Photodynamic Therapy

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Photosensitization of Singlet Oxygen and In Vivo Photodynamic Therapeutic Effects Mediated by PEGylated $W_{18}O_{49}$ Nanowires**

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The use of various nanomaterials, including gold nanostructures,[1] carbon nanomaterials,[2] palladium nanosheets,[3] copper sulfide nanoparticles,[4] and copper selenide nanoparticles,[5] in photothermal therapy (PTT) for the treatment of cancers and malignant tumors has been intensively investigated in recent years.[6–9] The absorption of tissue-penetrating near-infrared (NIR) light is a prerequisite for PTT reagents.[10,11] To achieve synergistic therapeutic effects, chemotherapeutic drugs and photosensitizers are sometimes immobilized on the surface of the nanomaterials.[12–14] In addition to the above-mentioned nanomaterials, transition-metal-oxide nanostructures are of particular interest, owing to their tunable localized surface plasmon resonance (LSPR). Recently, $W_{24}O_{68}$ nanorods were demonstrated to possess a strong LSPR that extends from the red edge to the NIR region of light.[15] Apart from nanorods, tungsten oxide nanowires were also developed for their use as NIR light absorbers and thus as PTT reagents for in vitro and in vivo cancer therapy.[14,16] In an earlier study, tumor suppression observed upon 980 nm light irradiation of cancer cells with internalized PEGylated tungsten oxide nanowires (PEG-$W_{18}O_{49}$ NWs) was solely attributed to result from photothermal heating.[16] Furthermore, we previously reported that spherical metal nanoparticles (NPs; such as Au, Ag, and Pt NPs) are able to sensitize the formation of singlet oxygen under visible light.[17] Moreover, internalized spherical Au NPs are able to sensitize the formation of singlet oxygen upon irradiation with 532 nm light, and consequently cause apoptosis.[18] Very recently, Au nanorods and decahedral Ag nanoparticles were also reported to sensitize the formation of singlet oxygen, leading to in vitro photodynamic cancer therapy upon irradiation with NIR light.[19] The LSPR band gap of tungsten oxide nanowires at 980 nm is approximately 1.26 eV, which is higher than the energy band gap of singlet oxygen ($\approx$0.97 eV), thus facilitating the LSPR-mediated energy transfer from the surface of the tungsten oxide nanowires to molecular oxygen to form singlet oxygen.

In this study, we report the ability of PEG-$W_{18}O_{49}$ NWs to sensitize the formation of singlet oxygen upon excitation with NIR light (980 nm). We further demonstrate that internalized PEG-$W_{18}O_{49}$ NWs are able to kill cancer cells and destruct solid tumors in mice by irradiation with 980 nm light.

Ultrathin PEG-$W_{18}O_{49}$ NWs were prepared according to reported procedures.[16] The as-prepared monoclinic, crystalline $W_{18}O_{49}$ NWs (average dimensions: length $\approx$ 50 nm, thickness $\approx$ 1 nm, width $\approx$ 4 nm, and interplanar d-spacing $\approx$ 0.378 nm for the (010) lattice) are identical to those used in our previous report[16] (see Figure S1 in the Supporting Information for structure identification using TEM and XRD, and inset of Figure 1a for the high-resolution TEM image with d-spacing). In order to obtain water-dispersible $W_{18}O_{49}$ NWs, they were PEGylated in a similar way as previously reported;[16] FT-IR spectra confirmed the successful PEGylation (see Figure S2). The UV/Vis/NIR spectrum in Figure 1a shows the extended NIR absorption of PEG-$W_{18}O_{49}$ NWs up to 1200 nm, covering the first and second biological windows.[20] The molar extinction coefficient of PEG-$W_{18}O_{49}$ NWs is approximately $0.5 \times 10^7\,M^{-1}\,cm^{-1}$ at 980 nm (see Figure S3), which is two to three orders of magnitude higher than that of conventional organic dyes and photosensitizers. In addition to the strong NIR absorption, we observed that PEG-$W_{18}O_{49}$ NWs can sensitize the formation of singlet oxygen upon photoexcitation, which is evident by the observation of phosphorescence from singlet oxygen at approximately 1264 nm (see Figure 1b). The corresponding excitation spectrum (see Figure 1a) shows that singlet oxygen can only be formed upon excitation with NIR light (875–1100 nm), but not with visible light and short-wavelength NIR light. Figure 1a also shows that the spectra of absorption and singlet oxygen excitation do not match. The mismatch clearly indicates that PEG-$W_{18}O_{49}$ NWs do not follow the Kasha–Vavilov rule.[21] For most organic compounds, the internal conversion occurs very efficiently from upper electronic excited states (S_n) to the lowest excited state (S_1). In such cases, photophysical and photochemical reactions always occur from the lowest electronic excited states (either S_1 or T_1), and are therefore independent of the excitation wavelengths, that is, they follow the Kasha–Vavilov rule.[21] Inorganic nanomaterials, such as metal nanoparticles and nanorods, contain many different LSPR states. Moreover, the LSPR excited states do not conjugate with each other, that is, internal conversions among different LSPR excited states are very inefficient, and different LSPR excited states may have their own photophysical and photochemical properties. Con-

