Tumor pH-responsive metastable-phase manganese sulfide nanotheranostics for traceable hydrogen sulfide gas therapy primed chemodynamic therapy

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Abstract

Manganese-based nanomaterials have piqued great interest in cancer nanotheranostics, owing to their excellent physicochemical properties. Here we report a facile wet-chemical synthesis of size-controllable, biodegradable, and metastable $\gamma$-phase manganese sulfide nanotheranostics, which is employed for tumor pH-responsive traceable gas therapy primed chemodynamic therapy (CDT), using bovine serum albumin (BSA) as a biological template (The final product was denoted as MnS@BSA). The as-prepared MnS@BSA can be degraded in response to the mildly acidic tumor microenvironment, releasing hydrogen sulfide (H$_2$S) for gas therapy and manganese ions for magnetic resonance imaging (MRI) and CDT. In vitro experiments validated the pH-responsiveness of MnS@BSA at pH 6.8 and both H$_2$S gas and •OH radicals were detected during its degradation. In vivo experiments showed efficiently tumor turn-on $T_1$-weighted MRI, significantly suppressed tumor growth and greatly prolonged survival of tumor-bearing mice following intravenous administration of MnS@BSA. Our findings indicated that MnS@BSA nanotheranostics hold great potential for traceable H$_2$S gas therapy primed CDT of cancer.

Key words: manganese sulfide, hydrogen sulfide, gas therapy, chemodynamic therapy, nanotheranostics.

Introduction

Cancer nanotheranostics provide a new solution for cancer management using nanotechnology to integrate medical imaging and therapy of cancer 1-7. Magnetic resonance imaging (MRI) is a classical noninvasive medical imaging that benefits high spatial resolution without ionizing radiation and tissue depth limits, which is widely employed in MRI-based nanotheranostics 8-12. For example, Gadolinium (Gd)-nanomaterials and iron oxide nanoparticles have been developed for longitudinal ($T_1$) and transverse ($T_2$) MR contrast agents, respectively 13-18. Recently, manganese-based MR contrast agents, such as manganese sulfide (MnS) 19-21, manganese oxide 22, manganese dioxide 23-27, manganese carbonate 28, and manganese iron 29-30, have been widely explored in the field of cancer.
theranostics. The MnS are mainly included three crystal formations: α-phase, β-phase and γ-phase. The α-phase MnS is highly stable and non-degradable, raising the potential long-term toxicity that hampers its further biomedical application. On the contrary, γ-MnS is metastable and can be degraded in the acidic microenvironment and release manganese ions (Mn²⁺). Therefore, the development of γ-MnS-based nanotheranostics is promising for tumor pH-responsive T₁-weighted MRI of cancer.

For cancer therapy, chemodynamic therapy (CDT) can convert hydrogen peroxide (H₂O₂) into the toxic hydroxyl radical (•OH) at tumor tissues, thus killing tumor cells. Recently, it has been reported that the MnO₂ shell was decomposed in tumor microenvironment to release Mn²⁺, which can catalyze H₂O₂ to produce •OH at the presence of HCO₃⁻ in physiological environments by a Fenton-like reaction manner as that catalyzed by iron ions. This process consumes glutathione (GSH) and enhances CDT effect simultaneously. Therefore, we proposed the released Mn²⁺ from tumor pH-responsive γ-MnS nanoparticles could be also used for CDT of cancer.

Additionally, gas therapy is an emerging therapeutic strategy based on the bioeffects of several kinds of gases, such as nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H₂S) and hydrogen (H₂). H₂S gas is an important endogenous bio-signaling molecule, similar to NO and CO, and exhibits concentration-dependent biological effects. It can be metabolized in mitochondria at nanomolar (nM) concentrations, while at micromolar (µM) concentrations, H₂S gas shows a substantial anti-proliferative effect on MCF-7 breast cancer cells. The anticancer effect of H₂S gas has also been reported in living organisms. However, traditional H₂S donors, such as Na₂S and NaH₂S, the H₂S gas release is too fast to maintain a long-term effect, while organic compounds, such as GYY4137, are too complicated to obtain. Therefore, the development of novel H₂S donors is highly desirable for cancer gas therapy.

The combination of CDT and H₂S gas therapy into a single nanopaltform can achieve enhanced anticancer effect. Because H₂S gas is a signal molecule that, like NO gas, can cause vasodilation and reduce vascular tension in solid tumors. Herein, we developed a facile wet-chemical method to synthesize size-controllable, biodegradable, and metastable γ-phase MnS using bovine serum albumin (BSA) as a template for tumor pH-responsive T₁-weighted MRI of cancer.

