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Mass spectrometry-based identification of S-nitrosocysteine *in vivo* using organic mercury assisted enrichment

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Abstract

Protein S-nitrosylation is considered as one of the molecular mechanisms by which nitric oxide regulates signaling events and protein function. The present review presents an updated method which allows for the site-specific detection of S-nitrosylated proteins *in vivo*. The method is based on enrichment of S-nitrosylated proteins or peptides using organomercury compounds followed by LC-MS/MS detection. Technical aspects for determining the reaction and binding efficiency of the mercury resin that assists enrichment of S-nitrosylated proteins are presented and discussed. In addition, emphasis is given to the specificity of the method by providing technical details for the generation of four chemically distinct negative controls. Finally it is provided an overview of the key steps for generation and evaluation of mass spectrometry derived data.

Keywords

cysteine modification; mass spectrometry; nitric oxide; protein S-nitrosylation

1. Introduction

Protein S-nitrosylation, the covalent addition of a nitric oxide equivalent to a reduced thiol, is considered as one of the mechanisms by which nitric oxide exerts its signaling effect *in vivo*. Experimental data has shown that S-nitrosylation alters the activity, the topology and affects protein-protein interaction *in vivo* [1–4]. However, important aspects such as the mechanism(s) by which S-nitrosylation occurs *in vivo*, the dependency of the modification on different isoforms of nitric oxide synthases (NOS), the factors that govern its selectivity and finally its relation with functionality or a phenotype, are under investigation. To gain insights for the above critical missing information the precise mapping and acquisition of *in vivo* S-nitrosoproteome(s) could be exceptionally valuable. To this end, an approach which

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enriches specifically for S-nitrosylated proteins present in complex biological samples would be useful. Moreover, mass spectrometry provides high sensitivity that is required for the identification of low abundant proteins within complex biological samples. Therefore, an enrichment approach coupled with mass spectrometry-based approaches would facilitate the identification of the *in vivo* S-nitrosoproteomes. Using the direct and specific chemical reactivity of organic mercury towards S-nitrosothiols we developed a mass spectrometry-based proteomic approach for the detection of protein S-nitrosocysteine *in vivo* [5]. In wild type mouse liver we precisely pinpointed 328 sites belonging to 192 proteins with the majority of them being novel targets. S-nitrosylated proteins in the liver are centered to key metabolic pathways indicating that S-nitrosylation represents a molecular mechanism for regulation of metabolism in the liver by nitric oxide. Moreover, using lysates from eNOS^{-/-} mice it was revealed a high dependency of liver S-nitrosoproteome from eNOS-derived nitric oxide. Structural analysis revealed that the *in vivo* S-nitrosoproteome in the liver has structural elements to accommodate different mechanisms for S-nitrosylation [5]. Using the same method we explored the S-nitrosoproteome in the thymus of S-nitrosogluthathione reductase knockout (GSNOR^{-/-}) and wild type mice [6]. It was documented for first time that the genetic deletion of S-nitrosogluthathione reductase, which metabolizes GSNO *in vivo* [7,8], results in an extended GSNO-dependent S-nitrosoproteome. Moreover, the study revealed a potential role of S-nitrosylation in the regulation of apoptotic cell death in the thymus gland.

In the current review an extended protocol for the synthesis of mercury resin, the preparation of biological samples and the overall performance of the method. Emphasis is given to generation of negative controls and the steps that are critical for optimal performance of the method.

2. Affi-Gel-10 mercury resin

2.1. Solutions and Reagents

Affi-Gel-10 support, 4 × 25 ml (Catalog number: 153-6046, Biorad, USA)

Anhydrous Isopropyl alcohol plus for HPLC, 99.9% (Catalog number EM-PX1838-1, VWR, USA)

Ethanolamine, minimum 98% (Catalog number: E9508-100 ML, Sigma-Aldrich)

N,N-Dimethylformamide, sequencing grade (Catalog number: MD-X17306, Fisher Scientific, USA)

p-amino-phenylmercuric-acetate (Catalog number: A9563-25G, Sigma-Aldrich, USA)

