Modulation of Hypoxia in Solid Tumor Microenvironment with MnO₂ Nanoparticles to Enhance Photodynamic Therapy

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Hypoxia not only promotes tumor metastasis but also strengthens tumor resistance to therapies that demand the involvement of oxygen, such as radiation therapy and photodynamic therapy (PDT). Herein, taking advantage of the high reactivity of manganese dioxide (MnO₂) nanoparticles toward endogenous hydrogen peroxide (H₂O₂) within the tumor microenvironment to generate O₂, multifunctional chlorine e6 (Ce6) loaded MnO₂ nanoparticles with surface polyethylene glycol (PEG) modification (Ce6@MnO₂-PEG) are formulated to achieve enhanced tumor-specific PDT. In vitro studies under an oxygen-deficient atmosphere uncover that Ce6@MnO₂-PEG nanoparticles could effectively enhance the efficacy of light-induced PDT due to the increased intracellular O₂ level benefited from the reaction between MnO₂ and H₂O₂, the latter of which is produced by cancer cells under the hypoxic condition. Owing to the efficient tumor homing of Ce6@MnO₂-PEG nanoparticles upon intravenous injection as revealed by T1-weighted magnetic resonance imaging, the intratumoral hypoxia is alleviated to a great extent. Thus, in vivo PDT with Ce6@MnO₂-PEG nanoparticles even at a largely reduced dose offers remarkably improved therapeutic efficacy in inhibiting tumor growth compared to free Ce6. The results highlight the promise of modulating unfavorable tumor microenvironment with nanotechnology to overcome current limitations of cancer therapies.

1. Introduction

Hypoxia, severe oxygen starvation, has been considered to arise in solid tumors due to irregular cancer cell proliferation and distorted blood tumor vessel development. It is also recognized as one of the characteristic hallmarks in solid tumors with insufficient oxygen supply, directly contributing to the malignant properties of cancers. It is known that hypoxia plays essential roles in tumor angiogenesis and cancer metastasis. Moreover, hypoxia would also lead to resistance to medical therapies, such as radiotherapy, chemotherapy, and photodynamic therapy (PDT), the latter of which is a noninvasive cancer treatment approach utilizing light to activate photosensitizers, generating singlet oxygen (SO) and reactive oxygen species in the presence of oxygen to kill tumor cells. To date, there have been various methods proposed to modify the hypoxic tumor microenvironment by promoting the oxygenation of tumors. For example, artificial blood substitutes such as perfluorocarbon-based oxygen carriers have been used to transport oxygen into the tumor. Such a method may still have limited efficiency, particularly toward tumor cells locating far from the intratumor blood vessels. In situ production of oxygen inside the tumor may be a more effective approach to overcome current limitations of cancer therapies.

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broken up under reduced pH could be used as a pH responsive drug carrier, showing enhanced drug release in the acidic tumor microenvironment.[34] Besides, MnO2 nanosheets based nanoprobes were also used as capping agents to block pores of mesoporous nanoparticles to obtain glutathione (GSH)-responsive drug delivery systems and GSH-activated magnetic resonance (MR) contrast agents (the released Mn2+ offers strong T1 MR contrast).[35] Moreover, on account of the unique reactivity of MnO2 nanoparticles with H2O2 to sustainably produce O2, a number of groups have reported that MnO2 nanoparticles or MnO2-containing upconversion nanocomposites upon intratumoral local injection could improve tumor oxygenation in vivo, so as to enhance the performance of radiation therapy.[32,35,36] Another recent study also uncovered that the chemotherapeutic efficacy could also be enhanced by MnO2 nanoparticles.[37] Furthermore, the MnO2 nanoparticles could be decomposed into Mn3+, which is water soluble and could be rapidly excreted, to avoid unwanted in vivo accumulation and long-term toxicity.[38] Despite those encouraging previous studies, the use of MnO2 nanoparticles by systemic administration to enhance in vivo PDT remains to be demonstrated to the best of our knowledge.

