Multifunctional WS$_2$@Poly(ethylene imine) Nanoplatforms for Imaging Guided Gene-Photothermal Synergistic Therapy of Cancer

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The combination of photothermal therapy (PTT) with gene therapy (GT) to improve PTT efficiency and thus eliminate cancer cells under mild hyperthermia is highly needed. Herein, multifunctional WS$_2$@poly(ethylene imine) (WS$_2$@PEI) nanoplatform has been designed and constructed for gene-photothermal synergistic therapy of tumors at mild condition. After a surface modification of WS$_2$ with a positively charged PEI, the as-prepared WS$_2$@PEI nanoplatform can not only act as an efficient survivin-siRNA carrier for GT but also exhibit remarkable near-infrared (NIR) photothermal effects for PTT. On the one hand, the photothermal effects induced by WS$_2$@PEI upon NIR irradiation can enhance the cellular uptake owing to the increase of the cell membrane permeability, which leads to the remarkable enhancement of silencing efficiency of survivin. On the other hand, the silencing of survivin can increase the apoptosis as well as reduce the heat resistance of cancer cells by downregulating the heat shock protein 70 expressions, which greatly enhance the sensitivity of cancer cells to PTT. As a result, compared to PTT or GT treatment alone, WS$_2$@PEI mediated synergistic GT/PTT therapy remarkably enhances in vitro cancer cell damage and in vivo tumor elimination.

1. Introduction

Cancer as one of the most devastating diseases in the world has caused extensive concern. However, the currently used single mode cancer therapy such as radiation therapy, chemotherapy, gene therapy (GT), and phototherapy is still far from satisfaction due to their many limitations, such as toxic side effects and limited therapeutic efficacy. For example, photothermal therapy (PTT), which converts photoenergy into heat to kill cancer cells under near-infrared (NIR) light irradiation, has increasingly attracted much attention because of its many advantages such as low cost, highly localized and specific tumor treatment, fewer side effects, and minimal trauma to tissues. Nevertheless, PTT alone may fail to eliminate the cancer cells because of the inevitable depth-dependent decline of laser intensity. What's more, some cancer cells with heat resistance are insensitive to laser irradiation along with the extension of irradiation time, which leads to the failure of PTT.[11,12] To eliminate the tumor by PTT, a high-power laser is usually required.[13,14] However, this may cause the unavoidable collateral damage to the surrounding normal tissue and exceed the tolerance of patients at high temperature, which greatly limits their clinical application for cancer treatment, especially in the head and neck cancer due to their anatomic sites and vital biological functions.[15,16] Therefore, it is highly desirable to develop a general strategy to improve PTT therapeutic effect under mild condition. To this end, the combination therapy is an embracing strategy to improve therapeutic efficiency, minimize side effects and suppress the heat resistance.[17–21] Among various types of therapies, gene therapy as one of major biological treatment in recent years has received extensive attention, which delivers the therapeutic nucleic acid into target cells as a drug to treat cancer cells.[22–26] For example, a small-interfering RNA (siRNA) can act as an effective vehicle to inhibit the expression of specific genes by preventing the translation of messenger RNA (mRNA) into protein.[27–29] Recent studies have shown that the thermal resistance of cancer cell is ascribed to the heat shock response triggered by PTT, which could prevent cancer cells from hyperthermia by suppressing apoptosis. By interfering the expression of the specific genes, it is possible to use siRNA to regulate the related genes expression, which could inhibit the heat shock response and thus make cancer cells more sensitive to PTT. Although a few reports on combination therapy of PTT and GT have been demonstrated recently,[15,19,30,31] few of them focus on...
increasing the sensitivity of cancer cells for PTT to realize the elimination cancer under mild hyperthermia.

