean up' protein mass fingerprints. In this study, individual pools of reduced recombinant human dual-specificity phosphatase 12 (hYVH1) were either *S*-nitrosylated or *S*-glutathionylated followed by the alkylation of any remaining free thiol with iodoacetamide (IAM). SNO thiols were denitrosylated with ascorbate and alkylated with a different alkylating agent, *N*-ethylmaleimide (NEM). The samples were mixed, proteolyzed, and the digest was added to a suspension of AuNPs. AuNP-bound peptides could be easily isolated by centrifugation and the addition of excess dithiothreitol (DTT) eluted bound peptides by thiol-exchange at the gold surface, thus allowing for mass spectrometric analysis of previously AuNP-bound peptides. In the resulting mass spectrum, SNO thiols appear as S-NEM, SG peptides as free thiols (reduced upon surface thiol-exchange) and free thiols appear as *S*-carbamidomethylated. The sterics of amino acid side chains surrounding disulfide-containing peptides was presumed to preclude their interaction with AuNPs, and this was evidenced in a separate experiment [38]. Interestingly, alkylated thiol-peptides seemed to retain enough thiol character to maintain their affinity for the AuNPs.

AuNPs were found to selectively enrich for thiol-containing peptides including both modified and unmodified as well as thioether (i.e. methionine), thus significantly simplifying the mass fingerprint and subsequently allowing the unambiguous simultaneous identification of sites of both protein *S*-nitrosylation and *S*-glutathionylation by tandem mass spectrometry (MS/MS), demonstrating for the first time the accomplishment of this feat in a single step [38].

CONCLUSION

Many variations of methods for the labeling, isolation and identification of *S*-modification sites are available, each with their own advantages and pitfalls. It is evident that the complete characterization of the *S*-nitrosoproteome and *S*-glutathioproteome is inextricably dependent upon the accuracy, throughput, and specificity of the available techniques, thus continuing research for the betterment of these techniques is a necessity.

ACKNOWLEDGEMENT

None.

CONFLICTS OF INTERESTS

None.

REFERENCES

[1] Greco, T. M.; Hodara, R.; Parastatidis, I.; Heijnen, H. F.; Dennehy, M. K.; Liebler, D. C.; Ischiropoulos, H. Identification of s-nitrosylation motifs by site-specific mapping of the s-nitrosoproteome in human vascular smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.,* **2006**, *103*, 7420-7425.

[2] Fang, K.; Ragsdale, N. V.; Carey, R. M.; MacDonald, T.; Gaston, B. Reductive Assays for S-Nitrosothiols: Implications for Measurements in Biological Systems. *Biochem. Biophys. Res. Commun.,* **1998**, 252, 535-540.

[3] Jaffrey, S. R.; Erdjument-Bromage, H.; Ferris, C. D.; Tempst, P.; Snyder, S. H. Protein S-Nitrosylation: A Physiolgical Signal for Neuronal Nitric Oxide. *Nat. Cell Biol.,* **2001**, *3*, 193-197.

[4] Kharitnov, V. G.; Sundauist, A. R.; Sharma, V. S. Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J. Biol. Chem.,* **1995**, 270, 28158-28164.

[5] Chen, S. C.; Huang, B.; Liu, Y. C.; Shyu, K. G.; Lin, P. Y.; Wang, D. L. Acute hypoxia enhances proteins' s-nitrosylation in endothelial cells. *Biochem. Biophys. Res. Commun.,* **2008**, *377*, 1274-1278.

[6] Steiner, D. R.; Gonzalez, N C.; Wood, J. G. Interaction Between Reactive Oxygen Species and Nitric Oxide in the Microvascular Response to Systemic Hypoxia. *J. Appl. Physiol.,* **2002**, *93*, 1411-1418.

[7] Gow, A. J.; Buerk, D. G.; Ischiropoulos, H. A novel reaction mechanism for the formation of s-nitrosothiol *in vivo*. *J. Biol. Chem.* **1997**, *272*, 2841-2845.

[8] Wink, D.; Darbyshire, J. F.; Nims, R. W.; Saavedra, J. E.; Ford, P. C. Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol.,* **1993**, *6*, 23-27.