Herein, we sought to utilize the compelling characteristics of MnO2 nanoparticles for modulation of tumor microenvironment by their reactivity toward H2O2 to overcome the tumor photodynamic resistance through in situ O2 generation. As-synthesized MnO2 nanoparticles stabilized by cationic polyelectrolyte poly(allylamine hydrochloride) (PAH) were conjugated with the photosensitizer, chlorine e6 (Ce6), coated with anionic polymer polyacrylic acid (PAA), and then further conjugated with amino terminated polyethylene glycol (PEG-NH2) via amide bonds to increase the nanoparticle water solubility and physiological stability.[39] Owing to in situ generated O2 from the reaction between MnO2 and H2O2, our Ce6@MnO2-PEG nanoparticles exhibit high in vitro PDT efficacy even under oxygen-deficient atmosphere, in which the photoxicity of free Ce6 is greatly reduced. As vividly illustrated by in vivo T1-weighted MR imaging and confirmed by ex vivo biodistribution measurement, Ce6@MnO2-PEG after systemic intravenous (i.v.) injection shows efficient tumor retention as well as rapid kidney filtration, the latter of which is likely attributed to the MnO2 nanoparticles, whose sizes, charges, and surface properties were finely controlled by polymer coating and PEGylation. Herein, Ce6 was activated in advance using 1-(3-(dimethylamino) propyl)-3-ethy carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) in dimethyl sulfoxide (DMSO), and then added into the aqueous solution of PAH-coated MnO2 nanoparticles at different weight ratios (Ce6:MnO2 = 0.1:1, 0.2:1, 0.5:1, 1:1, and 2:1) under ultrasonication and stirred at room temperature for 4 h. After removal of excess Ce6, UV–vis spectra of these samples were recorded to determine the Ce6 loading capacity (Figure 1b) and Supporting Information Figure S2). The Ce6 loading achieved a relatively high ratio of ≈45.81% (Ce6:MnO2, w/w) at the Ce6:MnO2 feeding ratio of 2.1(w/w), and the obtained Ce6-conjugated PAH-coated MnO2 (Ce6@MnO2-PAH) nanoparticles prepared under this condition were used for further experiments.

After Ce6 conjugation, the zeta potential of MnO2 nanoparticles decreased from ≈55.6 mV for MnO2-PAH to ≈20.3 mV for Ce6@MnO2-PAH, but remained to be positively charged (Figure 1e). Next, surface modification was applied onto the as-made Ce6@MnO2-PAH nanoparticles by a layer-by-layer (LBL) polymer coating method. Utilizing the electrostatic interaction, an anionic polymer PAA was used to coat Ce6@MnO2 nanoparticles by simple mixing. After removing excess PAA, amino-terminated PEG (Mr ≈ 5000) was then conjugated to carboxyl groups on the Ce6@MnO2-PAH-PAA surface via EDC-induced amide formation, obtaining PEGylated Ce6@MnO2 (Ce6@MnO2-Ce6) nanoparticles. The zeta potential of those nanoparticles switched from +20.3 to −23.4 mV after PAA coating and increased to −6.5 mV after PEGylation, indicating successful LBL coating of polymers on MnO2 nanoparticles (Figure 1b). The sizes of those nanoparticles, however, increased from ≈30 to ≈100 nm, as illustrated by both diameter light scattering (DLS) (Figure 1f) and TEM imaging (Figure 1g). The increase of nanoparticle sizes could be attributed to the partial aggregation of MnO2 nanoparticles formed during Ce6 conjugation and LBL polymer coating. Despite the increase of nanoparticle sizes after modification, the final product, Ce6@MnO2-PEG nanoparticles, exhibited great dispersity in various physiological solutions (Figure 1f, inset), in marked contrast to as-made

2. Results and Discussion

The procedure for the synthesis of Ce6@MnO2-PEG is illustrated in Figure 1a. MnO2 nanoparticles were first produced according to the literature method.[38] In brief, manganese permanganate (KMnO4) was reduced to MnO2 nanoparticles in the presence of cationic polyelectrolyte PAH, giving stable PAH-coated MnO2 (MnO2-PAH) nanoparticles with a dark brown color. As illustrated by UV–vis spectra (Figure 1b), the characteristic KMnO4 peaks (315, 525, and 545 nm) disappeared after this reaction, while a new broad absorbance band around 300 nm appeared, which should be resulted from the surface plasmon band of colloidal manganese dioxide.[40] X-ray photoelectron spectroscopy (XPS) was applied to investigate the chemical state of manganese element in the as-made MnO2-PAH sample (Figure 1c). The two characteristic peaks at 654.2 and 642.4 eV, which corresponded to the Mn (IV) 2p3/2 and Mn (IV) 2p1/2 spin–orbit peaks of MnO2, respectively,[41] evidenced that KMnO4 was reduced into MnO2 by PAH. Also, X-ray diffraction spectrum of the product indicated successful formation of MnO2 and its orthorhombic crystal structure (Supporting Information Figure S1). As revealed by transmission electron microscope (TEM) (Figure 1d), as-made MnO2-PAH nanoparticles showed average sizes at ≈30 nm.

