MRI Contrast Agents

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Reduction/Dissolution of a β-MnOOH Nanophase in the Ferritin Cavity To Yield a Highly Sensitive, Biologically Compatible Magnetic Resonance Imaging Agent**

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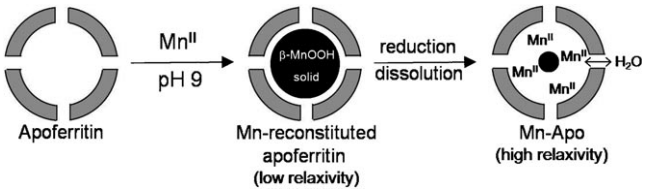
The limited sensitivity of magnetic resonance imaging (MRI) is the major drawback to thorough involvement of this method in molecular imaging applications. Therefore, the search for high-sensitivity MRI agents continues to be an active line of research in probe development.[1] Several nanocarriers have been considered for delivering a sufficient number of contrast agents to the targeting sites and pursuing their visualization in MR images. For instance, Lanza, Wickline et al. reported solid–lipid microemulsions that can be loaded with 10^5 amphiphilic Gd chelates and allow the detection of targets that are present at concentrations of hundreds picomoles per liter.[2] Later, Mulder, Nicolay et al. reported several successful examples of targeting probes using micelles and liposomes loaded with Gd complexes bearing lipidic chains.[3] Although, such particles display excellent sensitivity, some concern exists over their use "in vivo", regarding the metabolic fate of the amphiphilic Gd complexes and uptake of the circulating particles by macrophages. Both drawbacks are overcome if the particle consists of endogenous systems such as ferritin and the loaded agent has a well-tolerated excretion profile. Some years ago we reported on the use of Gd-loaded apoferritin[4–6] that displays a relaxivity of about 600–800 $mM^{-1}s^{-1}$ per apoferritin unit. The system is extremely well tolerated, and it is not taken up by macrophages. The attainable relaxation enhancement of Gd-loaded apoferritin is limited by the number of Gd-HPDO3A (Prohance) units (8–10) that that can be entrapped in the inner cavity of the protein with this procedure, based on H^+-controlled disassembly/reassembly of the apoferritin vesicle. Thus, to tackle the task of a further marked relaxation enhancement of the apoferritin-based system, one needs to design a new approach for the loading procedure.

Over the years, a number of studies have been devoted to understanding how iron ions enter the ferritin cavity and organize into ferri–oxy–hydroxy chains.[7,8] It has also been reported that other metal ions can be stored in large numbers inside the cavity.[9,10] Among them manganese ions have been shown to enter the protein to yield nanosized crystals of β-MnOOH.[11] Thus, a method is available to entrap a large number of paramagnetic ions inside the apoferritin cavity. However, to improve the T_1 relaxation enhancement capabilities it is necessary to solubilize the metal ion payload so that Mn^{II} aqua ions can transfer their paramagnetism to water molecules that freely exchange between the inner and outer compartment of the protein. Manganese is an essential metal, and biological systems have developed efficient routes to control its homeostasis. However, when administered as $MnCl_2$ it displays an LD_{50} as low as 0.22 $mmol\,kg^{-1}$.[1] Thus the possibility of administering Mn^{II} ions sequestered in the apoferritin cavity should guarantee a well-tolerated system from which Mn release would occur on a timescale compatible with its cellular homeostasis processes.

Manganese-reconstituted apoferritin (Scheme 1) was prepared by incubating iron-free horse spleen apoferritin at pH 9 in the presence of $MnCl_2$ solution under air (see Supporting Information) by following a previously reported procedure.[11]



Scheme 1. Mn-Apo preparation.

Transmission electron microscopy images showed that Mn ions form discrete mineral cores (MnOOH) within the ferritin shell (Figure 1). Experimental evidence for formation of the Mn core was also acquired by means of UV/Vis spectrophotometry, as the increase in absorbance in the 400–500 nm range correlates with formation of the MnOOH mineral.[11] Overall the incubation methodology at basic pH leads to a loading of about 3000–4000 Mn atoms per apoferritin unit.

