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Detection, identification and mapping of iron anomalies in brain tissue using X-ray absorption spectroscopy

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This work describes a novel method for the detection, identification and mapping of anomalous iron compounds in mammalian brain tissue using X-ray absorption spectroscopy. We have located and identified individual iron anomalies in an avian tissue model associated with ferritin, biogenic magnetite and haemoglobin with a pixel resolution of less than 5 μm. This technique represents a breakthrough in the study of both intra- and extra-cellular iron compounds in brain tissue. The potential for high-resolution iron mapping using microfocused X-ray beams has direct application to investigations of the location and structural form of iron compounds associated with human neurodegenerative disorders—a problem which has vexed researchers for 50 years.

Keywords: iron; brain; Alzheimer's disease; neurodegenerative disease; magnetite

1. INTRODUCTION

Iron is an essential element for living organisms; however, in some circumstances it can be toxic. In humans, disruption of normal iron metabolism and excess accumulation of iron in brain tissue have been strongly linked to virtually all neurodegenerative disorders, such as Alzheimer's, Huntington's and Parkinson's diseases (e.g. Beard *et al.* 1993; Markesbery 1997). Although anomalous iron concentrations appear to be a major factor in these diseases, current methods for assaying iron in diseased tissue rely mainly on staining techniques which only reveal the presence of Fe^{3+} and have relatively poor spatial resolution. In addition, these methods do not identify the iron compounds that are present (Perl & Good 1992). Though recent work by Smith *et al.* (1997) and Sayre *et al.* (2000) has made progress in identifying the presence of more toxic Fe^{2+}, and shown that redox-active iron is closely associated with Alzheimer's plaques and tangles, progress in our understanding of the role of iron in neurodegenerative diseases has been slow since this association was first described by Goodman over 50 years ago (Goodman 1953). This is primarily owing to problems associated with imaging small-scale iron compounds *in situ*.

Further complications arise because the increases in iron levels associated with these diseases do not necessarily correlate with increases in the iron storage and transport proteins, ferritin and transferrin (Loeffler *et al.* 1995; Fisher *et al.* 1997). This may be an indication of increased iron loading in the ferrihydrite core of the ferritin protein, the sequestration of iron compounds within plaque material (Sayre *et al.* 2000; Dobson & Batich 2004) or the presence of nanoscale biogenic magnetite, a ferrimagnetic iron oxide (Dobson 2001).

In many organisms, biomineralized magnetite nanoparticles form a vital component of geomagnetic field sensing systems (Blakemore 1975; Kirschvink *et al.* 1985). In the case of mammals, while magnetite almost certainly plays a central role in magnetoreception, and has been linked to structures in the mammalian central nervous system, its presence has yet to be confirmed by structural (diffraction) analysis *in situ* (Walker *et al.* 1997; Diebel *et al.* 2000). As with neurodegenerative tissue, the primary reason for this is that the particles are very small compared with the resolution of the imaging methods which are normally used on tissue sections.

In order to overcome the problems associated with the identification and mapping of iron compounds

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in brain tissue *in situ*, we have developed a novel technique using iron edge area scanning along with X-ray absorption near edge spectroscopy (XANES).

The synchrotron-based techniques presented here have the potential to provide, for the first time, the ability to characterize and map anomalous iron compounds *in situ* within histological brain tissue sections. This information is vital to our understanding of the role of iron compounds in neurodegenerative diseases.

2. EXPERIMENTAL DETAILS

An avian tissue model (homing pigeon—*Columba livia*) was selected for these initial studies, and samples were taken from sections of the mid-brain region. Tissue samples were removed from the cranium and fixed by immersion for 24 h in Karnovsky's solution (glutaraldehyde and paraformaldehyde, both of which do not form acids, pH = 7.2–7.4). Samples were then cut into sections of approximately 5 mm^2, with a thickness of 45–50 μm using a TPI 1000 Vibratome with Teflon-coated, stainless steel blades, exposed to osmium tetroxide (OsO_4) for 15 min, dehydrated and embedded in TAAB 812 resin (Marivac Ltd). Samples were sealed at 48 °C overnight, between two sheets of Kapton thermoresistant plastic, for analysis. We have demonstrated that these Kapton films are free of iron within the detection limit of X-ray fluorescence at the Advanced Photon Source's (APS) Materials Research Collaborative Access Team (MRCAT) beamline. All glassware and materials (vials, slides, Kapton, etc.) used in this preparation procedure were placed in an ultrasonic bath for at least 30 min and cleaned in 4% HCl. All solutions were purified to EM grade or filtered with a Whatman 42 2.5 μm filter. All chemicals were also magnetically cleaned using NdFeB magnets.