To formulate MnO2 nanoparticles for biomedical PDT applications, the photosensitizer, Ce6, was conjugated onto those nanoparticles, whose sizes, charges, and surface properties were finely controlled by polymer coating and PEGylation. Herein, Ce6 was activated in advance using 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) in dimethyl sulfoxide (DMSO), and then added into the aqueous solution of PAH-coated MnO2 nanoparticles at different weight ratios (Ce6:MnO2 = 0.1:1, 0.2:1, 0.5:1, 1:1, and 2:1) under ultrasonication and stirred at room temperature for 4 h. After removal of excess Ce6, UV–vis spectra of these samples were recorded to determine the Ce6 loading capacity (Figure 1b and Supporting Information Figure S2). The Ce6 loading achieved a relatively high ratio of ≈45.81% (Ce6:MnO2, w/w) at the Ce6:MnO2 feeding ratio of 2.1(w/w), and the obtained Ce6-conjugated PAH-coated MnO2 (Ce6@MnO2-PAH) nanoparticles prepared under this condition were used for further experiments.
MnO₂-PAH without PEGylation, which although water-soluble, would rapidly precipitate in the presence of salts.

As demonstrated in many previous studies, hypoxia, a character of the tumor microenvironment, has been shown to contribute to the resistance to PDT since oxygen is an essential requirement in the process of PDT. Since MnO₂ is known to be an excellent catalyst to trigger decomposition of H₂O₂ into H₂O and O₂, herein, we tested the dissolved O₂ in H₂O₂ solutions by an oxygen probe (JPBJ-608 portable Dissolved Oxygen Meters, Shanghai REX Instrument Factory) after various concentrations of Ce6@MnO₂-PEG nanoparticles were added. As expected, addition of Ce6@MnO₂-PEG would trigger the rapid generation of oxygen in the H₂O₂ solution, which in the absence of MnO₂ nanoparticles was quite stable (Figure 2a). We then wondered whether oxygen produced from H₂O₂ in the presence of Ce6@MnO₂-PEG would be favorable for light-induced SO generation by Ce6. The SO generation produced from Ce6 and Ce6@MnO₂-PEG under laser irradiation in the presence of 100 × 10⁻⁶ M H₂O₂, a concentration relevant to the tumor microenvironment, was then measured by a singlet oxygen sensor green (SOSG) probe, whose quenched fluorescence will be recovered by generated SO during PDT (Figure 2b). Due to the quenching effect of Ce6 by MnO₂ nanoparticles (Supporting Information Figure S3), the SO level produced by Ce6@MnO₂-PEG without H₂O₂ was lower than that produced by free Ce6. Interestingly, after addition of H₂O₂, MnO₂ nanoparticles could quickly react with H₂O₂ to produce sufficient O₂, resulting in the remarkable enhancement of light-induced SO generation by Ce6@MnO₂-PEG. In contrast, no appreciable difference in SO production by Ce6 was observed after H₂O₂ was added. Therefore, it is expected that the PDT efficiency of Ce6@MnO₂-PEG would be significantly improved inside the microenvironment with a substantial level of H₂O₂.

MnO₂ is known to be stable under neutral and basic pH, but would be decomposed into Mn²⁺ and O₂ under reduced pH. Since Mn²⁺ with five unpaired 3d electrons is a great T₁-shortening agent in MR imaging, MR imaging of Ce6@MnO₂-PEG solutions after incubation in buffers with different pH values (6.5 and 7.4) for 6 h was conducted. Obvious concentration dependent brightening effect was observed in T₁-weighted MR images of Ce6@MnO₂-PEG samples at pH 6.5, while the signals of Ce6@MnO₂-PEG at pH 7.4 appeared to be rather weaker (Figure 2c). The relaxivity (r₁) of Ce6@MnO₂-PEG at pH 7.4 was very low at 0.780 mM⁻¹ s⁻¹, attributed to the high valence (IV) of manganese and shielded paramagnetic centers inaccessible to water molecules. Importantly, the r₁ value
measured at pH 6.5 increased dramatically from the initial value to 6.528 mM$^{-1}$ s$^{-1}$, owing to the decomposition of MnO$_2$ into paramagnetic Mn$^{2+}$ (Figure 2d) and being consistent to the r1 value of other Mn$^{2+}$-containing nanoprobes.\(^{[37]}\) The color of MnO$_2$ disappeared after incubation at pH 6.5 for 6 h, suggesting the complete decomposition of those nanoparticles (Supporting Information Figure S4). Therefore, Ce6@MnO$_2$-PEG may act as a pH-responsive MR imaging agent, which is particularly useful for tumor imaging considering the acidic tumor microenvironment.

