Original Article

Identifying Double Bond Positions in Phospholipids using Liquid Chromatography-Triple Quadrupole Tandem Mass Spectrometry based on Oxygen Attachment Dissociation

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ABSTRACT

Lipids, a class of biomolecules, play a significant role in the physiological system. In this study, gas-phase hydroxyl radicals (OH•) and atomic oxygen (O) were introduced into a collision cell of a triple quadrupole mass spectrometer (TQ-MS) to determine the double bond positions in unsaturated phospholipids. A microwave-driven compact plasma generator served as a OH•/O source. The reaction between OH•/O and the precursor ions passing through the collision cell generates the product ions corresponding to the double bond positions in the fatty acyl chain. This double bond position specific fragmentation process initiated by attachment of OH•/O to the double bond of fatty acyl chain is characteristic to oxygen attachment dissociation (OAD). A TQ-MS incorporating OAD, in combination with liquid chromatography, realizes a high throughput analysis of the double bond positions in complex biomolecules. It is important to know the position of double bonds in lipids, as...
these molecules may exhibit widely different functionalities based on the position of double bonds. The assignment of double bond positions in a mixture of eight standard samples of phosphatidylcholines (phospholipids with choline head group) with multiple saturated fatty acyl chains have been successfully demonstrated.

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**INTRODUCTION**

Lipids are structurally diverse molecules that can be differentiated based on distinct head group, alkyl chain, position of double bond, and geometries such as stereospecific numbering (sn), cis-trans isomerization, and other alkyl chain modifications. Lipids with same molecular mass may exhibit completely different biological functions, depending on the position of the double bond in the fatty acyl chain.\(^1\) While a conventional collision induced dissociation (CID) based liquid chromatography–tandem mass spectrometry (LC-MS/MS) is an effective analytical technique to characterize the head group, alkyl chain length, and stereospecific numbering,\(^2,3\) it is still challenging to identify the position of the double bond in complex lipid samples containing numerous unsaturated species. To address the limitation of the conventional LC-MS/MS in the structural analysis of lipids, several complementary techniques have been proposed.\(^4-6\) In 2018, we developed a novel radical-induced MS/MS technique called oxygen attachment dissociation (OAD)-MS/MS, which allows double bond-specific dissociation upon the attachment of oxygen.\(^7\) OAD-MS/MS utilizes the interaction between the precursor ion and neutral radical species of hydroxyl radical (OH•) and/or atomic oxygen (O) generated by microwave discharge of water vapor and enables the assignment of the position of double bond. The methylene bridges adjacent to the double bond are selectively fragmented by OH• and/or O upon the oxidation of the double bond. These fragmented ions are useful for understanding the detailed structure of lipids in complex biological matrices. In contrast to other similar double bond-specific
fragmentation techniques such as ozone-induced dissociation (OzID), OAD-MS/MS is more laboratory-safe and operationally simple, because ozone, a strong oxidant, can be hazardous at even low concentrations. Additionally, the fragmentation efficiency of OAD-MS/MS can be higher than that of OzID under high vacuum (< 0.1 Pa). In fact, an ion mobility cell filled with high ozone pressure (≈ 10 Pa) was used for OzID in conventional LC-MS/MS for realizing high throughput analysis. In contrast, OAD-MS/MS can be coupled to a conventional collision cell of a triple quadrupole mass spectrometer (TQ-MS) under high vacuum (< 0.1 Pa), which significantly reduces the price and size of the equipment. In our previous study, OAD-MS/MS was demonstrated using matrix-assisted laser desorption/ionization (MALDI) ion trap time-of-flight mass spectrometry (IT-TOF MS). However, the relatively long reaction time (> 100 ms) required to detect the fragmented ions made the coupling to the conventional LC-TQ-MS system inefficient. To couple OAD-MS/MS to the conventional LC-TQ-MS system, the O/OH density inside the reaction cell should be enhanced around ten-fold, because the flight time of ions passing through the collision cell is practically less than 10 ms. To achieve this, we applied a microwave-driven inductively coupled plasma (ICP) radical source to OAD-MS/MS in this study. In our previous study, a microwave-driven capacitively coupled plasma (CCP) radical source was utilized for OAD-MS/MS. As reported by Shimabukuro and co-workers, the microwave-driven ICP source generates higher flux of radicals than the microwave-driven CCP source at high vacuum (< 0.1 Pa). Since the gas pressure of the collision cell is equivalent to the operating pressure of the microwave-driven ICP (≈ 0.1 Pa), the latter can be directly connected to the collision cell without differential pumping. This significantly improves the transport efficiency of the generated radical species into the collision cell. Herein, we coupled LC-TQ-MS and OAD-MS/MS (using ICP) and utilized this system for the determination of the position of double bond in unsaturated phospholipids.

