

Original Article

Thioaptamer conjugated single-wall carbon nanotubes in human breast cancer targeted photothermal therapy *in-vivo* and *in-vitro*

Zhengquan Han¹, Xiao Han¹, Zian Wang¹, Shiwu Wu², Rongsheng Zheng¹

¹Department of Medical Oncology, The First Affiliated Hospital of Bengbu Medical College, Anhui 233003, China;

²Department of Pathology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233000, Anhui, China

Received September 22, 2015; Accepted January 6, 2016; Epub January 15, 2016; Published January 30, 2016

Abstract: Objective: To prepare thioaptamer conjugated single-wall carbon nanotubes (SWNT-PEG-TA) and investigate their effect in human breast cancer targeted photothermal therapy *in-vivo* and *in-vitro*. Methods: An ultrasonic cell disruptor was used to prepare nanoscale single-wall carbon nanotubes (SWNT). SWNT-PEG with good water solubility was obtained by non-covalent modification of SWNT with polyethylene glycol (PEG). A thermoelectric thermometer was used to detect the temperature of SWNT-PEG-TA solution after irradiating with 808 nm laser of different energy. The condition of SWNT-PEG-TA after irradiating with 1 W/cm² power density of laser for 5 minutes was detected by a near-infrared thermal imager. Cell counting kit (CCK-8) method was used to detect the effect of SWNT-PEG on cell viability, as well as the effect of SWNT-PEG and SWNT-PEG-TA on cell viability under 1 W/cm² laser irradiation. SWNT-PEG and SWNT-PEG-TA were labeled with isothiocyanates dye (FITC) and were then incubated with cells, which were observed under a confocal microscope for cell uptake. In a subcutaneous breast cancer model in mice established, 24 hours after injecting SWNT-PEG and SWNT-PEG-TA through caudal vein, mice imaging was conducted by using a near-infrared thermal imager during 808 nm laser irradiation on the back. Then, the tumor volume was monitored. Results: The temperature of SWNT-PEG-TA solution after irradiating with 1 W/cm² laser for 5 min reached up to 62.4±3.8 °C. More SWNT-PEG-TA fluorescence was observed by confocal microscopy than SWNT-PEG. No significant effect on cell viability was found for cells treated by 0-10 µg/mL SWNT-PEG for 12 and 24 h. After irradiating by 1 W/cm² laser for 5 min, the viability of cells treated by SWNT-PEG-TA decreased remarkably compared with those treated by SWNT-PEG. After injecting equal quantities of SWNT-PEG and SWNT-PEG-TA through caudal vein, the temperature in tumor region reached up to 44.9 and 63.1 °C, respectively, under near-infrared thermal imaging. Further, evident tumor ablation was observed 30 days after the photothermal therapy with SWNT-PEG-TA. By contrast, no significant inhibitory effect on tumor growth was found after the photothermal therapy with SWNT-PEG. Conclusion: SWNT-PEG-TA prepared in this study can target cell surface actively and effectively and has a good cell-killing effect under 808 nm laser radiation, playing a good anti-cancer effect in *in-vivo* tumor treatment.

Keywords: SWNT, thioaptamer, breast cancer, photothermal therapy

Introduction

Single-wall carbon nanotube (SWNT) was believed to be closed tubular structures made of curled graphitic layers [1]. Since all atoms were exposed on the surface, the surface area was fairly high. Aromatic hydrocarbon molecules could be attached on it by π - π stacking force [2, 3]. In recent years, based on the unique size, morphology, structure and physical feature of carbon nanotube, potential biological applications had become a research hotspot [4]. SWNT had strong light absorption

in the near infrared region. Thus, under near-infrared laser radiation, the light energy absorbed could be converted into heat, generating photothermal effect. This effect could be used in photothermal therapy for tumor. Compared with traditional surgery, chemotherapy and radiotherapy, photothermal therapy was believed to be one of the safest approaches for treating tumor [5, 6]. In 2012, it was reported by Dai Hongjie research group that when SWNT was used as biological carriers with multifunctional properties or near-infrared reagents, under 2 W/cm² 808 nm laser radi-

