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Light and heavy ion beam analysis of thin biological sections

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ABSTRACT

The application of ion beam analysis (IBA) techniques to *thin biological sections* (ThBS) presents unique challenges in sample preparation, data acquisition and analysis. These samples are often the end product of expensive, time-consuming experiments, which involve many steps that require careful attention. Analysis via several techniques can maximise the information that is collected from these samples. Particle-induced X-ray emission (PIXE) and Rutherford backscattering (RBS) spectroscopy are two generally non-destructive IBA techniques that use the same MeV ions and can be performed simultaneously. The use of heavy ion PIXE applied to thick samples has, in the past, resulted in X-ray spectra of a poorer quality when compared to those obtained with proton beams. One of the reasons for this is the shorter probing depth of the heavy ions, which does not affect thin sample analysis. Therefore, we have investigated and compared 3-MeV proton and 36-MeV carbon ion beams on 7-μm thick mouse brain sections at the ANSTO Heavy ion microprobe (HIMP). The application of a 36-MeV C^{4+} ion beam for PIXE mapping of ThBS on thin Si_3N_4 substrate windows produced spectra of high quality that displayed close to a nine-times gain in signal yield (Z^2/q) when compared to those obtained for 3-MeV protons for P, S, Cl and K but not for Fe, Cu and Zn. Image quality was overall similar; however, some elements showed better contrast and features with protons whilst others showed improved contrast with a carbon ion beam. RBS spectra with high enough counting statistics were easily obtained with 3-MeV proton beams resulting in high resolution carbon maps, however, the count rate for nitrogen and oxygen was too low. The results demonstrate that on thin samples, 36-MeV C^{4+} will produce good quality PIXE spectra in less time; therefore, carbon ions may be advantageous depending on which element is being studied. However, these advantages may be outweighed by the inherent disadvantages including increased ion beam damage, the necessity of very high ion energies resulting in higher neutron fields.

1. Introduction

PIXE spectroscopy is a valuable technique with a long tradition in the analyses of biological systems including animal models [1–3], cells [4–7], cardiovascular disease [8,9], tissues surrounding metallic implants [10–12], brains suffering from Alzheimer's disease [13–15] and other organs [16–18]. Elemental analysis through μ-PIXE experiments has the potential to provide new insights into the progress of diseases, which may not only provide improved treatment options for diseases, but may also lead to a greater understanding of the function and mechanisms through which elements interact with the body and influence tissue biochemistry [19,20]. Whilst PIXE shares its role with several other techniques, such as laser-ablation inductively coupled plasma mass spectrometry (ICP-MS), it is particularly suited to the study of trace elements in biological tissue samples due to its quantitative imaging capabilities at a higher spatial resolution of around 1–10 μm together with low 1–10 $\mu g\, g^{-1}$ detection limits [21] compared to 15–50 μm spatial resolution of ICP-MS [22].

PIXE is undoubtedly a powerful technique; however, it requires careful interpretation of the data in order to account for changes in sample composition, fitting of relevant spectral features and correction of possible self-absorption effects [23]. One method to account for overlapping spectral features from different elements

Abbreviations: ThBS, thin biological section; GM, grey matter; CM, cerebral malaria; ML, molecular layer; FTIR, Fourier transform infrared; WM, white matter.

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is to perform a least-squares fit to every spectrum in each pixel of the image data. This is very time consuming and can be avoided by applying the *Dynamic Matrix Analysis* approach, as used by Geo-PIXE [23].

The ANSTO HIMP routinely uses a wide range of ions, such as H, He, C, Cl, Br and I ions over a large range of energies (3–120 MeV) [24]. Previous experiments on thick biological samples by Orlic and coworkers [1] used heavy ions such as 9-MeV He^{2+} and 20-MeV C^{4+} for PIXE experiments, which resulted in less sensitive elemental detections when compared to those obtained with 3-MeV H^+. This was postulated to be caused by a dominating Bremsstrahlung background obscuring some of the trace element lines [1]. However, the decrease in sensitivity can also be traced back to the shorter range of the heavy ion beams, which results in a smaller amount of material being probed. Hence, PIXE studies have been conducted on ThBS in order to investigate relative elemental sensitivities to analysis with 3-MeV H^+ and 36-MeV C^{4+} beams.

2. Experimental

The samples used for this analysis come from a larger set of samples that have been aimed at investigating biomolecular and elemental distribution changes of the mouse brain, during cerebral malaria (CM) and with antimalarial quinine treatment for CM, with the approval of the University of Sydney Animal Ethics Committee. Details of the mouse brain tissue preparation are published elsewhere [2]. Tissue samples were initially imaged via Fourier-transform infrared (FTIR) spectroscopy and subsequently mapped for elemental variations by PIXE. A tissue thickness of 7-μm was decided as a compromise between several factors. Cryosectioning in the 7–10 μm was easier to manage whilst thicker sections began to curl, which made it extremely difficult to position a specific region of the sample (i.e. cerebellum) onto the active area of the Si_3N_4 membrane substrate (Silson Ltd., Northampton, England). The substrate frame had dimensions of 1×1 cm^2 whilst the active area was 5×5 mm^2. This substrate was chosen because of low elemental interference and its ability to be used in a variety of microscopies [25]. Serial sections were collected for staining via haematoxylin and eosin. Thicker sections would be expected to increase the PIXE yield, but would result in decreased histology qualtiy due to staining of multiple overlapping layers of cell. Results of the quinine treatment experiment will be reported in a separate publication following completion of the data analysis. Formalin fixation of the tissues was avoided as it has been demonstrated to alter the distribution of elements in the brain [26,27].

