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“Magnetic resonance imaging contrast enhancement *in vitro* and *in vivo* by octanuclear iron-oxo cluster-based agents”

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Abstract

A water-soluble octanuclear cluster, [Fe₈], was studied with regard to its properties as a potential contrast enhancing agent in magnetic resonance imaging (MRI) in magnetic fields of 1.3, 7.2 and 11.9 T and was shown to have transverse relaxivities $r_2 = 4.01$, 10.09 and 15.83 mM s⁻¹, respectively. A related hydrophobic [Fe₈] cluster conjugated with 5 kDa hyaluronic acid (HA) was characterized by ⁵⁷Fe-Mössbauer and MALDI-TOF mass spectroscopy, and was evaluated in aqueous solutions *in vitro* with regard to its contrast enhancing properties [$r_2 = 3.65$ mM s⁻¹ (1.3 T), 26.20 mM s⁻¹ (7.2 T) and 52.18 mM s⁻¹ (11.9 T)], its *in vitro* cellular cytotoxicity towards

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Appendix A. Supplementary Data

Supplementary data to this article, including additional experimental details, infrared and EDS spectra, TGA plot, TEM image, gel filtration chromatogram, MALDI-TOF spectra of Tyr-HA and Fe8-Tyr-HA, can be found online at <https://doi.org/10.1016/j.jinorgbio.2018.06.005>

A-549 cells and COS-7 cells and its *in vivo* enhancement of T₂-weighted images (4.7 T) of a human breast cancer xenografted on a nude mouse. The physiologically compatible [Fe₈]-HA conjugate was i.v. injected to the tumor-bearing mouse, resulting in observable, heterogeneous signal change within the tumor, evident 15 minutes after injection and persisting for approximately 30 minutes. Both molecular [Fe₈] and its HA-conjugate show a strong magnetic field dependence on r_2 , rendering them promising platforms for the further development of T₂ MRI contrast agents in high and ultrahigh magnetic fields.

Keywords

MRI; paramagnetic contrast agent; molecular relaxivities; polynuclear iron complex; *in vivo* study; breast cancer.

1. Introduction

Over the past three decades, research in magnetic resonance imaging (MRI) has grown into a multidisciplinary field, spanning fundamental research to clinical diagnosis [1–3]. The major reasons for this growth are its non-invasive, ionizing radiation-free nature and its ability to perform 3D visualization [4].

Voxel brightness in MRI is governed by four parameters: local bulk magnetic susceptibility, local proton density, proton longitudinal (spin-lattice) relaxation time, T₁, and proton transverse (spin-spin) relaxation time, T₂. Pulse sequences that emphasize natural differences between the T₁ and/or T₂ times are available, often providing sufficient contrast to visualize the desired objects [5]. However, when these differences are not sufficiently differentiated, image contrast can be further improved by the artificial manipulation of T₁ and T₂ in the tissues to be imaged. This is achieved by the administration of physiologically acceptable, paramagnetic inorganic materials, which act as contrast enhancing agents (CAs). CAs shorten the T₁ and T₂ times of protons – paramagnetic resonance enhancement (PRE) [6] -- in tissues accessible to the CA, thus improving the contrast between types of tissue, radically emphasizing anatomic and pathologic features of concern [7].

Targeted CAs offer another dimension of molecular specificity to the abundant anatomical and functional information that MRI already provides [8, 9]. Target binding provides the pharmacodynamic effect of increasing the relaxivity of the CA, and therefore the MR signal. For example, using a targeted CA at a field strength of 1.5 T yielded images of contrast quality previously achieved only at 3.5–7.0 T with the non-targeted counterpart [10]. Other examples of the advantages of targeted CA in cancer diagnosis and detection have been shown with folate receptor (FR)-targeted compounds, octeotide, Herceptin, and others [11–13]. Also, CAs which contain an aptamer targeting moiety provide a single system where optimization of magnetic properties, pharmacokinetics and biodistribution can all be addressed at the same time [14]. The benefits and disadvantages of targeted CAs have been reviewed, and even though they yet remain to be introduced to the clinic, the increased relaxivity achieved by target binding outweighs the high cost and the synthetic and regulatory complications [15].

Gadolinium (Gd) chelates, with seven unpaired electrons and slow electron-spin relaxation, have been the most widely used positive CAs (brightening the voxels in their proximity) having a significant T_1 -shortening effect [5, 7, 16–17]. For negative contrast (darkening of the voxels), dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs), which induce large local field inhomogeneity, thus shortening the T_2 of protons in their vicinity, are generally used [18–23]. Despite the success of Gd^{3+} -chelates as T_1 -CAs, the discovery of *nephrogenic systemic fibrosis* (NSF), associated with agents involving acyclic chelates [24], has challenged the widespread use of these MRI CAs [25–29]. Besides NSF, recent in-depth studies have demonstrated Gd phosphate precipitation in mouse embryonic 3T3-L1 fibroblast cells grown in media containing Gd-based CAs [30] and more seriously, Gd accumulation in the brain of cadavers of patients with a history of Gd-based MRI-CA injections [31].

While Gd-chelates remain the principal MRI-CAs in clinical use, their potential toxicity issues have launched a search for CAs with alternative metal-ions [32–34]. Early efforts to develop Mn^{2+} -based MRI-CAs showed accumulation of metal ions in the brain [35, 36]; nevertheless, several Mn-based CAs have been the subjects of recent pre-clinical investigations [37–39]. Natural products with high paramagnetic ion content have also been investigated [40]. Unlike Gd, iron is a natural cellular constituent and often a cofactor for enzymes and receptors, having the advantage of excretion through the normal biochemical pathways of iron metabolism [41], although an excess of iron can still be toxic [42, 43]. Iron is also inexpensive and the most abundant transition metal on Earth. Consequently, mononuclear Fe^{3+} complexes have been studied as hepatobiliary MRI-CAs for a long time [44–46]. Dextran coated SPIONs of sizes between 5 and 50 nm [47] have been used for MR imaging of liver cancer [21–23]. A variety of methods have been developed that allow control of surface properties and size of iron oxide nanoparticles for use as MRI-CAs [48]. It should be noted that in spite of the growing number of new magnetic clusters in the literature, the application of polynuclear transition-metal complexes (to be differentiated from superparamagnetic nanoparticles, mentioned above) in MRI contrast enhancement has been less explored, possibly due to their water-insolubility and ligand lability.

Herein we present a preliminary evaluation of the MRI-CA properties of the novel $[Fe_8(\mu_4-O_4(\mu-4-R-pz)_{12}Cl_4)]$ ($[Fe_8]$; pz = pyrazolato anion, $C_3H_3N_2^-$) family of complexes and we propose them as the basis for further development of improved CAs (Fig. 1). We discuss the *in vitro* water-proton relaxation properties induced by these paramagnetic clusters in a directly water-soluble form, $R = CH_2CH_2OH$ (**1**), and compare these results with the corresponding values of gadoversetamid (2-[bis[2-[carboxylatomethyl]-[2-(2-methoxyethylamino)-2-oxoethyl]amino]ethyl]amino]acetate; gadolinium(3+), brand name Optimark™), a clinically approved Gd-based CA (in spite of its known instability *in vivo* [24]). We also describe the *in vitro* and *in vivo* evaluation of a prototypical targeted MRI-CA consisting of a conjugate of $[Fe_8]$ and a tyramine-functionalized hyaluronic acid ($[Fe_8]$ -Tyr-HA, **2**) in which $[Fe_8]$ is the hydrophobic variant with $R = H$. In compound **2**, HA is a vector targeting the CD44 proteoglycan, which is overexpressed in ovarian, breast and other cancer cells (*vide infra*).

