Research Article

Helena Svobodová, Jana Hlinková, Pavol Janega, Daniel Kosnáč, Barbora Filová, Marcel Miglierini, Ľubor Dlháň, Hermann Ehrlich, Dušan Valigura, Roman Boča, Štefan Polák, Štefan Nagy, and Martin Kopáni*

Deposits of iron oxides in the human *globus pallidus*

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Abstract: Samples taken from the human brain (*Globus Pallidus*) have been investigated by physical techniques such as light microscopy, scanning electron microscopy, transmission electron microscopy, Mössbauer spectroscopy and SQUID magnetometry. SEM-EDX/TEM investigation reveals multielemental composition of hematite and magnetite nanocrystals with sizes ranging from 40 nm to 100 nm and hematite microcrystals from 3 μm to 7 μm. Room temperature Mössbauer spectra show quadrupole doublets assigning to hematite and ferrihydrite. SQUID measurements of temperature dependence of the mass magnetic susceptibility between $T = 2 - 300$ K at DC field $B_0 = 0.1$ T, the field dependence of the mass magnetization taken at the fixed temperature $T_0 = 2.0$ and 4.6 K and the zero-field cooled and field cooled magnetization experiments (ZFCM/FCM) confirm a presence of ferrimagnetic phases such as maghemite and/or magnetite with hysteresis loops surviving until the room temperature. Differences between these measurements from the point of view of iron oxides detected can indicate important processes in human brain and interactions between ferritin as a physiological source of iron and surrounding environment.

Keywords: human *Globus Pallidus*, iron oxides, Mössbauer spectroscopy, SQUID magnetometry

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1 Introduction

Iron is the most important metal with relatively high concentration in some regions of the brain. It catalyzes reactions forming reactive oxygen species and is one of the major factors associated with neurodegenerative diseases [1]. In humans, a large amount of the total iron content (66%) is present in the molecules of haemoglobin, which contains four Fe^{2+} ions. It accumulates in brain cells in the form of the much less reactive Fe(III) iron – ferritin - primarily an iron storage protein situated in the cytoplasm of the cells and in lesser amounts in the blood circulation. This protein shows spherical morphology with a diameter of 12 nm. The core of ferritin consists of 6 nm Fe(III)-oxide particle stored in the form of ferrihydrite (5 $Fe_2O_3 \cdot 9\ H_2O$). Several studies show that the core physiological ferritin is composed of nanoparticles of ferrihydrite, magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) and hematite (α-Fe_2O_3). Under some conditions iron can accumulate and create particles. This process - biomineralization influences properties of cells and tissues and supports some of their biological functions. Physiochemical properties of particles reflect conditions under biomineralization taking place. Under conditions prevailing in human body, the formation of an amorphous state or minute crystalline phase is expected. The deposits of iron oxides in the human brain are well documented over last decades. Ferrimagnetic components like magnetite Fe_3O_4 and/or

***Corresponding Author: Martin Kopáni:** Institute of Medical Physics, Biophysics, Informatics and Telemedicine, Faculty of Medicine, Comenius University, 813 72 Bratislava, Slovakia; Email: martin.kopani@fmed.uniba.sk
Helena Svobodová, Jana Hlinková, Daniel Kosnáč, Barbora Filová: Institute of Medical Physics, Biophysics, Informatics and Telemedicine, Faculty of Medicine, Comenius University, 813 72 Bratislava, Slovakia
Pavol Janega: Institute of Pathological Anatomy, Faculty of Medicine, Comenius University, Bratislava, Slovakia
Marcel Miglierini, Ľubor Dlháň: Slovak University of Technology, FEI and FCHPT, Bratislava, Slovakia
Hermann Ehrlich: Institute of Experimental Physics, TU Bergakademie Freiberg, Freiberg, Germany
Dušan Valigura, Roman Boča: Department of Chemistry, Faculty of Natural Sciences, University of SS Cyril and Methodius, Trnava, Slovakia
Štefan Polák: Institute of Histology and Embryology, Faculty of Medicine, Comenius University, Bratislava, Slovakia
Štefan Nagy: Institute of Materials and Machine Mechanics, Slovak Academy of Sciences, Bratislava, Slovakia

maghemite (γ-Fe_2O_3) particles are iron oxide components in human brain tissue under physiological and pathological conditions [2]. Iron oxide particles can directly be visualized under scanning electron microscope. The diffraction patterns obtained from transmission electron microscope then bring information on which kind of iron-oxide minerals are present in an individual single crystal. Information about the oxidation and spin states of iron deposits in the samples under study is obtained from Mössbauer spectroscopy. This, however, reflects the presence of iron in the whole sample where iron atoms can exist in different oxidation and spin states.

