

Impact of surface coating and particle size on the uptake of small and ultrasmall superparamagnetic iron oxide nanoparticles by macrophages

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Purpose: Magnetic resonance imaging (MRI) using contrast agents like superparamagnetic iron oxide (SPIO) is an extremely versatile technique to diagnose diseases and to monitor treatment. This study tested the relative importance of particle size and surface coating for the optimization of MRI contrast and labeling efficiency of macrophages migrating to remote inflammation sites.

Materials and methods: We tested four SPIO and ultrasmall superparamagnetic iron oxide (USPIO), alkali-treated dextran magnetite (ATDM) with particle sizes of 28 and 74 nm, and carboxymethyl dextran magnetite (CMDM) with particle sizes of 28 and 72 nm. Mouse macrophage RAW264 cells were incubated with SPIOs and USPIOs, and the labeling efficiency of the cells was determined by the percentage of Berlin blue-stained cells and by measuring T_2 relaxation times with 11.7-T MRI. We used trypan blue staining to measure cell viability.

Results: Analysis of the properties of the nanoparticles revealed that ATDM-coated 74 nm particles have a lower T_2 relaxation time than the others, translating into a higher ability of MRI negative contrast agent. Among the other three candidates, CMDM-coated particles showed the highest T_2 relaxation time once internalized by macrophages. Regarding labeling efficiency, ATDM coating resulted in a cellular uptake higher than CMDM coating, independent of nanoparticle size. None of these particle formulations affected macrophage viability.

Conclusion: This study suggests that coating is more critical than size to optimize the SPIO labeling of macrophages. Among the formulations tested in this study, the best MRI contrast and labeling efficiency are expected with ATDM-coated 74 nm nanoparticles.

Keywords: ultrasmall superparamagnetic iron oxide, cultured mouse macrophage cells, surface coating, particle size, MRI

Introduction

The use of nanoparticles for cellular imaging is among the most important clinical breakthrough of the past decade. In particular, superparamagnetic iron oxide (SPIO) nanoparticles enhance contrast in magnetic resonance imaging (MRI), which allows clinicians to monitor anatomical, physiological, and molecular changes during the evolution of a disease or treatment. Following intravenous injection, these nanoparticles accumulate in macrophages residing in the liver, bone marrow, and spleen, as well as tumors and sites of inflammation. Accordingly, applications include the detection of inflammatory diseases, in vivo stem cell tracking,¹ hyperthermia therapy,² lymph node detection,³ and anticancer drug delivery.⁴

The current applications for SPIO nanoparticles are limited because these relatively large particles, with an average diameter of 80 nm, are rapidly internalized by the

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mononuclear phagocytic system of the liver and the spleen. This problem is currently addressed by the development of ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles (<50 nm).⁵ In vivo studies recently showed that USPIO nanoparticles of 35 nm in diameter have a longer half-life in the circulation system, allowing the labeling of macrophages migrating to remote areas.^{6,7} These studies opened the door to a number of new applications for molecular imaging because macrophages migrate and accumulate at sites of inflammation,⁸ autoimmune neuritis,⁹ renal ischemia,¹⁰ and solid organ transplant rejection.¹¹ The use of USPIO agents has allowed for the direct visualization of macrophage infiltration of carotid atheroma in clinical study.^{12,13} The surface coating of USPIO nanoparticles also influences their stability and cellular uptake by macrophages.⁶ Dextran-based coatings are preferred because of their low toxicity and they are biodegradable.¹⁴ For instance, dextran-coated SPIO nanoparticles and ferucarbotran are approved for liver MRI. Moreover, carboxydextran-coated SPIO nanoparticles have undergone clinical trials for MRI evaluation of lymph node metastasis;^{15,16} however, the impact of USPIO nanoparticles size and dextran coating composition on the uptake by macrophages has not been determined.

