Near-Infrared Light Responsive Nanoreactor for Simultaneous Tumor Photothermal Therapy and Carbon Monoxide-Mediated Anti-Inflammation

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ABSTRACT: Photothermal therapy (PTT) is an effective treatment modality with high selectivity for tumor suppression. However, the inflammatory responses caused by PTT may lead to adverse reactions including tumor recurrence and therapeutic resistance, which are regarded as major problems for PTT. Here, a near-infrared (NIR) light-responsive nanoreactor (P@DW/BC) is fabricated to simultaneously realize tumor PTT and carbon monoxide (CO)-mediated anti-inflammatory therapy. Defective tungsten oxide (WO$_3$) nanosheets (DW NSs) are decorated with bicarbonate (BC) via ferric ion-mediated coordination and then modified with polyethylene glycol (PEG) on the surface to fabricate PEG@DW/BC or P@DW/BC nanosheets. Upon 808 nm NIR laser irradiation, the DW content in P@DW/BC can serve as not only a photothermal agent to realize photothermal conversion but also a photocatalyst to convert carbon dioxide (CO$_2$) to CO. In particular, the generated heat can also trigger the decomposition of BC to produce CO$_2$ near the NSs, thus enhancing the photocatalytic CO generation. Benefiting from the efficient hyperthermia and CO generation under single NIR laser irradiation, P@DW/BC can realize effective thermal ablation of tumor and simultaneous inhibition of PTT-induced inflammation.

INTRODUCTION

Photothermal therapy (PTT) that utilizes near-infrared (NIR) light-absorbing agents to convert photoenergy into heat for cells thermal ablation is regarded as a minimally invasive and highly efficient treatment approach for tumor management.$^{1–3}$ Owing to the good controllability of NIR light (e.g., power density, duration, and range) and the negligible toxicity of photothermal agents (PTAs) in the dark, PTT can eliminate tumor cells specifically without harming normal tissues, which is a promising alternative to traditional tumor therapy.$^{4,5}$ However, because of the hyperthermia induced by PTAs, the most possible cellular death mode after PTT is necrosis, which is characterized by rupture of the plasma membrane, release of cellular contents, and in turn an introduction of inflammation.$^{6,7}$ Although inflammation is a common defensive response of the body to external stimuli, it has been shown that the therapy-induced inflammation might cause severe adverse effects, including tumor regeneration, metastatic dissemination, and therapeutic resistance.$^{8–11}$ Therefore, effective alleviation of inflammatory responses caused by PTT is of great significance for tumor treatment.

Carbon monoxide (CO), a colorless and odorless gas, is increasingly appreciated as a crucial signaling molecule and has been proved to hold substantial therapeutic potentials in cytoprotection, hypertension management, bacterial inhibition, and chemosensitization.$^{12–15}$ Moreover, CO has also been confirmed as an effective agent for anti-inflammation.$^{16–18}$ In light of this, combining CO with PTT might be a feasible way to reduce the therapy-induced inflammation. However, because of the great affinity between CO and hemoglobin, the direct use of gaseous CO is risky and lacks tumor selectivity. Therefore, developing advanced strategies to realize in situ CO generation in tumor tissues is necessary for the combination between PTT and CO. Recently, photocatalytic CO$_2$ reduction has been confirmed as a promising way to realize in vivo CO generation.$^{19,20}$ By regulating external light irradiation, the CO generation can be accurately adjusted, which makes it an attractive method for in situ CO production. However, current studies mainly depended on a visible-light-responsive photocatalyst to convert internal CO$_2$ to CO in vivo.$^{19,20}$ The limited penetration ability of visible light in biological tissues severely prevents the CO production in deep tissues. Moreover, the concentration of internal CO$_2$ is always relatively low, and the strong dependence on internal CO$_2$ also seriously hinders the efficient CO$_2$ photoreduction in vivo. Thus, developing novel photocatalytic CO generation systems to overcome these problems is an urgent need.

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Figure 1. Schematic illustration of P@DW/BC NSs for simultaneous tumor PTT and anti-inflammation. (A) Preparation process for P@DW/BC NSs. (B) Selective accumulation of P@DW/BC NSs in tumor after intravenous injection via the EPR effect. (C) P@DW/BC NSs-mediated PTT for tumor inhibition. (D) Heat-triggered BC decomposition to produce CO₂. (E) P@DW/BC NSs-mediated photocatalytic CO₂ reduction to produce CO. (F) Elimination of PTT-caused inflammation by CO.

