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Gold Nanoparticle Enrichment Method for Identifying *S*-Nitrosylation and *S*-Glutathionylation Sites in Proteins

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Abstract: We present a simple method by which gold nanoparticles (AuNPs) are used to simultaneously isolate and enrich for free or modified thiol-containing peptides, thus facilitating the identification of protein *S*-modification sites. Here, protein disulfide isomerase (PDI) and dual specificity phosphatase 12 (DUSP12 or hYVH1) were *S*-nitrosylated or *S*-glutathionylated, their free thiols differentially alkylated, and subjected to proteolysis. AuNPs were added to the digests, and the AuNP-bound peptides were isolated by centrifugation and released by thiol exchange. These AuNP-bound peptides were analyzed by MALDI-TOF mass spectrometry revealing that AuNPs result in a significant enrichment of free thiol-containing as well as *S*-nitrosylated, *S*-glutathionylated, and *S*-alkylated peptides, leading to the unequivocal assignment of thiols susceptible to modification.

The pioneering work of Stamler et al.[1] showed that NO• can modulate cellular signaling not only through the guanylate cyclase/cGMP pathway but also by *S*-nitrosylating proteins and small molecular weight thiols. A large volume of subsequent work has implicated *S*-nitrosylation of proteins in the regulation, subcellular compartmentalization, and degradation of proteins. Therefore, identification of the *S*-nitrosoproteome is an area of interest in the elucidation of reactive nitrogen species-(RNS)-mediated signaling pathways in health and disease.[2−7]

To date, ∼1000 plant, animal, and prokaryotic thiol proteins have been identified as potential *S*-nitrosylation targets.[2] However, the chemical characteristics of *S*-nitrosylated proteins, such as their low S−NO bond energy, photosensitivity, and susceptibility to reduction by ascorbate, thiols, thiol reducing agents, and Cu (I), complicate their facile isolation and detection.

Significant progress in the identification of the *S*-nitrosoproteome was made by introduction of the "biotin-switch" assay by Jaffrey et al.[8] Free protein-thiols are first blocked with an alkylation agent, followed by reduction of the protein *S*-nitrosothiols with ascorbate. In the final step, these newly formed thiols are reacted with thiol-specific biotinylating agents, resolved by gel electrophoresis and detected by Western blot. Recent improvements to minimize ascorbate side reactions in the "biotin-switch" assay include the use of *S*-NO stabilizers[5] or the replacement of ascorbate with nondisulfide reducing, denitrosylating agents, such as sinapinic acid.[9]

The saturation-labeling differential gel electrophoresis (DIGE) methods employing cysteine-specific fluorophores[10−13] have also been developed to identify both the thiol proteome and *S*-nitrosoproteome. The DIGE approach employs a pair of fluorescent maleimide-conjugated cyanine dyes to specifically label free thiols in multiple protein samples. Mixtures of differentially labeled proteins are resolved on the same 2D electrophoresis gel, and computer-aided analysis of the intensity differences between fluorophores compares the amount of *S*-nitrosylated (or thiolated) proteins.

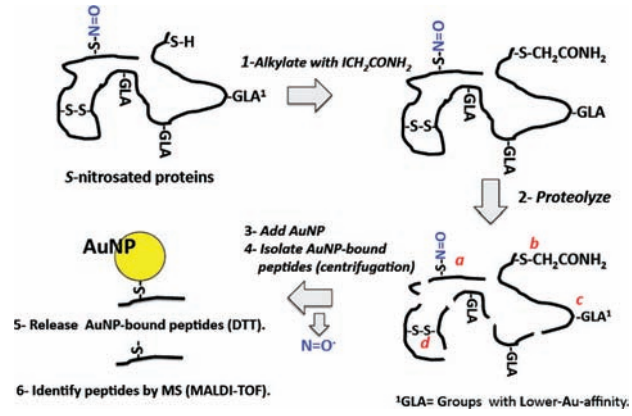
Recently, the "biotin-switch" method has also been used to directly isolate *S*-nitrosylated peptides from proteolyzed tissue lysates by capturing the biotinylated peptides on avidin beads for subsequent identification by LC-MS/MS techniques.[14,15]

Initially our aim was to use AuNPs to identify protein *S*-nitrosylation sites by taking advantage of the recently demonstrated property of AuNPs of reacting with SNO-proteins yielding NO• and AuNP-protein thiolates.[16]

The experimental strategy of this method is outlined in Scheme 1. Free thiols are first alkylated with iodoacetamide (IAM). The protein is proteolyzed, and AuNPs are introduced to the digest. The *S*-NO-peptides (Scheme 1a) are expected to react with the AuNPs to release NO• and form AuNP-thiolate peptides. Alkylated peptides (Scheme 1b) do retain some thiol character and are likely to have a higher affinity than the rest of the amino acid side chains (i.e., GLA-peptides, Scheme 1c). We predict that the sterics of the bulky residues surrounding disulfide-containing peptides (Scheme 1d) will preclude their interaction with AuNPs; however, less bulky glutathionylated peptides should interact with AuNPs yielding gold-thiolates.