Iron oxides are present in human brain and in animals. Magnetostatic bacteria, tuna, salmon and bird's head contain magnetite for magnetotaxis and magnetic navigation. Lepidocrocite in chitons because of the mechanical strength, goethite in limpets and ferrihydrite in sea cucumbers can also be found.

The aim of this study is to examine to properties of the tissue from the perspective of distribution, structure, magnetic properties, oxidation and spin states of iron oxides in human *globus pallidus* with light and electron microscopy, Mössbauer spectroscopy and SQUID magnetometry.

2 Experimental

Postmortem brain tissue samples from male humans *globus pallidus* of different age (sample 1 – 61 y, sample 2 – 65 y, sample 3 – 69 y, sample 4 - 85 y) were routinely obtained at autopsy to prepare tissue sections for the pathology diagnosis. Tissues were taken from four individuals with no clinical findings of any motor abnormalities, iron metabolism pathologies, movements involving limbs, face, tongues. All procedures were conducted in accordance with the Declaration of Helsinki. Special attention was paid to avoid manipulations with iron instruments.

2.1 Light microscopy

The samples for light microscopy investigation were fixed in 10% formaldehyde for 24 hours and embedded in paraffin blocks, cut by a microtome into 5 μm thin sections, and mounted on gelatin-coated slides. Afterwards sections were stained for general morphological purposes by haematoxylin and eosin and for iron Fe (III) detection by Perls' method. Tissue sections were then covered by cover glass and the samples were examined under the light microscope Eclipse E50i (Nikon).

2.2 Scanning and transmission electron microscopy (SEM/TEM)

The samples for electron microscopy investigation were fixed in 3% fixation solution of glutardialdehyde buffered by phosphate for scanning electron microscopy. Samples from the autopsy were dehydrated in graded acetone and subjected to critical point drying of CO_2. Specimens were mounted on glass stubs and coated with 30 nm layer of gold in ion sputtering apparatus (SCD 050, BALZERS). Samples were analyzed by scanning electron microscope EVO LS 15 (ZEISS) with the accelerating voltage of 15 kV. Simultaneous EDX line analysis was performed with AMETEK (EDAX) EDS Element Silicon Drift Detector. The time period of spectrum collection was 200 s with the energy range 0.160 to 9 keV.

The samples from the autopsy intended for TEM investigation were fixed in 3% solution of glutardialdehyde (SERVA, Heidelberg, Germany) for two hours and buffered by phosphate (pH 7.2 – 7.4). After dehydration of the tissue by alcohol, the samples were embedded into Durcupan ACM (Fluka AG, Busch, Switzerland) as recommended by the manufacturer and cut by ultramicrotome with a glass knife (C. Reichert, Wien, Austria). The thickness of the samples was 200 nm. Uncontrasted ultrathin sections were mounted on nickel grids and investigated by transmission electron microscope JEOL 840B (Jeol, Japan) with acceleration voltage of 150 kV. In order to determine the iron oxide phase, chemical analysis EDX KEVEX 3205-1200 (Kevex, Valencia, USA) was applied. For phase identification the International Centre for Diffraction Data (ICDD) for hematite ICDD card no. 33-0664, ferrihydrite ICDD card no. 29-0712 and magnetite ICDD card no. 190629 were used.

2.3 Mössbauer spectroscopy

Mössbauer spectra were collected at room temperature in the constant acceleration mode using a spectrometer equipped with ^{57}Co/Rh gamma source (Ritverc). Isomer shift values are quoted with respect to a room temperature Mössbauer spectrum of a calibration α-Fe foil with the thickness of 12.5 μm (Goodfellow). Spectra of all unfixed samples were recorded at high velocity (± 11 mm/s) to look for possible presence of iron oxides. Because no traces of sextets were revealed, spectra were subsequently taken at low velocity range (± 4 mm/s) to enhance the resolution.

