Structure-property relationships in manganese oxide - mesoporous silica nanoparticles used for T1-weighted MRI and simultaneous anti-cancer drug delivery

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The extremely low longitudinal relaxivity ($r_1$) of manganese oxide has severely impeded their substitution for cytotoxic gadolinium-based contrast agents for safe clinical magnetic resonance imaging (MRI). Here, we report on a synthetic strategy of chemical oxidation/reduction reaction in-situ in mesopores, followed by hydrogen reduction, for the fabrication of non-toxic manganese oxide/MSNs-based MRI-T1 contrast agents with highly comparable imaging performance to commercial Gd-based agents. This strategy involves a “soft-templating” process to prepare mesoporous silica nanoparticles, in-situ reduction of MnO$_2$ by the “soft templates” in mesopores and heat treatment under reducing atmosphere, to disperse manganese oxide nanoparticles within mesopores. This special nanostructure combines the merits of nanopores for maximum manganese paramagnetic center accessibility for water molecules for enhanced MRI performance and encapsulation/sustained release/intracellular delivery of drugs. The synthesized manganese oxide/MSNs were successfully assessed as a high performance contrast agent for MRI-T1 both in intro and in vivo, and meanwhile, was also demonstrated as an effective anti-cancer drug delivery (doxorubicin) vehicle, therefore, a family of manganese-based theranostics was successfully demonstrated based on the manganese oxide/MSNs composite.

1. Introduction

Compared to other representative imaging modalities (e.g., X-ray computed tomography, ultrasound, positron emission tomography, single-photon-emission computed tomography), magnetic resonance imaging (MRI) combines the merits of non-invasive diagnostic mode, superb spatial/anatomical resolution and the capacity for quantitative evaluation of disease pathogenesis [1–9]. However, the limited sensitivity and high toxicity of its contrast agents (CAs) cast a shadow on its farther clinical applications [3,4]. The progress of organic/inorganic synthetic chemistry enables the developments and innovations of CAs for MRI in the past decades, in which T$_1$-positive agents of paramagnetic species (producing hyperintense regions on the MR image) and T$_2$-negative agents of superparamagnetic materials (producing hypointense regions on the MR image) are the two typical representatives and have been widely studied. Although superparamagnetic nanoparticles, such as iron oxide nanoparticles, give a signal-decreasing effect (T$_2$-negative agents), the high susceptibility and low degradability of crystallized nanoparticles, however, still limits their extensive clinical applications, in addition to the limited resolution for certain hypointense regions present in many lesions such as hemorrhage and blood clots [8,10,11].

Gd$_3^+$-based small molecule chelates are the representative and preferential CAs for T$_1$-positive MRI because of their high attainable sensitivity, and are playing key roles in disease-diagnosis in current clinical applications (e.g. Magnevist$^\text{®}$ ($r_1 \approx 3.4$ mM$^{-1}$s$^{-1}$)) [3,4]. However, U.S. Food and Drug Administration (FDA) has warned that Gd$_3^+$-based CAs are associated with nephrogenic systemic fibrosis (NSF) in patients with impaired kidney function, hypersensitivity reactions and nephrogenic fibrosing dermopathy (NFD). Therefore, searching for alternatives to Gd$_3^+$ chelates has been urgently recommended and encouraged [12–14].
Manganese is the essential element for physical metabolism and its homeostasis has been efficiently controlled by biological systems. Importantly, Mn (II) ions could be used as the MRI CAs thanks to the five unpaired electrons with long electronic relaxation time [15,16]. However, it is very difficult to design and synthesize highly stable Mn (II) complexes with high sensitivities for clinical applications, which could be partially solved by recently developed nanotechnologies through building manganese-based nanoparticulate systems, such as MnO, Mn2O4, Mn3O4@SiO2, MnO@mesoporous SiO2, and even hollow MnO nanoparticles [17–23]. Unfortunately, the longitudinal relaxivity \( r_1 \) of these nanoparticles were still very low, largely due to that the entrapped paramagnetic centers were largely shielded within the nanocrystal lattices, resulting in the greatly reduced chances of interactions between manganese paramagnetic centers and water molecules [17–23]. Previous results have demonstrated that the accessibility of the paramagnetic centers to water molecules plays a key role in improving the efficiency of nanoparticles as \( T_1 \)-weighted MRI CAs [24]. It is noteworthy that the traditional coating of a silica layer on the surface of manganese oxide nanoparticles could cause the significant reduction of the \( r_1 \) values because of the shielding of the surface active manganese ions from the water molecules by these coating silica. What is responsible for the shortening in the \( T_1 \) relaxation times [19]. The challenge now lies in the enhancement of the accessibility of the manganese paramagnetic centers to water molecules, which is the key issue to be addressed to design highly efficient manganese-based MRI CAs.