Zinc wire gridlines were embedded under the Kapton film in order to provide an orientation system for overlaying the X-ray images with images which can be obtained subsequently using other microscopy techniques.

Samples were mounted on an x–y stage capable of 100 nm resolution. Each specimen was screened for iron content by monitoring the X-ray fluorescence with incoming X-ray energy above the K absorption edge for iron (7112 eV), and comparing that with the fluorescence below the iron absorption edge. The difference between these two values is proportional to the iron concentration within the sample. By rastering the x–y stage, an iron concentration map is obtained. The high intensity of X-rays at the APS synchrotron allows a detection limit of less than 1 ppm.

An area map of each sample was first conducted at 500 μm pixel resolution. Owing to the high sensitivity of the synchrotron X-ray fluorescence, one single nanometre-scale particle is detectable in a 500 μm diameter spot. This allows rapid (approx. 1 h) screening of samples for anomalously high iron concentrations that may correspond to magnetite, ferritin or concentrations of haem iron. The areas of high iron concentration were then further analysed at higher resolution using progressively smaller spot sizes: 20 μm then 5 μm.

Iron anomalies identified during this higher resolution scan were analysed by XANES. Information obtained by this method was compared with standards measured for magnetite, ferritin, haemosiderin and haemoglobin, as well as published results (Mackle *et al.* 1991; Rohrer *et al.* 1990). All samples, with the exception of ferritin, were dried and dispersed. Ferritin was measured in suspension. For biological standards we used a bacterial culture of *Magnetospirilum magnetotacticum* (ATCC), horse spleen ferritin, pigeon haemoglobin (lyophilized powder) and the haem-part of cytochrome-c (Sigma-Aldrich) (figure 1). Magnetite, maghaemite and haematite were synthesized at the University of Florida and confirmed by X-ray diffraction.

3. RESULTS

Using a microfocused X-ray beam, small-scale iron anomalies have been identified in rastered iron fluorescence scans of avian brain tissue samples. Figure 2 shows an iron edge area scan of an avian brain tissue section from the mid-brain region in which major anomalous iron peaks were identified. XANES scans were analysed for each of the major anomalies observed. Based on a comparison with standards, the anomalies were determined to arise from ferritin, magnetite and haemoglobin. The two insets in figure 2 represent the iron fluorescence signal as a function of position within the two marked portions of the tissue sample. The shadow area of the lower image is an X-ray transmission image obtained from the same data set used to generate the X-ray fluorescence maps. Subsequent light microscopy examination of the tissue samples did not reveal any observable radiation-induced degradation during the several hours of beam exposure time. Individual anomalies (peaks) were determined to arise from high concentrations of magnetite and ferritin, as seen in the figure.

An investigation of the effects of sample preparation techniques—specifically tissue fixation, embedding in resin and sealing in Kapton, as described in §2—indicates that these technique do not affect the iron compounds. Spectra for synthetic magnetite standards taken before and after processing are virtually identical (figure 3), demonstrating that magnetite is not altered as a result of chemical processing.

Previous studies of the effects of tissue fixation on iron biominerals also indicate that this process has a very minimal effect (primarily owing to the formation of formic acid from formalin) on the iron biominerals in tissue samples (Chua-anusron *et al.* 1997). In addition, earlier studies of potential iron contamination sources in tissue indicate that the presence of magnetite contamination is unlikely in this case—particularly as all the chemicals used were magnetically cleaned and filtered (Dobson & Grassi 1996). Although trace metal contamination is still possible, it can be distinguished from ferritin and biogenic magnetite using the XANES techniques described here.

