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MALDI mass spectrometry imaging of proteins exceeding 30 000 daltons

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Since its introduction 10 years ago by Caprioli and associates, MALDI mass spectrometry imaging has enabled spatial analysis of drugs, lipids, peptides, and polypeptides. In polypeptides, the detectable mass range is limited to small proteins with a mass less than 25 kDa. This is a limitation, as many proteins, including cytokines, growth factors, enzymes, and receptors have molecular weights, exceeding 25 kDa. In the present work, we report the development of a novel strategy to observe higher mass proteins up to 30 kDa.

Material/Methods: We investigated the development of sample preparation methods based on hexafluoroisopropanol (1,1,1,3,3,3-hexaluoro-2-propanol) and 2,2,2-trifluoroethanol solvents for protein solubilization optimized for high-mass proteins.

Results: We were, for the first time in mass spectrometry imaging, able to detect to proteins up to 70 kDa directly from tissue. These developments indicate future avenues by which the sensitivity of protein mass spectrometry imaging can be further improved. We applied these developments to ovarian cancer and demonstrate that protein are similar to that which can be obtained using 2D gel based analyses.

Conclusions: Increasing the possibility of detecting proteins and high-mass proteins is key for developing direct tissue proteomics and especially any potential functional investigation. These data will open the door of a novel step in mass spectrometry imaging.

key words: **MALDI MS imaging • high masses • proteins • profiling • hexafluoroisopropanol (1,1,1,3,3,3-hexaluoro-2-propanol) • 2,2,2-trifluoroethanol**

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Background

After 10 years of continuous development MALDI mass spectrometry imaging (MSI) has become a powerful and versatile tool for analyzing different classes of endogenous and exogenous molecules [1,2]. It has become an established technology for imaging drugs and their metabolites [3,4]; molecular images of peptides and small proteins up to 25 kDa can be routinely obtained [2,5–8]; and recently, major improvements have also been reported for imaging lipids [9–13]. However, proteins exceeding 25 kDa are not routinely detected by MALDI MSI. This represents a real methodologic limitation, as many classes of proteins with important biological activities – such as most cytokines, growth factors, enzymes, receptors, proproteins, and neuropeptide precursors – are larger than 25 kDa. Two recent articles reported sample preparation methods that enable MSI of higher mass proteins [14,15]. The first method uses extensive water washing procedures to deplete abundant soluble proteins followed by automated application of a matrix solution containing a high percentage of organic solvent. This sample preparation allowed detection of a 28-kDa integral crystalline lens membrane protein [14]. In the second approach, a matrix application protocol using Triton X-100 was shown to help detect proteins ranging from *m/z* 25 000 to 50 000 [15].

These observations suggest several lines of reasoning for the lack of sensitivity for higher mass proteins, which can be exploited for the further development of sample preparation methods. The use of organic solvents or Triton X-100 suggests higher mass proteins may not be detected, because they are not efficiently solubilized in the matrix solution and consequently, are not extracted from the tissue. The detection of higher mass proteins after depletion of abundant soluble proteins suggests competition with the abundant proteins for incorporation into the matrix crystals and/or ionization in the MALDI process (suppression effects [16]) may be another reason limiting routine detection of higher mass proteins.

MALDI predominantly generates singly charged protein ions, so a high-mass protein ion will be detected at high *m/z*. The technology typically used for protein MSI, a time-of-flight mass spectrometer equipped with a micro-channel plate (MCP) detector, is not well-suited to the detection of high *m/z* ions. The initial impact of an ion onto the detector releases some electrons, which are then amplified through the MCP to generate the signal. The MCP detection process is known to favor lower *m/z* ions because the ion to electron conversion efficiency is nonlinearly dependent on velocity; exhibiting a threshold velocity below which no signal is obtained, then increasing rapidly with increasing velocity before reaching a plateau in which detector response is insensitive to increasing velocity [17].