Herein, we developed WS$_2$ nanoparticles-based platform as a new paradigm of gene carrier that doubles as a photothermal agent for combined GT/PTT therapy (Scheme 1). First, the nanoscaled transition metal chalcogenides WS$_2$ are employed as PTT agents, which could efficiently convert the light into heat and thus induce hyperthermia to tumor tissues.[18] Moreover, this kind of material has been widely employed as carriers for loading various drugs or compounds due to its special structure and large surface area.[32] Therefore, after being modified with poly(ethylene imine) (PEI), these nanoparticles may also act as gene delivery carrier for gene therapy. Therefore, WS$_2$ nanoparticle is anticipated to be an ideal multifunctional nano-platform for combined GT/PTT of cancer. Next, we choose survivin as the target gene in cancer cells. Survivin, as a member of the family of apoptosis inhibitors (IAPs), could suppress cancer cells apoptosis by blocking caspase activation.[33,34] Thus, the disruption of survivin induction pathways will enhance the caspase activation, which leads to increase in apoptosis of cancer cells and decrease in tumor growth. As a result, the downregulation of survivin may reduce the heat resistance of cancer cells and consequently make the cell sensitive to heat at relative mild temperature. Therefore, WS$_2$@PEI nanoparticles mediated synergistic GT/PTT therapy is desirable to enhance the efficacy of PTT and inhibit tumor growth at mild hyperthermia. Apart from its potential for cancer treatment, this nanoplatform may also act as contrast agent for photoacoustic (PA) and X-ray computed tomography (CT) imaging owing to its efficient absorption of NIR light and strong X-ray attenuation ability. Taking together, the as-obtained nanocomplex shows potential as the versatile nanoplatform for the dual-modal imaging guided synergistic GT/PTT of tumors under mild condition.

2. Result and Discussion

WS$_2$ quantum dots (QDs) were prepared via a liquid exfoliation method according to our previous reports.[18,35] Transmission electron microscopy (TEM) and dynamic light scattering (DLS) were employed to obtain the information about morphology and size of the as-made WS$_2$ QDs. As shown in Figure 1a, the as-prepared WS$_2$ QDs exhibited a uniform size with an average diameter of about 3 nm. To make the WS$_2$ QDs to be a gene nanocarrier, PEI as a commonly used positively charged polymer was chosen to modify the surface of WS$_2$ QDs for loading and delivery of negatively charged siRNA through the charge–charge interactions.[23,24,36] According to the previous reports,[37] we used the thiol chemistry to decorate the surface of WS$_2$ by coating the lipoic acid-PEI (LA-PEI) that has a disulfide group on the PEI terminal. To make sure LA-PEI was successfully modified on the surface of WS$_2$ QDs, DLS and Fourier transform infrared (FTIR) spectroscopy were employed to characterize the WS$_2$ QDs before and after the surface modification. As shown in Figure 1b, after LA-PEI modification, the diameter of the as-prepared WS$_2$@PEI nanoparticles increased from 3 to 8 nm and the surface charge of nanoparticles changed from $-26.4$ to $+49.9$ mV, suggesting that WS$_2$@PEI has the potential as a gene delivery carrier by incorporating the negative-charged siRNA.
The modification of polymer on its surface was further confirmed by FTIR as shown in Figure 1c, in which the two peaks at around 1649 and 1405 cm\(^{-1}\) were attributed to the internal vibrations of the amide group of PEI. LA-PEI modified WS\(_2\) QDs exhibited well stability in different physiological solutions including H\(_2\)O, phosphate buffered saline (PBS), saline, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) as shown in Figure S1a (Supporting Information). We also measured the stabilities of WS\(_2\)@PEI in different physiological solutions including PBS and FBS by DLS as shown in Figure S1b,c (Supporting Information). The average size of as-prepared NPs stored in PBS and FBS do not show much change after 24 h. Because of the strong absorption in the NIR region of WS\(_2\)@PEI (Figure S2, Supporting Information), we next evaluated the photothermal effects of as-made WS\(_2\)@PEI with different concentrations upon 808 nm NIR laser irradiation (1 W cm\(^{-2}\), 10 min). The ultrapure water was used as the control (Figure 1d). The temperature of WS\(_2\)@PEI nanoparticles samples increased more rapidly with the increase of irradiation time or the concentration of WS\(_2\)@PEI. For instance, the temperature of WS\(_2\)@PEI with the concentration of 300 \(\mu\)g mL\(^{-1}\) increased from 25 to 53 °C after NIR irradiation for 10 min, while the water only rose about 2 °C under the similar conditions. This result suggests that the as-synthesized WS\(_2\)@PEI has the remarkable potential to be applied as the PTT agent.