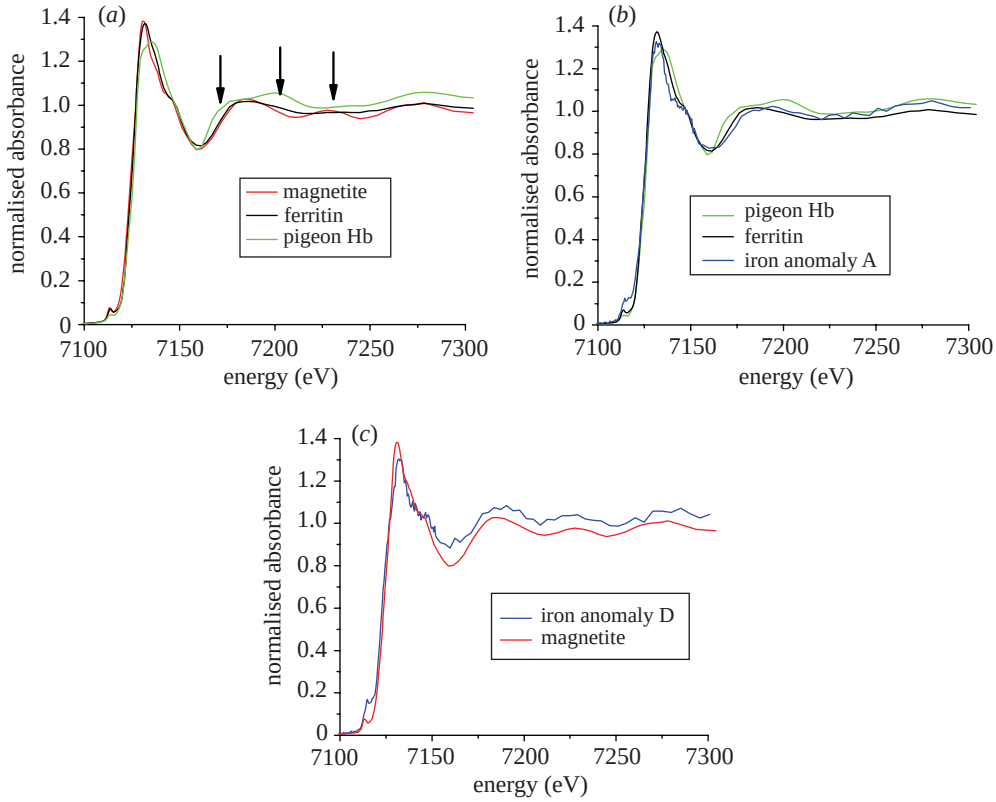

Figure 1. XANES spectra showing a comparison of (*a*) reference spectra from synthetic magnetite and horse spleen ferritin and pigeon haemoglobin; (*b*) reference spectra of ferritin and haemoglobin compared to that from iron anomaly A, showing good fit with ferritin; and (*c*) reference spectrum of magnetite and that from iron anomaly D, showing good fit.

