Chapter 18

Utilization of MALDI-TOF to Determine Chemical-Protein Adduct Formation *In Vitro*

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Abstract

Biological reactive intermediates can be created via metabolism of xenobiotics during the process of chemical elimination. They can also be formed as by-products of cellular metabolism, which produces reactive oxygen and nitrogen species. These reactive intermediates tend to be electrophilic in nature, which enables them to interact with tissue macromolecules, disrupting cellular signaling processes and often producing acute and chronic toxicities. Quinones are a well-known class of electrophilic species. Many natural products contain quinones as active constituents, and the quinone moiety exists in a number of chemotherapeutic agents. Quinones are also frequently formed as electrophilic metabolites from a variety of xeno- and endobiotics. Hydroquinone (HQ) is present in the environment from various sources, and it is also a known metabolite of benzene. HQ is converted in the body to 1,4-benzoquinone, which subsequently gives rise to hematotoxic and nephrotoxic quinone–thioether metabolites. The toxicity of these metabolites is dependent upon their ability to arylate proteins and to produce oxidative stress. Protein tertiary structure and protein amino acid sequence combine to determine which proteins are targets of these electrophilic quinone–thioether metabolites. We have used cytochrome *c* and model peptides to view adduction profiles of quinone–thioether metabolites, and have determined by MALDI-TOF analysis that these electrophiles target specific residues within these model systems.

Key words: BQ, MALDI-TOF, NAC-BQ, Post-translational modifications, Protein adduction, Quinone–thioether

1. Introduction

Protein adduction, or chemical-mediated post-translational modifications, typically refers to protein covalent binding by electrophilic xenobiotics and/or their metabolites. Such reactive electrophilic intermediates are frequently formed during the process of chemical elimination, from xenobiotics, such as therapeutic

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drugs and environmental contaminants. Since many xenobiotics are lipophilic in nature, the body seeks ways in which to convert them into more water soluble metabolites to facilitate renal excretion. It is during this metabolic conversion that reactive intermediates are inadvertently generated. Biological reactive intermediates can also be formed during normal cellular metabolism (1). For example, advanced glycation endproducts (AGEs) are formed from the reaction of reducing sugars with amino groups of proteins, resulting in the formation of reactive dicarbonyl compounds, such as methylglyoxal (2, 3). Additionally, reactive intermediates formed as by-products of cellular metabolism include superoxide anion, hydroxyl radical, and nitric oxide. The generation of these reactive intermediates can result in the subsequent formation of additional endogenous electrophiles, such as 4-hydroxynonenal (4, 5). Because these reactive intermediates tend to be electrophilic in nature, they can interact with a variety of tissue macromolecules, initiating processes that may produce acute tissue injury or chronic disease (1). Many of these reactive intermediates are known to modify specific thiols on sensor proteins, such as glutathione S-transferase zeta and thioredoxin, and thus inactivate the proteins (6, 7). However, other reactive intermediates can modify proteins on amino acid residues distant from their catalytic site, thereby promoting a toxicological response by interfering with critical protein–protein interactions and disrupting cellular signaling pathways (8, 9).

Quinones represent an extensive class of electrophilic xenobiotics that can form covalent adducts with proteins, and can produce toxicological effects via such adduction. Quinones are also capable of redox cycling, and consequently producing reactive oxygen species (10) and subsequent oxidative stress (11–13). 1,4-Benzoquinone (BQ) is a reactive electrophile formed via the metabolism of benzene (a low molecular weight hydrocarbon and an environmental pollutant) to HQ, or from HQ directly. Because of the electrophilic nature of BQ, several GSH molecules can be sequentially conjugated to BQ via the free cysteinyl sulfhydryl present in GSH (10, 14–16). This conjugation results in the formation of conjugates with varying degrees of GSH including mono-, bis-, tris-, and tetra-substituted GSH conjugates of HQ (17). Subsequent metabolism of these conjugates via the mercapturic acid pathway ultimately yields the corresponding *N*-acetylcysteine conjugates, such as 2-(*N*-acetylcystein-*S*-yl)hydroquinone (NAC-HQ). These quinone–thioether metabolites cause extensive renal proximal tubular cell necrosis and effects are mediated via their ability to generate ROS and their protein-arylation capabilities (18–20). Although the compounds studied represent metabolites of known environmental toxicants, the quinone moiety also exists as the pharmacophore in many chemotherapeutic drugs. In this instance, the ability of these drugs to

covalently bind to tissue macromolecules is a desired property of these cytotoxicants. In contrast, the covalent binding of electrophilic drug metabolites is also of importance to the pharmaceutical industry because reactive drug metabolites may contribute to unwanted drug-induced toxicities. Thus, the pharmaceutical industry often assays for the ability of candidate drugs to undergo protein covalent binding as an index of reactive intermediate formation, which can ultimately assist in the further development of otherwise attractive drug candidates (9).