Rabbit polyclonal anti-lysozyme antibody (Catalog number: Ab391, Abcam, USA)

2.2. General Description

Affi-Gel 501 has been used previously as an affinity chromatography method to isolate proteins with reduced cysteine residues. It is an organomercurial derivative of Affi-Gel-10 N-hydroxysuccinimide activated agarose gel. It is prepared by reacting Affi-Gel-10 with p-amino-phenylmercuric-acetate in isopropyl alcohol/dimethylformamide, followed by blocking of unreacted succinimide groups with ethanolamine. A revised procedure to generate Affi-Gel 501 for the enrichment of S-nitrosocysteine peptides and proteins is described in details below.

2.3. Manufacturing Procedure

1. One hundred ml of Affi-Gel 10 (agarose beads), shielded from light, are thawed for 30 minutes at room temperature.

2. The vials containing Affi-gel-10 are shaken gently until a uniform suspension is formed and the slurry is transferred onto a Buchner funnel.
3. The gel is washed with 300 ml of anhydrous isopropyl alcohol while it is gently mixed. Note that in all steps the gel should not be allowed to dry. In the case the gel gets dry it should be washed extensively with anhydrous isopropyl alcohol while being mixed to remove the air bubbles that are formed. In the case that insoluble to isopropyl alcohol agarose clumps are formed the synthesis process should stop and a new gel must be used.
4. Para-amino-phenyl-mercuric acetate (2.10 gr) is dissolved in 30 ml of N,N-dimethylformamide with stirring for 15–20 minutes at room temperature. It is critical that the mercury powder is fully dissolved before coupling to agarose beads.
5. Anhydrous isopropyl alcohol, 100 ml are added to the gel and the slurry is transferred into a clean dark bottle where mercury solution is added. The bottle is placed on a stirrer, set at low speed, for 4 hours at room temperature. *Note: coupling under anhydrous condition is preferable since there is no hydrolysis of the active esters in the absence of water. The only reaction which will take place is the one between the ester and the amino group of p-amino-phenyl-mercuric acetate.*
6. Unreacted groups that remain are blocked by the addition of 1 ml of ethanolamine to the gel slurry followed by stirring for 1h at room temperature.
7. The gel slurry is then transferred onto a Buechner funnel and washed with 300 ml of diethylformamide with gentle mixing.
8. The gel is washed with 1200 ml of anhydrous isopropyl alcohol while it is gently mixed.
9. After the final wash, the gel is suspended in 200 ml of anhydrous isopropyl alcohol and the slurry is stored into a dark bottle at 4°C. Resin should be used within 1–2 months.

2.4. Testing the reaction and binding efficiency of the organomercury resin

2.4.1 Reaction efficiency

1. A liver homogenate solution (prepared as described below) at concentration of 1 mg/ml is used. Protein suspension is treated with 2 μ M GSNO for 30 min at room temperature. *Note: if acetone precipitation is the preferable method for desalting the biological sample, protein concentration should not exceed 2 mg/ml since concentrated protein pellets become practically insoluble after acetone precipitation.*
2. Protein suspension is precipitated with 3 volumes of chilled acetone for 30 minutes at –20 °C.
3. Proteins are pelleted by centrifugation at $3500 \times g$ for 25 minutes at 4 °C.
4. The supernatant is discarded and the pellet is washed extensively with chilled acetone before being resuspended into blocking buffer (please see section 3.1 below) at final protein concentration of 0.5 mg/ml. Any insoluble material is removed by centrifugation at $3500 \times g$ for 5 minutes and transfer of the supernatant into clean tubes.
5. Blocking of reduced thiols and preparation of protein suspension for reaction with mercury resin are described in details at section 3.4.1.