The AuNP-bound peptides are harvested by centifugation and exchanged off of the AuNP surface by incubation with an excess of small molecular weight thiols such as dithiothreitol (DTT). The released peptides are then analyzed by mass spectrometry, and target peptide/

Scheme 1. AuNP-Enrichment Method for Identifying *S*-Nitrosylation Sites in Proteins



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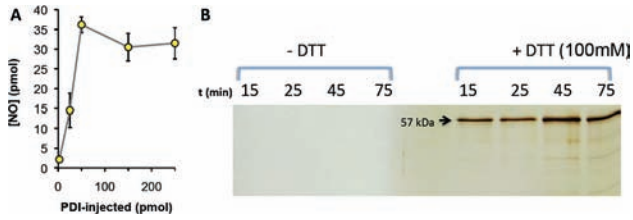


Figure 1. (A) AuNP-mediated release of NO• from SNO-PDI. Increasing amounts of SNO-PDI in 25 μL of phosphate buffered saline were added 1:1 to a constant amount ($\sim 5.3 \times 10^9$) of AuNPs in argon-purged, septa-sealed vials (2.0 mL). A 500 μL aliquot of the headspace was then injected into the NOA to quantify NO•. Error bars represent s.d. ($n = 3$). (B) SDS-PAGE of AuNP supernatants. AuNPs were isolated by centrifugation, washed, and incubated for the times indicated with SDS-PAGE sample buffer ± DTT (100 mM). The AuNPs were collected by centrifugation, and the supernatants were resolved by electrophoresis. Protein was visualized by silver stain.

protein assignment can be done by comparison to *in silico* digestion of known protein sequences or bioinformatic database mining.

Experimental Validation of Scheme 1. (i) AuNPs demonstrate a saturable capacity to release NO• from SNO-proteins. In order to confirm the ability of AuNPs to denitrosylate SNO-proteins, we mixed increasing amounts of *S*-nitrosylated protein disulfide isomerase (SNO-PDI)[17] with citrate-capped AuNPs (10−12 nm)[18] in sealed vials.

After 1 min, the headspace was removed and injected into a Sievers chemiluminescent NO• analyzer (NOA) for quantification of NO• release. These experiments clearly indicate that NO• is released upon interaction of AuNPs with SNO-PDI. NO-release reaches a plateau indicating that the AuNP surface becomes saturated with protein above ~50 pmol of PDI (Figure 1A).

(ii) Exchange of AuNP-bound proteins by small molecular weight thiols. AuNPs in the vials from (i) above were isolated, washed, and incubated with SDS-PAGE sample buffer with or without DTT. SDS-PAGE analysis of the supernatants indicated that PDI was only detectable in the DTT-containing samples; thus thiol-bound PDI can be released from AuNPs by thiol exchange within 45 min (Figure 1B).

(iii) AuNPs selectively enrich thiol-peptides from *S*-nitrosylated PDI and hYVH1 digests, enabling the identification of *S*-nitrosylation sites. Protein disulfide isomerase (PDI) is an ~57 kDa endoplasmic reticulum (ER) resident thiol oxidoreductase catalyzing isomerization and rearrangement of disulfide bonds thus ensuring the proper folding of nascent proteins.[19] PDI is also secreted from cells where it associates with the cell surface[20,21] and has previously been shown to be susceptible to *S*-nitrosylation *in vitro* and *in vivo*.[17,22]

PDI structural domains are arranged in an ***a*−*b*−*b*′−*a*′** topology, where the active site domains, ***a*** and ***a*′**, contain the vicinal thiols C36XXC39 and C379XXC382 respectively. In addition, the ***b*** and ***b*′** domains each contain an additional free thiol at positions C295 and C326 respectively. Previous studies have shown that ***a*′** active site thiols are susceptible to oxidation such that the native enzyme performs its catalytic function in a 1/2 oxidized state (i.e., ***a*** thiols reduced; ***a*′** thiols oxidized).[17,23]

hYVH1 (or DUSP12) is a member of the cysteine-based protein tyrosine phosphatase (PTP) superfamily and has been shown to be a cell survival phosphatase in response to various cellular insults.[24,25] This 37.5 kDa protein is constructed of an amino terminal catalytic domain containing four free thiols, C16, C28, C102, and the active site nucleophile C120. Unique to this protein is a carboxy-terminal zinc-binding domain containing seven zinc-coordinating thiols.[26]