Survivin has been reported to play a crucial role in cell apoptosis. And it always overexpressed in many kind of cancer cells.\(^{[33]}\) The silencing of survivin can trigger the cancer cell apoptosis and thus may enhance the sensitivity to PTT treatment. To this end, WS\(_2\)@PEI nanoparticles are also anticipated to be a nanovector for the delivery of survivin-siRNA to realize the synergistic GT/PTT therapy of cancer and obtain better therapeutic outcomes. To study the binding ability of survivin-siRNA to the as-prepared WS\(_2\)@PEI nanoparticles, we mixed WS\(_2\)@PEI and survivin-siRNA at different N/P ratios and zeta potentials was recorded to monitor the changes of its surface charges. As shown in Figure 1e, after being bound with siRNA, the surface charges of WS\(_2\)@PEI reversed, and the zeta potentials changed from +27.0 to −17.2 mV when the N/P ratio varied from 12.5 to 1.24. These results indicated survivin-siRNA was successfully bound on the surface of WS\(_2\)@PEI. The study on gene binding capability of WS\(_2\)@PEI was further carried out by 1% agarose gel electrophoresis assay (Figure 1f, up panel). The mobility of siRNA was completely retarded by the WS\(_2\)@PEI at the N/P ratio higher than 5.03. In contrast, bare siRNA could not be retarded. Moreover, after introducing the heparin, siRNA could be released from WS\(_2\)@PEI completely (Figure 1f, middle panel). These results indicated that WS\(_2\)@PEI is a promising carrier for gene loading and release. Meanwhile, WS\(_2\)@PEI can also protect siRNA from degradation induced by RNase. Figure 1f (down panel) exhibited the...
band for free siRNA sample was missing after treating with RNase, which revealed that the siRNA was degraded by the RNase. While, the WS$_2$@PEI-siRNA complexes still showed visible bands, indicating that WS$_2$@PEI can prevent the siRNA from degrading. Therefore, WS$_2$@PEI could be employed as both efficient gene nanocarrier and PTT agents for GT/PTT synergistic therapy of cancer.

Encouraged by its well photothermal effects and efficient gene delivery property, we next evaluated their combinational therapeutic effect on cancer cell killing. We first evaluated the cytotoxicity of WS$_2$@PEI in BEL-7402 cells and HeLa cells by using cell counting kit-8 (CCK-8) assay. After they were exposed to WS$_2$@PEI at different concentrations (0, 12.5, 25, 50, and 100 $\mu$g mL$^{-1}$) for 24 h, no obvious cytotoxicity of WS$_2$@PEI was observed in both cells, even at a high concentration up to 100 $\mu$g mL$^{-1}$, where the cell viabilities of BEL-7402 cells and HeLa cells were still higher than 98% (Figure 2a,b). In addition, we evaluated the cytotoxicity of WS$_2$@PEI in the dark and the results revealed that no obvious cytotoxicity was observed in both cells (Figure S3, Supporting Information). These results indicated the good biosafety of WS$_2$@PEI. Then we measured the in vitro anticancer efficacy of monotherapy (GT or PTT) or combination therapy (GT+PTT) in BEL-7402 cells and HeLa cells by using CCK-8 assay. All the PTT or PTT+GT were carried out under mild condition (<45 °C). As shown in Figure 2a,b, WS$_2$@PEI alone and NIR alone treatments have no influence on the cell viability, while WS$_2$@PEI-siRNA (GT) group and WS$_2$@PEI + NIR (PTT) group decreased the cell viability to 60% and 53.8% for BEL-7402 cells and to 45% and 41% for HeLa cells, respectively, when the concentration of WS$_2$@PEI and siRNA was 100 $\mu$g mL$^{-1}$ and 76 $\times$ 10$^{-9}$ m. This result indicated that WS$_2$@PEI nanoparticles have potential as both photothermal agents and gene carrier. But, the monotherapy (neither GT nor PTT) is not good enough for totally eliminating the cancer cells. Next, we evaluated the synergistic effect caused by WS$_2$@PEI mediated GT+PTT combinational therapy. It was found that the cell viability substantially decreased to 13% in BEL-7402 cells and 12.4% in Hela cells under the same experiment condition, confirming the considerably synergistic effect of GT+PTT in vitro. To further demonstrate the anticancer

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**Figure 2.** Photothermal effect of WS$_2$@PEI-siRNA on BEL-7402 cells and HeLa cells at an irradiation power intensity of 1 W cm$^{-2}$. BEL-7402 cells a) and HeLa cells b) cell viability after different treatments for 24 h. c) CA and PI staining images of BEL-7402 cells and HeLa cells after different treatments for 24 h (green: live cells; red: dead cells).
efficacy of monotherapy or combinational therapy on BEL-7402 cells and HeLa cells, the microscopy images of live–dead cell staining were obtained after different treatments (Figure 2c). The intuitive visual results further indicated that combination therapy significantly enhanced the cancer cell killing.

