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Novel Advances in Shotgun Lipidomics for Biology and Medicine

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Abstract

The field of lipidomics, as coined in 2003, has made profound advances and been rapidly expanded. The mass spectrometry-based strategies of this analytical methodology-oriented research discipline for lipid analysis are largely fallen into three categories: direct infusion-based shotgun lipidomics, liquid chromatography-mass spectrometry-based platforms, and matrix-assisted laser desorption/ionization mass spectrometry-based approaches (particularly in imagining lipid distribution in tissues or cells). This review focuses on shotgun lipidomics. After briefly introducing its fundamentals, the major materials of this article cover its recent advances. These include the novel methods of lipid extraction, novel shotgun lipidomics strategies for identification and quantification of previously hardly accessible lipid classes and molecular species including isomers, and novel tools for processing and interpretation of lipidomics data. Representative applications of advanced shotgun lipidomics for biological and biomedical research are also presented in this review. We believe that with these novel advances in shotgun lipidomics, this approach for lipid analysis should become more comprehensive and high throughput, thereby greatly accelerating the lipidomics field to substantiate the aberrant lipid metabolism, signaling, trafficking, and homeostasis under pathological conditions and their underpinning biochemical mechanisms.

Keywords

Alzheimer's disease; bioactive lipids; lipidomics; mass spectrometry; metabolic syndrome; shotgun lipidomics

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1. Introduction

Lipids are an essential component of any biological systems, which play many crucial functions, including cellular barriers, membrane matrices, signaling, and energy depots. Cellular lipids are highly complex and very dynamic. Tens to hundreds of thousands of possible lipid molecular species as predicted could be present in the cellular lipidome at the level of attomole to nanomole of lipids per mg of protein [1-3]. These lipid species possess different polar head groups and various aliphatic chains differentially connected to the head groups, which are the foundations to classify this large body of lipid species into a limited number of classes and subclasses, respectively [4, 5]. The aliphatic chains are varied with different lengths (i.e., different numbers of carbon atoms), various degrees of unsaturation, different locations of double bonds, potential branches, etc. These varieties constitute the large number of individual lipid molecular species.

In addition to these aforementioned diversities and complexes, many other factors could make the body of cellular lipids even more variable. For example, cellular lipid molecular species and composition are varied from species, cell types, cellular organelles, subcellular membranes, and membrane microdomains (e.g., caveola and/or rafts) [6-8]. Also, the body of cellular lipid species and their composition are dynamically changing as the environment is perturbed [9] or a consequence of life cycle [10-12].

The entire collection of chemically distinct lipid species in a cell, an organ, or a biological system has been referred to as a lipidome [13]. By analogy to other “omics” disciplines, studying lipidomes in a large scale and at the levels of intact molecular species by utilizing analytical chemistry technologies and tools has been referred to as lipidomics, which is suddenly bloomed with different definitions [9, 14], demonstrations of technologies [9, 15], and biological applications [9, 16, 17] in 2003. The emergency of this discipline in the era is likely due to that the investigators in the lipid field have clearly recognized that the metabolism of lipid molecular species or between individual lipid classes is interwoven [2] and the metabolism of the entire lipidome should be investigated in a systems biology approach [18]. The development of necessary technologies for lipidomics also greatly catalyzes the emerging of this field.

Lipidomics as a disciplinary field is through the analysis of lipid structures, mass levels, cell functions, and interactions in a spatial and temporal fashion to determine the dynamic changes of lipids during physiological or pathological perturbations, and life cycles. Recent studies in lipidomics have largely focused on five areas [19]. These include (1) identification of novel lipid classes and molecular species; (2) development of quantitative methods for analysis of lipids in cells, tissues, or biological fluids at the level of attomole to femtomole per mg of protein; (3) pathway analysis that clarifies metabolic adaptation in health and disease and biomarker analysis that facilitates diagnosis of disease states and determination of treatment efficacy; (4) tissue mapping of altered lipid distribution present in complex organs; and (5) bioinformatics approaches for the automated high-throughput processing and molecular modeling with lipidomics data. Accordingly, lipidomics plays an essential role in defining the biochemical mechanisms underlying lipid-related disease processes through

determination of alterations in cellular lipid signaling, metabolism, trafficking, and homeostasis.

Regardless of that many other modern technologies (e.g., nuclear magnetic resonance, fluorescence spectroscopy, high performance liquid chromatography (HPLC), and microfluidic devices) have been used in lipidomics research [20], mass spectrometry (MS), largely due to the development of new types of instruments and techniques, has been widely used in lipidomics and greatly accelerated the progress of lipidomics [21]. In electrospray ionization (ESI), ions are generated in an electrospray ion source after a stream of lipid solution is pushed into the ion source chamber by a mechanical force. Depending on whether lipid analysis conducted with the lipid solution delivered to the ion source chamber is under a constant lipid concentration condition, these ESI-MS based lipidomics strategies can be classified into two major categories: (1) direct infusion in which the concentration of lipid solution delivered to the ion source is constant and (2) HPLC delivered in which the concentration of lipid solution is constantly changing. These platforms are termed “shotgun lipidomics” and “LC-MS based lipidomics”, respectively, in literature. These approaches have previously been extensively reviewed [19, 22-24]. In this article, only the fundamentals and applications of shotgun lipidomics approach are discussed and updated.

1.1. Shotgun lipidomics

Direct infusion was originally used to efficiently deliver lipid samples and to avoid difficulties of lipid analysis from alterations in concentration, chromatographic anomalies, and ion-pairing alterations in early 1990 [25-28]. Around 2004, the platforms developed based on this strategy were separately named as “shotgun lipidomics” by Han and Gross [29] and Ejsing et al. [30]. Since then, this technology has become one of the widely used approaches in lipidomics, particularly for high-throughput analysis of lipids [19, 29, 31-34].

The most basic device to deliver lipid solution for direct infusion is a syringe pump. This device is relatively low cost and can be constructed to deliver a few microliters of solution per minute. Generally, the higher the flow rate delivered with this device, the more stable flow can be achieved. A tightly sealed, high quality glass syringe is always recommended for this purpose. Its major weakness is that automation of lipid analysis is difficult to achieve with this delivery system. Clogging of the delivery capillary line occurs frequently, even if the samples are prepared carefully. Moreover, the requirement of a relatively high flow rate for stabilizing the ion current makes the consumption of a large volume of lipid solution, thus a large amount of source biological materials.

Robotic nanoflow ion sources (e.g., NanoMate device) make the direct infusion automated and eliminates all the aforementioned limitations by using a syringe pump, i.e., substantial reduction of potential sample clogging and dramatic reduction of the sample size and cross-contamination [33, 35]. A basic procedure for utilization of NanoMate device has been provided in detail [33, 36]. Infusing 5 to 10 μL of sample solution is sufficient for nearly an hour of analysis with a stable spray, thereby guaranteeing high reproducibility, sequential runs of a series of requested mass spectra, and accurate quantitation [33, 35]. A major drawback of utilizing this device is its relatively high cost (e.g., higher prices of the device itself and the associated chips relative to the cost of an LC system including the columns). A

major concern of handling small volume lipid samples is the solvent evaporation during long automated analysis [33]. Inclusion of less volatile solvents such as isopropanol has been proved to be helpful in improving solvent preservation, lipid solubility, and ionization efficiency [37]. Sealing of the sample plates with thin aluminum foil has also been found useful to minimize solvent loss [33].

It should be recognized that loop injection (delivering sample solution with an LC system, but without an LC column) is also used in lipidomics and carries some features of direct infusion [38]. Since this sample delivery method is unable to maintain a constant lipid concentration as solvent is pushed through the sample loop, this method does not belong to the category of shotgun lipidomics, although a multiple-injection method to maintain the constant concentration has been practiced [38]. In contrast, direct infusion of individual fractions collected after LC separation (including those from a solid phase extraction (SPE) column) falls to the category of shotgun lipidomics due to its maintenance of a constant concentration of the delivered lipid solution.

1.2. Features of shotgun lipidomics

The principles of shotgun lipidomics are the maximal utilization of the unique chemical and physical properties of lipid classes, subclasses, and individual molecular species to facilitate identification and quantification of a cellular lipidome directly from organic extracts of biological samples on a large scale and in a high-throughput fashion [29]. These principles of shotgun lipidomics can only be achieved under the constant concentration of lipid solution. This is a key feature of shotgun lipidomics since a few factors that influence identification and quantification of individual lipid species can be eliminated under a constant concentration condition. First, interactions between lipid species are maintained constant under a constant concentration condition, therefore, the contribution of individual lipid species to the ion current in an ESI source is constant, thereby leading to a constant ratio of ion peak intensities between lipid species of a class as demonstrated in Figure 1 despite changes of ionization conditions [39]. Such a constant ratio is essential for ratiometric comparison (see below) between ion peaks and can be achieved under different experimental conditions (Figure 1), on different MS instruments, and in different laboratories if a constant concentration condition can be maintained and the solution is analyzed in the low concentration region to avoid occurrence of lipid aggregation [40]. Second, also due to the constant interactions between lipid species under a constant concentration condition, ion suppression between each other within a lipid class or between lipid classes is constant. Third, lipid aggregation, which is a big concern for lipid quantification [40], can be well controlled and minimized.

This unique feature of shotgun lipidomics (i.e., MS analysis is performed under a constant lipid concentration) allows researchers to have virtually unlimited time to improve mass spectral signal/noise ratio, to perform detailed tandem mass spectrometric (MS/MS) mapping with multiple fragmentation techniques (including precursor-ion scanning (PIS) and neutral loss scanning (NLS)), to conduct multi-stage MS/MS analyses, and to ramp different instrumental variables (e.g., collision energies, collision gas pressure, ion-mobility parameters, etc.). Performing these experiments is obviously the limitation typically

encountered from the concentration changing or time constraints present in the “on the fly” analysis during chromatographic elution.

Another major feature of shotgun lipidomics is that a full mass spectrum can be acquired to display the molecular ions of all the species of a lipid class of interest. This feature of shotgun lipidomics makes these molecular species easily visualized and allows scientists to perform PIS of the particular fragment ions and/or NLS of the interested neutrally lost fragments on the individual molecular species of a lipid class or a category of lipid classes for their identification and quantitation without time constraints. Each suite of these scans determines the molecular identity of individual molecular ion by recognizing that majority of lipid species represent combinations of a handful of building blocks [3, 31]. Moreover, this feature of shotgun lipidomics makes these molecular species easily quantified through direct comparison with their selected internal standard(s) (i.e., **ratiometric comparison**) after ^{13}C deisotoping. Because of minimal source fragmentation and selective ionization (which is largely determined by the charge property of the polar head group) in an ESI ion source, the response factors for the species of a polar lipid class are essentially identical under certain experimental conditions [40]. Hence, it is feasible to quantitate individual molecular species of a polar lipid class through direct comparison of ion peak intensities with that of a selected internal standard in the identical mass spectrum.

1.3. Classification of shotgun lipidomics

Based on the unique features described above, at least three different approaches of shotgun lipidomics are developed and well documented in the literature, including tandem MS-based shotgun lipidomics, high mass accuracy-based shotgun lipidomics, and multi-dimensional MS-based shotgun lipidomics (MDMS-SL). This subsection provides a brief description of these approaches. It should be pointed out that ion mobility-based shotgun lipidomics has been developed and greatly advanced recently (see refs. [41-44] for recent reviews). However, the application of this approach is mainly in identification of lipid species, including isomers, rather than in quantification. Moreover, as a separation tool, the ion mobility technology has been equally applied after LC separation [44, 45]. Therefore, discussion of this approach is not included in this article. The review articles cited above could be consulted to the readers who are interested in this approach.

1.3.1. Tandem mass spectrometry-based shotgun lipidomics—Because individual lipid species of a polar lipid class possess a common head group, one or more characteristic fragment ion(s) yielded from the head group are usually detected from the class of lipid species after collision-induced dissociation (CID). If such a characteristic product ion of individual species is produced because of a neutral loss of a common fragment, then a tandem MS mass spectrum in the NLS mode can be performed to detect all these lipid species lost the neutral fragment [46]. Similarly, if such a characteristic product ion of individual species represents the common fragment ion present in the product-ion mass spectra of individual lipid species of the class, then monitoring this fragment ion in the PIS mode is also able to visualize all these lipid species [46].

Following this line of reasoning, Brugger and colleagues have first developed a shotgun lipidomics strategy to “specifically isolate” individual lipid species of a polar class of interest by utilizing a class-specific NLS or PIS with a triple quadrupole mass spectrometer [47]. After the double filtering process of NLS or PIS with a triple quadrupole instrument, the obtained signal-to-noise ratios of the ion peaks corresponding to individual lipid species of the class are generally enhanced (typically with over an order of magnitude). In this strategy, two or more internal standards for each class of lipids of interest are usually added to individual sample during lipid extraction. The purpose of these spiked internal standards is to correct multiple factors as originally described [47] in addition to quantification because those factors apparently lead to variations of ion intensities of individual lipid species depending on their fatty acyl chain lengths and/or unsaturation. In this strategy, the acquired NLS or PIS mass spectrum “specifically” displays all endogenous lipid species of the class of interest along with the added internal standards. Thus, ion intensities of all these species above the limit of detection are determined from the spectrum. A calibration curve could be established based on the ion intensities of those spiked internal standards through least square regression of their ion intensities *vs*. one or more variations (e.g., the number of total carbon atoms or the degree of unsaturation) present in fatty acyl chains of spiked internal standards. Quantification is then achieved after comparison of the ion intensity of a species with the calibration curve after considering the regressed variable(s) of the lipid species.

This approach of shotgun lipidomics has been widely employed for lipidomics profiling of biological samples because of its simplicity and robustness [47-52]. A workflow of this approach has been clearly illustrated [53]. The characteristic fragments, which can be used for profiling individual lipid class, particularly in plant lipidomics, have been comprehensively tabulated in a protocol provided by Welti and colleagues [54]. As emphasized above, the key to be successful with this approach is the specificity of the characteristic fragment.

1.3.2. High mass accuracy-based shotgun lipidomics—As advanced in instrumentation, commercially-available hybrid type mass spectrometers (e.g., quadrupole-time of flight (Q-TOF) or Q-Exactive (i.e., quadrupole-OrbiTrap)) possess not only an improved duty cycle that increases the detection sensitivity, but also very high mass resolution and mass accuracy [55, 56]. These instruments can thus allow scientists not only to sensitively acquire full mass spectra of lipid samples of interest in the survey scan mode, but also to rapidly conduct product-ion MS analysis of lipid species in a small mass window (e.g., one or a couple of mass units) step-by-step to determine all the fragments in an entire mass region of interest [37, 57-59]. The high mass resolution and accuracy inherent in these instruments provides accurate measurement of the masses of individual molecular ions as well as fragment ions (e.g., 0.1 amu or higher). Such measurement of the fragments and molecular ions guarantees elimination of any possible false positive identification.

Following this line of reason, Shevchenko's laboratory at the Max-Planck Institute has initiated an approach of shotgun lipidomics based on high mass accuracy/resolution mass spectrometers (e.g., Q-TOF) [57-59]. In their initial strategy, the fragments corresponding to either fatty acyl constitutents or head group moieties are acquired after CID. Thus, any

interesting PIS and/or NLS can be extracted from the acquired data array of the product-ion mass spectra to serve the purpose for identification of a specific class of lipids. Thus, a name of multi-PIS (NLS) high mass accuracy shotgun lipidomics was given to such a strategy at the time. At least one internal standard is added during sample preparation. Quantification in this strategy is achieved through a comparison of the sum of the intensities of identified fragments of an ion to that of a pre-selected internal standard. A schematic workflow and detail description of this approach for lipid analysis have been provided by Ekroos and colleagues [33, 60].

Lately, this strategy has been evolved to acquire full mass spectra of lipid extracts in the negative- and positive-ion modes in the presence of ammonium acetate in the infused solution by using either Q-Exactive or Fusion instruments [61-63]. In this strategy, identification of lipid species is achieved from analysis of product-ion mass spectra and quantification is conducted by using full mass spectra from which ion intensities between individual lipid species of interest and their corresponding internal standard(s) are compared. A particular term of data-dependent acquisition shotgun lipidomics has been given to this strategy [37]. Other variations of this shotgun lipidomics approach, termed “top-down lipidomics”, “bottom-up shotgun lipidomics”, or “MS(All)” method, have also been developed [58, 61, 62, 64].

In the case of multi-PIS high mass accuracy shotgun lipidomics, identification of individual lipid species is achieved from bioinformatic reconstruction of the fragments from the extracted PIS or NLS. Specific software programs, termed LipidProfiler and LipidInspector, have been developed to process these PIS and/or NLS data sets [58, 59]. In the case of high mass resolution/accuracy-based shotgun lipidomics, software packages such as LipidXplorer [65] and ALEX [66] have been separately developed to process full mass spectral and product-ion analysis data.

1.3.3. Multi-dimensional MS-based shotgun lipidomics—Multi-dimensional MS-based shotgun lipidomics (MDMS-SL) [3, 31, 67, 68] maximally exploits the unique chemistry and physics inherent in discrete lipid classes or subclasses for analysis of lipids at very low-abundance levels of molecular species and including regioisomers. Specifically, differential hydrophobicity, stability, and reactivity of different lipid classes and subclasses are exploited during the sample preparation (i.e., a multiplexed extraction approach) [69].

The differential charge properties of different lipid classes (which are predominant with the head groups of polar lipid classes) are exploited to selectively ionize a certain category of lipid classes under multiplexed experimental conditions to separate many lipid classes in the ion source (i.e., intrasource separation) [39]. Figure 2 illustrates a workflow of individual lipid extract for global analysis of different lipid classes after “intrasource separation”, which has been demonstrated in Figure 3.

It has been recognized that the majority of cellular lipid species are the combination of a few building blocks such as polar head groups, fatty acyl constituents, and sphingoid backbones [31, 70]. Moreover, these building blocks, corresponding to the fragment ions or the neutrally-lost fragments, can be determined by using PIS and NLS, respectively, in a mass-

ramp fashion [29, 31]. MDMS-SL extensively exploited this structural characteristics of lipid species to effectively and thoroughly identify individual lipid species including isobaric and isomeric species [31, 71]. Accordingly, the informative fragment ion(s) from either the head group or resulted from the neutral loss of the head group are used to identify the lipid class of interest, and PIS or NLS of fatty acyl chains is used to identify the individual molecular species present within the class. Mapping of these building blocks of a lipid class or a category of lipid classes yields a two-dimensional mass spectrum [31]. Some examples of the PIS and/or NLS for the analyses of the building blocks of each lipid class with MDMS have been summarized [3].

