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15 December 2021

Mn-based MRI agent

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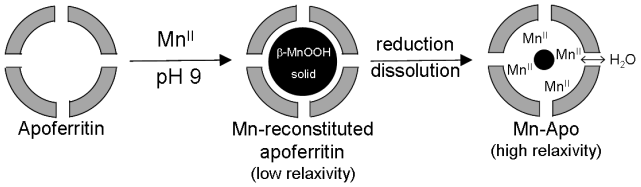
Reduction/Dissolution of the inorganic β-MnOOH nanophase in the ferritin cavity to yield a high sensitiv, biologically compatible MRI agent**

*Ferenc Krisztián Kálmán, Simonetta Geninatti-Crich and Silvio Aime**

The limited sensitivity of MRI is the major drawback to the thorough involvement of this modality in Molecular Imaging applications. Therefore the search for high sensitivity agents continues to be an active line of research in probe development laboratories[1]. Several nanocarriers have been considered in order to deliver a sufficient number of contrast agents to the targeting sites and to pursue their visualisation in MR images. For instance Lanza and Wickline reported solid-lipid microemulsions that can be loaded with 10^5 amphiphilic Gd-chelates and allow the detection of targets that are present at hundreds picomolar concentration[2]. Afterwards Mulder and Nicolay reported several successful examples of targeting probes using micelles and liposomes loaded with Gd-complexes bearing lipidic chains[3]. Although, such particles display an excellent sensitivity, some concern exists for their use "in vivo", either for the metabolic fate of the amphiphilic Gd-complexes as well as for the macrophages uptake of the circulating particles. Both drawbacks are overcome if the particle consists of endogenous systems such as ferritin and the loaded agent has a well tolerated excrection profile. Some years ago we reported on the use of Gd-loaded apoferritin[4-6] that displays a relaxivity of ca. 600-800 $mM^{-1}s^{-1}$ per apoferritin. 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 (eight to ten) that is possible to entrap in the inner cavity of the protein with this procedure, based on H^+-controlled deassembly/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 of the loading procedure.

Along the years, a number of studies have been devoted to understand the way through which 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 high number inside the cavity[9,10]. Among them it has been shown that manganese ions enter the protein to yield nanosized crystals of β-MnOOH[11]. Thus, a method is available to entrap a high 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 in order to have Mn^{II} aqua ions able to transfer their paramagnetism to water molecules that freely exchange between the inner and outer compartment of the protein. Manganese is an essential metal and the biological systems have developed efficient routes to control its homeostasis. However, when administered as $MnCl_2$ it displays a LD_{50} as low as 0.22 mmol/kg[1]. Thus the possibility of administering Mn^{II} ions sequestered in the apoferritin cavity should guarantee for a well tolerated system from which the Mn release would occur on a time scale compatible with its cellular homeostasis processes.

The Mn-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. Schematic representation of the Mn-Apo preparation.

TEM images showed that Mn-ions form discrete mineral cores (MnOOH) within the ferritin shell (Figure 1). Experimental evidence for the formation of the Mn-core was also acquired by means of UV-Vis spectrophotometry as the increase of absorbance in the 400-500 nm range correlates with the formation of the MnOOH mineral[11]. Overall the incubation methodology at basic pH leads to a loading of ca. 3-4 thousands manganese per apoferritin.

The Mn^{III} to Mn^{II} reduction inside the inner cavity can be carried out by means of several reagent endowed with $E_0 < 1.5$ V. It has not yet been definitely established how the core reduction takes place. Two mechanisms have been forwarded: 1) diffusion of the small reductant reagents through the three-fold channels and direct reaction at the mineral surface[12,13]or 2) electron transfer through the protein shell[15,16]. As reduction agents we have used two well known aminopolycarboxylic ligands, i.e. NTA and TETA whose oxydation typically occurs via N-dealkylation[17-19]. The treatment of Mn-reconstituted Apoferritin with NTA and TETA ligands allows to get the two tasks, i.e. elimination of any externally bound Mn ion and reduction of the internally loaded Mn^{III} to Mn^{II}. The obtained product named Mn-Apo contains manganese only in its internal cavity whose speciation (Mn^{II} vs Mn^{III}) is dependent upon the extent of the reductive treatment.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

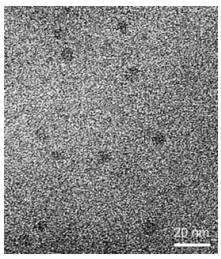

Figure 1. TEM image of Mn reconstituted apoferritin.

