Published in final edited form as:

Nanotoxicology. 2018 September ; 12(7): 677–698. doi:10.1080/17435390.2018.1472309.

Quantitation of Cell-associated Carbon Nanotubes: Selective Binding and Accumulation of Carboxylated Carbon Nanotubes by Macrophages

RUHUNG WANG^{1,2}, MICHAEL LEE JR^{2,#}, KARINA KINGHORN^{2,\$}, TYLER HUGHES^{2,†}, ISHWAR CHUCKAREE², RISHABH LOHRAY¹, ERIK CHOW³, PAUL PANTANO², and ROCKFORD DRAPER^{1,2,*}

¹Department of Biological Sciences, The University of Texas at Dallas, 800 West Campbell Road, Richardson, TX 75080, USA.

²Department of Chemistry and Biochemistry, The University of Texas at Dallas, 800 West Campbell Road, Richardson, TX 75080, USA.

³Department of Bioengineering, The University of Texas at Dallas, 800 West Campbell Road, Richardson, TX 75080, USA.

Present Affiliation: Section on Eukaryotic Transposable Elements, Cell Regulation and Development Group, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

\$ Present Affiliation: University of North Carolina at Chapel Hill, School of Medicine, Department of Cell Biology and Physiology, Chapel Hill, NC 27599.

† Present Affiliation: Washington University School of Medicine, Department of Pathology and Immunology, Campus Box 8118, 660 S. Euclid Avenue, St. Louis, MO 63110, USA.

Abstract

To understand the influence of carboxylation on the interaction of carbon nanotubes with cells, the amount of pristine multi-walled carbon nanotubes (P-MWNTs) or carboxylated multi-walled carbon nanotubes (C-MWNTs) coated with Pluronic® F-108 that were accumulated by macrophages was measured by quantifying CNTs extracted from cells. Mouse RAW 264.7 macrophages and differentiated human THP-1 (dTHP-1) macrophages accumulated 80–100 times more C-MWNTs than P-MWNTs during a 24-hour exposure at 37 °C. The accumulation of C-MWNTs by RAW 264.7 cells was not lethal; however, phagocytosis was impaired as subsequent uptake of polystyrene beads was reduced after a 20-h exposure to C-MWNTs. The selective accumulation of C-MWNTs suggested that there might be receptors on macrophages that bind C-MWNTs. The binding of C-MWNTs to macrophages was measured as a function of concentration at 4 °C in the absence of serum to minimize the potential interference by serum proteins or temperature-dependent uptake processes. The result was that the cells bound 8.7 times more C-

* Address correspondence to Prof. Rockford Draper, Department of Biological Sciences, FO31, The University of Texas at Dallas, 800 West Campbell Road, Richardson, TX 75080, USA. Tel: 972 883 2512. draper@utdallas.edu.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the manuscript.

MWNTs than P-MWNTs, consistent with the selective accumulation of C-MWNTs at 37 °C. In addition, serum strongly antagonized the binding of C-MWNTs to macrophages, suggesting that serum contained inhibitors of binding. Moreover, inhibitors of class A scavenger receptor (SR-As) reduced the binding of C-MWNTs by about 50%, suggesting that SR-As contribute to the binding and endocytosis of C-MWNTs in macrophages but that other receptors may also be involved. Altogether, the evidence supports the hypothesis that macrophages contain binding sites selective for C-MWNTs that facilitate the high accumulation of C-MWNTs compared to P-MWNTs.

Keywords

Nanotoxicology; Carbon Nanotube; Macrophages

Introduction

Carbon nanotubes (CNTs) come in two basic varieties, multi-walled (MWNT) or single-walled (SWNT), and have diverse applications in composite materials, energy storage, sensors, and biomedicine (Paradise and Goswami 2007; Liu et al. 2009; Schnorr and Swager 2010; Jariwala et al. 2013). MWNTs dominate the CNT market because they are the least expensive to synthesize and are used in more large-scale products. The global increase in the production of CNTs and incorporation into consumer products is cause for concern because of consistent reports that CNTs can be toxic [for reviews, see (Donaldson et al. 2010; Shvedova et al. 2010; Johnston et al. 2010; Petersen et al. 2011; Kunzmann et al. 2011; Petersen and Henry 2012; Jackson et al. 2013; Allegri et al. 2016)]. Of particular note is evidence that MWNTs initiate chronic inflammatory responses that cause pulmonary fibrosis in lab animals, similar to asbestos, and could therefore potentially cause mesothelioma. Moreover, MWNTs were recently found in the lungs of asthmatic Parisian children not known to have been exposed to a source of MWNTs, suggesting that MWNTs may be a previously unrecognized air pollutant (Kolosnjaj-Tabi et al. 2015).

Macrophages are a key intermediary in CNT pathology and the molecular mechanisms of how CNTs interact with macrophages to influence inflammatory responses have been widely studied; however, there remain significant knowledge gaps and conflicting literature reports, especially regarding the interaction of CNTs with cells and receptors. One open question is whether there are macrophage CNT receptors. We are not aware of any literature where macrophage receptors for any CNT types have been clearly demonstrated with direct binding assays, an obvious knowledge gap. Numerous groups, including ours, have studied the accumulation of various CNTs by cells and organisms over time using optical and physical methods (Petersen et al. 2008; Wang et al. 2009; Holt et al. 2011; Wang et al. 2011; Gao et al. 2011; Zhang et al. 2012a; Boyer et al. 2013; Li et al. 2013; Pondman et al. 2014; Murali et al. 2016; Wang et al. 2016; Mortimer et al. 2016). However, measuring cell surface-bound CNTs is challenging because it is difficult to quantify the small amounts of CNTs bound only to surface receptors [for review, see (Pantano et al. 2012)]. Nevertheless, there are good reasons to believe that various CNTs can interact specifically with macrophage surface components because known downstream consequences of CNT exposure, such as cytokine release, often involve macrophage receptors. A computational

study based on x-ray data suggested that (5,5) armchair SWNTs may bind to internal hydrophobic pockets of toll-like receptors (TLRs), consistent with observations that CNTs trigger downstream cytokine responses that could be initiated from TLR occupation (Turabekova et al. 2014). Graphene oxide, which shares surface structural features with carboxylated MWNTs (C-MWNTs), has been implicated indirectly in binding to TLR4 on macrophages because graphene oxide induces autophagic and necrotic responses that depend on TLR4 and downstream effectors (Chen et al. 2012; Qu et al. 2013). However, Qu et al. found that C-MWNTs did not cause a necrotic response, suggesting that TLR4 was not a C-MWNT receptor.

A further complication in identifying CNT receptors is the wide variety of covalent surface chemistries available that may modulate CNT binding, uptake, and processing by cells. The effects of covalent CNT surface modifications on many different biological endpoints, such as toxicity, induction of cytokines, and protein binding, have been widely studied [for recent reviews, see (Zhao and Liu 2012; Battigelli et al. 2013; Kim et al. 2014; Bilalis et al. 2014; Zhang et al. 2015)]. Given the large number of possible modifications, it is not surprising that there are multiple effects on biological endpoints, inferring that there could be multiple effects on potential receptors. For example, studies of MWNTs carrying a variety of covalent functionalities noted that different MWNT types were accumulated by macrophages to different levels and elicited different levels of pro-fibrogenic cytokines and growth factors, although no data on direct binding to specific receptors was presented (Li et al. 2013). In another example, the accumulation of MWNTs labelled with a fluorescent dye by murine macrophages was enhanced by increased MWNT oxidation and the accumulation was inhibited by dextran sulfate, a class A scavenger receptor (SR-A) antagonist, suggesting that SR-As were involved, but no direct evidence for binding to SR-As was presented (Singh et al. 2012). In addition, a combinatorial library of covalently modified MWNTs was screened for effects on different signaling pathways (Zhang et al. 2015). In one study, the MWNT library was screened for induction of autophagy and evidence was presented that C-MWNTs induced autophagy by a mechanistic target of the rapamycin (mTOR)-dependent pathway whereas a different covalently modified MWNT activated a mTOR-independent pathway. These results were interpreted to suggest that C-MWNTs bound to IGF-1 receptors (mTOR-dependent autophagy) while the other modified MWNTs bound to IFNA2 receptors (mTOR-independent autophagy); however, there was no direct evidence for binding of either MWNT type to the predicted receptors. The same group also noted that C-MWNTs enhanced the differentiation of the mouse myoblast C2C12 cell line to muscle cells, which was attributed to the binding of C-MWNTs to the bone morphogenetic protein receptor 2 (BMP2) (Zhang et al. 2012b). Supporting this idea was evidence of the proximity of C-MWNTs to BMP2 on the cell membrane by a FRET assay. Although indirect, this evidence bolsters the idea that C-MWNTs bind to a specific receptor on myoblasts and elicit a signaling cascade. In summary, alterations in pathways elicited by different MWNT covalent surface modifications point to the involvement of different receptors, but direct evidence for binding to a targeted receptor is often weak or absent.

In this paper, we observed that mouse RAW 264.7 macrophages and human differentiated THP-1 (dTHP-1) macrophages accumulated 80–100 times more C-MWNTs than pristine MWNTs (P-MWNTs) after 24 h at 37 °C when the MWNTs were suspended in cell culture

medium with the triblock copolymer Pluronic® F-108 (F108). A549 human lung fibroblasts also accumulated more C-MWNTs than P-MWNTs, although at lower amounts than macrophages, suggesting that the selective accumulation of C-MWNTs over P-MWNTs is not specific to macrophages. In addition, macrophages accumulated more carboxylated SWNTs (C-SWNTs) than pristine SWNTs (P-SWNTs), evidence that the effect is not restricted to MWNTs. The selective accumulation of C-MWNTs and C-SWNTs compared to P-MWNTs and P-SWNTs suggests that cells express binding sites that preferentially bind carboxylated CNTs (C-CNTs) compared to pristine CNTs (P-CNTs). To measure binding, macrophages were incubated with C-MWNTs or P-MWNTs in medium without fetal bovine serum (FBS) at 4 °C, conditions that avoid any interference by cell processes active at physiological temperature or by FBS proteins. There was strong binding of C-MWNTs to cells whereas the binding of P-MWNTs was not significant, results consistent with the selective accumulation of C-MWNTs at 37 °C. Similar binding results were seen with C-SWNTs and P-SWNTs. These data provide strong evidence for receptor sites on cells that bind C-CNTs but not P-CNTs. It was also observed that the binding of C-MWNTs and C-SWNTs to cells was reduced when the medium contained FBS, suggesting that one or more FBS components inhibited binding. Further, in studies of the direct binding of C-MWNTs to the cell surface of macrophages, SR-A antagonists (dextran sulfate and fucoidan) reduced the binding of C-MWNTs by about 50% and reduced the accumulation by 30%, suggesting that SR-As contribute to the binding and endocytosis of F108-coated C-MWNTs in macrophages, but that other receptors may also be involved.

