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Analysis of Lipids from Crude Lung Tissue Extracts by Desorption Electrospray Ionization-MS and Pattern Recognition

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Abstract

A method is described using Desorption Electrospray Ionization (DESI) Mass Spectrometry (MS) to obtain phospholipid mass spectral profiles from crude lung tissue extracts. The measured DESI-mass spectral lipid fingerprints were then analyzed by unsupervised learning principal components analysis (PCA). This combined approach was used to differentiate the effect(s) of two vaccination routes on lipid composition in mice lung. Specifically, the two vaccination routes compared were intra-nasal (i.n.) and intra-dermal (i.d.) inoculation of the *Francisella tularensis* Live Vaccine Strain (Ft-LVS). Lung samples of control and LVS-inoculated mice were quickly extracted with a methanol/chloroform solution and the crude extract directly analyzed by DESI-MS, with a total turnaround time of less than 10 min per sample. All the measured DESI-mass spectra (in both positive and negative ion mode) were compared *via* PCA which resulted in clear differentiation of mass spectral profiles of i.n.-inoculated mice lung tissues from those of i.d.-inoculated and control mice lung tissues. Lipid biomarkers responsible for sample differentiation were identified *via* tandem MS (MS/MS) measurements or by comparison with mass spectra of lipid standards. The DESI-MS approach here described provided a practical and rapid means to analyze tissue samples without extensive extractions and solvent changes.

Keywords

DESI; mass spectrometry; lipids; lung tissue

Introduction

Finding an effective vaccine for *F. tularensis* is especially relevant due to the ever present threat of bioterrorism, as *F. tularensis* is listed as a possible bio-weapon by the CDC[1]. It is

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known that different inoculation routes of the *F. tularensis* live vaccine strain (Ft-LVS) in mice can dictate the efficacy of the vaccine, and consequently, the protection imparted to the host[2;3]. Specifically, studies with mice involving LVS vaccination followed by challenges with virulent strains of *F. tularensis* found that the intra-nasal (i.n.) Ft-LVS inoculation confers more protection than intra-dermal (i.d.) Ft-LVS inoculation. The basis for this difference is not clearly understood as immunological responses to a vaccine result from the coordinated interplay of many biochemical and physiological processes. Elucidation of the molecular, as well as physiological, information as to the effect of vaccines in host organisms can lead to a better understanding of their mechanism of operation and a rational design of vaccines and their inoculation.

To this end, lipid analyses of tissues have yielded information about the immune response to a vaccine, because the majority of lipids provide the framework for cellular organelles and membranes, and as such, lipids are also involved in cellular signaling. For example, lipid composition differs between organelle membranes, cellular types, and tissues types[4], as well as between lung tissues from different mammals[5;6], and between different disease states[7;8]. More specific examples include an observed decrease of pulmonary surfactants like phosphocholines associated to *Pseudomonas aeruginosa* colonized in cystic fibrosis patients' lungs[9] and changes in lipid profiles in response to changes in physiological states due to an immune response to an allergen in asthma patients[10].