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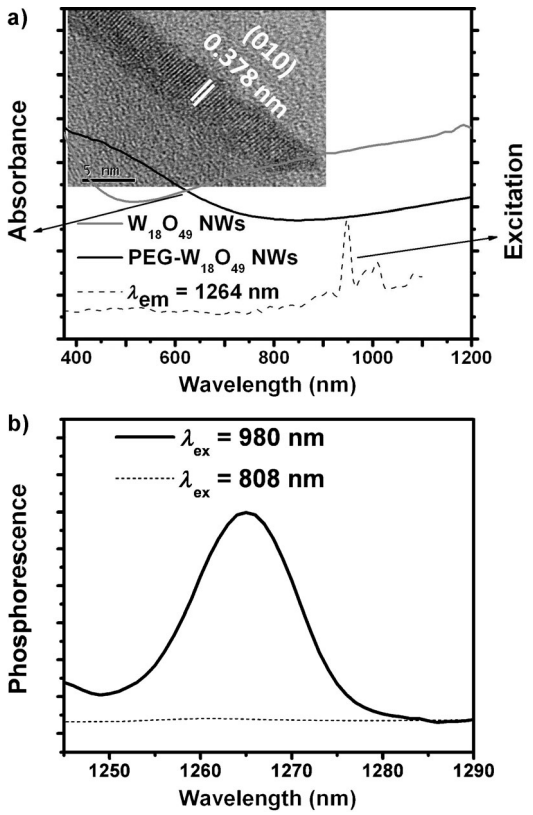

Figure 1. a) UV/Vis/NIR absorption spectrum of $W_{18}O_{49}$ nanowires before (grey solid line) and after (black solid line) PEGylation and excitation spectrum (black dashed line) for the phosphorescence of singlet oxygen (λ_{em} = 1264 nm) for PEG-$W_{18}O_{49}$ NWs. Inset: HR-TEM image of a single PEG-$W_{18}O_{49}$ nanowire. b) Phosphorescence of singlet oxygen caused by sensitization of PEG-$W_{18}O_{49}$ NWs at 808 and 980 nm. A long pass filter of 1000 nm was used to remove the stray and scattering light from the excitation wavelength.

sequently, the photoexcitation of inorganic metal nanostructures at different wavelengths will lead to different photophysical and photochemical behavior, as they do not follow the Kasha–Vavilov rule.[17,19] Figure 1b shows a very distinct phosphorescence of singlet oxygen at approximately 1264 nm upon excitation with 980 nm light, whereas excitation with 808 nm light does not result in any noticeable phosphorescence of singlet oxygen. The quantum yield for the sensitized formation of singlet oxygen by PEG-$W_{18}O_{49}$ NWs at an excitation wavelength of 980 nm is 0.29 (see the Supporting Information for measurement details).

In order to investigate the photodestruction capabilities of PEG-$W_{18}O_{49}$ NWs and also to differentiate the photodynamic from the photothermal effects, HeLa cells internalized with PEG-$W_{18}O_{49}$ NWs were irradiated using continuous-wave lasers with light of two different wavelengths, that is, 808 nm (200 mW cm^{-2}; 20 min) and 980 nm (200 mW cm^{-2}; 16 min). The irradiation times were adjusted according to the absorbance at 808 and 980 nm, so that the amount of photons that are absorbed by PEG-$W_{18}O_{49}$ NWs are the same at both 808 and 980 nm. When higher light intensities (808 and 980 nm, 500 mW cm^{-2} for 5 min) were used, more cellular death was observed in the absence of internalized PEG-$W_{18}O_{49}$ NWs (see control experiments shown in Figure S4). To avoid error sources from cellular death induced by direct laser heating, we set the light intensities in our experiments to 200 mW cm^{-2}, which is below the skin-tolerance threshold of approximately 360 mW cm^{-2} at 980 nm set by the American National Standards Institute (ANSI).[24] Furthermore, the intensity is 3.5 times lower than the previously reported intensity used for PTT of cancer cells with PEG-$W_{18}O_{49}$ NWs (980 nm, 720 mW cm^{-2}),[16] as well as two to three orders of magnitude lower than the intensities (1–22 W cm^{-2}) used in most studies of nanomaterials-mediated PTT.[22,23]

