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Microwave pumped high-efficient thermoacoustic tumor therapy with single wall carbon nanotubes

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ABSTRACT

The ultra-short pulse microwave could excite to the strong thermoacoustic (TA) shock wave and deeply penetrate in the biological tissues. Based on this, we developed a novel deep-seated tumor therapy modality with mitochondria-targeting single wall carbon nanotubes (SWNTs) as microwave absorbing agents, which act efficiently to convert ultra-short microwave energy into TA shock wave and selectively destroy the targeted mitochondria, thereby inducing apoptosis in cancer cells. After the treatment of SWNTs (40 μg/mL) and ultra-short microwave (40 Hz, 1 min), 77.5% of cancer cells were killed and the vast majority were caused by apoptosis that initiates from mitochondrial damage. The orthotopic liver cancer mice were established as deep-seated tumor model to investigate the anti-tumor effect of mitochondria-targeting TA therapy. The results suggested that TA therapy could effectively inhibit the tumor growth without any observable side effects, while it was difficult to achieve with photothermal or photoacoustic therapy. These discoveries implied the potential application of TA therapy in deep-seated tumor models and should be further tested for development into a promising therapeutic modality for cancer treatment.

1. Introduction

Over the past decade, single wall carbon nanotubes (SWNTs) with unique intrinsic property had been intensively explored for biological and biomedical applications. One intrinsic property of SWNTs is their ability to cross cellular membranes without eliciting cytotoxicity [1], so as a unique quasi one-dimensional material, they have been explored as novel delivery vehicles for drugs [2], proteins [3], and so on. Another intrinsic property of SWNTs is their strong optical absorbance in the near-infrared (NIR) region [4,5], which makes SWNTs appropriate to be used as agents for both photothermal [6,7] and photoacoustic therapy [8,9].

In photoacoustic therapy, SWNTs with unique photophysical properties improve therapeutic efficacy by allowing for selective targeting and destructing cancer cells. The SWNTs absorb the pulsed laser and transfer its energy into a strong shock wave that results in a firecracker-like explosion at a nanoscale, leading to the death of cancer cells from mechanical damage [8]. Furthermore, an earlier study has demonstrated the accumulation of SWNTs in the mitochondria of target cells [10]. Therefore mitochondria targeting SWNTs with a pulsed laser beam kill cancer cells mainly by triggering cell apoptosis that is initiated with the mitochondrial damage through the depolarization of mitochondria and the subsequent release of cytochrome C [9].

Photoacoustic therapy could efficiently kill cancer cells and inhibit the tumor growth in the subcutaneous xenograft tumor model. The single most important advantage of photoacoustic therapy as it largely eliminates the risk of toxicity and drug resistance [11]. However, due to the limited penetrative ability of laser, the application of the photoacoustic therapy was found to be particularly challenging in *in-vivo* deep-seated orthotopic tumor model. A new theranostic technique based on a microwave-pulse induced thermoacoustic (TA) has recently evoked a keen interest among researchers.

TA employs microwave pulse for the thermoelastic excitation thus generating a strong TA shock wave with deeply penetrative power [12–14]. Although there has been much exploratory research on TA biomedical application [15–17], including efforts aiming at TA effect enhancement [18–21], the application of TA effect in tumor therapy has not been reported. The development of

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ultra-short microwave pulse generator was a breakthrough in excitation efficiency, because it with high-peak-power could significantly enhance the shock wave derived by TA effect [22]. Therefore, it is reasonable to assume that the exogenous microwave absorbing agents irradiated by ultra-short microwave would further enhance the TA effect. It is now known that the SWNTs could accumulate in mitochondria of cancer cells, and it also could absorb microwave energy [23,24], then transform into the TA shock wave [21]. Herein, we reported a new bioapplication of SWNTs in tumor therapy which named as TA therapy. It was based on the TA shock wave of mitochondria-targeting SWNTs induced by ultra-short microwave to destroy mitochondria and then trigger cancer cell apoptotic pathways. In this study, we investigated the TA therapy *in vitro* and *in vivo*. More importantly, we established orthotopic liver tumor mice as deep-seated tumor models for investigating the anti-tumor effect of TA therapy in deep-seated tumors. Benefiting from the deep penetration ability of microwave in biological tissues [25], it could potentially be effective in treating deep-seated tumors. To the best of our knowledge, this is the first report investigating TA therapy with SWNTs induced by ultra-short microwave *in vitro* and *in vivo*. In particular, this work reveals its therapeutic potential in treating orthotopic deep-seated tumors, while this was difficult to achieve with phototherapy. Our results could motivate further clinical research on TA effect of SWNTs induced by ultra-short microwave as a novel therapeutic modality for cancer.