By means of HPLC (Figure 2). it has been shown that most of Mn-Apo is under the monomeric form as established by the coincidence of the peak of Mn-Apo with that of the native apoferritin.

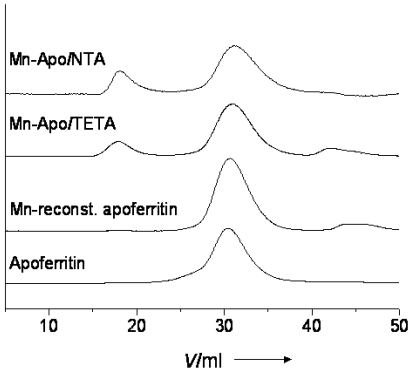

Figure 2. HPLC size exclusion chromatograms of native apoferritin compared with Mn-reconstituted apoferritin (without any reductive treatment) and with two samples of Mn-Apo treated for 4h with NTA and TETA, respectively. A volume of 200 μl of 1 μM protein solution was injected in the column using Hepes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

The occurring complexation and redox reactions are represented by the following equations[17]:

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

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

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

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

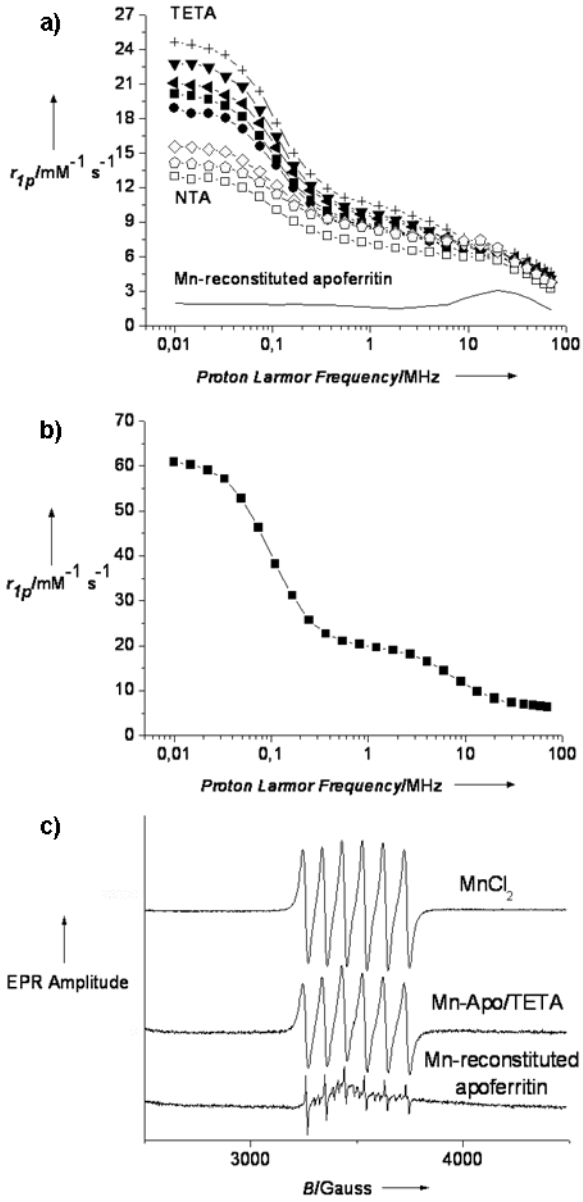

Figure 3. **a)** NMRD profiles, recorded at 25 °C, of Mn-reconstituted apoferritin (solid line); Mn-Apo obtained from the treatment with NTA (1:1 with respect to the total Mn concentration); reaction times: 5 (□), 60 (◊) and 180(⌂) minutes, respectively; Mn-Apo obtained from the treatment with TETA (1:1 with respect the total Mn concentration); reaction times: 5 (■), 60 (●), 180 (▼), 240(◄), and 420(+) minutes. **b)** NMRD profiles, recorded at 25 °C, of a 1 mM solution of $MnCl_2$. (The relaxivity values presented in Figure 3a and 3b are normalized to 1 mM) **c)** EPR spectra recorded at RT of Mn-reconstituted apoferritin (bottom); Mn-Apo (obtained from the treatment with TETA, 1:1 with respect the total Mn concentration, 420'(middle) and of a $MnCl_2$ solution(top). (The $Mn^{II,III}$ concentration of all EPR samples was 1 mM.)

