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FORUM REVIEW ARTICLE

Proteomic Approaches to Analyze Protein Tyrosine Nitration

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Abstract

Significance: The conversion of protein-bound Tyr residues to 3-nitrotyrosine (3NY) can occur during nitrative stress and has been correlated to aging and many disease states. Proteomic analysis of this post-translational modification, using mass spectrometry-based techniques, is crucial for understanding its potential role in pathological and physiological processes. ***Recent Advances:*** To overcome some of the disadvantages inherent to well-established nitroproteomic methods using anti-3NY antibodies and gel-based separations, methods involving multidimensional chromatography, precursor ion scanning, and/or chemical derivatization have emerged for both identification and quantitation of protein nitration sites. A few of these methods have successfully detected endogenous 3NY modifications from biological samples. ***Critical Issues:*** While model systems often show promising results, identification of endogenous 3NY modifications remains largely elusive. The frequently low abundance of nitrated proteins *in vivo*, even under inflammatory conditions, is especially challenging, and sample loss due to derivatization and cleaning may become significant. ***Future Directions:*** Continued efforts to avoid interference from non-nitrated peptides without sacrificing recovery of nitrated peptides are needed. Quantitative methods are emerging and are crucial for identifying endogenous modifications that may have significant biological impacts. *Antioxid. Redox Signal.* 19, 1247–1256.

Introduction

Nitrative stress leads to 3-nitrotyrosine formation

UNDER CONDITIONS OF nitrative stress, particularly when nitric oxide combines with superoxide to form peroxynitrite, protein Tyr residues can undergo nitration to 3-nitrotyrosine (3NY), and increases in 3NY have been correlated to aging and numerous disease states, including cardiovascular disease, neurodegeneration, and autoimmune diseases (26). Tyr nitration can alter the protein structure and function and may play a role in redox signaling (2, 11, 30).

To understand the biological impact of Tyr nitration, protein targets must be identified, most often by mass spectrometry (MS)-based proteomics. Because many reviews of nitroproteomic methods exist (2, 7, 9, 16, 24, 45, 47), including several dedicated to MS techniques (4, 28, 35, 36), the present review will focus on recent improvements in the field.

Some considerations for the development of nitroproteomic methods

The field of nitroproteomics has long been dominated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by Western blotting with antibodies against 3NY and analysis of in-gel-digested spots by MS. This remains the most sensitive technique available, routinely indicating endogenous nitration. However, it is laborious and suffers from problems with specificity, background signals, and reproducibility, due in part to variations among antibody batches and suppliers (2, 16, 46). Antibodies may be either too specific, depending on the antigen used to raise them, or not specific enough, as cross-reactivity can occur, for instance, with the Trp derivative 5-hydroxy-6-nitrotryptophan (25). Methods for enrichment by immunoprecipitation can suffer from some of the same limitations. Furthermore, 2D-PAGE can be biased against proteins on the extremes of size, isoelectric point, and hydrophobicity. Finally, unambiguous identification of nitrated proteins and residues is difficult because a single gel spot may contain multiple proteins, and tandem mass spectrometry (MS/MS) data are often not obtained for the tryptic peptides containing 3NY (2, 16).

Multidimensional chromatography, either on-line or offline, can partially automate the prefractionation process for 3NY proteomics. However, specifically offline prefractionation can be time-consuming and generate large amounts of samples for MS analysis, thus requiring significant instrument

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time. Due to the large dynamic range required for proteomic analysis, as well as concerns about column capacity and ion suppression of low-abundance species by an overwhelming matrix of high-abundance species, detection of low endogenous levels of 3NY [on the order of one to five residues per 10,000 Tyr residues in samples under inflammatory conditions (30)] depends upon sufficient simplification and/or purification of the sample.

To address these limitations, many methods for chemical derivatization of 3NY have been developed, mostly to enrich 3NY-containing proteins and/or peptides. These methods often involve several derivatization steps, almost always including the reduction of 3NY to 3-aminotyrosine (3AY) by sodium dithionite (SDT) (40), followed by reaction of the aromatic amine with a labeling reagent, along with one or more cleaning steps. Derivatization methods also present opportunities for relative and absolute quantitation using isotopic labeling and/or fluorogenic tagging. Some derivatization strategies have focused on simplification and avoidance of sample losses that inevitably accompany derivatization and cleaning.

The reduction of 3NY to 3AY can be a challenging step. The most popular method uses SDT (40), but concomitant 3AY and Tyr sulfation can interfere with MS analysis (12, 13). An alternative method using thiols and heme yields 3AY as a single product but is more sensitive to conditions and difficult to automate (3, 12, 13). An electrochemical 3NY reduction has not yet been widely applied in the field of nitroproteomics (2). 3AY may also form inadvertently during sample processing, as observed for the *Arabidopsis thaliana* proteome (21), and may even form *in vivo* (2). These phenomena should be accounted for in proteomic database searches.

Labeling the 3AY aromatic amine with high specificity is crucial, due to the very large excess of aliphatic amine groups (N-termini and Lys ε-amino groups) over low-abundance, endogenous 3NY sites in biological samples, approximated to be *ca.* 28,000-fold molar excess (1). Some groups have attempted to exploit the lower pKa of the aromatic amine, which is 4.75 in the free amino acid (40), relative to aliphatic amines (pKa *ca.* 8–10) to impart specificity in labeling. However, others observed that such pH-based selectivity is insufficient (1, 13, 48), and most methods have added steps to block reactive amines before the reduction of 3NY.

