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Dual Quinone Tagging for MALDI-TOF Mass Spectrometric Quantitation of Cysteine-Containing Peptide

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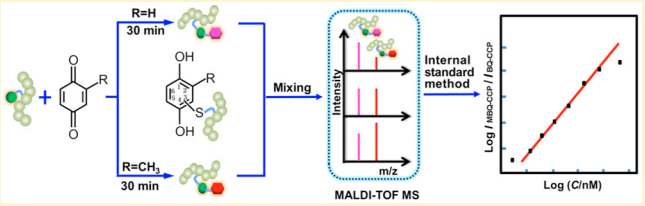
Supporting Information

ABSTRACT: A dual quinone tagging strategy is designed for quantitation of cysteine-containing peptide (CCP) with MALDI-TOF mass spectrometry. The quinone compounds can rapidly and specifically bind to the thiol group of cysteine residues by a Michael addition reaction, which is used to identify both CCP and the number of cysteine residues in CCP through the direct observation of untagged and tagged products. After reduced with DL-dithiothreitol, the intramolecular disulfide bond can also be identified. Using benzoquinone (BQ) and methyl-*p*-benzoquinone (MBQ) as dual tags and a peptide with an amino acid sequence of SSDQFRPDDCT (C-pep1) as a model target, respectively, the quantitation strategy is performed through the intensity ratio of MBQ-tagged C-pep1 to BQ-tagged C-pep1 as the internal standard. The logarithm value of the intensity ratio is proportional to C-pep1 concentration in a range from 5.0 to 5000 nM. The limit of detection is as low as 2.0 nM. The proposed methodology provides a novel tool for rapid characterization, identification, and quantitation of biomolecules containing thiol reactive sites and has a promising application in the large-scale detection and analysis of cysteine-containing biomolecules.



Protein identification, which refers to the study of protein content in a biological sample, is one of the main tasks in proteomics. However, sample complexity and the tremendous dynamic range of proteins in living systems are big challenges in comprehensive proteomics.[1,2] Therefore, many techniques including multidimensional protein identification technology and affinity selection of specific targets or residues such as immobilized metal ion affinity chromatography for phosphoproteome,[3−5] lectin affinity for glycoproteins,[6,7] and covalent chromatography[8,9] and isotope-coded affinity tag[10−12] for cysteine-containing peptides have been developed to reduce the sample complexity or to extract target proteins for comprehensive proteomic characterizations. In mass spectrometric detection of peptides and proteins, reactive nucleophilic amino acids, such as lysine[13] and cysteine,[14] are generally chemically tagged to ease protein identification by peptide mass fingerprinting. In particular, the presence of cysteine residues in peptides can be valuable for improving the reliability of protein identification via peptide mapping.[15] Thus, a rapid, simple means for determining information about the cysteine content in peptide maps is significant for the reliable identification of proteins.

Protein cysteine residue is one of the most reactive and common targeted amino acids due to its high chemical reactivity, low abundance, and universal distribution in a variety of proteomes.[16−19] The thiol groups of cysteine residues not only frequently participate in enzymatic reactions but also are subject to a variety of covalent post-translational modifications.[20−23] The tagging of peptides via the thiol groups leads to a drastic reduction in sample complexity for mass spectrometry (MS)-based proteomics.[24,25] The most commonly reported tagging reagents include iodoacetyl compounds,[26−28] maleimides,[29,30] acryloyl derivatives,[31] and thiol−disulfide exchange reagents.[32] However, most of these tagging reactions often require a time-consuming sample preparation procedure. Recently, a practical alternative by electrochemical oxidation or photooxidation of hydroquinone has been developed to fulfill the online tagging in a soft ionization technique of electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) source.[24,25,33−35] However, these tagging technologies cannot be used for quantitative detection due to the uncontrollable tagging efficiency and the instrument dependence of these tagging methods. Accordingly, it is still a persistent challenge to design a rapid and controllable tagging strategy for quantitation of cysteine-containing peptide (CCP) with MALDI-TOF MS.