No noticeable amount of cellular death was observed when cells with PEG-$W_{18}O_{49}$ NWs were incubated in the dark (Figure 2a), whereas their irradiation with 808 and 980 nm laser light resulted in a significant amount of cellular death. The amount of cellular death induced by with 980 nm light is about 2.3 times higher than that with 808 nm light (55.5 % vs. 22 % cell death at a concentration of 200 μg mL^{-1}, see Figure 2a). When the average temperature was lowered from 37 to 4 °C, cellular death decreased dramatically (drop from 22 % to 11 %) for the cells irradiated with 808 nm light, but only a little (53 %) for the cells irradiated with 980 nm light, although the number of photons that were absorbed by PEG-$W_{18}O_{49}$ NWs were the same. At 4 °C, the amount of cellular death is about five times higher with 980 nm light compared with 808 nm light (see Figure 2b), suggesting that the pathways that cause cellular death might be different for the different irradiation wavelengths. From the excitation spectrum for the phosphorescence of singlet oxygen (Fig-

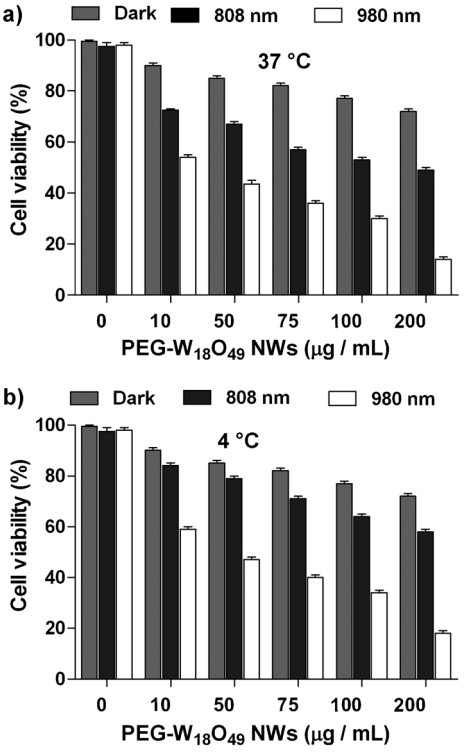

Figure 2. Cell viabilities of HeLa cells internalized with PEG-$W_{18}O_{49}$ NWs and treated in the dark and under photoirradiation conditions (808 and 980 nm) at a) 37 °C and b) 4 °C.

ure 1a), we know that PEG-$W_{18}O_{49}$ NWs can sensitize the formation of singlet oxygen with 980 nm light, but not with 808 nm light. Therefore, cellular death originating from irradiation with 808 nm light is probably a result of photothermal heating, whereas the photodynamic therapy (PDT) effect mediated by singlet oxygen is the only controlling factor responsible for the observed cellular death upon irradiation with 980 nm light. It is well understood that the PTT effect will be significantly suppressed by lowering the average temperature from 37 to 4 °C, because it only triggers apoptosis when the local cellular temperature reaches a threshold value and is maintained for a certain period of time, (e.g., 42–45 °C for 15–60 min or > 50 °C for 4–6 min).

To find further evidence supporting the existence of PDT effects upon irradiation with 980 nm light, the amount of reactive oxygen species (ROS) in HeLa cells with and without pretreatment with sodium azide (NaN_3) were measured. NaN_3 is a specific scavenger for singlet oxygen,[1,25] thus suppressing ROS levels inside cells when ROS originate from singlet oxygen. According to Figure 3a, the amounts of ROS generated by irradiation with 980 nm light are much higher than those generated by irradiation with 808 nm light. In addition, pretreatment of HeLa cells with NaN_3 drastically suppresses ROS levels in the case of irradiation with 980 nm light (see Figure S5 for flow cytometry histograms). The results shown in Figure 3a thus also point toward the conclusion that upon excitation with 980 nm light, cellular death is mostly the result of PDT effects mediated by singlet oxygen.

