

Supporting Information

Figure S1 (a) Raman Spectra of MWCNTs (black), MWCNT-OH (red) and MWCNT-COOH (blue). (b) FTIR spectra of MWCNTs (black), MWCNT-OH (red) and MWCNT-COOH (blue).

Figure S2 TEM images of ARPE-19 cell after incubation with (a) 50 $\mu\text{g/mL}$ MWCNTs exposed to cells for 24 h. Inset is the selected area of (a) at higher magnification. MWCNTs. Arrows indicate the presence of MWCNTs. (b) The 50 $\mu\text{g/mL}$ MWCNTs exposed to cells over 72 h. Arrows indicate the release of carbon nanotubes from cells.

Figure S3 Cell survival rate assay of ARPE 19 cells incubated with various amount of MWCNT, MWCNT-OH, MWCNT-COOH over (a) 24 h and (b) 48 h.

Figure S4 LDH assay for detection of ARPE-19 cells incubated with MWCNT, MWCNT-OH and MWCNT-COOH over 24 h and 48 h respectively.

Figure S5 Percentage change in ROS generation after incubated with MWCNTs, MWCNT-OH and MWCNT-COOH over (a) 24 h, and (b) 48 h.

The supporting information is available online at csb.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

Supporting Information

1 Reagents and materials

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12), Fetal bovine serum (FBS) and trypsin were all purchased from Invitrogen Corporation. Cell Counting Kit -8 (CCK-8) was from Dojindo Molecular Technologies, Inc.. Lactate Dehydrogenase (LDH) Release Test Kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay) was provided by Promega Corporation. Hoechst Staining Kit, Caspase 3 Activity Assay Kit and Reactive Oxygen Species (ROS) Assay Kit were supplied by Beyotime Biotechnology. All other chemicals were obtained from Sigma-Aldrich.

Multi-walled carbon nanotubes (MWCNTs), prepared by catalytic chemical vapor deposition (outer diameter of 10–30 nm, lengths of 10–30 mm, purity>95%), were purchased from Chengdu Organic Co. Ltd. (Chinese Academy of Science). MWCNTs were plasma modified at 100–500 kHz, 0.3–0.5 Torr and a power of 10–30 W for 30–60 seconds under acetic acid monomer or ethanol monomer to introduce –COOH or –OH groups into MWCNTs respectively. Samples were post-protected under N₂ for 10 mins after plasma treatment. MWCNTs base materials were dispersed in phosphate buffered saline (PBS, pH 7.4) at a concentration of 2 mg/mL (stock) and sonicated at 100 Hz for 30 min to prevent agglomeration of MWCNTs.

2 Cell culture

ARPE-19 cells purchased from ATCC (American Type Culture Collection) (Washington, DC, American) were kindly provided by Prof. Ling Hou (Wenzhou Medical College). ARPE-19 Cells were seeded in 96-well plates at the density of 5000 cells/well with DMEM/F12 medium containing 10% FBS and 50 µg/mL gentamicin [S1]. Cells were grown and maintained in a humidified atmosphere containing 5% CO₂ at 37°C and grown to 80% confluence. Various concentrations of MWCNTs (5 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL) were prepared in cell culture medium without serum prior to be used. After 24 h of cell attachment, the medium was replaced by low serum DMEM/F12 (containing 0.1% FBS) to prevent FBS interact with nanotubes [S2,S3]. Cells were subsequently cultured in the incubator with Freshly dispersed MWCNTs suspensions for a 24 h, 48 h and 72 h respectively. Cells without addition of MWCNTs were used as negative control throughout each assay.

3 Light-microscopic and TEM observations of cells exposed to MWCNTs

Cell morphologies were determined using a phase-contrast microscope. For TEM characterisation, cells were pre-fixed with 2.5% glutaraldehyde at 4°C overnight, rinsed 3 times in PBS, post-fixed with 1% osmium tetroxide for 1h followed by rinsing two times with deionized water. Samples were then dyed with 1% uranyl acetate at 37°C for 1h. Subsequently, samples were dehydrated using increasing concentrations (70%, 80%, 90%, 100%) of acetone followed by soaking in a acetone: ethoxyline (1:1) at 37°C for 1h and acetone: ethoxyline (1:4) at 37°C for overnight. Finally, samples were embedded in prue ethoxyline at 45°C.