2.1. Instrumental parameters

All PIXE and RBS experiments were run at the ANSTO heavy ion microprobe [24], which produces ion beams via a tandem Van de Graaff accelerator charged by a chain system. PIXE experiments were run with the same charge per pixel over a scan area of 1200×1200 μm^2 using a beam size of 5–8 μm. The Ge detector was positioned at 135° and a distance of 30 mm from the sample. A 105-μm thick Mylar filter was placed between the sample and the detector to prevent scattered ions from entering the detector. Additionally a RBS detector was placed at a scattering angle of 165° at 105 mm from the target. The detector area was limited by a 3-mm Al aperture.

2.2. Geo-PIXE image analysis

Geo-PIXE [28] (version 5.5) software (CSIRO Victoria, Australia) was used to extract quantitative results as well as elemental maps from the data. Geo-PIXE applies the *Dynamic Matrix Analysis* approach to extract elemental concentrations for each pixel.

3. Results and discussion

Fig. 1 shows the PIXE yields calculated with Geo-PIXE for 3-MeV H^+ and 36-MeV C^{4+} for a thick obsidian sample as used in Ref [1] and 7-μm thick tissue samples. Additionally the yield ratio per ion for C and H beams is shown for the two different sample types. The figure shows that for light elements up to Cl the yield increase for 36-MeV C^{4+} is close to a factor 36 compared to 3-MeV H^+ for the thick samples. For heavier elements, this increase quickly drops to below a factor of 20. This makes it more difficult to measure traces of the heavier elements in the presence of high concentrations of light matrix elements, as is the case in obsidian. For thin samples, however, the yield ratio per ion stays constant at a factor of 36. Hence, we have revisited the use of heavy ions for this investigation of thin mouse brain sections mounted on thin (500 nm) Si_3N_4 membranes, where this limitation does not apply.

3.1. RBS on ThBS produce high resolution images of carbon using 3 MeV H^+

With the use of a 3-MeV H^+ beam, simultaneous mapping of light elements with RBS was possible, while for 36 MeV C^{4+} ions, the count rate in the RBS detector was too low to be useful. Fig. 2A shows the RBS spectrum taken on one of the tissue samples. The lines mark the surface energies for the different elements. Since both the sample and the Si_3N_4 support were quite thin, backscattering from light target atoms appeared as well-spaced peaks. This made it easy to extract maps for light elements such as C, O and N. Both the Si and N appeared at energies below their respective surface energies, because they were originating from the Si_3N_4 film located below the sample. Fig. 2B displays RBS maps for the carbon and oxygen distribution in the mouse cerebellum. The carbon map corresponds precisely to the white matter (WM) layer of the cerebellum, which is composed of myelinated axons [26]; however, no distinction was possible between the grey matter (GM) and the molecular layer (ML). Traditionally the use of RBS on biological samples has been limited to mass thickness calculation and depth profiling [29,30] but when thin samples on Si_3N_4 membranes are analysed, spatial imaging of C and O becomes possible. This method of analysis could potentially detect carbon density differences associated with myelin loss and supplement magnetic resonance imaging techniques [31]. The simultaneously collected PIXE data could provide additional information regarding other elements that play a role in the same regions.

Mapping of C from the RBS spectrum works well because proton scattering on C has a strongly enhanced cross section over the

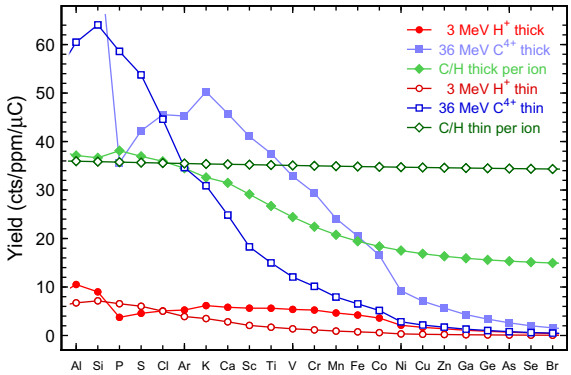

Fig. 1. Yield variation of thick obsidian vs. ThBS. The carbon/hydrogen yield ratio is almost constant in the thin samples. In thick samples, self absorption together with the difference in range of carbon and hydrogen results in a gradual drop in the yield ratio with increasing *Z*.