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Rapid detection and profiling of cancer cells in fine-needle aspirates

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There is a growing need for fast, highly sensitive and quantitative technologies to detect and profile unaltered cells in biological samples. Technologies in current clinical use are often time consuming, expensive, or require considerable sample sizes. Here, we report a diagnostic magnetic resonance (DMR) sensor that combines a miniaturized NMR probe with targeted magnetic nanoparticles for detection and molecular profiling of cancer cells. The sensor measures the transverse relaxation rate of water molecules in biological samples in which target cells of interest are labeled with magnetic nanoparticles. We achieved remarkable sensitivity improvements over our prior DMR prototypes by synthesizing new nanoparticles with higher transverse relaxivity and by optimizing assay protocols. We detected as few as 2 cancer cells in 1-μL sample volumes of unprocessed fine-needle aspirates of tumors and profiled the expression of several cellular markers in <15 min.

Microfluidics | nanoparticle | nuclear magnetic resonance

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e nsitive and quantitative technologies for molecular characterization of scant cells in easily accessible bodily sources (e.g., fine-needle aspirates (FNA), biopsies, whole blood, and other biological fluids) will have significant impact in life sciences and clinical practice (1, 2). If made available, such diagnostic platforms could be used for early detection/screening of cancer, comprehensive tumor characterization in patients, and targeted therapy based on personal responses to treatments (3, 4). The ideal detection technology would combine minimal sample processing with fast measurements, thus avoiding likely phenotypic/apoptotic changes of sampled cells. It would also allow for multiple biomarker detection in a single parent sample (multichannel detection) for accurate diagnosis (5). A number of sensors fulfilling some of these criteria have been developed based on optical (6, 7), electronic (8, 9), or magnetic detection (10, 11). The clinical utility of these systems, however, is limited, because they require lengthy sample purification or long assay times.

We recently developed a NMR-based sensor that offers fast detection of biological targets in native samples (12). Termined “DMR” for diagnostic magnetic resonance, the sensor measures the transverse relaxation rate ($R_2$) of water molecules in biological samples in which target molecules or cells of interest are labeled with magnetic nanoparticles (MNP).

Local magnetic fields created by the MNP accelerate the spin–spin relaxation of water protons, increasing the $R_2$ of samples (13) and thus providing a sensing mechanism. Because most biological objects have negligible magnetic susceptibilities, DMR measurements can be performed in unprocessed samples, allowing for fast assays. As proof-of-concept, we developed a prototype sensor (DMR-1) that detected bacteria, analytes, and abundant cancer cells (10^6 cells per mL) (12). Despite the underlying technological advantages, however, it has been difficult to achieve detection sensitivities (≈10^3 cells per mL) adequate for clinical applications.

The goal of the current study was to develop a DMR sensor with detection sensitivities and cellular profiling capabilities comparable with other standard methods (flow cytometry and Western blot analysis). This was achieved by developing (i) new magnetic nanoparticles with high transverse relaxivity, (ii) a NMR probe with improved signal-to-noise ratio (SNR), and (iii) an analytical DMR protocol that can quantify the expression level of molecular markers in tumor cells. The clinical utility of the system (DMR-2) was evaluated by using FNA (14, 15) from a panel of xenograft tumor models. We detected as few as 2 cells in 1-μL volumes and analyzed cells for growth factor expression in nonpurified samples. DMR-2 exhibited detection sensitivities as good as those achieved with clinical methods (e.g., flow cytometry and Western blot analysis), but the assay was performed in much shorter time (in <15 min) and with smaller sample size.

Results

Optimized Magnetic Nanoparticles (MNP) for DMR Assay. The overall DMR sensitivity for cell detection is determined by 2 different sensitivities. The first is the cell-concentration sensitivity that depends on the $R_2$ relaxivities of MNP. For a given cell concentration, MNP with high $R_2$ relaxivities will induce large $R_2$ changes. The second sensitivity is related to the signal-to-noise ratio (SNR) of the NMR system. With a high SNR, the sample volume for NMR detection can be reduced, thus lowering the cell detection threshold (equal to cell concentration × sample volume).

