Interfacial Electro-Epoxidation in Electrospray Ionization Mass Spectrometry for Simultaneous Analysis of Negatively and Positively Charged Unsaturated Glycerophospholipids

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ABSTRACT: In this study, we developed an AC-induced interfacial electro-epoxidation reaction for localization of double-bonds in negatively and positively charged forms of lipids simultaneously. An AC voltage plays multiple roles in this method, including initiation of the electrochemical epoxidation of double-bonds in both charge states of lipids, and protonation and deprotonation of lipid for detection in both ion modes. The advantages of simultaneous detection of negatively changed and positively charged unsaturated lipids, voltage-controlled electrochemical derivatization, and the low sample consumption allow its wide applications in a broad range of lipid-related research.

Introduction

Lipids are a diverse and ubiquitous group of biomolecules including fatty acids, triacylglycerols, glycerophospholipids, phenols, sphingolipids, sterols, eicosanoids.[1] They are the primary components of cell membranes and function crucially in cellular physiology such as energy storage, signal transduction, and metabolite transpot.[2, 3] Many studies have shown that altered lipid compositions are highly related with pathogenesis.[4, 5] For instance, human diseases such as obesity,[6] diabetes,[7] cardiovascular diseases,[8] cancers,[9] neurodegenerative disorders,[10] and autoimmune diseases[11] show aberrant lipid metabolism. Hence, it is highly desirable to fully characterize the compositions and structures of lipids for the sake of diagnosing diseases and identifying disease mechanisms.[12-15]

Elucidation of lipid structures has proven to be a challenge task not merely due to their large number of categories but also because of the diversity of lipid isomers including varied head groups and chain lengths, unsaturation levels, substitution positions as well as double-bond locations and geometries. The vast diversity and complexity of lipid structures has stimulated the development of new characterization methods with respect to sensitivity, selectivity and specialty. Mass spectrometry (MS) is often the choice for lipid analysis providing label-free detection, high sensitivity and structural characterization capability.[16] MS in combination with collision-induced dissociation (CID) is powerful in distinguishing lipid isomers bearing different head groups and chain lengths from specific fragment ions. However, the identification of the carbon-carbon double-bond positional isomers cannot be directly tackled by single stage or tandem MS. A few studies show that double-bond positional isomers may dramatically different roles in lipid physiology and pathology,[5, 17] thereby they draw extensive interest in the MS-based structural lipidomics community.

Mass spectrometric strategies for double-bond localization include (i) the use of hyphenated mass spectrometry methods such as ion mobility-mass spectrometry,[18] (ii) novel gas-phase fragmentation methods such as high energy collision-induced dissociation,[19] charge remote fragmentation,[20] and ultraviolet photodissociation,[21, 22] and (iii) derivatization methods yielding diagnostics peaks in CID. The advantages of chemical derivatization methods include easy operation and no requirements for special instrumentation, so they are potentially applicable to routine analysis. Chemical derivatization of carbon-carbon double bonds in lipids can proceed by ozonolysis,[23-26] methoxylation,[27, 28] methylthiolation,[29-31] olefin cross–metathesis,[32] Paternò–Büchi reaction,[17, 33-35] as well as epoxidation induced by plasma,[36-39] and offline meta–chloroperoxybenzoic acid.[40, 41]

Our group reported an on-demand electrochemical epoxidation method using a wire-in-a-capillary ESI emitter with a large orifice (LO-ESI emitter), which can electrochemically derivatize lipid double bonds for localization and analyze native lipids at different voltages in a single experimental run.[42] This method is particularly effective for positively charged lipids (e.g. PC and PE), because electro-epoxidation is initiated by positive DC voltages applied onto the ESI electrode. For negatively charged lipids, we convert the polarity by forming lithium ion adducts, functionalizing double bonds and performing the analysis in the positive ion mode.

In ESI-MS, charged droplets are conventionally produced under the excitation of a direct current (DC) high voltage. In the past two decades, two variants of ESI based on alternating current (AC)[43, 44] and pulsed DC[45, 46] high voltages have been developed. In a charging-discharging cycle, the AC and pulsed DC-ESI can generate successively positively and negatively charged droplets due to the polarity change, and protonated cations and deprotonated anions can be introduced and detected by MS when switching between the positive and negative ion modes. Simultaneous production of both positive and negative ions from a single spray emitter was also observed

in induced ESI-MS using contactless pulsed DC without changing the polarity of the applied potential.[47, 48] The feature of rapid polarity switching in AC and pulsed DC high voltage ESI motivates us to initiate on-demand electro-epoxidation of lipids using AC voltages.

