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Synergistic anticancer effect of RNAi and photothermal therapy mediated by functionalized single-walled carbon nanotubes

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ABSTRACT

Single-walled carbon nanotubes (SWNTs) are special nano-materials which exhibit interesting physical and chemical properties, presenting new opportunities for biomedical research and applications. In this study, we have successfully adopted a novel strategy to chemically functionalize SWNTs with polyethylenimine (PEI) through purification, oxidation, amination and polymerization, which were then bound by DSPE-PEG2000-Maleimide for further conjugation with the tumor targeting NGR (Cys-Asn-Gly-Arg-Cys-) peptide via the maleimide group and sulfhydryl group of cysteine, and finally hTERT siRNA was loaded to obtain a novel tumor targeting siRNA delivery system, designated as SWNT-PEI/siRNA/NGR. The results showed that SWNT-PEI/siRNA/NGR could efficiently cross cell membrane, induced more severe apoptosis and stronger suppression in proliferation of PC-3 cells in vitro. Furthermore, in tumor-bearing mice model the delivery system exhibited higher antitumor activity due to more accumulation in tumor without obvious toxicity in main organs. The combination of RNAi and near-infrared (NIR) photothermal therapy significantly enhanced the therapeutic efficacy. In conclusion, SWNT-PEI/siRNA/NGR is a novel and promising anticancer system by combining gene therapy and photothermal therapy.

1. Introduction

RNA interference (RNAi) is a powerful tool to suppress or silence the targeted genes in mammalian cells and model organisms [1–3]. Delivery of siRNA to target cells is complicated by the instability of siRNA, low uptake efficiency, and low bioavailability. Thus, more efficient siRNA delivery methods are required to realize the potential of siRNA to address basic biological questions and also provide therapeutic benefits. The challenge is how to make gene vectors safer and more effective. In recent years, accumulating studies have shown that single-walled carbon nanotubes (SWNTs) may be a new choice of gene vectors [4–6].

SWNTs have a lot of unique physical, chemical and biological properties, making this nano-material attractive for biomedical applications, including biosensor development [7,8], bio-electrochemistry [9], biomedical devices [10], deliveries of biomedical cargoes (including drugs [11], genes [1,12,13], proteins [14,15] etc.) into cells, and high optical absorbance of SWNTs in the near-infrared (NIR) region can be utilized for cancer photothermal therapy [11,16–19].

However, the surface of SWNTs is highly hydrophobic and insoluble in aqueous solutions and common organic reagents. Poor biocompatibility severely hindered the development of SWNTs in biomedical applications. Therefore, improving the biocompatibility of SWNTs continues to be a research hotspot with an aim to promote their biomedical applications. And another focus is to improve the biodistribution of the carrier at the tumor site. Therefore, to establish a tumor-targeting drug delivery system with covalently functionalized SWNTs to overcome all the existing obstacles has a wide range of scientific significance.

Since polyethylenimine (PEI) possess a high cationic charge density and allows the complexation of DNA [20,21] and siRNA [22,23], several PEI functionalized CNTs have been designed and tested for nucleic acid delivery [13,24]. More importantly, upon PEI complexation siRNAs are efficiently protected against nucleolytic degradation both in vitro in the presence of RNase and in vivo in the presence of serum nucleases [25]. Therefore, in this study we used branched PEI which was obtained by cationic polymerization of aziridine to modify and functionalize SWNTs.

It is reported that peptides containing the Asn-Gly-Arg (NGR) motif can selectively recognize tumor neovasculature and can be

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used, therefore, for ligand-directed targeted delivery of various drugs and particles to tumors or to other tissues with an angiogenesis component [26]. Thus, DSPE-PEG2000-Maleimide was incorporated in the delivery system in order to conjugate cysteine-containing NGR peptide for tumor targeting in this study.

A number of previous studies have shown some promising results using functionalized SWNTs for photothermal therapy [27]. Liu et al. [28] and Robinson et al. [29] separately reported that intravenous systemic administration of PEG-PMHC18-coated SWNTs and PEGylated phospholipids modified SWNTs followed by NIR laser irradiations, respectively, were able to inhibit tumor proliferation. Recently, our group reported that the SWNT-NGR-DTX drug delivery system showed higher efficacy than docetaxel in suppressing tumor growth in vitro and in vivo under NIR radiation [11]. However, combined siRNA and photothermal therapy using SWNTs has not yet been reported.

