Pluronic F108 Coating Decreases the Lung Fibrosis Potential of Multiwall Carbon Nanotubes by Reducing Lysosomal Injury

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Materials and Methods

Carbon nanotubes and chemicals. A powdered MWCNT stock was purchased from Cheap Tubes Inc. (Brattleboro, VT, USA). The starting raw material is also being referred to as as-prepared (AP) MWCNTs. Pluronic F108 was obtained from BASF (BASF Corporation, NJ, USA). Bronchial epithelial growth medium (BEGM) was obtained from Lonza (Mapleton, IL, USA), which is supplemented with a number of growth factors, including bovine pituitary extract (BPE), insulin, hydrocortisone, hEGF, epinephrine, triiodothyronine, transferrin, gentamicin/amphotericin-B and retinoic acid. Roswell Park Memorial Institute medium 1640 (RPMI 1640) was purchased from Invitrogen (Carlsbad, CA, USA). Low-endotoxin bovine serum albumin (BSA) and fetal bovine serum (FBS) were from Gemini Bio-Products (West Sacramento, CA, USA). Dipalmitoylphosphatidylcholine (DPPC), CA-074 methyl ester, z-VYAD-fmk and cytochalasin D were purchased from Sigma-Aldrich (St. Louis, MO, USA). Min-U-Sil was obtained from U.S. Silica (Frederick, MD, USA). All MWCNT stock solutions were prepared using pure deionized water (DI H_2O) with resistivity >18 Ω-cm.

MWCNT dispersion by BSA/DPPC and PF108. The dried tube powders were weighed in the fume hood on an analytical balance and stock solutions prepared in deionized H_2O at a concentration of 5 mg/mL in 4 mL glass vials. Three sets of tube dispersions were prepared for each of the media (RPMI 1640, BEGM and PBS). For BSA plus DPPC dispersal, both dispersants were added to the culture media or PBS at 0.6 mg/mL and 0.01 mg/mL, respectively, before the addition of a 5 mg/mL MWCNTs stock solution. To produce the highly dispersed PF108 tubes, ~33 mg powder was added to 12 mL of a 1% w/v PF108 aqueous solution. The

mixture was sonicated in a 16 mL glass vial by a Fisher Scientific Model 500 Scientific Dismembrator, using a 1/8" horn tip for 1 hour at 20 % amplitude (10-11 W) while being cooled in an ice bath. The resulting solution was centrifuged in a Beckmann Coulter SW41 rotor at 32,000 g for 30 minutes at 22 ℃ to remove large tube bundles and other impurities from the suspension. The upper 90 % of the supernatant was retained as a homogeneously dispersed (PF108-HD) stock solution. The concentration of the supernatant was determined by exploiting that the fact that the optical absorbance is linearly proportional to the MWCNT concentration. The formula used was:

$$Abs_{PostCentrifuged}/Abs_{AsSonicated}=Conc_{PostCentrifuged}/Conc_{AsSonicated},$$

where absorbance was measured at 660 nm (since all of the other variables are directly measured, the $Conc_{PostCentrifuged}$ is directly calculated from this formula).

An appropriate amount of each PF108-HD tube stock solution was added to the desired final concentrations to tissue culture media or PBS. To prepare a cruder and less homogeneous stock (PF108-C), we added 20 μL of each 5 mg/mL MWCNTs stock solution into 1 mL of a 1% PF108 containing tissue culture medium or PBS solution to a final of 100 μg/mL. These diluted suspensions were vortexed for 15 s (Fisher Scientific, Pittsburgh, PA, USA), sonicated for 15 min in a water sonicator bath (Branson, Danbury, CT, USA, 42 kHz frequency), and then vortexed for another 15 s to obtain the suspensions for *in vitro* and *in vivo* use.

Confocal microscopy to assess lysosomal damage and cathepsin B release, using Magic Red™. 1×10^5 primed THP-1 cells were seeded into each of an 8-well chamber and incubated with BSA-dispersed, PF108-C and PF108-HD tubes at 100 μg/mL in RPMI 1640 for 4 h. After fixation in 4 % paraformaldehyde for 1 h in PBS, cells were washed three times with PBS and stained with Magic Red™ (ImmunoChemistry Technologies) at 26 nM for 1 h. Following further washes with PBS, the cell membrane and nucleus were co-stained with 5 μg/mL Alexa Fluor633-conjugated wheat germ agglutinin (WGA) and Hoechst 33342, respectively, in PBS for 30 min. The chamber was visualized under a confocal microscope (Leica Confocal 1P/FCS) in the UCLA/CNSI Advanced Light Microscopy/Spectroscopy Shared Facility. High magnification images were obtained with the 100× objective. Cells without MWCNTs treatment were used as control. Cells treated with monosodium urate (MSU) crystals at 200 μg/mL were used as the positive control.

Assessment of NALP 3 inflammasome activation by small molecule inhibitors and gene knockdown. Differentiated THP-1 cells were seeded into 96-well plates at the density of 2.5×10^4 in 0.2 mL complete RPMI 1640 medium. The cells were-incubated with 10 μM CA-074 methyl ester (cathepsin B inhibitor) or 5 μg/mL z-VYAD-fmk (caspase-1 inhibitor) for 45 min. The medium was subsequently changed to fresh RPMI 1640 into which 100 μg/mL BSA-dispersed, PF108-C or PF108-HD tubes were suspended. Following the addition of one of the chemical inhibitors (CA-074 methyl ester or z-VYAD-fmk), the cells were incubated for a further 5 h and the supernatant collected to measure the IL-1β production by ELISA. For the NALP 3 and ASC deficient assay, $NALP3^{-/-}$ THP-1 cell and $ASC^{-/-}$ THP-1 cell were obtained from Invivogen (San Diego, California, USA). The cells were derived by siRNA knockdown of

NALP3 and ASC genes. The siRNA were delivered by lentiviral vectors. Cells were cultured in complete RPMI 1640 supplemented with 200 μg/mL HygroGold, and 100 μg/mL Normocin before exposure to 100 μg/mL BSA-dispersed, PF108-C and PF108-HD MWCNTs for 5 h. The supernatant was collected to measure the IL-1β production by ELISA.

Characterization of the MWCNT Suspension Stability Index in PBS or phagolysosome simulation fluid (PSF). A kinetic analysis of suspension stability was performed by monitoring the absorbance of the aqueous solution at 550 nm for different lengths of time. Typically, 1 mL of the BSA-dispersed, PF108-C or PF108-HD tube suspensions was prepared at a concentration of 50 μg/mL in either RPMI 1640, BEGM, PBS or PSF. The suspension stability index was determined by comparing the initial MWCNT absorbance at t = 0 to the absorbance at 1, 2, 3, and 24 h. The absorbance measurements were carried out using a SpectroMax M5e (Molecular Devices Corp., Sunnyvale, CA).

Preparation of phagolysosomal simulant fluid (PSF). Phagolysosomal simulant fluid (PSF) (pH 4.5) was prepared with the following chemical composition: 0.02 M potassium hydrogen phthalate, 142.0 mg/L sodium phosphate dibasic anhydrous (Na_2HPO_4), 6650.0 mg/L sodium chloride (NaCl), 71.0 mg/L sodium sulfate anhydrous (Na_2SO_4), 29.0 mg/L calcium chloride dihydrate ($CaCl_2$ $2H_2O$), 450.0 mg/L glycine ($C_2H_5NO_2$) and 4084.6 mg/L potassium hydrogen phthalate (1-(HO_2C)-2-(CO_2K)-C_6H_4).[1] Analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) to prepare the PSF.

