



# Distribution and chemical state analysis of iron in the Parkinsonian substantia nigra using synchrotron radiation micro beams

Ari Ide-Ektessabi <sup>a,\*</sup>, Takuo Kawakami <sup>b</sup>, Frank Watt <sup>c</sup>

<sup>a</sup> *International Innovation Center, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto 606-8501, Japan*

<sup>b</sup> *Department of Precision Engineering, Graduate School of Engineering, Kyoto University, Japan*

<sup>c</sup> *Department of Physics, Research Center for Nuclear Microscopy, National University of Singapore, Singapore*

## Abstract

Metallic elements and their organic compounds have dynamic regulatory functions in cells. Iron concentrations have been observed in the neuromelanin granules in the substantia nigra of brain tissues of patients with Parkinson's disease. Iron has been linked to cell death because of its potential to promote free radicals, leading to oxidative stress. In the present study, we have used synchrotron radiation X-ray fluorescence spectroscopy (SXRF) and Fe K-edge X-ray absorption near-edge structure (XANES) spectroscopy, to investigate distributions and chemical states of iron. The samples were brain tissues from monkeys which had been injected with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). SXRF analyses were performed for elemental mapping, using 7.16 keV energy beam. The chemical state analyses were performed between 7.16 and 7.12 keV energies. The lower limit was chosen to be slightly above the Fe<sup>2+</sup> absorption edge, in order to suppress the excitation of Fe<sup>3+</sup>. FeO (Fe<sup>2+</sup>) and Fe<sub>2</sub>O<sub>3</sub> (Fe<sup>3+</sup>) powders were used for XANES analyses as reference samples. The data were measured in fluorescence mode for the biological specimens and in transmission mode for the reference samples. The results for the Fe<sup>2+</sup>/Fe<sup>3+</sup> ratios from the neuromelanin granules showed significant variations, which were correlated with the level of iron concentration. Cells containing high level of iron had high level of Fe<sup>2+</sup>. With Fe<sup>2+</sup> having been suggested to potentially promote more free radicals than Fe<sup>3+</sup>, the high concentrations of iron may be the critical factor leading to cell death due to the presence of more free radicals. © 2003 Elsevier B.V. All rights reserved.

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## 1. Introduction

Parkinson's disease (PD) is characterized by a decrease in spontaneous movements, gait difficulty, postural instability, rigidity and tremor. PD is caused by the selective degeneration of neurons in the substantia nigra (SN) region of the brain, resulting in a reduced production of the neurotransmitter

\* Corresponding author. Tel./fax: +81-75-753-5259.

E-mail address: [h51167@sakura.kudpc.kyoto-u.ac.jp](mailto:h51167@sakura.kudpc.kyoto-u.ac.jp) (A. Ide-Ektessabi).

dopamine. The pathogenesis of the loss of the neurons in the SN is unknown, however, the increase in iron concentration in the SN has been reported by several research groups [1] using both human post-mortem brains and brain tissue from Parkinsonian animal models. Excessive transition metals would promote production of free radicals that cause oxidative damage and neuronal degeneration [2,3]. In order to understand the role of metals, especially iron, in the neurodegenerative disease, it is important to investigate distributions and chemical states of metallic elements in the tissues from sites of degeneration.

The distribution and metabolism of metallic elements have been studied by using spectroscopic methods such as energy dispersive X-ray electron microscopic analysis, laser microprobe mass analyzer and nuclear microprobe. These studies demonstrated the localization and distribution of iron in tissues from a patient with a neurodegenerative disease, but these techniques cannot analyze the chemical state of the iron without homogenization. Synchrotron radiation (SR) is an ideal X-ray source that provides continuum and a high intensity photon for the investigation of trace metallic elements contained in biological specimens. X-ray fluorescence (XRF) spectroscopy and X-ray absorption near-edge structure (XANES) spectroscopy are major techniques using SR. XRF using synchrotron radiation can determine the existence and distribution of ultratrace elements. XANES spectroscopy provides information on the valence state and binding structure of the absorbing elements. XANES spectroscopy has been used for chemical state analyses on a wide range of biological samples [4,5] and pathological specimens [6,7]. In our previous studies using SR microbeams, we used the X-ray analyses on tissue without destruction and staining, and demonstrated the distribution [8] and the chemical state [9] of iron in the tissue of the substantia nigra of the PD case, at the level of a single cell [1].

In the present study, we applied XRF and XANES to the SN of a monkey injected with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). X-ray analyses were performed without homogenization or isolation, which allowed histochemical analyses after X-ray analyses on the same tissue.

