Screening the Cytotoxicity of Single-Walled Carbon Nanotubes Using Novel 3D Tissue-Mimetic Models

Dania Movia,†,‡ Adriele Prina-Mello,†,§ Despina Bazou,†,¥ Yuri Volkov,†,§,* and Silvia Giordani†,‡,*

†Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN), ‡School of Chemistry, §§chool of Medicine, and ¥School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Ireland

SYNTHETIC CARBON ALLOTROPES, such as single-walled carbon nanotubes (SWNTs), represent a growing family of fascinating nanomaterials with outstanding properties that are relevant in many potential applications, ranging from synthetic materials to drug delivery systems.1 SWNTs’ unique properties (such as size, shape, and composition), however, raise concerns about their potential human toxicity,2,3 while their diversity from bulk materials poses significant challenges over traditional toxicity-screening approaches.4

The test system currently employed for assessing the toxicity of chemical substances was introduced over 80 years ago.4 Only a few changes have been incorporated over the past decades, thus leaving the screening procedures almost unchanged and the experimental protocols obsolete when testing nanomaterials.4,5 A three-step scheme has been recently proposed as a solution by Hartung,4 and it is described as follows: (1) define the limitations of current tools; (2) combine them in a strategic way; and finally (3) develop a new testing system that integrates the old set of testing protocols with modern technologies. Among the latest, Hartung proposed imaging techniques and robotized testing platforms (such as high-content screening and analysis (HCSA)), in silico methods (heatmaps and quantitative structure–activity relationship (QSAR)), advanced cell-culturing techniques (three-dimensional “organotypic” cell cultures), and “omics” technologies (genomics and proteomics).4 While HCSA6–12 and in silico methods13,14 are commonly recognized and exploited as important techniques for the assessment of nanomaterials toxicity, the use of three-dimensional (3D) in vitro cell models (that can better represent the 3D organization of tissues in vivo compared to the conventional 2D cell cultures15,16) is still at its infancy within the nanotoxicology field. 3D cell aggregates more closely resemble the in vivo situation with regard to cell shape and cellular environment,16 which can in turn regulate the gene expression and hence the biological behavior of cells.17 To the best of our knowledge, only a few studies used coculture systems18,19 and/or 3D cell cultures
for testing nanomaterial toxicity. However, no "organotypic" cell culture technique has been employed to date for the assessment of SWNT toxicity.

The purpose of the present study is to assess the cytotoxicity of high-pressure carbon monoxide (HiPCO) SWNTs using 3D cellular, tissue-mimetic models. A comparative approach determining the importance of the third dimension in cell culture is presented. The THP-1 cell line, a macrophage model, was employed to represent the resident phagocytic cells (i.e., monocytes and macrophages) located in the liver (Kupffer cells) that have the main function of removing pathogens, senescent cells, and external particles from the bloodstream. Since the liver is a preferential site of nanomaterial accumulation in humans and rats, the performed toxicity studies strongly advocate the pressing need for the redefinition of the established nanotoxicology protocols.

RESULTS AND DISCUSSION

Since the intrinsic toxicity of as-produced (r-SWNTs) SWNTs is reported to decrease with removal of toxic metal impurities and with reducing the length of the tubes, r-SWNTs were purified and oxidized following a previously reported procedure (Scheme 1).

Physicochemical Characterization. Complete physicochemical characterization of the tested material is required nowadays to enhance the validity of the performed toxicity studies. A thorough characterization of the impurities content, lengths, and electronic properties of p- and o-SWNTs has been previously reported, showing that p- and o-SWNTs are able to emit efficiently in the NIR despite the employment of strong chemical treatments that shorten the tubes and introduce defect sites on the nanotube surface. In this study, NIR-photoluminescence (PL) spectroscopy (Figure 1) and zeta potential measurements (Table 1) were carried out on p- and o-SWNTs dispersed in deionized (DI) water or RPMI 1640 cell media. Taking into account that (1) the dispersion grade of CNTs in the biological media can affect both their in vitro and in vivo toxicity, (2) bundles of SWNTs can be disrupted via noncovalent functionalization with surfactants and (3) in vitro cell responses seem to depend strongly on the surfactant employed, we introduced both a protein-based (bovine serum albumin, BSA) and a surfactant-based (Pluronic F68) methodology for dispersing p- and o-SWNTs in an aqueous/biological environment. BSA is a water-soluble globular protein that has been shown to adsorb on the CNT surface and to have an excellent dispersing capability even in in vitro conditions. BSA is characterized by a pH-sensitive tertiary structure, which strongly affects its capability of stabilizing CNT dispersions at varying pH. Better-dispersed SWNT solutions are achieved at pH ranging between 4 and 8, which was in agreement with our study where all SWNT solutions had a neutral pH (pH = 6–7). Pluronic F68 is a biocompatible, linear copolymer of isopropylene glycol repeating units, with properties very similar to a nonionic surfactant. Pluronic F68 is able to stabilize an aqueous dispersion of SWNTs and copolymers of the Pluronic family are successfully adopted in the toxicity testing of CNTs. SWNT dispersions in DMSO were used as reference. For consistency both p- and o-SWNTs were dispersed in DMSO.

