Bulgarian Chemical Communications, Volume 47, Number 4 (pp. 1061 – 1066) 2015

Bioconjugated carbon nanotubes for cancer targeting and near-infrared laser photothermal therapy

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Received April 4, 2015

Cancer targeting and selective photothermal destruction were achieved by using folate conjugated carbon nanotubes and a near-infrared continuous wave laser (800 nm). By functionalizing the nanotubes with folate, a photothermal agent was developed to selectively enter into the cancer cells and kill them through generating heat inside the cells under laser irradiation. The results of confocal fluorescence imaging showed that the functionalized nanotubes can specifically enter into the cancer cells via a folate receptor mediated pathway without significant internalization into normal cells. Under the irradiation of an 800 nm laser, cancer cells were selectively killed by the heat generation from carbon nanotubes inside the cancer cells, while the normal cells still remained alive. The photothermal technique combined with nanotubes and near infrared laser irradiation might afford a promising potential way for cancer targeting and selective therapy without causing toxicity and side effects.

Key words: Carbon nanotubes, cancer targeting, photothermal therapy, near-infrared laser

INTRODUCTION

Applying controlled and targeted therapeutics to cancer may provide a more efficient and less harmful solution to overcome the limitations of conventional techniques [1]. Over the past decade, there has been increasing interest in using nanotechnology for new potential therapeutics of cancer. Nanoparticles [2-4], nanoshells [5, 6], nanorods [7, 8], and recently nanotubes [9-11] have been shown to be applicable to cancer imaging and therapy. The subcellular size and unique physical properties of nanostructures make them very attractive for molecular transporters [11], anticancer drug delivery [12], and new therapeutic mechanisms [6, 13]. For active targeting to cancer cells, nanomaterials were functionalized with ligands such as antibodies, peptides, nucleic acid aptamers, carbohydrates, and small molecules [13], among which, folic acid (FA) is appealing as a small molecular ligand for targeting cancer cell membrane and allows for its use for specific cancer targeting [14].

In the attractive field of nanotechnology-based cancer therapy, nanostructures with unique photophysical properties have been considered as an interesting and promising approach for the destruction of cancer cells [5, 9, 10]. Single walled carbon nanotubes (SWNTs) are very suitable for these techniques due to their strong optical absorbance in the near infrared (700–1100 nm) region [15, 16]. This intrinsic property stems from the electronic band structures of the nanotubes. Strong optical absorbance originates from the electronic transitions from the first or second van Hove singularities [17]. The van Hove-like singularity in the density of states (DOS) moves towards the top of the valence band, enhancing the effective DOS near the Fermi energy and results in an increase in the electron–phonon interaction, thereby increasing the temperature of the nanotubes [18].

In the current work, we studied the cancer targeting of folate conjugated carbon nanotubes and the selective cancer destruction by using a cheap and simple near-infrared continuous wave (CW) laser (800 nm). By functionalizing the SWNTs with folate, we developed a photothermal agent that can selectively enter into the cancer cells that overexpress folate receptor on the surface of the cell membrane and kill them through generating heat inside cells under the excitation of CW laser. The uptake of folate-conjugated SWNTs into cells is investigated via a confocal fluorescence imaging route, and it is found that the functionalized SWNTs can enter into the cancer cells selectively via a folate-folate receptor-mediated pathway. Under the treatment of an 800 nm CW laser, cancer cells were selectively killed by the heat generation from carbon nanotubes inside cancer cells, while the normal cells still remained alive. These results demonstrated that biofunctionalized carbon nanotubes could be potential agents for cancer targeting and selective photothermal therapy.