To improve the DMR sensitivity, we first set out to increase the $R_2$ relaxivity of MNP. Because $R_2$ is proportional to $M^{-2}d^2$, where $M$ and $d$ are the magnetization and the diameter of MNP, respectively (16), high $R_2$ can be achieved by using materials with strong magnetization and by increasing the size of the magnetic core. On the other hand, MNP should still be small enough (<50 nm in hydrodynamic diameter) for optimal binding to cell surface and permeation into cells for intracellular marker targeting (17). Small MNP are also advantageous because they typically exhibit higher stability in isosmolar solution (no sedimentation) and superparamagnetic behavior to avoid spontaneous magnetic aggregation (18, 19). In addition, the MNP should be hydrophilic and biocompatible so as not to alter the expression profiles of incubated cells (20).

Review of reported and commercially available MNP indicated that most materials (blue circle in Fig. 1A) were not ideally suited for DMR assays because of their large size and/or low relaxivity. We therefore developed and optimized MNP for DMR assays. We opted to base on ferrite MNP (21–25) and doped the particles with Mn^{2+} (Mn-MNP) to increase overall magnetization (M). We also adopted a seed-growth approach to increase the metallic core size ($d$). Fig. 1B shows an example of the Mn-MNP with a core size of 16 nm. To obtain these particles, we first synthesized 10-nm core seeds by reacting Fe(acac)_3, Mn(acac)_2, and 1,2-hexadecanediol at high temperature (300 °C). By repeating the seed-mediated growth, small MNP seeds by reacting Fe(acac)_3, Mn(acac)_2, and 1,2-hexadecanediol at high temperature (300 °C). By repeating the seed-mediated growth, small MNP seeds were produced. Then, the Mn-MNP were further grown to the desired size using ferrite MNP. The resulting Mn-MNP exhibited a transverse relaxivity of 2.02·10^3 s^{-1}·mM^{-1} at 300 MHz and 30 °C. This relaxivity is 43 times higher than that of Fe(acac)_3 alone, indicating that Mn(acac)_2 and 1,2-hexadecanediol were successfully incorporated into the Mn-MNP.


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Because of reduced surface effects (e.g., spin-canting) in larger particles (Fig. S2), Mn-MNP assumed superior relaxivities with $r_2$ as high as $420 \text{s}^{-1}\text{mM}^{-1}\text{M}^{-1}\text{[metal]}$ (equal to $6.0 \times 10^{-11} \text{ s}^{-1} \text{[particle per milliliter]}^{-1}$). Furthermore, Mn-MNP were highly stable in isosmolar solution without sedimentation and magnetic aggregation. $d$, mean diameter of Mn-MNP. (B) Transmission electron microscope (TEM) images showed that all Mn-MNP ($d \leq 16 \text{ nm}$) had a narrow size distribution (Fig. S1 A and B) and were highly crystalline (Inset). (C) Both the X-ray powder diffraction (XRD) and the electron diffraction patterns (Inset) revealed a typical spinel structure of ferrite. The crystal sizes, measured by fitting the major peaks (311) in XRD, were in agreement with the TEM estimation (Fig. S1C), confirming the single domain nature of Mn-MNP. (D) All Mn-MNP were superparamagnetic at 300 K. The magnetization increased with particle size, possibly because of reduced surface effects (e.g., spin-canting) in larger particles (Fig. S2A). Thus, the $r_2$ relaxivity is the highest with the largest Mn-MNP ($d = 16 \text{ nm}$; Fig. S2B). CLIO, cross-linked iron oxide; MION, monocrystalline iron oxide; PION, poly crystalline iron oxide; CMD, carboxymethyl dextran-coated MNP.

NMR Probe with Higher SNR. Another important factor in determining the overall detection sensitivity is the SNR of a NMR setup. In the DMR-2 system, we improved the SNR by devising a NMR probe with higher filling factor. With these advantages, the sample volume for the DMR-2 was reduced by a factor of 10 (to 1 $\mu$L) compared with DMR-1. At the same measurement conditions (NMR electronics and MNP), DMR-2 achieved 10-fold enhanced mass-detection sensitivity over the DMR-1.

Scaling Cellular Measurements. In current clinical practice, FNA diagnoses are primarily reported as the presence or absence of malignant cells in a specimen. The capability to quantify and profile cancer cells, however, would likely improve diagnosis. For example, a critical issue when screening cancer cells is how to correlate the expression levels of tumor markers to the number of malignant cells in a given sample. Without this knowledge, one could either measure high expression in relatively few cells or low expression in many cells.