For example, if we are interested in analysis of choline lysoglycerophospholipid (lysoPC) species present in a biological lipid extract, we should have knowledge of the fragmentation pattern of lysoPC species. In fact, the patterns of lysoPC species as sodium or lithium adducts have been extensively characterized [72-74]. Specifically, the fragmentation pattern of sodiated lysoPC species contains fragment ions corresponding to the neutral losses of 59 (i.e., trimethylamine) and 205 amu (i.e., sodium cholinephosphate), respectively. These two fragment ions definitively determine the head group of these lysoPC species. Thus, the aliphatic chain of individual lysoPC species can be deduced from the *m/z* value. The connection of the acyl chain to the hydroxyl group of glycerol (i.e., regiospecificity) can be determined with the other two fragment ions at *m/z* 104 and 147 present in the fragmentation pattern, which correspond to choline and sodiated five-membered cyclophosphane, respectively. This is due to that the intensity ratio of the ions at *m/z* 104 and 147 is as 3.5 for the *sn*-1 acyl species and 0.125 for the *sn*-2 acyl species as previously determined [72, 74]. These differential intensity ratios of fragment ions present in different lysoPC regioisomers was well elucidated through charge-remote fragmentation pathways [73, 75]. Moreover, the fragment ion corresponding to the neutral loss of 205 amu (i.e., sodium cholinephosphate) from sodiated ether-linked lysoPC species is minimal as previously demonstrated [72]. This is due to the fact that less active protons at the C2 position are present in aliphatic chain of ether lysoPC species relative to those present in fatty acyl chains where the C2 protons are activated by the neighboring carbonyl group [73, 75]. Therefore, the peak intensity ratio present in NLS59 and NLS205 readily leads to identification of ether-containing lysoPC molecular species.

Based on such a fragmentation pattern, all lysoPC species including regioisomers present in any biological samples can be effectively, selectively, and sensitively detected and identified through NLS59, NLS205, PIS 104, and PIS147, which constitute a two-dimensional MS mapping of the building blocks of all lysoPC species present in a biological sample (e.g., mouse myocardium in Figure 3). It should be emphasized that although both NLS59 and NLS205 are specific to lysoPC species and sodiated lysoPC species in the mass region. However, any single tandem MS scan is not enough to definitively determine a detected ion as a lysoPC species. In other words, all the lysoPC species present in a biological sample can be detected by either NLS59 or NLS205, but a detected ion peak by either NLS59 or NLS205 may not represent a lysoPC species. However, combination of these scans along with others in two-dimensional mass spectrometric analysis should substantially increase the

specificity of such identification and the probability that an artificial ion peak can be detected by 2D MS analysis is negligible.

At its current stage of development, this platform can identify and quantify thousands of individual lipid species (including many regioisomers) of over 40 lipid classes in cellular lipidomes [3, 76], which represents > 95% of the total lipid mass levels of a cellular lipidome, directly from solvent extracts of biological materials from a limited amount of biological source materials (e.g., 10-50 mg of tissue, a million of cells, 200 μL body fluids, etc.) in an automated, unbiased, and relatively high-throughput manner [3, 35].

1.4. Advantages and drawbacks of shotgun lipidomics

1.4.1. Tandem mass spectrometry-based shotgun lipidomics—The advantages of this method include simplicity, efficiency, high sensitivity, ease of management, and less expensive instrumental requirements. All individual species in a particular lipid class can be detected in one MS/MS acquisition directly from a total lipid extract with any commercially available triple quadrupole (i.e., QqQ) type mass spectrometer. This shotgun lipidomics approach provides global determination of the species of any targeted class at the level of instrumentation sensitivity in a high-throughput fashion. Because of its great advantages, many laboratories adopted this strategy for analysis of lipids in a lipidomics scale [47-52].

A few concerns with this approach should be recognized, including: (1) the fatty acyl substituents of lipid species are not identified since the approach only targets to the class-specific head group fragments; (2) the detection with the so-called specific MS/MS scanning might not be entirely specific to the class or the category of classes of interest, whereas this non-specificity might lead to introduction of some artifacts; (3) some altered ionization conditions cannot be easily recognized during and after the experiments; and (4) accurate quantification of the detected lipid species might not be as simple as expected because of the differential fragmentation thermodynamics and kinetics manifest in individual lipid species within each lipid class.

1.4.2. High mass accuracy-based shotgun lipidomics—This shotgun lipidomics strategy provides efficient, broad, and sensitive measurement of lipid species in a high-throughput fashion. This approach could be conducted in an untargeted fashion to analyze any lipid species present in a cellular lipidome if the dynamic range of the instrument is permitted and the software packages are able to cover all those species. This technology has been applied to many biological studies [56, 77-81].

The concerns with this approach might include: (1) since quantification of lipid species in the multi-PIS (NLS) strategy is based on tandem MS techniques, it is better to include multiple (at least two) internal standards for each lipid class to cover the differential fragmentation thermodynamics/kinetics of different molecular species; (2) differential ionization responses of different species among a non-polar lipid class are well known and correction for these differential ionization responses in quantification of these species should be considered; (3) linear dynamic range of quantification largely depends on the used instrument under experimental conditions in either multi-PIS (NLS) or high mass accuracy

strategies; and (4) it might not be the best choice to analyze poorly ionized lipids, particularly those in low abundance through this approach.

1.4.3. Multi-dimensional mass spectrometry-based shotgun lipidomics—This approach overcomes the majority of the limitations of other shotgun lipidomics approaches and possesses some obvious advantages. Here are some examples. First, MDMS-SL exploits the mass spectrometer as a separation tool in addition to being an analyzer, thereby significantly minimizing the ion suppression effects. Second, MDMS-SL uses multi building blocks of individual molecular species to definitely identify its structure and their isomers and eliminates the presence of any artifactual species as present in the approach based on single PIS or NLS analysis. Third, MDMS-SL uses the peak contours in multi-dimensional space which facilitates refinements in quantitation through two-step quantification approach to extend the linear dynamic range. Fourth, MDMS-SL is ideally suited to exploit the distinctive chemical characteristics of many lipid classes. Prominent examples include the use of the $[M-2H+1]^{2-}$isotopologue approach for cardiolipin (CL) analyses [82], the use of fluorenylmethoxylcarbonyl (Fmoc) derivatization for the analysis of phosphoethanolamine-containing species [83], the use of specifically deuterated amine selective reagents for dynamic lipidomics through PIS analysis of the particular reagents [84], the use of alkaline hydrolysis to greatly enhance penetrance into the sphingolipidome [69], and the use of charge-remote fragmentation for fatty acidomics [76], among others.

There exist some limitations in MDMS-SL. First, in comparison to other shotgun lipidomics approaches, MDMS-SL is relatively low throughput and laborious because of the involvement of different procedures (e.g., derivatization) in multiplexed sample preparation. Second, MDMS-SL is unable to distinguish isomeric species, of which the fragmentation patterns are identical as for all shotgun lipidomics approaches. Finally, although MDMS-SL identifies and quantifies all individual species of a characterized lipid class in an unbiased fashion within the limits of instrumentation sensitivities, the approach is not ideal for identification and quantitation of species of an unknown or uncharacterized lipid class since identification of the building blocks of a lipid class has to be pre-determined.

2. Recent advances in shotgun lipidomics technology

Recognition of the limitations of shotgun lipidomics motivates the scientists in the field to improve and advance the technologies in this area, thereby making the strategies of shotgun lipidomics more applicable for analysis of lipids in a much sensitive, comprehensive, and efficient fashion. This section provides an overview of the advances related to shotgun lipidomics in recent years including extraction techniques, derivative methods, and bioinformatics developments. The improvement of extraction techniques greatly facilitates the high throughput demands of shotgun lipidomics. The development of derivatization methods greatly enhances ionization efficiency and accessibility of lipid classes which are present in very low abundance, hardly ionizable, or absent in sensitive building blocks for their analysis by MDMS-SL. The advancements in data processing and bioinformatics interpretation make the shotgun lipidomics more automation and tighter connection with biological studies.

On the other hand, we should also recognize that the recent advancements in commercially available mass spectrometers in parallel greatly facilitate the improvement of shotgun lipidomics strategies for their applications in biology and medicine. For example, the increased sensitivity and duty cycle of QqQ-type mass spectrometers effectively improve the low limit of detection and duration for analysis of lipids by using both tandem MS-based shotgun lipidomics and MDMS-SL; the improved mass accuracy and mass resolution instruments makes the high mass accuracy-based shotgun lipidomics even more powerful as evidenced with a very recent publication [61]. It can be foreseen that as instrumentation gets more advanced in the future, the power of shotgun lipidomics for biological and biomedical research should become more apparent. This part of the advances is obvious and thus not included in this section.

2.1. Novel methods of lipid extraction for shotgun lipidomics

Sample preparation is one of the key steps to the successful analyses of cellular lipidomes by MS. Different platforms for analysis of lipids may exploit different methods for sample preparation. For example, the methods employing direct infusion require much cleaner lipid extracts than those based on LC-MS since any contamination could yield significant ion suppression for lipid analysis after direct infusion, whereas the column could get rid of most of the contaminants. Traditionally, lipid samples from biological sources are extracted using a mixture of chloroform and methanol based on the Folch method [85] or the modified method of Bligh and Dyer extraction [86]. Unfortunately, regardless of how carefully the extractions are performed, a small amount of water and therefore inorganic residues (e.g., salts and glucose) are carried into the solvent extracts when the bottom solvent layer is taken out if these extraction methods are employed in addition to the use of very hazardous chloroform in the procedure. Moreover, automation of lipid extraction from the bottom solvent layer in comparison to taking the lipid solution from a top layer is also much difficultly achievable, although automation to process extracts from the Folch method has been practiced [60, 87]. Therefore, these practical issues have driven researchers in the lipidomics field to develop/establish novel lipid extraction techniques which are summarized in this subsection.

It should be emphasized that it is difficult to achieve a complete recovery of every lipid class by any known method of extraction. Any incomplete recovery could lead to an inaccurate measurement of the lipid content in a sample or an inconsistency in the results from different laboratories if the analytical methods are based on external calibration curves. To avoid this potential problem, at least one internal standard should be added for quantification of a lipid class of interest during the extraction procedure. Therefore, the effects of any incomplete recovery on analysis can be minimized. The differential recovery of individual molecular species of a lipid class of interest relative to the selected internal standard is a minor secondary effect.

2.1.1. MTBE method—One method developed to resolve the aforementioned difficulties is the one using methyltert-butyl ether (MTBE) [88]. In this extraction method, MTBE is present in the top layer against a lower aqueous phase. This method is now widely adopted in the lipidomics field.

The MTBE extraction method has recently been further improved for extraction of lipids from brain samples with mechanical homogenization utilizing ceramic beads to enhance high throughput and automation [89]. Another development based on this method is to utilize an intermediate fraction as well as the aqueous phase for total analysis of lipids and metabolites [90].

It seems that the extraction recoveries of many lipid classes examined by the MTBE method are comparable to those using chloroform. The 1-hour incubation period may be shortened by using low-power sonication [91] or a microwave oven [92]. It should be emphasized that (non)enzymatic lipid degradation/oxidation should be closely paid attention if sonication is used. Another drawback of the MTBE extraction method is that the organic phase contains quite a large amount of aqueous component. This may prolong the evaporation of the solvents prior to reconstituting the lipid solution. Moreover, the contamination of aqueous phase may cause complication of lipid analysis due to the presence of inorganic salts and other small molecules.

2.1.2. BUME method—Butanol saturated with water was previously used to disrupt the inclusion complexes of lipids in starch and give the best extraction recoveries of lipids from cereals [93]. This solvent combination was recommended for quantitative recovery of lysoglycerophospholipid (lysoGPL) species and for acylcarnitines [93]. Recently, modified versions of this system for lipid extraction were developed for global lipid analysis [94]. In the new version, a mixture of butanol:methanol (BUME) as initial extraction solvents was used [94]. Specifically, 300 μL of butanol:methanol (BUME) mixture (3:1, v/v) is first mixed with 10 to 100 μL of plasma sample to form an initial one phase extraction; 300 μL of heptane:ethyl acetate (3:1, v/v) is added to the vessel containing BUME mixture and mixed; and finally, 300 μL of 1% acetic acid is added to the extraction solution to form two phase separation.

We have further improved this BUME method in two aspects (Unpublished results). First, this method was extended to extract lipids from tissue samples. We found the profiles of all examined lipids extracted by this method were no apparently differences from those extracted by the modified Bligh-Dyer method. Second, the 1% acetic acid solution was replaced with 5 mM of LiCl solution considering that an acidic environment might lead to plasmalogen degradation. Again, we found this change did not lead to any apparent alterations in lipid profiles in comparison to those extracted with 1% acetic acid solution.

This BUME method may compensate the MTBE method with less salts/inorganic contaminants carried over in the organic phase. However, the organic phase contains the butanol component which is hard to be evaporated under common laboratory conditions (e.g., a stream of nitrogen). This latter issue could be resolved through diluting the extract to a solvent mixture to make the infusion solution for shotgun lipidomics.

2.2. New advances in identification and quantification of lipid species

As discussed in Subsection 1.3.3 and demonstrated in Figure 3, regardless of whether the lipid class is ion-suppressed by other abundant coexisting lipid class(es), identification and quantification can be readily conducted for the lipid class by MDMS-SL if this lipid class

can be sensitively ionized, and/or possesses sensitive and specific fragments which can be detected by NLS and/or PIS. This and many other examples indicate that the ion suppression occurred under a constant concentration does not prevent their identification and quantification as long as the linear dynamic range for analysis of these lipid classes is sufficient under the experimental conditions. Therefore, many recent efforts were made to enhance both ionization efficiency (to increase the linear dynamic range) and characteristic fragments for those lipid classes that are hardly ionizable, or do not possess sensitive and characteristic fragment ions (i.e., building blocks), or both through derivatization based on the unique chemistry of individual lipid classes.

It should be noted that in addition to the benefits from derivatization as mentioned above, introduction of a polar group through derivatization also makes the ionization of individual molecular species of a lipid class of interest depending on the introduced polar head group, but not (or less) depending on the aliphatic chain(s). In other words, introduction of a polar group can convert different ionization efficiencies of individual molecular species of a nonpolar lipid class to a polar lipid class of which ionization efficiencies of individual species are essentially identical under appropriately experimental conditions as shown in Figure 1 and extensively discussed previously [19, 29]. Therefore, all these advances in shotgun lipidomics are summarized in this section along with some other efforts on identification and quantification of individual lipid species in shotgun lipidomics.

2.2.1. Identification and quantification of 4-hydroxyalkanel species—4-Hydroxyalkenal species are a class of peroxidation products of polyunsaturated fatty acids (PUFAs) resulting from a variety of enzymatic or non-enzymatic reactions during diverse physiological and pathophysiological processes [95, 96]. For example, 4-hydroxy-2E-hexenal (4-HHE) and 4-hydroxy-2E-nonenal (4-HNE) are the products of n-3 and n-6 PUFAs, respectively, through a non-enzymatic peroxidation pathway; whereas 4-HNE and 4-hydroxy-2E,6Z-dodecadienal are generated enzymatically through a peroxidation pathway of n-6 PUFA ultimately produces 4-HNE and 4-hydroxy-2E,6Z-dodecadienal [97]. Therefore, the levels of these 4-hydroxyalkanel species are the indicator of oxidative stress of a biological system [98] and the increased levels of different 4-hydroxyalkanel species indicate the activation of different pathways and the sources of different substrates. Moreover, the generated 4-hydroxyalkanel species serve as “toxic second messengers” [99]. This is due to that these species reactively form covalent adducts with proteins and nucleic acids, thereby leading to inhibition of protein and DNA synthesis, dysregulation of enzyme activities, alteration in mitochondrial coupling, etc. [95, 100-102], and also easily diffuse from its origin site to propagate the oxidative injury.

4-Hydroxyalkanel species are present in low abundance, very reactive, unionizable, and absent in any characteristic building blocks. Therefore, analysis of these species indeed represents a challenge, particularly in a high throughput lipidomics fashion regardless of that numerous attempts to conquer this obstacle were made (see extensively cited references in [103]). However, our group has recognized the facile Michael adduct of carnosine (an endogenously existing dipeptide, β-alanyl-L-histidine) with 4-hydroxyalkenal species (e.g., 4-HNE) (Figure 4) [104], which can be exploited to quantitatively analyze 4-hydroxyalkanel species utilizing the MDMS-SL strategy [103].

Specifically, a stable isotope-labeled 4-HNE used as an internal standard is added during lipid extraction and the adduct of carnosine with 4-hydroxyalkanel species is finished in minutes. After working up the reaction solution, the lipid solution is directly infused to a mass spectrometer and analyzed for identification and quantification of 4-hydroxyalkanel species. It has been found that (1) ionization efficiency of the generated adducts by ESI-MS is substantially increased in comparison to native 4-hydroxyalkenal species in the positive-ion mode and (2) product ion MS analysis of carnosine-adducted 4-hydroxyalkenal species displays many abundant, informative, and characteristic fragment ions (Figure 5A-D). Therefore, these findings have been used to develop a sensitive, facile, shotgun lipidomics-based method for quantification of these compounds directly from chloroform extracts of biological samples in comparison to their stable isotope-labeled counterparts based on the MDMS-SL strategy (Figure 5E). This method has been used to determine the mass levels of 4-hydroxyalkenal species in various biological samples, including mouse heart, kidney, liver, and skeletal muscle [103] and assess their levels under pathophysiological conditions that lead to mitochondrial dysfunction such as treatment with low doses of rotenone [105], knockdown of tafazzin gene [106, 107], and disruption of peroxisome proliferator-activated receptor gamma coactivator 1 [108].

