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Nuclear microscopy measurement of copper in atherosclerosis – Sensitivity and limitations to spatial resolution

Reshmi Rajendran [a,b,*], Minqin Ren [a,b], Pan Ning [b], Benny Tan Kwong Huat [c], Barry Halliwell [b], Frank Watt [a]

[a] Centre for Ion Beam Applications, Department of Physics, National University of Singapore, MD7, 8 Medical Drive, Singapore 117597, Singapore
[b] Department of Biochemistry, National University of Singapore, MD7, 8 Medical Drive, Singapore 117597, Singapore
[c] Department of Pharmacology, National University of Singapore, MD7, 8 Medical Drive, Singapore 117597, Singapore

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Abstract

Nuclear microscopy studies at the Centre for Ion Beam Applications have indicated a link between iron, zinc and the development of atherosclerosis. In the present study, we have extended this study to copper, since copper is also capable of inducing free radical mediated damage. As copper in biological tissue occurs at the parts per million level and therefore close to the detection limits for PIXE analysis, we have substantially increased the beam intensity and scanning time to obtain adequate statistics and analytical sensitivity. The experiments were conducted on male New Zealand White rabbits, weighing approximately 2.5 kg and fed on high cholesterol diets for 8 weeks. Unlike iron concentrations, which were observed to be increased in the atherosclerotic lesion, our experiments show that copper is depleted in the lesion (1.9 ppm) compared with the adjacent artery wall (4.1 ppm). The concentration of copper present in the lesion is also much less than iron (approximately 30 times less on average), indicating that iron, rather than copper, is more likely to induce atherosclerosis through free radical mediated damage, purely on the basis of greatly reduced concentrations.

To obtain adequate statistics for copper, a 2.1 MeV proton beam focused to 2 μm with a relatively high beam current (from about 800 pA to 1 nA) was scanned over the sample for approximately 4 h. The effects of beam damage due to these high beam currents have been investigated and it was found that the tissue shrinks 10% each in the X and Y direction contributing to total area shrinkage of 20%. Despite tissue shrinkage problems, our results indicate that even with 1 nA beam currents, 4 h scan times and scan sizes of around 1×1 mm^2, we can still extract accurate trace elemental concentrations.

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Keywords: Atherosclerosis; Copper; Nuclear microscopy; PIXE; STIM; RBS

1. Introduction

Atherosclerosis is a systemic disease of the vessel wall that occurs in the aorta and in the carotid, coronary and peripheral arteries [1]. Iron and copper can theoretically both induce free radical mediated damage and thus promote atherogenesis. The possibility of iron generated free radicals leading to the progression of atherosclerosis has been investigated using nuclear microscopy [2]. Various other groups have also studied the role of iron in atherosclerosis [3–9]. However, studies on copper in atherosclerosis have been contradictory, with some studies claiming that copper deficiency leads to increased coronary risk [10] while others state that copper can induce oxidation of LDL *in vitro* [11]. Accurate copper analysis is difficult because of the minute amounts of copper present in tissue. In order to obtain adequate statistics for copper, we have

* Corresponding author. Present address: Department of Biochemistry, National University of Singapore, MD7, 8 Medical Drive, Singapore 117597, Singapore. Tel.: +65 6516 4953; fax: +65 6777 6126.
E-mail address: bchrr@nus.edu.sg (R. Rajendran).

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utilized nuclear microscopy with relatively high beam currents (around 1 nA). It has been shown that such high beam currents can cause damage to tissue [12–14], in the form of elemental loss, structural changes or tissue shrinkage. In order to investigate this, further tests were carried out. The aim of the present study was to map and quantify copper in unstained atherosclerotic tissue and to investigate the limits to the analytical sensitivity and spatial resolution at which copper can be measured.