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EXPERIMENTAL DETAILS

Single walled carbon nanotubes (SWNTs) with a diameter of 1-3 nm and an average length of 200 nm were made by a CVD approach, followed by purification and cutting process [19, 20]. Oxidized SWNTs were obtained by refluxing in 2.6 M nitric acid, and -COOH groups were formed on the side walls of the nanotubes [21]. Chitosan oligomers (CS) with molecular weight of 3000-5000, folate, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Sigma-Aldrich Corp. FITC fluorescence dye, dimethyl sulfoxide (DMSO), and 4-6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Corp. All other chemicals used in this work were of analytical grade.

The conjugation of chitosan on SWNTs was achieved by a simple noncovalent approach [22]. The SWNTs were sonicated for 1 h in a phosphate buffer saline (PBS) solution (0.5 mM) of chitosan (CS); the initial concentration of SWNTs was 1 mg/ml. The solution was centrifuged at 15000 g for 1 h to remove aggregated and large bundled nanotubes. The supernatant was collected and filtered through a 10 kDa MWCO membrane (Millipore Amicon) to remove excess chitosan, washed several times with water, and resuspended in PBS. For conjugation of folate and FITC on CS-SWNTs, folate (2.5 mM) was added to a solution of CS-SWNTs in PBS buffer (5 ml) at pH 7.4; to this solution 100 μl of FITC solution in DMSO (80 μM) was added, and then EDC (3.5 mM) and sulfo-NHS (3.5 mM) were added. The mixture was allowed to react overnight at room temperature with protection from light. The solution was subsequently dialyzed three times to ensure complete removal of excess unconjugated molecules. The remaining functionalized SWNTs (f-SWNTs) were dispersed in PBS buffer or DMEM cell medium for several days at room temperature to test the solution stability. UV-vis and fluorescence spectra were used to record the functionalization process. The concentration of final f-SWNTs was estimated via a concentration-dependent UV-vis spectroscopic route [23, 24].

Human hepatocellular carcinoma cells (Hep G2, an adherent cell line), used as the cancer cell model here, were cultured in DMEM without folate in cell medium. The folate-starved cells overexpress folate receptors on the cell membrane surface. Hep G2 cells were passed three rounds in the folate free medium before use to ensure overexpression of FA receptor on the cell surface. Normal cell model was constructed by culturing cells in DMEM medium with abundant free folate to block the folate receptors on the cell membrane surface. Both cancer and normal cell models were cultured in 6-well plates containing DMEM supplemented with 10% (v/v) fetal calf serum (FCS, Minghai Bio., China), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified air containing 5% CO_2. When the cells were grown to 80% confluence, nanotube solution was added to each well at a final concentration of 50 μg/ml. The incubations were carried out at 37 °C and in 5% CO_2 atmosphere for 5 h. After incubation, all cells were washed with PBS to remove excess SWNTs and placed in fresh medium before any assays described here.

For confocal imaging, the adherent Hep G2 cells were directly imaged by an Olympus FV-1000 laser scanning confocal microscope (LSCM). Before examination, Hep G2 cells were fixed with 4% paraformaldehyde for 15 min, and then washed three times with PBS buffer. The laser wavelength of excitation was 488 nm for fluorescence channel imaging and the detected wavelength was from 500 nm to 550 nm. The TD channel imaging was done using a 488 nm laser.

To measure the cell viability after various laser and nanotube treatments, cell samples were planted into 96-well plates and went through the microtiter tetrazolium (MTT) array. For test, 20 μl MTT (5 mg/ml, contained in 0.01 M PBS) was added into each well and incubated for 4 h at 37 °C, then the medium was replaced with DMSO (200 μl in each well). A492 was detected to measure the proliferative capacity of each group. The relative proliferation ratio of the treated groups was calculated based on the intensity compared to controls.

RESULTS AND DISCUSSION

Our starting materials were short oxidized CVD SWNTs with -COOH groups on the side wall, which were generated by refluxing in nitric acid. Fig. 1 shows a schematic drawing of SWNT functionalization. Oxidized SWNTs were firstly noncovalently functionalized with chitosan (CS), and then the CS-SWNTs were conjugated with folate and FITC. SWNTs functionalized by both folate and FITC were denoted as f-SWNTs. The final f-SWNTs were highly stable in PBS buffer, as well as in cell culture medium without aggregation even after 1 week. The unbound folate and FITC were removed from the solution by centrifugation and repeated filtering.