We further investigated the cell death mechanism after different treatments by using a flow cytometry (Annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) method). The flow cytometry images of the cells after different treatments were shown in Figure 3a. Annexin-V-FITC emission signal was plotted on the x-axis, while PI emission signal was plotted on the y-axis. Almost no apoptosis or necrosis cells were observed in the control group. After GT, the early apoptosis rate of cells reached 14.2% and the late apoptosis/necrosis rate was 17.61%, respectively, indicating the efficient delivery of siRNA into cancer cells which led to successful apoptosis of cancer cell. While, the combination group (GT+PTT) induced obviously higher cell apoptosis (about 46.6% of apoptotic/necrosis cells) than the GT alone group (about 31.8% of apoptotic/necrosis cells), further confirming the synergistic therapeutic effects of siRNA treatment and photothermal ablation. Downregulation of survivin expression in cancer cells clearly plays a key role for enhanced cell apoptosis in GT and GT+PTT group. We thus evaluated the survivin protein silencing efficiencies in BEL-7402 cells after different treatments by using Western Blot analysis. Figure 3b showed that the survivin protein expression level was more obviously downregulated by GT compared with control group and the silencing rate was about 46.2%. The results indicated that WS2@PEI nanoparticles could efficiently deliver survivin-siRNA into the cells and the survivin protein can also be silenced successfully. Moreover, the silence efficiency of survivin was greatly enhanced to 71.9% after GT+PTT treatment, indicating the PTT could enhance the survivin silence efficiency and thus induce more cancer cell apoptosis. Many recent studies have shown that cellular uptake of nanomaterials can be regulated by temperature. The mild local heat generated by nanomaterials could increase the fluidity of cell membrane.[38–41] Therefore, the photothermal effects induced by nanomaterials upon NIR irradiation could enhance the cellular uptake by increasing cell membrane permeability. We thus adopted inductively coupled plasma-mass spectrometry (ICP-MS) method to measure the amount of WS2@PEI nanoparticles in cells before and after 808 nm laser irradiation at the power density of 1 W cm–2 for 10 min. The result revealed that the photothermal effects could significantly enhance the intracellular uptake of WS2@PEI, which was much higher than those without laser irradiation (Figure 3c). To further confirm that the photothermal effects could promote the intracellular uptake of WS2@PEI, the dark-field scattering images of cells were used to visualize the accumulation of nanoparticles in the cells before and after 808 nm laser irradiation (Figure 3d). The results revealed that cells after laser irradiation accumulated more nanomaterials than the cells without laser irradiation, further confirming the photothermal effect could increase the cell membrane permeability and thus enhanced survivin-siRNA transfection efficiency. In addition, we also found that the protein expression level of HSP70 in the GT+TT group was obviously downregulated after NIR irradiation, while the protein expression of HSP70 in the PTT alone group was remarkably overexpression at the same condition. The heat shock and stress response triggered by PTT resulted in upregulating HSP70 expression in cancer cells, which could increase cell survival and heat stress tolerance by suppressing its apoptosis.[11,15,42] Downregulating HSP70 could thus reduce the heat resistance of cancer cells and consequently improve cell sensitivity to heat at relative mild temperature. In total, these results proved that combining survivin-siRNA with PTT could take their advantages both and finally result in well synergistic therapeutic effects and better therapeutic outcome compared to the monotherapy.