The aim of the present study is to determine the impact of particle size and surface coating on the cellular uptake and relaxing time of SPIO nanoparticles in mouse macrophages by MRI and microscopy. The USPIO (28 nm) and SPIO (72 and 74 nm) nanoparticles were coated with alkali-treated dextran (ATDM) or carboxymethyl dextran (CMDM).

Material and methods

Ultrasmall superparamagnetic iron oxides

We tested four types of SPIO and USPIO contrast agents. ATDM (surface voltage potential: -15 mV) was tested with particle sizes of 28 and 74 nm. CMDM (surface voltage potential: -24 mV) was tested with particle sizes of 28 and 72 nm. All SPIO and USPIO compounds were purchased from the Meito Sangyo Company, Ltd (Aichi, Japan).¹⁷

Cell culture of mouse macrophage RAW264 cells

The mouse macrophage cell line RAW264 was provided by the RIKEN BioResource Center (RIKEN Tsukuba Institute, Tsukuba, Ibaraki, Japan). RAW264 cells (1×10^6) were cultured in minimum essential medium (Sigma-Aldrich, Tokyo, Japan), 10% fetal calf serum (Nichirei Corporation, Tokyo, Japan), and 0.1 mM nonessential amino acids (Sigma-Aldrich) in 93 mm \times 21 mm Petri dishes

(IWAKI, Tokyo, Japan) with 18 mm square cover glasses (Matusnami Glass Ind, Ltd, Osaka, Japan). All cultures were incubated with 5% CO₂ at 37°C.

Measurement of SPIO and USPIO T₂ relaxation times

The contrast of proton MRI images depends on the relaxation times of the nuclear magnetization (T₁, longitudinal; T₂, transverse). Iron oxide nanoparticles primarily affect T₂ and work as negative contrast agents. A high intracellular concentration of SPIO and USPIO nanoparticles results in the reduction of T₂ relaxation time. Prior to the MRI experiment, the concentrations of USPIO particles were adjusted to 0.005 mg Fe/mL and prepared in five sample tubes with water samples serving as controls. MRI experiments for the three times measurements of the T₂ relaxation times were performed on an 11.7-T MRI scanner (Bruker BioSpin, Ettlingen, Germany) and used a volume RF coil with a 25 mm inner diameter for transmission and reception (m2m Imaging Corp, Cleveland, OH). T₂ mapping was performed using a multislice, multiecho spin-echo sequence (repetition time = 3000 ms; slice thickness = 1 mm; field of view = 25.6 \times 25.6 mm; matrix = 128 \times 128; slice orientation = transaxial; number of repetitions = 1) with echo times ranging from 10 to 100 ms in steps of 10 ms. T₂ maps were calculated from a single exponential fitting of MRI signal intensities at each echo time point.

Measurement of USPIO-labeling efficiency

The experiments were conducted to measure the T₂ relaxation times of the macrophages labeled with SPIO or USPIO nanoparticles. All USPIO particles were dissolved in the cell growth medium at a concentration of 0.3 mg Fe/mL, which is similar to the concentration used in another published report.¹⁸ Cells (2×10^6) were incubated with medium containing USPIO particles for 1, 2, or 4 h (same time points noted in a previous report)¹⁹ at 37°C under 5% CO₂. After incubation, the medium was removed by two washes with phosphate-buffered saline (PBS) followed by fixation of the cells for 30 min with 7.5% formalin. The presence of iron oxides in the RAW264 cells was detected by staining with Berlin blue (KFe(3)Fe(2)(CN)₆) (Wako Pure Chemical Industries, Ltd, Osaka, Japan)²⁰ for 20 min. After washing away the Berlin blue with PBS, the cell nuclei were counter-stained with nuclear fast red for 5 min. The nuclear fast red was removed by washing with PBS three times, and the cell specimens were fixed to the cover glass. For each experiment, the labeling

efficiency of each type of USPIO nanoparticle was assessed visually by three observers using a BZ-9000 microscope (Keyence Corporation, Osaka, Japan). The labeling efficiencies (percentages of SPIO/USPIO uptake) were qualitatively assessed from the microscope images in three independent experiments by estimating the number of iron-positive cells within five randomly selected 200 μm^2 fields.

