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Self-organized Mn²⁺-Block Copolymer Complexes and Their Use for *In Vivo* MR Imaging of Biological Processes

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Abstract

Manganese-block copolymer complexes (MnBCs) that contain paramagnetic Mn ions complexed with ionic-nonionic poly(ethylene oxide-*b*-poly(methacrylate)) have been developed for use as a T1-weighted MRI contrast agent. By encasing Mn ion within ionized polymer matrices, r_1 values could be increased by 250–350 % in comparison with free Mn ion at relative high fields of 4.7 to 11.7 T. MnBCs were further manipulated by treatment with NaOH to achieve more stable complexes (iMnBCs). iMnBCs delayed release of Mn²⁺ which could be accelerated by low pH, indeed by cellular uptake via endocytosis into acidic compartments. Both complexes exhibited good T1 contrast signal enhancement in liver following intravenous infusion. The contrast was observed in gallbladder due to the clearance of Mn ion from liver to biliary process. iMnBCs, notably, showed a delayed contrast enhancement profile in gallbladder, which was interpreted to be due to degradation and excretion of Mn²⁺ ions into the gallbladder. Intracortical injection of iMnBCs into the rat brain also led to delayed neuronal transport to thalamus. The delayed enhancement feature may have benefits for targeting MRI contrast to specific cells and surface receptors that are known to be internalized by endocytosis.

1. Introduction

Magnetic resonance imaging (MRI) is a powerful diagnostic modality that provides high anatomical resolution and three-dimensional images with excellent soft tissue contrast.¹ However, the use for MRI as a tool for molecular and cellular imaging is limited due to its relatively low sensitivity. To overcome this, a great deal of effort has been devoted to enhance sensitivity by creating more tissue contrast through the administration of MRI contrast agents. These agents work by shortening relaxation times of water protons depending on local concentration of the agents, thereby enhancing the contrast relative to

background tissue.² There are two main groups of MRI contrast agent. The first group predominantly increases longitudinal relaxation rate ($1/T_1$) and gives rise to increase in signal intensity. Most clinically employed agents based on paramagnetic gadolinium(Gd^{3+})-chelates belong to the class of T_1 contrast agents.³ Another group of contrast agents largely increase transverse relaxation rate ($1/T_2$) and lead to reduction of signal intensity. In addition to paramagnetic chelates, this class of contrast agents also include superparamagnetic iron oxide particles.^{4–6} Growth of MRI in medical imaging has led to extensive investigation into a development of new and potent contrast agents with high relaxivity and selectivity. In recent years, much effort has been paid to increase sensitivity by using macromolecular and nanocarriers to incorporate large quantities of contrast agent.⁷ These systems have been designed to deliver a high payload of contrast agent to target sites and tissues. A variety of nanocarriers have been reported including dendrimers, micelles, liposomes, carbon nanotubes, and silica-based particles, as well as biologically derived carriers such as ferritin, lipoproteins, and viral capsids.^{8–13} Dendrimer and macromolecular carriers have gained considerable interest due to flexible and precise control over their chemistry. These soft-particles, which provide an alternative to inorganic materials, are promising in terms of enhancing MRI relaxivities. In many cases, ionic relaxivities of Gd^{3+} -chelates that have been conjugated to nanocarriers are many fold higher than those of their small molecule counterparts. These enhancements could be partially attributed to slow tumbling rates ($1/\tau_R$) of the agents. However, some agents have shown remarkable enhancement in relaxivity by more than one order of magnitude. For example, gadolinium-carbon nanotubes or gadonanotubes have r_1 relaxivities of ca. $180 \text{ mM}^{-1} \text{ s}^{-1}$ at 1.5 T which is ~ 10 folds higher than free Gd^{3+} ion.^{9, 13} Thus, it is possible that there may be other unexplained factors that contribute to large enhancements of longitudinal relaxivity.