**EXPERIMENTS**

**Materials**
Standard phospholipids (Table 1) were purchased from Avanti Polar Lipids (Alabaster, AL). Each sample was dissolved in methanol, and the solution was diluted to the final concentration of 1 µM in 80% (20 mM ammonium formate) /20% (acetonitrile (ACN)/isopropanol (IPA) mixture, 1/1, v/v) solution. Each diluted solution was mixed with an equal volume into a single tube.

**Liquid chromatography**

Shimadzu Nexera LC system was used for the separation of the lipid mixture. Briefly, 5 µL solution of the lipid mixture was injected using an autosampler (SIL-30AC; Shimadzu, Japan) into a core-shell column (Phenomenex Kinetex™ 2.6 µm, C8 100 Å, 50 mm × 2.1 mm column) at a flow rate of 0.5 mL/min. The samples were eluted by step gradient of mobile phase A (20 mM ammonium formate aqueous solution) and mobile phase B (ACN/IPA mixture, 1/1, v/v). The elution was carried out at follows: 0 to 1 min, 20% B; 1 to 2 min, a linear gradient from 20% to 40% B; 2 to 25 min, a nonlinear (exponential) gradient from 40% to 92.5% B; 25 to 26 min, a linear gradient from 92.5% to 100% B; 26 to 35 min, 100% B to wash the column; 35 to 38 min, 20% B to re-equilibrate.

| Lipid name | Exact Mass |
|------------|------------|
| PC (18:0/18:0) | 789.625 |
| PC (18:1(9Z)/16:0) | 759.578 |
| PC (18:0/18:1(9Z)) | 787.609 |
| PC (14:1(9Z)/14:1(9Z)) | 673.468 |
| PC (16:1(9E)/16:1(9E)) | 729.531 |
| PC (18:1(6Z)/18:1(6Z)) | 785.593 |
| PC (18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z)) | 777.531 |
| PC (16:0/20:4(5Z,8Z,11Z,14Z)) | 781.562 |

**Table 1. Standard phospholipids used in this study.**
Tandem mass spectrometer and ICP source

All MS and MS/MS experiments were performed using a TQ-MS equipped with an electrospray ionization (ESI) source (LCMS-8030 plus, Shimadzu corporation Japan), as shown in Figure 1. An alumina tube (i.d. 4 mm) was connected to the collision cell to introduce OH• and O generated by a radical source based upon a microwave-driven ICP. A 0.3-mm-thick, 3.0-mm-wide copper ribbon wound around a 6 mm outer diameter 4 mm inner diameter alumina discharge tube (Nikkato Corp, Japan) served as the antenna for the 2.45 GHz power source (Tokyo Keiki Inc., TMEB101B00B) delivered via N-type connector as shown in Figure 1(C). A cylindrical Nd-Fe magnets (φ 26.9 mm × φ 14 mm × 64 mm) forms a magnetic field structure inside the alumina discharge tube with the region of the intensity greater than 875 G corresponding to the electron cyclotron resonance (ECR) condition at 2.45 GHz in the axial direction. Water vapor was introduced into the discharge tube at a flow rate controlled by a needle valve (US-916P-P6.35, Fujikin, Japan) below 1 sccm. Ultra-pure water (Milli-Q, Japan Millipore Co., Ltd., Tokyo) stored in a reservoir tank reached the inlet of the needle valve at room temperature. High quality H$_2$O (98 atom%) for some specific experiments was purchased from Taiyo Nissan Co. Ltd. The microwave power below 10 W was applied to the copper spiral antenna, and consequently, the microwave discharge of water vapor generated OH• and O. The ICP source does not expose any metallic parts from the water vapor contributing to reduce recombination of OH• and O at the metallic wall.

The gas pressures inside and outside the collision cell was maintained below 0.1 and 3×10$^{-3}$ Pa, respectively, which were within the normal range for TQ-MS. The presence of radical species inside the radical source was confirmed by optical emission spectroscopy using a compact optical spectrometer (Ocean optics USB 2000+ and Ocean optics Flame-S).