6. Protein suspensions at concentration equal to or lower than 1.5 mg/ml are added to an equal volume of activated mercury resin and rocked for 1h at room temperature. An aliquot is kept for the quantification of protein-SNO levels before reaction with mercury.
7. Unbound proteins are separated from the resin by centrifugation at $1000 \times g$ for 5 minutes at room temperature and transferred to a clean tube.
8. The unbound fraction as well as the input (see step 6) are assayed for protein S-nitrosocysteine content using reductive chemistries coupled to ozone based chemiluminescence detection [9].
9. The protein S-nitrosocysteine content is normalized with protein concentrations and the reaction efficiency is calculated based on: $\text{Reaction Efficiency} = (1 - \text{SNO}_{\text{unreacted}} / \text{SNO}_{\text{input}}) * 100$. Typically, the reaction efficiency is greater than 95%.

2.4.2 Binding efficiency—The determination of binding efficiency is the most precise measure of resin efficiency. A protein with a known number of reduced cysteines can serve as a model protein for the determination of binding efficiency. The number of reduced cysteines can be determined by alkylation of the protein followed by in solution digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. We have used mature hen egg lysozyme, which has 7 cysteine residues. The protein is first mildly pre-reduced with 5 mM dithiothreitol (DTT) for 10 minutes and LC-MS/MS analysis of lysozyme tryptic digest revealed that the cysteine 24 was the only site being alkylated after iodoacetamide (IAA) treatment. Also, using the mercury assisted peptide capture it was revealed that cysteine 24 was a unique site for S-nitrosylation on this protein. Therefore, hen egg lysozyme could serve as a model protein for determination of binding efficiency since the ratio of S-nitrosocysteine and protein concentration is known and it is 1:1. Organic mercury reacts directly and efficiently with S-nitrosothiols (Saville reaction). Mercury displaces thiol-bound nitric oxide and forms a relatively stable covalent bond with the thiol. The mercury thiol bond is more stable than thiol nitrogen bond in solution therefore Saville chemistry can be employed to identify proteins modified by S-nitrosylation. In addition, mercury-thiol bond is cleaved by reducing or strong oxidizing agents such as β -mercaptoethanol and performic acid respectively allowing for identification of modified proteins and peptides by LC-MS/MS. Importantly, the mercury resin was tested also for chemical reactivity towards other cysteine modifications such as disulfide bonds, alkylation, glutathionylation, sulfinic and sulfonic acid. Hen egg lysozyme was alkylated, glutathionylated, and oxidized after treatment with IAA, glutathione, H_2O_2 and performic acid respectively. Liquid chromatography tandem mass spectrometry was used to confirm the introduction of these modifications on lysozyme molecule. Then, a specific amount of modified protein was spiked in a liver homogenate and the samples were processed by mercury-assisted S-nitrosocysteine enrichment. As a positive control, a homogenate was spiked with S-nitrosylated lysozyme. No cysteine containing lysozyme peptides were identified in any of the samples containing alkylated, glutathionylated or oxidized lysozyme. Only in the sample spiked with S-nitrosylated lysozyme was identified a single peptide containing cysteine 24. These findings indicated that mercury resin is chemically reactive towards S-nitrosothiols but not towards other cysteine modifications.

The experimental process for the determination of binding efficiency was as follows:

1. Hen egg lysozyme (1 mg/ml) was exposed to 5 μM GSNO for 30 minutes at room temperature.

2. Protein was precipitated with 3 volumes of chilled acetone for 30 minutes at -20°C .
3. Protein was recovered in pellet by centrifugation at $3500 \times g$ for 30 minutes at 4°C .
4. Pellet was washed extensively with chilled acetone and resuspended into homogenization buffer (please see section 2.1).
5. Steps 2 and 3 were repeated and the pellet was resuspended in homogenization buffer.
6. Twenty micrograms of S-nitrosylated lysozyme were spiked into 3 mg of UV-illuminated liver homogenate (UV-illumination removes the nitroso group from protein S-nitrosocysteine, for details see below) and the mixture was processed for mercury assisted protein capture as described below. Another liver homogenate containing 20 μg of control (non GSNO-treated) lysozyme was processed in parallel serving as a control for non-specific interaction of lysozyme with mercury resin.
7. Protein S-nitrosylation content was quantified using reductive chemistries coupled to ozone-based chemiluminescence detection.
8. Mercury-based capture of modified proteins as well as washes and elution of bound proteins were performed as is described in details below.
9. Fifty micrograms from unbound and the bound fractions from both samples were resolved by SDS-PAGE electrophoresis and the presence of lysozyme revealed by western blot analysis using an anti-lysozyme antibody.
10. Densitometric analysis was employed to determine the amount of lysozyme present across different fractions. Isolated lysozyme loaded on the same gel at different amounts, was used to construct a standard curve. Based on calculations 97% of the S-nitrosylated lysozyme loaded onto the column was recovered in the bound fraction indicating that the binding efficiency was 97%.