In this study, we establish the SWNT-PEI/siRNA/NGR drug delivery system, using NGR peptide to target tumor, using DSPE-PEG2000 to increase the blood circulation time, using the mammalian cells membrane penetration properties of SWNTs to delivery hTERT siRNA into tumor cells, and using the photothermal therapy in NIR region to enhance the antitumor effects in vitro and in vivo. Quantum dots (QDs) are utilized to study the biodistribution of SWNT-PEI in tumor-bearing mice, and the toxicity of SWNT-PEI is also evaluated in vitro and in vivo.

2. Materials and methods

SWNTs were purchased from Chengdu Organic Chemicals Co. Ltd. (produced by CVD, purity >90%, Lot No. 1109). PC-3 human prostate cancer cell line was obtained from Chinese Academy of Sciences Cell Bank (Catalog No. TCHu158). hTERT siRNA was produced by Genepharma (Shanghai, China). CdSe/ZnS QDs was purchased from Wuhan Jiayuan Quantum Dots Co. Ltd. The reagents for Western blotting were gotten from Beyotime (Suzhou, China) and all other reagents were bought from Sigma–Aldrich Co. LLC.

2.1. Purification of single-walled carbon nanotubes

Pristine SWNTs were suspended in 12 M HNO_3, and stirred for 24 h at room temperature. The purified SWNTs were then collected on a membrane filter (Milipore, pore size 100 nm) and thoroughly rinsed with Mili-Q water and dried at 110 °C.

2.2. Oxidation of single-walled carbon nanotubes

Approximately 50 mg of purified SWNTs was heated to 110 °C under reflux in 50 ml of 4M HNO_3 for 2 h, cooled down at room temperature, followed by filtration on a membrane filter and multiple washes with Mili-Q water. The remaining solid was suspended in 1 M HCl in an ultrasonic bath for about 30 min, diluted by Mili-Q water and the washing steps were repeated. Consequently the purified SWNTs were decortated with carboxylic acid groups (SWNT–COOH), and then heated to dryness at 110 °C.

2.3. Amine-functionalized single-walled carbon nanotubes

SWNT-NH_2 was obtained after reaction of SWNT-COOH with ethylenediamine (EDA) and dicyclohexylcarbodiimide (DCC) for 24–48 h at 120 °C under reflux. After centrifugation, the yellow-colored supernatant was decanted and the remaining solid was washed with anhydrous ethanol to remove excess EDA, DCC and other by-products. SWNT-NH_2 was then obtained on a PTFE membrane filter (Millipore, pore size 100 nm). The remaining solid was dried under vacuum at room temperature.

2.4. Polymerization of SWNTs with PEI (SWNT-PEI)

SWNT-NH_2 was immersed in $SOCl_2$, and then aziridine (Caution: aziridine is toxic, carcinogenic, and teratogenic. Use only in a well-ventilated hood) and a certain amount of HCl were added. The mixture was left in an ultrasonic bath for about 30 min. The solution was heated to reflux for 24 h under nitrogen. The resulting product was thoroughly washed with copious $SOCl_2$ and sonicated in methanol. Finally the washed substrate was dried in vacuum at room temperature.

2.5. Characterization of SWNT-PEI

2.5.1. Transmission electron microscopy (TEM)

A small amount of pristine SWNTs, SWNT-COOH, and SWNT-PEI was added in water, respectively, and ultrasonication was performed to disperse particles well. A small drop of suspension was placed on grids. Finally, the dried samples were scanned by TEM (Tecnai G^2 20, FEI).

2.5.2. Fourier transform infrared (FT-IR) spectrum

The pristine SWNTs, SWNT-COOH and SWNT-NH_2 were milled with potassium bromide (KBr) and crushed to form a very fine powder in an agate mortar and pestle, then compressed into a thin pellet using an automated agate ball mill grinding system, respectively. Finally, the pallet was scanned by FT-IR Spectrometer (Nicolet iS10, Thermo)

2.5.3. Nuclear magnetic resonance (NMR) spectrum

SWNT-PEI and PEI were dissolved in D_2O (pH7.0) and characterized by ^1H NMR spectrum (Bruker DMX-500 spectrometer), respectively.