Viability of USPIO-labeled cells

We used trypan blue (Sigma-Aldrich) staining to measure cell viability. Viable cells are not stained by trypan blue, while dead and lysed cells are stained. All USPIO particles were dissolved in cell growth medium at a concentration of 0.3 mg Fe/mL, which is a lower concentration when compared to a sample test because the concentrations of SPIO and USPIO in macrophage cells were lower than sample SPIO and USPIO concentrations. Cells (2×10^6) were incubated in a medium containing USPIO particles for 1, 2, or 4 h at 37°C under 5% CO_2 . After incubation, the medium was removed with two washes of PBS and the cells were fixed with formalin. Cells were stained with trypan blue for 10 min and the viable and dead cells were counted under a microscope. The percentages of viable cells were qualitatively assessed by counting the number of trypan blue-positive cells within five randomly selected 200 μm^2 fields. The cell viability was calculated at least thrice and an average was taken.

Measurement of labeled cells using 11.7-T MRI

All USPIO nanoparticles were dissolved in cell growth medium at a concentration of 0.3 mg/mL. RAW264 cells (2×10^6) were incubated with medium containing USPIO particles for 30 min at 37°C under 5% CO_2 . After incubation, the medium was removed by two washes with PBS and the cells were fixed with formalin. The concentration of RAW264 cells was adjusted to 4×10^6 cells/mL and the cells (0.5 mL) were dissolved in 0.1% agarose gel (0.5 mL) to give a final concentration of 2.0×10^6 cells/mL. The sample temperature was maintained at approximately 23°C and the cells were imaged after settling into a pellet by gravity. For measurement of T_2 relaxation time, T_2 mapping was performed using a multislice multiecho spin-echo sequence (repetition time = 3000 ms; slice thickness = 1 mm; field of view = 25.6×25.6 mm; matrix = 128×128 ; slice orientation = transaxial; number of repetitions = 1) with echo times ranging from 10 to 100 ms in steps of 10 ms. T_2 maps were calculated from a single exponential fitting of MRI signal

intensities at each echo time point. The measurements of labeled cells using 11.7-T MRI was repeated thrice.

Statistical analysis

All statistical analysis was performed using Prism (version 5; GraphPad Software Inc, La Jolla, CA). One-way analysis of variance with the Bonferroni correction was applied in order to compare changes in percentages of SPIO and USPIO uptake, cell viability, and T_2 relaxation times for all SPIO/USPIO nanoparticles and labeled cells. $P < 0.05$ was considered significant.

Results

T_2 relaxation time measurements of USPIO nanoparticles

The MRI contrast provided by each type of nanoparticle formulation was compared by measuring the T_2 relaxation