It is well known that several synthetic block copolymers comprising polyelectrolyte blocks and nonionic blocks can undergo self-assembly with oppositely charged molecules to form polyion complexes (PICs).^{14–16} These PIC nanostructures have nonionic blocks protruded outward into aqueous media to provide polymer sheaths that aid in dispersion of the complexes. The utilization of PIC systems in drug delivery has been widely investigated and, indeed, several multivalent metal ions such as Ca^{2+} and Mg^{2+} have been shown to induce formation of nanostructured complexes with various classes of polyelectrolytes in aqueous solution.^{17–20} There have been few reports on the use of paramagnetic metal ions and poly electrolytes complexes for MRI contrast agents. Shen et al. was first to report the direct chelation of paramagnetic ions into copolymer backbone containing chelating moieties.²¹ Recently, our group has reported the formation of nanocomplexes between Mn^{2+} ions and graft copolymer complexes of poly(aminophosphonate)-co-poly(ethylene oxide).²² Direct interaction of Mn^{2+} ion and copolymer led to formation of nanosized particles, which were shown to have high r_1 s (at 60 MHz) of 14 to $18 \text{ mM}^{-1} \text{ s}^{-1}$. Moreover, they were found to be suitable carriers for anticancer drugs. Herein, we seek to utilize similar strategy to prepare nanocomplexes of paramagnetic ions with diblock copolymers, which have more defined molecular weight and structure. The purpose of this work is not only to improve upon MRI relaxivity of the agent, but also to exploit binding affinity of MRI agent within the complexes as a way to image biological processes in vivo.

Mn²⁺ was chosen due to its excellent MRI relaxivity, and because it is also biological relevant and displays interesting transport properties that have been exploited for imaging neural connections and activation in the central nervous system (CNS) and other tissues such as heart, pancreases, and tumors.^{23–30} Therefore, it would be useful to exploit and modulate controlled release of Mn²⁺ from such complexes by way of specific biological processes such as cellular internalization. To this end, the complexes were made pH-sensitive by treatment in alkaline solution to form oxide intermediates (Fig. 1). Mn²⁺ release characteristics and cellular cytotoxicity against mouse hepatocytes were then determined. Finally, biodistribution and the controlled release of Mn²⁺ in response to the uptake and *in vivo* transport of Mn²⁺ ions were also demonstrated in both mouse and rat models at 7 T and 11.7 T, respectively.

2. Results and discussion

2.1 Synthesis and characterization of nanocomplexes

It has been shown that several ionic block copolymers form complexes with oppositely charged molecules. Previous works by Bronich et al. explored the use of ionic complexation of PEG-PMA with Ca²⁺ to form cross-linked micelles that can be loaded with therapeutic agents such as cis-platinum and doxorubicin.^{19,31} Mn²⁺ spontaneously forms self-assembled nanosized complexes with PEG-PMA. Fabrication of the nanocomplexes was performed in one-pot and a two-step synthesis. Initial mixing of Mn²⁺ ions and block copolymers (Fig. 1) led to spontaneous formation of complexes through electrostatic interaction between Mn²⁺ cations and carboxylate anions. Stability of the complexes was enhanced by partially crosslinking carboxylate groups with ethylenediamine via EDC-mediated amide formation. The colloiddally stable complexes were only obtained by using PEG-*b*-PMA block copolymers. Attempts to form complexes with only PMA homopolymer were met with rapid increase in turbidity and subsequent aggregation and precipitation of complexes suggesting that PEG segment plays a crucial role in stabilizing nanoparticles and thus preventing interparticle agglomeration. Further characterization by energy filtered transmission electron microscopy (EFTEM) and electron energy loss spectroscopy (EELS) techniques revealed that Mn were bound in the complexes and closely associated with organic matrices as shown by the Mn and C map. The presence of a nitrogen peak suggested that an amide bond was formed after the crosslinking reaction (Fig. 2)

It is known that polyion complexes, even with a cross-linked core, are prone to initial fast release of the payloads in physiological environment. Indeed, MnBCs exhibit a rapid release of Mn even at pH 7.4. This may not be desirable in terms of developing targeted MRI agents, in which the agent is expected to target specific organ and site while minimizing non-specific signal background. To minimize premature release, retention of Mn was attempted by forming Mn oxide within the complexes. The MnBCs were treated with NaOH in mild alkaline solution (pH 9) resulting in more stable complexes (iMnBCs). Transition of Mn species within the complexes was monitored by UV-Vis spectroscopy. As shown in Fig. 3, UV-Vis spectra of MnBCs precursor did not show distinct adsorption above the wavelength of ~ 250 nm. Upon NaOH treatment and neutralization, there was a large

increase in adsorption at wavelengths between 300 to 400 nm indicating possible formation of manganese-oxyhydroxide (MnOOH) and subsequent formation of Mn oxide species.³²