**Experimental Section**

**Synthesis of MnS@BSA**

MnS@BSA were synthesized by gradually adding Mn(NO₃)₂ to a Na₂S solution. Typically, different amounts of BSA (2.5, 5, and 10 mg) was added into 400 µL of Na₂S (0.5 M). The volume was...
later set to 40 mL using deionized water and pH was adjusted to 7.4-7.8 using 0.5 M H2SO4. After 5 min of vacuuming to remove O2, the system was protected by N2. Then 2 mL of Mn(NO3)2 (0.05 M) was added to the mixed solution by a micro-injection pump in 30 min. A flesh pink product was produced, washed and concentrated by centrifugation (12000 rpm, 30 min). The obtained nanoparticles were named as MnS@BSA-2.5, MnS@BSA-5 and MnS@BSA-10. The MnS@BSA-10 with uniform size was used for further studies.

Levels of Mn2+ and H2S Released from MnS@BSA

For Mn2+, 400 μL of MnS@BSA (5.5 mM) was added into a 3500 Da dialysis bag with PBS buffer (50 mL, 10 mM) at pH 6.8 and 7.4, respectively. The solutions were kept stirring at 37 °C and 2 mL was removed for analysis at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 h. Mn element quantification was performed by using an ICP-AES. For H2S, 400 μL of MnS@BSA (5.5 mM) was added into a 3500 Da dialysis bag with HEPES buffer (50 mL, 10 mM) at pH 6.8 and 7.4, respectively. 1 mL of the solution was removed as a working solution for H2S analysis at 0, 1, 2, 5, 10, 20, 30, 60 min. H2S concentration was analyzed using a standard MB method. Briefly, 100 μL of the working solution, 100 μL of 1% \((w/v)\) Zn(OAc)2, 20 μL of DMPD (20 mM) and 20 μL of FeCl3 (30 mM) were mixed, kept at room temperature for 15-20 minutes, and the optical density (OD) at 663 nm was measured.

Hydroxyl Radical Catalyzed by MnS@BSA

MnS@BSA or MnCl2 was added into 10 μg/mL MB solution which containing 25 mM NaHCO3 and 10 mM H2O2. The OD at 665 nm of the above mixture solution was monitored by UV-Vis spectrophotometer at different time points from 0 to 60 min. For ESR detection, 25 mM of NaHCO3 were used as the solvent for H2O2 solution was monitored by UV-Vis spectrophoto-meter at different time points from 0 to 60 min. To assess the cytotoxicity of MnS@BSA, various concentrations of antioxidant lascorbic acid (AA, 0-80 μM) in 10 μM with different concentration of MnS@BSA ([Mn]=0-200 μM) were tested. The standard MTT assay was carried out to evaluate the cell viability.

Hydroxyl Radicals and H2S in Cells

\(5\times10^5\) of 4T1 cancer cells were digested and resuspended into 2 mL of DMEM and subcultured into \(\varphi 15\) confocal laser scanning microscopy (CLSM)-exclusive culture disks for another 12 h. Subsequently, the medium was removed and the disks were rinsed by PBS twice before adding 1 mL of DMEM containing 50-200 μM of MnS@BSA, 200 μM of MnCl2. Finally, the medium was removed and the fluorescence probe addition: 25 μM of non-fluorescent DCFH-DA was added to reduce hydroxyl radical for 30 min, then the fluorescence of DCF was observed on CLSM. For H2S, \(2.5\times10^5\) 4T1 cancer cells were subcultured into 24-well plates for 12 h. After incubated with 100 μM of the WPS-5 probe for 30 min, then treated with 0, 50, 100, 200 μM of MnS@BSA and 200 μM of NaS. Then added HEPES containing 100 μM of CTAB (pH 7.4). After 10 min, fluorescence images were acquired.

In vivo Imaging and Biodistribution

Tumor-bearing mice were scanned with a 3T clinical United Imaging 790 MRI scanner (United Imaging, Shanghai, China) before and after intravenous administration of 2.5 mg/kg of Mn (MnS@BSA or MnCl2). T1-weighted images were acquired by FSE sequence at 0, 1, 2, 4, 8, and 24 h and the following parameters were applied: TR=700 ms, TE=14.3 ms, Flip Angle=145 °, matrix size, 160 x 160, slice thickness, 1.5 mm. Signal intensities were measured in defined regions of interest (ROIs) with software named Image J. MRI were performed at 0, 1, 2, 4, 8, 24 h post-injection.