Inspired by the oxidation tagging of hydroquinone to CCP,[24,25,33−35] this work used benzoquinone and its derivative to design a dual quinone tagging strategy. The simple and rapid Michael addition reaction of the cysteine residues in CCP to benzoquinones was demonstrated and used for the identification of CCP and its cysteine residue number. By using

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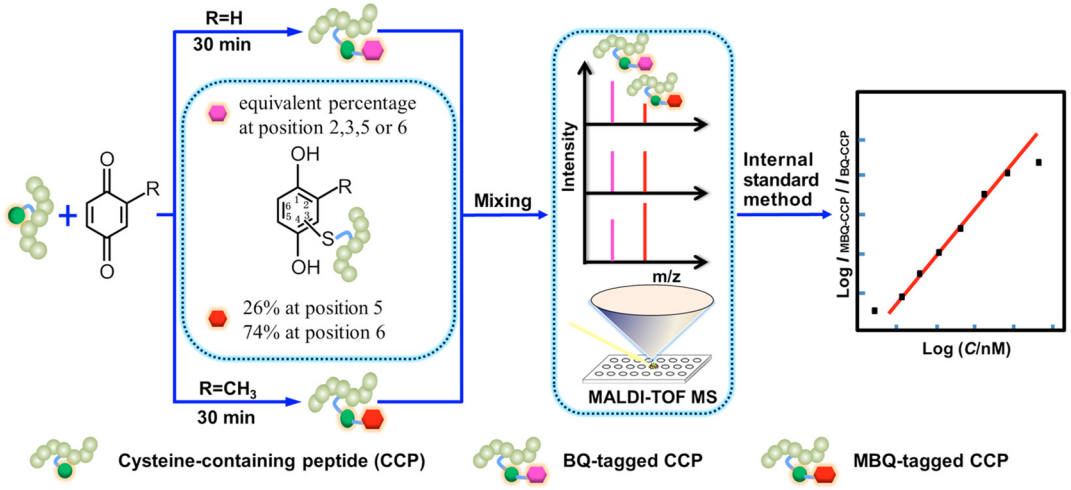
Scheme 1. Schematic Illustration of the Dual Tagging Strategy with Benzoquinones for MALDI-TOF MS Quantitation



benzoquinone (BQ)-tagged model target SSDQFRPDDCT (C-pep1) as an internal standard and methyl-*p*-benzoquinone (MBQ)-tagged C-pep1 to obtain the signal, a MALDI-TOF MS quantitation technique was proposed, which could be used for efficient detection of C-pep1 concentration with an internal standard method (Scheme 1). The logarithm value of the MS intensity ratio of MBQ-tagged C-pep1 to BQ-tagged C-pep1 showed a linear dependence on C-pep1 concentration in a range of 3 orders of magnitude. This method could quantitatively detect C-pep1 down to the nanomolar level. Because of its simplicity, quick speed, high sensitivity, and good versatility, the proposed strategy provides a new avenue for not only characterization and identification but also quantitation of biomolecules, including thiol reactive sites with MALDI-TOF MS, and has broad applications for analysis of cysteine-containing biomolecules.

EXPERIMENTAL SECTION

Materials and Reagents. Peptides SSDQFRPDDCT (C-pep1, Mw 1269.49), ACKCTCM (C-pep3, Mw 758.26), SSDQFRPDDGT (NC1, Mw 1223.51), and AEKTKEGVW (NC2, Mw 1046.54) were obtained from Sangon Biological Engineering Technology & Company Ltd. (Shanghai, China). Octreotide (C-pep2, $C_{49}H_{66}N_{10}O_{10}S_2$, MW 1019.24) was obtained from GL Biochem Ltd. (Shanghai, China). BQ, MBQ, *β*-casein, *β*-lactoglobulin A (*β*-LGA, from bovine milk, 90%), ammonium bicarbonate (NH_4HCO_3), DL-dithiothreitol (DTT), trifluoroacetic acid (TFA, ≥90%), and *α*-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich. Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany). Sequencing grade-modified trypsin was obtained from Promega (Madison, WI). All these reagents were used as received without further purification. Deionized water was prepared with a Milli-Q water purification system (Millipore, Milford, MA).