We herein describe MALDI-TOF based approaches to identify amino acid residues within peptides and proteins that are selectively adducted by reactive electrophilic quinone–thioethers. In order to decrease the analytical complexity inherent in the use of these compounds during mass spectral analysis, we utilized the mono-substituted NAC-HQ metabolite to investigate the adduction profile following reaction of this compound with several model peptides and proteins.

2. Materials

2.1. Sample Preparation

1. 1,4 BQ, *N*-1, and silver oxide (Aldrich, Milwaukee, WI).
2. HPLC-grade solvents including acetonitrile, acetic acid, methanol, and trifluoroacetic acid (TFA) (EMD Chemicals).
3. Shimadzu HPLC system (LC-10AS) with a UV–Vis spectrophotometric detector (280 nm) and an Ultrasphere ODS C18 column (5 μm packing, 10 mm × 25 cm, Beckman–Coulter).
4. 0.2 μm syringe filter (Whatman).
5. Rotovapor (Buchi) used in combination with a water aspirator pump (Cole–Parmer) for rotary evaporation during compound preparation.

2.2. Peptide Reaction

1. Ac-GAKKAG-OH (Ac-Gly-Ala-Lys-Lys-Ala-Gly-OH) can be purchased from American Peptide Inc, Sunnyvale, CA. Ac-QADGCAGPAG-OH (Ac-Gln-Ala-Asp-Gly-Cys-Ala-Gly-Pro-Ala-Gly) and Ac-QGADDEDDAG-OH (Ac-Gln-Gly-Ala-Asp-Asp-Glu-Asp-Asp-Ala-Gly-OH) can be purchased from Global Peptide, Fort Collins, CO. These custom peptides are >95% HPLC purity as characterized by HPLC and MS. The peptides come as lyophilized powders and are stored at –20°C immediately upon arrival.
2. Peptide reaction buffer(s): 10 mM Tris–HCl (Sigma) pH 7.5 and/or 50 mM ammonium acetate (Fisher Scientific), pH 6.
3. C18 packed tips (ZipTip) (Millipore, Inc).
4. Wetting solution: 100% acetonitrile (EMD chemicals).

5. Equilibration solution: 0.1% TFA in Milli-Q water (EMD chemicals).
6. Wash solution: 0.1% TFA in Milli-Q water (EMD Chemicals).
7. Elution solution: 0.1% TFA/50% acetonitrile (EMD Chemicals). These ZipTip solutions can be stored at room temperature and are stable for long periods of time.
8. A Shimadzu HPLC system (LC-10AS) with a UV–Vis spectrophotometric detector (280 nm) and an Ultrasphere ODS C18 column (5 μm packing, 10 mm × 25 cm, Beckman–Coulter).
9. α-Cyano-4-hydroxycinnamic acid (CHCA): 50% acetonitrile, 0.1% TFA in deionized water.
10. CHCA matrix (Sigma) and Voyager MALDI-TOF sample plate laser etched stainless steel, 100-position (Applied Biosystems).

2.3. Protein Reaction

1. Cytochrome *c* reaction buffer: 10 mM Tris–HCl (Sigma), pH 7.5, used with horse heart cytochrome *c* (Sigma).
2. Microcon 3,000 Da molecular weight cut-off centrifugal filter (Millipore).
3. 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid): 30–50% acetonitrile, 0.1% TFA in deionized water.
4. 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (ACROS organics) and Voyager MALDI-TOF sample plate laser etched stainless steel, 100-position (Applied Biosystems).

3. Methods

The basic scope of this chapter is to determine an electrophile-specific adduction profile using MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) analysis. By using quinones as our model electrophile and multiple *in vitro* reaction targets, including peptides and proteins, we should be able to identify the resulting quinone adduction profile on whole proteins, as well as determine the potential amino acid targets of these compounds with the use of small peptides. This will also help guide us in any quinone-specific post adduction chemistry that may be occurring.

MALDI ionization is used for the analysis of biomolecules, including peptides and proteins, where the matrix assists in desorption and ionization of the analyte. After the analyte is mixed with the appropriate matrix and spotted on the sample plate, the laser is used for ionization of the analyte. The ions are then separated based upon the time it takes for them to traverse the flight

tube to the detector. This is referred to as a time-of-flight mass analyzer (21, 22). By using MALDI-TOF analysis as our primary tool, we can achieve a projected quinone adduction profile on specified proteins and peptides, as well as determine accurate mass modifications of these compounds.

