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Distribution of cholesterol in the brain tissue as an example of TOF-SIMS analysis.

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ABSTRACT

TOF-SIMS is popular in biological sciences due to its ability to investigate the spatial distribution of molecules in tissue sections and other biological samples with a very high spatial resolution. Here we present an example of a strategy that determines the molecular distribution of cholesterol on the surface of a tissue section. In this chapter several sample preparation considerations for TOF-SIMS analysis are presented. Additionally, the influence of different sample coating methods and technical obstacles, which may appeared during analysis, will be discussed.

Keywords: TOF-SIMS, cholesterol, substance distribution, sample preparation.

ABBREVIATIONS

ACN	acetonitrile
DESI	Desorption Electrospray Ionization Mass Spectrometry
HCCA	α-Cyano-4-hydroxycinnamic acid
IMS	Imaging Mass Spectrometry
ITO glass slides	Indium Tin Oxide coated glass slides
MALDI	Matrix-assisted Laser Desorption/Ionization Mass Spectrometry
ME-SIMS	Matrix-Enhanced Secondary Ion Mass Spectrometry
Met-SIMS	Metal assisted Secondary Ion Mass Spectrometry
TFA	trifluoroacetic acid
TOF-SIMS	Time of Flight Secondary Ion Mass Spectrometry

INTRODUCTION

TOF-SIMS is an imaging technique, which allows the localization of molecules on the analyzed surface (for review see: [1,2]). Various molecules may be analyzed simultaneously in one experiment, using this method. Additionally, we may observe substances that were so far "invisible" due to the lack of specific imaging methods (e.g. lipids [3]). This is one of the reasons for the increased popularity of this method in biological sciences.

Briefly, SIMS employs a high-energy primary ion beam (liquid metal ion gun, such as Au^+, Ga^+, Bi^+, or more sophisticated ones, such as C_{60} cluster), to produce secondary ions from the analyzed surface. The secondary ions are generated through a collision cascade mechanism that has been widely described in literature. The exact mass of desorbed and ionized molecules is subsequently determined with a mass spectrometer, frequently a time of flight analyzer. The high kinetic energy of the primary ions substantially increases the internal energy of the secondary species. This in turn often leads to extensive fragmentation of the analyzed species, further complicating spectral analysis. It also limits the observed range of m/z values in the measurements (1 – 2000 m/z). On the other hand, an ion beam can be focused with very high precision, which makes TOF-SIMS an excellent tool in terms of spatial resolution in comparison to other IMS methods such as MALDI and DESI (spatial resolution of less than 10 nm for SIMS analysis has been reported [4]).

Here we would like to present a strategy for high spatial resolution cholesterol imaging in tissue sections. Potential obstacles, which may obscure the analytical result, will be discussed, together with other technical details.

EXPERIMENTAL

Chemicals

For TOF-SIMS analysis a stock solution of 1mg/ml of cholesterol (*Sigma-Aldrich*) in chloroform : methanol 1:1 (v/v) (Biosolve, the Netherlands), was used to prepare the appropriate analytical solutions. 10 mg/ml of α-Cyano-4-hydroxycinnamic acid (*Sigma-Aldrich*) in 50% ACN/H_2O/0.1% TFA was used to cover the analyzed sample. Additionally, hexane and ethanol were used during preparation of ITO glass slides (25x50x1.1-mm, R_s=4-8 Ω, Indium Tin Oxide coated slides (Delta Technologies, USA)) for the analysis.

ITO glass slides – washing

ITO slides were cleaned according to washing protocol before the samples were applied. Prior to this procedure, the conductive side of each slide was marked by cracking the slide's upper left corner. ITO glass slides were washed in plastic slide boxes that were filled with the appropriate solution (100 % hexane or 100 % ethanol (Biosolve, the Netherlands)). The filled boxes were placed in the beaker filled with water and immersed in a sonication bath. First, the slides were sonicated for 5 minutes with hexane. The hexane was removed and the boxes were filled with 99% ethanol, followed by sonication for 5 minutes. After that,

ethanol was removed and the slides were dried inside the open boxes in a vacuum desiccator for 20 min. Clean slides were stored in the closed boxes until further use.

Tissue sections

Ethical requirements. All experiments were performed in agreement with the respective Polish and European Council Directives (86/609/EEC), and were approved by the local Ethics Committee.

