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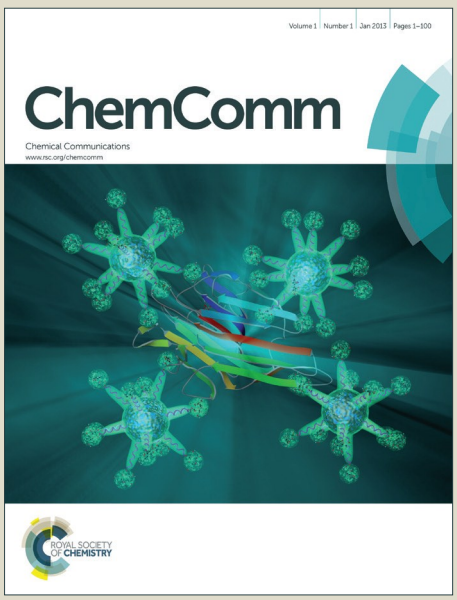

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Maleic Anhydride Proton Sponge as a Novel MALDI Matrix for the Visualization of Small Molecules (<250 m/z) in Brain Tumors by Routine MALDI ToF Imaging Mass Spectrometry

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A novel vacuum stable proton sponge, 4-maleicanhydridoproton sponge (MAPS), was prepared and applied as the matrix in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI) of an aggressive brain tumor tissue (glioblastoma multiforme). Ionic maps of lactate, 2-hydroxyglutarate and chloride anions (m/z 89, 147, 35, respectively) were obtained using a routine MALDI ToF mass spectrometer.

Changes in the metabolism of cells and tissues often precede morphological changes and pathological events in the establishment of cancer. Two major physiological differences between tumor cells and normally differentiated cells in brain tumors are an altered metabolism[1] and an altered abundance of certain ion channels.[2] A classical biochemical adaptation in cancer cells is the metabolic shift to aerobic glycolysis termed the "Warburg effect" in honor of its inventor Otto Warburg. The cancer cells are characterized by high glucose uptake, low oxygen consumption and elevated production of lactate regardless of oxygen availability.[4] Aerobic glycolysis is associated with a survival advantage as well as the generation of substrates such as fatty acids, amino acids, and nucleotides necessary for rapidly proliferating cells. During the last five years, the "Warburg Effect" gained a renaissance since the altered metabolism of tumor cells could be a target for a pharmacological intervention.[3]

Additionally, defects of tricarboxylic acid (TCA) cycle enzymes such as isocitrate dehydrogenase (IDH) have effects on the cellular redox state and promote carcinogenesis by affecting redox biology. In particular, the mutation IDH1 causes high cellular production of 2-hydroxyglutarate, which is defined as a so-called oncometabolite.[5-6] Ion channels play a special role in carcinogenesis.[7-8] Interesting ion channels in this context are the ClC Family Channels and the Sodium-Potassium-Chloride Cotransporter (NKCC). They regulate the intracellular chloride concentration in order to keep the cell in homeostasis. Although chloride is the major inorganic anion in the brain, it is often overseen because of low concentration in most mature neurons. Instead in the case of glioma brain tumor cells, Habela et al. showed an accumulation of intracellular chloride during the cell growth. Afterwards a release of chloride occurs during the cellular division.[9]

Therefore, the detection of low-molecular weight compounds in tumors is very important for the analysis of biological materials or tissue samples by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI), providing a better understanding of the principles of carcinogenesis. The information on the spatial localization of small metabolites in highly heterogeneous tissues, such as diffuse glioma, an aggressive brain tumor, provides crucial knowledge on metabolic characteristics in the tumor infiltrated tissues. Indeed, emerging results obtained by FT-ICR mass spectrometry have demonstrated that MALDI-MSI can be used to image low mass metabolites that are essential to understand the metabolism of cancer.[10-11]

Fast MALDI-MSI instruments will provide a supporting tool for diagnostics and decision-making during surgeries of tumor patients. Instruments, like the Bruker rapifleX™ Tissuetyper™ [12] are based on the MALDI-ToF MS technique and require the use of a robust matrix, which efficiently ionizes small mass molecules.

