MALDI-TOF MASS SPECTROMETRY IMAGING OF METALLOTHIONEIN IN CHICKEN EMBRYO

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Abstract: In last decades the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry imaging (MALDI-TOF MSI) has become an outstanding tool for detecting spatial distribution of different biomarkers in a variety of tissue samples. It utilizes the benefits of MALDI-TOF technique, which are rapid measurements of all mass spectra in a wide mass range and detection of analytes molecular weights. Moreover, the *in situ* identification of targeted biomarkers can be performed too. In our study, we focused on detection of metallothionein (MT) in chicken embryo. Metallothioneins are low-molecular weight proteins connected with cancer development and protection of organism against environmental pollution. Their main functions are detoxification of heavy metals, maintaining ion homeostasis and protection against the oxidative stress. According to our knowledge, nobody has done MALDI-TOF MSI of MT so far. Therefore, we have selected MT as our studied analyte not only because of this fact but also because a part of team IGA project is aimed on MT.

Key Words: zinc-binding proteins, MALDI-TOF MSI, chicken embryo

INTRODUCTION

Metallothioneins (MTs) are low-molecular weight proteins, usually around 6–7 kDa, where cysteines form at least one third of all amino acids and their thiol groups serve for coordination with divalent metal ions, especially Zn and Cu (Lynes et al. 2014). They are connected with cancer development, protection of the organism against environmental pollution effects and also with chemoresistance of cells. Their main functions are probably the detoxification of heavy metals, maintaining ion homeostasis and protection against the oxidative stress. MTs exist in all kind of mammalian cells. Four isoforms of human MT (MT-1, MT-2, MT-3, MT-4) were found so far (Pinter et al. 2015) and according to UniProt database there were found two chicken MTs (MT1 and MT3).

The matrix assisted laser desorption/ionization (MALDI) technique was introduced by Karas et al. in 1985 (Karas et al. 1985). Three years later, the same research group published a first study on the utilization of this ionization method for mass spectrometry of proteins (Karas, Hillenkamp 1988). Nowadays, it is routinely used for characterization of peptides, proteins and identification of bacteria. Because of its soft ionization of biomolecules, MALDI was found to be useful for mass spectrometry imaging of a variety of samples where information regarding the spatial distribution of molecules is needed. At the turn of the third millennium, MALDI mass spectrometry imaging (MALDI MSI, MALDI imaging) was first used for the determination of protein expression in mammalian tissues (Stoeckli et al. 2001). Usually, MALDI is used in combination with time-of-flight mass spectrometry (TOF MS), because it measures complete mass spectra over wide mass ranges at the same time (Caprioli et al. 1997). There also exist other types of mass spectrometers used with MALDI, such as Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR MS) or linear ion trap with orbitrap mass

spectrometers (LTQ Orbitrap MS) (Chen et al. 2014, Solouki et al. 1995, Strupat et al. 2009). Currently, the MALDI MSI technique is the subject of a comprehensive research to improve it in different ways – time of analysis (Bednarik et al. 2014, Prentice et al. 2015), spatial resolution (Korte et al. 2015), and sensitivity and detection of different analytes (Flinders et al. 2015, Wang et al. 2015). Information gained from MALDI MSI can be correlated with immunohistochemical images (Caldwell et al. 2006) or with images from other techniques such as magnetic resonance imaging (Acquadro et al. 2009) or laser ablation-inductively coupled plasma mass spectrometry/atomic emission spectrometry (Bianga et al. 2014). There exist several extensive reviews on recent progress in MALDI MSI and on the development of MALDI imaging techniques that are recommended to readers with interest in this field (Dreisewerd 2014, Rompp, Spengler 2013, Svatos 2010).

We have focused this work on optimizing the MALDI-TOF mass spectrometry imaging of metallothionein in formalin-fixed and paraffin-embedded (FFPE) chicken embryo samples. The results from this work will help us in future experiments with metallothioneins in different tissues.

MATERIAL AND METHODS

Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise.

Model organism

The fertilized eggs of Lenghorn hen (Integra a.s., Zabcice, Czech Republic) were incubated at 37 °C and relative humidity of 55% in the incubator (RCom 50 MAX, Gyeongnam, Korea). The experiment was performed with embryo in the 7th developmental day. In this day, the embryo was removed from the shell and was paraffinized according to a protocol (Berril 2002).