Figure 2. (A) SEM and TEM (inset) images of DW NSs. (B) SEM and TEM (inset) images of P@DW/BC NSs. (C) HRTEM image, STEM-HAADF image, and the corresponding element mapping images of P@DW/BC. (D) Hydrodynamic sizes and (E) ζ potentials of 1. DW NSs; 2. DW-PDA NSs; 3. DW/Fe³⁺ NSs; 4. DW/BC NSs; 5. P@DW/BC NSs. (F) PXRD pattern of P@DW/BC and JCPDS card of cubic WO₃. (G) Photographs of P@DW/BC NSs (1 mg mL⁻¹) dispersed in H₂O, PBS, and culture medium (containing 10% FBS). (H) UV–vis-NIR absorbance spectra of P@DW/BC NSs, DW NSs, and WO₃ atomic layers. (I) Thermal images of P@DW/BC solutions under 808 nm laser irradiation. (J) Heat curves of P@DW/BC solutions with various concentrations (Power density: 1 W cm⁻²). (K) Heat curves of P@DW/BC solution (100 µg mL⁻¹) under 808 nm laser irradiation with different power densities.
Keeping all these in mind, here, a defective tungsten oxide (WO\textsubscript{3}) (DW)-based NIR light responsive nanoreactor (termed as P@DW/BC) was fabricated for simultaneous tumor PTT and CO-mediated anti-inflammation. DW that was constructed by introducing oxygen vacancies higher than the reported critical density (7.3\%) in WO\textsubscript{3} can result in an intermediate band and achieve infrared (IR) light-driven CO\textsubscript{2} splitting to produce CO,\textsuperscript{21} making it an appealing material for \textit{in vivo} CO generation. In addition, DW also possesses strong and broad absorbance ranging from NIR to IR region, which shows great potential to be extended as a PTA for tumor thermal ablation.\textsuperscript{21} Moreover, bicarbonate (BC) was introduced to serve as a CO\textsubscript{2} releaser, which can quickly decompose into CO\textsubscript{2} upon heating to 40 °C or above.\textsuperscript{22,23} As illustrated in Figure 1, DW nanosheets (DW NSs) were first decorated with lipoic acid conjugated dopamine (LA-DPA) through the W−S bonds. Then, ferric ion (Fe\textsuperscript{3+}) was introduced to serve as a coordination center to bridge both DPA and bicarbonate (BC), where Fe\textsuperscript{3+} can coordinate with the two −OH groups on DPA in a bidentate manner and with BC in a monodentate manner.\textsuperscript{24,25} Finally, lipoic acid conjugated polyethylene glycol (LA-PEG) was engaged to improve the biocompatibility and dispersity of the nanosystem, thus providing PEG@DW/BC or P@DW/BC NSs. It was anticipated that after being injected intravenously, P@DW/BC could selectively accumulate in tumor tissues via the enhanced permeability and retention (EPR) effect. Then upon 808 nm laser irradiation, the DW content in P@DW/BC could conduct both photothermal conversion and CO\textsubscript{2} photoreduction to produce CO. Meanwhile, the generated heat was also supposed to trigger the decomposition of BC to produce CO\textsubscript{2} near the NSs, thus enhancing the CO generation by photocatalytic CO\textsubscript{2} reduction. Benefiting from the generated hyperthermia and CO upon single NIR laser irradiation, P@DW/BC was expected to effectively inhibit tumor growth by PTT and simultaneously weaken the PTT-induced inflammation by CO.

\section*{RESULTS AND DISCUSSION}

DW atomic layers were first prepared by annealing WO\textsubscript{3} atomic layers in a reducing atmosphere to create oxygen vacancies according to the literature method (Figure S1).\textsuperscript{21} Then the DW atomic layers were converted into DW NSs under sonication in water. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images in Figure 2A clearly illustrated the uniform nanostructure of the as-prepared DW NSs. The powder X-ray diffraction (XRD) pattern of DW NSs confirmed their crystal structure and all diffraction peaks can be indexed to cubic WO\textsubscript{3} (JCPDS...
Finally, to improve the particular, the photothermal conversion and also highlighted the controllability of heat generation. In the NIR laser. These results clearly implied the great light-concentration of P@DW/BC NSs as well as the power density irradiation, and the change depended closely on the ratio of oxygen vacancies and oxygen atoms was suggested the presence of abundant oxygen vacancies in DW NSs.