Six tumor-bearing mice were divided into two groups (n=3). After administration of 2.5 mg/kg of MnS@BSA through their tail veins. One group was euthanized after 4 hours, the other was euthanized after 24 hours. Tumors and major organs (heart, liver, spleen, lung, kidney and muscle) were obtained and washed with PBS. Each organ was immersed in 3 mL of nitric acid overnight, and then heated to 150 °C. The final volume was set to 1.5 mL. The concentration of Mn was measured by ICP-AES to estimate the in vivo bio-distribution of MnS@BSA.

In vivo Combination Therapy

The therapeutic effects of MnS@BSA were examined on 4T1-Luciferase mammary tumor xenograft on BALB/c mice. Tumor-bearing mice were randomly divided into seven groups: 5 mice per group, administered dose was 2.5 mg/kg MnS@BSA. The seven groups were: PBS, MnCl2, NaS, MnS@BSA -2.5, MnS@BSA -5, MnS@BSA -10 and MnS@BSA -10+AA. Their body weights and tumor volumes were measured in defined regions of interest (ROIs) with software named Image J. MRI were performed at 0, 1, 2, 4, 8, 24 h post-injection.
measured every two days to evaluate the therapeutic performance. FL imaging of mice were recorded on day 2, 4, 6, 8 and 14 post-treatments. Before FL imaging, 15 mg/mL of D-luciferase potassium salt was intraperitoneally injected at a dose of 10 μL/g, and bioluminescence imaging was performed at 10-15 min post-injection for tumor growth evaluation.

Results and Discussions

Preparation and Characterization of MnS@BSA

The α-MnS was commonly synthesized by the high temperature decomposition of manganese oleate in oil phase. It was not water-soluble and needed further surface modification for biomedical applications. In our case, MnS@BSA was synthesized by a wet-chemical method. Briefly, manganese source (MnCl₂) was slowly added to a mixture solution of Na₂S and BSA, which was vacuumed and protected with N₂ gas to obtain a pink solution, the typical color of γ-phase MnS. XRD pattern (JCPDS Card No.40-1289) further confirmed that MnS@BSA is γ-phase MnS. As shown in Figure 1B, MnS@BSA exhibited well-defined sphere. The size of MnS@BSA can be controlled from 300 to 150 nm by adjusting the amount of BSA from 2.5 to 10 mg. More BSA were added, the size of MnS@BSA were smaller (Figure S2). The MnS@BSA (10 mg of BSA) with the smallest size (~ 150 nm) was selected for the following experiments. As shown in the element mapping of MnS@BSA (Figure 1D), C, N and O elements were assigned to BSA, S and Mn elements were assigned to MnS. These results indicated that γ-phase MnS was successfully synthesized using BSA as template, which can improve its solubility and biocompatibility.

Hydroxyl Radicals and H₂S of MnS@BSA

The as-synthesized MnS@BSA can be easily decomposed and oxidized in aqueous solutions containing oxygen, especially under acidic conditions. As shown in Figure 2A and 2B, MnS@BSA was degraded much faster in the acidic solution than in neutral solution.
the neutral solution. These results are consistent with the levels of Mn$^{2+}$ released from MnS@BSA in PBS buffer at different pH values (Figure 2C). After 8 h, 98.3% of MnS@BSA was degraded in acidic solution, while only 57.3% of Mn$^{2+}$ was released at pH 7.4. Afterwards, the degradation of MnS@BSA in 4T1 cells was observed using bio-TEM imaging at 8 and 24 h post-incubation (Figure S3). A lot of MnS@BSA were observed inside 4T1 cells after 2 h incubation. After 24 h incubation, sphere MnS@BSA were hardly found in 4T1 cells. These results indicated that MnS@BSA could be degraded in 4T1 cells gradually. Meanwhile, the concentration of released H$_2$S from MnS@BSA under different pH conditions was measured using the methylene blue (MB) method. The H$_2$S gas from MnS@BSA was released much faster in the acidic solution than in the neutral solution (Figure 2D). The concentration of H$_2$S can reach up to 12 µM, which is able to kill cancer cells efficiently. These results suggested that pH-responsive MnS@BSA could be fully degraded in aqueous solutions at pH 6.8, followed by the release of Mn$^{2+}$ and H$_2$S gas, which promises for cancer diagnosis and treatment.