Methods

CNTs and other materials

Six different MWNT powders and two different SWNT powders were purchased from three manufacturers. The synthetic methods, product names, lot numbers, and the physicochemical properties associated with each CNT powder provided by the manufacturers are shown in Table 1. Four of the six MWNT powders, including two P-MWNT and two C-MWNT powders, were produced by NanoAmor (Nanostructured & Amorphous Materials, Inc., Houston, TX) using a Fe/Co/Ni-catalyzed chemical vapor deposition process. According to NanoAmor, all MWNTs had purities of >95% carbon, specific surface areas of >200 m²/g, and outer diameters of 10 – 20 nm and inner diameters of 5 – 10 nm. NanoAmor C-MWNTs were oxidized using sulfuric acid and potassium permanganate and were reported to have 1.9 – 2.1 wt% of carboxyl groups according to the manufacturer. The only difference between the NanoAmor products was that one set of P- and C-MWNTs had regular lengths of 10 – 30 µm and the other set of P- and C-MWNTs had shorter lengths of 0.5 – 2 µm. The majority of the work in this study was performed with suspensions prepared from the short P- and C-MWNT powders from NanoAmor. The regular-length NanoAmor MWNTs were used to prepare long and short MWNTs in suspension for comparison, as described later. The elemental analyses of the NanoAmor MWNT powders provided by the manufacturers were confirmed using a previously described combustion analysis technique (Braun and Pantano 2014). In brief, the combined carbon, hydrogen, and oxygen contents of the short P- and C-MWNT powders and the regular-length P- and C-MWNT powders were 99.78%, 99.96%, 99.02 %, and 98.05 %, respectively.

respectively, indicative of MWNT powders that are essentially metal-free. One more pair of short P- and C-MWNT powders, listed as NC3150™ and NC3151™ in Table 1, were purchased from NanoCyl (NanoCyl SA, Sambreville, Belgium). These MWNTs were produced by an Fe/Co-catalytic chemical vapor deposition process and according to the manufacturer, had high purity (>95% carbon content), an average outside diameter of ~9.5 nm, and short lengths of <1.0 μm, comparable to the short P- and C-MWNTs from NanoAmor. The NanoCyl C-MWNTs were carboxylated by a plasma method and were reported to have COOH contents of <8 wt%. The elemental analyses of the NanoCyl MWNT powders provided by the manufacturers were also confirmed using our combustion analysis technique (Braun and Pantano 2014) and the combined carbon, hydrogen, and oxygen contents of the P-MWNTs (NC3150™) and C-MWNTs (NC3151™) were determined to be 101.5% and 95.7%, respectively.

SWNT powders purchased from Carbon Solutions, Inc. (Riverside, CA), termed P2-SWNT and P3-SWNT by the manufacturer, were produced by a Ni/Y-catalyzed electric arc discharge method. For nomenclature consistency, pristine SWNTs (P2-SWNTs) will be referred to as P-SWNTs and carboxylated SWNTs (P3-SWNTs) will be referred to as C-SWNTs. Both the P-SWNT and C-SWNT powders were >90% in carbonaceous purity and the individual SWNTs in these powders were 0.5 – 3.0 μm in length and ~1.4 nm in diameter. The P-SWNTs are a purified pristine product that contains low functionality and the C-SWNT product was oxidized using nitric acid to contain 1.0 – 3.0 atomic % of carboxylic acid groups, per the manufacturer. The elemental analyses of these two SWNT powders were presented in our previous work (Braun and Pantano 2014). Caution: a fine particulate respirator and other appropriate personal protective equipment should be worn when handling dry CNT powders.

Pluronic® F-108 (F108), dextran sulfate (product #D6001), chondroitin sulfate (product #C9819), fucoidan (product #F563), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO) and were used as received. Non-functionalized polystyrene beads (Nanosphere™ 0.9 μm size standards, cat. No. 3900A) were purchased from Thermo Fisher Scientific (Waltham, MA).

Preparation of Pluronic® F-108 CNT suspensions

A stock F108 solution at 5 mM concentration was prepared by dissolving F108 powder in DI water purified using a Milli-Q system (Billerica, MA), filtered through a 0.22 μm membrane, and stored at 4 °C in the dark. All CNT suspensions were prepared with a freshly diluted and filtered 0.2 mM F108 solution. To reduce potential endotoxin contaminants that could lead to ambiguous toxicity results, all CNT powders were baked at 200 °C for 2 h (Nakata 1994) before F108 was added. The sonication, centrifugation, and dialysis protocols described in our previous works (Wang, 2013 #2423; Wang, 2016 #2834) were used with slight modifications to prepare F108-coated CNT suspensions, as summarized in Scheme 1.

The procedure shown in Scheme 1A was used to prepare CNT suspensions denoted as samples (a), (b), (g), (h), (i), and (j) in Table 2 with the corresponding short length CNT powders described under the Product Name column in Table 1 as Short MWNTs and Short MWNT-COOH by NanoAmor, NC3150™ and NC3151™ by NanoCyl, and P2-SWNT and

P3-SWNT by CSI. In general, 10 mL of 0.2 mM F108 solution was added to a glass vial containing 10 mg of baked CNT powder, and sonicated for 1 h at 37 kHz and 120 W. Sonicated CNT dispersions were centrifuged at 20,000 g for 5 min to remove CNT bundles, metal catalysts, and other impurities, as demonstrated in our previous work (Wang et al. 2013). Due to the lower purity of the SWNT powders, the supernatants of SWNT dispersions (samples (i) and (j) in Table 2) were centrifuged a second time at 20,000 g for 30 min. The supernatants were extensively dialyzed against freshly prepared 0.2 mM F108 solution at 4 °C for 3 d to remove F108 sonication by-products that are toxic using Float-A-Lyzer® G2 devices with a molecular weight cutoff of 100,000 Daltons (Spectrum Labs, Rancho Dominguez, CA), as described in our previous works (Wang et al. 2013; Wang et al. 2016).

To minimize any physiochemical differences between short and long MWNTs, except for their length, two suspensions were derived from the same powder comprising 10 – 30 µm long MWNTs using different sonication times and centrifugation procedures to produce F108-coated MWNTs of longer and shorter lengths, as shown in Scheme 1B. As an example, 10 mL of 0.2 mM F108 solution was added to 10 mg of baked pristine MWNT powder, listed as regular-length MWNTs in Table 1. The mixture was sonicated first at a reduced power of 60 W and 37 kHz for a shortened duration of 5 min to better preserve the length of the dispersed MWNTs. Half of the dispersion sample was centrifuged at 20,000 g for 5 min followed by dialysis. The other half of the dispersion was subjected to a second round of sonication at 120 W and 37 kHz for an extended duration of 90 min to facilitate the generation of dispersed MWNTs with shorter lengths. After sonication, the dispersion sample was centrifuged twice at 20,000 g for 10 min, followed by dialysis as usual. The two resulting MWNT suspensions are denoted samples (c) P-MWNT(L) and (e) P-MWNT(S), in Table 2. The same procedure was used with regular-length MWNT-COOH powder in Table 1 to generate two suspensions denoted as samples (d) C-MWNT(L) and (f) C-MWNT(S) in Table 2. Atomic force microscopy (AFM) analysis of these suspensions indicated that MWNTs of different lengths were produced by the procedure: specifically, the mean lengths of P-MWNT(L) and P-MWNT(S) were 807 and 139 nm, while the mean lengths of C-MWNT(L) and C-MWNT(S) were 554 and 199 nm, respectively (Table 2 and Figure S2B).

Characterization of CNT suspensions

The concentration of CNTs in each suspension was measured using the absorbance at 500 nm. As described in the previous section, degradation by-products of F108 produced during sonication of suspensions were removed by dialysis and removal was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by BaI₂ staining, as described in our previous work (Wang et al. 2013).

Dynamic light scattering (DLS) and zeta potential analyses were used as part of a quality control routine for the preparation of all CNT suspensions, as previously described (Wang et al. 2013; Wang et al. 2016) and detailed in the Supplemental Methods. During the preparation of CNT suspensions, routine measurements of CNT particle size distribution by DLS and zeta potential were taken at intermediate steps to ensure reproducible quality across different batches of prepared CNT suspensions. In addition, DLS and zeta potential

analysis were also performed periodically on CNT suspension stocks stored at 4 °C. All the CNT suspensions proved to be stable in storage for months, indicated by the lack of aggregates detected by DLS and constant zeta potential results (Table 2 and Figure S2A). Zeta potentials for C-CNTs were only slightly more negative than those for P-CNTs in water (Table 2). Zeta potential values were also determined for C-MWNTs, P-MWNTs, C-SWNTs, and P-SWNTs in cell culture medium with 10% serum. The values were less negative for each CNT type in medium with serum than in water, as expected due to the increase of salt and serum protein concentrations, and the C-CNTs still had slightly more negative zeta potentials than P-CNTs (Table 2).

CNT length analysis by AFM has been detailed in our previous publications (Wang et al. 2011) and similar protocols were used here (see Supplemental Methods) to determine particle lengths and distribution of the long and short P- and C-MWNT suspensions (samples (c) through (f) in Table 2). The length distributions of various MWNTs suspensions were plotted as histograms and shown together with the mean $\pm$ SD in Figure S2B.

Cell lines and cell culture

Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY). Ham's F-12K (ATCC® 30–2004) and RPMI 1640 (ATCC® 30–2001) media were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Trypsin-EDTA (0.5%) was purchased from Thermo Fisher Scientific and Accumax™ was purchased from Innovative Cell Technologies (San Diego, CA). Penicillin-streptomycin solution (100 U penicillin/0.1 mg streptomycin per mL) was purchased from Sigma Aldrich and used only in terminal cultures. Phorbol 12-myristate 13-acetate (PMA) and all other cell culture reagents were purchased from Sigma Aldrich.

Human lung epithelial carcinoma A549 cells (ATCC® CCL-185), Abelson murine leukemia virus transformed macrophage RAW 264.7 cells (ATCC® TIB-71), and human acute peripheral blood leukemia monocyte THP-1 cells (ATCC® TIB-202) were purchased from ATCC. A549 cells were cultured in Ham's F-12K medium, RAW 264.7 cells were cultured in DMEM, and THP-1 cells were cultured in RPMI-1640. All liquid media were supplemented with 1.5 mg/mL sodium bicarbonate, 10% (v/v) FBS, and 10 mM HEPES buffer at pH 7.4 and cells were grown in a 37 °C incubator with 95% air and 5% CO₂.

To induce monocyte-to-macrophage differentiation of THP-1 cells, a slight modification of the PMA-stimulation protocol described in detail by Daigneault et al. was used (Daigneault et al. 2010). Briefly, THP-1 cells grown in suspension to a cell density of 2×10^5 cells/mL were treated with 200 nM PMA (diluted in fresh regular culture medium) for 3 d under standard culture conditions. After the initial 3 d stimulus, THP-1 cells adhere to the culture dishes with an almost complete arrest in proliferation. To ensure complete differentiation, the PMA-containing media was removed and the differentiated THP-1 cells were washed gently and cultured in fresh medium without PMA for a post-PMA resting period of 2–3 d prior to being used in experiments. The THP-1 cells in suspension prior to differentiation are denoted as the un-differentiated THP-1 monocytes (undTHP-1) and the PMA-treated and

rested macrophage-like THP-1 cells that adhere to culture dishes are denoted as the differentiated THP-1 macrophages (dTHP-1) throughout this study.