Some considerations for MS of nitrated peptides and proteins

When matrix-assisted laser desorption ionization (MALDI) is used for ionization, 3NY photodegradation can occur with a loss of one or two oxygen atoms or reduction of 3NY to 3AY (6, 19, 20, 28, 34). While some studies use this characteristic pattern to aid in 3NY identification, it raises limits of detection because the MS signal intensity is shared among three or four peaks. Electrospray ionization (ESI) avoids this problem but does have some disadvantages, such as a lower tolerance for sample contaminants and matrix components. Regardless of the ionization source, both 3NY- and 3AY-containing peptides can have low ionization efficiency and/or poor MS/MS fragmentation (1, 13, 17, 19, 28, 44). 3AY may give deviating isotopic envelopes for fragment ions, possibly due to chemical rearrangements, which can interfere with protein database searching and prevent proper identification (13). Derivatization of 3AY can overcome these problems (13, 29). Removal of the nitro group, an electron predator, enables the use of electron capture and electron transfer dissociation as complementary fragmentation methods to improve sequence coverage (29); see Refs. (15, 22) for further discussion.

Multidimensional Chromatography

Technical and methodological advances have benefited nitroproteomic studies (2, 4, 5, 41). More recent developments are discussed below.

Combined fractional diagonal chromatography

Ghesquière *et al.* (12) applied combined fractional diagonal chromatography (COFRADIC) to nitroproteomics. Protein digests are separated by reverse-phase (RP) high-performance liquid chromatography (HPLC) and fractions collected over 1-min intervals. After reduction of 3NY to 3AY with SDT, samples are again fractionated using the same RP-HPLC conditions, and peaks showing a hydrophilic shift are collected for MS identification. While this method requires tedious fraction collection, it successfully identified six *in vivo* nitration sites in four different proteins from serum in a mouse model of sepsis, specifically female C57BL6/J mice injected intravenously with pathogenic *Salmonella enteritidis*. Likewise, 3NY sites were identified from *in vitro* nitration models, such as bovine serum albumin (BSA) treated with tetranitromethane (TNM) and Jurkat cell lysate exposed to peroxynitrite.

The authors also observed sulfated 3AY as a side product of reduction of Jurkat cell samples by SDT, where *ca.* 84% of peptides were partially sulfated. Thus, it is important to include sulfation when searching MS data against proteomic databases in experiments that use SDT.

Larsen *et al.* (18) combined COFRADIC with off-line nano-HPLC-MALDI-MS with tandem time-of-flight detection in a study that was limited to BSA nitrated *in vitro*, for which this method identified five 3NY sites. Advantages include the small amount of sample required and the potential for automation.

Fluorescent Labels

Incorporation of fluorescent moieties into nitropeptides or nitroproteins can provide a powerful tool for detection, with high signal-to-noise ratios, as well as for absolute and/or relative quantitation and for visualization in gels, Western blots, or tissue samples.

6-substituted 2-phenylbenzoxazoles

Several studies have demonstrated fluorogenic labeling of 3NY by reduction to 3AY with SDT followed by reaction with benzylamine (27, 38) or benzylamine derivatives, 4-(aminomethyl)benzenesulfonic acid (ABS) (37) or (3*R*,4*S*)-1-(4-(aminomethyl)phenylsulfonyl)pyrrolidine-3,4-diol (APPD) (8) (Fig. 1). A major advantage of this strategy is the selectivity for the *o*-aminophenol moiety of 3AY, which precludes the need to block other reactive amines in the peptide or protein. The products are highly fluorescent 6-substituted 2-phenylbenzoxazoles. For each reagent, three products have been characterized that differ in the substituent at the 6-position of the benzoxazole ring system but maintain similar fluorescent properties. Labeling can be followed by fluorescence detection and identification by MS. The method has also been applied for histochemical staining (39).