Indeed, the immobilizing process was found sufficient to slow Mn ions from being prematurely released. Fig. 4 shows the release of Mn²⁺ from the two different polymer complexes. MnBCs released Mn²⁺ quite rapidly in both at physiological pH and pH 4.5. However, there was an increase of Mn release at pH 4.5 relative to physiological pH, which was likely caused by protonation of the carboxylate group leading to weaker binding force between Mn ions and the polymeric carrier. The iMnBCs on the other hands are stable at physiological pH but release Mn slowly after exposure to pH 4.5. This behavior can be mainly attributed to acid-catalyzed hydrolysis of Mn oxide species into ionic Mn, which could then be released from the complexes through an ionic exchange processes. DLS revealed that there was no significant difference in hydrodynamic size between MnBCs and iMnBCs, which have dimensions of 150±30 and 170±20 nm, respectively. The amounts of Mn measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) also indicate that these two complexes have comparable Mn²⁺ contents of 6.13±0.74 % and 6.05±1.23 %, respectively.

2.2 Relaxivity measurements

Table 2 shows the experimentally obtained relaxivities of the nanocomplexes in comparison with other small molecules contrast agents. Longitudinal relaxivity (r_1) values of nanocomplexes are field-dependent, while r_1 s of MnCl₂, gadolinium(III) diethylene triamine pentaacetate (GdDTPA), and manganese(II) dipyrideroxyl diphosphate (MnDPDP) are constant across the measured fields. r_1 values of both GdDTPA and MnDPDP were found to be in good agreement with the literature.^{33, 34} MnBCs and iMnBCs have high r_1 values. At 4.7 T, MnBCs and iMnBCs have r_1 s of 40.2 and 8.55 mM⁻¹ s⁻¹ per Mn atom, respectively, exceeding those of previously reported manganese oxide and dendritic manganese chelates.^{35–38} Even at 11.7 T, both types of nanocomplexes still have significantly higher r_1 s. Possible explanations for prominent relaxivities are that the ionic and hydrophilic environment within nanocomplexes facilitates water-exchange rate with the complexed Mn ions as well as an outer sphere relaxation mechanism.^{39, 40} Upon transformation to iMnBCs, the relaxivities decreased by about 4–5 fold at all field strength. This reduction of the r_1 is likely because of transformation of water accessible Mn ion to less accessible Mn oxide intermediates. MR images were acquired using a 7.0 T MRI scanner to investigate the potential of the nanocomplexes as positive MRI contrast agents. As shown in Fig. 5a the bright contrast signals obtained from aqueous dispersions of the MnBCs were most prominent. Contrast signal generated from iMnBCs was shown to be comparable to that of MnCl₂. Simulated contrast signals were generated from the standard signal equation for the spin-echo pulse sequence (See supporting documents). Overall, the measured contrast enhancements were in good agreement (within 15%) with the values obtained from simulations. On comparing the enhancements across concentration ranges, it was found that MnBCs gave an approximately 200% higher signal than MnDPDP, an FDA approved Mn-chelates. As predicted, both MnBCs and iMnBCs were able to generate bright contrast signals exceeding those obtained from MnDPDP. Thus, in terms of relaxivity, these nanocomplexes are promising for use as sensitive macromolecular MRI contrast agents.

2.3 Cellular toxicity

The cellular toxicity of the nanocomplexes was assessed by *in vitro* cell proliferation MTS-based assay. Cellular toxicity in AML12 cells was shown to be dose and time-dependent (Fig. 6). MnCl_2 gave the highest toxicity followed by MnBCs and iMnBCs, respectively. At 4-hour incubation, significant cell death with survival of less than 80% was observed in cells treated with MnCl_2 at concentrations above ~ 0.8 mM, whereas neither MnBCs nor iMnBCs gave a significant decrease in survival. At a longer exposure time of 24 hour, significant toxicity of MnBC was observed at concentration above 0.5 mM. iMnBCs on the other hand did not cause noticeable loss in cell viability at either 4 or 24-hour incubation time. Although MnBCs exhibit fast release of Mn^{2+} ions, those complexes showed partial mitigation of toxicity. This might be attributed to Mn^{2+} ions still being associated with the complexes or lesser uptake as compared to MnCl_2 . In contrast to MnCl_2 and MnBCs, iMnBCs with their much better retention of Mn^{2+} did not cause significant toxicity to AML-12 cells at either short or long incubation times. This can be explained by the fact that iMnBCs release Mn^{2+} at a slow rate, and only up to $\sim 40\%$ of Mn^{2+} will be released, thereby limiting Mn-induced toxicity to the cells.