NIR-PL spectroscopy (Figure 1) was employed to clarify the stability of p- and o-SWNTs in RPMI 1640 cell media over time. NIR fluorescence is the optical property of SWNTs that is most sensitive to sample dispersion grade. Aggregation of isolated nanotubes prevents in fact the luminescence of SWNTs and reduces PL intensity. NIR-PL spectra showed that p- (Figure 1a) and o-SWNTs (Figure 1e) dispersed in RPMI 1640 media emitted more efficiently when the cell media was supplemented with BSA or Pluronic F68, respectively.
suggesting that better dispersions were achieved by addition of BSA and Pluronic F68. Shifts in the position of the NIR-PL peaks were shown when BSA or Pluronic F68 was added, thus confirming the interaction of such dispersing agents with the nanotubes' surface. In addition, the NIR-PL intensity of o-SWNTs (Figure 1e) was higher than that of p-SWNTs (Figure 1a). This result suggested that the more hydrophilic o-SWNTs were better dispersed in cell media than p-SWNTs. Interestingly, Pluronic F68 was less effective than BSA in stabilizing p- and o-SWNTs dispersions over time. The NIR-PL intensity of Pluronic F68-stabilized dispersions decreased significantly with time (Figure 1d, h), whereas BSA was capable of forming dispersions that were stable over 2 days (Figure 1c, g). This result found rationalization in the remarkable property of BSA to stabilize SWNT aqueous solutions even in the presence of monocationic (Na\(^+\)) and dication (Ca\(^{2+}\)) ions.\(^59\)

Similar to previously reported findings,\(^59\)–\(^62\) zeta potential measurements evidenced a negative surface...
TABLE 1. Surface Charges of p- and o-SWNTs before and after Dispersion in Supplemented RPMI 1640 Cell Media at Three Different Concentrations (120, 60, and 12 μg/mL)

<table>
<thead>
<tr>
<th>SWNTs</th>
<th>DMSO</th>
<th>BSA</th>
<th>Pluronic F68</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-SWNTs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>23.6</td>
<td>21.0</td>
<td>27.8</td>
</tr>
<tr>
<td>60</td>
<td>21.0</td>
<td>20</td>
<td>22.8</td>
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<tr>
<td>12</td>
<td>20</td>
<td>38.8</td>
<td>38.2</td>
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<tr>
<td>o-SWNTs</td>
<td></td>
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<td>120</td>
<td>40.2</td>
<td>58.4</td>
<td>38.2</td>
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<td>60</td>
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<td>12</td>
<td>38.2</td>
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a Surface charges were measured in DI water or in RPMI 1640 cell media.

b Dispersing agent added as described in the Experimental Section.

potential for both p- and o-SWNTs in DI water or in supplemented RPMI 1640 cell media at neutral pH (Table 1), with or without addition of dispersing agents (BSA or Pluronic F68). In DI water, o-SWNTs possessed increased absolute values of zeta potential when compared to p-SWNTs. This may be due to the negative surface charges associated with the dissociated carboxylic groups (COO–) on their surface63 or to the higher dispersibility of the more hydrophilic o-SWNTs in water (dispersions with zeta potential above -30 mV or below -30 mV correspond to stable solutions65). Yet the coexistence of the two phenomena is possible. A decrease in the absolute values of zeta potential was registered when both p- and o-SWNTs were suspended in RPMI 1640 cell media, suggesting that the dispersions stability decreased. Similar to what is observed with aqueous colloidal particles66 and to what is reported in previous studies on carbon nanotubes,59,60 our results are attributed to the high ionic strength of RPMI 1640 cell media that suppressed the electrostatic repulsion among tubes. Notably, p-SWNTs dispersions in RPMI 1640 cell media had higher zeta potential than o-SWNTs suspensions. As shown by NIR-PL data, this is not associated with a higher stability of p-SWNTs in RPMI 1640 media, whereas it can be explained by the passivation of the COO– attached onto the o-SWNT surface by the positively charged components (such as cations, proteins) of the cell media.

