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Localisation of adenine nucleotides in heat-stabilised mouse brains using ion mobility enabled MALDI imaging

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ABSTRACT

Information regarding the energetic state of tissue is important in a wide range of experimental fields, particularly in the study of metabolic stress, such as hypoxia or ischaemia. Metabolic stress leads to the degradation of adenine nucleotides in brain tissue, but little is known about any changes in the relative spatial distribution of these molecules. Ion mobility enabled MALDI imaging mass spectrometry has been employed to investigate the localisation of the adenine nucleotides ATP, ADP and AMP in mouse brain. The aim of the work is to develop a reproducible method for detecting changes in localisation and relative intensity of these nucleotides after metabolic stress. We demonstrate improved selectivity when ion mobility separation for the identification and localisation of the adenine nucleotides in tissue is employed. Tissue fixation methods have been evaluated to overcome rapid post-mortem degradation of adenine nucleotides such that biologically relevant localisation images can be obtained. These studies highlight the crucial importance of appropriate biological sample presentation in MALDI imaging experiments.

1. Introduction

The mammalian brain is very sensitive to metabolic stress [1]. These stresses can include reductions in the supply of oxygen (hypoxia) and glucose (hypoglycemia). There are a large number of potential incidents during which one or both these conditions can occur, including (but not limited to) strokes, heart attacks or epileptic seizures.

Information regarding the energetic state of tissue is important in a wide range of experimental fields, particularly in the study of metabolic stress. The energetic state of tissues or cells is often assessed by determination of adenine nucleotide (ATP, ADP and AMP) levels. This is commonly achieved using ultraviolet (UV)-HPLC, which provides quantitative data that can be used to indicate a broad spectrum of metabolic changes, but does not give specific localisation information. Metabolic stress leads to the degradation of adenine nucleotides in brain tissue [2], but little is known about changes in the relative distribution of these molecules. In healthy cells the ratio of adenine nucleotides is maintained with ATP in excess over both ADP and AMP [3]. During metabolic stress, these ratios change leading to an increase in the levels of AMP present.

There is a lack of information regarding the relative rates of change across the brain during metabolic stress. Determining the localisation of the adenine nucleotides in brain may provide more information into areas of the brain more vulnerable to ischaemia; such as those with a higher rate of metabolic activity. These areas may show the fastest decline of ATP levels during metabolic stress and slower recovery times. Establishing this information may be useful in explaining differential vulnerability and recovery of brain regions to metabolic stress and in establishing the potential for neuroprotective or neurorestorative therapies by improving the bioenergetic recovery of brain tissue after injury [4]. Adenine nucleotide localisation information could also be coupled with the localisation of cell-surface receptors for purines. ATP is released from tissue during ischaemia [5] and likely damages brain tissue through the activation of ATP receptors. Breakdown of ATP produces adenosine, which is believed to be protective through the activation of adenosine A_1 receptors. Accordingly, coupling this receptor localisation information together with the spatio-temporal profile of cellular adenine nucleotides could provide more insight into the outcome for brain tissue after metabolic stress.

MALDI imaging, pioneered by Caprioli et al. [6], is a useful technique for mapping the distribution of specific ions across surfaces, such as tissue sections. Originally developed for the analysis of peptides, MALDI imaging is now used in a wide range of application areas including imaging of proteins, drug compounds, lipids and metabolites in a range of tissue types. Some of the challenges faced by the imaging MS community using MALDI imaging experiments include sample preparation, spatial resolution, information content and selectivity. The selectivity of a MALDI imaging experiment is key to the detection of ions with low abundance or those

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subject to interference by endogenous ions of similar mass. FT-ICR instruments with high-resolution and mass accuracy have been used for MALDI imaging experiments to improve selectivity. These experiments reveal spatial details that would otherwise have been missed, but require very long acquisition times that can limit the usefulness of the experiment to small regions of interest [7]. A number of research groups have employed MS/MS fragmentation in order to improve the selectivity of the MALDI imaging experiment. With small molecules in particular, this can help to distinguish the ion of interest from matrix clusters formed in the MALDI experiment enabling specific localisation images to be obtained [8]. Whilst these tandem MS experiments improve the selectivity of the approach, only a limited number of analytes can be sequenced at each sampling position before the sample is consumed. At increased spatial resolutions this becomes more difficult since the available material at each position for study is decreased [9].

Ion mobility mass spectrometry (IMS) can be used to provide separation of ions on the basis of their molecular structure as well as their mass-to-charge ratio (m/z). Coupling ion mobility with MALDI imaging allows for the separation of ions according to size, shape and charge state. This has enabled different compound classes to be separated and classified, providing an advantage over MALDI imaging alone. MALDI-IMS-MS can be used to produce images with less interference from background ions of similar mass. This provides confidence that the biologically relevant distribution of an ion of interest is being observed and can distinguish the compounds of interest from the matrix, providing more precise localisation [9,10]. Ion mobility separation is performed on a millisecond timescale, allowing it to be incorporated easily within a MALDI imaging experiment without compromising the speed of data acquisition [11].

