Supporting Information:

Translocation of Functionalized Multi-Walled Carbon Nanotubes across Human Pulmonary Alveolar Epithelium: Dominant Role of Epithelial Type 1 Cells

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RESULTS

Evaluation TEER Data Using Electric Cell-Substrate Impedance Sensing (ECIS System)

Fig. S4 shows cell impedance values (a) compared to TEER values (b) of TT1 cells exposed to p(4VP). This result indicated that the measurement of TEER using Millicell®-ERS (Millipore, UK) is comparable to ECIS. Not only is the time scale of the changes very similar but so is the magnitude as a percentage of peak resistance values. Thus, we are confident that the more conventional measurement of TEER used in this study is satisfactory.

Raman Spectroscopy

After 7 days exposure Raman spectroscopy was employed to check the D/G ratio of the remaining p(4VP)-MWCNTs inside the cells. Raman spectroscopy provides a means to evaluate graphitic structure *in situ*, by measuring the characteristic I_D/I_G ratio; no change was observed, as might be expected for epithelial cells (Fig. S5i), although degradation of fMWCNTs has been observed in macrophages.[1]

MATERIALS

Multi-walled carbon nanotubes were purchased from Arkema (UK). Defined Cell Culture Medium (DCCM1) was purchased from Cadama (UK); PSG, NCS, rabbit polyconal IgG against EEA1, LAMP1 and ZO-1 were purchased from Invitrogen (UK). Alex Fluor®594 - goat anti rabbit IgG was obtained from Invitrogen, UK. The LAL Chromogenic Endotoxin Quantitation Kit was purchased from Thermo Scientific, UK. Transwell® plates with polyester membrane support, pore size of 0.4µm were purchased from Corning, UK. 4-vinyl-piridine, (4VP), and phosphate buffer saline solution (PBS) were purchased from Sigma Aldrich, UK. Cell proliferation reagent WST-1 and Cytotoxicity Detection kit PLUS were purchased from Roche (UK).

METHODS

p(4VP)-MWCNT Synthesis, Functionalisation and Shortening

The functionalization of MWCNTs (Arkema, UK) was carried out as described by Chen et al.[2] After the filtration-sonication cycle to remove any physically absorbed reactants. p(4VP)-MWCNTs were collected as dry powders by evaporating all the solvent. A suspension of the p(4VP)-MWCNTs at 2mg/ml was prepared using high performance liquid chromatography (HPLC) grade water and was bath sonicated (45 kHz, 80W, VWR International, USA) for 15 minutes. The individualized p(4VP)-MWCNTs were separated from the p(4VP)-MWCNTs aggregates by centrifugation. The p(4VP)-MWCNT aggregates were settled by centrifugation for 15 minutes at 10,000 g. The supernatant containing individualized MWCNTs was carefully decanted and the concentration of MWCNTs determined by UV absorbance and application of the Beer-Lambert law, $A = \varepsilon \times c \times d$, where A is the measured UV absorbance, ε is the extinction coefficient[3] (35.10 $mL \bullet mg^{-1} \bullet cm^{-1}$ for Arkema MWCNTs at 800 nm and d is the light path length (1 cm cuvette length, in this study). After the centrifugation separation process, the concentration of individualized p(4VP)-MWCNTs in HPLC water was determined. Three separate experiments were performed (n=3).

Characterisation of the p(4VP)-MWCNTs

The sample was observed using scanning electron microscopy (SEM). The p(4VP)-MWCNTs water dispersion was drop-cast onto a silicon substrate for characterisation of the length distribution using a LEO1525 Field Emission Gun Scanning Electron Microscope (FEG-SEM, Carl Zeiss Microscopy GmbH, UK). The SEM was operated in secondary electron mode with an accelerating voltage of 5 kV. The p(4VP)-MWCNTs were then shortened by tip-sonication for up to 5 h, using 750 Watt ultrasonic processors (VCX 750, Sonics & Materials, Inc., Newtown, USA), equipped with a 12.7 mm horn and operating at

225 Watt power input. The sonication horn was deeply inserted (to 1 cm height from the bottom) into 10 mL of the individualized p(4VP)-MWCNTs in a 15 mL vial, which was pre-immersed into a 500 mL beaker containing icy water for cooling purposes. Aliquots of 0.1 mL are taken at 0.5, 1, 2, 3 and 5 h for length distribution measurements by SEM in the same conditions mentioned above. Three separate experiments were performed (n=3).

