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.¹⁻⁴ 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.⁵⁻⁶ Moreover, hypoxia would also lead to resistance to medical therapies,⁷⁻¹³ such as radiotherapy, chemotherapy, and photodynamic therapy (PDT),¹⁴⁻¹⁸ 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.¹⁹⁻²² To date, there have been various methods proposed to modify the hypoxic tumor microenvironment by promoting the oxygenation of tumors.¹⁹⁻²²⁻³⁰ For example, artificial blood substitutes such as perfl uorocarbon-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 intra-tumor blood vessels. In situ production of oxygen inside the tumor may be a more effective approach to overcome tumor hypoxia. Recently, MnO₂ nanostructures have attracted considerable interests in bio-applications.¹¹⁻³⁷ By taking advantage of their high sensitivity to pH, MnO₂ nanosheets that can be rapidly
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 poly electrolyte 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 photocotoxicity 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 decomposition of MnO₂ nanoparticles into Mn³⁺ ions that are easily excreted. We further carry out in vivo PDT treatment for mice bearing 4T1 murine breast tumors. Notably, the therapeautic efficacy achieved by i.v. injection Ce₆@MnO₂-PEG and light exposure of tumors appears to be much better than that obtained with PDT using free Ce₆ at a 3.5-fold dose. Thus, our Ce₆@MnO₂-PEG nanoparticles would be a promising agent for tumor hypoxia modulation and imaging guided photodynamic cancer treatment with remarkably enhanced tumor inhibition efficacy.

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.[36] 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) 2p₁/₂ and Mn (IV) 2p₃/₂ 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 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 (Mₙ ≈ 5000) was then conjugated to carbonyl 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
MnO\textsubscript{2}-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 MnO\textsubscript{2} is known to be an excellent catalyst to trigger decomposition of H\textsubscript{2}O\textsubscript{2} into H\textsubscript{2}O and O\textsubscript{2}, herein, we tested the dissolved O\textsubscript{2} in H\textsubscript{2}O\textsubscript{2} solutions by an oxygen probe (JPBJ-608 portable Dissolved Oxygen Meters, Shanghai REX Instrument Factory) after various concentrations of Ce6@MnO\textsubscript{2}-PEG nanoparticles were added. As expected, addition of Ce6@MnO\textsubscript{2}-PEG would trigger the rapid generation of oxygen in the H\textsubscript{2}O\textsubscript{2} solution, which in the absence of MnO\textsubscript{2} nanoparticles was quite stable (Figure 2a). We then wondered whether oxygen produced from H\textsubscript{2}O\textsubscript{2} in the presence of Ce6@MnO\textsubscript{2}-PEG would be favorable for light-induced SO generation by Ce6. The SO generation produced from Ce6 and Ce6@MnO\textsubscript{2}-PEG under laser irradiation in the presence of 100 \times 10^{-6} M H\textsubscript{2}O\textsubscript{2}, a concentration relevant to the tumor microenvironment,\textsuperscript{[42]} was then measured by a singlet oxygen sensor green (SOSG) probe, whose quenched fluorescence will be recovered by generated SO during PDT\textsuperscript{[43]} (Figure 2b). Due to the quenching effect of Ce6 by MnO\textsubscript{2} nanoparticles (Supporting Information Figure S3), the SO level produced by Ce6@MnO\textsubscript{2}-PEG without H\textsubscript{2}O\textsubscript{2} was lower than that produced by free Ce6. Interestingly, after addition of H\textsubscript{2}O\textsubscript{2}, MnO\textsubscript{2} nanoparticles could quickly react with H\textsubscript{2}O\textsubscript{2} to produce sufficient O\textsubscript{2}, resulting in the remarkable enhancement of light-induced SO generation by Ce6@MnO\textsubscript{2}-PEG. In contrast, no appreciable difference in SO production by Ce6 was observed after H\textsubscript{2}O\textsubscript{2} was added. Therefore, it is expected that the PDT efficiency of Ce6@MnO\textsubscript{2}-PEG would be significantly improved inside the microenvironment with a substantial level of H\textsubscript{2}O\textsubscript{2}.

MnO\textsubscript{2} is known to be stable under neutral and basic pH, but would be decomposed into Mn\textsuperscript{2+} and O\textsubscript{2} under reduced pH. Since Mn\textsuperscript{2+} with five unpaired 3d electrons is a great T1-shortening agent in MR imaging, MR imaging of Ce6@MnO\textsubscript{2}-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@MnO\textsubscript{2}-PEG samples 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@MnO\textsubscript{2}-PEG samples at pH 6.5, while the signals of Ce6@MnO\textsubscript{2}-PEG at pH 7.4 appeared to be rather weaker (Figure 2c). The relaxivity (r1) of Ce6@MnO\textsubscript{2}-PEG at pH 7.4 was very low at 0.780 mM\textsuperscript{-1} s\textsuperscript{-1}, attributed to the high valence (IV) of manganese and shielded paramagnetic centers inaccessible to water molecules.\textsuperscript{[34]} 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 × 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 nanoparticles...
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 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 (t1/2α) and second (t1/2β) 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.