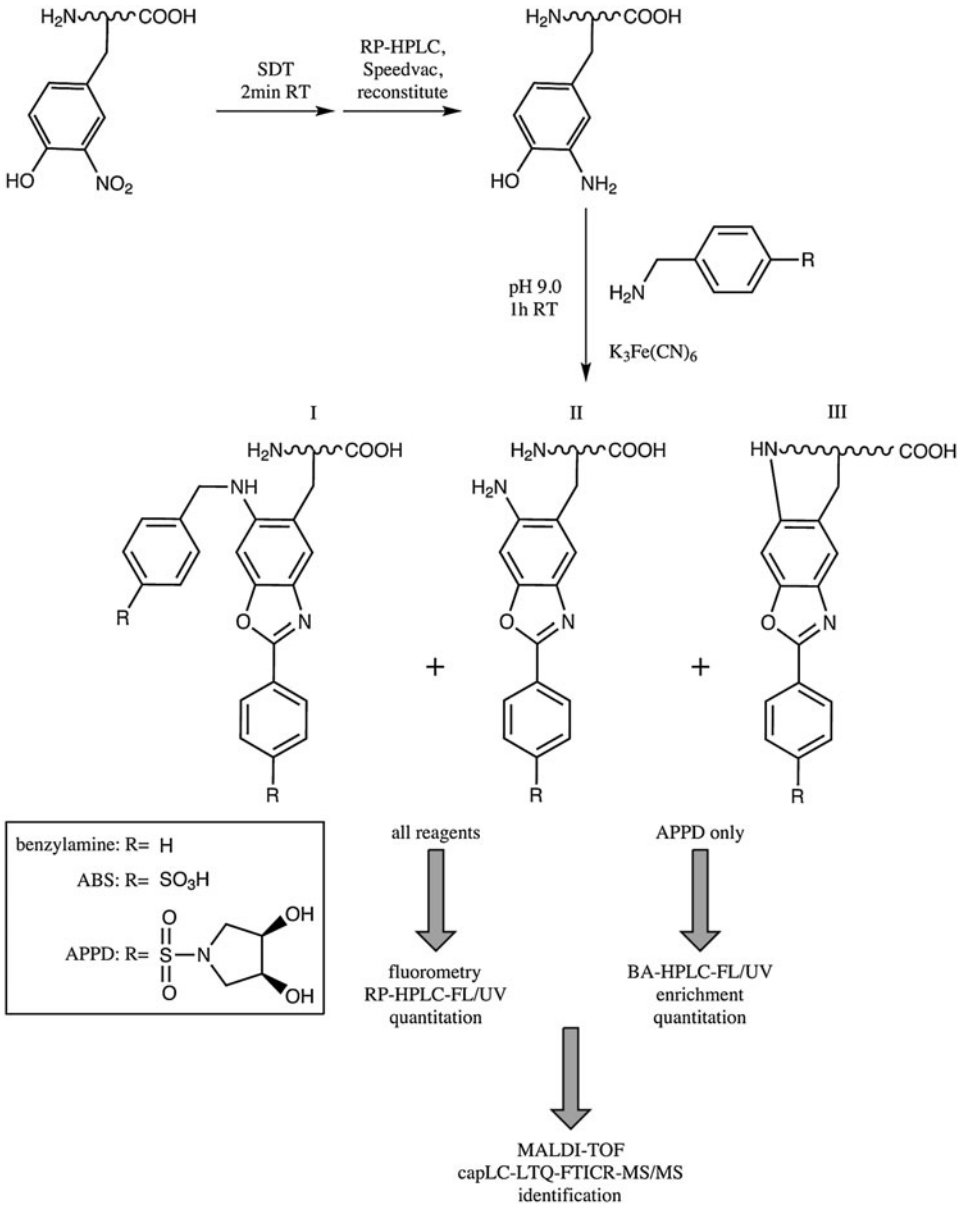

FIG. 1. Fluorogenic derivatization of 3NY to 6-substituted 2-phenylbenzoxazoles. Adapted and modified from Refs. (8, 37, 38). 3NY, 3-nitrotyrosine; SDT, sodium dithionite; RT, room temperature; RP, reverse phase; HPLC, high-performance liquid chromatography; BA-HPLC, boronate-affinity high-performance liquid chromatography; FL, fluorescence detection; UV, ultraviolet absorbance detection; APPD, (3*R*,4*S*)-1-(4-(aminomethyl)-phenylsulfonyl)pyrrolidine-3,4-diol; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; capLC, capillary liquid chromatography; LTQ, linear trap quadrupole (linear ion trap); FTICR, Fourier transform ion cyclotron resonance; MS/MS, tandem mass spectrometry.

For a synthetic nitropeptide, FSA(3NY)LER, tagged with ABS, the fluorescence quantum yield was determined as 0.77 ± 0.08 (37), and limits of detection by fluorescence spectrometry were measured as 12 and 40 pmol for the nitropeptide alone and in the presence of 100 μg digested proteins from C2C12 murine myoblasts, a model background matrix, respectively. Each of the reagents is amenable to isotopic coding for MS-based relative quantitation (27, 38).

Notably, 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan can also be labeled by this method, without reduction by SDT (37). The identity of the detected post-translational modification (PTM) can thus be deduced using controls not treated with SDT.

Affinity Labels

To avoid ion suppression of low-abundance nitropeptides, as well as to address chromatographic capacity limits, specific and efficient labeling of 3NY for affinity-based enrichment is a promising strategy for nitroproteomics. There has been great interest in developing antibody-free methods to overcome disadvantages of specificity, reproducibility, and throughput.

Boronate affinity

In the most recent development of a fluorogenic labeling strategy (27, 37, 38), Dremina *et al.* (8) describe a new benzylamine-derived reagent for derivatization and affinity enrichment of 3NY using boronate-affinity HPLC (BA-HPLC) (Fig. 1). The reagent APPD contains a benzylamine moiety for reaction with 3AY and a *cis*-diol moiety for pH-dependent coordination with boronic acid.

Both fluorescence spectrometry and BA-HPLC data (with fluorescence detection) can be used for quantitation of 3NY by this method, with limits of detection calculated as 19 nmol and 13 pmol, respectively, for a digest of rabbit phosphorylase *b* nitrated *in vitro* by peroxynitrite, alone or in a matrix of digested C2C12 murine myoblast proteins. Side product(s) can increase background and detection limits, but enrichment by BA-HPLC attenuates this problem.

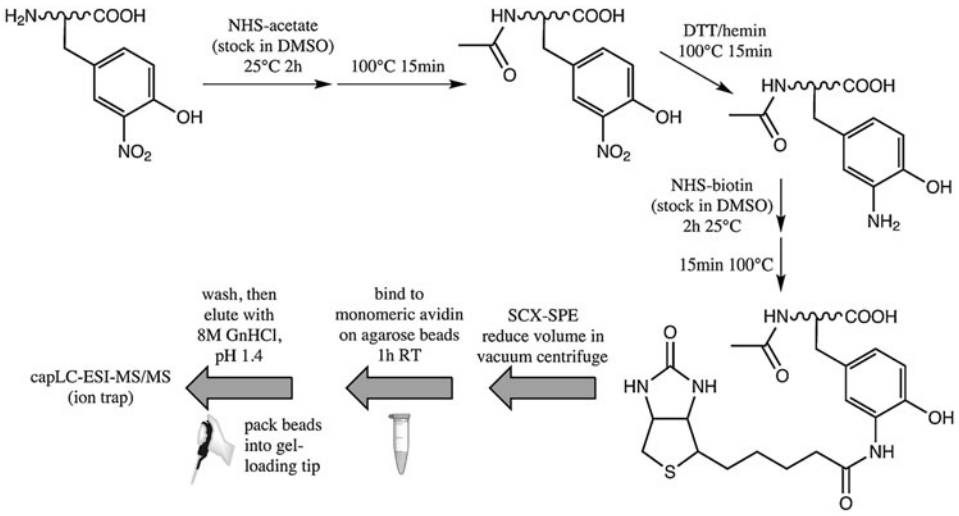

FIG. 2. Method for biotinylation of 3NY for affinity-based enrichment. This particular method minimizes sample cleaning steps. Adapted and modified from Ref. (1). NHS, N-hydroxysuccinimide; NHS-acetate, acetic acid NHS ester; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; NHS-biotin, biotinyl NHS ester; ESI, electrospray ionization; SCX, strong cation exchange; SPE, solid-phase extraction; GnHCl, guanidinium hydrochloride.