Selective Tagging of CCP. Benzoquinones, peptides including C-pep1, C-pep2, C-pep3, NC1, and NC2 were separately dissolved in deionized water to the required concentrations. The freshly prepared C-pep1, C-pep3, or DTT-reduced C-pep2 solution (5 μM, 100 μL) was mixed with benzoquinone (BQ or MBQ, 1 mM, 100 μL), incubated for different times (5, 10, 20, 30 min), and then deposited on the well of the MALDI target plate and dried at room temperature (RT) and atmosphere. For comparison, cysteine-free peptides such as NC1 and NC2 were mixed, respectively, with benzoquinones under the same conditions.

Protein Digestion and Tagging. After the desired protein (*β*-casein or *β*-LGA) was dissolved in the NH_4HCO_3 solution (25 mM, pH ∼ 8) to a final concentration of 1 mg mL^{-1} and denatured at 100 °C for 5 min, trypsin was added at an enzyme-to-protein ratio of 1:40 (w/w) to digest the protein at 37 °C for 16 h. Prior to use, 2 mM DTT was added into the digest of *β*-LGA and incubated at 37 °C for 1 h to break the disulfide bonds. To avoid the auto-oxidation of cysteine residue, the reduced sample was immediately used for subsequent tagging.

The tagging of the digested protein was fulfilled by adding 100 μL of 1 mM BQ or MBQ in 100 μL of the diluted digest to incubate at RT for 30 min. The tagged product could be directly deposited on the well of a MALDI target plate and dried at RT and atmosphere for MALDI-TOF MS analysis.

MALDI-TOF MS Analysis. After 1 μL of the tagged product was deposited on a MALDI plate and dried, 1 μL of saturated CHCA in 60% ACN (v/v) containing 0.1% TFA was introduced as a matrix. The MALDI-TOF MS experiments were performed on a 4800 Proteomics Analyzer (Applied Biosystems) equipped with the Nd:YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. MS data analysis was performed with Data ExplorerTM from Applied Biosystems. CCP were directly identified from one mass spectrum through matching a pair of peaks with an m/z shift of 108.09 or 122.12.

Quantitative Detection of C-pep1. To perform the quantitative detection, C-pep1 (100 μL) at different concentrations was separately mixed with an excess amount of MBQ (1 mM, 100 μL) and incubated for 30 min. Meanwhile, another C-pep1 solution at fixed concentration (200 nM) was mixed with an excess amount of BQ and incubated for 30 min, which was used as an internal standard. After the MBQ- and BQ-tagged peptides were mixed with a 1:1 volume, MALDI-TOF MS analysis was carried out for quantitative detection.

RESULTS AND DISCUSSION

Design of Strategy. Due to the quick binding of the thiol group of cysteine to benzoquinone and its derivatives via a Michael addition reaction, this work directly used for the first time BQ and MBQ as the tags of CCP for MALDI-TOF MS

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analysis. Different from the online tagging in MALDI analysis,[24] the use of BQ and MBQ avoided the need of TiO_2 nanoparticle as the photosensitizers to oxidize hydroquinone and generate BQ. To achieve MALDI-TOF MS quantitation, BQ was bound with a known amount of CCP to serve as the internal standard, and MBQ was tagged with CCP in the sample solution, and then their mixture was used for obtaining the mass spectrum in a single run (Scheme 1). The excessive BQ or MBQ could ensure all cysteine residues to be tagged by single BQ or MBQ. For BQ, the product was unique, while MBQ formed two tagged products at its positions 5 and 6, which was 26% and 74%, respectively.[33] The difference in binding positions did not affect the observation of MS peaks. The dual quinone tagging quantitation strategy could be performed by the dependence of the intensity ratio of MBQ-tagged CCP to BQ-tagged CCP on CCP concentration. Their logarithm values showed good linear calibration. Interestingly, the quinone tagging could also be used for identification of both CCP and the number of cysteine residues in CCP through the direct observation of MS peaks for untagged CCP and the tagged product.

