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Technological developments in lipidomics

Weimin Hou, Hu Zhou*, Fred Elisma*, Steffany A. L. Bennett and Daniel Figeys*

Abstract

Lipid analysis is a well-established field of research that focuses on one lipid or a few lipids. The recent developments in mass spectrometry technologies have enabled more comprehensive studies to be performed on lipids present in a sample. The move towards extensive lipid research has led to the coining of the term *lipidomics*, which is defined as the ensemble of lipids present in a sample. In this review, we will discuss the technical developments in the field of *lipidomics* and the current limitations of this nascent field.

Keywords: *lipidomics; systems biology; mass spectrometry; HPLC; identification; quantitation*

Lipids are broadly defined as any fat-soluble molecules which include a wide range of molecular structures. The classical view, however, of lipids as amorphous and benign membrane components has been shattered. We now know that lipids play many important roles in cells such as cellular structural support, energy storage and signal transduction. Also, recent research points to the vital roles that lipids may have in the brain, in Alzheimer diseases [1], in cancer [2, 3], in inflammation and cardiovascular diseases [4], in male fertility [5] and in other diseases. Glycerophospholipids, for example, form the largest lipid subclass by mass, and they are important components of biological membranes in which they modulate membrane trafficking. Furthermore, some of their metabolites, such as platelet-activating factors (PAFs), are powerful intracellular signalling molecules [6–9]. These metabolites can induce a broad range of biological responses [10], including the progression of neurodegeneration [11–13]. Although the study of lipids is not new, the global quantitative study of them is more recent. The term *lipidomics* was thus coined to define the global study of the lipid components of cells, tissues or organisms.

The interest in lipidomics has grown steadily from the first paper in 2003 [14] to a steady rate of about 12 original papers per year not including reviews. Thus far, 170 papers on lipidomics (including reviews) have been published. Obviously, this is a far cry from the 25 000 papers on genomics and 13 000 papers on proteomics in PubMed.

Lipidomics is definitely attracting more attention, and developments in technology are starting to overcome various challenges and limitations to better understand the lipidome. For example, up until now, it has been very difficult to get an extensive view of the lipidome. Past studies could only focus on a single lipid, a few selected lipids, or the total lipid with no information on its individual species. Fortunately, recent technology developments in mass spectrometry (MS) have made the characterization and quantitation of lipids feasible. The growing attention on lipid research is also seen from the initiatives underway of the LIPID Metabolites And

*These authors contributed equally to this work.

Corresponding author. Daniel Figeys, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5. Tel: +(613) 562 5800 ext 8674; Fax: +613-562-5655; E-mail: dfigeys@uottawa.ca.

Weimin Hou is a graduate student in the Chemistry department at the University of Ottawa focusing on lipidomics technologies.

Hu Zhou is a postdoctoral fellow at the Ottawa Institute of Systems Biology of the University of Ottawa focusing on proteomics and lipidomics technologies.

Fred Elisma is a research bioinformatician at the Ottawa Institute of Systems Biology of the University of Ottawa focusing on the development of tools for proteomics and lipidomics.

Steffany A. L. Bennett is an associate professor at the University of Ottawa. She studies lipid mediators of neurodegenerative disease.

Daniel Figeys is a professor in the Canada Research and the director of the Ottawa Institute of Systems Biology of the University of Ottawa focusing on proteomics and lipidomics technologies and application.

Pathways Strategy (LIPID MAPS consortium) [15], and The European Lipidomics Initiative (ELIfe) [16] as well as other emerging tools that other groups are putting in place to study the lipidome.

Although lipidomics is a very promising field of study, certain challenges still remain. First, in contrast with genomics and proteomics, there is no information that can predict the number of individual lipids present in an organism. Second, despite its rapid advancement, current technology still cannot exhaustively map lipidomes; thus, an emphasis on developing mapping techniques is needed. Third, the structural identification of lipids by MS, via the gas phase fragmentations of lipid ions, is complicated; thus, a means to solve this problem is vital for structural identification. Fourth, given the diversity in lipid classes, it is not possible to accommodate all classes with a common method for extraction, chromatography and detection. In the next section, we review recent developments in the field of lipidomics with an emphasis on phospholipids and including technology and informatics, and further discuss the current issues and challenges that need to be addressed.

STRUCTURAL DIVERSITY: A CHALLENGE FOR LIPIDOMICS

The structural diversity of lipids, and the fact that their structure cannot be predicted *a priori*, is a serious challenge to the field of lipidomics. Eukaryotic cells provide an excellent example of the structural complexity of lipids. Lipid can be broadly classified under eight classes: Fatty acyls, sterol lipids, prenol lipids, saccharolipids, polyketides, spingolipids, glycerolipids and glycerophospholipids. Glycerophospholipids and sphingomyelins (SMs) are two common structural classes of phospholipids. Typical glycerophospholipids (Figure 1) consist of a glycerol backbone, a radyl moiety (acyl ester, alkyl ether or vinyl ether) at the *sn-1* (stereospecific number) position, an acyl ester at the *sn-2* position and a polar head group attached to the *sn-3* position through a phosphodiester linkage. The common polar head groups in glycerophospholipids include choline, ethanolamine, inositol, serine and glycerol. Correspondingly, the glycerophospholipid classes are: the phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs), phosphatidylserines (PSs) and phosphatidylglycerols (PGs), with each class containing a different type of polar head group, while phosphatidic acids (PAs) have

