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A Uniform DHB Layer as Matrix for MALDI-FTICR MS-Based Lipidomics

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Notes

The authors declare no competing financial interest.

Abstract

A very uniform 2,5-dihydroxybenzoic acid (DHB) layer was first constructed, and used as matrix of matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR MS) for quickly exploring the changes of lipids in biological systems. Lipids extracts from biological samples were dissolved in chloroform and deposited onto the DHB layer. Benefiting by the insolubility of DHB in chloroform, the uniform matrix crystals were still maintained, and more importantly, the lipids analytes were distributed homogenously on the layer, which significantly increased the reproducibility of analysis using MALDI-FTICR MS. Taking advantage of the benefit of high resolution of FTICR MS and the fragment ions obtained by MS/MS, lots of lipids were identified. This method was used for exploring the changes of lipids in drug resistant tumor cells compared with paired drug sensitive tumor cells. The principle component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were employed for discovery of the changed lipids. This method, characterized by the simplicity and the speediness, demonstrated a new promising approach for lipidomics study.

1. Introduction

Lipidomics has been attracting the attention of scientists, as accumulating evidence suggests that many diseases are connected with the dysregulation of lipid metabolism.[1] Therefore it is important to explore the changes of lipids in biological systems. Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) has been used for lipid analysis. For example, Gross *et al.* developed a MALDI-TOF MS-based approach for the rapid analyses of cellular glycerophospholipids, in which through utilizing specific suites of solvent and alkali metal cation and 9-amimoacridine as matrix, the highly selective ionization of different lipid classes was achieved.[2] In addition, Richards *et al.* utilized metal oxide as matrix for analysis of lipids extracts from bacterial and algal sources demonstrating the application of the technique as a rapid screening methodology.[3]

Although MALDI MS represents a useful technique, the disadvantage of rather poor reproducibility, mainly originating from heterogeneity of the matrix-analyte co-crystal, limits the application of MALDI MS in quantitative analysis of lipids. In many cases, the best MALDI MS performance is usually achieved only at certain locations (sweet spots) of the matrix-analyte co-crystal. A uniform matrix-analyte co-crystal minimizes the need to search for sweet spots, and more importantly, it avoids the variability of signal intensity across different locations on the target surface, and greatly improves spot-to-spot reproducibility.

To obtain a uniform crystallization for quantification of MALDI MS, binary matrices (such as the combination of 2,5-dihydroxybenzoic acid/N,N-dimethylaniline[4,5]) and nanoparticles (NPs, most commonly, Ag,[6] SiO2,[7] TiO_2,[8] and Fe_3O_4[8,9] and even DHB nanoparticle[10]) were employed, and their spots on the probe of MALDI MS appeared more homogenous than traditional MALDI MS

matrices; However, their capabilities for lipid analysis needed further investigations. In addition, faster crystallization method, that was, the matrix and analyte were prepared in a volatile solvent and/or the MALDI probe was dried at elevated temperature and/or reduced pressure was proposed to obtain a uniform crystallization.[11] In our group, a uniform matrix-analyte co-crystal was realized for the quantitative analysis of plasma lysophosphatidylcholines (LPCs) with the assist of polystyrene (PS) colloidal spheres.[12] PS spheres have superior mono-dispersed property, and can self-assemble to form uniform photonic crystals, on which the analyte was deposited and distributed evenly. Due to the uniform co-crystal of matrix-analyte, the spot-to-spot reproducibility on the MALDI probe was satisfied with standard derivation (SD) lower than 4.1%. However, recently we found that, to maintain the uniform surface of photonic crystals, in the solvent of analyte, at least 50 percent water was needed. In this case, the PS assisted MALDI MS method for quantitative analysis of lipids was limited since most lipids were water-insoluble. Therefore, a supplementary method was urgently needed.

In this work, a uniform matrix-analyte co-crystal was tactfully obtained for MALDI-FTICR MS analysis of lipids, and this system was utilized for exploring the changes of lipids in drug resistant tumor cells compared with paired drug sensitive tumor cells. Through combining the benefit of FTICR MS with statistical interpretation of data, lots of lipids, in particular the changes of lipids in drug resistant tumor cells, were identified, which demonstrated a new methodology for lipidomics study.

2. Experimental

2.1 Chemicals and reagents

Standard lipids, including 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine

(16:0/20:4 PC), L-α-phosphatidylethanolamine (PE, egg, Chicken), L-α-phosphatidylinositol (PI, soy) and N-palmitoyl-D-erythro-sphingosylphosphorylcholine (d18:1/16:0 SM) were purchased from Avanti Polar Lipids (Birmingham, AL). HPLC-grade methanol (MeOH), ethanol (EtOH), isopropanol (IPA), acetonitrile (CH_3CN), chloroform ($CHCl_3$), formic acid as well as ammonium formate were purchased from Sigma or Fisher Scientific (Pittsburgh, PA). The matrix 2,5-dihydroxybenzoic acid (DHB) was purchased from Lancaster (Morecambe, UK). Ultra-pure water was from Milli-Q purification system (Millipore Corporation, USA). All of the above materials were used as received without further purification.