To measure cell number by DMR, we exploited a previously characterized phenomenon of low-grade phagocytosis of nontargeted MNP by tumor cells (28, 29). When mammalian cells were incubated with unmodified MNP (MNP-Ø) for 5 min, linear and cell number-dependent $R_2$ changes ($\Delta R_2$) are observed (Fig. 3A). Interestingly, these changes were similar across a wide variety of cell types. The results were fitted to $\Delta R_2^0 = r_2^0 N$, where $r_2^0$ is the polydimethylsiloxane). After polymer curing, the tubes were retracted to open up fluidic channels (see Methods for details). The entire bore of the solenoid thus can be filled with sample, resulting in $>350\%$ enhancement in NMR signal level (Fig. 2B). Compared with the lithographically patterned and metal-plated planar coils of the DMR-1 system (12), these solenoidal coils produced more homogeneous radio-frequency magnetic fields (27) and had less electrical resistance. With these advantages, the sample volume for the DMR-2 was reduced by a factor of 10 (to 1 $\mu$L) compared with DMR-1. At the same measurement conditions (NMR electronics and MNP), DMR-2 achieved 10-fold enhanced mass-detection sensitivity over the DMR-1.
The measured relaxivity per cell (cellular relaxivity) for MNP-Ø and N is the number of cells in the DMR detection volume. The cellular relaxivities ($r_2^N$) were statistically identical ($P > 0.99$) among different tumor cell lines, making them a universal measure for estimating cell number. By applying this method to xenograft FNA samples (HCT116; Fig. 3B), we could estimate cell numbers in experimental samples. We further defined the expression level of a targeted marker as $\Delta r_2^{Ab}/\Delta r_2^{Ø} = (r_2^{Ab} - N)/(r_2^{Ø} - N) = r_2^{Ab}/r_2^{Ø}$, where $\Delta r_2^{Ab}$ and $r_2^{Ab}$ are $R_2$ changes and the cellular relaxivity, respectively, with a marker-specific MNP. The DMR-2 assay, therefore, can report the cellular expression level of a specific marker.

**Optimized Assay Protocol with High Specificity.** Samples obtained from FNA, biopsies or blood are inherently complex in composition and variable in cell number. For a given detection method to be clinically useful, it has to be highly specific and robust under different sample conditions. To improve the specificity of the DMR assay, we first determined the level of interference from noncancerous cells. Using HER2/neu-specific Mn-MNP (Mn-MNP-Her2) as a model system, we monitored the time-dependent cellular binding/uptake of particles in cancer cells and host cells, specifically leukocytes (Fig. 4A). As expected, $T_2$ (equal to $1/R_2$) changes increased in both cell types with longer incubation time, reflecting the well-known uptake kinetics in phagocytic cells (30). However, at shorter incubation times, the relative specificity of MNP to cancer cells was still high with negligible MNP binding to host cells. These observations led to an assay protocol that maximized cancer cell detection while minimizing the effect of host cells.

Compared with the previous method (30-min incubation) (12), this protocol was 6 times faster and improved the specificity >10-fold. We further quantified the differences in cellular binding of cancer-targeted MNP between malignant and host cells. Mn-MNP-Her2 binding to cancer cells was much more avid, as verified by both fluorescent microscopy (Fig. 4B) and flow cytometry (Fig. 4C). The mean fluorescent intensity of MNP-targeted cancer cells was $>10^6$ times higher than those from leukocytes. DMR measurements resulted in similar differences with host cells having cellular relaxivities $\approx 10^{-6}$ s$^{-1}$[cell per microliter]$^{-1}$, whereas cancer cells had cellular relaxivities $\approx 10^{-3}$ s$^{-1}$[cell per microliter]$^{-1}$. The magnetic moment per cell, estimated from the relaxivities, were $10^{-7}$ and $10^{-10}$ emu for cancer and host cells, respectively. The magnetic fields from these cells can be measured by various magnetometers including Hall probes (31, 32), magnetoresistive elements (10, 11), and superconducting quantum interference devices (33). It is thus conceivable to combine DMR with a magnetic reader that can detect and sort cells according to cellular magnetic moments.