Crystal violet cell proliferation assay, lactate dehydrogenase (LDH) release cell viability assay, and enzyme-linked immunosorbent assay (ELISA) for quantifying cytokine secretion

A standardized cytotoxicity assay based on cell proliferation described previously in our MWNT and SWNT toxicity work was used for cytotoxicity measurements with A549 and RAW 264.7 cells exposed to various CNT suspensions (Wang et al. 2011). CNT suspensions were first diluted in freshly prepared 0.2 mM F108 solution to a concentration twice the desired CNT concentration to be tested. The diluted CNT suspension samples were then mixed 1:1 in equal volumes with a 2X concentrated medium appropriate for the cells under study with 20% FBS and 20 mM HEPES buffer. The result is medium at the same concentration as usual with 10% FBS, 10 mM HEPES buffer, and 0.1 mM F108. 3×10^4 A549 or 4×10^4 RAW 264.7 cells/well were seeded in 48-well plates and incubated at 37 °C overnight before the regular cell culture media was replaced with freshly prepared control or test media and incubated for 24 h. At the end of the incubation, cells were washed 3 times with fresh media, 2 times with PBS, air-dried, and fixed with 4% (w/v) paraformaldehyde (PFA) in PBS. Cell proliferation was determined using a crystal violet assay, as described in our previous work where it was also demonstrated that CNTs do not interfere with the assay (Wang et al. 2011).

Since the monocyte-to-macrophage differentiation of THP-1 cells induced by PMA treatment arrests proliferation (Daigneault et al. 2010), potential cytotoxic effects of CNT suspensions on differentiated THP-1 cells were assessed using a LDH release assay. LDH is present normally in the cytosol of healthy cells, whereas cells with impaired plasma membrane integrity allow LDH to leak into the medium. The LDH assay was also used with RAW 264.7 cells to validate the cytotoxicity test results obtained using the crystal violet assay. After validation, it was not considered necessary to do LDH assays with A549 cells. In general, 8×10^4 dTHP-1 or 1×10^4 RAW 264.7 cells/well were seeded in 96-well plates and incubated in regular culture media at 37 °C overnight to allow the cells to adhere to the plates. The media was replaced the next day with freshly prepared control or various CNT test media and incubated for 24 h. After incubation, the supernatants of treated wells were collected and assayed for the presence of LDH in the media, a marker for plasma membrane damage. A Thermo Scientific™ Pierce™ LDH cytotoxicity assay kit was used and the assay was performed following the manufacturer's instructions with an additional centrifugation step to remove CNTs from the media after the incubation. Briefly, cells in one of two duplicate wells treated identically were lysed by adding the Lysis Buffer provided in the kit to the well while cells in the other well received water. Thus, the total LDH produced by the cells was acquired from the media in the well that received Lysis Buffer. The released LDH was determined from the media in the duplicate well that received water such that released LDH but not cytosolic LDH was present in the medium. The plate was then incubated at 37 °C for 45 min to allow complete lysis of the cells exposed to Lysis Buffer. The plate was shaken on an orbital shaker for ~2 min before the supernatants from each well were collected and centrifuged at 20,000 g and 4 °C for 30 min. The supernatants were carefully

transferred to new centrifuge tubes leaving cells, lysed cell debris, and CNTs in the pellets. This additional centrifugation step was necessary to minimize the presence of CNTs in the supernatant that has previously been shown to interfere with the absorbance readout of many colorimetric cytotoxicity assays, including the LDH assay (Wang et al. 2012; Forest et al. 2015). To measure LDH in the supernatants of the centrifuged samples, aliquots were transferred to a new plate, mixed with the Reaction Mix provided in the kit, incubated for 30 min at room temperature, and the colorimetric reaction stopped by adding a Stop Solution, also included in the assay kit. The absorbance at 490 nm and the background absorbance at 680 nm were measured using a BioTek Synergy 2 Multi-Mode microplate reader (Winooski, VT). The amount of LDH in media of cells treated with Lysis Buffer is considered as the total LDH produced by the cells; the amount of LDH in the media of cells treated with MilliQ water is the LDH released from the cells. The level of % LDH released is calculated as the quotient of the amount of released LDH divided by the total amount of LDH multiplied by 100%, a commonly used index of cytotoxicity.

Methods for induction and quantitation of human tumor-necrosis factor α (hTNF- α) and human interleukin 1β (hIL- 1β) secretion in dTHP-1 cells using an ELISA kit are described in detail in the Supplemental Methods.

Phagocytosis activity of RAW 264.7 cells measured by the uptake of polystyrene beads

The manufacturer-provided properties of the polystyrene beads (NIST certified) included a diameter of $0.903 \pm 0.012 \mu\text{m}$, a density of 1.05 g/cm^3 , a solid concentration of 1%, a refractive index at 589 nm of 1.59, a pH of 5–8, no surface functionalization, and no detectable preservatives or surfactants. DLS and zeta potential analyses were used to determine the hydrodynamic diameter (HDD) and zeta potential of the beads, as detailed in the Supplemental Methods. The HDD of the beads diluted in water and measured at 25°C was $0.910 \pm 0.017 \text{ nm}$, consistent with the value provided by the NIST certification. The HDD of beads when diluted in cell culture medium with 10% FBS at 37°C to emulate cell culture conditions was $1.165 \pm 0.028 \mu\text{m}$ with a polydispersity (PDI) of 0.360 ± 0.075 and a zeta potential of $-10.0 \pm 0.6 \text{ mV}$, indicating that the beads were stably dispersed with a uniform size.

To measure bead phagocytosis, 2×10^4 RAW 264.7 cells/well were seeded on glass coverslips in 4-well plates and incubated at 37°C under regular cell culture conditions for 24 h before the experiment. Control and test samples included filtered MilliQ water, 0.2 mM F108 solution alone, and 200 $\mu\text{g/mL}$ of P-MWNTs or C-MWNTs suspended in 0.2 mM F108. Each sample was mixed with an equal volume of 2X culture medium supplemented with 20% FBS to give a constant 10% FBS in all media, and 0.1 mM F108 with or without 100 $\mu\text{g/mL}$ of either P- or C-MWNTs in the final test media. Cells were incubated in 0.4 mL/well of control or test media for 2 or 20 h at 37°C . After the incubation, the cells were washed 3 times with warm fresh culture medium in the absence of F108 and MWNTs. Cells were returned to 37°C for a 30-min chase, washed one more time with warm fresh medium, and exposed to 25 $\mu\text{g/mL}$ of non-functionalized 0.9 μm polystyrene beads in fresh medium for 2 h at 37°C . The concentration of beads used was empirically determined to balance having too many or too few beads in the cells to count. After the incubation with beads, cells

were washed 3 times with warm fresh medium and 2 times with warm PBS to remove excess non-phagocytosed beads, fixed with 4% PFA for 15 min at room temperature, rinsed briefly with MilliQ water, and then mounted in 8 μ L of FluoMount fluid per coverslip on glass slides. For qualitative analysis of the phagocytic activity of the cells, phase contrast cell images with 60 $\times$ magnification were acquired using a Nikon Eclipse TE2000 microscope equipped with an Andor Zyla camera and operated with *NIS-Elements AR 4.40.00* software. 40 $\times$ phase contrast cell images, acquired using Nikon Eclipse TS100 microscope and DS-Fi2 camera under the control of *NIS-Elements D 4.50.00* software, were used for quantitative assessment. The phagocytic activity of control and test cells were determined based on multiple images per sample where the total number of cells and phagocytosed beads were counted and recorded using *ImageJ Cell Counter* software. The mean number of beads per cell is indicative of the phagocytic activity of the cell under the experimental condition. The Student's T-Test was applied to compare the mean beads/cell of each pair of the control and a test group where the difference with $P < 0.05$ was considered statistically significant.

Accumulation of CNTs in A549, RAW 264.7, and THP-1 cells at 37 °C

The following procedure was used to detect the accumulation of P- and C-CNTs by A549, RAW 264.7, and THP-1 cells at 37 °C for 24 h. In general, CNT suspensions were first diluted in freshly prepared 0.2 mM F108 solution to twice the desired final CNT concentrations specified in the experiment. The diluted CNT suspension samples were then mixed 1:1 with a 2X concentrated medium appropriate for the testing cells supplemented with 20% FBS and 20 mM HEPES buffer such that the prepared testing media was at 1X medium concentration, 10% serum, and 10 mM HEPES. For cells adherent to culture dishes, 3×10^5 A549, 4×10^5 RAW 264.7 or 1×10^6 dTHP-1 cells/well were seeded in 6-well plates and incubated in regular culture media at 37 °C overnight to allow the cells to adhere to the plates. The culture media was removed the next day and 2 mL of freshly prepared control media that contained no CNTs or test media that contained a CNT suspension at a specified concentration was added to each well. For monocytic undTHP-1 cells cultured in suspension, 1 mL of the 2X control or testing media was placed in a well followed by the addition of 2×10^6 cells suspended in 1 mL of 2X media to initiate the incubation. Cells were incubated in control or test media at 37 °C for up to 24 h, as described in each experiment. At the end of the incubation, for the adherent cells, the control and test media were removed by aspiration and the cells were washed 3 times with fresh culture media followed by 2 washes with PBS. Cells were then lifted off the well using 0.5 mL Accumax™, transferred to a centrifuge tube, and the well was rinsed with 1.5 mL PBS that was subsequently added to the tube to make a final cell suspension of 2 mL/well/tube. For the undTHP-1 cells that grow only in suspension, 2 mL of the cell suspension was transferred to a microcentrifuge tube. Removal of control and test media and multiple washes with culture media and PBS were accomplished by multiple centrifugations at 1,000 g for 5 min at 4 °C. The cells after the last PBS wash were suspended in 2 mL PBS for further processing. Three aliquots of cell suspension, 100 μ L each, were used to determine cell counts in each sample using a Beckman Coulter Particle Counter (Miami, FL) and the cells in the remaining 1.7 mL cell suspension were collected by centrifugation at 1,000 g for 5 min at 4 °C. The cells in the pellet were lysed in 200 μ L of cell lysis buffer that contained 0.25 M Tris-HCl (pH 6.8), 8%

(w/v) SDS, and 20% (v/v) 2-mercaptoethanol. To ensure complete lysis of the cells, the lysate samples were heated in a boiling water bath for 2 h and then stored at 4 °C. The amounts of CNTs in the cell lysate samples were determined using a previously established SDS-PAGE method (Wang et al. 2009; Wang et al. 2011), described in a later section.

Surface binding of CNTs to RAW 264.7 macrophage cells at 4 °C

To detect and compare the association of P-MWNTs, C-MWNTs, P-SWNTs, and C-SWNTs with the surface of RAW 264.7 cells in the absence of endocytic or phagocytic activity, 5×10^5 RAW 264.7 cells/well were first seeded in 6-well plates and incubated in regular culture media at 37 °C overnight. In order to incubate cells at low temperature outside of the 37 °C CO₂ incubator, the regular culture media was replaced with a modified DMEM that contained no sodium bicarbonate and supplemented with 10% FBS and 10 mM HEPES buffer. The 6-well plates were then placed on a shallow ice-water bath and incubated in a 4 °C cold room for 30 min. A 2X concentrated medium without sodium bicarbonate and supplemented with FBS and HEPES buffer was pre-chilled to 4 °C before mixing 1:1 with a CNT suspension such that the final test media contained CNTs at the desired concentration. After chilled down to 4 °C, cells were incubated with the pre-chilled control medium that contained no CNTs or test medium that contained a CNT suspension at the specified final CNT concentration for 1 h at 4 °C. Because phagocytosis and endocytosis are blocked at low temperature, CNTs in the test medium are free to interact with cell surface components without subsequently entering the vacuolar compartment of the cells. After incubation, cells were washed, harvested, and the subsequent procedures for cell counting and lysate preparation were as described in the previous section. The amounts of cell-surface bound CNTs in the cell lysate samples were determined using the SDS-PAGE method (Wang et al. 2009; Wang et al. 2011), described next.