Male Wistar rats obtained from a local distributor (HZL, Warsaw, Poland), weighing 150-200 g were used in the experiments. Animals were sacrificed by decapitation. Brains were removed, wrapped in an aluminum foil and immediately frozen in liquid nitrogen. The entire procedure took no longer than 2 minutes. 10 μm sagittal rat brain tissue sections were cut using a cryomicrotome (Microm International GmbH, Walldorf, Germany). Sections were thaw-mounted on the ITO coated glass slides and stored at -80^{0}C until analysis. Prior to IMS analysis the slides were defrosted and dried in a vacuum desiccator at room temperature for at least 10min and covered with the appropriate matrix, when needed.

Gold coating

Samples were coated with gold prior to TOF-SIMS analysis using a Quorum Technologies (New Haven, UK) SC7640 sputter coater equipped with a FT7607 quartz crystal microbalance stage and a FT690 film thickness monitor**. 1nm** of gold layer was deposited on the sample surface. This method is referred to as metal assisted SIMS (MetA-SIMS).

Matrix coating

10 mg/ml of HCCA matrix in 50% ACN/H_2O/0.1% TFA was applied on the glass slide by an ImagePrep (Bruker, Bremen, Germany) vibrational nebulisation system. The entire process was divided into three phases: matrix deposition (the spray power 70%), incubation (45s), and drying (90s). The procedure was repeated until an even coating was obtained (ca. 16 times). Covering the sample surface with MALDI matrix prior SIMS analysis is referred to as matrix-assisted Secondary Ion Mass Spectrometry (ME-SIMS).

TOF-SIMS

SIMS measurement were performed on a Physical Electronics TRIFT II (Physical Electronics, USA) time of flight secondary ion mass spectrometer (TOF–SIMS) equipped with an Au liquid metal ion gun tuned for 22 keV Au^+ primary ions. Data were acquired analyzed and visualized with the WinCadence software (ver. 4.4.0.17) suite. Selected spectra were visualized using mMass – an open source mass spectrometry tool [5].

Tissue handling considerations

It is important to maintain the integrity of the spatial distribution of a given compound in the tissue during the entire analytical protocol. Therefore, proper storage and sample handling during preparation is necessary. After tissue removal, it is advisable to wrap it in an aluminum foil. This will preserve the shape of the tissue and prevent its adhesion to the sides of the LN_2 Dewar tank. It is important to immerse the wrapped tissue into the liquid

nitrogen very slowly (from 30 to 60 seconds) to prevent it from cracking (not to plunge it) [6].

ITO glass slides have to be carefully prepared before thaw-mounting the tissue sections on their surface. Possible ITO surface contaminants should be removed by extensive cleaning, as described earlier, prior to tissue mounting. These slides may be used only once, as there is no efficient method for tissue removal from their surface.

Before cutting, the tissue should be removed from the -80^{0}C freezer and placed in the cryomicrotome chamber to adjust its temperature to -20^{0}C. Tissue or organ may be mounted on the microtome sample holder with a drop of pure water. It is important to avoid any polymer-based embedding media, because they easily ionize, and may act as significant ion suppressors [6]. If necessary, gelatin may be used for embedding purposes.

Sections with a thickness of 10 - 20 μm are usually employed for IMS analysis. They are easy to handle, dry fast, and provide sufficient material for the analysis. Since they are not very thick, there is no danger of long and uneven drying, which could result in cracking or tissue distortion. Before cutting, especially with a new blade, it is advisable to clean it with, e.g. methanol to remove substances such as mechanical processing oils, which may contaminate the tissue surface during the sectioning process.

Prepared tissue slices mounted on the ITO glass should be stored at -80^{0}C. It is important to prevent water condensation on the tissue while taking the glass slides out of the freezer. Glass slides should be placed immediately in a desiccator to remove water and adjust the temperature of the tissue to the room temperature. It is also possible to store the glass slides in Petri dishes sealed with parafilm [7].

RESULTS AND DISCUSSION

Cholesterol is one of the main substances that control the fluidity of cellular membranes. It is implicated in many important processes in the brain, such as synaptogenesis, and myelin formation. Improper regulation of this substance was linked to Alzheimer's disease [8].