2. Experimental

2.1. Materials and methods

The following reagents were obtained from commercial sources and used as received: phosphate buffered saline (PBS), anhydrous ferric chloride, triethylamine, pyrazole, ferrozine, hydroxylamine, trichloroacetic acid (TCA), sodium acetate, ammonium citrate, and tris(hydroxymethyl)aminomethane (Tris). Tetrahydrofuran (THF) was distilled over CaCl_2 , while anhydrous CH_2Cl_2 was used as received. Complexes $[\text{Fe}_8]$ and **1** were prepared as previously described [49–51]. The tyramine (Tyr)-modified HA (Tyr-HA, MW = 5 kDa; average 13.4 disaccharide units), Tyr-HA, was also prepared by a procedure described previously [52]. Approximately 12% of the pendant carboxylic groups of the HA chain were condensed with Tyr, forming Tyr-HA via an amide bond (Scheme 1); e.g., on average, 1.6 Tyr units per 5 kDa HA chain. This level of substitution is sufficiently low to still preserve strong CD44 binding. Additional experimental details, along with an instrumentation list, are provided in Supplementary Information.

2.2. Proton relaxation times

Water proton relaxation times were determined by a Bruker Avance 500 MHz (11.9 T), a Bruker Avance 300 MHz (7.2 T) and a SpinMaster 55 MHz (1.3 T) instruments at 298 K. Longitudinal and transverse relaxation times (T_i , $i = 1$ and 2) were determined by spin-inversion recovery experiments ($180^\circ\text{-}\tau\text{-}90^\circ$) and the Carr-Purcell-Meiboom-Gill (CPMG, $90^\circ\text{-}\tau/2\text{-}180^\circ\text{-}\tau/2$) pulse sequences, respectively. Relaxivity values, r_1 and r_2 , were determined by best-fit plots of T_i^{-1} vs. concentration of compound **1**, according to $T_i^{-1} = T_{i,0}^{-1} - r_i[\text{Fe}_8]$. Fresh solutions were prepared prior to use for T_1 and T_2 measurements. To determine the relaxivity of the paramagnetic cluster in aqueous solution, 5.0 mM stock solution of **1** in deionized water was prepared and was successively diluted to concentrations in the range of 0.7 – 5.0 mM. For **2**, a 1.1 mM stock solution was to prepare diluted aliquots in the 0.1 – 1.1 mM range. For each concentration, T_1 and T_2 experiments were run in triplicate, using three independently prepared sets of fresh solutions, in order to obtain nine values. Relaxivities, r_1 and r_2 , were determined from the slope of plots generated from the mean of the nine values of $1/T_1$ or $1/T_2$ vs. CA concentration.

2.3. MRI of phantoms

MRI phantoms were obtained using 3 mL solutions in 5 mL glass vials supported on a Teflon base. The MRI experiments were performed with phantoms containing varying concentrations of Fe₈-based CAs, using gadoversetamide as a standard comparison for gadolinium-based CAs, and one phantom with deionized water (blank). All images and scans were acquired using standard T_1 -weighted and T_2 -weighted multi-echo spin-echo imaging protocols at room temperature in a scanning suite at 15–18 °C. For each sample, five 5mm slices were obtained with 1 mm space in between slices at the smallest field-of-view available for the sequence using 512×512 imaging matrices. Measurements of the image integrated signal intensity of equal circular Regions-of-Interest (ROIs) within the center slices image of each sample, the image of deionized water (blank) and the background were obtained. For T_1 weighted images the TE = 9–20 ms, TR was varied from 100 ms to 3,000 ms, and the excitation angle was 90 degrees. For T_2 weighted images TR =

3,000 ms, TE was varied from 30 ms to 120 ms in the multi-echo sequence, and the excitation angle was 90 degrees. T_1 and T_2 were determined by measuring the MR image integrated intensity changes as a function of the repetition time TR and the echo time TE, respectively. These relaxation times were calculated using least squares fitting of the image intensities versus time curves obtained from the phantom images.

2.4. Synthesis of [Fe₈]-Tyr-HA conjugate **2**

Tyramine-functionalized HA (Tyr-HA) was coupled to [Fe₈] via its phenolic groups, forming the new Fe₈-Tyr-HA conjugates (**2**). A solution of Tyr-HA (0.050 g, $\sim 10^{-2}$ mmol) in 10 mL of deionized water was prepared. To this solution, [Fe₈] (0.018 g, $\sim 1.2 \times 10^{-2}$ mmol) in the minimum amount of THF was added drop wise with stirring at room temperature. The solution was stirred for approximately 10 min and the solvent was removed using a rotary evaporator. The dark brown solid product was rinsed with CH₂Cl₂ to remove any excess [Fe₈] and vacuum dried in a desiccator.

2.5. Solubility and stability study of **2**

The stability of **2** was evaluated under physiologically relevant conditions. It was prepared as a stock solution (130 μ M) in water and then diluted to 3.3 μ M in water and also in pH 7.4, 25 mM Tris, 0.1 M NaCl buffer containing 10% fetal bovine serum. Four separate sets of the final solutions were prepared. The solutions were then monitored by UV-Vis spectroscopy every 15 minutes for 2 hours and then every hour for 24 hours at 25 °C temperature. See Supplementary Information for experimental details.

2.6. Cell culture

A-549, COS-7 and MDA-MB-468 cells were maintained in accordance with ATCC protocols. The cells were cultured in 75 cm² flasks with phenol red DMEM (Sigma, D6429) containing 1% of glutamine and 4.5 g/mL of glucose and sodium pyruvate, and supplemented with 10% FBS (HyClone), 1% of antibiotic solution prepared with 11 mg/mL streptomycin and 7 mg/mL penicillin (Calbiochem®, EMD Biosciences Inc.) in a humidified incubator with 5% CO₂ for A-549 and COS-7 and without CO₂ for MDA-MB-468 at 37 °C. All experiments were conducted after 3 to 10 cell passages.

2.7. Cell viability assay

A stock solution of 90 μ M of **2** was prepared in nanopure-quality water. A-549 and COS-7 cells at >80% confluence were seeded (5.0×10^4 cells/mL) into 96-well plates with 100 μ L of DMEM per well. After 24 h, cells were incubated with 30 μ M of **2** in a total volume of 200 μ L of DMEM for 48 h. Next the viability of the cells was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega). 1 h before completing the incubation time, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and phenazine methosulfate (PMS) solution was added to each well (333 μ g/ml MTS + 25 μ M PMS). After 1 h, the absorbance of each well was measured at 492 nm in a Multiskan FC, Thermoscientific plate reader spectrophotometer. Cells treated with 150 μ M linoleic acid were used as positive control

(cytotoxic agent, low viability), and 1 mg/mL α -lactalbumin and untreated cells were negative controls (no cytotoxicity, 100% viability).