2.4 Thalamo-cortical Mn^{2+} tracing experiment

It has been shown that Mn^{2+} ion enables *in vivo* tracing of CNS axon tracts by MRI. Upon entry into neurons, Mn^{2+} ions are transported along axonal microtubules to the synaptic cleft, where they are released and trans-synaptically relayed to other neurons.²⁴ Here we investigate whether slow release of Mn^{2+} from iMnBCs can lead to a delay in neuronal tracing. *In vivo* transport of Mn^{2+} along axons from the sensory S1 brain region to thalamus was clearly visible. Fig 7 shows that the increase of signal intensity in the thalamus region on the side of the animal injected with iMnBCs was slower than for the side injected with MnBCs. In contrast, there was no significant difference between the rate of signal increase for animals injected with MnBCs and MnCl_2 . For Mn^{2+} to be transported along axon tracts, either Mn^{2+} ions or the particles must be taken up by neurons. MnBCs, which exhibit fast release of Mn^{2+} even at neutral pH, presumably released a large portion of Mn^{2+} extracellularly before being taken up by neurons. Thus, the Mn^{2+} tracing behavior of MnBCs was not different from that of MnCl_2 . iMnBCs, which do not appreciably release Mn^{2+} prior to uptake, were likely internalized into intracellular acidic compartments, thus releasing free Mn^{2+} to be transported. Approximately two hours elapsed before any appreciable increase in signal intensity was observed in thalamus area after injection of iMnBCs. This long delay is likely caused slow uptake of particles by neurons, or by the majority of particles being taken up and processed by microglia or astrocytes, which could then release Mn^{2+} into the extracellular matrix. This released Mn^{2+} could then be internalized into neurons and transported along axons to the thalamus. In view of Mn^{2+} being widely employed in tracing neural circuitry, our present observations show the advantage of developing a vehicle whereby slow-released Mn^{2+} carriers might extend the range of tracing experiments that can be performed.

2.5 In vivo distribution and MR imaging of mouse abdomen

Contrast enhancement of MnBCs and iMnBCs relative to MnCl_2 was investigated in a mouse model with the aim of assessing contrast in various organs. The dose of Mn^{2+} ion was 20 μmol or 1.1 mg Mn^{2+} per kilogram body weight, similar to previous studies that were done on SiO_2 -coated MnO .⁴¹ The agent solution was injected into C57BL/6J mice (25–30 g of body weight) and images were acquired for up to 2 h after infusion. The time courses of T_1 -weighted MRI enhancement of different organs were determined after IV infusion of the agents. Figure 8A and 8B show images from a slice containing gallbladder (circle), liver, and kidney. Time courses of signal intensity changes are shown for MnCl_2 , MnBCs, and iMnBCs in Figure 8C, 8D, and 8E, respectively. All intensities were normalized to the pre-infusion values. Within a few minutes after injection of MnCl_2 and MnBCs, bright contrast signals in gallbladder could be readily detected. In Gallbladder, the contrast signals increase by a factor of 2.5–3.0. The large enhancement is due to clearance of Mn^{2+} from the liver and concentration of the Mn^{2+} in the gallbladder prior to excretion into the intestines. Unlike MnCl_2 and MnBCs, injection of iMnBCs did not immediately cause large contrast enhancement in the gallbladder. In this case, contrast enhancement did not start until about 10 minutes after injection, indicating that iMnBCs particles must first get processed by the liver (possibly endocytosed by Kupffer cells) and the particles then dissolved resulting in release of Mn^{2+} . The Mn^{2+} ions are then excreted through bile duct leading to the positive contrast in the gallbladder. The fact that contrast enhancement in gall bladder happened soon after particles were taken up by liver (plasma half-life of approximately 12 min) indicates that transport of Mn^{2+} from liver into the gallbladder should be quite faster than the rate of particle digestion. Indeed, imaging of such processes could not be performed using commercial agent GdDTPA. The delay in release of Mn^{2+} from iMnBCs may open possibilities to better characterize the rate of transport from liver to bile using MRI.