TOF-SIMS analysis allows a given substance to be identified by its mass (m/z), characteristic isotopic pattern, unique fragmentation and location in the examined tissue. The monoisotopic mass of cholesterol is 386.35 Da. The SIMS mass spectrum of this substance shows two characteristic peaks: one at m/z 369.35 ($[M\text{-}OH]^+$), and the second at 385.34 ($[M\text{-}H]^+$) (see Figure 1.). These peaks were used for cholesterol identification, as well as for semi quantitative analysis based on peak integrals, and to image the distribution of this molecule in the tissue section.

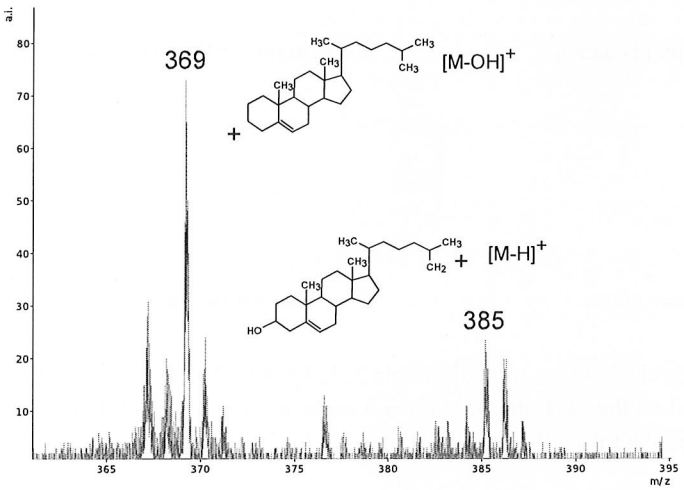

Figure 1. SIMS spectrum of cholesterol standard (100pmol/µl, raster size: 150x150 µm/3min). Characteristic peaks – 369 and 385 m/z.

The influence of sample preparation

TOF-SIMS analysis is extremely sensitive to the sample preparation. The spectral response is highly dependent on the ionization technique used: ME-SIMS with matrix, or conventional TOF-SIMS without matrix. This can be clearly observed in figure 2, where the series of different cholesterol dilutions were prepared and measured with and without HCCA matrix (Figure 2).

One µl of each cholesterol solution (0; 1; 25; 50; 75, and 100 nmol/ml) was applied on the clean ITO glass slides. Each spot was measured for 3 minutes on the area of 150 x 150 µm. The dependence between concentration of the solution and the integral for each characteristic peak was established. Figure 2 presents such plots for the peak at m/z 369 from the characteristic cholesterol fragment $[M\text{-}OH]^+$). Concentrations, at which the peaks characteristic for cholesterol may be easily distinguished from the background (their signal to noise ratio is above 3), are marked with red spots. It is clearly seen that the instrument response is totally different in these two cases.

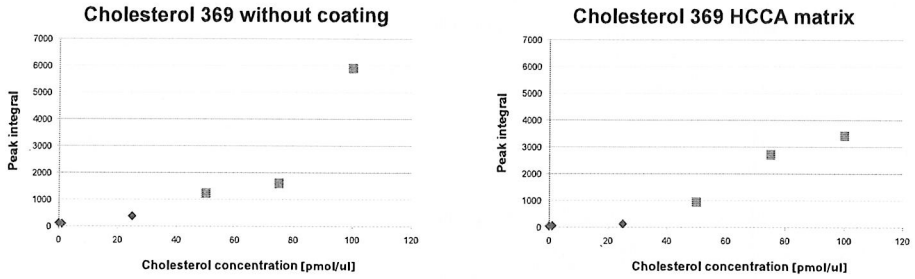

Figure 2. The series of various dilutions of cholesterol (0; 1; 25; 50; 75, and 100 pmol/μl of deposited sample), measured on the ITO glasses with and without HCCA matrix. Red marks indicate peaks easily distinguished from the background.

The results indicate that for any type of sample preparation and measurement (different matrix, different sample coating (such as gold coating), different ion gun (for example Bi, C_{60}, Au), the **concentration – intensity** curve should be prepared to find the limit of detection (LOD) and the limit of linearity (LOL) of instrument response for a given species. It is important to make sure that the further measurements from the tissue samples are obtained within the linear range of instrument response.