2.8. *In vivo* Studies

Imaging studies were conducted in the Small Animal Imaging Facility (SAIF) of MD Anderson, under a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Female nude mice (Harlan) were implanted in their mammary fat pads with human MDA-MB-468 breast tumor cells, according to our previous protocols [53, 54]. Depending on the maturity of the mice and the extent of development of the mammary tree, usually the fat pads immediately inferior to those most superior were injected, either on one side of the tree or on both. Once the orthotopic tumors had grown to at least 5 mm in their longest diameter (~ 3 weeks), these xenografted mice were MR imaged on a 4.7 T scanner. To both confirm intravenous (i.v.) injection of the Fe₈-Tyr-HA and to probe the plasma kinetics of its clearance, mice were scanned dynamically using a fast spoiled gradient echo sequence (TE = 1.6 ms, TR = 76 ms, 30° excitation angle). To assess tumor localization of this CA, anatomic imaging sequences were utilized: T₂-weighted (TE_{eff} = 70 ms, TR = 4150 ms), and T₂*-weighted (TE_{min} = 3.06 ms, TE = 3.64 ms, TR = 1200 ms, 30° excitation angle). MRI scans were obtained immediately prior to and after administration of Fe₈-Tyr-HA, either by direct intra-tumoral or i.v. injection. Application of ultrasound gel on these superficial tumors minimized air-induced artifacts on the MR images.

3. Results and Discussion

We have previously studied the physical chemistry of octanuclear ferric complexes of the general formula [Fe₈(μ_4 -O)₄(μ -4-R-pz)₁₂X₄] -- pz = pyrazolato anion; X = Cl, Br, NCS -- with various R-substituents at the pyrazole 4-position: R = H, Cl, Br, I, CH₃, (CH₂)_{2,3}Cl and (CH₂)_{2,3}OH (Fig. 1) [49, 50, 51]. The parent compound, with R = H and X = Cl, has an effective magnetic moment of 7.0 B.M. at ambient temperature, resulting from the antiferromagnetic coupling of eight high-spin Fe³⁺ centers [49]. The almost spherical shape (no dipole moment) and surface defined by carbon, hydrogen, and halogen atoms of these molecules render them highly hydrophobic. More recently, however, we have also reported the synthesis of two new variants of these octanuclear complexes with R = -CH₂CH₂OH (**1**) and -CH₂CH₂CH₂OH, which, because of their hydrophilicity, render the octanuclear complex directly soluble in water [50]. In aqueous solutions, **1** forms two types of H-bonded super-clusters consisting of ~ 13 and ~ 90 [Fe₈] units [50]. The fact that a well-resolved ¹H-NMR spectrum can be recorded for paramagnetic **1** implies that its electron spin relaxation time, T_{ie}, is shorter than the NMR timescale of 10⁻¹¹- 10⁻¹³ s. This is also consistent with typical T_{ie} values of 10⁻¹⁰ s reported for high spin Fe(III) compounds [7]. In aqueous media, hydrolysis of their terminal Fe-Cl bonds leads to the formation of H-bonded aggregates with dimensions of 5–7 nm (~ 13 Fe₈ molecules) and 40–60 nm (~ 90 Fe₈ molecules), as determined by DLS [50].

3.1. Relaxivity of complex 1

The proton longitudinal relaxation rate (R₁) and transverse relaxation rate (R₂) of aqueous solutions of **1** were measured in three different magnetic fields in the 0.7 – 5.0 mM range,

limited by the solubility of **1**. The plots (Fig. 2 and S1) show the expected linear relationship with the concentration of the paramagnetic cluster. For **1**, the slope of the least-square fit lines indicates unremarkable longitudinal relaxivity values (r_1 , Table 1) and marginal field dependence within experimental error. In contrast, the transverse relaxation rates (R_2) are faster -- significantly higher transverse relaxivities (r_2 , Table 1) -- and show a dependence on the magnetic field. The R_2 value of the 5.0 mM solution at 11.9 T was treated as an outlier, as no measurements could be conducted at higher concentration to examine whether it represents an inflexion point of the line.

The ratio of r_2/r_1 is an indicator of relaxometric properties of an imaging agent, and it is used to classify a given type of MRI CA. In general, for mononuclear paramagnetic chelates, r_2/r_1 ratios range between 1 and 2, whereas for SPIONs, typical values are > 3 and can be as large as 50 in the case of superparamagnetic colloids [55, 56]. Gd complexes, with r_1 values ranging from 4–16, are used primarily as T_1 -agents [57], whereas the SPIONs are T_2 -agents with a $r_1 \sim 30 \text{ mM}^{-1} \text{ s}^{-1}$ and $r_2 \sim 100 \text{ mM}^{-1} \text{ s}^{-1}$ [58]. The relaxivity r_2/r_1 ratios of compound **1** range from 5.9 (at 1.3 T) to 20.8 (at 11.0 T), suggesting the Fe_8 system as a T_2 -contrast agent.

The magnetic field strength is one of the factors influencing relaxation properties. Relaxation rates are described by the Solomon–Bloembergen–Morgan theory, which determines that the correlation time, τ_{Ci} , is dominated by the most rapid one among three contributing factors: T_{ie} (electron-spin relaxation times of the metal ion), τ_R (re-orientational correlation time of the metal-proton vector), and τ_m (rate of water-proton exchange) [59]; the former is magnetic field-dependent, influencing the overall τ_{Ci} , and hence the relaxation rates. Consequently, the increase of r_2 values of **1** is tentatively attributed to a slower electron spin relaxation of the $[\text{Fe}_8]$ cluster with increasing magnetic field. In the case of Gd-chelates, T_{ie} may be the limiting factor below 0.5 T, but not at 1.5 T. Instead, $1/\tau_{Ci}$ is dominated by τ_R above 1.5 T. In the present case, **1** has a shorter T_{ie} (10^{-11} – 10^{-13} s, *vide supra*) than mononuclear Fe(III), Mn(II) and Gd(III) complexes ($T_{ie} = 10^{-8}$ – 10^{-10} s), resulting in a different influence on τ_{Ci} and ultimately on the relaxation rates. The longitudinal relaxivity of **1** is practically invariable, whereas its transverse relaxivity increases with increasing magnetic field strength. The low r_1 values are attributed to the inter-cluster H-bonded aggregation of $[\text{Fe}_8]$ units (previously reported [50]), which render the Fe-ions in the interior of the aggregate inaccessible to H_2O molecules. On the other hand, the magnetic field dependence of r_2 values are attributed to the increased population of the closely-spaced [49] higher spin-states at increasing magnetic fields. These results indicate that contrast enhancement by compound **1** will improve in T_2 -weighted images acquired in stronger magnetic field scanners, while T_1 -weighted ones will not benefit from a stronger field. Three other octanuclear Fe(III) complexes have been studied with regard to their relaxivities, alas in narrower concentration ranges and in only one or two magnetic field strengths [60–62]. The r_1 values of these three complexes (1.1 – $5.4 \text{ mM}^{-1} \text{ s}^{-1}$) are higher than those of **1**, however, their r_2 values (3.1 – $8.4 \text{ mM}^{-1} \text{ s}^{-1}$) are all lower than those of **1**.

3.2. Phantoms

Phantom imaging allows the visualization of the CA effect. Imaging of **1** at four concentrations (5 mM, 2.5 mM, 1 mM and 0.5 mM), and of deionized water as control, allowed estimation of the r_1 and r_2 . For comparison, solutions of the same four concentrations of gadoveretsamide were also imaged. Both T_1 - and T_2 -weighted images were obtained. For the T_1 -weighted sequence, scans were run using five repetition times, $TR = 100, 200, 400, 600, 800$ and 2000 ms (Fig. 3). For the T_2 -weighted multi-echo sequence, scans were run with four different echo times, $TE = 30, 60, 90$ and 120 ms (Fig. 4). For **1**, the calculated r_1 and r_2 (Table 1) from these phantom images at 1.5 T are in good agreement with those determined by relaxometry at 1.3 T (see previous section). Visual inspection of the phantom images confirms that at 1.5 T, gadoveretsamide is superior to **1** as a positive (T_1) contrast agent, but of approximately of the same efficiency as a negative (T_2) contrast agent, in accordance with expectation based on the respective r_1 and r_2 values (Table 1).