3. Protein and peptide capture using the mercury resin

3.1. Solutions and Reagents

All the reagents used were of analytical grade. Homogenization and loading buffers are prepared, filtered through a $0.22 \mu\text{m}$ filter and stored at room temperature for a period of one month. Detergents and denaturing agents are added before use.

- Blocking buffer: 250 mM Hepes, 1 mM DTPA, 0.1 mM neocuproine, pH 7.7, 2.5% SDS
- Equilibration buffer: 50 mM NaCl, 50 mM MES, 1 mM DTPA, pH 6.0
- Loading buffer: 250 mM MES, 1 mM DTPA, pH 6.0, 1% SDS
- Homogenization buffer: 250 mM Hepes, 1 mM DTPA, 0.1 mM neocuproine, pH 7.7, 1% TritonX-100

3.2. Preparation and activation of mercury columns

Special caution should be taken in order to avoid contamination of the sample with particles originated from the plastic columns and tubes. These particles get ionized during electrospray ionization (ESI) preventing the detection of low abundant peptides that exist in the biological sample. For protein capture, where the eluted proteins are separated on a gel,

disposable plastic columns can be used (for example PD-10 columns) since during protein electrophoresis any contaminant (detergent based or plastic particles) migrates faster than proteins and therefore the sample is cleaned. However, for peptide capture the use of glass columns with disposable filter is recommended. The filter is replaced after five uses. The process for preparation and activation of mercury resin containing columns is described below:

1. Conical tubes (15 and 50 ml) are washed with 50% acetonitrile ACN with rocking for 30 minutes followed by extensive washes with water.
2. Disposable PD-10 columns containing 10 ml of 50% (ACN) are rocked for 30 minutes at room temperature followed by extensive washes with water. The glass columns are extensively washed with water.
3. A desired volume of gel slurry, equilibrated at room temperature for 20 minutes, is poured into each column and the storage buffer (isopropyl alcohol) is allowed to flow through the column.
4. Gel is washed with 10 bed volumes of isopropanol and if necessary is mixed gently to remove air bubbles.
5. The gel is washed with 20 bed volumes of water. In this step the resin will swell, however, the volume should not be readjusted.
6. Resin is mixed again gently to remove air bubbles.
7. The resin is activated by washing with 20 bed volumes of 0.1 M NaHCO₃ pH 8.8 followed by 20 bed volumes of equilibration buffer.

Note: Activated resin should not be incubated with equilibration buffer for a long time. If the resin is not used immediately (within the next 60 minutes), it is preferable that the resin stays in activation buffer. Use equilibration buffer just before use.

3.3. Tissue homogenization

Homogenization process differs depending on the type of tissue. For soft tissues such as brain and liver a single homogenization step is enough to extract the proteins whereas for tissues such as heart and lung two steps are required. Sonication of the tissue should be avoided since the provided energy may break the sulfur-nitrogen bond eliminating protein S-nitrosylation. Also, the biological stability of protein S-nitrosocysteine is affected by light; therefore it is recommended that in all steps the tubes containing the samples are covered with aluminum foil.