Biotin

The high-affinity interaction of biotin with avidin and streptavidin makes it an attractive labeling moiety. As reviewed elsewhere (2, 24), several groups have developed methods for biotinylation followed by enrichment on immobilized avidin or streptavidin.

The method of Abello *et al.* (1), summarized in Figure 2, reduces the need for cleaning steps and pH changes, thus minimizing sample losses. Aliphatic amines are acetylated by acetic acid N-hydroxysuccinimide ester (NHS-acetate), 3NY is reduced to 3AY using the dithiothreitol/hemin system, and a large excess of biotinyl N-hydroxysuccinimide ester (NHS-biotin) completes the derivatization. Following both N-hydroxysuccinimide (NHS)-based reactions, heating in a bath of boiling water degrades the remaining reagent and reverses any undesirable O-acetylation or O-biotinylation. The single cleaning step required, a strong cation exchange solid-phase extraction (SPE) used to remove the remaining biotin reagent, has poor recovery. After cleaning, the biotinylated peptides are enriched on a monomeric avidin resin, washed, and eluted by guanidinium hydrochloride under acidic conditions.

The peptide angiotensin II (Ang II) was nitrated *in vitro* by peroxynitrite and spiked into a tryptic digest of BSA as a model system. Although the peptide was successfully labeled and recovered, the SPE cleaning step is a major source of sample loss. Therefore, the authors propose future work to perform all derivatization reactions on intact proteins, such that size-based separation methods (*e.g.,* gel filtration or dialysis) can be used instead.

Metal chelation

A new adaptation of immobilized metal affinity chromatography (IMAC) has been developed by Lee *et al.* (19) for the enrichment of 3NY-containing peptides (Fig. 3). Primary amines are blocked by acetylation with sulfo-NHS-acetate before SDT reduction of 3NY to 3AY, after which the latter undergoes Schiff base formation and reductive amination with a large excess of pyridine-2-carboxaldehyde and sodium cyanoborohydride to give peptides that are bispyridinylated at the original nitration sites, with small amounts of monopyridinylation. Desalting steps are required following each of these reactions. The labeled peptides now chelate nickel (II) attached to magnetic agarose beads. After binding and washing, peptides are released with imidazole or ethylenediaminetetraacetic acid, desalted, and identified by MALDI-MS with time-of-flight detection.

This approach was applied to synthetic nitrated Ang II and to BSA nitrated by TNM. The authors demonstrate detection of as little as 100 fmol of nitropeptide spiked into a matrix of 10 μg HeLa cell lysate. As absolute signal intensity values are not presented with the MS data, it is unclear whether significant peptide losses occur during derivatization and cleaning steps. Also, while the mass shifts are generally in accord with expected values for each reaction step, all of the observed m/z values for Ang II and its derivatives appear higher than the corresponding theoretical values, with mass errors of 580–1080 ppm, a phenomenon also seen in the data of Kim *et al.* (17) (described below).

Fluorine affinity

Some of the same researchers have also developed a strategy to derivatize 3NY with highly fluorinated moieties for enrichment by fluorinated SPE (FSPE) (17) (Fig. 4). After acetylation of aliphatic amines with sulfo-NHS-acetate and reduction of 3NY to 3AY with SDT, peptides are tagged with *N*-succinimidyl 3-(perfluorobutyl)propionate, with cleaning steps after each reaction. Labeled peptides are enriched using FSPE, and the eluates are analyzed by MS.

Using this strategy for synthetic nitrated Ang II mixed with its non-nitrated counterpart, successful derivatization and enrichment are demonstrated by MALDI-MS. Interestingly, the absolute MS intensity for the tagged peptide is much greater after FSPE than before, and, while not discussed in the article, this may be an indication of ion suppression in the crude sample that is overcome by the affinity purification step.

However, as in the IMAC study (19), there are systematic deviations of observed m/z values from the theoretical values, especially for nitrated Ang II and its derivatives, which show large mass errors of +580–1038 ppm. The mass shifts after each reaction are generally as expected, except for $\Delta m/z = 46.1$ between native and nitrated Ang II (the expected value is 45.0). Inadequate instrument calibration is one possible explanation for this combination of phenomena, but the article does not discuss it. Also, there are some additional peaks in

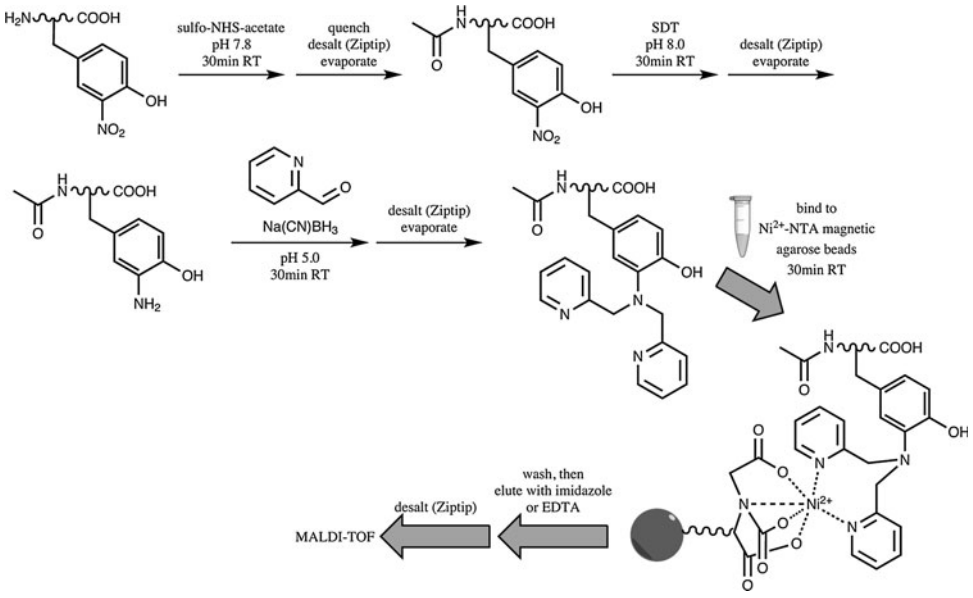

FIG. 3. Immobilized metal affinity chromatography method for enrichment of 3NY-containing peptides, by bispyridinylation of 3NY. Adapted and modified from Ref. (19). NTA, nitrilotriacetic acid; EDTA, ethylenediaminetetraacetic acid.