3.3. Characterization of Fe₈-Tyr-HA (2)

The four labile chloride terminal ligands of [Fe₈] allow its coupling to other moieties containing functional groups that can be metathetically exchanged for chloride, such as phenols. Hyaluronic acid (HA) is an unbranched polysaccharide distributed extensively throughout connective, epithelial and neural tissues, whose extra-cellular binding receptors include the cell surface glycoprotein CD44, a main receptor for HA [64–68]. Taking a cue from the well-known affinity of iron for phenolate ligands (*e.g.*, transferrins), we prepared tyramine-functionalized HA, Tyr-HA.

An EDS spectrum of the solid Fe₈-Tyr-HA conjugate (Fig. S2) showed the presence of iron and traces of chlorine in the conjugate, which was confirmed by an elemental analysis showing a 7.21% Fe-content. TGA analyses were performed for [Fe₈], Tyr-HA and Fe₈-Tyr-HA (**2**): A sample of [Fe₈] was decomposed to FeO after heating beyond 400 °C under air, while Tyr-HA was decomposed, leaving only 4% w/w of residue upon heating beyond 600 °C. Finally, a sample of Fe₈-Tyr-HA was decomposed leaving a 17% w/w residue, attributed to 4% from the Tyr-HA plus 13% from the Fe₈ components, respectively. The latter matched satisfactorily with the 11.2% FeO residue calculated based on the elemental analysis of the same sample (Fig. S3). The combined elemental analysis and TGA results suggest that approximately 84% of Tyr-HA functionalized oligosaccharides are attached to an [Fe₈] unit, *e.g.*, the composition of **2** is approximately 16% Tyr-HA and 84% Fe₈-Tyr-HA. The IR spectrum showed the diagnostic Fe-O stretch of Fe₈-cluster at 477 cm⁻¹ in an area where the spectrum of iron-free Tyr-HA is featureless (Fig. S4).

Deconvolution of the 80 K ⁵⁷Fe-Mössbauer spectrum (Fig. 5) revealed three components: Site A (blue trace, $\delta = 0.44$ mm s⁻¹, $E_Q = 0.43$ mm s⁻¹) attributed to the four iron centers of the Fe₄O₄-cubane core; site B (red trace, $\delta = 0.36$ mm s⁻¹, $E_Q = 0.83$ mm s⁻¹) attributed to outer Fe centers of Fe₈ coordinated to the phenolic oxygen atoms of tyramines; and site C (green trace, $\delta = 0.37$ mm s⁻¹, $E_Q = 1.89$ mm s⁻¹) attributed to outer Fe centers coordinated to μ -O-atoms, resulting from the hydrolysis of Fe-Cl bonds and forming bridges between adjacent Fe₈-units. The above spectral deconvolution assumed that 50% of total iron occupies A sites, resulting in occupation of sites B and C of 20% and 30%, respectively.

Based on the Mössbauer spectrum interpretation, the following average structural model (Scheme 2) is proposed for the Fe₈ content of **2**: Three Fe₈-units are linked by three μ -O atoms allowing the remaining six outer iron sites to be coordinated by phenolic O-atoms of tyramines. The assignment of the deconvoluted spectral features to sites A, B and C is based on previous experimental and theoretical studies of [Fe₈] and related complexes [69]. Scheme 2 depicts the types of bonding leading to clustering of Fe₈-Tyr-HA units.

In the solid state, Fe₈-Tyr-HA formed spheres ranging in diameter from 100 to 200 nm, as determined by TEM images (Fig. S5). In aqueous solution (7.5 mg Fe₈-Tyr-HA in 1.5 mL) the average size of particles was 143.7(4) nm (with a polydispersity index, PDI = 0.19), as determined by DLS. The particle size distribution remained invariant in the 4.5 to 9.5 pH range, with a sharp increase observed at pH = 10 (Fig. S6). In contrast, the ζ -potential of particles varied from -29 to -45 over the same pH range, consistent with gradual deprotonation of pendant carboxylic groups of HA at increasing pH (Fig. S7). Gel filtration chromatography estimated a 15.9 kDa mass for **2** in buffered aqueous solution, suggesting the presence of a three-ligand aggregate (Supplementary Information and Fig. S8). MALDI TOF spectroscopic results are also consistent with the formation of aggregates (Supplementary Information and Fig. S9).

3.4. Solubility and stability study of conjugate **2**

The ferrozine assay showed that **2** was reasonably water soluble and yielded relatively high iron concentrations. Conjugate **2** reached a maximal soluble iron concentration of 5.51 ± 0.43 mM or 0.17 ± 0.015 mM compound concentration in water. The compound is extremely stable in water. At 3.3 μ M it does not demonstrate any UV-Vis spectral changes even after 24 hours in solution (S10). The stability of the compound at the same concentration was monitored under physiologically relevant conditions (pH 7.4, 25 mM Tris buffer, 100 mM NaCl, 10% fetal bovine serum (FBS)) every fifteen minutes for two hours and then every hour up to 24 hours [Fig. 6]. The shoulder at 280 nm, attributable to the Tyr-HA, remains virtually constant for 12 hours. Between the 12 and 24 h time points the absorbance increases either due to slow binding to biomolecular species in the FBS or a gradual speciation change because of partial dissociation of Tyr-HA and compound aggregation as discussed in the analysis of the gel filtration chromatographic data (Supplementary Data). This FBS-induced aggregation will obviously affect the relaxivity, therefore contrast enhancement, achieved *in vivo* compared to those determined *in vitro*. No new absorbance shoulders or maxima appear during the spectral changes.

3.5. Relaxivity of complex **2**

The longitudinal and transverse relaxivities, r_1 and r_2 , of **2**, determined in a similar manner as those of **1** (Fig. S11, S12) are listed in Table 1. Its r_1 values are low compared to **1** significantly and much lower compared to Gd-based agents [57]. This is tentatively attributed to the trapping of Fe₈ units in the interior of the Fe₈-Tyr-HA conjugate, limiting the exchange of water molecules in its solvation sphere with bulk water. In contrast, the r_2 values of **2** are higher than both **1** and Gd-agents, showing a stronger magnetic field dependence than **1**. In fact, the r_2 values reached at higher field place **2** in the regime of nanoparticulate SPIOs [58]. The spectacular difference between the r_2 values of **1** and **2** is

attributed to the slower tumbling of the latter due to its polymeric nature (high molecular weight).

3.6. *In vitro* cytotoxicity evaluation of Fe₈-Tyr-HA (2)

The cellular cytotoxicity of conjugate **2** was examined by evaluating its ability to induce cell death in A-549 cells, which overexpress CD44, and COS-7 cells, which do not. The determination of *in vitro* cytotoxicity was designed to be a first-order assessment of potential toxicity anticipated *in vivo* when used as a CA. Tumors may have an enhanced permeability and retention (EPR) effect and are thereby able to uptake significantly larger solutes, and retain them due to dysfunctional lymphatic drainage, than most normal tissues *in vivo* [70, 71]. The HA moiety on conjugate **2** would also facilitate higher CD44-mediated interaction with the conjugate. Nonetheless, using the MTS assay, conjugate **2** even at as high a concentration as 30 μ M demonstrated no cytotoxicity against either cell line (Fig. 7), an encouragement for *in vivo* applications of the compound because it was not expected to be significantly toxic.