Cellular transmission electron microscopy. THP-1 cells were exposed to 50 μg/mL BSA-dispersed, PF108-C or PF108-HD tubes suspended in RPMI 1640 for 24 hr. Harvested cells were fixed with 2 % glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) and washed. After post-fixation in 1 % OsO_4 in PBS for 1 hr, the cells were dehydrated in a graded ethanol series, treated with propylene oxide, and embedded in Epon. 50–70 nm thick sections were cut on a Reichert-Jung Ultracut E ultramicrotome and picked up on Formvar-coated copper grids. The sections were stained with uranyl acetate and Reynolds lead citrate and examined on a JEOL 100 CX transmission electron microscope at 80 kV in the UCLA BRI Electron Microscopy Core.

Mouse exposure and toxicological assessment. Eight-week-old male C57Bl/6 mice were purchased from Charles River Laboratories (Hollister, CA). All animals were housed under standard laboratory conditions according to the institutional as well as the NIH guidelines for care and treatment of laboratory animals. These experiments are approved by the Chancellor's Animal Research Committee at UCLA and include standard operating procedures for animal housing (filter-topped cages; room temperature at 23 ± 2 ℃; 60% relative humidity; 12 h light, 12 h dark cycle) and hygiene status (autoclaved food and acidified water). Animal exposures to BSA/DPPC dispersed, PF108-HD and PF108-C tubes were carried out by oropharyngeal aspiration as previously described.[2] There were 6 animals in each group. Briefly, the animals were anesthetized by intraperitoneal injection of 100 μL ketamine (100 mg/kg)/xylazine (10 mg/kg). With the anesthetized animals held in a vertical position, 50 μL PBS suspension containing 2 mg/Kg of each of the tube types was instilled at the back of the tongue to allow pulmonary aspiration. Control animals received the same volume of PBS with BSA (0.6 mg/mL) and DPPC (0.01 mg/mL) or 1% PF108. The positive control was comprised of animals receiving 5 mg/kg crystalline silica (Min-U-Sil). The mice were sacrificed 40 h or 21 days later and

bronchoalveolar BAL fluid and lung tissue collected. The fluid was used to measure TGF-β1, IL-1β and PDGF-AA levels. Lung tissue was stained with Masson's trichrome.

Sircol assay for total collagen production. The right cranial lobe of each lung in the 21 day experiment was suspended in PBS at 50 mg tissue per ml and homogenized for 60 seconds with a tissue homogenizer (Fisher Scientific). 1 % Triton X-100 was added to the samples and incubated for 18 h at room temperature. Acetic acid was added to each sample to a final concentration of 0.5 M and incubated at room temperature for 90 minutes. Cellular debris was pelleted by centrifugation and the supernatant analyzed for total protein using a BCA Assay kit (Pierce/ThermoFisher Scientific) according to manufacturer's instructions. The Sircol Soluble Collagen Assay kit (Biocolor Ltd., Carrickfergus, UK) was used to extract collagen from duplicate samples using 200 μL of supernatant and 800 μL Sircol Dye Reagent according to the manufacturer's instructions. Similar prepared collagen standards (10–50 μg) were run in parallel. Collagen pellets were washed twice with denatured alcohol and dried before suspension in alkali reagent. Absorbance at 540 nm was read on a plate reader (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA). Data were expressed as μg of soluble collagen per mg of total protein.

ELISA to quantify TGF-β1, PDGF-AA and IL-1β levels. The TGF-β1 concentration in the BEAS-2B culture medium and the BALF was determined through the $E_{max}^{®}$ ImmunoAssay System (Promega, Madison, WI, USA) according to the manufacturer's instruction. A 96-well plate was coated with monoclonal anti-TGF-β1 and the captured growth factor detected by

polyclonal anti-TGF-β1 conjugated to horseradish peroxidase. Absorbance was measured at 450 nm using a plate reader (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA, USA). PDGF-AA activity in the BALF was assessed by the Quantikine ELISA kit from R&D Systems (Minneapolis, MN) following the manufacturer's instructions. The standard growth factor dilution series or 50 μL of fluid were pipetted into the anti-PDGF-AA pre-coated wells for antigen capture. After removal of the unbound growth factor by washing, an enzyme-linked anti-PDGF-AA monoclonal was added. Following washing to remove unbound secondary antibody, a substrate solution was added at 1:250 dilution for 30 minutes for color development. After termination of the color development, colometric intensity was measured at 450 nm in a plate reader (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA, USA). The IL-1β activity in the THP-1 culture supernatant and the BALF was determined by an OptEIA™ (BD Biosciences, CA) ELISA kit according to the manufacturer's instructions. Briefly, a 96-well plate was coated with monoclonal anti-IL-1β and the immobilized growth factor detected by polyclonal anti-IL-1β conjugated to horseradish peroxidase. Absorbance was measured at 450 nm using a plate reader (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA, USA). TGF-β1, PDGF-AA and IL-1β concentrations were expressed as pg/mL.

Confocal Raman microscopy. Raman analysis was performed using backscattering geometry in a confocal configuration at room temperature in a Renishaw inVia Raman microscope system equipped with a 514.5 nm Ar laser. Laser power and beam size were approximately 2.5 mW and 1 μm, respectively, while the integration time was adjusted to 15 s. For cell sample preparation, THP-1 cells were cultured on sterile glass cover slips overnight and then exposed to BSA-dispersed, PF108-C and PF108-HD MWCNTs for 24 h. Cells were washed three times in PBS

and fixed with 4% paraformaldehyde in PBS for 30 min. The cells were scanned under the Raman microscope following three further washes in PBS.

Determination of comparative surface area for interaction of FITC with the surfaces of MWCNT in the presence or absence of BSA and PF108. Fluorescein isothiocyanate (FITC) was added to PBS at 50 μg/mL, followed by the addition of or PF108-dispersed tubes at 10 μg/mL. After bath sonication for 15 min, the relative fluorescence units (RFU) of FITC in all these solutions were measured on a fluorescence spectrometer (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA, USA) at Ex 485 nm and Em 520 nm. The percentage of available surface on MWCNTs was calculated by the following formula: [3]

$$Available\,Surface(\%) = \frac{RFU_0 - RFU_i}{RFU_0 - RFU_{AP}} X100\%$$

where RFU_0 is the original RFU of FITC in PBS, RFU_i is the RFU of FITC after adding the dispersed tubes, RFU_{AP} is the RFU of FITC after the addition of AP-MWCNTs without dispersants in PBS. The available surface of AP-MWCNTs without dispersion in PBS was considered as 100%. The same procedure was repeated with phagolysosomal simulant fluid (PSF) to obtain the available surface of tubes in PSF.

Statistical Analysis. Mean and standard deviation (SD) were calculated for each parameter. Results were expressed as mean ± SD of multiple determinations. Comparisons of each group

were evaluated by two-side Student's t-test. A statistically significant difference was assumed to exist when p was < 0.05.

