High resolution and dynamic imaging of biopersistence and bioreactivity of extra and intracellular MWNTs exposed to microglial cells

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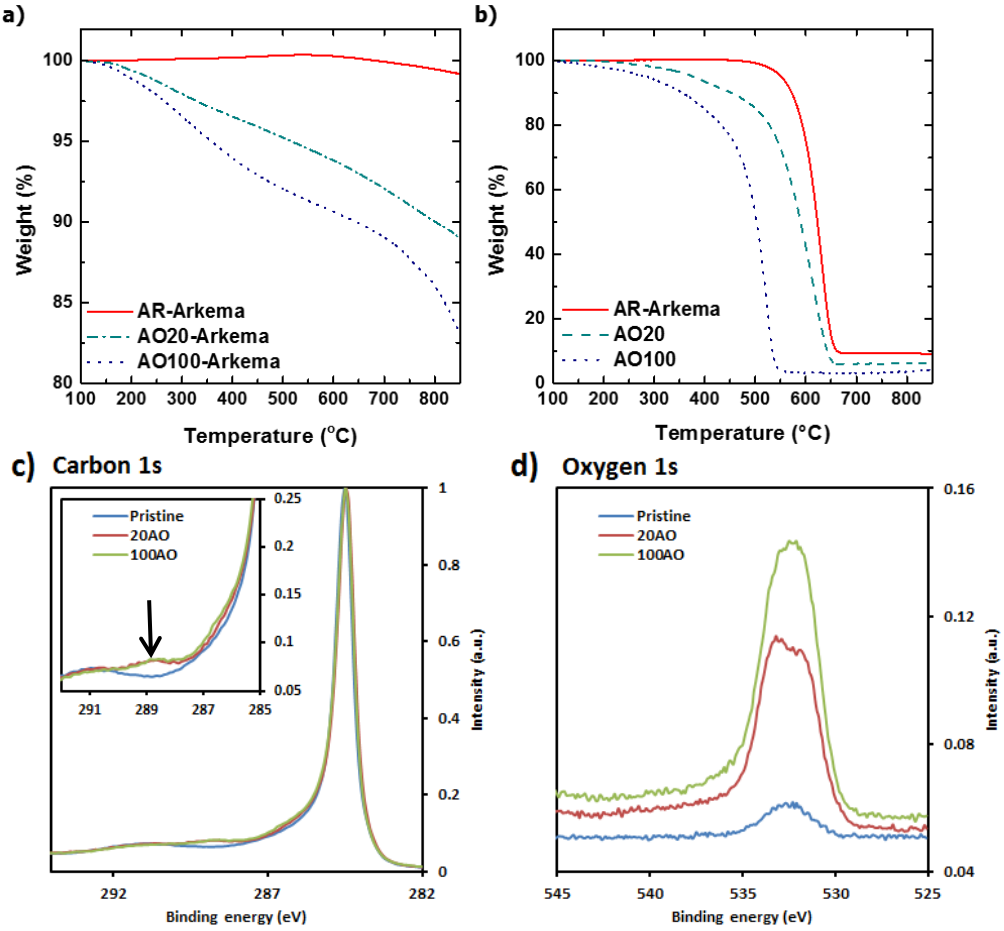
Supplementary Methods

TGA. TGA of acid oxidised (AO) MWNTs was performed to identify the alterations in thermal stability after oxidation treatment, as an indicator of the functional group content of the MWNTs. TGA was carried out on a Perkin-Elmer Pyris 1 machine (PerkinElmer Inc., UK). For a typical measurement, 2.0 ± 0.2 mg sample was heated to 100 °C, under N_2 atmosphere (60 mL/min), and held isothermally for at least 30 minutes to remove residual water and/or solvent; the temperature was then increased from 110 °C to 850 °C at a constant ramping rate of 10 °C/min under either flowing air or N_2 (60 mL/min).

XPS. X-ray photoemission spectra (XPS) were recorded on a K-Alpha™$^+$ XPS system (Thermo Scientific, UK) equipped with an Al Kα X-ray source (hν = 1486.7 eV) and operating at a base pressure of < 5×10^{-9} mbar. The X-ray spot size was ~400 μm^2. Data were collected with a pass energy of 20 eV and a step size of 0.1 eV. MWNT samples were dried and mounted on carbon tape, and a monoatomic argon flood gun was used for charge compensation.