High Content Screening and Analysis of 2D Cell Cultures. As per Hartung’s guidelines,4 we chose the HCSA system as the ideal tool for simultaneously detecting changes in many cellular properties, thus producing a comprehensive set of data that provide guidance on the toxic response of a whole organism.65 Cell viability, cell membrane permeability, lysosomal mass/pH, and nuclear staining intensity changes are the parameters commonly used for evaluating the cellular cytotoxicity of chemical compounds,66 drugs, and nanomaterials.167,168 Three different concentrations (120, 60, and 12 μg/mL) of p- and o-SWNTs were tested on populations of 20,000 cells.

Figure 2. Graphical response intensity tables (heatmaps) of multiparametric HCSA data for 2D cell cultures exposed to p- and o-SWNTs for 24 and 72 h. Colorimetric gradient tables reflect the cytotoxicity evolution by showing the cell viability (left column), cell membrane permeability, lysosomal mass/pH, and nuclear intensity (right column) changes. Colors range from dark green (values lower than 15% of maximum value measured) to bright green (30%), yellow (50%), bright orange (60%), dark orange (75%), and finally to red (values higher than 75% of maximum value). Heatmap values are normalized to the percentages of the positive control, and Z score is calculated as described in the Statistical Analysis section. Data represent three independent experiments performed in triplicate samples.

Doses are therefore expressed as 12 × 10–4, 6 × 10–4, and 1.2 × 10–4 μg/cell, respectively. No significant changes in cellular responses were detectable when 2D cell cultures were exposed to solutions containing equivalent amounts of DMSO, BSA, or Pluronic F68 for 72 h (Figure S3).

Cell Viability. Time-dependent effects of p- and o-SWNTs on the cell viability are shown in Figure 2. Both p- and o-SWNTs were demonstrated to influence considerably the cell viability, causing a significant decrease in cell count at all doses and for all nanotube dispersions after 24 and 72 h.

Cell Membrane Permeability. A significant increase in the cell membrane permeability was registered after a 72 h exposure to BSA-stabilized p-SWNT dispersions at all employed concentrations (Figure 2). These results indicate that the cytoplasmic membrane was compromised, while it remained unaffected after 24 h of exposure to DMSO- and Pluronic F68-stabilized dispersions of p-SWNTs at all examined time points. Significant changes were found in cells incubated with o-SWNTs, irrespective of the dispersing agent used (BSA, DMSO, or Pluronic F68) or the time point.

Lysosomal Mass/pH. No significant changes in the lysosomal mass/pH were seen in p- or o-SWNT-treated cultures (Figure 2).
Nuclear Intensity. As for the lysosomal mass/pH changes, marginal or nondetectable changes in the nuclear intensity were seen in all treatments with the exception of the $12 \times 10^{-4} \mu g/cell$ dose, after 24 and 72 h exposure to p- and o-SWNTs, and of the $6 \times 10^{-4} \mu g/cell$ dose in 2D cell cultures treated with DMSO-stabilized p-SWNT dispersions.

In summary, HCSA data consistently showed a considerable cytotoxic response after exposure to p- and o-SWNTs in a 2D cell culture of "macrophage-like" THP-1 cells. In particular, our HCSA data indicated that SWNTs can cause cell stress no matter the purity grade, the reactive groups present on the SWNT surface, the dispersing agent used, and/or the stability of the dispersion. Even though contradictory cytotoxicity results have been shown on the cellular response of macrophages in 2D cell models exposed to SWNTs, our results were in agreement with some studies recently reported in the literature. Additionally, although metal impurities seem to play a key role in the toxicity of SWNTs, recent studies did identify the CNTs themselves as the principle cause of cytotoxicity in macrophages, rather than the catalysts. Concentrations of p- and o-SWNTs tested in 2D cell cultures (120, 60, and 12 µg/mL, equivalent to $12 \times 10^{-4}, 6 \times 10^{-4}$, and $1.2 \times 10^{-4} \mu g/cell$) were relatively high, but consistent with published studies. In recent in vitro studies on macrophage responses, SWNT concentrations varied from 0.3–10 µg/mL, to $1–50 \mu g/mL$, 15–60 µg/mL, 3–150 µg/mL, 50–200 µg/mL, and finally up to 0.12–0.5 mg/mL. Also in the above referenced reports contradictory cytotoxicity responses have been detected (Table S11).