Taking into account the relaxivities of the samples treated with NTA and TETA and the relaxivities of the $MnCl_2$, the amount of the free Mn^{II} aqua ions in the inner cavity of apoferritin can be determined. Free Mn^{II} has been estimated to be 20-25% in the case of NTA treatment and 30-40% in the case of TETA of the entrapped manganese in the protein shell, respectively. The other part of the metal is in MnOOH form. 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 may rely on the complete oxidation of the polyaminocarboxylate molecule that has entered the cavity thus yielding to products characterized by being not longer coordinating agents.

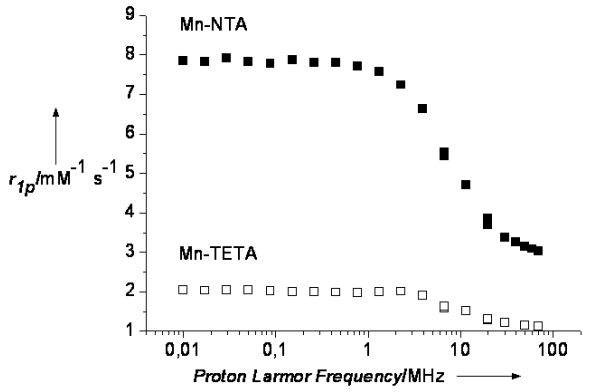

Figure 4. NMRD profiles, recorded at 25°C, of Mn-NTA and Mn-TETA complexes. (The values are normalized to 1 mM concentration.)

The stability of the Mn-Apo was assessed by measuring its NMRD profiles 1-2 weeks after the preparation. The results show that there was not release of Mn^{II} which is confirmed by the absence of the relaxivity hump at 30-35 MHz. Actually the prompt formation of Mn^{II} macromolecular adducts is observed upon the addition of a small aliquot of Mn^{II} ions containing solution. Thus, it has been found that Mn-Apo may 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} at 20 MHz, 25 °C per Mn 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 it displays a relaxivity (per apoferritin particle) that is almost one order of magnitude higher than that shown by Gd-loaded apoferritin. This achievement has been obtained thanks to the accumulation of a large number of manganese ions in its inner cavity.

Finally, the recently raised concern on the relationship between Gadolinium and Nephrogenic Systemic Fibrosis[26-28], calls for the introduction of paramagnetic complexes alternative to the Gd-based ones that are currently used in the clinical practice. Manganese is an essential metal in living systems and it may represent a viable alternative to Gd as cells have set-up well established storing/excretion pathways for controlling its homeostasis. Furthermore, Mn-Apo can be proposed in the diagnosis of a variety of liver diseases involving an alteration in the hepatic iron storing capabilities 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 conjugate low toxicity with high efficacy. The outer surface of Mn-Apo can be easily functionalized in order to endow it with targeting capabilities and design Mn-probes characterized by very high sensitivity. Apoferritin does not stimulate any immune reaction thus allowing for the design of probes that may stay in circulation for a time sufficiently long for specific targeting needs typical of Molecular Imaging diagnostic procedures.