3.1. Preparation of Compounds for In Vitro Reaction

1. *N*-Acetylcysteine (NAC) is dissolved in 1% acetic acid in water (~25 ml). BQ is dissolved in a mixture of 50% acetonitrile:water (~60 ml). NAC is added dropwise to the BQ solution.
2. The mixture is stirred for 15 min and the solution is extracted twice with three volumes of ethyl acetate to remove residual BQ and HQ formed by reduction. The solution is rotary evaporated down to 10–15 ml and the aqueous phase is lyophilized (see Note 1) (17).
3. The resulting product is purified by HPLC after dissolving it in 1% acetic acid in water (200 mg/ml) and injecting aliquots (100 µl). The sample elutes isocratically at 10 min using acetic acid:methanol:water (1:20:80 v/v.), at a flow rate of 3.0 ml/min, over 35 min and monitored at 280 nm.
4. These compounds must be in their oxidized forms in order for them to adduct nucleophilic protein residues, and so we manually oxidize them from the HQ conjugate to the BQ conjugate. NAC-HQ is dissolved in deionized distilled water with 0.1% TFA at a concentration of 50 mg/ml. Approximately 5 mg of silver is added and the solution is vortexed for 1 min. The solution is filtered through a 0.2 µm syringe filter.
5. The solution is then purified by HPLC. The mobile phase is acetic acid:methanol:water (1:10:90 v/v.) Aliquots of oxidized 2-(*N*-acetylcystein-*S*-yl)benzoquinone (NAC-BQ) (50 µl) are injected into the HPLC system and separated isocratically at 3.0 ml/min.
6. The product elutes at 12.4 min. The yellow product is then reanalyzed by HPLC using the previously described method and gives rise to a single UV-absorbing peak (see Note 2).

3.2. Reaction of Compounds with Peptides to Determine Amino Acid Targets and Accurate Adduct Masses

1. N-terminal protected peptides are used to ensure that the compound will not bind to the peptide N-terminus. Different peptides are used with various amino acid targets to differentiate among likely targets of these compounds. Examples include: (1) Ac-GAKKAG-OH (Ac-Gly-Ala-Lys-Lys-Ala-Gly-OH), (2) Ac-QGADDEDDAG-OH (Ac-Gln-Gly-Ala-Asp-Asp-Glu-Asp-Asp-Ala-Gly-OH), and (3) Ac-QADGCAGPAG-OH (Ac-Gln-Ala-Asp-Gly-Cys-Ala-Gly-Pro-Ala-Gly). These peptides are used to determine specificity of different amino acids (Lys and Cys) towards our quinone–thioether compounds. Specific residues within these peptides, including Lys and Cys,

have been shown to be targets of various other electrophiles (4, 23). Additionally, residues with less nucleophilic regions, such as carboxy groups, may also be targets, so we have included a peptide with several Asp and Glu residues.

2. The peptides are dissolved in various buffers. The solubility of the peptides is sequence dependent and involves hydrophobicity and hydrogen bonding. These selected peptides will solubilize readily in water; however, if the peptide is extremely acidic, the pH can be raised to increase solubility and if the peptide is extremely basic, the pH can be lowered to increase solubility. Peptides (1) and (2) from above are likely soluble in any aqueous solvent regardless of pH, because they contain a high charge distribution. Peptide (3) contains a Cys residue and these residues are susceptible to oxidation and polymerization, so it can be dissolved in a solvent system that is slightly acidic. A small amount of dithiothreitol (DTT), reducing agent, can be added to increase stability of these residues. Because we are using these peptides for protein adduction studies, the solvents we use here may be considerably different than many suggested solvents for these types of peptides.
3. These buffer conditions can vary greatly depending upon the compound in use for protein adduction reactions. Dissolve peptide (1) in 10 mM Tris–HCl pH 7.5, peptide (2) in 50 mM ammonium acetate pH 6, and peptide (3) in water (see Note 3).
4. Following solubilization of these peptides, they are aliquoted for storage and use. They can be stored at −20°C and can be thawed for individual use. Each aliquot will contain 100 μg. One aliquot can be divided into a control sample and a treated sample. The treated sample will be reacted with NAC-BQ at a 1:10 molar ratio of peptide:NAC-BQ. NAC-BQ will be weighed out and added as a dry powder to the solubilized peptide and reacted for 30 min to 1 h at room temperature.
5. Following the reaction, the excess NAC-BQ is removed. Because of the small molecular weight of the peptides, only C18 packed tips (ZipTip) can be used for NAC-BQ removal or HPLC analysis can also be done to remove the excess compound.
6. The C18 packed tips are typically used for desalting and concentrating peptides or small proteins before MALDI-TOF analysis. These C18 pipette tips can hold up to 10 μl of volume, but 5 μl can be used as to not waste too much sample. First, ensure the final sample solution has a pH <4, so add 0.5 μl TFA to the 5 μl sample volume. Depress the pipettor plunger to a dead stop and aspirate wetting solution into the tip. Dispense to waste and repeat. Aspirate equilibration solution.