1. Tissue is thawed quickly in light protected glass vial containing 2 ml of homogenization buffer with 1x protease inhibitors and chopped into small pieces using a TissueMizer (Tekmar, Mason, Ohio) with power set at 70%. For brain, liver and thymus tissues this step can be omitted.
2. Tissue is homogenized into chilled lysis buffer (10x of the original weight of frozen tissue) containing 1x protease inhibitors using a Teflon pestle and a Jumbo Stirrer (Fisher Scientific, Pittsburgh, PA). The process consists of three cycles of 20 strokes with half minute break in between the cycles. The homogenization process takes place on ice.
3. The homogenate is split into Beckman polyallomer tubes (Beckman Instruments, CA) and the volume is adjusted with lysis buffer (w/o inhibitors) to 5 ml per tube and centrifuged at 13000 × g for 30 minutes at 4°C (Sorval RC 5B Plus)

4. Supernatant is transferred carefully into new tubes trying to avoid the lipid layer on the top and the pellet is discarded.

Note: it is critical that the sample has the least contamination with lipids because their polar surface could represent a scaffold for nonspecific interactions between agarose and proteins.

5. Protein concentration is determined by Bradford assay using BSA as standard protein
6. The homogenate is aliquoted in amber tubes and placed at -80°C until use.

3.4. Samples preparation and generation of negative controls

The mercury assisted capture of protein S-nitrosocysteine is described in three basic steps:

1. Blocking of reduced thiols
2. Reaction with organic mercury compounds
3. Mass spectrometry based identification of modified proteins and their site of modification(s)

3.4.1. Blocking of reduced thiols

- 1 Protein aliquots are pulled out from -80°C and left to thaw in the dark.
- 2 Protein concentration is adjusted at 0.5 mg/ml with homogenization buffer and protein mixture is split into different tubes covered with aluminum foil.
- 3 Generation of negative controls: Four chemically distinct negative controls were employed for testing the specificity of the method, namely, UV-photolysis, reductive chemistries, metal catalyzed reduction by ascorbate and thiol-bound NO displacement by HgCl_2 . All four treatments resulted in greater than 99% elimination of protein S-nitrosocysteine as determined by reductive chemistries coupled to ozone-based chemiluminescence. Below are described the experimental conditions for the generation of all four negative controls. Typically it is recommended to use two negative controls per sample tested.

UV photolysis: S-nitrosothiols are sensitive to light and particularly to UV-light. Therefore, UV-illumination is considered an effective treatment for elimination of protein S-nitrosocysteine present in biological samples [10]. The time of exposure to UV-light depends on the protein S-nitrosocysteine content of the tissue. For example, the brain and the liver tissues, which have lower levels compared to the heart tissue, require 5 minutes of exposure whereas the heart requires about 10 minutes of exposure. On the other hand, prolonged exposure to UV light induces protein oxidation and protein cross-linking. The inclusion 0.1 M mannitol within the protein homogenate during photolysis reduces protein oxidation and prevents for the most part protein cross-linking. Inclusion of a low concentration of MMTS during photolysis can be beneficial as well. MMTS could block the reduced thiols (existing and newly formed by UV-photolysis of S-nitrosocysteine) and thus could prevent their further oxidation due to exposure to UV-light. The technical details about eliminating protein S-nitrosocysteine by UV-photolysis in liver homogenates are the following:

- To the protein homogenate 0.1 M mannitol and 5 mM MMTS are added, rocked at room temperature for 5 minutes and transferred into borosilicate glass vials
- The vials are placed on the top of an ice bucket and proteins are illuminated under a conventional UV-transilluminator for appropriate time.

Reducing agents: S-nitrosocysteine is labile under reducing conditions therefore reducing agents such as DTT and β -mercaptoethanol can be employed for the generation of negative controls. In addition to S-nitrosocysteine, disulfide bonds and reversibly oxidized thiols are reduced in the presence of DTT or β -mercaptoethanol. Therefore, reducing agents increase artificially the pool of reduced thiols in a biological sample. Since organic mercury has chemical reactivity against reduced thiols as well it is critical that these artificially generated sites for reaction are efficiently blocked in the next step.

- A sample is exposed to 5 mM DTT for 15 minutes at room temperature with rocking. If β -mercaptoethanol is used its concentration should be 15 mM since it is not such an effective reducing agent as DTT.

Copper/Ascorbate: S-nitrosothiols undergo reduction in the presence of ascorbate and the process becomes more efficient in the presence of transition metals such as copper and iron.