2.2 Cell culture and harvest

Human ovarian cancer cell line A2780 and its paired drug resistant cell line A2780/T (multi-drug-resistant variant) were obtained from Pujingkangli Science & Technology (Beijing, China). A2780 cells were cultured in RPMI 1640 (Life Technologies, Grand Island, USA) supplemented with 10% FBS (Life Technologies, Grand Island, USA), 100 μg/mL streptomycin and 100 units/L penicillin. A2780/T cells were cultured in DMEM (Corning, Manassas, USA) supplemented with 1 mg/L paclitaxel, L-glutamine (4 mmol/L), glucose (4.5 g/L), 10% FBS, 100μg/mL streptomycin and 100 units/L penicillin. Before use, A2780/T cells were seeded in medium without paclitaxel. Cells with approximately 90% confluence were treated with trypsin and harvested by centrifugation (5 min at 1,000 rpm) and washed three times using PBS (1×). A small aliquot was used for protein assay (BCA protein assay). The cell pellets were stored at -80 ℃ until use.

2.3 Lipid extraction

For cell pellet lipid assays, Bligh and Dyer method was used to extract lipids.[13] In brief, a

pellet containing 0.2 mg total protein was resuspended in 100 μL of water before it was transferred into 400 μL of Chloroform-methanol (2:1, v:v). After vortexing for 1 min and centrifugation (10,000 g, 5 min, room temperature), the lower phase were collected and dried with N_2. The samples were stored at -80℃ until MS analysis. The lipids extracts were re-suspended in 50 μL of $CHCl_3$, and then 1.5 μL of the solution was deposited on the DHB layer for MALDI-FTICR MS analysis.

2.4 Preparation of matrix: a uniform DHB layer

A Uniform DHB Layer was constructed by using μMatrix spotter machine (Hudson Surface Tech., Gyeonggi-do, South Korea). The standard 384-spot metal MALDI probe was bought from Bruker (Bruker Daltonics, Bremen, Germany). DHB was dissolved in ethanol with the concentration at 30 mg/mL. 1.5 mL of the matrix solution was loaded onto the printing head of the machine and started to print at the specified position of MALDI probe. Temperature was set at 70 ℃ to accelerate the solvent evaporation. The printing cycle was set at 4. The MALDI probe was then removed from the machine and cooled at room temperature before used.

DHB (60 mg/mL) was dissolved in chloroform-methanol (2:1, v/v), and 2.5 μL of the matrix solution were manually dropped on the MALDI probe, by which a uniform DHB layer could be also obtained. Both uniform DHB layers can be used as matrix of MALDI-FTICR MS for analysis of lipids.

2.5 MALDI-FTICR mass spectrometry

MALDI-FTICR Mass Spectrometry analysis was performed with a Bruker solariX mass spectrmeter equipped with a 9.4T superconducting magnet and equipped with SmartBeam™ laser optics. Mass calibrations were performed externally using DHB and a peptide mixture

(angiotensin II, substance P, bombesin, and ACTH clip 1-17) (Sigma, St. Louis, MO) as mass standards. External ion accumulation was used in both positive and negative ion mode over a mass range of 200–1800 m/z with a resolution of 200,000 at m/z 200. Data acquisition had been optimized using a random-walk routine under fuzzy logic control so that 600 laser shots were acquired per sample using solariXcontrol software.

For MALID imaging, spectra were acquired across the entire sample section with a SmartBeam II laser operating at 1000 Hz, a laser spot size of 25 μm, and a raster width of 200 μm. Data were processed using FlexImaging 3.0 software (Bruker Daltonics).

2.6 UPLC-ESI MS/MS

The changed lipids identified by MALDI-FTICR MS, if commercially available, were further investigated by ultra performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). I-class Acquity ultra performance liquid chromatography, (Waters) and API 4500 QTRAP mass spectrometer (Applied Biosystems) were used. Both the nebulizer and desolvation gases were nitrogen. Typical operating parameters were set as follows: curtain gas (CUR) 25, collision gas (CAD) medium, ion source gas 1 (GS1) 45, ion source gas 2 (GS2) 50, electrospray voltage 5500 and a temperature at 500 ℃. The mobile phase A was isopropanol/acetonitrile/formic acid (90:10:0.1, v/v/v) containing 10 mM ammonium formate. The mobile phase B was acetonitrile/water/formic acid (70:30:0.1, v/v/v) containing 10 mM ammonium formate. A CSH C18 column (1.7 μm, 2.1 mm ID×100 mm, Waters) was used for separation of lipids. The column was maintained at 55 ℃. The UPLC separations were 20 min/sample using the following scheme: 1) 0 min, 70% B; 2) 2 min, 57% B; 3) 2.1 min, 50% B; 4) 12 min, 46% B; 5) 12.1 min, 30% B; 6)18 min, 1% B; 7) 18.1 min,