Zeta Potential of the p(4VP)-MWCNTs

The 300 and 700nm p(4VP)-MWCNTs were suspended at 10μg/ml in deionised water and Defined Cell Culture Medium (DCCM1) and their surface charge density was measured using a Zetasizer Nano (Malvern, UK). Experiments were performed in triplicate (n=3) with 10 readouts on each experiment.

Endotoxin Assay

Although the thermal activation process guaranteed the sterility of the activated p(4VP)-MWCNTs,[2] after synthesis the p(4VP)-MWCNT were handled in a laminar flow cabinet to maintain sterility. An endotoxin assay was performed using LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, UK). All the glassware used for the MWCNT functionalization, washing steps and LAL assay were used after oven sterilization at 120°C for at least 2 h. Three separate experiments were performed (n=3).

Raman Spectroscopy

The p(4VP)-MWCNTs were recovered from the cell lysate by filtering through Vivacon® 500 centrifugal concentrators (2000 MWCO membrane) followed by a brief sonication of the membrane in 500 μl Millipore deionized water (18.2 Ω.cm at 25°C). The MWCNTs were then washed twice through the concentrators and dropped cast onto glass slides. Control p(4VP)-MWCNT samples were treated in an identical manner. Once the samples were dried,

Raman spectra (1000 - 1800 cm^{-1}) were collected on a LabRam Infinity Raman spectrometer, using a 532 nm laser (scan time 15 seconds, averagely 2 scan cycles). The peaks that are most pertinent for observation of sp^3 bonding are the D and G peaks which are located at approximately 1350 and 1580 cm^{-1} respectively. The relative intensities of these peaks provide a direct indication of the level of disorder present within the crystalline structure of the MWCNT system. The D/G ratio was determined from the ratio of integrated areas under the Raman bands at around 1350 cm^{-1} (D-band) and 1580 cm^{-1} (G-band) respectively. Average values and standard deviations were obtained from fifteen independent measurements (n=15).

Evaluation TEER Data Using Electric Cell-Substrate Impedance Sensing (ECIS System)

TT1 cells were seeded at 30,000 cell per well on the E-Plate L8 (ACEA Biosciences, USA) and they were left to reach confluence for 72h in the incubator at 37^{o}C with 5% CO_2 before the MWCNT exposure with real-time monitoring of cell impedance using iCELLigence system (ACEA Biosciences, USA). The cell free wells with the same volume of medium were used as a baseline control. After 72h the cells were continuously exposed to p(4VP) for 24h under the same conditions. Three separate experiments were performed and the data were presented as mean±SD (standard deviation). These data were compared with the result obtained from TEER measurement using Millicell®-ERS (Millipore, UK) as mention above to evaluate the TEER measurement.