Dispense to waste and repeat. Bind peptides to C18 pipette tip by aspirating and dispensing the sample seven to ten cycles for maximum binding. Aspirate wash solution into tip and dispense to waste and repeat once. In order to elute the peptides, dispense 1–4 μl of elution solution into a clean vial using a standard pipette tip. Aspirate and dispense eluant through C18 pipette tip at least three times without introducing air into the sample.

7. HPLC analysis can be performed to remove excess NAC-BQ from peptide reactions. Aliquots of NAC-BQ reacted peptides (100 μg) were injected into the HPLC system and the sample was eluted with a linear gradient of trifluoroacetic acid:acetonitrile:water (0.1:1:98.9 to 0.1:40:59.9 v/v) at a flow rate of 3 ml/min over 35 min. These aliquots are collected in glass vials, lyophilized to dryness, and can be stored at −20°C until ready for use. Typically, the aliquots are then dissolved in deionized, distilled water and used for MALDI-TOF analysis.
8. The reacted and purified peptide sample(s) is free of any salts or excess NAC-BQ that would interfere with MALDI-TOF analysis. The NAC-BQ adduct will still remain on the peptide.
9. For MALDI-TOF analysis, a matrix solution will be mixed with the peptide solution at a 1:1(v/v) ratio. The peptide solution that is mixed with matrix will be pure based on the above procedures using C18 tips or HPLC for purification. Ideal final protein concentration for peptides and proteins is 0.1–10 μM. For peptides and proteins of less than 10 kDa, CHCA is used as the matrix. This matrix can then be premixed with the peptide solution in a small Eppendorf tube (see Note 4). It is important that neither the matrix nor the peptide precipitates when the two solutions are mixed. The mixture (1–2 μl) can then be applied to the sample support (MALDI-TOF plate) and the sample is allowed to air evaporate. The dried sample is quite stable and can be stored at room temperature for several days to several weeks. An example of a MALDI-TOF spectrum following this type of reaction is shown in Fig. 1.

3.3. Reaction of Compounds with Pure Protein for Whole Protein Analysis to Determine Adduct Profile

1. This purified compound (NAC-BQ) can also be used in a reaction with pure cytochrome *c* from horse heart to determine adduction profile by NAC-BQ on cytochrome *c* and any resulting post-adduction chemistry as a result of NAC-BQ adduction. The data generated from peptide studies with NAC-BQ can be used as a guide to project the correct amino-acid targets and mass modifications.
2. Horse heart cytochrome *c* (1 mg) is dissolved in 10 mM Tris–HCl, pH 7.5 (see Note 5). The cytochrome *c* solution