- A sample is exposed to 0.5 mM ascorbate/0.005 mM CuSO_4 (both prepared fresh in water) for 30 minutes at room temperature with rocking.

Mercury chloride: S-nitrosothiols react fast and efficiently with mercury salts and a relatively stable covalent bond is formed between thiol and mercury.

- A sample is exposed to 10 mM mercury chloride (prepared as 200 mM stock in water) for 30 minutes at room temperature with rocking.
- 4 Protein suspensions are mixed with 3 volumes of chilled acetone and precipitated for 30 minutes at -20°C .
 - 5 Proteins are then pelleted by centrifugation at $3500 \times g$ for 15 minutes at 4°C .
 - 6 Supernatants are discarded and pellets are extensively washed with chilled acetone (at least five washes with 5 ml acetone each wash).
 - 7 Pellets are reconstituted in 1–2 ml of 5% SDS-containing blocking buffer.
 - 8 Protein suspensions are transferred to clean tubes, diluted to 2.5% SDS using blocking buffer without SDS at a final protein concentration of 0.5 mg/ml and rocked for 2–3 minutes at room temperature.

Note: It is critical that protein pellets are entirely soluble before the addition of MMTS. If not, protein suspensions are heated at 50°C for five minutes prior to MMTS addition to fully solubilize protein. Prolonged heating of the proteins at 50°C increases their solubility but eliminates S-nitrosocysteine. Therefore, if after five minutes at 50°C there is insoluble material then samples are centrifuged at $4500 \times g$ for 5 minutes at room temperature and the supernatant is transferred carefully to clean tubes while the insoluble pellet is discarded.

- 9 MMTS at final concentration of 40 mM is added in each tube.
- 10 Samples are vortexed for 30 seconds, and then are placed for 35 minutes at 50°C with frequent vortexing (every 5 minutes).
- 11 Proteins are then precipitated with 3 volumes of chilled acetone for 30 minutes at -20°C .
- 12 Proteins are pelleted by centrifugation at $3500 \times g$ for 15 minutes at 4°C .
- 13 Pellets are extensively washed with chilled acetone (see step 8), reconstituted in blocking buffer, transferred to clean tubes and steps 11–12 are repeated. *It is*

critical that protein pellets are entirely soluble before proceeding to the next step. Otherwise follow note of step 8.

- 14 Proteins pellets are resuspended into sufficient volume of loading buffer to achieve 1–1.5 mg of protein per ml of resin, and then are loaded onto the columns.

3.4.2. Reaction with organic mercury resin

1. Protein suspension reacts with organic mercury, shielded from light, for one hour at room temperature (the columns are covered with aluminum foil).
2. The resin is washed with 50 bed volumes 50 mM Tris-Cl pH 7.5, 0.3 M NaCl, 0.5 % SDS.
3. The resin is then washed with 50 bed volumes 50 mM Tris-Cl pH 7.5, 0.3M NaCl, 0.05% SDS.
4. Wash the column with 50 bed volumes 50 mM Tris-Cl pH 7.5, 0.3 M NaCl, 1% TritonX-100 and 1M Urea.
5. Followed by 50 bed volumes 50 mM Tris-Cl pH 7.5, 0.3 M NaCl, 0.1% TritonX-100, 0.1 M Urea.
6. Finally the resin is washed extensively with at least 200 bed volumes of H₂O to remove residual detergent and denaturing agents. *During the washes with water the resin is mixed gently 4–5 times to facilitate more thorough removal of detergent or denaturing agents.*
7. Bound proteins are eluted with 10 ml of 50 mM β -mercaptoethanol in water.
8. Samples are stored at –80 °C until use.
9. For in column digestion, steps 7–8 are omitted. After the final wash with water, it is added an equal to bed volume of 0.1 M NH₄HCO₃ containing 1 ug/ml Trypsin Gold (Promega, Madison, WI).
10. Protein digestion takes place for at least 16 hours at room temperature in the dark.