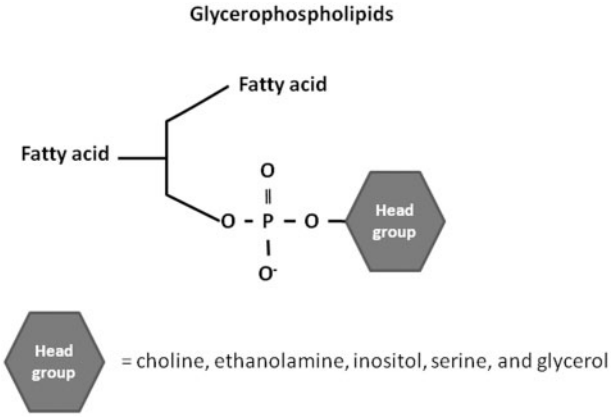

Figure 1: Diagram of the structure of glycerophospholipids, a sub-class of lipids.

a hydrogen rather than a polar head group attached to the phosphate. Lyso variants of glycerophospholipids can also be formed if a radyl moiety is missing at either the *sn-1* or *sn-2* position. PAFs are a special subclass of PCs, with an ether alkyl at the sn-1 position and an acetyl at sn-2 position. SMs are the PC analogues in sphingolipids, which contain a phosphocholine group bonded to a ceramide; the latter consists of sphingosine (2-amino-4-octadecene-1,3-diol) plus a fatty acid linked to sphingosine via an amide linkage. Although the most common lipids in eukaryotes are mentioned above, many other lipid structures exist.

In addition to a variety of polar head groups, phospholipids contain fatty acyl moieties with different chain lengths and different numbers of double bonds on different sites. The identification of a glycerophospholipid involves determining its polar head group, its radyl groups at both sn-1 and sn-2 positions, and, ideally, the locations of (possibly multiple) carbon–carbon double bonds on each radyl moiety. Unfortunately, due to the extraordinary structural diversity of phospholipids, the complete identification of all species in lipid extracts obtained from cell or tissue samples has not yet been accomplished. The full identification of species thus remains a challenging task, especially considering the fact that some lipid species are present in low abundance.

MS-BASED TECHNIQUES IN LIPIDOMIC RESEARCH

MS is a powerful technique for lipid analysis. The main advantages of MS are its abilities to separate and characterize charged ionized analytes in the gas phase

according to their mass-to-charge ratios (*m/z*). It can also provide structural information by fragmenting the lipid ions by collision-induced dissociation (CID). The various techniques used to record these fragmentation reactions are called tandem MS, or MS/MS or MS^n, and are described later in this review. These attributes lead to unparalleled selectivity, sensitivity and the ability to provide structural information for components in complex mixtures.

Typically, a mass spectrometer is composed of three major sections: (i) an ion source; (ii) a mass analyser that measures the *m/z* ratio of the ionized analytes; and (iii) a detector that records the ion signal corresponding to each *m/z* value. For a long time, MS was restricted to analysing small and volatile lipids. In the late 1980s, two 'soft' ionization techniques were developed (no fragmentation in the ionization process itself) for generating ions of intact biomolecules {electrospray ionization (ESI) [17] and matrix-assisted laser desorption/ionization (MALDI) [18]}.

These two techniques allow high mass and non-volatile compounds such as intact lipids to be amenable to mass spectrometric analyses. ESI produces gas-phase ions from molecules in a solution, and is easily directly coupled to liquid chromatography. This technique is currently the most frequently used in lipidomic research. MALDI, on the other hand, allows the production of intact gas-phase ions from samples embedded in a dry, crystalline matrix via laser pulses rather than ionizing directly out of solution. There are several basic types of mass analysers used in lipidomic research, including the Paul ion trap, the linear quadrupole, time of flight, Fourier transform ion cyclotron resonance (FT-ICR-MS) and Orbitrap. These analysers can be used as stand alone instruments, or they can be coupled into complex multistage instruments. The commonly coupled instruments are, the quadrupole-linear ion trap and the quadrupole-time-of-flight and linear ion trap-Orbitrap.

Ion trap mass spectrometer

Ion trap mass spectrometers (both the 3D 'Paul traps' and the newer 2D linear traps) are routinely used in MS laboratories. Ion trap mass spectrometers are typically coupled to HPLC through an ESI interface. The ion trap mass spectrometer can capture or trap ions that accumulate over a user-selected time, and then subject these ions to MS, MS/MS and even MS^n analyses. Ion trap mass spectrometers can also rapidly generate MS and MS/MS spectra. Moreover, ion trap MS is relatively inexpensive, and it provides good sensitivity. Although their use in lipidomics is not widespread, Larsen and co-workers [19] used ion trap mass spectrometers to characterize phospholipids up to MS^4. Ion trap mass spectrometers, however, have several disadvantages for lipidomics research. Low-mass accuracy and low-dynamic ranges often result from the ion trap's limited resolving powers, space charging effects and its low-duty cycle caused from the overhead time required to trap and manipulate ions for MS^n experiments. The development of a 'linear' or a 'two-dimensional ion trap', linear trap quadrupole (LTQ) or linear ion trap (LIT), can partly expand dynamic range and increase resolution [20].

Triple quadrupole

The triple quadrupole mass spectrometer has been the work-horse for studying small molecules mainly because of its high performance for quantitation, its ability to perform precursor ion scans and neutral loss scans and (in MRM mode, see subsequently) its exquisite sensitivity for identifying specific small molecules. Its characteristics are well suited for lipid analysis.

Briefly, a quadrupole is composed of four precisely matched parallel metal rods. Direct-current (dc) and radio-frequency (rf) potentials are applied to these electrodes and produce a high-frequency oscillating electric field; so, the mass separation is accomplished by the oscillating motion of ions in this electric field [21]. Ions of a specific *m/z* value pass through the geometry of quadrupole rods with a set of given dc and rf potentials. Usually, a mass spectrum is obtained by changing both the dc and rf potentials while keeping their ratio constant. In triple quadrupole instruments, three quadrupoles are arranged sequentially. Q1 and Q3 are operated by both dc and rf potentials, whereas Q2 is operated with only the rf potential [21]. The rf-only Q2 allows all ions to pass through, and the Q2 also serves as a total ion containment region and a collision cell. Because ions in the range 0–100 eV can be transmitted through quadrupoles, the MS/MS fragmentations in triple-quadrupole instruments are performed via low-energy fragmentation processes [21]. Triple quadrupole instruments can perform tandem MS/MS experiments as follows: precursor ions of a user-selected *m/z* are transmitted by the first quadrupole (Q1), fragmented via CID in the second quadrupole (collision cell, Q2), and then the resulting product ions are separated in Q3.