Receptor-mediated endocytosis through clathrin-coated pits is the most common pathway of endocytosis [25]. It provides a means for the

selective and efficient uptake of macromolecules and particles that may be present at relatively low concentrations in the extracellular medium.

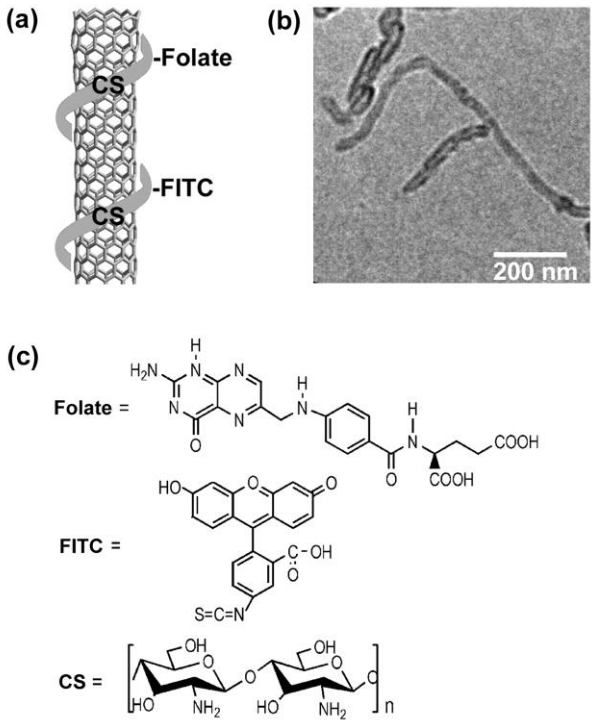

Fig. 1. (a) Schematic drawing of the functionalization of SWNTs with CS, folate and FITC. (b) TEM image of oxidized SWNTs. (c) Molecular structures of folate, FITC and CS.

Cells have receptors for the uptake of many different types of ligands, including hormones, growth factors, enzymes, and plasma proteins [26]. Herein, we conjugated the SWNTs with a kind of folate, which can specifically bind to folate receptors that overexpressed on the surface of cancer cells membrane. The selective internalization and uptake of SWNTs into Hep G2 cells were recorded by confocal imaging (Fig. 2). After incubating the cells together with 50 μg/ml FA-SWNTs for 3 h, strong fluorescence was observed in the cytoplasm of cancer cells, indicating that SWNTs entered into the cancer cells. However, for normal cells where the folate receptors on the surface of the cell membrane were blocked with free folate before incubation with FA conjugated nanotubes, just a weak fluorescence was observed in the cytoplasm. These results demonstrated that cancer cells were able to specifically internalize folate conjugated SWNTs via a folate receptor-mediated pathway while the normal cells were not.

The selective uptake of f-SWNTs inside cancer cells is due to the specific receptor-mediated interaction between the folate and the folate receptor on the cell membrane. Fig. 3 shows the magnified confocal images of the cell membrane of normal and cancer cells incubated with f-SWNTs. Bright green spots were observed on the surface area of the cell membrane of cancer cells, indicating the specific binding of f-SWNTs on the cancer cell membrane. For normal cells, no green spots were seen to show the specific binding.