the final product spectrum that are not explained. Among them is a small peak near 1100 *m/z*, which could represent 3AY-Ang II (1104 *m/z*), but the absence of annotation for this peak precludes a definitive determination. At the characteristically low ionization efficiency of the 3AY-peptide, even a small peak can represent a significant amount of peptide that was not tagged.

The decrease in absolute intensity of the MALDI peak for the non-nitrated, acetylated peptide following subsequent steps probably results from sample losses due to cleaning, or perhaps due to reaction conditions (*e.g.*, possible side products or peptide degradation). These losses can become very important when looking for low-abundant PTMs, like 3NY.

The method was able to tag, enrich, and detect 100 pmol of nitrated Ang II in a background of 10 μg of a BSA tryptic digest. More notably, 28 endogenously nitrated peptides from 28 proteins were identified in the Huh7 human hepatoma cell line, and the 3NY sites were confirmed by MS/MS with manual validation. Some of the peptide spectra show XCorr scores [cross-correlation values generated by the SEQUEST algorithm (10)] that are lower than ideal, possibly indicating a poor fit to the theoretical spectra for the assigned peptide sequences. The most intense peaks in the MS/MS spectra are assigned, but only one spectrum shown has backbone cleavages on both sides of the tagged Tyr, which is stronger evidence for nitration site assignment. Not all of the Lys residues are acetylated, even though the text lists this modification as a requirement for acceptance of the identification. In contrast, all of the N-termini are acetylated in the identified peptides. For two of the identifications, the nitration site is located in a specific functional domain.

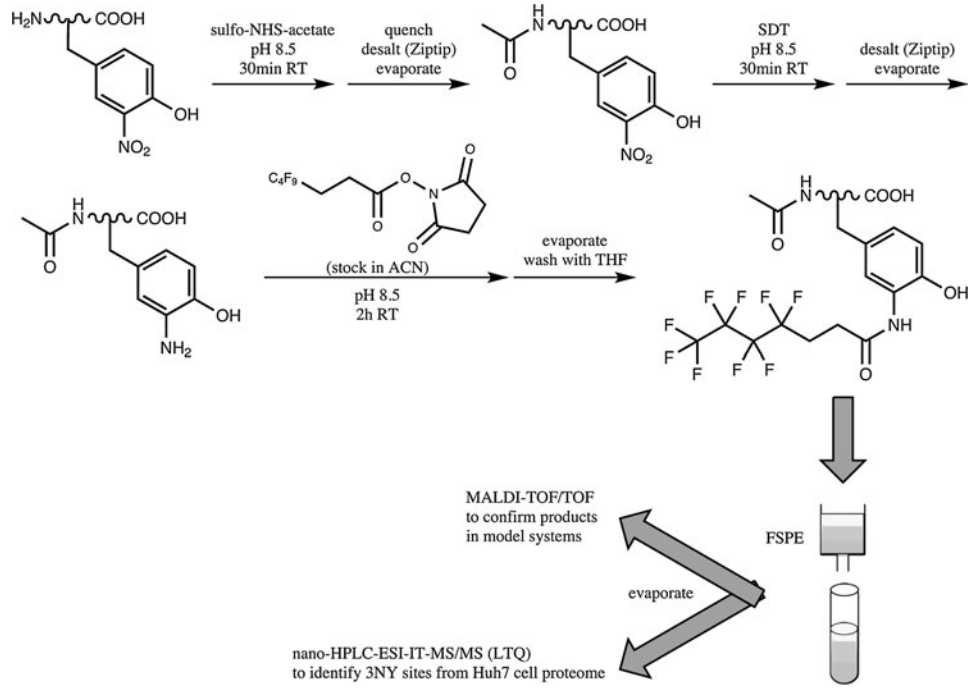

FIG. 4. Method for fluorination of 3NY for purification by FSPE. Adapted and modified from Ref. (17). ACN, acetonitrile; THF, tetrahydrofuran; TOF/TOF, tandem time-of-flight; FSPE, fluorinated solid-phase extraction; IT, ion trap.

Solid-Phase Chemistry

Several methods use covalent reactions between the peptide of interest and a solid-phase reagent, followed by chemical cleavage to recover the peptide with a vestigial tagging moiety that can be identified during MS analysis. Recent studies have endeavored to improve upon the earlier methods of Zhang *et al.* (48) and Nikov *et al.* (23).

Chemoprecipitation using solid-phase active ester reagent

To simplify the derivatization procedure and avoid sample losses incurred by cleaning steps, Prokai-Tatrai *et al.* (29) developed a chemoprecipitation technique in which amine groups are blocked by reductive methylation, 3NY is reduced to 3AY, and the latter reacts with a solid-phase active ester reagent (SPAER) on glass beads, followed by washing and gentle acid-catalyzed hydrolysis to remove the peptides, which retain a 4-formylbenzoylamido group (Fig. 5). By solution methods, the dimethylation step requires clean-up by SPE, but a one-pot method using solid-phase reagents is also reported. Importantly, dimethylation prevents nonselective labeling of amine groups while retaining their ability to ionize for facile detection in positive-mode HPLC-ESI-MS/MS.