Quantitation of CNTs extracted in cell lysates by SDS-PAGE

To measure CNTs in cell lysates, a SDS-PAGE method described previously (Wang et al. 2009; Wang et al. 2011; Wang et al. 2016) for quantifying MWNTs and SWNTs extracted from mammalian cells and zebrafish embryos was used. In brief, aliquots of cell lysate and known amounts of CNT suspension standards of the same CNT type were mixed with 2X SDS sample loading buffer to a final buffer concentration of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 62.5 mM Tris-HCl and boiled for 3 min. Samples at various dilutions and volumes were subsequently loaded into the wells of a SDS polyacrylamide gel composed of a 4% stacking gel on top of a 10% resolving gel. An electric current was applied at a constant 100 V for 2 h. CNTs in the lysates bind SDS in the sample loading buffer to become negatively charged, migrating toward the anode upon electrophoresis. Even though the individual SWNTs have a mean diameter of <2 nm, the large aspect ratio of CNT particles prevents them from sieving through the pores in a 4% polyacrylamide gel mesh. Thus, CNTs accumulate at the bottom of the sample loading well during electrophoresis and form a sharp band. Following electrophoresis, optical images of the gels were obtained using a flatbed scanner (HP Scanjet G3110), and the pixel intensity of each band was quantified using *ImageJ* software. The known amount of standard CNTs loaded in lanes and their corresponding pixel intensities form a linear calibration curve that was used to determine the unknown amount of CNTs in cell lysates, based on the pixel

intensities of lysate bands in the same gel. A representative SDS-PAGE gel with C-SWNT bands and their corresponding pixel intensities used to construct a standard calibration curve for C-SWNT is shown in Figure S4.

The optical scan technique for measuring CNT content in gel bands was validated by a Raman large area scan technique, detailed in the Supplemental Methods. As an example, quantitation of C-SWNTs based on optical pixel intensities of the gel bands (Figure S4C) was independently verified with Raman spectroscopy that detects CNTs by their distinctive scattering peak at 1595 cm^{-1} (termed the G-mode). The corresponding Raman scattering signal (Figure S4D) is proportional to C-SWNT amounts in the gel bands, indicated by the linear calibration curves shown in Figure S4E. The Raman scan method for CNT quantitation is detailed in the Supplemental Methods section.

Rapid assay for relative quantitation of surface-bound and accumulated C-MWNTs in RAW 264.7 cells

Quantitation of surface bound and cell associated C-MWNTs in studies with SR-A antagonists required a large number of assays that exceeded the capacity of the SDS-PAGE method described in the preceding section. Therefore, a more rapid assay was developed that measured the amount of C-MWNTs associated with cells in multi-well plates by the absorbance of C-MWNTs at 500 nm. The number of cells in each well was subsequently assessed by the absorbance of crystal violet that bound to the cells at 590 nm, which is proportional to cell number. The relative amount of C-MWNTs per cell is provided by the A_{500}/A_{590} ratio. To measure the relative amount of C-MWNTs bound to RAW 264.7 cells in the rapid assay, 1×10^5 cells/well were seeded in 48-well plates and incubated at 37°C under regular cell culture condition for 24 h prior to the experiment. Cells were first chilled to 4°C in serum-free media on ice water for 30 min before exposure to C-MWNTs for 1 h at 4°C , conditions that allow binding, but not phagocytosis, of C-MWNTs in the absence of serum interference. After the incubation at 4°C , excess un-bound C-MWNTs in the media were removed by washing the cells 3 times with 0.5 mL/well fresh medium and 2 times with PBS. Cells in the 48-well plate were air dried and then fixed to the bottom of the wells by incubating in 4% PFA for 15 min at room temperature. After fixation, cells were rinsed briefly with MilliQ water and air dried overnight to allow complete dehydration. The next day, the average absorption intensity of each well at 500 nm was measured by scanning the plate with a microplate reader where 25 data points were acquired per well. The total amount of surface-bound C-MWNTs in cells within a well is proportional to the average A_{500} of the well.

The rapid assay was also used to estimate the accumulated C-MWNTs in cells after a 24-h exposure to C-MWNTs for comparison with the SDS-PAGE method of C-MWNT quantitation (Figure S8). 4×10^4 RAW 264.7 cells/well were seeded in 48-well plates and incubated at 37°C for 24 h before incubation in media that contain C-MWNTs at 37°C for the next 24 h. After the incubation, excess C-MWNTs were removed by washing with fresh medium and PBS and cells were air dried, fixed with PFA, rinsed with water, and dehydrated as described above. The plate was scanned at 500 nm wavelength and the average A_{500} is proportional to the amount of accumulated C-MWNTs in the cells within the well.

For the rapid assays, the number of cells in a well was estimated using a crystal violet assay, as described in our previous work (Wang et al. 2011). Briefly, after the plate was scanned at 500 nm, either for the 4 °C surface-bound or the 37 °C accumulated C-MWNTs, 0.4 mL of 0.1% crystal violet dye dissolved in 10% acetic acid was added to each well for 15 min at room temperature, allowing the dye to bind to nucleic acids in cells fixed in the well. Excess unbound dye was washed away with water and the bound dye was eluted with 400 µL/well of 10% acetic acid. 100 µL of the elution was transferred to a well in a 96-well plate and the amount of crystal violet dye in the elution was determined by scanning the 96-well plate to acquire the crystal violet dye specific absorption intensity at 590 nm, avoiding potential interference from other cell components and MWNTs retained in the 48-well plate. The relative amounts of cell-associated C-MWNTs in a well (represented by the average A_{500}) was normalized to the number of cells in the well (represented by the crystal violet intensity, A_{590}) to give a value (A_{500}/A_{590}) denoted as the C-MWNTs binding or accumulation index, depending on the C-MWNT exposure condition.

The rapid assay described above was used to compare the effects of SR-A antagonists on C-MWNT binding to RAW 264.7 cells at 4 °C in the absence of serum (Figure 9). The same assay was used to compare the accumulated C-MWNTs by RAW 264.7 cells after a 24-h exposure to various concentrations of C-MWNTs in the media at 37 °C (Figure S8). The adaptation of the rapid assay for assessing accumulated C-MWNTs was validated by comparing C-MWNT accumulation data acquired using the rapid assay (Figure S8) to data acquired using the legacy SDS-PAGE method under identical experimental conditions (replotted in Figure S8 from data in Figure 6). As shown in Figure S8, the shape of the accumulation curve as a function of C-MWNT concentration is essentially independent of the assay.

Results

Enhanced accumulation of C-MWNTs compared to P-MWNTs in epithelial and macrophage cell lines

The accumulation of P-MWNTs and C-MWNTs [CNT suspension samples (a) and (b) in Table 2] was surveyed and compared in several cell lines after a 24-h exposure at 37 °C (Figure 1). MWNTs were extracted from cells and quantified after SDS-PAGE by measuring the pixel intensity of the MWNT bands compared to known standards, as described in the Methods. For each cell line, the background signal from cells exposed to F108 in the absence of MWNTs was minimal regardless of the cell type. A549 human adenocarcinoma lung epithelial cells accumulated 6 times more C-MWNTs than P-MWNTs. RAW 264.7 mouse macrophage cells accumulated about the same amount of P-MWNTs as A549 cells, but C-MWNTs were accumulated over 100 times more than P-MWNTs. Human THP-1 monocytes, prior to differentiation into macrophages, had minimal accumulation of either MWNT type. However, after differentiation, dTHP-1 cells accumulated P-MWNTs at 281 fg/cell compared to 22,057 fg/cell for C-MWNTs, a 79-fold difference. These data indicated that several cell lines preferentially accumulated C-MWNTs compared to P-MWNTs, especially macrophages. The possibility that P-MWNTs, but not C-MWNTs, adversely

affected cell health, and that sick cells were thus unable to take up as many MWNTs, was examined next.

P-MWNTs and C-MWNTs are minimally toxic to macrophages

The toxicity of P-MWNTs and C-MWNTs was measured with two different assays. First, the ability of cells to proliferate was assessed after a 24-h exposure to different MWNT concentrations using a crystal violet assay that is not affected by the presence of CNTs (Wang et al. 2011). Controls were cells exposed to F108 alone and there was no significant decline in cell numbers for RAW 264.7 or dTHP-1 cells with either P-MWNTs or C-MWNTs (Figure 2A). LDH release from potentially damaged cells was measured by an assay that removes CNTs prior to LDH quantitation so that CNTs do not interfere with the assay (Wang et al. 2012; Forest et al. 2015). There was very little LDH release from either RAW 264.7 or dTHP-1 cells (Figure 2B). These data suggest that the low uptake of P-MWNTs is not caused by a toxic response specific for P-MWNTs that impairs cell growth or that damages the plasma membrane. In addition, the release of the pro-inflammatory cytokines hIL-1 β and hTNF- α by dTHP-1 cells exposed to P-MWNTs and C-MWNTs was compared. The results (Figure S1) indicated that there was moderate hIL-1 β and hTNF- α release in response to exposure to either P-MWNTs or C-MWNTs, although there was strong release in the positive control treated with lipopolysaccharide (LPS). The cytokine data argue that differences in the uptake of P-MWNTs and C-MWNTs cannot be attributed to a difference in macrophage activation that triggers cytokine release. The influence of MWNT length, type, and manufacturer on the difference in P-CNT and C-CNT accumulation was examined next.

The effect of CNT length, diameter, synthetic method, and manufacturer on CNT accumulation by cells

In addition to the carboxyl functionalization, the possibility that other differences among CNTs, such as length, diameter, synthetic method, and manufacturer, may also affect CNT accumulation was addressed. First, matching pairs of long and short P-MWNTs and C-MWNTs suspensions were made from MWNTs powders that were initially 10 – 30 μ m long [listed as regular-length MWNTs and regular-length MWNTs-COOH powders in Table 1] by sonication at different powers and times as described in Scheme 1B. These MWNT suspensions are listed in Table 2 as CNT suspension samples (c) through (f). The HDD was determined by DLS, which showed that the P-MWNTs and C-MWNTs sonicated for longer times at higher powers had smaller HDD's than those sonicated at less power and for less time (Figure S2A). Direct analysis of lengths by AFM provided average length values for P-MWNTs (L) of 807 nm and for C-MWNTs (L) of 554 nm compared to P-MWNTs (S) of 139 nm and C-MWNTs (S) of 199 nm, verifying that the different preparations had significantly different lengths (Figure S2B). However, accumulation studies using RAW 264.7 cells with long and short P-MWNTs and C-MWNTs demonstrated that P-MWNTs had low accumulation in the cells regardless of length and that both long and short C-MWNTs accumulated to high levels (Figure 3).