Supplementary Figures

Fig. S1

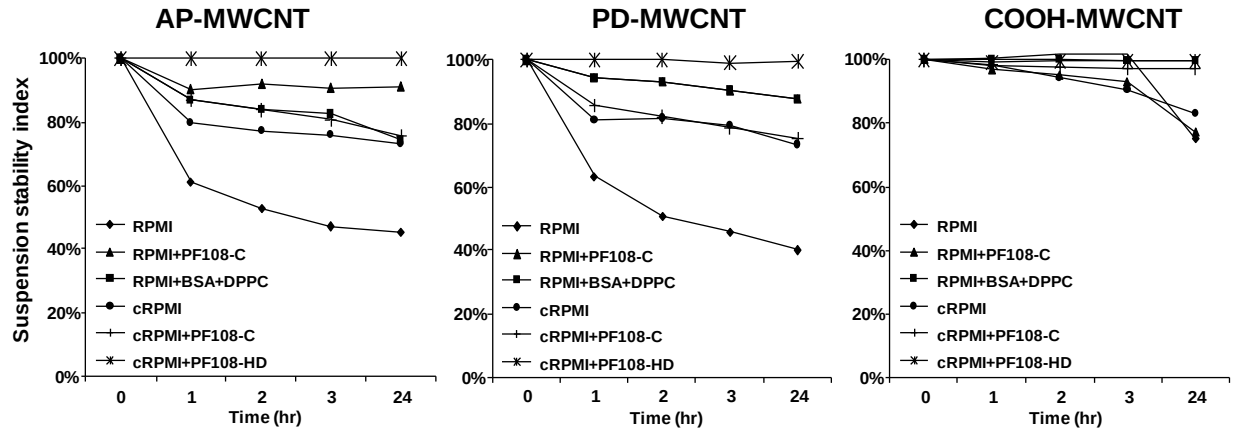


Figure S1. Suspension stability index of differently dispersed tube in RPMI 1640 medium. The afferent tube dispersions were added to RPMI 1640 at 50 μg/mL. The stability index was determined by comparing the initial MWCNT absorbance at t = 0 to the absorbance at 1, 2, 3, and 24 h. The absorbance measurements were carried out at λ = 550 nm in a SpectroMax M5e (Molecular Devices Corp., Sunnyvale, CA). PF-C: PF108-crudely dispersed; PF108-HD: homogeneously dispersed tubes.

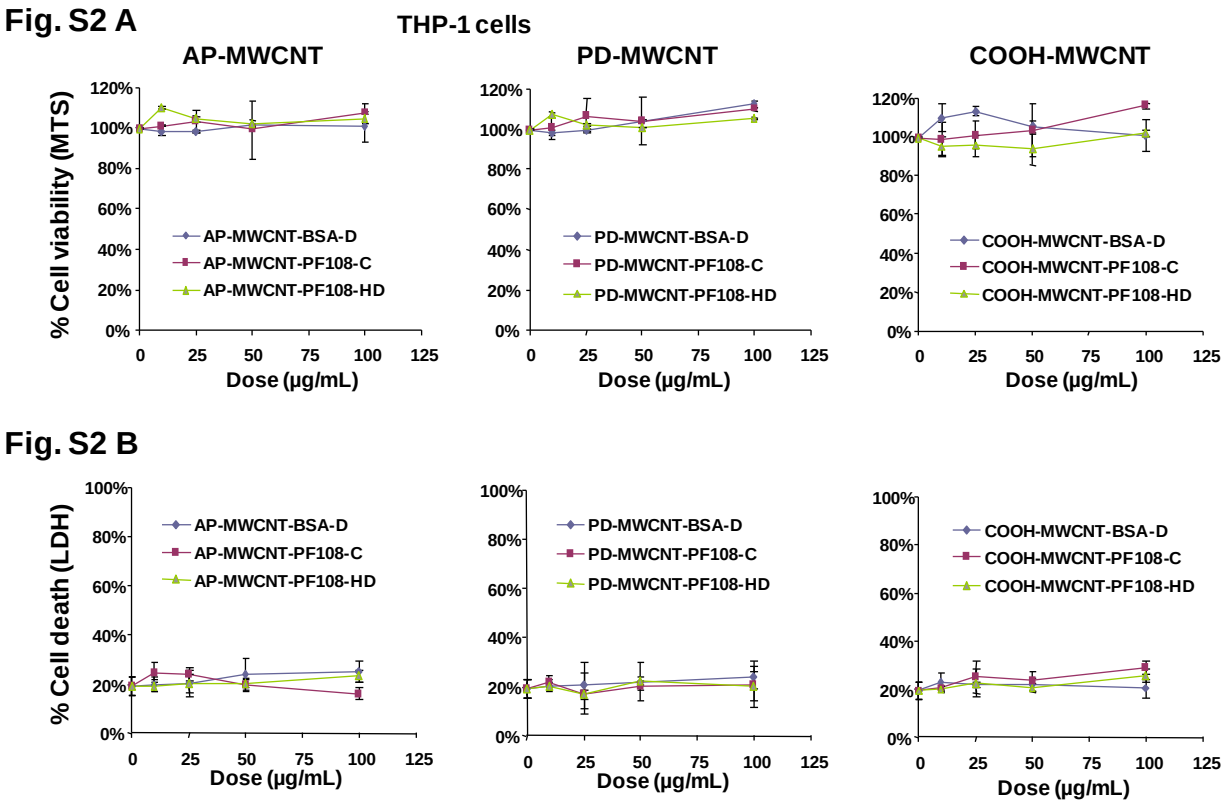


Figure S2. THP-1 cell viability and cytotoxicity assessment by MTS and LDH assays. (A) 2×10^4 cells are plated in 96-well plates and grown overnight. After exposure to BSA-dispersed, PF108-C and PF108-HD tubes at 12.5-100 µg/mL for 24 h, the cells were incubated with the MTS reagent for 1 h. Afterwards, the cells were centrifuged at 2000 g for 10 min, and 80 µL of the supernatant transferred to a new plate. The plate was read at 490 nm in a UV-vis spectrometer (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA, USA). All the MTS values were normalized according to the control (no MWCNT exposure), which represents 100% cell viability. (B) Similar to the experiment in A, cell supernatants from control and exposure experiments were collected and assayed for LDH activity. Data are representative of three separate experiments with at least three wells per treatment. BSA-D: BSA-dispersed; PF108-C: crudely dispersed; PF108-HD: homogeneously dispersed.