3.7. *In vivo* evaluation Fe₈-Tyr-HA (2)

In vivo pilot experiments in four mice demonstrated that the lead HA-Tyr-Fe₈ conjugate was physiologically compatible in the dose ranges administered (consistent with the cell viability studies), although these doses were largely limited by solubility and considerations of optimal injection volumes. A nude mouse bearing orthotopic human MDA-MB-468 breast tumor xenografts [53, 54], which overexpress CD44, were scanned using T₂-weighted and T₂*-weighted anatomic imaging sequences before and after administration of conjugate **2**. Figure 8 illustrates clear visibility of CA following direct (intratumoral) injection.

Mice were also scanned dynamically using a fast spoiled gradient echo sequence following i.v. injection of the Fe₈-Tyr-HA to both confirm injection and to initially assess plasma clearance pharmacokinetics. Clear negative contrast in circulating blood viewed in the inferior vena cava (Fig. S13), appeared rapidly (~1 min) post-injection, with strong signal attenuation still persisting at ~50% of maximum attenuation for more than 10 min following injection.

Next, having established the feasibility of detecting signal attenuation following systemic administration of the Fe₈-Tyr-HA, a mouse also bearing an MDA-MB-468 breast xenograft was injected i.v. with the CA via a tail vein catheter. A comparison of T₂-weighted scans (Fig. 9), acquired before and ~45 min after injection of 7 mg of compound **2** in 200 μ L PBS, revealed heterogeneous signal change within the tumor that was already evident by 15 minutes after injection (not shown) and that was increasingly conspicuous with time over this window of observations. The observation that the CA was largely cleared from the blood stream while the tumor contrast enhancement was maximized, corroborated the conclusion that the Fe₈-Tyr-HA targeted the tumor.

4. Conclusions

Mononuclear paramagnetic metal-ions with no magnetic anisotropy and large energy separation between spin-states, such as Gd³⁺, Mn²⁺ and Fe³⁺, have long electron-spin

relaxation times (T_{1e}) and are therefore ideal proton relaxation enhancers. On the other hand, paramagnetic exchange-coupled polynuclear complexes, with their closer-spaced spin-states, show relatively faster electron-spin relaxation and are not optimal promoters of proton-spin relaxation. Not surprisingly, the former ions have attracted most of the attention with regard to their potential in MRI contrast enhancement. Complex **1**, which gave well-defined ^1H -NMR spectra and therefore is believed to have a $T_{1e} < 10^{-8}$ s, falls into the latter category. However, polynuclear complexes may have other advantages over mononuclear ones, such as higher spin-states populated at ambient temperature and chemical stability under physiological conditions, while still able to promote proton nuclear relaxation with an observable MRI contrast.

The clinically approved Gd-complexes show a decrease in relaxivity with increasing magnetic field strength in the 1.5 – 7.0 T range, which is a potential drawback for their application in even larger fields, as the newer 3 T and 4.5 T clinical MRI scanners are becoming more common in practice [72, 73]. The scenario is even worse in case of small animal scanners that usually operate at ultrahigh 4.7 T - 7 T, or even higher magnetic fields [74, 75]. On the contrary, an opposite trend is observed in the Mn(II)-oxo clusters [76]. The $[\text{Fe}_8]$ -based CAs described here show the same trend of improving contrast enhancement with increasing magnetic field, which, considering the current trend towards higher-field clinical MRI scanners, places them in an advantageous position for future development. The low r_1 values of **1** and **2** have been tentatively attributed to clustering of $[\text{Fe}_8]$ units, preventing the close approach of H_2O molecules to interior Fe-ions. In future development, this can be remedied by increasing the steric bulk of new $[\text{Fe}_8]$ variants that will prevent clustering. Because the Fe_8 complexes are amenable to chemical manipulation, which can modify their chemical, magnetic, and pharmacokinetic properties, we anticipate that improved variants of **1** will be available soon. It should be emphasized that the octanuclear complex **1** is well-defined molecule (e.g., not an iron oxide nanoparticle), and therefore not comparable with SPIOs in a straightforward manner. Nevertheless, the r_2 values of both **1** and **2** are in the same range as SPIOs.

With these thoughts, we have proceeded with the development of physiologically compatible conjugates of $[\text{Fe}_8]$ clusters with targeting ligands. The HA conjugates described here represent the first effort in that direction. The 5 kDa HA of **2** is at the low end of the HA molecular weight range of 5 – 150 kDa. Because the longer HA chains are known to have higher affinity for CD44, we project that analogues of **2** employing larger HAs will allow contrast enhancement of tumors with lower doses of the administered CA. This may be further improved by optimization of the $[\text{Fe}_8]/\text{HA}$ ratio in the conjugate. The small extent of Fe_8 dissociation from Tyr-HA, currently a drawback, can be remedied by increasing the percentage of Fe-coordinating Tyr groups in the Tyr-HA vector.

In summary, we have prepared an HA conjugate of the paramagnetic $[\text{Fe}_8]$ MRI module and shown that the T_2 relaxation properties of this conjugate are greatly improved compared to those of the unconjugated module. It is expected that further optimization of the conjugate composition -- e.g., by varying the length of the HA chain, degree of functionalization, and steric bulk of the $[\text{Fe}_8]$ units -- will lead to CAs with superior contrast enhancing properties. Work in that direction, along with detailed biological evaluation, is in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5.

CA	contrast agent
CPMG	Carr-Purcell-Meiboom-Gill
DLS	dynamic light scattering
DMEM	dulbecco's modified eagle medium
EDS	energy dispersive spectroscopy
EPR	enhanced permeability and retention
FBS	fetal bovine serum
HA	hyaluronic acid
IACUC	Institutional Animal Care and Use Committee
MALDI-TOF	matrix assisted laser desorption ionization-time-of-flight
MRI	magnetic resonance imaging
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NSF	nephrogenic systemic fibrosis
PBS	phosphate buffered saline
PDI	polydispersity index
PMS	phenazine methosulfate
PRE	paramagnetic resonance enhancement
ROI	region of interest
SAIF	small animal imaging facility
SPION	superparamagnetic iron oxide nanoparticle

TCA	trifluoroacetic acid
TE	echo time
TEM	transmission electron microscopy
TR	repetition time
Tyr	tyramine

