

Supporting Information

Physicochemical Determinants of Multi-Walled Carbon Nanotube Bacterial Cytotoxicity

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Materials and Methods (Additional Details)

MWNT Characterization (TEM, TGA, EDX, and SEM). TEM imaging on a Tecnai F20 (Philips Electron Optics, Eindhoven, Netherlands) provided a visual means of characterizing the purity and structural characteristics of individual nanotubes. To perform TEM imaging, we dispersed the MWNT sample in ethanol and dried it on 200-mesh copper grids coated with carbon-Formvar (Electron Microscopy Science). A 200 kV accelerating voltage provided optimal image quality. Thermo-gravimetric analysis (TGA) (SETSYS 16/18) was executed from 200°C to 1000 °C at a heating rate of 10°C/min. Energy dispersive X-ray spectroscopy (EDX) was used to determine the elemental composition of residues from the TGA analysis. Finally, SEM imaging of the MWNTs on a XL30 microscope (FEI) yielded information on the bulk aggregation state of the samples. Sample preparation included dispersal of the MWNTs in 0.1 µg/mL EtOH, sonication for 10 minutes, and deposition onto a silicon wafer.

Metabolic Activity of Bacterial Cells. We verified the cell viability obtained in our membrane damage assays using a parallel test for cell metabolic activity. A redox-based metabolic activity marker, 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) (Sigma-Aldrich), enabled direct epifluorescent microscopic enumeration of viable *E. coli* cells. Cells were deposited on a MWNT-coated membrane and a bare membrane (as described above) and incubated for 30 minutes in a saline solution at 37°C. Membrane coupons were then transferred to a growth medium (M63 minimal medium with 1 mg/mL glucose) and incubated for 30 minutes. CTC (1.5 mL, final concentration 5 mM) was then added onto the membrane coupons, and the coupons were incubated in the dark at 37°C for 1h. Finally, the cells were counter-

stained with DAPI for 5 minutes. This time, the percentage of metabolically active cells was determined by the ratio of cells stained with CTC (red fluorescent formazan) to those stained with DAPI.

Quantification of Plasmid DNA Adsorption to MWNTs. p-DNA was extracted and purified with Qiagen plasmid kits (Midi25, Cat #12143). 90 μg of p-DNA were added to a clean glass bottle containing 20 mL (final volume) of 0.9 % (0.154 M) NaCl solution with 20 $\mu\text{g}/\text{mL}$ of MWNTs (final concentration). Bottles were placed on a shaker (200 rpm) and incubated for 1 hr and 24 hr at 37°C. After incubation, the solution was filtered through a 0.22- μm low protein binding Millex membrane (Millipore) and the concentration of p-DNA in the filtrate was analyzed by ND-1000 spectrophotometer (NanoDrop). During the experiments, the purity of p-DNA was assured by examining the ratio of UV absorption at 260 nm to that at 230 nm (> 1.8). The sorption data from 1 hr and 24 hrs was very similar, but for consistency in experimental design we used the 1 hr adsorption data. The p-DNA solution concentration reported in our study is a corrected value that accounts for the amount of p-DNA adsorbed to MWNTs.

Efflux of Intracellular RNA. For RNA quantification, cells were harvested at exponential growth phase and incubated with 20 $\mu\text{g}/\text{mL}$ of a MWNT sample in 50 mL of diluted LB medium (1:4 v/v) for 2 hours. After the incubation, cell suspensions were immediately centrifuged at 25,000 g for 15 minutes. To slow natural degradation of RNA, we added 50 μL of β -mercaptoethanol per 10 mL of the supernatant. We performed RNA isolation/purification in the supernatant using an RNeasy kit (Qiagen) and measured RNA concentrations with a ND1000 spectrophotometer (NanoDrop). The purity of RNA was evaluated by the ratio of UV absorbance at 260 nm to 280 nm (>2.0). All samples were maintained at 4°C during RNA sampling and isolation.