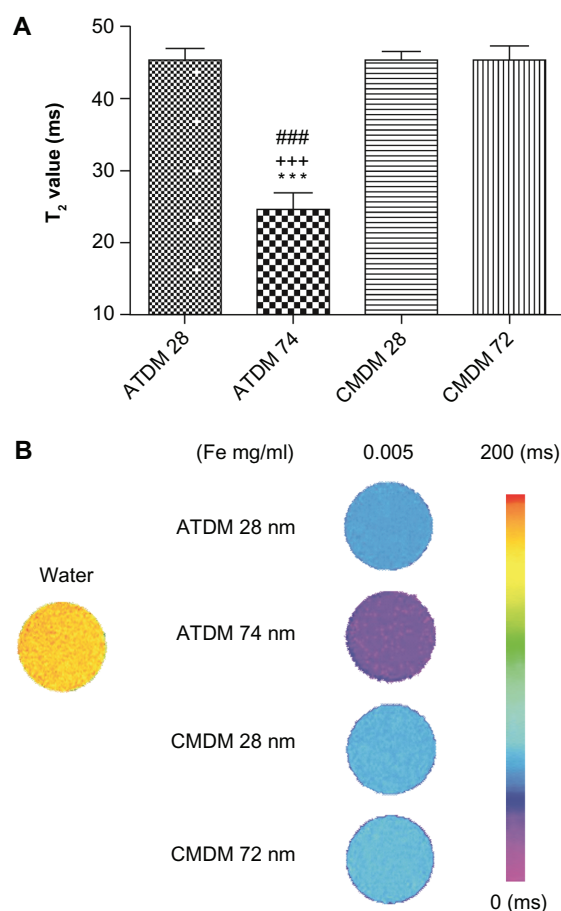


Figure 1 Relaxation time of two SPIO/USPIOs. (A) T_2 maps of control sample (127.5 ± 0.5 ms) for ATDM and CMDM, 28, 72, and 74 nm, at 0.005 and 0.01 mg/mL. The color scale represents T_2 values from 0 ms to 200 ms. (B) Graphical representation of T_2 relaxation time of each of the nanoparticles.

Notes: *** $P < 0.001$, significant difference vs ATDM 28 nm. *** $P < 0.001$, significant difference vs CMDM 28 nm. #### $P < 0.001$, significant difference vs CMDM 72 nm.

Abbreviations: SPIO, superparamagnetic iron oxide; USPIO, ultrasmall superparamagnetic iron oxide; ATDM, alkali-treated dextran magnetite; CMDM, carboxymethyl dextran magnetite.

time. At 0.005 mg Fe/mL, T_2 relaxation time of the 74 nm ATDM-coated nanoparticles was lower than for the other three formulations (Figure 1A). Since a low T_2 relaxation time translates into a high MRI negative contrast, these data suggest that ATDM-coated SPIO nanoparticles would provide the best MRI images among these formulations (Figure 1B).

Evaluation of in vitro USPIO labeling efficiency by microscopy

Microscopy analysis was used to compare the labeling efficiency of each nanoparticle formulation in terms of

