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Lipidomics: a mass spectrometry based, systems level analysis of cellular lipids

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Abstract

Lipidomics is a logical outcome of the history and traditions of lipid biochemistry and advances in mass spectrometry are at the heart of a renaissance in understanding the roles of lipids in cellular functions. Our desire to understand the complexity of lipids in biology has led to new techniques that allow us to identify over 1000 phospholipids in mammalian cell types and tissues. Improvements in chromatographic separation and mass spectrometry have positioned us to determine not only the lipid composition (i.e., parts list) of cells and tissues, but also address questions regarding lipid substrates and products that previously overwhelmed traditional analytical technologies. In the decade since lipidomics was conceived much of the efforts have been on new methodologies, development of computer programs to decipher the gigabytes of raw data, and struggling with the highly variable nature of biological systems where absolute quantities of a given metabolite may be less important than its relative change in concentration. It is clear that the technology is now sufficiently developed to address fundamental questions about the roles of lipids in cellular signaling and metabolic pathways.

Introduction

Genomic and proteomic innovations revealed the need to explore metabolic processes at the system level and lead inevitably to the development of lipidomics. Our laboratory initiated efforts to develop a lipidomics platform in the late 1990s. A driving force was the recognition that cells generate phosphatidic acid, a lipid second messenger, via multiple pathways. Thin layer chromatography and high performance liquid chromatography (HPLC) were proving insufficient to adequately address questions of sources of lipid molecular species. The focus of the work was to integrate changes in cellular lipids into the larger network of cell surface receptor signaling pathways. Much of the early efforts were designed to define how pattern changes in cellular lipids influenced the cellular response to G protein coupled receptor activation. Thus, lipidomics began with a focus on identifying lipid species that act as cellular messengers and how these molecules integrate signaling and metabolic processes of cells.

As originally conceived *computational lipidomics* was a mass spectrometry based profiling approach that includes the resolution, detection, and identification of lipid species [1,2]. However, it was intended to be more than comprehensive lipid analysis and to include a systems-biology approach to the study of lipids, their interaction with other molecules, their

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cellular functions, and determination of pattern changes in membrane lipid composition following signal transduction events or other important biological processes [3–5]. Characterization of lipid species by MS has evolved with advancements in instrumentation and technology. The variety of ionization methods used in the current instrumentation has the ability to generate gas phase ions from nonvolatile samples and has expanded the capabilities for detection and analysis of a wide range of lipids of all sizes and structures, described in recent reviews [6,7]. The many facets of lipidomics reflect both the diversity of lipid species in biology and the plethora of functions mediated by lipids in physiology and disease. Owing to lipidomics technology a precise phospholipid composition of *E. coli* was recently reported [8], the critical role of lipids in HIV replication was unveiled [9], and the spatial and temporal differences in phospholipid composition during embryo implantation were revealed [10]. Using lipidomics technology to examine phospholipid composition of liver extract in a hypercholesterolemia study potential biomarkers were recently identified [11]. Other uses that further illustrate the diversity of applications include differentiating roles of two diacylglycerol kinase isoenzymes in lipid metabolism [12], defining lipid changes in brain regions of a mouse model of Parkinson's disease [13] and use of lipid MS as a screen for development of inhibitors of phospholipases [14].

Mass spectrometric techniques for glycerophospholipid identification and quantitation

The two predominant methods for phospholipid identification and quantitation are shotgun lipidomics and LC/MS. These approaches have distinct strengths and weaknesses, but can be used most effectively in combination.

Identification of lipids by collision-induced dissociation

Tandem mass spectrometry (MS/MS or MS^2) is an essential tool in the identification of glycerophospholipids. In excess of 1000 phospholipids are present in mammalian cell types. This complexity leads to isobaric inter-class species (i.e. 34:0 PC and 34:1 PS in positive-ion mode), which are inseparable by direct infusion MS analysis. An even more complicated situation arises when samples have intra-class isobaric compounds (i.e. 38:4 PI, which can be composed of 18:0/20:4, 18:1/20:3, or 16:0/22:4 fatty acid combinations, to name a few examples). Mixtures of isobaric species such as these are extremely difficult to separate by any MS^1 chromatographic technique.