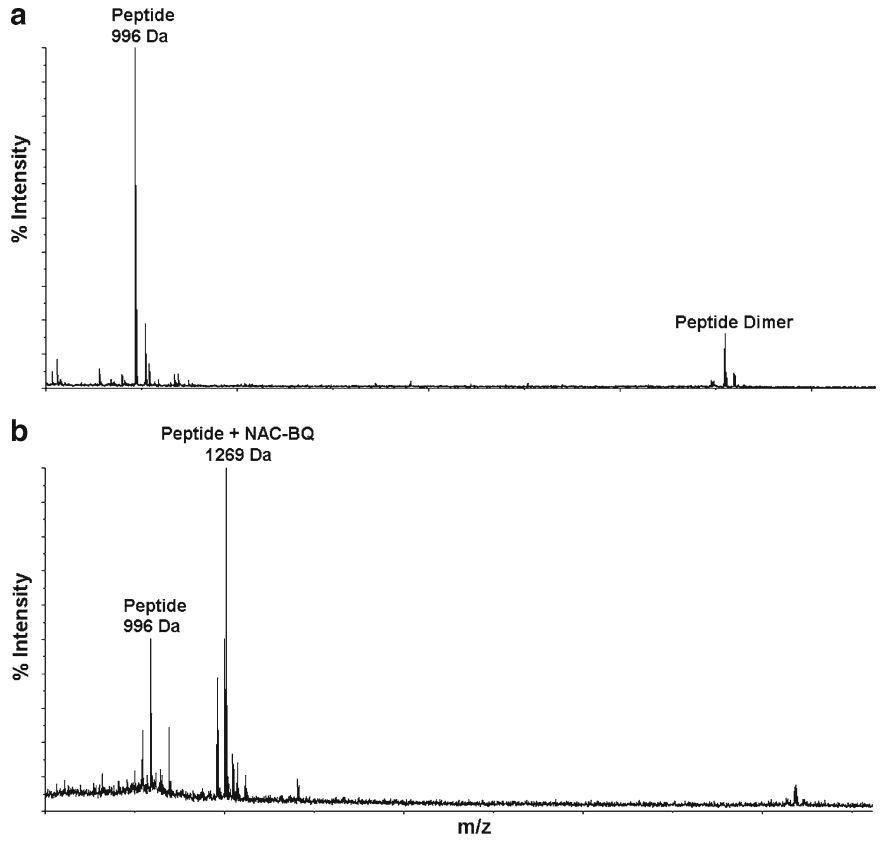

Fig. 1. MALDI-TOF analysis following reaction of NAC-BQ with Ac-QADGCAGPAG-OH. (**a**) MALDI-TOF analysis of control peptide in 10 mM Tris–HCl, pH 7.5. (**b**) The peptide was reacted with BQ at a ratio of 1:10 for 30 min at room temperature while rotating. At the end of the 30 min incubation, the sample was then HPLC-purified to remove excess NAC-BQ and lyophilized to dryness. The sample was then reconstituted in 10 mM Tris–HCl, pH 7.5. The resulting MALDI-TOF spectrum shows addition of NAC-BQ to the cysteine residue on the peptide, corresponding to one addition of NAC-BQ at 1,269 *m/z*.

is aliquoted (100 µl) prior to NAC-BQ reaction for use as a control sample.

3. The cytochrome *c* solution is reacted with dry NAC-BQ at a 1:10 molar ratio at room temperature for 30 min to 1 h (see Note 6).
4. The mixture is filtered once through a Microcon 3,000 Da molecular weight cut-off centrifugal filter for 20 min at 13,000 × *g* to remove excess NAC-BQ. The reaction mixture is then washed with 1 ml of 10 mM Tris–HCl, pH 7.5, and centrifuged as above.
5. Once completed, the filter is turned upside down in a new filter tube and centrifuged for 2 min at 2,000 × *g* to collect the remaining protein solution that was on the filter. Measure the volume to determine the protein concentration, as all of the NAC-BQ-reacted protein and the native protein should

remain on top of the filter because these have a mass of greater than 3,000 Da. The unreacted NAC-BQ should pass through the filter as it has a molecular mass of 269 Da. By collecting the solution remaining on the filter, this will be the solution containing the reacted protein and will be used for further analysis.

6. When using high salt buffers or any type of denaturing agent, the protein will need to be further purified using C18 packed tips. The protocol for using these tips with proteins is the same as described previously with the peptide.
7. For MALDI-TOF analysis, a matrix solution will be mixed with the protein solution at a 1:1(v/v) ratio. Ideal final protein concentration for peptides and proteins is 0.1–10 μM. For proteins of greater than 10 kDa, sinapinic acid is used as the matrix. This matrix can then be premixed with the protein solution in a small Eppendorf tube (see Note 4). It is important that neither the matrix nor the protein precipitates when the two solutions are mixed. The mixture (1–2 μl) can then be applied to the sample support (MALDI-TOF plate) and the sample is allowed to air evaporate. The dried sample is quite stable and can be stored at room temperature for several days to several weeks. An example of a MALDI-TOF spectrum following this type of reaction is shown in Fig. 2.

3.4. Detailed Program Method and Laser Use for Pure Protein/Peptides (22)

1. MALDI-TOF spectra are taken on Applied Biosystems Voyager DE-STR instrument with a 2-m flight path in the positive ion mode. The instrument is equipped with a nitrogen laser operating at 337 nm.
2. Open the Voyager Control Panel and click the load/eject button to load the sample plate (see Note 7).
3. Load a standard instrument setting file (.bic). Peptide spectra can be acquired in reflectron mode (for higher resolution) over the mass range 700–5,000 Da. Choose a file that corresponds to this setting. Additionally, this setting should include the appropriate matrix for peptide use which corresponds to α-cyano-hydroxycinnamic acid. Whole protein spectra can be acquired in linear mode over the mass range 8,000–40,000 Da and the matrix was selected as sinapinic acid for this whole protein acquisition (21) (see Note 8).
4. Save the parameters and manually turn on the high voltage and let it warm for 30 min.
5. Set the laser intensity to 1,300–1,500, set the desired sample position, and begin acquisition (see Note 9).
6. A good peak should correspond to signal intensity roughly between 5,000 and 40,000 (see Note 10).
7. The spectra can be sequentially stacked when analyzing peptide and whole protein samples.