The influence of sample coating

TOF-SIMS is very often considered as a technique, which does not demand any special sample preparation, except mounting of the tissue section on the ITO glass. However, for some molecules, application of the MALDI matrix such as HCCA (ME-SIMS), or covering the surface with a metal layer such as gold (MetA-SIMS) may increase the secondary ions yield for the analyzed molecules [9,10] (this is a more pronounced effect when atomic primary ions are employed). It seems that such sample pretreatment increases the generation of intact secondary ions, which may substantially extend the range of the TOF-SIMS analysis. Figure 3 shows the spectrum obtained from an untreated brain tissue section, the same tissue covered with MALDI matrix and the tissue coated with gold. In the latter case, gold coating increased the signal ca. 60 times, which enhanced the image quality during cholesterol analysis in the tissue. Each measurement lasted for 3 minutes and was taken from the area of 150 x 150 μm of the cortex.

A similar effect is observed for cholesterol standards on the glass. Application of MALDI matrix dramatically improved the spectra quality as reported numerous times in the ME-SIMS literature (data not shown).

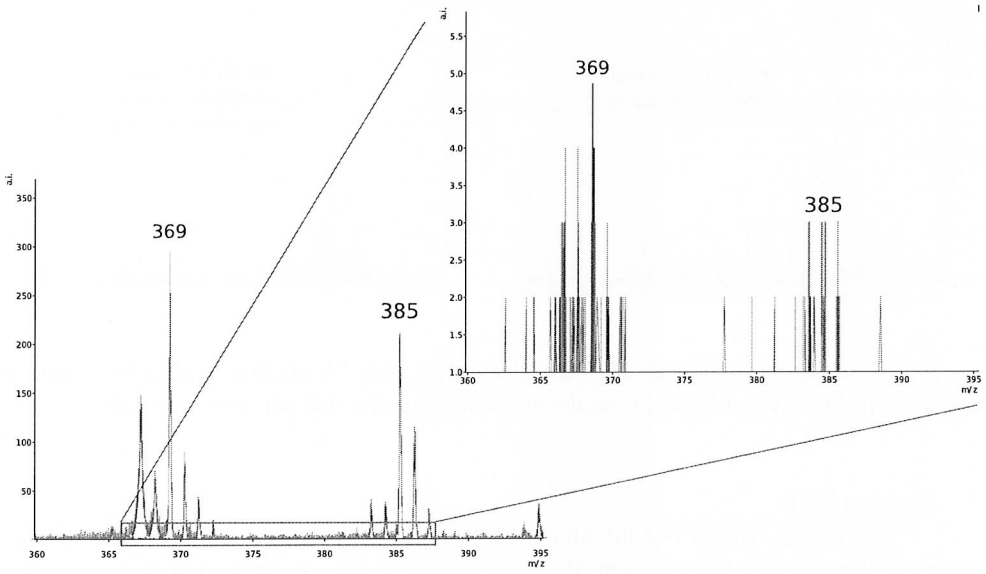

Figure 3. The influence of sample cover on the intensities of cholesterol peaks (green – gold coating, red - HCCA matrix coating, blue – tissue without coating).

Tissue surface chemical complexity

The effect of the complexity of the chemical environment of the analyte (chemical matrix such as tissue) may exert a great influence on the ionization efficiency during IMS measurements. The simplest way to check the role of this effect in the case of a particular substance is just to deposit a small amount of a substance standard on the tissue surface and perform the measurements. In the following case 1µl of cholesterol standard at a concentration of 75 pmol/µl was deposited on the tissue surface. The measurements were done following the same protocol as employed for the neat standard on ITO - each measurement lasted for 3 minutes on the area of 150 x 150 µm.

The effect of ion suppression caused by the tissue may be clearly seen, when the spectra obtained from the clean glass slide spotted with 75 pmol/µl of cholesterol standard and covered with HCCA matrix, are compared with the spectra from the tissue spotted with cholesterol and prepared in the same way. It is impossible to measure the cholesterol spectra under such circumstances. Measuring the whole spotted area (mosaic mode, the field of 3.5mm x 3.5mm, the raster size 218.75 µm, and the measurement time for each raster: 3 sec) strengthens the signal. A similar effect was observed for both characteristic cholesterol ions.