Statistical Analysis. Statistical analysis was performed for each of the toxicity assessment methods to determine means, variance, and statistical significance. The power procedure at $\alpha = 0.05$ was used to calculate the minimum samples required to satisfy the t-test for mean difference. Box plots were used to determine the quartiles. Two sample t-tests were performed to measure statistical significance between the control group and the MWNT samples. Statistical significance was set to $p < 0.05$, and any data with p -values lower than 0.05 were clearly indicated.

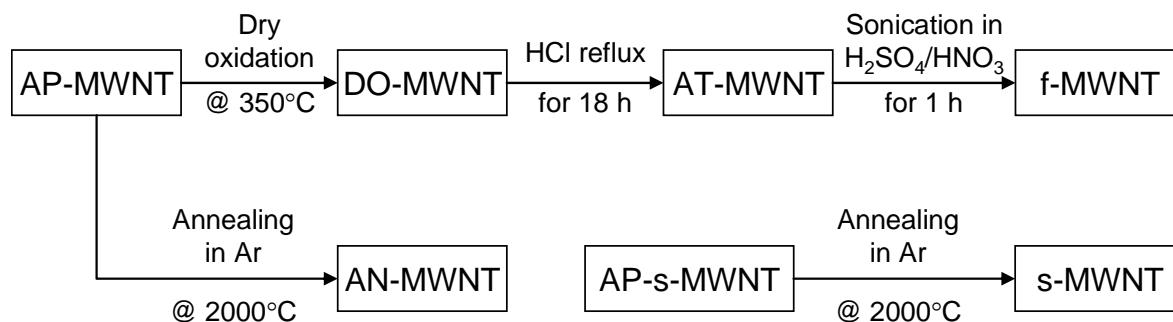


FIGURE S1. Preparation protocol for MWNT samples. The commercial as-prepared MWNT (AP-MWNT) was dried in air at 350°C for 6 h (DO-MWNT) and then refluxed in 10 M HCl solution for 18 h (AT-MWNT). The functionalization of AT-MWNT was performed by sonication in a mixture of H₂SO₄ and HNO₃ (1:3 v/v) for 1 h at room temperature (22°C). In parallel, AP-MWNT was placed at 2000°C for 12 h in Argon to anneal the structural imperfections (AN-MWNT). The short MWNT (s-MWNT) was prepared by annealing of the as-prepared short MWNT.