To determine whether the manufacturer source of the MWNTs influenced accumulation by RAW 264.7 cells, P-MWNTs and C-MWNTs from NanoCyl [CNT suspension samples (g)

and (h) in Table 2], were compared to MWNTs from NanoAmor [CNT suspension samples (a) and (b) in Table 2]. P-MWNTs regardless of manufacturer had low accumulation whereas C-MWNTs from both manufacturers accumulated to high levels (Figure 4A). In addition, accumulation measured at MWNT concentrations of 10 and 100 $\mu\text{g/mL}$ showed that C-MWNT accumulation was dose dependent (Figure 4A). As with NanoAmor MWNTs, neither P-MWNTs nor C-MWNTs from NanoCyl affected the proliferation of A549 or RAW 264.7 cells (Figure S3A).

The common denominator enhancing accumulation appears to be MWNT carboxylation. To see if this trend could be extended to SWNTs, the accumulation of P-SWNTs and C-SWNTs [CNT suspension samples (i) and (j) in Table 2] by A549 and RAW 264.7 cells were compared (Figure 4B). P-SWNTs were not significantly accumulated at either 100 or 200 $\mu\text{g/mL}$ by A549 cells; however, C-SWNTs at 200 $\mu\text{g/mL}$ were accumulated more than P-SWNTs by A549 cells, trends similar to P-MWNTs and C-MWNTs with this non-macrophage cell line. These parallel observations continued with RAW 264.7 cells where there was low accumulation of P-SWNTs but a high accumulation of C-SWNTs that was dose dependent (Figure 4B). The quantitation of cell-associated SWNTs by the SDS-PAGE method was verified by an independent technique based on the distinctive SWNT Raman scattering signal, as described in the Supplemental Methods and Figure S4. Thus, both the SDS-PAGE optical scanning and the Raman spectroscopy techniques provided evidence for the selective accumulation of C-SWNTs over P-SWNTs in macrophages. P-SWNTs were not significantly toxic to RAW 264.7 cells, although proliferation was mildly reduced by C-SWNTs at 200 $\mu\text{g/mL}$, the highest concentration tested (Figure S3B). We previously noted that the toxicity of this preparation of C-SWNTs correlates with the presence of oxidative debris fragments, not with the intact C-SWNTs themselves (Wang et al. 2011). Thus, the differential toxicity of P-SWNTs is not likely a factor in its low accumulation compared to C-SWNTs.

The effect of MWNT accumulation on subsequent phagocytosis of polystyrene beads by RAW 264.7 macrophages

The failure of cells to accumulate P-MWNTs could be explained if the P-MWNTs somehow inactivated phagocytosis, which would impair the subsequent uptake of P-MWNTs. Similarly, C-MWNTs might stimulate phagocytosis, resulting in high C-MWNT accumulation. To test for these possibilities, RAW 264.7 cells were first incubated with P-MWNTs or C-MWNTs (100 $\mu\text{g/mL}$) for 2 h or 20 h, washed, and then exposed to 0.9 μm polystyrene beads for 2 h, which are internalized by phagocytosis and can be counted in the cells. A relatively low concentration of beads was used in the medium to avoid a high number of beads in the cells, making them easier to count. For qualitative and quantitative assessments of the effects of MWNT accumulation on subsequent uptake of polystyrene beads, cell images were acquired and analyzed with phase-contrast microscopy. After a 2 h exposure to MWNTs, there was a mild reduction in beads/cell for cells exposed to P-MWNTs and a ~2-fold reduction in beads/cell for cells exposed to C-MWNTs (Figure 5). After a 20 h exposure to MWNTs, there was still only a mild reduction in beads/cell exposed to P-MWNTs, but a 6-fold decline in beads/cell after exposure to C-MWNTs (Figure 5). Typical images of RAW 264.7 cells exposed to polystyrene beads under the conditions of

this experiment are shown in Figure S5. These data are evidence that the uptake of P-MWNTs for up to 20 h does not strongly inhibit phagocytosis, nor does the uptake of C-MWNTs stimulate phagocytosis. Instead, the unexpected result was that the accumulation of C-MWNTs for 20 h strongly inhibits subsequent phagocytosis of beads by RAW 264.7 cells. The reason for this is not clear, but the ingestion of high C-MWNT amounts might sequester so much membrane in phagolysosomes that subsequent phagocytosis is inhibited.

Evidence for C-MWNT receptors on macrophages

The high accumulation of C-CNTs compared to P-CNTs by RAW 264.7 and dTHP-1 macrophages suggests that there may be receptors on the cell surface that selectively bind C-CNTs. If so, and if the receptors can be saturated as the concentration of CNTs in the medium increases, then the accumulation would be expected to plateau at higher CNT concentrations. When RAW 264.7 cells were incubated at 37 °C for 24 h with increasing doses of C-MWNTs between 0 and 200 µg/mL, uptake was linear to 100 µg/mL and then began to plateau (Figures 6 and S8). This is consistent with receptor-mediated uptake, but the interpretation could be complicated by the fact that exposure to C-MWNTs occurred at 37 °C for 24 h. At physiological temperature, internalized C-MWNTs could be lost from the cells by degradation (Kagan et al. 2014) or by recycling (Orecna et al. 2014). In addition, high cell loads of C-MWNTs appear to impair subsequent phagocytosis (see Figure 5). These factors could influence accumulation, which is a balance between the rate of uptake and the rate of loss. Therefore, binding studies were done with cells at 4 °C, a temperature at which phagocytosis does not normally occur (Silverstein et al. 1977). To verify that C-MWNT accumulation was blocked at 4 °C, the accumulation of C-MWNTs at 37 °C and 4 °C was compared as a function of time and it was evident that very little accumulation occurred at the low temperature (Figure S6). To measure cell surface binding, RAW 264.7 cells were incubated with 100 or 200 µg/mL MWNTs at 4 °C for 1 h in medium with 10% FBS and the cell-associated MWNTs were quantified (Figure 7A). Binding of P-MWNTs at 100 µg/mL was near background and increased slightly at 200 µg/mL. In contrast, dose-dependent binding of C-MWNTs was readily observed. The experiment was repeated with P- and C-SWNTs, and the results were similar: P-SWNT binding was low and dose-dependent, C-SWNT binding was evident (Figure 7B). These results directly support the conclusion that there are abundant binding sites on the surface of macrophages for C-MWNTs and C-SWNTs, but not for their pristine counterparts.

Serum inhibits the binding of C-MWNTs to the surface of macrophages

Although performing binding studies at 4 °C reduced potential complications to interpreting the data, FBS was still present in the medium, which introduces another set of complications. For example, CNT binding could be affected by FBS components binding to cells, or to the CNTs themselves, forming a protein corona that influences binding. Therefore, the binding of C-MWNTs and C-SWNTs to RAW 264.7 cells was tested at 4 °C as a function of FBS concentration in the medium (Figure 8). In the absence of FBS, the binding of C-MWNTs and C-SWNTs was remarkably higher than when FBS was present. As little as 1% FBS reduced the binding signal by more than 50% and binding was greatly reduced by FBS at 5%. The strong inhibition of binding by FBS suggests that a component of the FBS interacts with C-CNTs to reduce the binding capacity of C-CNTs, or

alternatively the FBS component binds the cell surface and inhibits C-CNT binding. The latter possibility is interesting because it suggests that a FBS component could be a competitive inhibitor of C-CNT binding, which might aid identifying the receptor(s) specific for C-CNTs in macrophages.

Evidence that SR-As are involved in C-MWNT binding to cells

SR-As are involved in the binding of numerous anionic ligands to macrophages (Canton et al. 2013; Zani et al. 2015) and have been implicated by indirect evidence in the binding of CNTs to cells (Hirano et al. 2008; Dutta et al. 2007; Gao et al. 2011; Singh et al. 2012). To see whether macrophage SR-As might be involved in C-MWNT binding, the effect of known SR-A binding antagonists fucoidan and dextran sulfate on C-MWNT binding was assessed. Chondroitin sulfate, an anionic polysaccharide that is not an SR-A antagonist, was used as a control. SR-A antagonists were first incubated with cells in the absence of C-MWNTs to avoid potential interactions of the antagonist with the C-MWNTs, an approach used by others (Gao et al. 2011; Singh et al. 2012). When RAW 264.7 cells were exposed to C-MWNTs (100 µg/mL) in medium at 4 °C in the absence of serum and in the presence of increasing amounts of fucoidan or dextran sulfate, the amount of C-MWNTs bound to the cells declined and leveled off to a maximum of about 50% compared to cells not exposed to the antagonists (Figure 9). Chondroitin sulfate had no effect on C-MWNT binding. These data suggest that there may be at least two types of receptors involved in C-MWNT binding, about half may be SR-A receptors sensitive to classical SR-A antagonists, and the other half may not be.

If SR-A antagonists inhibit binding of C-MWNTs to the cell surface, they should also inhibit the accumulation of C-MWNTs by cells at physiological temperature. To assess this, cells were first exposed to serum-free medium containing either no additions, with increasing amounts of dextran sulfate (100, 300, or 500 µg/mL), or with a single concentration of chondroitin sulfate (500 µg/mL) for 30 min followed by a 3 h incubation at 37 °C with C-MWNTs (200 µg/mL) in the presence of media. The amount of C-MWNTs accumulated was reduced by about 30% when dextran sulfate was present, but was not affected by chondroitin sulfate (Figure S7), supporting the idea that C-MWNT accumulation is partially mediated by SR-As.

Discussion

Numerous lines of evidence suggest that cultured cells, especially macrophages, accumulate more C-CNTs compared to P-CNTs and that the accumulation is due to an inherent chemical property of the C-CNTs:

1. Using an assay that quantifies CNTs extracted from cells, human epithelial A549 cells accumulated 6 times more C-MWNTs than P-MWNTs over a 24 h period. Murine macrophage RAW 264.7 cells accumulated 100 times more C-MWNT than P-MWNTs. Another macrophage line, human dTHP-1 cells, accumulated almost 80 times more C-MWNTs than P-MWNTs whereas its undifferentiated precursor monocytes (undTHP-1) did not over accumulate C-MWNTs. The latter observation is consistent with the possibility that differentiation of THP-1

monocytes into macrophages upregulates the receptor(s) responsible for the high accumulation of C-MWNTs. The accumulation of C-MWNTs was also dose and time dependent.