We also explored fast leukocyte depletion protocols to further enhance cancer cell detection, particularly in the whole blood where leukocytes are highly abundant. First, we investigated which leukocyte fraction would associate cancer-targeted MNP. Flow cytometry analysis revealed that nonspecific MNP binding/uptake was mostly by monocytes, neutrophils, NK cell and B cells (Fig. 4C). In repeat experiments, we removed these cells on the basis of CD11b (monocytes, neutrophils, NK cells) and B220 (B cells) expression. For the remaining host cells (mostly T cells), the MNP binding was...
negligible (Fig. 4D). Combining shorter incubation times and negative cell selection, the specificity of cancer-targeted MNP over host cells in unpurified native samples is thus >10^4, which could be highly advantageous when profiling rare cells (<10 cells in 1 mL of blood) such as circulating tumor cells (4).

**Tumor Cell Detection in FNA.** To evaluate the above optimized DMR-2 assay, we obtained FNA from xenograft tumors in mice. Aliquots of freshly obtained aspirates were incubated with antibody-modified MNP for 5 min, washed to remove excess particles, and measured by the DMR-2 system in 1-μL samples (See Methods for details).

We first determined the detection sensitivity of DMR-2 by targeting the HER2/neu receptor on samples from BT474 xenografted breast cancers as a model system. The number of MNP per cell, quantified by ICP-AES (inductively coupled plasma atomic emission spectroscopy) was ～5 × 10^5 (see Methods). When the same magnetically labeled cells were measured with the DMR sensor, a linear R2 change (R2 > 99%) was observed at different cell concentrations (Fig. 5A). The relaxivity per cell (cellular relaxivity), obtained by fitting the titration curve, was ～2.3 × 10^-3 s^-1[μmol]^-1; the number of MNP in a cell, estimated from the cellular relaxivity was ～10^5, which is in good agreement with the ICP-AES measurement. The detection limit was nearly at the single cell level (～2 cells in 1-μL detection volume), far surpassing the sensitivities of our previous results (～10^5 cells in 10-μL samples) (12) and other clinical methods (e.g., cytology and histology; Fig. 5B).

We next determined how the DMR-2 measurements compared with flow cytometry and Western blot analysis (Fig. 5C). The same samples were subjected to DMR measurements, flow cytometry and Western blot analysis. The R2 changes (ΔR2HER2/ΔR2Ø) from DMR (requiring ~10^3 cells) showed good agreement (R2 > 98%) with both flow cytometry (requiring ~10^5 cells) and Western blot analysis (requiring ~10^7 cells), demonstrating the analytical capability of DMR-2 technology. Importantly, the DMR-sensor required fewer cells than either of the other 2 approaches and gave results in a fraction of the time (~15 min).

In the absence of a single ubiquitous cancer marker, one strategy to minimize false-negative results is to profile putative cancer cells for multiple markers (“signature”). DMR can be adapted for such a multitarget detection scheme, because it can perform measurements on few cells in small sample volumes and in parallel. As proof-of-concept, we chose to detect 3 targets: EpCAM (CD326), EGFR (ERBB1, CD126), and HER2/neu (ERBB2, CD340). The maximum R2 changes from 3 markers were then used to calculate and display aggregate malignancy scores for a given FNA (Fig. 5D). For overall tumor detection, DMR assays yielded false-negative rates of 57–72% for single targets and 28% for 2 targets, whereas the false-negative rate was negligible for 3 targets. The DMR assay can be expanded beyond these 3 targets to profile complex cancer signatures.

**Discussion**

DMR is a promising biosensor technology with unique advantages in a clinical setting. The technology requires minimal sample preparation, can perform assays in turbid/obscure media, can be adapted to profile different targets (DNA, protein, metabolites, cells), and has the capability for high-throughput operation. Here, we built on our earlier proof-of-principle experiments and developed a new-generation (DMR-2) system that employs MNP with high r2 relaxivity and a new NMR probe. Together, these improve-
be further applied to monitor malignancy progression, metastases, and the presence of key cytokines. With such capabilities, DMR can be adapted to probe intracellular markers, signaling pathways, targets or to interrogate complex cancer signatures. For example, it can be applied to monitor malignancy progression, metastases, or therapy resistance (35–37) in personalized patient care.