References

1. Stark DD, Bradley WG, Mosby CV, Magnetic Resonance Imaging 3 Sub Edition. John Wiley & Sons, NY, 1999.
2. Merbach AE, Toth E (eds), The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging. John Wiley & Sons, NY, 2001.
3. Hashemi RH, Bradley WG, Lisanti CJ, MRI the basics. Williams & Wilkins, Baltimore, MD, 2010.
4. Storey P, In: Magnetic Resonance Imaging - Methods and Biologic Applications, Part I, P. V. Prasad (ed). Humana Press Inc., Totowa, NJ, 2006, pp. 3–57.
5. Caravan P, Strategies for increasing the sensitivity of gadolinium based MRI contrast agents. Chem Soc Rev. 35 (2006) 512–523. [PubMed: 16729145]
6. Clore GM, Iwahara J, Theory, Practice, and Applications of Paramagnetic Relaxation Enhancement for the Characterization of Transient Low-Population States of Biological Macromolecules and their Complexes. Chem Rev. 109 (2009) 4108–4139. [PubMed: 19522502]
7. Bertini I, Luchinat C, Parigi G, Ravera E, NMR of Paramagnetic Molecules, 2nd Ed. Elsevier, Amsterdam, NL, 2017.
8. Boros E, Gale EM, Caravan P, MR imaging probes: design and applications. Dalton Trans. 44 (2015) 4804–4818. [PubMed: 25376893]
9. Cheng Z, Al Zaki A, Hui JZ, Muzykantov VR, Tsourkas A, Multifunctional Nanoparticles: Cost Versus Benefit of Adding Targeting and Imaging Capabilities. Science. 388 (2012) 903–910.
10. Neumaier CE, Baio G, Ferrini S, Corte G, Daga A, MR and iron magnetic nanoparticles. Imaging opportunities in preclinical and translational research. Tumori. 94 (2008) 226–233. [PubMed: 18564611]
11. Li X, Du X, Huo T, Liu X, Zhang S, Yuan F, Specific Targeting of Breast Tumor by Octreotide-Conjugated Ultrasmall Superparamagnetic Iron Oxide Particles Using a Clinical 3.0-Tesla Magnetic Resonance Scanner. Acta Radiol. 50 (2009) 583–594. [PubMed: 19449236]
12. Chen TJ, Cheng TH, Hsu SC, Cheng TL, Liu GC, Wang YM, Targeted Herceptin-dextran iron oxide nanoparticles for noninvasive imaging of HER2/neu receptors using MRI. J Biol Inorg Chem. 14 (2009) 253–260. [PubMed: 18975017]
13. Meir R, Henning TD, Boddington S, Tavri S, Arora S, Piontek G, Rudelius M, Corot C, Daldrup-Link HE, Breast Cancers: MR Imaging of Folate-Receptor Expression with the Folate-Specific Nanoparticle P1133. Radiol. 255 (2010) 527–535.
14. Somasunderam A, Thiviyathan V, Tanaka T, Li X, Neerathilingam M, Lokesh GL, Mann A, Peng Y, Ferrari M, Klostergaard J, Gorenstein DG, Combinatorial selection of DNA thioaptamers targeted to the HA binding domain of human CD44. Biochemistry. 49 (2010) 9106–12. [PubMed: 20843027]
15. Klostergaard J, Parga K, Raptis RG, Current and Future Applications of Magnetic Resonance Imaging (MRI) to Breast and Ovarian Cancer Patient Management. P R Health Sci J. 29 (2010) 223–231. [PubMed: 20799509]
16. Toth É, Helm L, Merbach AE, Relaxivity of MRI contrast agents. Top Curr Chem. 221 (2002) 61–101.
17. Lauffer RB, Paramagnetic metal complexes as water proton relaxation agents for NMR imaging: theory and design. Chem Rev. 87 (1987) 901–905.

18. Shen Z, Chen T, Ma X, Ren W, Zhou Z, Zhu G, Zhang A, Liu Y, Song J, Li Z, Ruan H, Fan W, Lin L, Munasinhe J, Chen X, Wu A, Multifunctional Theranostic Nanoparticles Based on Exceedingly Small Magnetic Iron Oxide Nanoparticles for T₁-Weighted Magnetic Resonance Imaging and Chemotherapy. *ACS Nano*. 11 (2017) 10992–11004. [PubMed: 29039917]
19. Lu Y, –J Y, Xu, Zhang G–B, D. Ling, Wang M–Q, Zhou Y, Wu Y–D, Wu T, Hackett MJ, Kim BH, Chang H, Kim J, Hu X–T, Dong L, Lee N, Li F, He J–C, Zhang L, Wen H–Q, Yang B, Choi SH, Hyeon T, Zou D–H, Iron oxide nanoclusters for T₁ magnetic resonance imaging of non-human primates. *Nat Biomed Eng*. 1 (2017) 637–643. [PubMed: 31015599]
20. Kuznik N, Wyskocka M, Iron(III) Contrast Agent Candidates for MRI: a Survey of the Structure-Effect Relationship in the Last 15 Years of Studies. *Eur J Inorg Chem*. (2016) 445–458.
21. Barrow M, Taylor A, Murray P, Rosseinsky MJ, Adams DJ, Design considerations for the synthesis of polymer coated iron oxide nanoparticles for stem cell labelling and tracking using MRI. *Chem Soc Rev*. 44 (2015) 6733–6748. [PubMed: 26169237]
22. Li L, Jiang W, Luo K, Song H, Lan F, Wu Y, Gu Z, Superparamagnetic Iron Oxide Nanoparticles as MRI contrast agents for Non-invasive Stem Cell Labeling and Tracking. *Theranostics*. 3 (2013) 595–615. [PubMed: 23946825]
23. Laurent S, Forge D, Port M, Roch A, Robic C, Vander L, Muller R, Magnetic Iron Oxide Nanoparticles: Synthesis, Stabilization, Vectorization, Physicochemical Characterizations, and Biological Applications. *Chem Rev*. 108 (2008) 2064–2110. [PubMed: 18543879]
24. Baranyai Z, Pálkás Z, Uggeri F, Maiocchi A, Aime S, Brücher E, Dissociation Kinetics of Open-Chain and Macrocyclic Gadolinium(III)-Aminopolycarboxylate Complexes Related to Magnetic Resonance Imaging: Catalytic Effect of Endogenous Ligands. *Chem Eur J*. 18 (2012) 16426–16435. [PubMed: 23139193]
25. Kuo PH, Gadolinium-Containing MRI Contrast Agents: Important Variations on a Theme for NSF. *J Am Coll Radiol*. 5 (2008) 29–35. [PubMed: 18180006]
26. Ersoy H, Rybicki FJ, Biochemical safety profiles of gadolinium-based extracellular contrast agents and nephrogenic systemic fibrosis. *Magn Reson Imaging*. 26 (2007) 1190–1197.
27. Abu-Alfa AK, The Impact of NSF on the Care of Patients With Kidney Disease. *J Am Coll Radiol*. 5 (2008) 45–52. [PubMed: 18180009]
28. Marckmann P, Skov L, Rossen K, Dupont A, Damholt MB, Heaf JG, Thomsen HS, Nephrogenic Systemic Fibrosis: Suspected Causative Role of Gadodiamide Used for Contrast-Enhanced Magnetic Resonance Imaging. *J Am Soc Nephrol*. 17 (2006) 2359–2362. [PubMed: 16885403]
29. Ghio A, Soukup JM, Dailey LA, Richards J, Deng Z, Abraham JL, Gadolinium exposure disrupts iron homeostasis in cultured cells. *J Biol Inorg Chem*. 16 (2011) 567–575. [PubMed: 21267611]
30. Feng M, Fan Y–Z, Ma X–J, Li J–X, Yang X–G, The gadolinium-based contrast agent Omniscan promotes in vitro fibroblast survival through in situ precipitation. *Metallomics*. 7 (2015) 1103–1110. [PubMed: 25867453]
31. McDonald RJ, McDonald JS, Kallmes DF, Jentoft ME, Murray DL, Thielen KR, Williamson EE, Eckel LJ, Intracranial Gadolinium Deposition after Contrast-enhanced MR Imaging. *Radiology*. 275 (2015) 772–782. [PubMed: 25742194]
32. Wang Y, Li W, Zhou S, Kong D, Yang H, Wu L, Mn₁₂ single-molecule magnet aggregates as magnetic resonance imaging contrast agents. *Chem Commun*. 47 (2011) 3541–3543.
33. Viswanathan S, Kovacs Z, Green KN, Ratnakar SJ, Sherry AD, Alternatives to Gadolinium-Based Metal Chelates for Magnetic Resonance Imaging. *Chem Rev*. 110 (2010) 2960–3018. [PubMed: 20397688]
34. Zhang Q, Gorden JD, Beyers RJ, Goldsmith CR, Manganese(II)-Containing MRI Contrast Agent Employing a Neutral and Non-Macrocyclic Ligand. *Inorg Chem*. 50 (2011) 9365–9373. [PubMed: 21888331]
35. Gallez B, Baudalet C, Geurts M, Regional Distribution of Manganese Found in the Brain After Injection of a Single Dose of Manganese-Based Contrast Agents. *Magn Res Imag*. 16 (1998) 1211–1215.
36. Crossgrove J, Zheng W, Manganese toxicity upon overexposure. *NMR Biomed*. 17 (2004) 544–553. [PubMed: 15617053]