MALDI-TOF mass spectrometry imaging

Preparation of tissue samples

FFPE chicken embryo was cut into 10 μm thin slices using microtome Leica SM2010 R (Baria s.r.o., Prague, Czech Republic) and slices were mounted onto ITO (indium-tin oxide) glass slides (Bruker Daltonik GmbH, Bremen, Germany). The conductivity of surface was checked by ohmmeter. Deparaffinization and antigen retrieval were performed according to the protocol by Casadonte et al. (Casadonte, Caprioli 2011). Position of tissue slices was marked by at least three teaching marks by white pencil corrector. Then the glass slides with samples were scanned by Epson Perfection V500 Office (Epson Europe B.V., Amsterdam, Netherlands) with resolution 2400 DPI. MALDI matrix was sprayed onto the glass slides with samples by Bruker ImagePrep (Bruker Daltonik GmbH, Bremen, Germany). 2,5-dihydroxybenzoic acid (DHB) (Sigma-Aldrich, St. Louis, MO, USA) was used as MALDI matrix. DHB was prepared in concentration of 30 mg.ml^{-1} in 50% methanol and 0.2% trifluoroacetic acid (TFA). MALDI matrix mixtures were thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Berlin, Germany) for 2 minutes at 50% of intensity at room temperature. The samples were ready for analysis after drying.

Mass spectrometry imaging

The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany). Softwares flexControl 3.4 and flexAnalysis 2.2 were used for data acquisition and processing of mass spectra and software flexImaging 3.0 was used for analysis of MSI data. Firstly, scanned images of tissue slices were loaded into flexImaging 3.0 and MALDI adapter with glass slides was loaded into mass spectrometer. Then, the position of MALDI adapter was taught according to white teaching marks on glass slides in the way, that MALDI adapter was moved in flexControl to a position of teaching marks and on each teaching mark the position was pointed manually in flexImaging by mouse pointer – thus the mass spectrometer was taught about the position of tissue slices. Next, regions of acquisition were highlighted by mouse pointer in flexImaging and raster spot width was chosen (100 μm). Before MALDI MSI, a measuring method was determined and mass spectrometer was calibrated on a mixture of peptide and protein calibration standards (Bruker Daltonik GmbH, Bremen, Germany). The laser power was set

to 65%. MALDI MSI was performed in the linear positive mode in the m/z range 2–20 kDa. The MS spectra were acquired by averaging 1600 sub spectra from a total of 1600 laser shots per raster spot. After selection of MALDI MSI automatic method the MALDI MSI started. When it finished, the mass spectra were automatically loaded into flexAnalysis, where they were processed (baseline substraction was performed), and finally the processed spectra were automatically loaded into flexImaging.

Mass spectrometry imaging

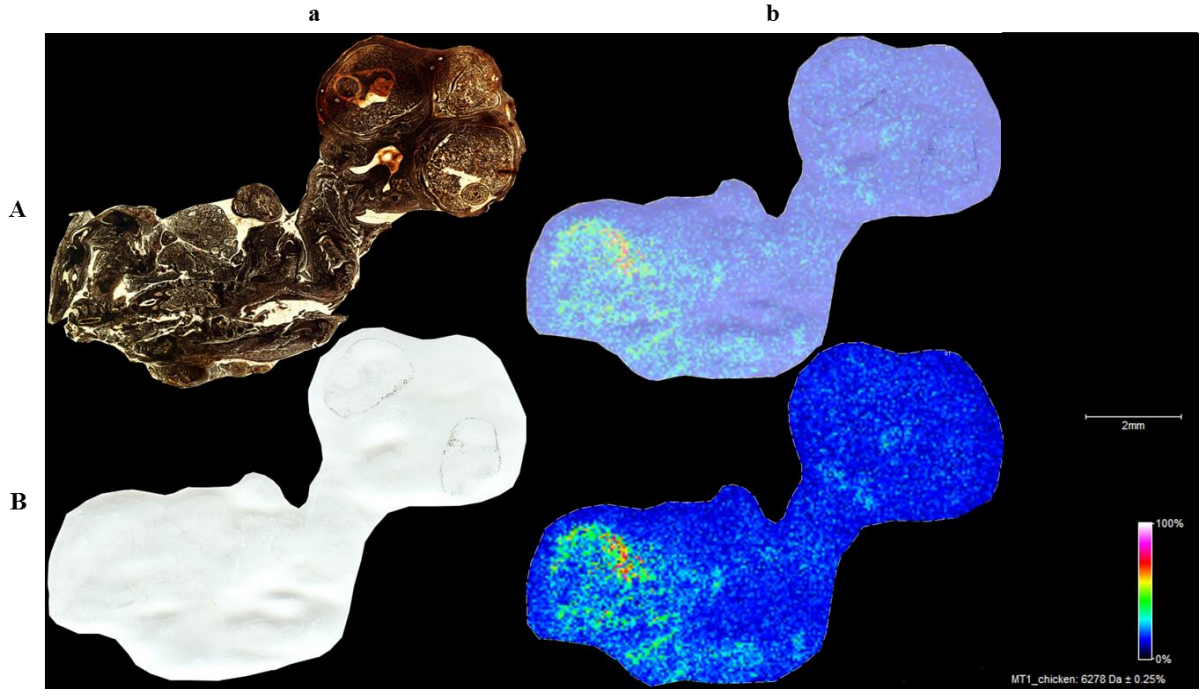
In flexImaging, the final preparation of MSI images was made by selecting peak of chicken metallothionein 1 (MT1) – the molecular weight of chicken MT1 was chosen according to UniProt database (www.uniprot.org). From a peak molecular weight was made a mass filter in a format "(molecular weight + atomic weight of hydrogen) ± 0.25%". Finally, images of tissue slices with used mass filters of selected peaks were used for preparation of final MALDI MSI images, which were made in GIMP 2.8 (www.gimp.org).

