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X-ray CT guided fault-free photothermal ablation of metastatic lymph nodes with ultrafine HER-2 targeting $W_{18}O_{49}$ nanoparticles

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ABSTRACT

Designing high accuracy in the diagnosing and fault-freely eliminating lymphatic metastasis of breast malignancy, to avoid the invasiveness and complications caused by traditional assays, is of great therapeutic importance. To this end, theranostic $W_{18}O_{49}$ nanoparticles targeting to human epidermal growth receptor 2 (HER-2) over-expressed breast malignancy were synthesized via polyol method. By taking advantage of their high X-ray attenuating and photothermotherapy potency, lymph nodes in the mice bearing HER-2 positive metastasis could be clearly distinguished under CT guidance and selectively eliminated by laser ablation. The therapeutic efficacy was further confirmed by the significantly extended survival period. These finding evidenced the potential of these nanoparticles for imaging guided photothermal ablation of HER-2 positive breast malignancy.

1. Introduction

Breast cancer is the leading cause of cancer related death in females, which accounting for almost 14% of the total cancer death in 2008 [1]. Over the past 25 years, an up-surging mortality rate could be observed in developing Asian countries [2]. Compared with that of primary tumor, few patients with metastatic breast tumor were completely cured post the therapy [3]. For metastatic tumor, substantial adverse effect and drug-resistance caused by radiation therapy and chemotherapy have been frequently observed, and seeking for potential treatment for metastatic carcinoma is of great clinical importance and challenging. Detailed investigations have shown that metastasis of breast malignancy occurs primarily through the lymphatic pathway [4]. Thus, the status of lymph node plays a pivotal role in clinical prognosis. Among various kinds of surgical associated assay, axillary dissection has been taken as a routine part for breast cancer treatment over 100 years [5]. However, significant morbidity post the axillary dissection could be observed [6]. One of these severe complications after surgery is lymphedema, which results in chronic inflammatory changes (e.g. lymph vessel fibrosis and psychological distress) and occurs up to 30% of patients [7]. Thus, sentinel lymph node (SLN) biopsy, featuring much less invasiveness, has been taken as a promising alternative in clinical trial [4]. Though SLN biopsy has been demonstrated to determine axillary nodal status accurately, the time-consuming feature and unacceptable false negative rate (10%–19%) have limited their application [8]. In addition, routinely used blue dyes (e.g. methylene blue) or radioactive trackers (e.g. 99m-Tc) in SLN mapping have been hindered by long term color residue and radiation damage to the tissue [9]. In addition, SLN biopsy assay still needs to expose SLN for detection, and the diagnostic accuracy greatly depends on the operator's experience. Thus it is impossible to totally eliminate the complications induced by surgical operation [10]. Therefore, finding a potential, facile method featuring fault-free determination and selective elimination of lymphatic metastasis will be extremely beneficial for patients.

Recently, tungsten oxide nanoparticles (TONPs) have been reported as a potent tool in cancer therapy [11,12]. Due to their strong

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localized surface plasmon resonance (LSPR) property and high X-ray attenuating potency, TONPs integrating computed tomography (CT) contrast and photothermotherapy (PTT) capability was gained [13,14]. Unfortunately, little work has been reported concerning direct facile synthesis of water soluble TONPs within 10 nm, which merit from their significantly enhanced tumor penetration and reduced liver uptake [15]. In this study, we synthesized ultrafine $W_{18}O_{49}$ NPs via polyol method with excellent water dispersity. Compared with traditional PTT candidates (e.g. Au nanorod), our platform merits from the following aspects: (1) Higher cost-effectiveness and facile synthesis procedure which can be easily scaled up; (2) Optimal size for lymphatic imaging (<10 nm) with respect to previous reported [16]; (3) 1064 nm laser irradiation rendered PTT allowing for deeper skin penetration and higher body tolerance [17].

Since HER-2 (Human Epidermal growth factor Receptor 2) over-expression has been highly correlated with breast malignancy prognosis in clinic [18], the as-obtained $W_{18}O_{49}$ NPs were further armed with HER-2 antibody (abbreviated as WOHA) and challenged with lymphatic metastasis animal model. Thus, with the CT guidance, metastatic lymph nodes could be accurately localized and photo-thermally eliminated fault-freely. We hope this agent will play an active role in the clinical trial.