The chemistry of the matrix is a fundamental parameter in the MALDI-MSI of small molecules in tissue sections because it has an important effect on the intensity of MS signals. The matrix must meet a number of requirements simultaneously. These are strong absorbance at the laser's wavelength, vacuum stability, solubility in solvents compatible with analytes and the ability to promote analyte ionization that is strongly correlated to the reactivity of the matrix in gas phase.[13-14]

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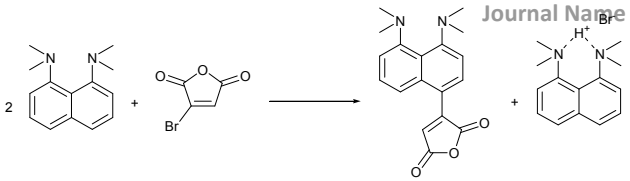
The detection of small molecules using classical matrices is very problematic because the ionization of the matrix and related clustering processes lead to strong background signals in the low mass range of the spectrum and because of susceptibility to in-source fragmentation of small molecules.[15] For this reason the search for new MALDI matrices that enhance the detection of low-molecular weight compounds remains an important aspect of small molecule MSI.

9-aminoacridine has been successfully used for MALDI-MSI of protic analytes, as well as for tissue imaging of endogenous compounds such as nucleotides and phospholipids in the negative-ion mode.[16] Organic salts such as N-(1-naphtyl) ethylenediamine dinitrate have also been employed as a matrix to analyze especially oligosaccharides, peptides, metabolites and explosives.[17] High molecular weight matrices such as porphyrin derivatives were used in the detection of water-soluble vitamins.[18] Reactive matrices were used as derivatizing agents to detect small molecules in high mass ranges such as [(E)-4-(2-cyano-2-carboxyvinyl) phenyl]boronic acid, 2,4-dinitrophenylhydrazine (DNPH) and 4-dimethylamino-6-(4-methoxy-1-naphthyl)-1,3,5-triazine-2-hydrazine (DMNTH).[19-20]

Here we present to our knowledge the first matrix suitable for the MALDI ToF MS-based MSI analysis of small molecules in a range as low as m/z 35 Da. The potential of the matrix is presented exemplarily on human diffuse glioma tissue sections.

A classical matrix used in MALDI-ToF analysis of small molecules is 1,8-bis(dimethylamino)naphthalene (DMAN), which belongs to a class of compounds called proton sponges since these highly alkaline amine bases can deprotonate the analytes in solution.[21] In general, proton sponges are characterized by both a destabilizing effect between lone electron pairs at two neutral substituted diamines, and by the hydrophobic shielding of these basic centers. The hydrophobic shielding of the basic centers and the N···H···N hydrogen bonds are responsible for the extremely low rate of protonation and deprotonation of these compounds; however, it has no influence on their high thermodynamic basicity.[22-23]

The equilibrium between the ion-pair and the associated base-acid complex in the liquid phase is characterized by the pK_a and the pK_b values of the base or the acid in the solution in accordance with the Brønsted-Lowry concept. This equilibrium is reflected in the crystal phase, and the amount of observed ions is manifested by the ion pair/ base-acid complex equilibrium. Shroff et al. suggested a new term for this process: Matrix-assisted ionization/laser desorption (MAILD).[24] Through the condensed phase ionization an improvement of small compound signals was observed, and for this reason DMAN is a very useful matrix for the detection of small molecules. Unfortunately, DMAN is highly unstable under vacuum conditions and is therefore not suitable in MSI experiments.[25-26]



Scheme 1. Synthesis of a 4-maleicanhydridoproton sponge (MAPS) using the Tyler´s reaction. The reaction of DMAN and bromomaleic anhydride leads to the formation of 4,5-bis(dimethylamino)naphthalen-1-yl)furan-2,5-dione, a 4-maleicanhydridoproton sponge or "MAPS".

In this work DMAN was modified by adding a maleic anhydride moiety in the para position as recently described by Tyler et al., obtaining 3-(4,5-bis(dimethylamino)naphthalen-1-yl)furan-2,5-dione, a 4-maleicanhydridoproton sponge or "MAPS" (Scheme 1).[27]

The chemical structure was confirmed by ^{1}H-NMR (Figure S1) and by ESI-MS (figure S2). In spite of structural modification, the deprotonating function of the proton sponge only changes the pK_a of MAPS in acetonitrile to a slightly lower basic value as compared to the parent proton sponge (pK_a DMAN= 18,62 and pK_a MAPS= 18,00).[27-28]

This shift in basicity does not influence the efficiency of identifying acidic compounds in MALDI MS procedure as shown in figure S3. Citric acid was detected, in stoichiometric amounts, showing the similar deprotonating properties as DMAN. Moreover a complete suppression of matrix ions in presence of analyte was observed. Azahelicene, an alternative MAILD matrix, also showed matrix ion suppression in presence of acidic compounds.[29]

Moreover the vacuum stability of the matrix was tested over time. A vacuum-stability curve was calculated (Figure S4), which shows that MAPS is more stable than DMAN and as robust as 2,5- dihydroxybenzoic acid (DHB), a classical MALDI matrix very durable in high vacuum conditions.