Mesoporous nanomaterial systems have been extensively introduced to nano-biomedicine for controlled drug release, cancer diagnosis and anti-cancer chemotherapy, etc [25–30]. Previous results have shown that water molecules can diffuse anisotropically in mesoporous silica with the fastest diffusion component along mesoporous channel [31]. Importantly, ordered mesoporous materials provide an ideal platform to disperse paramagnetic centers as much as possible because of their very large surface areas, diverse pore structures, tunable pore sizes and satisfactory biocompatibility [24]. Considering these two factors, if the manganese paramagnetic centers were distributed evenly within a mesopore system, such a pore system could enable water molecules diffuse freely in mesopores with greatly increased accessibility to interact with manganese paramagnetic centers, which may result in the improved longitudinal relaxivity. Furthermore, the nanoparticles are the ideal drug reservoirs to combine the merits of chemotherapy and disease-diagnosis in one simultaneously.

Herein, we wish to report on a synthetic strategy (oxidation/reduction reaction combined with heat treatment, O/R-HT) to prepare manganese-based theranostics with high longitudinal relaxivity for MRI and high surface area/pore volume for chemotherapy (e.g. doxorubicin) by dispersing manganese oxide nanoparticles into mesopores. This synthetic strategy takes the advantages of the strong oxidation and reduction potentials of MnO2 ions and organic molecules, respectively, for the in-situ dispersion of manganese oxide nanoparticles within mesopores. The advantages of this material system are: (1) Highly dispersed manganese oxides nanoparticles in the penetrating mesopore system ensures the high water-accessibility to manganese paramagnetic centers, and (2) Large surface area and pore volume of mesopores make take up a large amount of therapeutic agents within the pore system possible. Importantly, this synthetic strategy (O/R-HT) could be extended as the general protocol to fabricate diverse manganese-based mesoporous theranostics, which has been demonstrated by introducing OR/-HT process to block copolymer-templated SBA-15-type ordered mesoporous silica nanoparticles (MSNs).

### 2. Materials and methods

#### 2.1. Materials

Sodium hydrosulphate (Na2S2O4), cetyltrimethyl ammonium bromide (C14TAB), oligoethylene oxide (EO) surfactant Pluronic P123 (EO20PO70EO20, Mw = 5800), 3-aminopropyltriethoxysilane (APTES) and fluorescein isothiocyanate (FITC) were purchased from Sigma–Aldrich. Potassium permanganate (KMnO4), tetraethyl orthosilicate (Si(OCH2CH2O)4, or TEDS), hydrochloric acid (HCl, 36–38%), ammonium hydroxide aqueous solution (NH4OH, 25%–28%), absolute ethanol (C2H5OH) was obtained from Sinopharm Chemical Reagent Co. Anti-cancer agent doxorubicin hydrochloride (DOX-HCl) was purchased from Beijing Huafeng United Technology Co., Ltd. Phosphate buffer solution (PBS) was purchased from Shanghai Ruicheng Bio-Tech Co., Ltd. All chemicals were used as received without further purification.

Deionized water was used in all experiments.

#### 2.2. Synthesis of Mn-MSNs

The synthetic procedure for Mn-MSNs involves three steps. Firstly, MSNs were prepared by the typical sol-gel process combined with “soft-templating” method. Briefly, 0.28 g of NaOH was dissolved into 480 mL of deionized water, followed by adding 1.0 g of C14TAB. The mixture was stirred vigorously and the temperature was raised to 80 °C. 5 mL of TEOS was added dropwise into the solution and the reaction was continued for another 2 h to give rise to a white precipitation. The product was collected by centrifugation and washed with plenty of deionized water and ethanol several times. The sample was dried under vacuum at the room temperature for further use. Secondly, chemical oxidation-reduction reaction was activated by the treatment of as-prepared MSNs in KMnO4 aqueous solution. Briefly, 100 mg of as-prepared MSNs were dispersed into 10 mL of deionized water under the ultrasonic treatment. Then 10 mL of KMnO4 aqueous solution (0.1 M) was added into MSNs dispersions dropwise under the magnetic stirring. The mixture was then transferred into water bath (40 °C) and the reaction was continued for another 4 h under the magnetic stirring in the dark. The product was collected by centrifugation and washed with plenty of water under ultrasonic treatment to remove unreacted MnO2 ions. After the sample was dried at 80 °C, the remaining surfactant (C14TAB) was removed by calcination in air at 550 °C for 6 h. Finally, the sample was heat treated in reducing atmosphere. Briefly, the as-prepared manganese oxide dispersed MSNs were treated in mixed H2 (5% volume percentage) and Ar (95% volume percentage) gases at 500 °C for 4 h.

#### 2.3. Synthesis of Mn-SBA-15

The synthetic strategy was based on the O/R-HT process. Briefly, 0.4 g of ZrOCl2·8H2O and 1.25 g of P123 were dissolved into 100 mL of HCl aqueous solution (2 M) in sequence. The mixture was stirred at 35 °C in water bath for 3 h until the solution turns clear. 2.8 mL of TEOS was added dropwise into above clear solution and the reaction was lasted for 24 h. The product was collected by centrifugation, washed thoroughly with water and dried under vacuum at the room temperature. The subsequent KMnO4 treatment (0.005% v/v KMnO4) was similar to the preparation of Mn-MSNs. After the treatment, manganese oxide loaded SBA-15 nanoparticles (Mn-SBA MSNs) were treated in mixed H2 (5% volume percentage) and Ar (95% volume percentage) gases at 500 °C for 4 h.