2. Experimental section

2.1. Chemicals

Tungsten hexachloride (WCl_6), Poly acrylic acid (PAA, Mw = 2 KDa) and diethylene glycol (DEG), Ethyl dimethylaminopropylcarbodiimide (EDC) and sulfo-NHS were all purchased from Sigma Aldrich. All the chemicals were of analytical grade and used as received without further purification.

2.2. Synthesis of $W_{18}O_{49}$ NPs

500 mg WCl_6 and 200 mg PAA were dissolved in 100 mL DEG, and the suspension was allowed to react under N_2 protection at 60 °C for 30 min. The mixture was turned from colorless to golden yellow. Then the temperature was elevated to 180 °C and kept for another 30 min with a final color of emerald. After that, the suspension was allowed to cool down to room temperature and 50 mL deionized water was added into the mixture to induce precipitation. The as-obtained $W_{18}O_{49}$ NPs were collected by centrifugation and washed thoroughly with deionized water three times to remove the residue. Conjugation of the anti-HER-2 monoclonal antibody (Millipore, clone TA-1) was performed by activating the carboxyl groups of the $W_{18}O_{49}$ NPs as we previously described [19]. Briefly, 150 mg EDC, 90 mg sulfo-NHS and 30 μL anti-HER-2 antibody (1 mg/mL) were added into ice cooled 50 mL PBS suspension containing 100 mg $W_{18}O_{49}$ NPs. The mixture was allowed to react for 5 h at 4 °C under vigorously stirring. Then, the as-obtained WOHA NPs were harvested by three cycles of centrifugation and washing with PBS to remove unbounded antibody and excess reactant. The purified WOHA NPs were re-suspended in PBS and stored at 4 °C for application.

2.3. Characterization

Morphology of the as-synthesized $W_{18}O_{49}$ NPs was investigated by transmission electron microscopy (TEM, JEOL TEM-100) and high resolution TEM (JEOL, TEM-2100). The X-ray diffraction (XRD) pattern was recorded in the 2θ range of 20–60°on a Hitachi X-ray diffractometer using Cu k1 radiation (λ = 1.54056 Å) at 40 kV and 200 mA. Surface plasmon resonance properties were explored using a UV–Vis-NIR spectrophotometer (Shimadzu UV-3600). Surface element composition was investigated by X-ray photoelectron spectroscope (XPS, ThermoFisher K-Alfa) with a focused monochromatic Al X-ray (1486.6ev) source. Fourier transform infrared spectroscopy (FT-IR) was performed through mixing lyophilized dry powder of the as-obtained NPs with KBr (5 mg: 1 g W/W) and pressed it to a plate on a Bruker IFS 66V vacuum-type spectrometer.

2.4. Cell lines

MDA-MB-435 (HER-2 (+), MM435) and MDA-MB-453 (HER-2 (−), MM453) human breast malignancy were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and selected for a highly aggressive ability to spontaneously metastasize from orthotopic primary tumor transplant to lymphatics. Both MM435 cells and MM453 cells were transfected with the firefly luciferase vector to generate the MM435/*Luc*$^+$ and MM453/*Luc*$^+$ cell line. The MM435/*Luc*$^+$ cells and MM453/*Luc*$^+$ cells were cultured in DMEM supplemented with 10% fatal bovine serum (FBS, Gibco).

2.5. In vitro cytotoxicity assay (MTT assay)

Cytotoxicity of $W_{18}O_{49}$ NPs was tested via a MTT colorimetric assay. Human alveolar basal epithelial cell line A549 was seeded in 96-well plates at a density of 10^5 cells/well in 100 μL of Iscoves modified Dulbecco's medium and incubated overnight at 37 °C in 5% CO_2 atmosphere. The medium of each well was replaced with 100 μL fresh medium containing various concentrations of the $W_{18}O_{49}$ NPs. All concentrations were tested in five replicates. After 24 h and 48 h incubation, the medium was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS, PH 7.0), followed by adding 20 μL of MTT solution (2.5 mg/mL in PBS). The cells were then incubated for another 4 h at 37 °C. After that, the medium was aspirated. The collected cells were re-suspended in 200 μL DMSO, and the absorbance of each well at 490 nm was measured using a iMark Enzyme mark instrument (BIO-RAD Inc., USA).