37. Zhu J, Gale EM, Atanasova I, Rietz TA, Caravan P, Hexameric Mn^{II} Dendrimer as MRI Contrast Agent. *Chem Eur J*. 20 (2014) 14507–14513. [PubMed: 25224391]
38. Phukan B, Patel AB, Mukherjee C, A Water-Soluble and Water-Coordinated Mn(II) Complex: Synthesis, Characterization and Phantom MRI Image Study. *Dalton Trans*. 44 (2015) 12990–12994. [PubMed: 26135518]
39. Haedicke IE, Li T, Zhu YLK, Martinez F, Hamilton AM, Murrell DH, Nofiele JT, H. –L. M. Cheng, T. J. Scholl, P. J. Foster, X. –A Zhang, An enzyme-activatable and cell-permeable Mn^{III}-porphyrin as a highly efficient T₁ MRI contrast agent for cell labeling. *Chem Sci*. 7 (2016) 4308–4317. [PubMed: 30155077]
40. Córdova-Fraga T, de Araujo DB, Sanchez Elias J TA Jr., Carneiro AAO, Brandt-Oliveira R, Sosa M, Baffa O, *Euterpe Olerácea* (Açaí) as an alternative oral contrast agent in MRI of the gastrointestinal system: preliminary results. *Magn Res Imaging*. 22 (2004) 389–393.
41. Bulte J, Kraitchman D, Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed*. 17 (2004) 484–499. [PubMed: 15526347]
42. Carbonell T, Rama R, Iron, Oxidative Stress and Early Neurological Deterioration in Ischemic Stroke. *Curr Med Chem*. 14 (2007) 857–874. [PubMed: 17430141]
43. Valerio LG Jr., Mammalian Iron Metabolism. *Toxicol Mech Meth*. 17 (2007) 497–517.
44. Lauffer RB, Vincent AC, Padmanabhan S, Villringer A, Saini S, Elmaleh DR, Brady TJ, Hepatobiliary MR contrast agents: 5-substituted iron-EHPG derivatives. *Magn Reson Med*. 4 (1987) 582–590. [PubMed: 3613958]
45. Reimer P, Schneider G, Schima W, Hepatobiliary contrast agents for contrast-enhanced MRI of the liver: properties, clinical development and applications. *Eur Radiol*. 14 (2004) 559–578. [PubMed: 14986050]
46. Schwert DD, Richardson N, Ji G, Radüchel B, Ebert W, Heffner PE, Keck R, Davies JA, Synthesis of Two 3,5-Disubstituted Sulfonamide Catechol Ligands and Evaluation of Their Iron(III) Complexes for Use as MRI Contrast Agents. *J Med Chem*. 48 (2005) 7482–7485. [PubMed: 16279808]
47. Koenig SH, Kellar KE, Theory of 1/T₁ and 1/T₂ NMRD profiles of solutions of magnetic nanoparticles. *Magn Res Med*. 34 (1995) 227–233.
48. Lee N, Hyeon T, Designed synthesis of uniformly sized iron oxide nanoparticles for efficient magnetic resonance imaging contrast agents. *Chem Soc Rev*. 41 (2012) 2575–2589. [PubMed: 22138852]
49. Baran P, Boca R, Chakraborty I, Giapintzakis J, Herchel R, Huang Q, McGrady JE, Raptis RG, Sanakis Y, Simopoulos A, Synthesis, Characterization, and Study of Octanuclear Iron-Oxo Clusters Containing a Redox-Active Fe₄O₄-Cubane Core. *Inorg Chem*. 47 (2008) 645–655. [PubMed: 18078337]
50. Das S, Chakraborty I, Skachkov D, Ahmadi M, Ishikawa Y, Baran P, Raptis RG, Water-Soluble Derivatives of Octanuclear Iron–Oxido–Pyrazolato Complexes – an Experimental and Computational Study. *Eur J Inorg Chem*. (2012) 3704–3711.
51. Raptis RG, Georgakaki IP, Hockless DCR, First Example of a Ferric/Oxygen-Cubane; an Octanuclear Complex of T-Symmetry, Stable Over Five Oxidation States. *Angew. Chem., Int. Ed*. 38 (1999) 1632–1634.
52. Kim KS, Park SJ, Yang J–A, Jeon J–H, Bhang SH, Kim B–S, Hahn SK, Injectable hyaluronic acid–tyramine hydrogels for the treatment of rheumatoid arthritis. *Acta Biomater*. 7 (2011) 666–674. [PubMed: 20883838]
53. Auzenne EJ, Klostergaard J, Mandal PK, Liao WS, Lu Z, Gao F, Bast RC Jr, Robertson FM, McMurray JS, A phosphopeptide mimetic prodrug targeting the SH2 domain of STAT3 inhibits tumor growth and angiogenesis. *J Exp Therap Oncol*. 10 (2012) 155–162. [PubMed: 23350355]
54. McMurray JS, Mandal PK, Liao WS, Klostergaard J, Robertson FM, The consequences of selective inhibition of signal transducer and activator of transcription 3 (STAT3) tyrosine705 phosphorylation by phosphopeptide mimetic prodrugs targeting the Src homology 2 (SH2) domain. *JAK-STAT*. 1 (2012) 263–273. [PubMed: 24058783]
55. Weissleder R, Ross BD, Rehemtullah A, Gambhir SS, *Molecular imaging: Principles and Practice*, People's Medical Publishing House, USA, (2010) pp 390–392.