Meanwhile, negligible Fe3+ (less than 6%) and CO2 (less than 56.5%) were observed in the XPS spectra in Figure S4, which indicated the successful introduction of Fe3+. Encouraged by the successful CO2 generation by P@DW/BC NSs, then the hyperthermia-induced CO2 production ability of P@DW/BC NSs was studied. LA-PEG modified DW NSs (termed as P@DW NSs) that without the introduction of BC on the NSs were served as control material. As illustrated in Figure 3A, P@DW/BC and P@DW NSs (200 μg mL−1) aqueous solutions were irradiated with/without 808 nm laser (1 W cm−2, 4 min) and then centrifuged to obtain the supernatants. Afterward, calcium hydroxide (Ca(OH)2) and bromothymol blue (BTB) solution were added to the supernatants for CO2 detection and pH value evaluation, respectively. CO2 was supposed to react with Ca(OH)2 in water to yield CaCO3 precipitation. As shown in Figure 3B, whether P@DW/BC, P@DW, or P@D + Laser (L) group showed almost no precipitation after the addition of Ca(OH)2. However, for P@DW/BC + L group, precipitation of CaCO3 with gray-white color was clearly observed after the addition of Ca(OH)2, demonstrating the successful generation of CO2 by P@DW/BC under irradiation. Additionally, the release ratio of CO2 was calculated to be 95% for P@DW/BC NSs after 4 min of laser irradiation, indicating the effective conversion of BC to CO2 in vitro. Furthermore, considering that the generated CO2 could dissolve in water, yielding carbonic acid to acidify the solution, acid-base indicator dye BTB was then utilized for the pH value evaluation of the supernatants. The color of the BTB solution changed from yellow to green and then to blue when the pH value of the solution increased from 6.0 to 7.0 and then to 7.6. As shown in Figure 3C, after the addition of BTB, only the supernatant from P@DW/BC + L group showed a yellow color, while other three groups were green (the same color as BTB added to deionized water). This clearly indicated the relatively low pH value of supernatant from P@DW/BC + L group, further confirming the thermal-induced CO2 release by P@DW/BC under irradiation. Additionally, the pH values of P@DW/BC and P@DW solutions after laser irradiation were also directly monitored by a pH meter. As displayed in Figure 3D, with the prolongation of the irradiation time, the pH value of P@DW/BC + L group decreased gradually, which revealed a dependence of the CO2 producing amount on irradiation time. While for P@DW + L group, the pH value showed negligible change during the irradiation, indicating the lack of CO2 production ability of P@DW NSs. Moreover, compared with P@DW + L group that increasing the concentration of the NSs had minimal effect on the pH value, P@DW/BC + L group exhibited an obvious pH value decrease with the increase of the concentration (Figure 3E), which provided strong evidence for the thermal-induced CO2 release ability of P@DW/BC NSs.

As shown in Figure 2H, the same as DW NSs, P@DW/BC NSs exhibited strong absorbance in the NIR region, indicating their good potential as efficient NIR-absorbing agents. In view of this, the photothermal conversion properties of P@DW/BC NSs were first studied. The temperature changes of P@DW/BC solutions under 808 nm laser irradiation were monitored by utilizing an IR camera. As shown in Figure 2I–K, the temperature of P@DW/BC solutions increased rapidly under irradiation, and the change depended closely on the concentration of P@DW/BC NSs as well as the power density of the NIR laser. These results clearly implied the great light-to-heat conversion ability of the as-prepared P@DW/BC NSs and also highlighted the controllability of heat generation. In particular, the photothermal conversion efficacy (η) of P@DW/BC NSs was calculated to be 39.1% (Figure S8), which was higher than or comparable to many of the reported PTAs (Table S1). Additionally, the photothermal effect of P@DW/BC NSs was almost unchanged during five irradiation OFF/ON cycles (Figure S9), confirming the good photothermal stability of P@DW/BC NSs.

