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Looking for biogenic magnetite in brain ferritin using NMR relaxometry

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ABSTRACT: Mammalian cellular iron is stored inside the multisubunit protein ferritin, normally taking the structure of a ferrihydrite-like mineral core. It has been suggested that biogenic magnetite, which has been detected in the brain and may be related to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, could initially form in ferritin. Indeed, as ferritin is present in the brain, the ferrihydrite core could be a precursor for biogenic magnetite formation – particularly in cases where the normal functioning of the ferritin protein is disrupted. In this work, NMR relaxometry was used to detect magnetite inside samples of ferritin extracted from normal and Alzheimer-diseased brains. The method was first calibrated with different fractions of horse spleen ferritin and synthetic magnetite particles. The relaxometry results suggest that the proportion of iron contained in brain ferritin in the form of well-crystallized magnetite instead of ferrihydrite must be $< 1\%$, which is much less than that reported for 'magnetite-like' phase in recent transmission electron microscopy studies of similar samples. Consequently, the magnetization of this 'magnetite-like' phase must be very low compared with that of magnetite. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: magnetite; brain; ferritin; magnetoferritin; relaxometry

INTRODUCTION

Biogenic magnetite (Fe_3O_4), a ferrimagnetic iron oxide containing both Fe(III) and Fe(II), has been detected in the human brain and may be associated with neurodegenerative diseases.[1–5] Although the origin and role of magnetite in the brain are unknown, the ferrihydrite core of the iron storage protein ferritin may act as a precursor for its formation. Recently, transmission electron microscopy (TEM) analysis of ferritin cores associated with neurodegenerative brain tissue [Alzheimer's disease (AD) and progressive supranuclear palsy] has revealed the presence of wüstite and 'magnetite-like' structures within the protein shell.[6] However, the exact proportion of brain ferritin containing magnetite is not known.

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Abbreviations used: AD, Alzheimer's disease; TEM, transmission electron microscopy; NMRD, nuclear magnetic relaxation dispersion; BBS, borate-buffered saline; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Ferritin is the principal iron storage protein, not only in the human body (and brain), but in virtually all organisms. It is comprised of 24 protein subunits forming a spherical shell 12–13 nm in diameter.[7] Iron is stored in an 8 nm diameter inner cavity as a ferrihydrite-like mineral core ($5Fe_2O_3{\cdot}9H_2O$), which is a superparamagnetic antiferromagnet at room temperature.[7,8]

In an attempt to detect magnetite within the ferritin protein, NMR longitudinal and transverse relaxation times (T_1 and T_2) at different magnetic fields were measured for aqueous solutions containing human brain ferritin.

From a qualitative point of view, ferrihydrite and magnetite particles should have a similar effect on water proton relaxation – owing to their magnetic moment, water T_1 and T_2 are shortened. However, the magnetic moment of a ferrimagnetic magnetite particle is significantly greater than the moment associated with the antiferromagnetic ferrihydrite-like crystal contained inside ferritin. Consequently, at the same iron concentration in an aqueous solution, the effect of magnetite particles on T_1 and T_2 is more than 200 times larger than the effect of ferrihydrite-containing ferritin.[9,10]

The evolution of $1/T_1$ with external magnetic field, called the nuclear magnetic relaxation dispersion

(NMRD) profile, is a fingerprint of the magnetic particles causing relaxation. Its shape will be completely different for ferrihydrite- or magnetite-containing ferritin, because the relaxation mechanism of water protons in the presence of these particles is different.[11–13] Similarly, transverse relaxation properties of magnetite and ferritin are easily distinguishable. NMRD profiles were used to detect the presence of magnetite in the aqueous samples of control and AD human brain ferritin.

EXPERIMENTAL

Samples

Stable aqueous solutions of commercial and extracted ferritin and of magnetoferritin were used. Horse spleen ferritin (ref. F4503) was obtained from Sigma Chemical (Bornem, Belgium). The ferritin protein in the horse spleen ferritin sample was found to have a hydrodynamic protein size of 13 nm and an average loading factor of about 2600 iron atoms per protein. Magnetoferritin (a synthetic magnetite-containing ferritin) was kindly provided by Mann and co-workers.[14,15] Sinerem is a commercial MRI contrast agent from Guerbet (Roissy, France). These particles are composed of a magnetite core (4–6 nm) embedded in dextran with an overall particle size of 20 nm.