Tagging of C-pep1. To verify the efficient tagging of CCP by benzoquinones, BQ and MBQ, SSDQFRPDDCT containing one cysteine residue was employed for monitoring of the selective tagging reaction with MALDI-TOF MS (Figure 1).

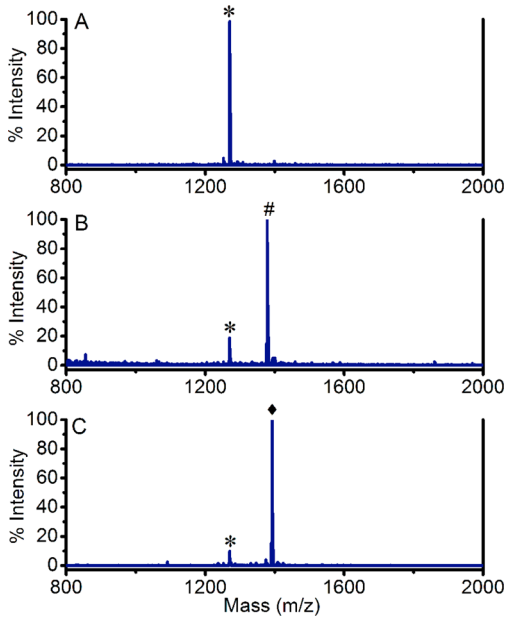

Figure 1. Mass spectra of (A) C-pep1, (B) mixture of C-pep1 and BQ, and (C) mixture of C-pep1 and MBQ with CHCA as matrix. * protonated C-pep1, # protonated BQ-tagged C-pep1, and ♦ protonated MBQ-tagged C-pep1.

The original C-pep1 showed only one peak at m/z of 1270.50 (Figure 1 A), which corresponded to the protonated form of C-pep1, while the mass spectrum of the mixture of C-pep1 and BQ exhibited two peak distributions, C-pep1 at 1270.50 and a new peak at 1378.62 (Figure 1B). The difference of 108.12 corresponded to the presence of single BQ, which was tagged to the free cysteine residue contained in C-pep1. Moreover, with the increasing reaction time, the C-pep1 peak quickly decreased and completely disappeared at the reaction time of 30 min, while the peak of BQ-tagged C-pep1 increased (Figure S1 of the Supporting Information), indicating the complete tagging of C-pep1. These phenomena were observed in the MALDI-TOF MS monitoring of the mixture of MBQ and C-pep1 (Figure 1C and Figure S1 of the Supporting Information), which showed a new peak at 1392.43. Thus, both BQ and MBQ could be efficiently bound with cysteine residue. The tagging site in the peptide was further verified with a positive-ion MS/MS method. The MS spectra showed the binding of BQ or MBQ with cysteine residue (Figure S2 and Tables S1–S3 of the Supporting Information).

To demonstrate the specificity of benzoquinones to tag the cysteine residue, cysteine-free peptides, SSDQFRPDDGT (NC1) and AEKTKEGVW (NC2), were used to react with benzoquinones. After mixing them with BQ or MBQ, no BQ- or MBQ-tagged peak was observed (Figure 2), illustrating that the BQ or MBQ was linked to only cysteine residue.