There are three scan modes possible for tandem MS using triple quadrupole instruments: (i) a product ion scan done for a selected precursor ion; (ii) a precursor ion scan is done for a user-selected product ion; and (iii) a neutral loss scan is performed to reveal precursors that fragment by ejecting a neutral fragment of molecular mass selected by the user. In addition, multiple reaction monitoring (MRM) records signals arising from fixed (not scanned) user-selected *m/z* values for both precursor and product ions, and provides the most accurate and precise quantitative analyses currently possible. In the product-ion scan mode, Q1 is fixed to transmit only the precursor ions with a user-selected *m/z* value. These precursor ions are then fragmented by CID in the collision cell (Q2), and the resulting product ions are displayed as a spectrum by scanning Q3. Product ion scanning has been previously used for identifying unknown lipids such as SMs [22] and sulfatides [23]. In contrast, the precursor ion scan function of a triple quadrupole can focus on sub-groups of ions carrying specific structural moieties that appear in whole (or in part) in characteristic product ions. In this mode, Q3 is fixed only to transmit specific product ions with a user-selected *m/z* value, and Q1 is scanned to detect all the precursor ions that generate this fragment. Finally, in neutral loss scanning mode, the mass spectrometer again provides a precursor ion spectrum, but this time it corresponds to specific sub-sets of lipids that fragment via loss of a neutral moiety with a user-specified molecular mass. In neutral loss scanning, both Q1 and Q3 are scanned in a synchronized manner with a constant *m/z* difference between the two analysers. Precursor ion and neutral loss scans have been used to detect sub-sets of lipids that contain a specific functional group, such as phospholipids [24–26]. In MRM mode, a series of user-specified precursor-fragment pairs is cycled to the detector by the triple quadrupole. Q1 is fixed to select one precursor ion, and Q3 is also fixed to select the product ion specified for that precursor. MRM has been used for the quantitation of specific lipid molecules that yield known product ions [27, 28]. Although triple quadrupole mass spectrometers are unique in that they can perform product ion, precursor ion and neutral loss scanning, they also have several disadvantages; most notably, low-resolving power and medium mass accuracy and a very low duty cycle for the scanning modes (though not for MRM). The low mass accuracy makes it much more difficult to unambiguously identify the lipid. Furthermore, triple quadrupole mass spectrometer is at a disadvantage for full-scan analysis owing to duty cycle constraints relative to ion traps and time-of-flight (TOF) mass spectrometers. Alternatively, hybrid instruments in which the third quadrupole (Q3) is replaced by a TOF, named QQ-TOF [24, 29] and QTOF, can provide very good mass accuracy and resolving power for product ions, but they cannot perform precursor and neutral loss scans. Finally, a hybrid instrument, based on replacing Q3 with a LIT, named QQ-LIT (or Qtrap), can perform precursor and neutral loss scans as well as multiple-stage MS/MS (MS^n) [30].

Imaging MS by MALDI-TOF in lipidomics

MALDI-TOF MS has become a very promising approach for lipidomics studies, particularly for the imaging of lipids from tissue slides. Briefly, in MALDI-TOF MS, the sample of interest (often a tissue slide) is mixed or coated with a solid matrix (usually a simple aromatic compound) that has a specific absorption spectrum. The sample is introduced into a vacuum chamber, and a pulsed laser that emits light at a wavelength within the absorption range of the matrix is focused on the region of interest. The matrix then absorbs the light from the pulsed laser and is rapidly vaporized. The resulting vaporized plume rapidly expands in the vacuum, and the analytes (e.g. lipids) are carried along. During this process, the lipid acquires a single charge. The lipid ions are then rapidly accelerated over a short distance by applying a strong electric field. All the ions, therefore, achieve an essentially identical kinetic energy, so that ions of different *m/z* (and thus different mass) have different velocities. The ions are then introduced into a TOF mass spectrometer consisting of a long field free flight-tube maintained under sufficiently high vacuum so that no ion collisions with background gas molecules can occur. Because no external forces (e.g. via electric fields) are applied, the lipid ions travel through the flight-tube with the mass-dependent velocities that they acquired during the brief initial acceleration. Therefore, by measuring the time required for the lipid ions to traverse this tube, their *m/z* values can be deduced. In applications devoted to mapping the lipid profiles across a tissue slide, the process is then repeated by moving the laser beam across the slide.

Interestingly, such MALDI-TOF approaches have been used in lipidomics to investigate the spatial

distribution of the levels of lipid species in tissue samples [31–33]. In MALDI imaging MS, the tissue sample is frozen and cut into thin slices (usually 5–14 μm thickness), then placed onto a MALDI sample target and coated with matrix. MALDI imaging is normally performed using a N_2 UV laser (337 nm) [32] for lipids, but infrared (IR) lasers (for example, Nd:YLF, 249 nm) have also been used for analysing phospholipids from rat brain [34]. So far, 2,5-dihydroxybenzoic acid (DHB) has been the most frequently used matrix for lipids [34–39], but other matrices, for instance, 2,4,6-trihydroxyacetophenone (THAP) have also been utilized [40]. Recently, a new matrix coating system, called the oscillating capillary nebulizer (OCN), has been introduced. This system sprays small droplets of matrix aerosol onto the sample surface for improved matrix homogeneity, and it reduces the matrix crystal size and controls solvent effects [35]. Most papers to date have used MALDI-TOF mass spectrometer for lipid imaging [35, 37, 40–43]. However, hybrid TOF instruments, such as TOF-TOF [44], QIT-TOF [39] and ion mobility-TOF [34, 37, 38] have also been utilized in some instances. Secondary ion MS (SIMS)-TOF is another powerful method for imaging lipid distribution. This technique does not require matrix, and instead of laser irradiation, it uses high-energy particle bombardment with a continuous beam of highly focused, energetic ion such as Bi_3^+ and Ga^+ [31, 45, 46]. Methods to couple MALDI-TOF MS and thin-layer chromatography (TLC) for lipid analysis were developed by several research groups [47–53]. We expect that MALDI imaging (using TOF or hybrid mass spectrometers) will be an increasingly important tool for studying the lipidome in view of its ability for rapid screening of lipid distributions in tissue slides. The detailed identification of the lipids and the sensitivity of the approach, however, still need to be addressed.