Encouraged by the efficient photothermal conversion of P@DW/BC NSs, then the hyperthermia-induced CO2 production ability of P@DW/BC NSs was studied. LA-PEG modified DW NSs that without the introduction of BC on the NSs were served as control material. As illustrated in Figure 3A, P@DW/BC and P@DW NSs (200 μg mL−1) aqueous solutions were irradiated with/without 808 nm laser (1 W cm−2, 4 min) and then centrifuged to obtain the supernatants. Afterward, calcium hydroxide (Ca(OH)2) and bromothymol blue (BTB) solution were added to the supernatants for CO2 detection and pH value evaluation, respectively. CO2 was supposed to react with Ca(OH)2 in water to yield CaCO3 precipitation. As shown in Figure 3B, whether P@DW/BC, P@DW, or P@D + Laser (L) group showed almost no precipitation after the addition of Ca(OH)2. However, for P@DW/BC + L group, precipitation of CaCO3 with gray-white color was clearly observed after the addition of Ca(OH)2, demonstrating the successful generation of CO2 by P@DW/BC under irradiation. Additionally, the release ratio of CO2 was calculated to be 95% for P@DW/BC NSs after 4 min of laser irradiation, indicating the effective conversion of BC to CO2 in vitro. Furthermore, considering that the generated CO2 could dissolve in water, yielding carbonic acid to acidify the solution, acid-base indicator dye BTB was then utilized for the pH value evaluation of the supernatants. The color of the BTB solution changed from yellow to green and then to blue when the pH value of the solution increased from 6.0 to 7.0 and then to 7.6. As shown in Figure 3C, after the addition of BTB, only the supernatant from P@DW/BC + L group showed a yellow color, while other three groups were green (the same color as BTB added to deionized water). This clearly indicated the relatively low pH value of supernatant from P@DW/BC + L group, further confirming the thermal-induced CO2 release by P@DW/BC under irradiation. Additionally, the pH values of P@DW/BC and P@DW solutions after laser irradiation were also directly monitored by a pH meter. As displayed in Figure 3D, with the prolongation of the irradiation time, the pH value of P@DW/BC + L group decreased gradually, which revealed a dependence of the CO2 producing amount on irradiation time. While for P@DW + L group, the pH value showed negligible change during the irradiation, indicating the lack of CO2 production ability of P@DW NSs. Moreover, compared with P@DW + L group that increasing the concentration of the NSs had minimal effect on the pH value, P@DW/BC + L group exhibited an obvious pH value decrease with the increase of the concentration (Figure 3E), which provided strong evidence for the thermal-induced CO2 release ability of P@DW/BC NSs.

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(100 μg mL⁻¹) were dispersed in water that boiled in advanced to remove the dissolved CO₂. Then CO probe and PdCl₂ were added and the mixtures were irradiated with 808 nm laser (1 W cm⁻²). As displayed in Figure 3G, P@DW/BC solution showed distinct fluorescence enhancement in 5 min, demonstrating the effective CO generation upon laser irradiation. The conversion ratio of CO₂ to CO was detected to be 0.021%. However, for P@DW solution, negligible fluorescence enhancement was observed during the same period of laser irradiation (Figure 3H), which was owing to the insufficient CO₂ supply for the photocatalytic reaction. Furthermore, the CO generation abilities of P@DW/BC and P@DW NSs in living cells were also investigated. Mouse colon cancer (CT26) cells were coincubated with P@DW/BC or P@DW NSs in the same concentration (100 μg mL⁻¹) and then irradiated with 808 nm laser (1 W cm⁻²). The intracellular CO generation was evaluated by using the same CO probe described above. As shown in Figure 3I, with the extension of the illustrating time, both P@DW/BC and P@DW NSs treated cells showed obvious fluorescence enhancement, indicating the successful CO generation in the cells. However, compared with P@DW that can only use the endogenous CO₂ for photocatalytic CO generation, P@DW/BC that can realize hyperthermia-induced CO₂ generation showed much stronger CO production ability in the cells. Specifically, the fluorescence intensity of P@DW/BC treated cells enhanced over 40 times after 6 min irradiation, which was significantly higher than that in P@DW treated ones (about 25 times) (Figure 3J). Such results fully demonstrated the successful CO generation by P@DW/BC NSs in living cells and also proved the effective enhancement of CO generation by the thermal-induced CO₂ release strategy.

Motivated by the good performance of P@DW/BC NSs above, then the cytotoxicity of P@DW/BC NSs was tested in CT26 cells. As shown in Figure 4A, no significant cytotoxicity was induced by P@DW/BC NSs at tested concentrations after 24 h, where the cell viabilities were still higher than 92% even the NSs concentrations were up to 200 μg mL⁻¹, suggesting the low toxicity of the P@DW/BC NSs in dark. In sharp contrast, cells after the treatment of P@DW/BC NSs upon 808 nm laser irradiation (1 W cm⁻², 6 min) were killed remarkably in which only less than 9% of the cells were still
alive after 24 h (Figure 4B), suggesting the efficient tumor cell thermal ablation by P@DW/BC NSs. Additionally, the low cytotoxicity in the dark condition and good PTT effect of P@DW/BC NSs were also visually confirmed by live/dead cell staining assay as displayed in Figure 4C, which further proved the great potential of P@DW/BC NSs as safe and efficient PTAs for tumor PTT.