2.4 SQUID magnetometry

The superconducting quantum interference device (SQUID) magnetometer (MPMS-XL7, QuantumDesign) has been used in recording magnetic functions: temperature dependence of the mass magnetic susceptibility $\chi_\rho(T; B_0)$ at small DC field B_0 = 0.1 T transformed to the product function χT, and the field dependence of the mass magnetization $M_\rho(B; T_0)$ taken at the fixed temperature T_0 = 2.0 and 4.6 K. Also, the zero-field cooled and field cooled magnetization experiments (ZFCM/FCM) were conducted along with the search for the hysteresis at low as well as elevated temperatures. The unfixed samples were raw lyophilized powders encapsulated into a gelatin made container as a sample holder.

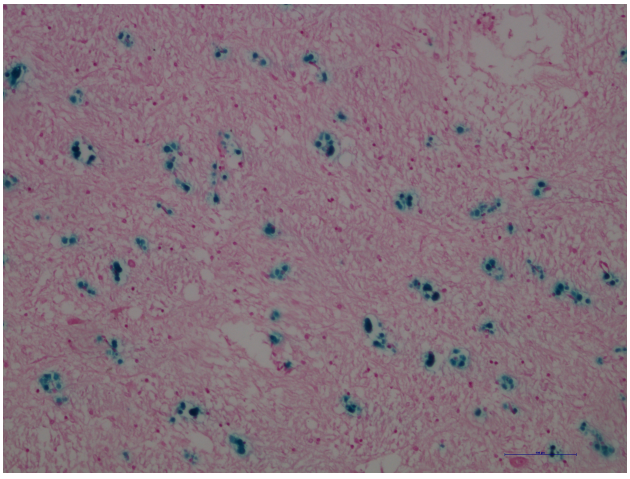

Figure 1: Human brain, *globus pallidus*. Blue dyed deposits correspond to the presence of Fe(III) probably around glial cells. Scale bar = 100 µm.

3 Results and discussion

3.1 SEM-EDX-TEM experiments

Iron (ferritin and/or hemosiderin) accumulation is observed in places with high metabolic activity around glial cells and depends on age [3]. The ageing of cells is associated with the loss of ability of the cells to maintain a variety of factors that include iron homeostasis, transport, metabolic rate, distribution and utilization of iron-binding proteins. The inevitable consequence of ageing is an increase of iron in specific brain regions that are preferentially targeted in neurodegenerative diseases. Different structure of ferritin core was found in healthy physiological and in pathological conditions. Changes in the structure of ferritin may grow from ferritin iron cores to iron oxide (nano)particles in tissues and cells [4].

Our findings revealed Fe(III) positive globular structures in samples located probably around glial cells with sizes ranging from 3 µm to 10 µm (Figure 1). During the ex-vivo examination of tissues and cells it is crucial to stabilize the sample. For this purpose, the fixation in formalin is used. This process of formalin fixation following with embedding tissue in paraffin can alter the concentration of iron compared with that observed in fresh tissue. Multiple studies revealed comparable levels between fixed and fresh tissue [5–8]. On the other hand, some authors argue that iron used in samples is not tightly bound within the fixed tissue and fixation leads to leach iron and its reduction [9, 10].

The results of iron depositions near glial cells agree with the results of other studies [11, 12]. It was observed that many spheroid structures in *globus pallidus* containing ferric iron regardless of the presence of diseases or different conditions are associated with iron mediated oxidative stress [13, 14]. This view is supported by multielemental composition of spheroid structures measured by EDX microanalysis [15, 16]. Our EDX-SEM investigation of complexes reveals multielemental composition with various amounts of O, Na, Si, P, S, Cl, K, Ca, Au and Fe. All iron-oxygen rich particles contained also phosphorus and sulfur (Figure 2). Elements like Ca, Fe, P, and S were detected in agreement with our results. The presence of gold in the samples is a side result of preparation. Our findings reveal the presence of Na and Si. These elements come probably from gelatin-coated slides and from their natural occurrence in human brain. They are vital elements present in human brain. Function of silicon in human brain is still unknown. Aluminum is a chemical element which can influence hematite formation and its structure. Ions can preferentially promote formation of some iron oxides and can influence their morphology, structure, size and magnetic properties.

