In-situ TiO$_{2-x}$ decoration of titanium carbide MXene for photo/sono-responsive antitumor theranostics

Dong-Yang Zhang$^{1,2,†}$, Hengke Liu$^{2,†}$, Muhammad Rizwan Younis$^{2,†}$, Shan Lei$^2$, Yunzhi Chen$^1$, Peng Huang$^{2,*}$ and Jing Lin$^{1,2,*}$

Abstract

Background: Sonodynamic therapy (SDT) has emerged as a noninvasive therapeutic modality that involves sonosensitizers and low-intensity ultrasound. However, owing to the rapid recombination of charge carriers, most of the sonosensitizers triggered poor reactive oxygen species (ROS) generation, resulting in unsatisfactory sonodynamic therapeutic effects.

Results: Herein, a photo/sono-responsive nanoplatform was developed through the in-situ synthesis of TiO$_{2-x}$ on the surface of two-dimensional MXene (titanium carbide, Ti$_3$C$_2$) for photoacoustic/photothermal bimodal imaging-guided near-infrared II (NIR-II) photothermal enhanced SDT of tumor. Because of several oxygen vacancies and smaller size (~10 nm), the in-situ formed TiO$_{2-x}$ nanoparticles possessed narrow band gap (2.65 eV) and high surface area, and thus served as a charge trap to restrict charge recombination under ultrasound (US) activation, resulting in enhanced sonodynamic ROS generation. Moreover, Ti$_3$C$_2$ nanosheets induced extensive localized hyperthermia relieves tumor hypoxia by accelerating intratumoral blood flow and tumor oxygenation, and thus further strengthened the efficacy of SDT. Upon US/NIR-II laser dual-stimuli, Ti$_3$C$_2$@TiO$_{2-x}$ nanoplatform triggered substantial cellular killing in vitro and complete tumor eradication in vivo, without any tumor recurrence and systemic toxicity.

Conclusion: Our work presents the promising design of photo/sono-responsive nanoplatform for cancer nanotheranostics.

Keywords: Titanium carbide, Oxygen deficient titanium dioxide, Photoacoustic imaging, Photothermal therapy, Sonodynamic therapy
Introduction

Sonodynamic therapy (SDT), a type of non-invasive tumor modality, offered promising advantages than conventional tumor treatment, such as high tissue penetration, high spatiotemporal selectivity, and non-invasiveness [1–6]. In SDT, sonosensitizers triggered reactive oxygen species (ROS) production under ultrasound (US) stimulation, leading to selective tumor cell killing with minimal damage to nearby healthy cells [7–11]. Hence, several organic and inorganic sonosensitizers have been developed, however, organic sonosensitizers are mainly suffer from high skin phototoxicity [12, 13]. Alternatively, inorganic sonosensitizers with negligible phototoxicity and enhanced chemical stability, respectively, have shown great promise for tumor SDT. Especially, titanium dioxide (TiO₂) as an inorganic sonosensitizer, has been widely employed, but the rapid recombination (50±30 ns) of US-mediated charge carriers (electron–hole pair) endows TiO₂ with poor ROS quantum yield, resulting in limited SDT efficacy [14]. Previous reports suggested that the presence of oxygen deficiencies within TiO₂ or the integration of TiO₂ with noble metals could improve the SDT activity [15–18], however, because of the large size TiO₂ NPs, the adsorption of oxygen molecules onto the surface of TiO₂ is remarkably poor, leading to unsatisfactory ROS yield. Thus, an optimal design engineering of inorganic sonosensitizers is desirable to overcome these limitations, promoting high SDT performance under US activation. Moreover, as the SDT reaction is exclusively dependent on tissues oxygen, the continuous sonodynamic ROS generation induced severe tumor hypoxia due to oxygen depletion, and hence, restricts the overall SDT efficacy. Therefore, the integration of SDT with other non-invasive tumor treatment modalities, which may complement SDT effects, is greatly needed.