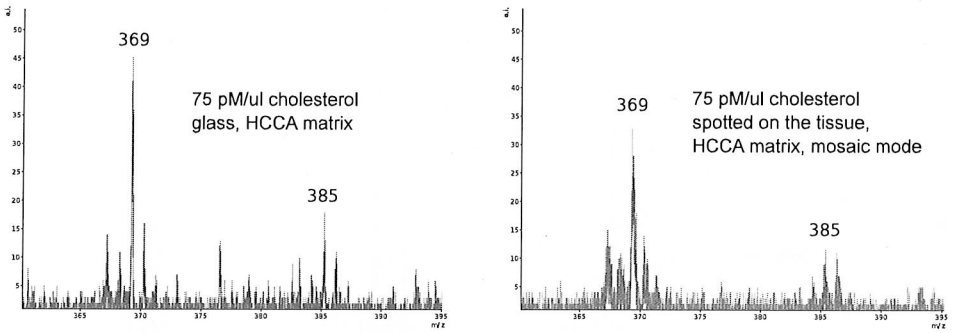

Figure 4. Spectra obtained from the clear glass with 75 pmol/μl cholesterol standard covered with HCCA matrix, and from the tissue spotted with standard (mosaic mode).

Brain analysis

Our results showed that the MetA-SIMS with gold yields the best results in case of cholesterol analysis. Figure 5 presents the image of the frontal – sagittal rat brain section. The tissue slice was prepared by careful brain sectioning, followed by mounting the slices on a cleaned ITO glass followed by a 1 nm gold coating. Raster size during measurement was 11mm x 11mm, that has been analyze in the mosaic mode with 64x64 tiles (raster size 171.9 μm x 171.9 μm) that were measured for 5 seconds each.

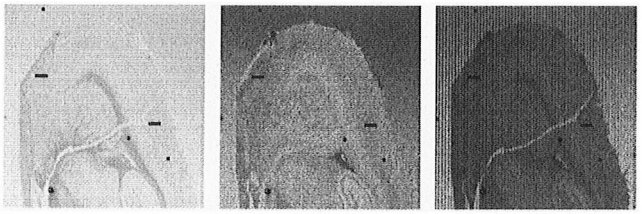

Figure 5. Analysis of cholesterol distribution in the frontal – sagittal rat brain section. Distribution of cholesterol pseudomolecular ion at 385 m/z, and characteristic fragment at 369 m/z, as well as total ion image are shown.

CONCLUSION

TOF-SIMS is demonstrated to be a useful tool to study the distribution of ions, small molecules (especially drugs), and lipids in biological samples. Different ways of measuring

cholesterol as a standard and as an endogenous molecule in the tissue, showed the importance of sample preparation.

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REFERENCES

[1] Chughtai, K.; Heeren, R. M. A. (2010) *Chem Rev , 110*, 3237–3277.

[2] van Hove, E. R. A.; Smith, D. F.; Heeren, R. M. A. (2010) *J Chromatogr A, 1217*, 3946–3954.

[3] Touboul, D.; Brunelle, A.; Laprévote, (2011) O. *Biochimie, 93*, 113–119.

[4] Kaletaş, B. K.; van der Wiel, I. M.; Stauber, J.; Lennard J Dekker; Güzel, C.; Kros, J. M.; Luider, T. M.; Heeren, R. M. A. (2009) *Proteomics , 9*, 2622–2633.

[5] Strohalm, M.; Kavan, D.; Novák, P.; Volný, M.; Havlíček, V. (2010) *Anal Chem 82*, 4648–4651.

[6] Schwartz, S. A.; Reyzer, M. L.; Caprioli, R. M. (2003) *J Mass Spectrom, 38*, 699–708.

[7] Altelaar, A. F. M.; Luxembourg, S. L.; McDonnell, L. A.; Piersma, S. R.; Heeren, R. M. A. (2007) *Nat Protoc, 2*, 1185–1196.

[8] Di Paolo, G.; Kim, T.-W. (2011) *Nat Rev Neurosci, 12*, 284–296.

[9] Delcorte, A.; Bour, J.; Aubriet, F.; Muller, J. F.; Bertrand, P. (2003) *Anal Chem, 75*, 6875–6885.

[10] Wu, K. J.; Odom, R. W. (1996) *Anal Chem, 68*, 873–882.