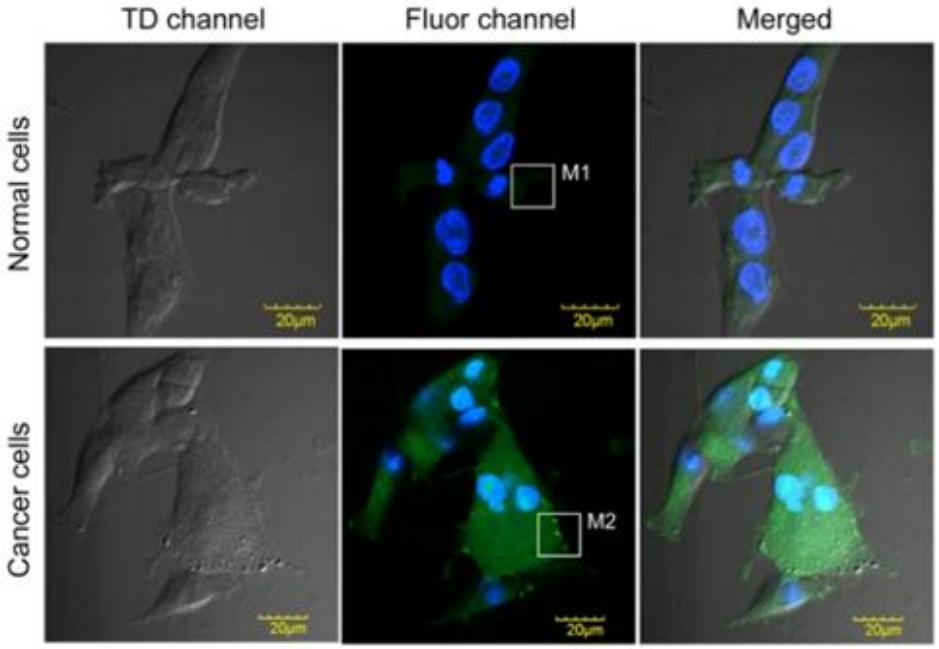

Fig. 2. Multi-channel confocal microscopy images of normal and cancer cells incubated with 50 μg/ml bioconjugated carbon nanotubes for 3 h. The magnified images of M1 and M1 are shown in Fig. 3.

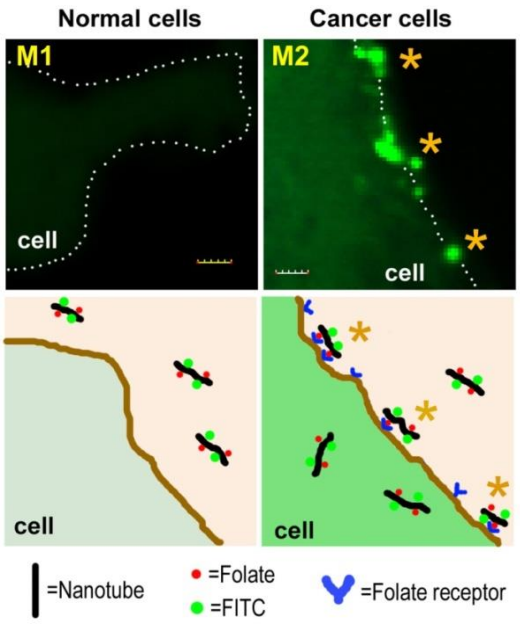

Fig. 3. Confocal images and representative schematic illustrations showing the selective targeting of folate conjugated carbon nanotubes to cancer cells through the specific receptor-mediated interaction. Both normal and cancer cells were incubated with 50 μg/ml bioconjugated carbon nanotubes for 3 h. The yellow stars indicate the specific binding of f-SWNTs to the cell membrane. M1 and M2 were magnified from Fig. 2, the scale bar is 2 μm.

To quantitatively analyze the targeting rate, we used flow cytometry to record the fluorescence intensity of cells, which denoted the relative amount of f-SWNTs in the cells (Fig.4). The targeting capability was defined as the ratio of FA-SWNTs in cancer cells and in normal cells. Strong fluorescence was observed in cancer cells incubated with f-SWNTs, whereas in untreated cells and normal cells with f-SWNT incubation, the fluorescence intensity was much lower (Fig.4). This clearly confirmed the high selective targeting of f-SWNTs to cancer cells. The targeting capability is about 6 times higher at a concentration of f-SWNTs of 50 μg/ml.