Fig. S3A

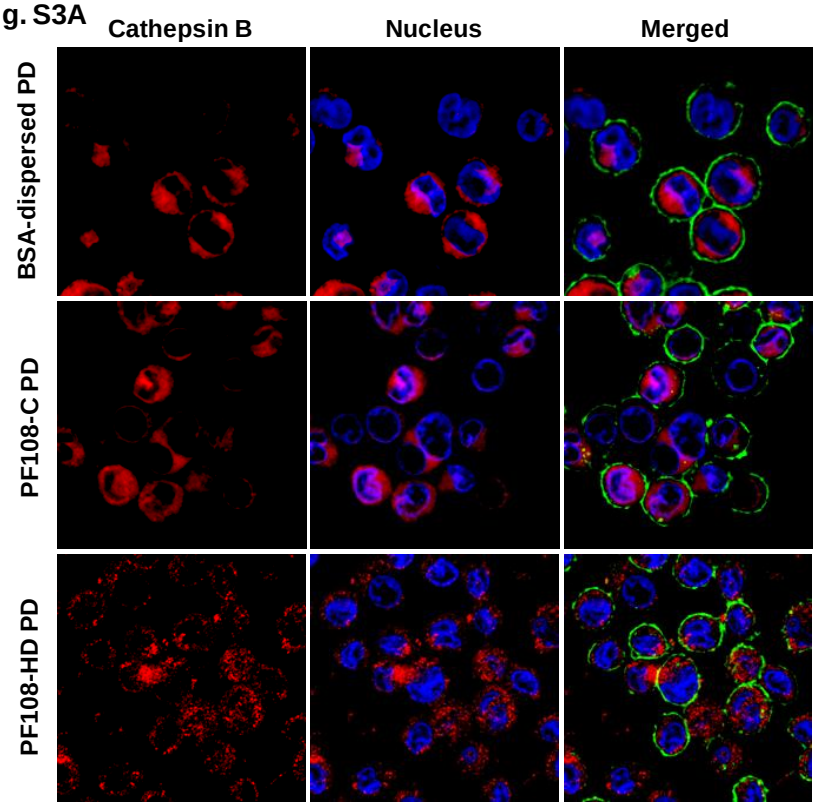


Fig. S3B

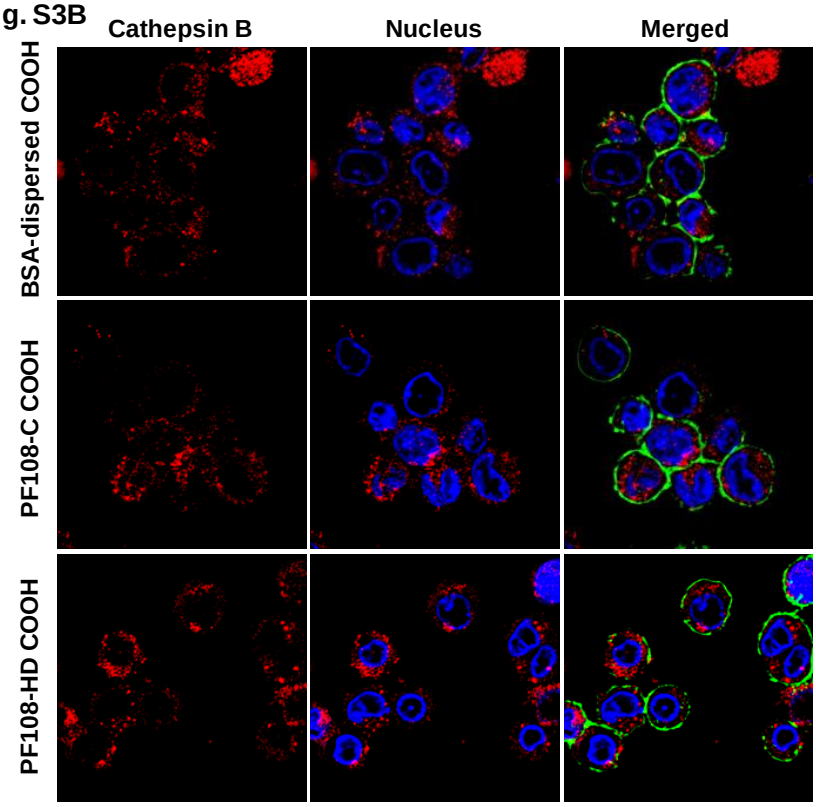


Figure S3. Confocal microscopy to study lysosomal damage and cathepsin B release by dispersed PD and COOH-MWCNTs in THP-1 cells stained with Magic Red™. This is identical to the experiment in Figure 3. THP-1 cells were exposed to BSA-dispersed, PF108-C and PF108-HD PD (A), and COOH-MWCNTs (B) at 100 μg/mL for 4 h in complete RPMI 1640. After fixation and permeabilization, cells were stained with Magic Red™ (ImmunoChemistry Technologies), wheat germ agglutinin 633 and Hoechst 33342 dye, followed by visualization under a confocal 1P/FCS inverted microscope as described in the supporting Material and Method section.

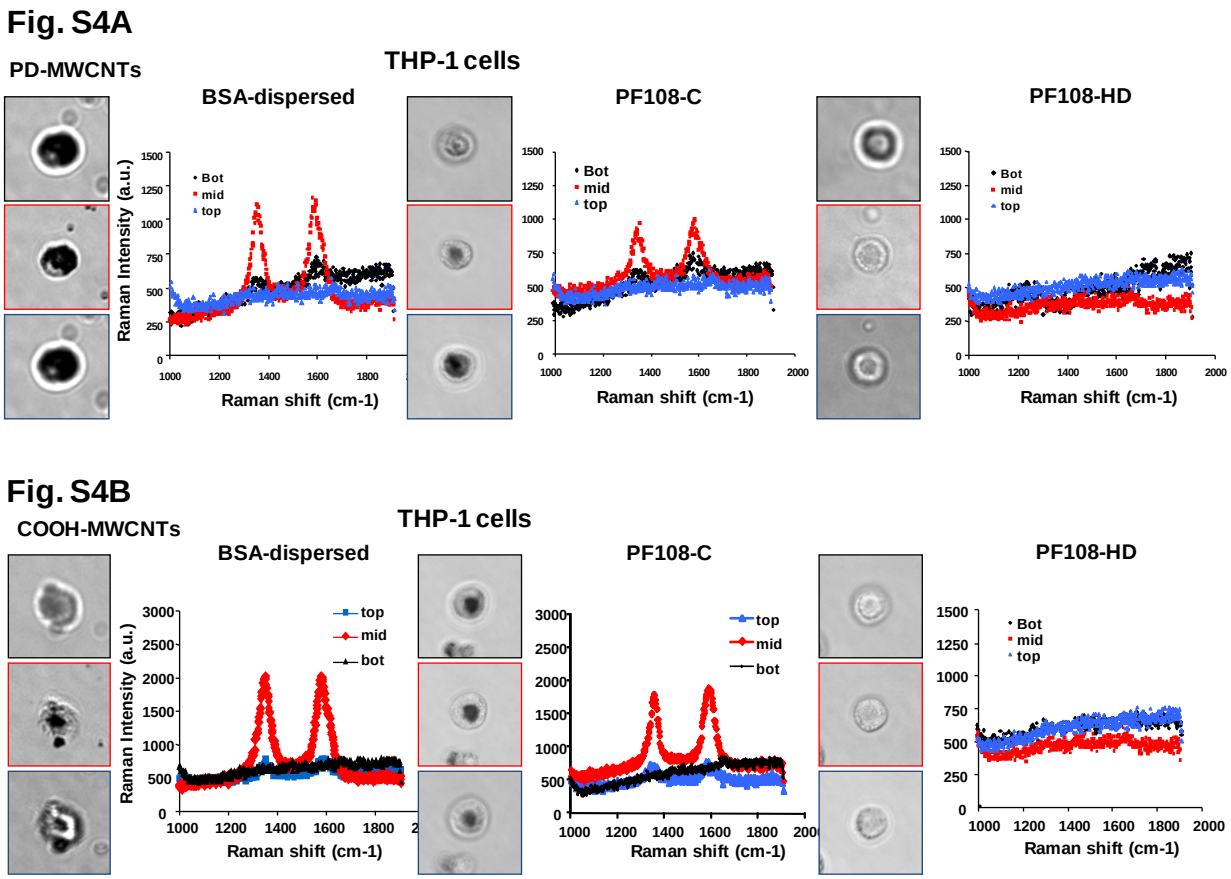
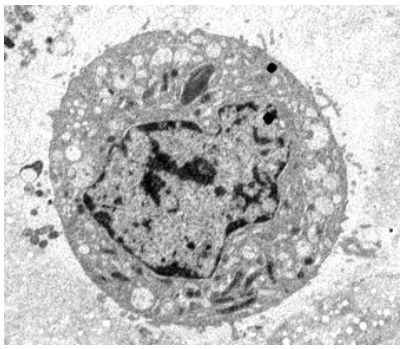