Motivated by the effective combination therapy in vitro, the combination therapeutic effect of GT/PTT in vivo was then investigated. When the tumor volume reached approximately 75 mm3, Balb/c nude mice bearing BEL-7402 tumors randomly divided into six groups with four mice each group: a) PBS as the control group, b) Laser group, c) WS2@PEI group, d) WS2@PEI-siRNA group (GT), e) WS2@PEI+Laser group (PTT), and f) WS2@PEI-siRNA+Laser group (GT+PTT). After intratumoral injection of WS2@PEI, the mice in (b), (e), and (f) group mice were subsequently irradiated by the 808 nm laser with a power density of 1 W cm–2 for 10 min. Then the temperature of the tumor was monitored by an NIR thermal camera (Figure 4a,b). Under 808 nm laser irradiation, the temperature of the nanomaterials-injected mice rapidly increased to ~43 °C, which was capable of inducing mild hyperthermia and promoted survivin-siRNA transfection to kill cancer cells. In contrast, the temperature of the mice injected with PBS increased by only ~3 °C under 808 nm laser exposure, indicating that the WS2@PEI nanoparticles have a promising potential to be an efficient PTT agent for photothermal ablation of tumors. Similarly, the relative tumor volume change in each group was recorded every three days. As shown in Figure 4c–e, the control groups (a, b, and c group) exhibited no obvious tumor growth inhibition, demonstrating the negligible toxicity of WS2@PEI or laser to the mice. The tumor growth in group d (GT) was obviously inhibited after injection with WS2@PEI-siRNA and the tumor growth inhibited ratio reached 59.7%, indicating the survivin-siRNA delivered by the WS2@PEI could inhibit tumor growth. While, in group e (PTT), the tumor growth can also be suppressed after irradiation with 808 nm laser and the tumor growth inhibited ratio reached 66.3%. But the tumor recurred very quickly on the 12th day after the PTT treatment since mild hyperthermia was not good enough to eliminate the tumors. In remarkable contrast, the tumor growth in group f (GT+PTT) reached 91.7% inhibition ratio and the tumor was not resumed until the final of the experiment, which indicated that the combination therapy of GT and PTT could not only inhibit the growth of tumors but also efficiently suppress the recurrence of tumor. All these data revealed that the preeminent anticancer efficacy can be achieved by the combination of GT and PTT. These results were further confirmed by hematoxylin and eosin (H&E) stained of tumor slices. As shown in Figure 4f, tumors treated with PBS, WS2@PEI nanoparticles or 808 nm laser alone showed no obvious damage to tumor cells. And monotherapy (GT or PTT) killed part of tumor cells. The most tumor cells were damaged only after the combinational treatments (GT+PTT). These results suggested that the WS2@PEI-siRNA nanocomplexes are promising agent for siRNA-enhanced PTT tumor suppression and thoroughly preventing their resuming.
To examine the silencing efficacy of survivin-siRNA in the BEL-7402 tumor models, survivin protein expression in tumor tissues was analyzed by Western Blot and immunohistochemistry after different treatments. As shown in Figure 5a,b, the protein expression level of survivin in tumor tissue treated with GT was significantly downregulated compared to the control group. These results demonstrated that our nanocarrier effectively delivered survivin-siRNA into cancer cells. And we also found that survivin protein expression level in the GT+PTT group was significantly downregulated compared with the GT alone group. This indicated photothermal effects triggered by NIR laser irradiation could promote the siRNA transfection which has been reported. We also analyzed the impact of survivin on the HSP70 protein expression in vivo by Western Blot and immunohistochemistry. As shown in Figure 5a,b, the HSP70 proteins expression level increased significantly in the
tumors tissues after treatment with PTT, but the HSP70 proteins expression level decreased significantly in the tumors tissues after treatment with GT+PTT. The results suggested that silencing of survivin can block the upregulation of HSP70 proteins expression level and thus enhanced sensitivity of cells to PTT. These results were well in agreement with the in vitro experiments.

A well nanodelivery system should not only exhibit well therapy efficiency but also possess optimal imaging capabilities to realize theranostic.\(^\text{[43]}\) To precisely monitor the therapeutic response and avoid the associated side effects on normal healthy tissues, WS\(_2@\)PEI nanoparticles with high NIR absorption and strong X-ray attenuation ability are expected to be an efficient contrast agent for both PA and CT imaging. CT is one of most commonly used imaging tools for medical diagnosis featured with high-resolution 3D structure details of whole body. Therefore, we next applied WS\(_2@\)PEI nanoparticles as the CT imaging contrast agent in vivo (Figure 6a). 200 \(\mu\)L of WS\(_2@\)PEI nanoparticles solution (15 mg mL\(^{-1}\)) were intravenously administrated to the tail of BEL-7402 tumor-bearing mice and imaged at different time points (0.5, 1, 2, and 4 h) after the i.v. administration, respectively. Before administration of WS\(_2@\)PEI nanoparticles, weak CT signals were detected in the liver, spleen, and tumor region. In contrast, the remarkably enhanced contrast in liver and spleen was detected after i.v. administration, demonstrating the gradual accumulation of
Figure 5. The protein expression levels of survivin and HSP70 of tumors from different groups are determined by Western Blot a) and immunohistochemistry b), respectively. Statistical analysis was performed using the one-way ANOVA: **p < 0.01.