Even though several analytical techniques like nuclear magnetic resonance (NMR) spectroscopy[11], liquid chromatography[12] and thin layer chromatography[13] have been used to profile lipids in biological samples, mass spectrometry (MS) offers unique advantages in the analysis of lipids[14;15]. Mass spectrometry provides sample speciation of lipid type and composition through multi-stage MS analyses, or when combined with LC or TLC [16;17;18]. The recent development of ambient ionization techniques like Desorption Electrospray Ionization (DESI) has enabled the analysis of samples in their native state (or very little sample preparation)[19;20;21;22] allowing for rapid high throughput measurements. In DESI, samples are deposited onto a solid surface and solvent from a pneumatically assisted electrospray source is directed towards the sample at a certain angle. The ionized solvent upon impinging the sample surface both dissolves and ionizes analyte molecules. The droplets containing the dissolved analyte are splashed off the surface by other droplets and towards the mass spectrometer inlet, with subsequent droplet desolvation yielding ionized gas phase molecules. DESI has the ability to be a high-throughput technique, as no sample injections are required and samples can be pre-spotted onto a surface and analyzed quickly in series[23;24]. Selectivity of the DESI sampling/ionization step can be changed by the judicious choice of solvents, additives and reactants[25;26;27]. As a result, DESI-MS analysis has been applied to a wide range of samples and analytes including lipids[28], intact proteins[29;30], forensic samples[31], metabolites[21;32;33], bacteria[34;35], and plant material[36]. DESI-MS has also been applied to the direct analysis of tissues including imaging, using computer controlled XY motion stage to move the sample and to enable data collection of the mass spectral images[37;38;39]. Moreover, DESI-MS has been shown to be less susceptible to suppression effects in the analysis of mixtures and to high salt concentrations[40], making it suitable for the rapid analysis of crude samples. In the present study, DESI-MS in conjunction with pattern recognition data analysis are applied to the analysis of crude lipid extracts from mouse lung tissues. The study here presented demonstrates the utility of the DESI-MS approach for the high throughput acquisition of characteristic and reproducible lipid profiles for pattern recognition.

Materials and methods

Chemicals

Solvents used were HPLC grade methanol and water (Burdick and Jackson, Muskegon, MI). Acetic acid and chloroform were used without further purification (Sigma-Aldrich, St. Louis, MO). Standards were used to optimize DESI experimental parameters and relative quantitation of lipids. Phospholipid standards used were 1-Palmitoyl-2-Stearoyl-*sn*-Glycero-3-Phosphocholine (GPCho 14:0/16:0), and 1-Stearoyl-2-Docosahexaenoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (GPGro 18:0/20:6) (Avanti Polar Lipids, Alabaster, AL) and used to tune the DESI-MS system. All lung tissues were obtained from mice (ICR strain). Phosphate buffered saline (PBS) was prepared using (all from Sigma-Aldrich) NaCl (130 mmol/L), KCl (3 mmol/L), Na₂HPO₄ (10 mmol/L), KH₂PO₄ (1.5 mmol/L) and adjusted at pH 7.4.

Mice Treatment and Lung Tissue Sample preparation

Mice were either inoculated intradermally with 50 μ L of a solution containing 2×10^5 cfu/mL Ft-LVS or PBS, or anesthetized with ketamine-xylazine and inoculated intranasally with 25 μ L of PBS or PBS containing 4×10^5 cfu LVS. Mice were euthanized by pentobarbital overdose 6 days following exposure and lungs were collected and frozen immediately on dry ice and maintained at -80°C for up to 12 hours before processing. The inoculation experimental design is summarized in Table 1.

Samples analyzed included controls: lungs from non-inoculated mice (untreated/control, $n=4$), lungs from mice inoculated *via* i.d. with vaccine saline solution (phosphate buffer solution, PBS) but without the LVS (referred to as i.d. PBS, $n=4$), lungs from mice inoculated *via* i.n. saline solution but without the LVS (i.n. PBS, $n=4$), lungs from mice inoculated with LVS *via* i.d.(i.d. LVS, $n=4$), and lungs from mice inoculated with LVS *via* i.n. pathway (i.n. LVS, $n=4$).

The crude lipid component was extracted from tissue samples (~200 mg) using chloroform: methanol (2:1) as described by Folch *et al* [41]. Briefly, about 200 mg of lung tissue were sonicated in 1.2 mL of 3:1 chloroform:methanol mixture. The lipid fraction contained in the chloroform layer was then used directly for all DESI-MS analyses. The overall experimental procedure is summarized in Figure 1. It is worth noting that in the analysis of multiple samples, the DESI-MS approach would be even faster as several spots can be deposited onto the probe. In the direct infusion ESI-MS method, extra time is consumed rinsing and filling the syringe with the new solution, unless a Flow Injection Analysis (FIA) approach is implemented.