[9] Liu, X.; Miller, M. J. S.; Joshi, M. S.; Thomas, D. D.; Lancaster, J. R. Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci U.S.A..,* **1998**, *95*, 2175-2179.

[10] Rafikova, O.; Rafikov, R.; Nudler, E. Catalysis of s-nitrosothiols formation by serum albumin: the mechanism and implication in vascular control. *Proc. Natl. Acad. Sci. U.S.A.,* **2002**, *99*, 5913-5918.

[11] Ramachandran, N.; Root, P.; Jiang, X. M.; Hogg, P. J.; Mutus, B. Mechanism of transfer of no from extracellular s-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. *Proc. Natl. Acad. Sci. U.S.A.,* **2001**, *98*, 9539-9544.

[12] Benhar, M.; Forrester, M. T.; Stamler, J. S. Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nat. Rev. Mol. Cell Biol.,* **2009**, *10*, 721-732.

[13] Benhar, M.; Thompson, J. W.; Moseley, M. A.; Stamler, J. S. Identification of s-nitrosylated targets of thioredoxin using a quantitative proteomic approach. *Biochemistry* **2010**, *49*, 6963-6969.

[14] Zhang, Y.; Hogg, N. The mechanism of transmembrane s-nitrosothiol transport. *Proc. Natl. Acad. Sci. U.S.A.,* **2004**, *101*, 7891-7896.

[15] Spadaro, D.; Yun, B. W.; Spoel, S. H.; Chu, C.; Wang, Y. Q.; Loake, G. J. The Redox Switch: Dynamic regulation of protein function by cysteine modifications. *Physiol. Plant.*, **2010**, *138*, 360-371.

[16] Gallogly, M. M.; Mieyal, J. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr. Opin. Pharmacol.,* **2007**, *7*, 381-391.

[17] Shelton, M.; Mieyal, J. J. Regulation by reversible s-glutathionylation: molecular targets implicated in inflammatory diseases. *Mol. Cells,* **2008**, *25*, 332-346.

[18] Fratelli, M.; Demol, H.; Puype, M.; Casagrande, S. ; Villa, P. ; Eberini, I. ; Vandekerckhove, J. ; Gianazza, E.; Ghezzi, P. Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics,* **2003**, *3*, 1154-1161.

[19] Casagrande, S.; Bonetto, V.; Fratelli, M.; Gianazza, E.; Eberini, I.; Massignan, T. ; Salmona, M. ; Chang, G. ; Holmgren, A. ; Ghezzi, P. Glutathionylation of human thioredoxin: a possible crosstalk between the glutathione and thioredoxin systems. *Proc. Natl. Acad. Sci.* U.S.A., **2002**, 9745-9749.

[20] Fratelli, M.; Demol, H.; Puype, M.; Casagrande, S. ; Eberini, I. ; Salmona, M. ; Bonetto, V. ; Mengozzi, M. ; Duffieux, F.; Miclet, E.; Bachi, A.; Vandekerckhove, J.; Gianazza, E.; Ghezzi, P. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human t lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **2002**, *99*, 3505-3510.

[21] Jaffrey, S. R.; Fang, M.; Snyder, S. H. Nitrosopeptide Mapping: a novel methodology reveals s-nitrosylation of dexras1 on a single cysteine residue. *Chem. Biol.,* **2002**, *9,* 1329-1335.

[22] Kallakunta, V. M. ; Staruch, A. ; Mutus, B. Sinapinic acid can replace ascorbate in the biotin switch assay. *Biochim. Biophys. Acta.,* **2010**, *1800*, 23-30.

[23] Wang, X.; Kettenhofen, N. J.; Shiva, S.; Hogg, N.; Gladwin, M. T. Copper dependence of the biotin switch assay: modified assay for measuring cellular and blood nitrosated proteins. *Free Radical Biol. Med.,* **2008**, *44*, 1362-1372.

[24] Kozarova, A.; Sliskovic, I.; Mutus, B.; Simon, E. S.; Andrews, P. C.; Vacratsis, P. O. Identification of redox sensitive thiols of protein disulfide isomerase using isotope coded affinity techology and mass spectrometry. *J. Am. Soc. Mass. Spectrom.,* **2007**, *18*, 260-269.

