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Sensitive Biological Detection with a Soluble and Stable Polymeric Paramagnetic Nano Cluster

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Abstract

We describe the design, synthesis and biological characterization of manganese oxocluster-based “single molecule magnets (SMMs)”. We demonstrate that polymeric micellar nanoparticles can serve as a carrier and help to stabilize delicate SMM molecules from breaking down easily and thus prevent their property loss. Concentrating thousands of Mn-clusters per micelle provided a high ionic and per-particle relaxivity allowing sensitive MR imaging *in vivo*. This reports one of the earliest examples of *in vivo* imaging of a rationally designed polymeric micelles that features SMM.

Molecular nano-magnets are organic molecules with a single or multiple metal ions having unpaired electrons in their outer orbitals.¹ These molecules are often referred as “single molecule magnets” (SMMs) as they show superparamagnetic behavior under a certain blocking temperature at the molecular scale exhibiting magnetic properties of purely molecular origin. The prerequisites for SMM behavior are (i) a very high spin state and (ii) a large magnetic anisotropy.² Lis *et. al.* described one of the earlier examples of SMM comprising a cluster of twelve manganese ions with acetate ligands, often called Mn₁₂-acetate [[MnIII/IV]₁₂O₁₂(CH₃CO₂)₁₆(H₂O)₄].³ In this dodecanuclear mixed-valence manganese carboxylate clusters eight of the manganese ions are in the +3 oxidation state (spin S=2) and four are in the +4 state (Spin S=3/2). Such clusters are thus able to obtain a magnetically coupled large ground state of S = 10 and are responsible for many frequency dependent unfamiliar magnetic relaxation effects, properties^{4–5} and application.^{6–10}

Vasovist® (MS-325), which is a gadolinium-based (gadofos-veset trisodium) agent has been approved for peripheral vascular and coronary artery disease. However, in light of the recent FDA warning about an association between gadolinium (Gd)-based contrast agents and nephrogenic systemic fibrosis (NSF), the use of the lanthanide Gd is now more regulated.¹¹ The intrinsic property of Mn₁₂ is somewhere between paramagnetic complexes and superparamagnetic nanoparticles. Recently, they have been explored as contrast agents for biological imaging. However, their poor stability and solubility in water are major drawbacks for successful translation to preclinical applications. A few approaches were proposed including binding the clusters to polystyrene beads and an emulsion-assisted self-assembly method for dispersing stearic acid modified Mn₁₂.¹² While these were viable approaches to use SMM as a T2 weighted (darkening contrast) MRI agent, a more biocompatible, robust methodology is required to use it as a T1 weighted agent (brightening contrast) to diagnose vascular disease. We hypothesized that the induced magnetic moment of Mn₁₂ clusters in the applied field would accelerate the relaxation of surrounding water

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SUPPORTING INFORMATION. Description of experimental methods and analytical measurements. This material is available free of charge via the Internet at <http://pubs.acs.org>.

protons. Clearly, a more biocompatible approach is the unmet need by restricting the agent within a size range of <20nm to impede in the blood stream for delayed extravascular leakage and prolonged retention. This will enable the rapid acquisition of high resolution MRA's using clinical MRI machines. Towards this aim, we developed and characterized a new "soft" nano-magnet comprising the Mn_{12} and a well-defined polymeric micellar nanoparticle (Poly-SMM) for sensitive MRI detectability. This strategy offers several advances over the existing reports particularly the poor inherent aqueous stability to address the inherent insolubility issues with the parent Mn_{12} molecules. Our objective was to develop a platform comprising multiple copies (in hundreds) of Mn_{12} clusters, which presented the metal ions directly on the surface for increased interaction with surrounding water. The shortened relaxation of the surrounding water protons provides T1-weighted contrast agent. For the carrier nanoparticles, a stable polymeric system with a sub 20 nm particle size for eventual biomedical application in extravascular imaging and drug delivery was developed. Our approach followed a co-self-assembly of amphiphilic diblock copolymer and a well known surfactant to restrict the particle sizes within 20 nm.