Figure 6. In vivo CT and PA imaging in BEL-7402 tumor-bearing mice. a) CT images of BEL-7402 tumor-bearing mice before and after i.v. injection of WS$_2$@PEI nanoparticles solution (15 mg mL$^{-1}$, 200 μL) at different time points (0.5, 1, 2, and 4 h). L: liver, S: spleen, T: tumor, B: bladder. b) PA images of tumor before and after i.v. injection with WS$_2$@PEI nanoparticles (2 mg mL$^{-1}$, 200 μL) at various time points (0.5, 2, 5, and 24 h).
WS$_2$@PEI nanoparticles by reticuloendothelial system. What’s more, the contrast signal was also enhanced in bladder, indicating that WS$_2$@PEI nanoparticles with ultrasmall sizes might be cleared out through renal excretions and thus minimized their potential side effects. Additionally, the obtained images after 1 h administration also indicated highly enhanced contrast signal in the tumor site, suggesting the obviously passive targeting ability of WS$_2$@PEI nanoparticles to tumor site. Besides the application for CT imaging, these WS$_2$@PEI nanoparticles could also be used as PA imaging contrast agents because of their high NIR absorption. Due to its advantage of high spatial resolution to soft tissues and real-time guiding, PA is greatly desirable to cross-sectional “listening” of entire tumors. To estimate PA imaging capability in vivo, BEL-7402 tumor-bearing mice were intravenously (i.v.) disposed with 200 $\mu$L of WS$_2$@PEI nanoparticles (2 mg mL$^{-1}$), and then cross-sectional PA images of tumor were collected at different time points (0.5, 2, 5, and 24 h) as shown in Figure 6b. Before the administration of WS$_2$@PEI nanoparticles, weak PA signals in the tumor region were detected, which arise from the tumor blood. After i.v. injection of WS$_2$@PEI nanoparticles, the PA signals obviously increased, demonstrating the gradual accumulation of WS$_2$@PEI nanoparticles in tumor region. After injection for 5 h, the PA signal became very obvious and lasted as long as 24 h. This result clearly proved that WS$_2$@PEI nanoparticles have potential to be dual model imaging contrast agent for both CT and PA imaging.

We also investigated the potential side effects of nanomaterials for all of tumor-bearing mice after different treatments. During our experiments, diet and activity of all mice were normal and no obvious body weight loss among these groups was observed after different treatments (Figure S4, Supporting Information). In order to further evaluate the potential side effects of these treatments, we studied the serum biochemical indicators and hematology indicators. The results revealed no obvious differences of the blood biochemical and hematology indicators in the treatment groups and control groups (Figures S5 and S6, Supporting Information). In addition, to evaluate the potential damage to these major organs, including heart, liver, spleen, lung, and kidney, we investigated the post-mortem histopathology by H&E stain. As shown in Figure 7, no obvious morphological differences were observed among these groups. These results revealed that the WS$_2$@PEI nanoparticles exhibited well biocompatibility.

3. Conclusion

In general, we have successfully constructed a multifunctional nanoplatform based on WS$_2$@PEI-siRNA nanocomplex for CT/PA imaging guided gene/photothermal synergistic therapy. The as-fabricated WS$_2$@PEI-siRNA nanocomplex displayed well CT/PA imaging enhancement and prominent GT/PTT synergistic therapeutic effects, facilitating to precisely locating and thoroughly realizing the eradication of tumor. Moreover, numerous toxicity experiments confirmed that the as-fabricated WS$_2$@PEI nanoparticle has no obvious toxicity in vitro and in vivo, indicating that WS$_2$@PEI nanoparticles possessed

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*Figure 7. Representative H&E sections of heart, liver, spleen, lung, and kidney after different treatments.*
well biocompatibility. Overall, the as-prepared WS$_2$@PEI-siRNA nanocomplex offers a new possibility in exploring the versatile nanoplatform for GT enhanced PTT of tumor under mild condition.

4. Experimental Section

Materials: Commercial tungsten disulfide (WS$_2$, 99.8%) and LA were obtained from Alfa Aesar Company and used without further purification. Sulfuric acid (H$_2$SO$_4$, 95.0%–98.0%, analytical reagent) and poly(ethylene imine) (PEI) were purchased from Beijing Chemical Corporation (Beijing, China). DMEM and Roswell Park Memorial Institute 1640 (RPMI-1640) were purchased from HyClone, USA. PBS was obtained from Gibico BRL (Grand Island, NY). CCK-8 was purchased from BOSTER Co., Ltd (Wuhan, China). Calcein-AM (CA)/PI double stain kit was purchased Yeasen Biotechnology Co., Ltd (Shanghai, China). Western and IP Cell lystate, β-actin antibody and bicinchoninic acid (BCA) Protein Assay Kit and BeyoECL Plus were purchased from Beyotime Institute of Biotechnology Corporation (Nanjing, China). HSP70 antibody and survivin antibody were purchased from Proteintech Group Inc. Co., Ltd (Wuhan, China). Annexin V-FITC apoptosis detection kit and whole cell lysis assay kit were purchased KeyGen BioTECH Co., Ltd. (Nanjing, China). The siRNA duplexes of survivin-siRNA (sense: 5'-CACCCGAUCUCAUCUATT-3', anti-sense: 5'-UCAAAUGAGAGCCUGGT-3') were purchased from GenePharm Co. Ltd., (Shanghai, China). Male Balb/c mice at age of 4–6 weeks were purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). Ultrapure water was used throughout.