In order to confirm the role of singlet oxygen more accurately, HeLa cells were treated with singlet oxygen sensor green (SOSG) before photoirradiation. The nonfluorescent SOSG is a selective sensor for singlet oxygen,[26] but is inert to hydroxyl radical as well as superoxide, and will become fluorescent upon reaction with singlet oxygen.[26] According to the results shown in Figure 3b, HeLa cells internalized with PEG-$W_{18}O_{49}$ NWs show higher amounts of singlet oxygen upon irradiation with 980 nm light than upon irradiation with 808 nm light. Moreover, the mean fluorescence intensities of SOSG upon exposure to 808 nm light are similar to those in the absence of internalized PEG-$W_{18}O_{49}$ NWs, thus indicating that photoirradiation with 808 nm light does not generate singlet oxygen inside HeLa cells (see Figure S6 for flow cytometry histograms).

The ability to sensitize the formation of singlet oxygen does not guarantee that a photosensitizer can be used as an efficient PDT reagent for the in vivo destruction of solid tumors. As a proof-of-concept study, we carried out in vivo experiments in order to evaluate the PDT and PTT effects of PEG-$W_{18}O_{49}$ NWs to destruct B16F0 melanoma tumors in mice. The PEG-$W_{18}O_{49}$ NWs were directly injected into the tumor sites, and the tumors of two groups of tumor-implanted C57BL/6J mice immediately exposed to light from two different diode lasers, that is, 808 nm light (200 mW cm^{-2}; 11 min) and 980 nm light (200 mW cm^{-2}; 9 min), respectively (Figure 4a–d). Photoirradiation of tumors injected with PEG-$W_{18}O_{49}$ NWs with 808 nm light results in a significant elevation of the temperature from 35 to 45 °C, whereas no noticeable temperature rise was observed as a result of

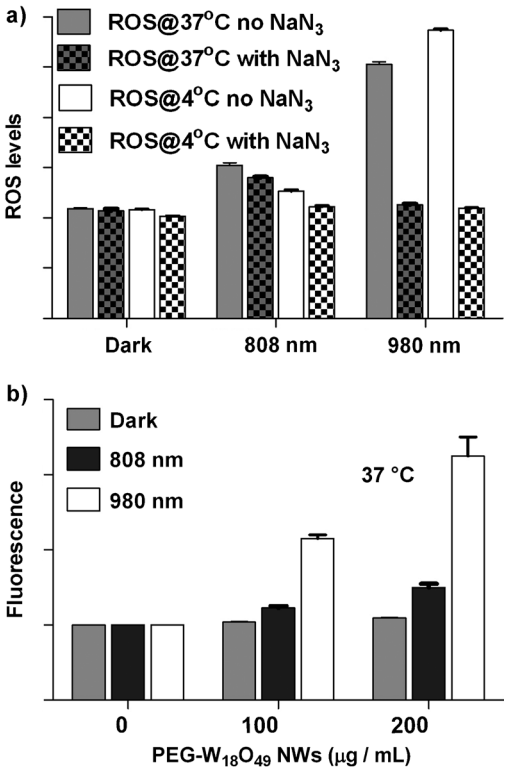

Figure 3. a) ROS generation monitored by DCF fluorescence using flow cytometry for HeLa cells internalized with PEG-$W_{18}O_{49}$ NWs, followed by photoirradiation with and without NaN_3 pretreatment. Concentration of PEG-$W_{18}O_{49}$ NWs: 200 μg mL^{-1}. b) Fluorescence levels of SOSG as a function of the amount of PEG-$W_{18}O_{49}$ NWs determined from HeLa cells fed at 37 °C for 4 h in the dark, followed by photoirradiation under different conditions. DCF = 2′,7′-dichlorofluorescein.

photoirradiation with 980 nm light. The overall profiles of the temperature rise upon photoirradiation of tumor sites are shown in Figure 4e. The results are consistent with our expectation that photoirradiation with 808 nm light will result in a pure PTT effect, whereas photoirradiation with 980 nm light will mostly create a PDT effect, in combination with a very small PTT effect.