Being activated by an external light source, photothermal therapy (PTT) is an ideal non-invasive treatment modality, which induced irreversible destruction of tumor cells via localized hyperthermia generated by photothermal agents [19–22]. As photothermal agents with NIR-I light absorption have been actively developed, NIR-I tumor PTT is well-established, while PTT of deep-seated tumors at second biowindow (NIR-II) is yet limited. Moreover, though NIR-I laser light excitation endows PTT with sufficient penetration depth, an inhomogeneous thermal distribution as well as tumor self-regulation limit the complete photothermal eradication of deep-seated tumors, favoring tumor recurrence and metastasis [23]. Previously, Xia et al. demonstrated photothermal enhanced photodynamic therapy (PDT) of Hela tumor in vivo under low power single NIR-I laser activation [24]. They suggested that localized hyperthermia could not only activate PDT by the controlled release of indocyanine green, but also relieve PDT-induced tumor hypoxia, leading to accelerate photodynamic tumor killing. Recently, our group have shown that PTT under both NIR-I and NIR-II laser excitation, could enhance multimodal tumor therapies such as starvation therapy, chemodynamic therapy, and immunotherapy [25–28]. Notably, it has been reported that PTT could complement SDT as mild hyperthermia accelerates intratumoral blood flow and tumor oxygenation, which promote sonodynamic ROS generation by relieving tumor hypoxia [29–31]. Whereas, under US activation, SDT
also complements PTT by ROS-mediated tumor cell killing as well as replenishment of thermally resistant deeply localized tumors due to the high penetration depth of US [32]. Considering the prominent features of individual PTT and SDT, their integration would be highly advantageous to overcome their inherent limitations and achieve ultimate therapeutic effects.

Two-dimensional MXene nanomaterials, such as titanium carbide (Ti$_3$C$_2$), are widely used in biomedical applications [33–40]. Particularly, Ti$_3$C$_2$ with good absorption in NIR-II region, excellent photothermal performance, and low toxicity, has become a potential photothermal therapeutic agent. Furthermore, multimodal imaging capacity of Ti$_3$C$_2$ provides an opportunity to monitor nanomaterials biodistribution as well as effectively guide the tumor treatment in vivo. Owing to the promising biomedical characteristics, Ti$_3$C$_2$ is an ideal candidate to couple with sonosensitizer to trigger PTT enhanced SDT of deep-seated tumors at second biowindow. In this study, a Ti$_3$C$_2$-based 2D nanotheranostic hybrid is constructed in-situ for duplex photoacoustic (PA)/photothermal imaging-guided synergistic PTT/enhanced SDT at NIR-II biowindow (Scheme 1). In brief, the multifunctional nanohybrid (Ti$_3$C$_2$@TiO$_2$$_x$) is developed by a hydrothermal method via in-situ growth of TiO$_2$$_x$ nanoparticles with several oxygen defects onto Ti$_3$C$_2$ nanosheets. The resultant Ti$_3$C$_2$@TiO$_2$$_x$ were further modified with polyethylene glycol (PEG), defined as TTP, showing good biocompatibility and aqueous dispersibility in various physiological solutions. The as-obtained TTP not only exhibits good photothermal performance, but also demonstrates superior ROS production under US excitation than TiO$_2$ due to high surface area and the presence of multiple oxygen vacancies, promoting enhanced separation of charge carriers (electron–hole pairs). A remarkably higher therapeutic efficacy was achieved under dual-modal PA/PT imaging guidance both in vitro and in vivo, without any obvious toxic effects after intravenous administration. This study provides a promising strategy of photo-sonoinduced theranostics of deeply localized tumors.

**Material and methods**

**Preparation of Ti3C2@TiO2-x-PEG (TTP)**

Ti$_3$C$_2$ nanosheets were obtained following the reported literature [36]. Then, 2D Ti$_3$C$_2$@TiO$_2$ hybrid was fabricated according to the previous report with slight modification. 100 mg of Ti$_3$C$_2$ was dispersed in 3.0 M hydrochloric acid (15 mL) containing 0.04 M ammonium fluoride. Above dispersion was transferred to the Teflon stainless steel autoclave and kept at 200 °C for 12 h. After centrifugation and washing with deionized (DI) water, the as-collected Ti$_3$C$_2$@TiO$_2$ precipitate was added to hydrize hydrate solution (25 mL, 50 wt%) under
stirring. Next, the suspension was further transferred to the Teflon stainless steel autoclave and kept at 200 °C for 12 h. The final Ti3C2@TiO2-x hybrid was obtained by centrifugation and washing with DI water.