Investigation with scanning electron microscopy after removal of cover glass showed isolated, irregular iron-rich particles (Figure 2). Biomineralization process, as an origin of iron oxides, in the brain is the interaction between iron and the surrounding environment. It takes place in the human body - environment with low temperature and pressure. Under these conditions the formation of an amorphous state or minute crystalline phase is expected. Conditions prevailing in living organism (low temperature and pressure, almost neutral pH) may also cause the formation of metastable phases, and subsequent transformation on stable phases [17]. The final product of biomineralization

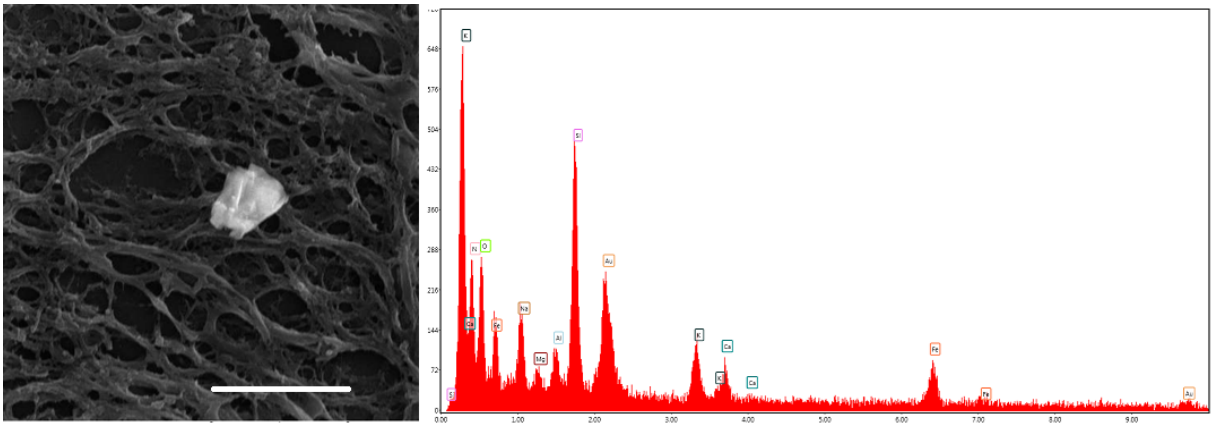

Figure 2: Human brain, *globus pallidus*. Left – Iron-rich particle. Right – EDX spectrum of this particle showed O, Na, Si, P, S, Cl, K, Ca, Au and Fe. Scale bar = 10 µm. SEM.