## 2. Experimental

### 2.1. Materials and sample preparation

Brain tissue sections encompassing the substantia nigra were obtained from primate models which were unilaterally injected with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine) [10]. MPTP caused a PD like syndrome. The tissues are unstained and freeze-dried. Sections of 10  $\mu\text{m}$  in thickness were cut out and mounted on a sub-micron self supporting pioloform film. The pioloform film has no trace elements.

### 2.2. X-ray analyses

X-ray analyses using synchrotron radiation on the brain tissues and reference samples were performed at beam line 39XU of Spring-8, Japan Synchrotron Radiation Research Institute (JASRI). Synchrotron radiation from the storage ring (8 GeV, maximum current 100 mA) was monochromated with a Si(111) double crystal monochromator. Incident photon beams were restricted by a set of  $x$ - $y$  slits and a pinhole. Incident beam size was about 10  $\mu\text{m}$  in diameter. Measurements were performed in vacuum.

XRF analyses were performed for elemental mapping. Fluorescent X-rays were collected by a solid-state detector (SSD). Fe K-edge XANES analyses were performed in the energy range of 7.100–7.160 keV at 0.5 eV intervals. The data were measured in fluorescence mode for biological specimens and in transmission mode for the reference samples. Incident and transmitted photon flux was monitored with an air-filled ion chamber. Fe K-edge fluorescent X-rays were also collected by a SSD.

### 2.3. Chemical state analyses procedures

XANES spectroscopy is based on the sensitivity of X-ray absorption to the valence state and neighboring atoms of the absorbing elements. If the incident energy near the absorption edge is chosen properly, selective excitation of specific chemical species will occur. XANES spectra of FeO ( $\text{Fe}^{2+}$ ) and  $\text{Fe}_2\text{O}_3$  ( $\text{Fe}^{3+}$ ) (reference materials)

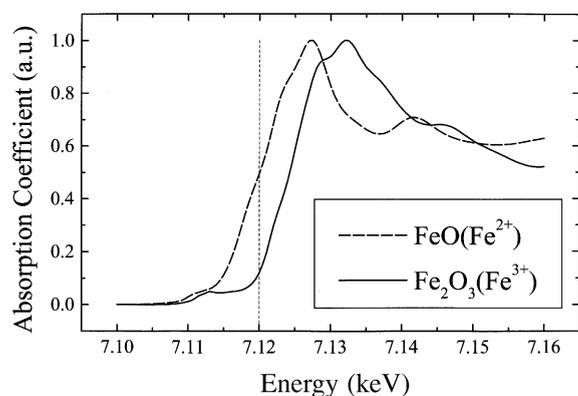


Fig. 1. X-ray absorption fine structure spectra of FeO ( $\text{Fe}^{2+}$ ) and  $\text{Fe}_2\text{O}_3$  ( $\text{Fe}^{3+}$ ).

are shown in Fig. 1. At energies above the absorption edge, such as 7.160 keV, both  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  are excited. On the other hand, at energies near the absorption edge, such as 7.120 keV,  $\text{Fe}^{2+}$  is selectively excited and the excitation of  $\text{Fe}^{3+}$  is suppressed. Since fluorescent X-rays are emitted in proportion to the excitation of the absorbing elements, XRF imaging that distinguishes the chemical state is obtained because of the sensitivity of the X-ray absorption coefficient to the chemical state.

In this study, chemical state imaging was obtained using the following procedure. First, XRF imaging was performed with the incident X-ray energy at 7.160 keV and at 7.120 keV. Then the