2.2.2. Fatty acidomics (global analysis of cellular lipids containing a functional group of carboxylic acid)—There are a great number of cellular lipid species containing a functional group of carboxylic acid. A few classes among this category include non-esterified fatty acids (NEFA) including branched and cyclic fatty acids, modified fatty acids including oxidized (e.g., eicosanoids and docosanoids), nitrosylated fatty acids, halogenated fatty acids, etc. [5], bile acids, etc. These lipid species play many essential roles in the biological systems (e.g., providing energy sources, serving as signaling molecules, and being the major structural components in complex lipids of cellular membranes) [109]. Because of their structural diversity and as presented in low to very low abundance of the majority of these species in nature, identification and quantification of these lipid species are apparently a huge challenge. In practice, in spite of the great efforts made to analyze these lipids by very different approaches, only a subgroup of these lipids were analyzed at a time in each method based on their structural similarity. Global analysis of all of these lipid species is only a dream, but never being executed to the best of our knowledge prior to the development of fatty acidomics. This term is referred to as the global analysis of all of cellular lipid species containing a functional group of carboxylic acid, including identification of chain length, double bond number and locations, modified group(s) and locations, branch chain position, etc., and quantification of these identified species including different isomeric species [76].

This technology was developed based on a concept that introduction of a permanent charge site to the commonly-carried carboxylic acid of these lipid species through an amide coupling through an amidation reaction (Figure 6) should be very helpful for analysis of these lipid species. This concept has previously been applied for analysis of eicosanoid species after LC separation and called “charge reverse” to improve the sensitivity [110]. Lately, this technique has also been used for analysis of NEFA [111, 112]. The features of this concept include: (1) enhancing the ionization efficiency because of the permanent

charge, (2) yielding lots of informative structural fragments because of the introduced stable amide bond and due to charge-remote fragmentation nature in mass spectrometry [113, 114], and (3) applying the sensitive and characteristic fragment ions for identification and quantification of these lipid species in a MDMS-SL approach.

In practice, we have tested a variety of charge-carried reagents and found all the derivatives with these reagents could yield informative charge-remote fragmentation patterns regardless of the different chemical and physical properties of the reagents [76]. Moreover, all these informative fragmentation patterns could be effectively used for structural elucidation of lipid species containing a carboxylate group. Essentially, the derivatives have allowed us to identify all the species containing a carboxylic acid including their isomers [76].

Intriguingly, quantification of all of identified lipid species containing a carboxylic acid including isomers can also be achieved without chromatographic separation since charge-remote fragmentation of the derivatives of fatty acids provides very distinct fragmentation pattern of individual species. Therefore, a mixture of those isomers can be determined from simulation of the tandem MS mass spectrum of the mixture of the isomers with the tandem MS mass spectra of individual isomers.

For example, there are three different isomers of 18:1 fatty acid (FA) due to their different locations of double bonds (i.e., 18:1(n-7), 18:1(n-9), and 18:1(n-12) FA species). The mass levels of these isomers in a biological system provide very different biological meanings. The content of 18:1(n-7) FA indicates the degree of a biosynthesis pathway contributing to the system. In this pathway, palmitic acid (16:0 FA) first goes desaturation through Δ9 desaturase activity to form 16: 1(n-7) FA which then elongates to 18:1(n-7) FA through an elongase activity. The level of 18:1(n-9) FA suggests the contribution of Δ9 desaturase activity to convert 18:0 FA to 18:1(n-9) FA. The level of 18:1(n-12) reflects the uptake of fatty acids from the diets containing plant products.

Tandem MS mass spectra of these 18:1 FA isomeric derivatives with N-[4-(aminomethyl)phenyl]pyridinium (AMPP) display very distinct fragmentation patterns (Figure 7A-C). Specifically, the intensities of the abundant fragment ions at *m/z* 169 and 183 relative to their individual molecular ion intensities are essentially identical. Thus, these two ions can be used for screening the presence of these isomers at this *m/z* position in the mass spectra of PIS169 and PIS183, and quantifying the total content of their mixture relative to the selected internal standard. Furthermore, the fingerprints of the fragment ions between *m/z* 190 and 430 are very different from each other (Figure 7A-C), representing the sequential loss of individual methylene groups at the carboxylic end after charge-remote fragmentation and the ions at *m/z* 323, 295, and 253, which separately characterize the double bond positions of 18:1 FA isomers, respectively. Therefore, a tandem MS mass spectrum of any mixture of these 18:1 FA isomers (e.g., Figure 7D-E) can be simulated with those fragmentation patterns of individual 18:1 FA isomers shown in Figure 7A-C. For example, mixed ratios of 0.343±0.003/0.330±0.015/0.327±0.011, 0.203±0.014/0.419±0.008/0.378±0.006, and 0.063±0.004/0.639±0.011/0.298±0.015 with all correlation coefficients (γ^2) of > 0.99, which match very well with authentic mixtures of 0.33/0.33/0.33, 0.20/0.40/0.40, and 0.06/0.60/0.34, respectively, have been obtained from

simulation of tandem MS mass spectra shown in Figure 7D-E. The determined composition of these FA isomers in the mixture, in combination with the determined content of the total mixture, thus allows one to measure the content of individual 18:1 FA isomers.

This approach can be applied for determining the mass levels of any individual species in FA isomeric mixtures if different fragmentation patterns of the derivatized FA isomers are present. In fact, different fragmentation patterns of FA isomers including many types of eicosanoid isomers as examined are truly present [76]. Very different fragmentation patterns of nitrosylated FA species were present [76], likely due to the involvement of nitrosyl group in the fragmentation process in addition to the charge-remote fragmentation. By using fatty acidomics, the unique fragmentation pattern of the branched, saturated FA species (e.g., phytanic acid) allows one not only to readily identify the location of the methyl branches, but also to determine the possible existence of any unbranched isomeric FA species. In summary, as a powerful addition to lipidomics tools, fatty acidomics could be widely used to identify and quantify the lipid species containing a carboxylic acid group, thereby greatly accelerating identification of the biochemical mechanisms underlying numerous pathological conditions.

In addition to the advantages of fatty acidomics discussed above, there exist at least three other advantages with this strategy. First, the ion peak intensity of a mixture of derivatized FA isomers is addable, making the detection of the ion much easier. Second, since the ionization efficiencies of the derivatized FA species are predominant with the derivatized reagent carrying the charge, the contribution from the aliphatic chain component is minimal and thus, less internal standards are needed for quantification of all lipid species containing a carboxylic acid group. Third, lipid species carrying a positive charge are rare in nature, thus the derivatized FA species can be readily isolated/enriched with a cation exchange column if necessary.

2.2.3. Identification and quantification of lipid species containing a hydroxyl group—There are many lipid classes, subclasses, and individual molecular species containing a hydroxyl functional group, including diacylglycerol (DAG), monoacylglycerol (MAG), *N*-acylethanolamine (NAE) (e.g., anandamide), oxysterol, and so on. These classes of lipid species are relatively less polar and thus, their ionization efficiencies are low in addition to their low abundance in nature. Following the same line of reasoning as aforementioned, derivatization of this hydroxyl functional group to introduce a polar or charge-carried group as in fatty acidomics should greatly facilitate the analysis of these classes of lipids. For example, derivatization of thin layer chromatography-purified DAG fraction with *N*-chlorobetainyl chloride has been conducted [115]. Unfortunately, the study is unable to identify acyl chains of DAG species or resolve 1,2- and 1,3-DAG regioisomers, and extra effort on pre-isolation of DAG fraction is required.

The recent advance in resolving all of these concerns has been the strategy through derivatization of the hydroxyl group with dimethylglycine (DMG) developed by our group [116]. Specifically, characterization of the derivatized authentic lipid species by product-ion ESI-MS analysis has initially been performed and characteristic fragment features are determined. We have found that an abundant fragment ion at *m*/*z* 110 corresponding to

lithiated DMG and an abundant product ion corresponding to the neutral loss of 103 Da (i.e., DMG) are resulted from all derivatized lipid species [116, 117]. Therefore, these two fragment features can be used for screening the presence of these lipids (including isomers) and quantifying the total content of the ion peak represented in comparison to their corresponding internal standards.

From the comparison between product ion mass spectra of lithiated *sn*-1,2- and 1,3-DMG-DAG species, we have also found that the mass spectra of 1,3-DMG-DAG displayed an extra moderate fragment ion peak corresponding to the neutral loss of 87 amu from the molecular ions (i.e., the loss of DMG as an aldehyde). Thus, the neutral loss of 87 amu is specific to lithiated 1,3-DMG-DAG species and NLS of 87 amu can be used to discriminate 1,3-DAG from 1,2-DAG isomers.

Moreover, the fragment features associated with aliphatic chains and regioisomers have also been determined. For example, the product-ion mass spectra of lithiated DMG-DAG species have showed a pair of fragment ion peaks corresponding to the loss of an individual FA chain as an acid or lithium salt, allowing us not only to identify the FA constituents of DAG species, but also to determine the position of each fatty acyl chain of 1,2-DAG species (i.e., regisoisomers) and differentiate the 1,2- and 1,3-DAG isomers [116].

Based on these fragment features of authentic DAG species after derivatization, DAG species present in biological lipid extracts can be identified by MDMS-SL. Figure 8 shows such an analysis of DAG species present in the lipid extract of mouse liver. For example, an ion peak at *m*/*z* 686 is present in both NLS103 and PIS110 spectra corresponding to a 34:1 DAG species (34 carbon atoms and one double bond in the FA chains). The presence of this ion in NLS87 spectrum indicate the presence of 1,3-DAG from a part or all of the ions. After quantification by using NLS87 for 1,3-DAG species in comparison to the 1,3-di15:0 DAG internal standard, and using NLS103 for the total DAG mass of the ion peak, we have determined that there are 10% of 1,3-isomer and 90% of 1,2-isomer present in this 34:1 DAG isomeric mixture. The NLS spectra of naturally occurring FAs indicate that the ion peak at *m*/*z* 686 is crossed with NLS254 (i.e., 16:1 FA), NLS256 (i.e., 16:0 FA), NLS282 (i.e., 18:1 FA), and NLS284 (i.e., 18:0 FA) spectra. After baseline correction [118] and ^{13}C de-isotoping [40] of the ion peak at *m*/*z* 686, it has been determined that the 34:1 DAG species contain 2.5% of 18:0-16:1 DAG and 97.5% of 16:0-18:1 DAG species.

This strategy can be used for analysis of oxysterol isomers as previously demonstrated [117]. Similarly, identification and quantification of MAG and NAE can also be conducted. Figure 9 showed the informative and characteristic product-ion mass spectra of representative DMG-derivatized MAG and NAE species (Unpublished data), indicating that MDMS-SL can be applied for analysis of these species directly from lipid extracts after DMG derivatization.

2.2.4. Identification and quantification of lysoglycerophospholipid species—It is well known that lysoGPL species possess diversified cellular functions, including serving as extracellular mediators, induction of cellular proliferation, involvement in the development of nervous and vascular systems, transduction of intracellular signal, and

suppression of apoptosis [119-123]. Accurate measurement of the aberrant lysoGPL species under pathological conditions is very important, allowing for delineation of biosignatures for diseases, and providing not only deep insight into the biochemical mechanisms underpinning the diseases, but also biomarkers for diagnosis and prognosis of the diseases at their earliest stages.

Identification and quantification of these lysoGPL species in a lipidomics fashion are limited because of their low abundance and difficulty to be recovered during lipid extraction after organic solvent extraction [124, 125] regardless of great efforts which have been made on analysis of some particular lysoGPL classes [126-129]. Recently, a simple isolation and enrichment strategy through SPE utilizing hybrid SPE cartridges from Sigma-Aldrich Chemical Co. (St. Louis, MO) has been exploited for the lysoGPL species present in aqueous phase [130]. The principle of Hybrid SPE to bind GPL species is based on the Lewis acid-base interaction between Hybrid SPE Zirconia ions and GPL species [131]. The extracted lysoGPL species are analyzed by MDMS-SL. Specifically, characterization of individual lysoGPL classes including lysophosphatidic acid (lysoPA), lysoPC, lysoPE, lysophosphatidylglycerol (lysoPG), lysophosphatidylinositol (lysoPI), and lysophosphatidylserine (lysoPS) has revealed at least two specific and informative fragment ions of each lysoGPL class, which have been used for definitive identification and quantification of individual lysoGPL species from the aqueous-phase recovered lipid solutions in combination with regular organic lipid extracts. The fatty acyl chain information can then be derived after definitive identification of the polar head group of each species. It should be noted that although discrimination of regioisomers of lysoGPL species has not been conducted in the study due to the limited availability of regioisomeric lysoGPL species commercially, this task could be achieved through extensive elucidation of the differential fragmentation patterns of such kinds of isomers as previously demonstrated for the classes of lysoPC and lysoPE [72, 74].

This established approach has been applied for quantitative analysis of the different content of individual lysoGPL species present in the liver of *ob/ob* mice from their wild type littermates. Figure 10 comparatively has showed some representative tandem MS mass spectra of these lysoGPL classes between the lipid extracts of the liver from wild type and *ob/ob* mice. It is revealed that significant changes of many lysoGPL species of these classes are present in *ob/ob* mouse liver in comparison to their wild type controls. Specifically, the content of the majority of lysoPI, lysoPE, lysoPC and lysoPG species is significantly increased whereas the content of lysoPA and lysoPS species is less increased in *ob/ob* mouse liver compared with that of the controls. For example, the lysoPI content is changed from 404.6 ± 33.2 in wild type mouse liver to 624.2 ± 21.5 pmol/mg protein in *ob/ob* mouse liver. The significant accumulation of all lysoGPL classes indicates the lipotoxicity under obesity conditions. These results are consistent with the fact that lysoGPL species inhibit fatty acid oxidation in the liver and reduce energy expenditure, thereby leading to diet-induced obesity with a high fat diet as previously demonstrated [132].

2.2.5. Identification and quantification of monomethyl- and dimethylphosphatidylethanolamine—

N-monomethyl- and *N*,*N*-dimethyl-phosphatidylethanolamine (M*M*ePE and D*M*ePE, respectively) are the intermediates for *de*

novo synthesis of choline glycerophospholipid (PC) in which PE species are sequentially methylated through catalysis with an enzyme, i.e., phosphatidylethanolamine *N*-methyltransferase (PEMT) [133]. The levels of M*M*ePE and D*M*ePE are usually present in low abundance in animal tissues including the liver although the contents of these lipid classes are relatively high in yeast. The changed levels of these lipid classes may indicate alterations in the PEMT activity or the metabolic pathway of PC synthesis, which may be associated with pathological conditions [134-136].

For sensitive and efficient analysis of M*M*ePE and D*M*ePE along with analysis of PC and PE species, Ejsing and colleagues have developed a new "mass-tag" strategy to methylate M*M*ePE and D*M*ePE, and PE species with deuterated methyl iodide (CD_3I) to generate PC molecules with different deuterated degrees at the quaternary amine with a mass offset of 3, 6 and 9 amu, respectively [137]. This methodology allows characterizing M*M*ePE and D*M*ePE, and PE species as endogenous PC with specific mass offsets, since all of the investigated species have a phosphocholine head group and equal ionization efficiency. Thus, it is possible to accurately quantify PC, M*M*ePE, D*M*ePE, and PE species using only PC and/or PE internal standards.

We have further optimized this strategy at the steps of both mass-tag and analysis [138]. Specifically, the reaction conditions including pH condition, temperature, and reaction time have been optimized to greatly improve the yield, reduce the utilization of a large amount of toxic methyl iodine, and eliminate any potential side reactions. During the analysis, the principles of MDMS-SL have been applied for the analysis with lithium adducts of PC species [74]. Thus, all of individual molecular species of PE, M*M*ePE, D*M*ePE, and PC species including fatty acyl chains and regioisomers have been identified and quantified at the same time [138]. Figure 11 illustrated a representative two-dimensional MS analysis of the head group building blocks of PE, M*M*ePE, and D*M*ePE species from an equimolar mixture of these species.

By using this approach, alternations of PC, M*M*ePE, D*M*ePE, and PE species in the liver of streptozotocin-induced diabetic mice at the very early stage (one week after streptozotocin injection) in comparison to their controls have been investigated [138]. It has been found that the total amounts of the measured PC, M*M*ePE, D*M*ePE, and PE lipid species do not show significant changes between the diabetic and control groups although PC species tend to decrease and PE species tend to increase in the diabetic liver samples. However, the analysis with the improved methodology has demonstrated the significant remodeling of fatty acyl chains in these lipid classes with a reduction of the levels of 16:1 FA and increases in the amounts of 18:2, 18:1, and 18:0 FA chains in diabetic mouse liver [138].

2.2.6. Enhancement of ionization and characteristic fragments through methylation of glycerophospholipid head groups for analysis of glycerophospholipid classes for shotgun lipidomics—A couple of laboratories have believed that enhancing ionization efficiencies of molecular species of different GPL classes could better analyze the species of these lipid classes to reduce ion suppression of the lipid classes present in low abundance by those co-existing in high abundance in shotgun lipidomics [139, 140]. Moreover, it is natural that enhancement of ionization efficiencies of

the lipid classes of interest definitely yields much broader linear dynamic range through increases in the low limits of detection. It should be emphasized that the maximal limit of a linear dynamic range in MS analysis of lipids is already capped with the concentration at which lipids begin aggregation [40].

The researchers have achieved this purpose through methylation of primary amines as well as the hydroxyl group of phosphate(s) by using diazomethane directly provided or *in situ* generated [139, 140] as previously described [141]. Through this strategy, the formed trimethyl amine among the classes of PE and phosphatidylserine (PS), which possesses a fixed positive charge site, yields much higher ionization efficiencies in comparison to their original primary amine. Moreover, methylation of the phosphate among the lipid classes of PC, PE, PS, sphingomyelin (SM), and phosphatidylinositol (PI) turns the groups carrying a negative charge at the phosphate site into its neutral form, which further enhances the ionization efficiency if analysis of these lipid classes is performed in the positive-ion mode. After methylation, the fragments resulted from their head groups are structurally informative and characteristic, and thus, these fragment ions can be employed for sensitive identification and quantification of these lipid classes [139, 140]. For example, all of molecular species of phosphoinositides including PI, PI phosphate (PIP), PIP_2, and PIP_3, which are usually present in low abundance and difficult to be analyzed can be readily ionized for identification and quantification after methylation of those phosphate groups [140]. It should be noted that diazomethane is very hazardous, which should be used cautiously.

Indeed, through labeling the primary amine, phosphate, carboxylate groups of these lipid classes with light and heavy methyl group(s) (i.e., CH_3 and CHD_2, respectively), comparative quantification of their individual molecular species between different groups of samples could also be achieved as demonstrated [140, 142]. Moreover, this strategy could also be extended for sensitive analysis of other lipid classes containing a phosphate group such as phosphatidylglycerol, phosphatidic acid, cardiolipin, and their lyso counterparts.