Mn_{12} -acetate was synthesized similar to Lis *et. al.*³ Briefly, $Mn(CH_3CO_2)_2 \cdot 4H_2O$ was dissolved in a mixture of 60% acetic acid and water. To this, a finely ground $KMnO_4$ was added in small amounts over the course of about 2 minutes until dissolved. The final solution was removed from the stir plate and allowed to remain undisturbed for 3 days, to observe the crystalline growth of long black rectangular rods. For the synthesis of polymeric micelles, an amphiphilic diblock-*co*-polymer polystyrene-*b*-polyacrylic acid¹³ (PS₈-*b*-PAA₄₀₀, $M_n \times 10^{-3}$: 0.8-*b*-29.3 polydispersity index: PDI=1.18, 0.33 micromoles, 6.0 mg, 0.5 mole%) was co-self assembled with polyoxyethylene (20) sorbitan monooleate (polymer: polysorbate=6:1). The mixture was briefly bath sonicated at ambient temperature for 2 minutes until a transparent suspension was achieved. The formation of the micelles was confirmed by dynamic light scattering (DLS) measurement, which revealed a particle size of 16 ± 4 nm with polydispersity 0.021. Zeta (electrophoretic potential) values ($\zeta = -12 \pm 3$ mV) were negative, confirming a predominant occupancy of the surface by the carboxylic acid groups of the hydrophilic polyacrylate segment of the amphiphilic copolymer. Polysorbate was purposefully chosen as a co-surfactant to impart stability to the micelles after the attachment of the oxoclusters and to restrict the particle diameter within 20 nm with low polydispersity. From an initial 1:6 ratio of block copolymer and polysorbate concentrations, we varied the ratio to optimize the concentration of components in the mixture (Supporting information Figure 1S). Our attempt to use a different co-surfactant as polyethylene glycol octadecyl ether (PEGOE) resulted in aggregated particles with particle sizes over 200 nm. (Supporting information Figure 2S) We presume that the attachment of Mn_{12} -acetate to the polymeric micelles was driven by ligand substitution reactions owing to the presence of the more acidic carboxylates of PAA ($pK_a \sim 4$) [$Mn_{12}acetate + 16RCO_2H \rightarrow (Mn_{12}O_{12}(O_2CR)_{16}(H_2O)_4 + 16MeCO_2$; R=acrylic]. The ligand exchange reaction did not greatly alter the hydrodynamic diameter of the post conjugated micelles. Unbound Mn clusters were removed by exhaustive dialysis against infinite sink of water using 10kDa MWCO cellulosic membrane. The particles were characterized by transmission electron microscopy (TEM), atomic force microscopy (AFM), scanning electron microscopy (SEM), energy dispersive x-ray spectroscopy (EDX) and inductively coupled plasma resonance-optical emission spectroscopy (ICP-OES). DLS measurements revealed the number-averaged hydrodynamic diameter as 17 ± 4 nm with low electro-phoretic potential values ($\zeta = -10 \pm 5$ mV). As evident from the polydispersity indexes, (PDI: 0.17) these particles were produced with narrow distribution. The anhydrous state properties were determined by AFM (Figure 1A) and TEM (Figure 1B–C) analyses by drop depositing the aqueous suspension of the Poly-SMM over a glass slide. The particle height was calculated to be 14 ± 6 nm. UV-Vis spectroscopy confirmed the absorbencies at ~ 520 nm, corresponding to the presence of multiple Mn_{12} atoms in oxo clusters and charge transfers from the inner Mn ions towards