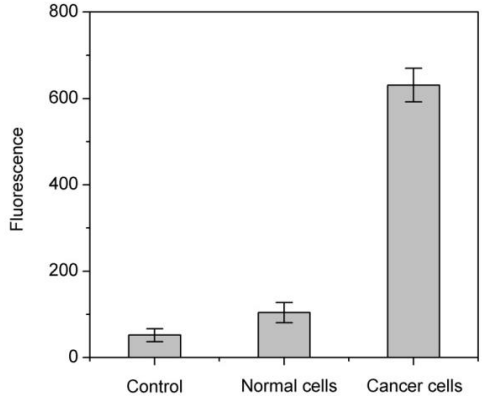

Fig. 4. Analysis of the targeting capability of folate conjugated carbon nanotubes to cancer cells.

To investigate the destructive effect of SWNTs combined with laser excitation on living cancer cells both normal and cancer cells incubated with SWNTs were exposed to an 800 nm near-infrared CW laser for 5 min. From the confocal images of cancer cells with SWNT uptake before and after laser treatment, as shown in Fig.5, one can clearly see the cracks of cancer cells due to the photothermal effect of SWNTs inside cells under laser excitation. Before laser treatment, the cancer cells have a clear dividing line at the edge of the cell membrane. After laser treatment, it is hard to distinguish the cytoplasm of the different cells since all cancer cells show a cracked and atrophic form. In the confocal images of normal cells treated with f-SWNTs and laser irradiation, normal cells survived after the same laser irradiation due to the low uptake of f-SWNTs. This result clearly shows the high selectivity of f-SWNTs on the photothermal destruction of cancer cells.

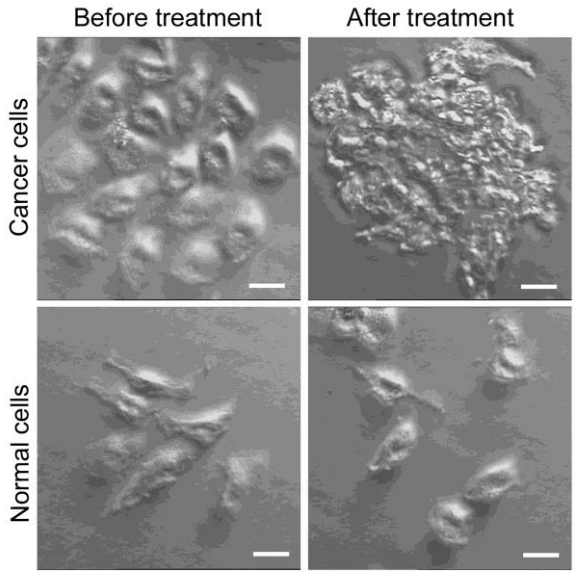

Fig. 5. Targeted cancer therapy by using carbon nanotubes and NIR laser. Both normal and cancer cells were incubated with 50 μg/ml nanotube for 3 h, followed by an 800 nm NIR laser irradiation for 5 min at a power density of 2.5 W/cm^2.

The selective destruction of cancer cells was further quantitatively analyzed through measuring the cell viability of cells with a MTT method. As shown in Fig.6, the cell viability of cells only with 50 μg/ml f-SWNTs (NTs) or 5 min laser treatment (laser) shows no obvious differences from the control cells (Control). Yet, the cancer cells with both f-SWNTs and laser treatment showed a remarkable decrease in cell viability. These results clearly demonstrated that the bioconjugated cancer nanotubes might act as potential agents for cancer-targeted near-infrared photothermal therapy.