Contrast enhancements in liver and kidney were also detected. Whereas MnBCs behaved much like MnCl_2 , contrast enhancement in the liver enabled by the particles exceeded that of MnCl_2 (80% vs 50%). This is likely due to uptake of MnBCs by liver prior to fully release of Mn^{2+} . This observation suggests that MnBCs might still serve as a useful MRI contrast agent for liver imaging at low dose of Mn^{2+} . Increases of signal intensities in kidneys of animals injected with MnBCs were similar to those observed with MnCl_2 . As expected, kidney enhancements were limited in the case of iMnBCs since the release of Mn^{2+} ion was slow enough such that liver took up most iMnBCs. To confirm this, we next assessed whether iMnBCs can minimize uptake of Mn^{2+} into heart and choroid plexus. Indeed, as shown in Fig. 9, injection of MnCl_2 led to ~ 45 % and 20% enhancements in heart and choroid plexus, respectively. These significant enhancements were due to free Mn^{2+} ions being taken up quite rapidly into cells of these organs.^{42–44} However, only minimal enhancement was observed with injection of iMnBCs in both organs. This indicates that there was very little release Mn^{2+} from iMnBCs into the blood stream. Combining results of the contrast signal enhancements from different organs, we envision that the iMnBCs are suitable to be developed into a targeted imaging agent to allow enhancement of all tissues while background from the non-targeted agent is low.

3. Experimental

3.1 Materials

Manganese (II) chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 99%), Ethylenediamine hydrochloride (99%), and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 99%) were purchased from Sigma Aldrich. PMA-b-PEG block copolymers with narrow molecular weight distribution (Polydispersity index of 1.15) were purchased from Polyscience Inc. (Vancouver, CA) and were dissolved in deionized water with pH adjusted to 7 using 1 N NaOH, and stored at 4 °C prior to use. Deionized water was obtained through the MilliQ A10 Synthesis water purification system (EMD Millipore, MA).

3.2 Synthesis of manganese(II)-block copolymer nanocomplexes

To fabricate the complexes, block copolymer solution (2.0 mg/mL, 12 mL) was filtered through a 0.45- μm syringe-driven filter unit (Millex-GS, Millipore) and added to a 20-mL vial equipped with a magnetic stir bar. Manganese chloride solution (100 mg/mL in DI water) was added slowly into the polymer under stirring. The amount of charged manganese ion was calculated based on stoichiometric ratio of cation to anion by using two carboxylate groups per one manganese ion. Upon adding manganese solution, an increase of turbidity of the mixture solution indicated formation of complexes. EDC hydrochloride (7.76 mg) was dissolved in DI water and added slowly to the preformed electrostatic complexes to activate part of carboxylate group. After 10 min, Ethylenediamine hydrochloride (2.72 mg) was added to crosslink between two carboxylate groups forming an amide bond through carbodiimide-mediated amidation.⁴⁵ The reaction was taken to completion overnight at room temperature. The mixture solution containing crosslinked complexes was transferred into a dialysis cassette (molecular weight cut off = 10,000 g mol^{-1}) and dialyzed against 2 L of DI water for 3 days, with two changes of water daily. The final complexes were recovered by freeze-drying.

To immobilize Mn^{2+} inside polymeric matrices as well as to afford pH-sensitive complexes (iMnBCs), NaOH was used to create Mn oxide intermediates within the particles. Mn oxides are rather stable in basic pH condition but dissolve at acidic pH. This would make it likely that a low pH condition could dissolve the iMnBCs. To fabricate the iMnBCs, 10 mg of the MnBCs (2 mg/mL) in DI water were reacted with 1 N NaOH (150 μL) which pH was raised to ~ 10, which were accompanied by change of, color from colorless to brown solution. The mixtures were allowed to react at room temperature for 1 hour before adding 1 N HCl (100 μL) to adjust pH to 7. The color of the solution changed from brown to dark-brown upon adding HCl. The complexes were centrifuged through membrane and washed with DI water 3 times and recovered by freeze-drying.