2.2.7. Identification of double bond location(s) of fatty acyl chains—Fatty acids (FAs) play many essential roles in a biological system including providing energy sources, serving as signaling molecules, and being the major structural building blocks of complex lipids of cellular membranes [109]. The different locations of the double bond(s) form the isomers of an unsaturated FA in which the chain length and the number of double bonds are identical. The composition of unsaturated FA isomers reflects the dietary history and FA biosynthesis while the temporal changes of this composition display the metabolic rate of the biological system [109]. The altered composition of these FA isomers not only indicates the physiological responses of FA metabolism after a perturbation, but also affects the membrane fluidity, its function, and the environment for membrane proteins, which ultimately leads to the pathogenesis of diseases [34, 143-145]. Regardless of that great progress has been made for identification of double bond locations in lipidomics, such as ozonolysis [146-149], the challenges for identification and quantification of FA isomers have recently attracted the scientists in lipidomics to conquer the obstacles by using different approaches.

Similar to the fatty acidomics for global analysis of fatty acid species including isomers as described above, MDMS-SL has also been performed to identify the location of double bonds after derivatization of fatty acids with AMPP [112]. Specifically, the locations of proximal double bonds in AMPP-derivatized fatty acids are identified by diagnostic fragment ions resulting from the markedly reduced 1,4-hydrogen elimination from the proximal olefinic carbons. Additional fragmentation patterns resulting from allylic cleavages further substantiated the double bond position assignments. The application of this approach for the analysis of fatty acids in human serum has demonstrated the existence of n-3 and n-6 isomers of linolenic acid (18:3 FA). The investigators have revealed that the isomeric ratio of n-6 *vs*. n-3 18:3 FA is remarkably different in esterified neutral lipids from their nonesterified moieties [112].

In another recent study [150], the presence of FA isomers has been identified based on the intensity changes of the specific fragment ions with varying CID conditions (e.g., collision energy and collision gas pressure) following the principle of MDMS-SL [31]. In the study, the underlying mechanism of the changed fragment ion intensities of FA isomers with varying CID conditions has also been investigated. It has been found that the differential interactions between the negative charge carried by the fragment ion and the electron clouds of the double bonds are the causal factor. This novel method has also been applied for identification and quantitation of free FA isomers present in the mouse plasma samples and extended for identification and quantification of the double bond isomers of FA chains present in GPL species of biological samples by multi-stage tandem MS (MS^3) [150].

A novel method by coupling Paternò–Büchi reaction (Figure 12) with tandem mass spectrometry has been developed for determination of double bond locations in various types of lipid species including NEFA, GPL, and lysoGPL [151]. In this method, the reaction has been facilitated by UV irradiation of a nanoelectrospray plume entraining the lipids of interest and acetone. The reaction products have been directly identified on-line after CID through rupture of oxetane rings formed in the reaction detection of diagnostic ions specific to the double bond location. The method has been applied for identification of double bond locations of different lipid mixtures [151].

Finally, another novel method by employing electron impact excitation of ions from organics or electron-induced dissociation techniques has been developed [152]. This method allows the scientists to identify the location of double bond(s) present in individual fatty acyl chain of phospholipid species in addition to identification of other structural information based on the generated fragmentation patterns of intact phospholipids. The researchers have demonstrated the application of this method for identification of intact PC species present in egg yolk lipid extracts [152].

To this end, it should be recognized that a reaction strategy involving functional group selective modification of the *O*-alkenyl-ether double bond within plasmenyl ether containing lipids using iodine and methanol has been developed [153]. Utilizing this strategy, plasmalogen species can be readily distinguished from their plasmanyl counterparts.

2.3. Processing and interpretation of shotgun lipidomics data

The unique strategies used to acquire MS data make the data processing and interpretation different from those obtained from the analysis by LC-MS. A few new developments in these areas should greatly facilitate the advances of shotgun lipidomics, which are summarized in this section.

2.3.1. New software tools for data processing

2.3.1.1. LipidBlast – in silico tandem mass spectral library: LipidBlast is a library containing tandem mass spectra in the product ion mode created *in silico*, validated to a great extent, and maintained by the Fiehn laboratory at University of California-Davis. LipidBlast contains a total of 212,516 tandem mass spectra for 119,200 different lipids in 26 lipid classes [154]. This library is freely available for commercial and noncommercial use at http://fiehnlab.ucdavis.edu/projects/LipidBlast/. The *in silico* MS/MS library has been created based on the following steps as described [154].

First, the developers of the library have defined the structures to be included and subsequently exhaustively *in silico* generated all possible structures. To this end, they have imported approximately half of all the LipidBlast compound structures from Lipid MAPS database or generated using Lipid MAPS tools [155]. This part includes 13 lipid classes of the most common GPL and glycerolipid [155]. Because Lipid MAPS database does not cover many bacterial and plant lipids, the developers of LipidBlast have generated an additional 54,805 compounds from 13 additional lipid classes using the combinatorial chemistry algorithms provided by ChemAxon Reactor11 (JChem v.5.5, 2011; http://www.chemaxon.com/) and SmiLib12 to yield a total of 119,200 compounds

Next, the developers of LipidBlast have experimentally acquired MS/MS spectra on different platforms and theoretically interpreted structural class-specific fragmentations and rearrangements. They have performed MS/MS measurements in the product ion mode of over 500 highly diverse GPL and glycerolipid standard compounds containing different numbers of carbon atoms and double bonds from different lipid classes. In addition, they have selected MS/MS spectra from approximately 300 publications for those lipid classes of which the pure standards are unavailable. They have analyzed the fragmentations and rearrangements for individual lipid class, including the precursor ions of $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M-H]^-$, $[M-2H]^{2-}$, $[M]^+$, and $[M+Li]^+$, and product ions, as well as their relative ion abundances. They have found that the examined lipids show predictable MS/MS spectra, with the dominant fragmentations being the loss of the polar head groups, the product ions resulting from the losses of acyl or alkyl chain from precursor ions, and the product ions corresponding to the fatty acid fragments (best observed in the negative-ion mode as $[FA-H]^-$). They have observed many other specific fragments and rearrangements that are subsequently added to the rule-based generation of MS/MS mass spectra in LipidBlast.

Third, the developers of LipidBlast have generated characteristic fragmentations and heuristic modeling of ion abundances for possibly detectable adduct ions of lipid species of individual lipid class based on the rules observed above. Specifically, they have created the LipidBlast MS/MS library by extending the obtained knowledge about fragmentations and

ion abundances from the lipid standards to the thousands of *in silico*–generated lipid structures. Heuristic methods to model precursor and product ions, including their relative ion abundances for individual lipid class are used. For each individual precursor ion, the characteristic losses and specific fragment ions together with their accurate masses and molecular formulas are calculated. The libraries are created according to the observed ion intensities from standards by the corresponding instruments, considering the association of different relative ion intensities with specific types of mass spectrometers. Finally, all MS/MS spectra with the nomenclatures of lipid species, adduct name, lipid class, accurate precursor mass, accurate mass fragment, heuristic modeled abundance, and fragment annotation are generated as electronic files.

Fourth, the developers of LipidBlast have rigorously validated the *in silico*–generated MS/MS mass spectra. They have performed evaluations to detect false positives and false negatives, using decoy database searches and MS/MS analysis of authentic lipid standards measured in-house and from the literature. The search parameters and detailed statistics are available at the website given above.

Finally, the developers of LipidBlast have demonstrated the applications of the library for high-throughput lipid identification [154]. They have analyzed lipid extracts of the human plasma using a low-resolution mass spectrometer. Using LipidBlast, they have structurally annotated a total of 264 lipids. The data set has been cross-checked with manual peak annotations and data available from Lipid MAPS. Using accurate-mass LC-MS/MS, they have annotated a total of 523 lipid molecular species. Similar numbers of plasma lipids have been obtained in comparison to those previously published [156, 157].

The developers of the library have concluded that LipidBlast could be successfully applied to analyze MS/MS data from over 40 different types of mass spectrometers and used with other available search engines and scoring algorithms, which represents a paradigm shift in lipidomics because it is not feasible to chemically synthesize all metabolites or natural products as authentic standards for library generation or quantification purposes. Moreover, the current array of MS/MS mass spectra for plant, animal, viral, and bacterial lipids in LipidBlast could be readily extended to many other important lipid classes.

2.3.1.2. LipidSearch: LipidSearch is a software tool (made available by Thermo Fisher Scientific) developed jointly by Prof. Ryo Taguchi and MKI (Tokyo, Japan). It is a powerful new tool for automatic identification and relative quantification of cellular lipid species from large amounts of mass spectrometric data obtained from both LC-MS and shotgun lipidomics approaches. A lipid database containing over 1.5 million lipid ions and their predicted fragment ions is associated with the software. It supports a variety of instruments and a number of acquisition modes, including PIS, NLS, and product ion analysis.

The software tool provides two different identification algorithms. First, a group-specific algorithm identifies lipids based on the polar head groups or fatty acids using a combination of PIS and NLS from lipids mixtures. Second, the comprehensive identification algorithm for product ion scans is used to discriminate each lipid by matching the predicted fragmentation pattern stored in the database.

The identified lipids are quantified by detecting their precursor ions from the full MS scans or integrating extracted ion areas from LC-MS chromatograms. The accurate peak areas are calculated by denoising and smoothing the peak profiles prior to separating any partially overlapped peaks. The quantified results are compared using t-test statistics. Since the software is newly developed and is still in premature stage, broader validation is still needed to demonstrate its power for identification and quantification of lipid species. The software package can be purchased from Thermo Fisher Scientific Co.

2.3.1.3. Other software packages for shotgun lipidomics: Multiple programs and/or software packages were developed based on the principles of shotgun lipidomics, including LIMSA [158], LipidProfiler [58], LipidInspector [59], AMDMS-SL [3], LipidXplorer [65], and ALEX [66]. These tools were developed based on the different platforms of shotgun lipidomics. LIMSA, which is available through the website (www.helsinki.fi/science/lipids/software.html), serves as an interface to process data from individual full MS and tandem MS spectra. The software packages of LipidProfiler and LipidInspector deal with the multiple PIS and NLS data; whereas LipidXplorer and ALEX process the data sets acquired from high mass accuracy/high mass resolution instruments (e.g., Orbitrap). The AMDMS-SL program is developed to identify and quantify individual lipid species from the data obtained from the MDMS-SL approach.

2.3.2. Simulation of lipidomics data for bioinformatic interpretation—The majority of the advances in bioinformatics in lipidomics have largely focused on lipid identification and quantification as described above. Interpretation of altered lipids in both content and composition determined by lipidomics resulted from adaptive or pathological changes in lipid metabolism with biological functions is still lagged [159]. Thus, development of bioinformatic and systems biology approaches to link the changes of cellular lipidome to alterations in the biological functions, including the enzymatic activities that are involved in the biosynthesis of the altered lipid classes and molecular species remains challenging. Such development should significantly advance the understanding of the roles of lipids in biological systems and of the biochemical mechanisms underpinning lipid changes [160]. In fact, some efforts have been made in this area and the outcome is apparently very attractive.

One of the attempts for the aforementioned purpose has been the recent development in dynamic or steady state simulation of the obtained lipidomics data [161-164]. Specifically, the lipid classes and individual molecular species involved in the biosynthesis of a particular lipid class are clustered and the known biosynthesis and/or remodeling pathways are utilized to simulate the ion profiles of the lipid class of interest. Through simulation based on the known pathways, a best match between the simulated and determined ion spectra is achieved. Owing to the large set of lipidomics data, numerous parameters involving the biosynthesis pathways can be derived from the simulation. These parameters are largely associated with biological functions through the model used for simulation, and thus can be used to quantitatively interpret the pathways involved in adaptive changes in lipid metabolism after any perturbation.

For instance, the pathways involving cardiolipin (CL) synthesis and remodeling species are well known. In eukaryotes, the cascade of CL synthesis and remodeling (Figure 13) is initiated from phosphatidic acid (PA), which reacts with CTP to generate cytidine diphosphate-diacylglycerol (CDP-DAG). CDP-DAG condensates with glycero-3-phosphate to yield phosphatidylglycerol (PG) phosphate which dephosphates to produce PG. Then, immature CL species are synthesized from the condensation of PG species with CDP-DAG species. Once synthesized, the immature CL species undergo remodeling to their mature species from available donor acyl chains (i.e., *sn*-2 acyl chains of PC and PE, or acyl CoA). Thus, this synthesis/remodeling process can be dynamically simulated by using the determined compositions of PG, PC, PE, and acyl CoA to best match with those of determined CL species [161, 162]. From simulating the specific distributions of CL acyl chains, the coordinated activities of the enzymes (e.g., phospholipase(s), acyltransferase(s), and/or transacylase(s)) involving CL remodeling can be assessed [161, 162]. Naturally, when applying this dynamic simulation approach to interrogate alterations in CL species under patholo(physio)logical conditions, the pathways involving the distributions of complex tissue-specific CL molecular species and underlying alterations in acyl chain selectivity in health and disease can be accrued. Moreover, as a side advantage of simulation, all of the CL molecular species, including isomeric species, can be readily recapitulated, and the existence of a variety of very low abundance CL molecular species, many of which cannot be accurately determined using currently available technologies, can be predicted.

In a similar example of TAG simulation, the well-known TAG biosynthesis pathways, in combination with the comprehensively-determined lipidomics data, have been exploited to determine the contributions of individual TAG synthesis pathways to TAG pools, in order to recapitulate the enzymatic activities involving TAG biosynthesis [164]. The simulation was based on the known TAG biosynthesis pathways of DAG reacylation with acyl CoA [165] (Figure 14), but recognizing that DAG species could be produced from different pathways. These include, but are not limited to, (i) the dephosphorylation of PA (DAG_{PA}), (ii) the reacylation of MAG species (DAG_{MAG}), which could result from multiple sources including dephosphorylation of lysoPA, TAG/DAG hydrolysis with lipase activities, and reacylation from glycerol [165, 166], and (iii) to a minimal extent, the hydrolysis of PI and PI polyphosphate, or even other GPL species through phospholipase C activities (DAG_{PI}). This approach has been extensively validated by the comparison of individual simulated TAG species with those obtained from lipidomics analysis [164]. The simulated K_1 to K_3 constants represent the relative contributions of the different DAG pools resulting from the PA, MAG, and PI pathways to TAG synthesis. Similar to the case of CL, the bioinformatic simulation of TAG profiles not only represents a powerful tool to determine altered TAG biosynthesis pathways under pathophysiological conditions, but also provides best prediction of TAG molecular species present in the biological system, which are unachievable utilizing the currently available analytical approaches.

This type of bioinformatic simulation has also been applied to the PC and PE biosyntheses [163]. From these studies, it is clearly demonstrated that through simulation, the enzymatic activities involving the lipid biosynthesis/remodeling can be assessed, the insights into the

biochemical mechanisms underpinning altered lipids under patho(physio)logical states can be provided, and broader identification of individual lipid species compared to those determined by MS can be achieved. However, it should also be recognized that although the clustered simulation is very useful and powerful, and provides the foundation for analysis of the entire lipidome, simulation approaches or models for analysis of a more comprehensive network for lipidomics are still warranted.

3. Representative applications of shotgun lipidomics in biology and medicine

Determining altered lipid states after a stimulus and revealing the mechanism(s) responsible for the changes of lipid homeostasis between states become the major task in lipidomics. To validate the revealed underlying mechanism(s), lipidomics serves as a powerful tool to recapitulate these changes with model systems (e.g., animals, cells, plants, etc.) through gene manipulation. Moreover, lipidomics may also be very useful in aid of the development of drugs for therapeutic intervention to the altered lipid homeostasis based on the revealed pathogenic mechanism(s) as well as of testing the efficacy of any developed drugs. Finally, as a member of the “omics” family, lipidomics can always be exploited for biomarker discovery and development at any levels such as organelle, cells, organs, and entire body systems. For example, changes of CL species in content, composition, or their combination in animals, which can sensitively reflect the efficiency of electron transport chains in mitochondria, represent a fine biomarker of mitochondrial function [167].

The applications of shotgun lipidomics for biological and biomedical research are too many to discuss them in the article. Many shotgun lipidomics-focused review articles of applications for biology and medicine should be consulted [8, 32, 34, 54, 63, 168-173]. Herein, only some representatives in different areas of lipidomics, particularly those recently conducted in the authors' laboratory are given below to show the power of this strategy not only in revealing the altered cellular lipidomes after a patho(physio)logical perturbation, but also in providing insights into the underlying biochemical mechanisms responsible for the changes.

3.1. Shotgun lipidomics in Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia in the aging population, but there is no effective therapy established to delay or reverse its onset or progression. AD is characterized clinically by progressive cognitive impairment [174] and neuropathologically by the appearance of diffusible amyloid-beta (Aβ), neuritic plaques, and intraneuronal neurofibrillary tangles [175]. Many hypotheses addressing the causes responsible for AD were investigated. These include Aβ cascade, tau deposition, oxidative stress, inflammation (i.e., arthritis-of-the brain hypothesis), metabolic disorder, and acetylcholine signaling defects, among others. Although tremendous progress has been made toward understanding AD, particularly early-onset AD, the true biochemical mechanism(s) underlying the pathogenesis of the late-onset AD still remain unknown. To date, the only known major genetic risk factor for late-onset AD, including both familial and sporadic and accounting for over 95% of total cases, is the ε4 allele of apolipoprotein E

(apoE4) [176, 177]. During the last decade, multiple large-scale AD clinical trials have failed, including multiple strategies mostly focused on targeting the amyloid precursor protein (APP) secretases, or its cleavage product Aβ, highlighting our incomplete knowledge of both cognitive impairment and the pathogenesis of AD [178-180].

By using MDMS-SL which uniquely allows substantiating the cell population of analyzed brain samples [181], we have determined the altered lipid levels of pure gray and white matter from different brain regions of subjects at the earliest clinically-recognizable stage of AD [182, 183]. Shotgun lipidomics analysis has revealed three marked changes of lipid levels in brain samples examined. The uncovered changes include a significant loss of plasmalogen content [182], a significant increase and compositional change in ceramide [183], and a substantial loss of sulfatide (ST) [183]. The loss of plasmalogen content is likely associated with oxidative stress [145] which is commonly manifest in AD [184]. The increased content of ceramide may be linked with inflammation and neuronal cell death since ceramide mediates many cell stress responses, including the regulation of programmed cell death (i.e., apoptosis) [185, 186] and cell aging (i.e., senescence) [187]. ST is a class of myelin-specific glycosphingolipids, i.e., sulfated galactosylceramides, in the CNS. The loss of ST may be connected to the white matter lesions occurred in AD [188, 189].