Human brain ferritin was extracted from one Alzheimer's disease case (female, aged 88 years) and one non-demented case (female, aged 80 years). Freshly frozen tissue, primarily frontal and temporal lobe, trimmed with a ceramic knife to avoid potential iron contamination from stainless-steel autopsy scalpels, was used in the extraction procedure.[16] Acid-washed glassware and non-metallic tools, such as ceramic knives, were used throughout the procedure to avoid iron contamination. Doubly distilled water was magnetically cleaned before use by standing containers on strong rare earth (NdFeB) magnets for 2 days to allow any magnetic particles to be pulled out of suspension. Tissue was thawed, placed in an acid-washed and cleaned borosilicate glass vessel and homogenised in a Class 3 cabinet with a Teflon homogeniser in doubly distilled water. The homogenate was then heated at 70–75 °C for 10 min. At this stage the ferritin was in solution and the homogenate was transferrred on to ice and cooled rapidly before being centrifuged for 30 min at $10\,000 \times g$ and 4 °C and the supernatant retained. The pH of the supernatant was adjusted to 4.6 with 1 mol/L acetic acid and the homogenate was recentrifuged to remove material precipitated by the low pH. The supernatant pH was adjusted to 7.2 and buffered with borate-buffered saline (BBS). Ferritin was then precipitated by the slow addition of 40% (w/v) $(NH_4)_2SO_4$ and recentrifuged. The precipitate was recovered and dialysed against several changes of BBS before being concentrated and applied to a Sepharose column before reconcentration with Amicon ultrafiltration cells with a variety of membrane sizes. For the control sample, the ultrafiltration process was used in place of the column fractionation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining was used to confirm the presence of both heavy- and light-chain ferritin in the final product.

The iron concentration of the samples was measured by inductively coupled plasma atomic emission spectroscopy.

T_1 and T_2 measurements

Relaxation time measurements were performed on Bruker (Karlsruhe, Germany) PC110, PC120, PC140 and mq 60 instruments working at a proton Larmor frequency (ν_0) of 10, 20, 40 and 60 MHz, respectively (a magnetic field of 1 T corresponds to a proton Larmor frequency of 42.6 MHz). A Bruker AMX 300 (300 MHz) spectrometer was used for high-field measurements.

T_1 NMRD profiles were recorded at 37 °C from 0.01 to 10 MHz on a Spinmaster fast field cycling relaxometer (STELAR, Mede, Italy). The uncertainty in the relaxation times was $< 3\%$.

T_2 was obtained at 37 °C with a Carr–Purcell–Meiboom–Gill sequence, with an echo time of 1 ms. The repetition time was always longer than $5T_1$. The monoexponential fits were good, thereby providing no evidence of a multi-exponential behaviour.

RESULTS

Phantoms and limit of detection

Magnetite nanoparticles of $\sim$6 nm have a maximum effect on the proton relaxation time of water at 2–3 MHz.[9] Phantoms were measured in order to look at this characteristic feature and determine the concentration threshold for the observation and identification of magnetite when iron oxides are expected to represent the main magnetic contribution. The NMRD profiles of different mixtures of horse spleen ferritin and magnetite particles (Sinerem), with a total iron concentration of 100 mmol/L, are shown in Fig. 1. The upper curve corresponds to the solution with the highest proportion of magnetite (4.25%) and the lower curve to a sample containing ferritin only. As the effect of magnetite on relaxation times is much more significant than that of ferritin, a fraction of magnetite as small as 0.34% produces a curve that is distinct from that of pure ferritin and above 1% the relaxation peak characteristic of magnetite can be observed.

This demonstrates that our method is sensitive to the presence of very small amounts of magnetite in ferritin samples, partly because the magnetite-induced NMRD

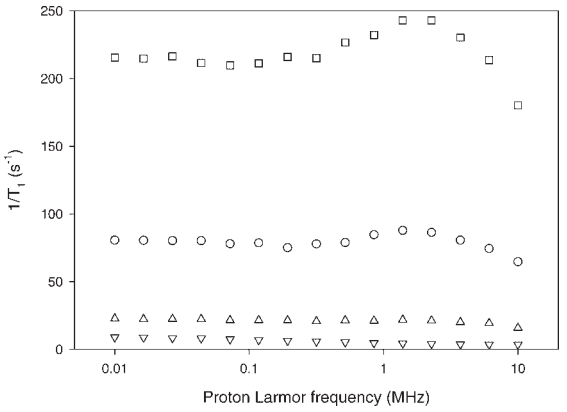

Figure 1. NMRD profiles at 37 °C of mixtures of horse spleen ferritin and magnetite particles (Sinerem) with decreasing proportions of magnetite particles (Sinerem) [0% (▽), 0.34% (△), 1.13% (○), 4.25% (□)] for a total iron concentration of 100 mmol/L

curve has a maximum in $1/T_1$ at ~2–3 MHz, a maximum that is never observed for horse spleen ferritin.