In this work, we report an AC-induced electrochemical mass spectrometric strategy for characterization of carbon-carbon double-bond positions in unsaturated lipids in both positive and negative ion modes. Negatively and positively charged forms of lipids are electro-epoxidized simultaneously in the positive half cycle. Negatively and positively charged lipids are detected individually in the positive and negative half cycle by MS.

Methods

Materials. Acetonitrile (ACN), water, chloroform, methanol, hydrochloric acid, oleic acid and ammonia chloride were purchased from Sigma-Aldrich (St. Louis, MO). All lipids standards were purchased from Avanti Polar Lipids (Alabaster, AL). All reagents were used without additional purification.

Mass spectrometry. An Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA) was used for all the nanoESI-MS/MS Analysis. The following MS parameters were used for all data acquisition. Samples were ionized though the application of 1.5~3 kV AC voltage (at peak, 100 Hz square wave). S-lens RF level was set to be 67.6% in the positive ion mode and 60% in the negative ion mode. Capillary temperature was set at 275 °C in both ion modes. Full MS scans were acquired over m/z range 200~1000 in the positive ion mode and 100~500 in the negative ion mode. In both ion modes, the resolution was set at 60000. Microscan number was set at 1 and maximum injection time was set at 500 ms. Tandem mass spectra were obtained via collision-induced dissociation (CID) with a normalized collision energy of 30%.

LO-ESI emitters. Emitters were pulled from single-channel borosilicate glass capillary tubes (1.5 mm O.D., 0.86 mm I.D., #1B160-6, World Precision Instruments, Sarasota, FL) by using a P-1000 micropipette puller (Sutter Instrument Company Co., Novato, CA). Typical program for pulling the large orifice tip used in this work is: Ramp 490; Heat 525; Pull 0; Velocity 3; Time 250; Pressure 500. The average orifice of the fabricated nanoESI emitters was around 60 μm (Figure S1).

LO-ESI-MS/MS Analysis. A platinum wire was inserted into the LO-ESI emitter to provide efficient electrical contact with the solution. Alternating current (AC) high voltages were applied to the electrode inside the emitter by using a homemade tunable alternating current power supply. The AC power supply consisted of a wave function generator (Model DS345, 30 MHz, Stanford Research Systems, Sunnyvale, CA), a power amplifier (Model 609D-6, 0 ~ ±4kV, Trek, Lockport, NY) and a digital storage oscilloscope (DSO5102P, 100MHz, 1GSa/s, Hantek Electronic Co., Ltd, Qingdao, China) for the monitoring of output voltages. The LO-ESI emitter is placed 5 mm in front of the mass inlet.

Results and Discussion

AC induced on-demand electro-epoxidation for localizing C=C bonds in negatively charged lipids. The LO-ESI set-up is shown in Figure 1 and the instrument parameters were the same as used in our previous work.[42] The voltage is supplied by an external AC power

supply instead of the DC power incorporated in the mass spectrometer. Oleic acid (FA 18:1(Δ9)) was selected as a proof-of-concept for demonstrating the feasibility of the AC-LO-ESI-MS strategy for negatively charged lipids.
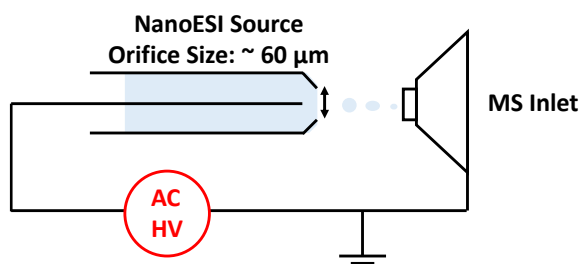

Figure 1. The configuration of AC-LO-ESI source for on demand electro-epoxidation of lipid double-bonds.