In addition to identifying tagged derivatives of three synthetic nitropeptides in a BSA tryptic digest matrix (>200-fold molar excess of unmodified over nitrated peptides), 32 unique tagged peptides were identified from 12 different proteins in a tryptic digest of human plasma samples that were nitrated *in vitro* by TNM, some of which were previously found nitrated in aging and disease states. Eight contaminating, unmodified peptides from plasma proteins were also identified. This is attributed to nonspecific adsorption to the beads, which the authors propose to address by more thorough washing with organic solvents.

Building upon this work, Guo *et al.* (14) developed a method for relative quantitation by using isotopically labeled formaldehyde for reductive methylation, which did not alter the chromatographic properties of the products. This method was successfully applied to two model systems: synthetic nitropeptides spiked into a matrix containing the tryptic digest of human serum albumin and the model protein ubiquitin nitrated *in vitro* and spiked into a matrix of human plasma (14).

Quantitation

An important strategy for quantitation of 3NY consists of using isotopically coded reagents for differential labeling of samples from two (or more) experimental conditions and mixing these samples before MS analysis, thus allowing for quantitative comparisons of ion peak areas. While this is largely applied to relative quantitation, there are ways to achieve absolute quantitation by including an isotopically labeled internal standard. Isobaric tagging strategies have also emerged, which maintain a single *m/z* value for precursor ions but yield specific reporter ions after fragmentation, with the latter used for quantitation. These include tandem mass tags (TMT) (42) and isobaric tags for relative and absolute quantitation (iTRAQ) (32).

Isobaric tags for relative and absolute quantitation

The iTRAQ method was developed based on labeling primary amines for profiling whole proteomes (32). By acetylating primary amines with NHS-acetate, then reducing 3NY to 3AY with SDT, and finally labeling 3AY with iTRAQ reagents, with some cleaning procedures required, Chiappetta *et al.* (6) have adapted the iTRAQ methodology for identification and quantitation of 3NY sites (Fig. 6). Nano-HPLC-ESI-MS/MS was performed in the precursor ion scanning mode for specific reporter ions, which enabled the use of the mass spectrometer's duty cycle for analyzing only peptides of interest and thus enhanced the limit of detection without the need for affinity purification/enrichment.

This method was applied to BSA nitrated *in vitro* by TNM, alone or in the presence of 30 mg of *Escherichia coli* protein extract, and to bovine milk proteins nitrated *in vitro* with TNM. For the latter, nine 3NY sites were identified, which

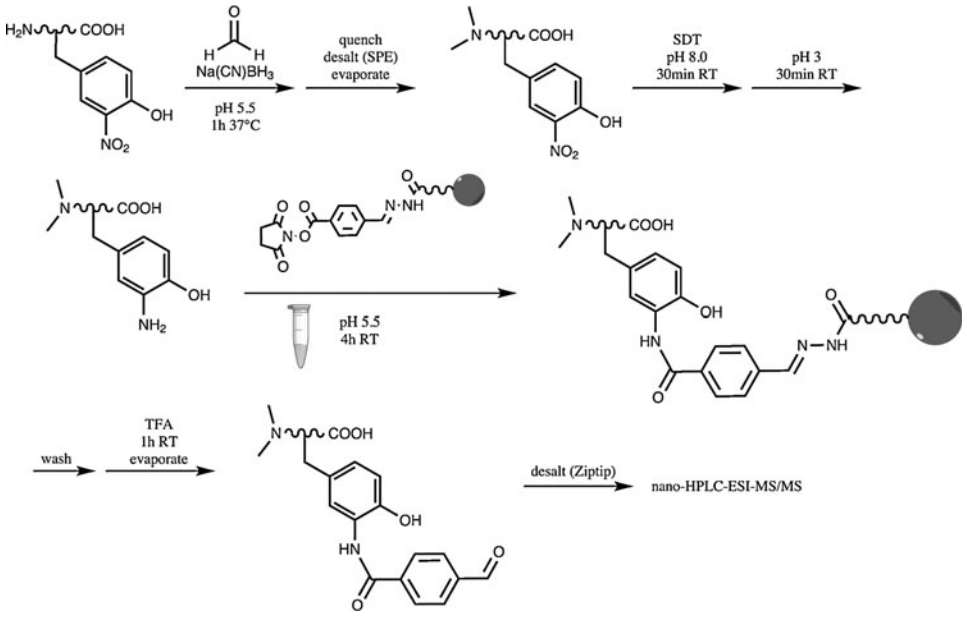

FIG. 5. SPAER method for enrichment of 3NY by chemoprecipitation. This represents the solution-phase method, but a solid-phase method was also tested. Adapted and modified from Ref. (29). Incorporation of isotopic labels for relative quantitation is further described in Ref. (14). SPAER, solid-phase active ester reagent; TFA, trifluoroacetic acid.

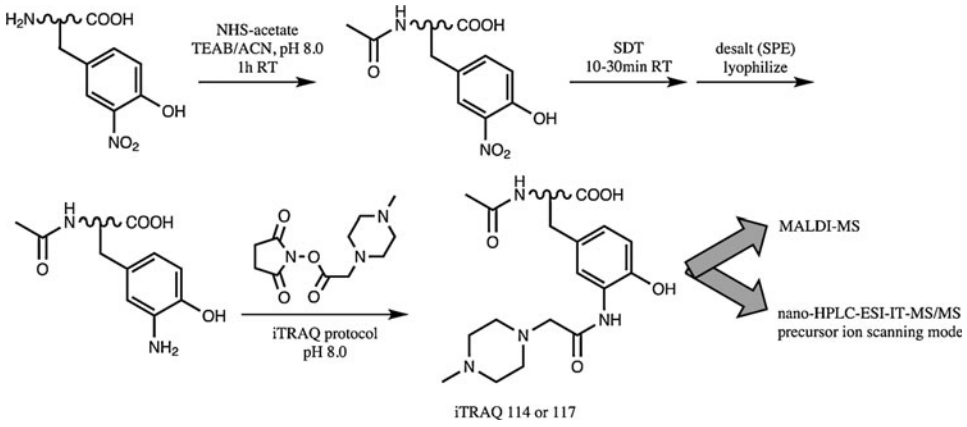

FIG. 6. Relative quantitation of 3NY with an adaptation of the iTRAQ method. Adapted and modified from Ref. (6). TEAB, tetraethylammonium bicarbonate; iTRAQ, isobaric tag for relative and absolute quantitation.

were found in both high- and low-abundance proteins, with accurate relative quantitation for seven of them.