High resolution and high mass accuracy mass spectrometers

The accurate and precise measurement of a lipid's molecular mass helps to unambiguously identify the lipid. To date, the Fourier transform mass spectrometer (FTMS) provides the highest resolution and mass accuracy available from commercial instruments. FTMS is able to produce high resolutions and mass accuracies because of its ability to trap ions in a strong magnetic field under very high vacuum. Furthermore, it is easily coupled with HPLC-ESI making it suitable for analysing lipid mixtures. However, FTMS instruments are costly, and their biggest disadvantage lies in the difficulty of its operation and maintenance. The magnetic field, for example, is achieved through a superconducting magnet that requires continuous cooling by liquid helium, and the trap only functions when a very high vacuum is maintained. Therefore, time, cost and manual labour are considerations for this method of lipid analysis.

The Orbitrap is a new type of commercial mass spectrometer based on an oscillating electric field. The common characteristics between the FT and the Orbitrap mass spectrometers are high mass accuracy and resolving power. The Orbitrap, however, is less expensive and much easier to operate than the FT-MS. The hybrid instruments, such as LIT-FT and LIT-Orbitrap, can yield high-quality mass spectra of lipids and have been used for lipidomic identification and quantification [54, 55]. Bruker recently introduced the MaXis, a high resolution TOF instrument with a sub-p.p.m. mass accuracy. It is likely that the current versions of Orbitraps and other high-resolution mass spectrometers such as the MaXis will see widespread application instead of the FTMS in biological laboratories because of their lower cost and ease of use.

Identification of phospholipids by ESI-MS/MS

An extensive network of proteomic laboratories already exists around the world. These research groups could easily participate in lipid research because many of the techniques used for small molecules and proteomics are readily applicable to lipidomics. The coupling of HPLC with ESI-MS/MS, for example, is the most commonly used technique for lipidomic analysis [56–58].

Lipidomics also has its own peculiarities. Phospholipids are sensitive to oxidation, light and enzymes such as lipases. Care must therefore be taken for the proper handling and storage of the samples prior and during their analyses. In proteomics, it is not unusual to use an autosampler and load 96 samples. This technique, however, is not appropriate for lipidomics studies because it leads to lipid degradation over time. Also, during the ESI process, phospholipids can either acquire positive or negative charges that allow them to be studied in positive or negative ESI-MS/MS. Some phospholipids will

not acquire a charge by ESI and therefore cannot be analysed by this technique. Furthermore, lipids predominantly acquire only a single charge which simplifies the interpretation of the MS and MS/MS spectra.

Information on the molecular weight of each lipid species can be determined from a survey scan. The greater the mass accuracy, the more accurate is the prediction of the atomic composition of the lipid. In a second stage, an individual lipid can be subjected to CID. The fragmentation patterns generally contain information that can help elucidate the structure of the lipids such as the polar head group and fatty acyl moieties.

Precursor ion and neutral loss scans are also useful for detecting moieties that are characteristic of lipid sub-classes. PCs, lysophosphatidylcholine (LPC), PEs, PSs and SMs can be detected in positive ESI-MS/MS [59–63]. The fragmentation of protonated PC, LPC, and SM ions yield a peak at $m/z = 184$ which is the diagnostic fragment for the phosphocholine head group. A precursor ion scan of $m/z = 184$ would therefore highlight the PC containing lipids out of all the lipids present. Of further interest, $[M+H]^+$ ions of PCs appear at even m/z values (the closest integer to accurate mass), while protonated SMs exhibit odd m/z values [59, 60]. The fragmentation of protonated PE yields a peak at [M+H–141]+, which corresponds to the neutral loss of the polar head group (PE). Similarly, PS ions ($[M+H]^+$) can be identified by a peak at [M+H–185]+ in a tandem mass spectrum, arising from the loss of the polar head group (phosphoserine).

Overall, strategies that combine survey and precursor ion scans ($m/z = 184$) with neutral loss scans (−141 and −185) can help to unambiguously determine the atomic composition and head groups present in a lipid. Although positive ESI-MS/MS has been widely used for lipid studies, one of its limitations is that little information pertaining to the two fatty acyl constituents can be obtained from the fragmentation pattern. Alternatively, instead of protonation, negative ESI-MS/MS and positive ESI-MS/MS with metal ion adduction have been employed to elucidate the structures of lipid species.

