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A new potential radiosensitizer- multi-walled carbon nanotubes modified by ammonium persulfate

Research Article

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Abbreviations: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, (MTT); American Type Culture Collection, (ATCC); Dulbcco's Modifed Eagle Medium, (DMEM); infrared, (IR); phosphate buffer solution, (PBS); pure MWCNTs, (p-MWCNTs); single-stranded DNA, (ssDNA)

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Summary

Here we prepare carbon nanotubes modified with ammonium persulfate, very short carbon nanotubes with 50-100 nanometer length was obtained, and the higher Z potential of 52 mV was detected, these supporting the successful modification. HeLa cells were irradiated with γ rays via adding or absent above functionalized carbon nanotubes (f-WCNTs) into cell culture medium with different concentration and radiation dosage. Confocal microscopy images and fluorescence-labeled DNA detection verified the successfully pure multi-walled carbon nanotubes (p-WCNTs) and f-WCNTs penetrated into cells. Compared with pure radiation, by MTT test, f-WCNTs induced cell death markedly with about 8.7 times higher than former one under little dose of radiation; meanwhile, no obvious toxicity was observed both in p-WCNTs and f-WCNTs without of radiation exposure. We hypothesized that large amount of hydroxyl and carbonyl organs on the surface of very short f-WCNTs changed into free radicals result from radiations led cell damage. These implied that f-WCNTs could be regarded as a new radiosensitizer.

I. Introduction

As is well known, radiotherapy plays a very important role in cancer treatment, and the cure effect strictly relied on the intrinsic radiosensitivity of target cancer cells. Besides of very fewer sensitive examples, such as ataxia telangiectasia (Maria et al, 2008) cells, most malignancy cells are of moderate radiosensitivity, even some of them are radioresistant (Kaliberov et al, 2007). In order to achieve better treatment effect, radiosensitizers (Bump et al, 1982; Cuneo et al, 2007; Kaliberov et al, 2007; Kinsella et al, 2007; Lally et al, 2007; Specenier et al, 2007; Zhang et al, 2007) are frequently used in vivo or in vitro performances. Radiosensitizer introduce a higher radiotherapy effect mainly through capturing electrons in target volume under irradiations to prevent them from recombination with radiation-injured samples (Bump et al, 1982).

In recent years, ability of carbon nanotubes (Mattson et al, 2000; Kueltzo and Middaugh, 2003; Pantarottoet al, 2003; Savic et al, 2003; Pantarotto et al, 2004) to transport across cell membranes has received significant interest. Because of its' non-toxic property, carbon nanotubes have been used as a new tool to deliver poorly penetrating drug, peptides, peptidomimetics, protein or small organic probe molecules into cancerous cells (Mattson et al, 2000; Kueltzo and Middaugh, 2003; Pantarottoet al, 2003; Savic et al, 2003; Pantarotto et al, 2004; Shi Kam et al, 2004). However, there is not any studies report that carbon nanotubes have been used in radiobiology experiments as radiosensitizer.

In present study, we describe preparation of MWCNTs modified with ammonium persulfate. The modified carbon nanotubes are not only able to readily enter cervix cancer cells (HeLa cells), but also to effectively kill the cancerous cells under low dose of γ radiations. Furthermore, both pure MWCNTs (p-MWCNTs) and f-MWCNTs themselves appear non-toxic for HeLa cells. With the increasing radiation dosage and

concentration of f-MWCNTs in growth medium, there is a significant decrease of the cell survivals.