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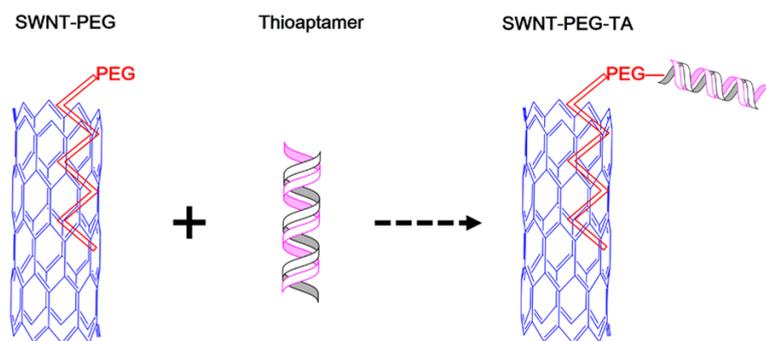


Figure 1. The synthesis of SWNT-PEG-TA.

tion, tumor cells could be killed [7]. Yurgel et al. reported the condition when antibody-modified SWNT was used in *in-vitro* photothermal therapy of tumor cells [8].

Aptamer was a segment of oligonucleotide molecules (RNA or DNA) which could be bound to target molecules tightly and selectively with high affinity. They were screened out from oligonucleotide molecules synthesized randomly by Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Aptamer, with special three-dimensional conformations folded by secondary and tertiary structure formation, could interact with target molecules with high affinity and specificity [9]. Mann et al. prepared thioaptamer (TA) conjugated nanoliposomes by modifying the surface of long-circulating liposomes with TA. TA could identify E-selectin (ES; it was expressed selectively in inflamed vessels in patients with advanced tumor) specifically. An animal experiment suggested that TA-nanoliposomes could be gathered in the site of allografted breast cancer effectively through intravenous injection [10]. Breast cancer was a malignant tumor commonly found in female and its incidence was on the rise [11]. Thioaptamer conjugated single-wall carbon nanotubes (SWNT-PEG-TA) prepared in this study was used in breast cancer targeting photothermal therapy and the targeting property of SWNT-PEG-TA was investigated by near-infrared imaging.

Materials and methods

Major reagents

SWNT was purchased from Carbon Nanotechnologies Inc. (CNI, the U.S.), fluorescent dye isothiocyanate (FITC) and 4',6-Diamidino-2-phenylindole (DAPI) from Shanghai Qcbio Science & Technologies co., Ltd., DSPE-PEG₂₀₀₀-

NH₂ from Sigma (the U.S.), and CCK-8 kit from Dojindo China CO., Ltd. Thioaptamer (TA) was synthesized by Takara Bio (Dalian) and its nucleotide sequence was (5'-CGCTCGGATCGATAAGCTTCGATCCACTCTCCCG TTCACTTCTCCTCACGTACCGGATCC-TCTAGAGCACTG-COOH-3').

Major instruments

Confocal laser scanning microscope Leica TCS SP5-II was purchased from Leica (Germany), multifunctional microplate reader BioTek SynergyTM 4 from BioTek (the U.S.), thermocouple thermometer Fluke 714 from Fluke Corporation (the U.S.) and thermal infrared imager (R500) from NEC-AVIO (Japan).

Cell culture

Human breast cancer HCC1937 cells were purchased from Shanghai cell bank (CAS) and were epithelium-like cells adhering to the wall. They were cultured in RPMI-1640 (GIBCO; art. No. 31800022; with 10% qualified fetal bovine serum) with 5% CO₂ at 37°C.

Preparation of SWNT-PEG-TA

SWNT was put into deionized water and ultrasonically oscillated for 30 min. Then, adequate DSPE-PEG₂₀₀₀-NH₂ was added. The mixture was stirred properly and unattached DSPE-PEG₂₀₀₀-NH₂ was removed by ultrafiltration centrifugation. The remaining solution was resuspended in deionized water. SWNT-PEG-NH₂ obtained was then mixed with TA-COOH. During this time, N-ethyl-N'-(3-(dimethylamino)propyl) carbodiimide and N-Hydroxysuccinimide (NHS) were added while stirring. After reaction for 6 hours at room temperature, the resultant solution was dialyzed by using an activated dialysis bag for 4 days until free organic ions were removed. The SWNT-PEG-TA obtained at last was stored in a refrigerator at 4°C.