Unambiguous structural characterization of the glycerophospholipids requires an understanding of the fragmentation processes involved in each lipid type. Hsu and Turk have recently completed an in-depth study of the major phospholipid classes [15–22]. Product ions arising from both positive and negative-ion mode fragmentatio n processes have been investigated yielding a wealth of information on fatty acid, lysolipid and headgroup-related fragments expected for each lipid type. In addition to the predominant diacyl phospholipids, fragmentation processes for ether- and vinyl ether-containing phospholipids have also been reported [21]. When chemically pure samples are analyzed, the *sn*-1 and *sn*-2 fatty acid composition can be determined following analysis of the lysolipid fragment ratios.

Shotgun lipidomics

Important innovations in ESI intrasource separation of lipids by direct infusion MS without prior chromatographic separation was described by Han and Gross over the last several years [23–27]. This approach, now termed "shotgun lipidomics", has gained popularity [28–31]. Cell extracts are analyzed by direct infusion MS using precursor ion scans (PIS) and neutral loss scans (NL) to identify key lipid fragments. Using this method, lipid class (headgroup identification) is accomplished using PIS and/or NL scans in positive- and/or negative-ion

modes (see Table 1). The fatty acid content of individual lipids is then identified by PIS analysis in negative-ion mode. For example, 38:4 PI (18:0/20:4 PI) would be identified by a precursor ion scan of 241 *m/z* in negative ion mode (PI headgroup) as well and PIS scans of 283 *m/z* (18:0 FA) and 303 *m/z* (20:4 FA). In its current form, shotgun lipidomics now widely utilizes automated nanospray techniques. This facilitates extended analysis of low volume samples that would not be practical using other analytical methods. Overall, this technique is excellent for identifying the major pools of phospholipids (ca. 90% of the total phospholipid pool by mass). However, this technique in its current form is not ideal for identifying trace level phospholipids and there are significant limitations in the ability to achieve absolute quantitation except for the most abundant species.

HPLC/MS lipid identification and quantitation

The application of ESI-MS as a soft ionization technique, originally developed for macromolecules [32], was an important breakthrough in the analysis of glycerophospholipids. Although shotgun or direct infusion mass spectrometry offers some advantages for analysis of phospholipids from complex mixtures there are limitations in its use. The presence of isobaric species, ion suppression, and exact lipid identification requires a different analytical approach. Some of these problems can be solved by interfacing HPLC with on-line ESI-MS. Initial separation of phospholipids by class can be achieved by normal phase LC/MS [33–36] resulting in less ion suppression, high ionization yield and increased sensitivity for minor components. A gradient as well as isocratic elution can be applied [35,36]. An important factor for lipid quantitation is the use of internal standards which have a similar instrumental response to the one of the analytes since it depends on the head group chemistry, acyl chain length and degree of unsaturation. [37]. The use of several standards per class ensures greater number of minor species identified in a complex lipid extracts as this relaxes the requirement for low lipid concentrations needed for linearity [35,37].

A combination of reverse phase HPLC and MS allows detailed analysis of individual molecular species with a high precision in a focused approach applicable for some limited categories of molecules, including polyphosphoinositides [38–41]. Another useful technique based on LC/MS is the focused analyses of specific groups of phospholipids by way of employing headgroup specific scans [39,40].

The practical results from comprehensive lipidomics profiling of different cells or tissues is the discovery of novel lipid species previously not identified. The strategy for MS based novel lipid identification and characterization is presented in Fig. 1. Based on this strategy we have recently identified a unique ether phosphatidylinositol species during lipidomics profiling of human cirrhotic liver (unpublished data). The application of lipidomics profiling have led to discovery of N-acyl phosphatidylserine in mouse brain [42,43] and n-acyl phosphatidylethanolamine and phosphatidylserylglutamate in *E. coli* [44,45].