The novel matrix MAPS was applied in MALDI-MSI using cryo-sections of diffuse glioma brain tumors, an aggressive brain tumor, resulting in the localization of oncometabolites using a routine MSI setup. MAPS was applied onto the diffuse glioma sections via a vibrational spray (ImagePrep™ device, Bruker, Bremen). MALDI imaging experiments with 70 µm per pixel of lateral resolution were performed using a MALDI ToF mass spectrometer (ultrafleXtreme™, Bruker, Bremen). Afterwards, MALDI-MSI results were compared with those of haematoxylin and eosin stained (H&E) tissues (Figure 1) by a pathologist.

Figure 1a shows two subsequent glioma sections, optical and haematoxylin & eosin-stained (H&E) pictures, respectively, in which cancerous and non cancerous areas are present. The appearance of deprotonated $[M-H]^-$ compounds such as lactate (Figure 1c) and 2-hydroxyglutaric acid (Figure 1d), at m/z 89 and 147 respectively, as well as chloride ions (Figure 1b) was observed predominantly in the glioma area of tissue. Thereby, a clear localization of the tumor was obtained.

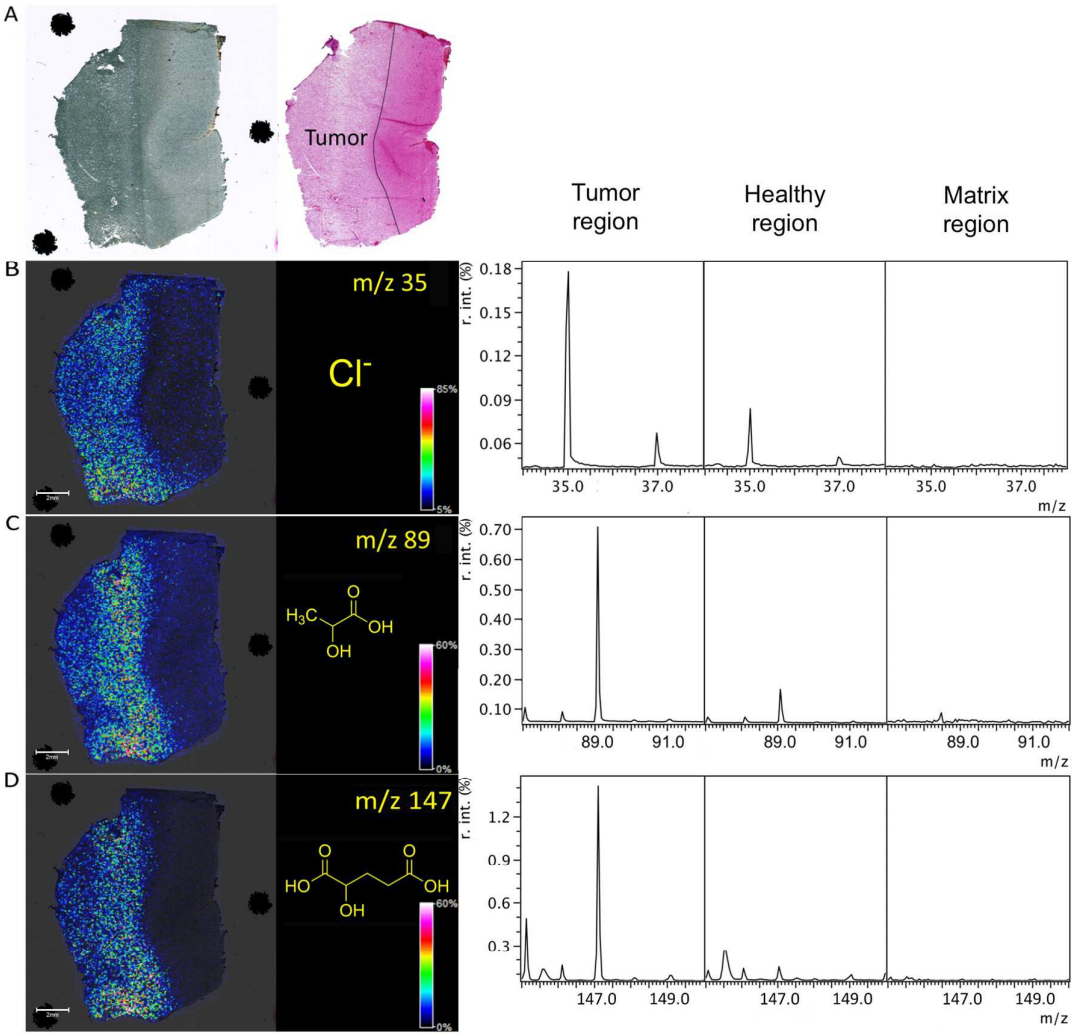


Fig. 1. MALDI Imaging results of a human diffuse glioma tissue section using the novel matrix MAPS. a) Optical (left) and H&E stained (right) sections were graded by a pathologist. Subsequent MALDI-Imaging showed the spatial distribution and regions of interesting spectra corresponding to chloride ions (b), lactate (c) and 2-hydroxyglutarate (d) in the tissue sections. Cancerous regions could be clearly distinguished from healthy tissue. Variations in ionic relative intensity between tumor, healthy and matrix regions were observed.

The same results were observed in other glioma samples (see Figure S5). The detection of tissue specific signals without any interference from matrix was observed as shown in the two dimensional (2-D) density plots in figure S6. Comparing low-mass 2-D density plots of matrix and cancerous region showed a clear difference in number and positions of bands is observed.

These results showed that the new matrix MAPS fits perfectly in MALDI-MSI experiments for visualizing cancer tissue through its deviating metabolism and cellular redox state. Moreover our results confirm biological results such as the "Warburg effect", overproduction of 2-HG, and intracellular accumulation of chloride in glioma cells.[30] This would be not possible using classical matrices such as DHB (Figures S7 and S8). The identification of lactate and 2-HG was confirmed by exact mass experiments using an FT-ICR mass spectrometer (Table 1).

Using the new matrix MAPS in combination with a standard MALDI-MSI setup the elevated occurrence of lactate (Warburg effect) in cancerous tissue could be clearly confirmed. This result is very important because Warburg effect is typical of most of tumor tissues e.g. breast cancer[31], lung cancer and colon cancer[32]. Therefore MAPS could be useful for analyzing other kind of tumors by MSI.