To improve the solubility and biocompatibility of Ti3C2@TiO2-x, 20 mg of DSPE-PEG dissolved in chloroform (20 mL) was added dropwise into Ti3C2@TiO2-x ethanol solution (2 mg/mL, 20 mL) under ultrasonication. The product was obtained by evaporating the solvent and then resuspended in phosphate buffered saline (PBS), and finally stored at 4 °C for future use.

In vivo toxicity
BALB/c female mice (n ≥ 3) were intravenously (i.v.) injected with TTP nanohybrids (4 mg/mL, 200 μL) or PBS (200 μL). The body weight of mice was recorded every 3 days. The blood and primary organs (kidneys, lungs, spleen, liver, heart) were acquired after 30 days post-injection. The blood was used to perform hematoxylin and eosin (H&E). All animal experiments were conducted as per the approved institutional guidelines for the care of laboratory animals of Shenzhen University.

Photoacoustic/thermal imaging and biodistribution of nanohybrids in vivo
PA signals of the varying concentrations of TTP nanohybrid in solution were recorded by a photoacoustic imager. For in vivo NIR-II PA imaging, 4T1 tumor-bearing mice (n = 3) were i.v. injected with TTP nanohybrids (2 mg/mL, 200 μL), and the PA signals were monitored at different time points (0, 1, 2, 4, 8, and 24 h).

4T1 tumor-bearing BALB/c nude mice were i.v. injected with TTP nanohybrid (2 mg/mL, 200 μL). After 4 h post-injection, the tumors were irradiated by a 1064 nm laser and the real-time temperature changes were monitored with a thermal imager.

4T1 tumor-bearing nude mice (n = 3) were i.v. injected with TTP nanohybrid (2 mg/mL, 200 μL). After 24 h post-injection, the mice were euthanized to collect the primary organs. The primary organs were digested by hydrochloric acid, and the organs were stained by hematoxylin and eosin (H&E). All animal experiments were conducted as per the approved institutional guidelines for the care of laboratory animals of Shenzhen University.

In vivo treatment
4T1 tumor-bearing BALB/c nude mice with ~50 mm³ tumor volume, were randomly divided into 6 groups (n ≥ 5) as follows: (i) PBS, (ii) laser + US (iii) TTP nanohybrid, (iv) TTP nanohybrid + US, (v) TTP nanohybrid + laser, (vi) TTP nanohybrid + laser + US. After 4 h post-injection, the tumors were treated with laser irradiation (1064 nm, 0.8 W/cm², 10 min) or/and US (1 W/cm², 5 min). The tumor volumes and body weights of mice were recorded every 2 days. The tumor volume was calculated based on the formula: V (mm³) = AB²/2, where A and B are the maximum length (mm) and the minimum width (mm) of the tumor, respectively. The body weights of mice were recorded every 2 days. The mice were sacrificed at 14th day post-treatment to collect tumors, serum, and primary organs. The tumors were photographed, weighed, and stained by Terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) assay. The serum was used to determine the levels of liver and kidney function indicators, while the primary organs and tumor were stained by H&E for histopathological investigations.