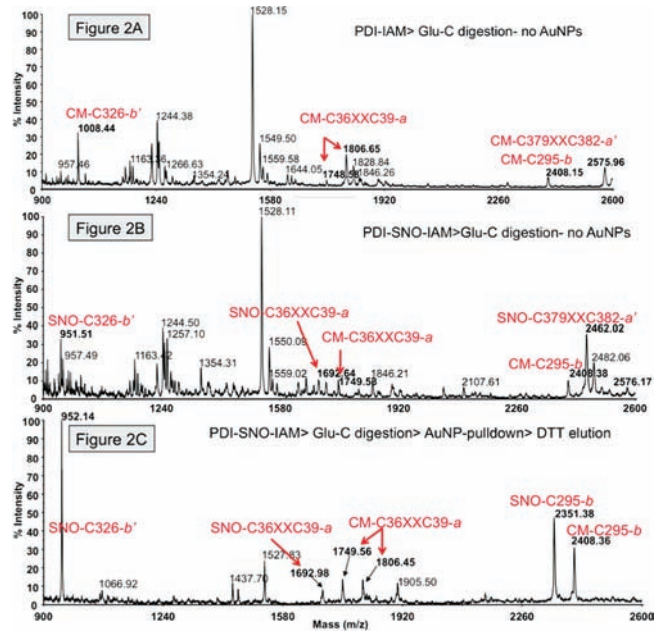


Figure 2. Mass fingerprint analysis of PDI glutamic peptides. (A) Fully reduced PDI (2 μM) was reacted with 10 mM IAM for 1 h, exchanged into 50 mM ammonium bicarbonate using Zeba Desalt spin columns, and proteolyzed by a 10:1 ratio of PDI/Endoproteinase Glu-C. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to *in silico* digests as previously described.[27] (B) Fully reduced PDI was *S*-nitrosylated by exposure to 1 mM *S*-nitrosoglutathione (GSNO),[17] then alkylated, proteolyzed, and analyzed as above. (C) Fully reduced PDI was *S*-nitrosylated, alkylated, and proteolyzed as above. The digest was added to 1.3×10^{10} AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed 3×, and AuNP-bound peptides were released by incubation with 100 mM DTT for 10 h. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above. All Cys-containing peptide masses are bold. The Cys contained within each peptide, along with their respective domain and modification, are all labeled in red. Selected parent ion masses were confirmed by postsource decay (PSD) as described[27] (Supporting Information).

Other recent findings show both domains undergo redox regulation;[27] however their susceptibility to *S*-nitrosylation has not been demonstrated. Of these, only thiols within the catalytic domain and the vicinal C312XC314 pair are detectable in tryptic mass fingerprints. Remaining thiols reside in small, unresolvable peptide fragments owing to the rich lysine and arginine content within the zinc-binding domain.

In fully reduced PDI and hYVH1, all detected thiols could be carbamidomethyl-(CM)-labeled with IAM (Figures 2A and S7).

When fully reduced PDI or hYVH1 were *S*-nitrosylated before alkylation, all detected thiols were partially protected from CM labeling (i.e., labeled as SNO-peptides in Figures 2B, 2C, S8, and S10). This strongly suggests the formation of pools of *S*-nitrosylated thiols in both PDI and hYVH1, as they resisted alkylation by IAM. The *m*/*z* values for these peptides correspond to free thiol peptide masses due to S−NO bond lability during ionization at 317 nm in MALDI-MS analyses (Figures 2B and 3B).[28]

AuNP-pulldowns of the peptide digests effectively enriched for the target thiol-containing peptides, resulting in overall simplification of the mass fingerprints and subsequent annotation. Both free thiol and CM-labeled peptides were observed in PDI and hYVH1 digests, allowing for detection of both *S*-nitrosylated and free thiol peptide pools (Figures 2C and S10).