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Mn-based MRI agent

Ferenc Krisztián Kálmán, Simonetta Geninatti-Crich and Silvio Aime*

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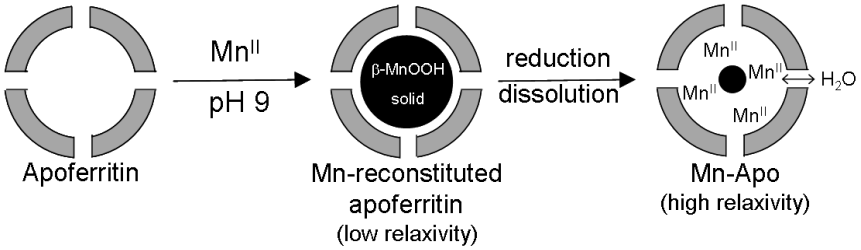
Reduction/Dissolution of the inorganic β-MnOOH nanophase in the ferritin cavity to yield a high sensitivy, biologically compatible MRI agent



An innovative approach to the design of MRI Contrast Agents has been pursued through the entrapment of Mn^{II} aqua ions inside the Apoferritin cavity. This task has been addressed by partial reduction/dissolution of the previously formed β-MnOOH. The reductive treatment has allowed to generate an apoferritin-based nanocarrier containing up to 300-400 Mn^{II} aqua ions. This yielded to the remarkable relaxivity value (per apoferritin) of 4000-7000 $mM^{-1}s^{-1}$.

Supporting Information

Experimental Section

Preparation of Mn-reconstituted apoferritin: The iron-free horse spleen apoferritin (Sigma-Aldrich) was reconstituted in the presence of $MnCl_2$ solution (Fluka, Mn Standard) at pH = 9.0, under air. To avoid the fast oxidation of the Mn^{II} ion, the apoferritin and Mn^{II} were added into a N_2-saturated AMPSO (N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid) (Fluka, ≥ 99.0 %) solution. The protein and the Mn(II) solution were added to give a 1×10^{-6} and 3×10^{-3} M concentration (3000 Mn^{II}/apoferritin), respectively. After 1 week reaction time, the samples were concentrated using Vivaspin centrifugal concentrators (50000 MWCO), treated with NTA (2-(bis(carboxymethyl)amino)acetic acid) (Fluka, ≥ 99.0%) or TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid) (Fluka, ≥ 97%) aminopolycarboxylates (in 1 to 1 metal to ligand ratio) to reduce Mn^{III} as well as to eliminate MnOOH particles and the bound Mn^{II} ions from the outer surface of the protein shell. The reductive treatment was carried out at pH = 7.4 in HEPES buffer (Sigma-Aldrich) at different reaction time (0 – 7 h). To wash out the soluble species the samples were dialysed using cellulose membrane (Sigma-Aldrich, M.W. 12000). The dialysis was carried out at pH = 7.0 and the solution (2 l) was changed after 4 hours and continued overnight. After the dialysis, samples were centrifuged (7000 rpm) to eliminate the aggregated and precipitated materials. At the end of this process the concentration of the protein (Bradford method, BSA standard) and the metal was measured. The metal concentration in a given volume was determined by adding the same volume of HCl 37% and left at 120 °C overnight. By measuring the water proton relaxation rate of these solutions, it is possible to determine its concentration.

TEM: High-resolution transmission electron microscopy analyses were performed on a JEOL JEM 3010-UHR, operating at 300 kV. Samples were dispersed on a lacey carbon Cu grid.

Proton Nuclear Magnetic Relaxation Dispersion (NMRD) $1/T_1$ profiles: Data were measured at 25 °C over a continuum of magnetic field strengths from 0.00024 to 0.47 T (corresponding to 0.01-20 MHz proton Larmor Frequency) on a Stelar field-cycling relaxometer (Stelar, Mede, Italy), under complete computer control with an absolute uncertainty of 1%. Data points from 0.47 T (20 MHz) to 1.7 T (70 MHz) were collected on a Spinmaster spectrometer (Stelar, Mede, Italy) working at variable field.

Chromatography: The high-performance liquid chromatography (HPLC) characterization of Mn-apo, prepared as described above, was carried out on an Amersham Akta Purifier chromatographic system equipped with an Amersham Superose 6 size-exclusion column (XK 16/30, G.E. Healthcare). The experimental workup was carried out in an isocratic condition at 1.0 ml/min, using an aqueous mobile phase containing Hepes 5 mM and NaCl 0.14 M (pH = 7.4).

EPR: X-band CW-EPR spectra were recorded on a Bruker (EMX) spectrometer equipped with a cylindrical cavity operating a 100 kHz field modulation. Spectra were recorded at RT in a 50 µl capillary tube using 10 mW of incident microwave power and a modulation amplitude of 3 G.