Bioimaging TOF-SIMS: localization of cholesterol in rat kidney sections

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Abstract Here, we show the localization of a whole organic molecule in biological tissue using time-of-flight secondary ion mass spectrometry (TOF-SIMS). Rat kidneys were sectioned by cryoultramicrotomy and dried at room temperature. The samples were covered with a thin silver layer and analyzed in an imaging TOF-SIMS instrument equipped with a Ga^+-source. The cholesterol signal showed a high concentration in the nuclear areas of the epithelial cells and a lower concentration over areas representing the basal lamina of renal tubules. A more diffuse distribution of cholesterol was also found over areas representing the cytoplasm or plasma membrane of the epithelial cells.

Keywords: Cholesterol; Localization; Kidney; TOF-SIMS; Silver matrix

1. Introduction

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is a technique allowing identification and localization of unknown molecules at sample surfaces [1]. It has several advantages over alternative methods, e.g., its sensitivity to all elements, detection of all isotopes, excellent spatial resolution (100 nm), and simultaneous imaging of the surface distribution of detected elements and molecules [2]. The properties of TOF-SIMS make it an attractive method for analysis of biological samples, e.g., tissue sections, and several attempts in that direction have been made. The development in this area until 1997 has been excellently reviewed [3]. However, SIMS is based on the outcome of the impact of a high energy primary ion onto a target, and the yield of large molecular fragments, necessary for the identification of the original molecule, seems to be limited [3]. Results presented in recent years are still at the level of localizing molecular fragments like phosphocholine ($m/z = 184$) [4], vitamin A fragment-1 [5], or OH and CN [6].

Recently, it was found that the yield of large fragments or molecular ions can be enhanced by using silver or gold as a matrix during secondary ion formation [7].

The deposition of a 2 nm thick layer of gold or silver on thin films of organic molecules results in a 2-fold yield enhancement for metal-quasimolecular ions of organic molecules and a 1–10-fold increase for the characteristic fragments of such organic molecules [8,9].

We have previously been able to show subcellular localization of molecular ions of cholesterol by imaging organic TOF-SIMS using silver imprints of freeze-dried cells [10].

Another successful approach yielding larger fragments is the use of low energy primary ions like gold clusters [11] but according to our experience, silver coating gives a higher yield.

The aim of the present study was to reinvestigate the localization of cholesterol in rat kidney using imaging TOF-SIMS. For preparation of kidney sections, we chose the cryoultramicrotome technique described by Tokuyasu [12] in order to get plane sections that can be covered with an even metal layer.

Using cryoultramicrotome sections of rat kidney, sputter-coated with a silver layer, we were able to localize molecular ions of cholesterol with subcellular resolution. The results point to exciting new possibilities making TOF-SIMS images of tissues by using modern histological preparation techniques and metal matrix systems for controlled ionization of organic molecules.

2. Materials and methods

2.1. Animals and tissue preparation

Male Sprague–Dawley rats (300 g) were sacrificed by terminal bleeding. The kidney was rapidly dissected out, cut into 1 mm pieces and fixed in 2% formaldehyde, 2.5% glutaraldehyde and 0.05 M cacodylate buffer. The samples were fixed for 15 h and infiltrated with 2.3 M sucrose for 48 h to achieve cryoprotection.

2.2. Cryosectioning

The tissue samples were mounted on specimen pins, vitrified by plunge-freezing into liquid propane at −170 °C and stored in liquid nitrogen until they were sectioned. With cryoprotection, samples will remain vitrified at higher temperatures and can be cryosectioned at −80 °C resulting in good sections with few cracks. Ultrathin cryosections (0.4 μm) were cut with a Reichert Ultracut E equipped with an FC 4E cryounit. The sections were picked from the knife surface with a wire loop containing a small drop of 2.3 M sucrose solution. The sections, thawed on the drop, were then applied to cover glasses, rinsed with a drop of saline which was finally removed by a rapid rinse in distilled water. Thus, salt precipitation was avoided at the surface of the tissue section during air drying. The dry samples were stored in a desiccator before sputter coating with silver. Some sections were stained for light microscopy during 1 min in a 1% solution of toluidine blue/azan.

2.3. Silver coating

The dry tissue sections were placed in an Emitech 550X sputter coater equipped with a silver target. The most important properties of an efficient silver matrix for cationization of organic molecules is the thickness of the metal layer, which should not exceed a few nanometers [9]. In the present study, we found that the optimal coating differed between samples. Thus, a series of different coating time was made for each sample, to empirically determine the optimal coating conditions.

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Normally, sputtering was performed during 45 s at an argon partial pressure of 0.28 bar and a current of 25 mA.

