R_2 enhancement by formation of a tungsten-iron alloy crystal in the apoferritin cavity

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Introduction: A fundamental challenge in molecular MRI is the sensitivity to delivered contrast agents (1). Thus, there is a critical need to increase the relaxivity of current contrast agents to detect sub-nanomolar concentrations *in vivo*. Natural nanoparticles are attractive agents due to its biodegradability and delivery properties (2). Ferritin, a 12nm iron carrier protein has been used as a natural contrast agent. However, the protein in its native form possesses a weakly magnetic crystal core that has a relaxivity of ~ 1-10$mM^{-1}s^{-1}$ Previous groups have developed various synthesis methods to increase its effectiveness and to load the protein core with highly magnetic iron oxide crystals (3, 4). In order to increase per-ion and per-particle relaxivity, one way of enhancing the magnetic properties for particles that are small enough to contain of a single magnetic domain, less than ~30nm, is to create an alloy of different magnetic metals (5). In this work we formed an alloy crystal in the interior of the apoferritin cavity in an effort to enhance R_2 and increase the process yield. Although tungsten is diamagnetic, its inclusion in the crystal formed a formed a tungsten-iron alloy, with a per-particle relaxivity of 433,651$mM^{-1}s^{-1}$ and per-iron of 27,666$mM^{-1}s^{-1}$ and a percent yield increase of 200% compared to that of magnetoferritin.

Methods: *Particle Synthesis:* A 2µM Apoferritin solution (Sigma Aldrich) was buffered in 0.05M MES at pH 8.5, 48mM $FeCl_2$ (Sigma Aldrich) and 48mM sodium Tungstate Dihydrate (Sigma Aldrich) were de-aerated for 15 minutes with N_2. The solution was kept at a temperature of 55 to 60°C. We added 125µl of $FeCl_2$ to the apoferritin solution every 10 minutes for a total of 20 additions, after the 10th addition 125µl of Sodium tungstate was added every 5 minutes after every $FeCl_2$ addition. Samples were dialyzed against 0.15M NaCl, and filtered using a magnetic column (Miltenyi Biotec), and eluted into 0.15 NaCl buffer. As a protein control, 2µM bovine serum albumin (Thermo Scientific) was used instead of apoferritin. Total protein concentration was obtained with a Bradford assay, and inductively coupled plasma – optical emission spectroscopy (ICP-OES) was used to measure metal concentrations. *Relaxometry:* The particle relaxivity was measured using a 1.5T Bruker Minispec relaxometer. Bruker's curve-fitting tool was used to find the corresponding T_2 values (Inter-pulse τ = 10ms, 200 points) and T_1 values (pulse separations ranging from 5 to 20000ms, 4 scans, 10 points) of samples suspended in a 1% agarose gel. *Electron Microscopy:* Particle samples were adsorbed on Cu-C grids and transmission electron microscopy (TEM) images were obtained using a Philips CM12 electron microscope. High Resolution Electron Microscopy (HREM) images were obtained using a Philips CM200-FEG TEM/STEM. *Electron Spin Resonance:* EPR was performed with a X-band spectrometer (Bruker ESP300E) with 5mW power, 25G modulation and at a temperature of 5K under liquid helium.

Results and Conclusions: Loading the apoferritin core with an alloy of tungsten and iron resulted in an increased per-iron and per-particle relaxivity (R_2) of 27,666$mM^{-1}s^{-1}$ and 433,651$mM^{-1}s^{-1}$ respectively, (see Figure 1). This synthesis procedure along with the addition of a diamagnetic metal increased the nanoparticle yield after filtration by 200% when compared to magnetoferritin. Also, ICP-OES indicated that ~724 Fe ions and 7,454 tungsten ions are present within the protein. TEM showed the formation of electron dense metallic cores of mixed composition with diameters ranging from 5-7.5nm which are larger than native ferritin and magnetoferritin (Figure 2). HREM also showed that the crystal structures in the core are formed in a multi-twinned fashion each direction with lattice spacing of 2.5Å corresponding to magnetite (Figure 3). Electron spin resonance showed that the newly synthesized W-Fe alloy nanoparticles had less Fe(III) in its cores compared to magnetoferritin. The presence of Fe(III) in the cores was confirmed by the typical iron peak at g=4.3. By contrast, $FeCl_2$ (a Fe(II) state) did not show paramagnetic signal in the spectrum (Figure 3). We conclude that the magnetic properties (R_2) of magnetoferritin and the % yield can be strongly enhanced by the addition of a diamagnetic metal into the synthesis to form an alloy crystal in the apoferritin cavity.

		R_2 $mM^{-1}s^{-1}$	R_1 $mM^{-1}s^{-1}$
W- Magnetoferritin	Fe	80	27666
	W	8	2687
	Particle	1260	433651
	Volumetric /nm³	1.93	479
Magnetoferritin particles	Fe	0.07	78
	Particle	407	404,045
	Volumetric /nm³	0.35	351.23

Figure 1. *W-magnetoferritin alloy, and magnetoferritin relaxivities based on Fe,W, particle concentrations and volume of particle. For transverse (R_2) and longitudinal (R_1) relaxivities a CPMG and na IR pulse sequence was used respectively.*

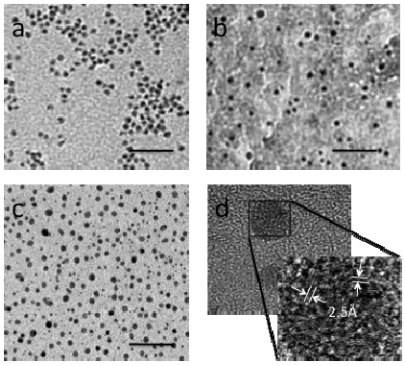

Figure 2. *TEM images of (a) Native ferritin, (b) W- magnetoferritin, (c) Magnetoferritin. Scale bars are 50nm (d) HREM of W- magnetoferritin showing lattice fringes and multi-twinned crystal formation with lattice spacing of 2.5Å in each direction.*

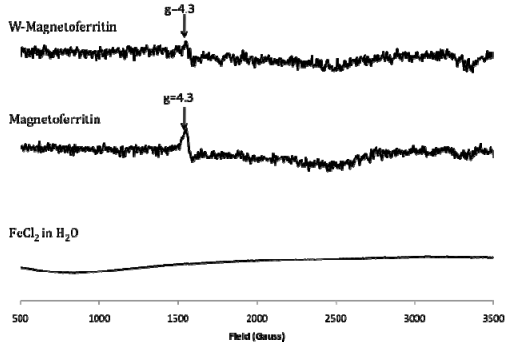

Figure 3. *EPR Spectrum of $FeCl_2$ in dH_2O showing no Fe(III) (bottom). Magnetoferritin showing characteristic peak at g= 4.3 (middle),W-Magnetoferritin alloy decreased Fe(III) signal when compared to magnetoferritin (bottom) .*

References: (1)Cassidy P.J. et al. J.R.Soc.Interface. (2005) 2, 133-144. (2) Cormode D.P. et al. Advanced Drug Delivery Reviews. 62 (2010) 329-338. (3) Bulte JW. et al. Magn Reson Imaging (1994);4:497-505 (4) Clavijo Jordan V. et al, MRM (2010) (5) Jun Y-W. Accounts of Chemical Research (2008) Vol 41. No.2