RESULTS AND DISCUSSION

Microwave discharge of H$_2$O

First, we analyzed the composition of ions and neutral reactive species generated by the
microwave discharge of water. For this, the third quadrupole mass filter (Q3) was scanned from \( m/z \) 2 to 500 in the microwave discharge of water, without injecting the sample. Figure 2 shows the mass spectrum obtained by Q3 scan at a scan speed of 1,000 unit/s. Since no ions were observed when the microwave discharge was turned off (data not shown), it was concluded that the observed ions originated from the products of the microwave discharge. Abundant \( \text{H}_2\text{O}^+, \text{H}_3\text{O}^+, \text{NO}^+, \) and \( \text{O}_2^+ \) were observed at \( m/z \) 18.0, 19.0, 30.0, and 32.0, respectively. Ions observed between \( m/z \) 43 and 48 may be attributed to minor contaminants. Species such as \( \text{NO}^+ \) and the minor contaminants originate because of the microwave discharge of background residual gas (i.e., air and impurities) inside the radical source. To avoid introducing these ions into the collision cell, ion deflection magnets can be placed at the exit of the radical source. In this study, the target precursor ions for OAD-MS/MS are only singly charged positive ions. Therefore, the population of the observed ions is negligible in OAD-MS/MS because of the Coulombic repulsion among them.

Figure 3 shows the optical emission spectrum of the microwave discharge of water. The optical emission spectrum gives a rough estimate of the neutral reactive species generated by the discharge. The optical emission spectroscopy was performed in a separate experimental setup, disconnected from the mass spectrometer. The microwave discharge of water contained \( \text{H}^•, \text{O}, \) and \( \text{OH}^• \). The emission signal from \( \text{OH}^• \) was observed in the ultraviolet region at 309 nm \( (\text{A}^2\Sigma^+ - \text{X}^2\Pi) \), while those from O was observed at 845 nm. In addition, the microwave discharge resembles the atomic hydrogen spectrum up to Balmer gamma and delta lines (410 and 434 nm), which correspond to the higher excited levels of hydrogen atoms. Since the generated radicals were transported from the radical source to the collision cell via the alumina tube (Figure 1), the radicals would be cooled to room temperature owing to the collision with the inner surface of the alumina tube. These low-temperature \( \text{OH}^• \) and O species can initiate the OAD, as reported in our previous study.\(^7\) Low-temperature \( \text{H}^• \) radical, on the other hand, does not play any significant role in the dissociation of double bond.\(^7\)
OAD-MS/MS of phospholipid using TQ-MS

To demonstrate the utility of OAD-MS/MS for the analysis of phospholipid, PC 18:1/16:0 was used as a model and infused by syringe pump (0.5 mL/min) and ionized by ESI (3.5 kV capillary voltage). The precursor ion was isolated in Q1 and fragmented in the collision cell (q2) containing OH•/O. The product ions were scanned in Q3. The scan speed of Q3 was set to be 1,000 unit/s. Figure 4(A) shows the OAD-MS/MS spectrum [M+H]+ of the model phospholipid, PC 18:1/16:0. As in the case of previously reported MALDI-QIT-TOF based OAD-MS/MS result,7 OAD performed on TQ-MS/MS provided the abundant fragment ions at m/z 622.4, 650.4, 664.5, and 693.4, which are formed by oxidation of the C=C double bond accompanied by C–C bond cleavage adjacent to the oxidation site. The proposed structures of the fragmented ions are shown in Scheme 1. As a result, the present TQ-MS/MS based method is useful for the identification of double bond position in lipid acyl chain.

Next, we focused on the fragment ion at m/z 693.4, which is observed as odd nominal mass, although other fragments are appeared as even nominal mass. To simplify the peak assignment, we considered the so-called nitrogen rule, in which peak pair with odd and even nominal mass (in this case, a peak pair of ions at m/z 664.5 and 693.4). According to the rule, the ion at m/z 693.4 can be considered to be formed by the oxidative C–C bond cleavage with subsequent NO• attachment (Scheme 1). Unlike in the case of O and OH•, NO• does not attach to C=C double bond. In contrast, NO• can attach to the alkoxy radical, which is considered as the intermediate for the oxidative C–C bond cleavage and the corresponding reaction rate was reported as $4.4 \times 10^{-11}$ cm$^3$/molecule$^{-1}$s$^{-1}$.14 In consequence, we conclude that the ion at m/z 693.4 has -ONO group, as shown in Scheme 1. For comparison, OAD-MS/MS was performed using the same radical source in the MALDI-QIT-TOF MS used in our previous study (Figure 4(B)).7 However, the peak at m/z 693.4 for the nitrogen adduct was not observed in this experiment, indicating that the background nitrogen gas was contributed from the atmospheric ESI source and/or solvent.