2. When MWNTs in the SDS-PAGE bands were quantified by a laser scanning Raman technique, which is based on a different physical principle than the optical flatbed scanner method, the results were similar: More C-MWNTs were accumulated than P-MWNTs. Thus, the high accumulation of C-MWNTs is not likely to be an artifact of one measurement method.
3. Neither C-MWNTs nor P-MWNTs adversely affected the ability of cells to grow nor did they damage the plasma membrane, evidence that the low accumulation of P-MWNTs is not the consequence of cell damage inflicted by P-MWNTs. However, the accumulation of C-MWNTs by RAW 264.7 cells did suppress the subsequent phagocytosis of polystyrene beads, especially after a 20-h exposure to C-MWNTs, suggesting that there was a physiological consequence to accumulating high amounts of C-MWNTs. The reason why phagocytosis is impaired after C-MWNT accumulation is unknown, but it is possible that sequestration of so much membrane in phagolysosomes that contain the C-MWNTs hinders subsequent phagocytosis.
4. Both C-MWNTs and P-MWNTs elicited a moderate release of the pro-inflammatory cytokines TNF- α and IL-1 β by dTHP-1 cells, but there was little difference between them, despite differences in binding and internalization. This suggests that the difference in accumulation between the two MWNT types does not originate from a difference in macrophage activation. Moreover, tests for possible LPS presence in the CNT preparations were negative. Nevertheless, all CNT powders were baked to inactivate LPS prior to suspension preparation arguing that LPS contamination is not a source of the selective accumulation of C-MWNTs.
5. Short and long C-MWNTs were accumulated by cells to the same extent, although the longest MWNTs studied were not as long as those involved in frustrated phagocytosis (Donaldson et al. 2010). Further, neither MWNT synthetic method nor manufacturer affected the common observation that more C-MWNTs than P-MWNTs were accumulated by cells, indicating that differences in these parameters do not underlie the accumulation difference.
6. C-SWNTs were also accumulated more than P-SWNTs, evidence that neither the number of nanotube walls nor nanotube diameter are critical parameters in C-CNT uptake.
7. DLS data of C-CNTs and P-CNTs showed very little difference in the particle size distribution, suggesting that the high accumulation of C-CNTs is not likely due to a difference in the aggregation states of C-CNTs and P-CNTs.

The evidence summarized in the previous paragraph suggests that cells may have receptors that selectively bind C-CNTs compared to P-CNTs. The binding of C-CNTs to cells was tested under two experimental conditions. First, RAW 264.7 cells were chilled to 4 °C and

incubated in medium with serum that contained different concentrations of C-MWNTs or P-MWNTs, washed, and the cell-associated CNTs were quantified. Short and long C-MWNTs and P-MWNTs were also compared in a separate experiment. The results indicated a dose-dependent association of C-MWNTs with cells, but much lower association of P-MWNTs with cells. Similar results were found with C-SWNTs and P-SWNTs, indicating that both types of C-CNTs appeared to bind cells. These experiments were performed at low temperature to avoid any influence of temperature-dependent activities on the results, such as phagocytosis, cell signaling, or other energy-dependent functions. However, FBS was present in the medium, which could influence the binding. Therefore, binding was tested with MWNTs in medium at low temperature and in the absence of FBS. C-MWNTs were again bound by cells, but surprisingly at much higher amounts than in the presence of FBS. The fact that inhibition of binding by FBS was dose dependent further suggests that FBS contains binding inhibitors. The results reported here lead to the hypothesis that macrophages have binding sites for F108-coated C-CNTs but not their pristine counterparts. A549 cells also accumulate a statistically significant amount of C-CNTs, although not as much as macrophages, suggesting the binding sites for C-CNTs may be present at reduced levels or affinities on cells other than macrophages.

Because SR-As have been implicated in CNT binding to cells in other studies (Hirano et al. 2008; Dutta et al. 2007; Gao et al. 2011; Singh et al. 2012), the effect of SR-A antagonists (fucoidan and dextran sulfate) on C-MWNT binding to RAW 264.7 cells was tested at low temperature and in the absence of serum, which revealed that binding was reduced by the antagonists by about 50%. The accumulation of C-MWNTs by RAW 264.7 cells at 37 °C was also reduced, but not completely inhibited. These data provide mechanistic insight into the receptors used by C-MWNTs and suggest, but do not prove, that about half of the binding to macrophages is via SR-A receptors, while half may be mediated by one or more different receptors.

The C-CNT receptors appear to be highly sensitive to the presence of carboxyl groups on C-CNTs. The wt% of the carboxyl groups present in the C-CNTs used in this study was more than 1% but less than 8%, depending on the manufacturer and type of CNT. There was a slight difference in zeta potential between C-CNTs and P-CNTs in water, with C-CNTs being more negative, as expected. In medium with FBS, the zeta potential of C-CNTs was significantly reduced, remained somewhat more negative than P-CNTs, and it is not clear how the receptors distinguish between C-CNTs and P-CNTs. However, the distribution of carboxyl groups on oxidized CNTs is not believed to be uniform; rather, there tend to be more carboxyl groups at defect sites and especially at CNT ends (Wong et al. 1998; Chen et al. 1998; Sun et al. 2002; Zhang et al. 2003). Thus, charge differences localized at C-CNT ends could be higher than would appear by overall zeta potential values and may underlie the receptor recognition of C-CNTs.

The inhibition of CNT binding to cells by serum was unexpected and was observed for both C-MWNTs and C-SWNTs. One explanation is that one or more serum components interact with C-CNTs and interfere with binding to cells. Another explanation is that a serum component binds to the cells and interferes with C-CNT binding. The latter possibility is particularly interesting because one predicted scenario is that a serum component competes

for binding with C-CNTs to the same receptor. If so, identifying the inhibiting serum component may facilitate a more refined identification of C-CNT receptors, an approach that is under investigation.

Conclusions

As far as we are aware, this paper presents the first direct evidence for the existence of cell surface receptor(s) that bind C-CNTs under conditions where neither cell physiological activity nor serum components are present to confound the interpretation of the results. The enhanced binding of C-CNTs to cells is consistent with their increased accumulation at 37 °C. The approach described here is a model by which complexities of CNT interaction with cells can be further explored. For example, serum inhibited the binding of C-CNTs to cells, either because serum components interacted with the C-CNTs or with the cells, or both, and it may be possible to parse the effects of serum components. For another example, the binding of C-MWNTs to RAW 264.7 cells was reduced by about 50% in the presence of SR-A antagonists at low temperature and in the absence of serum, suggesting that half of the C-MWNT binding to macrophages may be via SR-A receptors, while half may be mediated by different receptor(s). It may be possible to identify the other C-CNT receptor(s) by adding back individual FBS components to screen for factors that interfere with C-CNT binding using the rapid binding assay at 4 °C. Ultimately, identifying CNT receptor(s) could lead to the rational engineering of safer CNTs and other carbon nanomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the National Institute for Environmental Health Sciences (Grant R15-ES023666; P.P., R.D.), the National Cancer Institute (Grant R15-CA152917; R.D., P.P.), and the University of Arizona/Semiconductor Research Corporation Engineering Research Center for Environmentally Benign Semiconductor Manufacturing (Grant ERC425-048; R.D., P.P., R.W.) for supporting this work. The authors are grateful to the Undergraduate Research Fund of the University of Texas at Dallas School of Natural Sciences & Mathematics for support of undergraduate students. The authors also are grateful to Dr. Carole Mikoryak for reading the manuscript and to Dr. Vasanth Murali for early work on Raman microscopy that was not included in the manuscript.

REFERENCES

- Allegri M, Perivoliotis DK, Bianchi MG, Chiu M, Pagliaro A, Koklioti MA, Trompeta A-FA, Bergamaschi E, Bussolati O, Charitidis CA. 2016 Toxicity determinants of multi-walled carbon nanotubes: The relationship between functionalization and agglomeration. *Toxicol Rep* 3:230–243. [PubMed: 28959543]
- Battigelli A, Menard-Moyon C, Da Ros T, Prato M, Bianco A. 2013 Endowing carbon nanotubes with biological and biomedical properties by chemical modifications. *Adv Drug Del Rev* 65:1899–1920.
- Bilal P, Katsigiannopoulos D, Avgeropoulos A, Sakellariou G. 2014 Non-covalent functionalization of carbon nanotubes with polymers. *RSC Adv* 4:2911–2934.
- Boyer PD, Holt BD, Islam MF, Dahl KN. 2013 Decoding membrane-versus receptor-mediated delivery of single-walled carbon nanotubes into macrophages using modifications of nanotube surface coatings and cell activity. *Soft Matter* 9:758–764.
- Braun EI, Pantano P. 2014 The importance of an extensive elemental analysis of single-walled carbon nanotube soot. *Carbon* 77:912–919. [PubMed: 25110357]

- Canton J, Neculai D, Grinstein S. 2013 Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol* 13:621–634. [PubMed: 23928573]
- Chen G-Y, Yang H-J, Lu C-H, Chao Y-C, Hwang S-M, Chen C-L, Lo K-W, Sung L-Y, Luo W-Y, Tuan H-Y, Hu Y-C. 2012 Simultaneous induction of autophagy and toll-like receptor signaling pathways by graphene oxide. *Biomaterials* 33:6559–6569. [PubMed: 22704844]
- Chen J, Hamon MA, Hu H, Chen Y, Rao AM, Eklund PC, Haddon RC. 1998 Solution properties of single-walled carbon nanotubes. *Science* 282:95–98. [PubMed: 9756485]
- Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. 2010 The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS ONE* 5:e8668 (10 pp).
- Donaldson K, Murphy FA, Duffin R, Poland CA. 2010 Asbestos, carbon nanotubes and the pleural mesothelium: A review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol* 7:5 (17 pp). [PubMed: 20565709]
- Dutta D, Sundaram SK, Teeguarden JG, Riley BJ, Fifield LS, Jacobs JM, Addleman SR, Kaysen GA, Moudgil BM, Weber TJ. 2007 Adsorbed proteins influence the biological activity and molecular targeting of nanomaterials. *Toxicol Sci* 100:303–315. [PubMed: 17709331]
- Forest V, Figarol A, Boudard D, Cottier M, Grosseau P, Pourchez J. 2015 Adsorption of lactate dehydrogenase enzyme on carbon nanotubes: How to get accurate results for the cytotoxicity of these nanomaterials. *Langmuir* 31:3635–3643. [PubMed: 25768724]
- Gao N, Zhang Q, Mu Q, Bai Y, Li L, Zhou H, Butch ER, Powell TB, Snyder SE, Jiang G, Yan B. 2011 Steering carbon nanotubes to scavenger receptor recognition by nanotube surface chemistry modification partially alleviates NF κ B activation and reduces its immunotoxicity. *ACS Nano* 5:4581–4591. [PubMed: 21595480]
- Hirano S, Kanno S, Furuyama A. 2008 Multi-walled carbon nanotubes injure the plasma membrane of macrophages. *Toxicol Appl Pharmacol* 232:244–251. [PubMed: 18655803]
- Holt BD, Dahl KN, Islam MF. 2011 Quantification of uptake and localization of bovine serum albumin-stabilized single-wall carbon nanotubes in different human cell types. *Small* 7:2348–2355. [PubMed: 21626688]
- Jackson P, Jacobsen NR, Baun A, Birkedal R, Kuhnelt D, Jensen KA, Vogel U, Wallin H. 2013 Bioaccumulation and ecotoxicity of carbon nanotubes. *Chem Cent J* 7:154 (21 pp). [PubMed: 23369571]
- Jariwala D, Sangwan VK, Lauhon LJ, Marks TJ, Hersam MC. 2013 Carbon nanomaterials for electronics, optoelectronics, photovoltaics, and sensing. *Chem Soc Rev* 42:2824–2860. [PubMed: 23124307]
- Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Aschberger K, Stone V. 2010 A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. *Nanotoxicology* 4:207–246. [PubMed: 20795897]
- Kagan VE, Kapralov AA, St. Croix CM, Watkins SC, Kisin ER, Kotchey GP, Balasubramanian K, Vlasova II, Yu J, Kim K, Seo W, Mallampalli RK, Star A, Shvedova AA. 2014 Lung macrophages “digest” carbon nanotubes using a superoxide/peroxynitrite oxidative pathway. *ACS Nano* 8:5610–5621. [PubMed: 24871084]
- Kim J-E, Kang S-H, Moon Y, Chae J-J, Lee AY, Lee J-H, Yu K-N, Jeong DH, Choi M, Cho M-H. 2014 Physicochemical determinants of multiwalled carbon nanotubes on cellular toxicity: influence of a synthetic method and post-treatment. *Chem Res Toxicol* 27:290–303. [PubMed: 24405247]
- Kolosnjaj-Tabi J, Just J, Hartman KB, Laoudi Y, Boudjemaa S, Alloyeau D, Szwarc H, Wilson LJ, Moussa F. 2015 Anthropogenic carbon nanotubes found in the airways of parisian children. *EBioMedicine* 2:1697–1704. [PubMed: 27014740]
- Kunzmann A, Andersson B, Thurnherr T, Krug H, Scheynius A, Fadeel B. 2011 Toxicology of engineered nanomaterials: Focus on biocompatibility, biodistribution and biodegradation. *Biochim Biophys Acta - Gen Subjects* 1810:361–373.
- Li R, Wang X, Ji Z, Sun B, Zhang H, Chang CH, Lin S, Meng H, Liao YP, Wang M, Li Z, Hwang AA, Song TB, Xu R, Yang Y, Zink JI, Nel AE, Xia T. 2013 Surface charge and cellular processing of

covalently functionalized multiwall carbon nanotubes determine pulmonary toxicity. *ACS Nano* 7:2352–2368. [PubMed: 23414138]