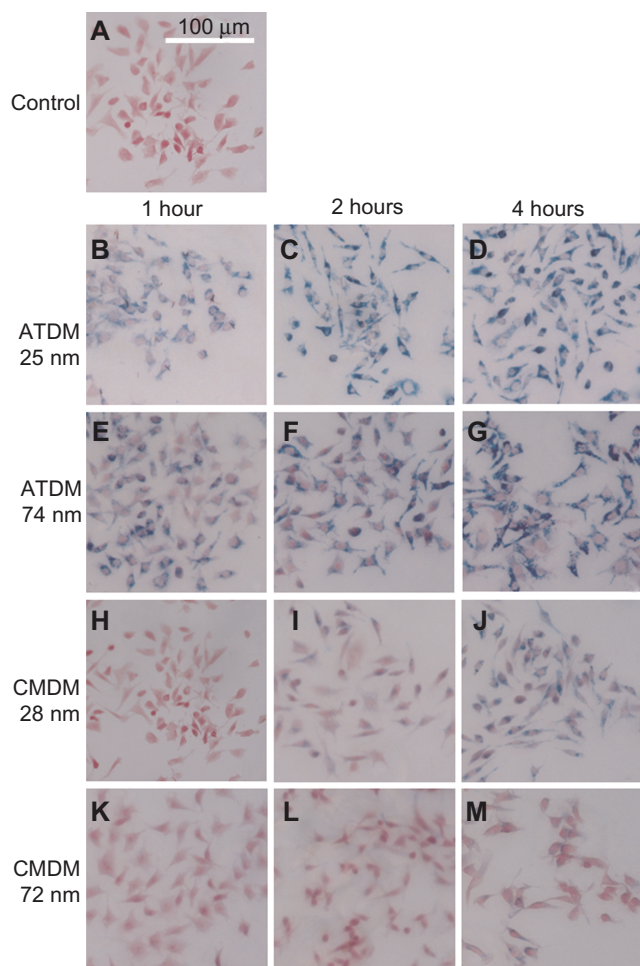


Figure 2 SPIO/USPIO labeled macrophage cells. Microscopy images of labeled cells by ATDM and CMDM. Iron particles are stained blue, and RAW264 macrophage cell are stained red. (A) Unlabeled control cells; (B) ATDM 28 nm labeled cells at 1 h administration; (C) ATDM 28 nm labeled cells at 2 h administration; (D) ATDM 28 nm labeled cells at 4 h administration; (E) ATDM 74 nm labeled cells at 1 h administration; (F) ATDM 74 nm labeled cells at 2 h administration; (G) ATDM 74 nm labeled cells at 4 h administration; (H) CMDM 28 nm labeled cells at 1 h administration; (I) CMDM 28 nm labeled cells at 2 h administration; (J) CMDM 28 nm labeled cells at 4 h administration; (K) CMDM 72 nm labeled cells at 1 h administration; (L) CMDM 72 nm labeled cells at 2 h administration; (M) CMDM 72 nm labeled cells at 4 h administration.

Abbreviations: SPIO, superparamagnetic iron oxide; USPIO, ultrasmall superparamagnetic iron oxide; ATDM, alkali-treated dextran magnetite; CMDM, carboxymethyl dextran magnetite.

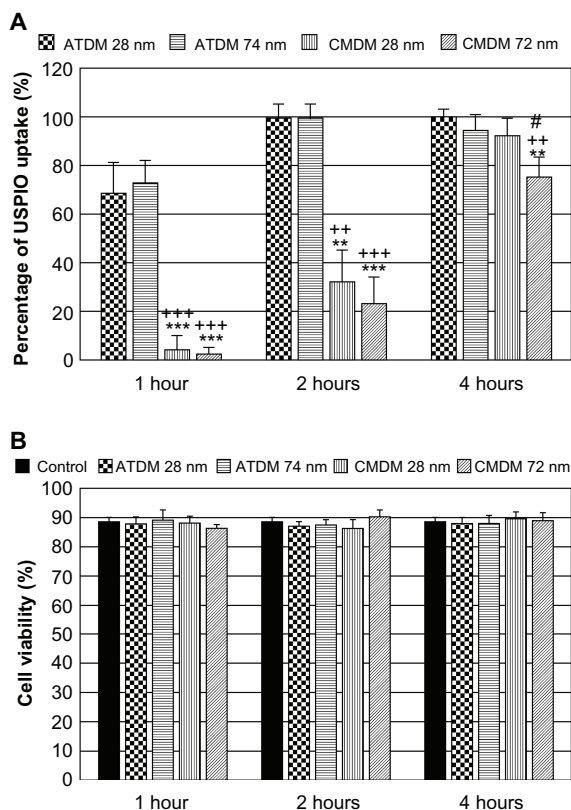


Figure 3 Labeling efficiency and viability in macrophage cells after SPIO/USPIO administrations. (A) Percentage of SPIO/USPIO uptake after SPIO/USPIO administration at 1 to 4 h. (B) Cell viability after each SPIO/USPIO culture.

Notes: $^{**}P < 0.01$, significant difference vs ATDM 28 nm. $^{***}P < 0.001$, significant difference vs ATDM 28 nm. $^{**}P < 0.01$, significant difference vs ATDM 74 nm. $^{***}P < 0.001$, significant difference vs ATDM 74 nm. $^{#}P < 0.05$, significant difference vs CMDM 28 nm.