Furthermore, considering that P@DW/BC NSs can simultaneously achieve tumor PTT and CO generation under single 808 nm laser irradiation, whether the generated CO can effectively mitigate the inflammatory responses caused by PTT was investigated. Before that, previously reported PTAs, PEG conjugated tungsten sulfide (P@WS2) NSs were prepared to serve as control material without CO generation ability (Figure S10).34,35 As was expected, although the photothermal cytotoxicities of P@WS2 and P@DW/BC were controlled basically the same (Figure 4E), the inflammatory reactions after the treatments were quite different. The treatment of laser irradiation, P@WS2, or P@DW/BC exhibited negligible effect on the secretion of TNF-α and IL-6 (Figure 4F,G), indicating these treatments would induce negligible inflammatory responses. However, the secretion of TNF-α and IL-6 were dramatically increased after the treatment of P@WS2 + L, indicating the remarkable pro-inflammatory responses caused by PTT. On the contrary, the expression levels of TNF-α and IL-6 were minimally affected by P@DW/BC + L, suggesting the inflammatory responses caused by PTT were effectively inhibited. Apart from these, immunocytochemistry staining was also performed to visualize the expression levels of TNF-α and IL-6 in macrophages. As shown in Figure 4H, compared with P@WS2 + L group that induced obvious overproduction of TNF-α and IL-6, the treatment of P@DW/BC + L exhibited limited effect on the expression of TNF-α and IL-6.

Figure 5. (A) In vivo IR thermal images of mice and (B) temperature increase curves of tumors after the treatments of PBS or P@DW/BC upon 808 nm laser irradiation (1 W cm⁻²). (C) CO contents in tumor tissues of mice after various treatments. n = 3, ***p < 0.001. Relative expression of (D) TNF-α and (E) IL-6 in sera of mice at 24 h after various treatments, n = 3, ***p < 0.001. Representative immunofluorescence images for (F) TNF-α, (G) IL-6, (H) VEGF, and (I) COX-2 in tumor tissues of mice at 24 h after various treatments. Scale bar: 50 μm. (J) Relative tumor volume of mice during the experiments (n = 6). (K) Survival graph of mice from various groups (n = 5). Representative pictures of (L) H&E staining and (M) Ki67 immunofluorescence staining of tumor tissues of mice on the 19th day. Scale bar: 100 μm.
which further verified that the CO generated by P@DW/BC can effectively eliminate the inflammation triggered by PTT.

After confirming the feasibility of P@DW/BC NSs for in vitro PTT and anti-inflammation, the possibility of P@DW/BC NSs for in vivo applications was then investigated. CT26 xenograft BALB/c mice were used as animal models for the assessments. Cy5.5-labeled P@DW/BC NSs were first prepared and injected intravenously to mice to investigate the biodistribution of the NSs in vivo using a small animal imaging system. It can be seen that the fluorescence signal at tumor tissue increased gradually in the first 12 h and kept at a high level even at 24 h after the injection (Figure S11). This clearly demonstrated the selective accumulation of P@DW/BC in tumor tissue and 12 h post injection was chosen as the appropriate time point for laser irradiation. As shown in Figure SAB, the temperature of tumor area increased remarkably to over 42 °C after 8 min of 808 nm laser illustration (1 W cm⁻²), which was capable of ablating tumor cells by moderate hyperthermia. In marked contrast, the tumor temperature of mice injected with PBS just increased by less than 2 °C after the same irradiation. These results confirmed that P@DW/BC NSs can be effective agents for in vivo tumor PTT.

Then, the CO content in tumor tissues after various treatments were also monitored.²⁰ It can be seen that the treatment of P@WS₂, P@WS₂ + L, or P@DW/BC alone minimally affected the CO content in tumor (Figure S5C). However, for mice treated with P@DW/BC + L, the CO content in tumor increased remarkably and was detected to be nearly 6 times as high as that in PBS group. This fully demonstrated the successful CO generation by P@DW/BC NSs under 808 nm laser irradiation in vivo. Notably, P@DW/BC NSs also exhibited better CO generation ability than P@DW NSs in vivo (Figure S12), proving that the thermal-induced CO₂ release strategy can also promote in vivo CO generation.