PEs, PIs, PSs, PGs, PAs and their lyso variants can all be detected by negative ESI-MS [59–62, 64–68]. In comparison to positive ESI tandem MS, negative ESI tandem MS can generate fragmentation patterns with a wealth of structural information on these phospholipid species, including fragments which correspond to polar head groups and fatty acyl constituents. Fragmentation of PE anions ($[M-H]^-$) generates a fragment at $m/z = 196$, relating to the dehydrated glycerol phosphoethanolamine head group. When deprotonated PIs ($[M-H]^-$) are subjected to CID, a wide variety of product ions can be observed along with a peak at $m/z = 241$. These results correspond to the dehydrated PI head group. Whereas, PS anions ($[M-H]^-$) can be identified by observing a peak at [M+H–87], and these anions correspond to the neutral loss of the serine head group. The fragment anions contain useful information on their polar head groups. More importantly, however, negative ESI-MS/MS generated fragment anions that correspond to the two fatty acyl moieties ($R1COO^-/R2COO^-$) for the glycerophospholipids ionized in negative mode. Fragments corresponding to the lyso variants of these glycerophospholipids ($[M-H-Y-RCOOH]^-$/$[M-H-Y-RCH=C=O]^-$), (resulted from the loss of one fatty acyl group) can also be detected. These fragments provide critical information for characterizing the fatty acyl constituents at the *sn*-1/*sn*-2 positions. Therefore, negative ESI-MS/MS is a very powerful technique for elucidating the structures of glycerophospholipids.

The fragmentation of lipids can be modified by changing the counter ion present in solution. In particular, metal ions have been commonly used for adducting phospholipids which include Li+, Na+ and K+ [22, 62, 69, 70]. Reports show that other metal ions such as Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} can be used to adduct phospholipids [71]. The fragmentation pattern of glycerophospholipids that form adducts with certain metal ions is much richer than the fragmentation of the corresponding protonated ions in positive ESI-MS/MS. The fragmentation of sodiated PCs and SMs both generated a sodiated five-member cyclophosphane at $m/z = 147$ and a product ion the results of the loss of trimethylamine at $[M+Na-59]^+$. Both fragments are the diagnostic fragments of the phosphocholine head group. Of notable importance, product ions arising from the two fatty acyl moieties, e.g. [M+Met–R1COOH]+ and [M+Met–R2COOH]+, can be generated from these metal adducted glycerophospholipids ions. Phospholipids adducted with different metal ions normally generate different fragmentation patterns from one another. The complementary structural information obtained through ionization via metal ion adduction, especially structural

information on the two acyl constituents, could be used to unambiguously identify the lipid species of interest. Chloride adducts have also been reported for the identification of PCs in negative ESI-MS/MS, where the product ions corresponding to the two fatty acyl moieties have been generated [72].

It is apparent that a series of strategies that combine survey MS scans with product ion and precursor ion scans can be employed for studying the lipidome. These combined strategies rely on the use of different types of mass spectrometers. Although each type of mass spectrometer has its strengths and limitations in terms of scanning capabilities, resolution, mass accuracy, dynamic range and sensitivity, promising results from studies show that lipidomics would clearly benefit from more research on how to extract structural information by MS.

Characterization of fatty acyl constituents

Phospholipids generate fragments that are characteristic of their polar head groups when they are subjected to CID fragmentation. Therefore, the polar head groups of glycerophospholipids are relatively easy to identify. The biggest challenge in identifying a diacyl-glycerophospholipid with mass spectrometric techniques is the characterization of the two fatty acyl moieties attached at the *sn*-1/*sn*-2 positions on the glycerol backbone (Figure 2). At least three pieces of information pertaining to the fatty acyl groups are needed to identify a glycerophospholipid: (i) the total lengths of the two fatty moieties and the total number of unsaturated carbon double bonds on the acyl moieties; (ii) the assignment of the two fatty acyl moieties to the sn-1 or sn-2 position; and (iii) the localization of polyunsaturated carbon double bonds on each acyl moiety.

The molecular mass of a phospholipid can be determined by MS with different accuracy and precision depending on the nature of the instrument used, but the mass is at least always correct to the nearest integer. Provided that the polar head group of the lipid species is identified (as discussed above), then the total length of the two fatty acyl chains and the total number of double bonds can be determined. However, a number of combinations of different fatty acyl moieties with different numbers of unsaturated double bonds can all add up to the same molecular mass. Therefore, the length of each fatty acyl chain and the number of double bonds on each moiety also need to be determined. Using negative ESI-MS/MS,

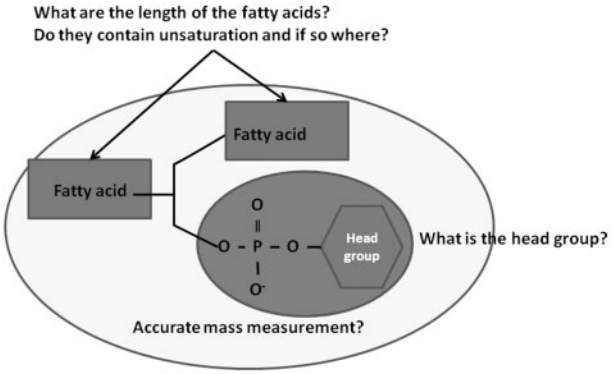

Figure 2: Characterization of lipids that can be achieved (partially or fully) by mass spectrometry. The accurate mass allows the unambiguous atomic composition determination, the head groups can be determined by precursor and neutral loss scans, the lengths and degrees of unsaturation of the side chains can be determined through positive ionization with metal ion adduction and MS/MS or by negative ionization, and the location in the acyl chains of unsaturation can be achieved using OzESI-MS.

a variety of peaks can be observed which include the two carboxylate anions ($R1COO^-/R2COO^-$), and the two carboxylate anions (R_1COO^-/R_2COO^-) and the fragments produced from the loss of one of the fatty acyl moieties ($[M-H-Y-RCOOH]^-$/$[M-H-Y-RCH=C=O]^-$). These fragments provide critical information about the mass of the two fatty acyl constituents and allow the length of each fatty acyl moiety and the total number of double bonds on each acyl chain to be unambiguously determined [65–68, 73]. Some of the metal adducted glycerophospholipid ions that form in positive ESI-MS/MS generate product ions like $[M+Met-R_1COOH]^+/[M+Met-R_2COOH]^+$ that can also be used to determine the structures of the two fatty acyl moieties [71].