70% B; 8) 20 min, 70% B. All the changes are linear, and the flow rate was set to 400 μL/min. Positive ion MRM mode was used for quantitative analysis of PIs, PEs, PCs and SMs. Monitoring ions were at m/z 837.5 (the parent ion) -577.5 (the product ion) for PI(16:0/18:1), 863.5-603.5 for PI(18:1/18:1), 865.5-605.5 for PI(18:1/18:0), 887.5-627.5 for PI(18:0/20:4), 703.6-184.0 for SM(d18:1/16:0), 813.6-184.0 for SM(d18:1/24:1), 768.5-627.5 for PE(38:4), 782.4-184.0 for PC(36:4), 806.4-184.0 for PC(38:6) and 782.4-184.0 for PC(40:7).

2.7 Multivariate data analysis

Raw MALDI-FTICR MS data were processed by DataAnalysis 4.1 software. Mass were calibrated and a default approach was used to assign peaks. No additional processing was done before the Fourier transformation. The resulting peak list, including the values of m/z and their corresponding intensities, was exported as CSV files. These data files were processed with Metaboanalyst 2.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) (accessed October 2014).[14,15] Unsupervised principal component analysis (PCA) and supervised partial least square-discriminant analysis (PLS-DA) were carried out to apply statistically driven models in order to determine latent variables indicative of hidden relationships between the observed data. If a separation between the two cell line groups was observed in the PCA scores plot, PLS-DA was performed to highlight the differences. The potential different features (the values of m/z) that significantly discriminated between groups were selected and subjected to further characterization of the molecular formula.

2.8 Structural identification

High resolution MS spetra were used to distinguish different lipids, and the identification of lipids was achieved by precisely matching mass with free online databases, including Lipidmaps

(http://www.lipidmaps.org) (accessed October 2014) and HMDB (http://www.hmdb.ca) (accessed October 2014).[16-18] The mass error was set at 2 ppm. MS/MS spectra obtained by collision induced dissociation (CID) were further used for confirmation of structure of lipids.

3. Results and discussion

3.1 Construction of a very uniform DHB layer and a uniform DHB-analyte co-crystal for MALDI-FTICR MS

Among the vast majority of commercially available matrix, 2,5-dihydroxybenzoic acid (DHB) was the widely accepted matrix compound for the analysis of lipids for it providing an excellent signal-to-noise (S/N) ratio for the peaks of the analyte of interest and providing a weaker background than cinnamic acid derivates that tend to significant matrix cluster generation.[19,20] Also, the carboxylic acids in matrix DHB ($pK = 2.97$) was capable of triggering the generation of H^+ adducts and inhibiting the Na^+ adducts in the positive ion mode.[21] However, one obvious drawback of DHB as MALDI matrix was its tendency to form large needle-like crystals (shown in Fig. 1A). The crystal was not evenly distributed, leading to poor spot-to-spot reproducibility. To change the DHB crystal morphology, μMatrix spotter machine was adopted in this study. The machine was equipped with piezoelectric materials which could rapidly propel picoliter matrix solution onto the MALDI probe to print a homogeneous matrix layer (shown in Fig. 1B).

DHB (10-40 mg/mL) was prepared in fast evaporating solvent (ethanol or methanol) and loaded onto the machine, and different printing cycles (2-10 cycles) were tested. It was observed that the morphology of DHB layer mainly depended on the concentration of DHB used. Low concentration (10 or 20 mg/mL) of DHB generated thin and sparse crystal surface, while high concentration (40 mg/mL or above) of DHB generated grainy crystal surface. Eventually DHB

with the concentration at 30 mg/mL and 4 printing cycles were used to construct a homogeneous surface (shown in Fig. 1B). On the DHB layer, 1.5 μL of the lipid solution, chloroform used as the solvent, was deposited. Benefiting by the insolubility of DHB in chloroform, the lipid solution did not destroy the uniform matrix layer, and a homogeneous matrix-analyte co-crystal was formed (Fig. 1C). The co-crystal was further investigated by scanning electron microscopy (SEM), and it was found that the co-crystal surface presented as the porous structure, and no large chunk of needle-like DHB crystal was observed (Fig. 1D). On the contrast, if the solvent of lipid was changed to methanol or others in which DHB was soluble, the matrix layer was totally destroyed and formed a coffee ring-like shape (data not shown).

Moreover, it was found that when DHB (60 mg/mL) was dissolved in chloroform-methanol (2:1, v/v), and 2.5 μL of the matrix solution were manually dropped on the MALDI probe, a uniform DHB layer could be generated. After manually dropped 1.5 μL of the lipid solution (dissolved in chloroform), the DHB layer still remained homogeneous (shown in Fig. 1E). The result of SEM showed that the DHB crystals with the size around 30 μm were neatly arranged, presenting a relatively uniform structure (shown in Fig. 1F). Although the micro-morphology observed by SEM was different, further experiments showed that its function in desorption and ionization of lipids were almost identical with the DHB layers constructed by μMatrix spotter machine. This provided an alternative approach to construct a very uniform DHB layer for MALDI-FTICR MS.