Results and discussion
Synthesis and characterization
The development of TTP nanohybrid was carried out in several steps. In brief, bulk Ti3C2 powder was first exfoliated via ultrasonication by using TPAOH as an intercalating agent, resulting in the formation of 2D Ti3C2 nanosheets with an approximate lateral dimension of 150 nm (Fig. 1A). Next, TiO2 NPs were grown in situ onto the as-obtained 2D Ti3C2 nanosheets by hydrothermal method [41]. Compared to the usually prepared TiO2 NPs (~100 nm) [15, 42, 43], in situ fabricated TiO2 NPs were much smaller (~10 nm) in diameter as revealed by TEM (Fig. 1B). Considering an inverse relationship between the NPs size and their surface area, the as-obtained TiO2 NPs with high surface area could facilitate higher surface adsorption of oxygen molecules and the separation of electron–hole pairs, promoting higher ROS generation [44, 45]. Finally, an engineering of in-situ produced Ti3C2@TiO2 was performed through hydrazine hydrate reduction method [46], which induced multiple oxygen defects within TiO2 NPs, resulting in the formation of Ti3C2@TiO2-x nanohybrid. Notably, no apparent aggregation or morphological change was seen after modifications as shown in Fig. 1C, D. The selected area electron diffraction (SAED) pattern of Ti3C2@TiO2-x (Fig. 1E, inset) indicated the crystalline nature of TiO2, whereas the lattice spacings of 0.352 nm were assigned to (101) plane of anatase TiO2 [47]. Meanwhile, energy dispersive X-ray spectroscopy (EDS) indicated the presence of titanium, oxygen, and carbon elements in Ti3C2@TiO2-x (Fig. 1F, G and Additional file 1: Fig. S1A, B). Compared to Ti3C2 (20 nm), ~40–80 nm of height increases were noticed for Ti3C2@TiO2 and Ti3C2@TiO2-x indicating in situ production and the decoration of TiO2 onto Ti3C2 (Additional file 1: Fig. S2). The X-ray diffraction (XRD) spectrum of TiO2-x@Ti3C2 was well-indexed
with TiO₂ (JCPDS no. 21–1272) and Ti₃C₂ (JCPDS no. 32–1383) as shown in Additional file 1: Fig. S3. The elemental composition and oxygen vacancies were further confirmed by XPS spectroscopy. Additional file 1: Fig. S4A–E and Fig. 1H, I verified the presence of titanium (25.92%, 15.7%), oxygen (52.47%, 25.17%)
and carbon (21.62%, 44.74%) in Ti3C2@TiO2-x hybrid, respectively, while O 1 s peak is negatively shifted from 529.9 (Ti3C2@TiO2) to 529.2 eV in Ti3C2@TiO2-x nano-hybrid (Fig. 1H, I), which is attributed to the existence of oxygen vacancies within Ti3C2@TiO2-x, favoring highly efficient ROS production [48, 49]. The quantitative analysis also indicated a prominent decrease in O/Ti ratio from 2 to 1.6 after reduction (Additional file 1: Fig. S4F), implying the creation of oxygen vacancies in Ti3C2@TiO2-x. Such a thorough characterization proved the successful fabrication of in-situ 2D Ti3C2@TiO2-x nanohybrid with desired oxygen deficiencies to accelerate ROS generation.

Later, Ti3C2@TiO2-x was surface functionalized with DSPE-PEG, which imparts an aqueous solubility and biocompatibility. Notably, the surface PEGylation did not induce any observable morphological change in the resultant TTP and Ti3C2@TiO2-PEG as shown in Additional file 1: Fig. S5A. B. FT-IR spectra of TTP showed the presence of –CH2 and C–O bonds at 2870 and 1100 cm−1, respectively, which are assigned to the DSPE-PEG modification (Additional file 1: Fig. S6), while the amount of PEG loaded onto Ti3C2@TiO2-x hybrid was about 3% as quantitatively determined by TGA (Additional file 1: Fig. S7), indicating the successful surface grafting of Ti3C2@TiO2-x hybrid by PEG. The hydrodynamic diameter of TTP was about 170 nm as determined by DLS (Additional file 1: Fig. S8), whereas, the surface zeta potential of Ti3C2@TiO2-PEG and TTP was about −7.8 and −11.2 eV, respectively, suggesting limited protein binding and longer blood circulation (Additional file 1: Fig. S9) [50]. Owing to PEG grafting, TTP and Ti3C2@TiO2-PEG exhibited good stability in different physiological solutions such as PBS, Dulbecco’s modified eagle medium (DMEM), and fetal bovine serum (FBS) as shown in Additional file 1: Fig. S10A, B. Meanwhile, no notable change was recorded in the hydrodynamic diameter of TTP and Ti3C2@TiO2-PEG under different physiological solutions for 3 days, verifying the excellent colloidal stability of TTP (Additional file 1: Fig. S10C, D). UV–vis/NIR absorption spectroscopy indicated the broadband absorption of TTP in NIR-II region without any particular absorption peak (Fig. 1I), which is solely ascribed to the presence of 2D Ti3C2 sheets, suggesting the potential of as-designed TTP hybrid for dual-modal PT/PA imaging and PTT at second biowindow.