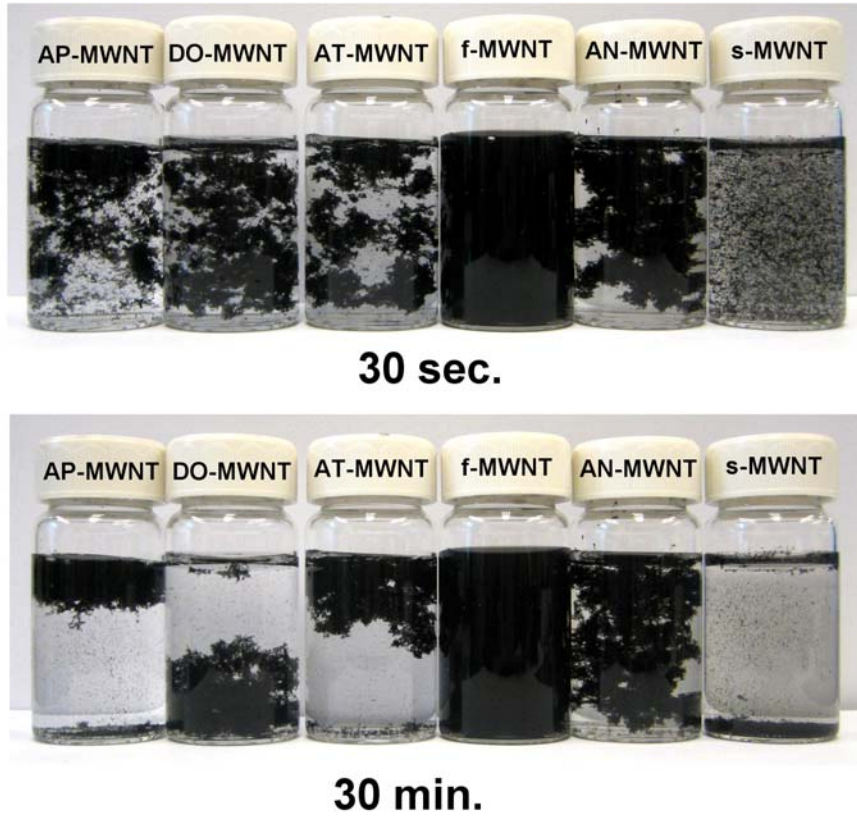


FIGURE S2. Qualitative assessment of the dispersion and settling characteristics of the various MWNT samples in 0.9 % (0.154 M) NaCl solution. All MWNT samples (20 $\mu\text{g}/\text{mL}$) were sonicated in a sonication bath for 15 minutes and then placed on the bench.

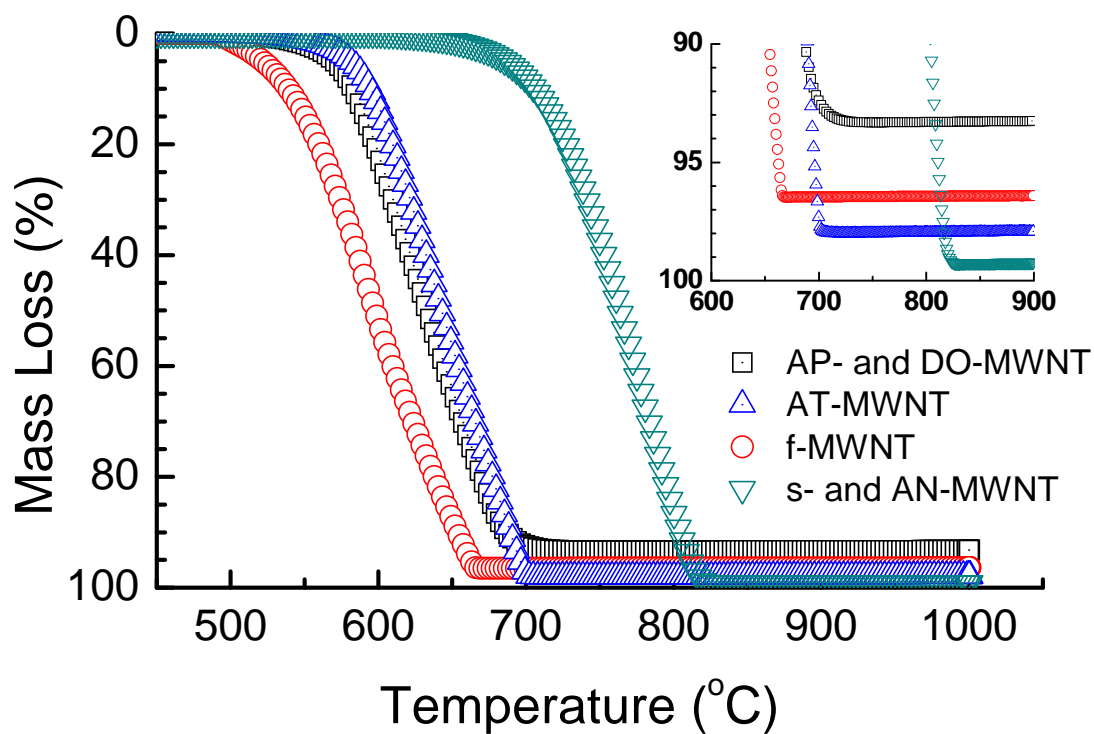


FIGURE S3. Mass loss curves from thermo-gravimetric analysis (TGA) of MWNT samples. During the experiments, the initial mass of each MWNT sample was approx. 20 mg, and temperature was increased from 200°C to 1000°C at a rate of 10°C/min. The TGA profile of DO-MWNT was very similar to that of AP-MWNT, while the AN-MWNT profile matched closely to s-MWNT curve (data not shown).