Optical microscopy

Deparrafinized and stained chicken embryo slice was covered by cover slip. The sample was placed by coverslip down and the immersion oil was used. The objective (PlanFLN; Mag. 100x; NA 1,3; F.N. 26.5) and the magnification lens 1.6x were used, and the total magnification was 1600x. The inverted research fluorescence microscope Olympus IX71S8F-3 (Olympus Corporation, Tokyo, Japan) was used. The image was captured by Olympus Camera DP73 and processed by Olympus Stream Basic 1.7 Software. The image resolution was 4800 x 3600 pixels. The parameters for the ambient light images were: exposure time – 2.2 ms and ISO 200.

RESULTS AND DISCUSSION

*Figure 1 Spatial distribution of metallothionein MT1 (6277 Da) in chicken embryo. (**Aa**) A picture of slice of stained chicken embryo from optical microscope. (**Ba**) A scanned picture of slice of FFPE chicken embryo slice. (**Ab**) A scanned picture of slice of FFPE chicken embryo merged with results from MALDI-TOF MSI of chicken metallothionein MT1. (**Bb**) Results from MALDI-TOF MSI of chicken metallothionein MT1. Higher intensities of metallothionein MT1 mass peak have brighter color in the mass spectrometry image. The size of a raster spot was 100 µm x 100 µm. MALDI-TOF MSI was performed in linear positive mode in the m/z range 2–20 kDa. As matrix was used 2,5-dihydroxybenzoic acid (DHB).The mass spectra were acquired by averaging 1600 sub spectra from a total of 1600 laser shots per raster spot. See more details in "material and methods" section.*



MALDI-TOF mass spectrometry imaging was used to obtain spatial (2D) distribution of metallothionein in chicken embryo. For MALDI MSI are mainly used cryo-sectioned frozen tissue samples because there are no other interferences for MALDI-TOF mass spectrometry, but FFPE tissue samples can be used too – researchers are optimizing the methods for their measuring because there exist large collections of different FFPE tissue samples used in clinical research (De Sio et al. 2015). We wanted to optimize the method of deparaffinization and antigen retrieval (Casadonte, Caprioli 2011) for our future research.

Results from MALDI-TOF MSI are shown in Figure 1. A chicken metallothionein MT1 with molecular weight of 6277 Da was detected. The highest amounts of MT1 were found in lower section of chicken embryo (Figure 1Bb). In comparison with optical image of chicken embryo (Figure 1Aa) these data show that MT1 is probably expressed mainly in chicken liver. This was expected because the expression of MT is connected with detoxification of organism. Therefore, in future experiments we will focus also on detection of MT in chicken embryo's organs in connection with exposure to different heavy metals, which can induce higher expression of MT.

CONCLUSION

A MALDI-TOF mass spectrometry imaging of metallothionein in chicken embryo revealed, that in normal growing conditions the expression of chicken metallothionein MT1 in chicken embryo occurs mainly in liver. It was demonstrated, that MALDI-TOF mass spectrometry imaging can be used for detection of metallothioneins in deparaffinized formalin-fixed and paraffin-embedded tissue sample slices. This is promising for future research.

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REFERENCES

Acquadro E., Cabella C., Ghiani S., Miragoli L., Bucci E. M., Corpillo D. 2009. Matrix-Assisted laser Desorption Ionization Imaging Mass Spectrometry Detection of a Magnetic Resonance Imaging Contrast Agent in Mouse liver. *Analytical Chemistry*, 81(7): 2779–2784.

Bednarik A., Kuba P., Moskovets E., Tomalova I., Krasensky P., Houska P., Preisler J. 2014. Rapid Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Imaging with Scanning Desorption Laser Beam. *Analytical Chemistry*, 86(2): 982–986.

Berril M. 2002. Histology Protocols. Retrieved 7.9.2015, from https://www.trentu.ca/biology /berrill/histology/histology_protocols.htm.