Methods

Synthesis of Mn-MNP. Iron (III) acetylacetonate [99.9%, Fe(acac)3], manganese (II) acetylacetonate [Mn(acac)2], oleylamine (70%), 1-octadecene (95%), 1,2-hexadecanediol (90%), chloroform (99%), and NaHCO3 were purchased (Fisher Scientific) and used as received. Isopropanol (99.5%), hexane (98%, DMSA) and dimethyl sulfoxide (99.9%, DMF) were purchased (Sigma–Aldrich) and used without further modification. Isopropanol (99.5%), hexane (98.5%), ethanol (99.5%), and NaHCO3 were purchased (Fisher Scientific) and used as received.

First, we synthesized 10-nm Mn-MNP as a seed for the subsequent syntheses. Fe(acac)3 (4 mmol, 1.4 g), Mn(acac)2 (2 mmol, 0.5 g), 1,2-hexadecanediol (10 mmol, 2.9 g), oleic acid (6 mmol, 1.9 mL), oleylamine (6 mmol, 2.8 mL), and 1-octadecene (20 mL) were mixed by vigorous stirring under N2 flow (1 h). The mixture was then heated and kept at 200 °C for 2 h. Subsequently, the temperature was quickly elevated to 278 °C. The solution turned from dark brown to black at this point. After reflux, the mixture was cooled to room temperature, and iso-propanol (80 mL) was added. Mn-MNP were collected by centrifugation (1,811 × g, 15 min) and dispersed in hexane. Mn-MNP in a powder form was obtained by adding excess ethanol to Mn-MNP in hexane and collecting precipitates.

To make 12-nm Mn-MNP through the seed-mediated growth, 10-nm Mn-MNP (100 mg) were dissolved in hexane (10 mL) along with the same amount of metal acetylacetonates, 1,2-hexadecanediol, oleic acid, oleylamine, and 1-octadecene as described above. The mixture was heated and kept at 100 °C for 1 h under N2 flow to remove hexane. The mixture was heated again and kept at 200 °C for 2 h. Finally, the temperature was increased to 300 °C, and the mixture was refluxed for 2 h before being cooled down to room temperature. The precipitate was collected by the same washing and isolation procedure. In a similar manner, 16-nm Mn-MNP were synthesized by using 12-nm seeds as a seed.

We characterized the morphology, structure, composition, and magnetic properties of Mn-MNP using a transmission electron microscope (TEM; JEOL 2100), an X-ray powder diffractometer (XRD; R3000; Rigaku), an inductively coupled plasma atomic emission spectrometer (ICP-AES; Activa-S; HORIBA Jobin Yvon) and a vibrating sample magnetometer (VSM; EV-5; ADE Magnetics), respectively.

Surface Modification of Mn-MNP and Antibody Conjugation. We first added ethanol to 16-nm Mn-MNP in hexane and centrifuged (1,811 × g, 10 min) the mixture. The precipitate (~150 mg) was homogeneously redispersed in 10 mL of chloroform, and triethylamine (50 μL) was added. We then added DMSA (50 mg in 10 mL of DMSO) to the Mn-MNP solution. The mixture was shaken for 6 h at 37 °C and gradually turned heterogeneous. After centrifugation (1,811 × g, 10 min), the precipitate was washed with chloroform to remove excess DMSA, and dispersed in 10 mL of ethanol. DMSA (50 mg in 10 mL of DMSO) was added again to Mn-MNP in ethanol, and the whole process was repeated. The final precipitate (Mn-MNP-DMSA) was dispersed in 10 mL of H2O. Ultimately, the particles were terminated with sulfhydryl (–SH) functional groups. The number of sulfhydryl group per Mn-MNP was ~50 as determined by Ellman's reagent (Pierce Biotechnology).
Monodisperse antibodies to cell surface markers (anti-HER2/neu: Herceptin; Genentech; anti-EGFR: Erbitux; Imclone Systems; anti-EpCAM: MAAB9601; R&D Systems) were purchased and used without modification. To conjugate antibody, we first rendered them sulfhydryl active by attaching a maleimide functional group. We dissolved antibody (6 mg/ml) in 1 mL of PBS buffer, adjusted pH to 8.2 by adding 0.1 M NaHCO3, and added 1 mg of sulfo-SMCC. The mixture was then incubated for 30 min at 37 °C. The sulfhydryl-active antibody was purified with PD-10 desalting column (GE Healthcare Bio-Sciences) and immediately combined with Mn-MNP-DMISA (5 mg/mL). The mixture was shaken for 6 h at 4 °C, followed by purification by using Sephadex G-100 (DNA grade; GE Healthcare Bio-Sciences). The number of antibodies per Mn-MNP was ~10 as determined by bichinonic acid assay (BCA protein assay kit; Pierce Biotechnology).