Characterization of the photothermal effect of SWNT-PEG-TA

In this study, five aliquots of 5 µg/mL SWNT-PEG-TA solution were placed into five 1.5 mL EP tubes. They were irradiated by 0.1, 0.5, 1, 1.5 and 2 W/cm² 808 nm laser for 5 min, respectively. Then, the temperature of these

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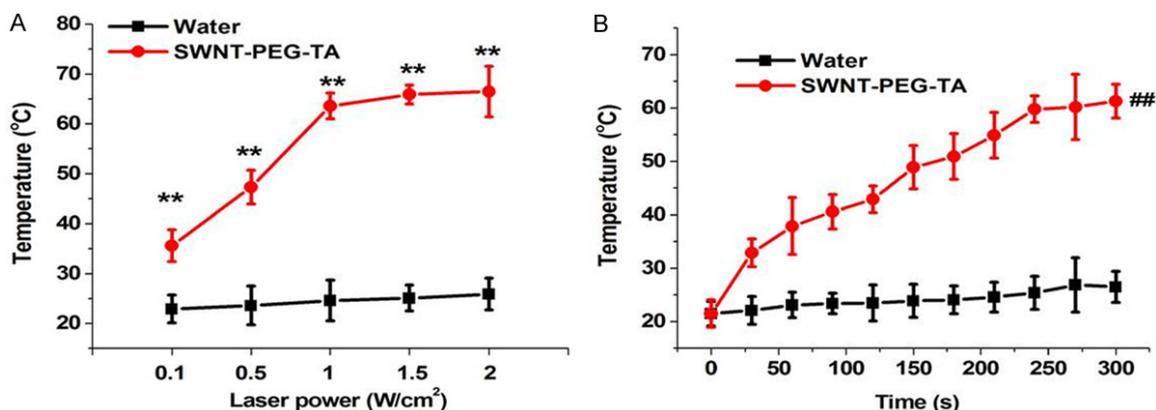


Figure 2. The temperature curve of SWNT-PEG-TA solution after irradiation. A. Effect of laser power on the temperature change of water and SWNT-PEG-TA solution. B. Effects of time on the temperature change of water and SWNT-PEG-TA solution. Compare to water, **P<0.01; ##P<0.01.

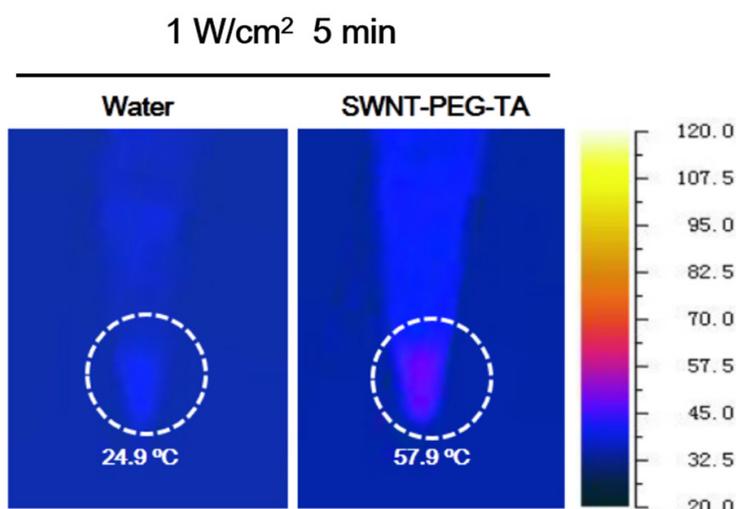


Figure 3. The NIR thermal imaging of SWNT-PEG-TA solution compare to water.

solutions was detected by a thermocouple thermometer. After that, the laser with the most efficient power was used for solution irradiation. The temperature of solution was measured every 30 min and the temperature variation curve was thus prepared. Finally, thermal imagery was conducted for equivalent amount of water and SWNT-PEG-TA solution with an infrared thermal imager to observe the photothermal effect of SWNT-PEG-TA directly.