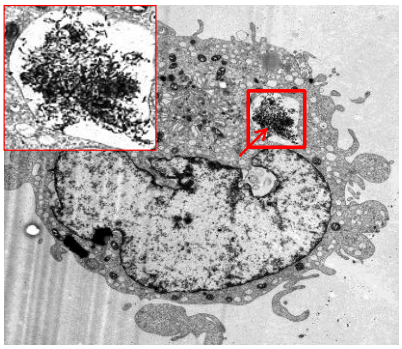
Figure S4. Confocal Raman analysis of THP-1 cells viewed under a light optic microscope to show differential cellular uptake of dispersed (A) PD and (B) COOH-MWCNTs. This experiment was performed identical to the experiment in Figure 4A. Raman microscopy was performed at the top, middle, and bottom cellular planes to compare the signal intensities of the beam focused at these levels.

Fig. S5A

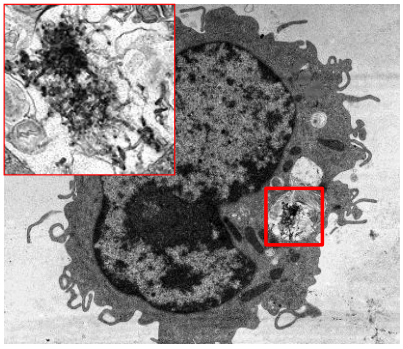
Control THP-1 cell



BSA-dispersed PD-MWCNTs



PF108-C PD-MWCNTs



PF108-HD PD-MWCNTs

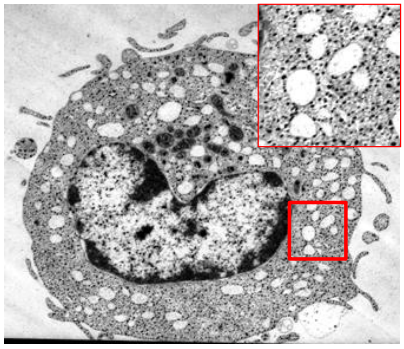
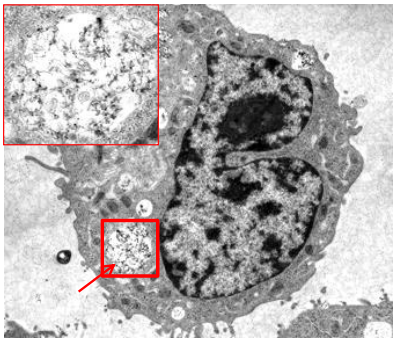
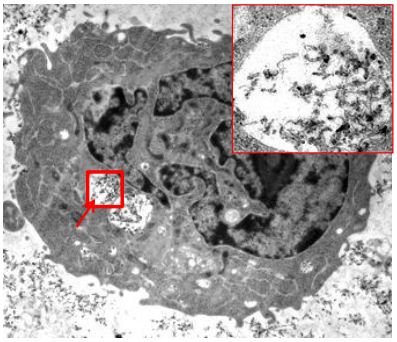


Fig. S5B

BSA-dispersed COOH-MWCNTs



PF108-C COOH-MWCNTs



PF108-HD COOH-MWCNTs

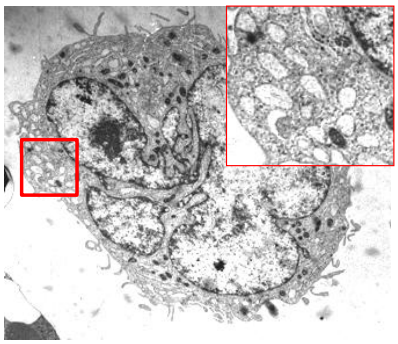


Figure S5. Representative TEM images to show the subcellular localization of dispersed tubes in THP-1 cells treated with (A) PD or (B) COOH-MWCNTs. This experiment is identical to the experiment in Figure 4B. The arrows point to tubes localized in membrane-lined vesicles. While there was good uptake of BSA-dispersed and PF108-C tubes, there was little cellular uptake of PF108-HD PD- and COOH-MWCNTs.