20 μM Oleic acid dissolved in acetonitrile/water (80/20, v/v) with 10 mM NH_4Cl was loaded into a LO-ESI emitter with a 60 μm orifice, and the mass spectrometer was operated in the negative ion mode. Upon the application of 3 kV voltage (at peak, 100 Hz square wave) to the platinum wire in the emitter, a typical stable Taylor cone was formed at the tip of the emitter, and the depronated acid at m/z 281.24 was dominant in the mass spectrum (Figure 2a) without any apparent peaks corresponding to the FA epoxides, showing no electrochemical epoxidation of C=C bonds when using the stable Taylor cone. Once the AC voltage was switched to 2 kV, a new peak at m/z 297.24 was detected with 65% relative abundance of the deprotonated acid at m/z 281.24 (Figure 2b), while a metastable Taylor cone could be observed. An exact 16.00 Da mass increase from m/z 297.24 above the deprotonated peak at m/z 281.24 corresponds to one oxygen atom. The results demonstrate the occurrence of electro-epoxidation of the C=C bond in oleic acid only for the metastable Taylor cone. Upon CID of the epoxide at m/z 297.24, two diagnostic ions at m/z 155 and 171 were observed in the tandem mass spectrum (Figure 2c). The diagnostic peaks have the signature 16 Da mass difference, and their masses indicate that the double-bond position at Δ9 in oleic acid. Other fragment ions at m/z 279.23 and 253.25 correspond to the losses of water and carbon dioxide, respectively, showing the carboxylic substructure of oleic acid.
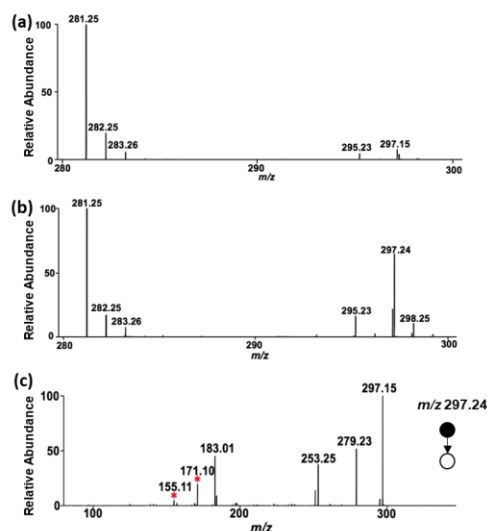
Figure 2. Full mass spectra of oleic acid (FA 18:1(Δ9)) at (a) 3 kV and (b) 2 kV (AC voltage, peak to peak, square wave, 100 Hz); (c) tandem spectrum of epoxidized oleic acid, the red stars indicate the two diagnostic ions to locate double-bond positions.

We also investigated the feasibility of the AC-LO-ESI strategy in the analysis of a mixture of multiple negatively charged lipids. A mixture solution of 20 μM oleic acid and 20 μM vaccenic acid (FA 18:1(Δ11)) was dissolved in acetonitrile/water (80/20, v/v) with 10 mM NH4Cl. Upon the application of a 2 kV AC voltage, only one epoxidized peak was observed at m/z 297.24, which came from electrochemical epoxidation of oleic acid and vaccenic acid. The fragments of the epoxide at m/z 297.24 generated two groups of diagnostic ions, i.e., the ions at m/z 155 and 171 for oleic acid and the ions at m/z 183 and 199 for vaccenic acid (Figure S2). In addition, the ion intensity ratios of each group of diagnostic ions were found to be linearly correlated with the concentration ratios (R=0.9984, Figure S3), indicating no mutual interference and also concentration-independent epoxidation. All these results prove the success of the AC-LO-ESI-MS strategy for double-bond localization of negatively charged lipids. In addition to direct analysis of negatively charged unsaturated lipids, the AC-LO-ESI-MS strategy maintains the advantages of the DC-LO-ESI-MS strategy such as voltage-controlled chemical derivatization and low sample consumption.