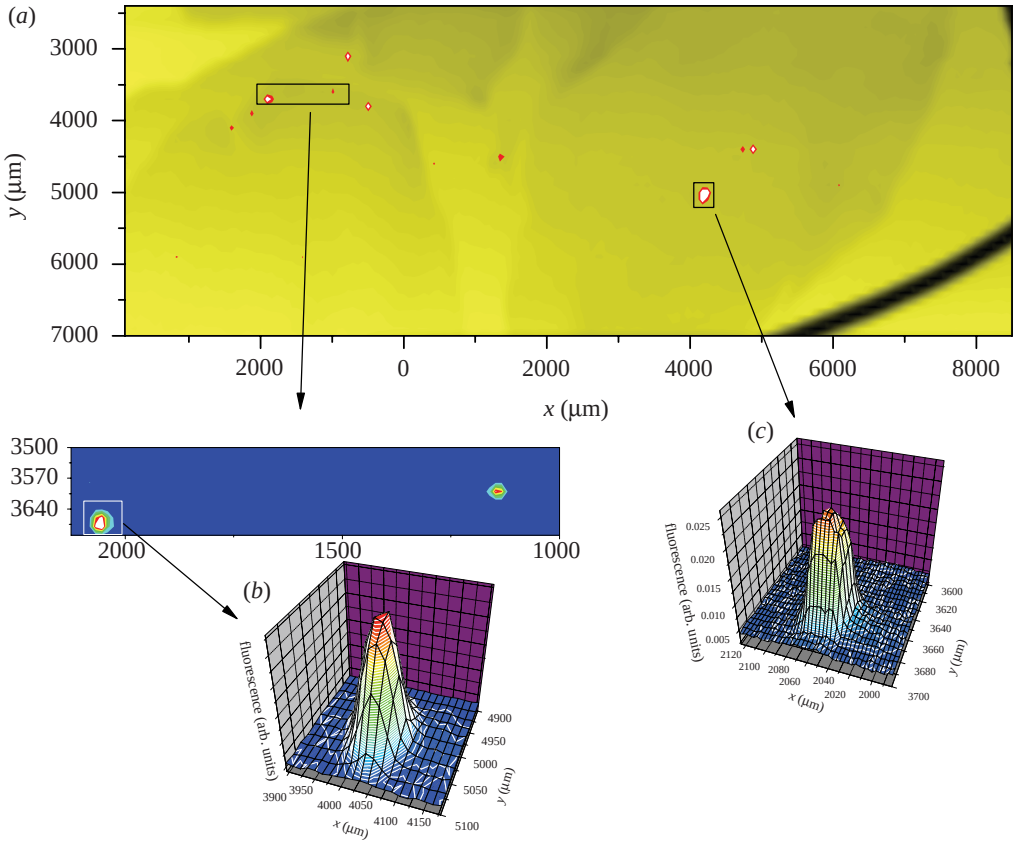

Figure 2. Maps of the coronal section of a pigeon brain consisting of (*a*) X-ray transmission map (black–yellow) with superimposed iron fluorescence map (red–white) with 500 μm resolution; (*b*) iron fluorescence map at 20 μm resolution, with 5 μm resolution wireframe image inset of iron anomaly A consisting primarily of ferritin-like iron compounds; and (*c*) iron fluorescence wireframe map at 5 μm resolution of iron anomaly D, consisting primarily of magnetite.

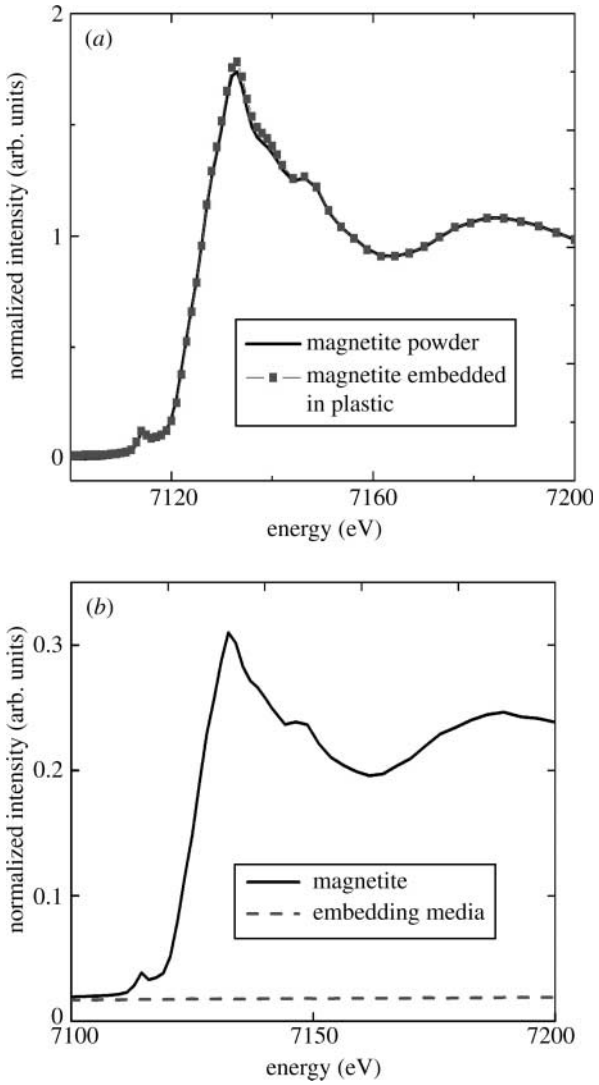

Figure 3. (*a*) Normalized XANES spectra comparing synthetic magnetite standards before and after chemical processing, embedding and baking in Kapton. (*b*) Relative XANES spectra showing the lack of background iron signal from the Kapton film compared with the signal from a standard magnetite sample.