Supporting Figures

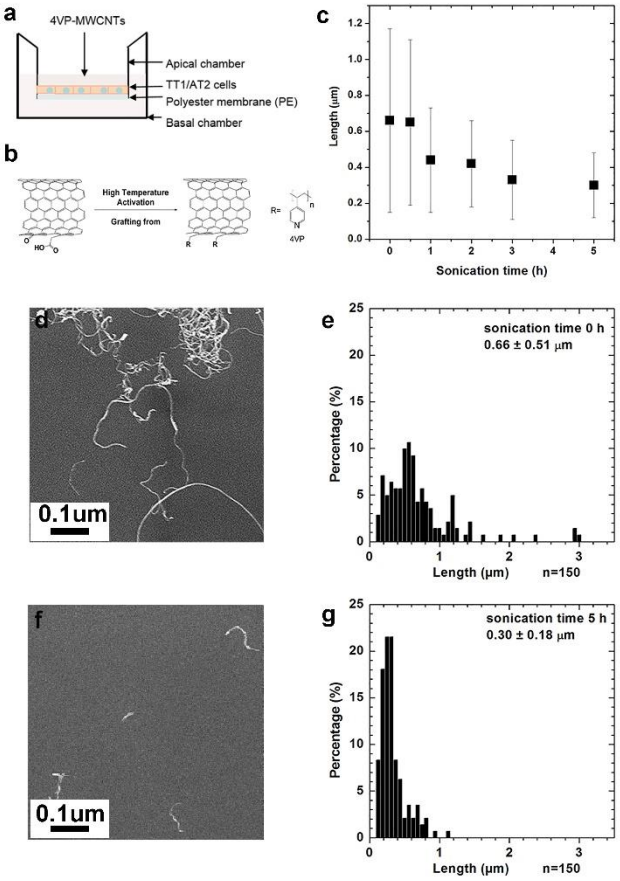

Figure S1. Model study, functionalization and shortening process of p(4VP)-MWCNTs. (a) The diagram shows the mono-culture model of TT1 or AT2 cells used in this study. Cells were grown on the support membrane of polyester (PE) with a pore diameter of 0.4 μm. (b) The MWCNTs synthesized by CVD were functionalised with 4-vinyl pyridine (4VP) via thermochemical grafting. The 4VP(R) is hydrophobic in non-polar solvents and becomes cationic in acidic aqueous solution. (c) The mean length of the p(4VP)-MWCNTs as a function of sonication time. (d-e) A representative SEM image of individual p(4VP)-MWCNTs before sonication, and the corresponding length distribution (e). (f-g) A representative SEM image of p(4VP)-MWCNTs after sonication for 5 h and the corresponding length distribution (g).

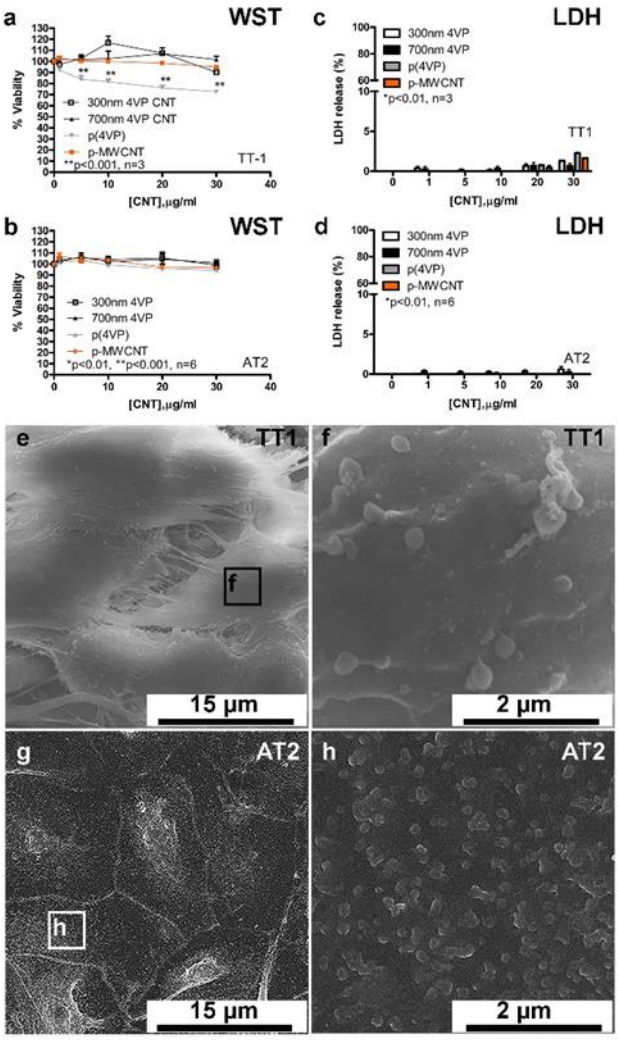

Figure S2. Effect of p(4VP)-MWCNTs on TT1 (n=3) and AT2 cell (n=6) viability. (a-d) The cytotoxic effects of p(4VP)-MWCNTs compared with non-functionalised MWCNT (p-MWCNTs) and p(4VP) were analysed following 24h p(4VP)-MWCNT exposure using WST (a-b) and LDH (c-d) assays, respectively. None of the p-MWCNTs, 300 and 700nm p(4VP)-MWCNTs showed significant toxic effects ($p>0.05$) on the cell viability of TT1 (a) and AT2 (b) cells. A significant toxicity of p(4VP) was observed on TT1 cells, while, no toxic effect was observed on AT2 cells. An insignificant release ($p>0.05$) of LDH was observed on TT1(c) and AT2 (d) exposed to p-MWCNTs, 300 and 700nm p(4VP)-MWCNTs suggesting no effect on cell membrane integrity. (e-h) Non-treated TT1(e-f) and AT2 (g-h) cells (observed using SEM and f, h show the higher magnification images of e and g).