In vitro experiments were then carried out to determine whether our nanoparticles would be an effective agent for PDT. First, Ce6@MnO$_2$-PEG exhibited no obvious adverse effects to the viabilities of 4T1 cells at tested concentrations from 1.5 to 100 $\times$ 10$^{-6}$ M for 24 h, as evaluated by the methyl thiazolyl tetrazolium (MTT) assay (Figure 3b). It has been reported that cellular uptake efficiency of molecules, such as Ce6, could be greatly enhanced after being loaded onto nanoparticles.\(^{[44]}\) In our experiments, 4T1 murine breast cancer cells were incubated with Ce6@MnO$_2$-PEG and Ce6, respectively, at the same Ce6 concentration for different periods of time. After removal of excess agents, cells were washed for three times before being stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and imaged under a confocal fluorescence microscope (Figure 3c). Notably, significantly higher Ce6 fluorescence was observed in cells incubated with Ce6@MnO$_2$-PEG, compared to those treated with free Ce6, despite the partial quenching of Ce6 after it was loaded onto nanoparticles. To quantitatively determine cellular uptake of Ce6, cells were lysed and solubilized by an acidic buffer, in which MnO$_2$ nanoparticles could be decomposed and Ce6 fluorescence would be recovered. However, based on the fluorescence intensities of Ce6 measured from cell lysated samples, we clearly found that Ce6@MnO$_2$-PEG showed obviously higher uptake by 4T1 cells compared to free Ce6 (Supporting Information Figure S5).

Since the aberrant metabolism of cancer cells could lead to significantly elevated cellular concentrations of H$_2$O$_2$, we hypothesized that Ce6@MnO$_2$-PEG nanoparticles might be able to trigger the decomposition of endogenous H$_2$O$_2$ produced by cancer cells, and thus generate O$_2$ in situ to enhance the efficacy of PDT under hypoxia conditions. We thus examined the PDT efficiency by incubating 4T1 cells with Ce6@MnO$_2$-PEG nanoparticles or free Ce6 in either N$_2$ or O$_2$ atmosphere without exogenous H$_2$O$_2$ added. Cells were then exposed to 661 nm light at the power density of 5 mW cm$^{-2}$ for 30 min. After further incubation for 24 h, their viabilities were measured by the MTT assay. As shown in Figure 3d, both Ce6 and Ce6@MnO$_2$-PEG nanoparticles showed comparable levels of phototoxicities to 4T1 cells in the O$_2$ atmosphere. In contrast, once the experiment was conducted in the N$_2$ atmosphere, the cancer cell killing efficiency of Ce6@MnO$_2$-PEG nanoparti-
cles remained to be rather high, while that of free Ce6 showed significantly reduced phototoxicity to cancer cells owing to the insufficient oxygen supply. Therefore, Ce6@MnO2-PEG appears to be an efficient PDT agent to kill cancer cells even in the hypoxic environment.

After demonstrating the capability of Ce6@MnO2-PEG to react with H2O2 for the production of O2 and enhancement of PDT efficiency in vitro, we then would like to use these nanoparticles for in vivo cancer treatment in a mouse tumor model. To understand the in vivo behaviors of Ce6@MnO2-PEG, MR imaging was applied to examine tumor-bearing mice before, 5 min, and 24 h after i.v. injection of Ce6@MnO2-PEG (10 mg kg⁻¹) (Figure 4a). No significant MR contrast showed up for mice immediately after injection of Ce6@MnO2-PEG, owing to the factor that MnO2 before being decomposed into Mn²⁺ is not an efficient T1-MR contrast agent (Figure 2c,d). The MR signal intensity at the tumor site showed approximately threefold positive enhancement (Figure 4b) at 24 h postinjection (p.i.), suggesting high tumor accumulation of those nanoparticles via the enhanced permeability and retention (EPR) effect, as well as the gradual decomposition of MnO2 into Mn²⁺ in the mildly acidic tumor microenvironment. Furthermore, strong T1 signals were also observed in the kidneys but not in the mouse liver (Figure 4c,d), suggesting possible renal clearance of Mn²⁺ ions decomposed from MnO2 nanoparticles.