AC induced on-demand electro-epoxidation for localizing C=C bonds in positively charged lipids. Regardless of the successful localization of double bonds in negatively charged lipids, the half positive duration in each AC cycle is still idle for MS detection, which opens an additional window to examine positively charged lipids. A positively charged phosphatidylcholine (PC 18:1(Δ6)-18:1(Δ6)) was used as the model analyte to test the feasibility of the AC-LO-ESI strategy for identification of C=C bonds in positively charged lipids. PC 18:1(Δ6)-18:1(Δ6) (20 μM) was dissolved in acetonitrile/ water (80/20, v/v) containing 10 mM HCl and loaded into a LO-ESI emitter with a 60 μm orifice. Upon the application of 3 kV AC voltage, only the protonated PC peak at m/z 786.67 was observed (Figure 3a) when the mass spectrometer was operated in the positive ion mode. When a low AC voltage of 2 kV was applied instead, the mass spectrum in Figure 3b demonstrates the formation of +16 Da and +32 Da products at m/z 802 and m/z 818, respectively. Upon CID, the tandem mass spectrum of the mono-epoxidized product at m/z 802 clearly shows the presence of two diagnostics peaks at m/z 618 and 634 with a 16 Da mass shift (Figure 3c), which indicates the double bond located at Δ6 position.

Furthermore, we compared electrochemical epoxidation efficiency of PC (defined as the intensity ratio of the mono-epoxide at m/z 802.67 to the intact PC at m/z 786.67) induced by AC and DC voltages. As shown in Figure S4, the AC strategy offers slightly superior epoxidation efficiency over the DC strategy. In this case, the result demonstrates that the AC strategy offers comparable effectiveness to the DC strategy in identifying double bond positional isomers based on electrochemical epoxidation of C=C bonds.

A mixture solution of 20 μM PC 18:1(Δ6)-18:1(Δ6) and 20 μM PC 18:1(Δ9)-18:1(Δ9) in acetonitrile/water (80/20, v/v) containing 10 mM NH4Cl and 1 mM HCl was also tested by the AC-LO-ESI strategy. Upon the application of a 2 kV AC voltage, mono- and di-epoxidized peaks were observed at m/z 802.67 and 818.68, respectively. The fragment of the epoxide at m/z 802.67 generated two

groups of diagnostic ions, i.e., the ions at m/z 618 and 634 for PC 18:1 (6)-18:1 (6) and the ions at m/z 660 and 676 for PC 18:1 (9)-18:1 (9) (Figure S5). In addition, a good linear relationship between the ion intensity ratios of each group of diagnostic ions and the concentration ratios (R=0.9984) was observed (Figure S6), indicating no mutual interference and also concentration-independent epoxidation.
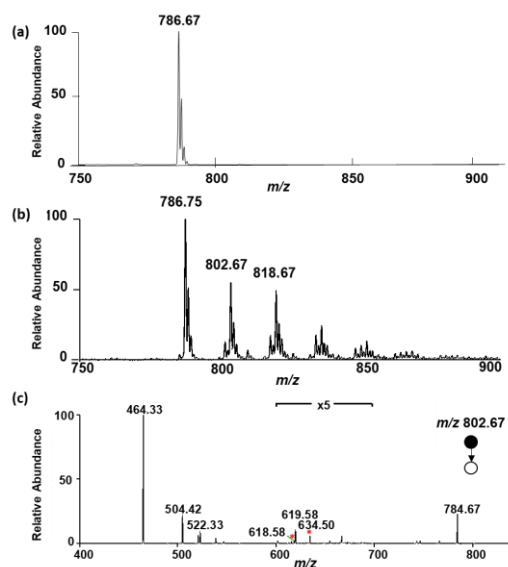

Figure 3. Full mass spectra of PC 18:1(Δ6)-18:1(Δ6) at (a) 3 kV and (b) 2 kV (AC voltage, peak to peak, square wave, 100 Hz); (c) tandem spectrum of epoxidized PC 18:1(Δ6)-18:1(Δ6). the red stars indicate the two diagnostic ions to locate double-bond positions.