Abbreviations: SPIO, superparamagnetic iron oxide; USPIO, ultrasmall superparamagnetic iron oxide; ATDM, alkali-treated dextran magnetite; CMDM, carboxymethyl dextran magnetite.

the percentage of macrophages labeled within 200-mm² fields, and the rate of culture saturation over time. Overall, the ATDM-coated nanoparticles provided a more efficient labeling of the macrophages than the CMDM-coated nanoparticles (Figure 2). Quantitative analysis revealed that more than 60% of the macrophages were labeled by ATDM-coated nanoparticles after 1 h, compared to <10% with CMDM-coated nanoparticles, regardless of the particle size (Figure 3A). This profile was maintained after 2 h. However, the efficiency of ATDM-coated nanoparticle labeling had reached 100%, and CMDM-coated nanoparticles were detected only in 20%–30% of macrophages. After 4 h, all nanoparticle formulations had a labeling efficiency superior to 95%, except for 20%–30% for CMDM-coated 28 and 72 nm nanoparticles. Figure 3B shows that none of the nanoparticle formulations affected the viability of the macrophages. Altogether, these data suggest that the use of

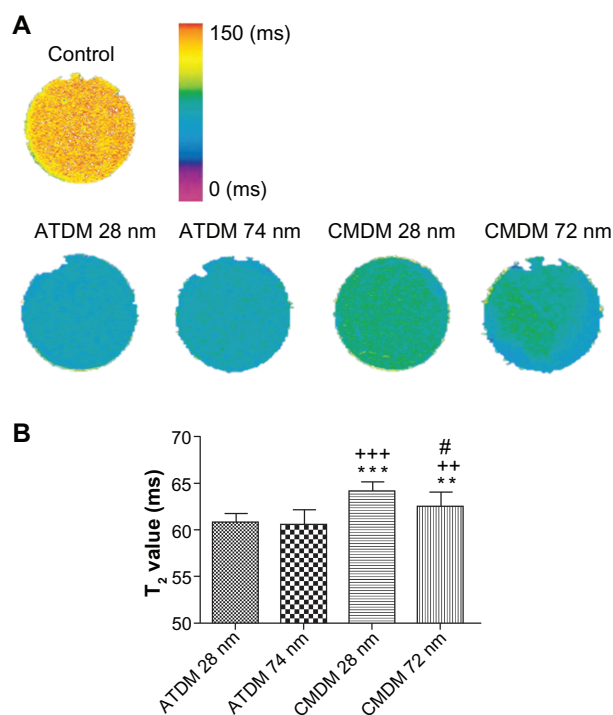


Figure 4 T₂ relaxation times of labeled cells evaluated by MRI. **(A)** T₂ map of control and SPIO/USPIO labeled cell pellets at 30 min cell culture. The color scale represents T₂ values from 0 ms to 150 ms. **(B)** T₂ values of cells labeled by the four SPIO/USPIO. **Notes:** ***P* < 0.01, significant difference vs ATDM 28 nm. ****P* < 0.001, significant difference vs ATDM 28 nm. ***P* < 0.01, significant difference vs ATDM 74 nm. ****P* < 0.001, significant difference vs ATDM 74 nm. #*P* < 0.05, significant difference vs CMDM 28 nm

Abbreviations: MRI, magnetic resonance imaging; ATDM, alkali-treated dextran magnetite; CMDM, carboxymethyl dextran magnetite; SPIO, superparamagnetic iron oxide; USPIO, ultrasmall superparamagnetic iron oxide.

USPIO nanoparticles (28 nm), instead of SPIO nanoparticles (72 and 74 nm), does not improve the labeling efficiency of macrophages. In contrast, ATDM coating provides a more rapid and efficient labeling of most cells for at least 4 h, which is an asset for MRI imaging.

Magnetic resonance imaging of labeled mouse macrophage cells

The T₂ relaxation time of each nanoparticle formulation was measured following a 30-min labeling period of the macrophages to determine the impact of cellular uptake. Figure 4A shows the different MRI signal intensities obtained for the four types of nanoparticles. Quantitative analysis revealed significantly higher T₂ values for the CMDM-coated particles than the ATDM-coated particles (Figure 4B). Among the ATDM-coated particles, USPIO particles generated an even brighter signal change than the SPIO particles. These data suggest that ATDM-coated USPIO particles would offer the best MRI image contrast than all four formulations.