3.2 The uniform DHB layer as matrix of MALDI-FTICR MS for analysis of lipids

The uniform DHB layer as matrix of MALDI-FTICR MS for analysis of lipids was investigated. Fig. 2 showed mass spectra of MALDI-FTICR MS analyzing lipids extracts from cell pellets. When the DHB layer was used as matrix for positive ion detection, PCs, LPCs, SMs, TAGs, PEs,

PAs, etc, were detected. Among of them, PCs were dominated. As a major constituent of cell membrane, PCs possessed a quaternary ammonia group (permanent positive charge), which was sensitively detectable in positive ion mode. This was the reason why the positive ion spectra of cell extracts were dominated by PC signals. When the DHB layer was used as matrix for negative ion detection, Pas, PEs, PSs and PIs were detected.

Taking advantage of the benefit of high resolution of FTICR MS and the fragment ions obtained by MS/MS, lots of lipids were identified. Fig. 3 showed mass spectra of MS/MS for several lipids. In positive ion detection mode, a neutral loss (NL) of 98 or 43 indicated the presence of a PA (Fig. 3A) or PE (Fig. 3C), respectively. PE also gave rise to another typical NL of 141(Fig. 5C). A NL of 59 indicated the presence of SM or PC (Fig. 3B), which also gave rise to a characteristic fragment ion at m/z 184, the polar head group of PC or SM. In negative ion detection mode, the fatty acid chain in sn-2 position of PI was easily dissociated (Fig. 3D).

The MALDI-FTICR MS reproducibility of spot-to-spot on the DHB layer was then tested. The laser irradiated different locations of the matrix-analyte co-crystal, and the mass spectra of lipids extracts from cell pellets were shown in Fig. 4. Undoubtedly, a satisfied spot-to-spot reproducibility was obtained (shown in Fig. 4A). Mass spectrometry imaging was also used to further investigate the reproducibility of MALDI-FTICR MS analysis. Fig. 4B showed the morphology of DHB-analyte co-crystal (B1) and mass spectrum imaging of representative ion with m/z at 695.4631 (B2), 728.5208 (B3), and 768.5527 (B4), respectively. From the images, it seemed that these compounds were distributed relatively homogenously across the whole spot, which ensured the spot-to-spot reproducibility.

3.3 MALDI-FTICR MS for quickly exploring the changes of lipids in drug resistant tumor cells

Lipid extracts from drug resistant tumor cells (A2780T, 6 samples) and paired drug sensitive tumor cells (A2780, 6 samples) were analyzed by MALDI-FTICR MS. The MS data for 4 different locations for each sample were acquired, and total 48 MS spectra were integrated and divided into 2 groups for statistic analysis. PCA and PLS-DA score plots were shown in Fig. 5. As can be seen in Fig. 5, the specimens gathered closely according to cell type, and a dramatic shift in the score plots was also observed for different cell lines in both PCA and PLS-DA plots, indicating that lipids were obviously changed in drug resistant tumors cells. Combining the results of PCA and PLS-DA, the main changed lipids included 2 PAs, 13PCs, 2PEs, 6 PIs, 2 SMs and 1 GM, and the detailed information was listed in Table 1. The most changed lipids come from the PCs and PIs. But the changes were observed to be inconsistently altered, where some PCs and PIs were up-regulated, and some of them were down-regulated.

Furthermore，correlation analysis showed that the changed lipids obtained by MALDI-FTICR MS were significantly correlated with those obtained by UPLC-ESI MS (shown in Fig. 6, $p = 0.0036$, $R^2 = 0.85171$), suggesting that this approach, a uniform DHB layer-assisted MALDI-FTICR MS, is effective for exploring the changes of lipids in biological systems.

Based on the changed lipids identified, pathway analysis using MetPA software showed that two pathways, glycerophospholipid metabolism and glycosylphosphatidylinositol (GPI)-anchor biosynthesis, might be involved in tumor cells for drug resistance. Glycerophospholipids were main components of cell membrane, and our data showed that the altered glycerophospholipid metabolism might change the composition of cell membrane to resist the interference of

exogenous drug. Recent research showed that phosphatidylinositol 3-kinase (PI3K) played an important role in paclitaxel-resistance of ovarian carcinoma cells,[22,23] and the glycosylphosphatidylinositol(GPI)-anchor biosynthesis might be a response to the PI3K enzyme activity. The mechanism underlying these perturbation and the enzymes and genes involved in this process required further study.