- Liu Z, Tabakman S, Welscher K, Dai H. 2009 Carbon nanotubes in biology and medicine: In vitro and in vivo detection, imaging and drug delivery. *Nano Res* 2:85–120. [PubMed: 20174481]
- Mortimer M, Petersen EJ, Buchholz BA, Orias E, Holden PA. 2016 Bioaccumulation of multiwall carbon nanotubes in *tetrahymena thermophila* by direct feeding or trophic transfer. *Environ Sci Technol* 50:8876–8885. [PubMed: 27398725]
- Murali VS, Wang R, Mikoryak CA, Pantano P, Draper RK. 2016 The impact of subcellular location on the near infrared-mediated thermal ablation of cells by targeted carbon nanotubes. *Nanotechnology* 27:425102 (15 pp).
- Nakata T 1994 Destruction of challenged endotoxin in a dry heat oven. *PDA J Pharm Sci Technol* 48:59–63.
- Orecna M, De Paoli SH, Janouskova O, Tegegn TZ, Filipova M, Bonevich JE, Holada K, Simak J. 2014 Toxicity of carboxylated carbon nanotubes in endothelial cells is attenuated by stimulation of the autophagic flux with the release of nanomaterial in autophagic vesicles. *Nanomedicine* 10:939–948. [PubMed: 24566271]
- Pantano P, Draper RK, Mikoryak C, Wang R. 2012 Electrophoretic methods to quantify carbon nanotubes in biological cells In: D'Souza F, Kadish KM, eds. *Handbook of Carbon Nanomaterials*. Singapore: World Scientific Publishers, 84–107.
- Paradise M, Goswami T. 2007 Carbon nanotubes - Production and industrial applications. *Mater Design* 28:1477–1489.
- Petersen EJ, Henry TB. 2012 Methodological considerations for testing the ecotoxicity of carbon nanotubes and fullerenes: Review. *Environ Toxicol Chem* 31:60–72. [PubMed: 21994158]
- Petersen EJ, Huang Q, Weber JWJ. 2008 Bioaccumulation of radio-labeled carbon nanotubes by *eisenia foetida*. *Environ Sci Technol* 42:3090–3095. [PubMed: 18497171]
- Petersen EJ, Zhang L, Mattison NT, O'Carroll DM, Whelton AJ, Uddin N, Nguyen T, Huang Q, Henry TB, Holbrook RD, Chen KL. 2011 Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. *Environ Sci Technol* 45:9837–9856. [PubMed: 21988187]
- Pondman KM, Sobik M, Nayak A, Tsolaki AG, Jäkel A, Flahaut E, Hampel S, ten Haken B, Sim RB, Kishore U. 2014 Complement activation by carbon nanotubes and its influence on the phagocytosis and cytokine response by macrophages. *Nanomed Nanotechnol Biol Med* 10:1287–1299.
- Qu G, Liu S, Zhang S, Wang L, Wang X, Sun B, Yin N, Gao X, Xia T, Chen J-J, Jiang G-B. 2013 Graphene oxide induces toll-like receptor 4 (TLR4)-dependent necrosis in macrophages. *ACS Nano* 7:5732–5745. [PubMed: 23734789]
- Schnorr JM, Swager TM. 2010 Emerging applications of carbon nanotubes. *Chem Mater* 23:646–657.
- Shvedova AA, Kagan VE, Fadeel B. 2010 Close encounters of the small kind: Adverse effects of man-made materials interfacing with the nano-cosmos of biological systems. *Annu Rev Pharmacol Toxicol* 50:63–88. [PubMed: 20055698]
- Silverstein SC, Steinman RM, Cohn ZA. 1977 Endocytosis. *Annu Rev Biochem* 46:669–722. [PubMed: 332066]
- Singh RP, Das M, Thakare V, Jain S. 2012 Functionalization density dependent toxicity of oxidized multiwalled carbon nanotubes in a murine macrophage cell line. *Chem Res Toxicol* 25:2127–2137. [PubMed: 22994501]
- Sun YP, Fu K, Lin Y, Huang W. 2002 Functionalized carbon nanotubes: Properties and applications. *Acc Chem Res* 35:1096–1104. [PubMed: 12484798]
- Turabekova M, Rasulev B, Theodore M, Jackman J, Leszczynska D, Leszczynski J. 2014 Immunotoxicity of nanoparticles: A computational study suggests that CNTs and C60 fullerenes might be recognized as pathogens by Toll-like receptors. *Nanoscale* 6:3488–3495. [PubMed: 24548972]
- Wang G, Zhang J, Dewilde AH, Pal AK, Bello D, Therrien JM, Braunschut SJ, Marx KA. 2012 Understanding and correcting for carbon nanotube interferences with a commercial LDH cytotoxicity assay. *Toxicology* 299:99–111. [PubMed: 22634321]

- Wang R, Hughes T, Beck S, Vakil S, Li S, Pantano P, Draper RK. 2013 Generation of toxic degradation products by sonication of Pluronic^R dispersants: Implications for nanotoxicity testing. *Nanotoxicology* 7:1272–1281. [PubMed: 23030523]
- Wang R, Meredith NA, Lee M, Jr., Deutsch D, Miadzevskaya L, Braun E, Pantano P, Harper S, Draper R. 2016 Toxicity assessment and bioaccumulation in zebrafish embryos exposed to carbon nanotubes suspended in Pluronic(R) F-108. *Nanotoxicology* 10:689–98. [PubMed: 26559437]
- Wang R, Mikoryak C, Chen E, Li S, Pantano P, Draper RK. 2009 Gel electrophoresis method to measure the concentration of single-walled carbon nanotubes extracted from biological tissue. *Anal Chem* 81:2944–2952. [PubMed: 19296592]
- Wang R, Mikoryak C, Li S, Bushdiecker D, 2nd, Musselman IH, Pantano P, Draper RK. 2011 Cytotoxicity screening of single-walled carbon nanotubes: detection and removal of cytotoxic contaminants from carboxylated carbon nanotubes. *Mol Pharm* 8:1351–61. [PubMed: 21688794]
- Wong SS, Joselevich E, Woolley AT, Cheung CL, Lieber CM. 1998 Covalently functionalized nanotubes as nanometre-sized probes in chemistry and biology. *Nature* 394:52–55. [PubMed: 9665127]
- Zani I, Stephen S, Mughal N, Russell D, Homer-Vanniasinkam S, Wheatcroft S, Ponnambalam S. 2015 Scavenger receptor structure and function in health and disease. *Cells* 4:178. [PubMed: 26010753]
- Zhang J, Zou H, Qing Q, Yang Y, Li Q, Liu Z, Guo X, Du Z. 2003 Effect of chemical oxidation on the structure of single-walled carbon nanotubes. *J Phys Chem B* 107:3712–3718.
- Zhang T, Tang M, Kong L, Li H, Zhang T, Zhang S, Xue Y, Pu Y. 2012a Comparison of cytotoxic and inflammatory responses of pristine and functionalized multi-walled carbon nanotubes in RAW 264.7 mouse macrophages. *J Hazard Mater* 219–220:203–212.
- Zhang Y, Mu Q, Zhou H, Vrijens K, Roussel MF, Jiang G, Yan B. 2012b Binding of carbon nanotube to BMP receptor 2 enhances cell differentiation and inhibits apoptosis via regulating bHLH transcription factors. *Cell Death Dis* 3:e308 (12 pp).
- Zhang Y, Wu L, Jiang C, Yan B. 2015 Reprogramming cellular signaling machinery using surface-modified carbon nanotubes. *Chem Res Toxicol* 28:296–305. [PubMed: 25536342]
- Zhao X, Liu R. 2012 Recent progress and perspectives on the toxicity of carbon nanotubes at organism, organ, cell, and biomacromolecule levels. *Environ Int* 40:244–255. [PubMed: 22244841]

Figure 1. Accumulation of P-MWNTs and C-MWNTs by A549 epithelial cells, RAW 264.7 macrophages, undifferentiated THP-1 (undTHP-1) monocytes, and differentiated THP-1 (dTHP-1) macrophages.

MWNTs suspended in 0.2 mM F108 [CNT suspension samples (a) and (b) in Table 2] were mixed with an equal volume of 2X medium containing 20% FBS to give a final MWNT concentration of 100 $\mu\text{g/mL}$ and 10% FBS. The cells were then incubated in medium that contain either F108 alone (F108), P-MWNTs (P-M), or C-MWNTs (C-M) at 37 °C for 24 h. MWNTs were extracted from cells and quantified by the SDS-PAGE method. Numbers above the bars are the average fg MWNTs/cell. Data is the average of 3 experiments $\pm$ SEM. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ by comparing P-M to C-M treated groups.

Figure 2. Cytotoxicity of P-MWNTs and C-MWNTs in RAW 264.7 and dTHP-1 macrophages. MWNTs suspended in 0.2 mM F108 [CNT suspension samples (a) and (b) in Table 2] were mixed with an equal volume of 2X medium containing 20% FBS to give a final MWNT concentration of 50, 100, or 200 μg/mL in 10% FBS. Exposure to F108 alone is the untreated control, P-M is P-MWNTs, and C-M is C-MWNTs. Cells in 48-well plates were incubated at 37 °C under standard cell culture conditions for 24 h prior to the experiment. (A) Cell proliferation after a 24-h exposure to F108 alone or MWNTs was determined by the crystal violet assay where the proliferation of control was set as 100%, and (B) membrane

integrity was assessed using the LDH release assay where the total LDH detected in the corresponding cell lysate was set as 100%. See Methods for assay details. Data is the average of quadruple samples in 3 independent experiments $\pm$ SD.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Accumulation of P-MWNT(L), P-MWNT(S), C-MWNT(L), and C-MWNT(S) in RAW 264.7 macrophages.