Mass Spectrometry

All measurements were performed with a Quadrupole Ion Trap Mass Spectrometer (LCQTM-Classic, Thermo/Finnigan/Electron/Fisher, San Jose, CA) in either the positive or negative ionization modes. Mass analysis parameters were set as follows: Mass range 500 – 1000, accumulation time of 150 ms, and Automatic Gain Control (ACG) turned off (to achieve high S/N measurements). For tandem MS measurements (MS/MS), *q*-fragmentation was set at 0.25, collision energy varied between 30–40%, and fragmentation time 100 ms. All ESI measurements were performed with a nano-ESI source with a capillary with od/id 360 μ m/75 μ m and tip id of 30 μ m (from New Objective Cat No. FS360-75-30-N-20-C12). Samples were infused *via* a syringe pump at a flow rate of 5 μ L/min and the ESI voltage set at 4 kV. All DESI measurements were performed with a home-build DESI source[35] with the following parameters: angle between spray source and sample surface was $\alpha = 55^\circ$, and between sample surface and the mass spectrometer inlet was $\beta = 7.3^\circ$. Nitrogen nebulizing

gas was used with a head pressure of 250 psi. For positive and negative ion DESI, a methanol/water solvent (1:1) with 0.1% acetic acid at a flow rate of 7 $\mu\text{L}/\text{min}$ was used.

Data Processing and Pattern Recognition

Only the lipid profiles obtained via DESI-MS analysis of crude extracts were used in the pattern recognition analysis. Principal Components Analysis (PCA) was performed with the Pirouette software (version 3.11, Infometrix, Bothell, WA). Each mass spectrum was collected as a set of raw intensities and nominal m/z values. The data were normalized to total intensity and mean centered and both score and loading plots were generated. The loadings or eigenvector coefficients indicate the magnitude of the contribution of a variable (in this case the mass spectral peaks or phospholipids' m/z values) to the composition of the projected scores. Each mass spectrum is represented as a single point on the scores plot, and mass spectra are grouped based on similarities in their intensity versus m/z profiles. Mass-to-charge ratio values that are closer to the origin of the loading plot are common to all of the spectra.

Results and discussion

Comparison between DESI-MS and direct infusion ESI-MS of crude lipid extracts

In the development of a rapid analysis of lipids in crude lung tissue extracts, positive ion mode DESI-MS and (direct infusion) ESI-MS were compared. Figure 2 shows the mass spectra obtained from each technique of lung tissue lipid extract from i.n. LVS inoculated mouse. Mass spectral lipid profiles were obtained in both analyses with similar signals relative intensities and m/z values, most corresponding to lipid biomarkers; however, the DESI-MS analysis was carried out in less than 10 min total analysis time (sample extraction, preparation and data acquisition), while the ESI-MS analysis (direct infusion) required over 30 min total analysis time. The analysis time for the ESI-MS analysis (shown in Figure 1) is a result of manual sample loading, solvent removal and syringe infusion. The longer analysis time required in ESI-MS mainly resulted from the incompatibility of the extraction solvent (chloroform/methanol) with the MS vacuum system (*i.e.*, chlorinated compounds). Even though the time for direct infusion ESI-MS analysis can be decreased by implementing microarray ESI technologies [42;43], the removal of the chlorinated solvent used in lipid extraction is a required step as they are incompatible with the MS vacuum system. In this sense, the DESI-MS analysis has a built-in solvent removal step during sample deposition onto the DESI-probe (glass slide), making its measurement independent of the original sample solvent. This sample drying step in DESI (solvent evaporation) is much shorter (30 s for a 1 μL sample) than the time required to evaporate enough sample (about 100 μL) for ESI analysis. In addition, direct ESI-MS analysis of the crude undiluted extract (*i.e.*, the same one used in the DESI-MS analysis) contaminated the MS system (heated capillary inlet in the LCQTM system) causing severe sample carry over between measurements. As a result, additional time was spent removing the extraction solvent and re-suspending the sample in an ESI-compatible solvent (*e.g.*, methanol/water with 0.1 % acetic acid). The DESI-MS analysis of the undiluted extract did not cause system contamination or carry over problems between samples, increasing the sample analysis throughput. As a result, all subsequent measurements on crude lipid extracts from mice lung tissues were performed by DESI-MS. The high sample throughput achievable using DESI-MS provided a key advantage when acquiring data for the pattern recognition analysis, as many replicate measurements must be made to obtain a statistically significant data set.