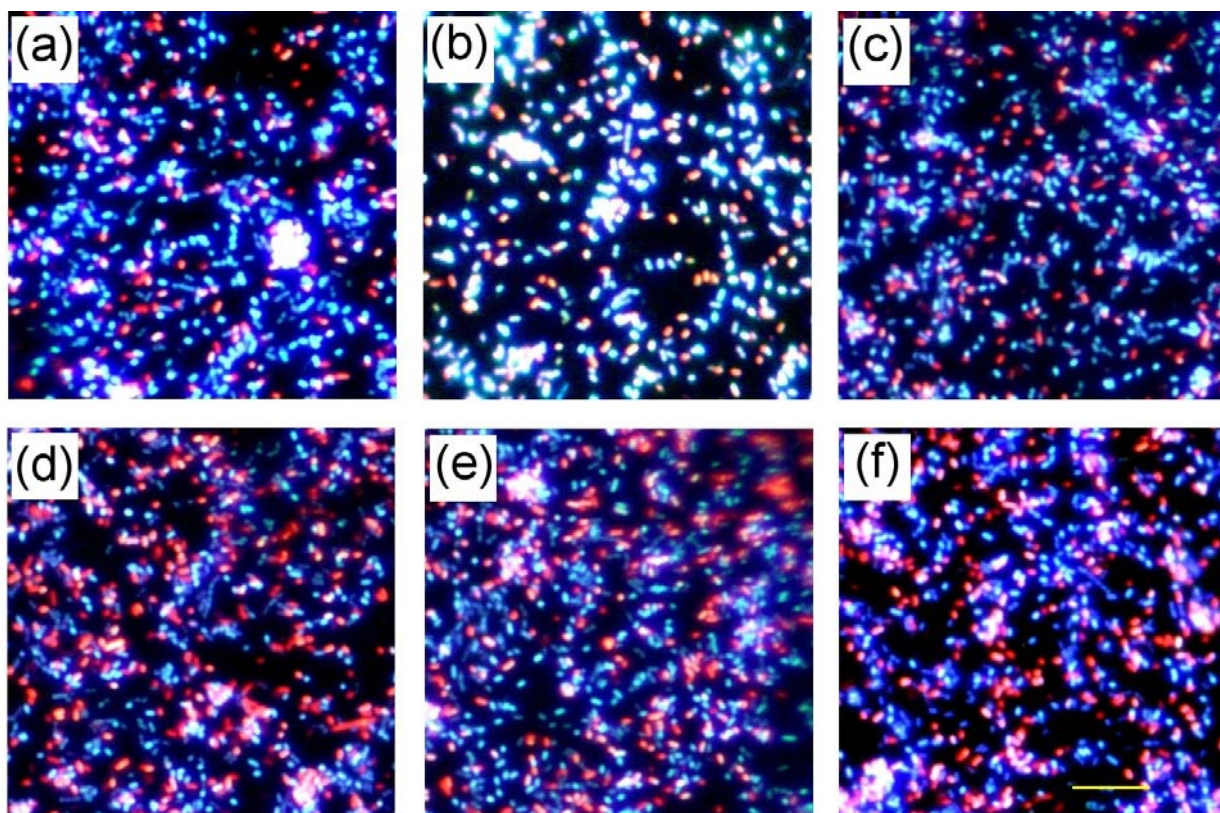


FIGURE S4. Representative images of cells stained with DAPI (blue) and PI (red) on filters coated with (a) AP-MWNT, (b) DO-MWNT, (c) AT-MWNT, (d) f-MWNT, (e) AN-MWNT, and (f) s-MWNT. The bar represents 20 μm .