2. Materials and methods

Rabbits on high fat diet develop atherosclerotic lesions [15]. The seven rabbits chosen for our study were male New Zealand White rabbits of the same age, weighing approximately 2.5 kg and fed on high cholesterol diets (standard Guinea Pig and Rabbit diet + 1% cholesterol) for 8 weeks to induce atherosclerosis. After sacrifice, the aortic arch was removed and cut into three segments [16]. Residual blood was removed from the inner artery wall by flushing these segments with deionised water, and the blocks of tissue were then flash frozen in liquid nitrogen. The tissue was stored at −80 °C, and transported on dry ice where necessary. Special care was taken to prevent thawing and refreezing and subsequent ice crystal damage. Serial sections of 14 μm thicknesses were cut using a Leica CM3050S cryostat set at −17 °C with the knife at −22 °C. Sections were picked up on gelatin coated glass sides for Hematoxylin and Eosin staining to identify regions of interest. Adjacent unstained sections were picked up on freshly made pioloform film mounted on nuclear microscopy target holders for STIM, RBS and PIXE analysis.

2.1. Experimental set up

The nuclear microscopy experiments were carried out at the Centre for Ion Beam Applications, CIBA, at the National University of Singapore. A 2.1 MeV proton beam was focused to a 1 μm spot size. Data from the three techniques of proton induced X-ray emission (PIXE) for trace elemental analysis, Rutherford backscattering spectrometry (RBS) to measure the concentration of matrix constituents of the sample and scanning transmission ion microscopy (STIM) to identify regions of interest in the specimen, were simultaneously collected. Previously, these techniques have successfully mapped and measured iron and zinc in atherosclerotic tissue and established a link between these trace elements and atherogenesis [17]. Though PIXE allows simultaneous detection of multiple elements with high quantitative accuracy, its analytical sensitivity has a lower limit of about 1 ppm in biological material such as tissue sections. Hence copper, which is present only at ppm amounts in tissue, is at the threshold of detection for PIXE. The required statistics for PIXE was obtained by using high beam currents (up to 1 nA) for a longer (4 h) scan time, compared to previous studies measuring iron and zinc where currents of 100–300 pA were used for less than an hour. In order to prevent high dead time in the data acquisition (DAQ) system throughout the run, the STIM detector was connected to the DAQ system only during an initial short time phase (around 30 min) using currents of around 100 pA. The current was increased from 100 pA (for the STIM measurements) to 1 nA (for the PIXE and RBS analysis) by opening up the object apertures, thereby increasing the beam spot size from 1 μm to around 3 μm. In order to avoid possible localised heating effects, the scan speed was increased to 1 ms per PIXEL over the 256×256 scan size, and limited to this value because very fast scan speeds can cause hysteresis in the magnetic scanning system leading to reduced resolution. List mode data acquisition was employed for these long runs, so that trace elemental data could be extracted from different regions of the scanned area.

3. Results and discussion

3.1. Copper analysis

For analysis of copper, the areas chosen were those with average sized lesions and minimum calcification. Calcium pileup occurs at 7.4 keV, which is between the K-α X-ray peaks of iron and copper, making automatic extraction of the copper data from the PIXE spectrum more difficult. All scans encompassed the lesion/artery wall interface so as to include both lesion and the normal artery wall for comparative off line analysis. Elemental concentration analyses were carried out for both the lesion and artery wall. Contrary to that observed for iron [2], copper was higher in the healthy artery wall compared to the lesion (see for example Fig. 1). Among the seven animals analysed, copper had an average concentration of 4.1 ppm in the healthy artery wall compared to 1.9 ppm in the lesion.

3.2. Sensitivity

In the experiments conducted to analyse copper, a 2.1 MeV proton beam focused to 1 μm with high beam currents from about 800 pA to 1 nA was scanned over an average area of 1×1 mm^2 for ≈4 h. To maximize the X-ray detection efficiency, the XYZ target manipulator was set to the 45° position [15]. The PIXE detector with an area of 65 mm^2 was placed as close to the sample as possible (16 mm) in order to increase the solid angle (0.2539 sr) and thus increase count rate. A 300 μm Perspex filter with a 1.5 mm central hole was used to reduce the X-ray counts from the lower mass elements, thereby reducing dead-time and pile up and optimizing the system for the detection of trace elements from Ca to Zn [18].