Magnetoferritin and brain ferritin

The NMRD profiles of ferritin extracted from a normal brain and from an Alzheimer's diseased brain are shown in Fig. 2. The curve of magnetoferritin ([Fe] = 0.04 mmol/L) gives a reference for magnetite-containing ferritin and is typical of relaxation induced by magnetite particles, with the maximum at ~2 MHz. However, the profiles of normal brain ferritin and AD brain ferritin show a monotonous decrease of $1/T_1$ with increasing magnetic field, without any peak in the relaxation. These results suggest that $< 1\%$ of iron in brain ferritin is present as magnetite particles. The broad dispersion observed for human ferritin samples is due

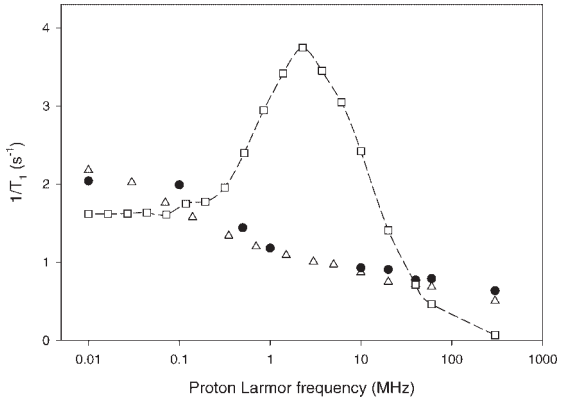

Figure 2. NMRD profiles at 37 °C of magnetoferritin (□, 0.04 mmol/L), ferritin extracted from a normal brain (△, 3.95 mmol/L Fe) and an AD brain (●, 8.88 mmol/L Fe)

Table 1. Transverse relaxation rate of magnetoferritin and brain ferritin samples at 20, 60 and 300 MHz (all the rates have been normalized for an iron concentration of 10 mmol/L)

	$1/T_2$ (s^{-1})		
	20 MHz	60 MHz	300 MHz
Normal brain ferritin	4.30 ± 0.22	7.70 ± 0.38	27.8 ± 1.4
AD brain ferritin	2.46 ± 0.12	5.10 ± 0.26	37.2 ± 1.9
Magnetoferritin	2900 ± 140	2880 ± 140	3480 ± 170

first to the contribution of 'normal' ferritin, but can also contain a contribution from some diamagnetic proteins present in the samples that affects the relaxation at low field and disappears at higher field.

Transverse relaxation

The transverse relaxation of the samples was also investigated: $1/T_2$ of the different samples is given in Table 1 for three different magnetic fields (20, 60 and 300 MHz). These values have been normalized to an iron concentration of 10 mmol/L. The behaviour of magnetoferritin appears completely different from that of brain ferritin: the rate is very large and varies by only 20% on passing from 20 to 300 MHz. Meanwhile the rates for brain ferritin increase with increasing magnetic field, without any sign of saturation, typical of transverse relaxation induced by magnetite particles.[10] The rates for ferritin at 60 MHz are 400 times smaller than those for magnetoferritin and vary by a factor of 3.6 between 60 and 300 MHz, compared with 4.3 for horse spleen ferritin.[17,18] The data are typical of ferritin-induced transverse relaxation and confirm that the majority of the cores are not composed of magnetite.

DISCUSSION

Our results suggest that the percentage of brain ferritin proteins containing well-crystallized magnetite is $< 1\%$ in these samples. However, the proportion of *in vivo* brain ferritin containing poorly crystallised (and therefore less magnetic) magnetite-like material may be higher. The crystallinity of magnetite is closely related to the way in which it forms.[8] Since the formation of magnetite may originate from disrupted iron oxidation inside the ferritin protein at the surface of the core, the gradual inclusion of a magnetite-like structure originating as a poorly structured ferrihydrite may lead to iron cores with a complex and disordered nature. It should be noted, however, that magnetite which has been observed in most biological systems is highly ordered and chemically pure,[19]

although this is mainly based on the observation of larger biogenic magnetite particles (i.e. tens of nanometres or larger).

In the case of pathological ferritins, high-resolution TEM and electron diffraction analysis supports the idea that the cores are composed of a variety of crystal structures.[6] Poor crystallinity and a complex core structure would drastically reduce the magnetic properties and therefore raise the NMR magnetite detection limit. Quintana *et al.*[6] interpreted their diffraction data using three phases of iron cores: ferrihydrite, magnetite-like (up to 30% of the particles) and wüstite. This last mineral, like ferrihydrite and unlike magnetite, is antiferromagnetic and therefore about 100 times less 'magnetic' than highly crystalline magnetite. Wüstite cores would therefore have similar NMR properties to ferrihydrite crystals. The magnetite-like phase, which did not show all the diffraction lines of standard synthetic magnetite, may be an intermediate phase between ferrihydrite, wüstite and well-ordered magnetite. Our NMR data suggest that the magnetic properties of this phase are closer to those of ferrihydrite and wüstite than to those of well-ordered magnetite. For these compounds, the NMR method described in this paper provides an interesting complementary method of characterization to TEM diffraction.

It should also be noted that the magnetite concentration (as a proportion of total iron) could be higher *in vivo* than in the extracted ferritin. As the extraction process is designed to isolate the ferritin protein, magnetite inclusions that are independent of the protein will not be included in the extract. For example, our experimental procedure does not select iron compounds related to other proteins present in the brain, such as neuromelanin, lipofuscin and hemosiderin.[20] The last compound, which is a partially denatured form of ferritin, is not soluble and was probably eliminated during centrifugation.

On this basis, the quantity of magnetite in extracted ferritin is less than or equal to the quantity in the whole brain. A next step would be to apply the relaxometry method directly to brain tissues, although there would be some experimental difficulties.

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