MWNT stability. The stability of pristine and AO MWNTs in cell medium was assessed by measuring the percentage of MWNTs remaining in suspension as a function of settling time. MWCNT suspensions were prepared at a concentration of 10 μg/ml in complete medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal bovine serum (Sigma, UK), 8 mM L-glutamine (Sigma, UK), 100 U/L penicillin and 100 μg/mL streptomycin (Sigma, UK)). After ultrasonication for five minutes at room temperature (RT), suspensions were measured using UV absorption at 800 nm to determine the MWNT concentration. Suspensions were then left to settle, and UV absorption measurements were made at 0.5, 1, 2, and 24 hours.



e)

MWNT Functionalisation	TGA weight loss under N_2 (%)	TGA weight loss in air (%)	XPS oxygen at%	Raman D:G ratio
Pristine	1	9	2	1.14
20AO	10	6	8	1.19
100AO	16	4	10	1.3

Figure S1. TGA and XPS measurements of pristine and AO MWNT samples (a,b) TGA profile of pristine and acid oxidised (AO) Arkema MWCNTs under N_2 and air atmosphere. Continuous weight loss from 100 °C to 850 °C was observed for both 20AO and 100AO MWNT samples under N_2 atmosphere (a). This loss was attributed to combustion of the oxygen-containing functional groups generated during oxidation, as well as amorphous carbon debris. 20AO MWNTs displayed a higher percentage of weight loss compared to 100AO MWTNs, losing 10 wt% and 16 wt% respectively, as tabulated in (e). The residue remaining after TGA in air (b) indicates the metal content (oxides), which decreased with increasing severity of oxidation: 9 wt%, 6 wt%, and 4 wt% remained in pristine, 20AO and 100AO MWNT samples respectively. **(c,d) XPS spectra** of the carbon 1s peak (c) and oxygen 1s peak (d), both normalised using the maximum intensity of the carbon 1s peak. The extra intensity on the high-energy tail of the carbon 1s peak (c, inset) indicates the presence of oxygen-containing functional groups (including C-OH, C=O, COOH) in all samples. In addition, both acid oxidised MWNT spectra display increased intensity at ~289 eV compared to pristine MWNT (c, arrow), indicating that AO MWNT contain higher concentrations of carboxylic acid groups. The amount of oxygen, indicated by the integrated O1s intensity, increased with severity of oxidation (d). **(e) Summary of TGA, XPS and Raman characterisation of MWNTs**, showing the variations in % weight loss of thermally unstable species (attributed to both functional groups and debris), estimated oxygen at% measured by XPS, and the D:G ratio which indicates defect density.

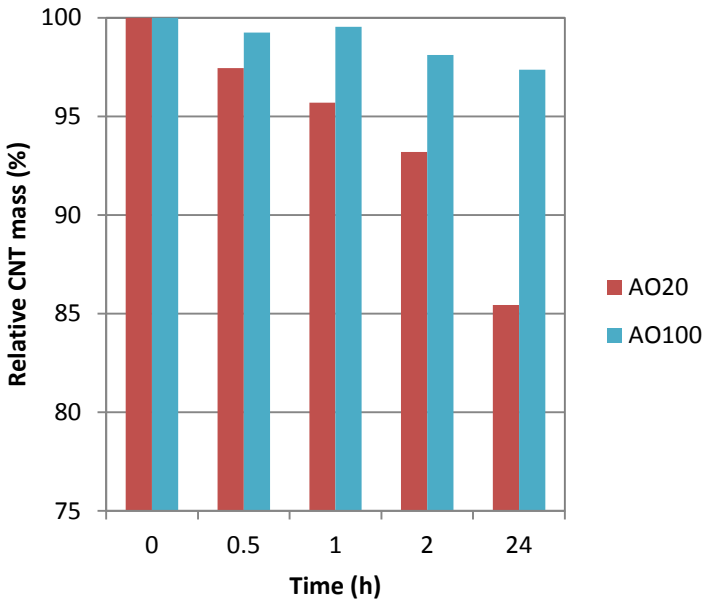


Figure S2. Stability of AO MWNTs in complete cell medium as a function of settling time, measured by UV absorption. The percentage of CNTs contributing to a UV absorption at 800 nm decreases as a function of settling time for both 20AO and 100AO MWNTs. After 24 hours, the remaining 15% of 20AO MWNTs and 3% of 100AO MWNTs have most likely aggregated and fallen out of suspension. It is worth noting that this method is not sensitive to any MWNT aggregates which remain in suspension, as this population will still contribute to the UV absorption signal. The stability of pristine MWNTs was not quantified, as they settle before the UV measurement could be acquired, rapidly yielding a clear, sedimented solution due to the initial high degree of aggregation and hydrophobicity of these MWNTs.