4. Conclusions

In this study, a uniform DHB layer-assisted MALDI-FTICR MS-based approach was developed for the rapid analysis of lipids extracts from cell pellets. When the lipids extracts (dissolved in chloroform) were deposited on the uniform DHB layer, benefiting by the insolubility of DHB in chloroform, the uniform DHB layer was still remained, and gave rise to a uniform matrix-analyte co-crystal, which greatly improved the spot-to-spot reproducibility. This method could be employed for quickly exploring the changes of lipids in complex biological system, and through combining the benefit of high resolution of FTICR MS with statistical interpretation of data, lots of lipids, in particular the changes of lipids, were identified. Since MALDI-FTICR MS analyses can be performed at very high rates in comparison to other MS technologies, this approach provides an extremely sensitive and high-throughput platform for lipidomics study. Compared with UPLC-ESI MS-based method for lipidomics study, the uniform DHB layer as matrix for MALDI-FTICR MS-based method is higher throughput and more convenient, which presented an alternative approach for lipidomics study.

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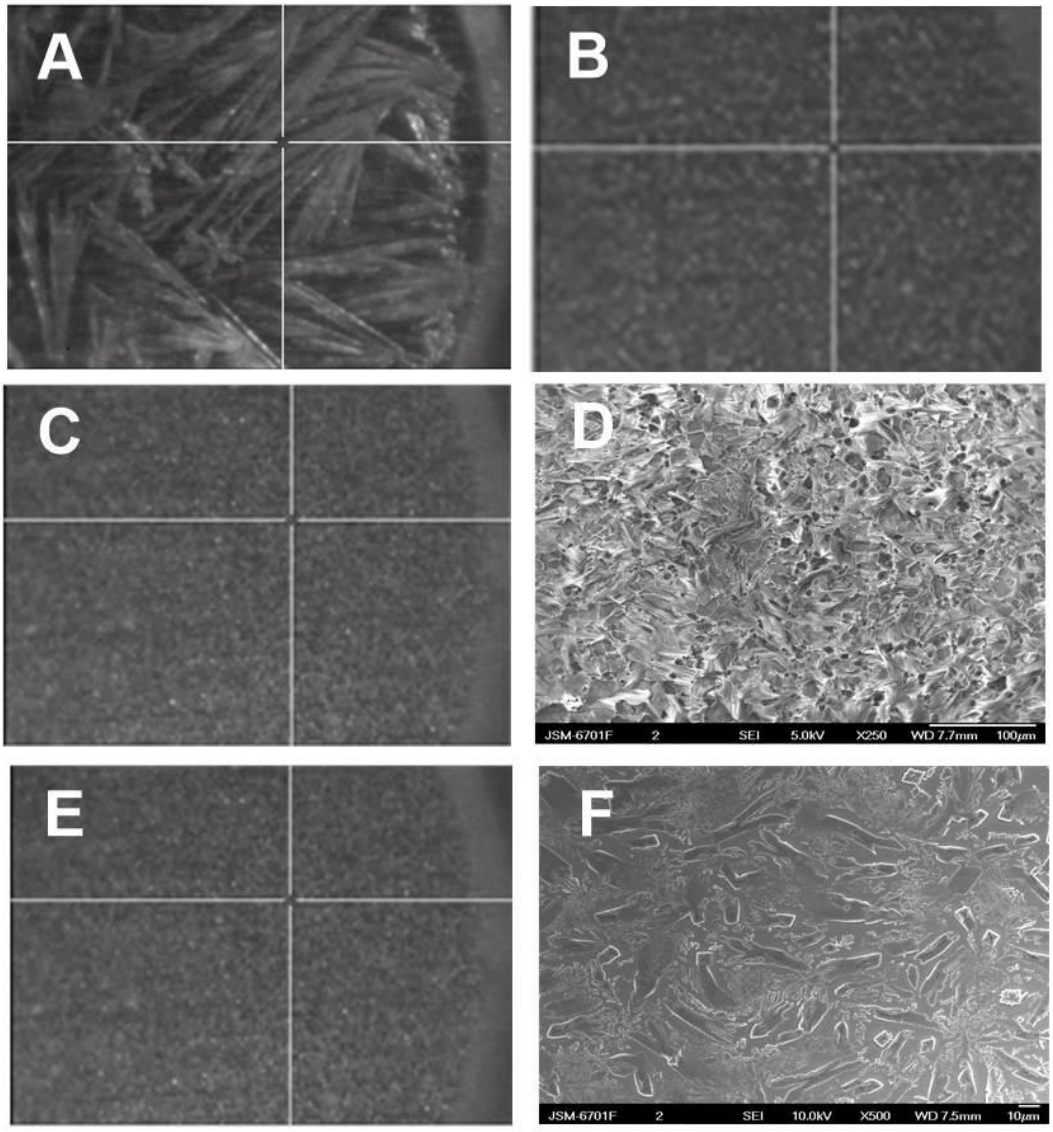

Fig. 1 Morphologies of DHB crystal or DHB-analyte co-crystal. (A) DHB with concentration at 30 mg/mL, ethanol as solvent, was manually dropped on the MALDI probe; (B) DHB with concentration at 30 mg/mL, ethanol as solvent, was printed on the MALDI probe by μMatrix spotter machine; (C) On the DHB layer, shown in Fig. 1B, 1.5 μL of the analyte, chloroform as solvent, was deposited; (E) 2.5μL of DHB with concentration at 60 mg/mL, chloroform–methanol (2:1, v/v) as solvent, was manually dropped on the MALDI probe, then 1.5 μL of the analyte, chloroform as solvent, was deposited. A, B, C and E were obtained by the camera equipped in the MS instrument. (D) and (F) were high resolution images of C and E, respectively, obtained by scanning electron microscopy (SEM JEOL 6701).