In-vitro test on the targeting property of SWNT-PEG-TA

SWNT-PEG-TA was labeled by FITC by physical absorption. FITC-labeled SWNT-PEG and SWNT-

PEG-TA were cultured with breast cancer cells for 2-3 hours. Afterwards, the mixture was washed by PBS to remove extracellular nanocomposites and dyes and was observed under confocal microscopy for fluorescent signal in cells.

Cytotoxicity test on photothermal therapy in-vitro

First at all, we studied the cytotoxicity of different concentrations of SWNT. Cells were treated by 0-10 µg/mL SWNT for 12 and 24 hours. Then, CCK-8 reagent was added and the absorbance at 450 nm (OD_{450 nm} value) was detected with a microplate reader. Cell viability (%) = (OD in experimental group-OD in blank group)/(OD in control group-OD in blank group) × 100%. After that, a CCK-8 kit was used to detect the impact of SWNT-PEG and SWNT-PEG-TA on cell viability under 808 nm laser radiation (1 W/cm², 5 min).

Infrared thermal imaging and tumor photothermal therapy in-vivo

Breast cancer cells in logarithmic growth phase were collected and made into cell suspension (10⁶ cells/ml), 200 µL of which was injected intravenously into the back of female Balb/c nude mice. After aseptic treatment, these mice were fed in an animal house. When tumor volume reached up to 150 mm³, follow-up studies

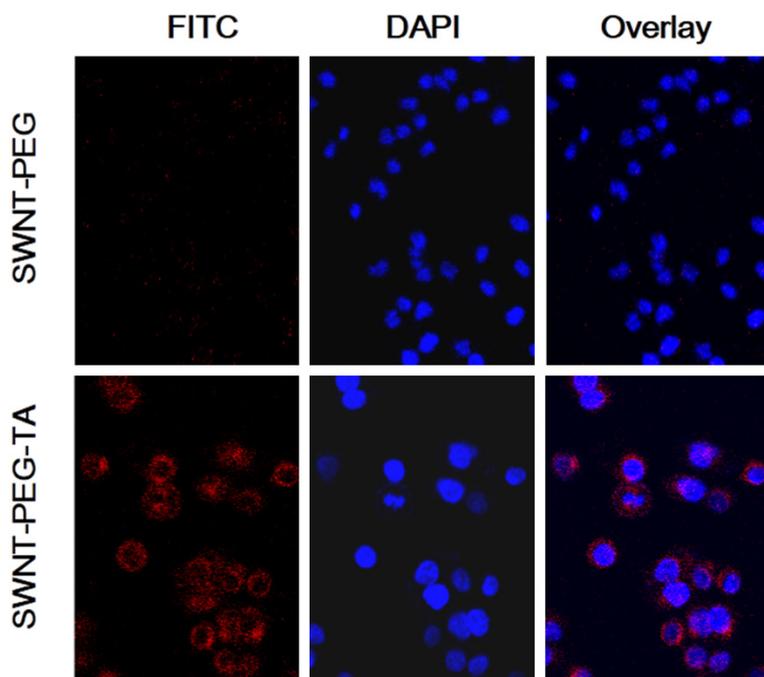


Figure 4. The uptake of SWNT-PEG-TA by breast cancer cells. Column 1: FITC channels showing the red fluorescence from FITC labeled SWNT-PEG-TA and SWNT-PEG distributed in cytoplasm. Column 2: DAPI channels showing the blue fluorescence from DAPI stained nuclei. Column 3: Merged channels of FITC and DAPI channels.

were continued. Tumor volume = Length × width × width ÷ 2.

Tumor-bearing mice were randomized into two groups (7 mice per group). And equivalent amount of SWNT-PEG and SWNT-PEG-TA were injected via their caudal vein. After 24 hours, the tumor region was irradiated with an 808 nm laser (1 W/cm²) for 5 min and whole body imaging was conducted by using a thermal imager.