We then quantitatively measured Mn²⁺ levels in the mouse body by the ex vivo inductively coupled plasma atomic emission spectroscopy (ICP-AES) method. The blood circulation of Ce6@MnO2-PEG was then studied after i.v. into tumor-bearing mice. Blood samples were extracted from mice at various time points p.i. and then measured by ICP-AES to determine the concentrations of Mn²⁺ in the blood. As shown in Figure 4e, the blood levels of Ce6@MnO2-PEG decreased gradually over time in accordance with a two-compartment model. The first (t₁/₂(α)) and second (t₁/₂(β)) phases of circulation half-lives were calculated to be 0.62 ± 0.04 h and 7.65 ± 0.09 h, respectively, by secondary exponential fitting. The fairly long circulation time of Ce6@MnO2-PEG in the blood should be favorable for effective tumor accumulation via the EPR effect. After 24 h, above mice were sacrificed to take out main organs for biodistribution study. After dissolving all collected organs and tissues by chloroazotic acid, ICP-AES was also used to determine Mn²⁺ levels in those samples (Figure 4f). Besides high Mn²⁺ levels in the tumor and kidneys as observed by MR imaging, significant accumulation of Ce6@MnO2-PEG nanoparticles was observed in reticuloendothelial systems (RES) including the liver and spleen. Taken MR imaging and biodistribution results together, we could conclude that MnO2 nanoparticles, which have low T1 contrast in their intact form, after macrophage clearance by RES organs would be gradually decomposed into Mn²⁺ and subsequently excreted from the body via kidney filtration. The high efficiency of tumor retention together with rapid renal clearance behaviors of such MnO2 nanoparticles is particularly promising for effective and
safe cancer-targeted therapy. Notably, no obvious toxicity to the treated animals was observed by histological examination conducted 14 d after i.v. injection of Ce6@MnO₂-PEG (Supporting Information Figure S6).

To confirm that MnO₂ nanoparticles would indeed have the ability to regulate hypoxia environment within tumors, a hypoxyprobe (pimonidazole) immunohistochemical assay was performed for tumor slices extracted at different time points post treatment with Ce6@MnO₂-PEG nanoparticles. Cell nuclei, blood vessels, and hypoxia areas were stained with DAPI (blue), anti-CD31 antibody (red), and antipimonidazole antibody (green), respectively, for outcomes of the immunofluorescence staining assay. Compared to tumors from untreated mice, the tumors from mice after i.v. injection with Ce6@MnO₂-PEG nanoparticles showed significantly decreased tumor hypoxia, as evidenced by the greatly weakened pimonidazole-stained (green) hypoxic signals (Figure 5a). The quantitative analysis of hypoxia positive areas recorded from more than ten micrographs for each group further illustrates that i.v. injection of Ce6@MnO₂-PEG could greatly suppress tumor hypoxia, whose level was found to be rather low at 24 h p.i. (Figure 5b). Therefore, owing to the reactivity between H₂O₂ and MnO₂ to effectively generate O₂, the overall tumor oxygenation status could be remarkably elevated after i.v. injection of Ce6@MnO₂-PEG nanoparticles, favorable for effective in vivo PDT cancer treatment to overcome the hypoxia-associated photodynamic resistance.
Next, the efficacy of Ce6@MnO₂-PEG for in vivo cancer PDT was evaluated with the mouse 4T1 tumor model. Balb/c mice bearing subcutaneous 4T1 tumors were divided into four groups: Group 1: PBS; Group 2: Ce6@MnO₂-PEG without laser irradiation (MnO₂: 10 mg kg⁻¹; Ce6: 4.5 mg kg⁻¹); Group 3: Ce6@MnO₂-PEG with laser irradiation, and Group 4: free Ce6 with laser irradiation (16 mg kg⁻¹). After receiving various treatments, the tumor sizes were measured by a digital caliper every 2 d. While Ce6@MnO₂-PEG injection without laser irradiation showed obvious suppressive effect on tumor growth (Figure 5c and Supporting Information Figures S7 and S8). PDT treatment with i.v. injection of Ce6@MnO₂-PEG plus 661 nm laser irradiation (5 mW cm⁻², 1 h) resulted in greatly delayed tumor growth inhibition (Figure 5c), reaching an efficacy much better than that offered by PDT with free Ce6 at a 3.5-fold overdose. Microscopy images of hematoxylin and eosin (H&E) stained tumor slices further revealed that the PDT triggered by Ce6@MnO₂-PEG exerted serious damages to tumor cells while cells in control groups of tumors largely retained their regular morphology with typical membrane and nuclear structures (Figure 5d). It is thus obviously that Ce6@MnO₂-PEG, even at a much reduced dose, appears to be a powerful PDT agent superior to free Ce6, owing to the increased Ce6 uptake in the tumor and reversed tumor hypoxia, highly effective PDT cancer treatment is realized with Ce6@MnO₂-PEG, showing greatly improved efficacy even under a largely reduced dose. Considering the gradual decomposition of MnO₂ nanoparticles, the easy kidney