Furthermore, whether the CO generated by P@DW/BC NSs can effectively reduce the PTT triggered inflammation was studied. The levels of the cytokines in sera of mice after various treatments were first evaluated. As shown in Figure SDE, the treatment of P@WS₂ or P@DW/BC alone showed minimal effect on the cytokine levels of TNF-α and IL-6 in sera of mice, indicating both of them induced negligible pro-inflammatory responses. However, after the treatment of P@WS₂ + L, the levels of TNF-α and IL-6 in sera of mice increased obviously, which was attributed to the inflammatory responses caused by PTT. In striking contrast, the levels of TNF-α and IL-6 in sera of P@DW/BC + L group showed no significant difference when compared with that of the PBS group, suggesting the superior anti-inflammation effect of CO. In addition, the expression of TNF-α and IL-6 in tumor tissues after the treatments was also investigated by immunofluorescence staining. Figure SF,G show that P@DW/BC + L induced much lower expression of TNF-α and IL-6 in tumor tissue when compared with P@WS₂ + L, which verified the good anti-inflammatory effect of CO. Moreover, the expression of vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) in tumor tissues after various treatments were also investigated. The inflammatory responses are supposed to trigger the expression of them, which could diminish the therapeutic efficacy and create enhanced environment for tumor recurrence.²⁵,⁴⁰ It can be seen that different from PTT alone (P@WS₂ + L) that triggered the promotion of VEGF and COX-2 in tumor, the combination of PTT with CO (P@DW/BC + L) resulted in largely weakened expression of them in tumor (Figure S1H), which clearly demonstrated that the CO-mediated anti-inflammation was beneficial for effective tumor therapy.

Afterward, the in vivo antitumor efficacy of the samples was studied. CT26-tumor-bearing mice were randomly divided into five groups and treated with PBS, P@WS₂ (100 μL, 9 mg mL⁻¹), P@DW/BC (100 μL, 10 mg mL⁻¹), P@WS₂ + L (1 W cm⁻², 8 min), and P@DW/BC + L, respectively. It should be pointed out that the dosage of P@WS₂ NSs injected to mice can achieve nearly the same photothermal effect as P@DW/BC NSs in vivo (Figure S13). As shown in Figure S1J, the treatment of P@DW/BC or P@WS₂ NSs alone showed nearly no effect on tumor suppression. Although both the treatments of P@DW/BC + L and P@WS₂ + L obviously inhibited the tumor growth at the beginning (almost the first 4 days), the tumor volume in P@WS₂ + L group increased rapidly after 1 week while the tumor volume in P@DW/BC + L group kept nearly unchanged even after 19 days. Specifically, the tumor inhibition rate of P@DW/BC + L group reached over 96% and was obviously higher than that of P@WS₂ + L group (74%) at the end point of the experiment (Day 19). Moreover, the tumor tissues of mice after various treatments were also collected for hematoxylin-eosin (H&E) staining and Ki67 immunofluorescence staining fluorescence. The results in Figure S1LM showed that the treatment of P@DW/BC + L triggered much stronger cellular damage and resulted in much weaker cell proliferation when compared with P@WS₂ + L, further validating the synergistic effect of CO and PTT for tumor inhibition. Importantly, CO-mediated anti-inflammation also greatly improved the survival rate of mice after PTT. It was found that over 60% of mice died in the P@WS₂ + L group, while 100% of the mice in P@DW/BC + L group were still alive after 60 days (Figure S1K), demonstrating the combination of CO with PTT was also beneficial for the long-term treatment of tumors.

Moreover, the potential systemic toxicity of P@DW/BC was evaluated. No abnormal body changes of mice were observed during the treatment (Figure S14). Meanwhile, blood biochemistry analysis (Figure S1S) and H&E staining of major organs (Figure S16) of mice on the 19th day also suggested that no acute side effect was caused after the treatment. Both of these indicated the good biosafety of P@DW/BC NSs in vivo.