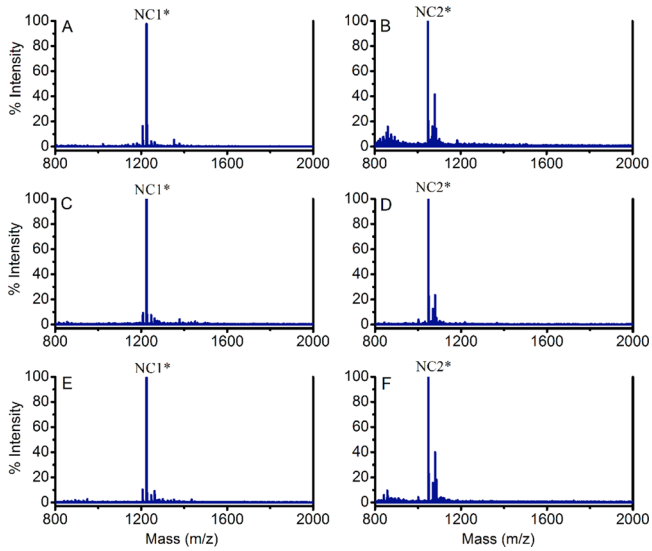

Figure 2. Mass spectra of (A) NC1, (B) NC2, (C) mixture of NC1 and BQ, (D) mixture of NC2 and BQ, (E) mixture of NC1 and MBQ, and (F) mixture of NC2 and MBQ with CHCA as matrix. * protonated NC1 and NC2.

Tagging of Peptides Containing Several Cysteines. Since lots of proteins contain many cysteine residues, the peptides containing an intramolecular disulfide bond (C-pep2) and three cysteines (C-pep3) were utilized to examine the count of cysteine units in peptides. Before treatment, C-pep2 showed only one peak at 1019.22, and no tagging of BQ or MBQ was observed (Figure 3, panels A, C, and E). After the intramolecular disulfide bond was reduced with DTT, the MS spectrum showed a peak at 1021.24 (Figure 3B), which was two protons more than C-pep2, indicating the opening of the intramolecular disulfide bond. The opened disulfide bond could be tagged by one or two benzoquinones, which could be observed at 1129.49 and 1237.51 for BQ and 1143.51 and 1265.54 for MBQ, respectively (Figure 3, panels D and F). As discussed above, the intensity of these peaks depended on the reaction time. These results demonstrated that the disulfide bond was chemically inactive for the Michael addition reaction, while the addition reaction occurred on the free thiol group, which provided a method for identification of intramolecular disulfide bond via a reduction process with DL-dithiothreitol.

When the peptide contained several cysteines, for example, three cysteines in ACKCTCM (C-pep3), upon mixing with BQ or MBQ, the singly, doubly, and triply tagging signals occurred

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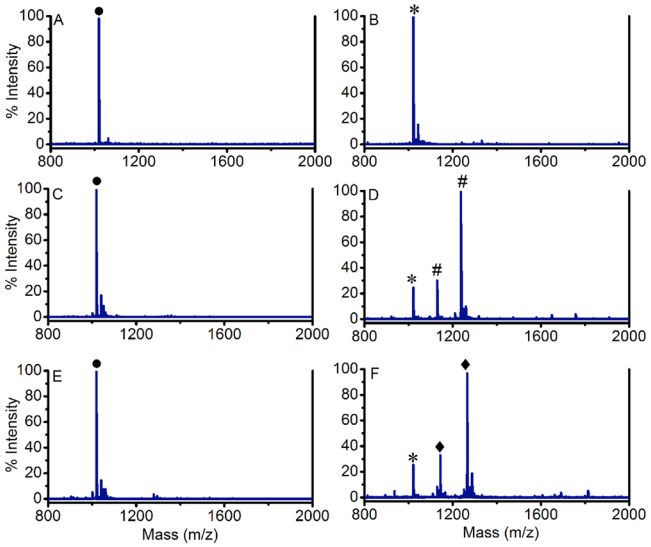

Figure 3. Mass spectra of (A) C-pep2, (B) reduced C-pep2, (C) mixture of C-pep2 and BQ, (D) mixture of reduced C-pep2 and BQ, (E) mixture of C-pep2 and MBQ, and (F) mixture of reduced C-pep2 and MBQ with as CHCA matrix. ● protonated C-pep2, * protonated C-pep2 after reduction with DTT, # protonated BQ-tagged reduced C-pep2, and ♦ protonated MBQ-tagged reduced C-pep2.

