

## Mini-Review: Enhancement of Maldi-MS Imaging on Sphingolipids

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Matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI) is a powerful technology for studying the distribution of proteins, lipids, and chemicals within the organisms, including plants and animals. The major advantage of MSI is its ability to produce a simultaneous localization and identification of a parent molecule and its metabolites without labeling and without any prior knowledge. MSI has been extensively employed to detect the differentiated pattern of lipids in various organs in different diseases, such as brains in Alzheimer's disease and kidneys in chronic kidney diseases. However, the major obstacle of using MSI in lipid detection is its poor detection sensitivity of some classes of lipids due to ion suppression effects [1]. Especially when phosphatidylcholines (PCs), the major components of all eukaryotic plasma membranes, is presented in the sample, their quaternary ammonium will intensely decrease the ion yields for all other lipids in positive ion mode MSI measurements [2]. Although these ion suppression effects are usually less significant in the negative ion mode, plenty of lipid classes cannot be analyzed at all or demonstrate only low ionization efficiencies in negative ion mode MALDI measurements [e.g., PCs, di- and triacylglycerols (DAGs/TAGs)].

Several solutions have been proposed to solve this problem. For the instrumentation approach, a "MALDI-2" technique is introduced. A secondary MALDI-like ionization processes are initiated by a post-ionization laser beam intersecting the expanding analyte-matrix plume [3]. This is achieved by intercepting the particle plume within nitrogen cooling gas environment with a pulsed ultraviolet (UV) laser. This technique increases the detection intensities of several classes of membrane lipids up to two orders of magnitude higher, especially for cholesterol, phosphatidylethanolamine (PE), plasmalogens (PE-O), phosphatidylserine (PS), and neutral glycosphingolipids (GSLs) such as galactosylceramide (GalCer) because they are usually difficult to be imaged by conventional MALDI-MSI [3]. The major drawback of this modification is the requirement of a complex instrumentation which is not commercially available.

From the approach of matrices, apart from the conventional matrix, 2,5-dihydroxybenzoic acid (DHB), new matrices are developed to "unmask" certain lipid classes, such as GSLs, in complex mixtures [4-7] where most of the detected species are sodium and potassium adducts. Several new matrix solutions are also developed to maximize the detection of [M-H]<sup>-</sup> ganglioside species, such as 2,6-dihydroxyacetophenone [DHA]/ammonium sulfate 3 mM for extracted gangliosides and DHA/ammonium sulfate 125 mM/heptafluorobutyric acid [HFBA] 0.05% for imaging applications dissolved in 50% ethanol [8].

Owing to the fast growth of nanomaterials, applications of nanoparticles with mass spectrometric analyses become more popular. This approach offers several advantages over mass spectrometry using conventional

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organic matrices in many ways, including high absorption coefficient, excellent stability and biocompatibility, ease of preparation and chemical modification [9]. In addition, the mass spectra are simplified because chemical noise by the matrix is minimized [10]. High quality images of the regional distribution of several lipid classes can be obtained as nanoparticle matrix layer is stable, reproducible, and homogenous [11].

Initially, nanoparticles with iron oxide have been used to detect the distributions of PC and GalCer in positive ion mode and sulfatides in negative ion mode [12,13]. However, the detection of sulfatides with nanoparticles is unexpectedly weaker than that with DHB. For this reason, several groups tried to develop a new matrix system with nanoparticles for MSI analyses to obtain higher spatial resolution and sensitivity. The core metals focus on gold, silver, and their mixture [14-16]. Nano silver particles (AgNPs) and gold nanoparticle (AuNPs) have been demonstrated to be useful for detecting neutral lipids (e.g., TAGs, cholesterol) as [M+Au]<sup>+</sup> or [M+Ag]<sup>+</sup> adducts [11,17,18] and ganglioside distribution [19].

Gold nanoparticles (AuNPs), which was modified with alkylamine, have been used to visualize the distribution of GSLs in biological tissue sections. AuNPs are known to ionize GSLs with high sensitivity [15,20]. Application of AuNPs allows sphosphatidyl inositol (PI), sulfatides, and gangliosides species (GM1, GM2, GM3, GD1, and GD3) to be detected with high sensitivity in negative ion mode [18]. Application of AuNPs to the analysis of mouse brains achieved the desired visualization of minor components of GSLs [18]. On the other hand, AgNPs have also been shown to be effective when analyzing neutral lipids such as cholesterol, ceramides, DAGs, and TAGs in positive ion mode [11]. Unfortunately, these nanoparticle matrices also induce a higher thermal load during the laser-induced desorption than classical MALDI matrices and this higher load can cause fragmentation of more complex lipids (e.g., for TAGs) [21]. In addition, these additional steps also result in a loss of the biomolecules, especially for the low abundant lipids.