Fabrication of NMR Probes. To embed solenoidal microwires along with microfluidic channels, we adopted a molding technique for device fabrication. First, an insulated wire (diameter ~160 µm) was wound around a polyethylene tubing (diameter ~960 µm) to make a solenoidal coil. The coil-wound tubes were then placed on a custom-machined metal mold, and the mold was filled with polydimethylsiloxane (PDMS; Sylgard 184; Dow-Corning). After PDMS cure, the tubes were withdrawn to open up fluidic channels; the solenoidal coils were retained in PDMS supports. The device thus had a filling factor ~1, which lead to high NMR signal levels.

DMR Measurements. The NMR probe was mounted on a printed circuit board along with RF amplifiers and fluidic fixture. To obtain a complete NMR system, the board was packed into a miniaturized DMR setup as previously described (12). All DMR measurements were performed at the polarizing magnetic field of B0 = 0.5 T generated by a portable permanent magnet. Transverse relaxation rate (R2) was measured on 1-µL sample volumes, by using Carr–Purcell–Meiboom–Gill pulse sequences with the following parameters: echo time, 4 ms; repetition time, 6 s; the number of 180° pulses per scan, 500; the number of scans, 8. For ΔR2 calculation, we took R2 differences between a MNP-targeted and a cell-number-matched control sample. All measurements were done in triplicate, and data were displayed as mean ± standard error.

Fluorescent Detection of Endogenous Cells. Leukocytes (105 cells per milliliter) prelabeled with CFSE (carboxyfluorescein diacetate succinimidyl ester) were matched control sample. All measurements were done in triplicate, and data were collected on a LSRII (BD Biosciences) and analyzed with FlowJo v.8.5.2 (Tree Star, Inc.).

Xenograft Tumor Model. Tumor cells were cultured to ~70% confluence, detached, and suspended in protease-free PBS solution. For breast cancer cell lines (BT474 and MCF7), ~106 cells were mixed with 150 µL of Matrigel (#356230; BD Sciences) per tumor site. The mixture was then orthotopically implanted into the mammary fat pads of female C57BL/6 nude mice (The Jackson Laboratories) that had a 17T external solenoidal coil (6.72 mg/Innovative Research Inc.) s.c. implanted 7 days before tumor cell inoculation. For other cell lines, ~105 cells per tumor were s.c. implanted on the back of female C57BL/6 nude mice; the prostate cancer cells (LnCaP) were implanted into a male mouse (COX7, MGH breeding colony). Mice were housed and maintained under aseptic conditions according to guidelines set by the Institutional Animal Care and Use Committee.

FNA Protocols. When the tumor reached ~1 cm in size, they were aspirated by using 22-G needles. After the aspiration, the needle was washed in cell-dissociation solution (Cellstripper; Mediatech) to dislodge cells. Four needle washes per tumor were combined and then equally divided into five 1-ml aliquots. Four aliquots were incubated with unmodified or target-specific Mn-MNP (HER2/neu, EGFR, and EpCAM) under identical conditions (5 min at 37 °C with 50 µg/mL [Fe+Mn]). Subsequently, excess Mn-MNP were removed by triple washes via centrifugation (200 × g, 5 min) and cell pellets were resuspended in PBS (1 mL). As a control, the fifth aliquot was prepared in a similar manner but without MnMNP incubation.

ICP-AES Measurements to Determine Particle Number per Cell. Needle aspirates of a breast tumor (BT474) were incubated with Mn-MNP conjugated with HER2/ neu antibodies (37 °C, 5 min). Reference samples were prepared without MNP targeting. All samples were triple-washed with centrifugation (200 × g, 5 min) and dissolved in iCP-grade hydrochloric acid and 3% H2O2 (Fisher Scientific). Metal (Fe and Mn) concentrations were then analyzed by ICP-AES. From the result, the number of Mn-MNP was estimated, assuming that each particle had 2.2 × 106 metal atoms.

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