Reduction of Mn^{III} to Mn^{II} inside the inner cavity can be carried out by means of several reagents endowed with $E_0 <$ 1.5 V. It has not yet been definitely established how core reduction takes place. Two mechanisms have been put forward: 1) diffusion of the small reductants through the threefold channels and direct reaction at the mineral surface[12,13] and 2) electron transfer through the protein shell.[15,16] As reducing agents we have used two well-known

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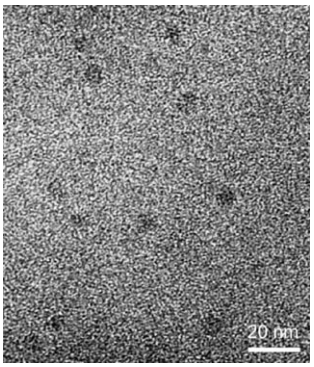

Figure 1. TEM image of Mn-reconstituted apoferritin.

aminopolycarboxylic ligands, that is, nitrilotriacetic acid (NTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) whose oxidation typically occurs through N-dealkylation.[17–19] Treatment of Mn-reconstituted apoferritin with NTA and TETA ligands allows two tasks to be accomplished, that is, elimination of any externally bound Mn ions and reduction of the internally loaded Mn^{III} to Mn^{II}. The resulting product, named Mn-Apo, contains manganese only in its internal cavity, the speciation (Mn^{II} vs. Mn^{III}) of which is dependent on the extent of reductive treatment.

Size-exclusion HPLC (Figure 2) showed that most of the Mn-Apo is in the monomeric form, as established by the coincidence of the peak of Mn-Apo with that of native apoferritin.

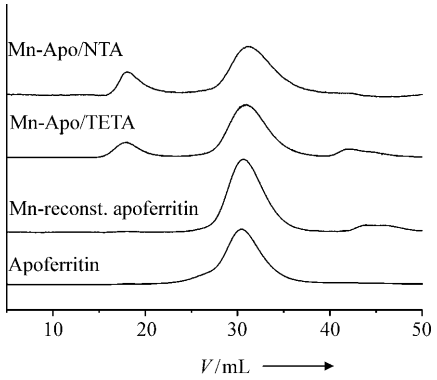

Figure 2. HPLC size-exclusion chromatograms of native apoferritin compared with Mn-reconstituted apoferritin (without any reductive treatment) and with two samples of Mn-Apo treated for 4 h with NTA or TETA. A volume of 200 μL of 1 μM protein solution was injected into the column with 5 mM Hepes buffer and 0.14 M NaCl (pH 7.4) as aqueous mobile phase.

The occurring complexation and redox reactions are represented by Equations (1) and (2).[17]

$$MnOOH + H_xL^{(y-x)-} \rightarrow MnL^{(y-3)-} + 2\,OH^- + (x-1)\,H^+ \quad (1)$$

$$L^{y-} + 2\,MnOOH + 5\,H^+ \rightarrow L_{ox}{}^{(y-1)-} + 2\,Mn^{2+} + CO_2 + HCHO + 3\,H_2O \quad (2)$$

From the literature on ferritin,[20–23] it is known that the release of Fe^{II} ions formed after reductive treatment from the protein inner cavity is slow or impossible in the absence of specific chelators. The Mn-Apo system synthesized in this work behaves analogously.

Figure 3a shows the NMR dispersion (NMRD) profiles of the Mn-reconstituted apoferritin on treatment with NTA or TETA. Both profiles display the characteristic dispersions of Mn^{II} aqua ion (at 0.02 and 0.8 MHz). The NMRD profile of the Mn^{II} aqua ion is also shown for comparison (Figure 3b).