2.5.4. Thermogravimetric analysis (TGA)

TGA was performed by scanning from 25 °C to 850 °C under nitrogen at a heating rate of 20 °C/min by using a Pyris1 TGA (PerkinElmer).

2.5.5. Ex vitro treatment under 808 nm laser radiation

Temperature change was investigated during continuous radiation to the solutions of SWNT-PEI/siRNA/NGR at different concentrations (SWNT ≈ 35, 15, 7.5 μg/ml) by an 808 nm laser at 1.5 W/cm^2.

2.6. Preparation of SWNT-PEI/siRNA/NGR delivery system

SWNT-PEI was dissolved in 5% glucose (RNase Free) and then 10 μl of DSPE-PEG-Maleimide (1 mg/ml) was added and kept in ultrasonic bath for about 2 h. After centrifugation, the supernatant was collected.

For preparation of SWNT-PEI/siRNA complexes, aqueous solution of SWNT-PEI was added slowly into hTERT siRNA (sense: 5′-CACGGUG ACCGACGCACUGTT-3′; antisense: 5′-CAGUGCGUCGGUCACCGUGTT -3′) [30] solution and incubated 30 min at room temperature with gently vortexing in the cell medium (RPMI-1640, Gibco).

SWNT-PEI/siRNA complexes were loaded onto 1% agarose gels. Electrophoresis was performed at 120 V for 15 min, then visualized by Goldview staining and images were obtained from a GelDoc 2000 imager system (Bio-Rad, Munich, Germany).

SWNT-PEI, siRNA, SWNT-PEI/siRNA complexes was diluted to a certain concentration, respectively, and analyzed with an UV–Vis spectrophotometer (UV-3600, Shimadzu, Japan).

SWNT-PEI/siRNA/NGR delivery system was obtained after adding NGR (CNGRCK2HK3HK11, NGR: maleimide (mol/mol) = 1: 20 [31]) into SWNT-PEI/siRNA complex solution and stirring 8 h at room temperature.

After SWNT-PEI/siRNA/NGR delivery system was prepared, NGR (FITC labled), SWNT-PEI/siRNA and SWNT-PEI/siRNA/NGR (FITC labeled) samples were subjected to ultracentrifugation (60,000 r/min) for 1 h, and then the fluorescence intensity in the supernatant was detected to investigate the conjugation efficiency of NGR peptide.

2.7. Cellular uptake

PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin in an incubator (MCO-15AC, Sanyo) at 37 °C in which the CO_2 level was maintained at 5%.

PC-3 cells were plated into chambered cover-slides 24 h prior to incubation at a density of 1×10^5 cells/well with FITC, SWNT-PEI/siRNA (Red fluorescence labeled) and SWNT-PEI/siRNA (Red fluorescence labeled)/NGR(FITC labeled) were added to the above plate wells. The concentration of SWNTs and siRNA were ~10 μg/ml and ~150 nM, respectively. The cells were incubated at 37 °C for 1 h, 2 h and 4 h, respectively.

The transfected cells were washed twice with PBS and then fixed in 4% formalin for about 15 min, and then washed with Mili-Q water to remove formalin. The cells were imaged by a fluorescence microscope (Zeiss LSM 510).

2.8. Cell growth inhibition assay

PC-3 cells were plated into 96-well plates and treated with SWNT-PEI, SWNT-PEI/NGR, SWNT-PEI/siRNA or SWNT-PEI/siRNA/NGR to investigate cytotoxicity at 24 h, 48 h and 72 h, separately (SWNT ≈ 10 μg/ml, siRNA = 150 nM). For laser irradiation experiment, the cells were cultured and treated in the same way. After 4-h treatment, the cells were then subject to 808 nm NIR radiation for 1 min. After 24-h culture, cell viability was measured by sulforhodamine B assay (SRB).