at 867.27, 975.29, and 1083.30 (Figure 4B) or 881.26, 1003.27, and 1125.30 (Figure 4C) along with the untagged C-pep3 at

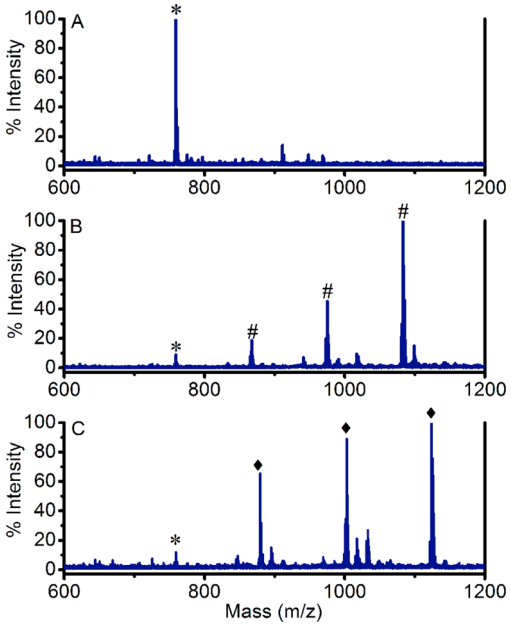

Figure 4. Mass spectra of (A) C-pep3, (B) mixture of C-pep3 and BQ, and (C) mixture of C-pep3 and MBQ with CHCA as matrix. * protonated C-pep3, # protonated BQ-tagged C-pep3, and ♦ protonated MBQ-tagged C-pep3.

759.25 (Figure 4A). It should be noted that the difference in binding positions could show up at the same m/z when the same number of BQ or MBQ was tagged to C-pep3. Furthermore, the peak intensity ratios of triply to doubly tagged, doubly to singly tagged and singly tagged to untagged C-pep3 increased with the increasing time. Therefore, both BQ and MBQ were possibilities for tagging the three residues at a sufficient reaction time, providing a convenient tool for counting cysteine residues in peptides, which was very important in protein identification.

Tagging for Cysteine-Containing Protein. The good tagging property of benzoquinones makes the tagging method promising to be applied in selective analysis of CCP in protein digest. Using β-lactoglobulin A, a cysteine-containing protein, as a model, the robustness of the tagging method was demonstrated. Both the tagging processes with BQ and MBQ showed three CCPs in the digest (Figure 5 and Table S4 of the

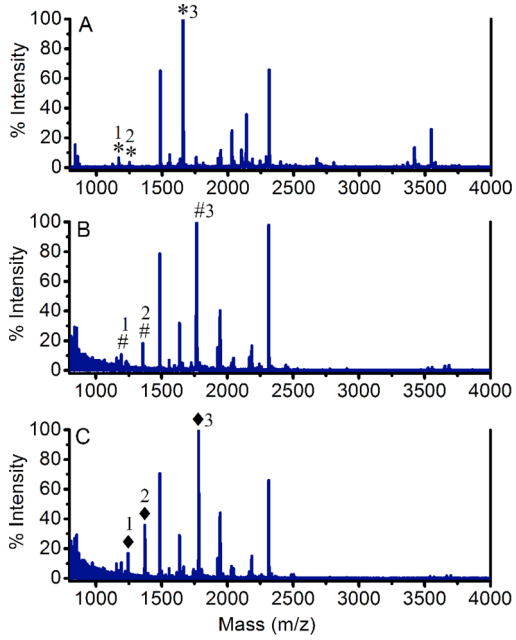

Figure 5. Mass spectra of (A) β-LGA digest, (B) mixture of β-LGA digest and BQ, and (C) mixture of β-LGA digest and MBQ with CHCA as matrix. * protonated CCP, # protonated BQ-tagged CCP, and ♦ protonated MBQ-tagged CCP.