4 Cell viability assay

Influence MWCNT based materials on cell viability were evaluated using CCK-8 assay. Typically, ARPE-19 cells were seeded into a 96-well plate at a density of 5000 cell/ well and incubated for 24 h. The medium was aspirated and cells were washed one time with PBS. Thereafter, cells were exposed to three types of MWCNTs (MWCNTs, MWCNT- OH and MWCNT-COOH) dispersed in low serum medium at 5 µg/mL to 100 µg/mL for 24, 48, 72 h. Each treatment condition was done in triplicate. After cells were exposed for a period, supernatant were removed and cells were rinsed three times, followed by addition of 100 µL cell culture medium with 10 µL of the CCK-8 solution to each well of the plate. The plate was then incubated for 3 h in the incubator. The absorbance was measured using a Microplate Reader at 450 nm.

5 LDH release assay

ARPE-19 cells (1×10^5 cells/ml) were plated in a volume of 100 μ L into a 96-well plate and were incubated for 24 h. Nanotubes suspended in culture medium were subsequently introduced. The plate was centrifuged at 250 g for 5 min after exposure. Supernatant of each well was subsequently transferred to a fresh 96-well flat bottom plate. The LDH release was measured as described in the manufacturer's instructions. Each treatment condition was repeated for three times.

6 Oxidative stress studies

ROS was used to evaluate the effect of nanobutes on oxidative stress. ROS generation was measured by two methods: Microscopic confocal laser imaging and Fluorometric analysis. For Microscopic confocal laser imaging, cells were grown on coverslips and were incubated for 72 h in the presence or absence of nanotubes. Cell images were taken using a laser confocal microscope. For Fluorometric analysis, five thousand cells were seeded in 96-well plate and allowed to adhere to the well bottom for 24 h. Then the medium was replaced with MWCNT-based materials with different concentrations. Cells were incubated with DCFH-DA (10 μ M) for 30min in the incubator after a predetermined time. The medium containing DCFH-DA was aspirated and the wells were washed with PBS three times to remove the uncombined probe and followed by the addition of 100 μ L fresh culture medium. Fluorescence intensity was measured using microplate reader on excitation wavelength at 488 nm and emission wavelength at 525 nm.

7 Apoptosis and necrotic studies

Apoptotic and necrotic cells were qualitatively analyzed by Hoechst Staining Kit. In a typical experiment, ARPE-19 cells (2×10^5 cells/mL) were plated into a 6-well plate. After 24h incubation, cells were exposed to nanotubes. The medium was subsequently aspirated and cell fixed liquid was added. Original solution was then drawn and the wells were washed with PBS thrice followed by the addition of Hoechst 33258 dyeing liquid. Sample was remained with dye for 5m. After washing by PBS thrice, cells were observed using a fluorescence microscope. Apoptotic and necrotic cells were quantitatively determined using a Caspase 3 Activity Assay Kit.

8 Statistical analysis

Statistical analysis was executed using SPSS 18.0. All the experimental data were reported as the mean $\pm$ SD. Values were compared by multi-sample comparison tests after performing ANOVA. The significance level was set at $p < 0.05$.