Mass spectra of crude lipid extracts from mouse lung tissue

Observed phospholipids signals in lung samples were tentatively identified by tandem mass spectrometry (MS/MS; collision induced dissociation, CID). In cases where the ion intensity

was below the threshold needed for MS/MS measurement and identification, only a tentative assignment was made (among the many possibilities) based on the observed m/z value of published lung phospholipid profiles and matches with lipid databases (e.g., lipidomics web site www.lipidmaps.org maintained by the LIPID MAPS consortium and the Nature Publishing Group). A quick survey of all lipid profiles obtained indicates that differences observed between all mass spectra collected were mainly due to changes in the relative intensities of the phospholipids detected (A list of general structures, names and abbreviations for glycerophospholipids detected in this study is presented in the supplementary information section in Table 1S). Figure 3 shows representative negative ionization mode DESI-mass spectra for two crude extracts of mouse lung tissues, i.d. LVS and i.n. LVS inoculated mice. These mass spectra are characteristic of a complex mixture with the observed m/z values corresponding to known glycerophosphoglycerol (GPGro) and glycerophosphoinositol (GPIs) lipid signals, as would be expected from these acidic phospholipids. For example, two signals were analyzed by MS/MS and found most likely to be 16:0/16:0-GPGro at m/z 721 and 16:0/18:1-GPGro at m/z 747 (corresponding tandem mass spectra are shown in Figure 1S in the supplementary material section). For the tandem mass spectrum of the ion at m/z 721 (16:0/16:0-GPGro, Figure 1S(a)), characteristic GPGro fragment ions observed include the losses of the *sn*2 fatty acyl chain as the ketene (m/z 483) and as the neutral carboxylic acid (m/z 465) [14]. A single carboxylate anion at m/z 255 indicates the most likely the presence of a palmitatyl unit at both *sn*1 and *sn*2 positions. Also, the intense signal at m/z 391 corresponds to the ion $[M-H-R_2COOH-74]^-$, observed to be particularly intense when CID is performed in a quadrupole ion trap [44], as it was done in this study. A similar fragmentation pattern is observed for the precursor ion at m/z 747 (Figure 1S(b)), showing the carboxylate anion signals for the *sn*1 and *sn*2 fatty acyl side chains (tentatively C16:0 and C18:0, respectively). The exact assignment of these two chains to each position cannot be ascertained by their signal intensities, as other instrumental and structural factors are known to influence this ratio [14]. Similarly, the MS/MS analysis of the ion at m/z 885 yielded characteristic fragmentation consistent with 18:0/20:4-GPIs [14] (see Figure 2S in supplementary material). The MS/MS analyses of remaining signals in the lipid profiles obtained were either too weak for meaningful interpretation or most likely due to a mixture of lipids since their tandem mass spectra had multiple fatty acyl fragments (R_nCOO^-).

Representative positive ionization mode DESI-mass spectra are shown in Figure 4. Peak intensities and spectrum complexity were lower in the positive ion mode, in agreement with previously published work on DESI MS of complex lipid mixtures [28]. Tandem mass spectrometry was performed on several ions, that yielded a large fragment ion due to the loss of 59 u (trimethylamine, $(CH_3)_3N$), indicating that most of the signals observed resulted from $[M+Na]^+$ GPCho species. Moreover, several of these MS/MS analyses yielded information to assign a tentative structure. For example, both ions at m/z 754 and 756 correspond to 16:0/16:1-GPCho and 16:0/16:0-GPCho, respectively, and their tandem mass spectra (MS^2 and MS^3 analyses) are shown in Figure 3S (supplementary material).