The specific and substantial loss of ST content in early stages of AD has particularly caught our attention. MDMS-SL analysis has demonstrated a virtually total loss (∼ 90%) of ST in gray matter of all examined regions. The reduction of ST content in white matter of subjects varies from 35 mol% in cerebellum to 58 mol% in temporal cortex. Intriguingly, the depletion of ST levels does not significantly go further in either gray or white matter with the progression of AD severity, likely because of the achieved balance between utilization (degradation) and biosynthesis of ST.

Mechanistic study revealed that ST metabolism is tightly associated with apoE metabolism and is dependent on its expression levels, turnover rates, and isoforms [183, 190-193]. It is particularly exciting that this novel metabolism pathway of ST [181] connects the majority of the risk factors for AD, including apoE, retinoic acid receptor, ATP-binding cassette protein A1 (ABCA1), LDL receptor-related protein, white matter lesions, endocytotic dysfunction, lysosomal fibrillogenesis, etc. [189, 194-199]. Moreover, our *in vitro* work has showed that ST enhances Aβ binding, particularly that of Aβ42, to apoE lipoproteins [200].

Furthermore, MDMS-SL has been used to determine the altered content and composition of lipids in brain samples of multiple AD animal models which transgenically express APP mutants including APPsw, PDAPP, APPsw/PS1, and APP/PS1/Tau transgenic mice [201-205]. It has been found that the levels of ST in all of AD animal models examined are specifically reduced [192] (also Han, unpublished data). For example, it has been demonstrated that the ST levels in brain tissues are reduced beginning at approximately 6 months of age in PDAPP mouse brain cortex and at 9 months of age in APPsw transgenic mouse cortex relative to their respective littermate controls. These findings indicate that the reduction of ST levels is tightly associated with the development of Aβ deposition in the animal models.

Another recent study in this research area was the determination of lipidomic changes of postmortem brain samples of subjects at the stage of preclinical AD by MDMS-SL to explore the hypothesis that alterations in ST, ceramide, and PE plasmalogen are present at this stage. Preclinical stage of AD refers a stage of cognitively normal at death, but with AD neuropathology which usually occurs 10 to 30 years earlier than the earliest clinically-recognizable stage of AD [206, 207]. It was found that (1) ST levels are significantly lower in subjects with pre-clinical AD compared to those without AD neuropathology; (2) the levels of PE plasmalogens are marginally lower at this stage of AD; and (3) changes of the ceramide levels are undetectable with the available samples [206]. This lipidomics study clearly indicates that cellular membrane defects are present at the earliest stages of AD pathogenesis and suggest that the loss of ST is among the earliest events of AD development, while alterations in the levels of PE and ceramide occur relatively later in disease development.

Finally, the MDMS-SL approach has also been applied to measure levels of over 800 species of GPL, SM, ceramide, TAG, cholesterol, and cholesteryl ester in plasma of AD individuals to explore potential lipid biomarkers for diagnosis of AD [208]. Using plasma from 26 AD (17 with mild and 9 with moderate AD) and 26 controls, it was found that SM levels are significantly lower and ceramide levels are higher in AD patients compared to the controls. Furthermore, it was also found that the rank of the changed mass levels of SM and ceramide species is strongly correlated with the rank of AD severity ($p < 0.004$), which is consistent with other reports [209-211]. The higher plasma ceramide levels [208, 212] are in line with other studies with different samples, including the middle frontal cortex [213], white matter [183], and CSF [209] from AD subjects compared to normal controls. Taken together, the findings across methods and sample varieties (brain tissue, CSF, and plasma) strongly suggest that the sphingolipid pathway is perturbed in AD.

In summary, these studies based on lipidomics analysis have not only revealed an important event occurred at the very early stages of AD development, but also provided the deep insights into the underlying biochemical mechanism leading to the lipid changes and the potential causal factors of AD pathogenesis. Moreover, the studies have further led us to reveal the critical role of ST in apoE-mediated Aβ transport, metabolism, and clearance [191, 214], thereby providing a potential drug target for treatment of AD.

3.2. Shotgun lipidomics for drug screen

As well known, an altered cellular lipidome with a stimulus is generally involved in multiple changes of lipid synthesis/metabolism pathways and networks, and needs many coordinative regulations at the levels of genome, transcriptome, and proteome. Whether the altered lipidomes under a patho(physio)logical condition can be recovered to normal after the treatment with a therapy would represent the cure of these networks and coordination. Therefore, one of the tasks in lipidomics is to use the tools to screen effective drugs of a disease.

As an example, idiopathic pulmonary fibrosis (IPF) is a fatal lung disease of unknown etiology with liver damage and the survival duration from the onset of symptoms ranges from four to five years [215, 216]. The current standard treatment of IPF is to use

glucocorticoids. Such treatment leads to many adverse effects including hepatic steatosis [217] and insulin resistance [218] in addition to its questionable effectiveness. Thus, it is critical and urgent to find new drug(s) for treatment of IPF. Recently, the application of baicalin (BAI), a flavonoid compound, for treatment of IPF has been caught great attention [219]. It is also known that BAI possesses the anti-inflammatory, anti-oxidative, and scavenging free radicals [220-223]. Whether BAI treatment is able to correct IPF-induced hepatic lipid changes becomes a focal point regarding its efficacy for treatment of IPF.

In a recent study [224], shotgun lipidomics has been employed to demonstrate the efficacy of BAI treatment to the IPF-induced hepatic lipid disorder in an IPF animal model induced by bleomycin as previously established [225]. Shotgun lipidomics has revealed a significant lipid disorder in the liver of the IPF animal model, including CL, PC, PE, PG, PI, PS, lysoCL, and lysoPC species, some sphingolipids, and TAG as examined [224]. The significantly altered CL content and composition strongly indicate mitochondrial dysfunction in the liver of the animal model. The main feature was the remarkable increases in the levels of immature CL species such as tri18:2-16:0 and tri18:2-16:1, representing the disregulation of CL remodeling.

Treatment of IPF animals with BAI has not only cured the lung complication as evidenced with histological studies, but also virtually corrected all the hepatic lipid disorder as demonstrated by shotgun lipidomics analysis [224]. Specifically, after administration with BAI, the majority of these lipid classes at the molecular species levels have became normal in comparison to the control group. Furthermore, BAI treatment has greatly improved the fat abundance and composition with increases of those species containing polyunsaturated fatty acids such as docosahexaenoic acid.

In contrast to the BAI treatment, treatment of the model with dexamethasone (one of the commonly used glucocorticoids for treatment of IPF) did not improve IPF, but led to tremendous alterations in hepatic lipidomes and accumulation of TAG content [224]. In summary, the study has showed that BAI is a potential drug for treatment of IPF and that MDMS-SL is a powerful tool for drug screening. Moreover, lipidomics studies also allow us uncover the biochemical mechanisms responsible for the altered hepatic lipidomes of the IPF animal model and following its treatments.

3.3. Shotgun lipidomics for neurotoxicity and injury induced by anesthetics

Numerous studies showed the adverse effects of general anesthetics on the developing brain using preclinical models [226-228], thus, raising public concern regarding the safety of anesthetics used in child surgery in many cases for a prolonged period (e.g., > 8 hours). Severe neuronal cell death is obvious [226-228]. However, the underlying mechanism(s) responsible for the neurotoxicity and injury, ultimately leading to cell death remain elusive.

Sevoflurane is a volatile anesthetic that has been widely used in general anesthesia because of its less adverse effects on the developing brains of animal models compared to those structurally similar anesthetics such as isoflurane [229]. However, extensive animal model studies have also showed that this anesthetic agent could cause neuronal apoptosis and behavioral dysfunctions [230-232].

Recently, MDMS-SL has been employed to determine whether altered lipid homeostasis and signaling as the potential adverse effects of sevoflurane on developing brains are present after infant monkeys are exposed to a clinically-relevant concentration of this anesthetic agent for a prolonged period and whether lipidomics analysis can provide insights into the biochemical mechanism underpinning its induced neurotoxicity if existing [233]. Intriguingly, MDMS-SL analysis has revealed substantial alterations in lipid content and composition of multiple lipid classes and molecular species in brain frontal cortex of infant monkeys exposed to sevoflurane for 9 hour. Consistently, Fluoro-Jade C staining has revealed more degenerating neurons after sevoflurane exposure along with the higher levels of cytokines in comparison to the controls [233].

For instance, MDMS-SL has uncovered that the mass levels of PE species are remarkably reduced in the sevoflurane group (indicated with red arrows, Figure 15). The majority of the reduced PE species have been identified to be plasmalogen species. It is believed that plasmalogens possess antioxidant properties in the body and is more susceptible to reactive oxidative species [145, 234]. The determined increases in lysoPE levels are consistent with those formed from degradation of the reduced PE species, strongly supporting the causal reason of plasmalogen loss as an antioxidant. Following the same line of evidence, the level of HNE, a known product of lipid peroxidation and oxidative stress, as measured by the method described in Section 2.2.1, was found significantly higher in the treatment group relative to the controls 9.67 ± 2.75 *vs*. 5.40 ± 2.02 nmol/mg protein). This finding not only further evidences the existence of oxidative stress and the peroxidation-induced plasmalogen degradation, but also suggests a potential mechanism underlying neurotoxicity and cell death after prolonged sevoflurane exposure.

Shotgun lipidomics has also showed that the levels of PS (indicated with a blue arrow, Figure 15) and PG are significantly reduced in the brain of infant monkeys after sevoflurane exposure. Functionally, PS is involved in the activation of several signaling pathways that are associated with neuronal survival, neurite growth, and synaptogenesis. The significant reduction of PS content along with the reduced mass levels of PE clearly indicates the disruption of GPL homeostasis and the changes of the integrity, orientation, permeability, and functions of plasma membranes, which in turn, could lead to subsequent neuronal dysfunction and/or degeneration. Whether the loss of PS is a consequence of PE mass reduction or the deactivation of PS synthase after sevoflurane exposure remains elusive.

The reduction of PG content after sevoflurane exposure is intriguing, since PG largely exists in mitochondrial membrane. This finding along with the synthesis of PS in mitochondria might suggest that mitochondria could be the major target of sevoflurane exposure, leading to the increased oxidative stress including the elevation of HNE. Although it is not yet known how general anesthetics induce apoptosis, impairment of mitochondrial function is generally recognized as a pathological center to the processes leading to apoptosis.

3.4. Shotgun lipidomics for metabolic syndrome

Metabolic syndrome including obesity, hypertension, dyslipidaemia, and hyperglycemia has already become epidemic in the developed countries, which leads to a dramatic increase in insulin resistance, liver steatosis, type 2 diabetes, and atherosclerosis. It is well known that

lipid disorder which has been called “lipotoxicity” is tightly associated with metabolic syndrome [235-238]. As the nature of lipidomics as a lipid analysis-based discipline, its applications for studying the origin of metabolic syndrome are very broad. A few recent review articles [239-242] should be consulted if the readers are interested in this area of lipidomics work. Below only a few representative examples from the authors' laboratory are given.

For instance, the connection of mitochondrial dysfunction with metabolic syndrome is well recognized (see refs. [243-245] for recent reviews). But the impact of mitochondrial complex I malfunction on obesity is not fully understood despite the tight link of decreased complex I activity with obesity [246-248]. By performing MDMS-SL and employing muscle-derived cell C2C12 as a model, we have demonstrated that treatment of the cells with a very low dose of rotenone leads to the profound deposition of intracellular TAG in a time- and dose-dependent fashion [105]. Mechanistic studies have showed that rotenone treatment induces mitochondrial stresses (including decreased mitochondrial oxygen consumption rate (OCR), increased ratio of NADH/NAD^+ (i.e., reductive stress), and changes of mitochondrial metabolite profiles); activates mitochondrial metabolite shuttling into cytoplasm for fatty acid synthesis; and induces TAG synthesis and deposition. The significance of the findings from the study is in multiple folds. First, it is implicated that deactivation of complex I proteins due to mutation, even very modest, could chronically lead to lipotoxicity. To this end, the incidents of mitochondrial complex I mutations are high since this cluster of proteins is very complex, containing 45 subunits in mammals and some mutations crucial to the complex I function are lethal [249]. Second, individuals who are chronically exposed to the toxins targeting complex I function have the potential to elicit obesity. For example, the widely used herbicide atrazine [250], which inhibits complex I, may contribute to the increasing prevalence of obesity. Third, mitochondrial dysfunction has already been postulated as a major causal factor for fat accumulation [251-253].

It is well known that lysoGPL species possess numerous biological functions [254]. However, the role of these lipotoxic metabolites in development of obesity is not well defined. By using a newly-developed shotgun lipidomics method (see Section 2.2.4 and Figure 10), we have clearly demonstrated the accumulation of the majority of the classes of these lipids except lysoPA and lysoPS in the liver of *ob/ob* mice in comparison to the controls [130]. Further lipidomics studies have also revealed that the mass levels of both lysoPC and lysoPE in the liver of *ob/ob* mice relative to their controls are accumulated in an age-dependent manner (Han, unpublished observation). Specifically, the difference of total amount of lysoPC and lysoPE between *ob/ob* and WT mice was 1.7 and 1.4-fold, respectively, at 16 weeks of age *vs.* 1.2 and 1.4-fold at 12 weeks. In general, the significantly elevated lipotoxic lipid levels at 16 weeks of age have been believed to cause the inhibitory action on insulin signaling [255], since the insulin level and the ratio of insulin *vs.* glucose in *ob/ob* mice are apparently increased starting at around 13 weeks of age [256], which indicated a transition from a normal insulin state into an insulin-resistant state. Although further conclusive study is needed, it seems that the observations suggest an association of lysoGPL accumulation with the development of insulin resistance.

In another representative study, MDMS-SL has been used to determine the circadian changes in lipid abundance of GPL, TAG, and many other lipid classes in mouse liver and dissect its clock/feeding dependency [257]. Lipidomics analysis of lipid extracts from the livers of wild type and clock-disrupted mice (i.e., *Per1/2* null mice) fed either *ad libitum* or exclusively during the night has been conducted in a temporal manner. It was found that a similar fraction of lipids (∼17%), particularly those of TAG species, oscillated in both mouse strains, but in completely different phases. In contrast to the lipidomics findings, RNA analysis by real-time quantitative PCR has revealed that the expression levels of several master lipid regulators and enzymes involved in TAG metabolism retained their circadian expression in clock-disrupted mice. These contradictive observations has led us to examine the effects of feeding-time on the phase changes and the levels of hepatic TAG in both wild type and *Per1/2* null mice. Remarkably, upon nighttime restricted feeding, wild type mice exhibits a sharp decrease (∼50%) in hepatic TAG levels. The findings indicate that both circadian clocks and feeding-fasting cycles play a prominent role in the regulation of circadian TAG accumulation and total TAG levels in the liver. The study suggests that the mealtime may have a significant effect on accumulation of fat in the liver, thus contributing the development of non-alcohol fatty liver disease and metabolic syndrome. Alternatively, the findings should have important implications for the potential treatment of metabolic diseases.

4. Summary and perspective

Since the emerging of lipidomics discipline around 2003, the advanced analytical technologies, including soft ionization methods in mass spectrometry, separation science such as ultra performance LC and nanomaterials, and computational technology have driven this field greatly to expand to essentially any biological and biomedical research. Among the development of these analytical technologies and strategies used in lipidomics, direct infusion-based shotgun lipidomics gets more matured, advanced, and popularly applied. It is particularly noteworthy that the power of the MDMS-SL technology becomes obvious as it advances to comprehensively cover cellular lipidomes including those bioactive lipid classes and individual species, and mathematically interprets the contributions of individual pathways to the aberrant lipid metabolism and homeostasis under pathological conditions through bioinformatic modeling.

As advances in the technologies for lipidomics, interweaving of this discipline with other fields becomes apparent. The applications involving lipidomics have been just like rocket shooting. From biomarker development for early diagnosis and prognosis of diseases to phenotyping gene regulation, from determining lipid changes with a perturbation to identifying the underlying mechanism(s) responsible for these changes which could lead to the development of drug targets, and from serving as a tool for drug screening to testing the efficacy of any developed drugs, lipidomics plays more and more key roles. The given examples herein only provide a little flavor for the delicious meal which is not covered in the article.

Regardless of the tremendous advances made in recent years in shotgun lipidomics (lipidomics in general), a few areas of progress on the technology are still desirable. First, it

is clear that further increases in the coverage of any platforms for truly comprehensive analysis of the cellular lipidome, particularly for those of very low abundance species and in an automated, high throughput manner, remain to be a big challenge. Conquering the obstacles should bring us to a new era of this discipline since interpretation of lipidomics data should not only extensively consider different pathways within a network of an individual lipid category, but also expand the influence from other categories of lipids and consider the inter-relationship between the networks. Second, it seems also clear that integration of lipidomics investigations with other omics including genomics and proteomics research is critical to fully understand and validate the molecular mechanisms underpinning the altered lipid content and composition after a stimulus. Third, although dynamic lipidomics through simple stable isotope labeling has been well practiced [84, 258, 259], more complex studies in a fluxomics scale to reveal the metabolic reaction rates remain open. Fluxomics in lipids should provide the comprehensive understanding of the roles of lipids in biological systems in health and their alterations during disease processes. Finally, although it has been demonstrated the usefulness and powerfulness of bioinformatics for data interpretation based on simulation or dynamic modeling [161-164], these attempts are only the isolated studies on different clustered pathways. Similar modeling or novel approaches for analysis of a more comprehensive network or even ideally for the entire cellular lipidome are still warranted.