Bianga J., Bouslimani A., Bec N., Quenet F., Mounicou S., Szpunar J., Bouyssiere B., Lobinski R., Larroque C. 2014. Complementarity of MALDI and LA ICP mass spectrometry for platinum anticancer imaging in human tumor. *Metallomics*, 6(8): 1382–1386.

Caldwell R. L., Gonzalez A., Oppenheimer S. R., Schwartz H. S., Caprioli R. M. 2006. Molecular assessment of the tumor protein microenvironment using imaging mass spectrometry. *Cancer Genomics & Proteomics*, 3(5): 279–287.

Caprioli R. M., Farmer T. B., Gile J. 1997. Molecular imaging of biological samples: Localization of peptides and proteins using MALDI-TOF MS. *Analytical Chemistry*, 69(23): 4751–4760.

Casadonte R., Caprioli R. M. 2011. Proteomic analysis of formalin-fixed paraffin-embedded tissue by MALDI imaging mass spectrometry. *Nature Protocols*, 6(11): 1695–1709.

Chen B. M., Lietz C. B., Li L. J. 2014. In Situ Characterization of Proteins Using Laserspray Ionization on a High-Performance MALDI-LTQ-Orbitrap Mass Spectrometer. *Journal of the American Society for Mass Spectrometry*, 25(12): 2177–2180.

De Sio G., Smith A. J., Galli M., Garancini M., Chinello C., Bono F., Pagni F., Magni F. 2015. A MALDI-Mass Spectrometry Imaging method applicable to different formalin-fixed paraffin-embedded human tissues. *Molecular Biosystems*, 11(6): 1507–1514.

Dreisewerd K. 2014. Recent methodological advances in MALDI mass spectrometry. *Analytical and Bioanalytical Chemistry*, 406(9–10): 2261–2278.

Flinders B., Morrell J., Marshall P. S., Ranshaw L. E., Clench M. R. 2015. The use of hydrazine-based derivatization reagents for improved sensitivity and detection of carbonyl containing compounds using MALDI-MSI. *Analytical and Bioanalytical Chemistry*, 407(8): 2085–2094.

Karas M., Bachmann D., Hillenkamp F. 1985. Influence of the wavelength in high-irradiance ultraviolet-laser desorption mass-spectrometry of organic-molecules. *Analytical Chemistry*, 57(14): 2935–2939.

Karas M., Hillenkamp F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10000 Daltons. *Analytical Chemistry*, 60(20): 2299–2301.

Korte A. R., Yandeau-Nelson M. D., Nikolau B. J., Lee Y. J. 2015. Subcellular-level resolution MALDI-MS imaging of maize leaf metabolites by MALDI-linear ion trap-Orbitrap mass spectrometer. *Analytical and Bioanalytical Chemistry*, 407(8): 2301–2309.

Lynes M. A., Hidalgo J., Manso Y., Devisscher L., Laukens D., Lawrence D. A. 2014. Metallothionein and stress combine to affect multiple organ systems. *Cell Stress & Chaperones*, 19(5): 605–611.

Pinter T. B. J., Irvine G. W., Stillman M. J. 2015. Domain Selection in Metallothionein 1A: Affinity-Controlled Mechanisms of Zinc Binding and Cadmium Exchange. *Biochemistry*, 54(32): 5006–5016.

Prentice B. M., Chumbley C. W., Caprioli R. M. 2015. High-speed MALDI MS/MS imaging mass spectrometry using continuous raster sampling. *Journal of Mass Spectrometry*, 50(4): 703–710.

Rompp A., Spengler B. 2013. Mass spectrometry imaging with high resolution in mass and space. *Histochemistry and Cell Biology*, 139(6): 759–783.

Solouki T., Marto J. A., White F. M., Guan S. H., Marshall A. G. 1995. Attomole biomolecule mass analysis by matrix-assisted laser-desorption ionization fourier-transform ion-cyclotron resonance. *Analytical Chemistry*, 67(22): 4139–4144.

Stoeckli M., Chaurand P., Hallahan D. E., Caprioli R. M. 2001. Imaging mass spectrometry: A new technology for the analysis of protein expression in mammalian tissues. *Nature Medicine*, 7(4): 493–496.

Strupat K., Kovtoun V., Bui H., Viner R., Stafford G., Horning S. 2009. MALDI Produced Ions Inspected with a Linear Ion Trap-Orbitrap Hybrid Mass Analyzer. *Journal of the American Society for Mass Spectrometry*, 20(8): 1451–1463.

Svatos A. 2010. Mass spectrometric imaging of small molecules. *Trends in Biotechnology*, 28(8): 425–434.

Wang X. D., Han J., Yang J. C., Pan J. X., Borchers C. H. 2015. Matrix coating assisted by an electric field (MCAEF) for enhanced tissue imaging by MALDI-MS. *Chemical Science*, 6(1): 729–738.