The advantages of the use of ion mobility enabled MALDI imaging over conventional MALDI MS and MS/MS imaging data has been demonstrated, with the distribution of an anticancer drug in rat whole body tissue sections being much more clearly identified [12]. Ion mobility separation allowed the distribution of the drug under investigation to be separated from interference by endogenous ions. Other examples of the use of IMS to improve the selectivity of imaging mass spectrometry experiments have also been presented [11,13–17].

Here, we focus on the use of IMS to improve the selectivity of a MALDI imaging experiment for the detection of adenine nucleotides in mouse brain. Post-mortem changes in the levels of adenine nucleotide take place rapidly following death, owing to the activity of ATPase enzymes in the tissue, and the absence of oxygen and glucose necessary for ATP synthesis. Standard tissue handling and sample preparation for an imaging mass spectrometry experiment requires immediate snap freezing of the tissue in liquid nitrogen following dissection. Snap-freezing of tissue is widely used to reduce enzymatic activity in tissues, but as the tissue thaws enzymatic activity will be recovered and this can lead to further ATP degradation during sample preparation as we have described previously [2].

Previous work by Benabdellah et al. has demonstrated the potential applicability of MALDI imaging experiments for the localisation of primary metabolites in rat brain, including adenine nucleotides, by using 9-aminoacridine (9-AA) as a matrix compound [18]. The identification of primary metabolites was confirmed using MS/MS experiments. Despite successful identification of ATP in tissue sections, localisation images of ATP across the brain section were not obtained. This problem was attributed to the low sensitivity of the approach to ATP, but may also be related to the rapid post-mortem turnover of ATP. Work by another group has employed MALDI imaging to identify changes in the localisation of adenine nucleotides in mouse hippocampus following a kainate-induced seizure [19]. The potential post-mortem degradation of the adenine nucleotides was discussed, with in situ freezing offered as a potential method to prevent this happening [20]. The severity of the seizures suffered by the kainate-treated mice meant that this was impractical. These previous studies indicate the potential of using MALDI imaging for assessing the distribution of adenine nucleotides and their changes during times of metabolic stress, however it is clear that post-mortem changes in these compounds presents a significant experimental challenge.

Traditional tissue fixation procedures do not lend themselves towards imaging MS experiments. Although there are examples of successful protein imaging experiments using formalin-fixed paraffin-embedded (FFPE) tissues, on-tissue enzymatic digestion prior to analysis is required, which would be less suitable for small molecules such as adenine nucleotides [21]. Ethanol washes could be employed as a temporary tissue fixation method with the additional advantage of washing away salts and other contaminants from the tissue surface [22].

A mass spectrometry compatible heat stabilisation technique for use in tissue fixation has been developed recently by Denator AB (Stablilizor™, Denator AB, Sweden). Enzymes are inactivated after sampling by heating the tissue to 95 °C rapidly using a combination of heat and pressure under vacuum. This procedure raises the temperature quickly, homogenously and reproducibly and prevents downstream sample degradation which is commonly encountered with other techniques such as snap-freezing [23]. The applicability of this technique for MALDI imaging experiments has been demonstrated, showing retention of a marker in treated mouse brain sections that was otherwise lost in untreated sections [24]. Further work highlighted the fact that heat treatment at the point of excision also prevented degradation of proteins in tissues. Some issues with the quality of tissues following heat treatment were noted, however, which could effect the precision of spatial information obtained during MALDI imaging [25].

The work described here aims to evaluate the potential of mobility enabled MALDI imaging for the detection and localisation of adenine nucleotides in mouse brain samples. Sample preparation methods, including the use of ethanol washes and heat-stabilisation, were evaluated as tissue fixation approaches to prevent rapid degradation of the adenine nucleotides following death.

2. Materials and methods

2.1. Materials

9-Aminoacridine hydrochloride (9-AA), adenosine-5-triphosphate (ATP) disodium salt hydrate and adenosine-5-monophosphate (AMP) disodium salt standards were purchased from Sigma–Aldrich. Ethanol was purchased from Fischer Scientific at the highest purity grade available and used without further purification. Water was purchased from J.T. Baker at the highest purity grade available and used without further purification.

2.2. MALDI-MS analysis of standards

Standards of ATP, ADP and AMP were prepared at 1 mg mL^{-1} in distilled water. 9-AA was prepared at 10 mg mL^{-1} in 70% EtOH with 0.1% TFA. Each adenine nucleotide solution was diluted 1:1 with 9-AA matrix solution as described. 1 μL was spotted onto a stainless steel target plate and allowed to dry prior to MALDI-MS analysis.

2.3. MALDI-MS and mobility enabled MALDI-MS/MS experiments

Data were acquired by means of a Synapt G2 HDMS mass spectrometer operated in MALDI negative ion mode from 100 to 600m/z with and without mobility separation. The TOF mass analyser was