Mass fingerprints of PDI revealed that the vicinal thiols in the ***a***-domain (C36 and C39) and those in the ***b*** (C295) and ***b*′** (C326) domains are susceptible to *S*-nitrosylation. The absence of the ***a*′**-

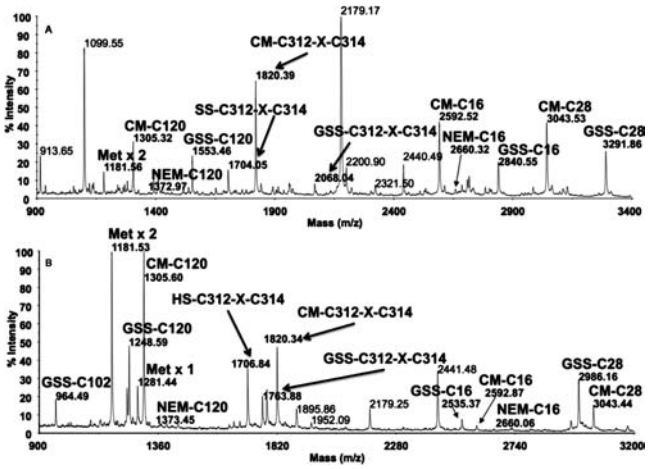
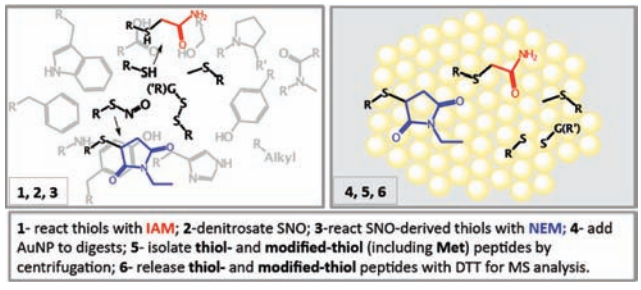


Figure 3. Mass fingerprint analysis of differentially alkylated hYVH1 and glutathionylated tryptic peptides. (A) Digest mixture. (B) AuNP enrichment. Samples were processed as described in the text.

Scheme 2. Modified AuNP-Enrichment Method



domain vicinal thiols in the AuNP-pulldowns (Figure 2C) and their presence in the solution digests (Figure 2B) is in agreement with previous studies indicating that the vicinal thiols in the ***a′***-active site domain are more susceptible to oxidation in comparison to their ***a***-domain counterparts.[17,23] Furthermore, the absence of ***a′***-domain thiols in the AuNP-pulldowns suggests that, as predicted, AuNPs do not interact with disulfide-linked peptides (Scheme 1d).

The fact that CM-labeled peptides were pulled down by AuNPs raised the possibility that thiols and thioethers, i.e. Methionine (Met), retain a higher affinity for gold in comparison to other functional groups in proteins, even upon chemical modification by alkylation or thiolation.

Therefore, a simple modification of the AuNP-based enrichment technique (Scheme 2) should permit discrimination between sites of *S*-nitrosylation, *S*-glutathionylation, and free thiols.

To test this, separate batches of hYVH1 were either partially *S*-nitrosylated or partially *S*-glutathionylated. The remaining free thiols were alkylated with IAM. The SNO-thiols were denitrosylated with ascorbate and differentially alkylated with a second thiol-reactive agent, *N*-ethylmaleimide (NEM). The samples were mixed, proteolyzed, and subjected to AuNP-pulldowns. The peptides were released by thiol exchange and identified by MS (Figure 3).

The mixed peptide solution (Figure 3A) indicated the presence of *S*-glutathionylated, *S*-CM, and *S*-NEM peptides. The reduced peak intensities of the NEM-modified peptides are indicative of ion suppression in mixed digests (Figure S9). As predicted, the AuNP-pulldown significantly enriched for free thiol, alkylated thiol, and Met-containing peptides.

This model thiol-modification/AuNP-enrichment study indicates that, upon partial *S*-nitrosylation of hYVH1, C16 and C120 are *S*-nitrosylated. On the other hand, C16, C28, C102, C312, C314, and the active site C120 are susceptible to glutathionylation. In addition, this technique was able to enrich for Met-containing peptides.

In summary, we have for the first time demonstrated that thiols and thioethers retain a higher affinity for gold in comparison to other functional groups in proteins, even upon chemical modification by alkylation or thiolation. We have adopted this property of AuNPs to devise a simple, novel method by which AuNPs can be used to isolate, detect, and enrich for *S*-modified peptides in a single step. When combined with thiol modification and mass spectrometry, our AuNP-based method can unambiguously identify sites of protein modification.

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Supporting Information Available: Experimental details and MS/MS analysis of selected peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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