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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, [Fe_8], 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 [Fe_8] cluster conjugated with 5 kDa hyaluronic acid (HA) was characterized by ^{57}Fe-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 A-549 cells and COS-7 cells and its *in vivo* enhancement of T_2-weighted images (4.7 T) of a human breast cancer xenografted on a nude mouse. The physiologically compatible [Fe_8]-HA conjugate was i.v. injected to the tumor-bearing mouse, resulting in observable, heterogeneous signal change within the tumor, evident 15 min after injection and persisting for approximately 30 min. Both molecular [Fe_8] and its HA-conjugate show a strong magnetic field dependence on r_2, rendering them promising platforms for the further development of T_2 MRI contrast agents in high and ultrahigh magnetic fields.

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, T_1, and proton transverse (spin-spin) relaxation time, T_2. Pulse sequences that emphasize natural differences between the T_1 and/or T_2 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 T_1 and T_2 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 T_1 and T_2 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

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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, octreotide, 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 outweigh 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 preclinical 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 hepatobilliary 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 $[Fe_8(\mu_4\text{-}O)_4(\mu\text{-}4\text{-R-pz})_{12}Cl_4]$ ($[Fe_8]$; 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*).

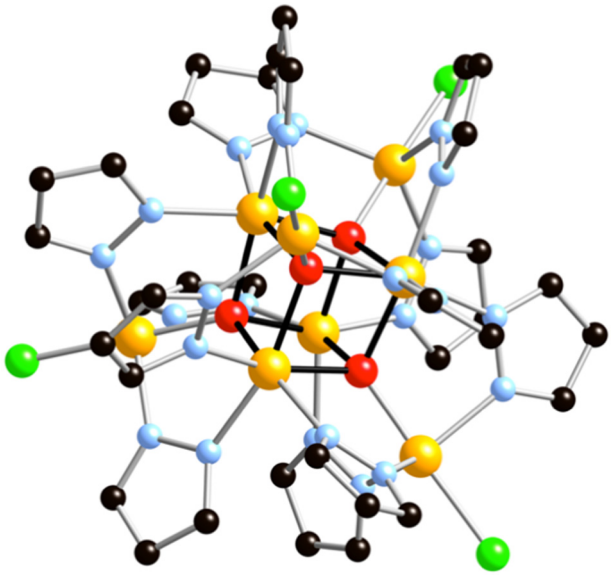

Fig. 1. Ball-and-stick diagram of $[Fe_8(\mu_4\text{-}O)_4(\mu\text{-}4\text{-R-pz})_{12}Cl_4]$ (R = H, $[Fe_8]$; R = CH_2CH_2OH (**1**), not shown). Color coding: Yellow, Fe; red, O; blue, N; green, Cl; and black, C. H-atoms not shown for clarity.

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 $[Fe_8]$ 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°-τ-90°) and the Carr-Purcell-Meiboom-Gill (CPMG, 90°-τ/2–180°-τ/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 $T_i^{-1} = T_{i,0}^{-1} - r_i[Fe_8]$. 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.