#### 2.4. Synthesis of FITC conjugated Mn-MSNs and Mn-SBA-15

Briefly, 15 mg of fluorescein isothiocyanate (FITC) was reacted with 100 μL 3-amino propyltriethoxysilane (APTES) in 5 mL of absolute ethanol under dark conditions for 24 h. Subsequently, 20 mg of Mn-MSNs (or Mn-SBA-15) were reacted with FITC-APTES stock solution (1 mL) under dark conditions for 24 h. The FITC grafted nanoparticles were collected by centrifugation and washed with ethanol several times to remove the unreacted FITC-APTES. The product was finally dried under vacuum at room temperature in the dark.

#### 2.5. Confocal laser scanning microscopy (CLSM) observations of the particle-endocytosis by MCF-7 cells

FITC-Mn-MSNs (or FITC-Mn-SBA-15) were suspended in culture media (RPMI 1640) by 30 min sonication in water bath with the concentration of 50 μg/mL. MCF-7 cells were seeded in a CLSM-special cell culture dish until the cell density reaches 50–60% in 5% CO2 at 37 °C. The RPMI 1640 culture media containing FITC-Mn-MSNs (or FITC-Mn-SBA-15) was applied into the seeded cells and co-incubated for 6 h followed by nuclei staining using DAPI (Cell Apoptosis DAPT Detection Kit, KeyGEM). After incubation, the cells were washed three times using PBS. To quench the extracellular FITC fluorescence, trypsin blue was added to the culture medium (200 μg/mL) for 10 min co-incubation. After thoroughly washing to remove trypsin blue, the cells were viewed with a CLSM (FV 1000, Olympus, Japan). To determine the intracellular location of the nanoparticles, Lyso-Tracker Red (lysotracker Red Kit, Beyotime Institution of Biotechnology) was employed to stain the lysosomes of MCF-7 cells after staining nuclei by DAPI. Three-dimensional fluorescence
2.6. CLSM observations of the intracellular release of DOX from DOX-Mn-MSNs

DOX-Mn-MSNs were suspended into RPMI 1640 cell culture media with the DOX concentration of 10 μg/mL, followed by adding the nanoparticle-suspensions into a CLSM-specific culture dish of seeded MCF-7 cells with a cell density of 60–80%. After the co-incubation for different time intervals (2 h, 4 h and 6 h), the cell was washed with PBS for three times and stained by DAPI. The fluorescence images of cells were obtained by a CLSM (FV 1000, Olympus, Japan). Propidium iodide (PI) staining of dead cells was performed to directly observe the therapeutic efficiency of DOX-loaded Mn-MSNs by CLSM. 100 μL of PI working solution was diluted into 4 mL of RPMI 1640 cell culture media, followed by adding 100 μL diluted PI solution into 36 h-incubated cells. After the co-incubation for 15 min, the cells were observed by CLSM (FV 1000, Olympus, Japan).

2.7. Bio-TEM images of nanoparticle-endocytosed cells

The Mn-MSNs (or Mn-SBA-15) were suspended into RPMI 1640 cell culture (100 μg/mL) by sonication for 30 min, followed by introducing nanoparticle-suspensions into seeded MCF-7 cells. After incubation for 24 h, the cells were washed by PBS for three times and detached by incubation with 0.25% trypsin for 5 min. The cells were collected by centrifugation at 5000 rpm/minute for 2 min. Then, the cells were fixed by glutaraldehyde at room temperature after the removal of incubation medium. The fixed cells were rinsed with propylene oxide, embedded in EPOMK12 and polymerized in the oven subsequently at 37 °C for 12 h, 45 °C for 12 h and 60 °C for 48 h. Ultrathin sections of approximately 70 nm thick were cut with a diamond knife on a Leica UC1 ultramicrotome and transferred to the copper grid. The images were viewed on JEM-1230 electron microscopy.

2.8. In vitro and in vivo MRI assay

The in vitro and in vivo MR imaging experiments were performed on a 3.0 T clinical MRI instrument (GE Sigma 3.0 T). For in vitro MRI test, the manganese concentrations of Mn-MSNs dispersed in water were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). T1-weighted Fast-recovery spin-echo (FR-FSE) sequence is described as follows: TR 1000, 2000, 3000 and 4000, Slice = 3 mm, Space = 0.5 mm, Fov = 20, Phase fov = 0.8, Freq × Phase = 384 × 256, Nex = 2, ETL = 2. For in vivo MRI assay, walk 256 cells were implanted subcutaneously into SD female mouse (5 × 106 cell/site) to establish a tumor model. MRI assay were performed when the tumor size reached about 2 cm × 2 cm × 2 cm (body weight = 210 g). For Mn-MSNs, in vivo MRI was carried out at selected time intervals before and after intravenous injection of Mn-MSNs PBS suspensions (dose: 2.8 mg Mn per kg of mouse body); while for Mn-SBA-15, in vivo MRI was conducted via intratumoral injection of Mn-SBA-15 suspensions (dose: 0.27 mg Mn per kg of mouse body). Animal procedures were in agreement with the guidelines of the Institutional Animal Care and Use Committee.