Two-dimensional isotopic coding

Building on previous work (43), Tsumoto *et al.* (44) developed a method to increase the ionization efficiency of tryptic nitropeptides, prevent their photodecomposition during MALDI experiments, and incorporate isotopic labels (Fig. 7). Due to the two-dimensional nature of this isotopic labeling strategy, with the first dimension applied to all peptides and the second dimension applied only to 3NY, both non-nitrated and nitrated peptides are quantitatively compared between two samples in a single experiment. This feature was used to quantify the degree of nitration achieved by *in vitro* methods for specific Tyr residues. Although not explicitly discussed, it could also be very useful to control for protein expression levels, an important concern for comparative proteomic profiling of PTMs. Any 3NY labeling strategy that includes a step to block aliphatic amines could likely be adapted to take advantage of this feature.

Following acetylation of aliphatic amines with light or heavy acetic anhydride, 3NY is reduced to 3AY by SDT for subsequent tagging with 1-(6-methyl[D_0/D_3]nicotinoyloxy) succinimide, with appropriate cleaning procedures, followed by MS analysis.

Two model systems were used, synthetic nitrated Ang II and BSA nitrated *in vitro* with peroxynitrite. Ionization efficiency of tagged peptides was greatly improved compared to their 3NY- or 3AY-containing counterparts, recovering to about the same level as the acetylated, non-nitrated peptide. Since no absolute intensities are shown for the mass spectra, it is not possible to compare samples for peptide losses during derivatization and cleaning steps.

Combined precursor isotopic labeling and isobaric tagging

Robinson and Evans (31) developed the method of combined precursor isotopic labeling and isobaric tagging (cPILOT) to multiplex as many as 16 samples, using the previously reported isobaric tagging methods TMT (42) and iTRAQ (32) (Fig. 8). This allows for simultaneous comparison of numerous biological samples, which is more cost efficient. After acetylating amines with light or heavy NHS-acetate to give duplexed samples, SDT reduces 3NY to 3AY, and sets of either TMT or iTRAQ reagents complete the derivatization, with up to 16-plex experiments possible

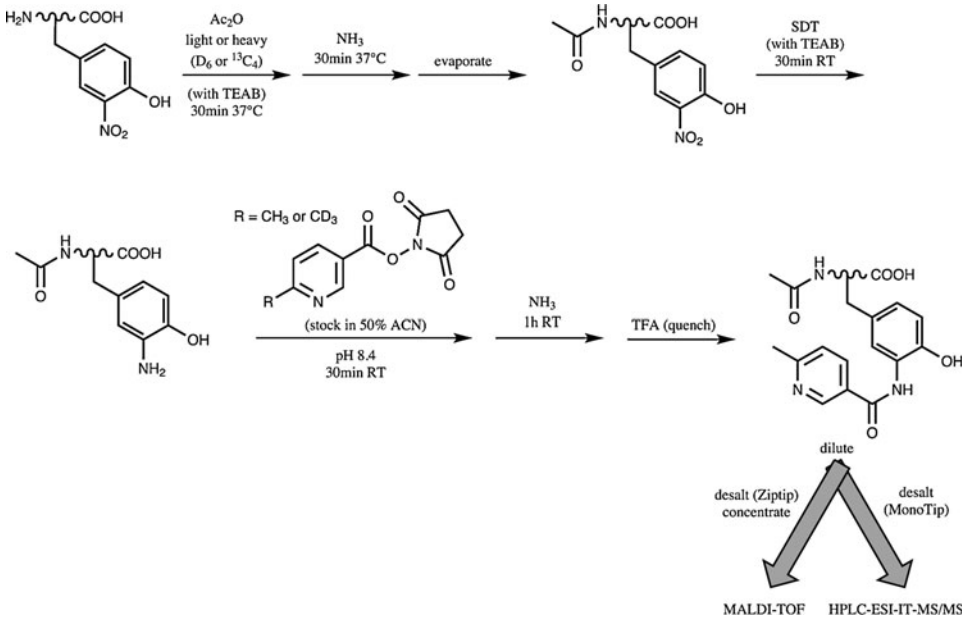

FIG. 7. Method for isotopic coding of 3NY for quantitation. Adapted and modified from Ref. (44). Ac_2O, acetic anhydride.