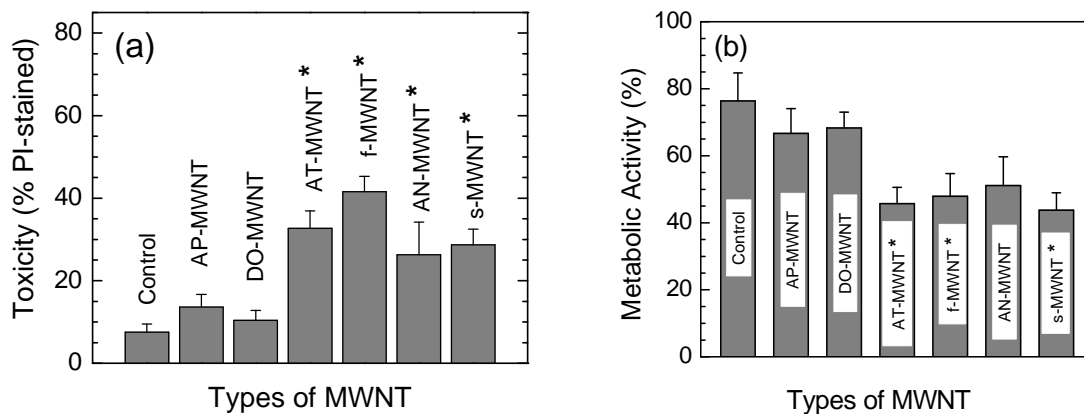


FIGURE S5. Summary of fluorescence-based toxicity assays following *E. coli* cell contact with MWNT-coated filters. (a) Live/Dead test and (b) metabolic activity test. For Live/Dead and metabolic activity tests, samples were prepared by filtration of cell suspensions through MWNT-coated filter and incubation at 37°C in 0.9 % (0.154 M) NaCl solution for 30 min. For control, cells were filtered through 0.45 μm PVDF membrane (without MWNTs), then incubated as described. The asterisks (*) indicate statistical significance of MWNT sample at a 95% confidence level ($p\text{-value} < 0.05$).

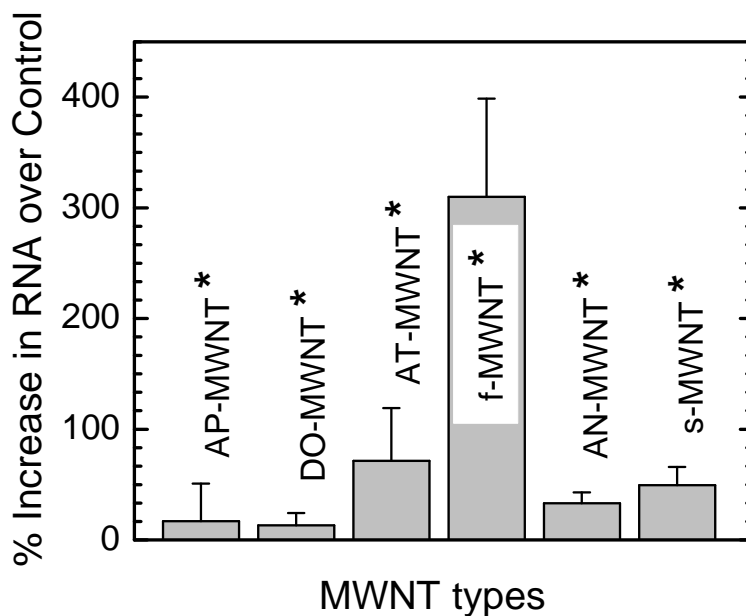


FIGURE S6. Percent increase in concentration of RNA in the supernatant of cells incubated with MWNT samples compared to that of the control (without MWNTs). RNA concentration of the control was 21.4 ± 2.6 ng/mL. For RNA quantification, cells were harvested at exponential growth phase and incubated with $20 \mu\text{g/mL}$ of each MWNT sample in 50 mL of diluted LB medium (1:4 v/v) for 2 hours. After the incubation, cell suspensions were immediately centrifuged at 25,000 g for 15 minutes. To slow natural degradation of RNA, we added $50 \mu\text{L}$ of β -mercaptoethanol per 10 mL of the supernatant. We performed RNA isolation/purification in the supernatant using an RNeasy kit (Qiagen) and measured RNA concentrations with a ND-1000 spectrophotometer (NanoDrop). All samples were maintained at 4°C during RNA sampling and isolation. The asterisks (*) indicate statistical significance of MWNT sample at a 95% confidence level (p -value < 0.05).