Supporting Information). The result proved that the tagging method could selectively tag CCP in protein digest and enhanced the identification accuracy of β-lactoglobulin A.[25]

Dual Quinone Tagging Strategy. To achieve the quantitative detection, the tagging reaction must be complete and the product should possess sufficient stability. With the use of C-pep1 as the model target, the reaction time and pH of tagging buffer were optimized. At room temperature, the tagging products obtained at weak acidic, neutral, and weak alkaline pH showed the same MS peak (data not shown), indicating the pH of tagging buffer did not affect the tagging reaction. After incubation at room temperature for 20 or 30 min, the MS spectra for both BQ and MBQ tagging showed only the peak of tagged C-pep1 (Figure S1, panels E–H, of the Supporting Information), indicating the complete reaction. Therefore, 30 min was selected for the dual quinone tagging quantitative analysis.

The stability of the tagging products was verified by storage or incubation with another benzoquinone at room temperature. After incubation for 30 min, which was enough for the MS analysis, both BQ-tagged C-pep1 and MBQ-tagged C-pep1 did not show any change (Figure 6), indicating good stability and the tags could not be substituted by other BQ derivatives.

The inhomogeneous crystallization of analytes in MALDI matrices often produce "sweet spots" on the sample plate,

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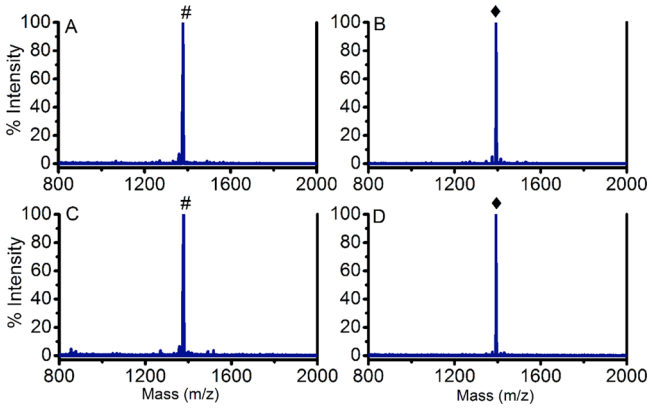

Figure 6. Mass spectra of (A) BQ-tagged C-pep1, (B) mixture of BQ-tagged C-pep1 and MBQ, (C) MBQ-tagged C-pep1, and (D) mixture of MBQ-tagged C-pep1 and BQ after storage or incubation for 30 min. # protonated BQ-tagged C-pep1 and ♦ protonated MBQ-tagged C-pep1.

which results in poor reproducibility and hinders the quantitative application of MALDI-TOF MS. The absolute peak intensity of MBQ-tagged C-pep1 showed great change in different experiments, which led to the deviation up to 50% (Figure S3 of the Supporting Information). Thus, this work used BQ-tagged C-pep1 as the internal standard to compare the peak intensity of MBQ-tagged C-pep1. At the same concentration of MBQ-tagged C-pep1 for several repeated detections, the peak intensity ratio of MBQ-tagged C-pep1 at 1392.62 to BQ-tagged C-pep1 at 1378.43 in single MS spectrum showed the same value (Figure S3 of the Supporting Information), proving the prerequisite for successful quantitative analysis.

The components of sample did not affect the MALDI-TOF MS quantitation with the proposed dual quinone tagging strategy. After different concentrations of β-casein digest, which does not contain the CCP, were added in the mixture of BQ- and MBQ-tagged C-pep1, although the absolute intensity of BQ- or MBQ-tagged C-pep1 became lower with the increasing concentration of β-casein digest, no obvious change of the peak intensity ratio was observed (Figure S4 of the Supporting Information). Thus, the dual quinone tagging strategy could be used for accurate quantitation with MALDI-TOF MS analysis.