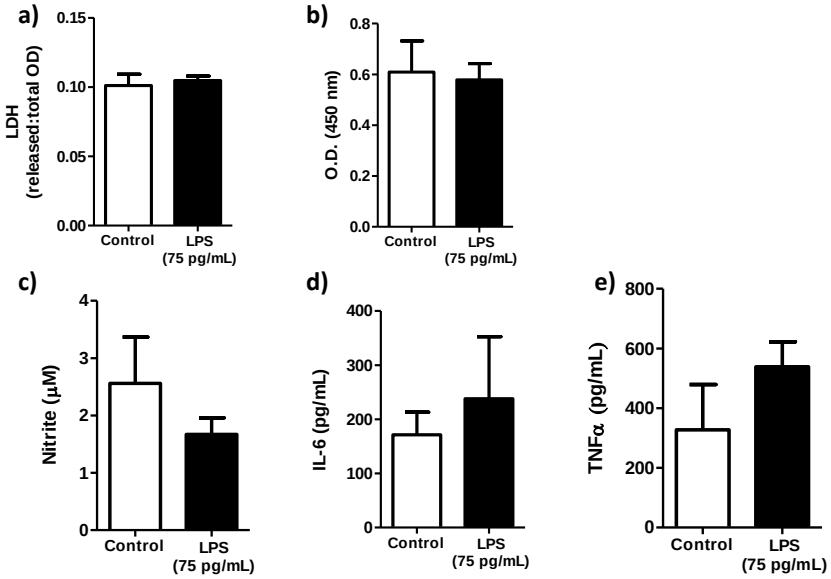


Figure S3. Microglia cell viability and activation following low endotoxin treatment. N9 microglia cells were treated with low level (75 pg/mL) LPS for 24hr, after which cell viability was assessed through an LDH release assay (a) and an MTS assay (b). Microglia cell activation was assessed after a similar treatment through quantification of nitrite production by a Griess assay (c) and IL-6 (d) and TNFα (e) release through an ELISA. Results represent mean ± standard error of the mean of at least three independent experiments. Statistical significance was examined through an unpaired t-test.