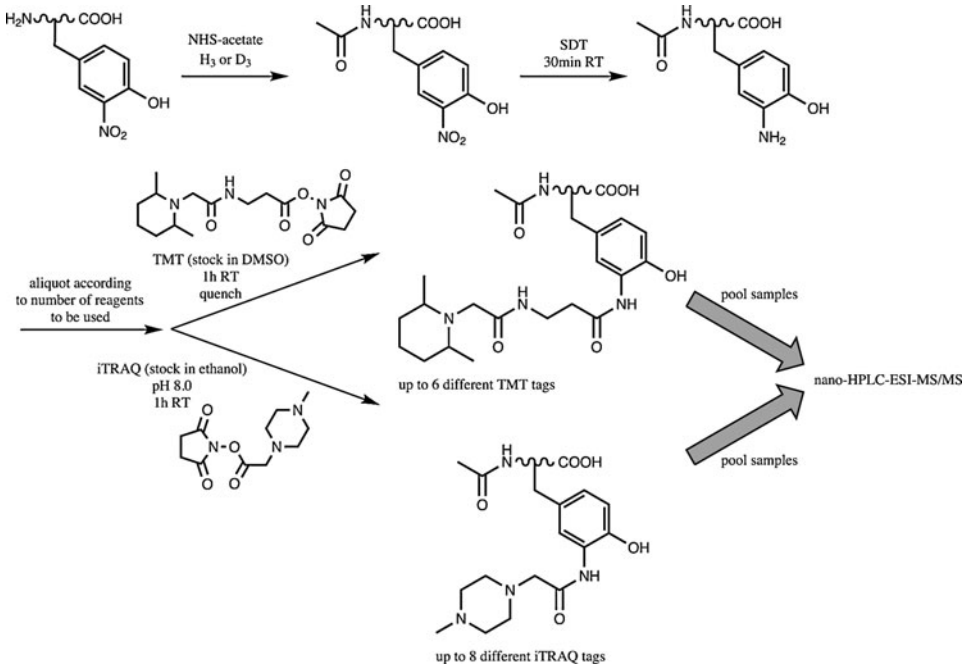

FIG. 8. Multiplexed method for relative quantitation of 3NY in up to 16 samples. Adapted and modified from (31). TMT, tandem mass tags.

in theory and no cleaning steps required until immediately before MS.

Using BSA nitrated *in vitro* by peroxynitrite as a model, a 12-plex experiment (duplexed acetylation combined with 6-plex TMT) showed overlap in the precursor ion isolation (i.e., insufficient resolution), causing inaccurate quantitation (31). For investigation of unknown, *in vivo* nitration sites in the mouse spleen proteome, scaling back to 4-plex still resulted in some overlap, but the relative quantitation was accurate nevertheless. Notably, five endogenous nitration sites were identified from five unique proteins. Possible improvements include enrichment strategies before labeling to increase proteomic identifications and an increase in the mass shift for the first dimension of isotopic labeling to overcome the problem of precursor ion overlap.

Conclusions

Despite many successful proof-of-concept studies with model systems, detection of endogenous 3NY remains a significant challenge. At present, most endogenous 3NY assignments are still based on 2D-PAGE/Western blot and immunoprecipitation methods (2). Of the recent studies covered in this review, only three (12, 17, 31) successfully identified *in vivo* nitration sites, for a total of 39 sites in 37 different proteins. Other successful studies (33, 49) did not derivatize 3NY, but used prefractionation and advanced HPLC-MS equipment, most notably capillary columns that were 65 cm in length, compared to 10–15 cm for most other studies.

This difficulty may be due to a combination of very low levels of endogenous 3NY and sample losses during derivatization and cleaning steps. Many studies (1, 6, 8, 14, 17, 19, 44) rely on the disappearance of mass spectral peaks for the starting materials to demonstrate a complete conversion for each reaction. However, this is not a truly quantitative technique, especially when MALDI is used, and furthermore this data cannot account for sample losses (*e.g.*, due to adsorption on plastic or glassware) or undetected side products.

Successful nitroproteomics requires selective separation and/or purification of nitropeptides from the matrix of non-modified peptides to reduce ion suppression and remain within the limits of column capacity for HPLC-MS/MS. This must be done with a minimum of sample losses, especially considering the low abundance of 3NY *in vivo* and the small amounts of biological material that are often available from precious specimens. Indeed, several of the most successful methods to date have either avoided 3NY derivatization completely (33, 49) or kept it to a minimum (12).

Acknowledgments

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Abbreviations Used

2D-PAGE = two-dimensional polyacrylamide gel electrophoresis
3AY = 3-aminotyrosine
3NY = 3-nitrotyrosine
ABS = 4-(aminomethyl)benzenesulfonic acid
Ac_2O = acetic anhydride
ACN = acetonitrile
Ang II = angiotensin II
APPD = (3*R*,4*S*)-1-(4-(aminomethyl)phenylsulfonyl) pyrrolidine-3,4-diol
BA-HPLC = boronate-affinity high-performance liquid chromatography
BSA = bovine serum albumin
capLC = capillary liquid chromatography
COFRADIC = combined fractional diagonal chromatography
cPILOT = combined precursor isotopic labeling and isobaric tagging
DMSO = dimethyl sulfoxide
DTT = dithiothreitol
EDTA = ethylenediaminetetraacetic acid
ESI = electrospray ionization
FL = fluorescence detection
FSPE = fluorinated solid-phase extraction
FTICR = Fourier transform ion cyclotron resonance
GnHCl = guanidinium hydrochloride
HPLC = high-performance liquid chromatography
IMAC = immobilized metal affinity chromatography
IT = ion trap
iTRAQ = isobaric tag for relative and absolute quantitation
LTQ = linear trap quadrupole (linear ion trap)
MALDI = matrix-assisted laser desorption ionization
MS = mass spectrometry
MS/MS = tandem mass spectrometry
NHS = N-hydroxysuccinimide
NHS-acetate = acetic acid N-hydroxysuccinimide ester
NHS-biotin = biotinyl N-hydroxysuccinimide ester
NTA = nitrilotriacetic acid
PTM = post-translational modification
RP = reverse phase
RT = room temperature
SCX = strong cation exchange
SDT = sodium dithionite
SPAER = solid-phase active ester reagent
SPE = solid-phase extraction
TEAB = tetraethylammonium bicarbonate
TFA = trifluoroacetic acid
THF = tetrahydrofuran
TMT = tandem mass tags
TNM = tetranitromethane
TOF = time-of-flight
TOF/TOF = tandem time-of-flight
UV = ultraviolet absorbance detection