Pristine and carboxylated MWNTs of longer (L) and shorter (S) lengths suspended in 0.2 mM F108 were prepared by procedures outlined in Scheme 1B. The four suspensions prepared are shown in Table 2 as samples (c) – (f). Each sample was mixed with an equal volume of 2X medium containing 20% FBS to give a final MWNT concentration of 100 µg/mL in 10% FBS. RAW 264.7 cells were exposed to media that contain F108 alone (F108), pristine long MWNTs (P-M(L)), pristine short MWNTs (P-M(S)), carboxylated long MWNTs (C-M(L)), or carboxylated short MWNTs (C-M(S)) at 37 °C for 24 h. MWNTs were extracted from cells and quantified by the SDS-PAGE method. Data is the average of 5 independent experiments $\pm$ SD.

Figure 4. Accumulation of P- and C-CNTs of different diameters, types, and manufacturers by A549 epithelial cells and RAW 264.7 macrophages.

Pristine and carboxylated MWNTs and SWNTs suspended in 0.2 mM F108 were mixed with an equal volume of 2X medium containing 20% FBS to give a final CNT concentration of 10, 100, or 200 μg/mL and 10% FBS. Cells were incubated in media with indicated CNT suspension sample at 37 °C for 24 h. After incubation, CNTs were extracted from cells and quantified by the SDS-PAGE method. (A) RAW 264.7 cells were exposed to P-M (P-MWNTs) and C-M (C-MWNTs) from NanoAmor [CNT suspensions (a) and (b) in Table 2] or from NanoCyl [CNT suspensions (g) and (h) in Table 2] at 10 or 100 μg/mL. (B) Epithelial A549 and macrophage RAW 264.7 cells were exposed to P-S (P-SWNTs) and C-S (C-SWNTs), both purchased from CSI [CNT suspensions (i) and (j) in Table 2], at SWNT

concentrations of 100 or 200 $\mu\text{g/mL}$ for A549 cells and 10 or 100 $\mu\text{g/mL}$ for RAW 264.7 cells. Data is the average of quadruple samples in 2 independent experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. Phagocytosis of polystyrene beads in RAW 264.7 cells with accumulated P-MWNTs and C-MWNTs.

Raw 264.7 cells seeded in 4-well plates on glass coverslips were incubated at 37 °C under standard cell culture conditions for 24 h. Test samples included 0.2 mM F108 solution alone and 200 µg/mL of P-MWNTs or C-MWNTs suspended in 0.2 mM F108 [CNT suspensions (g) and (h) in Table 2]. Control medium (Cntl) contained no F108 or MWNTs. Test media contained a test sample mixed with an equal volume of 2X medium to give a 0.1 mM F108 alone (F108), 100 µg/mL P-MWNTs (P-M) or 100 µg/mL C-MWNTs (C-M) in 0.1 mM F108 solution. Cells were incubated in control or test media for 2 or 20 h at 37 °C, washed, then exposed to 25 µg/mL of non-functionalized 0.9 µm polystyrene beads for 2 h at 37 °C. After the incubation, cells were washed thoroughly, fixed with 4% PFA and mounted on glass slides. Phase contrast images were acquired and the cell count and the number of beads within cells were counted using ImageJ Cell Counter software. Data is the average of 14 images analyzed per sample, with an average of 128 ± 9 cells per image, acquired from 3 independent experiments $\pm$ SD. Numbers in the bars are the average number of beads per cell and the error bars are SD. * is for $P < 0.05$ and ** is for $P < 0.005$, compared to the control in the absence of F108 and MWNTs.

Figure 6. Accumulation of C-MWNTs by RAW 264.7 macrophages as a function of C-MWNT concentration in media.

C-MWNTs suspended in 0.2 mM F108 [CNT suspension sample (b) in Table 2] were mixed with an equal volume of 2X medium containing 20% FBS to give a desired final C-MWNT concentration and 10% FBS. RAW 264.7 cells were exposed to C-MWNTs at 50, 100, 150, or 200 $\mu\text{g/mL}$ for 24 h at 37 °C. After incubation, C-MWNTs were extracted from cells and quantified by the SDS-PAGE method. Data is the average of duplicates in 3 independent experiments $\pm$ SEM.

Figure 7.

Surface binding of P-MWNTs, C-MWNTs, P-SWNTs, and C-SWNTs to RAW 264.7 cells at 4 °C. Pristine and carboxylated MWNTs and SWNTs were suspended in 0.2 mM F108, listed in Table 2 as (a) P-MWNT, (b) C-MWNT, (i) P-SWNT, and (j) C-SWNT. Each suspension was mixed with an equal volume of 2X medium containing 20% FBS to give a final FBS concentration of 10% and CNT concentrations of 100 or 200 µg/mL, as indicated. RAW 264.7 macrophage cells were incubated (A) with P- and C-MWNT suspensions (P-M for P-MWNTs and C-M for C-MWNTs) or (B) with P- and C-SWNT suspensions (P-S for P-SWNTs and C-S for C-SWNTs) for 1 h at 4 °C. After incubation, surface-bound CNTs were extracted and quantified by the SDS-PAGE method. Numbers above the bars are the

average fg CNTs/cell. Ctrl is without treatment and F108 is with the surfactant alone. Data is the average of 3 independent experiments $\pm$ SD. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ compared to F108 alone in the absence of CNTs.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8. FBS inhibits C-MWNT and C-SWNT binding to RAW 264.7 cells at 4 °C.

200 µg/mL C-MWNTs and C-SWNTs suspended in 0.2 mM F108 [CNT suspensions (b) and (j) in Table 2] were mixed with an equal volume of 2X medium supplemented with 2 to 40% FBS to give final FBS concentrations of 1 to 20% and a constant CNT concentration of 100 µg/mL. RAW 264.7 macrophages were pre-chilled to 4 °C then incubated with the test media at 4 °C for 1 h, as in Figure 7 except that various amounts of FBS were included in this assay. Surface-bound C-CNTs were extracted and quantified using the SDS-PAGE method. Data is the average of 3 independent experiments ± SD. Cell culture media supplemented with 10% FBS, indicated by the arrow, was the standard FBS amount used for cell culture in this paper, unless specified otherwise.

Figure 9. Effects of SR-A inhibitors on C-MWNT binding to RAW 264.7 cells at 4 °C. RAW 264.7 cells in 48-well plates were incubated at 37 °C under standard cell culture conditions for 24 h prior to the experiment. Test cells were treated with either fucoidan, dextran sulfate, or chondroitin sulfate in serum-free media and in the absence of C-MWNTs for 30 min at 4 °C and then washed. Test media were prepared by mixing 200 µg/mL C-MWNTs in 0.2 mM F108 [CNT suspension (h) in Table 2] with an equal volume of 2X serum-free medium to give a C-MWNTs concentration of 100 µg/mL followed by the addition of fucoidan, dextran sulfate, or chondroitin sulfate at various concentrations, as indicated in the figure. Control medium contained the same 100 µg/mL C-MWNTs but no inhibitor was added. Cells were incubated with either the control or test media for 1 h at 4 °C. After the incubation, the relative amounts of surface-bound C-MWNTs were measured using the rapid assay described in the Methods section. The amount of C-MWNTs bound to control cells in the absence of any reagent was set to 100%. Data is the average of quadruple samples in 3 independent experiments $\pm$ SEM.

Scheme 1.

Preparation of Pluronic® F108 coated CNT suspensions by sonication, centrifugation, and dialysis.

Table 1.

Properties of CNT materials provided by manufacturers.

CNT Type	Manufacturer	Synthetic Method	Product Name (Stock/Lot No.)	Surface Functionality	Content of -COOH (wt%)	Length (µm)	Diameter / OD/ID (nm)	Purity (% carbon)	SSA ² (m ² /g)	Product Description
MWNT	Nanostructured & Amorphous Materials (NanoAmor)	CCVD	MWNTs (1205Y1)	-	-	10–30	10–20/ 5–10	> 95	> 200	Regular-length MWNTs
			MWNTs-COOH (1269Y1F)	-COOH	1.9–2.1	10–30	10–20/ 5–10	> 95	> 200	Regular-length MWNTs-COOH functionalized, dispersible
			Short MWNTs (1236Y1S)	-	-	0.5–2	10–20/ 5–10	> 95	> 200	Short-length MWNTs (easily dispersible)
			Short MWNTs-COOH (1256Y1F)	-COOH	1.9–2.1	0.5–2	10–20/ 5–10	> 95	> 200	Short-length MWNTs-COOH functionalized, dispersible
	Nanocyl SA (NanoCyl)	CCVD	NC3150 TM (100426)	-	-	< 1.0	9.5	> 95	-	Research grade short thin MWCNT 95+%C purity
SWNT	Carbon Solutions, Inc (CSI)	Electric Arc Discharge	NC3151 TM (120828)	-COOH	< 8.0	< 1.0	9.5	> 95	-	Research grade short thin MWCNT 95+%C purity & surface modified COOH
			P2-SWNT (02–419)	-	-	0.5–3	1.4	> 90	-	Purified, low functionality
			P3-SWNT (03-A010)	-COOH	1.0–3.0	0.5–3	1.4	> 90	-	Purified, high functionality

¹OD/ID: Outside Diameter/Inside Diameter

²SSA: Specific Surface Area

Table 2. Preparation procedure parameters, particle size, and zeta potential analysis of Pluronic® F108 coated CNT suspensions.

CNT Suspension	CNT Product Name		CNT Type	Manufacturer	Sonication at 37 kHz	Centrifuge at 20,000 rcf	HDD ¹ (nm)	PDI ²	Zeta Potential (mV) Water Medium + 10% FBS	
	(a)	Short MWNTs							-19.5 ± 2.3	-2.2 ± 0.8
(b)	C-MWNT	Short MWNTs-COOH	MWNT	NanoAmor (NA)	120 W, 60 min	5 min	89 ± 1	0.18	-20.4 ± 0.3	-4.8 ± 0.4
(c)	P-MWNT(L) ³	MWNTs	MWNT	NanoAmor (NA)	60 W, 5 min	5 min	216 ± 20	0.41	-	-
(d)	C-MWNT(L) ³	MWNT-COOH			120 W, 90 min	10 + 10 min	200 ± 30	0.36	-	-
(e)	P-MWNT(S) ⁴	MWNTs					84 ± 1	0.21	-	-
(f)	C-MWNT(S) ⁴	MWNT-COOH	MWNT	NanoCyl (NC)	120 W, 60 min	5 min	71 ± 1	0.22	-	-
(g)	P-MWNT	NC3150™					118 ± 2	0.22	-22.9 ± 0.6	-1.1 ± 0.3
(h)	C-MWNT	NC3151™	MWNT				86 ± 7	0.23	-26.3 ± 0.9	-3.8 ± 0.2
(i)	P-SWNT	P2-SWNT	SWNT	Carbon Solution, Inc (CSI)	120 W, 60 min	5 + 30 min	150 ± 2	0.38	-19.0 ± 0.7	-1.1 ± 0.4
(j)	C-SWNT	P3-SWNT					88 ± 2	0.49	-25.0 ± 0.3	-2.5 ± 0.3

¹ HDD: Hydrodynamic diameter

² PDI: Polydispersity index

³ (L): Longer lengths

⁴ (S): Shorter lengths