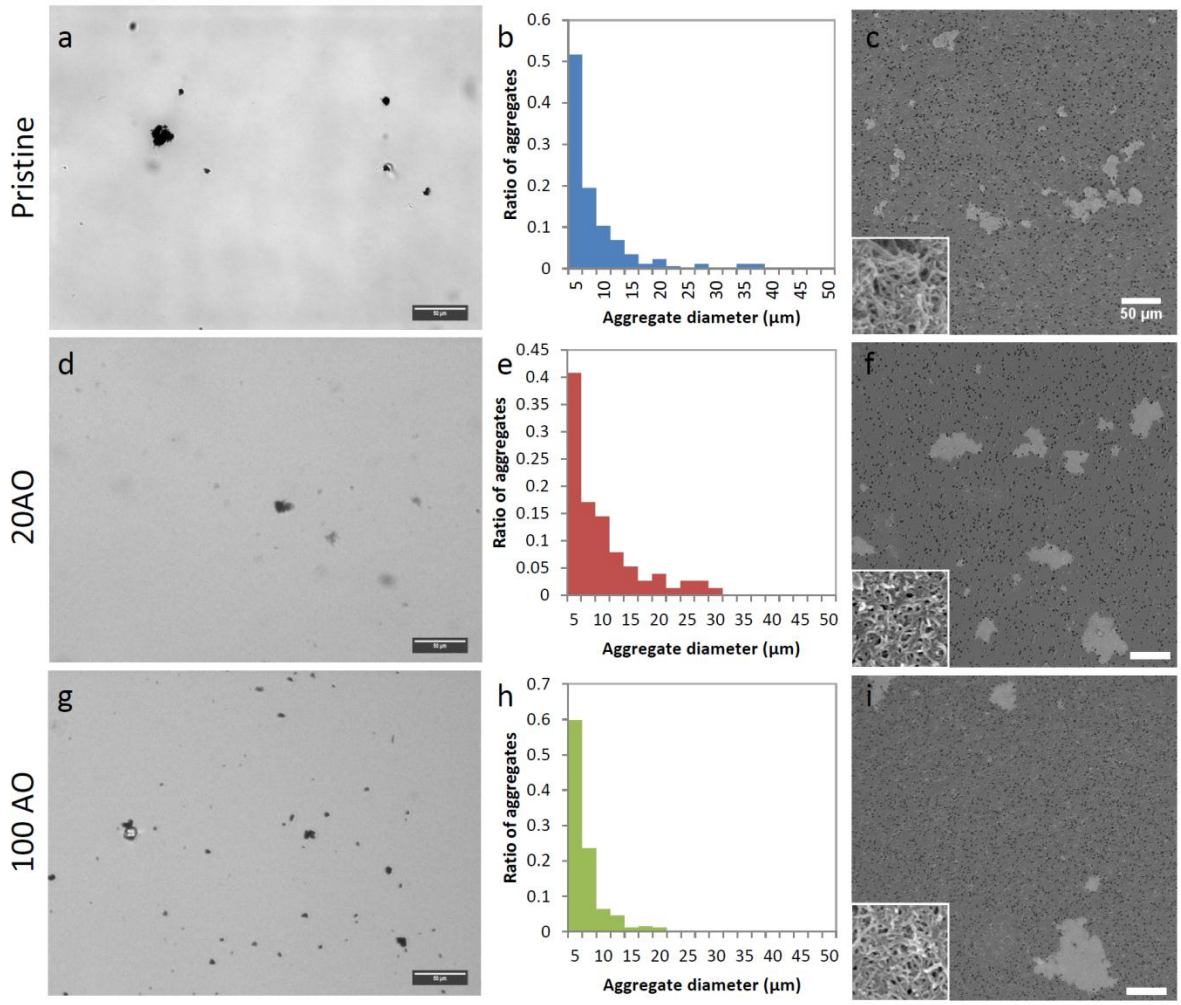


Figure S4. Characterisation of MWNT aggregates by light microscopy and SEM imaging. (a,d,g) Light micrographs of pristine, 20AO and 100AO MWNT aggregates respectively, with (b,e,h) corresponding histograms of aggregate diameters measured from ~100 aggregates in light micrographs. Light micrographs were recorded from samples of 10 µg/mL MWNTs suspended in complete medium. (c, f, i) SEM samples were prepared by filtering 1 mL of the same samples through PTFE filter membranes with 100 nm pore diameters. Samples were then washed by filtering 1 mL DIW through the same membrane, before drying and sputter coating with Au for SEM imaging (secondary electron mode). Scale bars 50 µm, inset displays a magnified 1 µm × 1µm region within a filtered aggregate.

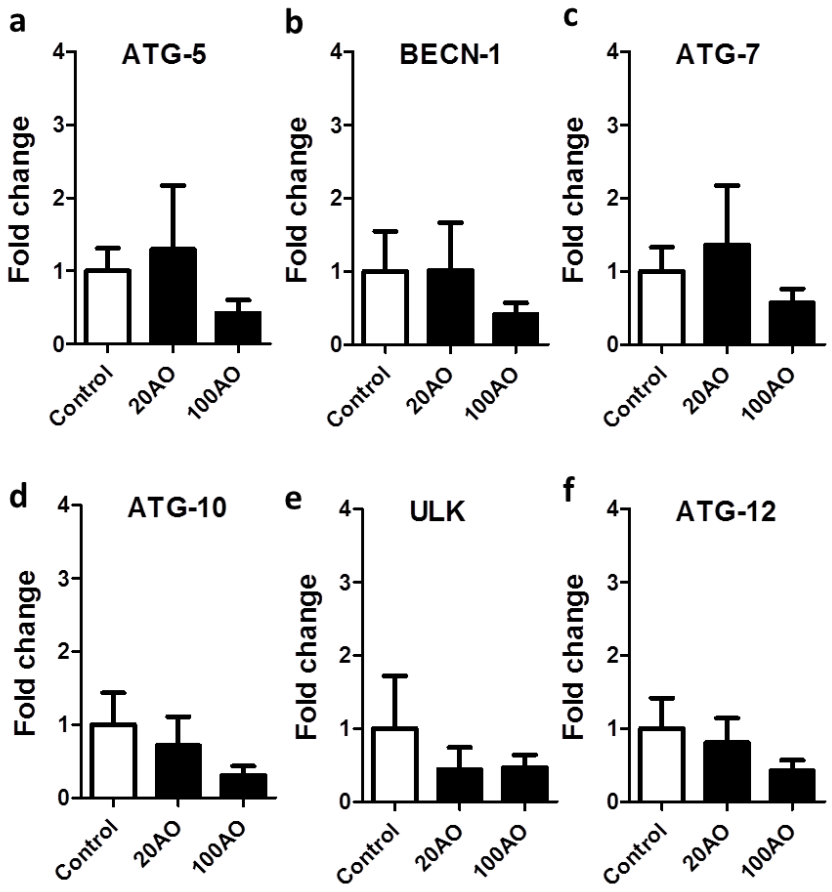


Figure S5. Effect of acid oxidised MWNT on autophagy-related gene expression. The expression of autophagy related genes was quantified utilising qRT-PCR following treatment of N9 microglia with 20AO and 100AO MWNT for 24hrs. Exposure of N9 microglia did not result in the induction of genes normally associated with autophagy induction. Results are displayed as mean ± standard error of the mean of three independent experiments. *** denotes $p < 0.005$ *vs.* control as determined by a one-way ANOVA with Tukey's *post-hoc* test. Probe design utilised for qRT-PCR can be found in Pienaar *et al.* 2015.