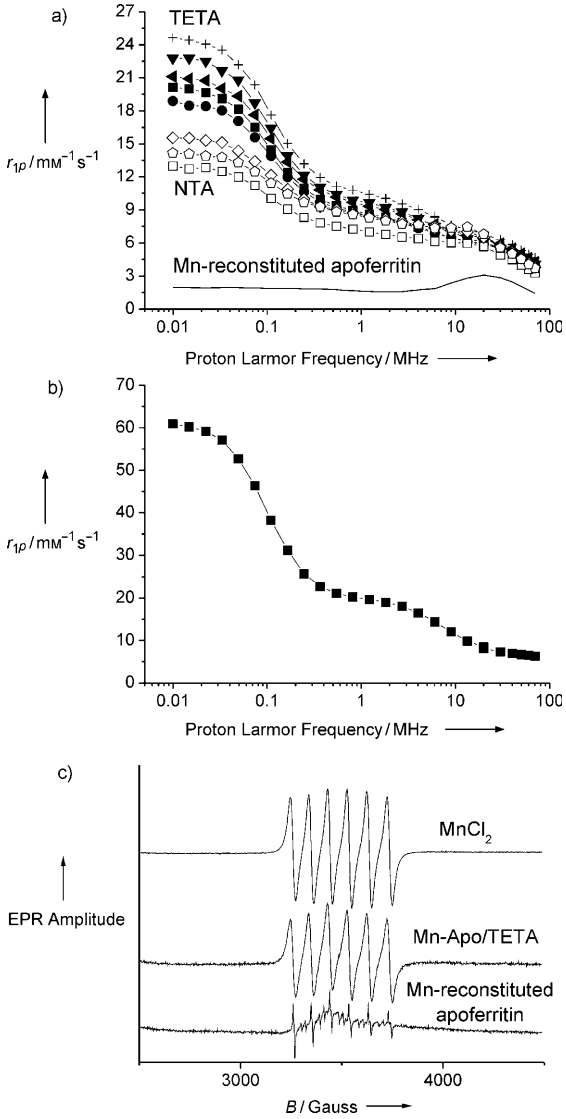

Figure 3. a) NMRD profiles, recorded at 25 °C, of Mn-reconstituted apoferritin (solid line); Mn-Apo obtained by treatment with NTA (1:1 with respect to the total Mn concentration) for reaction times of 5 (□), 60 (◇), and 180 min (⬠); Mn-Apo obtained by treatment with TETA (1:1 with respect the total Mn concentration) for reaction times of 5 (■), 60 (●), 180 (▼), 240 (◀), and 420 min (+). b) NMRD profiles, recorded at 25 °C, of a 1 mM solution of $MnCl_2$. The relaxivity values displayed in (a) and (b) are normalized to 1 mM. c) Room-temperature EPR spectra of Mn-reconstituted apoferritin (bottom); Mn-Apo (obtained by treatment with TETA (1:1 with respect to the total Mn concentration) for 420 min (middle); and an $MnCl_2$ solution (top). The total Mn concentration in all three EPR experiments was 1 mM.

The formation of Mn^{II} aqua ion on reduction of the MnOOH core was confirmed by acquiring an EPR spectrum that shows the characteristic six-line pattern (Figure 3c). As shown in Figure 3a the NMRD profiles depend on the duration of treatment of the Mn-Apo samples with NTA and TETA ligands. The observed changes are indicative of differences in manganese speciation that are the result of incomplete reduction/elimination processes. The number of $Mn^{II,III}$ ions per apoferritin molecule also differs for NTA and TETA: 1090 ± 90 $Mn^{II,III}$ ions after TETA treatment and 570 ± 60 after NTA treatment. The different behavior shown by the two reagents reflects the complexity of the redox/complexation/elimination processes involved in formation of the Mn-Apo system.

By taking into account the relaxivities of the samples treated with NTA and TETA and the relaxivity of $MnCl_2$, the amount of the free Mn^{II} aqua ions in the inner cavity of apoferritin can be determined. Free Mn^{II} was estimated to account for 20–25 and 30–40% of the total amount of manganese entrapped in the protein cage on NTA and TETA treatment, respectively. The remaining Mn is likely mainly in the form of unchanged MnOOH. The EPR data are in good agreement with these estimates (Figure 3c).