The feasibility of using MS to identify the positions of the two fatty acyl substituents on the glycerophospholipid backbone is still under debate. For instance, deprotonated diacyl-glycerophospholipids, PEs, PGs and PCs, were found to have a higher tendency for losing the fatty acyl moiety at the *sn*-2 than at the *sn*-1 position; thus they produce a higher abundance of $R2COO^-$ anions than $R1COO^-$ anions in a tandem mass spectrum [64, 65, 68, 74]. Some researchers have proposed to determine the positions of the two fatty acyl chains for phospholipid species on the basis of the abundance ratio of $R2COO^-$ and $R1COO^-$ anions. However, these

abundance ratio results from the fragmentation of deprotonated PIs and are affected by the collision energy applied [67]. Another attempt to identify the positions was made using positive ESI-MS/MS of glycerophospholipids adducted with certain metal ions [70, 71]. Studies showed that relative intensities of the peaks corresponding to the loss of the fatty acyl moiety at *sn*-1 position ($[M+Met\text{-}R_1COOH]^+$) and at *sn*-2 position ($[M+Met-R_2COOH]^+$), respectively, could help determine the positions of the two fatty acyl chains [70, 71, 75]. However, because the chain length, the number of unsaturated double bonds of the fatty acyl moieties, and the applied collision energy all play a role in the efficiency of the cleavage of fatty acyl moieties [67], the broad applicability of this type of approach is still under debate.

Another technical challenge in lipidomics is to develop a method for locating the unsaturation and polyunsaturation sites in fatty acyl chains. Recently, a novel approach was reported that used ozone electrospray MS (OzESI-MS) to distinguish lipid species that differed only in the sites of double bonds. In this method, the polyunsaturated double bonds are cleaved by ozonolysis during ESI, resulting in a pair of fragments diagnostic of the positions for double bonds [76, 77]. The accurate location of double bonds in lipids still remains a challenge for lipidomics. Therefore, more research is needed to solve this problem.

Lipid separation

Lipid extracts can be analysed directly by electrospray MS without front end separation [59, 60, 72, 78]. However, direct infusion is limited by ionization suppression of lipid species that have low ionization efficiencies, especially if they are present in low abundance in lipid extracts [79, 80]. Also, a single peak in a survey scan can represent several isobaric lipid species [59]. Moreover, CID of precursor ions can generate a mixture of fragmentation patterns of several lipid species making the identification process extremely difficult or impossible. Consequently, only a few lipid species can be identified by direct infusion ESI-MS.

Instead, most reports on ESI-MS of lipids rely on online or offline separation of lipids. The common techniques used for lipid separation include TLC and normal phase (NP) and reversed phase (RP) liquid chromatographies (LC) [61, 73]. TLC and NP LC generally separate phospholipids based on the polarity of their head groups; whereas, RP liquid chromatography (RP-HPLC) separates phospholipids based on the hydrophobicity of their fatty acyl chains. Typically in RP-HPLC separation, the order of elution in a class of lipids is related to the length of the fatty acyl chain, such that lipids with the shorter fatty acyl chains elute faster than those with longer ones. Furthermore, the higher the number of double bonds, the faster the lipid elutes in comparison to the saturated form. Phospholipids with the same molecular mass but with different pairs of fatty acyl chains normally cause problems for direct infusion ESI-MS/MS. Nonetheless, these problems are resolved by applying RP-HPLC using a C_{30}-derivatized silica column so that the phospholipids can be unambiguously identified [73]. Despite the concerns of potential sample loss during LC separation (NP and RP) and oxidation during TLC separation [81], ESI-MS/MS integrated with frontend HPLC separation has become the method of choice in MS-based lipidomics. For example, the recent introduction of ultra-high performance LC (UHPLC), which provides enhanced peak capacity to lipidomics (with peak width down to 3 s), promises to greatly improve the separation of complex lipidomic samples [29].

Lipid quantification

In infusion ESI-MS and in the absence of ionization suppression effects, the mass spectrometric peak intensity is proportional to the concentration of a lipid species in a mixture. In contrast, in LC-ESI-MS, the chromatographic peak area is proportional to the amount of a lipid species in a complex mixture. Therefore, mass spectrometric techniques can identify lipids as well as measure the abundance of each lipid species in a complex mixture [59, 62, 72, 82, 83]. It should be kept in mind that different lipid species usually have different mass spectrometric responses [80]. Furthermore, the instrumental responses are significantly different for different class of phospholipids. In a mass spectrum, the relative signal intensities of the ions of different lipid species do not directly represent their molar abundances. A lipid species with a higher peak intensity than that of another could actually be present in a lower concentration. Therefore, it is not feasible to use MS directly to estimate the relative abundance of lipids belonging to different phospholipid classes, because of the great difference in the ionization efficiency pertaining to their polar head groups [81]. Estimations of the relative abundances of lipid species

within a specific phospholipid class is less problematic if the responses of all the lipid species are properly normalized with respect to the appropriate internal standards added to the lipid mixture to be analysed [81, 84, 85].

Absolute quantification of a specific lipid species relies on the availability of a (possibly synthetic) lipid standard. An isotopically labelled version of the target lipid is preferable, although an unlabelled lipid with acyl groups not observed in nature can also be used. As a result of difficulties in obtaining suitable internal standards, absolute quantification of lipid species is usually applicable to a limited number of targeted lipids in a complex mixture [63, 82]. The feasibility of using isotopically labelled lipid standards for quantification is based on the assumption that the isotope-labelled lipid standard and its normal counterpart have identical physiochemical properties. They should, therefore, have identical response in mass spectrometers.