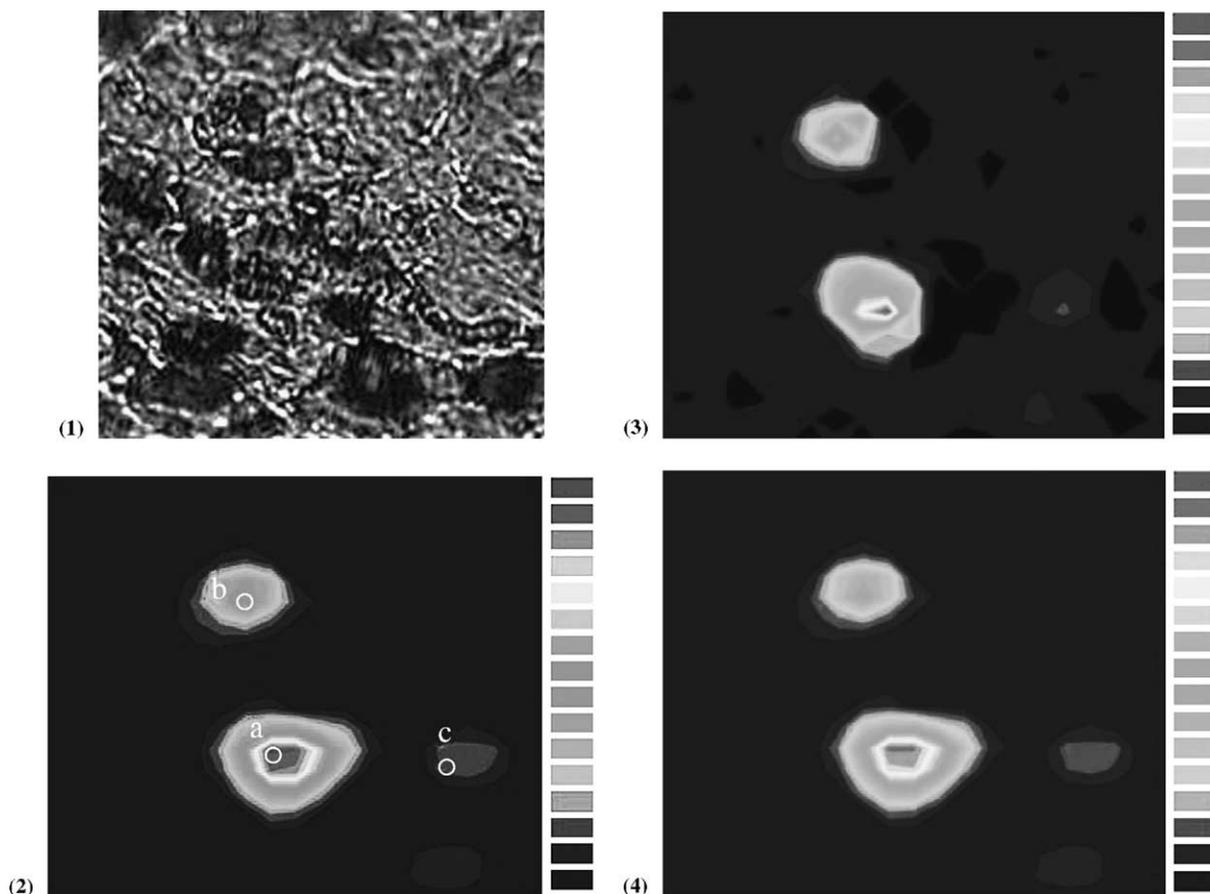


Fig. 2. XRF imaging of substantia nigra tissue from a monkey injected with MPTP. The measurement area was  $80 \times 80 \mu\text{m}$ , and was divided into  $20 \times 20$  pixels. (1) Optical micrograph of the measurement area. Note that the black oval shapes are voids in the sample, not the cells. (2) X-ray fluorescence imaging of yield of iron. (3) Chemical state imaging of  $\text{Fe}^{2+}$ . (4) Chemical state imaging of  $\text{Fe}^{3+}$ .

yields of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  were derived from the XRF yields of each point using the absorption coefficients at incident energies of 7.160 and 7.120 keV.

### 3. Results

#### 3.1. XRF imaging for chemical state analyses

XRF analyses were performed on substantia nigra tissues of monkeys, which were injected with MPTP. The optical microscopic photograph and XRF imaging of iron in the SN tissues are shown in Fig. 2(1) and (2), respectively. The XRF images matrices consist of  $20 \times 20$  pixels of  $4 \mu\text{m}$  size. A color scale represents the X-ray fluorescent yields in each pixel. In the Fig. 2(1), melanized neurons (neuromelanin) can be observed as pigmented granules. Iron concentrations are detected in the neuromelanin granules. Chemical state imaging, which separates  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  concentrations, was performed in the same area of the SN tissue (Fig. 2(3) and (4)). Distributions of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  were relatively well distinguished in the SN tissue. Iron components in the melanized neurons were mixed state of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ . In order to investigate the chemical state in detail, we measured the XANES spectra.