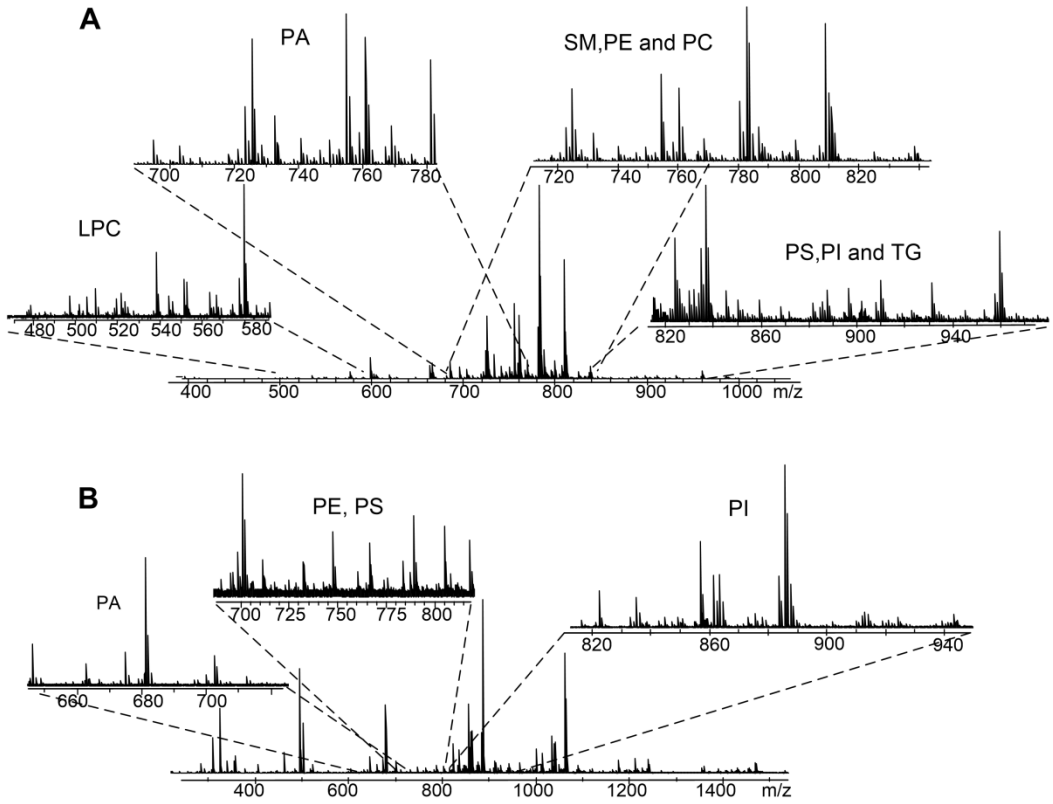

Fig. 2 Mass spectra of lipids extracts form cell pellets by MALDI-FTICR MS in positive mode (A) and negative mode (B), a uniform DHB layer used as matrix.