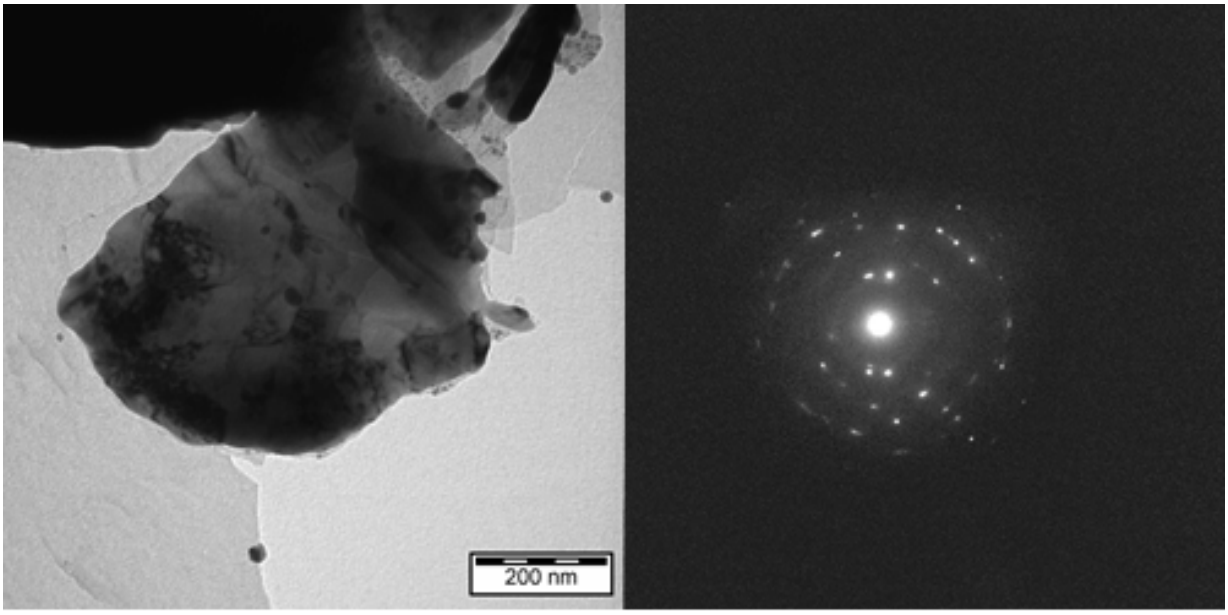

Figure 3: TEM records of the Human brain, *globus pallidus*. Irregular micrometer-sized hparticle (left) with diffraction pattern corresponding to hematite - α-Fe_2O_3 (right). Scale bar 3 = µm.

depends on many other factors such as chemical elements and compounds, temperature, pH [18], the presence of reactive oxygen species (ROS) and time. Selective electron diffraction in TEM shows the presence of crystalline material with irregular structures (Figure 3).

The lattice parameters a = 5.0016 Å, b = 5.0016 Å, c = 13.6202 Å of rhombohedral crystal system (space group $R\bar{3}c$) correspond to hematite and lattice parameters a = 8.3990 Å, b = 8.3990 Å, c = 8.3990 Å of cubic crystal system correspond to magnetite (space group $Fd\bar{3}m$). Some particles were regular, non-homogeneous, solid punctuate, and sporadically exhibited hexagonal shape. The shape of smaller particles was more regular, with the size around 2 µm. On the surface of some particles small deposits of unknown origin have been seen. Information on hematite particles in human brain are sporadic [19] and relate only to indirect magnetic method [20]. Finding of hematite microparticles might be caused by the fact that these particles are dominant iron oxide particles in those samples. Conditions prevailing in *globus pallidus* of human brain (temperature, pH, microenvironment and the presence of oxygen radicals) may favor hematite formation over other iron oxides compounds [21, 22].