Peptide elution

1. After the completion of protein digestion the resin is washed with 40 bed volumes of 1 M NH₄HCO₃ plus 0.3 M NaCl. *Note that during the washes with NH₄HCO₃ based buffers, CO₂ is formed which disturb the integrity of the bed affecting the flow within the resin. In this case, the resin is mixed gently and is allowed to settle before being washed with the next buffer.*
2. The resin is washed with 40 bed volumes 1M NH₄HCO₃
3. Followed by 40 bed volumes 0.1 M NH₄HCO₃
4. The resin is cleaned from salts by washes with at least 200 bed volumes of water. *In this step the resin is mixed gently 3–4 times during the washes.*
5. Bound peptides are oxidatively released by incubation with 1% performic acid (equal to bed volume) for 45 minutes at room temperature and collected into glass tubes. *Performic acid is synthesized by reacting 1% formic acid and 0.5% hydrogen peroxide for at least 60 minutes at room temperature with rocking in a glass vial shielded from light*

6. Eluates are stored at -80°C overnight followed by lyophilization and re-suspension into 300 μl of 0.1% formic acid. Peptide suspension are transferred to low retention tubes (Axygen, Union City, CA).
7. The volume is reduced to about 30 μl by speed vacuum.
8. Any remaining contaminant (mainly broken resin) is removed by centrifugation at $16000 \times g$ for 20 minutes at 4°C and recovery of the peptides in the supernatant.
9. Twenty μl of peptide suspension is transferred to an HPLC vial and submitted for MS/MS analysis.

3.4.3. Mass spectrometry based identification of modified proteins and their sites of modification

1. The protein fractions obtained from mercury resin assisted protein capture are concentrated using 10 kDa cut off filters initially and 10 kDa microconcentrators next until final volume of about 20 μl .
2. Proteins are loaded on NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) and electrophoresed in MOPS running buffer (Invitrogen, Carlsbad, CA) for approximately 2 cm.
3. Gels are stained with Colloidal blue (Invitrogen, Carlsbad, CA). Densitometric analysis is performed to verify that the intensities of UV-treated negative control is less than 90% of the untreated samples, otherwise samples are not processed.
4. Stained gels are processed by in-gel trypsin digestion or stored in 2 % acetic acid for up to 1 month.
5. Each lane was cut into 3 slices and individually digested with trypsin according to the standard protocol [11].
6. Tryptic peptide digests were analyzed by hybrid-Orbitrap mass spectrometer (Thermo Electron, San Jose, CA) as previously described [12].

4. Generation and evaluation of SEQUEST peptide assignments

The post mass spectrometry analysis has been described previously [12]. In brief, from the raw files, which contain the MS/MS spectra are generated the DTA files. DTA files are submitted to Sorcerer Sequest for database search and generation of potential sequence-to-spectrum peptide assignments. Finally, Scaffold is used to validate protein identifications and perform manual inspection of MS/MS spectra. For protein capture experiments the requirements are two unique peptides minimum per protein with confidence set at 99% and 80% for protein and peptide respectively. For validation of peptide capture experiments the false discovery rate (FDR), which is the number of peptides that hit to the reverse database is determined. False discovery rate serves as a general indicator of biological sample quality. To determine FDR they are used the SEQUEST X_{corr} assigned to every peptide by Scaffold software. Empirically defined X_{corr} thresholds are applied to filter cysteine-acid and non-modified peptides independently so the FDR is 5%. In addition, the number of cysteine containing peptides identified in both negative and untreated samples is used to calculate the false identification rate (FIR), which is a major determinant of method specificity. Notably, for all organs tested the FIR was below 5%. Finally, for peptide capture experiments, since protein identification is based on a single peptide all cysteine-containing spectra are manually inspected based on previously defined criteria [5].

Therefore, the current methodology identified independently proteins as well as sites of modification by S-nitrosylation. To construct the final list, the two independent lists are

compared side by side and they are accepted only the proteins for which a site(s) of modification have been identified too.

Abbreviations

ACN	acetonitrile
DTPA	Diethylene-triamine-pentaacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MMTS	<i>S</i> -Methyl methanethiosulfonate
SDS	sodium dodecyl sulfate

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