Issues in mass spectrometry-based data analysis and quantification

Independent of the analytical method used, the rate limiting step in lipidomics is still the data analysis that can impede the screen of large sets of samples. Nonetheless, the *sine qua non* of scientific research is quantitation, and state-of-the-art lipid data analysis has been greatly transformed by the rise of algorithms, tools, and standards for use in quantification. Generally, this is accomplished through the judicious use of ESI-LC/MS for high quality separation of lipid extractions along gradients that allow for area under curve (AUC) peak integrations, or by use of headgroup-specific mass scanning techniques. The workflow in a typical quantitative analysis system is summarized in Figure 2.

There are several complications that must be dealt with in order to accomplish a comprehensive lipidomics analysis. Readers are referred to a detailed overview of the data analysis methodologies currently in use in the field [46], though several challenges for future improvements exist. Here, we focus on a few common features of state-of-the-art quantification systems currently in use. At the beginning of the data analysis pipeline, more than one strategy exists for automated ESI-MS/MS identification [30,47], but none is ideal. Once full- or headgroup-specific-scans by ESI-MS have been acquired, visualization of the raw data is a critical step to ascertain quality control, to error check AUC integrations, which may often be semi-automated, and to ensure that physical separation on LC columns has been accomplished as intended. Instrument manufacturer software is currently inadequate, and eve n open source solutions [48] may require considerable customization for use with complex lipid mixtures. This is followed by the use of background baseline corrections and signal-to- noise criteria in most analysis systems [e.g., 48]. As the ionization efficiency varies across carbon number, degrees of unsaturation and headgroup composition under normal ESI MS conditions, robust quantitation methods take this factor into account. When appropriate (e.g., for lipid classes with large heterogeneity of ionization efficiency), multiple internal standards per class are typically used [35,37]. Particularly when analyzing classes with large numbers of species, deisotoping is essential to accurate quantitation, and algorithms to accomplish this exist [49]. The application of such methods in portions of the spectrum where lipid species exist at nearly every *m/z* or where there are severe isobaric overlaps are still a major challenge. In some samples (e.g., headgroup scanning conducted with a large number of heterogeneous acyl chain lengths and number of double bonds, or direct inject MS), relative quantitation within similarly ionizable class groupings is reported. Such profiling analyses have the greatest potential to be informative when the lipid subclass of interest can be measured over the broadest number of identified spesies possible and with sufficient numbers of replicates to draw robust conclusions.

Association of measured lipid changes with biological pathways of interest is an emerging area in systems biology. Various methodologies are in active states of development and use [47, 50]. The number of lipids simultaneously measured continues to grow and the appreciation of increased complexity of lipid species is likely to increase dramatically as emerging techniques better define positional specificity of double bonds on fatty acids [51]. The need for application of modern methods to limit the false discovery rate [52] in high-dimensional statistical comparisons is also essential. With thousands of lipid analytes per experiment, such issues are as important in lipidomics as in other omics endeavors.

Conclusions

The combination of highly sensitive ESI-based mass spectrometric techniques and the ability to identify and quantitate thousands of lipid species has made mass spectrometry an essential tool for lipid biochemistry. Results from lipidomics profiling provides insights into the roles of lipids in cellular networks and is being used to identify prognostic or diagnostic markers of disease progression. An extensive database on the lipid composition of macrophages, mass spectra, CID-fragmentation spectra, and useful resources for lipidomics research can be found at the LIPID MAPS website, http://www.lipidmaps.org/.