2.9. DOX loading and in vitro release of DOX from DOX-loaded Mn-MSNs under different pH values (pH = 7.4, 6.0 and 5.0)

5 mg of Mn-MSNs was dispersed into DOX PBS solution (6 mL, 0.5 mg/mL). After stirring for 24 h under dark conditions, the DOX-loaded Mn-MSNs were collected by centrifugation. To evaluate the DOX loading content, the supernatant DOX solution was collected and the residual DOX was measured by UV−vis spectra at a wave number of 480 nm. For in vitro DOX release assay, 2 mg of DOX-Mn-MSNs were encapsulated into a dialysis bag and put into 15 mL PBS solutions under different pH values (pH = 7.4, 6.0 and 5.0). Then, the releasing process was performed in a shaking table with a shaking speed of 142 rpm at 37 °C, and monitored by UV−vis adsorption spectra at a time-course.

2.10. In vitro cytotoxicity

The in vitro cytotoxicity was assessed by the typical 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. To evaluate the cytotoxicity of DOX-loaded Mn-MSNs against cancer cells, MCF-7 cells were seeded in a 96-well plate at a density of 2 × 103 per well and cultured in 5% CO2 at 37 °C for 24 h. Free DOX and DOX-loaded Mn-MSNs were dispersed into the culture media (RPMI 1640) with the equivalent DOX concentration, and the cells were incubated in 5% CO2 at 37 °C for 36 h. The concentrations of DOX were 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20 μg/mL, respectively. At the end of the incubation, the culture media were removed and 100 μL of MTT solutions (dissolved in RPMI 1640 with a final concentration of 0.8 mg/mL) were added. The media were then replaced with 100 μL of dimethyl sulfoxide (DMSO) per well, and the absorbance was monitored by a microplate reader (BioTek ELx800) at the wavelength of 490 nm. The cytotoxicity was expressed as the percentage of cell viability compared to untreated control cells. The in vitro cytotoxicity of blank Mn-MSNs nanoparticles were also assessed by similar MTT protocol except for the different initial cell density (1 × 104) in 96-well plate. The concentrations of Mn-MSNs were 0.625, 1.25, 25, 50, 100, 200, 300 and 400 μg/mL, respectively.

2.11. In vivo drug distribution experiment

Balb/c nude mice were inoculated with 100 μL of phosphate buffered saline (pH = 7.4) containing 1 × 107 MCF-7/ADR cells by subcutaneous injection in the left flank. Experiments were performed at day 10 after inoculation when the tumor volume reached approximately 100 mm3. Tumor-bearing mice were randomly assigned to the following three treatment groups (n = 5): saline control group, free DOX group (5 mg/kg) or DOX-loaded Mn-MSNs group (5 mg/kg DOX). In each treatment group, mice were sacrificed at 24 h after intravenous drug administration (n = 5). Heart, liver, spleen, lung, kidney and tumor were collected and weighted. Following this, one part of the tissues were frozen in 0.1% embedding medium (SLEE, Germany) at −20 °C. Frozen sections of 20 μm thickness were prepared with a cryotome cryostat (SLEE, MEY, Germany) and stained with 300 nM DAPI (Sigma) at room temperature. Subsequently, the sections were observed under a confocal microscope (FV 1000, Olympus, Japan). The other part of tissues were homogenized with lysis buffer. The tissue samples were collected into bottles and centrifuged at 15000 rpm for 10 min at 4 °C. The amount of DOX in supernatant of each sample was determined using a microplate reader (Infinite F200, TECAN, Austria). The data were normalized to the tissue weight.

2.12. Characterization

Transmission electron microscopy (TEM)/Scanning transmission electron microscopy (STEM) images were obtained on a JEM-2100F electron microscope operated at 200 kV. Scanning electron microscopy (SEM) images were obtained on a HITACHI S-4800 microscope. UV−vis spectra were recorded on a UV-1601PC Shimadzu UV−vis spectrophotometer. X-ray diffraction (XRD) pattern was collected using a Rigaku D/MAX-2200 PC X-ray diffractometer with Cu target (40 kV, 40 mA). Nitrogen adsorption-desorption isotherms at 77 K were measured on a Micromeritics Tristar 3000 system. Electron spin resonance (ESR) spectra was recorded on a EMX-8/2.7 spectrometer. Inductively coupled plasma atomic emission spectrometry (ICP-AES) test was conducted on VISTA AX (Varian company, America).