II. Experimental

The stable aqueous suspensions of purified, shortened, and functionalized carboxylic acid nanotubes is obtained by oxidation and polishing (Chen et al, 1998; Liu et al, 1998; Sano et al, 2001) of laser-ablated raw multi-walled carbon nanotubes (purchased from Shenzhen Nanotech Port Co. Ltd.). In order to eliminate metal catalysts, the carbon nanotubes was afterward dispersed in 6 M HCl under ultrasonic agitation, washed with sodium hydroxide solution and deionized water to neutrality and dried. The purified MWCNTs are suspended in 500 mL concentrated H_2SO_4/HNO_3 (V/V=3:1) solution and sonicated in a water bath for 24 h at 35-40 °C. Centrifugation (7000 rpm, 5 min) removed larger unreacted impurities from the resultant suspension to afford a stable suspension of MWCNTs. The cut nanotubes are recovered by filtration with polytetrafluoroethylene membrane with a pore size of 0.22 μm and rinsed with deionized water. Subsequently, they are then further polished by suspension in a 4:1 mixture of concentrated H_2SO_4/30% aqueous H_2O_2 and stirring at 70 °C for 30 min. After filtering and washing again, the resulting MWCNTs can be relatively dispersed in water, this resulting material was regarded as p-MWCNTs. Then, 50mg p-MWCNTs are added into 10ml deionized water, sonicated for 10 minutes. Thereafter, ammonium persulfate is added into upper solution with the terminated concentration of 0.5M, and stirring for 48 hours at 50 °C. The rinse and filtration process is repeated as described above, at the end, we get f-MWCNTs.

All of the tested materials are conducted in sterile phosphate buffer solution (PBS), and kept the cells from contaminant. HeLa cells purchased from American Type Culture Collection (ATCC) are cultured in Dulbcco's Modifed Eagle Medium (DMEM, Invitrogen-Gibco), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells are incubated in a humidified atmosphere of 5% CO_2-95% air at 37 °C in a 75 cm^2 flask, and supplied with fresh medium every three days.

Incubation of cells is done by adding PBS of the p-MWCNTs, f-MWCNTs into the culture medium (concentration ranges from 0 to 50μg/mL in the culture medium), and the incubation duration is always 4 h. After incubation, the cells are washed with PBS and resuspended in fresh culture medium.

All confocal images are taken immediately after the incubation and washing steps except for the radiation experiment and cell viability assay. The cell suspension (20 μL) is dropped onto a glass-bottomed dish and image by a Zeiss LSM 510 confocal microscopy.

Cells are randomly divided into three groups: two are adding p-MWCNTs, f-MWCNTs into cell culture medium, respectively; and another group is regarded as control with no other materials added into. Cells are irradiated with ^{60}Co γ rays with dosage range from 0 to 6 Grays (Dose rate was 1Gray per minute).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) is used to determine cell survival in a quantitative colorimetric assay. Various dehydrogenase enzymes in active mitochondria, forming a blue-colored insoluble product, cleave its tetrazolium ring formazan. The HeLa cells are incubated with MTT (5 mg/mL) added to the culture medium for 4 h at 37 °C. The medium is then aspirated and the formazan product is dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 490 nm with control of 650 nm. The results are expressed as a percentage of control culture viability.

III. Results

To examine the dispersion state of the MWCNTs in water solutions, one drop of the water solution with MWCNTs (1 mg/mL) is dropped on a silicon-oxide substrate for scanning electron microscope analysis, the image reveal mostly short (about 100 nm-1 μm) p-MWCNTs and 30-100nm f-MWCNTs with diameters of 30 nm corresponding to mostly isolated individual MWCNTs (**Figure 1a, b**). No significant amount of particles is observed on the substrate, suggesting good purity of short MWCNTs in water solution. In pure water, the suspension of the black MWCNTs is stable for extending periods of time and does not agglomerate, which is likely relative to amount of shortened MWCNTs. This phenomenon is in accordance with what reported in literatures (Liu et al, 1998; Sano et al, 2001). In PBS containing ~0.2 M salt, the suspension of MWCNTs is less stable and start to aggregate after 2 h. We use Zsizer 3000HS (Malvern Instruments Ltd, UK) to obtain the ζ potential of p- and f-MWCNTs, which is 42 and 54mV. This indicates the more negatively charged groups existed on the surface of f-MWCNTs than that of p-MWCNTs (**Figure 1c, d**). Furthermore, infrared (IR) analysis shows that both carbonyl and hydroxyl peak number of f-MWCNTs are higher than that of p-MWCNTs (**Figure 1e, f**). These groups will change into free radicals in aqueous atmosphere when exposed to ion radiation, and consequently induce cell damage (Albano, 2006).