Simultaneous identification of double bonds in negatively and positively charged lipids by AC induced electro-epoxidation. The above success of the AC-LO-ESI-MS strategy for applications to lipids which ionize to give either negatively or positively charged ions urges us to further undertake the simultaneous identification of both the negatively and positively charged forms of lipids in a single run by taking full advantage of duration time in an AC cycle. The whole workflow follows in four directions (Figure 4). In the +3kV and −3 kV half cycle, lipids of both polarities in their native forms were detected by the mass spectrometer. The protonated species in the positive ion mode and the deprotonated species in the negative ion mode were successively detected and fragmented for the characterization of head group lipid isomers. Electrochemical epoxidation of C=C bonds in both positively and negatively charged lipids was induced in the 2 kV half cycles. The positively charged epoxidized lipid was detected by the mass spectrometer in the same +2 kV half cycle, followed by the detection of the negatively charged epoxidized lipid in the next -2 kV half cycle.
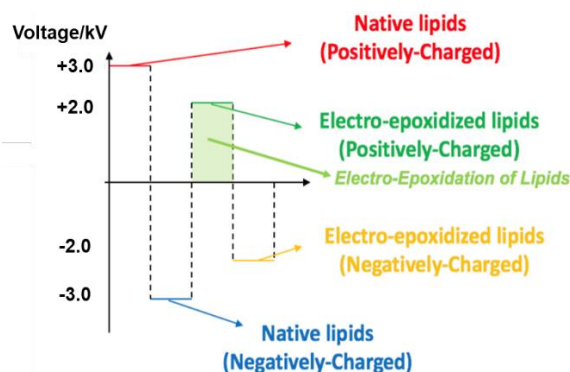
Figure 4. Lipid analysis workflow using AC-LO-ESI for the detection of positively and negatively charged forms of lipids.

Two lipids, positively charged PC 18:1(Δ6)-18:1(Δ6) and negatively charged oleic acid FA 18:1(Δ9), were used for investigating the feasibility of simultaneous identification in one run. A mixture of 20 μM PC 18:1(Δ6)-18:1(Δ6) and 20 μM FA 18:1(Δ9) was prepared in acetonitrile/water (80/20, v/v) containing 10 mM NH4Cl and 1 mM HCl, and then introduced to the LO-ESI emitter. When a stable electrospray was initiated at an AC voltage of 3 kV, the mass spectrometer detected the protonated PC at m/z 786.67 in the positive ion mode and the deprotonated FA at m/z 281.24 in the negative ion mode. The spectra are nearly the same with comparable intensities to the spectra of pure lipid standard analysis (Figure 2a, 3a). As the AC voltage was decreased to 2 kV, the mono- and di-epoxides of PC at m/z 802.67 and 818.67, respectively, were observed in the positive ion mode (Figure S7a), and the epoxidation peak of FA with +16 Da shift at m/z 297.24 were thereafter detected in the negative ion mode (Figure S7b). The signal intensities of the epoxides were comparable to those in the pure lipid standards (Figure 2b and 3b), demonstrating no mutual interference in the simultaneous identification. In addition, diagnostic ion pairs with a signature 16 Da mass separation can also be detected in the tandem mass spectra using CID fragmentation (Figure S6c and S6d), confirming the positions of C=C bonds in FA and PC. Limits of detection (LOD) for the simultaneous identification of PC 18:1(Δ6)-18:1(Δ6) and FA 18:1(Δ9) were further evaluated. 1 μM was found for both lipids (Figure S8, S9) at 3 times signal/noise level. These results demonstrate the feasibility of the AC-LO-ESI strategy in simultaneous identification of both positively and negatively charged lipids in a single trial.

When we investigated the simultaneous identification of lipids using AC-LO-ESI method, we found the chloride ions in the solvent system plays an important role in the electro-epoxidation and mass spectrometric detection. In DC-LO-ESI method, chloride ions from 10 mM hydrochloric acid were sufficient for electrochemical epoxidation, and also beneficial to the detection of native and epoxidized PC ions in the protonated forms. However, excess protons suppressed the signals of the native and epoxidized FA in the deprotonated forms. A mixture of hydrochloric acid and ammonium chloride were found a good balance between the epoxidation and MS detection of lipids of both polarities. A further investigation of the ratio of the two chloride sources suggested an optimal mixture of NH4Cl (10 mM) and HCl (1 mM) for simultaneous lipid detection in both ion modes.

Conclusion

In summary, we have developed a mass spectrometric strategy of AC-induced epoxidation for double-bond localization in lipids using both positively and negatively charged unsaturated lipids simultaneously. The application of AC voltage allows simultaneous detection of lipids in both ion modes, but also the electrochemical epoxidation of both lipids in the positive half cycles. This method has the following features: i) the online electro-epoxidation allows structural characterization with no external apparatus or pre-analysis treatment of samples; ii) simultaneous detection of lipids in both ion modes simplifies the workflow for the complex sample analysis and requires a small sample consumption. The unique feature of locating double bonds in both ion modes simultaneously and the simple setup enable its wide application in many lipid-related fields.

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