Cytotoxicity Assays of 3D Cell Culture Models in Situ in the Ultrasound Standing Wave Trap (USWT). Trypan Blue Exclusion Assay. The live/dead cytotoxicity assay was performed in order to (1) validate the data obtained by the Trypan Blue exclusion assay, and (2) investigate whether the cell death was localized to the edges of the 3D aggregates, where cells were in direct contact with SWNTs. Quantification of cell death based on the epifluorescent images (Figure 3c, d) revealed no significant cytotoxic effects of the three differently stabilized p- and o-SWNT dispersions when compared to the negative control (cell death ranged between 20% and 44%). In parallel, the distribution of live and dead cells in SWNTs-treated 3D aggregates (Figure 4d) was similar to the negative control (Figure 4c) for all time points examined. Figure 4 shows representative results for a p-SWNT BSA-stabilized dispersion; consistent results were obtained for all the other SWNT samples.

Cytotoxicity Assays in 3D Cell Culture Models in Culture. In order to determine whether the different cellular responses observed in the 2D and 3D cellular models could be somehow associated with the different activation state of the THP-1 cells in the employed models (adherent "macrophage-like" cells in the 2D cell model and suspended monocytes in 3D cell aggregates), 3D cell aggregates of PMA-activated THP-1 cells were initially formed in the USWT, subsequently recovered from it, and exposed to p- and o-SWNTs in 24-well
plates. Confocal microscopy analysis confirmed that PMA induced the activation of THP-1 cells within the 3D aggregates (Supporting Information).

Trypan Blue Exclusion Assay. Nontreated (negative control) 3D aggregates remained 80% viable over 24 h (similarly to the results obtained for 3D aggregates...
in situ in the USWT). Exposure to p- and o-SWNTs did not significantly impair cell viability, with the exception of the DMSO-stabilized p-SWNT dispersion (Figure 5a, b).

Flow Cytometry of Live/Dead Cells. p-SWNTs and o-SWNTs did not cause any detectable cytotoxic outcome when compared to the nontreated aggregates (Figure 5c, d). The accompanying representative flow cytometric graphs are shown in the Supporting Information (Figure S1).

Live/dead staining analysis of 3D cell aggregates by confocal microscopy (Figure 6) further confirmed the flow cytometric data. Figure 6b and d are representative of all the SWNT dispersions tested in 3D cell aggregates in culture.

In summary, cell viability analysis (Figure 3 and Figure 5) consistently showed no detectable cytotoxic response or cell stress in 3D cell aggregates exposed to p- and o-SWNTs in situ in the USWTs or in culture. Furthermore, epifluorescent microscopy analysis provided evidence that cell death had a random distribution within SWNT-treated 3D aggregates in situ in the USWT (Figure 4) and in culture (Figure 6), and it was not localized to the areas where cells were in direct contact with the SWNTs.