3.3 Characterization of nanocomplexes

The amount of Mn in the complexes was quantified using ICP-AES performed on a SPECTRO ACROS ICP 165 (Spectro Analytical Instruments, Germany). Effective hydrodynamic sizes were measured via dynamic light scattering using Malvern Zetasizer NanoZS particle analyzer (Malvern Instruments Ltd) at a wavelength of 633 nm from a 4.0-mW, solid-state He-Ne laser at a scattering angle of 173° and at 25 ± 0.1 °C. Intensity

average diameters were calculated with the Zetasizer Nano 4.2 software. For DLS analysis, the complexes were dispersed in DI water at a concentration of 0.5 mg mL^{-1} , and the dispersion was vortexed for 1 min and filtered through a $1.0\text{-}\mu\text{m}$ Teflon filter directly into a polystyrene cuvette for analysis.

To investigate whether nanoparticles contained Mn, energy filtered transmission electron microscopy (EFTEM) and electron energy loss spectroscopy (EELS) were performed. These techniques allow one to examine nanoscale chemical composition, and determine the atomic ratio of manganese to carbon in the nanoparticles. To prepare specimens for electron microscopy, the particles were mixed with DI water at a 1:50 ratio. Carbon film of thickness 5 nm to 10 nm was evaporated onto cleaved mica, floated on the surface of DI water, and collected onto lacey carbon coated EM copper grids. Then $3 \mu\text{L}$ to $5 \mu\text{L}$ of solution (water + nanoparticles) was adsorbed onto each grid for 2–5 min and wicked off using filter paper. EFTEM images as well as EELS spectra were recorded by means of a Tecnai TF30 electron microscope (FEI Inc.) equipped with a Tridiem Imaging Filter (Gatan Inc.), operating at an accelerating voltage of 300 kV. EFTEM images, binned to 512×512 pixels and with 2.6 nm/pixel , were acquired with integration times of 16–40 seconds with a slit width of 20 eV. Manganese was imaged using a pre-edge energy window of 625 eV (background) and a post-edge energy window of 650 eV (signal), and carbon was imaged using a pre-edge energy window of 265 eV (background) and a post-edge energy window of 290 eV (signal). The background under the Mn $L_{2,3}$ and C K edges was removed by fitting an inverse power law (red dotted line in Fig. 3e). After the background subtraction, Mn $L_{2,3}$ and C K maps were calculated by dividing the net core-edge signals by the respective inelastic cross sections and by the total beam current.

3.4 Relaxivity measurements

T_1 relaxation times of the nanocomplexes and MnCl_2 were measured on an 11.7T/31-cm horizontal bore magnet (MagneX Scientific Ltd., Abingdon, UK), which was equipped with a 12-cm gradient/shim set (Resonance Research Inc., Billerica, MA, USA) and interfaced to a Bruker Avance console (Bruker BioSpin, Billerica, MA, USA). A 90-mm volume coil was used to transmit and receive. A T_1 saturation recovery sequence ($\text{TR} = 200, 642.6, 1211.7, 2010.4, 3360.2, 10,000 \text{ ms}$; $\text{TE} = 8.63 \text{ ms}$) was used to measure T_1 . The images were fitted with a 3-parameter function to calculate T_1 values using Bruker TopSpin built-in analysis program. The specific relaxivities (r_1) of the nanoparticles were measured as follows. Samples were prepared at five different concentrations, and T_1 values were measured for each concentration, which were then used for r_1 calculations, respectively. Relaxivity was determined from the slope of concentration-dependent T_1 changes. The Mn concentrations were based on the molar concentration of manganese atom measured by ICP-AES.

3.5 Cellular cytotoxicity assessment

Cell proliferation assay (MTS assay, Promega) was used to measure cytotoxicity of nanocomplexes. AML mouse hepatocyte cells were seeded into a 96-well plate at a density of 5,000 cells/well. The particles were suspended in PBS and added to the wells at varying concentrations. The same concentrations of MnCl_2 solutions were used and compared with

nanocomplexes. After 24-hour incubation, MTS assays were performed according to the manufacturer protocol.

3.6 In vitro release studies of manganese ions

To determine the release of Mn from the complexes in physiological media, 20 mg of complexes (equivalent to 1.4 mg Mn) were dispersed in 2 mL of DI water and placed in a 10,000 MWCO dialysis cassette (Fisher Scientific). The cassette was placed in a 200-mL beaker and 148 mL of PBS as the receptor medium was added to the beaker. Aliquots of 10 mL were taken from the receptor medium and placed in separate scintillation vials at each time point. At each time, 10 mL of fresh medium was added to the beaker to retain a constant volume. The Mn in each aliquot was measured by ICP-AES. Comparative release study was performed on MnCl_2 using similar procedure.