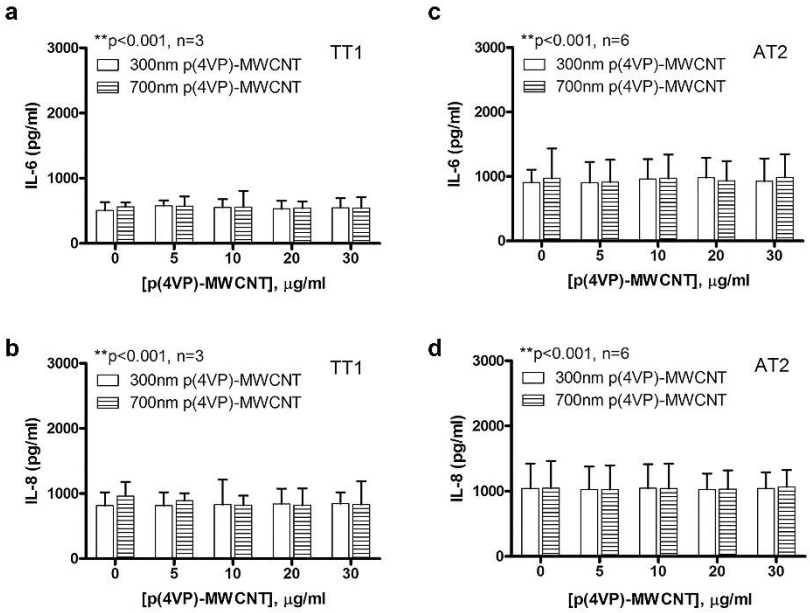

Figure S3. Effects of p(4VP)-MWCNTs on inflammatory mediator release, IL-6 and IL-8, on TT1 (n=3) and AT2 cells (n=6). (a) A non-significant release of IL-6 was observed following 24h exposure of TT1cells to 300 and 700nm p(4VP)-MWCNTs following 24h exposure ($p>0.001$, n=3). (b) No significant release of IL-8 was observed with TT1 cells following 24h MWCNT exposure (n=3). (c-d) similar trends of IL-6 (c) and IL-8 (d) release were observed with AT2 cells following 24h exposure to 300 and 700nm p(4VP)-MWCNTs, respectively.

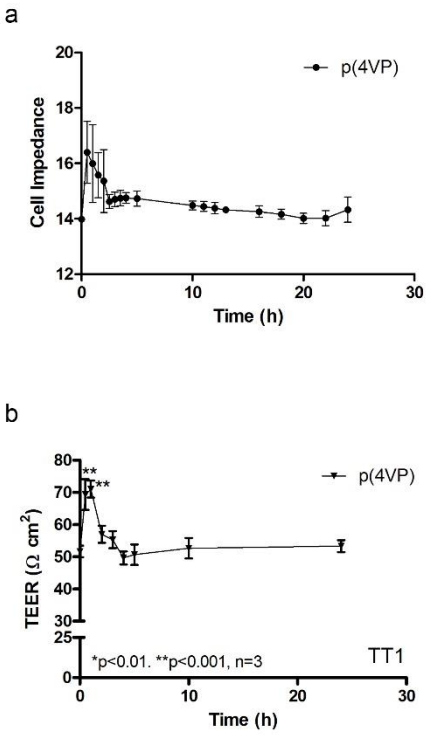

Figure S4. Cell impedance and TEER profiles of TT1 exposed to p(4VP) for 24h. (a) Cell impedance measured using ECIS system (n=3). (b) TEER measured using Millicell®-ERS (n=3). This result indicated that measurement of TEER is comparable to ECIS. Not only was the time scale of the changes much the same but also the magnitude as a percentage of peak resistance values was the same. This confirmed that the conventional measurement of TEER used in this study is satisfactory.