The STIM detector, which was placed at an off-axis angle of 15° [19], was disconnected after the first half hour, after adequate structural information had been collected, thus decreasing the dead time of the data acquisition system and further increasing the count rate from the PIXE detector. The RBS detector, which was placed at a

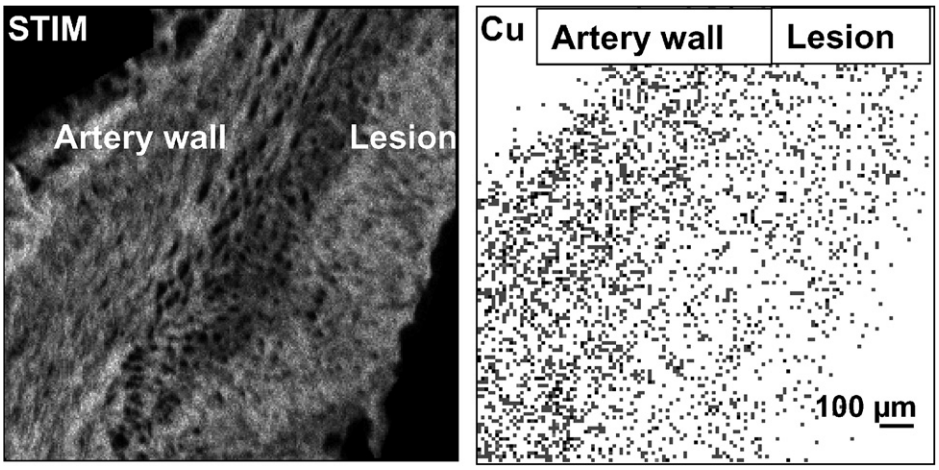

Fig. 1. The STIM map shows the structure and the corresponding copper map indicates that copper is increased in the artery wall compared to the adjacent lesion. The copper map was obtained from 2.1 MeV proton beam focused to about 2 μm scanning an area of 1.2×1.2 mm^2 at a scan speed of 1 msec with a current of 1 nA for 4 h and 34 min.

backward angle and used to measure charge and the composition of the matrix, had a sold angle of 0.08201 sr. At these experimental parameters, we were able to map and quantify copper down to a few parts per million, the lowest value measured being 1.2 ± 0.4 ppm.

3.3. Limitations to spatial resolution

When organic samples are exposed to high beam currents analytical errors can arise from changes in the sample due to beam damage. It has been calculated that a 3 MeV proton beam with a current of 1 nA focused down to a 1 μm size scanning over a 100×100 μm^2 area scan size can deposit an energy of approximately 2×10^6 J/g in a 1 μm thick nylon foil [20]. Extrapolating this, we would expect a 2.1 MeV proton beam with a beam current of 1 nA focused down to 2 μm size scanning over 200×200 μm^2 area over a 15 μm thick tissue section to deposit $\approx 0.02 \times 10^6$ J/g. Some problems that might arise due to high beam currents are sputtering of the specimen, elemental distortion, surface topography variation, and tissue shrinkage. The beam can damage the specimen by sputtering from the surface, caused by displacement of atomic nuclei and nuclear transmutations within the material [14]. This in turn, can affect the elemental concentration as sputtering can remove different elements from specimen materials at different rates [21]. In addition to sputtering, disturbances at the atomic level could also alter the surface topography of uniformly bombarded areas.