Fig. S6A

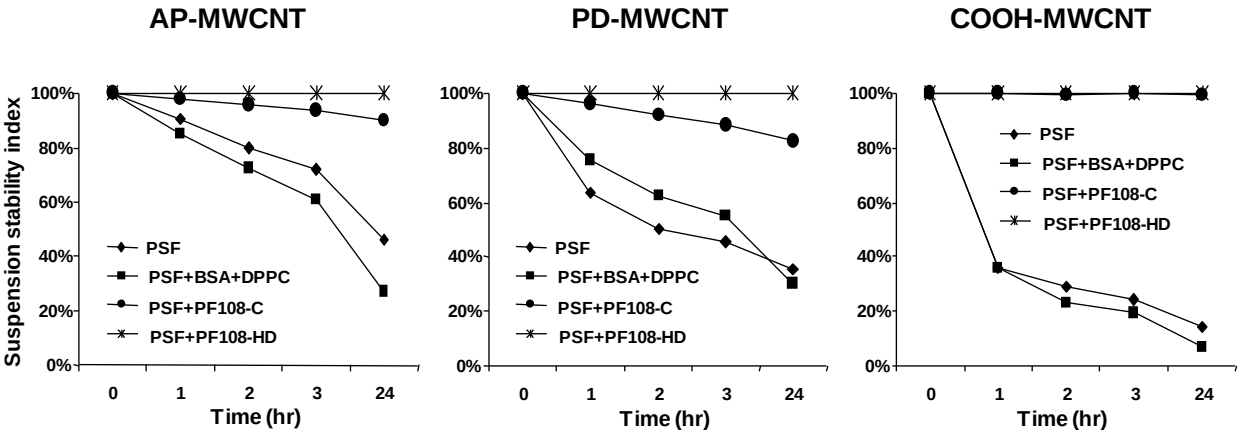


Fig. S6B

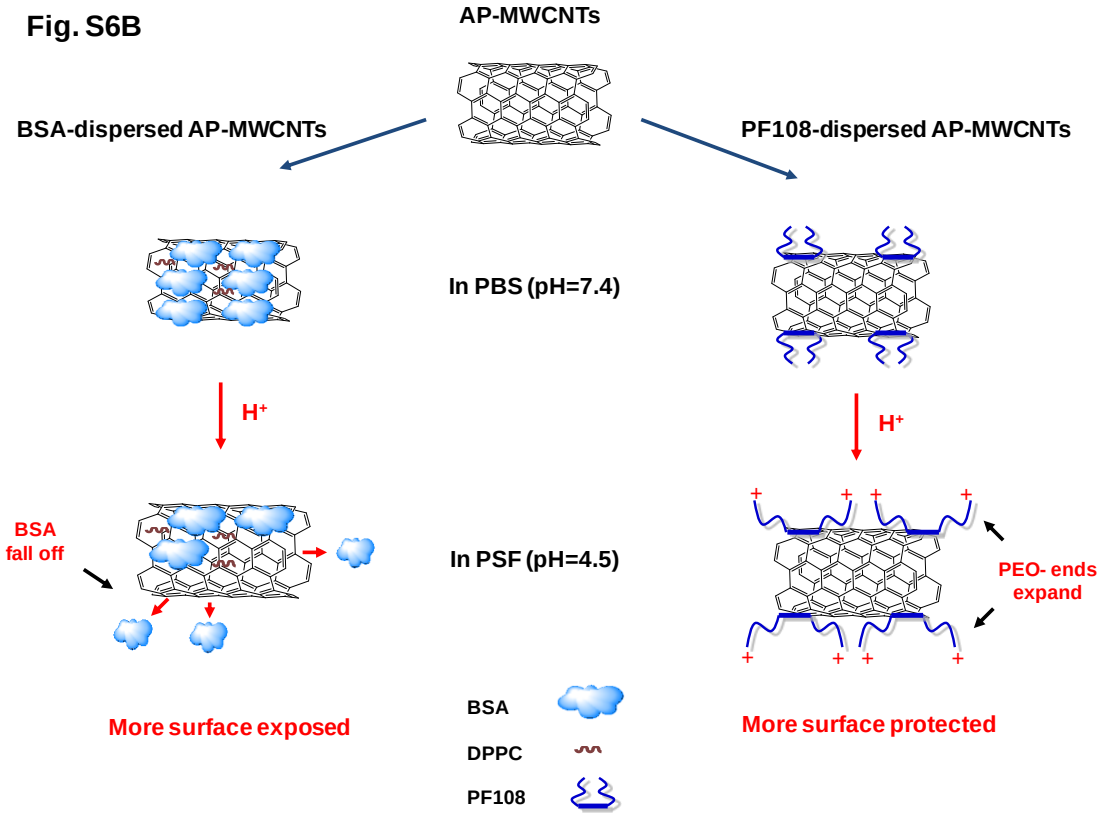


Figure S6. Suspension stability index of the differently dispersed tubes in phagolysosomal simulant fluid (PSF) as well as a schematic to explain the results. (A) The suspension stability index of the dispersed tubes was assessed after addition to PSF at 50 µg/mL. The stability index measurement was conducted as described in Figure S1. (B) Schematic to illustrate the different mechanisms of BSA- and PF108-dispersed tubes in PBS and PSF.

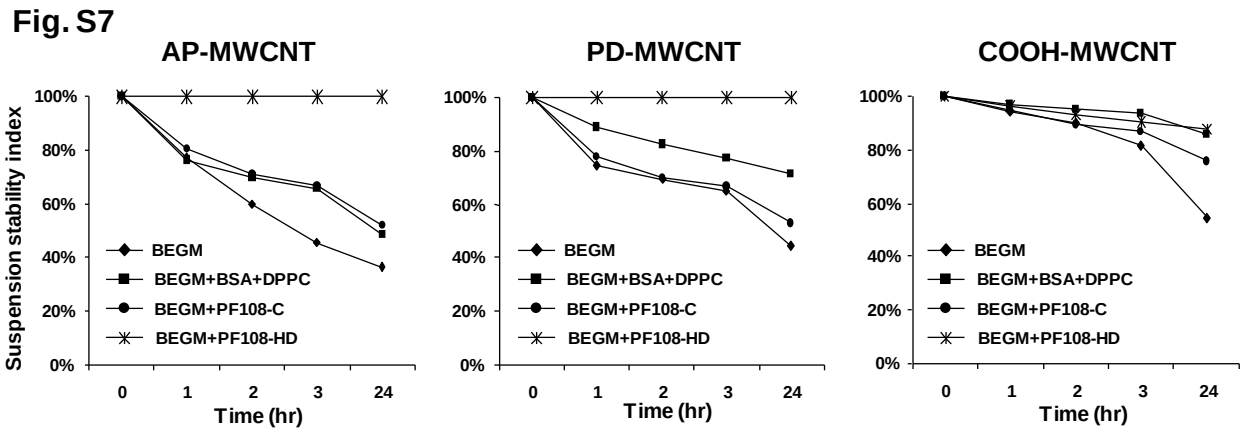


Figure S7. Suspension stability index of the dispersed tubes in BEGM. Assessment of the suspension stability index of the differently dispersed tubes after their addition to BEGM at 50 μg/mL. The stability index measurement was conducted as described in Figure S1.