Manganese element is a trace element in living organisms, which may catalyze Fenton-like reactions in physiological environment to produce highly toxic •OH radicals. The Mn$^{2+}$ and HCO$_3$- in the blood constitute the catalysts for Fenton reactions, which can consume enriched H$_2$O$_2$ in tumor microenvironments to generate •OH radicals. In order to evaluate the Fenton-like reactivity of MnS@BSA, MnS@BSA was added into MB solution containing HCO$_3$- and H$_2$O$_2$, as shown in Figure 2E. The degradation of MB indicated the concentration dependency of manganese concentrations. Both MnCl$_2$ (used as a control) and MnS@BSA produced •OH that could degrade MB at a similar rate (Figure S4). A unique quadruple peak of •OH was observed in electron spin resonance (ESR) spectra (Figure 2F), but was not observed in control groups. All results indicated that released Mn$^{2+}$ ions from MnS@BSA can generate a lot of •OH under physiological conditions.
In vitro Combination Therapy

Next, in vitro experiments of MnS@BSA were performed to evaluate its combination cancer therapeutic effect and cytotoxicity. As shown in Figure 3A, cell viability showed a concentration-dependent therapeutic effect on 4T1 cells when incubated with Na2S, MnCl2 and MnS@BSA (10-200 µM), which is in agreement with the live/dead cell staining results (Figure S5). The therapeutic effect of MnS@BSA on tumor cells can be attributed to the bioeffects of both H2S gas and •OH radicals. The mortality of 4T1 cells caused by H2S gas and •OH radicals was calculated in Figure 3B. As one can see that 65-69% of cytotoxicity was induced by •OH radicals, which suggested •OH radicals were more potent than H2S gas at all tested concentrations. Interestingly, Na2S, MnCl2 and MnS@BSA showed negligible cytotoxicity on MCF10A cells (a control cell line) as evidenced by the maintained cell viability at ~75% at all tested concentrations (10-200 µM) (Figure S6). The different cytotoxicity between 4T1 and MCF10A cells was attributed to the levels of H2O2 inside cells. Indeed, the H2O2 concentration of 4T1 cells was 2.2 times higher than that of MCF10A (Figure 3C), followed by HeLa and A375 cells. This higher concentration of H2O2 allowed for the enhanced •OH generation for responsive CDT treatment of 4T1 cells but no other cell lines. Therefore, MnS@BSA elicited an improved therapeutic effect on tumor cells with high levels of H2O2. To further demonstrate the therapeutic mechanism, reductive L-ascorbic acid (AA) was used to neutralize •OH radicals at cell level. By adding 10 µM of AA to different concentrations of MnCl2 (Figure 3D) or MnS@BSA (Figure 3E), 4T1 cell viability could increase by 10-21%. In particular, with the increase of AA, toxic effects of •OH radicals produced by MnS@BSA were gradually decreased (Figure 3F). These results indicated that therapeutic effects of MnS@BSA were attributed to the combination effects of •OH radicals and H2S gas.

![Figure 3.](http://www.thno.org)
In order to directly observe the generation of •OH radicals \textit{in vitro}, 4T1 cells were incubated with different concentrations of MnS@BSA for 4 h. Then the probe of oxidative stress, 2’,7’-dichlorofluorescin diacetate (DCFH-DA), was added. After deacetylates with intracellular esterase to form the non-fluorescent 2’,7’-dichlorofluorescin (DCFH), DCFH would be further oxidized into bright green fluorescent dye, 2’,7’-dichlorofluorescein (DCF) by reactive oxygen species (ROS) including •OH radicals\textsuperscript{36, 49}, which can be observed by confocal laser scanning microscopy (CLSM). As expected, the fluorescence intensity of DCF was elevated as the amount of MnS@BSA increased from 50 to 200 µM, which suggested the increase of oxidative stress strongly depended on the concentration of MnS@BSA (Figure 3G). These results are consistent with the results of Fenton-like reaction mentioned above. Similarly, free MnCl\textsubscript{2} also could induce the generation of ROS. Meanwhile, H\textsubscript{2}S gas released in 4T1 cells was observed by adding a Washington State Probe-5 (WSP-5) H\textsubscript{2}S fluorescent probe (Figure 3H). It is based on 2-pyridyl disulfide fluorescent, which release the fluorophores and turn on the fluorescence by tandem nucleophilic substitution-cyclization reaction. WSP-5 probe can selectively and rapidly react with H\textsubscript{2}S in cells to generate a green fluorescence signal\textsuperscript{50}. The fluorescence intensity of WSP-5 was increased in accordance to the added concentration of MnS@BSA. The same effect was also evidenced in the Na\textsubscript{2}S group, further indicating that MnS@BSA can release H\textsubscript{2}S gas inside cells. All these results proved that MnS@BSA can generate •OH radicals and H\textsubscript{2}S in 4T1 cells, promising for combined CDT and H\textsubscript{2}S gas therapy.