Using the USWT we successfully fulfilled the necessity of reproducing one of the most important features of tissues in vivo, i.e., its three-dimensional architecture, bridging the gap between in vitro cell models and complex in vivo studies and offering added value to the in vitro prediction of SWNT toxicity. THP-1 cells spontaneously form aggregates of a few cells when cultured in suspension; these small spheroids can be recovered for experimentation. The use of 3D cell aggregates formed by the USWT satisfied, however, the necessity of developing a standardized protocol.
that can be used to form well-defined and reproducible 3D aggregates of any cell type and/or of various cell types (cocultures). Since macrophages are not found as agglomerates in tissues, but dispersed with other cells, the USWT is the way toward the creation of 3D cell models of macrophages that are more representative of the human physiology. 3D cell aggregates formed by USWT give rise to a more “tissue-mimetic” cell model compared to conventional 3D cellular models. The USWT avoids in fact the introduction of polymeric 3D-cell-growth substrates while allowing intercellular adhesive interactions to occur among cells. Additionally, it permits monitoring the cytotoxic response of cells to SWNTs without any interference by sedimentation processes. To this end, the different cytotoxic responses between the 2D and 3D cell models (both in situ in the USWT and in culture) presented here are most likely associated with the addition of the third dimension in the cell culture models and the extensive cell–cell interactions occurring in the 3D cell aggregates. It is a well-established fact that enhanced cell adhesion contacts commonly result in the increased population viability, in contrast to the loss of adhesion, or anoikis, leading to apoptosis. In addition, some intrinsic limitations of in vitro models, including the methods of exposing cells, do not allow accurately reproducing the physiologically coordinated responses among various cell types in tissues and organs, and the delivery of SWNTs in one single dose does not reflect in vivo realistic conditions, where SWNTs are likely to accumulate gradually and at different concentrations due to different penetration thresholds through the different cellular layers. We acknowledge that the different toxicity thresholds between the 2D and 3D cell models could also be
associated with a decreased capability of penetration of p- and o-SWNTs within the 3D cell aggregates. Further studies are necessary, therefore, to clarify the cellular mechanisms involved in the response of 3D cell models to SWNTs.

**Cytokine Expression.** Indirect postexposure quantification of TNF-α and IL-6 expression levels by enzyme-linked immunosorbent assay (ELISA) allowed us to quantify the extent of inflammatory protein secretion related to the cell stress-induced signaling cascade and transduction activity. ELISA assays revealed a high secretion of pro-inflammatory cytokines (IL-6 and TNF-α) in 2D cell cultures following exposure to p- (Figure 7a–f) and o-SWNTs (Figure 7g–i) after 24 and 72 h exposure.

In contrast, no significant changes in IL-6 and TNF-α secretion were detected in 3D cell aggregates exposed to p- and o-SWNTs in situ in the USWT (Figure 8a, c, e, g) or in culture (Figure 8b, d, f, h) after 24 h exposure, which is in concert with the above-described data on comparative SWNT cytotoxicity in 2D and 3D models. Control experiments were carried out to ensure that potential adsorption of proteins (such as cytokines) onto p/o-SWNTs surface was not influencing the quantification of the concentrations of IL-6 and TNF-α secreted by THP-1 cells exposed to such nanomaterials (data shown in the Supporting Information).

**CONCLUSIONS**

Our study showed that p- and o-SWNTs were cytotoxic in 2D but not to the same extent in 3D cell culture models. As there is great uncertainty when conducting toxicological studies of CNTs (mainly due to the accuracy of the toxicity protocols and of the technologies employed until now), our study clearly demonstrated the importance of 3D cell culture models. The ability to rapidly and reproducibly form uniformly shaped and sized 3D aggregates by the USWT is undoubtedly of high interest and potential in the screening of the toxicity of SWNTs and other nanomaterials. In addition to the opportunities to more closely imitate realistic 3D tissue microenvironments, these 3D models can help in identifying and establishing necessary chemical modifications that are required to make such nanomaterials more biocompatible for a range of biological and medical applications.

**MATERIALS AND METHODS**

Chemicals and solvents were purchased from commercial sources (Sigma-Aldrich, Fisher Scientific, Invitrogen, and Calbiochem). As-produced HiPCO SWNTs (r-SWNTs) were purchased from Unidym (lot # R0546). Purification and Oxidation of r-SWNTs. r-SWNTs were purified and oxidized following the procedure reported in previous studies.31,32 (Scheme 1).

**p-SWNT and o-SWNT Dispersions.** NIR-photoluminescence spectroscopy, zeta potential measurements, and toxicity tests were carried out on dispersions of p- and o-SWNTs in RPMI 1640 cell media (GIBCO, Bio Sciences Ltd., Ireland), with or without 100% bovine serum albumin (BSA) (Sigma-Aldrich) or 10% Pluronic F68 (Saff Biosciences). Dry powder p-SWNTs and o-SWNTs were weighted and added to a 1:1 mixture of sterile DMSO and sterile DI water at a concentration of 2 mg/mL (stock solutions). Stock solutions were sonicated (sonic bath, 4 h) and aliquoted in three vials of 100 μL to prepare the working solutions as follows: (1) 5 μL of sterile BSA was added (BSA-stabilized dispersion); (2) 5 μL of Pluronic F68 was added (Pluronic F68-stabilized dispersion); (3) the stock solution was left unchanged (DMSO-stabilized dispersion). A 1.9 mL portion of RPMI 1640 cell media (supplemented with 2 mM l-glutamine, 10% FBS, and 10 mg/mL penicillin–streptomycin) and incubated at 37 °C and 5% CO2. At 60% confluence, cells were diluted in modified RPMI 1640 media at concentrations appropriate for each experimental procedure. The passage number was restricted between 5 and 15.