Fig. S8A

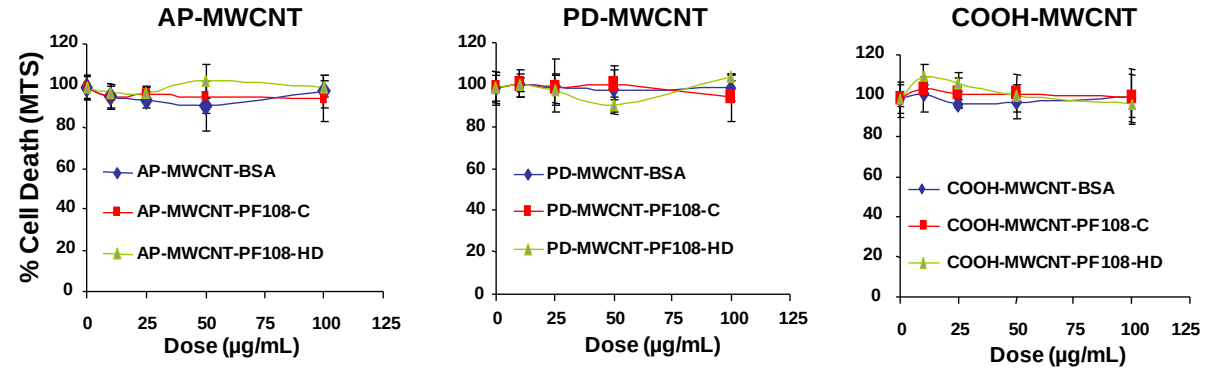


Fig. S8B

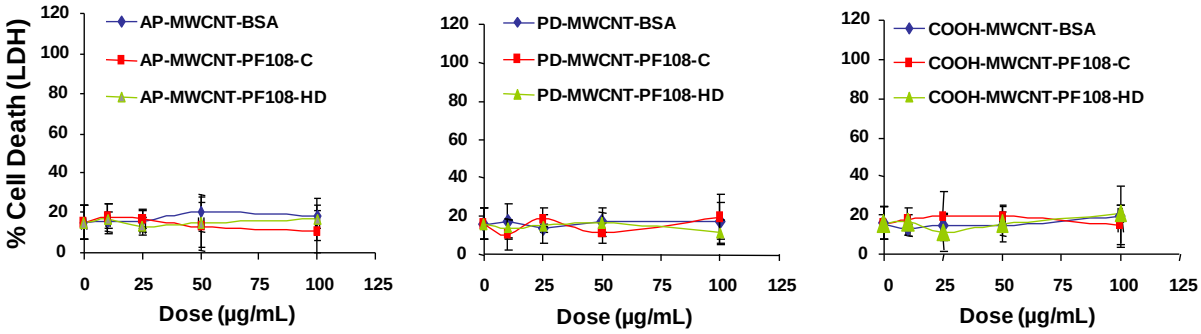


Figure S8. BEAS-2B Cell viability and cytotoxicity assessment by (A) MTS and (B) LDH assays. 1.5×10^4 cells were plated in 96-well plates and grown overnight. This experiment was performed identically to the experiment in Figure S2.

Fig. S9

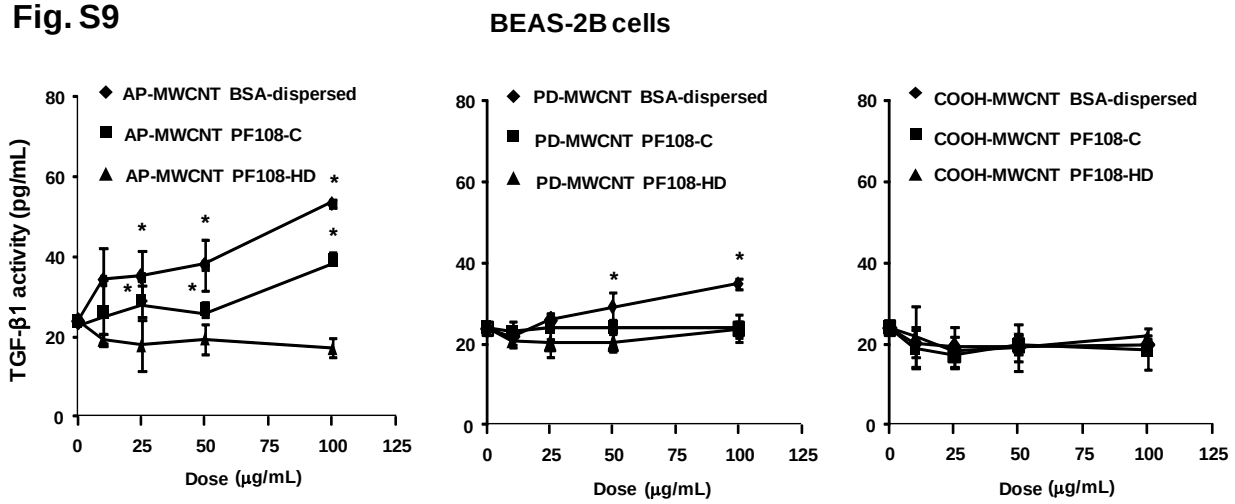


Figure S9. Comparison of the profibrogenic effects of the different dispersal methods for AP, PD and COOH-MWCNTs in BEAS-2B cells. BEAS-2B cells were treated for 24 h with the MWCNTs and the supernatants collected to measure the TGF-β1 production by ELISA. * denotes a *p* value <0.05, comparing control to MWCNT-exposed cells.

Fig. S10

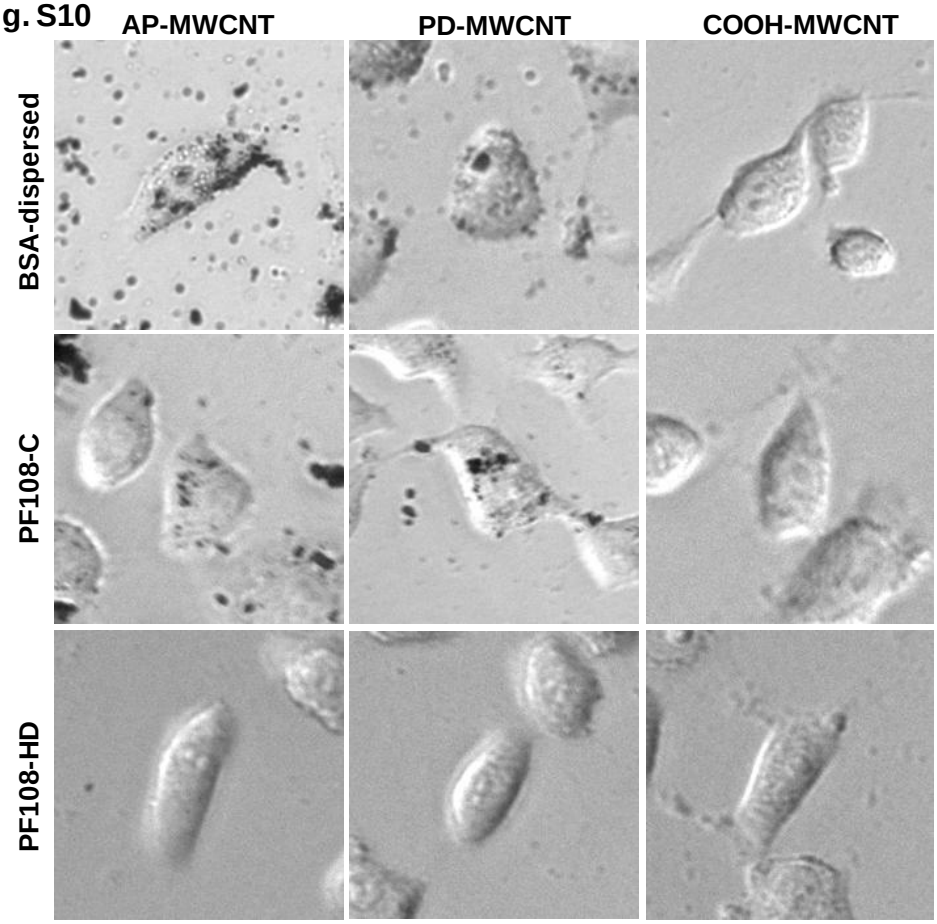


Figure S10. Cellular uptake of the dispersed tubes in BEAS-2B cells. Representative light optical images looking at cellular uptake of BSA-dispersed, PF108-C and PF108-HD tubes. BEAS-2B cells were treated with the AP, PD, and COOH-MWCNTs for 24 h and then observed by a phase contrast microscopy (Carl Zeiss, Inc. Peabody, MA, USA).