\textit{In vivo Imaging and Biodistribution}

The released Mn\textsuperscript{2+} ions from MnS@BSA are not only a catalyst for the Fenton-like reaction, but also a contrast agent for tumor pH-responsive \(T_1\)-weighted MRI. Since MnS@BSA is pH-responsive in acidic solutions, its longitudinal relaxivity (\(r_1\)) was measured under different pH conditions, as shown in Figure S7. The \(T_1\) relaxivity of MnS@BSA, \(r_1\), was calculated to be 23.67 mM\textsuperscript{-1}s\textsuperscript{-1} in a pH 6.8 buffer at 2 h. Encouraged by the high \(r_1\) value of MnS@BSA at pH 6.8, we performed \textit{in vivo} tumor pH-responsive MRI of MnS@BSA on 4T1 tumor-bearing mice after intravenous (i.v.) injection with MnCl\textsubscript{2} as a control. Tumor signal of 4T1-bearing mice treated with MnS@BSA clearly increased over time, while the tumor signal kept no change for mice treated with MnCl\textsubscript{2} (Figure 4A and B). The tumor to normal ratio (TNR) of mice treated with MnS@BSA reached to a peak number of 2.26 at 2 h post-injection (Figure 4C). The mice treated with MnCl\textsubscript{2} showed negligible MR contrast enhancement. These results were further confirmed by the biodistribution study of Mn element at 4 h post-injection (Figure 4D). Since Mn\textsuperscript{2+} can be gradually excreted through metabolism, we also monitored Mn levels in excrements (Figure 4E). At 48 h post-injection, Mn concentration in mice was reduced to 0.02%, suggesting that MnS@BSA could be fully excreted within one day. These results suggested MnS@BSA could be fully cleared from the body of mice and exempt from the risk of potential long-term toxicity.
**In vivo Combination Therapy**

Finally, *in vivo* MnS@BSA-induced therapeutic effect was studied on 4T1-luc tumor-bearing mice. Mice were randomly divided into 5 groups: (i) saline, (ii) MnCl₂, (iii) Na₂S, (iv) MnS@BSA, (v) MnS@BSA+AA. The tumor volumes were monitored every 2 d during 2 weeks and normalized to their initial size in Figure 5A. The saline control group showed fast tumor growth, whereas the MnS@BSA treated group exhibited higher tumor suppression as compared with MnCl₂ and Na₂S treated group. The MnS@BSA+AA treated group exhibited obvious tumor regrowth after day 11, due to the neutralization of •OH radicals by AA. These results suggested the combination effect of CDT/gas therapy over any single modality treatment solely. For all groups, mice showed no noticeable body weight change, suggesting no systemic toxicity of MnS@BSA (Figure 5B). Meanwhile, the survival of mice administered with MnS@BSA was greatly prolonged (Figure 5C). More importantly, no obvious toxicity was induced by MnS@BSA, as evidenced by results of blood biochemistry, hematology analysis, and acute toxicity measurement of all major organs (Figure S8-10). Furthermore, all tumor growth was also monitored using bioluminescence imaging during treatment period. As shown in Figure 5D, the tumor luminescence was located on right hind of all groups in the whole treatment process. Especially, only the tumor of MnS@BSA group showed obviously suppression effect, but tumors of other groups kept growth. After different treatments, H&E staining images of all treatment groups were taken (Figure 5E). Tumors of saline, MnCl₂ and Na₂S groups show more chromatin and large nuclei, indicated slight effect of tumor cell proliferation. But for MnS@BSA treated group, the tumor slices shown that the chromatin was pyknotic or even absent in the slice. MnS@BSA-AA group can consume some •OH to reduce the oxidative damage, which show more chromatin than MnS@BSA group. The tumors treated with CDT/gas combination therapy showed much higher damage than other groups.

**Conclusions**

In conclusion, we have developed a tumor pH-responsive metastable-phase MnS@BSA for combined CDT and H₂S gas therapy. The as-prepared metastable-phase MnS@BSA is able to response mildly acidic microenvironment and can release Mn²⁺ for Fenton-like reaction to generate •OH at the presence of endogenous H₂O₂ of tumor cells. The MnS@BSA can also generate H₂S to benefit gas
therapy of cancer. Furthermore, the size-controllable MnS NPs can be used as an MRI contrast agent for treatment monitoring owing to its high r1 relaxivity of Mn2+ ions. This work provides a new design strategy of a nanotheranostic agent for traceable H2S gas therapy ydrogen primed CDT. Our findings open new horizons for the biomedical applications of manganese-based cancer theranostics.

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Supplementary Material

Supplementary figures and tables. http://www.thno.org/v10p2453s1.pdf

Competing Interests

The authors have declared that no competing interest exists.

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