To visualize the interaction of p-MWCNTs, and f-MWCNTs with cells, the HeLa cells are incubated with these nanomaterials (50μg/mL) for 4 h at 37 °C. After the cells are carefully washed with PBS and digested by steapsin, and a fresh culture medium is added. Subsequently, the cells are observed directly in glass-bottomed dishes under confocal microscope. **Figure 2a, b** show the cells with dark cytoplasm and apparent nuclei free of MWCNTs, indicative of intracellular and not extracellular localization of the MWCNTs.

To further verify the cellular uptake of both p-MWCNTs and f-MWCNTs, negatively charged single-stranded DNA (ssDNA) labeled with 6-carboxy fluorescein is bound to the sidewall of MWCNTs via hydrophobic interaction. The dispersive complexes of the MWCNTs/ssDNA are dialyzed for 2 h with constant stirring in PBS to eliminate free ssDNA. The confocal images indicate that the stable complexes of MWCNTs/ssDNA in PBS reveal green fluorescence, which confirms the ssDNA can be strongly absorbed on sidewall of MWCNTs. We then study the interactions of these resulting complexes with the HeLa cells. **Figure 2c** shows that these complexes appear to uniformly accumulate in the cytoplasm in the HeLa cells after internalization, and not adhering to the cells extracellularly, which further confirm the intracellular uptake of the complexes. As negative-control experiment, the cells are incubated with a solution that contained only fluorescently labeled ssDNA. No fluorescence of the cells is detected, which means that the MWCNTs can traverse the cell membranes and transport adsorbed ssDNA into the cells.

Having discovered the ability of p-MWCNTs and f-MWCNTs to enter cells, we further seek to examine their potential toxicity due to the delivery of these nanomaterials into cells. Toward this end, the p- and f-MWCNTs are used as the toxic control assay, and the cell survival is conducted by the observed MTT experiments. The cells are separately incubated with pure DMEM culture medium (contain 10% BSA), p-MWCNTs and f-MWCNTs for 4 h, and rinsed with sterile PBS. Subsequently, these cells are transferred into 96 bores board. After 48 h incubation, the MTT is added into the each bore in the board. After 5 h, the clear solution into each bore is obtained by centrifugation, and respectively transferred into the other 96 bores board. As show in **Figure 3**, along with increasing the concentration of p- and f-MWCNTs in the incubation solution (concentration ranges from 0 to 50μg/mL in the culture medium) survival rate of the HeLa cells is stable at about 95%, which is