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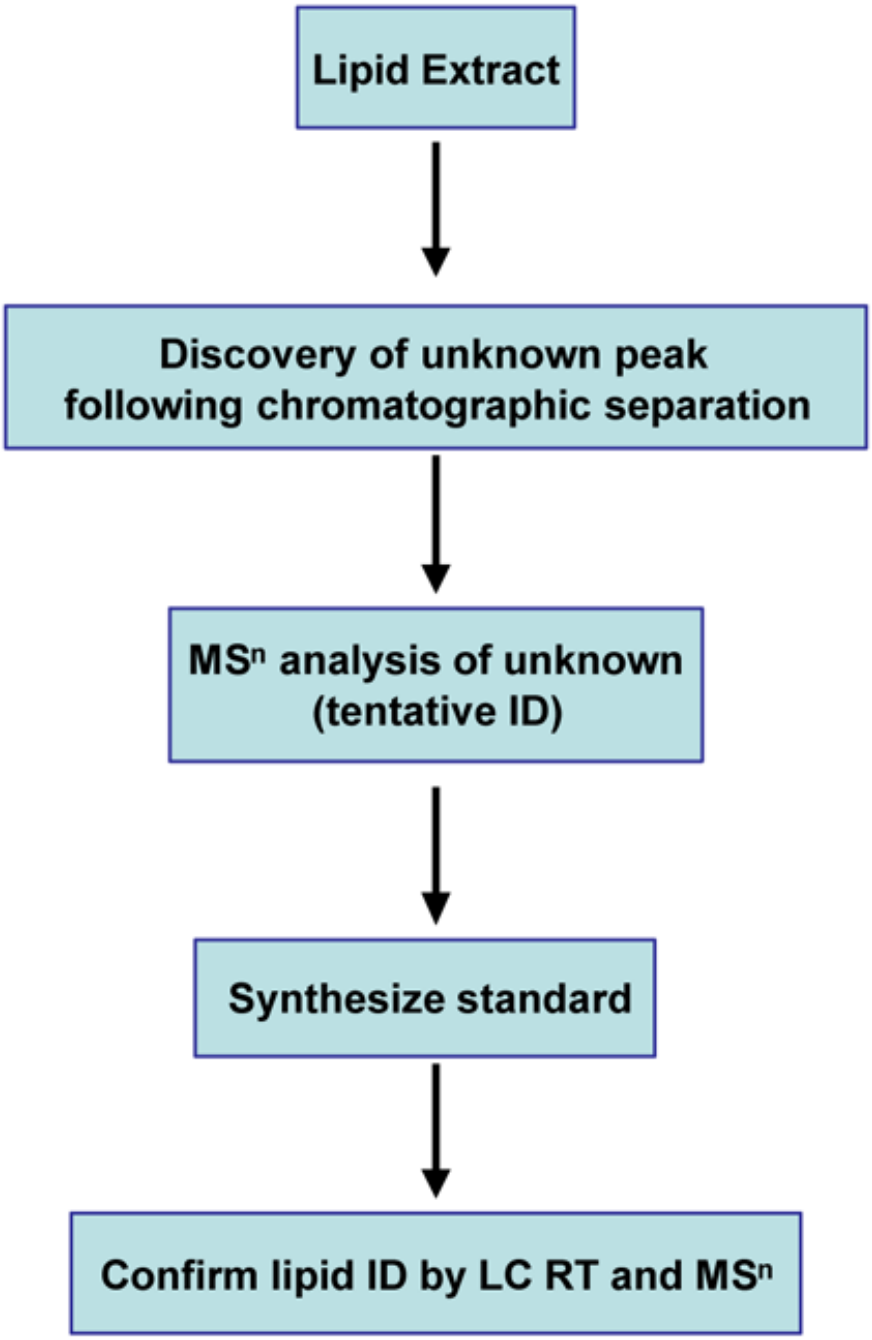

Figure 1.
Strategy for MS-based novel lipid identification. Glycerophospholipids from biological extracts are separated by HPLC chromatography. Unknown peaks are subjected to MS/MS or MS^n analysis to tentatively identify lipids. Synthetic standards are then used to confirm the lipid identity using HPLC retention time (RT) and MS^n fragmentation patterns.