3.7 In vivo MRI studies of contrast enhancement by nanocomplexes administered intravenously via tail vein in mice

To obtain the biodistribution of relaxation effects of the nanocomplexes in the mouse body, The particles were infused through the tail vein, and the contrast enhancements was observed by T_1 -weighted MRI. A total of 6 adult male B6 mice (25–30 g) were used in this study. MRI was performed before and immediately after infusion. Each animal received particles at the dose of 1.1 mg Mn^{2+} per kg body weight. For tail vein injections, animals were initially anesthetized by 4% isoflurane (3:3:2 air: nitrogen: oxygen mixture), and kept on 1.5–2.5% isoflurane during the infusions. Injections were performed slowly over 2 min. MRI was performed immediately after and at varying times after injections. For MRI scans, mice were placed in a custom made plastic holder. The anesthesia was maintained at 1.5–2% using a nose cone and rectal temperature was maintained at $37 \pm 1^\circ\text{C}$ by a heated water bath. No abnormalities were observed after injection in all the mice. MRIs for the tail vein injection were acquired on a 7T/21cm horizontal bore Bruker Biospec System. A Fast Low Angle SHot (FLASH) sequence gated to respiratory motion was applied to acquire images. The echo time (TE) was 2.559 msec, and repetition time (TR) was approximately 127 msec and the excitation flip angle was 60° to achieve T_1 -weighting. Each set consisted of 16 slices with no gap and a FOV of $3.84 \times 2.56 \times 1.2$ cm, matrix $128 \times 192 \times 16$. For comparison, similar experiments were carried out using MnCl_2 and GdDTPA.

3.8 Intracortical injection of particles for Mn^{2+} Thalamo-cortical tracing

Three male Sprague-Dawley rats received brain injections of MnBCs, iMnBCs, and MnCl_2 (10 mM Mn^{2+}). Injections were made to the somatosensory cortex in the forepaw area (AP 0.0 mm, ML 3.8 mm, and DV 2.9 mm), according to the Paxinos and Watson atlas.^{46, 47} Animals were initially anesthetized with 5% isoflurane (3:3:2 air: nitrogen: oxygen mixture), then positioned in a stereotactic frame (Benchmark Digital Stereotaxic, myNeuroLab.com) and kept on 2–3% isoflurane during the surgery. After exposing the skull, two small burr holes were drilled above the S1 cortex. The injection device consisted of a 30-gauge needle cemented to a polyethylene tube (PE-20), which then connected to a Hamilton microsyringe. The needle was placed at the proper coordinates in the stereotactic frame. A total of 0.2 μL solution was injected into each hemisphere at a speed of 0.2 $\mu\text{L}/\text{min}$, which was controlled by a micro-injection pump (Cole-Parmer 74900). Five minutes after

injections, the needle was slowly removed. The burr holes were sealed with bone wax and the incision was sutured. MRI was performed immediately after stereotactic injections to make sure solutions were delivered to the proper sites. A series of images were acquired to measure the transportation of manganese from the S1 cortex to thalamus. Brain images were acquired on an 11.7T/31 cm horizontal bore magnet. A Magnetization Prepared Rapid Gradient Echo (MP-RAGE) sequence was used. Sixteen coronal slices with FOV= 2.56 × 2.56 cm, matrix 256 × 256, thickness = 0.5 mm (TR = 4000 ms, Echo TR/TE = 15/5 ms, TI = 1000 ms, number of segments = 4, and averages = 8) were used to cover the area of interest at 100 µm in-plane resolution in 34 min.

4. Conclusions

In this work, an aqueous-based synthetic route for Mn-based polymeric nanoparticle for potential MRI contrast agents has been developed. These nanocomplexes have high r1 relaxivities with potential applications in high-field MRI. Two types of particles were synthesized with different Mn release behaviours. The first type of particles released Mn²⁺ rapidly. The second type of particles was designed to display delayed Mn release, based upon a formation of oxide intermediate. We have successfully exploited the delayed release behaviour of these nanocomplexes to image two biological processes: 1) Mn²⁺-neuronal tracing in the brain, and 2) an uptake into liver with subsequent transport of Mn²⁺ ion to gallbladder. Based on our previous work, the iMnBCs offers the third approach that low pH-triggered release of Mn²⁺ can be achieved. The other two approaches are the use of Mn³⁺-transferin complexes and MnO nanoparticles, respectively.^{48, 49} Slow and delayed release of MRI contrast that can be triggered by the low pH environment of the endosomal/lysosomal pathway, should be very useful for developing molecular imaging agents with improved sensitivity and specificity. Overall, we envision that flexibility in surface modification chemistry of polymeric nanoparticles with tissue-specific ligands could lead to target other complex biological processes such as inflammation and uptake by cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Schematic illustration of Mn²⁺-block ionomer complexes