28 tumor-bearing mice were divided into 4 groups (7 mice per group) with normal saline, SWNT-PEG-TA, SWNT-PEG + Laser and SWNT-PEG-TA + Laser, respectively. During 28 days of treatment, the tumor volume of mice was measured every three days.

Statistical analysis

All measurement data was expressed by mean ± S.D. in this study. Comparison between samples was conducted by independent sample t test. P≤0.05 indicated that there was significant difference, while P≤0.01 indicated that there was extremely statistically significant difference. Statistics analysis was performed

by the application of a statistical software SPSS13.0.

Results and analysis

Synthesis of SWNT-PEG-TA

SWNT was unidimensional. PEG could be attached to its surface by Van der Waals force or hydrogen bond force. Next, stable nanocomposite SWNT-PEG-TA was formed by the attachment of amido bond to carboxylated phosphorothioate ester. The process of synthesis was shown in **Figure 1**.

Photothermal effect of SWNT-PEG-TA

As shown in **Figure 2A**, five aliquots of 5 μg/mL SWNT-PEG-TA solution were placed into 1.5 ml EP tubes. After irradiation with 0.1, 0.5, 1, 1.5 and 2 W/cm² 808 nm laser for 5 min, their temperature reached up to 35.5±3.6, 47.1±3.2, 62.8±2.1, 64.6±1.5 and 65.2±4.9°C, respectively. By contrast, the temperature of water with the same volume irradiated with the same laser was about 26.5°C. Next, 1 W/cm² laser was used to irradiate SWNT-PEG-TA and water with the same volume. The temperature of solution was measured every 30 seconds and the results were shown in **Figure 2B**. The temperature of SWNT-PEG-TA solution increased gradually and reached up to the maximum 62.4±3.8°C after 5 min, while that of water remained to be lower than 27°C. Finally, thermal imagery was conducted for the same amount of water and SWNT-PEG-TA solution by using an infrared thermal imager. Results presented in **Figure 3** demonstrated the high-efficient photothermal effect of SWNT-PEG-TA more directly.

In-vitro study on the targeting property of SWNT-PEG-TA

FITC-labeled SWNT-PEG and SWNT-PEG-TA were incubated with breast cancer cells for 2-3 hours, respectively. Then, extracellular dyes were washed off and the fluorescence of FITC was observed by confocal microscopy. Results,

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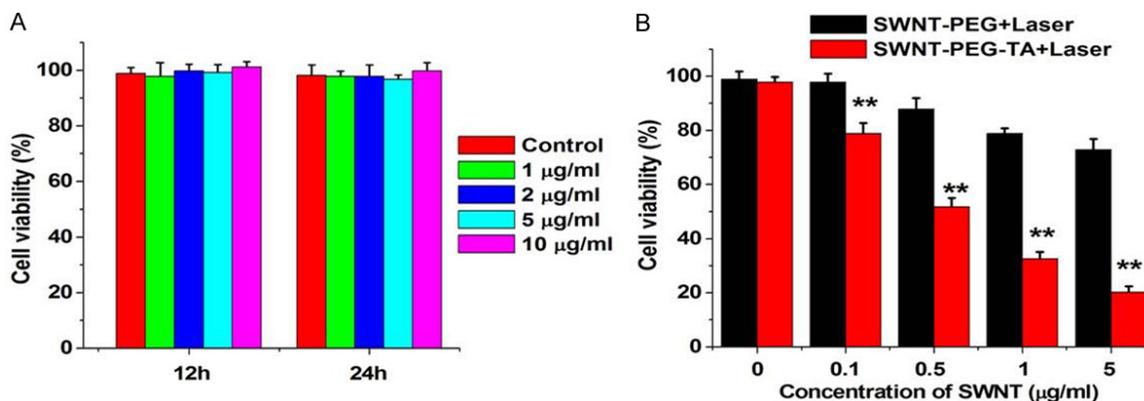


Figure 5. The photothermal effect of SWNT-PEG-TA on cell viability. A. The cell viability of SWNT-PEG at the concentration from 1 µg/mL to 101 µg/mL at 12 h and 24 h without laser; B. The cell viability of SWNT-PEG-TA and SWNT-PEG at different drug concentration under laser irradiation. Under laser irradiation. **P<0.01.