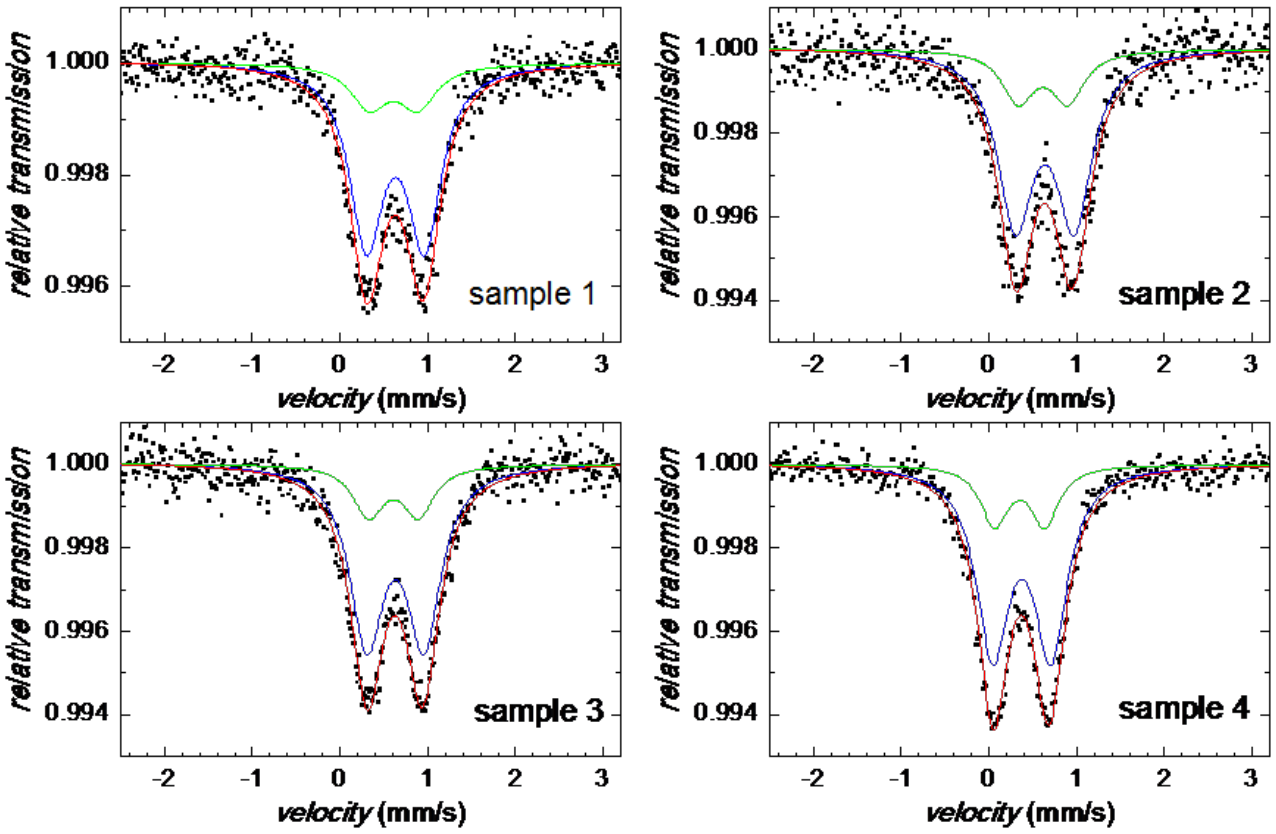

Figure 4: Mössbauer spectra for samples **1** through **4**.

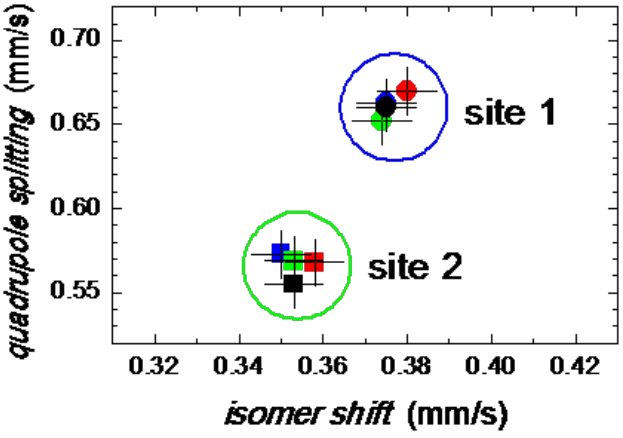

Figure 5: Hyperfine parameters of the Mössbauer spectra for samples **1** through **4**.

3.2 Mössbauer spectra

Two-line absorption spectra in Figure 4 are characteristic for a non-magnetic material. In fact, iron-containing nanoparticles exhibit superparamagnetic behaviour due to dynamic spin relaxation process of their magnetic moments at room temperature. These spectra were fitted with two doublets. One of them, with average isomer shift (< *IS* >) and quadrupole splitting (< *QS* >) values of (0.376±0.007) mm/s and (0.661±0.014) mm/s, respectively, represents Fe^{3+} nuclei in the high-spin state.

The second atomic site, also Fe^{3+}, is characterized by < *IS* > = (0.354±0.007) mm/s and < *QS* > = (0.566±0.014) mm/s. All samples exhibit very similar hyperfine parameters that are almost equal for the individual doublets in the frame of experimental error as demonstrated in Figure 5. Both spectral components show rather large line widths which indicate that the structure of the particles is highly disordered and/or distribution in their size is observed. Typical *IS* and *QS* values for ferrihydrite core of hemosiderin and ferritin are of 0.35 mm/s and 0.71 mm/s, respectively [23]. Thus, the observed doublets can be assigned to hematite and ferrihydrite.

3.3 SQUID magnetometry

Magnetometry reflects the presence of magnetic species such as paramagnetic Fe^{2+} centers of the hemoglobin and Fe^{3+} of the oxyhemoglobin, traces of other metal ions such as Cu^{2+}, air dioxygen, and the ferrihydrite contained

in ferritin. All these species produce a minor response with respect to the diamagnetism of the organic tissue that forms the brain mass. Some iron-oxide deposits are antiferromagnetic materials, *e.g.* FeO, α-FeOOH, and α-Fe_2O_3. However, their nanoparticles could bring magnetic response due to the presence of uncompensated magnetic moments at their surfaces. The ferrimagnetic materials, such as γ-Fe_2O_3 and Fe_3O_4, generate a strong paramagnetic response that comes into competition with the above mentioned substantial diamagnetism and weak paramagnetism. Existence of these ordered ferrimagnetic phases in the human brain under physiological and pathological conditions has already been reported elsewhere [2, 24]. Investigation of magnetic properties of human *Globus Pallidus* by SQUID magnetometry confirmed presence of ordered magnetic phases with the hysteresis loops surviving event at the room temperature [25, 26].