Preparation of WS$_2$ QDs: The WS$_2$ QDs was synthesized via a facile exfoliation method according to the previous reports.\cite{18,35} First, grinding miller was used to make commercial WS$_2$ bulk grinded for about 3–4 h. For intercalation, the obtained ground WS$_2$ powder (40 mg) dispersed in about 40 mL of sulfuric acid (H$_2$SO$_4$, 95.0%–98.0%) was then set in the water bath at the temperature of 90 °C for 1 d. After intercalation, the intercalated WS$_2$ sample was collected by centrifugation and washed repeatedly with purified water to remove residual sulfuric acid. Finally, 30 mL of deionized water was used to disperse the obtained intercalated WS$_2$ sample for further ultrasonication for 3 h to obtain WS$_2$ QDs solution. Then the WS$_2$ QDs was collected by centrifugation in 12 000 rpm. The as-prepared WS$_2$ QDs sample was redispersed in pure water and stored at 4 °C for next experiments. The yield of the as-obtained WS$_2$ QDs is calculated to be about 70% based on W atoms.

Surface Modification of WS$_2$ QDs with LA-PEI: LA-PEI was prepared via the previous reported method.\cite{18} First, 150 mg of PEI ($M_w$ = 1.8 kDa) and LA with two molar equivalent were dispersed in 10 mL of water and acetonitrile, respectively. Then the PEI solution was adjusted to pH 7.4. Then 50 molar equivalent 1-ethyl-3-(3-dimethylamino) propyl) carbodiimide hydrochloride and N-hydroxysuccinimide were added toward to LA solution with stirring. After stirring, PEI solution was mixed with this LA solution and incubated overnight. The product was dialyzed with nuclease-free water at various N/P ratios (N/P = 0, 1.24, 2.48, 3.72, 5.03, 6.28, 7.52, 10.0, 12.5) and incubated for 30 min at room temperature. For the heparin release assay, WS$_2$@PEI-siRNA complex incubated with heparin for 20 min at room temperature. The weight ratio is 1.5 of siRNA/heparin. In order to value the protection of siRNA by WS$_2$@PEI nanoparticles, 2 μL of RNase (1 μg μL$^{-1}$) was applied to digest 1 μg of siRNA formulated with WS$_2$@PEI nanoparticles at 37 °C for 1 h. The loading efficiency and release behavior of WS$_2$@PEI-siRNA, as well as protection of siRNA by WS$_2$@PEI were determined by 1% agarose electrophoresis at 120 V for 20 min.\cite{29,45}

In Vitro Cytotoxicity Study: BEL-7402 cells and HeLa cells were maintained in RPMI-1640 and DMEM supplemented with 10% FBS, respectively. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$. Cell viability was evaluated with the CCK-8 method. BEL-7402 cells and HeLa cells were seeded in 96-well culture plates and cultured overnight in an incubator, respectively. Then, the medium was removed and cells were treated with WS$_2$@PEI and WS$_2$@PEI-siRNA with or without 808 nm laser (1 W cm$^{-2}$ power for 10 min) and then further cultured for 24 h, respectively. The final concentration of WS$_2$@PEI was 0, 12.5, 25, 50, and 100 μg mL$^{-1}$, respectively. And the final concentration of siRNA were 0, 8 × 10$^{-9}$, 16 × 10$^{-9}$, 32 × 10$^{-9}$, and 76 × 10$^{-9}$ μM, respectively. Then, CCK-8 (10 μL per well) solution was added in each well and incubated at 37 °C for 1 h. The absorbance was measured at 450 nm by a microplate reader.

Calcein-AM and Propidium Iodide Staining: BEL-7402 cells and HeLa cells were seeded in the six well plates. Then, the medium was removed and cells were treated with WS$_2$@PEI and WS$_2$@PEI-siRNA with or without 808 nm laser (1 W cm$^{-2}$ power for 10 min), respectively. The final concentration of WS$_2$@PEI and siRNA were 100 μg mL$^{-1}$ and 76 × 10$^{-9}$ μM, respectively. After 24 h of different treatments, the cells digested by 0.25% pancreatic enzyme were then washed twice with PBS and stained with CA–PI for 15 min. The luminescence microscopy imaging was conducted by luminescence microscope.