Table 1. Formula, measured and theoretical mass values of the metabolites identified from glioma tissue analyzed by MALDI-FT-ICR MS.

Formula $[M-H]^-$	m/z exp	m/z theor	Error (ppm)	Proposed metabolite

$[C_3H_6O_3\text{-}H]^-$	89.024421	89.024418	0,03	Lactate
$[C_5H_8O_5\text{-}H]^-$	147.02989	147.029897	-0,05	2-HG

Additionally, an increased level of 2-HG and chloride ions could be shown in the same tissue for the first time via MALDI-MSI. In particular 2-HG ionic maps allowed the phenotypic detection of the IDH1 mutation in glioma, which is diagnostically relevant for pathologist to establish the grade of tumor.

The detection of all these cancer tissue specific metabolic changes allows for the fast and unambiguous distinction between healthy and cancerous tissue. It will also help to further analyze and more deeply understand the process of carcinogenesis.

Observing the overall average mass spectrum of figure S8a, MAPS allows for the detection of multiple other compounds which provide valuable information for investigating the metabolic changes in tumors and the biological activity of new chemotherapeutics, opening an alternative imaging application in drug development process.[33] Furthermore, MAPS can be applied to diverse tissue types, where it provides the spatial information on e.g. pharmacologically active small molecules, even down to the size of single elemental ions.

Concerning approaches where high-resolution imaging is required, MAPS provides the presented high sensitivity along with the properties of producing a thin film when applied on tissues, rather than a crystalline structure as e.g. in case of DHB (Figure S9). This fact removes the limitations due to matrix crystal size or crystal distributions and opens the doors for novel high-resolution MALDI-based imaging methods.

Conclusions

In conclusion, the chemical modification of the common DMAN MALDI matrix by insertion of a reactive moiety (maleic anhydride) in para position opens new application possibilities in the analysis of small molecules by MALDI MSI in biological tissue samples such as tumors, without the requirements for further derivatization steps.

The vacuum stability of the proton sponge was improved and therefore localization of small molecules using a conventional MALDI ToF mass spectrometer was made possible.

The new MAPS matrix is a valid tool for Imaging of the "Warburg Effect" and tumor metabolites opening a new way to understand the chemical basis of cancer. With MALDI-MSI using MAPS as matrix, we were able to identify regions within tumors carrying a mutation in the IDH1-gene without the need for specific antibodies. This could provide predictive information for pathological analysis. Furthermore, MAPS can detect chloride anions involved in growth of glioma tumor cells. Moreover, the electrophilic maleic anhydride moiety opens an alternative chemical synthetic route towards a new series of vacuum-stable proton sponge based molecules available for MALDI Imaging MS of small molecules and inorganic anions.

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