Multivariate Analysis of Lipid Mass Spectral Profiles

All lung samples, i.n. LVS, i.d. LVS inoculated and controls, were analyzed by DESI-MS in both positive and negative ionization modes and their lipid profiles compared *via* Principal Components Analysis (PCA). Principal components analysis results (both score and loading plots) using negative and positive ionization mode DESI-mass spectra of crude extracts of mouse lung tissue are shown in Figure 5 and Figure 6, respectively. From score plots in Figures 5(a) and 6(a) a clear differentiation is observed between the lipid profiles from i.n. LVS and i.d. LVS mouse samples using both ionization modes. In addition, for both positive and negative ionization modes, the i.d. LVS samples were grouped with the control samples

analyzed, indicating that the i.d. vaccination of *F. tularensis* LVS had no measurable effect on the lipid profiles of mouse lung tissues and thus are indistinguishable from control samples. Principal component score and loading plots from these data were also found to be reproducible, as the same components causing the differentiation between the groups were obtained in 3 separate experiments performed over a period of 3 days. It is also evident that better differentiation between sample groups was achieved when the negative ionization mode DESI-mass spectra were compared *via* PCA (Figure 5a) than when positive ionization mode DESI-mass spectra were compared (Figure 6a). In the negative ionization mode PCA score plot there is no mass spectral feature differentiating the i.d. LVS and control samples; however, the DESI mass spectra of i.n. LVS samples were clearly differentiated from control and i.d. LVS inoculated samples.

Overall, differentiation of these samples *via* PCA of DESI-mass spectra resulted from differences in the relative ratios of lipid biomarkers, and not on the appearance of specific or unique lipid biomarkers. Specifically, the PCA loading plot derived from the analysis of the negative ionization mode DESI-mass spectra (Figure 5b) revealed that samples treated with i.n. LVS showed an increase of the ion at m/z 885 (most likely $[M-H]^-$ of 18:0/20:4-GPIs) and a decrease of the signal corresponding to the lipid 16:0/18:1-GPGro at m/z 747.

Similarly, the PCA loading plot resulting from the analysis of positive ionization mode DESI-mass spectra of i.n inoculated samples show that levels of 16:0/18:1-GPCho ($[M+Na]^+$, m/z 782); 18:2/18:2 or 16:0/20:4-GPCho ($[M+Na]^+$, m/z 804); 18:1/18:2-GPCho ($[M+Na]^+$, m/z 806); and 18:0/18:2 or 18:1/18:1-GPCho ($[M+Na]^+$, m/z 808) had increased signal intensities relative to control and i.d. inoculated samples (pertinent signals circled in Figure 6b). In negative ionization mode DESI-MS analysis, PCA classification along with loading plots provide evidence for an increase of the phosphoinositol (PI) species 18:0/20:4-GPIs (m/z 885) in lung tissues of mice treated with i.n. LVS over control and i.d. LVS samples.