3. Results and discussion

3.1. Design, synthesis and structural characterization of Mn-MSNs

As shown in Fig. 1, the overall nanostructural fabrication process involves the synthesis of MSNs templated by “soft templates” such as surfactants or block copolymers, oxidation/reduction (O/R) reaction between organic molecules within MSNs and MnO2 in aqueous solution to in situ form manganese oxide nanoparticles within mesopores and meanwhile the removal of the remaining surfactants, and the heat treatment of nanoparticles under reducing atmosphere. We first chose MCM-41-type ordered MSNs, typically templated by cationic surfactant-cetyltrimethyl ammonium bromide (C16TAB), to demonstrate our hypothesis because their pore and particulate sizes can be facilely controlled and tuned from 2 to 10 nm and 60–110 nm, respectively [24]. Most importantly, the biocompatibility of MSNs has been well-demonstrated and their applications in nano-biomedicine have been widely explored [32,33]. The synthetic procedure for MSNs was based on the NaOH-catalyzed hydrolysis/condensation of tetraethyl orthosilicate (TEOS) in the presence of C16TAB [24]. Then, the surfactant-containing MSNs were dispersed into KMN04 aqueous solutions (0.05 M) and stirred for 4 h at 40 °C in the dark (See experimental section). After the calcination (350 °C, 6 h) to remove the remaining surfactants (C16TAB), the manganese oxide loaded MSNs (designated as Mn-MSNs) were treated in H2/Ar atmosphere (500 °C, 4 h). The design and synthetic target for this nanoparticulate system is to disperse manganese paramagnetic centers within mesopores as thoroughly as possible, making the water-accessibility of magnetic centers as high as possible. It is known that the typical synthetic process for mesoporous materials employs “soft templates” such as surfactants as the pore-making/directing agents, which are in the form of micelles and evenly distributed
Fig. 1. Schematic representation for the preparation of manganese oxide dispersed MSNs (Mn-MSNs) by the O/R-HT strategy. The “soft templates”, typically employed for synthesizing mesoporous materials and evenly be restricted within mesopores, could react in-situ with potassium permanganate (KMnO₄) by chemical oxidation-reduction (O/R), by which manganese oxide nanoparticles are formed and retained within nanopores (Step A). The relaxivity of manganese oxide within mesopores could be enhanced by heat treatment under reducing atmosphere (Step B). Finally, water molecules could diffuse freely into mesopores, and interact with manganese paramagnetic centers.

Fig. 2. Morphological, structural and in vitro MRI characterizations of Mn-MSNs after H₂ reduction. TEM (a) and STEM (a₁) images of Mn-MSNs, EDS elemental mappings of Mn (a₂), Si (a₃) and O (a₄) elements on a Mn-MSNs. Plots of b) $T₁^{-1}$ and c) $T₂^{-1}$ versus Mn concentrations for Mn-MSNs before and after H₂ reduction (inset: T₁- and T₂-weighted MRI results obtained from aqueous suspensions of Mn-MSNs before and after H₂ reduction).
within mesopores before they are removed to create the pores [34,35]. We used potassium permanganate (KMnO4), a cheap and environmental-friendly compound with very high oxidation potential, to oxidize the surfactants in the mesopores by a simple O/R reaction under a mild condition, by which manganese species could be generated in-situ within mesopores with high dispersity. Importantly, the final valence of manganese element could be facilely tuned by optimizing synthetic parameters such as H2 thermal treatment [36,37].

Fig. 2 shows the morphology, structural and chemical characteristics of Mn-MSNs after H2 reduction. The Mn-MSNs exhibits spherical morphology, high dispersity and two-dimensional hexagonally ordered pore array (Fig. 2a and Figs. S1, S2 and S4). To further investigate the distribution of manganese paramagnetic center within mesopores, EDS elemental mapping of Mn, Si and O elements in Mn-MSNs was conducted (Fig. 2a1–a4 and Fig. S3). The result shows that the manganese element is homogeneously distributed in the whole mesoporous silica matrix without the accumulation at the particle surface, demonstrating the pore-confined formation and deposition of manganese oxide nanoparticles within mesopores. Inductively coupled plasma atomic emission spectrometry (ICP-AES) results show that the manganese element content in Mn-MSNs is 5.94% (w/w), which could be facilely tuned by varying the initial synthetic parameters such as MnO4/C0 concentrations, reaction time and temperature. The Mn-MSNs nanoparticles exhibit small-angle X-ray diffraction (SAXRD) peaks at 2.5, 4.3 and 5.0° that are characteristic of (100), (110) and (200) planes of the typical