**2D Cell Culture Models.** THP-1 cells were seeded in 96-well plates at a concentration of 20,000 cells/well (final volume: 200 μL/well) using a Matrix WellMate (ThermoFisher Scientific, USA) and activated with PMA 0.5 mM (Sigma-Aldrich) for 72 h to induce differentiation into adherent macrophages and stop their natural proliferation. After removing the cell media, adherent THP-1 cells were exposed to p- and o-SWNT dispersions. The final volume of solution added was 200 μL/well. Three different doses of p- and o-SWNTs were tested: 120, 60, and 12 μg/mL, corresponding to 12 × 10−4, 6 × 10−4, and 1.2 × 10−4 μg/cell, respectively. The anticancer agent paclitaxel (1.7 μM) (Sigma-Aldrich) was used as a positive control. Cells were exposed to SWNTs for 24 and 72 h.

**Cytopathology of 2D Cell Cultures**

- **Fluorescent staining of 2D cell cultures:** Supernatants were collected from each well for postexposure assays, and multiple washings with phosphate-buffered saline (PBS) were carried out in order to completely remove SWNTs from wells and avoid any potential interaction with the staining dyes. The multiparameter Cytopathology Kit HCSA reagent kit (ThermoFisher, USA) was used.65,66 Staining and quantification was performed according to previously reported protocols.65,66 This kit allows the detection of changes in many cellular properties.65,66 Briefly, changes in cell viability are directly correlated to the toxic effects of the material tested. Following a toxic insult, cells may also respond with changes in nuclear intensity, and an increased nuclear intensity is generally associated with the cell nuclei collapse and cell stress.66 Changes in the cell membrane permeability are often associated with an

**Physicochemical Characterization.** NIR-PL studies were carried out on p- and o-SWNT dispersions in RPMI 1640 cell media in a LOT ORIEL NS1 NanoSpectrolyzer (Applied Nanofluorescence, USA) at an excitation wavelength (λexc) of 683 nm over a period of 1 h with an interval of 3.6 min. Measurements were also carried out at λmax values of 638 and 785 nm with similar results (data not shown). Zeta potential measurements were performed on p- and o-SWNTs in DI water or in supplemented RPMI 1640 cell media by a Zetasizer Nanoseries Nano-ZS (Malvern Instruments, UK). Measurements were carried out in six replicates for each solution. Data are represented as average ± standard deviation. Cell Culture. Human monocytic leukemia (THP-1) cells were obtained from the American Tissue Culture Collection (ATCC, USA). Briefly, THP-1 cells were cultured in suspension in modified RPMI 1640 media (supplemented with 2 mM l-glutamine, 10% FBS, and 10 mg/mL penicillin–streptomycin) and incubated at 37 °C and 5% CO2. At 60% confluence, cells were diluted in modified RPMI 1640 media at concentrations appropriate for each experimental procedure. The passage number was restricted between 5 and 15.

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ongoing toxic or apoptotic response, and the loss of cell membrane integrity is a common feature of marked cytotoxicity.\textsuperscript{90} Finally, external agents can interfere with the normal cell physiology by affecting the mass, the number, or the function of organelles such as lysosomes.\textsuperscript{90}

**Imaging and HCSA analysis:** Measurements were carried out using the InCell 1000 automated fluorescent microscope system (GE Healthcare, USA) and its associated analysis software (In Cell Analyzer System, GE Healthcare, USA). 96-well plates were read on In-Cell Analyzer 1000 using three detection channels (461, 509, and 599 nm) with three different excitation filters (\(\lambda \text{Ex} < 503\) nm, \(\lambda > 509\) nm, and \(\lambda > 599\) nm). Ten random microscopical fields were sequentially acquired by the IN-Cell 1000 automated microscope system at a magnification of 10×. Acquired images for each exposure time and dose were then analyzed by the In-Cell Analyzer software according to previously reported procedures.\textsuperscript{90,96,98}

**Heatmaps:** Multiparameter HCS data are presented as cluster heatmap graphical tables to highlight the potential differences in cellular responses among SWNT dispersions, concentrations, and exposure times.\textsuperscript{90} For each SWNT solution the changes in cytotoxicity-associated parameters are presented in colorimetric gradients at the three different concentrations tested (Figure 2).