As shown in Figure 5a, the mice treated with 980 nm light have the smallest tumor sizes (0.009 % of initial tumor size) at day 10, compared to mice treated with 808 nm light (≈30.2 %) and doxorubicin-treated mice (≈36 %). During the course of the therapy, PDT-treated mice showed scar formation, and later on (day 28), the skin at the irradiation site peeled off and new skin was developed. The body weights of the mice in all treatment groups did not show any noticeable changes during the course of the therapy (see Figure S10 in the Supporting Information). Moreover, the average half-life of 29 days for mice irradiated with 980 nm light is far higher than that of doxorubicin-treated mice (20 days) and that of untreated mice (18 days; see Figure S11). The activity of caspase-3 in tumor tissue dissected from different treatment groups immediately after photoirradiation indicates a higher number of apoptotic cells in the histological sections of mice irradiated with 980 nm light compared with mice irradiated with 808 nm light (see Fig-

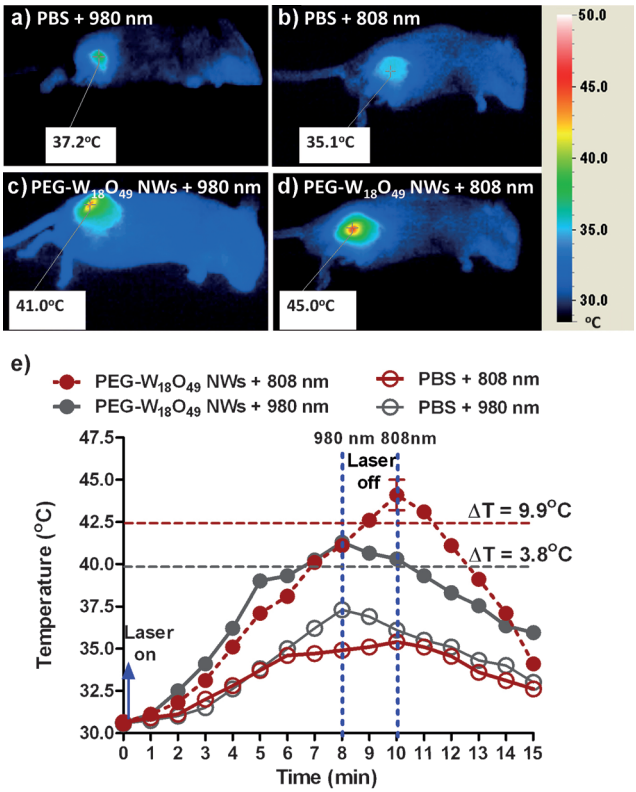

Figure 4. a)–d) Photothermal images of mice under different conditions as labeled in the figures. e) Temperature rise profiles as a function of irradiation time for conditions (a)–(d). Concentration of PEG-$W_{18}O_{49}$ NWs injected into the tumor: 15 mg kg^{-1}. The dashed blue lines are the time points where the lasers were turned off.