Fig. 3. FITC-Mn-MSNs are internalized by MCF-7 cells. The MCF-7 cells were incubated with the nanoparticles (FITC-Mn-MSNs, 50 μg/mL) for 6 h. After incubation, the cell nucleus were stained by DAPI (blue), and the extracellular fluorescence was quenched by trypan blue and the endocytosed particles with FITC-label (green) inside cells were detected by CLSM (a1–a3); Three-dimensional fluorescence reconstructions of nanoparticle-endocytosed MCF-7 cells (b1–b3) to demonstrate the internalization of nanoparticles within cancer cells; Three-dimensional fluorescence reconstructions of nanoparticle-endocytosed MCF-7 cells after nucleus and lysosomes double-staining (c1–c4), to demonstrate the intracellular location of Mn-MSNs nanoparticles (nuclei (c1): blue fluorescence of DAPI staining; lysosomes (c2): red fluorescence of Lyso-tracker red staining; Mn-MSNs (c3): green fluorescence of FITC grafting). The yellow fluorescence signal in overlay image (c4), which comes from the superposition of the red and green, suggests that some Mn-MSNs have entered lysosomes while the rests are in the cytoplasm; selected bio-TEM images of MCF-7 cells after co-incubation with Mn-MSNs nanoparticles for 24 h showing (d1) Mn-MSNs uptaken by MCF-7 cells by endocytosed process, (d2) Mn-MSNs trapping in vesicles inside the cell and (d3) endosomal escape of nanoparticles into the cytoplasm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
MCM-41-type mesoporous materials, respectively (Fig. S5a). The SAXRD results indicate that Mn-MSNs possess ordered mesoporous arrays. The wide-angle XRD patterns (WAXRD, Fig. S5b) exhibit no apparent diffraction peaks for crystallized manganese oxide nanoparticles, demonstrating the absence of large manganese oxide particles formed on the material matrix during the synthetic process. Nitrogen adsorption-desorption technique was employed to characterize the pore structures of Mn-MSNs after the H2 reduction, which shows that the isotherms of Mn-MSNs exhibit the typical Langmuir IV type hysteresis loop, indicating the presence of well-defined mesopores (Fig. S6a). In addition, Mn-MSNs are highly porous with a surface area of 665 m²/g, pore volume of 0.99 cm³/g and pore size of 2.4 nm (Fig. S6b), indicating that the mesopore system of MSNs remain open and penetrable after the deposition of manganese oxide species.

3.2. Mn-MSNs as the highly efficient theranostics for T1-weighted MR imaging and simultaneous anti-cancer drug delivery

To assess the effectiveness of Mn-MSNs as MRI CAs, the magnetic resonance relaxivity of the nanoparticles was measured using a clinical 3.0 T human clinical scanner. The in vitro longitudinal relaxation rate (1/T1) and transverse relaxation rate (1/T2) as a function of the manganese ion concentrations of Mn-MSNs before and after H2 reduction were evaluated. As shown in Fig. 2b and c, Mn-MSNs have an r1 value of 0.45 mM⁻¹s⁻¹ and r2 value of 9.1 mM⁻¹s⁻¹ before the heat treatment. However, the specific relaxivity of Mn-MSNs was found to significantly increase after H2 reduction. The r1 and r2 values of Mn-MSNs after H2 reduction reached 2.28 and 15.9 mM⁻¹s⁻¹, 5.1 and 1.7 times higher than their original values before H2 reduction, respectively. It can be inferred that the increased relaxivities of the Mn-MSNs after H2 reduction

![Fig. 4. In vivo MR imaging capability of Mn-MSNs after H2 reduction. A time-course (0, 5, 30, 60 min) of the signal enhancement in T1-weighted MRI of tumor (a1–a4), liver (c1–c4) and kidney (d1–d4) of a tumor-bearing mouse after intravenous injection of Mn-MSNs; b1–b4: magnified images of corresponding circled area in a1–a4.]

Y. Chen et al. / Biomaterials 33 (2012) 2388–2398 2393
are mainly due to the lowered valence of manganese ions under reducing atmosphere (e.g., Mn⁴⁺–Mn²⁺ transformation) [38], like transforming Fe₂O₃ into Fe₂O₄ under H₂/Ar atmosphere [27,28], which was further demonstrated by the higher hyperfine splitting (A) value after H₂ reduction as indicated in electron spin resonance (ESR) spectra of Mn-MSNs (Fig. S7) [39]. Importantly, this A value (2.28 mM⁻¹s⁻¹) is 6.2, 12.7, 17.5 and 19.0 times higher than those of 7 nm (0.37 mM⁻¹s⁻¹), 15 nm (0.18 mM⁻¹s⁻¹), 20 nm (0.13 mM⁻¹s⁻¹) and 25 nm (0.12 mM⁻¹s⁻¹) monodispersed MnO nanoparticles, respectively [19]. It could also be directly observed that the Mn-MSNs nanoparticles decreased both the longitudinal relaxation time (T₁) and the transverse relaxation time (T₂) as can be known from the changes of signal intensity with the increasing manganese concentrations (image insets of Fig. 2b and c), demonstrating that the prepared Mn-MSNs could functionalize as both the T₁- and T₂-weighted MR imaging CAs. The mesoporous silica channel is responsible for high dispersity of manganese oxide nanoparticles and fast rates in the diffusion of water molecules inside the mesopores, which plays a significant role in accounting for the achieved high relaxivity of such a manganese-based Mn-MSNs mesoporous system.