the outer ones. The concentration of manganese was analytically determined by ICP-OES. Based on an average of three formulations, the concentration of undiluted Poly-SMM was measured to be 4.7 mg/L, which equates to ~3000 Mn/particle nominally. EDX spectroscopy (Figure 1D) further confirmed the presence of Mn, as evident from the occurrence of primary and secondary emission lines at 0.637 keV and 0.649 keV respectively. The magnetic susceptibility measurements described by vibrating sample magnetometer (VSM) revealed that the predominant phase of the material is paramagnetic in nature. (Figure 1E) These uniquely constructed polymeric nanoparticles possess long shelf-life stability and retain the particle integrity for their further exploration in preclinical studies. The stability and stringency of Mn₁₂ ligands associated with the micelles were studied in a dissolution experiment. The release of the Mn complex was examined by UV spectroscopy at ~520 wavelength range and reveals a less than ~19% total release over 3 days against an infinite sink indicating a nominal loading efficiency of 81–83% with good retention in dissolution at 37 °C. The majority of the Mn was released during the first (11±1%) and second (7±2%) day of dissolution. Time-dependent stability of the particles at ambient temperature and within a physiological pH range 6–8 was studied and a minor variance was confirmed based on light scattering and electrophoretic potential measurements. To assess the blood and serum stability, a 'blood smear' assay was performed to study morphological changes in lymphocytes and blood clumping using conventional light microscopy under high-power field. As represented in Figure 2(A–B), no significant clumping or morphological alterations were observed in rodent blood treated with Poly-SMM (blood: NP = 4:1) (experimental details in the Supporting Information).

As discussed above, Poly-SMMs were purified by exhaustive dialysis against infinite sink of water prior to characterizing MR properties. The MR properties of serially diluted Poly-SMMs were characterized in aqueous suspension. Four dilutions of the particles were prepared in microcentrifuge tubes at approximately 1:0, 1:2, 1:4 and 1:8 Poly-SMMs in ultrapure water (0.2 μM), corresponding to 4.7 mM, 2.8 mM, 1.6 mM and 0.6 mM Mn. The nominal nanoparticle concentration was calculated from particle size determined by laser light scattering and the total volume of Mn cluster incorporated into Poly-SMMs adjusted for loss of metal during dialysis. The dilutions were scanned at room temperature on clinical MRI scanners (1.5T) with a transmit-and-receive birdcage head coil (Achieva, Philips Healthcare) to measure relaxation rates, r_1 and r_2 . A single slice inversion recovery sequence (i.e., the Look–Locker technique)¹⁴ was employed to calculate the ionic (per mM of Mn) and particulate (per mM Poly SMMs) r_1 relaxivities at 1.5T (resolution = $0.7 \times 0.7 \times 3 \text{ mm}^3$, 30 samples of the inversion recovery signal starting at 17ms and spaced at 7ms, with 10° sampling flip angle, TE=1.9ms, TR=4.14s, 4 averages). Similarly, r_2 relaxivity was measured using a multi echo–spin echo technique, resolution = $0.9 \times 0.9 \times 3 \text{ mm}^3$, 20 echoes at 4.4 ms intervals, TR=541 ms, 4 averages). The relaxivities (mean ± std.err.) for the poly-SMM based on Mn concentrations are $r_1 = 5.2 \pm 0.12 \text{ mM}^{-1}\text{s}^{-1}$ [Mn] and $r_2 = 10.7 \pm 0.24 \text{ mM}^{-1}\text{s}^{-1}$ [Mn] at 1.5T. The ionic relaxivity values of poly-SMM were found to be similar to commercially available gadolinium based contrast agents (e.g. magnevist).¹⁵ It is worthy to mention that the ionic relaxivity (r_1) of Poly-SMM is much higher than the previously reported procedures.^{6–7} Interestingly, a solution of Mn₁₂-acetate in acetic acid (excess) produced a $r_1 = 3.0 \text{ mM}^{-1}\text{s}^{-1}$, which is lower than Poly-SMMs. Particulate relaxivities were also calculated as $r_1 = 192 \pm 20 \text{ mM}^{-1}\text{s}^{-1}$ [Poly-SMMs] and $r_2 = 390 \pm 65 \text{ mM}^{-1}\text{s}^{-1}$ [Poly-SMMs] for 1.5T (Figure 2).