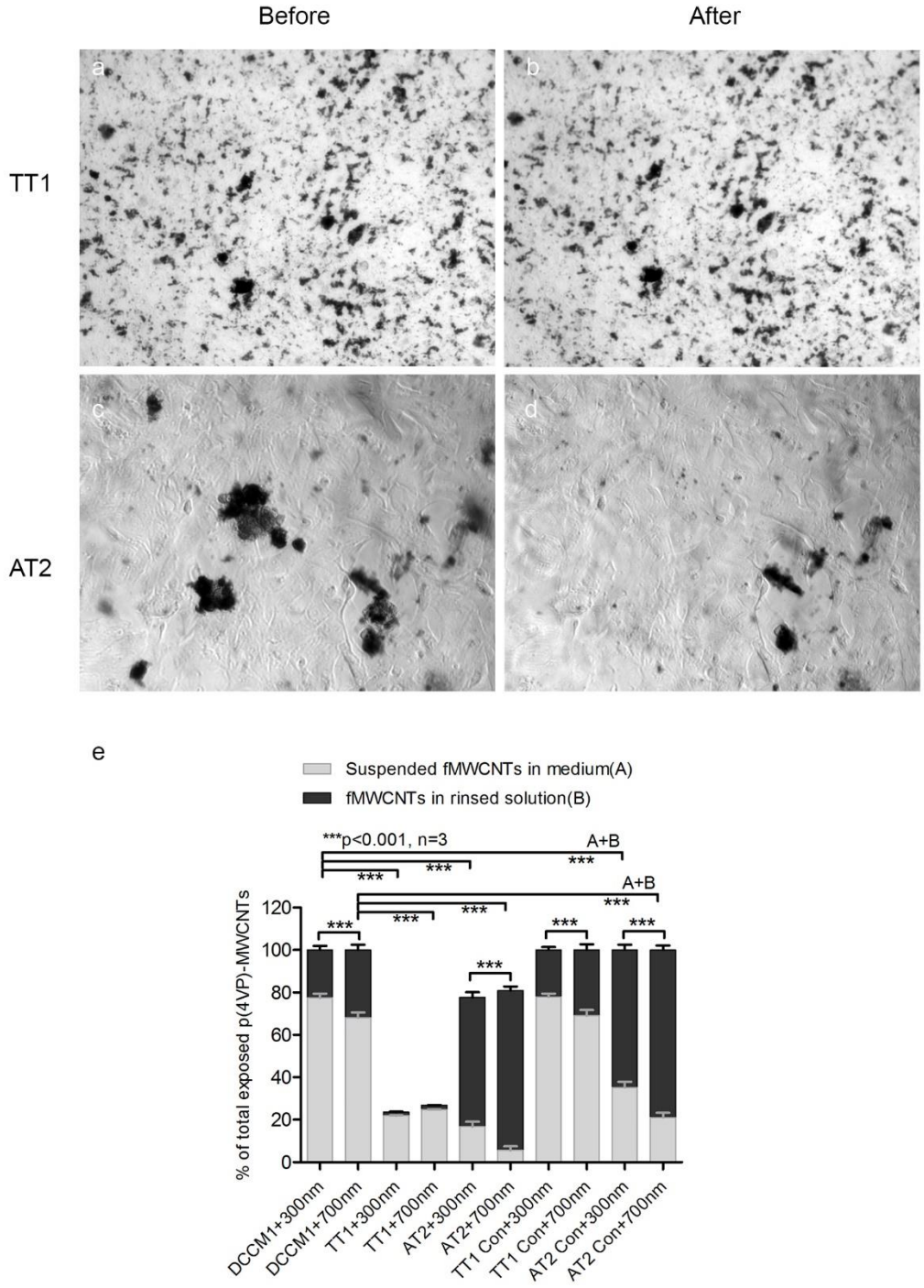

Figure S5. Barrier effect of AT2 secretions and degree of agglomeration/aggregation of 300nm and 700nm long p(4VP)-MWCNTs. (a-b) TT1 cells were exposed to 300nm long p(4VP)-MWCNTs for 24h; the cells were imaged before (a) and after (b) rinsing with PBS. (c-d) AT2 cells were exposed to 300nm long p(4VP)-MWCNTs for 24h; the cells were