It is known that excellent cell uptake of the CAs guarantees the efficient cell imaging by MRI for diagnosis and intracellular drug delivery for chemotherapy. The Mn-MSNs nanoparticles were covalently conjugated with the typical organic dye fluorescent dye–molecule–protein (FITC-Mn-MSNs) using simple silane coupling chemistry, to make them visible by confocal laser scanning microscopy (CLSM) for the observations of interaction and intracellular location of nanoparticles. Breast cancer MCF-7 cells were chosen as the modal cells, which were incubated with fluorescent nanoparticles for 6 h at 37 °C at a dose of 50 μg/mL. In order to distinguish the nanoparticles only attached to the membrane from those internalized by the cells, the extracellular FITC was quenched by trypan blue [40]. As shown in Fig. 3a1–a3, the green fluorescence punctuated spots lighting in the cytoplasm of the cells could be clearly observed, verifying the uptake of the nanoparticles by cancer cells. Three-dimensional confocal fluorescent image volumes of MCF-7 cells further demonstrate that the fluorescent signal is originated from the cytoplasm, rather than from the aggregates on the cell surface (Fig. 3b1–b3). To verify the intracellular location of Mn-MSNs nanoparticles, the lysosomes were labelled with red fluorescent lysotracker (Fig. 3c1–c4). After 6 h incubation, the green fluorescence of FITC-Mn-MSNs was co-localized with red lysotracker (yellow signal in Fig. 3c4), thus indicating that Mn-MSNs nanoparticles could enter lysosomes of MCF-7 cells while the rest nanoparticles are within the cytoplasm. However, the nanoparticles could not pass through the nuclear membrane as demonstrated by the absence of fluorescent signals of nanoparticles in nucleus. The distribution and possible uptake process of nanoparticles by cancer cells were further observed by bio-TEM after the ultrathin section of the cells. A large number of nanoparticles could be found in the cytoplasm, which maintains their spherical morphology, indicating that the prepared nanoparticles could be efficiently uptaken by cancer cells (Fig. 3d2–d3), in consistence with CLSM results. The endocytosis process, a typical non-specific procedure for the uptake of nanoparticles by cancer cells [41,42], could be observed from Fig. 3d4. Mn-MSNs were enclosed by the cell membrane to form vesicles, then internalized by cells (Fig. 3d3). The Mn-MSNs were further processed in endosomes and lysosomes and eventually released into the cytoplasm (Fig. 3d4).

The effectiveness of Mn-MSNs after H₂ reduction as the MRI CAs in vivo was assessed employing a 3.0 T scanner. Fig. 4 shows the Mn-MSNs contrast-enhanced T₁-weighted MRI of a tumor-bearing

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**Fig. 5.** (a) The release profiles of DOX from DOX-loaded Mn-MSNs at different pH values (pH = 7.4, 6.0 and 5.0; inset: digital pictures of the releasing media in 600 min of release). (b) CLSM images of MCF-7 cells incubated with DOX-loaded Mn-MSNs (DOX concentration: 10 μg/mL) for different time intervals (2 h, 4 h and 6 h; blue fluorescence [b1,b₂ and b₃]: nuclei stained with DAPI; red fluorescence [b₁₂,b₂₂ and b₃₂]: DOX molecules; [b₁₃, b₂₃ and b₃₃]: merged images of blue and red fluorescence; [b₁₄,b₂₄ and b₃₄]: fluorescence intensity of paned area in b₁₂,b₂₂ and b₃₂, respectively). (c) Viabilities of MCF-7 cells when incubated with free DOX and DOX-loaded Mn-MSNs at different concentrations as assessed by MTT protoless. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
mouse. A significant T1-weighted signal enhancement is clearly visible in the tumor site after 5 min tail vein injection (Fig. 4a1–a4 and b1–b4). The signal enhancement implies that Mn-MSNs could reach the tumor site, typically by the enhanced permeability and retention (EPR) effect [43], demonstrating the unique potential of the prepared nanoparticles for use as the T1-weighted MRI CAs for cancer diagnosis. In addition, the live signal enhancement is probably due to the phagocytosis of the Mn-MSNs nanoparticles by the reticuloendothelial systems (RES) (Fig. 4c1–c4). This is beneficial for the MR imaging of liver abnormalities such as liver cancer. A time-course of signal enhancement was clearly observed in T1-weighted imaging of kidney, demonstrating the excretion potential of nanoparticles by kidney system (Fig. 4d1–d4).

To evaluate the potential of Mn-MSNs as the drug delivery system for cancer chemotherapy, a typical anti-cancer chemotherapeutic agent doxorubicin (DOX) was chosen as the model drug to investigate the drug loading and intracellular release behavior. The drug loading amount on the support is 382 mg/g, as determined by UV–vis characterization. The in vitro drug release properties achieved at different pH values (pH = 7.4, 6.0 and 5.0) demonstrated the sustained and pH-responsive DOX release profile from DOX-loaded Mn-MSNs (Fig. 5a), which is determined by the electrostatic interaction between positively charged DOX molecules and negatively charged mesopores. This pH-responsive character is very important because pH values in different tissues and cellular compartments vary significantly. For instance, the tumor extracellular environment is significantly more acidic (pH ≈ 6.5) than blood (pH ≈ 7.4), and the pH values of endosomes and lysosomes are even lower (ca. 5.0–5.5) than cytosolic pH values [44–46].