[25] Archer, S. Measurement of Nitric Oxide in Biological Models. *FASEB J.,* **1993**, *7,* 349-360.

[26] Mannick, J. B.; Schonhoff, N.; Papeta, N.; Ghafourifar, P.; Szibor, M.; Fang, K.; Gaston, B. S-Nitrosylation of mitochondrial caspases. *J. Cell Biol.,* **2001**, *154*, 1111-1116.

[27] Iwakiri, Y.; Satoh, A.; Chatterjee, S.; Toomre, D. K.; Chalouni, C. M.; Fulton, D.; Groszmann, R. J.; Shah, V. H.; Sessa, W. C. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein s-nitrosylation and protein trafficking. *Proc. Natl. Acad. Sci. U.S.A.,* **2006**, *103*, 19777-19782.

[28] Ckless, K.; Reynaert, N. L.; Taatjes, D. J.; Lounsbury, K. M.; van der Vliet, A.; Janssen-Heininger, Y. In situ detection and visualization of s-nitrosylated proteins following chemical derivatization: identification of ran gtpase as a target for s-nitrosylation. *Nitric Oxide,* **2004**, *11*, 216-227.

[29] Aesif, S. W.; Anathy, V.; Havermans, M.; Guala, A. S.; Ckless, K.; Taatjes, D. J.; Janssen-Heininger, Y. M. W. In situ analysis of protein s-glutathionylation in lung tissue using glutaredoxin-1-catalyzed cysteine derivatization. *Am. J. Pathol.,* **2009**, *175*, 36-45.

[30] Aesif, S. W.; Janssen-Heininger, Y. M. W.; Reynaert, N. L. Protocols for the detection of s-glutathionylated and s-nitrosylated proteins in situ. *Methods Enzymol.,* **2010**, *474*, 289-296.

[31] Williams, J. G.; Pappu, K.; Campbell, S. L. S-Nitrosylation and nitric oxide-mediated guanine nucleotide exchange. *Proc. Natl. Acad. Sci. U.S.A.,* **2003**, *100*, 6376-6381.

[32] Stamler, J. S.; Toone, E. J.; Lipton, S. A.; Sucher, N. J. (S)NO Signals: translocation, regulation, and a consensus motif. *Neuron,* **1997**, *18*, 691-696.

[33] Nedospasov, A.; Rafikov, R.; Beda, N.; Nudler, E. An autocatalytic mechanism of protein nitrosylation. *Proc. Natl.,* **2000**, *97*, 13543-13548.

[34] Hamnell-Pamment, Y.; Lind, C.; Palmberg, C.; Bergman, T.; Cotgreave, I. Determination of site-specificity of s-glutathionylated cellular proteins. *Biochem. Biophys. Res. Commun.*, **2005**, *332*, 362-369.

[35] Lind, C.; Gerdes, R.; Hamnell, Y.; Schuppe-Koistinen, I.; von Lowenhielm, H. B.; Holmgren, A.; Cotgreave, I. A. Identification of s-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch. Biochem. Biophys.*, **2002**, *406*, 229-240.

[36] Palani, A.; Lee, J. S.; Huh, J.; Kim, M.; Lee, Y. J.; Chang, J. H.; Lee, K.; Lee, S. W. Selective enrichment of cysteine-containing peptides using spdp-functionalized superparamagnetic Fe_3O_4@SiO_2 nanoparticles: application to comprehensive proteomic profiling. *J. Proteome Res.,* **2008**, *7,* 3591-3596.

[37] Susumu, E. O.; Blanco-Canosa, J. B.; Medintz, I. L.; Dawson, P. E.; Mattoussi, H. Preparation of stable maleimide-functionalized au nanoparticles and their use in counting surface ligands. *Small*, **2010**, *6,* 1273-1278.

[38] Faccenda, A.; Bonham, C. A.; Vacratsis, P. O.; Zhang, X.; Mutus, B. Gold nanoparticle enrichment method for identifying s-nitrosylation and s-glutathionylation sites in proteins. *J. Am. Chem. Soc.,* **2010**, *132*, 11392-11394.

Received: October 13, 2010 Revised: March 15, 2011 Accepted: March 15, 2011