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Abbreviations

Aβ	amyloid-beta
AD	Alzheimer's disease
AMPP	N-(4-aminomethylphenyl)pyridinium
apoE	apolipoprotein E
APP	amyloid precursor protein
BAI	baicalin
BUME	butanol/methanol system
CDP-DAG	cytidine diphosphate diacylglycerol
CID	collision-induced dissociation
CL	cardiolipin
DAG	diacylglycerol or diglyceride
D*Me*PE	*N*,*N*-dimethylphosphatidylethanolamine
DMG	dimethylglycine
ESI	electrospray ionization
FA	fatty acyl or fatty acid
Fmoc	fluorenylmethoxylcarbonyl
GPL	glycerophospholipid
HNE	4-hydroxy-2E-nonenal
(HP)LC	(high performance) liquid chromatography

IPF	idiopathic pulmonary fibrosis
lysoGPL	lysoglycerophospholipid
lysoPA	lysophosphatidic acid
lysoPC	choline lysoglycerophospholipid(s)
lysoPE	ethanolamine lysoglycerophospholipid(s)
lysoPG	lysophosphatidylglycerol
lysoPI	lysophosphatidylinositol
lysoPS	lysophosphatidylserine
MAG	monoacylglcyerol or monoglyceride
MDMS	multi-dimensional mass spectrometry
MDMS-SL	multi-dimensional mass spectrometry-based shotgun lipidomics
M*Me*PE	*N*-monomethyl phosphatidylethanolamine
MS	mass spectrometric or mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl-tert-butyl ether
NAE	*N*-acylethanolamine
NEFA	non-esterified fatty acid(s)
NLS	neutral loss scan or scanning
PA	phosphatidic acid
PC	choline glycerophospholipid(s) including all subclasses (phosphatidylcholine, plasmenylcholine, and plasmanylcholine)
PE	ethanolamine glycerophospholipid(s) including all subclasses (phosphatidylethanolamine, plasmenylethanolamine, and plasmanylethanolamine)
PEMT	phosphatidylethanolamine *N*-methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIP	phosphatidylinositol phosphate
PIP_2	phosphatidylinositol diphosphate (or bisphosphate)
PIS	precursor-ion scan or scanning
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Q	quadrupole

QqQ	triple quadrupoles
Q-TOF	quadrupole-time of flight
SM	sphingomyelin
SPE	solid phase extraction
ST	sulfatide

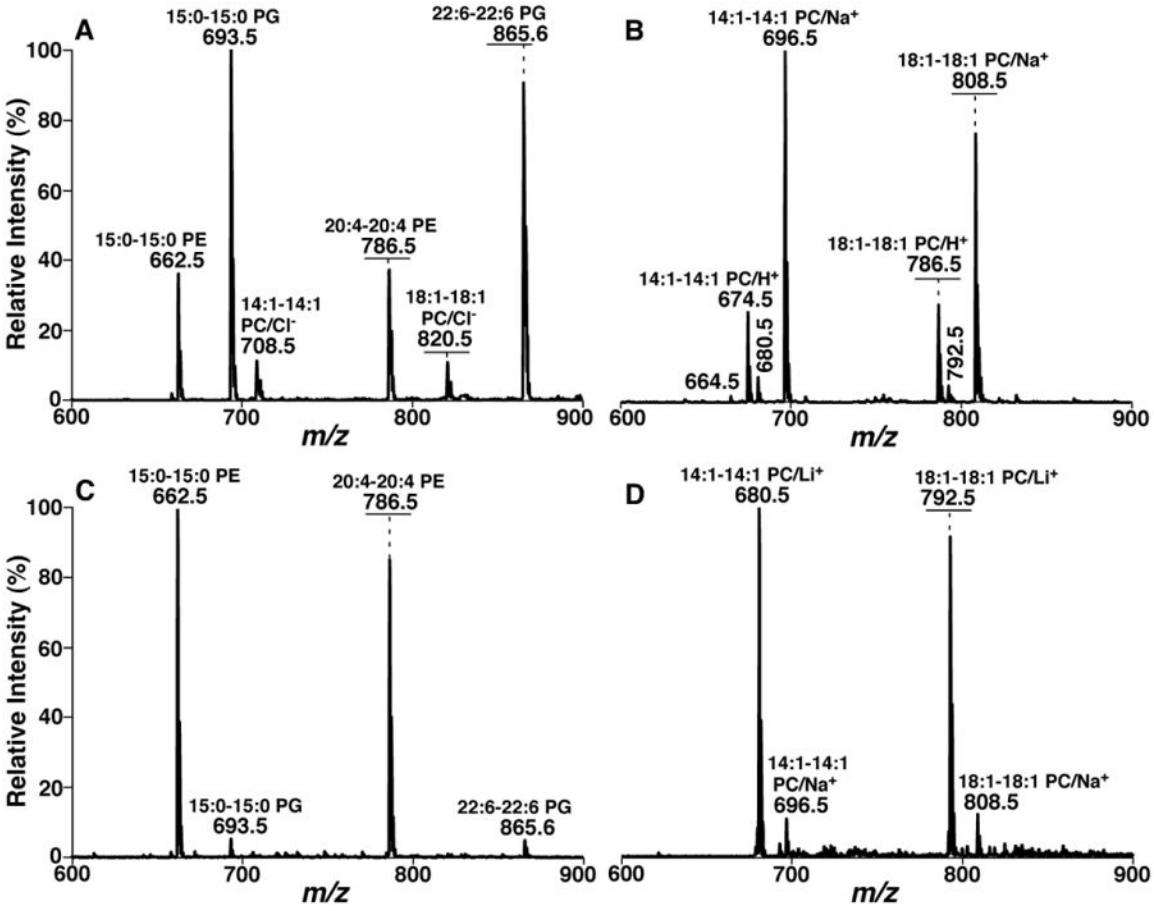

Figure 1.

The dependence of ionization efficiency on the charge propensities of analytes. A glycerophospholipid mixture was comprised of 1 pmol/μL each of di15:0 and di22:6 phosphatidylglycerol (PG) (representing anionic glycerophospholipids), 10 pmol/μL each of di14:1 and di18:1 phosphatidylcholine (PC) (representing charge neutral, but polar lipids), and 15 pmol/μL each of di15:0 and di20:4 phosphatidylethanolamine (PE) (representing weakly anionic lipids) species in 1:1 chloroform/methanol (v/v). The mixture was analyzed with a QqQ mass spectrometer (TSQ Quantum Ultra, Thermo Fisher Scientific) in the negative- (Panels A and C) or positive-ion (Panels B and D) mode after direct infusion in the absence (Panels A and B) or presence (Panels C and D) of 30 pmol/μL of LiOH in methanol. All the indicated molecular species were confirmed with product ion ESI-MS analysis. The horizontal bars indicate the ion peak intensities after ^{13}C deisotoping and normalizing the species to the one with less number of carbon atoms (i.e., the one with lower molecular weight) in each lipid class. (Modified from the ref [39] with permission from the American Society for Mass Spectrometry, Copyright 2006).

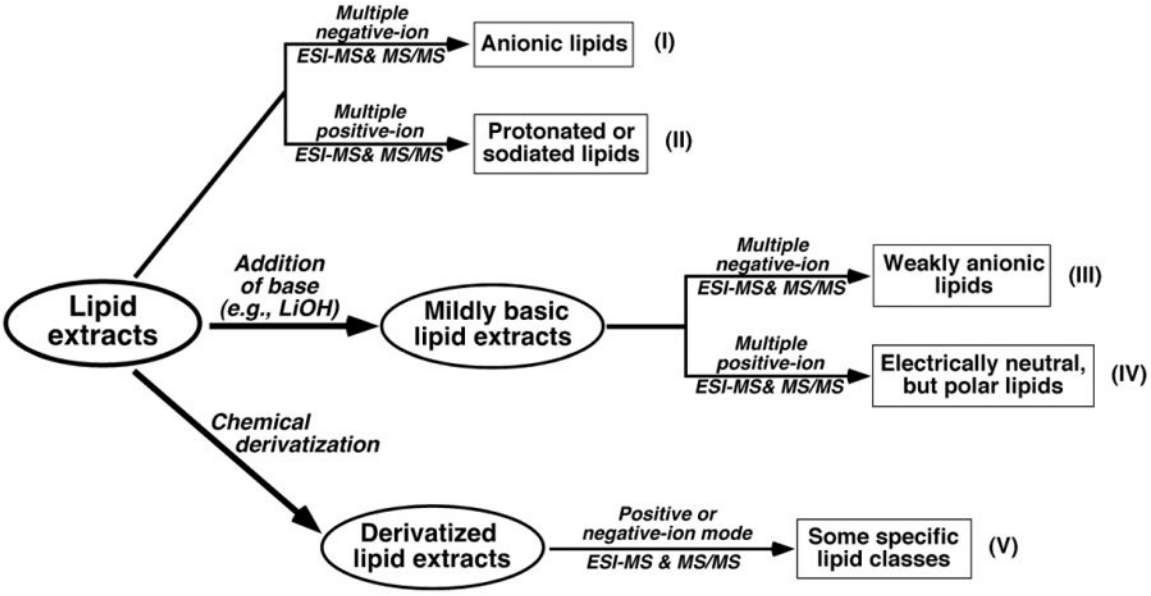

Figure 2.

Illustration of the workflow of global analysis of cellular lipidomes directly from a crude extract of a biological sample after intrasource separation or with derivatization. First, diluted lipid extracts are directly analyzed in the negative- and positive-ion modes to determine anionic lipids (Condition I) and individual species of some special lipid classes (e.g., sodiated choline lysoglycerophospholipid and protonated acylcarnitine) (Condition II), respectively. Then weakly anionic lipid classes such as ethanolamine-containing lipids (Condition III) and charge-neutral, but polar lipids (e.g., phosphocholine-containing lipid classes and triacylglycerols) (Condition IV) are determined in the negative- and positive-ion modes, respectively, after addition of a small amount of LiOH to the diluted lipid solution. Finally, small portions of lipid solution can be separately derivatized for analysis of some specific lipid classes such hydroxyalkenal, carboxylic acid-containing lipid classes, hydroxyl-containing lipid classes, etc. (Condition V). Some examples of analysis of these lipid classes after derivatization are given in Section 2.2. After intrasource separation or derivatization, analyzing individual species of a class of interest is achieved through building block analysis by MS/MS in an MDMS-SL manner, which is exemplified in Figure 2.

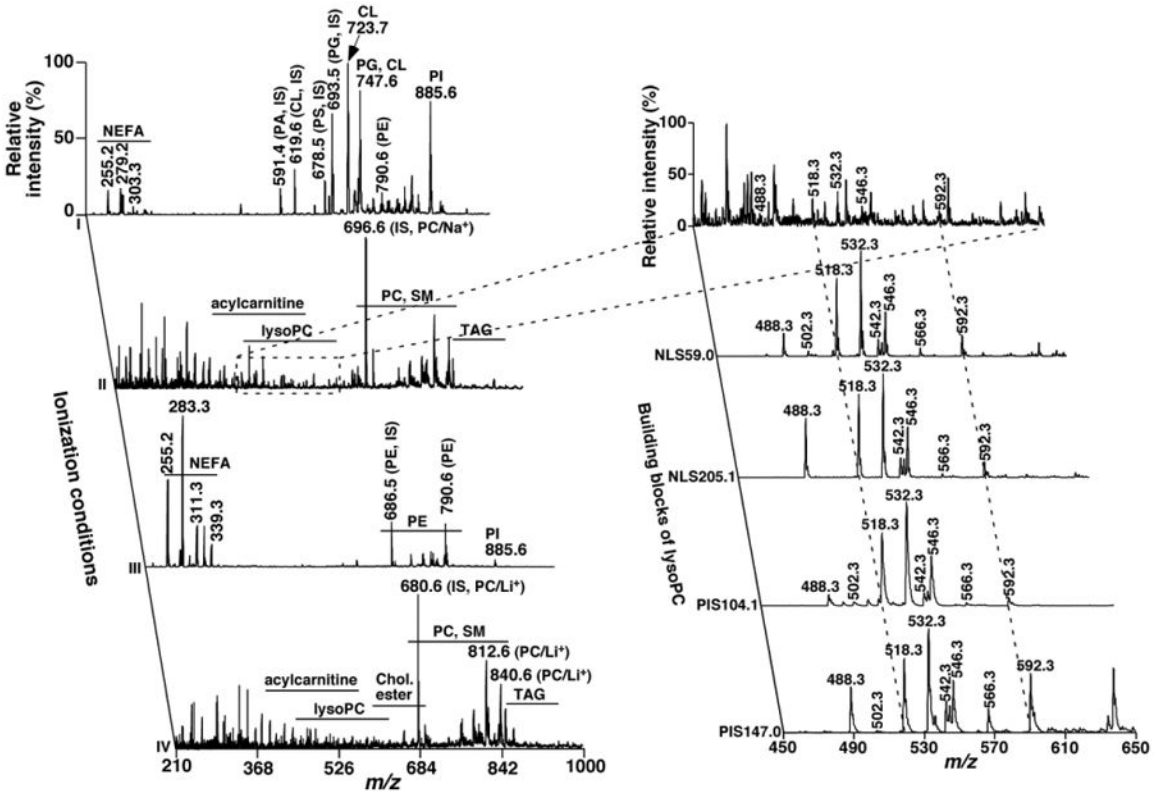

Figure 3.

Illustration of analyzing mouse myocardial lipid species by using multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) after intrasource separation. Mouse heart sample (~ 15 mg) was dissected and a lipid extract of the tissue was prepared by using a modified procedure of Bligh-Dyer extraction. A small portion of the lipid extract was diluted and directly analyzed under different mass spectrometric conditions (Conditions I and II of Figure 2) to selectively ionize anionic lipids (Top mass spectrum of left panel) or to form sodiated or protonated lipids for analysis of some special lipid classes from these kinds of adducts (Second mass spectrum of left panel), respectively. Another portion of the lipid extract was diluted and rendered to weakly basic with addition of a small amount of a base solution such as LiOH. This basic lipid solution was used to analyze weakly anionic lipids (e.g., free fatty acids, phosphatidylethanolamines (PE), and lysoPE, etc.) in the negative-ion mode (Condition III of Figure 2) (Third mass spectrum of left panel) and charge-neutral lipids (e.g., acylcarnitine, phosphatidylcholine (PC), lysoPC, triglycerides, cholesteryl esters, etc.) in the positive-ion mode (Condition IV of Figure 2) (Bottom mass spectrum of left panel). Each of these detected lipid classes was then identified and quantified separately by two-dimensional mass spectrometry. Right panel shows an example of analyzing individual choline lysoglycerophospholipid (lysoPC) species (which are present in very low abundance as shown in the second mass spectrum of left panel as acquired under Condition II) through the combined analyses of multiple neutral loss scans (NLS) and/or precursor-ion scans (PIS). These NLS and/or NLS spectra specifically detect the fragments (i.e., building blocks) resulted from individual lysoPC species including regioisomers. The rest portion of the lipid extract was used to analyze many other lipid classes (e.g., mono- and

diacylglycerols, eicosanoids, retinoic acids, 4-hydroxyalkanels, etc., which are present in very low abundance, or not ionizable, or not containing specific fragments for NLS/PIS analyses) through derivatization as described in the text (Section 2.2).

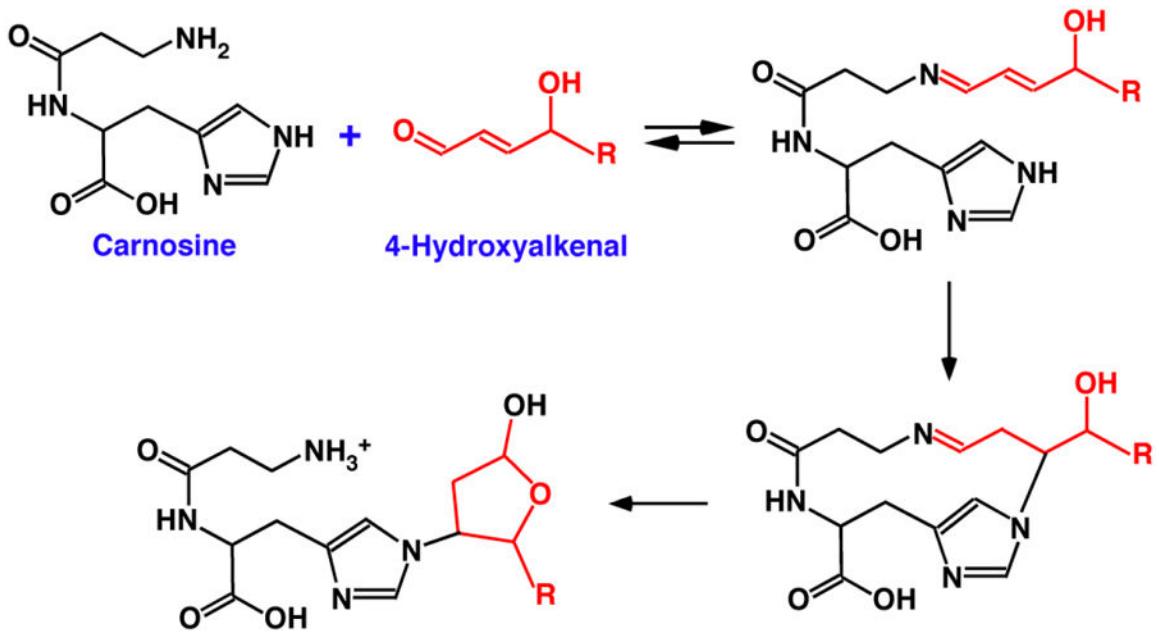

Figure 4.

Scheme of Michael adduct of carnosine with 4-hydroxyalkenal species followed by spontaneous hydrolysis. R represents any aliphatic moiety with or without double bond(s).