**Evaluation of photothermal and sonodynamic capacity**

Considering the good NIR-II absorption, the photothermal capacity of TTP was investigated. Figure 2A, B displayed significant temperature enhancement in TTP solution under 1064 nm laser irradiation (0.8 W/cm2) for 5 min, which is directly proportional to the irradiation time and TTP concentration, respectively. Furthermore, the calculated photothermal conversion efficiency (PCE, η) of TTP was about 35.8% (Additional file 1: Fig. S11A, B) [51], which is superior or even comparable to many previously reported photothermal nano-agents [52–55]. Notably, under 5 repetitive laser on/off cycles, no apparent temperature decrease was recorded as shown in Additional file 1: Fig. S12A. Moreover, the absorption spectrum and TEM morphological characterization did not show any particular change in TTP even after 1064 nm laser irradiation for 20 min, suggesting the excellent photothermal stability of TTP (Additional file 1: Fig. S12B, C). Inorganic TiO2 nanoparticles have been widely reported as sonosensitizers, capable of ROS production such as singlet oxygen (1O2) and hydroxyl radicals (·OH), etc. under US irradiation [17, 18, 42, 56, 57]. Therefore, electron spin resonance (ESR) spectroscopy was employed to determine the ROS generation by TTP under US activation. Figure 2C presented the characteristic (1:1:1) peaks of 1O2 after US stimulation of both Ti3C2@TiO2-PEG and TTP group, respectively. The 1O2 generation ability of TTP was further evaluated by using 1, 3-diphenylisobenzofuran (DPBF) probe. Figure 2D and Additional file 1: Fig. S13 showed time-dependent gradual decrease in DPBF absorbance at 410 nm by both Ti3C2@TiO2-PEG and TTP group, while the control group did not reduce the DPBF absorption at all. It is worth mentioning that TTP showed stronger 1O2 production capacity than Ti3C2@TiO2-PEG, which is possibly ascribed to the presence of several oxygen vacancies within TiO2-x. Notably, these oxygen vacancies...
Fig. 2 (See legend on previous page.)
played a vital role as they promoted effective separation of charge carriers (electron–hole pairs), and thus facilitate an enhanced interaction between charge carriers and surface adsorbed oxygen molecules, leading to enhanced ROS generation. Meanwhile, the concentration and US power-dependent $^1\text{O}_2$ production was also recorded by TTP as shown in Fig. 2E, F and Additional file 1: Fig. S14A, B. Besides $^1\text{O}_2$, the characteristic (1:2:2:1) peaks of 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) and ·OH adducts were appeared both in TiO$_2$@Ti$_3$C$_2$-PEG and TTP groups, indicating the generation of ·OH ions (Fig. 2G). Similarly, a ·OH indicator TPA, which emits fluorescence after reacting with ·OH under UV light, was also used to detect the ability of TTP to produce ·OH under US stimulation. As shown in Additional file 1: Fig. S15, a strong fluorescence signal is generated at 422 nm after Ti$_3$C$_2$@TiO$_2$-PEG and TTP activation by US. However, the control group and US irradiation alone showed much weaker fluorescence signal. The quantitative analysis suggested that TTP has a stronger ·OH production ability than Ti$_3$C$_2$@TiO$_2$-PEG. Similar to $^1\text{O}_2$, the production of ·OH is also dependent on TTP concentration and US power density (Fig. 2H, I). These results verified that TTP holds excellent $^1\text{O}_2$ and ·OH production capacity under ultrasonic stimulation. For better apprehension of the sonodynamic performance of Ti$_3$C$_2$@TiO$_2$-x, the band gap calculation was made by Kubelka–Munk function, which was about 3.32 and 2.65 eV for TiO$_2$ and Ti$_3$C$_2$@TiO$_2$-x, respectively (Fig. 2I). Such a narrow band gap of Ti$_3$C$_2$@TiO$_2$-x than TiO$_2$ NPs could be ascribed to the overlapping oxygen deficiency induced defect states with the band edge of the semiconductor. Thereby, Ti$_3$C$_2$@TiO$_2$-x served as a charge trap, and remarkably restricts the charge recombination (electron–hole pairs) under US excitation, promoting an enhanced sonodynamic ROS generation during SDT (Fig. 2K).