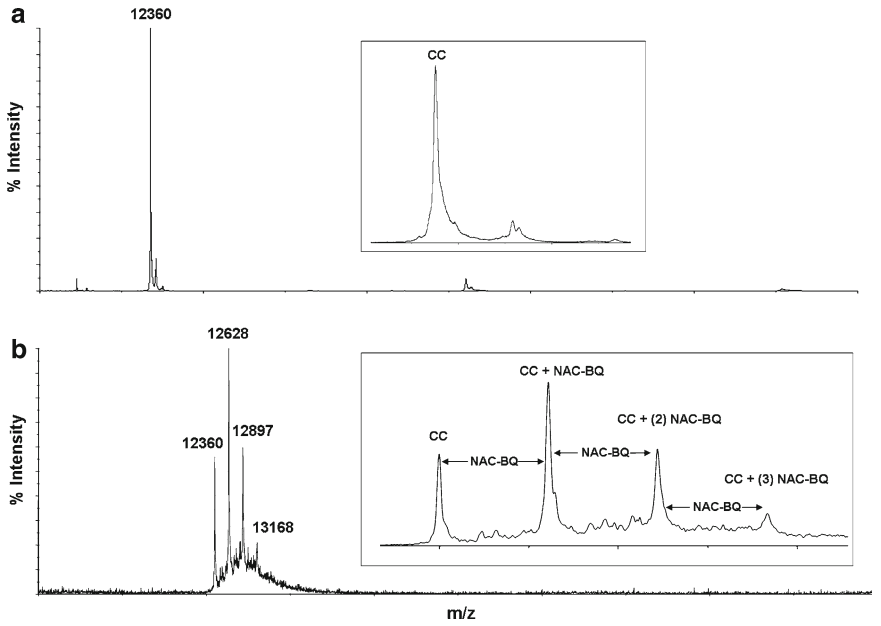

Fig. 2. MALDI-TOF whole protein spectra for cytochrome *c* reacted with NAC-BQ. (**a**) Control spectrum of cytochrome *c* in 10 mM Tris–HCl pH 7.5, where the peak at 12,360 *m*/*z* indicates native cytochrome *c*. (**b**) Cytochrome *c* was incubated in 10 mM Tris–HCl pH 7.5 and then reacted with a 1:10 molar ratio of NAC-BQ. The excess NAC-BQ was removed using Microcon 3,000 Da molecular weight cut-off centrifugal filters. The resulting MALDI-TOF spectrum shows several additions of 268 Da to cytochrome *c*, corresponding to one addition at 12,628 *m*/*z*, two additions at 12,897 *m*/*z*, and three additions at 13,168 *m*/*z*. The insets in (**a**) and (**b**) are magnified regions of each spectrum. Reproduced from (24) with permission from American Chemical Society.

8. Internal and external calibration can be applied to samples. Internal calibration standards typically include cytochrome *c*, bovine trypsin, and carbonic anhydrase. These standards should bracket the mass range of interest and should be of similar intensities as the samples (see Note 11). External calibration can also be applied to samples. This type of file can be acquired using a protein mixture (cytochrome *c* and myoglobin) and saved for use with real samples. Typically, these standards are spotted nearby the sample positions and applied to the actual samples. This calibration file must be created and saved in Data Explorer. Then, existing calibration files can be manually applied to real samples with each acquisition (see Note 12).
9. The Data Explorer Software program is used to analyze all MALDI-TOF spectra (see Note 13).
10. Keep the sample plate covered and protected at all times. Finger prints and scratches will interfere with good data acquisition.
11. Clean the sample plate after all sample positions have been filled and analyzed (see Note 14).