3. Conclusions
In summary, MnO₂ nanoparticles with photosensitizer (Ce6) conjugation and PEG coating are fabricated in this work as a new generation of imaginable photodynamic nanoagent. At the in vitro level, such Ce6@MnO₂-PEG nanoparticles are found to be highly effective to kill cancer cells by PDT, even under an oxygen deficient environment, owing to their ability to convert endogenous H₂O₂ produced by cancer cells into O₂. Upon systemic administration into mice, Ce6@MnO₂-PEG nanoparticles show efficient accumulation inside the tumor, in which MnO₂ nanoparticles are gradually decomposed into Mn²⁺ ions to offer a strong T1 MR contrast. In the meanwhile, the tumor oxygenation level is greatly enhanced as the result of MnO₂-triggered O₂ production from H₂O₂ existing within the tumor microenvironment. Owing to the enhanced Ce6 uptake in the tumor and reversed tumor hypoxia, highly effective PDT cancer treatment is realized with Ce6@MnO₂-PEG, showing greatly improved efficacy even under a largely reduced dose. Considering the gradual decomposition of MnO₂ nanoparticles, the easy kidney

Figure 5. In vivo PDT with Ce6@MnO₂-PEG nanoparticles. a) Representative immunofluorescence images of tumor slices after hypoxia staining. The nuclei, blood vessels, and hypoxia areas were stained by DAPI (blue), anti-CD31 antibody (red), and antipimonidazole antibody (green), respectively. b) The relative hypoxia positive area as recorded from more than ten micrographs for each group. c) Tumor volume growth curves of mice after various treatments (five mice for each group). Light irradiation (L⁺) was conducted by the 661 nm light at the power density of 5 mW cm⁻² for 1 h. d) H&E stained tumor slices from different groups collected 24 h after light irradiation. The scale bar is 100 µm.
filtration of MnO₂; as well as the no obvious in vivo short-term toxicity observed in this study, MnO₂ nanomaterials may be a unique type of safe nanoplatform promising for cancer therapeutics, particularly for enhancing cancer treatment outcomes via modulating the unfavorable tumor microenvironment.

4. Experimental Section

Materials: All chemicals were of analytical grade and used without further purification if not indicated otherwise. PAA (M₉ = 1800), PAH (M₉ = 15 000), T-(3-dimethylaminopropyl)propylyl-3-ethylcarboxylate hydrochloride (EDC), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and NHS were purchased from Sigma-Aldrich. Potassium permanganate (KMnO₄) and sodium hydrochloride (EDC), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and NHS were purchased from Sigma-Aldrich. Potassium permanganate (KMnO₄) and sodium hydrochloride (EDC) were added into mentioned solution under ultrasonication and kept at room temperature for 20 min. The activated Ce₆ was added into 5 mL of PAA solution (4 mg mL⁻¹), pH (pH = 7.4) and stirred at room temperature for 6 h before measuring the samples with UV–vis spectrophotometer. The concentration of Ce₆ was calculated from a standard curve for the determination of intracellular Ce₆. After incubation for 24 h, the standard thiazolyl tetrazolium (MTT, Sigma-Aldrich) test was conducted to measure the cell viabilities relative to the untreated cells.