9 Raman and FTIR spectroscopy

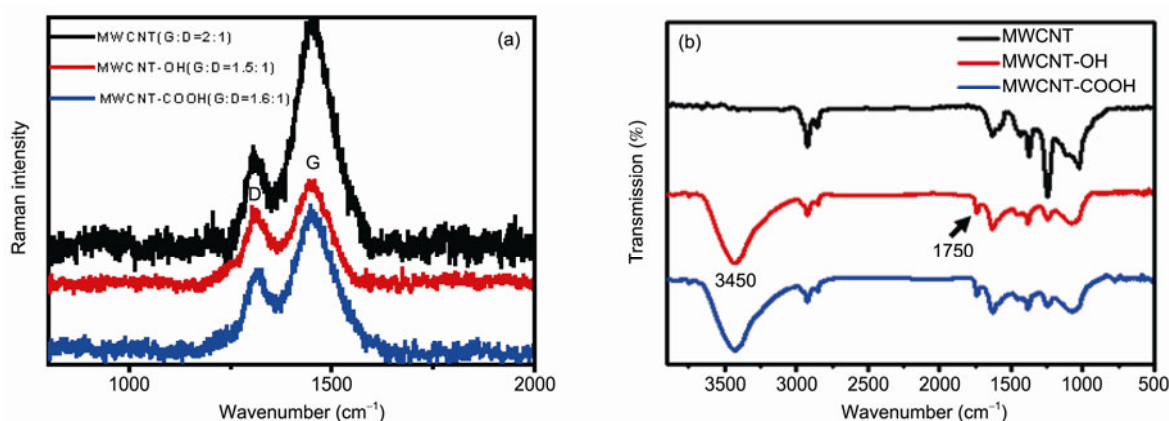


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10 Light-microscopy and TEM

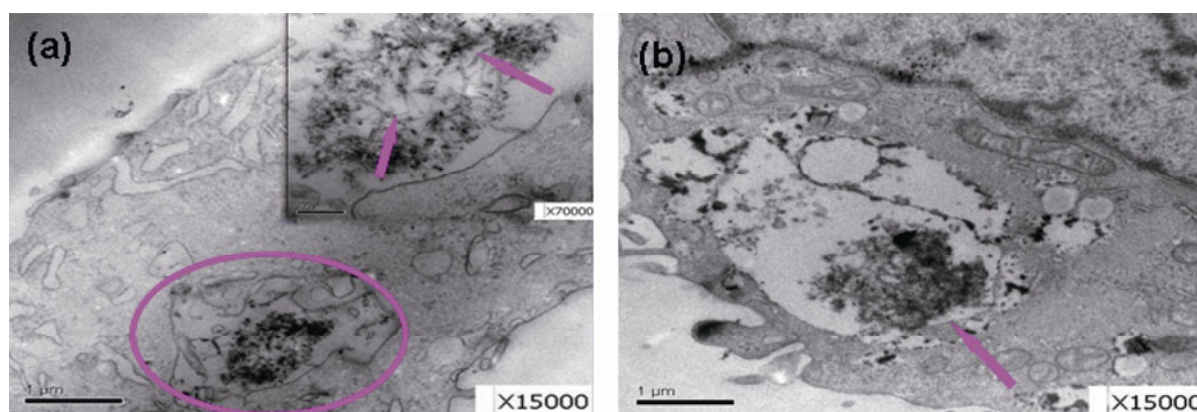


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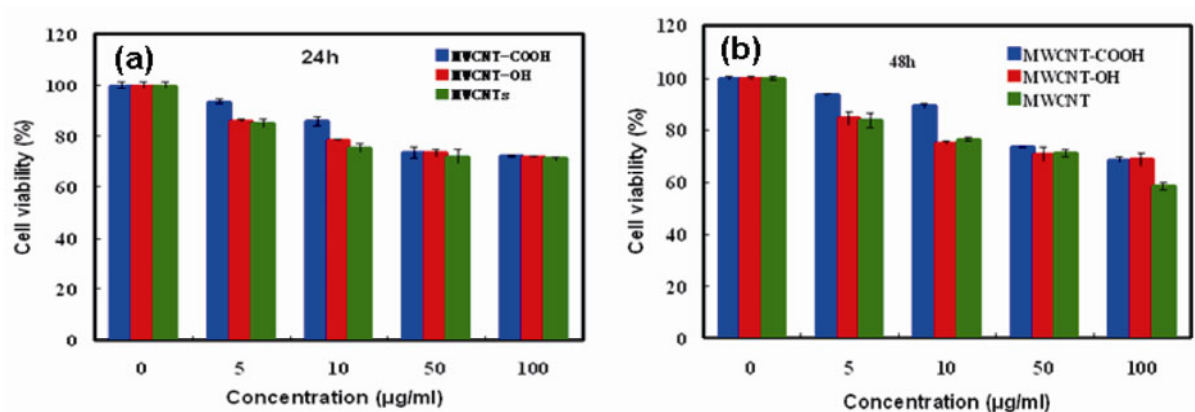