### 3D Cell Culture Model

A 1 mL amount of nonactivated THP-1 cells was transferred to an Eppendorf tube, centrifuged, and resuspended in supplemented, fresh RPMI 1640 cell media to a final concentration of \(1 \times 10^8\) cells/mL.

**Ultrasound standing wave trap and optical system:** USWT consisted of three features: a transducer (Ferroperm, Kvistgaard, Denmark) in a housing of radial symmetry, an aqueous phase, and a reflector that provided optical access from the top as previously described.\textsuperscript{79,92} The trap was driven with a function generator (Hewlett-Packard 33120A, UK). A fast, high-resolution XM10 camera (Soft Imaging System, SIS, GmbH, Munster, Germany) mounted on an Olympus BX51 M reflection epi-fluorescence microscope allowed observation in the direction of sound propagation (negative z-axis).\textsuperscript{92} Images were captured by a standard PC equipped with the Cell-D image acquisition and processing software (Soft Imaging System, SIS, GmbH).

**3D cell aggregates in situ in the USWT:** Cells were introduced into the USWT, the ultrasound was switched on for 10 min at 0.85 MPa (at which point the aggregate was mechanically robust), and 3D aggregate was formed (Figure 9a) as previously described.\textsuperscript{79} In this work, aggregates consisted of approximately 36,000 THP-1 cells, while their size was ca. 1.5 mm in diameter. p- or o-SWNTs dispersions (DMSO-stabilized, BSA-stabilized, and Pluronic F68-stabilized suspensions) at a concentration of 120 \(\mu\)g/mL, corresponding to approximately \(1.2 \times 10^{-4}\) \(\mu\)g/cell, were introduced into the USWT and surrounded completely the 3D aggregate (Figure 9c). After 24 h, 3D THP-1 cell aggregates had a closely packed morphology with few small empty spaces (Figure 9b and d). 3D cell aggregates were exposed to SWNT samples for 24 h in situ in the USWT and monitored by microscopy. Aggregates perfused with fresh, supplemented RPMI 1640 cell media or paclitaxel served as negative and positive controls, respectively.

**3D cell aggregates in culture:** In these series of experiments, THP-1 cells were treated with 2 \(\mu\)L of PMA (0.5 mM) to induce their differentiation into adherent macrophages. Following this, formation of 3D cell aggregates in the USWT proceeded as described above. However, aggregates were recovered from the trap after the 10 min period with a sterile 2 mL syringe (Plastipak, Becton Dickinson, UK), plated in 24-well plates (one 3D aggregate/well), and subsequently exposed to p- or o-SWNTs as described above. PMA-activated 3D aggregates had an average height of 25–30 \(\mu\)m.

### Cytotoxicity Analysis of 3D Cell Cultures

All SWNT dispersions were tested on 3D aggregates in duplicate both in situ in the USWT and in culture.

**Trypan Blue exclusion assay:** 3D aggregates were collected from the USWT and from the wells after exposure to p- or o-SWNTs, centrifuged, and resuspended in 10 \(\mu\)L of fresh RPMI 1640 cell media. A 10 \(\mu\)L amount of 0.4% Trypan Blue solution (Invitrogen, Molecular Probes, Oregon, USA) was then added. Blue-stained/dead cells and viable cells were counted, and percentage cell death was calculated as follows (eq 1):

\[
\% \text{ cell death} = \frac{\text{dead cells}}{\text{viable cells} + \text{dead cells}} \times 100 (1)
\]

It is important to note that the Trypan Blue exclusion assay does not interact with SWNTs.\textsuperscript{90}

**Live/dead cytotoxicity assay:** The live/dead cytotoxicity kit for mammalian cells (Invitrogen, Molecular Probes, Oregon, USA) was used. The kit is formed by two components: Calcein AM and ethidium homodimer-1 (EthD-1). SWNTs do not interact with the live/dead cytotoxicity assay.\textsuperscript{94}