56. Coroiu I, Relaxivities of different superparamagnetic particles for application in NMR tomography. *J Magn Magn Mater.* 201 (1999) 449–452.
57. Aime S, Cabella C, Colombatto S, Crich S, Gianolio E, Maggioni F, Insights into the use of paramagnetic Gd(III) complexes in MR-molecular imaging investigations. *J Magn Reson Imaging.* 16 (2002) 394–406. [PubMed: 12353255]
58. Wang Y–XJ, Hussain SM, Krestin GP, Superparamagnetic iron oxide contrast agents: physicochemical characteristics and applications in MR imaging. *Eur Radiol.* 11 (2001) 2319–2331. [PubMed: 11702180]
59. Sharp RR, Nuclear-spin relaxation in paramagnetic solutions when the electronic zero-field splitting and Zeeman interactions are of arbitrary magnitude. *J Magn Reson.* 100 (1992) 491–516.
60. Isaacman S, Kumar R, del Barco E, Kent AD, Canary JW, Jerschow A, Critical examination of Fe₈ as a contrast agent for magnetic resonance imaging. *Polyhedron.* 24 (2005) 2691–2695.
61. Cage B, Russwek SE, Shoemaker R, Barker AJ, Stoldt C, Ramachandran V, Dalal NS, The utility of a single-molecule magnet Fe₈ as a magnetic resonance imaging contrast agent over a broad range of concentration 26 (2007) 2413–2419.
62. Rodríguez E, Roig A, Molins E, Arús C, Quintero MR, Cabañas ME, Cerdán S, Lopez-Larrubia P, Sanfeliu C, In vitro characterization of an Fe₈ cluster as potential MRI contrast agent. *NMR Biomed.* 18 (2005) 300–307. [PubMed: 15912577]
63. Rohrer M, Bauer H, Mintorovitch J, Requardt M, Weinmann H–J, Comparison of Magnetic Properties of MRI Contrast Media Solutions at Different Magnetic Field Strengths. *Investig Radiol.* 40 (2005) 715–724. [PubMed: 16230904]
64. Lee SJ, Ghosh SC, Han HD, Stone RL, Bottsford-Miller J, Shen de Y, Auzenne EJ, Lopez-Araujo A, Lu C, Nishimura M, Pecot CV, Zand B, Thanappapras D, Jennings NB, Kang Y, Huang J, Hu W, Klostergaard J, Sood AK, Metronomic Activity of CD44-Targeted Hyaluronic Acid-Paclitaxel in Ovarian Carcinoma. *Clin Cancer Res.* 18 (2012) 4114–21. [PubMed: 22693353]
65. Lesley J, Hascall VC, Tammi M, Hyman R, Hyaluronan binding by cell surface CD44. *J Biol Chem.* 275 (2000) 26967–26975. [PubMed: 10871609]
66. Banerji S, Wright AJ, Noble M, Mahoney DJ, Campbell ID, Day AJ, Jackson DG Structures of the CD44–hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. *Nat Struct Mol Biol.* 14 (2007) 234–239. [PubMed: 17293874]
67. Toole BP, Hyaluronan–CD44 interactions in cancer: paradoxes and possibilities. *Clin Cancer Res.* 15 (2009) 7462–7468. [PubMed: 20008845]
68. Orian-Rousseau V, CD44, a therapeutic target for metastasising tumours. *Eur J Cancer.* 46 (2010) 1271–1277. [PubMed: 20303742]
69. Zueva EM, Sameera WMC, Piñero DM, Chakraborty I, Devlin E, Baran P, Lebruskova K, Sanakis Y, McGrady JE, Raptis RG, Experimental and theoretical Mössbauer study of an extended family of [Fe₈(μ₄-O)₄(μ-4-R-px)₁₂X₄] clusters. *Inorg Chem.* 50 (2011) 1021–1029. [PubMed: 21188980]
70. Fang J, Nakamura H, Maeda H, The EPR effect. Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Deliv Rev.* 63 (2011) 136–151. [PubMed: 20441782]
71. Danhier F, Feron O, Preat V, To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *J Control Release.* 148 (2010) 135–146. [PubMed: 20797419]
72. Varga-Szemes A, Kiss P, Rab A, Suranyi P, Lenkey Z, Simor T, Bryant RG, Elgavish GA, In Vitro Longitudinal Relaxivity Profile of Gd(ABE-DTTA), an Investigational Magnetic Resonance Imaging Contrast Agent. *PLoS ONE.* 11 (2016) e0149260. [PubMed: 26872055]
73. Caravan P, Farrar CT, Frullano L, Uppal R, Influence of molecular parameters and increasing magnetic field strength on relaxivity of gadolinium- and manganese-based T1 contrast agents. *Contrast Media Mol Imaging.* 4 (2009) 89–100. [PubMed: 19177472]
74. Terreno E, Delli Castelli D, Viale A, Aime S, Challenges for Molecular Magnetic Resonance Imaging. *Chem Rev.* 110 (2010) 3019–3042. [PubMed: 20415475]

75. Helm L, Optimization of gadolinium-based MRI contrast agents for high magnetic –field applications. *Future Med Chem.* 2 (2010) 385–396. [PubMed: 21426173]
76. Caravan P, Strategies for increasing the sensitivity of gadolinium based MRI contrast agents. *Chem Soc Rev.* 35 (2006) 512–523. [PubMed: 16729145]

Figure 1. Ball-and-stick diagram of $[\text{Fe}_8(\mu_4\text{-O}_4(\mu\text{-4-R-pz})_{12}\text{Cl}_4)]$ ($\text{R} = \text{H}$, $[\text{Fe}_8]$; $\text{R} = \text{CH}_2\text{CH}_2\text{OH}$ (**1**), not shown). Color coding: Yellow, Fe; red, O; blue, N; green, Cl; and black, C. H-atoms not shown for clarity

Figure 2.

Plot of $1/T_2$ versus concentration of $[\text{Fe}_8\text{O}_4(4\text{-OHCH}_2\text{CH}_2\text{-pz})_{12}\text{Cl}_4]$, **1**, in water, 298 K, at 1.3, 7.2 and 11.9 T.

Figure 3.

T₁-weighted imaging, scanned with varying TR; TE = 9.6 s, water blank and gadoversetamide reference (upper row), **1** (lower row), 18 °C, 1.5 T.

Figure 4. T₂-weighted imaging, scanned with varying TE; TR= 3000 s, water blank and gadoversetamide reference (upper row), **1** (lower row), 18 °C, 1.5 T.

Figure 5.
 ^{57}Fe -Mössbauer spectrum of **2** at 80 K. Site A, blue; site B, red; site C, green.

Figure 6. The stability of 3.3 μM conjugate **2** at pH 7.4 in 10% FBS monitored by changes in its UV-vis spectrum. Inset: Absorbance change at 280 nm over time.

Figure 7.

The cell viability of A-549 and COS-7 was examined in the presence of 30 μ M conjugate **2**, 150 μ M linoleic acid (LinOA) as a positive control, and 1 mg/mL α -lactalbumin (LA) as a negative control.

Figure 8.

Pre- (top panels) and post-injection (bottom panels) MR scans (4.7 T) of a mouse bearing MDA-MB-468 human breast tumors implanted bilaterally in mammary fatpads; the tumor on the left was injected intra-tumorally with $\sim 70 \mu\text{g}$ of $\text{Fe}_8\text{-Tyr-HA}$. Left panels- T_2 ; right panels- T_2^* . The arrow in the upper left panel indicates the presence of overlaying ultrasound gel; the arrow in the upper right panel indicates one of the bilateral tumors; arrow in lower right panel indicates the injection site of the CA.

Figure 9. T₂-weighted scans (4.7 T) of MDA-MB-468-bearing nude mouse before (left) and ~45 min after (right) i.v. injection of 7 mg of Fe₈-Tyr-HA. Setup was essentially identical to that for the experiment shown in Figure 8; blue arrow in left panel indicates ultrasound gel; red arrow in right panel indicates visual evidence of tumor accumulation of the CA.

Scheme 1.

Tyramine functionalization of hyaluronic acid.

Scheme 2.

Tentative modes of bonding leading to the aggregate formation

Table 1.Longitudinal and transverse relaxivities^a in aqueous solutions at 25 °C.

	r_1 (mM ⁻¹ s ⁻¹)			r_2 (mM ⁻¹ s ⁻¹)		$[r_2/r_1]$
	1.3 T	7.2 T	11.9 T	1.3 T	7.2 T	11.9 T
[Fe ₈ O ₄ (4-OHCH ₂ CH ₂ -pz) ₁₂ Cl ₄] (1)	0.68	0.75	0.76	4.01 [5.9]	10.09 [13.5]	15.83 [20.8]
Phantom imaging of 1 (at 1.5 T)	0.86	-	-	4.84 [5.6]	-	-
Fe ₈ -Tyr-HA (2)	0.09	0.12	0.12	3.65 [40.6]	26.20 [218.3]	52.18 [434.8]
Gadoversetamide (Optimark™)	3.8 ^b	3.8 ^c	-	4.2 ^b	4.7 ^c	-

^a For comparison to mononuclear Gd CAs, the relaxivity is reported in mM⁻¹ of the cluster (as opposed to mM⁻¹ of metal);^b At 1.5 T and 37 °C (from ref. 63);^c At 4.7 T and 37 °C (from ref. 63).