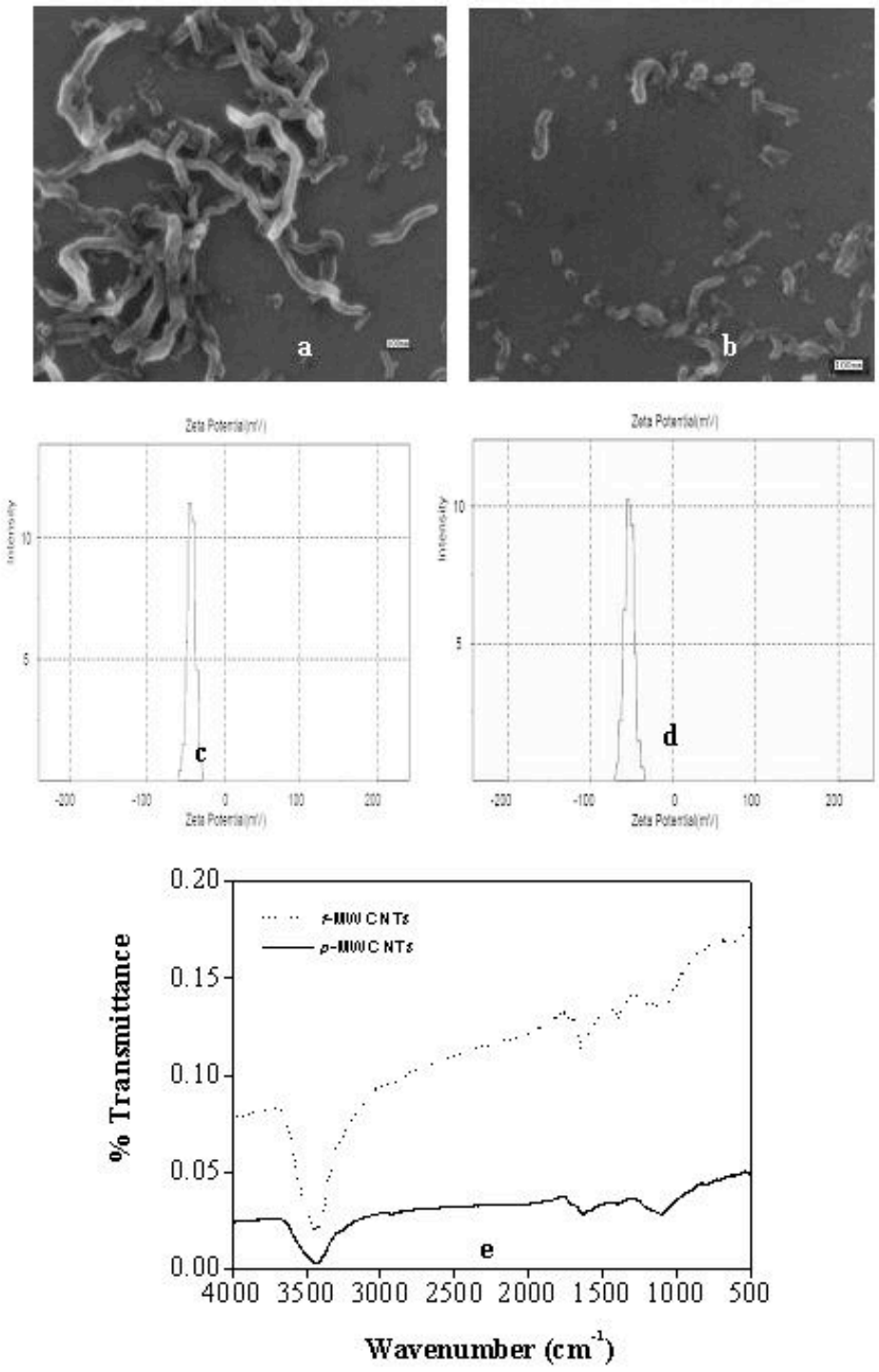

Figure 1. Physical and chemical properties of p-MWCNTs and f-MWCNTs, (**a**) SEM image of p-MWCNTs, (**b**) SEM image of f-MWCNTs, (**c**) ζ potential of p-MWCNTs, (**e**) ζ potential of f-MWCNTs, (**e**) IR spectrum diagram of p-MWCNTs and f-MWCNTs.

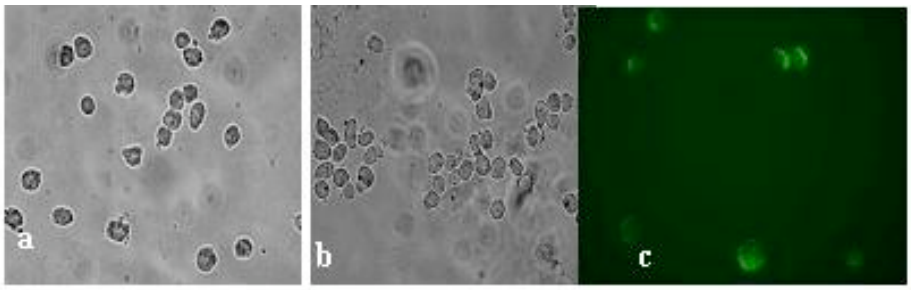

Figure 2. Confocal images of HeLa cells after incubation in solution of p-MWCNTs and f–MWCNTs: (**a**) after incubation in p-MWCNTs, (**b**) after incubation in f–MWCNTs, (**c**) after incubation in the MWCNTs/ssDNA complexes at 37 °C.