All our experiments were carried out with 2.1 MeV protons. Unlike keV and low MeV ions, sputtering yields for higher energy protons are relatively low, and so we would expect minimal damage due to sputtering in our experiments. This does not affect our measurements of copper as experimental evidence indicates that under normal bombardment of beams of protons, substantial amounts of the volatile elements hydrogen and oxygen can be lost in a few seconds, whereas carbon is generally stable as are most other elements [22]. Watt et al have shown that by scanning a 100 pA 2 MeV proton beam focused to a 1 μm spot size across a single cultured cancer cell in continuous raster mode, there is an approximate exponential loss of hydrogen and oxygen, while trace elements including copper were found not to change within experimental errors [12].

It has been recommended that the best way to assess the damage to the surface topography is to monitor the signals from the major elements [14]. In our present work, consistent counts were obtained for sulfur for equal intervals of charge, indicating that the elemental composition of the tissue remains unchanged, although hydrogen and oxygen were not monitored. The sulfur counts plotted against the charge collected in each 10% of the run are shown in Fig. 2. To understand changes to the structure of tissue, further tests were conducted. Comparative structural images before and after exposing the sample to high beam currents were obtained from the STIM maps procured at the beginning and end of the 4 h run (Fig. 3). The sensitivity of these measurements was increased by using a smaller scan size of 200×200 μm^2. Our results were consistent with

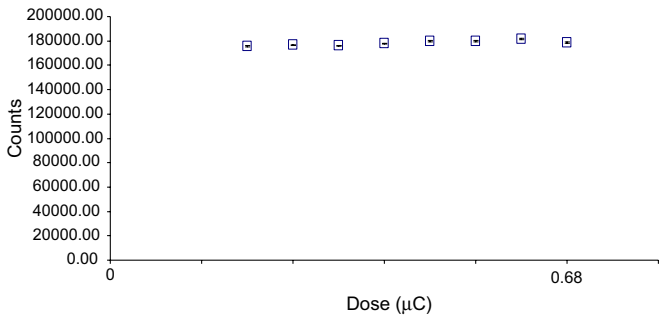

Fig. 2. Sulfur is shown in a consecutive manner in each 10% of the run and is a constant after the STIM detector is disconnected. By monitoring the signal from a major element like sulfur, we can infer that for elements of interest, changes to the tissue do not occur even after a 4 h run.

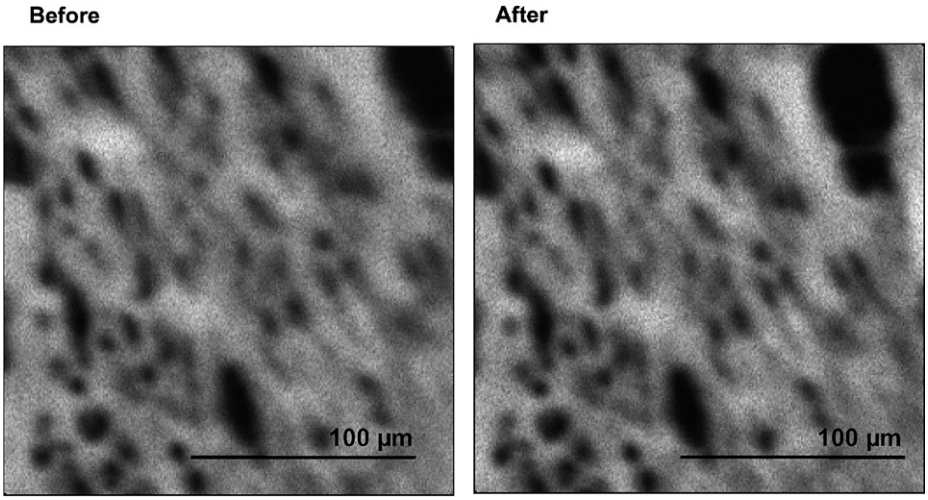

Fig. 3. STIM map showing the shrinkage of the tissue before and after a 4 h 12 min run with a beam current of 850 pA. The beam current shows average 10% shrinkage in each in the X and Y direction, contributing to total area shrinkage of 20%.