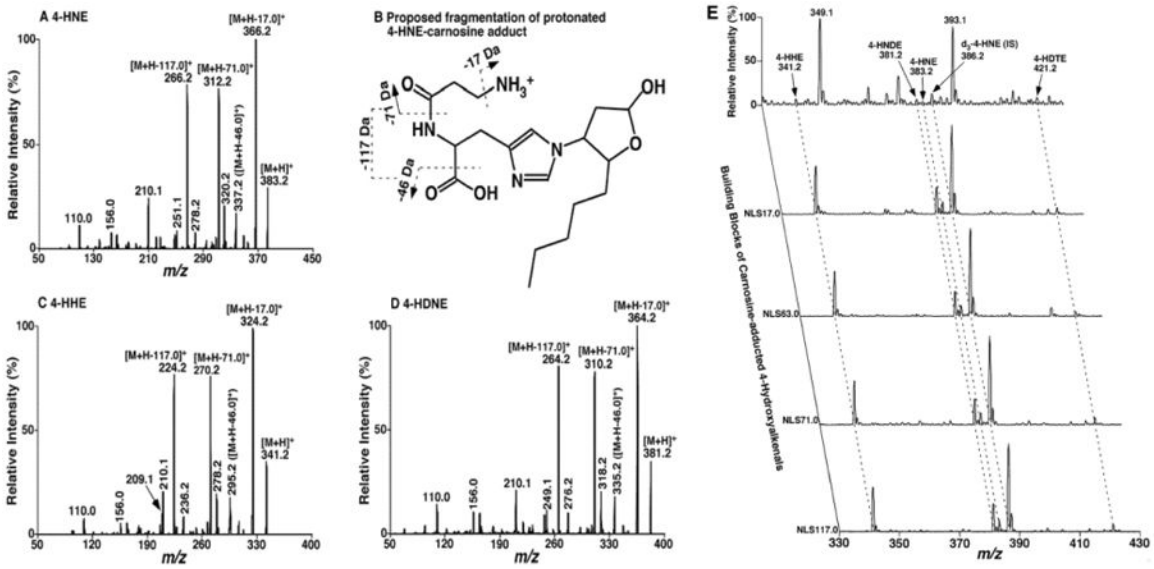

Figure 5.

Representative product ion and two-dimensional mass spectrometric analyses of carnosine-adducted 4-hydroxyalkenal species. Carnosine-4-hydroxyalkenal adducts were prepared by incubating individual 4-hydroxyalkenal species as previously described [103]. Product ion ESI-MS analyses of carnosine adducts of 4-hydroxynonenal (4-HNE, Panel A), 4-hydroxyhexenal (4-HHE, Panel C), and 4-hydroxynondienal (4-HNDE, Panel D) were performed on a QqQ mass spectrometer as previously described [103]. The fragmentation pattern of protonated 4-HNE-carnosine adduct was proposed (Panel B). Lipid extracts of mouse myocardium were prepared by a modified Bligh-Dyer method and derivatized with carnosine as described previously [103]. Two-dimensional mass spectrometric analyses of carnosine-adducts of 4-hydroxyalkenal species present in mouse myocardial lipid extracts comprised of their unique fragments (i.e., building blocks) derived from Panels A to D including NLS17.0, NLS63.0, NLS71.0, and NLS117.0 were conducted on a QqQ mass spectrometer (TSQ Vantage, Thermo Fisher Scientific) after direct infusion of the derivatized lipid extract at collision energy of 16, 27, 23, and 28 eV, respectively, and collision gas pressure of 1 mTorr. “IS” stands for internal standard.

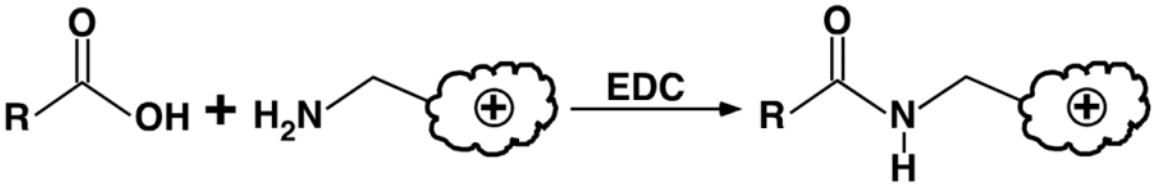

Figure 6.

Scheme of amidation reaction between aliphatic carboxylic acid and a primary amine carrying a positive charge. The reaction is catalyzed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

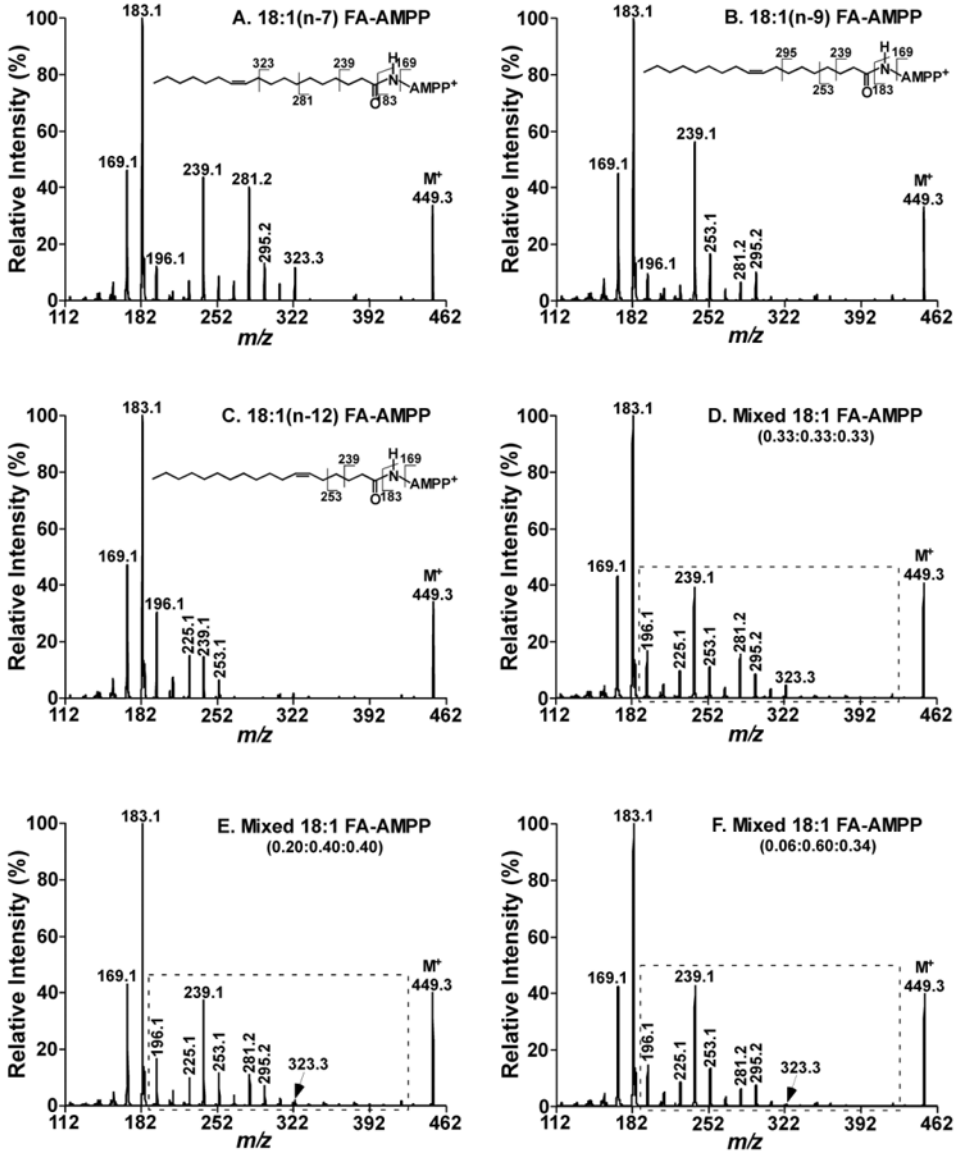

Figure 7.

Product ion ESI-MS analyses of 18:1 fatty acid isomers and their mixtures after derivatized with N-(4-aminomethylphenyl)pyridinium (AMPP). Derivatization of 18:1 fatty acid isomers and their mixtures with AMPP and product ion ESI-MS analyses of derivatized 18:1(n-7) (Panel A), 18:1(n-9) (Panel B), and 18:1(n-12) (Panel C) FA isomers, and n-7/n-9/n-12 18:1 FA isomer mixtures in a ratio of 0.33:0.33:0.33 (Panel D), 0.2:0.4:0.4 (Panel E), or 0.06:0.60:0.34 (Panel F) at collision energy of 40 eV and collision gas pressure of 1 mTorr were performed as described previously [76]. The majority of the abundant fragment ions after charge-remote fragmentation with AMPP was assigned and illustrated in the corresponding molecular structures. The signatures highlighted with the broken lined boxes were used to determine the composition of 18:1 FA isomers in the mixtures through multiple linear regression analysis of these signatures as the responses with the fragmentation patterns of individual 18:1 FA isomers (shown in Panels A to C) as the predictors.

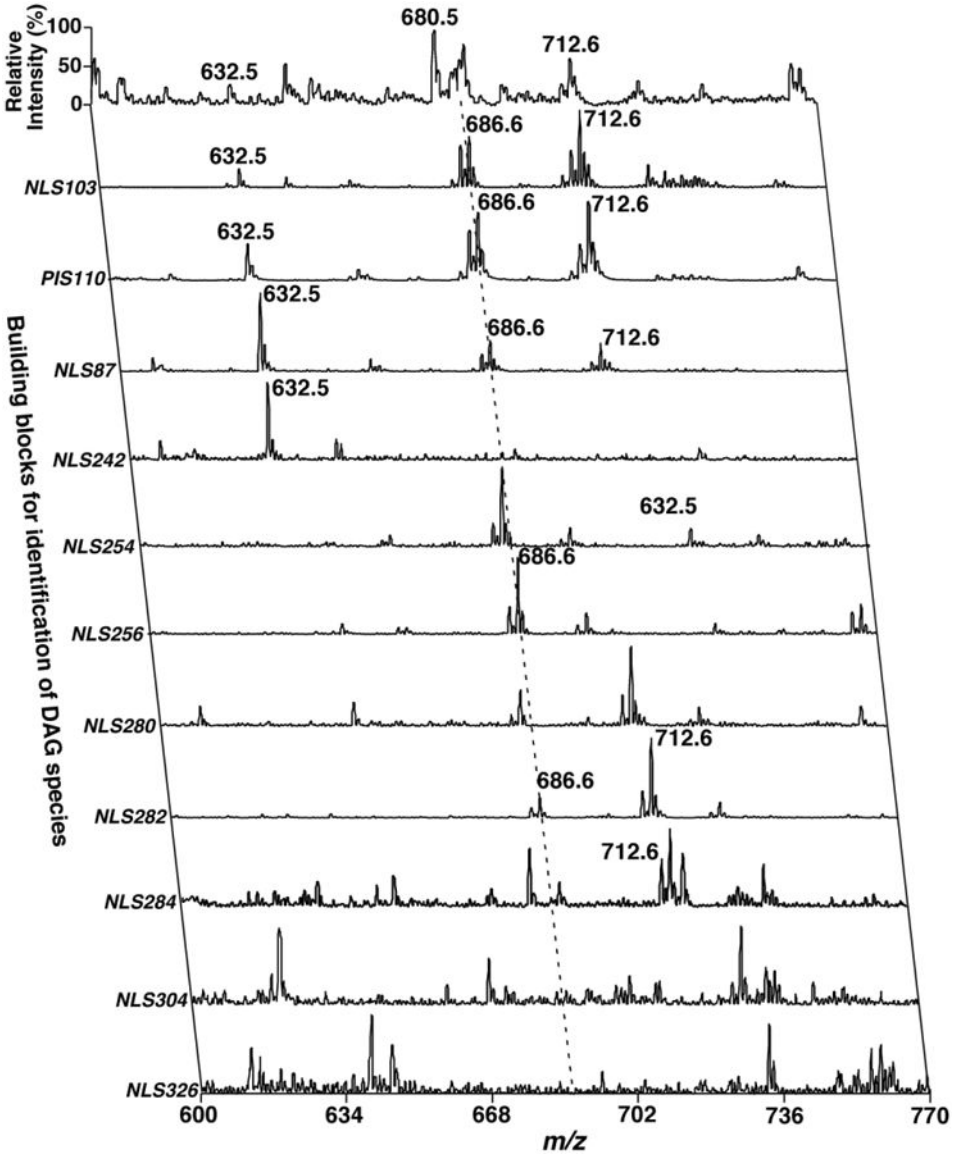

Figure 8.

Representative two-dimensional MS analysis of lithiated DMG-DAG species present in lipid extracts of mouse liver samples. All MS/MS scans (i.e., NLS103, PIS110, and NLS87) for characterization of the DMG head group and all NLS of FA chains from lithiated DMG-DAG species were performed as previously described [116]. Individual MS and MS/MS traces presented in two-dimensional mass spectrometric analysis were displayed after normalization of the base peaks in the spectra. Identification of individual DMG-DAG species from the two-dimensional mass spectrometry exemplified was discussed in the text.

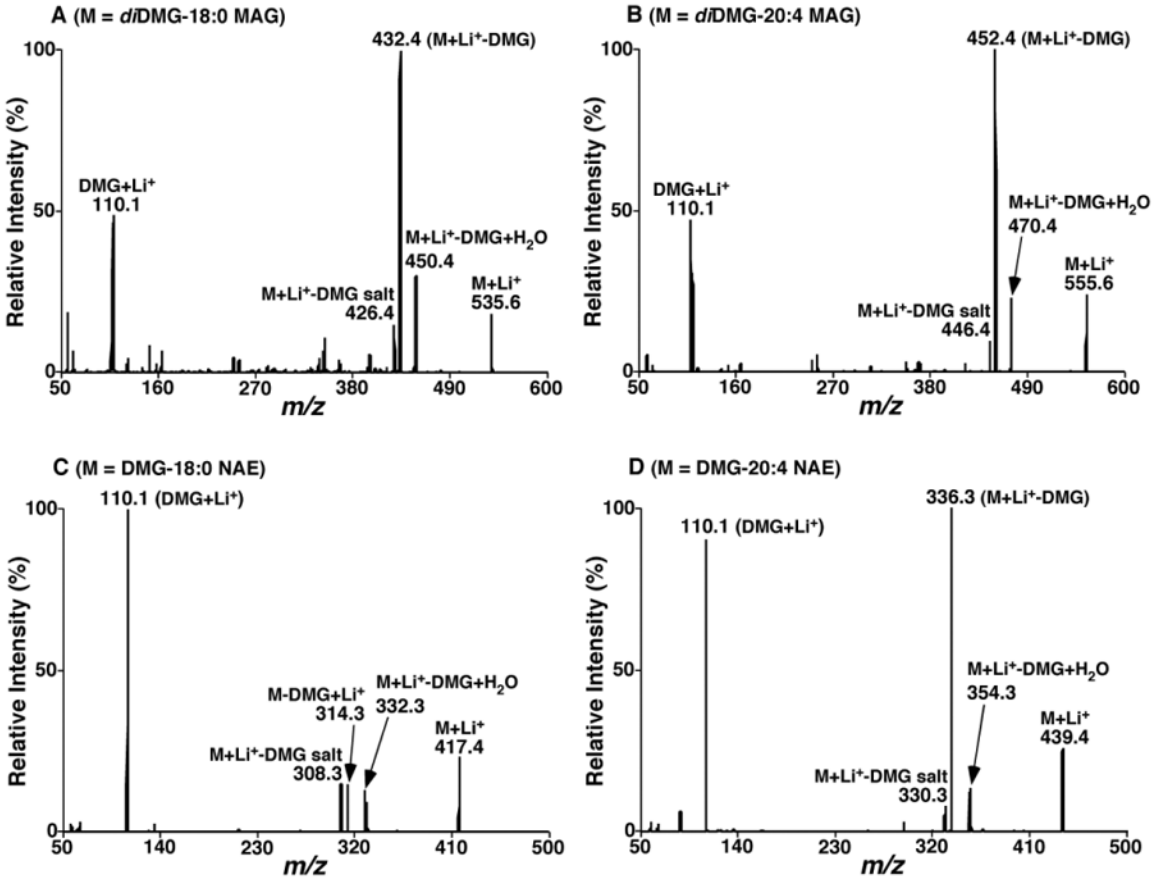

Figure 9.

Product ion ESI-MS analyses of representative lithiated dimethylglycine (DMG)-derivatized monoacylglycerol (MAG) and N-acylethanolamine (NAE) species. Derivatization of MAG and NAE species with DMG was conducted as described previously [116]. Product ion analyses of derivatized 18:0 (A) and 20:4 MAG (B), and 18:0 (C), and 20:4 NAE (D) species were performed after selection of the corresponding lithiated molecular ions by Q1, collision activation in Q2 with collision energy of 35 eV and collision gas pressure of 1.0 mTorr, and product ion detection by Q3. The majority of the fragment ions in the spectra were assigned.

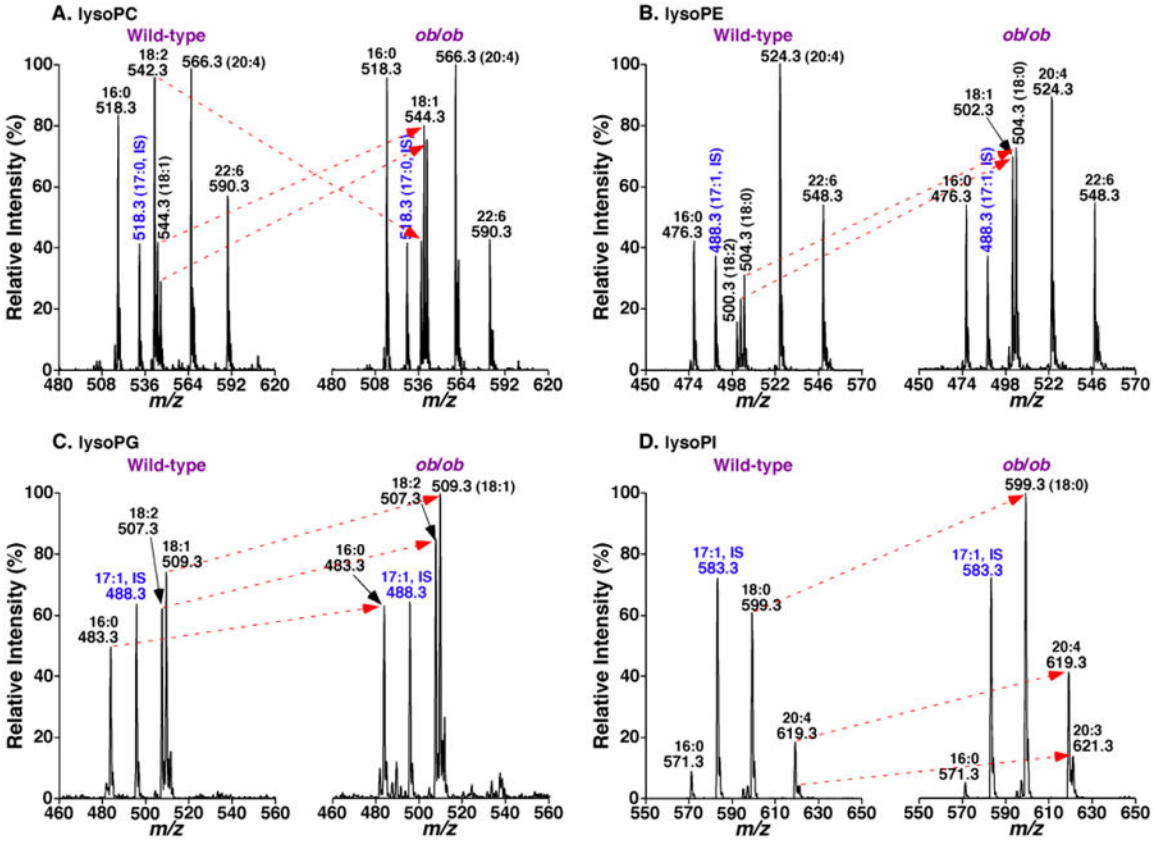

Figure 10.