In TOF analyzers, ion velocity is inversely proportional to the square root of the *m/z* ratio. Ions of high *m/z* may not be detected within the optimum detection-efficiency plateau and thus generate less detector signal. Furthermore, MCP detectors have a finite amplification potential within any single time-of-flight scan. Lower mass ions, detected first, can saturate the detector leading to even lower detection efficiencies for higher mass ions. In typical MALDI applications in proteomics, such as peptide fingerprinting and LC-MALDI, high-mass insensitivity is rarely a problem because complex mixtures of proteins with masses above 20 000 are not analyzed. The tissue sections analyzed by MALDI MSI can result in a significant background, which is more intense in the low-mass region but continues throughout the entire mass range [18]. If detecting a wide mass range this background, as well as peptide/protein ions of lower mass, can saturate the MCP detector and further decrease the detection efficiency for higher mass protein ions.

In the present paper, we have investigated the development of sample preparation methods based on hexafluoroisopropanol (1,1,1,3,3,3-hexaluoro-2-propanol, HFIP) [19,20] and 2,2,2-trifluoroethanol (TFE) solvents for protein solubilization optimized for high-mass proteins. Using these sample preparation procedures proteins, up to 70 kDa were detected. This opens the door of protein biomarkers tracking directly from tissue without any extraction keeping their tissue localization.

Material and Methods

Materials

Sinapinic acid (SA), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), trifluoracetic acid (TFA), 2,2,2-Trifluoroethanol (TFE), ethanol (ETOH), acetone, acetonitrile (ACN), chloroform, water CHROMASOLV plus for HPLC (H2O) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Samples

Rodent brains

Adult male Wistar rats weighing 250 to 350 g and adult, male, wild-type mice weighing 30 to 50 g (animal use accreditation by the French ministry of the agriculture No. 04860) maintained under standard care were used. Animals were killed by decapitation and immediately dissected to remove the brain, which was then flash frozen in liquid nitrogen and stored at –80°C.

Ovarian biopsies

Tissues, ascites, and cystic fluids were obtained with informed consent and institutional review board approval (CCPPRBM Lille: CP 05/83), from patients undergoing ovarian tumor resection at Hospital Jeanne de Flandre (Lille, France). Patient information was collected, including sex, age, treatment received before and after surgery, extent of surgery, current status (alive, alive with progressive disease, deceased, and cause of death), and survival from the time of original pathologic diagnosis. Samples were collected at the time of surgery, immediately frozen, and stored at –80°C until analysis.

Tissue preparation

Animal brains and ovarian biopsies

Thin 10- to 12-µm tissue sections were cut from frozen rat/mouse brains and ovarian biopsies using a Leica CM1510S cryostat (Leica Microsystems, Nanterre, France) and placed

onto ITO-coated conductive glass slides (Bruker Daltonics, Bremen, Germany). For ovarian biopsies, histopathologic diagnoses were performed by a pathologist, blinded to the original clinical diagnosis, from adjacent H&E-stained sections. Tissue sections were submitted to different washing steps to remove salts and abundant lipids. Each tissue section were first immersed in a bath of cold acetone [21] for 30 seconds followed by a bath of cold ETOH 95% [22] for 30 seconds, and finally immersed in chloroform (21) for 1 minute.

MALDI MSI and profiling

Five mL of a solution containing 10 mg/mL of SA in AcN/aqueous TFA 0.1% (7:3, v/v) was prepared. The solution was deposited on to the tissue using an automatic sprayer (ImagePrep, Bruker Daltonics). Molecular images were acquired on an UltraFlex II MALDI-TOF/TOF instrument (Bruker Daltonics) equipped with a Micro-Channel Plate (MCP) detector. The instrument is equipped with a Smartbeam laser and is controlled by FlexControl 3.0 (Build 158) software (Bruker Daltonics). All protein spectra were processed with FlexAnalysis 3.0 (Bruker Daltonics) using the Top Hat baseline subtraction method and 4 cycles of Gauss smoothing with a width of 2 m/z.

Mass spectrometry imaging datasets were recorded in positive ion, linear time-of-flight mode, and averaged at 1000 laser shots for each position. Analysis of higher mass proteins required higher laser fluencies. Typically, for these experiments, the laser offset was set to 30%, laser range 20%, laser fluence 70%, and the laser focus set to medium. The images were acquired following a raster of 200 by 200 µm and reconstructed using FlexImaging 2.1 (Build 15) (Bruker Daltonics).