4. Notes

1. NAC-HQ is weighed after lyophilization and stored in glass vials at −20°C. This compound is also stable at room temperature.
2. NAC-BQ is weighed and stored in glass vials at −20°C, although it will be stable for long periods of time at room temperature.
3. We found that Lys residues adduct NAC-BQ more efficiently when in a neutral to basic pH, such as 10 mM Tris–HCl, pH 7.5. Subjecting Lys residues to a neutral/basic pH, they are more nucleophilic, priming them for higher reaction efficiency with NAC-BQ. Glu residues adduct NAC-BQ when in a more acidic pH, such as 50 mM ammonium acetate, pH 6.0. This environment seems to stabilize a resonance structure that becomes protonated at a low pH and thus enables this residue to bind NAC-BQ via Michael addition (24). Cys residues adduct NAC-BQ in water much better than in a buffered system. Ideally, peptides containing Cys residues should be used with a mild reducing agent or kept at a lower pH because of disulfide formation. We observe that disulfide formation does occur; however, it is much more prevalent in basic conditions and seems to be not as probable in water. Additionally, we still are able to form NAC-BQ adducts on Cys residues using water as a solvent system, so we avoid using any buffering system. Furthermore, DTT and other reducing agents, interfere with NAC-BQ adduction because they likely scavenge the electrophilic compound and prevent it from binding to the Cys residue.
4. The matrix solution is usually made with a concentration of 20 mg/ml; however, it is good practice to make small volumes and try to use fresh matrix solutions whenever possible. The matrix solutions are saturated solutions. Following addition of the solvents to the matrix, the solution is vortexed and then allowed to settle for a few minutes and use the top matrix solution layer, free of undissolved matrix.
5. A buffering system is used that consists of 10 mM Tris–HCl, pH 7.5 and NAC-BQ adduct formation is much more efficient in this buffer than in water. This is likely because many of the NAC-BQ-adducts form on Lys residues and these residues are more nucleophilic in buffered systems.
6. The reaction can proceed for longer than 1 h; however, it has been shown that the *N*-acetylcysteine bond to the BQ ring is not stable in high salt or high pH conditions for periods of time exceeding 6 h. Therefore, sample preparation

time is critical for accurate adduct mass analysis. When the NAC-BQ compound is stable, it will form adducts with a mass of 268 Da. Following NAC-BQ adduction and thioether bond elimination due to microenvironment instability, the resulting adduct mass will be 105 Da. This mass is indicative of the BQ ring (24).

7. Multiple sample plate configurations may be stored in the computer, but only select the configuration that corresponds to the plate being loaded so that the positions can be navigated based on plate configuration.
8. The instrument can be set to incorporate a much broader range of masses from 1,000 to 100,000 Da. Because cytochrome *c* is being analyzed, a program is used that narrows the mass range window closer to the actual mass of cytochrome *c*.
9. The laser intensity becomes saturated at 1,800 and is higher for these samples at these concentrations. The laser intensity used is dependent upon the instrument laser, in addition to the type and concentration of sample being analyzed.
10. In order to optimize the acquisition, the laser intensity can be raised or lowered, and the laser position with the sample can be moved to a more desirable location.
11. In order to avoid overloading the signal with the calibration standards, work with the dilution factor of the standards.
12. External calibration is not as good as internal calibration; however, the closer the standard is to the sample spot, the better the calibration and less the error.
13. Basic features of Data Explorer include peak detection and peak labeling, graphics, resolution, baseline correction, noise reduction and smoothing, and calibration options.
14. Wash the sample plate with methanol and deionized water (v/v 1:1) and rinse well with deionized water. If sample or matrix residue persists, apply 0.1% detergent solution to sample plate and use a soft tooth brush to scrub the plate. Rinse well with deionized water. Allow the plate to dry completely before applying any samples.