CLSM was used to study the internalization of the DOX-Mn-MSNs into cancer cells and intracellular release of DOX (Fig. 5b11–b13, b21–b23 and b31–b33). A time-course (2 h, 4 h and 6 h) fluorescent intensity enhancement of DOX in cancer cells indicates the sustained and intracellular release of drugs from DOX-loaded Mn-MSNs (Fig. 5b14–b16). The delivery of DOX into the cancer cells led to growth inhibition and cell death. Mn-MSNs alone show very low cytotoxic effect (IC50 > 400 μg/mL, Fig. 5b), while DOX-loaded Mn-MSNs exhibit a dose-dependent cytotoxic effect, which was slightly higher than that observed with equivalent doses of free DOX in the range of high DOX concentration (Fig. 5c). The quantitative growth inhibition of MCF-7 cells was obtained when the cells were treated with either free DOX or DOX-Mn-MSNs, demonstrating that Mn-MSNs can be used as a drug delivery vehicle (Fig. 5f). Consistent with the in vitro cytotoxicity and CLSM results, the entrapped DOX from Mn-MSNs seems to exhibit the controlled or sustained release behavior in vivo from Mn-MSNs.

The in vivo therapeutic potential was assessed by intravenous injection of free DOX and DOX-loaded Mn-MSNs (DOX-Mn-MSNs) into tumor-bearing mice at a DOX dose of 5 mg/kg. Mice were sacrificed 24 h after the injection of drugs, and the major organs...
were harvested and homogenized for DOX extraction. The concentration of DOX in each organ was measured by fluorescence spectroscopy. In addition, the presence of red fluorescence from DOX in tumor tissue was directly observed by CLSM (Fig. 6a–c). The amount of DOX accumulated in tumor site for DOX-Mn-MSNs was significantly higher than that for free DOX, which was likely due to the accumulation of DOX-Mn-MSNs by the EPR effect (Fig. 6d and inset) [43,47]. The results indicated that Mn-MSNs could induce higher accumulation of DOX in tumor as the drug delivery system with passive tumor targeting property in vivo. However, DOX accumulation in the RES including liver and spleen is relatively high, implying further surface modification of the nanoparticles such as PEGylation is urgently needed for avoiding the capture of nanoparticles by RES to improve the blood circulation time [48].

3.3. Extending the O/R-HT synthetic strategy for SBA-15-type Mn-MSNs

It is known that a majority of mesoporous materials with diverse morphologies and structures can be synthesized by "soft-templat- ing" strategy using various organic molecules as templates, which are believed to react with MnO4/C0 ions similarly with C16TAB to generate a family of manganese-based ordered mesoporous theranostics. To demonstrate the general applicability of the developed O/R-HT process for manganese-based mesoporous theranostics, we chose another typical mesoporous silica system SBA-15, which was obtained using the oligo(ethylene oxide) surfactant Pluronic P123 (EO20PO70EO20, Mw ∼ 5800) as the pore-making agents [49]. To reduce the size of SBA-15-type MSNs for nanomedical requirements, SBA-15 MSNs were synthesized by our recently developed composite liquid crystal templating method for the bottom-up tailoring of nonionic surfactant-templated mesoporous silica nanomaterials by ZrIV [50]. Manganese oxide nanoparticles were introduced into mesopores of SBA-15 (Mn-SBA-15) employing the O/R-HT process by the oxidation of P123 using MnO4 ions (detailed structure characterization, see Fig. S10–S15). The in vitro MRI results show that Mn-SBA-15 MSNs have T1 and T2 values of 1.37 and 20.2 mM −1s−1, respectively, before H2 reduction, while these values of Mn-SBA-15 after heat treatment are both 2.3 times higher (Fig. 7a and b). In addition, Mn-SBA-15 MSNs also show excellent uptake by cancer cells (CLSM and bio-TEM results, see Fig. S16). The in vivo T1- and T2-weighted MRI images of the mouse tumor revealed bright signal enhancement and a dark signal void, respectively (Fig. 7c–f), in the region where the Mn-SBA-15 MSNs were intratumoral injected. The results verify that Mn-SBA-15 MSNs are uniquely potential as the dual CAs for both T1- and T2-weighted MR imaging.

4. Conclusions

In summary, we have developed a general synthetic strategy (O/R-HT) to obtain a highly efficient, cytotoxicity-free manganese-based MRI-T1 contrast agent by dispersing manganese oxide species within mesopores of mesoporous silica nanoparticles (Mn-MSNs). This process involves the preparation of MSNs and the
reduction of MnO$_4$ in-situ by “soft templates” within mesopores afterwards for the free water molecule access and interaction with the highly dispersed manganese oxide species, leading to significantly increased longitudinal relaxivity ($r_1$ values), highly comparable to commercial Gd-based CAS, for MRI, which was demonstrated both in vitro and in vivo. Furthermore, the large surface area and high pore volume of mesopores enable the prepared Mn-MSNs a sustained, pH-responsive and intracellular release of anti-cancer agents (doxorubicin) for chemotherapy. As most mesoporous materials are fabricated by using organic soft templates, we can expect that the present O/R-HT strategy can be used to produce various kinds of manganese-based ordered mesoporous theranostics.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.11.086.

References