#### 3.2. Fe K-edge XANES analyses in tissues

Fe K-edge XANES analyses were applied to selected points where high iron concentrations were detected in the tissues in order to analyze chemical states in detail. Measurement points are shown as (a)–(c) in Fig. 2(2). Fig. 3 shows XANES spectra in the tissues and those of reference samples (FeO and  $\text{Fe}_2\text{O}_3$ ). The ordinate and abscissa represent the absorption coefficient and incident X-ray energy, respectively. The spectra were normalized by the absorption jump. The absorption

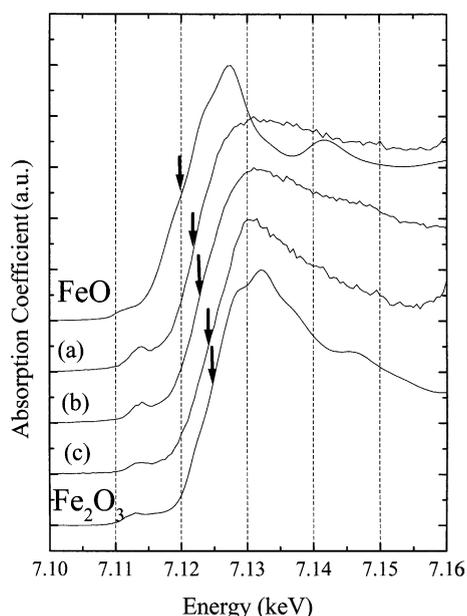


Fig. 3. Fe K-edge XANES spectra of FeO,  $\text{Fe}_2\text{O}_3$  and the measurement points (a), (b) and (c) in Fig. 2(1).

jump was defined as the difference between the highest and the lowest point in each spectrum. The absorption edge is defined at the half-height of the absorption jump.

From the spectra in Fig. 3, we can conclude that the chemical shifts of iron at the points of (a)–(c), which are neuromelanins with high iron concentration, are 2.0, 2.8 and 4.2 eV from the absorption edge of FeO ( $\text{Fe}^{2+}$ ). The chemical shift of  $\text{Fe}_2\text{O}_3$  ( $\text{Fe}^{3+}$ ) is 4.3 eV. The chemical shift of (c) is almost equivalent to that of  $\text{Fe}^{3+}$ . The chemical shift for (a) is the smallest of the measured points. This implies that the proportion of  $\text{Fe}^{2+}$  is the highest for this point.

In order to investigate the ratio of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , we used the chemical shifts. Table 1 shows the  $\text{Fe}^{2+}/\text{Fe}^{3+}$  ratio of each measurement point.

Table 1  
Chemical shift and  $\text{Fe}^{2+}/\text{Fe}^{3+}$  ratio of the points of (a), (b) and (c)

	FeO	$\text{Fe}_2\text{O}_3$	a	b	c
Absorption edge (keV)	7.1200	7.1243	7.1220	7.1228	7.1242
Chemical shift (eV)	0	4.3	2.0	2.8	4.2
$\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio			1.15	0.53	0.02

#### 4. Discussion

Chemical state analyses were applied to pathological tissues. Chemical state imaging which separates  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  was obtained with the following assumptions: (1) the absorption coefficient curves of different valence states of iron ( $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ) were represented by only iron oxides ( $\text{FeO}$  and  $\text{Fe}_2\text{O}_3$ ), (2) iron contained in the tissues is a superposition of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ . It should be noted that these assumptions are too simplified to represent the complex system which contains chlorine and other compounds of Fe. These assumptions can be justified if we consider the important role of oxides of iron in oxidation and reduction activities of brain. Under these assumptions, the chemical states of iron contained in the tissues were well distinguished.

XRF analyses revealed an excessive accumulation of iron in the neuromelanin granules of the SN of the monkey injected with MPTP. This result is in good agreement with previous studies, which show iron concentration in neuromelanin in tissues from PD cases [9,11]. In the present study, iron contained in the neuromelanin is shown to be mixed states of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ . However, the  $\text{Fe}^{2+}$  distribution in the granule was observed to be uneven, showing accumulation in the left side as seen in Fig. 2(3). This result implies the  $\text{Fe}^{2+}$  would accumulate locally in neuromelanin.

From XRF imaging (Fig. 2(2)), iron accumulation at point (a) is the highest level of the three measurement points, and then point (b). The point (c) is the lowest level of iron accumulation of the three points. Therefore, the  $\text{Fe}^{2+}/\text{Fe}^{3+}$  ratios (Table 1) correlate with the level of iron accumulation. The granules containing high iron accumulations have also high level of  $\text{Fe}^{2+}$ . The Fenton reaction [12] showed that  $\text{Fe}^{2+}$  could promote the generation of hydroxyl radical, which is one of the most reactive free radicals. Hence, according to the Fenton reaction, the neuromelanin which contains high iron accumulation, have the poten-

tial to promote the generation of free radicals, resulting in the degeneration of neurons.

In summary, we have analyzed the chemical state of iron in the brain tissue from monkeys that had been injected with MPTP to create the degeneration of neurons like PD. The chemical state imaging showed the difference of distributions of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ , and the chemical shifts revealed the chemical state in detail. This method allows us to know behaviors of transition metals inside and outside cells, and can be used widely for investigations in neurology and cell biology.

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