Combined, results obtained in this study parallel observations made by Wright and coworkers[45] in the analysis of neutrophils isolated from human plasma, where high levels of unsaturated GPIs species were found. Furthermore, since neutrophils are specialized cells in the immune system (phagocytes), the changes observed in phospholipid composition in this study could be related to an immune response in the lung tissue due to the i.n. LVS inoculation. In fact, the same study by Wright and co-workers[45] also reported a decrease in 16:0/16:0-GPCho in sputum of asthmatic subjects due to a dilution effect from infiltration of plasma lipoprotein into the airway lumen. This is also in agreement with results derived from the PCA and loading plots obtained from the analysis of positive ion DESI-mass spectra in this study (Figure 6b), where a decrease in 16:0/16:0-GPCho levels is observed in i.n. LVS samples versus control and i.d. LVS samples. Moreover, Heeley *et al* analyzed bronchoalveolar lavage fluid (BALF) from asthmatic volunteers after allergen challenge and reported an increase in 16:0/18:2-PCho, 16:0/18:1-GPCho, 16:0/20:4-GPCho, 18:1/18:2-GPCho, and 18:0/18:2-GPCho due to the influx of plasma components into the airway lumen[10]. Their observations also correlate with the observed increase in signal intensities of GPCho species (at m/z 's 783, 805, 807, and 809) for i.n. LVS inoculated mice in this study (Figure 6b). The mass spectra of i.d. LVS vaccinated samples are grouped with those from control samples, indicating that i.d. LVS inoculation does not appreciable affect the lipid environment in the mouse lungs.

Conclusion

A method was developed for the rapid profiling of lipid biomarkers from mouse lung tissue by DESI-MS and pattern recognition data analysis using PCA. Since DESI-MS analyzes the

sample on a solid substrate, solvent removal was built-in into the protocol, and as a result, issues with extraction solvent incompatibility with the MS vacuum system were avoided. This resulted in a rapid analysis time of 10 min/sample for the analysis of crude biological tissues in this study, demonstrating the suitability of this approach for the high throughput analysis of clinical samples (for example, for rapid screening of clinical samples like BALF or sputum from asthma patients[45]). The technique was applied to study the effect of different inoculation routes of the bacterium *F. tularensis* LVS in mice. The combined approach of DESI-MS and PCA was shown to successfully characterize the phospholipid profile in lung samples of *F. tularensis* LVS inoculated mice, with the ability to differentiate lung samples from subjects inoculated *via* intra-nasal and intra-dermal routes. Moreover, lipid signals responsible for sample differentiation (identified via MS/MS analysis) correlate with increase levels of neutrophils and lymphatic cells, commonly associated with an immune response. Although previous studies have quantitated lipids as crude mixtures, that is, without prior chromatographic separation, it remains to be seen if suppression effects often observed in ESI of lipid mixtures affect the accuracy of similar measurements using DESI, making the use of an internal standard. Regardless of possible suppression effects during the DESI process of lipid mixtures, the approach used in this study demonstrated the ability to differentiate small differences in relative sample lipid composition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure. 1. Mouse lung sample preparation procedure for DESI-MS and time comparison with infusion ESI-MS analysis.

Figure. 2.

Direct analysis of mouse lung tissue lipid extract (i.n. LVS inoculated) with (a) positive ion DESI-MS and (b) direct infusion positive ion ESI-MS. For the ESI-MS analysis, the sample solvent was changed from chloroform:methanol to 50% methanol water (1 % acetic acid) in order to obtain comparable results as in the DESI-MS analysis.

Figure. 3.

Negative ion mode DESI-mass spectra of crude extract of mouse lung tissue (a) i.d. LVS (b) i.n. LVS inoculated mouse lungs (tentative assignments of sn1 and sn2 groups were based on MS/MS analyses).

Figure. 4. Positive ion DESI mass spectra of crude extracts from (a) i.d. LVS and (b) i.n. LVS inoculated mouse lungs (tentative assignments of GPCho sn-1 and sn-2 groups were based on MS/MS analyses).

Figure. 5.
Principal component analysis of negative ion DESI-mass spectra of mice crude lung tissue
(a) Score plot and (b) Loading plot

Figure. 6.
Principal component analysis of positive ion DESI-mass spectra of mice crude lung tissue
(a) Scores plot (b) Loading plot.

Table 1

Mouse lung samples analyzed in this study.

Lung sample	inoculation route
id LVS/PBS	intra-dermal (id)
in LVS/PBS	intra-nasal (in)
id PBS	intra-dermal
in PBS	intra-nasal
Control	none