Fig. S11

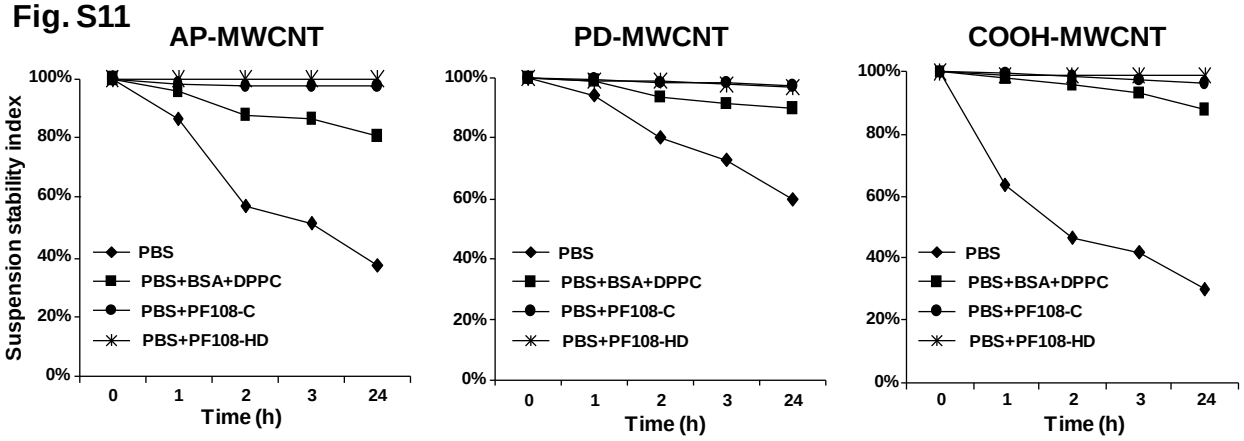


Figure S11. Suspension stability index of the differently dispersed tubes in PBS. The suspension stability index of the different tube dispersants at 50 μg/mL in PBS were determined by UV-vis spectroscopy of the supernatant as previously described by us. [4]

Fig. S12

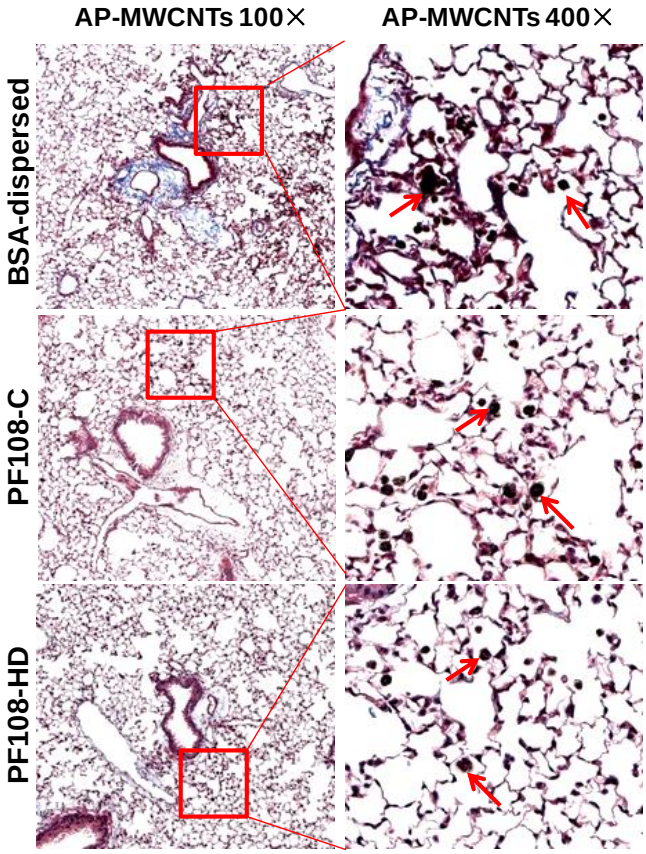


Figure S12. Cellular uptake of the differently dispersed AP-MWCNTs by AM in the mouse lung. The lung sections were obtained from the same experiment as in Figure 8 and stained with Masson's trichrome. Low (100×) and high (400×) magnifications are shown of representative regions in the lungs of AP-MWCNT exposed animals. All the BSA-dispersed, PF108-C and PF108-HD AP-MWCNTs were phagocytosed by AM after 21 days exposure.

Fig. S13

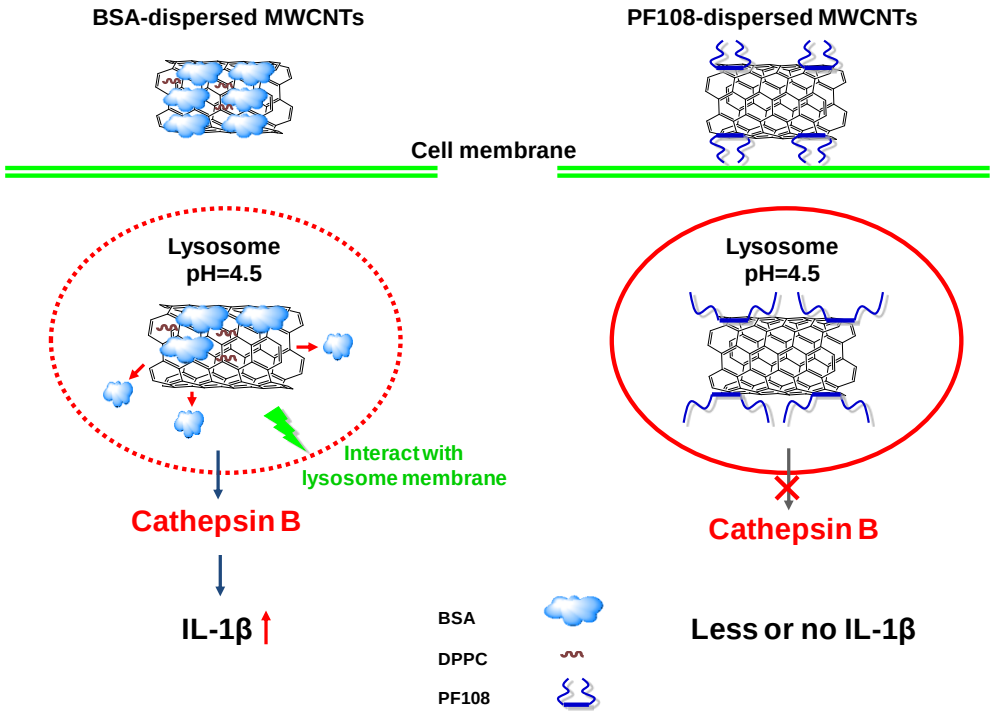


Figure S13. Schematic to explain the differences of BSA/DPPC versus PF dispersal on the behavior of MWCNTs. For BSA coated tubes, the surface coating is unstable in the acidic lysosomal environment, leading to the detachment, loss of suspension stability and interaction of the tubes surface with the lysosomal membrane. It is suggested that this leads to the lysosomal damage and cathepsin B release shown by confocal microscopy and subsequent activation of the NALP3 inflammasome and IL-1β production. In contrast, we propose that PF108 coating leads to passivation of the tube surface by providing protective brush-like layer that leads to steric hindrance. This keeps the tubes in suspension and prevents their interaction with the lysosomal membrane. This results in decreased bioreactivity and prevents the pro-fibrogenic effects.

Supplementary Results and Discussion

Endotoxin analysis of MWCNT. Endotoxin content of all the MWCNT as well as the BSA, DPPC, FBS or Pluronic F108 solution was determined to be < 0.5 U/mL.

Supplementary References

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