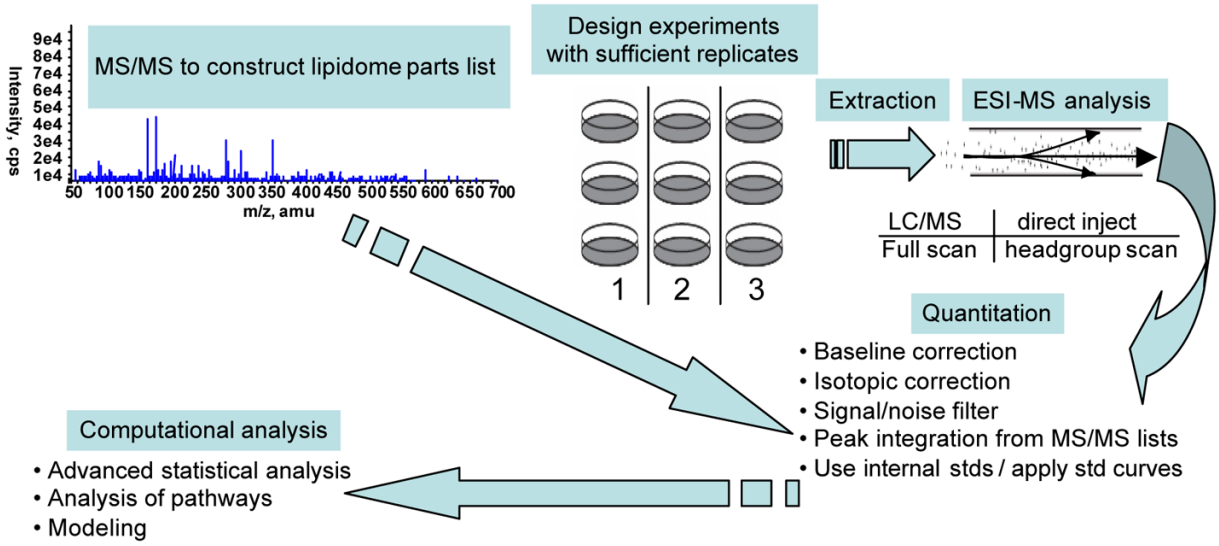

Figure 2.

Workflow in a quantitative lipid analysis determination. Fragmentation of member species are conducted to determine the molecular species. Varying degrees of quantitation (e.g., absolute or relative) can be performed on data coming from either full or headgroup-specific scans in systems with or without an LC column. A series of data processing steps including but not limited to baseline subtractions, deisotoping, and peak matching to lipids positively identified by MS/MS are standard elements in a typical lipidomics system. The sophisticated use of higher order statistical analysis, often multivariate by nature, is growing in applications and importance.

Table 1

Summary of MS/MS methods for phospholipid headgroup analysis

Lipid Class	Precursor Ion	MS/MS Mode	Fragment
PA	$[M-H]^-$	PIS, 153 *m/z*	glycerol phosphate -H_2O
PC	$[M+H]^+$	PIS, 184 *m/z*	phosphocholine
	$[M+Li]^+$	NL, 189 *m/z*	Li cholinephosphate
	$[M+Na]^+$	NL, 205 *m/z*	Na cholinephosphate
	$[M+Li/Na]^+$	NL, 59 *m/z*	trimethylamine
	$[M+Li/Na]^+$	NL, 183 *m/z*	phosphocholine
	$[M+Cl]^-$	NL, 50 *m/z*	methylchloride
PE	$[M-H]^-$	PIS, 196 *m/z*	glycerol phosphoethanolamine -H_2O
PG	$[M-H]^-$	PIS, 153 *m/z*	glycerol phosphate -H_2O
		PIS, 227 *m/z*	glycerol phosphoglycerol -H_2O
PI	$[M-H]^-$	PIS, 153 *m/z*	glycerol phosphate -H_2O
		PIS, 241 *m/z*	cyclic inositol phosphate
PS	$[M-H]^-$	PIS, 153 *m/z*	glycerol phosphate -H_2O
		NL, 87 amu	serine