It is crucial to know if *ex-vivo* post-mortem magnetic properties of iron correspond to *in-vivo* findings. Formaldehyde fixation causes chemical alterations of iron environment which can result in the alteration of magnetic properties of examined tissue *ex-vivo* and *in-vivo*. The process of tissue fixation is not fully understood. Fixation probably induces changes in tissues and cells such as dehydration, protein crosslinking and transmembrane water exchange [27]. It is known that fixation significantly reduces T_1 and T_2 relaxation times. Several studies revealed that the fixation in formaldehyde reduced T_2 relaxation time up to 80% and to 20% in T_1 [28, 29]. It seems that the reduction of relaxation times T_1 and T_2 is not only the result of formaldehyde fixation but also the result of post-mortem interval, temperature, fixation solution, embedding media [29–31]. Still, some authors found no change in *ex-vivo* magnetic susceptibility over time [32, 33].

Figure 6 demonstrates a typical record of the temperature dependence of the mass magnetic susceptibility between T = 2 – 300 K taken at low field B_0 = 0.1 T. The magnetic susceptibility on heating from 2 K decreases until 30 K when it stays almost constant. The product function, χT *vs* T, displays a considerably positive slope which indicates a presence of the ordered ferro-/ferrimagnetic phase instead of the paramagnetic one. (for a net paramagnetic phase, the Curie law, $\chi = C/T$, will be obeyed and then the product function, $\chi T = C$, would be a straight line with zero slope.)

The zero-field cooled magnetization and field-cooled magnetization (ZFCM/FCM) records are shown in Figure 7. This data confirms a kind of the long-range ordering that survives until room temperature. As the data were taken in the field decreasing mode, some remnant magnetization survives at the zero field.

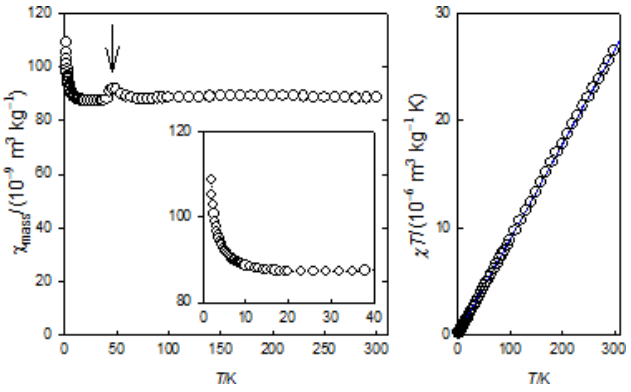

Figure 6: Magnetic susceptibility (left) and product function (right) for sample **1**. (Small hook at ca 50 K refers to the solidus-solidus transition of the dioxygen impurity).

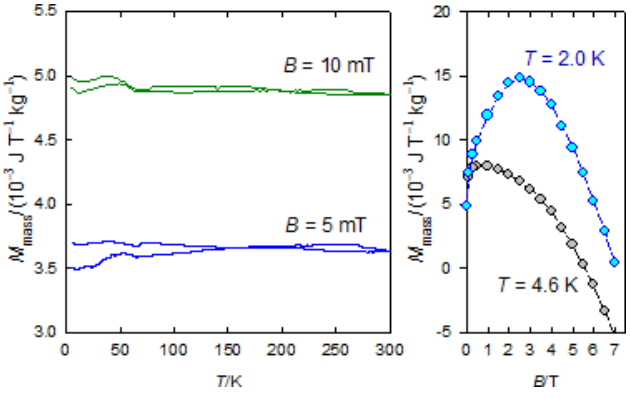

Figure 7: FCM/ZFCM curves (left) and a field development of the mass magnetization (right) for **1**.