The NMRD profiles of Mn^{II} complexes with NTA or TETA are characterized by a single dispersion at 5 MHz (Figure 4). The observed NMRD profiles of Mn-Apo does not show evidence for this feature, although we can not exclude that some Mn^{II} complexes with NTA or TETA are present inside the inner cavity of apoferritin, as their occurrence may be masked by the largely dominant contribution arising from Mn^{II} aqua ions. Alternatively, the absence of Mn^{II} complexes of NTA or TETA (or of their oxidation products) may be explained in terms of their facilitated release from the inner compartment of the protein. In the case of iron ions, it has been reported that small negatively charged complexes can be released, whereas Fe^{II} aqua ions remain confined in the aqueous cavity.[20,22,24,25] Another explanation for the lack of Mn^{II} complexes is based on complete oxidation of aminocarboxylate molecules that have entered the cavity to yield products that no longer act as coordinating agents.

The stability of Mn-Apo was assessed by measuring its NMRD profiles one to two weeks after preparation. The results showed no release of Mn^{II} occurred, as is confirmed by the absence of the relaxivity hump at 30–35 MHz. In fact, prompt formation of Mn^{II} macromolecular adducts is observed on addition of a small aliquot of a solution containing Mn^{II} ions. Thus, it was found that Mn-Apo can reach a very high relaxivity due to the presence of about 1090 Mn atoms in the cavity with an average relaxivity of 6.2 ± 0.3 $mM^{-1}s^{-1}$ per Mn ion at 20 MHz and 25 °C and in the range of ca. 4000–7000 $mM^{-1}s^{-1}$ per apoferritin molecule.

The Mn-Apo system shows markedly superior properties as MRI contrast agent, as its relaxivity per apoferritin particle is almost one order of magnitude higher than that shown by Gd-loaded apoferritin due to accumulation of a large number of manganese ions in its inner cavity.

Finally, the recently raised concern over the relationship between gadolinium and nephrogenic systemic fibrosis[26–28] calls for the introduction of alternative paramagnetic complexes to the Gd-based ones that are currently used in clinical practice. Manganese is an essential metal in living systems and may represent a viable alternative to Gd, as cells have set up well-established storage/excretion pathways for controlling its homeostasis. Furthermore, Mn-Apo can be proposed for the diagnosis of a variety of liver diseases involving alteration in hepatic iron-storage capability and ferritin-receptor expression (e.g., hepatocarcinoma, fibrosis, cirrhosis). The administration of Mn^{II} aqua ions well confined within the ferritin inner cavity appears an efficient method to combine low toxicity with high efficacy. The outer surface of Mn-Apo can be easily functionalized to endow it with targeting capabilities and to design Mn probes characterized by very high sensitivity. Apoferritin does not stimulate any immune reaction, and thus probes can be designed that may stay in circulation for a time sufficiently long for specific targeting needs typical of molecular-imaging diagnostic procedures.

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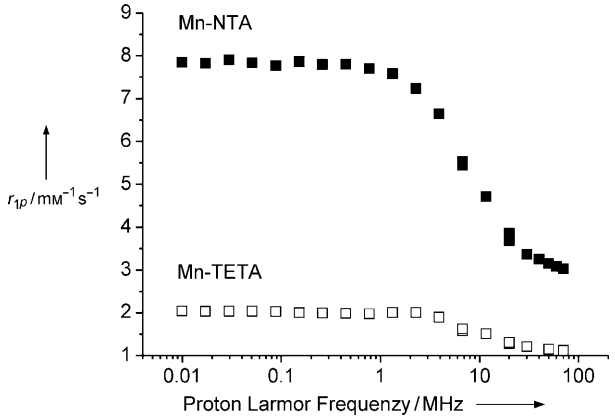

Figure 4. NMRD profiles, recorded at 25 °C, of Mn-NTA and Mn-TETA complexes. The values are normalized to 1 mM concentration.

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