In vivo MRI imaging was performed in a rat model (n = 3) to evaluate contrast using an intravenous tail vein dose of 2 mL/kg, 0.28 mmol of Mn/kg. MR imaging was performed pre and post intravenous administration of Poly-SMM into rat. The MRI signal enhancement of major clearance organs was monitored over a period of 2.5 h. (Figure 3) The significant enhancement of MR signal of the major organs including the liver, heart and kidney was

observed. At 2.5h after Poly-SMM injection, the MR signal corresponding to heart, liver and kidney were enhanced by 144%, 123% and 141% respectively. The enhancement of signal in heart even after 2h indicates that these particles may presumably have long circulatory half life and can be used eventually for coronary imaging. Furthermore, in a preliminary biodistribution studies, the parent particles incorporated with a water-soluble near infrared dye (ADS832WS, $\lambda_{\text{ex}} = 824 \text{ nm}$, $1.90 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$) was probed *in vivo* with optical imaging (IVIS). Bio-d of the polymeric nanoparticles was determined at 2h and 24 h post I.V. injection (1 ml /kg). Liver was found to be the predominant organ of the micelle accumulation as obvious from the measured fluorescence intensity. The other major clearance organs were kidney, lymph node and spleen. (Supporting information Figure 3S) The superior delineation of the reticuloendothelial (RES) organs (liver, spleen etc.) suggested that the particles apparently had distributed into major clearance organs, typical of nanoparticulate agents. More in depth clearance and PK studies will be required to fully understand their *in vivo* behavior.

In conclusion, we have taken a rational approach to polymeric nano-magnet design for better sensitivity and improved inherent properties for preclinical application. A fast synthetic route is chosen to co-self assemble amphiphilic polymers to produce sub 20 nm sized micelles presenting carboxylic acid groups on the surface. Mn_{12} -acetate, a well studied single-magnet molecule was chosen to embellish the polymeric shell by a ligand-exchange reaction mechanism. *In vitro* MR imaging studies revealed that these particles offered higher T1 relaxivity in comparison to naked Mn_{12} -acetate and were also more efficacious than the recently reported approaches. Finally, in a preliminary *in vivo* study we demonstrate that these particles can successfully be used for biological imaging in living subjects. Although more in depth studies are warranted to fully understand the temperature dependent magnetic susceptibility and the effect of co-surfactant in this polymeric system; to the best of our knowledge, this reports the first *in vivo* imaging of a rationally designed polymeric particle that incorporates 'single molecule magnets'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Synthesis and characterization of Poly-SMM: co-self assembly of amphiphilic diblock copolymer (PS-*b*-PAA) and sorbitan monooleate, sonication, 25°C, 2 min; (ii) Mn₁₂-acetate; (b); dialysis 10kDa cellulosic membrane, nanopure water (0.2μM). (A) AFM image of poly-SMM drop deposited over freshly cleaved mica; (B) TEM images of poly-SMM (scale= 100 nm); (C) an enhanced image of a single nanoparticle (scale= 20 nm); (D) EDX spectrum of Poly-SMM; (E) VSM magnetization and hysteresis graph; (F) dissolution of Mn₁₂-acetate from Poly-SMM over three days against infinite sink of

Figure 2.

Optical microscopy images of 'blood smear' (A) untreated (magnification: 20 $\times$) and (B) treated with Poly-SMM (magnification: 40 $\times$); (C) the image shows the T1-weighted MRI of the contrast agent diluted in water from lowest concentration tested to highest (as shown with arrow); relaxivity measurements of Poly-SMM: relaxivities, r_1 and r_2 , are calculated from the measured relaxation rates as a function of Poly-SMM concentration. The graphs are the data for longitudinal relaxation rate (r_1) and transverse relaxation rate (r_2) at 1.5T based on [Mn] (D) and [NP] (E).

Figure 3.

In vivo MR imaging of Poly-SMM. (A) signal intensity of the major organs before and after the administration of Poly-SMMs; (B) MRI images of major organs at baseline (A-C) and 2.5h; (D-F) at 1.5T indicating superior delineation after Poly-SMM administration.