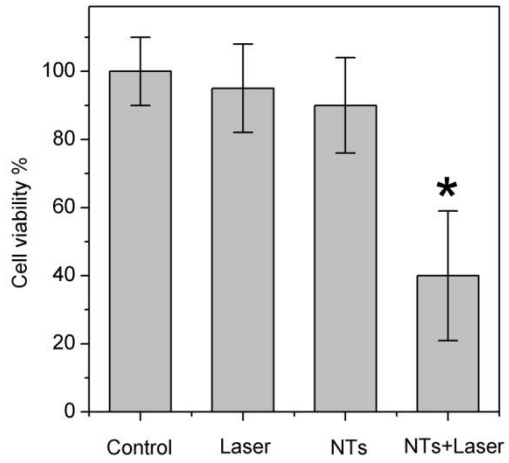

Fig. 6. Cell viability of cancer cells after different treatments: (Control) Control cells without any treatment; (Laser) Cancer cells irradiated with a 800 nm CW laser for 5 min in the absence of nanotubes; (NTs) Cancer cells incubated with 50 μg/ml nanotube for 3 h; (NTs+Laser) Cancer cells irradiated with the laser for 5 min in the presence of 50 μg/ml nanotube. Power density of 2.5 W cm^{-2} was used for all of these studies. Under a significance level of 5% (a = 0.05), the p values are shown as $p < 0.05$(*).

CONCLUSIONS

We studied the cancer targeting of folate conjugated carbon nanotubes and the selective cancer destruction by using a near-infrared continuous wave (CW) laser (800 nm). By functionalizing the SWNTs with folate, we developed a photothermal agent that can selectively enter into cancer cells that overexpress folate receptors on the surface of cell membrane and kill them through generating heat inside the cells under the excitation of a CW laser. The uptake of folate-conjugated SWNTs into the cells was investigated via a confocal fluorescence imaging route, and it is found that the functionalized SWNTs can enter into the cancer cells selectively via a folate-folate receptor-mediated pathway. Under the treatment of an 800 nm CW laser, cancer cells were selectively killed by the heat generation from carbon nanotubes inside the cancer cells, while the normal cells still remained alive. These results demonstrated that biofunctionalized carbon nanotubes could be potential agents for cancer targeting and selective photothermal therapy. Our current photothermal technique combined with nanotubes and near infrared laser might afford a promising potential way for cancer killing without causing toxicity and drug resistance since such a photothermal process is an instantaneous physical effect. These finds will be useful for the application of the structural properties of SWNTs for future therapeutic approaches.

Acknowledgements: *This work was supported by the Natural Science Foundation of Jiangsu Province (BK2012799), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20123218110008), Funding for Outstanding Doctoral Dissertation in NUAA (BCXJ12-07) and by the Fundamental Research Funds for the Central Universities.*

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БИО-СПРЕГНАТИ ВЪГЛЕРОДНИ НАНОТРЪБИ ЗА ОТКРИВАНЕ НА РАК И ФОТОТЕРМАЛНА ТЕРАПИЯ С ЛАЗЕР В БЛИЗКАТА ИНФРА-ЧЕРВЕНА ОБЛАСТ

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Постъпила на 4 април, 2015 г.

(Резюме)

Откриване на ракови клетки и селективното им фототермичното разграждане е постигнато чрез фолат-спрегнати въглеродни нанотръби и вълнов инфра-червен лазер (800 nm) с непрекъснато действие. Така е създаден фототермичен агент, който прониква селективно в раковите клетки и ги убива чрез генерирането на топлина в тях след лазерно възбуждане. Резултатите, получени чрез конфокално флуоресцентно изобразяване показват, че функционализираните нанотръби могат специфично да проникнат в раковите клетки чрез фолат-модифицирани рецептори без съществено проникване в нормалните клетки. При облъчването с лазер при 800 nm раковите клетки селективно се убиват от топлината, генерирана във въглеродните нанотръби, докато нормалните клетки остават живи. Фототермичният метод с нанотръби и облъчването с инфрачервен лазер може да разкрият обещаващ начин за откриване на рак и селективна терапия без предизвикана токсичност и странични ефекти.