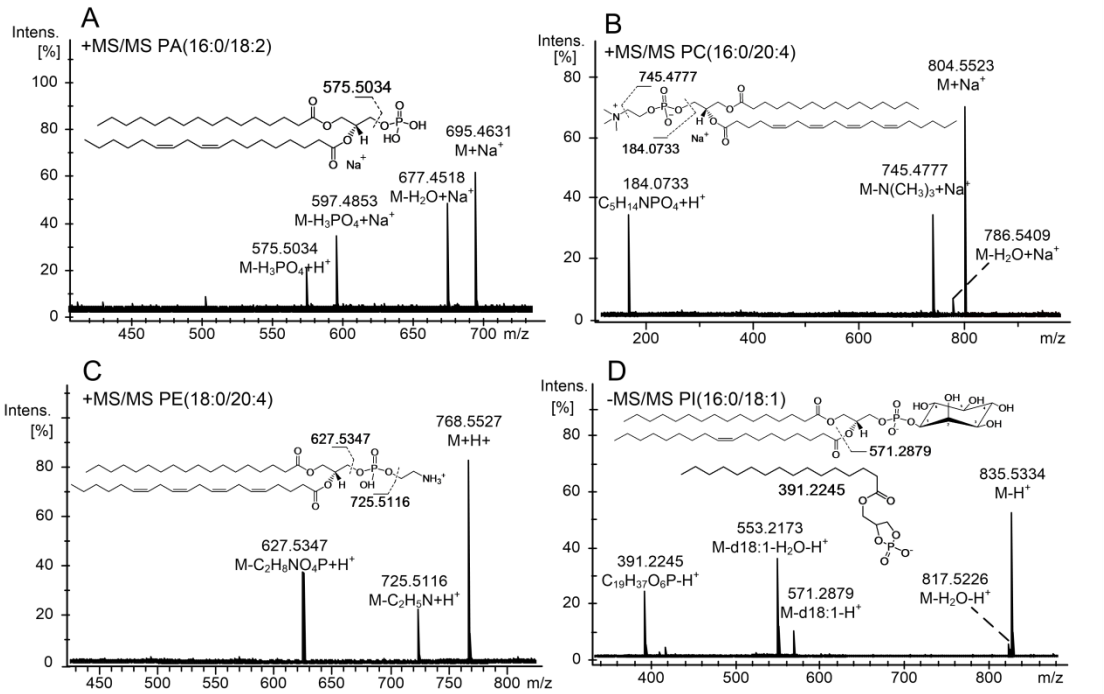

Fig. 3 MS/MS spectra obtained from PA (34:2) (A), PC (36:4) (B), PE (38:4) (C), and PI (16:0/18:1) (D) present in lipids extracts from cell pellets, acquired on a Bruker solariX MALDI-FTICR MS. Collision induced dissociation (CID) was used for acquiring MS/MS spectra, and argon was used as collision gas.

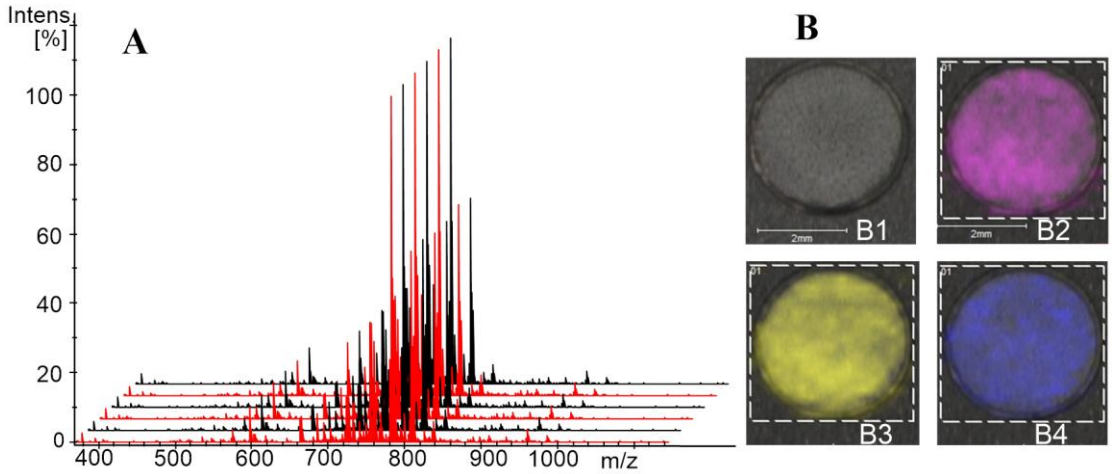

Fig. 4 The reproducibility of MALDI-FTICR MS analysis using a uniform DHB layer as matrix. (A) Representative mass spectra of lipids extracts from cell pellets when a laser irradiated different locations of DHB-analyte co-crystal. (B) The morphology of DHB-analyte co-crystal (B1) and mass spectrum imaging of representative ion with m/z at 695.4631 (B2), 728.5208 (B3), and 768.5527 (B4), respectively.