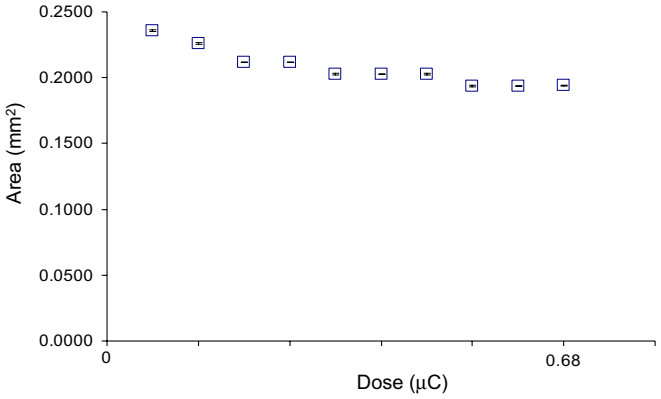

Fig. 4. The total area shrinkage in each 10% of the run is almost 20% and it follows an exponential trend, and is in agreement with previous studies.

those which showed 20% shrinkage in cells [12,13]. Fig. 4 shows the area shrinkage plotted against the charge collected sequentially for each 10% of the run over an total accumulated charge of 6.837 μC. Similar rates of shrinkage were observed in the X (89.7%) and Y (91.6%) direction. Our results, which exhibit an exponential trend, are in agreement with most models for mass or elemental loss under charged particle bombardment which predict some form of exponential decay [23]. These results also suggest that there is a limit to the spatial resolution when using high beam currents, as the extent of shrinkage from beam damage depends on the loading parameter which is measured in C/μm^2 [24].

4. Conclusions

As a follow up of iron and zinc studies in atherosclerosis using nuclear microscopy [17], copper in New Zealand white rabbits on a high fat diet, was mapped and quantified using nuclear microscopy with a slight modification of the experimental parameters. Our studies were the first of its kind to conduct comparative copper measurements in the lesion and the adjacent healthy artery wall in rabbits on a high fat diet and this was possible by extracting data from list mode files. Our experiments showed that, unlike iron, which was increased in atherosclerotic lesions, copper was decreased, with the seven animals having an average of 4.1 ppm in the healthy artery wall as opposed to 1.9 ppm in the lesion. These results are in agreement with other studies which state that copper deficiency and not excess leads to coronary artery disease [10]. Our nuclear microscopy measurements were able to measure copper with a sensitivity of a few parts per million, the lowest value measured being 1.2 ± 0.4 ppm. This is lower than the lowest limit reported by other studies on cholesterol fed New Zealand White rabbits, where values of 1.8 ± 0.2 μg/g wet tissue [25] and 1.49 ± 0.48 μg/g wet tissue [26] were reported in aortic tissue. These measurements were carried out by atomic absorption spectroscopy which is a destructive method and does not enable simultaneous identification of spatial location of the copper calculated.

In order to obtain adequate statistics for copper, a 2.1 MeV proton beam was focused to a beam spot size of 1 μm and continuously scanned across an average scan size of 1×1 mm^2 in raster mode for about 4 h. At these conditions, beam damage was investigated by examining structural damage and shrinkage with the help of STIM. Our results indicate that tissue *areal* shrinkage of about 20% does occur in an exponential manner, and this is in agreement with previous studies [12,13]. The X and Y direction showed similar rates of shrinkage (89.7%) and 91.6%). At these beam current densities over long periods of time, the resulting shrinkage therefore will affect the spatial resolution, smearing out the scan and reducing the effective resolution by around 10% of the scan size in the X and Y directions. The gradual shrinkage during the period of the scan can also affect the measurement of trace element

concentration if the area chosen for analysis is adjacent to an area which has significantly raised or lowered trace element levels: The ensuing overlap of this adjacent tissue will therefore distort the elemental concentrations extracted from the initial scanned region. Further tests have to be carried out to find out if tissue shrinkage does stop after the tissue is subject to a specific charge and if an optimum charge window can be identified for trace elemental quantification.

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