2. Experimental section

2.1. Materials

1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[amino/carboxy(PEG)2000] (PL-PEG-NH_2) was obtained from Avanti Polar Lipids Inc. (AL, USA); Cell-counting kit-8 was obtained from Dojindo Laboratories (Kumamoto, Japan); fluorescein isothiocyanate (FITC), propidine iodide (PI) and Rh123 were obtained from Sigma–Aldrich Corporation (MO, USA). Hoechst33258 purchased from Invitrogen life technologies, inc. All other chemicals used in this work were of analytical grade. All reagents were used without further purification.

2.2. Functionalization of SWNTs

Functionalization of SWNTs with PL-PEG-NH_2 was performed following the procedure described in earlier reports [7,9]. CoMoCAT SWNTs with an average diameter of 0.81 nm and length 500–1500 nm were sonicated in an aqueous solution of PL-PEG-NH2 (1 mg SWNTs, 1 mg PL-PEG-NH_2, 1 mL water) for 6 h. The mixture was then centrifuged at 10,000 g for 15 min, and the supernatant was collected. Excess phospholipids were removed by repeated filtration using 100 kDa filters (Millipore Corporation, Billerica, MA) and rinsing with phosphate-buffered saline (PBS).

2.3. Calculation of SWNTs absorption coefficient

The microwave properties of SWNTs were analyzed by a vector network analyzer. The experiment yields the real and imaginary parts of the dielectric for the sample. This data is then used to calculate the absorption coefficient according to the equation given below [18].

$$\alpha(\omega) = \frac{\omega}{c_0}\sqrt{\frac{\sqrt{\left(\varepsilon_r^2+\varepsilon_i^2\right)\left(\mu_r^2+\mu_i^2\right)}-(\varepsilon_r\mu_r-\varepsilon_i\mu_i)}{2}} \quad (1)$$

Here, c_0 is the speed of electromagnetic waves in vacuum (approximately 3×10^8 m/s), ε_r and ε_i are the real and imaginary parts of the complex relative permittivity, respectively. μ_r and μ_i is the real and imaginary component of the relative complex permeability at angular frequency.

2.4. Microwave-induced thermoacoustic signal of SWNTs

100 μL of deionized water and SWNTs with concentrations of 25, 50, 70 and 100 μg/mL were put into a silicon tube with a diameter of 0.8 mm to detect their microwave-induced TA signals, respectively. The experimental setup was designed according to Lou et al. [22] The microwave generator with a central frequency of 434 MHz transmitted ultra-short pulse microwave with the peak power of 20 MW and pulse duration of 10 ns. The operation was at typical pulse repetition frequencies in the range of 1–40 Hz. In the data acquisition system, a focused ultrasonic transducer (I10P6NF20, Doppler Ltd., China) with a central frequency of 10 MHz (100% bandwidth at 6 dB), a focal length of 20 mm, and an active element of 6 mm diameter was employed to detect the TA signals.

2.5. Preparation of a cell-like model

Alginate-polylysine-alginate microcapsules containing SWNTs were prepared using a water-in-oil-in-water double emulsion method. Firstly, 5 mL aqueous solution containing sodium alginate (1.5% w/w) and SWNTs (1 mg/mL) was briefly dispersed in an isooctane solution containing a lipophilic surfactant (Span 85, 2.0% w/w) by using a mechanical stirrer at 8000 rpm. After 15 min, 15 mL of 0.1 M $CaCl_2$ solution was added to form Ca-alginate microspheres. The emulsion formed was stirred for 1 h. Then, the microspheres were collected by filtration and washed three times with deionized water. Ca-alginate beads were suspended in a solution of polylysine (0.1% w/w) for 10 min, washed with deionized water and then immersed in alginate solution (0.05% w/w) for 10 min. Finally, the resulting microcapsules containing SWNTs were immersed in sodium citrate solution (0.05 M) for 6 min, washed with deionized water and stored at 4 °C for further experiment [8,11].

2.6. Cell culture

Mouse mammary tumor cell line EMT-6 was used in this study. Cells were cultured in RPMI 1640 (GIBCO) supplemented with 15% fetal calf serum (FCS), penicillin (100 units/mL), and streptomycin (100 μg/mL) in 5% CO_2/95% air at 37 °C in a humidified incubator. Mouse hepatoma cell line H22 was obtained from laboratory center of animals of Sun Yat-Sen University and passaged in the ascites of mice.

2.7. Cell death assays

Cytotoxicity *in vitro* was examined with a colorimetric tetrazolium salt-based assay using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Tumor cells growing in a 96-well microplate (5×10^3 per well, 100 μL) were irradiated by the pulse microwave at repetition frequency of 10–40 Hz with or without SWNTs incubation. OD450, the absorbance value at 450 nm, was read with a 96-well plate reader (INFINITE M200, Tecan, Switzerland) to determine the viability of the cells.

To assess the changes in nuclear morphology of apoptosis, cells (1×10^4 per well) were cultured in 35 mm glass bottomed dishes. Three hours after TA treatment under 40 μg/mL SWNTs and 40 Hz pulse microwave, the cells were stained with Hoechst 33258 for 10 min at room temperature and washed twice with PBS. The cell