The magnetization curves do not follow a usual course approaching saturation; due to the dominant diamagnetic signal from the host tissue, the mass magnetization rises to the maximum when it turns down since the latter component prevails in high fields

$$M_p(B) = M_f(B) - |\chi_{\text{dia}}|B \tag{1}$$

A presence of the remnant magnetization approves a search for the magnetic hysteresis that was positive; the corresponding hysteresis curves are drawn in Figure 8 for T = 20 and 200 K. Notice that the magnetic hysteresis reflects the presence of only ferrimagnetic phases such as γ-Fe_2O_3 or Fe_3O_4. The superparamagnetic behavior of the ferritin escapes above 15 K and it does not manifest itself in the recorded hysteresis curves above 20 K.

3.4 General remarks

From the above results, we concluded that samples taken from the human brain contain ferrimagnetic

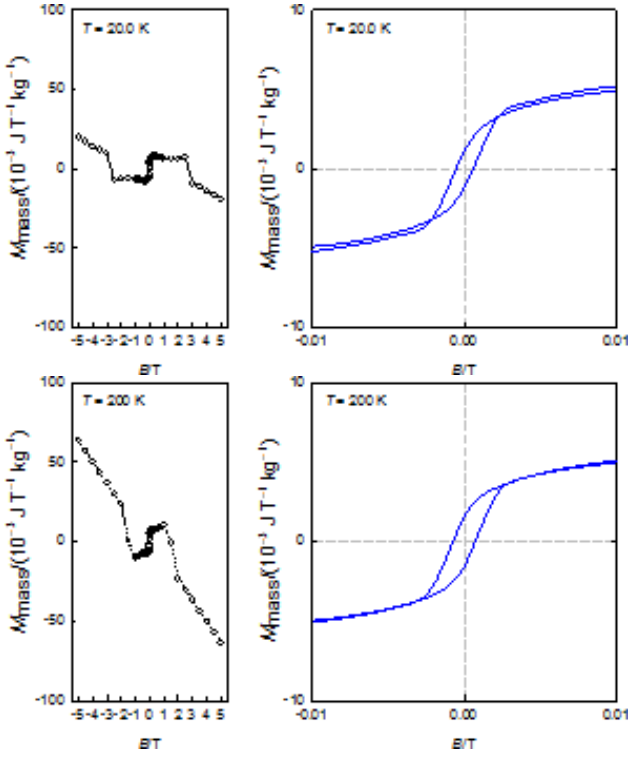

Figure 8: Hysteresis curves for the sample **1**

(maghemite γ-Fe_2O_3, magnetite Fe_3O_4) and antiferromagnetic (hematite α-Fe_2O_3, goethite α-FeOOH, wüstite FeO) iron oxides of different size, shape, and count. SEM/TEM experiments show the presence of nanometer–sized hematite and magnetite crystals and micrometer-sized hematite crystals. On the surface of some hematite particles small deposits of undisclosed origin are also seen. The presence of various iron oxides confirmed by Mössbauer spectroscopy and SQUID can be the result of the interaction between iron/ferritin and the surrounding environment. Yang *et al.* [34] found differences in the structure of the iron complexes in two different microenvironments: saccharide sulfate/hydroxyl/carboxylate microenvironment (chondroitin sulfate) and saccharide hydroxyl microenvironment (dextran). Iron in the former microenvironment is mainly in the form of hematite with a minor amount of ferritin-like structure ($Fe_2O_3 \cdot nH_2O$). However, iron in the saccharide hydroxyl microenvironment nucleated in the form of ferritin-like structure had a lower amount of hematite. Our previous study confirmed the co-localization of iron and sulphated and neutral polysaccharides in human *Globus Pallidus* which can lead to the formation of various iron oxides [35].

4 Conclusions

The samples taken from the human *Globus Pallidus* show a presence of various iron-oxide deposits of different size, shape, and count. Chemical composition of these particles is multielemental. Mössbauer spectroscopy shows that the observed doublets can be assigned mostly to hematite and ferrihydrite. Both spectral components show rather large line widths which indicate that the structure of the particles is highly disordered and/or distribution in their size is observed. SQUID magnetometry confirms a presence of ferromagnetic maghemite and/or magnetite. Still more measurements are needed to explain differences between the data. We propose that the interaction between ferritin as a physiological source of iron and surrounding environment is a crucial factor influencing the results of biomineralization.

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