High-mass protein profiling

Two sample preparation procedures were developed. In the first procedure, a micropipette was used to deposit a droplet of 10 mg/mL SA in pure HFIP followed by a droplet of 20 mg/mL SA in AcN: 0.1% TFA (7:3, v/v). In the second procedure, a droplet of TFE: 20% TFA (1:1, v/v) was deposited onto the tissue, then a droplet of 20 mg/mL SA in ETOH: 0.1% TFA (9:1, v/v), and finally a droplet of 10 mg/mL SA in AcN: 0.1% TFA (7:3, v/v).

High-mass protein MSI

Automatic spraying

A solution of 10 mg/mL SA in TFE: 10% TFA (1:1, v: v) was first deposited on to a rat brain tissue section using 20 cycles of spraying for 1.5 seconds and drying for 30 seconds. A solution of 10 mg/mL SA in ETOH: 0.1% TFA (9:1, v/v), which was deposited after a final solution of 5 mg/mL SA in AcN: 0.1% TFA (7:3, v/v) using the same spraying method. High-mass protein analysis also was performed after imaging low mass polar proteins by removing the matrix from the tissue section with a bath of MeOH: H_2O (1:1, v/v) for 1 minute, and then applying the high-mass matrix solution.

Automatic micro-spotting

A chemical inkjet printer (CHIP-1000, Shimadzu, Kyoto, Japan) was used to sequentially deposit arrays of nl-sized droplets of matrix solutions with a 300-µm pitch on to a rat brain tissue section. Fifteen nl of a solution of 10 mg/mL SA in TFE: 10% TFA (1:1, v/v) was first deposited onto the tissue, followed by 10 nl of a solution of 10 mg/mL SA in ETOH: 0.1% TFA (9:1, v/v), and finally by 15 nl of a solution of 5 mg/mL SA in AcN: 0.1% TFA (7:3, v/v).

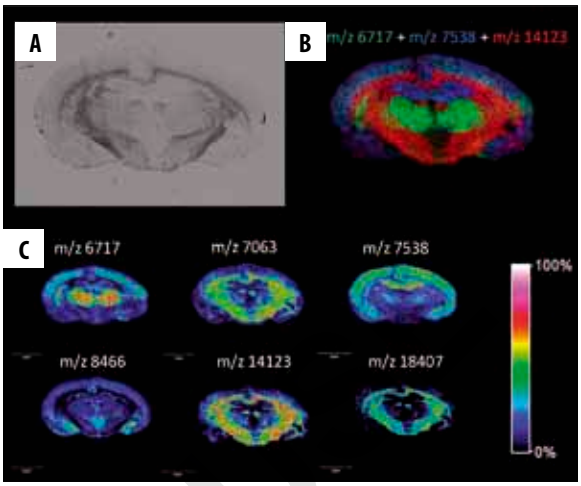

Figure 1. MALDI MSI of proteins obtained from a rat brain tissue section prepared by automatic spraying of the matrix solution 10 mg/ml SA in AcN: 0.1% TFA (7:3, v/v). (**A**) Optical image of the tissue section, (**B**) overlaid molecular images of protein ions detected at *m/z* 6717, 7538 and 14 123, (**C**) molecular images of protein ions detected at *m/z* 6 717, 7 063, 7 538, 8 466, 14 123 and 18 407.

Results

MALDI MSI of proteins

In MALDI MS as well as in MALDI MSI, proteins are typically analyzed using SA as matrix in a polar solvent such as AcN: 0.1% TFA. Figure 1 shows protein MSI measurements from a rat brain tissue section prepared using automatic spray deposit of the matrix solution 10 mg/mL SA in AcN: 0.1% TFA (7:3, v/v). These conditions generate a thin, homogenous layer of small crystals covering the tissue section and enabling images with specific distributions to be recorded (Figures 1A,C). Such sample preparation conditions are typically for high spatial fidelity MSI of proteins in tissue; however, only a restricted number of proteins and within a restricted mass range are typically observed, with most protein signals below *m/z* 25 000.