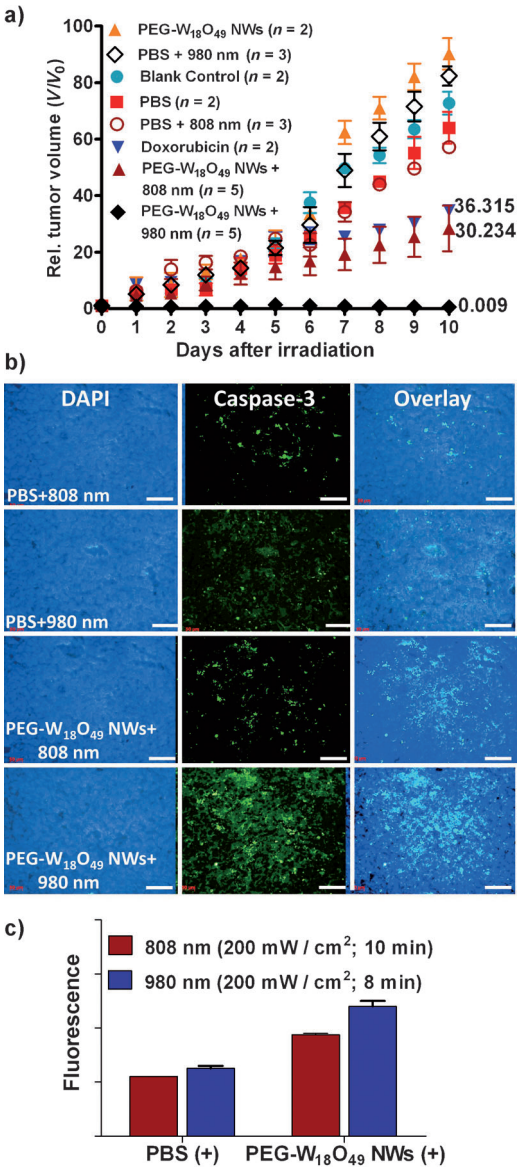

Figure 5. a) Relative tumor volume in eight different groups as a function of time (days 0–10); n = number of experiments. b) Caspase 3 activity in tumor tissue dissected from the treatment groups immediately after phototherapeutic treatment. Scale bars: 50 μm. DAPI = 4,6-diamidino-2-phenylindole. c) Apoptotic index represented as the fluorescence intensities of caspase 3; quantifying the whole tumor tissue area of various treatment groups.

ure 5b and c). The tumor and organs such as liver and spleen of mice injected with PEG-$W_{18}O_{49}$ NWs and PBS were also histologically examined after irradiation with 808 and 980 nm light. For mice internalized with PEG-$W_{18}O_{49}$ NWs and treated with both 808 and 980 nm light, necrosis of the tumor tissue was clearly observed, but liver and spleen were not damaged noticably (see Figure S12). These results suggest the successful destruction of tumor cells as a result of PDT effects of PEG-$W_{18}O_{49}$ NWs. To the best of our knowledge, this is the first demonstration of PDT effects mediated by PEG-$W_{18}O_{49}$ NWs upon excitation with NIR laser light (980 nm). PEG-$W_{18}O_{49}$ NWs with their extended NIR absorption of up to 1200 nm have great potential to serve as NIR absorbers in the second biological window, promising deep tumor tissue PDT and PTT treatments.

The synthetic process and working mechanisms responsible for the cellular death mediated by PEG-$W_{18}O_{49}$ NWs are summarized in Figure S13. Compared to PTT treatment, PDT treatment has several advantages: a) PDT does not require the intracellular temperature to reach a threshold temperature of 42 °C or to maintain it for a certain time; b) higher laser doses adopted in PTT treatments could pose several problems, such as reshaping and unwanted burning/damage of tissue; c) PDT treatment can be much more efficient at low laser doses, where nearly every singlet oxygen can cause damage to cancer cells.

In summary, we have developed PEGylated $W_{18}O_{49}$ nanowires, which can sensitize the formation of singlet oxygen and thus cause the complete destruction of solid tumors in mice upon irradiation with 980 nm light. To the best of our knowledge, this is the first reported example of a "nanomaterial-mediated photodynamic therapy" effect to completely destruct tumors in mice upon irradiation with NIR light. We showed the strong dependence of the phototherapeutic effect of $W_{18}O_{49}$ nanowires on the excitation wavelength. Under low-power laser irradiation (980 nm, 200 mW cm^{-2}), the major pathway responsible for cellular

death is the PDT-initiated apoptosis with a very small contribution from the PTT effect (53% PDT vs. 2.5% PTT in 55.5% total cellular death). When 808 nm light was used, cellular death is solely a result of the PTT effect. The existence of the PDT and PTT pathways was proven by the detection of ROS and heat-shock protein (HSP 70). We have also demonstrated that the extinction coefficients of PEG-$W_{18}O_{49}$ NWs, which are approximately $10^7\,M^{-1}cm^{-1}$ (e.g., $\approx 0.55\times10^7\,M^{-1}cm^{-1}$ at 980 nm), are about two to three orders of magnitude higher than the extinction coefficients of conventional organic photosensitizers. The ability to be excited by NIR light in order to exert both PDT and PTT effects is a very important and unique feature of the PEG-$W_{18}O_{49}$ NWs, because organic photosensitizers that can be activated by NIR light are very rare. Our in vivo experiments have also shown that the PDT effect mediated by PEG-$W_{18}O_{49}$ NWs is far more effective than their PTT effect and the effect of the anticancer drug doxorubicin in the destruction of tumors in mice. Therefore, PEG-$W_{18}O_{49}$ NWs are very good PDT and PTT reagents for the destruction of tumors without the need of additional chemotherapeutic drugs and organic photosensitizers.

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