Biochemical methods have also been tested to reduce ion suppression by enzymatic degradation of PCs. Phospholipase C (PLC) has been used to cleave PLs next to the phosphate moiety and eliminate the charged head group without impairing the hydrophobic membrane anchor (a DAG molecule for diacyl-PL precursors). Sparvero et al. [22] applied the on-tissue PLC digestion with additional chemicals to cross-linking of carboxyl/amino-containing molecules and demonstrated an improvement for detecting mitochondrial cardiolipins and brain gangliosides by MALDI-MSI at a lateral resolution of 50–200 μm in the negative ion mode [22,23]. However, the positive ion mode MALDI-MSI analysis of lipids following a pure PLC treatment was not reported in these two studies.

Vens-Cappell et al. [24] recently demonstrated a pure PLC treatment on the positive ion mode MALDI-MSI analysis of lipids. They performed an on-tissue PLC digestion on 16 μm-thick fresh-frozen murine brain

and kidney organ sections to obtain high contrast MALDI- (mass spectrometry) MS images of GSLs. PLC was simply diluted in water and adjusted to an enzymatic activity of 50 U/mL. 40  $\mu$ L of PLC solution was spotted onto the sections for 30 min at 37°C. The reaction was stopped by removing bulk liquid and air-drying. After having coated with DHB matrix with a sublimation/recrystallization protocol to produce average crystal sizes of about 2 to 3  $\mu$ m [3]. MALDI-MS imaging was performed with a modified Synapt G2-S mass spectrometer (Waters, Manchester, UK) using an effective focal spot size of  $\sim$ 5  $\mu$ m in diameter [3]. Each pixel was irradiated with 30 laser pulses.

By comparing with two adjacent coronal brain sections, one of which was PLC-treated, the MS images and corresponding mass spectra demonstrated an elevation in the ion abundance of GalCer(d18:1/C24:1) and the other nine detected GalCerlipofoms by a factor of about 10 [24]. The overall GalCer distribution was further confirmed by an immunohistochemical staining performed with the PLC-treated section after the MS measurements. In addition, the PLC treatment also demonstrated an increase in signals of cholesterol with about 5-times higher intensities. It is because phospholipid (PL) degradation will unmask ion species that display masses being near-isobaric to those of abundant PCs. For example, [GalCer (d18:1/C24:1)+Na]<sup>+</sup> ion signals at m/z 832.66 are often masked by the more intense signals of [PC (38:4)+Na]<sup>+</sup>, exhibiting an about 80 mDa lower mass.

One of the challenges is whether any diffusion of GalCer, cholesterol, or remnant molecular PLs occurs notably after incubating the sections with aqueous enzyme solution. Fortunately, there was no such diffusion to be observed, suggesting that the overall structural integrity of the cell plasma membranes remained widely preserved [24].

As DAG fragments and cleaved hydrophilic head groups were produced during PLC-treatment, the authors also examined whether these fragments were detected and interfere the final images. The results showed that these fragments were detectable in tissue with comparatively low signal intensities [24]. In contrast, the cleaved hydrophilic head groups were found in both reaction solution and a smeared distribution across the whole tissue sections.

In common, PLC solutions are generally buffered by using a HEPES buffer to obtain an optimal pH at about pH 7. It is also known that these buffers strongly interfere with the analyte/matrix crystallization to decrease the ion yields. Thus, such buffered PLC solution will significantly reduce the sensitivity of MALDI-MS analyses. To bypass such situation, PLC in this study was simply diluted in water, instead of a buffer [24]. A time-course study using mouse brain sections showed that 30 min of PLC incubation at 37°C was sufficient to provide a complete degradation of PCs and a concomitant maximization of GSL ion signals, suggesting that the tissue sections seem to retain an adequate endogenous content of salts to ensure the same PLC activity in an aqueous as well as in a buffered solution [24]. However, long incubation, such as overnight incubation for 16 hours, resulted in signal reduction for ions of other less concentrated PLs. This may be due to degradation of the PLC during long incubation.

Another useful finding is a demonstration of a signal enhancement for GSLs even when tissue sections have already been “pre-analyzed” by standard MALDI-MSI (e.g., to first record PC profiles). The authors just simply removed the matrix coating from the slide by rinsing with water and 0.1 M sodium acetate and then applied PLC and a second DHB matrix coating [24]. Then they performed a second scan on the same slide. The results demonstrated the signal enhancement for GSLs was still observed. These results suggest a possibility of this sequential approach although a slight reduction in the lateral resolution to approximately 20-30  $\mu$ m was observed. This phenomenon may be due to the repeated analyte extraction from the tissue by matrix sublimation and recrystallization and the matrix washing step.

In conclusion, this on-tissue PLC digestion has been demonstrated to successfully enhance MALDI-MSI of neutral GSLs by reducing ion suppression effects. This method is superior to the present methods as it is easily to be implemented without any sophisticated modification on the MALDI-MS or matrices. This method will be beneficial for future analysis on GSLs in human diseases.

### Conflict of Interest Statement

The author declared no competing interests.

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