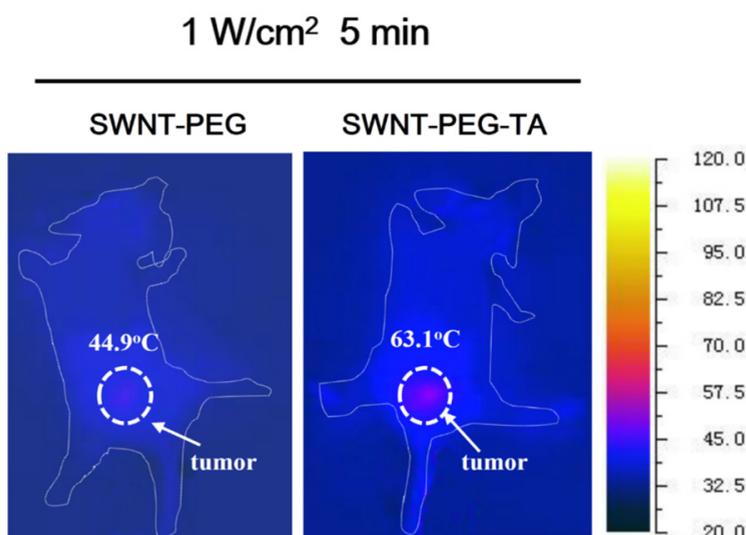


Figure 6. The photothermal effect of SWNT-PEG-TA *in-vivo*.

as presented in **Figure 4**, indicated that there was very few SWNT-PEG within cells, which was significantly fewer than the amount of SWNT-PEG-TA. It demonstrated that TA targeted the surface of breast cancer cells.

Cytotoxicity test of SWNT-PEG-TA in photothermal therapy

As shown in **Figure 5A**, treatment with 0-10 µg/mL SWNT-PEG for 12 and 24 hours had no obvious impact on cell viability. As shown in **Figure 5B**, the same concentration of SWNT-PEG and SWNT-PEG-TA both decreased cell viability under 808 nm laser radiation (1 W/cm², 5 min), but the inhibitory effect of the latter was more

significant (P<0.01). It was possibly due to the targeting property of TA which enabled more SWNT-PEG enter into cells and. Thus, these cells were killed under photothermal effect.

Photothermal effect of SWNT-PEG-TA *in-vivo*

The same amount of SWNT-PEG and SWNT-PEG-TA were injected into tumor-bearing mice through caudal vein. After 24 hours, the tumor region on the back of mice was irradiated with 1 W/cm² laser for 5 min. As shown in **Figure 6**, a red zone (circle in white) was found by infrared thermal imaging on the back of mice and was confirmed to be tumor region after being compared with the physical map. It demonstrated that SWNT-PEG and SWNT-PEG-TA gathered around the tumor region. However, more SWNT-PEG-TA gathered here and its photothermal effect was more significant with a final temperature of tumor region of 63.1°C. Thus, it was determined by infrared thermal imaging that SWNT-PEG-TA was of better *in-vivo* targeting property.

SWNT-PEG-TA in tumor photothermal therapy *in-vivo*

As shown in **Figure 7**, SWNT-PEG-TA alone had no significant impact on tumor growth. SWNT-PEG plus laser irradiation inhibited tumor