2.4. TOF-SIMS analysis

The silver-coated tissue was analyzed with a gridless reflectron-based TOF-SIMS instrument at the University of Münster using a pulsed primary beam of focussed 30 keV Ga^+ ions. High resolution TOF-SIMS images (lateral resolution <200 nm) were obtained with a mass resolution of $m/\Delta m = 4000$. The primary ion beam current was 0.12 pA.

TOF-SIMS spectra were recorded by scanning the primary ion beam over the analysis area and acquiring at each position mass spectra of the positive or negative ions leaving the surface. The recorded TOF-SIMS data were stored in raw data files, which contain complete spatial and spectral information.

3. Results

A TOF-SIMS mass spectrum in positive ion mode (Ga^+ ion desorption) of freeze sectioned rat kidney coated with silver is shown in Fig. 1. In the mass range, 320 Da $\leqslant m/z \leqslant$ 330 Da many ions of high intensity are present, representing Ag_3^+ isotopes cationized with low molecular organic fragments. The highest peak (322.8 Da) representing $Ag_3H_2^+$. In the range 490 Da $\leqslant m/z \leqslant$ 500 Da, the high intensity peaks represent silver cationized cholesterol (493.4 and 495.4 Da). Significant peaks of unknown origin are also found in the range 505 Da $\leqslant m/z \leqslant$ 515 Da. We have used the silver cationized cholesterol ion in order to localize cholesterol in histological sections of rat kidney.

A light micrograph of a kidney section is shown in Fig. 2. Proximal tubules are recognized by their minor luminary opening, basal nucleus and apical lysosomes. Distal tubules are recognized by their wide luminary opening, basal striation and apical location of the nucleus. A collecting duct is seen, with a low epithelium containing intercalated cells and principal cells.

Fig. 3 shows the localization of cholesterol in a serial section taken from the same series as the section shown in Fig. 2. The image shows the localization of the peak $m/z = 493$, representing silver-cationized cholesterol.

The cholesterol signal shows a high concentration in the nuclear areas of the epithelial cells and a lower concentration over areas representing the basal lamina of some tubules. A more diffuse distribution of cholesterol is also seen over areas representing the cytoplasm or plasma membrane of the epithelial cells. Further analysis of serial sections indicates that the nuclei that are enriched in cholesterol are located in the distal tubules.

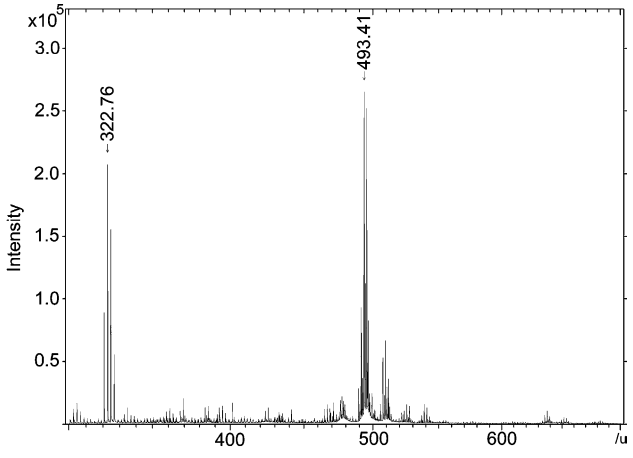

Fig. 1. TOF-SIMS mass spectrum in positive mode of a cryoultramicrotome kidney section mounted on a cover glass sheet.

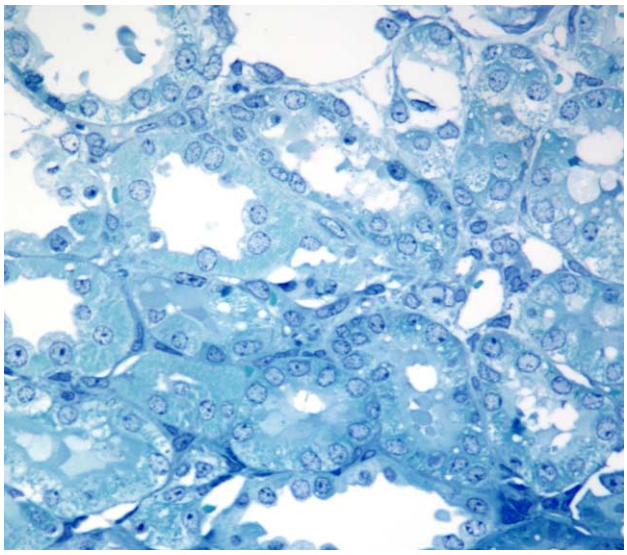

Fig. 2. Light micrograph of a cryoultramicrotome kidney section mounted on cover glass and stained with toluidine blue/azan.