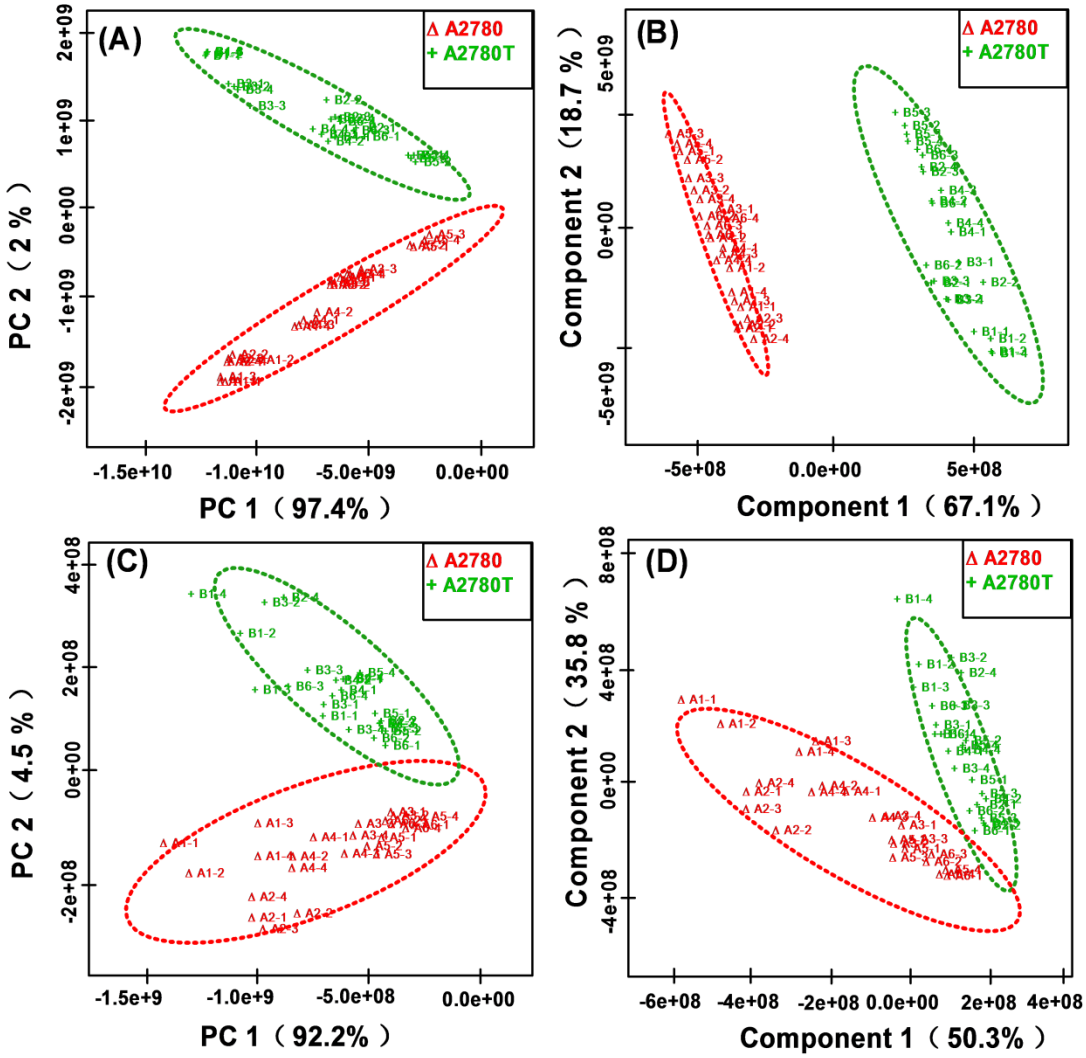

Fig. 5 Pattern recognition analysis for group A2780 (drug sensitive tumor cells) and A2780T (paired drug resistance tumor cells). (A) PCA model result in positive mode; (B) PLS-DA model result in positive mode; (C) PCA model result in negative mode; (D) PLS-DA model result in negative mode. The ovals indicate the 95% confidence interval for sample groupings.

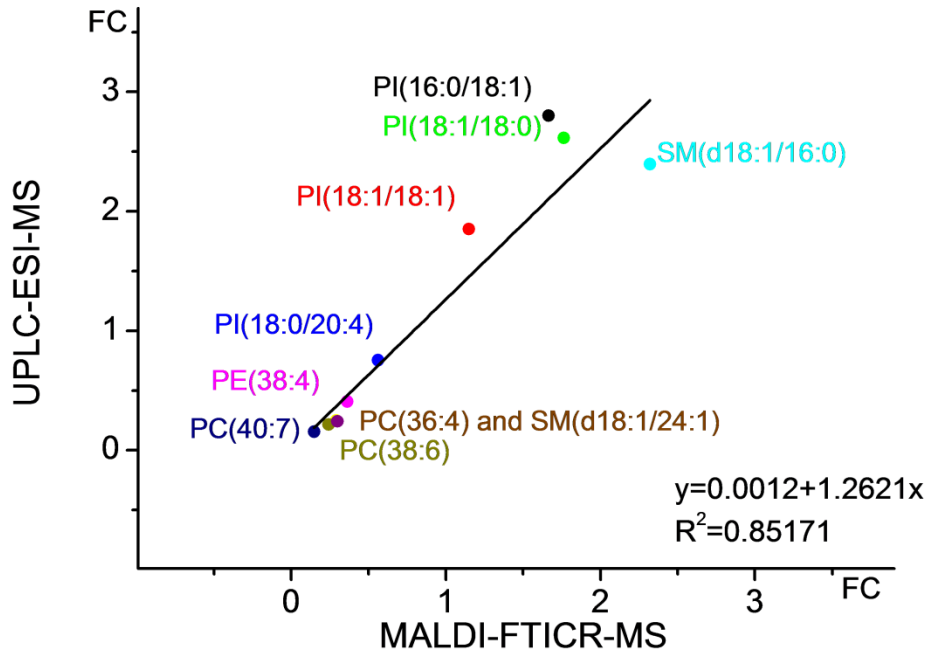

Fig. 6 Correlation of several changed lipids between the results obtained by MALDI-FTICR MS and UPLC-ESI MS (p = 0.0036, R^2 = 0.85171).