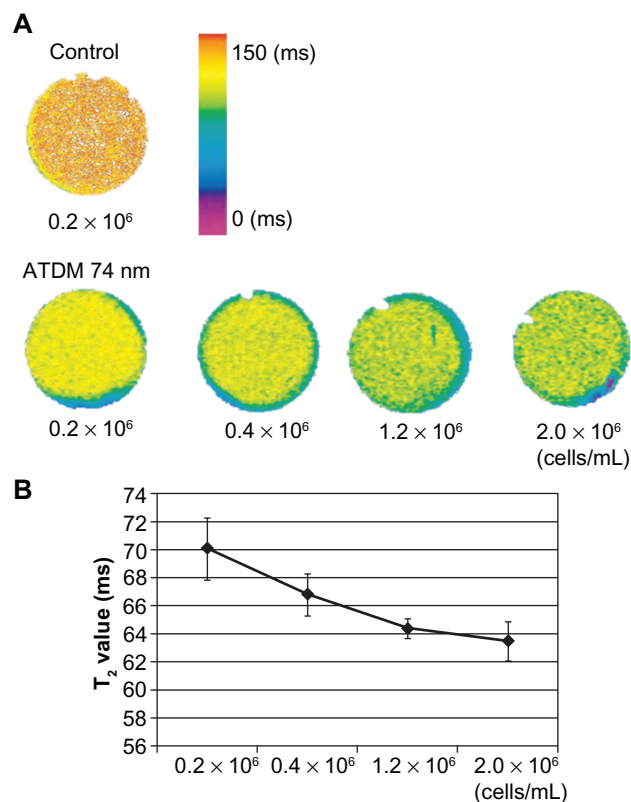


Figure 5 T₂ relaxation times of different labeled cell density using ATDM-74 nm. **(A)** T₂ map of control and ATDM 74 nm labeled cell pellets at 30 min; cell culture at different cell density. The color scale represents T₂ values from 0 ms to 150 ms. **(B)** T₂ values of labeled cells at different cell density. **Abbreviation:** ATDM, alkali-treated dextran magnetite.

Finally, the impact of cell density on the contrast quality of MRI images was tested by measuring the T₂ relaxation time of macrophages labeled with ATDM-coated 74 nm nanoparticles for 30 min. Figure 5A shows that the T₂ relaxation time decreases with increasing cell density. A linear relationship between relaxation time and cell density was observed for 0.4×10^6 macrophages per mL (Figure 5B). These data suggest that the ability of MRI negative contrast agent is increased at high cell density.

Discussion

The present study suggests that SPIO/USPIO labeling efficiency in cultured mouse macrophages was influenced by the particle size and surface coating. The labeling efficiencies of four SPIO/USPIOs in mouse macrophage cells could be visualized by microscopy and 11.7-T MRI.

Oude Engberink et al reported that SPIO particles with a diameter of 150 nm were more efficiently incorporated into mononuclear cells than USPIO particles with a diameter of 30 nm.¹⁹ Particles with larger diameters were more efficiently incorporated into macrophages.^{21,22} After 1 to 4 h of admin-

istration, there were no differences in labeling efficiencies between ATDM at 28 nm and at 74 nm (Figure 3A). In addition, there was no difference in labeling efficiencies by CMDM 1 h after administration. The diameter of SPIO used in previous studies was over 150 nm.^{21,22} The diameter of USPIO used as a control was below 30 nm.^{23,24} The culture time varied from 30 min²¹ to 6 h.⁸ In our experiment, the diameters of USPIO and SPIO particles that were compared were only about 30 and 70 nm and we show that particle size is not an important factor in the uptake of 28 and 74 nm iron nanoparticles.