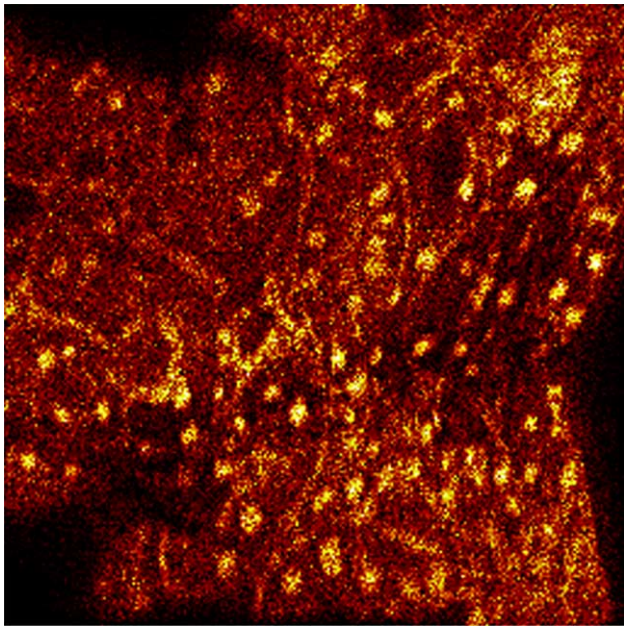

Fig. 3. Image prepared from TOF-SIMS raw data files, showing the distribution of the silver-cationized cholesterol ion with $m/z = 493$.

4. Discussion

The application of a silver layer as a matrix over the tissue sections before TOF-SIMS analysis yielded molecular ions of cholesterol and metallized cholesterol in quantities sufficient for imaging. The mechanism of sensitivity enhancement by gold and silver deposition on organic layers has been studied

by others [7–9]. Metallization of organic molecules and facilitated molecular desorption are two important mechanisms behind this effect.

For real-world samples, like tissue sections, a plane surface is important in order to avoid variations in the thickness of the metal coat sputtered over the surface. Cryoultramicrotomy yields plane sections over limited areas, but knife scratches and rifts are frequent artifacts that may disturb the formation of an even metal film. Another source of artifacts is the compression of sections during cutting [13]. However, this problem is partly overcome by thawing the sections before mounting on glass.

In comparison to the silver imprint technique used previously [14], the silver coating technique offers several significant advantages. The silver imprint technique produces a layer of organic material, on the silver matrix, that varies in thickness over the surface. The thickness of the organic imprint cannot be defined in advance, which makes the acquired images of the distribution of organic molecules difficult to interpret. Moreover, the pressure applied to the sample during imprinting may cause distortion of the cells.

The localization of cholesterol in cells has been thoroughly studied with specific probes like filipin [15] or perfringolysin O-toxin [16]. Results obtained with filipin and freeze fracturing [15] show a more marked response to filipin in the plasma membrane of most cells than in their intracellular membranes. Wide differences in the extent of labelling were found in the plasma membrane of different cell types. Within the intracellular membranes, a higher density of cholesterol was found in the Golgi membranes than in the endoplasmic reticulum, nuclear envelope and mitochondrial membranes. However, some interesting exceptions to this pattern were noted. In some cell-types, e.g., capillary endothelial cells and mammallian bladder cells, the nuclear envelopes show a pronounced response to filipin.

The heterogeneous labelling of the plasma membrane and the labelling of nuclear membranes, in some cells but not in others, has been confirmed in studies of rat kidney with filipin and freeze-fracture replica technique [17]. Much effort has been spent, trying to exclude the possibility that the heterogeneous labelling is due to uneven penetration of the probe into different parts of the sample [17]. A high concentration of cholesterol in the nuclear membrane was also found in the present study with the TOF-SIMS analysis of epithelial cells in the distal tubules. The main advantage of the TOF-SIMS method over previously used methods is that no probes are involved, which makes the analysis free of problems with probe reactivity and penetration.

The subcellular distribution of cholesterol-binding perfringolysin O-toxin in lymphoblastoid cells, studied by immunoelectron microscopy, also confirms the localization of cholesterol to the plasma membrane and intracellular vesicles [18]. The study also illustrates well the difficulties of using a large m.w. probe and tissue fixation, i.e., the results obtained regarding localization of cholesterol were dependent on the experimental protocol. However, all different approaches used gave essentially similar intracellular cholesterol labelling [18]. The intracellular localization of cholesterol presented in this study is thus considered stable with the fixation procedure used.

In conclusion, routine fixation and cryoultramicrotomy of rat kidney together with silver coating of the sections allows localization of cholesterol with imaging TOF-SIMS. Cholesterol was found enriched in the nuclear membranes of epithelial cells in the distal tubules, confirming previous results that have been questioned [17].

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