It has been shown that washing the tissue sections with organic solvents such as chloroform [21] and ETOH [22,23] before depositing the matrix provides spectra with a higher intensity and more protein signals in the mass range up to 20 000, and that Liam images can be obtained showing the specific location of the proteins. Without the organic wash, only a few proteins could be detected (Figure 2) that have a relatively low intensity and signal/noise ratio. In contrast, spectra acquired from tissue prewashed with organic solvents from an adjacent rat brain tissue section, show a high number of intense protein signals up to *m/z* 20 000 and a few protein signals with lower intensity above 20 000 *m/z* (Figure 2). This effect has been attributed to the removal

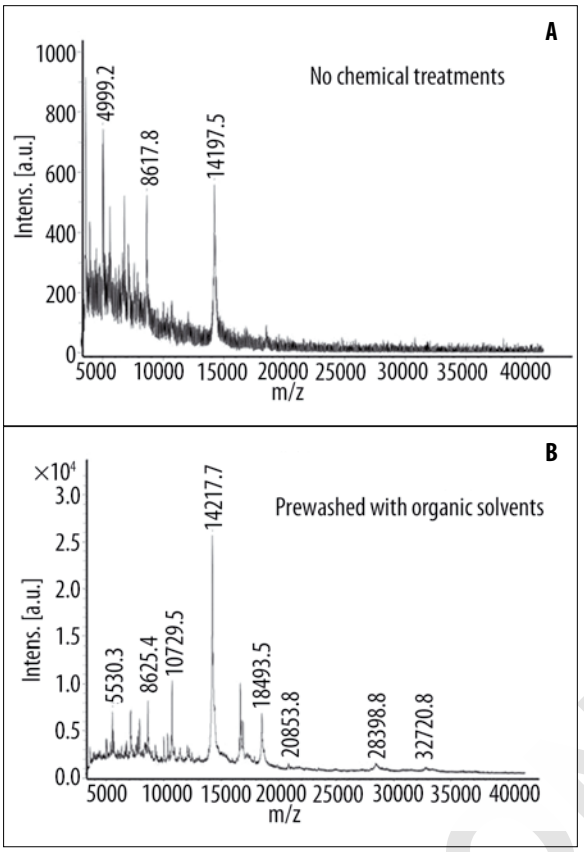

Figure 2. MALDI Mass spectra from rat brain tissue sections prepared by deposition of the matrix solution 10 mg/ml SA in AcN: 0.1% TFA (7:3, v/v). (**A**) unwashed tissue, and (**B**) tissue prewashed with successive baths of cold acetone, cold 95% EtOH, and chloroform prior to matrix deposition.

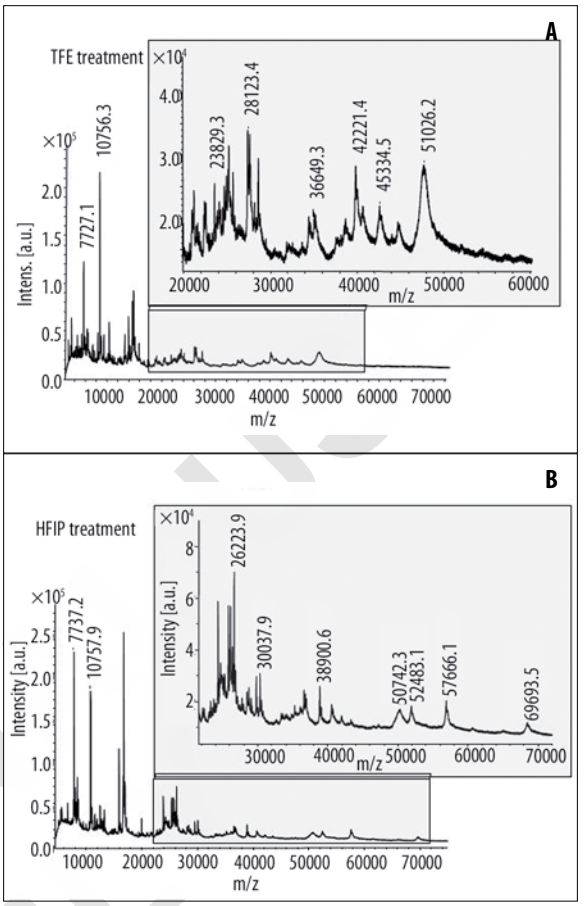

Figure 3. Mass spectra acquired from a rat brain tissue section after (**A**) TFE treatment and (**B**) HFIP treatment.

of salts and small organic compounds such as abundant lipids that lead to ion suppression effects [16]. Nevertheless, even with organic washes, protein signals above m/z 20 000 were either detected with very low intensity or not at all.