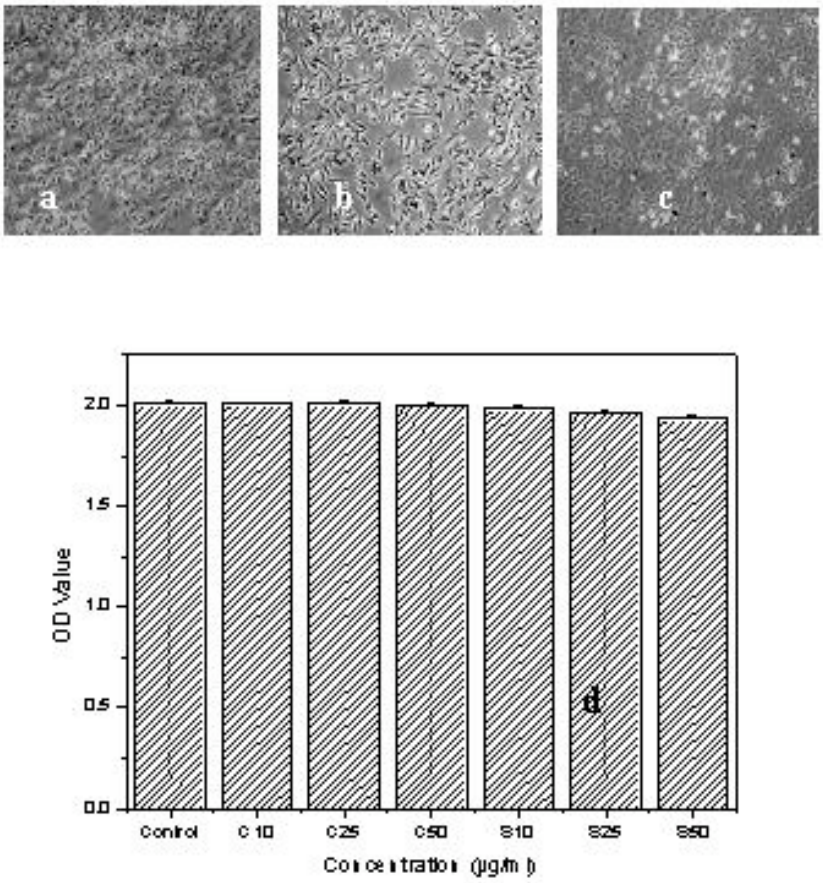

Figure 3. Confocal images of HeLa cells after incubation in solution of p-MWCNTs and f–MWCNTs: (**a**) after incubation in pure DMEM culture medium with 10% fetal bovine serum, (**b**) after incubation in p–MWCNTs, (**c**) after incubation in f–MWCNTs, (**d**) cell viability showed by OD value. $P<0.05$.

similar with that of control group, no significant changes of cell survival are observed. These results indicate that p- and f-MWCNTs themselves are little toxic to HeLa cells after the cells are incubated even in highly concentrated solutions.

The p- and f-MWCNTs are conducted under high pressure and high temperature, and that all of the tested materials are conducted in sterile PBS, and kept the cells from contaminant. Thus, the observed endotoxin is nearly nonexistent when these cells are exposed to the p- and f-MWCNTs in our experiment. Since these materials are immediately dispersed by sonication within a few seconds, the cell survivals are not likely influenced by the residual transition metal contaminants into the MWCNTs.

When absent of radiations, p- and f-MWCNTs have been verified no toxicity to HeLa cells, regardless of its concentration. After irradiation, cell viability was decreased along with the increase of radiation dosage and material concentration. For f-MWCNTs group, there is a sharp decrease, compared with p-MWCNTs and control group; and the cell survival of p-MWCNTs group is lower than that of control group. Cell viability is similar with our previous report (Yang et al, 2005), under 6 Gy γ radiation, there is a 40 percent cell survive with no MWCNTs addition, and the cell survival curves are linear-quadratic,

which is consistent with the classical low linear energy transfer radiation model. After further 72 hours incubation in fresh DMEM culture medium, there is an obvious repair effect in control group (**Figure 4d**), and this repair effect is not observed in p- and f-MWCNTs groups (**Figure 4e, f**), it indicates that the MWCNTs induces radiation damage is lethal attack to cells.