ICP-MS for W Element Quantification: Cellular uptake of WS$_2$@PEI nanoparticles was quantitatively studied via ICP-MS. For ICP-MS experiments, BEL-7402 cells were exposed to 25 μg mL$^{-1}$ WS$_2$@PEI nanoparticles with or without 808 nm laser (1 W cm$^{-2}$ power for 10 min). The cells were collected at different time (1, 3, 6, 9, 12, and 24 h) and were processed through a series of procedure to get the ICP-MS samples. A series of W standard solutions (0, 0.5, 1, 5, 10, 50, and 100 ppb) was prepared with 2% nitric acid solution. Both standard and test solutions were measured by ICP-MS.

Cells Dark-Filed Imaging: BEL-7402 cells were seeded in the six well plates. Then, the medium was removed and cells were treated with WS$_2$@PEI (25 μg mL$^{-1}$) with or without 808 nm laser (1 W cm$^{-2}$ power for 10 min), while the cells treated with medium were considered as control. After 6 h, the cells were then washed twice with PBS and fixed by 4% paraformaldehyde solution for 20 min. The dark-field imaging was conducted by a luminescence microscope OLYMPUS X73 integrated with dark-field accessories.

Western Blot Analysis: Total proteins were collected from treated BEL-7402 cells after treatments with different formulations. The results were analyzed by Western Blot. Proteins collected from BEL-7402 cells
implanted tumor tissues after treatment with different formulations were also analyzed by Western Blot.

Animal Model for CT and PA Imaging: To obtain the CT images in vivo, 200 μL of WS@PEI nanoparticles (15 mg mL⁻¹) were intravenously injected to the tail of Balb/c nude mice bearing BEL-7402 tumors and imaged on a small X-ray CT (Gamma Medica-Ideas) at different time intervals (0.5, 1, 2, and 4 h). After imaging, the filtered back projection method was implemented to obtain the reconstruction images and then analyzed by amira 4.1.2. system. Imaging parameters were as follows: field of view, 1024 pixels × 1024 pixels; effective pixel size 50 μm, tube voltage 80 kV, tube current 270 μA. Meanwhile, to acquire the PA images in vivo, 200 μL of WS@PEI nanoparticles (2 mg mL⁻¹) were intravenously injected to the tail of Balb/c nude mice bearing BEL-7402 tumors. After injection, the PA instrument (multispectral optoacoustic tomography inversion 128, ITtheramical, Germany) was used to collect the PA images at different time point (0.5, 1, 2, 5, and 24 h). The main parameters were as follows: the ten wavelengths for each slice from 700 to 900 nm; the region of interest is 20 mm.

In Vivo Gene and Photothermal Synergistic Therapy: All male Balb/c nude mice were conducted under protocols approved by the Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety (Institute of High Energy Physics, CAS). The Balb/c nude mice bearing BEL-7402 tumors model was established by subcutaneously injection of 1 × 10⁶ BEL-7402 cells in 0.1 mL PBS into the right hind legs right of the mice. The mice were used for in vivo experiments when the tumor volume reached 75 mm³ (volume = length × width²/2, measured with a vernier caliper). The mice then were separately randomly assigned to six groups and each group included four mice: (a) PBS; (b) Laser; (c) WS2@PEI; (d) GT; (e) PT; and (f) GT + PT (gene and photothermal combinational therapy). The BEL-7402 tumor-bearing mice were intratumorally injected with WS2@PEI-siRNA (The final concentration of WS2@PEI-siRNA is 2 mg mL⁻¹ and injection dose is 50 μL per mm³ tumor volume.) and then exposed to 808 nm laser (power density: 1 W cm⁻², 10 min). The tumor volume and mice weight were measured in the following per 3 d and the whole experiment lasted 21 d. At the end of the experiments, all mice were sacrificed, and the tumors and major organs were harvested and weighed to evaluate the therapeutic efficacy of different experiment groups.

Pathological and Immunohistochemical Analysis: Tumor and major organs including heart, liver, spleen, lung, and kidney were removed and were set in 4% paraformaldehyde solution for fixing, processed routinely into paraffin, sectioned at 8 μm thickness, stained with H&E. In survivin and HSP70 of tumor tissues immunohistochemistry, the slices were stained with the survivin antibody (1:100) and HSP70 antibody (1:100). Later, these slices were examined with a microscope (OLYMPUS X73, Japan).

Blood Biochemistry and Hematology Analysis: The blood sample was collected by removal of the eyeball in the different treated mice above. About 100 μL of the collected blood sample was placed into an anticoagulant tube (potassium ethylenediaminetetraacetic acid (EDTA) collection tube) for hematology analysis. For blood biochemistry analysis, 1 mL of collected blood sample was stand in room temperature for 3 h, which was centrifuged to remove blood cells. The obtained blood plasma samples were used for the biochemistry measure.

Supporting Information

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

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