Using an efficient labeling contrast agent is critical for cell imaging. The most commonly used cell-labeling method is to culture cells with a contrast agent. This method employs surface modification with cations,²⁵ virus transfection,²⁶ lipofectamine,²⁷ and poly-L-lysine²⁸ to increase the labeling efficiency. These positively-charged reagents are cocultured with negatively-charged USPIO to alter the USPIO into a positively-charged compound. Such a charge alteration increases the affinity of USPIO to negatively-charged cell membranes, thereby facilitating the intracellular uptake of the USPIO contrast agent. The mechanism through which the positively-charged contrast agent passes through the cell membrane remains unclear, however, it may include cell wall destruction, adhesion, and internalization. The ATDM-coated particles are more positively-charged compared to the CMDM-coated particles. Our experiment demonstrated a more efficient uptake of iron particles during approximately 0.5–4 h culture for more positively-charged ATDM (–15 mV) than for CMDM (–24 mV). Furthermore, *in vitro* 11.7-T MRI of labeled cells also demonstrated a shorter T_2 relaxation time for ATDM-labeled than for CMDM-labeled cells, although CMDM and ATDM had the same diameter (Figure 4B). The different charge and coating states of the surface potential may have influenced the uptake by cultured macrophages.

Surface coating can influence the cytotoxic nature of the nanoparticle. In a previous study, it has been shown that aminosilane- and dextran-coated nanoparticles did not affect the cell viability of mouse macrophage cells.²⁹ However, silane-coated SPIO nanoparticles negatively affected mouse macrophage cell viability in a dose-dependent manner.²⁹ Moreover, silica-coated SPIO nanoparticles, rather than dextran-coated nanoparticles, displayed dose-dependent cytotoxicity.³⁰ In our study, none of the nanoparticle formulations affected the viability of the macrophages (Figure 3B). Our dextran-coated nanoparticles are safer than nanoparticles

coated with aminosilane and silica.^{29,30} The different charge and coating states of the surface potential did not influence the cell viability of cultured macrophages in the four types of SPIO and USPIO contrast agents that we used.

In the *in vivo* imaging of labeled macrophages, a longer half-life of blood is critical for increasing accessibility to target organs and tissues.^{7,8,28,29} SPIO particles with a diameter of about 50 nm are rapidly incorporated into Kupffer cells in the liver and reticuloendothelial system in the spleen. Hence, the half-life of SPIO in the blood is as short as approximately 4–8 min.^{13,28} On the other hand, USPIO particles with a diameter below 30 nm are barely recognized by the reticuloendothelial system. The half-life of USPIO in blood is as long as approximately 24–36 h in humans and a few hours in rats.^{6,7,31} The current study shows that CMDM-coated nanoparticles label macrophages at a significantly lower rate than ATDM-coated nanoparticles, suggesting that they should remain soluble in blood circulation for a longer period of time. There are no differences in T_2 relaxation time of labeled cells between ATDM at 28 and 74 nm (Figure 4B); however, the T_2 relaxation time of contrast agent differs between ATDM at 28 and 74 nm (Figure 1). Hence, the shortened T_2 relaxation time of labeled cells cannot simply be explained by the difference in the labeling efficiency of cells. Also, no difference was noted in the T_2 relaxation time of contrast agent between ATDM at 28 and CMDM 28 nm (Figure 1), even though the T_2 relaxation times of ATDM labeled cells were shorter than that of CMDM labeled cells (Figure 4B). Thus, ATDM USPIO with a smaller diameter was more efficiently incorporated into cells than CMDM USPIO, thereby providing a more effective *in vivo* contrast agent for macrophages.

Conclusion

We conclude that the best contrast agent would be ATDM-coated 74 nm SPIO nanoparticles. Despite recent interest in smaller particles (USPIO), this study shows that coating plays a more important role than size to optimize MRI contrast, while increasing the nanoparticle labeling time.

Disclosure

The authors report no conflicts of interest in this work.

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