Recently, there has been increasing interests in relative quantitation of lipid species in different lipidomes, for example between wild and mutant, on basal and stimulated cells, different cell populations and on disease and normal samples [24, 60]. Generally, this approach requires the comparison of multiple HPLC-ESI-MS/MS analyses. Extraction and analytical processes can cause fluctuations in signal, which can be an issue for each sample individually processed. Fortunately, non-naturally occuring lipid standard can be spiked into all the samples at different stages of the sample processing, which facilitates the correction of the signal intensity. When the analytical strategy includes both positive and negative ESI-MS, a standard lipid species that is detected in both positive and negative modes (PEs and PSs), can be chosen as an internal standard for normalization [60].

Metabolic labelling of lipids with stable isotopes has been reported in quantitative lipidomics [24, 79, 84]. Appropriate, stable isotope labelled chemicals can be introduced into the growth medium for cell culture. Depending on the metabolic pathway, either all or a fraction of the lipids can be labelled with stable isotopes. For example, Ekroos *et al*. [24] cultured cells with ^{13}C-labelled glucose for 24 h. The lipid extracts from these cells were a mixture of isotopically labelled endogenous lipids, which were used as a comprehensive internal standard for quantitative profiling of phospholipids [24]. DeLong *et al*. [84] introduced D4-ethanolamine and D9-choline chloride into cell culture medium to distinguish the metabolic product PCs from different metabolic pathways. Wenk *et al*. [79] metabolically labelled mouse neuronal cells with [^{3}H]inositol to profile intracellular signalling lipids phosphoinositides. Undoubtedly, isotopic labelling is also a promising approach for the relative quantitation of lipidome.

Neutral lipids

All of the techniques described above deal with lipids that can either readily acquire a negative or positive charge. Unfortunately, many lipids have structures that do not readily permit the acquisition of a charge by ionization techniques such as ESI and MALDI. One solution for these lipids is to use atmospheric pressure chemical ionization (APCI) interfaces [86, 87]. This interface is also compatible with the online separation of lipids by HPLC. Most models of APCI interfaces rely on the generation of a corona discharge to create an environment appropriate for gas phase chemistry, which then leads to the generation of charged analytes compatible with MS. The basic design of an APCI interface consists of a capillary tubing for the transfer of the HPLC effluent. This capillary is surrounded by a concentric nebulizing gas, which then leads to the generation of a spray through a nozzle. The desolvation is often accelerated using a heated vaporizer tube that surrounds the HPLC capillary exit. A needle is present for the generation of a corona discharge, which then leads to the generation of ions from the molecular species present in the spray from the nozzle. The generated ions are then guided to the entrance of the mass spectrometer for analysis. Although, APCI has been used for specific lipid analysis, its application to global lipidomic studies is more recent [88]. As well, a variant of APCI termed electron-capture APCI (ECAPCI)/MS has also been recently used for lipidomic studies [89, 90].

NON-MS BASED TECHNIQUES IN LIPIDOMIC RESEARCH

Nuclear magnetic resonance

Although the field of lipidomics is dominated by MS, other techniques have also been introduced. In the early 1990s, Adosraku *et al*. [91] used proton nuclear magnetic resonance (NMR) to analyse lipid profiles of human erythrocytes. High resolution ^{31}P-NMR spectroscopy was also applied to

characterize phospholipid composition of tissues and body fluids [92]. Recently, a new approach named *two-dimensional ^{1}H-^{13}C heteronuclear single quantum coherence NMR* (2D HSQC NMR) was developed for global lipid profiling of mycobacteria [93]. Both ^{1}H and ^{31}P NMRs can directly analyse non-destructive lipid; however, because of their limited sensitivity relative to MS, they are only applied for very abundant lipids, such as cholesterol and phosphocholine [58].

BIOINFORMATICS FOR LIPIDOMICS

Lipids classification and databases

Due to the lack of a universally accepted lipid classification scheme, databases have been created which differ in their scope and organization. There are three particular databases, namely, LipidBank [Yasugi, 2002, 12058481; Watanabe, 2000], LIPIDAT [Caffrey, 1992, 1315624] and LMSD [Sud, 2007, 17098933]. These databases provide the user with a wide range of information about lipids. More information about these databases is found in Table 1, which lists several online resources that are available for lipids.

Recently, a US-based consortium called LIPID MAPS has proposed a classification schemes for lipids which classifies them into eight categories: (i) fatty acyls; (ii) glycerolipids; (iii) glycerophospholipids; (iv) sphingolipids; (v) sterol lipids; (vi) prenol lipids; (vii) saccharolipids; and (viii) polyketides [15]. Their proposition also includes a unique α-numeric 12-character lipid identifier, which provides information on the source database and the lipid category, class, subclass and number. This system allows a total of 1.68 million possible lipid identifications. The advantage of the LIPID MAPS system is its amenability to database storage and retrieval as well as bioinformatics manageability. The LIPID MAPS database contains information on more than 10 000 lipid structures, obtained from four major sources: (i) LipidBank and LIPIDAT that were manually curated; (ii) the LIPID MAPS consortium's core laboratories and their partners; (iii) lipids identified by LIPID MAPS experiments; and (iv) from computationally generated structures for appropriate lipid classes [94].