**In vitro combined PTT/SDT**

The in vitro cellular uptake of TTP was determined by ICP-MS. The cellular uptake mechanism of TTP was investigated by using various inhibitors. As presented in Additional file 1: Fig. S16, the cellular uptake of TTP was significantly reduced when incubated with chlorpromazine or at 4 °C, suggesting that TTP entered into cells through an energy-dependent and clathrin-mediated endocytosis pathway. As shown in Fig. 3A, the Ti content in 4T1 cells increases gradually with an increase in incubation time, indicating an efficient cellular internalization of TTP. While, MTT assay revealed no potential cytotoxicity of internalized TTP on 4T1 and human embryonic kidney (HEK293T) cells in dark, suggesting good biocompatibility (Fig. 3B and Additional file 1: Fig. S17). However, TTP substantially reduced the cellular viability of 4T1 cells under either 1064 nm laser (PTT) or US (SDT) activation, respectively. The cellular viability was reduced up to 45% and 39% by individual PTT and SDT treatment alone (Fig. 3C), whereas, the combined PTT/SDT triggered significant cellular killing, resulting in a dramatic decrease (16.5%) in cells viability, suggesting superior therapeutic killing performance of combined PTT/SDT treatment than either PTT or SDT alone. MTT findings were also further endorsed by live and dead assay (Fig. 3D) and Annexin V-FITC and PI staining (Fig. 3E) as much higher proportion of dead cells (apoptosis and necrosis) were observed after dual laser/ US treatment (Additional file 1: Fig. S18), verifying an extensive in vitro cellular killing by synergistic PTT/SDT.

Considering that $^1\text{O}_2$ and ·OH can be produced under ultrasonic stimulation in an aqueous solution. The ROS probe 2,7-dichlorofluorescein diacetate (DCFH-DA) was utilized to assess ROS production in vitro in 4T1 cells. Negligible green fluorescence was noticed in cells after treated with US, laser, or TTP alone, while an obvious green fluorescence was seen after simultaneous treatment with TTP and US (Fig. 3F), implying significant ROS production.

**In vivo biocompatibility**

Biosafety is an important prerequisite for the development of nanodrugs in biomedical field. Hence, the in vivo biocompatibility of TTP was evaluated by hemolysis assay, hematology analysis, and H&E staining of the major organs (heart, liver, spleen, lungs, and kidneys). Additional file 1: Fig. S19 showed no adverse effects of TTP on red blood cells, while all the hematological parameters, including liver (aspartate transaminase, AST, alanine aminotransferase, ALT), kidney function (blood urea nitrogen, BUN, creatinine, CRE), and other blood biochemical indicators as shown in Fig. 4A–F, were also in normal range like PBS-treated control mice. In addition, H&E analysis did not display any apparent inflammation in major organs (Fig. 4G). Moreover, like PBS-treated mice, no particular change was recorded in the body weight of TTP-treated mice even after one month (Additional file 1: Fig. S20). Collectively, these results verified the good biocompatibility and biosafety of TTP in vivo.

**In vivo biodistribution and duplex PA/PT imaging**

Prior to the determination of in vivo therapeutic potential of TTP, we assessed the in vivo biodistribution of TTP by PA imaging and ICP-MS, respectively. As expected, the PA signal intensity was positively correlated with the concentration of TTP (Additional file 1: Fig. S21). As can be seen in Fig. 5A, B, compared to pre-injection, the PA signal intensity is significantly enhanced in tumor tissues.
after post-injection, indicating an effective tumor accumulation of TTP. Importantly, the highest PA signal was found at 4 h post-injection, which provides an effective guidance and optimal treatment time for tumor PTT in vivo. Next, the Ti content in major organs of mice was detected by ICP-MS. Figure 5C displayed prominent accumulation of TTP in reticuloendothelial system e.g., liver and spleen, whereas, 5% ID/g of TTP was found in tumor tissues, which is advantageous for combined tumor PTT/SDT in vivo. Besides PA imaging
and ICP-MS, in vivo PT imaging was also performed to determine the temperature enhancement at tumor site by intravenously injected TTP under 1064 nm laser excitation for 10 min. The temperature of TTP-treated mice was abruptly increased from 28 to 52 °C, while a slight temperature change was noticed in PBS group (Fig. 5D, E). These results demonstrated the good photothermal performance of TTP in vivo.