Representative comparison of tandem MS mass spectra of lysoglycerophospholipid (lysoGPL) species present in lipid extracts of the livers between wild-type and *ob/ob* mice. Tandem mass spectra were acquired as described previously [130]. Each paired mass spectra were displayed after normalization to the internal standard (IS) peaks (i.e., the peaks corresponding to IS are equally intense in each paired spectra) for direct comparisons. The red arrows indicate the changes of some lipid species in *ob/ob* mice in comparison to controls. LysoPC, lysoPE, lysoPG, and lysoPI denote choline lysoglycerophospholipid, ethanolamine lysoglycerophospholipid, lysophosphatidylglycerol, and lysophosphatidylinositol, respectively.

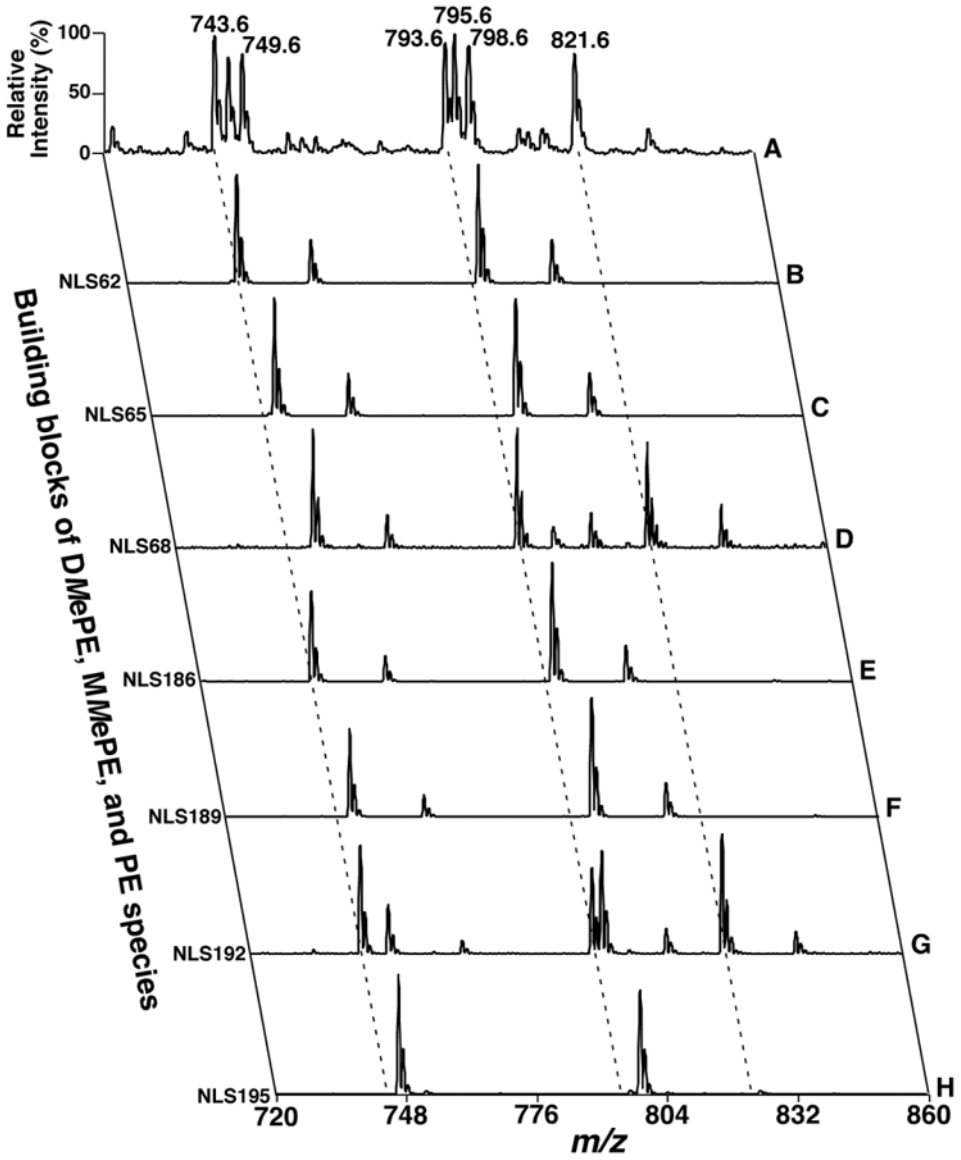

Figure 11.

Representative MS and neutral loss analyses of methylated D*M*ePE, M*M*ePE, and PE species. An equimolar mixture of di16:0 and di18:1 D*M*ePE, di16:0 and di18:1 M*M*ePE, and di16:0, di18:3, and 16:0-22:6 PE at 1 pmol/μl each was treated with CD_3I as described previously [138]. A survey scan (A), and NLS62.1 (B, for methylated D*M*ePE), NLS65.1 (C, for methylated M*M*ePE), and NLS68.1 (D, for methylated PE) performed at collision energy of 26 eV, and NLS186 (E, for methylated D*M*ePE), NLS189 (F, for methylated M*M*ePE), NLS192 (G, for methylated D*M*ePE and PE), and NLS195 (H, for methylated M*M*ePE) performed at collision energy of 33 eV were acquired as previously described [138].

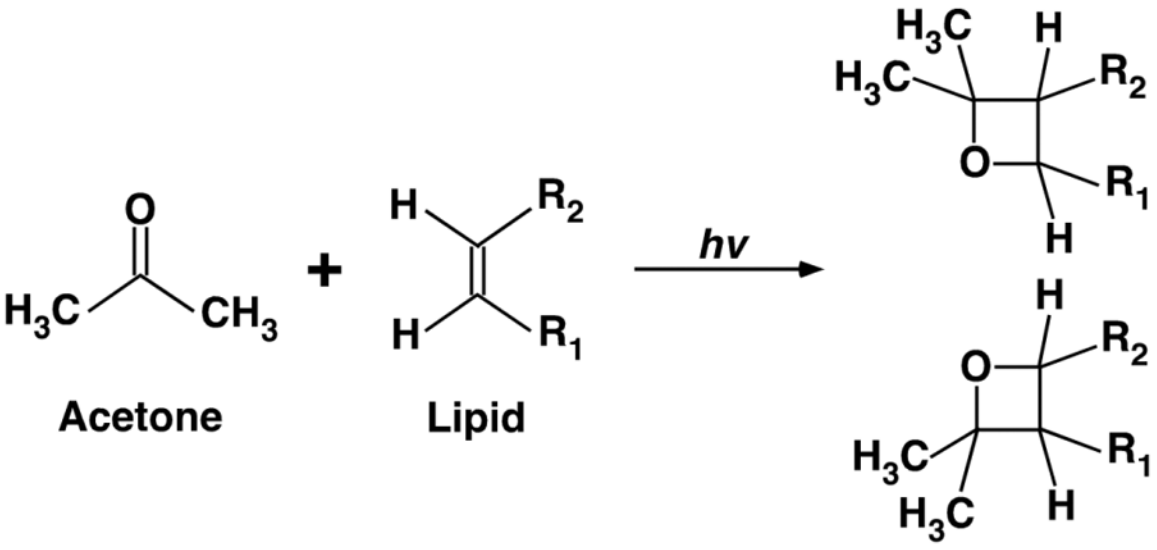

Figure 12.
Scheme of Paternò–Büchi reaction between acetone and a lipid species containing a double bond initiated with UV light (*hv*).

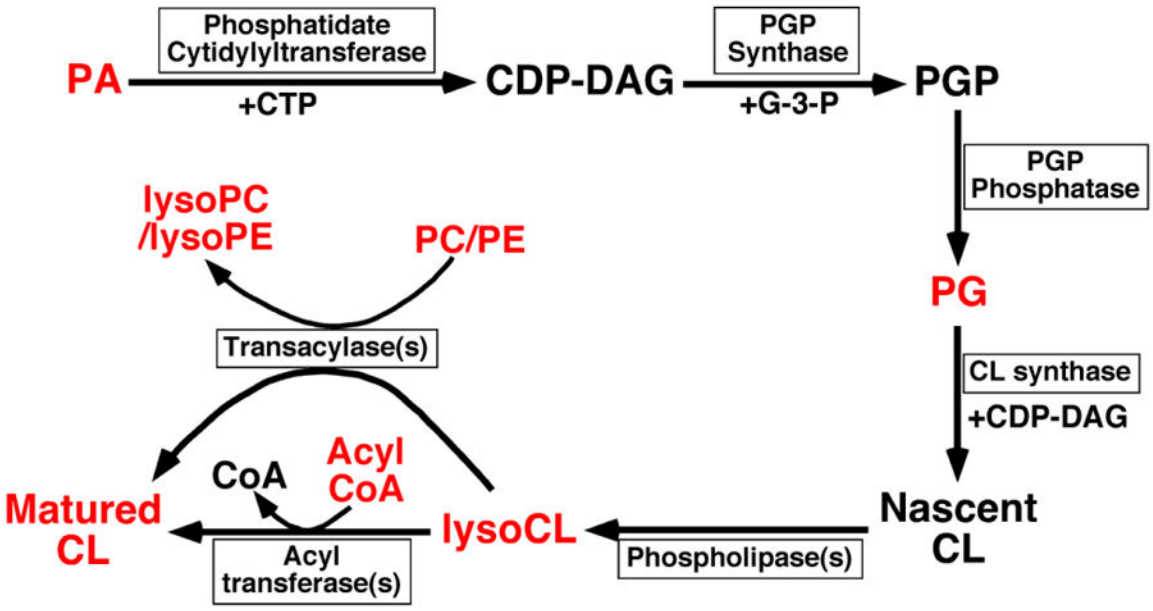

Figure 13.

Schematic illustration of cardiolipin biosynthesis and remodeling pathways. Cardiolipin (CL) biosynthesis is initiated from condensation of phosphatidic acid (PA) and CTP to produce cytidine diphosphate-diacylglycerol (CDP-DAG) through phosphatidate cytidylyltransferase activity. The produced CDP-DAG reacts with glycerol-3-phosphate to form phosphatidylglycerol phosphate (PGP) catalyzed by PGP synthase. PGP is dephosphated to generate phosphatidylglycerol (PG). Newly synthesized CL (immature CL) is formed by the condensation of PG and CDP-DAG catalyzed by CL synthase. Immature CL is then deacylated to form monolysoCL and then reacylated using acyl chains from acyl CoA or the *sn*-2 acyl chain of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species, leading to the formation of matured CL. The highlighted lipid classes with red indicate those that were determined by MDMS-SL.

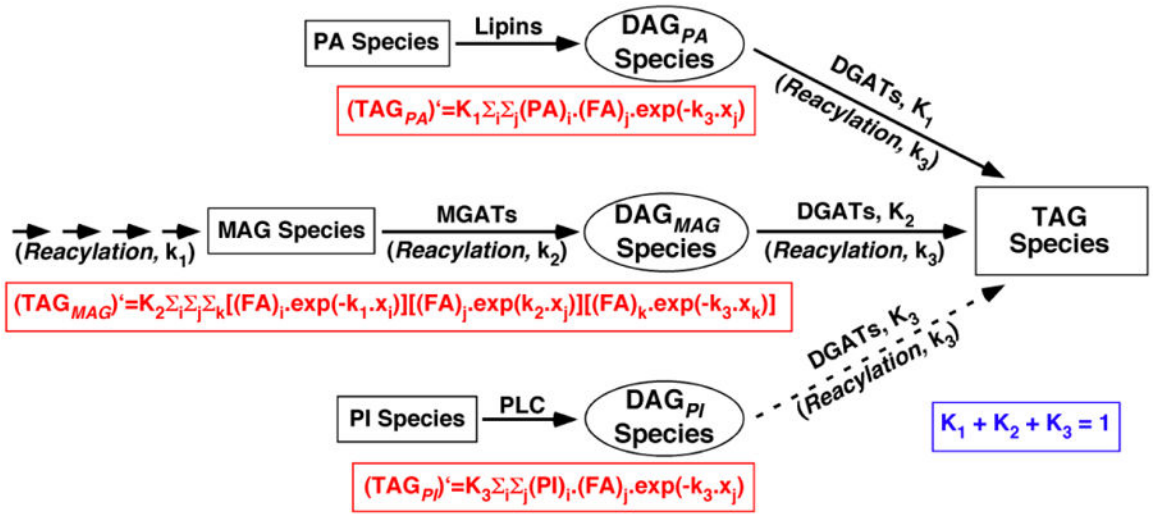

Figure 14.

Schematic illustration of triacylglycerol biosynthesis model for simulation of triacylglycerol ion profiles. Triacylglycerol (TAG) species are *de novo* synthesized with reacylation of diacylglycerol (DAG) species of different pools produced mainly through dephosphorylation of phosphatidic acid (PA) (DAG_{PA}) and reacylation of monoacylglycerol (MAG) (DAG_{MAG}) as well as, to a less degree (as indicated with a broken line arrow), through hydrolysis of phosphatidylinositol (PI) with phospholipase C (PLC) activities (DAG_{PI}). The contributions of these pathways to the TAG pools were determined through simulation of individual TAG ion profile with parameters of K_1, K_2, and K_3, respectively, which are the probabilities of individual DAG pools being reacylated to TAG. In addition, the parameters of k_1, k_2, and k_3 were used in the *sn*-1, 2, and 3 reacylation steps of TAG species in the forms of $\exp(-k_1.x_j)$, $\exp(k_2.x_j)$, and $\exp(-k_3.x_j)$, respectively, where k_1 and k_3 represented a simulated decay constant whereas k_2 represented a simulated enhancing constant, and x_j is the number of double bonds present in the corresponding FA chain. MGAT and DGAT denote MAG and DAG acyltransferases, respectively. The multiple arrows at the k_1 step indicate that MAG species could be generated from a variety of sources such as lysoPA dephosphatation, DAG hydrolysis, and glycerol acylation.

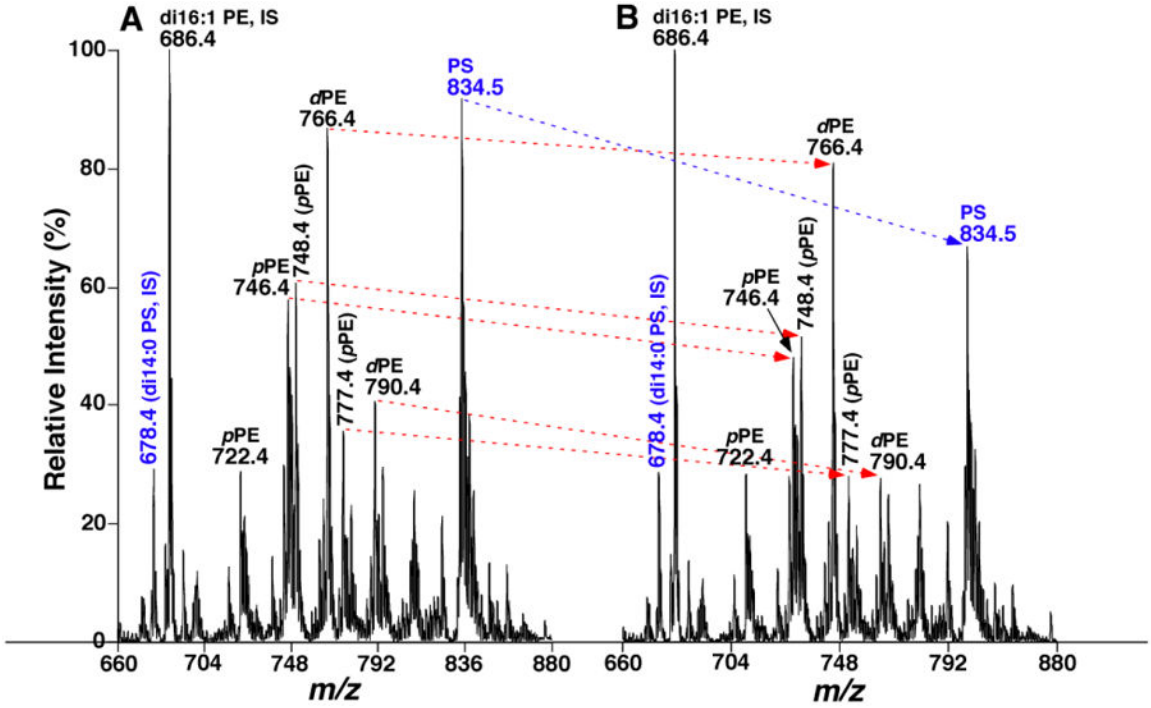

Figure 15.

Comparison of representative mass spectral analyses of ethanolamine glycerophospholipid (PE) and phosphatidylserine (PS) species present in the samples of brain from infant monkeys with or without exposure to sevoflurane. MDMS-SL analysis of lipid extracts from brain samples of infant monkeys with or without prolonged exposure to sevoflurane was performed in the negative-ion mode as previously described [233]. The narrows with red between the paired mass spectra indicated that the mass levels of individual PE species were significantly reduced in sevoflurane-exposed brain compared to those of the controls. The narrows with blue between the paired mass spectra indicated that the mass levels of individual PS species were significantly reduced in sevoflurane-exposed brain compared to those of the controls. The paired mass spectra are displayed after normalization to the internal standard (IS) peaks (i.e., the peaks corresponding to the IS are equally intense in each paired spectra) for direct comparisons.