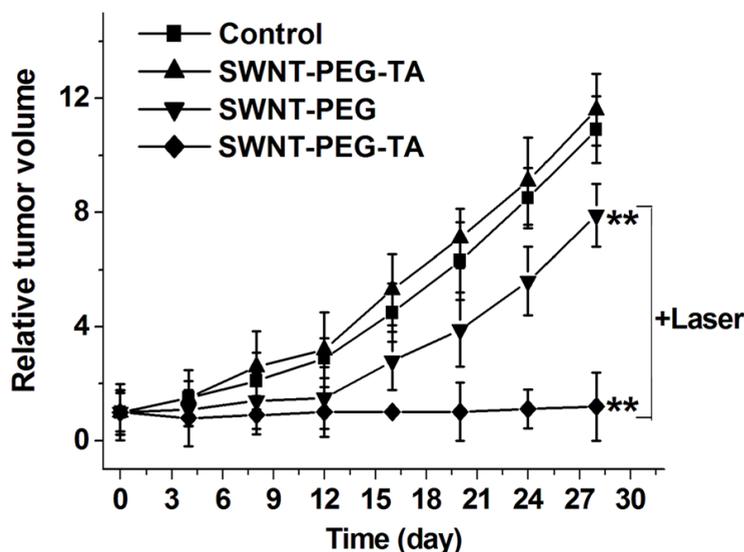


Figure 7. The effect of SWNT-PEG-TA in *in-vivo* tumor photothermal therapy. Compare to control and SWNT-PEG-TA without laser. ** $P < 0.01$.

growth to some extent, but the tumor continued to grow after 12 days' treatment. By contrast, SWNT-PEG-TA plus laser irradiation inhibited tumor growth all along. At the end of treatment cycle, the tumor was still very small.

Discussion

In these decades, SWNT were widely studied in nano-medical field because of its unique physical and chemical properties. It was about 1 nm in diameter and several hundred nanometers in length. Since the size of SWNT was almost the same as that of biomacromolecules and plenty of π - π conjugated system existed on the surface of carbon nanotubes, SWNT could be used as carriers of some biological macromolecules like aromatic hydrocarbon and drugs [12]. However, unmodified SWNT had poor water solubility and great toxicity. Therefore, surface modification was essential before entering into living organisms so as to improve its water solubility and decrease its toxicity [13]. Based on the action mode between soluble molecules and carbon nanotubes, the surface modification of SWNT can be generally classified into two types: (1) Covalent modification: After amination of carbon nanotubes, the amino could react with other organic molecules; or after attachment of a functional group-COOH by the oxidization of SWNT, SWNT was modified by a covalent bond formed between other mole-

cules and -COOH; (2) Non-covalent modification: SWNT bound with molecules with aromatic rings or others by π - π conjugation rather than covalence attachment. Liu Zhuang et al. attached polyethylene glycol (PEG) with hydrocarbon chain to the surface of carbon nanotubes by non-covalent force. Then, anti-cancer drug adriamycin was loaded by π - π stacking force, turning carbon nanotubes to water soluble drug carriers with low toxicity.

Owing to the modifiability of the surface of carbon nanotubes, Ruibin Li et al. functionalized oxidized SWNT with a glycoprotein antibody. The functionalized SWNT was then used to carry anti-cancer drug adriamycin and act on human chronic granulocytic leukemia K562R cell strain. It was found that the antibody recognized and targeted receptors on the surface of K562R cells [14]. In this study, by attaching PEG molecules via non-covalent absorption, we obtained SWNT-PEG with good biocompatibility and low toxicity. Then, we got SWNT-PEG-TA by covalent attachment of tumor-targeting TA to the amido bond (Figure 1).

In addition, SWNT, due to its strong absorbance in the near infrared region, had good photothermal effect and could be used in tumor photothermal therapy. Compared with traditional treatment, chemotherapy and radiotherapy, photothermal therapy was characterized by low toxicity and good effect under controllable energy. SWNT-PEG-TA prepared was both of light-thermal conversion ability and tumor targeting property. Results of *in-vitro* temperature test suggested that the temperature of SWNT-PEG-TA could reach up to 60°C and above under laser irradiation, which was sufficient to kill tumor cells. After injection of SWNT-PEG-TA through caudal vein, TA targeted the tumor region and increased the temperature of the tumor region to 60°C and above under laser irradiation. In this way, it played its role of tumor ablation.

In conclusion, SWNT-PEG-TA prepared in this study is of high biocompatibility, low toxicity,

high tumor targeting property and good effect in tumor photothermal therapy. It may become an ideal method of tumor treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Rongsheng Zheng, Department of Medical Oncology, The First Affiliated Hospital of Bengbu Medical College, Anhui 233003, China. E-mail: Zrs_doc@163.com

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