Sample preparation for high-mass profiling

In typical tissue proteomics experiments, protein extraction involves different steps to disrupt noncovalent interactions and separate the proteins from other molecules. The main interactions are noncovalent, including ionic, hydrogen bonds, and hydrophobic interactions. Hydrophobic interactions can be disrupted by using compounds or solvents that provide a competing hydrophobic environment or that disrupt protein structure (chaotropes). In polar solvents or pure water, a hydrophobic environment is created by detergents such as Triton X100 or SDS [24], and protein structures can be disrupted by chaotropes such as urea [25]. However, the high concentrations of detergents and chaotropes required for solubilization are mostly incompatible with MALDI mass spectrometry. Finally, denaturation of proteins by changing the solvent's pH has been shown to solubilize proteins with an efficiency close to that of SDS-based extraction [26]. With this background in mind, several procedures were investigated to improve protein solubilization, and thus, extraction and sensitivity.

Initial experiments involved using a micropipette to deposit a 10% TFA solution onto a rat brain tissue sections to denature the proteins. A matrix solution of 20 mg/mL SA in AcN: 0.1% TFA (7:3, v/v) was then deposited onto the tissue. Close examination of the mass spectra obtained with this sample preparation method revealed no notable improvements. In a second study, the MSI compatible detergent octyl beta-D-glucopyranoside [27] was investigated. A solution of 10% TFA (aq.) containing 1% detergent was deposited onto rat brain tissue sections and subsequently covered with the same SA matrix solution. The mass spectra recorded using this second protocol also did not show any notable improvement compared with those obtained without any such treatment.

The matrix solution normally used to extract proteins from the tissue is based on the polar solvents AcN and 0.1% TFA (aq.). AcN is a relatively hydrophobic solvent known to solubilize many proteins of intermediate hydrophobicity. It was then investigated if the hydrophobic solvents TFE [28,29] and HFIP [30], which have been used to extract membrane proteins [31–33] and high-mass proteins [34], could aid in detecting high proteins directly from tissue.

A solution of 20% TFA: TFE (1:1, v/v) was deposited onto the tissue section to extract the proteins from the tissue. In MALDI, co-crystallization of proteins with the matrix is crucial for their analysis. As SA is not soluble in TFE, another