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12 LDH release assay

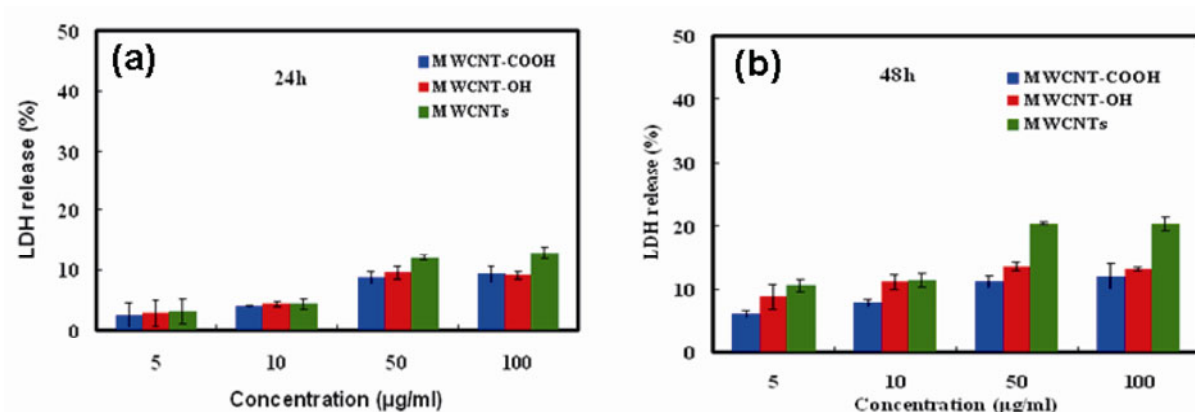


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13 Oxidative stress studies

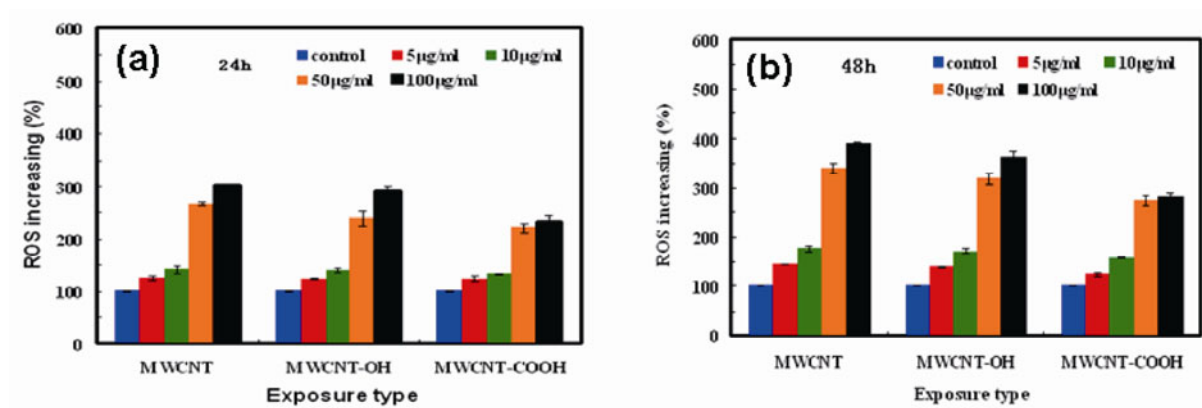


Figure S5 Percentage change in ROS generation after incubated with MWCNTs, MWCNT-OH and MWCNT-COOH over (a) 24 h, and (b) 48 h.

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