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References

1. Monks, T.J. (2006) Introduction. *Drug Metab Rev* **38**, 599–600.
2. Brouwers, O., Teerlink, T., van Bezu, J., Barto, R., Stehouwer, C., and Schalkwijk, C. (2007) Methylglyoxal and methylglyoxal-arginine adducts do not directly inhibit endothelial nitric oxide synthase. *Ann. N. Y. Acad. Sci.* **1126**, 231–234.
3. Kankova, K. (2008) Diabetic threesome (hyperglycaemia, renal function and nutrition) and advanced glycation end products: evidence for the multiple-hit agent?, *Proc. Nutr. Soc.* **67**, 60–74.
4. Carbone, D.L., Doorn, J.A., Kiebler, Z., and Petersen, D.R. (2005) Cysteine modification by lipid peroxidation products inhibits protein disulfide isomerase. *Chem. Res. Toxicol.* **18**, 1324–1331.
5. Sampey, B.P., Carbone, D.L., Doorn, J.A., Drechsel, D.A., and Petersen, D.R. (2007) 4-Hydroxy-2-nonenal adduction of extracellular signal-regulated kinase (Erk) and the inhibition of hepatocyte Erk-Est-like protein-1-activating protein-1 signal transduction. *Mol. Pharmacol.* **71**, 871–883.
6. Anderson, W.B., Board, P.G., and Anders, M.W. (2004) Glutathione transferase zeta-catalyzed bioactivation of dichloroacetic acid: reaction of glyoxylate with amino acid nucleophiles. *Chem. Res. Toxicol.* **17**, 650–662.
7. Go, Y.M., Halvey, P.J., Hansen, J.M., Reed, M., Pohl, J., and Jones, D.P. (2007) Reactive aldehyde modification of thioredoxin-1 activates early steps of inflammation and cell adhesion. *Am. J. Pathol.* **171**, 1670–1681.
8. Luo, J., Hill, B.G., Gu, Y., Cai, J., Srivastava, S., Bhatnagar, A., and Prabhu, S.D. (2007) Mechanisms of acrolein-induced myocardial dysfunction: implications for environmental and endogenous aldehyde exposure. *Am. J. Physiol. Heart. Circ. Physiol.* **293**, H3673–3684.
9. Baillie, T.A. (2006) Future of toxicology-metabolic activation and drug design: challenges and opportunities in chemical toxicology. *Chem. Res. Toxicol.* **19**, 889–893.
10. Ross, D. (2000) The role of metabolism and specific metabolites in benzene-induced toxicity: evidence and issues. *J. Toxicol. Environ. Health* **A 61**, 357–372.
11. Pagano, G. (2002) Redox-modulated xenobiotic action and ROS formation: a mirror or a window? *Hum. Exp. Toxicol.* **21**, 77–81.
12. Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G., and Monks, T.J. (2000) Role of quinones in toxicology. *Chem. Res. Toxicol.* **13**, 135–160.
13. Verrax, J., Delvaux, M., Beghein, N., Taper, H., Gallez, B., and Buc Calderon, P. (2005) Enhancement of quinone redox cycling by ascorbate induces a caspase-3 independent cell death in human leukaemia cells. An *in vitro* comparative study. *Free Radic. Res.* **39**, 649–657.
14. Ruiz-Ramos, R., Cebrian, M.E., and Garrido, E. (2005) Benzoquinone activates the ERK/MAPK signaling pathway via ROS production in HL-60 cells. *Toxicology* **209**, 279–287.
15. Person, M.D., Mason, D.E., Liebler, D.C., Monks, T.J., and Lau, S.S. (2005) Alkylation of cytochrome c by (glutathion-S-yl)-1,4-benzoquinone and iodoacetamide demonstrates compound-dependent site specificity. *Chem. Res. Toxicol.* **18**, 41–50.
16. Lindsey, R.H., Jr., Bender, R.P., and Osheroff, N. (2005) Effects of benzene metabolites on DNA cleavage mediated by human topoisomerase II alpha: 1,4-hydroquinone is a topoisomerase II poison. *Chem. Res. Toxicol.* **18**, 761–770.
17. Lau, S.S., Hill, B.A., Highet, R.J., and Monks, T.J. (1988) Sequential oxidation and glutathione addition to 1,4-benzoquinone: correlation of toxicity with increased glutathione substitution. *Mol. Pharmacol.* **34**, 829–836.
18. Peters, M.M., Jones, T.W., Monks, T.J., and Lau, S.S. (1997) Cytotoxicity and cell-proliferation induced by the nephrocarcinogen hydroquinone and its nephrotoxic metabolite 2,3,5-(tris-glutathion-S-yl)hydroquinone. *Carcinogenesis* **18**, 2393–2401.
19. Kleiner, H.E., Jones, T.W., Monks, T.J., and Lau, S.S. (1998) Immunochemical analysis of quinol–thioether-derived covalent protein adducts in rodent species sensitive and resistant to quinol–thioether-mediated nephrotoxicity. *Chem. Res. Toxicol.* **11**, 1291–1300.
20. Yoon, H.S., Monks, T.J., Walker, C.L., and Lau, S.S. (2001) Transformation of kidney epithelial cells by a quinol thioether via inactivation of the tuberous sclerosis-2 tumor suppressor gene. *Mol. Carcinog.* **31**, 37–45.
21. Kussmann, M., Lassing, U., Sturmer, C.A., Przybylski, M., and Roepstorff, P. (1997) Matrix-assisted laser desorption/ionization mass spectrometric peptide mapping of the neural cell adhesion protein neurolin purified by sodium dodecyl sulfate polyacrylamide gel

electrophoresis or acidic precipitation. *J. Mass Spectrom.* **32**, 483–493.

22. Voyager. (2004) Voyager Biospectrometry Workstation Training, Applied Biosystems, Forester City, CA.
23. Stewart, B.J., Doorn, J.A., and Petersen, D.R. (2007) Residue-specific adduction of tubulin by 4-hydroxynonenal and 4-oxononenal causes cross-linking and inhibits polymerization. *Chem. Res. Toxicol.* **20**, 1111–1119.
24. Fisher, A.A., Labenski, M.T., Malladi, S., Gokhale, V., Bowen, M.E., Milleron, R.S., Bratton, S.B., Monks, T.J., and Lau, S.S. (2007) Quinone electrophiles selectively adduct "electrophile binding motifs" within cytochrome c. *Biochemistry* **46**, 11090–11100.