2.6. In vitro X-ray attenuation study

Suspension containing equivalent concentration of $W_{18}O_{49}$ NPs and clinical iodine contrast agent iodixanol (in respective of tungsten and iodine concentration, 5, 26, 51, 76, 101 mM) were added into 24-well cell culture plate. CT images were collected using clinical CT Gemstone spectral 64-Detector CT (Discovery CT 750HD, GE Amersham Healthcare System, Milwaukee, WI), and the images were acquired at an X-ray voltage of 65 KVp, an anode current of 500 μA.

2.7. Assessment of targeting efficacy

Both $W_{18}O_{49}$ NPs and WOHA NPs were conjugated with fluorescein isothiocyanate (FITC) by labeling kit (BD Bioscience) according to the manufacturer's instructions. The as-obtained FITC labeled nanoparticles were harvested by centrifugation and re-suspended in PBS and diluted to 2 mg/mL. To verify the binding efficacy of WOHA NPs to MM435 cells with respect to $W_{18}O_{49}$ NPs, 200 μL freshly prepared PBS suspension of fluorescent NPs was added into confocal imaging specific petri-dish (Biotek) containing MM435 cells in a density of 2×10^5 cells/well in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. After 4 h incubation at 37 °C under 5% CO_2, the medium in each well was discarded, and the cells were subjected to three gentle washes by PBS suspension to remove unbounded NPs. Then lysosomes and nucleus of cells were highlighted by Lysotracker Red and DAPI (Invitrogen) according to manufacturer's instructions. After that, cells were washed with PBS to remove excess dye and fixed with formalin prior to test. To verify the potential role of HER-2 antigens, the HER-2 binding sites were blocked by incubation with 10 μg/mL HER-2 antibody for 40 min at 37 °C prior to culture with NPs. Then the media was removed and the cells were washed with PBS for at least three times. Staining was observed under a laser scanning confocal microscope (LSCM, Carl Zeiss, LSM510 Meta). Acquired images were further manipulated by Image Pro Plus software (Media Cybernetics).

Targeting potency of WOHA NPs was also quantitatively investigated. Both HER-2 positive MM435 cells and negative MM453 cells were brought into study. 200 μL PBS containing equivalent concentration of WOHA NPs and $W_{18}O_{49}$ NPs (1 mg/mL) was added into 6-well cell culture plate containing MM435 cells and MM453 cells with a density of 10^6 cells/well in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin. After incubation at 37 °C for 4 h, the cells were released (0.25% trypsin-EDTA, Gibco), re-suspended and washed with PBS for at least three times, and re-suspended into DMEM media containing 10% FBS. Then the precipitants were treated with NaOH suspension (10 M) at 80 °C, and the concentrations of tungsten content in each group were analyzed by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry, Agilent 7500ce), and all the tests were conducted in triplicate and averaged results with statistical significance ($p < 0.01$) were provided.

2.8. In vitro PTT

For in vitro PTT, MM435 cells were cultured with WOHA and $W_{18}O_{49}$ NPs under analogous conditions as above mentioned. After 4 h incubation, the media were discarded and the cells were subjected to laser irradiation (1064 nm, 0.5 W/cm^2) for 5 min after three washes with PBS suspension. To determine the viability of cells post treatment, cells were labeled with Calcein AM and PI (Live/Dead Kit, Invitrogen) according to manufacturer's instructions. Then, the cells were imaged directly with LSCM without formalin fixation.

2.9. CT imaging

For indirect CT lymphography in normal animal, we used a clinical Gemstone Spectral 64-Detector CT (Discovery CT 750HD, GE Amersham Healthcare System, Milwaukee, WI) to conduct the CT experiment. The pilot study for CT lymphography and identification of lymphatics and lymph node was performed in male adult New Zealand white rabbits weighing 3 kg. The animals were sedated with an intraperitoneal injection of a mixed solution of ketamine hydrochloride (50 mg/kg weight) and droperidol (30 mg/kg weight). After anesthesia, the rabbit were fixed on a rigid paper and supine position was adopted. Images of pre-scan were collected at an X-ray voltage of 65 kVp, an anode current of 500 μA. One bed position was scanned for