To validate the proposed structures of the fragmented ions (Scheme 1), the microwave discharge of H$_2$O was employed instead of H$_2$O for the OAD-MS/MS (Figure 4(C)). While the
peak at m/z 622.4 was unchanged, peaks at m/z 650.4, 664.5, and 693.4 were increased by 2.0 Da, indicating that these peaks correspond to the oxidized form, wherein $^{16}$O is replaced by $^{18}$O. This result supports our proposed structure of the fragmented ions shown in Scheme 1. Interestingly, the ONO-adducted ion observed at m/z 693.4 is increased only by 2.0 Da. If both the oxygen atoms of ONO were replaced by $^{18}$O, the mass increase should have been 4.0 Da. Therefore, the product ion of m/z 693.4 would be induced by NO• attachment to the oxidized C=C specific fragment of m/z 664.5. Since NO• is generated by the microwave discharge of the residual gas from atmospheric ESI source, not from H$_2^{18}$O, the -ONO adduct ion observed at m/z 693.4 may increase only by 2.0 Da.

Next, we utilized OAD-MS/MS for the LC-MS of the lipid mixture listed in Table 1. Auto-MS/MS mode (data dependent acquisition) with a cycle time of 1 s was used for this purpose. MS was performed in Q3 scan mode with a scan time of 0.5 s. Following the MS experiment, the ion with the highest intensity (> $1 \times 10^6$ cps (count per second)) was selected and simultaneously analyzed in OAD-MS/MS from m/z 500–1,000, with a scan time of 0.5 s. Figure 5(A) shows the total ion chromatogram (TIC, m/z 500–1,000). Each standard phospholipid injected was clearly separated by LC. Figure 5(B)-(J) shows OAD-MS/MS spectrum of each phospholipid obtained by single scan with a scan time of 0.5 s. While no product ions were observed for the saturated phospholipid of PC (18:0/18:0) (Figure 5 (B)), abundant diagnostic product ions were observed for all the unsaturated phospholipids (Figure 5 (C)-(I)). As observed in the OAD-MS/MS experiment using syringe pump (Figure 3), the triplet peaks (non-oxidized, oxidized, and nitric-oxidized ions) around the double bond are observed corresponding to the position of the double bond. Although the OAD-MS/MS spectrum of phospholipid with multiple saturated fatty acyl chain (Fig. 5 (H) and (I)) becomes complex owing to overlapping multiple triplet peaks, the spectrum can be interpreted by analyzing each triplet peak. Figure 4(B) shows that the accuracy of peak assignment can be further enhanced by comparing the results of the microwave discharge of H$_2^{16}$O and H$_2^{18}$O.

**CONCLUSIONS**
Phospholipids were analyzed with OAD-MS/TQ-MS coupled with LC, in order to identify the double bond positions in standard phospholipids. The ICP radical source generated OH• and O with higher fluxes and lower operation pressure compared to the previously developed CCP radical source. The new ICP radical source realized a successful direct connection to the conventional collision cell of TQ-MS without the differential pumping system. Similar to that observed in our previous study on OAD-MS/MS using MALDI-IT-TOF-MS,7) the methylene bridges adjacent to the double bonds were selectively oxidized and consequently fragmented by OH• and O. These fragmented ions can be used for the high throughput analysis of the double bond positions in the collision cell. OAD-MS/MS, in combination with LC, is a promising new analytical technique for understanding the detailed structure of lipids in complex biological matrices.
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Figure 1. (A) Schematic diagram of triple quadruple mass spectrometer modified for OAD-MS/MS. (B) Picture of OAD-MS/MS system. (C) Picture of the alumina discharge tube of radical source.
Figure 2. MS spectrum obtained by Q3 scan in the microwave discharge of water, without injecting the sample.

Figure 3. Optical emission spectra of microwave water ICP at $1.4 \times 10^{-1}$ Pa, 10 W: (A) visible light region and (B) ultraviolet region.
Figure 4. OAD-MS/MS spectrum of PC(18:1/16:0) with microwave discharge of water using (A) ESI-TQ-MS and (B) MALDI-QIT-TOF. (C) OAD-MS/MS spectrum with microwave discharge of $^{18}$O using ESI-TQ-MS.
Scheme 1. Proposed structure of fragment ions observed in Figure 4.

Proposed mechanism of NO₂ adduct formation.

[PC (18:1(9Z)/16:0) + H]^+

m/z: 760.58

m/z: 622.44

m/z: 650.44

m/z: 664.45

m/z: 693.45

Scheme 1. Proposed structure of fragment ions observed in Figure 4.
Figure 5. (A) TIC of phospholipid mixture and (B)-(I) OAD-MS/MS spectra of each phospholipid obtained by one single product ion scan with 0.5 s.