IV. Discussion

The ammonium persulfate oxidized and polished areas of the MWCNTs contain large amount of negatively charged carbonyl, hydroxyl groups along the sidewalls, such groups are of feasibility to be radioanalyzed to become hydroxylic and other free radicals under radiation. These free radicals are able to kill cancer cells directly, possibly including cell membrane, plasma, nuclei and other substructures. The unoxidized areas of the MWCNTs may still afford regions of appreciable hydrophobicity, and the DNA molecules can be strongly adsorbed on surface of MWCNTs presumably via hydrophobic interaction with sidewalls of the carbon nanotubes (Pantarotto et al, 2004; Shi Kam et al, 2004). The MWCNTs can nonspecifically associate with hydrophobic regions of the cell surface and internalize by endocytosis (Silverstein et al, 1977; Vida and Emr, 1995). The unoxidized areas can also afford regions to combine with special tumor cell antibody, which will introduce f-MWCNTs into tumor cells, thereafter lead a targeted radiosensitizing effect.

V. Conclusions

In conclusion, the ammonium persulfate functionalized MWCNTs are soluble, and can enter cancer cells, exhibiting dose-dependent cytotoxicity under radiation. Thus, because of the unique biocompatibility, and bioabsorption of the MWCNTs, it provides the basis for new classes of materials for enhancing the cancer cell radiosensitivity, though the targeting nature of MWCNTs is not discussed in present study. This indicates that MWCNTs are of possibility to be prepared for a new kind of radiosensitizer.

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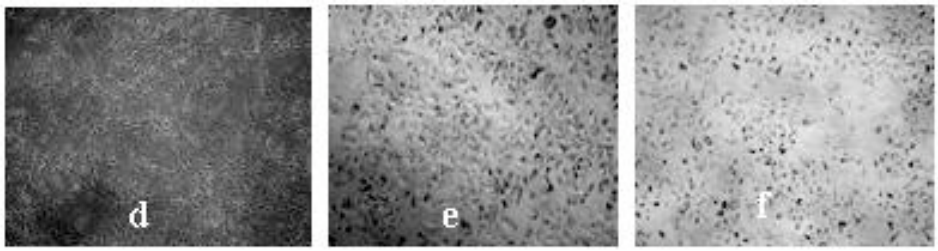

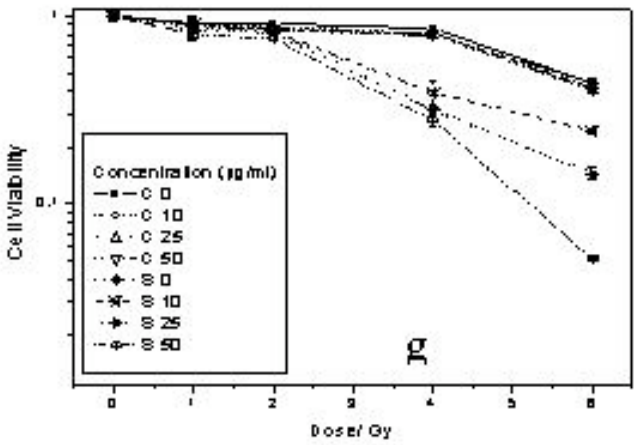

Figure 4. MTT analysis of HeLa cell survival ratio after gamma radiation. (**a**) 4Gy radiation; (**b**) 4Gy+25μg p-MWCNTs; (**c**) 4Gy+25μg f-MWCNTs; (**d**) 4Gy irradiation and 72 hours repair; (**e**) 4Gy+25μg p-MWCNTs and 72 hours repair; (**f**) 4Gy+25μg f-MWCNTs and 72 hours repair; (**g**) Cell violability curves. $p<0.05$.

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