To examine the cellular uptake of Ce₆@MnO₂-PEG nanoparticles, 4T1 cells were planted in 6-well plates (1 × 10⁴ per well) after adherent and then incubated with series concentrations of Ce₆@MnO₂-Peg. After incubation for 24 h, the standard thiazolyl tetrazolium (MTT, Sigma-Aldrich) test was conducted to measure the cell viabilities relative to the untreated cells.

Cellular Experiments: Murine breast cancer 4T1 cells were obtained from American Type Culture Collection (ATCC) and cultured at 37 °C under 5% CO₂. All cell culture related reagents were purchased from Invitrogen. All cells were cultured in normal RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

For cell cytotoxicity assay, cells were seeded into 96-well plates (1 × 10⁴ per well) until adherent and then incubated with various concentrations of Ce₆@MnO₂-PEG and free Ce₆ at various concentrations. After 2 h, the 96-well plates were placed in a transparent box, ventilated with N₂ or O₂ in advance, and then kept for 30 min before being exposed to 660 nm irradiation at a power density of 5 mW cm⁻² for 30 min along with N₂ or O₂ circulating. Then, cells were transferred into fresh media and further incubated for 4 h. The standard MTT test was then conducted to measure the relative cell viabilities.

Animal Model: Balb/c mice were purchased from Nanjing Peng Sheng Biological Technology Co. Ltd. and used under protocols approved by Soochow University Laboratory Animal Center. The 4T1 tumors were generated by subcutaneous injection of 1 × 10⁶ cells suspended in 200 μL PBS medium onto the back of each female Balb/c mouse. The tumor sizes reached about 120 mm³ in vivo experiments were then carried out.

In Vivo MR Imaging: 4T1 tumor-bearing mice were intraperitoneally (i.p.) injected with Ce₆@MnO₂-PEG at a dose of 10 mg kg⁻¹ (MnO₂ body weight). After 24 h, MR imaging was performed under a 3.0 T magnetic field with a special coil for small animal imaging (GE Healthcare, USA).

In Vivo Biodistribution: Three tumor-bearing mice were i.v. injected with Ce₆@MnO₂-PEG (MnO₂: 10 mg kg⁻¹). About 10 μL blood was extracted every time from each tail at indicated time points, weighted and then dissolved in digesting chloroazotic acid (HNO₃:HCl = 3:1) to analyze the amount of Mn²⁺ in the samples using ICP-AES.

24 h after i.v. injection with Ce₆@MnO₂-PEG, above mice were sacrificed. Major organs and tissues (the liver, spleen, kidney, heart, lung, intestine, stomach, skin, bone, muscle, and tumor) were collected, wet-weighted, and solubilized in chloroazotic acid under heating for 2 h. After diluting each sample with DI water to 10 mL, ICP-AES was used to measure Mn²⁺ concentrations in different samples. The baseline Mn²⁺ levels in different organs of untreated mice were also measured and subtracted.

In Vivo PDT: 4T1 tumor-bearing mice were divided into four groups (five mice in each group): (1) PBS alone; (2) Ce₆@MnO₂-PEG alone; (3) Ce₆@MnO₂-PEG + laser; (4) free Ce₆ + laser. The solution of Ce₆@MnO₂-PEG (200 μL, MnO₂: 1 mg mL⁻¹; Ce₆: 0.45 mg mL⁻¹) was i.v.
injected into the body of mice in groups 2 and 3 while 200 µL of Ce6 (1.6 mg mL⁻¹) was injected into that in group 4. After 24 h, the mice in groups 3 and 4 received laser irradiation of 661 nm (5 mW cm⁻²) for 1 h. Tumor sizes and body weight were monitored every 2 d for 2 weeks. The lengths and widths of the tumors were measured by a digital caliper. The tumor volume was calculated according to the following formula: Volume = Width² × length/2. Relative tumor volumes were calculated as V/V₀ (V₀ is the tumor volume when the treatment was initiated).

Immunohistochemistry: 4T1 tumor-bearing mice were i.v. injected with PBS or Ce6@MnO₂-PEG (200 µL, 1 mg mL⁻¹) tumor volume when the treatment was initiated).

PBS or Ce6@MnO₂-PEG (200 µL, 1 mg mL⁻¹) was injected into that in group 4. After 24 h, the mice in

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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