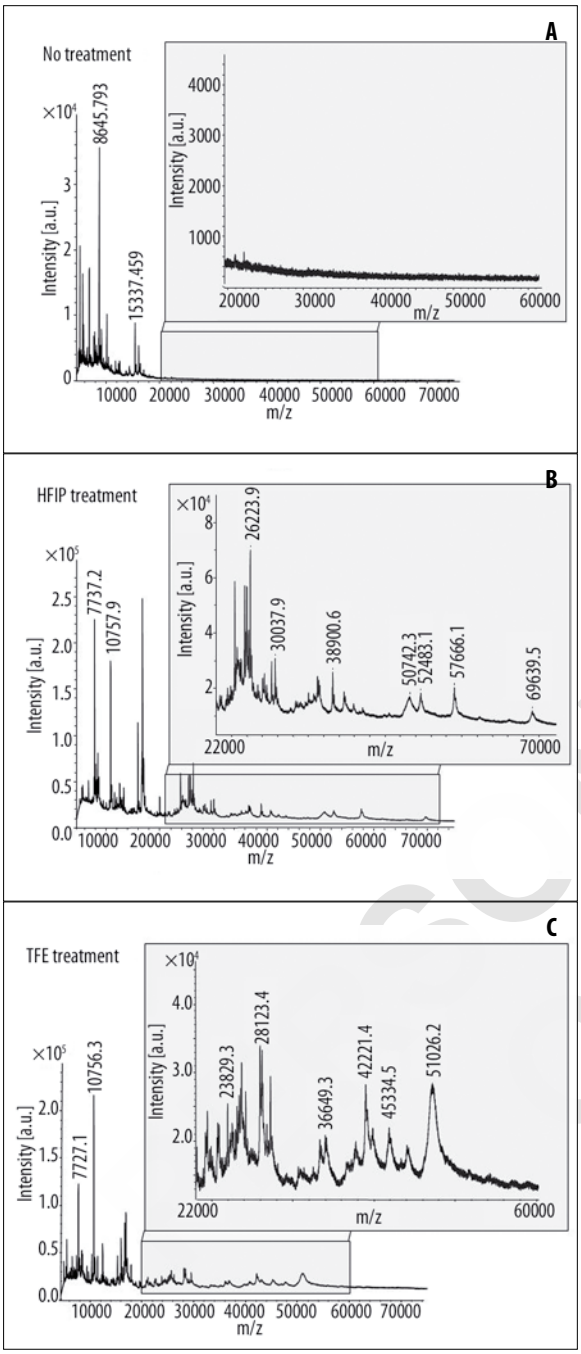

Figure 4. Mass spectra acquired from a rat brain tissue section after deposition of (**A**) SA in AcN: 0.1% TFA (7:3, v/v), and after removal of the first matrix coating followed by HFIP treatment (**B**) or TFE treatment (**C**).

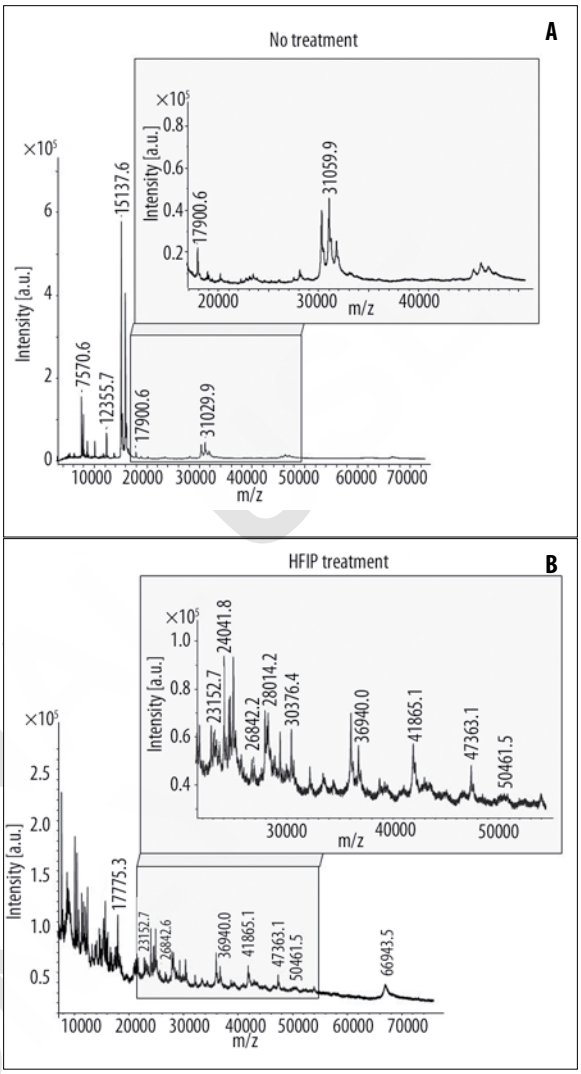

Figure 5. Mass spectra acquired from ovarian cancer tissue after deposition of (**A**) 20 mg/ml SA in AcN: 0.1% TFA (7:3, v/v), and after subsequent matrix removal and HFIP treatment (**B**).

solvent was used to dissolve the matrix. After the TFE treatment, 10 µL of the solution 20 mg/mL SA in ETOH: 0.1% TFA (9:1, v/v) was deposited onto the tissue to co-crystallize the proteins with the matrix. However, this led to formation of large matrix crystals and a dramatic increase in chemical noise in the mass spectra. Recrystallization of the large matrix crystals by addition of a third solution, 20 mg/mL SA in AcN: 0.1% TFA (7:3, v/v), produced small white matrix crystals containing higher mass proteins. Mass spectra recorded from rat brain tissue sections after the complete 3-step procedure contained protein signals up to *m/z* 50 000 with good S/N (Figure 3A). In comparison, spectra from an adjacent tissue section that was not prepared using the 3-step TFE procedure contained no ions above *m/z* 30 000.

The number of steps needed for denaturation, extraction, and incorporation of high-mass proteins in matrix crystals using TFE makes it quite impractical for MSI. The solvent HFIP is known for its extraction efficiency of hydrophobic proteins and was found to be an excellent solvent of the matrix SA; therefore, enabling direct co-crystallization with high-mass proteins. It was found that high-mass proteins could be detected by depositing the matrix solution 20 mg/mL SA in pure HFIP, followed by addition of the recrystallization solution 20 mg/mL SA in AcN: 0.1% TFA (7:3, v/v). This led to detecting the higher mass proteins, up to *m/z* 70 000, with higher sensitivity and higher reproducibility than were obtained using the TFE procedure (Figure 3B).