**In vivo synergistic PTT/enhanced SDT**

Finally, the therapeutic efficacy of synergistic PTT/enhanced SDT triggered by TTP was investigated. 4T1 tumor-bearing mice with a mean tumor volume of about 80 mm³ were randomly assigned to seven groups, and received an i.v. injection of either TTP (20 mg/kg) or PBS, respectively. The tumor volume of each mice undergo different treatment conditions, was recorded every two days, while after 14 days, all the tumors were dissected,
weighed, and photographed. As Additional file 1: d the tumor growth, whereas the combined PTT/SDT triggered complete tumor eradication. The in vivo therapeutic effects were further evaluated by TUNEL assay, which showed the highest green fluorescence intensity in the combined TPP + Laser + US treatment group (Fig. 6D), indicating substantial cellular apoptosis. Similarly, the H&E staining of tumor tissues verified the superior therapeutic effects as noticeable nuclear shrinkage and reduced number of cancer cells (Fig. 6E) were found in combined treatment than individual PTT or SDT alone. These results are in accordance with the in vitro findings. Remarkably, the body weight of the mice in each group was remained stable after different treatments (Fig. 6F). In addition, no significant lesions or damages were found in the major organs as confirmed by H&E staining (Additional file 1: Fig. S23) and blood biochemistry analysis (Fig. 6G, H), implying the negligible systemic toxicity of TTP in vivo.

**Conclusion**
In summary, TTP was prepared by the *in-situ* fabrication of ~10 nm TiO$_2$ NPs with multiple oxygen defects onto the Ti$_3$C$_2$ nanosheets, followed by PEG grafting, offering synergistic PTT/enhanced SDT under PA/PT bimodal imaging guidance in second biowindow. TTP possessed good colloidal stability, excellent photothermal conversion efficiency, and absorption in NIR-II region. Importantly, TTP with higher surface area and several oxygen-defects, facilitated higher surface adsorption of O$_2$ molecules as well as an efficient separation of charge carriers under US activation, promoting an enhanced interaction of charge carriers with surface adsorbed O$_2$ to trigger higher sonodynamic ROS generation than commonly reported TiO$_2$ NPs. Owing to good photothermal and enhanced US-stimulated ROS production capacity, TTP showed high therapeutic efficiency in vitro with appreciable biocompatibility, and demonstrated complete tumor elimination in 4T1 tumor bearing mice in vivo, because of synergistic photothermal enhanced SDT, which is far greater than single treatment modality. Our work demonstrated the design engineering of hybrid nanosystems for enhanced theranostics of deeply localized tumors in clinic.
Abbreviations
NIR-I: Near-infrared-I; ROS: Reactive oxygen species; PTT: Photothermal therapy; US: Ultrasound; SDT: Sonodynamic therapy; TiO2: Titanium dioxide; Ti3C2: Titanium carbide; PA: Photoacoustic; PEG: Polyethylene glycol; TPAOH: Tetrapropylammonium hydroxide; DSPE-PEG: 1,2-Distearyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)); TPA: Terephthalic acid; MTT: 3-(4,5)-Dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide; PI: Propidium iodide; TEM: Transmission electron microscope; XPS: X-ray photoelectron spectrometer; FT-IR: Fourier transform infrared; TGA: Thermo-gravimetric analysis; DLS: Dynamic light scattering; ICP-MS: Inductively coupled plasma mass spectrometry; TTP: Ti3C2@TiO2-x-PEG; PBS: Phosphate buffered saline; H&E: Hematoxylin and eosin; TUNEL: Terminal-deoxynucleotidyl transferase mediated nick end labeling; SAED: Selected area electron diffraction; EDS: Energy dispersive X-ray spectroscopy; XRD: X-ray diffraction; DMEM: Dulbecco’s modified eagle medium; PBS: Fetal bovine serum; O2: Oxygen.

Supplementary Information
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Additional file 1. Additional information includes part of material and methods, additional figures and tables.

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Authors’ contributions
YZ, HL, and WRY: Investigation, Data curation, Writing—original draft. SL and YC: Writing—review & editing. JL & PH: Conceptualization, Supervision, Funding acquisition, Writing—review & editing. All authors read and approved the final manuscript.

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Availability of data and materials
All data used to generate these results is available in the main text and supporting information.

Declarations
Ethics approval and consent to participate
All animal studies were approved by the Animal Ethics and Welfare Committee of Shenzhen University.

Consent for publication
All authors agree to be published.

Competing interests
The authors declare no conflict of interests.

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