4. DISCUSSION

The results presented here represent a major advance in the *ex vivo* analysis of iron in brain tissue sections. The primary advantages in using synchrotron radiation lie in the low limits of detection, the potential for relatively high-resolution imaging and the unambiguous identification of the specific iron compounds responsible for iron anomalies. We have demonstrated the ability of the focused beamline at the MRCAT to detect regions of interest with a 5 μm pixel resolution, over relatively large areas, in the time-span of a few hours, and to identify the iron compounds responsible for the anomalies, in this case, ferritin and biogenic magnetite.

In the experiments described here, data were collected using state-of-the-art microfocusing equipment in the Kirkpatrick–Baez (KB) configuration to define a beam size of approximately 5 μm diameter. The full beam size is typically several millimetres, so alternative methods using slits to obtain a small beam eliminate more than 99% of the beam strength. This results in long counting times and small signal-to-noise ratios. These disadvantages have been overcome in this study, allowing rapid, high-resolution analysis of large areas of tissue sections at sub-cellular resolution. The deposition of zinc grid lines on the sample substrate (figure 2*a*) will allow orientation for subsequent imaging with other techniques (i.e. confocal, light and electron microscopy) and the construction of composite maps of iron compounds, and cell and tissue structures. In addition, the technique can be adapted to other important metals which have been implicated in neurological disorders, such as aluminium and zinc.

Although the tissue sections were exposed to osmium tetroxide for only a short period of time, we cannot rule out the possibility of some oxidation. However, the presence of magnetite in the sections (which contains both Fe^{2+} and Fe^{3+}) indicates that the iron compounds are probably not oxidized. In order to further mitigate against potential effects in current and future studies, we no longer use OsO_4 or any other contrast agents.

In a previous study, Griffiths *et al.* (1999) concluded that the iron present in the substantia nigra of Parkinson's tissue and age-matched control tissues consisted of ferritin, based on a fitting of XAFS data collected using homogenized tissue and presumably much larger X-ray excitation volumes. Comparison of our results with those of Griffiths and others highlights the importance of the high spatial resolution, *in situ* analysis method presented here. In the present study, we found that there is a background XANES spectrum arising from a combination of iron compounds (primarily ferritin), however, there are large spikes caused by other dominant iron forms which can be caused by very small particles. Assuming a typical 1 mm^3 X-ray excitation volume, a 100 nm particle would only comprise a volume fraction of 10^{-12}, which could be a very small part of the overall iron signal, depending on the relative concentrations. Even a particle concentration of $10^6\times$the ferritin background means that the X-ray absorption signal from the particle would be only 1 ppm of the background signal. For our current 3 μm minimum beam size, that volume fraction becomes 9000, and the high concentration of iron in the particle assures that the particle X-ray absorption signal is detectable. In addition, the Griffiths study was carried out on homogenized tissue, which excludes the possibility of mapping iron anomalies to specific structures within the tissue sample.

5. CONCLUSIONS

The continued development of this technique should lead to major advances in mapping iron anomalies, and the related structural information, directly to cells and tissue structures in human brain tissue *in situ*. We are currently investigating the use of micro-EXAFS (extended X-ray analysis of fine structure) as an additional tool which can be used for the confirmation

of these results and further elucidation of ferritin core structures. At present this is done primarily by iron staining methods, and any information on the relationship between iron distribution and cellular structures obtained this way is limited. Iron staining also offers no information on the specific compounds of iron present. This can be vitally important as the form of iron (including its oxidation state) can determine whether it plays a detrimental or beneficial role in neurophysiological processes.

In addition, the technique described here can play an important role in investigations of magnetoreception in animals, as is demonstrated by these investigations of pigeon tissue. The construction of composite images using this and other microscopy techniques should allow for an unambiguous identification of the iron biominerals involved in magnetoreception and their relation to structures in the mammalian nervous system.

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