imaged before (c) and after (d) rinsing with PBS. Following rinsing, the fMWCNT in the conditioned medium and in the rinsed solutions were measured for fMWCNT concentrations and the percentage of fMWCNTs were cumulatively plotted in comparison to control. (e) Percentage of fMWCNTs observed in the 24h exposed medium (remaining suspended in the conditioned medium; gray) and in the rinsed solution (reflecting cell-associated or in secretions; black). These experiments are denoted as TT1+300nm, TT1+700nm, AT2+300nm, AT2+700nm (cell exposures). Data from the cell free system, where 24 hour conditioned medium from TT1 and AT2 was subsequently incubated with fMWCNTs for 24 hours (ie cell-free system) are shown in columns denoted TT1 Con-300nm, TT1 Con+700nm, AT2 Con+300nm, AT2 Con+700nm. The control study was performed using fresh DCCM1 medium (DCCM1+300nm, DCCM1+700nm). DCCM1 provided a stable suspension with 23-32% fMWCNT agglomerates/aggregates. AT2 cell secretions, but not TT1 cell secretions, significantly ($p<0.001$, $n=3$) increased fMWCNT agglomerates/aggregates to 65% (300nm) and 79% (700nm), respectively.

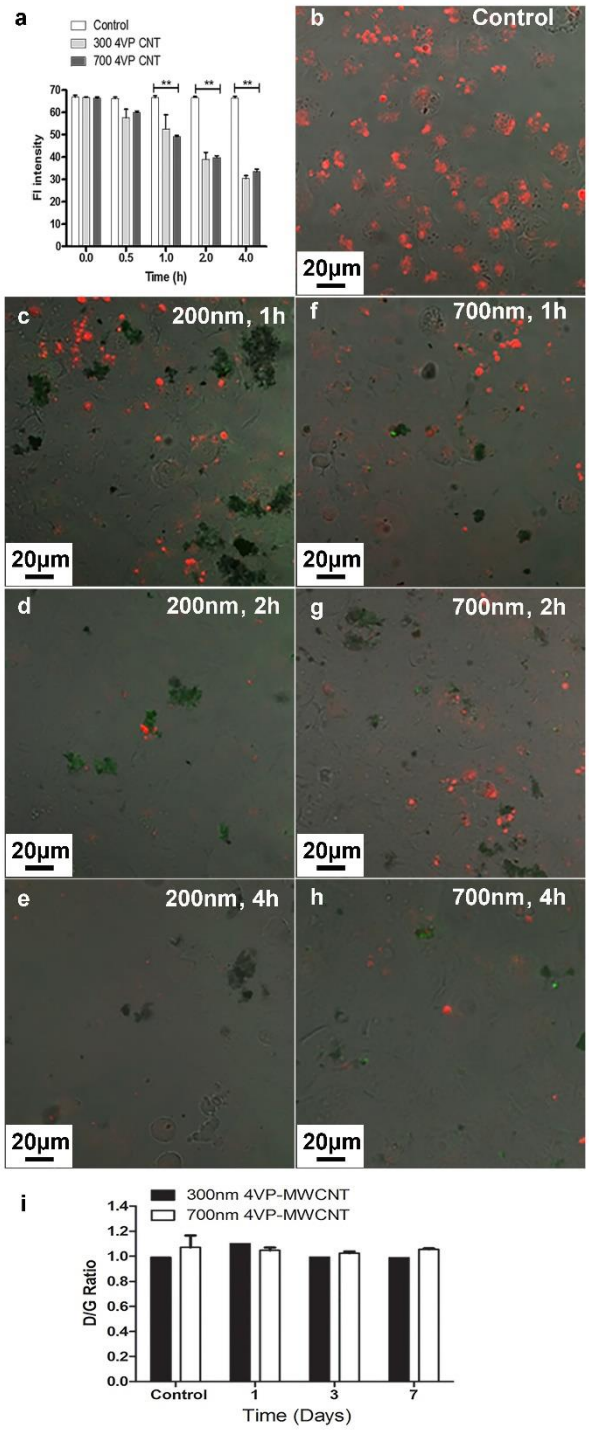

Figure S6. Penetration of the lysosomal membrane of TT1 cells and Raman spectroscopy following exposure to p(4VP)-MWCNTs. (a-h) The effect of p(4VP)-MWCNTs on the integrity of the lysosomal membrane. Acridine orange (red) was used to elucidate this effect and the intensity of the dye was analysed using confocal microscopy. A decrease in the red fluorescent intensity observed in TT1 cells (b-h) at T= 1h (c,f), 2h (d,g) and 4h (e,h) were

measured and illustrated in a. This reduction indicated a decrease in membrane integrity of the lysosomal compartment (**$p<0.001$, $n=3$). (i) The D/G ratio of the p(4VP)-MWCNTs did not change significantly ($p>0.05$) over 7 days exposure.

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