For 3D cell aggregates in situ in the USWT, 2 \(\mu\)L of EthD-1 (2 mM) and 200 \(\mu\)L of Calcein AM (20 \(\mu\)M) were added to 800 \(\mu\)L of nonactivated THP-1 cells (10\(^7\) cells/mL). After incubation at 37 °C/5% CO\(_2\) for 30 min, 3D cell aggregate formation and perfusion of SWNTs proceeded as described above. 3D aggregates were examined at low magnification (5×) at 1, 3, 7, and 24 h. Quantification of live/dead cells within each 3D cell aggregate was carried out using Image Pro7 software (Media Cybernetics Inc., USA) by applying advanced image filtering, object recognition, and counting algorithms on the collected fluorescent images. For 3D aggregates in culture, aggregates were collected from the wells, centrifuged, and resuspended in 800 \(\mu\)L of RPMI 1640 cell media. Then 2 \(\mu\)L of EthD-1 (2 mM) and 200 \(\mu\)L of Calcein AM (20 \(\mu\)M) were added. After incubation at 37 °C/5% CO\(_2\) for 30 min, cells were washed by multiple steps of centrifugation and the percentages of live/dead cells was quantified by flow cytometry (Facs Canto II, BD, USA).

**Confocal microscopy of 3D aggregates in culture:** 3D cell aggregates (PMA-activated) were formed in the USWT as described before, subsequently recovered and deposited in a 8-well glass-borosilicate chamber slide, and incubated for 24 h at 37 °C with or without p- or o-SWNTs. After 1 day in culture, 300 \(\mu\)L of staining solution (EthD-1 2 mM, Calcein AM 20 \(\mu\)M) was added to each well. After incubation in the dark for 45 min, aggregates were washed with sterile PBS. Specimens were immediately analyzed by a ZEISS 510 Meta confocal microscope (Carl Zeiss, Axiovert, Germany) equipped with a Zeiss LSM Image Browser. Two-channel qualitative confocal imaging was carried out by acquiring a series of Z-stack images.

### Cytokine Secretion of 2D and 3D Cell Models

The concentrations of natural human interleukin-6 (IL-6) and human tumor necrosis factor-alpha (TNF-\(\alpha\)) secreted by the exposed THP-1 cells were measured by ELISA assays (Duoset ELISA development kit, R&D Systems: human TNF-\(\alpha\)/TNFSF1A; human IL-6) and compared to the relevant controls. The assays were repeated in duplicate. The optical density of each well at 450 nm was determined by means of an Epoch microplate reader (Biotek, USA), calibrated against standards, and corrected by subtracting the optical aberration of the 96-well plastic plate. The cell count for 2D and 3D cell cultures was carried out by HCSA and Trypan Blue exclusion assay, respectively, in order to quantify the cytokine production as picograms per cell (pg/cell) or picograms per mL (pg/mL) at the different concentrations and time points.

### Statistical Analysis

A two-way analysis of variance (ANOVA) followed by a Bonferroni post-test analysis was carried out for all HCSA and cytokine assays (Prism; Graph-Pad Software Inc., USA). \(p < 0.05\) was considered statistically significant and was denoted with (*) in the graphs. \(p\) values are reported in Tables S1 –S10 (Supporting Information). HCSA and ELISA data, as well as the results obtained by Trypan Blue exclusion and live/dead assays, are presented as mean values (\(n_{\text{mean}} = 2\) ± standard error of the mean and normalized to the negative control. Due to the
large amount of information acquired by HCSA, a data mining and exploration platform was used (KNIME (http://KNIME.org, 2.0.3)) in combination with a HTS screening module (http://code.google.com/p/hts, 0.3.0) in order to screen and normalize all parameters under investigation as previously reported. All measured parameters were normalized using the percentages of the positive control. Z score was used for scoring the normalized values. These scores were summarized using the mean function as follows: Z score = (x-mean)/SD, as previously reported. Heatmaps (i.e., graphical illustration in a colorimetric gradient table format) were adopted as the most suitable schematic representation to report on any statistical significance and variation from normalized controls based on their Z score value. Heatmap tables illustrate the range of variation of each quantified parameter from the minimum (green), through the mean (yellow), to the maximum (red) value.

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Supporting Information Available: Tables of p values; summary in vitro studies on macrophages exposed to SWNTs; schematic representation of the protocol employed for the preparation of SWNT dispersions; flow cytometry graphs; changes in cellular parameters in 2D cell cultures after exposure to surfactants; characterization of 3D cell aggregates in culture; control experiments for the ELISA assays. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES

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