Analytical Performance. As the excessive BQ or MBQ was used for the tagging of analytes with sufficient reaction time, the dual quinone tagging quantitation strategy could be performed by the dependence of the intensity ratio of MBQ-tagged CCP to BQ-tagged CCP on CCP concentration. Using BQ-tagged C-pep1 with the fixed concentration as an internal standard, the peak intensity ratio of MBQ-tagged C-pep1 at 1392.62 to BQ-tagged C-pep1 at 1378.43 increased with the increasing concentration of C-pep1 (Figure 7). At a reaction time of 30 min, the plot of the logarithm value of peak intensity ratio versus the logarithm value of C-pep1 concentration showed linearity in the range from 5.0 to 5000 nM with a R value of 0.996. From the calibration curve and the noise detected for a blank sample, the limit of detection (LOD) was calculated to be 2.0 nM. The relative standard derivations for three repeated detection of C-pep1 at 5.0, 100, and 500 nM were 3.56%, 3.69%, and 2.72%, respectively, indicating acceptable precision. These results suggested that the dual quinone tagging quantitative strategy was potentially appropriate for quantification of cysteine-containing analytes.

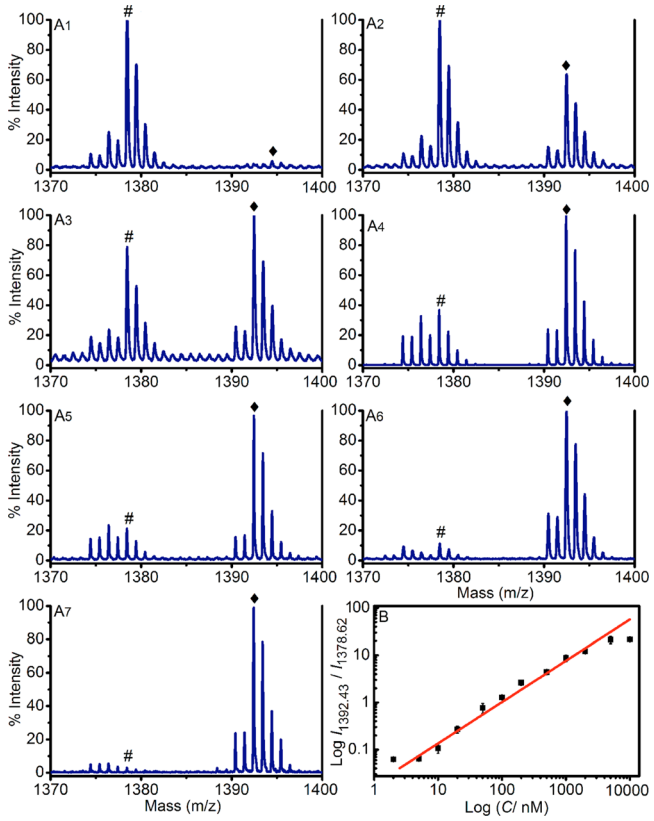

Figure 7. MALDI-TOF MS quantitation for detection of C-pep1 using 200 nM BQ-tagged C-pep1 as the internal standard. A1 to A7: mass spectra at 5.0, 50, 100, 200, 500, 1000, and 5000 nM C-pep1; B: calibration curve. The data are obtained from three independent experiments.

CONCLUSIONS

This work designs a simple and efficient dual tagging strategy for in situ labeling and MALDI-TOF MS quantitation of CCP. The quick Michael addition reaction of the thiol group in cysteine residues with benzoquinone or its derivatives and the specificity of this reaction are demonstrated. With the specific reaction, a method for identification of CCP and the number of cysteine residues is proposed. By using benzoquinone-tagged CCP as an internal standard and methyl-p-benzoquinone as the tag of CCP with unknown concentration, the MALDI-TOF MS quantitation of CCP is achieved. This novel method shows good analytical performance with high sensitivity, wide linear range, and acceptable precision. The proposed dual quinone tagging and MALDI-TOF MS quantitation provide a powerful tool for detection of cysteine-containing biomolecules and would facilitate the high-throughput study of biological processes.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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