Fig. 2. EFTEM and EELS analyses of manganese-block copolymer nanocomplexes. A) EFTEM of nanocomplexes B) manganese map C) carbon map C) overlay of manganese and carbon map D) EELS spectra obtained from the complexes. Scale bar = 200 nm.

Fig. 3.
UV-Vis absorption spectra of nanocomplexes solution during alkaline treatment

Fig. 4.

In vitro release profile of Mn²⁺ at A) pH 7.4 in PBS and B) pH 4.5 in ABS. The solution containing MnCl₂ and nanocomplexes (equivalent to 1.4 mg Mn²⁺) was placed in dialysis cassette. The amounts of Mn²⁺ that diffuse through membrane into receptor medium were measured by ICP-AES.

Fig. 5. T1-weighted MR Phantom images (at 7T) of the MnBCs, iMnBCs, MnCl_2 , and MnDPDP at various Mn^{2+} concentrations.

Fig. 6.
Cell viability of AML12 cells after incubation with various concentrations of MnBCs, iMnBCs, and MnCl2 at A) 4-hour and B) 24-hour incubation

Fig. 7.

MP-RAGE images of thalamocortical tract-tracing with injection of MnBCs, iMnBCs, and MnCl_2 . The transport of Mn^{2+} tracing from S1 region (arrowed in panel A and B) to thalamus (circles in panel C-H) after intracortical injection of MnBCs vs iMnBCs (A) and MnBCs vs MnCl_2 (B) was monitored and the increase of normalized signal intensities within thalamus area was plot as function of scan time (panel I). Significant delayed and slow tracing of Mn^{2+} was clearly noted in case of iMnBCs.

Fig. 8.

In vivo time-course MR imaging of mouse abdomen after intravenous infusion of the agents at 20 $\mu\text{mol Mn}^{2+}$ or Gd^{3+} per kg. A) contrast enhancements in liver and gall bladder (circled) B) contrast enhancements in liver (arrowed) and kidney (circled). Plot of evolution of signal intensities from each agents were shown in panels C–E. $GdDTPA$, at low dose of 10 μmol per kg, did not generate noticeable contrast enhancements in all organs.

Fig. 9. MR images of mouse pituitary gland (dashed rectangle) and heart (arrow) at 7 T after i.v. injection of MnCl₂ (A,C) and iMnBCs (B,D) after i.v. injection iMnBCs

Table 1

Characterization of nanocomplexes

Complexes	Molecular weight of PEG-PMA ^a (g mol ⁻¹)	Hydrodynamic size (nm)	PDI	Zeta Potential (mV)	% wt of Mn ²⁺ Mn in final complexes (%w/w)
MnBCs	2000–7000	150±30	0.133	-20.2±3.7	6.13±0.74
iMnBCs	2000–7000	170±20	0.148	-25.3±4.2	6.05±1.23

^a molecular weight of each block

Table 2

Relaxivities of MnBCs in comparison with MnCl₂, MnDPDP, and GdDTPA at different field strengths. For each agent, five different concentrations were prepared, and T₁ values were measured for each concentration, which were then used for r₁ calculations, respectively. Relaxivity was determined from the slope of concentration-dependent T₁ changes.

	Ionic R ₁ Relaxivities (mM ⁻¹ s ⁻¹)		
	4.7 T	7.0 T	11.7 T
MnBCs	40.2 ±2.71	21.7±2.20	16.1±2.31
iMnBCs	8.55±1.32	6.60±1.45	3.10±1.15
MnCl₂	7.00±1.20	6.70±0.90	7.12±0.85
MnDPDP	4.00±0.64	3.30±0.40	3.50±0.85
GdDTPA	4.50±0.55	4.50±1.12	4.20±0.74

*
in DI water at room temperature