■ CONCLUSIONS

In summary, an NIR laser responsive nanoreactor P@DW/BC was fabricated for simultaneous tumor PTT and anti-inflammation. Upon single 808 nm laser irradiation, P@DW/BC can realize both photothermal conversion and CO₂ photoreduction to produce CO. Moreover, the heat triggered by P@DW/BC can also lead to the decomposition of BC to produce CO₂ near the nanoreactor, thus enhancing the CO generation under laser irradiation. Owing to the good photothermal effect and CO generation in vivo, P@DW/BC can effectively suppress tumor growth and also reduce the inflammatory responses caused by PTT, which showed great therapeutic advantages compared with traditional PTAs. We hope that this study could provide new ideas for the application of CO in biomedical fields and also point out new directions for the combination of PTT and anti-inflammation therapy.
MATERIALS AND METHODS

Materials. Tungsten chloride (WCl₆) and oxalic acid (OA) were purchased from Adamas Reagent Co., Ltd. (Shanghai, China). LA-PEG (Mₙ = 5000) was obtained from Ponsure Biotech, Inc. (Shanghai, China). α-Lipoic acid and dopamine hydrochloride were acquired from Alfa Aesar chemical company (China). Cy5.5-PEG-SH (Mₙ = 2000) was provided by Shanghai ToYongBio Tech.Inc. (China). Ferric chloride-6H₂O (FeCl₃·6H₂O) and ammonium bicarbonate (NH₄HCO₃) were purchased from Sinopharm Group Co., Ltd. Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, and trypsin were obtained from BI Corp. Calcein-AM and propidium iodide were obtained from BI Corp. Thermo Fisher Scientific. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Thermo Fisher Scientific. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Thermo Fisher Scientific.

Preparation of DW NSs. DW atomic layers (50 mg) were dispersed in 30 mL of DI water for untrasonication for 2 h in an ice-bath. Then the mixture was centrifuged in 3000 rpm to move the large particles, thus providing DW NSs.

Preparation of P@DW/BC NSs. DW NSs aqueous solution (1 mg mL⁻¹, 20 mL) was added by LA-DPA (10 mg) and then stirred at room temperature for 6 h. After that, the mixture was centrifuged and washed with DI water and ethanol. The as-prepared DW-PDA NSs were then dispersed in ethanol. Next, FeCl₃·6H₂O (10 mg) was added to the DW-PDA ethanol solution, and the mixture was stirred overnight. Afterward, the mixture was centrifuged and washed with DI water and ethanol to obtain Fe³⁺ modified DW Fe³⁺ NSs. Subsequently, 1 mL of NH₄HCO₃ solution (5 mM) was added to the DW Fe³⁺ ethanol solution every 1 h under ice-bath, followed by 5 additions. Then, the products were centrifuged and washed with ice water to obtain DW/BC NSs. Moreover, LA-PEG (15 mg) was added to the DW/BC aqueous solution and the mixture was stirred at an ice-bath for 6 h. Finally, the mixture was washed with DI water to remove the excess LA-PEG, thus providing P@DW/BC NSs.

Preparation of P@WS₂ NSs. Bulk WS₂ (20 mg, 99.9%, 2 µm) was dispersed in 30 mL of water for untrasonication for 6 h in an ice-bath. Then the mixture was centrifuged and washed with DI water to obtain WS₂ NSs. For PEG modification, 10 mg of WS₂ NSs was mixed with 10 mg of LA-PEG in 30 mL of water. After the mixture was stirred at room temperature for 6 h, excess LA-PEG was removed by centrifugation and repeated water washing, thus obtaining PEG@WS₂ NSs with a hydrodynamic size of about 72 nm.

In Vitro Photothermal Effect. P@DW/BC NSs aqueous solution (1 mL) with various concentrations (0, 50, 100, and 150 µg mL⁻¹) were irradiated with 808 nm laser (0.5, 1, or 1.5 W cm⁻²). The temperature of the solution was recorded by an IR camera during the irradiation.

CO₂ Detection via Ca(OH)₂ and pH Value Evaluation via BTB. DI water was boiled in advance to remove the naturally dissolved CO₂. Then P@DW/BC or P@DW NSs (400 µg) were dispersed in the water, irradiated with 808 nm laser (1 W cm⁻², 4 min) and centrifuged (10 000 rpm, 15 min) at 25 °C. The supernatants were collected and added by 0.5 mL of Ca(OH)₂ aqueous solution (1 mg mL⁻¹). Finally, the mixture was centrifuged (3000 rpm, 5 min) to determine whether CaCO₃ precipitation was yield. For pH value evaluation via BTB, to the supernatants that were collected above was added BTB, and then photos of the mixtures were taken.