Table 1. Molecular masses of peaks found in MALDI MSI after using the HFIP high-mass protein sample preparation that are consistent with known ovarian cancer protein biomarkers.

Protein name	Molecular mass (Da) determined by MALDI MSI with high-mass procedure	Molecular mass (Da) protein identified in ovarian cancer	References
Tetranectin (CAA45860)	17 775	17 776	[36–39]
Neutrophil gelatinase-associated lipocalin precursor	22 576	22 571	[40]
Plasma retinol-binding protein precursor	22 986	22 990	[41]
Metalloproteinase inhibitor 1 precursor	23 152	23 153	[42]
Kallikrein 5 Precursor	26 842	26 838	[43]
Isoform 1 of Urokinase plasminogen activator surface receptor precursor	36 940	36 949	[44,45]

Figure 4 indicates that detecting high-mass proteins from tissue requires specific treatments using hydrophobic solvents. Smaller, polar proteins could be detected before high-mass protein analysis by sequential sample preparation and MSI analysis of the same tissue section. Rat brain tissue sections were first prepared using a solution containing 20 mg/mL SA in AcN: 0.1% TFA (7:3, v/v). After analyzing the tissue section, the matrix was removed by immersing the tissue in a bath of MeOH: H_20 (1:1, v/v) for 1 minute. The tissue was then dried, and the TFE or HFIP treatment applied. The spectrum obtained after the first matrix preparation, before TFE/HFIP treatment, included only low-mass proteins up to *m/z* 20 000 (Figure 4A). After removing the first matrix layer and HFIP/TFE treatment, high-mass proteins up to *m/z* 70 000 were obtained (Figure 4B,C). This strategy offers new perspectives for protein analysis and imaging from tissue sections by allowing, for the first time, measurement of small polar proteins followed by higher mass proteins.

Application in pathological proteomics: biomarker analysis

Recently, our group has applied MALDI MSI for biomarker research of ovarian cancer. MALDI MSI allowed detection of different markers, including the Cter fragment of the immunoproteasome 11s (m/z 9744) as a biomarker for serious cancers [35]. We tested high-mass sample preparation procedures on ovarian cancer biopsy samples to determine if additional proteins could be detected. This was performed as a second MSI experiment to retain the lower mass polar proteins provided by the standard sample preparation procedures. 10 µl of the solution 20 mg/mL SA in AcN: 0.1% TFA (7:3, v/v) was deposited onto an ovarian cancer biopsy tissue section. As shown in Figure 5A, many small, polar proteins were detected up to *m/z* 30 000 but few were found above this mass. After removal of this matrix using a bath of MeOH: H_2O (1:1, v/v), the HFIP procedure was applied. Figure 5B shows the MS spectrum obtained after HFIP treatment, the number of peaks detected in the *m/z* 20 000–50 000 range has clearly increased. Some masses are in line with ones detected in classic proteomics (Table 1). Future investigations will determine if the higher mass proteins released using the new sample preparation strategies include new candidate biomarker proteins.

Discussion

To date, protein MSI of tissue sections has been limited to lower mass proteins. In this study, we developed new samples preparation protocols by improving protein extraction from the tissue. It was demonstrated that highly hydrophobic solvents HFIP and TFE enabled high-mass proteins to be detected, even without the high-mass detector. These treatments led to detection of proteins up to *m/z* 70000. High-mass procedures can be implemented after a regular MSI experiment of small, polar proteins to increase the number of proteins detected and to expand the mass range of proteins included in the analysis. This was performed with rat brain tissue and with ovarian cancer biopsy tissue, and demonstrated that small, polar proteins (first MSI experiment) and higher mass proteins (second MSI experiment) can be sequentially analyzed in a single tissue. The higher mass proteins made available by these strategies increase the possibility of detecting new candidate biomarkers.

Conclusions

Targeted chemical treatments are needed to improve protein extraction from tissue and to incorporate them into the matrix crystals. Future work will concentrate on improving protein sensitivity and translating these sensitivities to MSI, in which the need to minimize protein delocalization can reduce protein extraction efficiency.

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