Lipid analysis software

The structures of large and complex lipids are difficult to draw. In the late 1980s, David Weininger [95] addressed this issue by initiating the Simplified Molecular Line Entry Specification (SMILES) project. The SMILES format represents the lipid structure as a compact graph with nodes as atoms and edges as bonds. However, the SMILES format does not include 2D coordinates, which makes the visual recognition and comparison difficult. In order to resolve this problem, the LIPID MAPS consortium developed multiple drawing programs and MS prediction tools which are coupled with a drawing tool [94]. The LIPID MAPS website contains at the moment six drawing programs which can

Table 1: Online resources for lipid classification and databases

Resource	URL	Country	Comments
LIPID MAPS	http://www.lipidmaps.org/	USA	Lipid classification scheme
LipidBank	http://lipidbank.jp/	Japan	Classify the lipid in 17 categories covering a wide variety of animal and plant
LIPIDAT	http://www.lipidat.ul.ie/	USA	A relational database of thermodynamic and associated information on lipid mesophase and crystal polymorphic transitions
LMSD	http://www.lipidmaps.org/data/structure/index.html	USA	Composed of structures and annotations of biologically relevant lipids
LMPD	http://www.lipidmaps.org/data/proteome/index.html	USA	Lipid-associated protein sequence with annotations from different sources (KEGG, UniProt, etc.)
Cyberlipid Center	http://www.cyberlipid.org/	France	Lipid database that includes isoprenoid-derived molecules
SphinGOMAP	http://sphingolab.biology.gatech.edu/	USA	Pathway map for sphingolipid biosynthesis
Lipid Library	http://www.lipidlibrary.co.uk/	UK	Composed of information about lipid chemistry, biology and analysis
KEGG	http://www.genome.jp/kegg/pathway.html	Japan	Manually drawn pathway maps that included fatty acid biosynthesis and degradation, sterol metabolism and phospholipids pathway
GOLD	http://gold.uni-graz.at/index.html	Austria	Composed of annotated pathway and curated data set

accurately depict the following lipid structures: fatty acyls, glycerolipids, glycerophospholipids, cardiolipins, sphingolipids and sterols. The layout of a LIPID MAPS drawing tool consists of a 'core' structure and several pull-down menus. Each pull-down menu allows the end-user to choose from a list of head groups and sn1 and sn2 acyl side chains. These lists represent the most commonly found molecules in mammalian cells. After specifying the details of the desired structure, the lipid is rendered in the web browser as a Java-based MarvinView applet. In addition, the end-user may visualize the structure with the Chemdraw ActiveX/Plugin.

Over the past years, different methods and programs have been developed for processing and identifying lipids from MS data. We can sort these programs into three categories: (i) programs that are available at no cost; (ii) open source programs; and (iii) programs available for purchase from private companies. One of the programs in the first category is called LipidNavigator (http://lipidsearch.jp/LipidNavigator.htm), which was developed by Mitsui Knowledge Industry in collaboration with the Taguchi laboratory at the University of Tokyo. LipidNavigator is a high-throughput web tool and automated system for phospholipids identification that used as an input various types of MS raw data.

Another program in the first category, called *TriglyAPCI*, was developed by Cvacka *et al*. [96] for interpreting APCI-MS of triglycerides. This software was developed using Microsoft Visual basic 6.0 and it works by first identifying each ion in the spectrum obtained from a LC/MS analysis as a fragment or a molecular adduct, then it searches for a relationship among the compounds and suggests possible triacylglycerol (TAG) structures.

Another team in Nashville under the supervision of Dr H. Alex Brown developed software for analysing large amount of data obtained by MS [97]. Their software makes use of the S-Plus version 3.3 for Windows programming suite. This software uses algorithms for data normalization, to statistically compare the different spectral patterns from different replicates. To achieve this information, the software sorts the data by converting the intensities to standard units (-1, 0, $+1$) and ranks them. In the next phase of the analysis, a Shewhart control chart is constructed for each peak to ensure that the analysis remains stable over a particular time period. In this way the software can compare the data obtained under different conditions. These computational analyses, therefore, allow the construction of lipid arrays that highlight the changes in lipids under different biological conditions.

In the open source category, we can find software such as SECD (Spectrum Extraction from Chromatographic Data) and LIMSA (LIpid Mass Spectrum Analysis) that can process both positive and negative ion mode data as well as perform lipid identification based on MS/MS spectra [98]. SECD was developed for the extraction of LC-MS data and used the NetCDF format for the input data. SECD displays the results as a pseudo-3D map where the retention time is the x-axis, m/z is the y-axis, and a grey scale represents intensity. Moreover, SECD software allows users to select a sub-region of the chromatogram. LIMSA is a dynamic library which can be used alone for batch processing; however, it can also be used in conjunction with other software, as it can take output from SECD as its input data. This software implements functionalities such as identification, deconvolution and quantification of lipids.

In addition, Katajamaa *et al*. [99, 100] have recently introduced a Java-based toolbox, platform-independent software called Mzmine. This data processing and graphing software implement algorithms for spectral filtering, peak picking detection, 2D plot visualization, alignment and normalization.

The last category (commercially available software) includes a software developed by MDS Sciex called Lipid Profiler that has been used in several lipidomics studies [101–103]. When combined with Analyst, (also developed by MDS Sciex), the software implements algorithms for isotope correction, identification and quantification of lipid species detected by multiple precursor ion scanning (MPIS) [24, 101].

CONCLUSIONS

Lipidomics, a sub-set of metabolomics, is nascent but has already shown promising discoveries. Currently, it is primarily focused on technological development, the identification and quantitation of lipids, lipidomic imaging of tissue slides and the development of bioinformatic tools. We expect other branches of lipidomics to form and new technologies to be developed in a similar way that proteomics emerged in its early days. We also expect that functional lipidomics will be a key component of the lipidomics field. For example, lipid microarrays that immobilize lipids onto a chip have been developed

to probe lipid–protein interactions in functional lipidomics [104]. Alternatively, proteins can also be immunopurified and the associated lipids analysed using lipidomic approaches. Lipids interact with protein, RNA and other biomolecules, and hence, the understanding of the lipid interactome will be vital for better understanding their roles in cells and diseases.

Key Points

- MS is central to the field of lipidomics.
- Novel approaches based on HPLC-ESI-MS/MS permit the quantitation of complex lipid mixtures.
- Novel approaches based on MALDI-MS imaging allow the imaging of lipid profiles in tissue sections.
- The identification and characterization of unsaturation of lipids are possible, but challenges still remain.
- The applicability of lipidomics to human disease is broad including: brain injury, cancer, inflammation, fertility and others.

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