In Vitro CO Detection. For in vitro CO detection, 1 mL of P@DW/BC or P@DW NSs (100 µg mL⁻¹) aqueous solution (in which the water was boiled in advance) were added by CO probe (5 µM) and PdCl₂ (5 µM). The mixtures were then irradiated with 808 nm laser (1 W cm⁻²) for 5 min. The fluorescent spectra of the mixtures were recorded (Ex: 490 nm).

Cell Culture. CT26 cells were cultured in 1640 medium containing 1% antibiotics (penicillin-streptomycin, 10 000 U/mL and 10% FBS. Macrophages (RAW 264.7) were cultured in DMEM medium containing 1% antibiotics (penicillin-streptomycin, 10 000 U/mL) and 10% FBS. These cells were grown at 37 °C with 5% CO₂ in a humidified atmosphere.

Intracellular CO Detection. For intracellular CO detection, CT26 cells were incubated with P@DW/BC or P@DW NSs (100 µg mL⁻¹) for 4 h. Then the culture medium...
was replaced by the fresh medium, and cells were irradiated with a 808 nm laser for 0, 2, 4, or 6 min. Finally, cells were cocultivated with a mixture of CO probe (1 μM) and PbCl₂ (1 μM) for 30 min, washed with PBS, and then observed via CLSM.

Cytotoxicity and Live/Dead Cell Staining Assay. CT26 cells were cocultivated with P@DW/BC NSs with various concentrations for 4 h. Then the culture medium was replaced by new medium. Cells were irradiated with/without 808 nm laser (1 W cm⁻²) for 6 min and then further cultured for 20 h. Afterward, MTT (20 μL, 5 mg mL⁻¹) was added to each well, and cells were further cocultivated for 4 h. Finally, the supernatant was replaced by 150 μL of DMSO and shaken well to measure the optical density (OD). The cell viability was determined by the following formula: cell viability (%) = OD(sample)/OD(control) × 100%. For the live/dead staining assay, cells were stained at 4 h post irradiation with calcein-AM and PI for 15 min and then observed by an inverted microscope.

Determination of Cell Cytokine Production. CT26 cells were cocultivated with P@DW/BC or P@WS₂ NSs for 4 h. Then the culture medium was replaced by new medium, and cells were irradiated with 808 nm laser (1 W cm⁻², 6 min). After that, the supernatants of the CT26 cells were transferred to the culture medium of macrophages, and the macrophages were incubated overnight. The supernatants of the macrophages were collected and analyzed using ELISA kits to evaluate the levels of TNF-α and IL-6. Besides, the macrophages that were incubated overnight were also collected, washed with PBS, fixed with 4% formaldehyde, and followed by the evaluation of the levels of intracellular expression of TNF-α and IL-6 via immunocytochemistry staining.

Animal and Tumor Models. The animal experiments were performed according to the guidelines for laboratory animals established by the Wuhan University Center for Animal Center Experiment/A3-Lab, and all study protocols were subject to approval by the Institutional Center of Wuhan University (Wuhan, China). Six-week-old female BALB/c mice were subject to approval by the Institutional Center of Wuhan Animal Center Experiment/A3-Lab, and all study protocols were performed according to the guidelines for laboratory animals approved by the Wuhan University Center for Laboratory Animals. The tumor and major organs were collected for histology and stained.

Blood samples of mice were collected for blood biochemistry and follow-up tests. Moreover, the tumor tissues of mice after various treatments at 24 h. Then the expression levels of TNF-α and IL-6 were analyzed using ELISA kits. Moreover, the tumor tissues of mice after various treatments at 24 h were also collected for immunohistochemistry staining to determine the expression of TNF-α, IL-6, VEGF, and COX-2.

In Vivo Anti-Inflammation Studies. Serum samples were isolated from mice after various treatments at 24 h. Then the expression levels of TNF-α and IL-6 were analyzed using ELISA kits. Moreover, the tumor tissues of mice after various treatments at 24 h were also collected for immunocytochemistry staining to determine the expression of TNF-α, IL-6, VEGF, and COX-2.

Systemic Toxicity Evaluation. On the 19th day, the blood samples of mice were collected for blood biochemistry analysis.

Statistical Analysis. Statistical analysis was performed using a two-tailed Student’s t test. All data were presented as means ± standard deviation (SD). The differences were considered to be statistically significant for a p value <0.05 (**p < 0.05, ***p < 0.01, ****p < 0.001).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.9b01342.

Preparation of DW nanosheets and other supporting figures and tables (PDF)

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Notes
The authors declare no competing financial interest.

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