Figure 4. In vivo MR imaging and behaviors of Ce6@MnO2-PEG nanoparticles. a) In vivo T1-weighted MR images (transverse section) of a tumor-bearing mouse taken before injection (upper), 5 min (middle), and 24 h post i.v. injection (bottom) of Ce6@MnO2-PEG. b) T1-weighted MR signals in the tumor before, 5 min, and 24 h postinjection of Ce6@MnO2-PEG. c) In vivo T1-weighted MR images (longitudinal section) of the same mouse taken before injection (left) and 24 h post i.v. injection (right) of Ce6@MnO2-PEG. d) T1-weighted MR signals in the kidney and liver before and 24 h postinjection of Ce6@MnO2-PEG in mice by measuring the concentration of Mn in blood at different time points postinjection. f) Biodistribution of Ce6@MnO2-PEG in mice after i.v. injection. The concentration of Mn was measured by ICP-AES. Error bars were based on standard deviation (SD) of three mice.
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 H$_2$O$_2$ produced by cancer cells into O$_2$. Upon systemic administration into mice, Ce6@MnO2-PEG nanoparticles show efficient accumulation inside the tumor, in which MnO2 nanoparticles are gradually decomposed into Mn$^{2+}$ ions to offer a strong T1 MR contrast. In the meanwhile, the tumor oxygenation level is greatly enhanced as the result of MnO2-triggered O$_2$ production from H$_2$O$_2$ 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
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 unless noted otherwise. PAA (Mₐ = 18000), PAH (Mₐ = 15000), 7-((3-diethylaminopropyl)propyl)-3-ethylcarbazolide (EDC), DMSO, 3-(4,5-dimethyl-2-3-diphenyltetrazolium bromide (MTT), and NHS were purchased from Sigma-Aldrich. Potassium permanganate (KMnO₄) and sodium chloride (NaCl) were obtained from Sinopharm Chemical Reagent CO., Ltd. (China) and used as received. Bovine serum albumin (BSA) and hydrogen peroxide (H₂O₂) 30 wt% solution were purchased from J&K chemical CO. Amino group terminated gelatin (PEG-NH₂, Mₐ = 5000) was obtained from Biomatrix Inc. Aqueous solutions were prepared with deionized (DI) water (18.2 MΩ cm) collected from a Milli-Q system. *Note: Different samples were exposed to the light with central wavelengths at 661 nm with the power density of 5 mW cm⁻². SOSG, which is highly sensitive to singlet oxygen, was employed here during the detection process. Typically, SOSG at the concentration of 2.5 × 10⁻⁴ M was introduced to measure SO generation by Ce6 (2 × 10⁻⁶ M) or Ce6@MnO₂-Peg under light irradiation in N₂ atmosphere. Experimental groups include free Ce6 and bare Ce6@MnO₂-Peg with and without H₂O₂ (100 × 10⁻⁶ M) added. The generated SO was determined by measuring recovered SOSG fluorescence (excitation = 494 nm).

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 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.

To examine the cellular uptake of Ce6@MnO₂-Peg nanoparticles, 4T1 cells were plated in 6-well plates (1 × 10⁶ per well). After adhesion, free Ce6 and Ce6@MnO₂-Peg were added into the wells at the concentration of 6 × 10⁻⁴ M (Ce6) and cultured for different time periods (0.5, 1, 2, and 4 h) before cells were washed with PBS and dissolved in lysis buffer containing 1% sodium dodecylsulfate, 1% Triton X-100, 40 × 10⁻³ M tris (hydroxymethyl) aminomethane (tris) acetate, 10 × 10⁻¹ M ethylenediaminetetraacetic acid, and 10 mm dithiothreitol (pH = 6.5) with brief sonication. After it was kept for 6 h, relative amount of intracellular uptake of Ce6 was recorded by measuring the fluorescence intensity of Ce6.

For photodynamic therapy, 4T1 cells seeded in 96-well plates were mixed with Ce6@MnO₂-Peg or 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 kept for 30 min before 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 24 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 0.3 μL PBS medium onto the back of each female Balb/c mouse. When the tumor sizes reached about 120 mm³, in vivo experiments were then carried out.

In Vivo MR Imaging: 4T1 tumor-bearing mice were intravenously 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 chlorozicotropic 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.

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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 = (Length × Width) / 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) with pimonidazole hydrochloride (60 mg kg⁻¹). After predetermined time, tumors were surgically excised 90 min after intraperitoneal injection with pimonidazole hydrochloride (60 mg kg⁻¹). Hypoxyprobe-1 plus kit (Hypoxprobe Inc.), which was reductively activated in hypoxic cells and formed stable adducts with thiol (sulfhydryl) groups in proteins, amino acids, and peptides. Anti-pimonidazole mouse monoclonal antibody conjugated to FITC (FITC-Mab1) binds to these adducts, allowing their detection by immunohistochemical methods. For immunofluorescence staining, OCT compound (Sakura Finetek) was used for frozen sections of the tumors. For detection of pimonidazole, tumor sections were incubated with mouse anti-pimonidazole antibody (dilution 1:200, Hypoxprobe Inc.) and Alex 488-conjugated goat anti-mouse secondary antibody (dilution 1:200, Jackson). Images were obtained by a confocal microscopy (Leica SP5).

**Supporting Information**

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

**Acknowledgements**

This work was partially supported by the National Basic Research Programs of China (973 Program) (2012CB932600), the National Natural Science Foundation of China (51525203, 51132006), a Jiangsu Natural Science Fund for Distinguished Young Scholars (BK20130005), Collaborative Innovation Center of Suzhou Nano Science and Technology, and a Project Funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Received: February 5, 2016
Published online: May 20, 2016
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